Advances in Ex Vivo Modified Cell Therapies

1. Development of an Optimized Lentiviral Transduction Process for *Ex Vivo* CD34+ Hematopoietic Stem Cell Gene Therapy Drug Product Manufacture

Saranya Elavazhagan[1], Maria del Mar Masdeu[1], Tomasz Zabinski[1], Lily Du[1], Florence Enjalbert[1], Valentina Pennucci[1], Batika Rana[1], Christopher Whiting[1], Chiara Recchi[1], Paul Heal[1], Adrian J. Thrasher[2], Denise A. Carbonaro-Sarracino[1], Jia Wolfe[1], Bobby Gaspar[1], Pervinder Sagoo[1]

[1]Discovery Research, Orchard Therapeutics Europe Ltd, London, United Kingdom,[2]UCL GOS, Institute of Child Health, London, United Kingdom

Gene therapy using transplantation of autologous *ex vivo* gene modified CD34$^+$ hematopoietic stem cells (HSC) as an approach to treat a range of monogenic disorders, is now recognized for its transformative potential through several clinical studies and regulatory approvals. Despite significant advances in the commercialization of gene therapies, the main barriers to patient accessibility are the current limited capacity for manufacture of GMP-grade lentiviral (LV) vectors, and the corresponding substantial costs incurred by production of gene and cell therapies. This is further compounded by the need to manufacture higher numbers of gene modified HSCs for adult patients such as those suffering from X-Linked Chronic Granulomatous Disease (X-CGD), and for indications with larger patient cohorts. Reducing vector requirements and cost of goods therefore presents a key challenge in commercializing gene therapies. Application of transduction enhancers enables the use of lower quantities of LV vector to achieve the same output of gene modified cells. Several enhancer compounds are already routinely applied in clinical gene and cell therapy manufacture to improve the viral transduction process at various cellular levels, such as viral attachment, vector entry, and genome integration. To develop an optimized protocol for LV transduction of HSCs, we have screened over 20 commercially available and novel candidate compounds for enhancement activity, when applied individually or in combination to target distinct viral transduction pathways. Our comprehensive survey of improvements in transduction efficiency (TE) and vector copy number (VCN) achievable by these enhancers was conducted with both scale-down high-throughput and clinical-scale transduction processes for HSC gene therapy drug product manufacture, using clinical-grade therapeutic LV vectors. The most potent enhancer combinations were then assessed for compatibility with other known transduction culture process modifications, to develop an optimized protocol for HSC transduction. Enhancer treated HSCs were subject to extensive *in vitro* and *in vivo* characterization, including RNAseq transcriptional profiling and competitive engraftment studies in mice. Here we describe J-Boost, a representative compound from a novel class of transduction enhancers (diblock copolymers, PCT/US20/56123) which facilitates viral entry. When used in concert with Protamine Sulphate (PS) and high-density cultures, J-Boost results in up to ~9 fold increases in VCN and ~4 fold increases in TE, enabling 50-70% reduction in LV vector for HSC transduction to achieve desired target drug product profiles. Transduction is further enhanced by use of Retronectin^{TM} coated culture vessels. Our results demonstrate that J-Boost/ PS enhancers are largely inert, inducing minimal alteration of HSC gene expression, HSC phenotype and multilineage progenitor function, while generating gene modified HSCs with comparable qualitative and quantitative HSC engraftment potential in NSG mice. Importantly, we show compatibility of this optimized transduction protocol with therapeutic LV vectors and manufacture methods currently in use for drug products under development for Beta-thalassemia, Mucopolysaccharidosis I (MPS I) and X-CGD. Demonstration of the safety, efficacy and comparability of this optimized transduction protocol validates its potential for clinical application and the achievable reduction in vector usage and manufacturing costs.

2. Non-Viral Integration of Large Cargo in Primary Human T Cells by CRISPR/Cas9 Guided Homology Mediated End Joining

Matthew J. Johnson, Beau R. Webber, Nicholas Slipek, Walker S. Lahr, Xiaohong Qiu, Blaine Rathmann, Miechaleen D. Diers, Bryce Wick, R Scott McIvor, Branden S. Moriarity

University of Minnesota, Minneapolis, MN

Adoptive cell therapy (ACT) using genetically engineered immune cells, such as CAR-T cells, holds tremendous promise for the treatment of advanced cancers. Current methods for the manufacturing of these cells rely on the use of viral vectors, greatly increasing manufacturing time, expense, and complexity. Furthermore, these viral vectors integrate in a non-site specific manner reducing functionality and raising safety concerns. Here we describe methods for efficient CRISPR-based, non-viral engineering of primary human T cells that overcome key limitations of previous approaches, namely DNA-induced toxicity and low efficiency integration of large genetic cargos. By synergizing temporal optimization of delivery, reagent composition, and integration mechanism, we achieve targeted knockin of cargo ranging from 1 to 3 kilobases at rates of up to 70% at AAVS1 (Figure 1), with post-editing cell viability of over 80%; efficiencies nearing those of viral vector platforms. Notably, approaches utilizing homology mediated end joining (HMEJ) and shorter homology arms (48bp) consistently outperformed those using longer 1kb homology arms and traditional homologous recombination. Off-target editing and integration were evaluated using GUIDE-seq and targeted locus amplification (TLA), respectively. As proof of concept, we engineered CAR-T cells and transgenic TCR T cells using a splice acceptor gene construct and gRNA specific to the TRAC locus, such that the CAR or transgenic TCR is expressed under the control of endogenous TRAC regulatory elements. Using this approach we consistently achieved integration rates of over 20% for CAR-T cells and over 25% for TCR transgenic T cells (Figure 2). Additional optimizations, including culturing cells with anti-CD3 and anti-CD28 antibodies immediately following electroporation, further increased integration, averaging 39% for CAR templates. Furthermore, we demonstrate that these cells remain highly functional, retaining low expression of exhaustion markers, excellent proliferation and cytokine production capacity, and

potent anti-tumor cytotoxicity equal to or better than cells generated using a viral vector. Most importantly, these methods result in minimal-to-undetectable off-target editing and are readily adaptable to cGMP compliant and clinical-scale manufacturing. This non-viral gene engineering protocol offers a realistic, near-term alternative to the use of viral vectors in the production of genetically engineered T cells for cancer immunotherapy, offering immense potential for reducing manufacturing time, cost, and complexity compared to viral vectors without compromising cell expansion or function, while potentially increasing safety and efficacy via targeted integration.

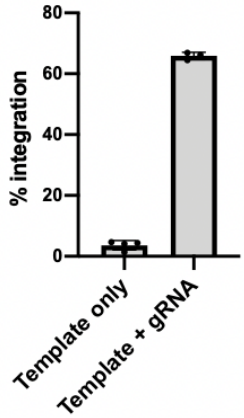


Figure 1. Expression of GFP in primary human T cells 8 days after transfection with Cas9 mRNA and a DNA minicircle template with 48bp of homology to AAVS1 encoding splice acceptor-GFP in the presence or absence of AAVS1 gRNA. GFP expression measured by flow cytometry.

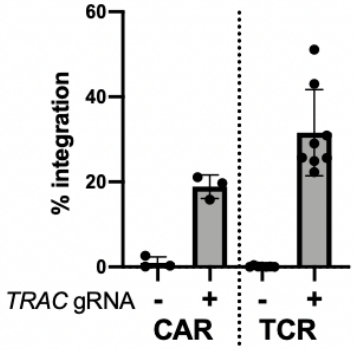


Figure 2. Expression of CAR or TCR in primary human T cells 7 days after transfection with Cas9 mRNA and a DNA minicircle template targeting TRAC and encoding CAR or TCR in the presence or absence of TRAC gRNA. Expression measured by flow cytometry.

3. Epigenetic Modulation of Aging to Increase CAR-T Cell Fitness

Benedetta Nicolis di Robilant, Rashmi Bankoti, Hazal Pektas, Lasse Jørgensen, Andreas Kongsgaard, Maddalena Adorno

Dorian Therapeutics, San Carlos, CA

Background: CAR-T cell therapy has had incredible clinical success in the treatment of hematological malignancies. However, very limited activity against solid tumors has been achieved so far, despite targeting a variety of antigens and tumor types. Here we show the impact of reducing cellular senescence in CAR-T cells to improve T cell fitness. Increasing evidence shows that immunosenescence is an important state of T cell dysfunction distinct from exhaustion; solid tumors strongly induce senescence as a key strategy to evade immune surveillance and sustain a suppressive tumor microenvironment. Notably, the *ex vivo* manufacturing process of CAR-T cells also induces senescence extremely quickly; 15 days of T cell expansion age cells 30 years, as measured by telomere length, T cells differentiation and CDKN2a mRNA levels. Here we show that rejuvenating T cells by blocking cellular aging at the epigenetic level targeting USP16 is a powerful strategy to improve T cell fitness and clinical outcomes. Methods and Results: T cell aging is a very early phenomenon upon T cell activation, and it is drastically accelerated during ex vivo CAR-T manufacturing, resulting in a huge reduction of their ability to expand and kill cancer cells. In line with this hypothesis, we demonstrated that reducing cellular senescence increases CAR-T cell functions both in vitro and in vivo. USP16 is a deubiquitinating enzyme responsible for the removal of ubiquitin moieties from histone H2AK119, increasing chromatin accessibility to pro-senescent programs. Here, we show that targeting USP16 by means of a shRNA co-expressed within a CD19 or a GD2.CAR construct reduces T cell aging and increases stem cell memory (Tscm) frequency during manufacturing, without affecting proliferation. USP16 modulation also results in increased killing, polyfunctionality, and expansion upon in vitro stimulation with tumor cells. Notably, the delay of cellular senescence induces long-lasting cellular fitness as T cells are less exhausted upon multiple tumor challenges. Finally, T cells rejuvenated by USP16 modulation, show a strong increase in anti-tumor activity in an in vivo model of leukemia and neuroblastoma. **Conclusions:** Preventing T cell senescence by modulating the expression of USP16 increases self-renewal and anti-tumor activity, significantly improving the efficacy of CAR-T therapy. Development of small molecules against USP16 could offer a viable solution to improve T cell fitness during manufacturing.

4. Effective and Efficient Intracellular Delivery Achieved with the Cell Squeeze® Technology Enables Rapid, Scaled, and Reproducible Production of Cell Therapies

David Chirgwin, Maisam Dadgar, Jason Murray, Claire Page, Scott Loughhead, Ipsita Roymoulik, Howard Bernstein

SQZ Biotechnologies, Watertown, MA

Personalized cellular therapies, especially in cancer, have grown in number over the last five years. Cell therapies require precise biological

engineering using cargos which are intracellularly delivered. However, conventional approaches for delivering materials into cells are limited in the types of biology they can engineer and have manufacturing challenges with regards to scale and reproducibility. The Cell Squeeze® technology uses microfluidic chips with constrictions to deform cells resulting in the effective intracellular delivery of molecules to cells while preserving cell health and function. This technique enables numerous opportunities to reproducibly generate new classes of cell therapies at scale. We have implemented the Cell Squeeze® process for cGMP and aseptic manufacturing. Our system is appropriate for use in a Grade B cleanroom utilizing a single-use sterile disposable kit containing multiple microfluidic chips. The Cell Squeeze® manufacturing system can accommodate up to 20 billion cells for processing in 2 minutes or less. Cell viability at manufacturing scale is >90% and the percent of the cell population delivered is >80%. This system is currently being used to manufacture drug product in a Phase 1 clinical trial using peripheral blood mononuclear cells (PBMCs) to generate antigen presenting cells as a cellular vaccine. To further enhance accessibility of SQZ Biotechnology cell therapies, we are also developing a point-of-care manufacturing system with the potential to manufacture cell therapies for same-day dosing. Our advances in cell therapy manufacturing will potentially enable multiple therapeutics across oncology, infectious disease, and immune disorders to be developed and delivered to a broad patient population.

5. Sequential CRISPR-Mediated Engineering and Clonal Banking for the Generation of Multiplexed Engineered Master Pluripotent Cell Lines for the Mass Manufacture of Off-the-Shelf Immune Cells Targeting Solid Cancers

Ramzey Abujarour, Lauren K. Fong, Fernanda R. Cugola, Nicholas Brookhouser, Chelsea M. Ruller, Berhan Mandefro, Janel Huffman, Karma Farhat, Tom T. Lee, Bahram Valamehr

Fate Therapeutics, San Diego, CA

Chimeric antigen receptor (CAR) T and NK cell therapies have shown great promise in treating blood malignancies, but tackling solid tumors is hindered by a shortage of targetable tumor-specific antigens, antigen escape, and poor activity of expanded patient-derived immune cells. While a multiplexed engineering approach can arm immune cells to address these obstacles, engineering patient- or donor-derived NK and T cells often leads to inconsistent, heterogeneous, and costly products. We describe here a platform to enable complex genetic engineering and precise targeting of independent loci through sequential rounds of CRISPR-mediated editing, single cell cloning, screening and banking of induced pluripotent stem cells (iPSCs) leading to the generation of multiplexed engineered master iPSC clones. Using this platform, we are developing a master iPSC bank that is uniformly engineered with four anti-tumor modalities. A core of three edits are first introduced and include: i) IL-15 receptor fusion protein (IL-15RF) for enhanced NK cell activity, ii) high affinity non-cleavable CD16 (hnCD16) for enhanced antibody-dependent cellular cytotoxicity, and iii) CD38 deletion for enhanced NK cell function. The fourth edit, CAR targeting the HLA-I related molecules MICA and MICB (MICA/B), is engineered next in a second locus for pan-tumor targeting. Challenges with building such a complex cellular therapy include genomic instability and off-target editing that could be introduced during CRISPR/nuclease-based engineering of multiple loci, and during the multiple rounds of single cell cloning, expansion, and banking. To reduce off-target edits that could be caused by simultaneously targeting two loci, we performed sequential engineering instead, targeting one locus at a time to build a more complex master iPSC line. In the first step of the engineering process, iPSCs were engineered with the three core anti-tumor modalities by targeting an IL-15RF-hnCD16 cassette into the CD38 locus, resulting in complete CD38 gene disruption. The CD38 gene was targeted with fully characterized gRNA and donor plasmid containing the IL15-RF-hnCD16 cassette. Engineered iPSCs were cloned from single cells and clones were screened and fully tested before and after banking. Out of 257 iPSC clones screened, 88 clones (34%) had the IL-15RF-hnCD16 transgenic cassette targeted specifically into the CD38 gene. Evaluation of genomic stability of the selected clones after banking revealed that 12 of the 15 selected clones maintained genomic stability as determined by G-banded karyotyping, and 10 clones out of 11 tested showed no genomic copy number variations as determined by a genome-wide SNP microarray analysis. Banked iPSC clones were further evaluated for pluripotency and propensity to differentiate into NK cells which were further tested for phenotype and function. The best performing clone was then selected for a second round of genetic engineering, whereas CAR-MICA/B is inserted into a safe harbor locus at the single cell level to create the final master iPSC clone containing the three core edits and the CAR-MICA/B. The generated engineered iPSC subclones are currently being screened for specific targeting of the CAR-MICA/B into the safe harbor locus and other critical quality attributes (including maintenance of pluripotency and genomic stability) to nominate the final iPSC master cell bank. This master cell bank will serve as the starting material for clinical investigation of FT536, an off-the-shelf pan-cancer targeting CAR NK cell immunotherapy with potential therapeutic application to multiple hematopoietic and solid tumors.

6. Generation of Engineered Tregs (EngTregs) from Umbilical Cord Blood Derived CD4+ T Cells via HDR-Dependent *FOXP3* Gene Editing

Shivani Patel[1], Yuchi Honaker[1], Su Jung Yang[1], Noelle P. Dahl[1], Warren Anderson[1], Samuel Scharffenberger[1], Iram Khan[1], Michelle Christian[1], Karen Sommer[1], David J. Rawlings[1,2,3]

[1]Center for Immunity and Immunotherapies and Program for Cell and Gene Therapy, Seattle Children's Research Institute, Seattle, WA,[2]Pediatrics, University of Washington School of Medicine, Seattle, WA,[3]Immunology, University of Washington School of Medicine, Seattle, WA

Regulatory T cells (Tregs) play a crucial role in peripheral immune tolerance and homeostasis. Adoptive transfer of Tregs is being pursued in clinical trials for autoimmune diseases and for bone marrow and solid organ transplantation. Current Treg trials utilize ex-vivo expanded thymic Tregs (tTregs) derived from either peripheral blood (PB) or umbilical cord blood (UCB); however, this approach must overcome technical challenges that include manufacturing adequate

cell doses and maintaining cell purity and Treg stability. We previously developed gene engineered Tregs (EngTregs) from PB-derived CD4+ T cells as an alternative source for Treg cell therapies. This approach utilizes co-delivery of a *FOXP3*-targeting designer nuclease and an rAAV homology-directed-repair (HDR) donor template designed to introduce a gene cassette containing MND promoter and cis-linked LNGFRt surface tag into the *FOXP3* locus. The resulting enforced and stable expression of FOXP3 reprograms HDR-edited cells to acquire a Treg-like phenotype and suppressive function in vitro and in vivo in xeno-GvHD mouse models. This approach permits clinical-scale production of highly purified EngTregs for potential therapeutic use. The naïve phenotype of CD4+ T cells in UCB suggests a potential proliferation and potency advantage over T cells in adult PB as a source for EngTregs production. Further, HDR editing in UCB T cells might provide the capacity to generate multiple cell doses for short-term allogeneic cell therapies. In the current study, we evaluated the feasibility of using UCB-derived CD4+ T cells to generate EngTregs. We found that UCB-derived CD4+ T cells could be edited at a high efficiency. Edited cells could also be efficiently enriched by the surface LNGFRt selection marker. Notably, the expansion methods optimized for PB-derived EngTregs (rapamycin treatment, high dose of IL-2 and G-rex culture) did not yield comparable cell viability or quantity for UCB-derived EngTregs. Therefore, we established alterative protocols for cell expansion; approaches that significantly improved the yield and viability of the UCB EngTregs products. In parallel with these studies, we also tested an alternative editing strategy designed to introduce a heterodimeric, chemically-induced signaling complex (CISC; that mimics IL-2 signaling in response to an exogenous dimerizer) upstream of the *FOXP3* gene. UCB derived EngTregs cells products (including LNGFRt+ or CISC+ EngTregs, respectively) exhibited high purity, stable FOXP3 expression, consistent expression of key Treg markers and limited expression of proinflammatory cytokines upon stimulation. Additionally, we tested whether UCB-derived EngTregs could suppress allogeneic effector T cells in the xeno GvHD mouse model. Our preliminary data showed that the UCB-derived EngTregs have suppressive capabilities against HLA-mismatched allogeneic effector T cells in NSG recipient mice. In summary, our data demonstrate a robust capacity to engineer UCB-derived T cells leading to generation of EngTregs that exhibit a stable Treg phenotype and function. Additional optimization of cell expansion is likely to provide cell yields that enable UCB to be a viable future cell source for EngTregs.

7. VOR33: A Clinic-Ready CRISPR/Cas9 Engineered Hematopoietic Stem Cell Transplant for the Treatment of Acute Myeloid Leukemia

John Lydeard[1], Michelle Lin[1], Chong Luo[1], Shu Wang[1], Amanda Halfond[1], Mark B. Jones[1], Julian Scherer[1], Dane Hazelbaker[1], Meltem Isik[1], Azita Ghdossi[1], Juliana Xavier-Ferrucio[1], Gary Ge[1], Elizabeth Paik[1], Gabriela Zarraga-Granados[1], Taylor Perkins[1], Matthew Li[1], Brent Morse[1], Siddhartha Mukherjee[2], Sadik Kassim[1], Tirtha Chakraborty[1]

[1]Vor Biopharma, Cambridge, MA,[2]Columbia University Medical Center, Columbia University, New York, NY

Introduction: AML is the most common form of adult acute leukemia, with median 5-year survival rate <30%. Allogeneic hematopoietic cell transplant (HCT) has long been the standard of care for high-risk patients (pts), with >3500 transplants performed annually in the US. There is unmet need for new treatments in ~40% of pts who relapse. With existing targeted therapies, cell surface marker expression between cancer and normal cells is not differentiated enough to limit "on-target, off-tumor" toxicity. Antigens (Ag) (eg, CD33) expressed on normal myeloid cells and/or progenitors (Levine et al. 2015) confer dose-limiting toxicity of Ag-directed therapies in AML. To unlock the full potential of targeted treatments, we create treatment-resistant hematopoietic stem cells (HSCs) by genetically ablating CD33 from healthy, HLA-matched (10/10) donor HSCs followed by HCT, creating a target Ag-negative hematopoietic system. The reconstituted hematopoietic compartment of pts receiving CD33-null cells will be resistant to cytotoxicity induced by Mylotarg™, an anti-CD33 monoclonal antibody conjugated with cytotoxic calicheamicin. Human CD33 null hematopoietic cells show no impairment of function and are resistant to CD33-targeted therapies (Borot et al. 2019; Humbert et al. 2019; Kim et al. 2018). Notably, this is consistent with natural genetic evidence of CD33 null humans with no deleterious phenotype (gnomad.broadinstitute.org/). Here, we describe the preclinical data and process scale-up of the CD33-null HSC graft (VOR33) for a first-in-human clinical trial. **Methods/Results:** The manufacturing process yielded clinically relevant doses of VOR33 (>3x10^6 viable CD34+ cells/kg) under GMP-like conditions with GMP-appropriate reagents. CD34+ cells, isolated from G-CSF and plerixafor mobilized peripheral blood leukapheresis products, were edited using CRISPR/Cas9 to disrupt *CD33* gene. At scale, we routinely achieved gene knockout of >70% (90% biallelic) with no loss of cell viability. Cells differentiated from VOR33 displayed normal myeloid markers, phagocytosis potential and induction of inflammatory cytokines equivalent to unedited (CD33+) control cells. Phenotypic and functional characterization revealed no difference in frequency of long-term HSCs in VOR33 vs unedited controls. Pharmacology studies using NOD/SCID-gamma mice, with VOR33 cells manufactured under GMP-like conditions, showed normal long-term engraftment (16-week bone marrow chimerism of 83.1±9.0% vs 87.9±7.3% in control group) and multilineage differentiation. In addition, we observed persistence of VOR33 gene editing and preservation of indel species distribution after 16 weeks, indicating no counterselection or clonal expansion of CD33-null cells. Importantly, we found loss of CD33 protein conferred

selective protection to VOR33-derived myeloid cells vs Mylotarg *in vitro* (>65-fold) and *in vivo* (>60-fold). In our GLP toxicology study of >40 tissues, we saw no tumorigenicity or notable changes in toxicology parameters. In-depth genotoxicity analyses were carried out with a subset of scaled-up manufactured lots of VOR33, including those used for toxicology and pharmacology studies. Deep sequencing of 2369 genomic sites, by homology-dependent and independent methods, revealed no off-target editing. **Conclusion:** These studies set the stage for initiation of, as well as evaluation of safety and efficacy in, a multicenter first-in-human clinical trial of VOR33 in pts with AML.

Cancer - Oncolytic Viruses

8. Preclinical Toxicology Assessment of an Oncolytic Measles Virus Armed with *H. pylori* Immunostimulatory Bacterial Antigen in Preparation for a Phase I Trial in Breast Cancer Patients

Kimberly Viker[1], Michael B. Steele[1], Ianko D. Iankov[1], Arun Ammayappan[1], Susanna C. Concilio[1], Brad Bolon[2], Nathan J. Jenks[1], Eleni Panagioti[1], Mark J. Federspiel[1], Minetta C. Liu[3], Kah Whye Peng[1], Evanthia Galanis[3]

[1]Department of Molecular Medicine, Mayo Clinic, Rochester, MN,[2]GEMpath, Inc., Longmont, CO,[3]Division of Medical Oncology, Mayo Clinic, Rochester, MN

Introduction: Despite recent therapeutic advances, metastatic breast cancer (MBC) remains incurable. Engineered measles virus (MV) strains based on the attenuated MV Edmonston vaccine platform have demonstrated significant oncolytic activity against solid tumors. *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is responsible for the robust inflammatory reaction in the gastroduodenal mucosa during infection. NAP attracts and activates immune cells at the site of infection inducing expression of inflammatory mediators. Our team engineered an MV strain expressing the secretory form of NAP (MV-sNAP) that exhibits anti-tumor and immunostimulatory activity in human breast cancer xenograft models. In this study, we investigated the biodistribution and toxicity of MV-sNAP in MV-susceptible transgenic Ifnarko™-CD46Ge mice. The primary objectives were to identify potential toxic side effects and to define the optimal equivalent dosage of MV-sNAP prior to proceeding with a Phase I clinical trial in MBC patients. **Methods:** Ninety-six, 5-to 6-week-old female Ifnarko™-CD46Ge mice were stratified into 6 groups with 2 cohorts (48 mice/cohort). Treatment with MV-sNAP (doses: 10^6 or 10^7 $TCID_{50}$/mL) or vehicle control was started on day 0 using either a single subcutaneous (SC) or intravenous (IV) injection (day 0) or via 3 repeated SC or IV injections on days 0, 14, 28. Body weight and clinical signs of toxicity were monitored daily, and hematology, plasma chemistry, plasma cytokines, gross pathology, and histopathology of major organs were analyzed on days 11, 12, 54 or 56 of the study. The immune response to MV-sNAP was assessed by a NAP-mediated ELISA virus neutralization test. Biodistribution of MV-sNAP was evaluated by qRT-PCR. **Results:** All mice survived to their respective endpoints, with no evidence of clinical toxicity. No significant difference between control and treated animals was observed in complete blood counts or plasma chemistry values including liver function at all time points. Low MV genome copy numbers of MV-sNAP were found on Day 11 in the inguinal lymph nodes (ILN) in 2 of 8 animals that received a single SC low dose injection, and in 3 of 8 animals that received a single SC high dose injection. In contrast, a single IV administration of MV-sNAP resulted in significant MV genome copy numbers in most tissues on day 11 in both dose groups. In animals given three SC MV-sNAP injections, the low-dose group exhibited expression only in the spleen and ILN in 1 of 8 animals on day 56. By day 12, a single IV administration of MV-sNAP resulted in a strong early immune response to MV antigens in all mice of both dose groups. High anti-MV titers were maintained by day 54 following three IV injections in all animals of both dose groups. MV-sNAP did not significantly increase circulating levels of pro-inflammatory cytokines. Histopathologic findings following administration of MV-sNAP showed asymptomatic minimal or mild hemorrhage in the lung following SC or IV administration (1-2/8 animals on day 11), and minimal leukocyte infiltration/inflammation at the SC injection site. These modest changes were interpreted to be non-adverse. **Conclusion:** Both SC and IV delivery of MV-sNAP were well tolerated, and no significant toxicity was observed in a relevant (i.e., MV-susceptible) engineered mouse model. This outcome supports the safety of the MV-sNAP platform for oncolytic virotherapy of MBC. Enrollment in a Phase I clinical trial of MV-sNAP in patients with MBC (NCT 04521764) started in November 2020.

9. Validating Secreted IFNβ as an In Vivo Biomarker of Intratumoral Replication of VSV-IFNβ-NIS

Lianwen Zhang, Michael B. Steele, Nathan J. Jenks, Alysha N. Newsom, Martha Q. Lacy, Kah Whye Peng, Stephen J. Russell

Mayo Clinic, Rochester, MN

VSV-IFNβ-NIS is an oncoytic vesicular stomatitis virus which is currently being evaluated in multiple clinical trials. Infection of target tissues can be monitored using SPECT or PET imaging to detect expression of the NIS transgene. IFNβ was encoded in the virus to limit its replication in normal tissues and to enhance the inflammatory/immune response in the infected tumor. However, we demonstrate here in murine models that IFNβ can also serve as a convenient soluble biomarker of VSV-IFNβ-NIS replication in vivo. We first evaluated the baseline level of IFNβ that is produced by innate immune cells upon intravenous administration of VSV. Infusion of 1e8 $TCID_{50}$ VSV-hIFNβ-NIS in tumor free C57BL mice induced a transient increase of endogenously secreted mIFNβ, between 32-64 pg/ml at 24h, and is undetectable at 48h. In contrast, the level of hIFNβ produced by VSV-hIFNβ-NIS infected cells in tumor free mice was 500-2000 pg/ml at 24h but is significantly higher in 5TGM1 tumor bearing animals (1000-65,000 pg/ml). The difference in tumor bearing versus tumor free mice becomes more significant at 48h where the virus continues to replicate and spread in the permissive 5TGM1 tumors. Varying doses of VSV-mIFNβ-NIS was also given intravenously to mice bearing 5TGM1 or MPC11 tumors, and blood levels of IFNβ were measured. Results indicate that the level of virally encoded IFNβ in tumor bearing mice is

dose dependent. There was a corresponding increase in median mIFNβ detected in the plasma of 5TGM1 mice given 1e8, 3e8 and 1e9 $TCID_{50}$ VSV-mIFNβ-NIS. Importantly, the level of IFNβ remained high over days 1-3, and started to decline from day 4, suggesting that secreted IFNβ could be a valuable biomarker to monitor the kinetics of viral infection in cells, followed by subsequent extinction of the infection and death of infected cells. We did not observe a secondary wave of infection in these immunocompetent animals. By day 7, there was no detectable levels of mIFNβ. Corresponding anti-VSV antibody titers are detectable by day 7, and plateaued by day 15. Interestingly, when we compared intratumoral versus intravenous administration of 3e8 $TCID_{50}$ of virus, we found overall higher levels of plasma mIFNβ in the intravenous group versus the intratumoral group, suggesting that systemic delivery of an oncolytic virus might be more beneficial to allow a more uniform seeding of the tumor parenchyma and infectious foci. In the MPC-11 plasmacytoma model in Balb/c mice, we saw a significantly higher level of plasma IFNβ, indicating a much more permissive tumor substrate for viral replication, and/or rapid death and release of cellular contents. In summary, our study confirms that virally encoded IFNβ produced by VSV-IFNβ-NIS infected cells can serve as a convenient and simple biomarker of viral replication in vivo, and that the level and profile (duration) of IFNβ in the plasma could serve as an early indicator of the relative permissiveness of the tumor substrate to the oncolytic virotherapy. These findings in the murine models are recently corroborated by early findings in the intravenous VSV-IFNβ-NIS Phase I trials in cancer patients.

10. Development of Novel Oncolytic Vector Based on Alternative Adenovirus Serotype 6 for Glioblastoma and Breast Cancer Therapy

Margarita Romanenko[1,2], Ivan Osipov[2], Sergei Kutseikin[2], Mariia Sizova[2], Anastasiya Paramonik[2], Antonina Grazhdantseva[3], Galina Kochneva[3], Julia Davydova[1], Sergey Netesov[2]

[1]Department of Surgery, University of Minnesota, Minneapolis, MN,[2]Novosibirsk State University, Novosibirsk, Russian Federation,[3]State Research Center of Virology and Biotechnology "Vector", Koltsovo, Russian Federation

The majority of adenovirus oncolytic agents are based on the adenovirus serotype 5 (Ad5), which is the most studied and the most used type of Ad in the field of gene therapy. However, there is a constant search for alternative Ad types, which are able to overcome the existing obstacles hampering the Ad5 application such as high seroprevalence and rapid clearance of viral particles after i.v. administration. Among many examined alternative Ad serotypes, adenovirus serotype 6 (Ad6) exhibits high oncolytic efficacy against many cancer types. Importantly, Ad6 was shown to have a lower seroprevalence and improved ability to escape from Kupffer cells when compared to Ad5. We recently demonstrated that wild-type Ad6 exhibits a dose-dependent cytotoxicity against glioblastoma cells *in vitro* and a significantly improved therapeutic effect in U87MG xenografts after intratumoral injection, which was comparable of that with Ad5 wild-type control vector. To further enhance the therapeutic efficacy of Ad6, we constructed a tumor-specific recombinant vector Ad6-hTERT-GMSCF with the E1 region controlled by the human telomerase reverse transcriptase promoter (hTERT) and the human granulocyte-macrophage colony-stimulating factor (GMCSF) transgene expressed from the Ad E3 region. The immunomodulatory activity of secreted GMCSF was evaluated after infection A549 lung adenocarcinoma cells with Ad6-hTERT-GMSCF by assessing the stimulation level of human erythroleukemia cells TF-1 proliferation. The oncolytic efficacy of Ad6-hTERT-GMSCF *in vitro* and *in vivo* was evaluated on both triple-negative breast cancer (MDA-MB-231) and glioblastoma multiforme (U87MG) cell lines. The insertion of hTERT promoter and GMSCF into the Ad6 genome did not significantly affect the oncolytic potential of Ad6 vector as it was demonstrated by the cell viability test. The *in vivo* efficacy of Ad6-hTERT-GMSCF in comparison to wild-type Ad6 and Ad5 vectors was evaluated using subcutaneous MDA-MB-231 or U87MG xenografts in SCID mice after performing injections three times (day 1, 3, and 5). The new recombinant Ad6-hTERT-GMSCF was able to significantly inhibit the tumor growth in both MDA-MB-231 and U87MG models. Importantly, the therapeutic effect of Ad6-hTERT-GMSCF was comparable with that of Ad5 and Ad6 wild-type control viruses. We are currently investigating the level of GMCSF in tumor xenografts. The oncolytic potential of a novel Ad6-hTERT-GMSCF vector needs further investigation in immunocompetent models. Further, additional genetic modification (e.g., fiber knob switching) may be beneficial for further enhancement of anticancer efficacy of Ad6-based recombinant vectors.

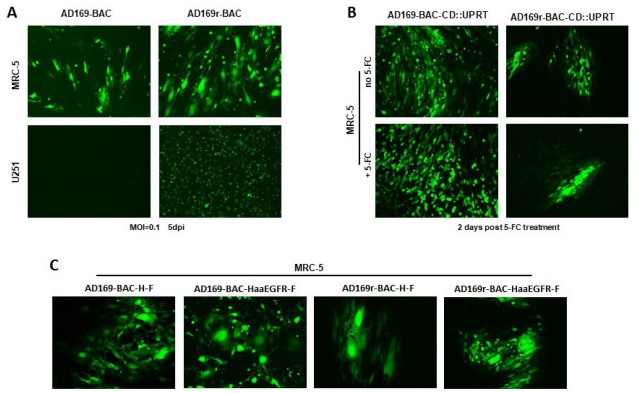
11. Human Cytomegalovirus Engineered for Glioma Therapy

Haifei Jiang, Rebecca Nace, Stephen Russell

Molecular Medicine, Mayo Clinic, Rochester, MN

Human cytomegalovirus (HCMV) is a 230nm diameter beta-herpesvirus with a ~235kb double-stranded linear DNA genome, an icosahedrally ordered nucleocapsid, a tegument layer and a complex envelope. HCMV naturally hijacks neutrophils and monocyte/macrophages for local spread, then infects endothelial cells and disseminates systemically in various hematological cell types. In the brain, HCMV infects glial cells and spares neurons. We are therefore interested to develop HCMV as an oncolytic platform for the treatment of glioma since it should infect both the tumor cells and tumor infiltrating macrophages and, if appropriately armed, may efficiently recruit cytotoxic T cells and NK from circulation to target CMV antigen positive cells within the glioma.To develop HCMV as a glioma-targeting vector we worked with the lab adapted strain AD169 and constructed a bacterial artificial chromosome (BAC) clone (AD169-BAC). Genome sequencing confirmed the expected sequence deletions between UL1-UL20 and of the UL/b′ region which in wtCMV encode protein and RNA products that combat T cell and NK cell activation promote virus latency in hematological cells. Vaccines based on lab adapted AD169 and Towne strains have good safety profiles in clinical trials. To enhance glioma cell targeting, we repaired the function of the gH/gL/UL128-131 glycoprotein complex by replacing the UL131 like sequence in the AD169 backbone with the UL131 ORF from Merlin strain (AD169r-BAC). Comparing AD169-BAC and AD169r-BAC, both efficiently infected and killed MRC-5 cells, but only AD169r-BAC spread in U251 glioma cell cultures (Fig A). Both viruses showed antitumor activity in U87 and U251 glioma xenograft models, although AD169r-BAC was more potent. We next knocked out the US1-US11 region which encodes proteins and RNAs

that inhibit MHC-I and MHC-II pathways and in vivo testing in U87 model suggested superior infectivity of these viruses. We next inserted the suicide gene CD::UPRT (cytosine deaminase fused to uracil phosphoribosyl transferase) into AD169-BAC and AD169r-BAC (Fig B), and in parallel inserted untargeted and EGFR-targeted measles F/H glycoprotein complexes into the same backbones (Fig C). We are currently testing the efficacy and mechanisms behind these recombinant AD169 viruses expressing suicide genes or fusogenic F/H complexes in subcutaneous and orthotopic glioma models. Our preliminary data suggest that HCMV could be developed as a unique anti-cancer oncolytic or gene therapy platform, and more therapeutic transgenes will be added.



12. Virulent Velogenic Newcastle Disease Virus Is More Oncolytic Than Attenuated and Lentogenic Newcastle Viruses

Ahmed Majeed Al-Shammari[1], Sarah Hassan[2], Aida Bara Allawe[3]

[1]Experimental Therapy Department, Mustansiriyah University, Iraqi Center for Cancer and Medical Genetic Research, Baghdad, Iraq,[2]Baghdad University, College of Veterinary Medicine, Baghdad, Iraq,[3]Department of Microbiology, College of Veterinary Medicine, Baghdad University, Baghdad, Iraq

Lentogenic LaSota strain, attenuated AMHA1 strain and AMHA2 virulent Iraqi strain of Newcastle disease virus (NDV) are three replication-competent, non-recombinant natural oncolytic Newcastle disease viruses evaluated in the current study for their anticancer effects against breast cancer cells. All these strains selectively replicate within tumor cells. AMHA1 is attenuated NDV virus after 7 years of passaging in embryonated chicken eggs, carrying avirulent lentogenic fusion protein motif. Iraqi virulent strain AMHA2 carry virulent F protein cleavage site motif. In vitro cytotoxicity assay, clonogenic assay, replication curve, quantitative real-time PCR assay for NDV mRNA and cytopathic effect studies revealed that virulent AMHA1 strain was more effectively infected, replicated, and killed human and mouse breast cancer cells, followed by attenuated AMHA2 and lastly lentogenic LaSota being less effective. The virulent AMHA2 strains were able to induce more powerful apoptotic response as studied by acridine orange-propidium iodide apoptosis assay in breast cancer cells followed by attenuated AMHA1, in contrast to LaSota NDV strain which produce less apoptotic effect yet still significant. Immunohistochemistry performed on human and mouse breast cancer cells revealed high NDV HN viral protein expression in cancer cells. To study the apoptosis mechanism induced by each strain, caspase-8 and caspase-9 protein expression were evaluated and found that caspase-9 showing higher expression induced by all three strains with association of caspase-8 which have lower expression. The use of more virulent strains in oncolytic virotherapy can be more efficient strategy for effective tumor treatment.

13. Generation and Characterization of Replication-Competent Oncolytic Foamy Virus Vectors

Karol Budzik, Rebecca Nace, Yasuhiro Ikeda, Stephen Russell

Molecular Medicine, Mayo Clinic, Rochester, MN

Simian Foamy Viruses (SFVs) are ancient, non-pathogenic retroviruses that infect a variety of nonhuman primates with sporadic cases of zoonotic transmission to humans. Similar to Gammaretroviruses replication of SFVs is limited to dividing cells, however, unlike Gammaretroviruses, SFVs have a remarkable ability to persist unintegrated at the centrosome of quiescent cells for weeks, waiting for nuclear membrane disintegration during cell division. This feature enables SFV to efficiently infect cells with long doubling times, such as human tumor cells, which have been reported to have doubling times between 25 to over 200 days. SFV's unique features make it a promising candidate for a useful human cancer therapy. Here, we describe the generation and characterization of oncolytic Simian Foamy Virus (oFV) vectors. oFV was generated by combining genome segments from 2 strains of the chimpanzee SFV - PAN1 and PAN2. oFV was then engineered to carry a reporter gene - GFP in place of the *bel-2* gene (oFV-GFP). Both the parental and the GFP-carrying virus replicated efficiently, albeit slowly, in a panel of human cancer cell lines *in vitro* and exhibited a clear oncolytic activity *in vivo.* A single dose of 10^7 IU of either vector potently controlled growth of orthotopic intraperitoneal ovarian cancer metastases and significantly prolonged survival of the treated mice. Subsequently, we armed oFV with a suicide gene, HSV1 Thymidine Kinase (TK), and tested *in vivo* in subcutaneous xenograft glioblastoma tumors engineered to express firefly luciferase in response to FV infection. Upon infection with oFV-TK and treatment with Ganciclovir, luminescence in the infected indicator tumors decreased, indicating accelerated death of the oFV infected cells. Our data show that oFV is a promising gene delivery platform and candidate for a cancer therapeutic.

14. An Oncolytic Adenoviral Vector Expressing an Anti-PD-L1 scFv Reduces Tumor Growth in a Melanoma Mouse Model

Maria Vitale[1,2], Eleonora Leggiero[2], Margherita Passariello[2], Anna D'Agostino[2], Lorella Tripodi[2,3], Claudia De Lorenzo[1,2], Vincenzo Cerullo[1,4], Lucio Pastore[1,2]

[1]Molecular Biology and Medical Biotecnology, University of Naples, Federico II, Napoli, Italy,[2]CEINGE, Naples, Italy,[3]European school of Molecular Medicine (SEMM), Naples, Italy,[4]3Division of Biopharmaceutics and Pharmacokinetics, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

Oncolytic virotherapy is an emerging therapeutic approach, based on replication-competent viruses, to selectively infect and destroy cancer cells, causing the release of tumor-associated antigens (TAA), therefore stimulating an antitumoral immune response. Indeed, oncolytic adenoviruses (Onc.Ads) can kill cancer cells in different ways, primarily by inducing immunogenic cell death. To increase the anticancer activity of Onc.Ads, it is possible to combine them with a strategy aimed at blocking tumor immune evasion. Programmed death ligand 1 (PD-L1) is mainly expressed on tumor cells surface; binding to its receptor, PD-1, expressed on CD8+ T cell inhibits their proliferation and antitumoral activity. As demonstrated with monoclonal antibodies, a PD-L1 antagonist can prevent the immune escape T cell-mediated of the tumor cells. We developed an Onc.Ad expressing a single-chain variable antibody fragment (scFV) against PD-L1 to combine blockage of PD-1/PD-L1 interaction with the antitumoral activity of Onc.Ads. We confirmed the expression and secretion of scFv anti-PD-L1 in the supernatant of infected cells by western blot analysis. B16-OVA cells (a mouse melanoma cell model) were then infected with Onc.Ad5Δ24-scFV-PD-L1 and treated with C57BL/6 splenocytes. We observed that treatment combination was significantly more effective in reducing cell viability. We then evaluated tumor progression *in vivo* in three groups of mice, inoculated with syngeneic B16-OVA melanoma cells, treated with either Onc.Ad5Δ24-scFV-PD-L1, Onc.Ad5Δ24 or mock treatment. We observed that treatment with Onc.Ad5Δ24-scFV-PD-L1was more effective in reducing melanoma growth compared to mice treated with Onc.Ad5Δ24. We will further immunologically characterize immune response induced by the combined treatment; however, these results suggest that Onc.Ad-induced expression of an immune checkpoint inhibitor is an effective and promising strategy.

Delivery Technologies and CRISPR for Therapeutics

15. CRISPR/Cas9-Mediated Targeted Gene Insertion Platform Achieves Durable, Normal Human Alpha-1 Antitrypsin Protein Levels in Non-Human Primates

Sean Burns, Jenny Xie, Adam Amaral, Darleny Lizardo, Carri Boiselle, Catherine Moroski-Erkul, Kathryn Walsh, Tenzin Yangdon, Elena Kollarova, Vinita Doshi, Riley Cole, Nikunja Kolluri, Shreelekha Jaligama, Kenneth Manning, Harini Sampath, Dohyun Kim, Palak Sharma, Trisha Das, Samantha Soukamneuth, Sucharitha Parthasarathy, Andrew Whynot, Richard Duncan, Lucinda Shaw, Matthew Roy, Michelle Young, Noah Gardner, Yuanxin Xu, Laura Sepp-Lorenzino, Jessica Seitzer, Anthony Forget

Intellia Therapeutics, Cambridge, MA

CRISPR/Cas9-based genome editing offers the potential to address genetic diseases at their source. Using this technology, we have created a liver-directed, modular genome editing platform to enable rapid therapeutic advancement for genetic diseases with high unmet need, by either reducing a disease-causing protein through knockout editing or enabling production of a functional protein through targeted gene insertion. We have pioneered the use of lipid nanoparticles (LNPs) to enable systemic, transient, and well-tolerated delivery of the CRISPR/Cas9 genome editing system. Using a hybrid approach combining LNP-encapsulated CRISPR/Cas9 components with an adeno-associated virus (AAV) for "promoterless" donor DNA template delivery, we achieve targeted gene insertion resulting in high levels of protein expression *in vivo* in both murine and non-human primate (NHP) models. Here, we illustrate the power of our platform for advancing genome editing to treat alpha-1 antitrypsin deficiency (AATD). In this disease, mutations in the *SERPINA1* gene lead to liver pathology due to aggregation of the alpha-1 antitrypsin (AAT) protein in hepatocytes, and lung pathology due to deficiency of the AAT protein in the lungs. Normal levels of AAT protein in humans are approximately 1,000-2,700 ug/mL, a high level that has been challenging to achieve using chronic protein augmentation therapy or traditional gene therapy approaches. By leveraging CRISPR/Cas9 to perform precise gene insertion downstream of the strong endogenous albumin promoter, we now demonstrate the sustained production of normal human levels of AAT protein in NHP, without impacting normal albumin levels. The AAT protein concentration rose quickly after a single administration, reaching the normal range by week 4, and remained stable within the normal range through week 14 in an ongoing study. The physiological levels of human AAT protein produced in this study are expected to be fully therapeutic to restore protease inhibition and protect the lungs in AATD. These results in NHPs build on our previous findings in a humanized mouse model of AATD in which we showed successful consecutive *in vivo* genome editing (*SERPINA1* knockout plus insertion) as an alternative approach to addressing both liver and lung manifestations of the disease. Moreover, they highlight the potential of our modular targeted gene insertion platform to produce durable, high-level expression of therapeutic proteins for diverse genetic diseases, beyond what has been achieved using traditional gene therapy approaches.

16. Direct rAAV-Mediated *In Vivo* Gene Editing of Hematopoietic Stem Cells

Ishani Dasgupta, Qiushi Tang, Terence R. Flotte, Allison M. Keeler

Horae Gene Therapy Center, Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA

Gene editing of hematopoietic stem and progenitor cells (HSPCs) has progressed to clinical stage and represents a tremendously promising platform for future gene therapy for hemoglobinopathies such as sickle cell disease (Hgb SS disease). However, there are inherent practical limitations to scaling up such approaches to make them accessible to global populations most affected by these disorders. Thus, *in situ* gene modification that enables a direct targeting of the HSPCs *in vivo* would be ideal. Most prior *in vivo* editing techniques involved systemic injection of the editing machinery focused on targeting the liver, thus taking advantage of the efficiency of rAAV-mediated liver gene transfer. In this study, we are employing a targeted delivery strategy of direct injection of rAAV encoding a transgene flanked by homology arms to initiate homology directed repair (HDR) mediated

gene editing of HSPCs in the bone marrow. To obtain stable transgene expression without adversely affecting endogenous gene expression, we decided to edit at the genomic safe harbor (GSH) site, AAVS1. First, we optimized the construct *in vitro* to ensure optimal transgene expression in target cells. We compared expression of the reporter GFP expressed by the CMV, EF1a and MND promoters. Human HSCs were isolated from cord blood by negative selection and enrichment of CD34+ cells, followed by electroporation with ribonucleoprotein complex containing AAVS1 guideRNA and Cas9. CD34+s cells were then transduced with rAAV6 expressing GFP from the different promoters flanked by AAVS1 homology arms. The MND promoter reported robust and long-term (up to 9 days) GFP expression in human HSCs *in vitro*. Additionally, the number of GFP expressing cells increased over time in culture suggestive of editing. Based on the *in vitro* results, we shortlisted the MND promoter for subsequent *in vivo* experiments. Since our objective is to target HSCs and cells of the hematopoietic lineage, it is necessary to assess whether the MND promoter is expressed in these cells of interest. To test this *in vivo,* firstly we established conditions for optimum engraftment and differentiation of human CD34+s in the mouse bone marrow. We then engrafted human HSCs, electroporated with CRISPR/Cas editing machinery and transduced with rAAV6 encoding GFP driven by MND promoter, flanked by AAVS1 homology arms into immunocompromised NBSGW (nonobese diabetic (NOD)-severe combined immunodeficiency (SCID)-gamma) mice. About 40-80% human chimerism (CD45+) and multilineage distribution of HSCs was observed in peripheral blood, bone marrow and spleen of both control and transduced mice. A low frequency of GFP+ cells of the myeloid lineage was obtained in the bone marrow of the mice subjected to editing. Next, to determine HDR-based editing efficiency *in vivo*, we injected rAAV6 encoding AAVS1 homology arms and GFP driven by the MND promoter directly into the bone marrow of engrafted NBSGW mice. Our ddPCR results confirm that a localized intraosseous injection concentrates the vector in the targeted niche, thereby specifically targeting the bone marrow and enhancing transduction of the desired cell types. Future studies using a HDR based editing approach are underway to establish an optimum method for *in vivo* editing of HSPCs.

17. CRISPR-Cas9 Genome Editing of Human CD34+ Cells at Gamma-globin Promoter to Induce Fetal Hemoglobin as Sickle Cell Disease Therapy

Varun Katta[1], Kiera O'Keefe[1], Cicera R. Lazzarotto[1], Thiyagaraj Mayuranathan[1], Jonathan Yen[1], GaHyun Lee[1], Yichao Li[1], Naoya Uchida[2], Shondra M. Pruett-Miller[3], John Tisdale[2], Akshay Sharma[4], Mitchell J. Weiss[1], Shengdar Q. Tsai[1]

[1]Hematology, St Jude Children's Research Hospital, Memphis, TN,[2]Molecular and Clinical Hematology Branch, National Heart, Lung and Blood Institute, Bethesda, MD,[3]Department of Cell and Molecular Biology, St Jude Children's Research Hospital, Memphis, TN,[4]Department of Bone Marrow Transplantation and Cellular Therapy, St Jude Children's Research Hospital, Memphis, TN

Sickle cell disease (SCD) affects nearly 100,000 Americans and millions of individuals worldwide. Patients with SCD are affected by pain crises, chronic anemia, multi-organ dysfunction, and early mortality. Remarkably, individuals that co-inherit SCD mutations and genetic variants that cause hereditary persistence of fetal hemoglobin (HPFH) are largely asymptomatic. Thus, a promising genome editing strategy to treat SCD is to induce fetal hemoglobin (HbF, $\alpha 2\gamma 2$) to replace abnormal sickle adult hemoglobin (HbS, $\alpha 2\beta^{s}2$). Recently, disruption of an erythroid-specific enhancer of BCL11A to elevate HbF ($\alpha 2\gamma 2$) was reported to be promising in an early clinical trial (Frangoul et al.). Another promising approach is to disrupt repressor-binding motifs for BCL11A or ZBTB7A proteins in the γ-globin gene promoters in HSCs.To compare the efficiency of editing BCL11A and ZBTB7A binding sites in the γ-globin gene (*HBG1* and *HBG2*) promoters and associated levels of HbF ($\alpha 2\gamma 2$) induction, we electroporated human primary $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) with Cas9-3xNLS ribonucleoproteins (RNPs). We observed high editing efficiencies (83.8%-97.9% indels) and transplanted edited HSPCs into immunodeficient NBSGW mice. 17 weeks post-transplantation, all hematopoietic lineages derived from RNP-treated donor HSPCs exhibited 63.5 to 92.7% indel mutations at the γ-globin promoter ZBTZ7A or BCL11A binding sites, indicating consistent, high-level editing of repopulating hematopoietic stem cells (HSCs). Editing of the BCL11A binding site resulted in HbF induction up to 31.8% in erythroid progeny, compared to <2% in erythroid progeny from unedited HSCs. Disruption of the ZBTB7A binding site at similar frequencies also resulted in erythroid HbF induction, although to a lesser extent (13-18%).To assess our approach in SCD patient cells, we edited plerixafor-mobilized $CD34^+$ HSPCs from one healthy donor and three adult individuals with SCD using Cas9-3xNLS RNPs targeting the BCL11A binding site in the γ-globin promoter. We attained consistently high indel rates ranging from 80.6% to 94.5% in both $CD34^+/CD90^-$ progenitor and $CD34^+/CD90^+$ HSC-enriched populations. 17 weeks following xenotransplantation of edited cells, we observed persistent high-level editing (49.3%-91.5%) of all HSC-derived lineages with HbF levels of 18.3% to 34.3% in human erythroid progeny compared to <5% in unedited controls. Single cell western blot revealed broad HbF induction, where 49-58% of edited erythroblasts showed γ-globin expression compared to controls (<6%). One concern is that Cas9-directed double strand breaks in the γ-globin promoters could result in the deletion of the intervening 4.9-kb region. By digital droplet PCR, we observed 20-25% frequencies of this 4.9 kb deletion in HSPCs prior to xenotransplantation and at 17 weeks post-engraftment. We are currently validating unintended genome wide activities identified by CHANGE-seq and *in silico* methods. In conclusion, our preclinical data suggests that *ex vivo* modification of autologous HSPCs via CRISPR-Cas mediated disruption of the BCL11A repressor binding site in the gamma-globin promoter genes can induce HbF to therapeutically relevant levels, and therefore, represents a promising genome editing cell therapy for SCD.

18. In Utero Lipid Nanoparticle Delivery of CRISPR Technology to Correct Hereditary Tyrosinemia Type 1

Kshitiz Singh[1], Rachel S. Riley[2], Meghana V. Kashyap V. Kashyap[1], Brandon White[1], Sourav K. Bose[1], Haiying Li[1], Rohan Palanki[2], Margaret M. Billingsley[2], Barbara E. Coons[1], John S. Riley[1], Philip Zoltick1[1], Kiran Musunuru[3], Michael J. Mitchell[2], William H. Peranteau[1]

[1]Center for Fetal Research, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Bioengineering, University of Pennsylvania, Philadelphia, PA,[3]University of Pennsylvania, Philadelphia, PA

According to the WHO, ~295,000 newborns die within 28 days of birth every year from congenital diseases many of which have a genetic origin. Many of these diseases can be diagnosed prenatally and pathology begins before or shortly after birth. These characteristics combined with normal fetal developmental properties including the small size, an immature immune system, and an abundance of proliferating progenitor cells in multiple organs highlight the potential of *in utero* gene therapy to treat select diseases. The development of safe, clinically-relevant prenatal delivery methods for gene therapy, including gene editing, is critical to the future application of *in utero* gene therapy. Lipid nanoparticles (LNPs) have emerged as an alternative, potentially safer, approach for nucleic acid delivery compared to traditional viral vector delivery. In this study, we use LNPs to prenatally deliver CRISPR technology in the mouse model of hereditary tyrosinemia type 1 (HT1) in which mutations in the *Fah* gene of the tyrosine catabolic pathway result in accumulation of upstream toxic metabolites and death by ~1 month of age. Specifically, we assess LNP-mediated delivery of adenine base editor (ABE) mRNA and SpCas9 mRNA to perform liver-directed gene editing to correct the *Fah* disease-causing mutation or silence the *Hpd* gene (to prevent accumulation of toxic metabolites) respectively and rescue the lethal phenotype. In an initial experiment, gestational day (E) 16 C57BL/6 fetuses were injected via vitelline vein with LNP containing GFP mRNA. Liver analyses by immunohistochemistry (IHC) and stereomicroscope 5 days post-injection demonstrated GFP expression supporting future LNP studies in the HT1 mouse model. *Fah*-/- fetuses were via the vitelline vein at E16 with LNPs containing either ABE mRNA and a *Fah*-targeting gRNA (LNP.ABE.Fah) or SpCas9 mRNA and an *Hpd*-targeting gRNA (LNP.SpCas9.Hpd). After birth, injected fetuses were cycled off NTBC with removal of the drug by 2 weeks of age. Fetuses injected with LNPs not containing gene editing RNA molecules served as controls. On-target editing efficiency in liver DNA at the time of sacrifice (at least 90 days after removal of NTBC) was assessed by next-generation sequencing. Phenotype correction was assessed by monitoring weight gain, survival at 90 days, and liver function tests. Finally, IHC of the liver was performed to assess FAH and HPD protein expression. On-target *Hpd and Fah* editing efficiencies in liver DNA 90 days after NTBC removal were 65.51+/-1.12% and 27.01+/-0.77% respectively. The high editing efficiency 90 days after removal of NTBC in both approaches is related to a known survival advantage conferred to edited cells. IHC analysis of experimental and control mice confirmed a reduction in HPD protein expression in mice injected with LNP.SpCas9.Hpd and increased FAH protein expression in recipients of LNP.ABE.Fah. Recipients of LNP.SpCas9.Hpd and LNP.ABE.Fah demonstrated liver function, weight gain and survival that was comparable to unedited *Fah*-/- mice maintained on NTBC and significantly improved compared to unedited *Fah*-/- mice in which NTBC was removed at 2 weeks of age. This study demonstrates the feasibility of using an mRNA-LNP delivery platform for therapeutic *in utero* adenine base editing and CRISPR-mediated nonhomologous end joining. It supports this approach as a treatment for HT1 and highlights its potential to treat other genetic liver diseases.

19. Correction of DMD Mutations in Human iPS-Derived Muscle Cells by Single-Cut CRISPR/Cas9-Based Gene Editing

Ziad Al Tanoury[1], Lingjun Rao[1], Riffat Ahmed[1], Yulan Ai[1], Ben Nixon[1], Theodore Lee[1], Phoebe Tsai[1], Cristina Rodriguez Caycedo[2], Yi-Li Min[1], Eric Olson[2], Alison McVie-Wylie[1], Tudor Fulga[1]

[1]Vertex Cell and Genetic Therapies, Watertown, MA,[2]Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX

Duchenne muscular dystrophy (DMD) is a fatal X-linked recessive disorder caused by mutations in the *DMD* gene encoding dystrophin, a protein essential for sarcolemma integrity and stability. Among more than 4,000 mutations identified in patients with DMD, the majority are deletions that cluster in "hot spot" regions. Mutations that delete single or multiple exons of the dystrophin gene can be corrected by reframing or exon skipping to restore the dystrophin open reading frame. We previously showed that single-cut CRISPR/Cas9-based gene editing can induce exon reframing or skipping events thereby restoring the synthesis of near full-length dystrophin in the skeletal muscle, diaphragm, and heart (Min et al., 2019). To validate this strategy in human cells, we used induced pluripotent stem cells (iPSCs) from DMD patients carrying dystrophin out-of-frame exon deletions and from corresponding siblings encoding wild type dystrophin (non-DMD). Corrected iPSC clonal lines were derived from DMD patient cells after gene editing using *Sp*Cas9 complexed with guide RNAs targeting exons 51 and 45 (generated in Dr. Olson's laboratory at UT Southwestern). iPS cells were then differentiated into cardiomyocytes (iCM) and skeletal muscle (iSKM) cells using previously established protocols (Chal et al., 2016; Lian et al., 2012). Beating iCMs and twitching iSKMs were observed as early as 6 and 15 days, respectively. Within 3-4 weeks of differentiation, cardiac (Troponin T) and skeletal muscle (Fast MyHCII) markers were observed in differentiated cells. As expected, no dystrophin was detected in iCMs or iSKMs derived from DMD iPSCs. However, restoration of dystrophin protein expression was evident in both iCMs and iSKMs derived from corrected iPSCs. Furthermore, similar to the non-DMD derived cells, dystrophin appeared to be correctly localized at the sarcolemma on skeletal muscle fibers in all edited clones. RT-PCR and Sanger sequencing confirmed exon skipping or +1 nucleotide insertions (i.e. reframing events) in iCMs differentiated from edited clonal lines. In summary, our data provides an important proof of concept for efficient correction of *DMD* mutations and restoration of dystrophin expression in human DMD patient-derived iPSCs by CRISPR/Cas9-based gene editing. Furthermore, our strategy offers an attractive platform for the development of relevant *in vitro* DMD models and drug screening strategies.

20. Cell-Based Delivery Strategies for Artificial Transcription Factors in Preclinical Animal Models

Peter Deng[1], David L. Cameron[1], Julian A. N. M. Halmai[1], Anna Adhikari[2], Nycole Copping[2], Jennifer J. Waldo[1], Jan A. Nolta[3], Jill L. Silverman[2], David J. Segal[4], Kyle D. Fink[1]

[1]Neurology, UC Davis, Sacramento, CA,[2]MIND Institute, UC Davis, Sacramento, CA,[3]Stem Cell Program, UC Davis, Sacramento, CA,[4]Biochemistry and Molecular Medicine, UC Davis, Davis, CA

Therapies based on DNA-modifying proteins such as zinc finger, transcription activator-like effectors, and CRISPR/Cas9 to regulate gene expression are becoming viable strategies to treat genetically linked disorders through manipulation of endogenous gene regulation. The identification of an effective delivery systems for these proteins *in-vivo* remain a major translational hurdle. In this work, we evaluate a mesenchymal stem/stromal cell (MSC)-based delivery system as a putative cell-based strategy for the secretion of DNA modifying proteins. MSCs are advantageous as a delivery system due to their favorable ease of culturing, immunomodulatory properties, and favorable clinical safety profile. Presently, we report the first the use of a Zinc Finger secreting MSC (ZF-MSC) in transgenic Angelman Syndrome (AS) mouse models. In our *in-vivo* work we evaluate two routes of administration for ZF-MSC - direct intracranial injection or access into the cerebral spinal fluid space. Secreted ZF protein from mouse ZF-MSC is detectable in the murine hippocampus 1-week following either intracranial or cisterna magna injection. This secreted ZF is able to activate the imprinted paternal Ube3a gene in a transgenic $Ube3a^{Yfp}$ reporter mouse at 1 and 3-weeks following either intracranial or cisterna magna injection of ZF-MSC. We detect high co-localization of secreted ZF protein within the CA1 and CA3 regions of the hippocampus in the $Ube3a^{Yfp}$ reporter mouse. ZF-MSC were detectable along cerebral spinal fluid rich regions such as the lateral ventricle, 3rd ventricle, and cerebellum following either route of administration. A significant increase in Yfp+ neurons are observable 1-week following intracranial ZF-MSC administration. A significant increase in $Ube3a^{Yfp}$ protein expression is observable in the hippocampus, midbrain, and cerebellum 3-weeks following a cisterna magna injection of ZF-MSC. An amelioration of motor deficits in rotarod and forepaw propulsion is observed 3-4 weeks following intracranial injection of ZF-MSC in the $Ube3a^{mat-/pat+}$ AS mouse. Overall the results of these studies demonstrate that ZF-MSC secrete functionally active ZF protein to activate paternal Ube3a in AS mouse models. This approach may provide a less-invasive, non-surgical means to deliver gene modifying therapies into the CNS through access of the cerebral spinal fluid injections.

21. A CRISPR-Mediated Strategy for *Mecp2* Gene Correction *In Vivo* as a New Treatment for Rett Syndrome

Mirko Luoni[1], Serena Giannelli[1], Simone Bido[1], Antonio Niro[1], Eleonora Conti[1], Vania Broccoli[2]

[1]San Raffaele Scientific Institute, Milan, Italy,[2]National Research Council (CNR), Milan, Italy

Rett syndrome (RTT) is a severe neurological disorder caused by loss-of-function mutations in Methyl-CpG binding protein 2 (*MeCP2*) gene. Gene therapy strategies aimed to restore MeCP2 function hold great therapeutic potential since as shown in murine models, RTT phenotype can be reversed. Despite this, MeCP2 expression is not homogenous among different cell types and tight regulation of its expression is crucial to avoid adverse effects. Indeed, the overexpression of MeCP2 can lead to severe neurological alterations clinically comparable to RTT. In this scenario, a gene replacement approach, aimed to restore MeCP2 function under the control of the endogenous regulatory elements, represents an ideal strategy to avoid regulatory problems. Thus, we conceived a homology-independent CRISPR-based approach to introduce a minimal *Mecp2* coding sequence upstream of the endogenous translation start site. *In vitro* analyses on primary neuronal cultures revealed a high efficiency of *Mecp2* repair, with over 30% of neurons that re-activate Mecp2 expression under the control of the endogenous regulatory elements. Next, we exploited the AAV-PHP.eB to validate the efficiency and the safety of this strategy *in vivo* in wild-type and symptomatic *Mecp2-null* mice. Molecular and phenotypic analyses confirmed the feasibility to repair in a site-specific manner *Mecp2* gene in the rodent brain, ameliorating the pathological phenotype of RTT mice without eliciting significant toxic reactions. This approach defines a novel therapeutic strategy with increased safety with respect to standard gene addition therapy strategies, holding important clinical implications for RTT.

Development of AAV Capsid Variants

22. A Novel Liver-Tropic AAV Capsid sL65 Shows Superior Transduction and Efficacy in Humanized Mice and Non-Human Primates

Jing Liao[1], Amy Bastille[1], Lauren Drouin[1], Matt Edwards[1], Dylan Frank[1], Laure Freland[1], Susana Gordo[1], Chih-Wei Ko[1], Noah Miller-Medzon[1], Elizabeth McCarthy[1], Nikhil Ramesh[1], Valerie Villareal[1], Jenisha Vora[1], Carmen Wu[1], Noel Walsh[1], John Xiong[1], Xiaohan Zhang[1], Shengwen Zhang[1], Marti Cabanes Creus[2], Leszek Lisowski[2], Nelson Chau[1], Kyle Chiang[1], Matthias Hebben[1]

[1]LogicBio Therapeutics, Lexington, MA,[2]Children's Medical Research Institute, The University of Sydney, Sydney, Australia

Engineering the Adeno-associated virus (AAV) capsid facilitates the generation of recombinant vectors displaying novel properties of interest, including tropism, potency, ease of production and lower recognition by preexisting antibodies. The resulting vectors could target a broader patient population and be effective at reduced dosage, with lower manufacturing cost. In addition to using cutting-edge technology to create highly diverse capsid libraries, innovative screening methods must be used to validate the libraries and to successfully identify human-tropic capsids. Previously, the Children's Medical Research Institute reported the identification of novel capsid variants exhibiting high transduction

and expression levels in human hepatocytes using a model of humanized FRG mice. Here, we further characterized these lead capsids with regards to manufacturability, seroprevalence in a panel of 200 human sera samples, and expression efficacy as measured by human Factor IX (hFIX) production in non-human primates (NHPs). Our experiments led to the identification of a novel capsid with outstanding properties related to both manufacturability and in vivo potency. When used to package a recombinant AAV genome bearing human Factor IX, this capsid sL65 produces high titers ($7x10^{11}$ vg per mL of cell culture from suspension HEK293 cells). In NHPs (*Macaca fascicularis*), the hFIX expression level delivered by sL65 was up to 4-fold higher when compared to vectors pseudotyped with benchmark capsids AAV8 and LK03. There was a tight correlation between plasma hFIX concentration, episomal copy number and mRNA level in the liver, indicating that the higher potency was induced by increased transduction and expression compared to AAV8 and LK03 capsids. Biodistribution of vectors was investigated at 6 weeks post dosing by droplet digital PCR. A strict liver tropism was observed for sL65. Interestingly, in situ hybridization staining revealed that the vector distribution was uniform in the liver while the LK03 serotype showed preferential transduction in the periportal area. None of the animals exhibited any vector-associated adverse events. Finally, we found 58% of the samples in a panel of 200 human sera had neutralizing titers < 1:10 and 67% < 1:30. In summary, these data confirm the promising potential of capsid sL65 as a new vector for human liver. With the high potency in a humanized in vivo system and non-human primates, high production yield and low prevalence of pre-existing antibodies in the human population, sL65 is an excellent candidate to overcome the current limitations of traditional AAV vectors including toxicity caused by high dosage, high manufacturing costs and low translatability from mouse studies to human trials.

23. Efficient Design of Optimized AAV Capsids Using Multi-Property Machine Learning Models Trained across Cells, Organs and Species

Shireen Abestesh, Ilke Akartuna, Alexander Brown, Megan Cramer, Farhan Damani, Jeff Gerold, Jorma Gorns, Jeff Jones, Helene Kuchwara, Jamie Kwasnieski, Sylvain Lapan, Kathy Lin, Elina Locane, Stephen Malina, Eryney Marrogi, Hanna Mendes Levitin, Patrick McDonel, Nishith Nagabhushana, Stephen Northup, Roza Ogurlu, James Oswald, Jakub Otwinowski, Chris Reardon, Chris Reardon, Cem Sengel, Amir Shanehsazzadeh, Sam Sinai, Michael Stiffler, Heikki Turunen, Flaviu Vadan, Adrian Veres, Anna Wec, Lauren Wheelock, Sam Wolock, Justin Yan, Eric Kelsic

Dyno Therapeutics, Cambridge, MA

While next-gen high-throughput assays now enable us to learn how capsid sequence changes affect capsid functionality, measuring and optimizing capsid properties in the most therapeutically relevant models, such as non-human primates (NHP), remains challenging. The efficiency of transduction in target organs is often much lower than ideal, and most of the sequence space is non-functional. Therefore, the chance of identifying an improved variant through random search is miniscule. To overcome these challenges, we investigated to what extent machine learning models can improve the efficiency of AAV capsid design, as defined by the probability that a designed variant will have improved function. We synthesized and barcoded libraries containing 803,041 designed sequence variants of 3 natural AAV capsid serotypes and measured their properties as delivery vectors in cell culture, and in vivo in mice and NHPs. We show that single-property machine learning models trained on these data can improve the efficiency of library design by at least several hundred fold. Furthermore, we demonstrate the value of multi-property models in several ways. Models trained on multiple properties in combination help overcome data sparsity and measurement error, thereby improving model accuracy and providing a more reliable interpretation of experimental results. Multi-property models also provide a coherent framework in which to connect information from experiments across cell lines, organs, and species to the most relevant outcomes in NHP studies, thereby reducing the high resource and ethical burdens of NHP experimentation. Finally, multi-property models learn representations of the capsid landscape that can better optimize these vectors across the multiple properties that are key to enhanced therapeutic efficacy, for example: i) increasing transduction of target cells, ii) minimizing off-target tissue biodistribution, and iii) improving manufacturability. With further refinement, multi-property machine learning models will enable the design of highly optimized AAV capsids that open new frontiers in delivery, toward realizing the full potential of gene therapy.

24. Risk-Adjusted Selection for Validation of Sequences in AAV Design Using Composite Sampling

Lauren Wheelock[1], Stewart Slocum[1,2], Jorma Görns[1], Sam Sinai[1]

[1]Dyno Therapeutics, Cambridge, MA,[2]Johns Hopkins University, Baltimore, MD

High-throughput DNA synthesis methods have unlocked massive potential in the design of novel AAV vectors for gene therapy. New computational techniques are needed to generate candidate sequences at the volume and quality necessary to take full advantage of these synthesis methods. Machine learning models trained on large datasets of AAV variants can predict the properties of unobserved sequences, allowing for screening of billions of candidates in-silico before selecting the best ones for experimental validation. A naive approach to pruning billions of candidates down to hundreds of thousands for synthesis would involve selecting the top-scoring sequences according to machine learning model predictions. However, model predictions are never perfectly accurate, and a more principled approach can improve the chances of discovering high-performing variants. A similar problem arises later in the pipeline: promising candidates from high-throughput experiments are selected for medium- to low-throughput validation by comparing their experimental measurements. Like machine learning models, experimental measurements are noisy and may be biased, and promising vectors from experiments in model organisms may not directly transfer to clinically valuable vectors in

humans. Hedging against the risk of model and experimental error in this context is critical for improving the chances that the validation set includes the variants with the most desired ground-truth properties. Here we demonstrate the utility of a novel method, Composite Sampling (CS), that allows for pruning candidate sequences by accounting for model uncertainty and experimental noise, in a manner that maximizes the chances of including the best candidates in our validation set. CS is a tunable computational method that allows the experimenter to modulate validation risk when faced with noisy model or experimental evaluation. This method can be used in any setting where samples can be scored according to a desirability and a diversity metric. Here we show our method's value for AAV capsid design. We use retrospective studies in experimental AAV datasets and simulation of multiple synthetic datasets to demonstrate that Composite Sampling is consistently better than greedy approaches in selecting high-performing sequences for the validation set. In comparison to current approaches, our method increases the probability that high-throughput screens of capsid libraries will yield vectors that are optimized for therapeutic applications.

25. A Comparison of Methods Used for the Determination of Full and Empty rAAV Particles

Bryan Troxell[1], I-Wei Tsai[1], Mark Rodgers[1], Kevin Mouillesseaux[2], Jack Jingi Ren[3]

[1]Analytical Development and Research Quality Control, StrideBio, Inc, Morrisville, NC,[2]Process Development, StrideBio, Inc, Research Triangle Park, NC,[3]Research Vector Production, StrideBio, Inc, Research Triangle Park, NC

The use of recombinant Adeno-associated virus (rAAV) as a biological vehicle for the delivery of therapeutic DNA into humans provides an unparalleled treatment for a range of diseases. Although the production of therapeutic biologics for treating human diseases has been established, the first FDA approved gene therapy for human use occurred within recent years. To produce rAAV at the appropriate scale for treatment, requires effective manufacturing and purification of capsids containing the target DNA from the complex matrix of the upstream harvest. These processes may introduce undesirable process and product-related impurities that impact the overall performance and quality of the final drug product. One such product-related impurity is the presence of improperly packaged or "empty" (hereafter referred to as empty) rAAV capsids. The collective data indicate that the level of empty capsids in final products may reduce the *in vivo* efficacy and result in adverse events following administration. Regulatory guidance suggests that accurate analytical methods be used early in the product development cycle of gene therapies to evaluate and monitor the level of empty capsids. Sedimentation velocity analytical ultracentrifugation (SV-AUC) is considered a primary analytical method for the determination of full and empty rAAV particles. Additional methods have been utilized for the determination of the full empty ratio. These methods include transmission electron microscopy (TEM), vector genome to capsid determinations, and more recently cryogenic electron microscopy (Cryo-EM) and charge detection mass spectrometry (CD-MS). Each of these methods offers advantages and disadvantages in terms of assay sensitivity, accuracy, linearity, robustness, and utility for in-process testing. Presented here is the comparison of these methods (notably SV-AUC and CD-MS) with an internally developed method to assess the full-to-empty ratio. A variety of parental and engineered proprietary capsids containing single-stranded and self-complementary vectors were assessed. Across multiple productions, SV-AUC, CD-MS, and our internal method were in good agreement for a variety of packaged DNA targets. SV-AUC and CD-MS are especially advantageous for the quantification of partially packaged DNA in rAAV, which tends to increase in abundance with self-complementary vectors. The internal method allowed for the timely measurement of full-to-empty particles at the cell lysate stage as well as purified material; however, the stage of sample purification as well as the packaged genome may impact which method is most suitable for determining levels of empty, partial, and full capsids. Results from this work suggest there is a benefit to using orthogonal methods to determine the level of empty particles.

26. A Tetracycline Enabled Self-Silencing Adenovirus (TESSA) Platform Delivers High-Quality, High-Titre, Multi-Serotype Recombinant Adeno-Associated Virus (AAV) Stocks

Maria Patricio, Weiheng Su, Qian Liu, David W. Brighty, Ryan Cawood

Oxgene, Oxford, United Kingdom

A significant challenge for Adeno-associated virus (AAV) -mediated gene transfer has been the capacity to produce recombinant AAV vectors of high quality, at high-titre and at large scale. We have addressed these challenges by developing a proprietary Tetracycline-Enabled Self-Silencing Adenovirus (TESSA) system that simultaneously provides Adenovirus (Ad) helper functions and encoded AAV Cap/Rep. When cells carrying an AAV ITR-flanked transducing genome are infected with TESSA-Cap/Rep, high-titre rAAV stocks are produced that are essentially free of contaminating TESSA vector. We have now examined the ability of TESSA to support production of adiverse range of AAV serotypes. The Capcoding region of AAV serotypes 1-9 were independently engineered into the E1 region of TESSA and the quality, physical and infectious titre of the rAAV produced by this system were characterised. For all rAAV serotypes, physical titres and infectious titres were invariably greater than that observed for conventional plasmid-based rAAV systems. For these serotypes, the ratio of full to empty particles is greatly improved. Moreover, for some AAV serotypes, the TESSA system delivered infectious titres 1000-fold greater than the plasmid-based helper-free systems. Importantly, TESSA-derived rAAV serotypes are competent for reporter gene and therapeutic gene transfer into a multiplicity of human cell types in vitro and a variety of model animal tissues in vivo. Therefore, we have developed a versatile system that can be applied to a range of naturally occurring AAV serotypes, and to experimentally engineered AAV capsids with novel tissue targeting phenotypes. The TESSA system provides unique opportunities for robust high-quality scale up of rAAV manufacture that will reduce costs and facilitate rapid progression to clinical application of novel rAAV-based gene therapies.

27. Next Generation AAV Drug Products: Enhanced Stability & Clinical Ease for High Titer Preparations

Lori B. Karpes, Tyler J. Peters, Mark Bailey, Michael Mercaldi, Eric Faulkner, Tim Kelly
Homology Medicines, Bedford, MA

Homology Medicines has developed a Plug-&-Play Process and Manufacturing Platform to develop and manufacture gene transfer and gene editing therapeutics for rare diseases, which utilizes our proprietary Clade F AAV capsids derived from hematopoietic stem cells (AAVHSCs). As our programs have progressed through development and into the clinic, we have increased focus on the Drug Product sciences which includes the thoughtful development of stabilizing formulations enabling high titers, easing of clinical storage and supply chains, and enhanced long-term stability. AAV preparations have long held a reputation as challenging not only for production, but for long-term stability at even low concentrations. However, here we demonstrate not only the stability of AAVHSCs in the liquid state, but also the impact of novel formulations on capsid stability. Our AAV preparations achieve titers in excess of 1E14 vg/mL and demonstrate stability for a minimum of one year at 2-8°C and more than six months at room temperature. Benefits of vector stability in the liquid state are a reduction in the need for -80°C-storage infrastructure and simplification of the clinical supply chain by enabling 2-8°C storage at the manufacturing site and clinical pharmacy. This work demonstrates the marked stability of our proprietary AAVHSCs as well as the impact of a well-developed formulation. Furthermore, this effort shows that by bringing a Drug Product focus to AAV product development, an organization can help support clinical and commercial success by providing long product expiries, smaller administration volumes, and 2-8°C supply chains.

28. Development of a Membrane-Based Affinity Matrix for Downstream Purification Process of Adeno-Associated Virus Vectors

Takuma Sueoka, Masakatsu Nishihachijo, Hisako Yaura, Shota Hirayama, Masahiro Aratake, Hiroyuki Watanabe, Kazunobu Minakuchi
KANEKA Corporation, Takasago, Hyogo, Japan

Adeno-associated virus (AAV) vectors are promising tools for gene therapy. Along with expanding market scale, reducing production costs is a key concern. Although the titer of AAV vectors in the upstream process has increased over the years, the downstream process is still outdated and should be improved. Affinity chromatography is a powerful tool for capture step of biologics production owing to its high selectivity. However, the chromatographic purification of AAV vectors from crude cell lysate is challenging due to high impurity contents in the loading material and damage to AAV transduction efficiency by contacting with low pH solution in the elution step. Tangential-flow filtration (TFF) is introduced prior to affinity chromatography to reduce the burden and to concentrate AAV vectors, but introduction of an additional process is ideally avoided to simplify the process and reduce production costs. Preventing damage from low pH denaturation increases overall AAV vector productivity in terms of a yield and quality. Herein, we developed a novel affinity matrix, which is composed of membrane-based matrix with a larger pore size than conventional beads resin. This enables a higher flow rate, resulting in a ten-fold shorter contact time than the conventional resin that compensates for the lack of the concentrating effect of TFF. This matrix provided a higher AAV vector yield in the elution pool than the beads resin owing to less contact with low pH conditions. Moreover, better cleanability was also shown likely due to less clogging inside the large pores. Our new affinity matrix intensifies the AAV vector affinity chromatography step and shows insight to develop a new platform of AAV vector downstream process.

Gene Therapy for Inborn Errors of Metabolism

29. Coadministration of AAV Expressing MDR3 (VTX-803) and ImmTOR Allows for Vector Re-Administration to Treat Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3) in Juvenile *Abcb4*$^{-/-}$ Mice

Nicholas D. Weber[1], David Salas-Gómez[2], Leticia Odriozola[2], Irene Ros-Gañán[1], Mirja Hommel[2], Takashi Kei Kishimoto[3], Jean-Philippe Combal[4], Gloria González-Aseguinolaza[2,5,6]
[1]Vivet Therapeutics S.L., Pamplona, Spain,[2]Division of Gene Therapy and Regulation of Gene Expression, Cima Universidad de Navarra, Pamplona, Spain,[3]Selecta Biosciences, Watertown, MA,[4]Vivet Therapeutics S.A.S., Paris, France,[5]Vivet Therapeutics, Pamplona, Spain,[6]Instituto de Investigación Sanitaria de Navarra (IdISNA), Pamplona, Spain

Liver-directed AAV gene therapy has been shown to be an effective modality for the correction of genetic disorders, such as hemophilia, in adult patients. However, gene therapy for many inborn errors of metabolism, such as progressive familial intrahepatic cholestasis type 3 (PFIC3), would be most effective if administered in infancy or early childhood, to prevent irreversible damage. PFIC3 is a rare monogenic disease leading to cholestasis, cirrhosis and ultimately liver failure, with generally an early onset that requires early treatment when diagnosed in children under 3 years of age. PFIC3 is characterized by a dramatic reduction in biliary phosphatidylcholine (PC) content due to mutations in the *ABCB4* gene, which codes for multidrug resistance protein 3 (MDR3) and is responsible for transporting PC across the canaliculi membranes of hepatocytes into the bile, where PC neutralizes bile acid toxicity. A key challenge for AAV gene therapy in pediatric PFIC3 patients is the potential for therapeutic benefit to wane over time as the non-replicating AAV vector is diluted due to hepatic cell proliferation in the growing child, and that AAV cannot be re-administered due to the formation of high titers of persistent neutralizing antibodies. Here we tested the effectiveness of tolerogenic ImmTOR nanoparticles carrying the immunomodulating drug rapamycin to enable repeated intravenous administration of an hepatotropic AAV vector carrying human *ABCB4* cDNA (VTX-803) in 2-week-old juvenile *Abcb4*$^{-/-}$ mice when ImmTOR is co-administered with the first

treatment of AAV. As a proof-of-concept study, we utilized a sub-therapeutic dose of VTX-803 in order to focus on the experimental outcomes of coadministration with ImmTOR and readministration. VTX-803 when co-injected with ImmTOR allowed for a successful readministration of VTX-803 alone two weeks later and resulted in a robust and stable correction of the disease phenotype lasting over 7 months, while repeat dosing of the vector alone (at a sub-therapeutic dose) or together with empty SVP did not exhibit a therapeutic effect. In males, a temporary therapeutic effect was observed following only a single treatment of VTX-803 with ImmTOR, but not observed with empty SVP, suggesting that ImmTOR could increase vector transduction and/or transgene expression. This effect was observed through 10 weeks post-treatment, after which time the effect was lost, highlighting the importance of repeat dosing especially in juvenile animals with growing livers where transgene-positive cells can be diluted over time. Vector treatment without ImmTOR resulted in the production of AAV-specific neutralizing antibodies (NAbs), while vector coadministration with ImmTOR prevented NAbs production. Thus, this provides further evidence that redosing AAV gene therapy can be achieved through coadministration with ImmTOR, which has the dual benefit of potentially improving AAV vector transduction and mitigating vector-specific immunogenicity.

30. Preclinical Evaluation of Combined Adeno-Associated Virus and Nanoparticle Delivery of piggyBac® Transposon System for Durable Transgene Expression in the Growing Neonatal Murine Liver

Jingjing Jiang, Bernard Kok, Xinggang Liu, Vananh Pham, David Ebeid, Mehul Dhanani, Sean Essex, Jivan Yewle, Brian Truong, Devon J. Shedlock, Joshua Rychak, Bruce F. Scharschmidt, Eric M. Ostertag, Julian D. Down

Poseida Therapeutics, San Diego, CA

Gene delivery via recombinant adeno-associated virus (rAAV) has been shown to be efficacious in pre-clinical models and clinical trials for a variety of genetic diseases. A major limitation of current rAAV-mediated gene therapy, however, is dilution of episomal rAAV and loss of transgene expression in rapidly dividing tissues as well as toxicity at higher rAAV doses. Loss of transgene expression and therapeutic efficacy are particularly limiting for gene therapy of infants and young children severely affected by metabolic and other disorders involving the liver. To address these issues, we have explored rAAV and/or novel nanoparticles (NP) as vectors for delivery of piggyBac® transposon and transposase to facilitate transgene integration in the host hepatocyte genome using ornithine transcarbamylase (OTC) deficiency as a disease model. A human OTC expression cassette in a piggyBac® transposon with liver-specific promoters was administered using rAAV with a liver-tropic capsid to neonatal wild-type and OTC deficient (Spf^{ash}) mice, with and without "Super" piggyBac (SPB), a hyperactive form of the transposase. As compared with transposon alone, concomitant rAAV delivery of SPB resulted in stable vector integration into the hepatocyte genome with durable and enhanced transgene expression for over 3 months according to vector copy number, bioluminescence imaging, IHC, human OTC mRNA and protein levels and mitigation of the OTC disease phenotype. We further demonstrated that replacing rAAV delivery of SPB with a NP formulation for efficient liver delivery of mRNA resulted in similar high levels of durable transgene expression (~30% hepatocytes). Genomic modification and clonality of transposed hepatocytes are being further evaluated from LM-PCR and integration site analysis. These findings collectively demonstrate the unique potential of piggyBac® technology for *in vivo* liver-directed gene therapy for infants and young children, as well as the versatility afforded by utilizing viral and/or non-viral delivery systems for single-treatment and permanent correction of OTC deficiency and other genetic diseases.

31. Targeting Aberrant Acylation as a Novel Approach for Treating Methylmalonic Acidemia (MMA) and Related Other Organic Acidemias

Sangho S. Myung[1], PamelaSara Head[2], Jessica L. Schneller[1], Samantha McCoy[1], Yong Chen[3], Marjan Gucek[3], Irini Manoli[1], Charles P. Venditti[1]

[1]NHGRI, NIH, Bethesda, MD,[2]NIGMS, NIH, Bethesda, MD,[3]NHLBI, NIH, Bethesda, MD

Organic acidemias (OAs), such as methylmalonic acidemia (MMA), are a group of clinically severe inborn errors of metabolism that typically arise from defects in the catabolism of amino- and fatty acids. The accretion of acyl-CoA species is postulated to cause intracellular toxicity and underlie the dysregulation of multiple intermediary pathways seen in the patients, such as the urea cycle and glycine cleavage system. Here, we explore an alternative pathophysiological consequence of impaired acyl-CoA metabolism: the accumulation of aberrant posttranslational modifications (PTMs) on enzymes in critical intracellular pathways. Using an MMA mouse model that recapitulates MMA-associated hepatic mitochondriopathy (Mmut-/-;TgMCKMmut), we surveyed PTMs in hepatic extracts with acyl-lysine antibodies and discovered widespread hyper-acylation. Next, we prepared affinity anti-acyl-lysine columns to enrich for modified proteins, and then performed mass spectrometry to characterize the PTM proteome. Excessive acylation of enzymes involved in glutathione, urea, arginine, tryptophan, valine, isoleucine, methionine, threonine, and fatty acid metabolism were detected in the MMA mice, and validated via immunoprecipitation analysis. We extended our analyses to other perturbed pathways, including the glycine cleavage system and mitochondrial replication, which we found to also be aberrantly modified in liver extracts from both MMA patients and Mmut-/-;TgMCKMmut mice as compared to respective controls The emerging pattern from our aggregate studies further supports a model where hyperacylation of key enzymes in pathways known to be dysregulated in MMA likely contributes to altered metabolism and identifies a new set of targets. With these new insights, we investigated the sirtuin (SIRT) family of enzymes as potential therapeutic agents given their known role as specialized deacylases. SIRT 1-7 were individually assayed for activity toward MMA specific acylations of modified protein substrate. An optimal SIRT emerged, but we noted that aberrant acylation also inhibited the functionality of our candidate enzyme, lowering enzymatic activity *in vitro*. Using rational mutagenesis, we created a "SuperSIRT" that was resistant to acylation-dependent inhibition, validated activity in vitro, cloned it behind a liver specific promoter (LSP), and packaged

with an AAV8 capsid. The resulting AAV8 LSP SuperSIRT or an AAV8 LSP EGFP control were then systemically delivered to juvenile Mmut-/-;TgMCKMmut and Mmut+/-;TgMCKMmut mice at a dose of $1e^{13}$ GC/kg, and followed by clinical, biochemical, and enzymatic analyses. After 1 month, the blood ammonia levels were significantly reduced in the treated mutant mice compared to the AAV8 LSP EGFP and untreated mutant control groups, while plasma methylmalonic acid levels remained unchanged. In hepatic extracts from the AAV8 LSP SuperSIRT treated Mmut-/-;TgMCKMmut mice, the aberrant acylation on key protein targets in the urea cycle and glycine cleavage pathway was reversed compared to GFP treated controls. In summary, our studies have identified a new PTM axis in patients and mice with MMA, which has allowed the development of a SuperSIRT gene therapy that could be used to treat all forms of MMA and might be extended to other disorders where aberrant acylation plays a role in disease pathophysiology, such as organic acidemias and fatty acid oxidation disorders.

32. AAV Liver Gene Therapy-Mediated Inhibition of FGF23 Signaling as a Therapeutic Strategy for X-linked Hypophosphatemia

Louisa Jauze[1], Volha Zhukouskaya[1], Severine Charles[1], Christian Leborgne[1], Agnes Linglart[2], Catherine Chaussain[3], Claire Bardet[3], Giuseppe Ronzitti[1]

[1]Genethon, Evry, France,[2]Univesite Paris Saclay APHP, Le Kremlin-Bicêtre, France,[3]Université de Paris, Montrouge, France

X-linked hypophosphatemia (XLH) is a rare disease due to increased fibroblast growth factor 23 (FGF23) secretion from bones which results in phosphate wasting in kidneys. Decreased circulating phosphate is the primary cause of severe skeletal deformities and short stature that greatly affect patients' quality of life. Conventional treatment for XLH requires life-long, repeated supplementation of phosphate and active vitamin D analogs and is associated with severe long-term side effects and poor compliance. Recently, the use of Burosumab (Crysvita®, Ultragenyx, and Kyowa Kirin), a monoclonal antibody for FGF23, has been proposed as an alternative treatment. Based on the central role of the overactive FGF23 pathway in the pathophysiology of FGF23, here we devised a liver-targeted AAV gene therapy strategy to inhibit FGF23 signaling and rescue bone pathology with a single injection. Secretion of an FGF23 competing factor (cFGF23) by the liver of a murine model of XLH led to the restoration of the impaired skeletal phenotype, significant reduction of osteomalacia and bone and joint alterations in Hyp-Duk mice. Our data provide proof-of-concept to the use of AAV liver gene therapy for the treatment of XLH, a prototypical disease associated to overexpression of soluble factors in tissues refractory to AAV gene therapy, thus expanding the reach of this therapeutic modality and providing novel options for the treatment of this disease category.

33. Comparison of Gene Addition Therapy in Genetically Distinct Mouse Models of Classical Phenylketonuria

Daelyn Y. Richards[1,2], Michael A. Martinez[1], Shelley R. Winn[1], Sandra Dudley[1], Cary O. Harding[1]

[1]Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR,[2]Department of Medical Education and Clinical Sciences, Washington State University Elson S. Floyd College of Medicine, Spokane, WA

Phenylketonuria (PKU) is a highly complex biochemical disorder with nearly 1,000 pathogenic alleles in the phenylalanine hydroxylase (*PAH*) gene that cause a wide spectrum of effects on PAH enzyme dynamics. Clinical trials examining gene addition therapy for classical phenylketonuria (cPKU) have launched with much excitement, yet there is a lack of preclinical data on therapeutic outcomes in genetically distinct animal models. The longstanding cPKU $Pah^{enu2/enu2}$ mouse contains a missense mutation p.F263S (c.835 T > C), resulting in normal production of aberrant non-functional PAH. It has long been hypothesized that the mutant PAH monomers in this model may interfere with wildtype (WT) monomers, impairing overall function of the PAH holoenzyme complex, a phenomenon known as the dominant negative effect. Recently we created the $Pah^{dexon1/dexon1}$ cPKU mouse that contains a deletion of *Pah* exon1 and is completely void of PAH protein, thus removing the variable of mutant monomer expression. In comparing overall PAH enzyme activity in heterozygous $Pah^{+/enu2}$ (N=6) and $Pah^{+/dexon1}$ (N=3) animals, we found that $Pah^{+/enu2}$ animals revealed markedly decreased enzyme activity (29 ± 5% WT activity) in comparison to $Pah^{+/dexon1}$ animals (58 ± 7% WT activity) with a $P < 0.0001$, and for the first time, indisputably confirmed the presence of a dominant negative effect caused by the mutant monomers produced from the Pah^{enu2} allele. This was further supported with liver-directed gene addition therapy studies comparing the two models. The murine liver-tropic adeno-associated virus serotype 8 was packaged with a Liver Specific Promoter driving expression of murine PAH (AAV2/8 LSPmPAH) and administered to 6-8 week old animals via retro-orbital injection that were harvested after two weeks for molecular analyses. All animals showed corrected blood Phe well below the therapeutic target range (below 360 μM), however they revealed stark differences in total PAH enzyme activity between $Pah^{enu2/enu2}$ and $Pah^{dexon1/dexon1}$ cPKU animals. Two $Pah^{enu2/enu2}$ cohorts of 3 males and 3 females (N=6 each) received medium dose (1×10^{11} vector genomes [vg]) or high dose (1×10^{12} vg) gene therapy. The total vector genomes per diploid liver genome (vg/dlg) ranged between 8-42 vg/dlg and 56-291 vg/dlg, respectively, resulting in enzyme activity of 7.7 ± 2.2% and 14.8 ± 3.3%, respectively. In single $Pah^{dexon1/dexon1}$ animals, low dose (1×10^{10} vg), medium dose (1×10^{11} vg) and high dose (1×10^{12} vg) gene therapy conferred total liver vector genome copies of 2.9 vg/dlg, 16.2 vg/dlg and 50 vg/dlg, which resulted in an astonishing 4.8%, 42%, and 96.1% WT PAH enzyme activity. While more studies are needed to include more $Pah^{dexon1/dexon1}$ animals at all doses and $Pah^{enu2/enu2}$ animals at low doses, this data reveals the profound impacts genotype can have on overall enzyme dynamics in the setting of gene addition therapy for PKU. A further advantage of the $Pah^{dexon1/dexon1}$ cPKU animal model for gene therapy development is the ability to perform immune-based molecular analyses to localize and quantify gene therapy delivered PAH enzyme, a feat that is impossible in the $Pah^{enu2/enu2}$ model. Liver immunohistology

of gene therapy treated *Pah*$^{dexon1/dexon1}$ animals revealed a dose dependent expression of PAH, which reveals critical knowledge that could be used to further optimize gene therapy development for PKU.

34. AAV8 Gene Therapy as a Potential Treatment in Adults with Late-Onset Ornithine Transcarbamylase (OTC) Deficiency: Updated Results from a Phase 1/2 Clinical Trial

Cary O. Harding[1], Maria Luz Couce[2], Tarekegn Geberhiwot[3], Wen-Hann Tan[4], Aneal Khan[5], Luis Aldamiz-Echevarria[6], George A. Diaz[7], Connie Lee[8], Ana Cristina Puga[8], Eric Crombez[8]

[1]Oregon Health & Science University, Portland, OR,[2]University of Santiago de Compostela, Santiago de Compostela, Spain,[3]University of Birmingham, Birmingham, United Kingdom,[4]Boston Children's Hospital, Harvard Medical School, Boston, MA,[5]University of Calgary, Calgary, AB, Canada,[6]Cruces University Hospital, Biocruces Bizkaia Health Research Institute, Barakaldo, Spain,[7]Icahn School of Medicine at Mount Sinai, New York, NY,[8]Ultragenyx Gene Therapy, Cambridge, MA

Introduction: OTC deficiency is an X-linked urea cycle disorder resulting in episodic hyperammonemia that can cause cumulative neurocognitive damage and even death. The current standard of care includes a proteinrestricted diet and nitrogen-scavenging agents, but there remains high unmet medical need with continued risk of hyperammonemic crises. DTX301, an AAV8 vector containing the *OTC* transgene, is currently under investigation for the treatment of OTC deficiency. **Methods:** CAPtivate (NCT02991144) is an ongoing global, multicenter, open-label phase 1/2 dose-escalation trial evaluating the safety and preliminary efficacy of DTX301 in adults with late-onset OTC deficiency. The primary endpoint is incidence of adverse events (AEs). Secondary endpoints are changes in the rate of ureagenesis and 24-hour plasma ammonia levels. Patients received a single IV infusion of DTX301 at the following doses: Cohort 1 (2 x 10^{12} Genome Copies [GC]/kg), Cohort 2 (6 x 10^{12} GC/kg), and Cohort 3 (1 x 10^{13} GC/kg). Study duration is 52 weeks followed by up to 4 years of long-term follow up. A partial responder is defined as a patient with a clinically meaningful and sustained increase in rate of ureagenesis with stabilization or improvement in ammonia control. A complete responder is defined as a patient who has also successfully discontinued ammonia-scavenging drugs and protein-restricted diet. **Results:** DTX301 dosing of 3 patients in each of cohorts 1, 2, and 3 is complete. Cohort 4 (1 x 10^{13} GC/kg with prophylactic oral steroid taper) is enrolling. No treatment-related serious AEs or dose-limiting toxicities were reported; all AEs were mild or moderate (grade 1, 2) during the study. Seven patients experienced treatment-emergent AEs (TEAEs) that were considered related to study drug. Five patients experienced asymptomatic ALT increases consistent with those seen in other AAV gene transfer clinical trials. ALT increases were managed and resolved with a protocol-specified tapering regimen of oral corticosteroids administered in outpatient setting. Other TEAEs considered related to study drug were photophobia, headache, hypertension, vector-induced hepatitis, and hypophosphatemia. Overall, 6 of 9 patients responded to DTX301: 3 patients were complete responders, and 3 patients were partial responders. All 9 treated patients maintained or improved ammonia control. Cohort 1 had one complete responder. Cohort 2 had 1 complete responder and 1 partial responder. Cohort 3 had 1 complete responder and 2 partial responders. The longest-treated responders from cohorts 1 and 2 are showing a durable response at 2.5 to 3 years after treatment and remain clinically and metabolically stable with good ammonia control. **Conclusions:** Data from CAPtivate indicate that DTX301 has an acceptable safety profile and may be a potential new therapy with long-term therapeutic benefit for patients with OTC deficiency. Followup of all patients is ongoing and enrollment in cohort 4 is nearly complete.

35. AAV-Mediated Delivery of MiRNA-34B/C Improves Liver Fibrosis

Pasquale Piccolo[1,2], Rosa Ferriero[1], Anna Barbato[1], Sergio Attanasio[1], Marcello Monti[1], Claudia Perna[1], Florie Borel[3], Patrizia Annunziata[1], Annamaria Carissimo[1], Rossella De Cegli[1], Severo Campione[4], Luca Quagliata[5], Luigi Terracciano[5], Chantal Housset[6,7], Jeffrey H. Teckman[8], Christian Mueller[3], Nicola Brunetti-Pierri[1,2]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy,[2]Department of Translational Medicine, Federico II University, Naples, Italy,[3]Department of Pediatrics and Horae Gene Therapy Center, UMass Medical School, Worcester, MA,[4]Pathology Unit, Cardarelli Hospital, Naples, Italy,[5]Molecular Pathology Division, Institute of Pathology, University of Basel, Basel, Switzerland,[6]Centre de Recherche Saint-Antoine, Sorbonne Université, INSERM, Paris, France,[7]Department of Hepatology, CRMIVB-H, Saint-Antoine Hospital, Assistance Publique-Hôpitaux de Paris, Paris, France,[8]St. Louis University School of Medicine, Cardinal Glennon Children's Medical Center, St. Louis, MO

Liver fibrosis is a major complication of chronic liver diseases and is orchestrated by an intricate molecular network. MiRNAs have been known to regulate several distinct processes involved in liver fibrosis. In this study, we found a novel miRNA cluster, including miR-34b and -c, to be upregulated in several forms of liver fibrosis both genetic and acquired. We found that murine hepatic miR-34b/c is upregulated in liver diseases due to ABCB4 deficiency and α-1 antitrypsin deficiency, disorders prone to the development of liver fibrosis. miR-34b/c upregulation was also found to occur in mouse models of liver fibrosis induced by thioacetamide and CCl_4. Mechanistically, miR-34b/c expression was dependent upon JNK-mediated phosphorylation of FOXO3 transcription factor on Ser574. Deletion of miR-34b/c resulted in early development of liver fibrosis and increased signaling of PDGF, a target of miR-34b/c. Moreover, miR-34b/c was effective in blunting TGF-β-mediated activation of human hepatic stellate cells, a key event in liver fibrosis development. Finally, we found that AAV-mediated hepatocyte-specific overexpression of miR-34b/c significantly ameliorated liver fibrosis in two independent mouse models of acquired liver fibrosis (thioacetamide and CCl_4).In conclusion, this study reveals a novel pathway involved in liver fibrosis that is potentially implicated in both genetic and acquired forms of hepatic fibrosis. Furthermore, it supports miR-34b/c as a novel therapy against liver fibrosis.

Genetic Blood and Immune Disorders

36. Follow-Up of a Phase I/II Gene Therapy Trial in Patients with Fanconi Anemia, Subtype A

Julian Sevilla[1], Paula Rio[2], Susana Navarro[2], Rebeca Sánchez-Domínguez[2], Jose C. Segovia[2], Wei Wang[3], Josune Zubicaray[1], Rosa Yañez[2], Jose A. Casado[2], Yari Gimenez[2], Francisco J. Roman-Rodriguez[2], Omaira Alberquilla[2], Eva Galvez[1], Eva Merino[1], Jordi Barquinero[4], Anne Galy[5], Nagore Garcia de Andoin[6], Ricardo Lopez[7], Albert Catala[8], Francois Lefrere[9], Marina Cavazzana[10], Gayatri Rao[11], Jonathan Schwartz[11], Roser M. Pujol[12], Jordi Surralles[12], Jean Soulier[13], Manfred Schmidt[3], Cristina Diaz de Heredia[4], Juan Bueren[2]

[1]Servicio Hemato-Oncología Pediátrica, Fundacion Biomédica Hospital Niño Jesús, Madrid, Spain,[2]Hematopoietic Innovative Therapies, CIEMAT/CIBERER/IIS-FJD, UAM, Madrid, Spain,[3]GeneWerk, GmbH, Heidelberg, Germany,[4]Hospital Val d'Hebron, Barcelona, Spain,[5]Genethon, Evry, France,[6]Hospital Universitario de Donostia, San Sebastian, Spain,[7]Hospital de Cruces, Bilbao, Spain,[8]Hospital San Joan de Deu, Barcelona, Spain,[9]Hopital Necker-Enfants Malades, Pari, France,[10]Hopital Necker-Enfants Malades, Paris, France,[11]Rocket Pharmaceuticals, Inc., New York, NY,[12]Servicio de Genética e Institut de Reserca / Departmento de Genética y Microbiología, IIB-Sant Pau, Hospital Sant Pau/ Universitat Autònoma de Barcelona/CIBERER, Barcelona, Spain,[13]Hôpital Saint-Louis and University Paris Diderot, Paris, France

Fanconi anemia (FA) is a monogenic inherited disorder mainly characterized by congenital abnormalities, childhood bone marrow failure (BMF) and cancer predisposition. Here we report the results of the 1-3 year follow-up of the eight evaluable FA-A patients corresponding to a phase I/II gene therapy trial. $CD34^+$ cells were mobilized with filgrastim and plerifaxor and collected following 2-3 apheresis procedures. $CD34^+$ cell-enriched fractions were transduced during a total period of 20-24h with the PGK-FANCA.Wpre* lentiviral vector and then infused without any pre-conditioning regimen. Nine patients age 3-7 years were infused with a range of $7.3x10^4$ to $1.9x10^6$ $CD34^+$ cells/kg. One patient was withdrawn from the clinical trial due to contamination of the medicinal product and bacteremia which was treated by antimicrobial therapy. Vector copy numbers (VCN) in colonies derived from the manufacturing products ranged from 0.2 to 0.9 VCN/cell. VCNs in patients' PB and BM after infusion of transduced cells showed slow but progressive engraftment of gene-corrected cells in six out of the eight evaluable patients, reaching stabilized values as high as 0.6 copies/cell through the end of the follow-up (3 years). Insertion site studies confirmed the safety of the LV with 1-3 years follow-up. VCN increases were associated both with a progressive MMC-resistance of BM progenitor cells and a reduction in the chromosomal instability in PB T cells exposed to diepoxybutane. Stabilized, and even improved PB cell counts have been observed in patients with higher levels of gene correction. Nevertheless, two out of the eight evaluable patients who were treated at advanced stages of the disease or infused with low numbers of corrected $CD34^+$ cells showed progressive evolution of BMF. Our results demonstrate that gene therapy of non-conditioned FA patients has the potential to prevent BMF, and support the infusion of corrected $CD34^+$ cells prior to the development of BMF. Based on these results, a global phase II clinical trial is currently ongoing under the sponsorship of Rocket Pharmaceuticals Inc., focused on the prevention of BMF by means of the infusion of higher numbers of corrected $CD34^+$ cells in early stages of the disease.

37. Liver Gene Therapy with Lentiviral Vectors Corrects Hemophilia A in Mice and Achieves Normal-Range Factor VIII Activity in Non-Human Primates

Michela Milani[1], Cesare Canepari[1,2], Tongyao Liu[3], Mauro Biffi[1], Fabio Russo[1], Tiziana Plati[1], Rosalia Curto[1], Susannah Patarroyo-White[3], Ilaria Visigalli[1], Paola Albertini[1], Eduard Ayuso[4], Christian Mueller[3], Andrea Annoni[1], Luigi Naldini[1,2], Alessio Cantore[1,2]

[1]San Raffaele-Telethon Institute for Gene Therapy, Milano, Italy,[2]Vita Salute San Raffaele University, Milano, Italy,[3]Sanofi, Waltham, MA,[4]INSERM UMR1089, University of Nantes, Nantes, France

Liver gene therapy with adeno-associated viral (AAV) vectors delivering a clotting factor transgene into hepatocytes has shown multi-year therapeutic benefit in adults with hemophilia. However, the mostly episomal nature of AAV vectors currently challenges application of AAV-vector mediated liver gene therapy to young pediatric patients. In contrast, lentiviral vectors (LV) integrate into the target cell chromatin and are maintained as cells divide. We previously developed LV that achieve stable and therapeutic levels of coagulation factor IX (FIX) transgene expression in the liver of adult mice, dogs and non-human primates (NHP) after systemic delivery. To evaluate LV-mediated liver gene therapy for hemophilia A, we generated LV expressing engineered versions of the human B-domain deleted Factor VIII (FVIII) by codon optimization of the transgene (coFVIII) and the inclusion of a non-structured XTEN polypeptide (coFVIII.XTEN), known to increase the half-life and secretion of the payload protein, in the B-domain region. We administered LV expressing FVIII, coFVIII or coFVIII.XTEN intravenously (i.v.) to newborn hemophilia A mice and observed long term FVIII activity up to 200% of normal and restoration of hemostasis in mice treated with LV encoding for engineered transgenes, with transgene output 10-20 fold higher for coFVIII compared to FVIII transgene and 10-fold higher for coFVIII.XTEN than coFVIII. We then set out to evaluate FVIII expression in NHP and produced large-scale batches of allo-antigen free and phagocytosis shielded (CD47 high) LV carrying coFVIII or coFVIII.XTEN. We administered 1e9 transducing units (TU)/Kg (n=2) or 3e9 TU/Kg (n=3) dose for LV.coFVIII.XTEN or 3e9 TU/Kg (n=2) or 6e9 TU/Kg (n=3) dose for LV.coFVIII. A corticosteroid immune-suppression regimen was applied from day -1/3 to day +7/9, since human FVIII is known to be highly immunogenic in NHP. Administration of LV via peripheral vein was well tolerated with no significant changes in body temperature. Self-limiting leukopenia and limited serum aspartate aminotransferases (AST) elevation were observed. Therapeutically relevant FVIII amounts were observed in all treated animals, with the target 60-100% of normal human FVIII activity achieved at 3e9 TU/Kg dose for the LV.coFVIII.XTEN treatment group. We monitored both anti-FVIII antibody

(Abs) formation, acute cytokine response to LV administration and T cell responses in the blood and the spleen of treated NHP. Upon corticosteroids discontinuation, all NHP developed anti-FVIII Abs, but 4/5 LV.coFVIII.XTEN treated NHP maintained LV-positive hepatocytes in the liver at the end of the study and their splenocytes did not respond to *ex vivo* FVIII stimulation. On the contrary, only 1/5 LV.coFVIII treated NHP maintained LV-positive hepatocytes in the liver and all of them showed splenocyte activation after FVIII stimulation *ex vivo*. Overall, our data show efficient and well tolerated gene transfer to the liver of NHP by LV, with an improved therapeutic index for the engineered FVIII.XTEN transgene, supporting further pre-clinical and potentially clinical development of this gene therapy strategy.

38. Towards Clinical Translation of Hematopoietic Cell Gene Editing for Treating Hyper-IgM Type 1

Valentina Vavassori*[1,2], Elisabetta Mercuri*[1], Genni Marcovecchio[1], Maria Carmina Castiello[1,3], Daniele Canarutto[1,2], Claudia Asperti[1], Aurelien Jacob[1], Luisa Albano[1], Elena Fontana[3,4], Eugenio Scanziani[5], Marina Radrizzani[1], Anna Villa[1,3], Pietro Genovese#[1], Luigi Naldini#[1]

[1]SR-TIGET, Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]CNR, Milan, Italy,[4]Humanitas, Milan, Italy,[5]Fondazione Unimi, Milan, Italy

HIGM1 is caused by mutations of CD40L, whose absence in CD4 T cells impairs signaling for B cell activation and Ig class-switching. Since unregulated CD40L expression leads to lymphoproliferation/lymphoma, gene correction must preserve its physiological regulation. Gene editing of either autologous T cells or hematopoietic stem cells (HSC) held promise for treating HIGM1. We developed a "one size fits all" editing strategy to insert a 5'-truncated corrective CD40L cDNA in the first intron of the native human gene, effectively making expression conditional to targeted insertion in the intended locus. By exploiting a protocol that preserves T stem memory cells (TSCM), we reproducibly obtained ~40% of editing efficiency in healthy donor and patients derived T cells, restoring regulated, although partial, CD40L surface expression that was sufficient to restore helper function on B cell co-cultures. To select, track and potentially deplete edited T cells, we coupled the corrective cDNA with a clinically compatible selector gene and confirmed that enriched T cells preserved their engraftment capacity in NSG mice. Unexpectedly, the presence of an IRES-linked downstream coding frame counteracted the shorter half-life of transcript from the edited locus, allowing replenishment of intracellular stores and surface translocation of physiological amounts of CD40L upon activation. We also tailored the *CD40L* editing strategy to human HSC, reaching up to 15-30% editing in HSC long term engrafting NSG mice, depending on the HSC source. We then modelled the therapeutic potential of both T cell and HSC gene therapy by infusing increasing proportions of WT murine cells, as surrogates of edited cells, in HIGM1 mice. Administration of functional T cells at clinically relevant doses in HIGM1 mice, preconditioned or not with different lymphodepleting regimens, achieved long term stable T cell engraftment and partial rescue of antigen specific IgG response and germinal center formation in splenic follicles after vaccination with a thymus dependent antigen. Remarkably, infusion of T cells from mice pre-exposed to the antigen, mimicking treatment of chronically infected patients, was effective even in absence of conditioning and protected the mice from a disease relevant infection induced by the opportunistic pathogen Pneumocystis murina. Transplantation of functional T cells admixed with HIGM1 T cells resulted in lower vaccination response, indicating competition between WT and HIGM1 cells and implying that increasing the fraction of corrected cells in the graft by selection would improve immune reconstitution. Concerning HSC gene therapy, transplanting 25% WT cells along with HIGM1 ones in HIGM1 mice - mirroring the editing efficiencies achieved in human HSC - rescued antigen specific IgG response and established protection from pathogen comparably to T cell therapy. These findings suggest that autologous edited T cells can provide immediate and substantial benefits to HIGM1 patients and position T cell as competitive strategy to HSC gene therapy, because of more straightforward translation, lower safety challenges and potentially comparable clinical benefits. We thus embarked in assessing GMP compliant reagents and protocols for T cell activation, culture and editing and developed a scalable manufacturing process. Optimization of clinical grade culture conditions allowed further increasing editing efficiency, total cellular yield and maintenance of TSCM thus paving the way to the design of a clinical trial.

39. A Phase 1/2 Study of Lentiviral-Mediated Ex-Vivo Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I (LAD-I): Interim Results

Donald B. Kohn[1], Gayatri Rao[2], Elena Almarza[3], Dayna Terrazas[1], Eileen Nicoletti[4], Augustine Fernandes[1], Caroline Kuo[1], Satiro De Oliviera[1], Theodore Moore[1], Ken Law[4], Brian Beard[4], Julian Sevilla[5], Cristina Mesa-Nunez[6], Claire Booth[7], Adrian Thrasher[7], Juan Bueren[6], Jonathan Schwartz[4]

[1]UCLA, Los Angeles, CA,[2]Rocket Pharmaceuticals, Inc., Cranbury, NJ,[3]Rocket Pharmaceuticals, Inc, New York, NJ,[4]Rocket Pharmaceuticals, Inc, Cranbury, NJ,[5]Pediatric Oncology Hematology and Stem Cell Transplant Department, FIB Hospital Infantil Universitario Niño Jesús, and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[6]Division of Hematopoietic Innovative Therapies, Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[7]Infection, Immunity, & Inflammation Department, UCL Great Ormond Street (GOS) Institute of Child Health, London, United Kingdom

Introduction: LAD-I is a rare disorder of leukocyte (primarily neutrophil) adhesion resulting from *ITGB2* gene mutations encoding for the β2-integrin component, CD18. Severe LAD-I (i.e., CD18 expression on <2% of PMNs) is characterized by severe infections, impaired wound healing, and childhood mortality. Although allogeneic hematopoietic stem cell transplant (alloHSCT) is potentially curative, utilization and efficacy are limited by donor availability and risk of graft-versus-host disease (GVHD). RP-L201-0318 (NCT03812263) is a phase 1/2 open-label clinical trial evaluating the safety and efficacy of RP-L201, consisting of autologous CD34+ cells transduced with a lentiviral vector (LV) carrying the *ITGB2* gene encoding for CD18 (Chim-CD18-WPRE) in severe LAD-I. **Methods:** Pediatric patients

≥ 3 months old with severe LAD-I are eligible. Peripheral blood (PB) HSCs are collected via apheresis after mobilization with granulocyte-colony stimulating factor (G-CSF) and Plerixafor and transduced with Chim-CD18-WPRE LV. Myeloablative busulfan conditioning is followed by RP-L201 infusion. Patients are followed for safety and efficacy (i.e., survival to age 2 and at least 1-year post-infusion, increase in PMN CD18 expression to at least 10%, PB vector copy number (VCN), decrease in infections/hospitalizations, and resolution of skin or periodontal abnormalities). **Results:** Four patients (ages 7 months to 9 years) have been treated with RP-L201 with follow-up ranging from 6 weeks to 12 months. RP-L201 cell doses ranged from $2.8x10^6$ to $6.5x10^6$ CD34+ cells/kg with VCNs from 1.83 to 3.8 copies/cell (liquid culture). No serious treatment-emergent adverse events were reported. Neutrophil engraftment was observed in ≤ 5 weeks. PB PMN CD18 expression in Patient 1 12-months post-treatment was 40% (sustained from 47% at 6-months, vs. < 1% at baseline), with PB VCN of 1.2. Skin lesions present at baseline resolved with no new lesions reported. Patient 2 PB PMN CD18 expression 6-months post-treatment was 23% with PB VCN at 0.75. Patients 3 and 4 PB PMN CD18 expression were 74% at 3-months and 59% at 6-weeks post-treatment, respectively. No new infections have been reported in patients post-infusion. **Conclusion:** These results demonstrate that RP-L201 leads to durable neutrophil CD18 expression and improved clinical course. Additional patient treatment is planned for early 2021.

40. Autologous Ex Vivo Lentiviral Gene Therapy for the Treatment of ADA-SCID

Claire Booth[1], Don B. Kohn[2], Kit L. Shaw[2], Jinhua Xu-Bayford[3], Elizabeth Garabedian[4], Valentina Trevisan[1], Denise A. Carbonaro-Sarracino[5], Kajal Soni[1], Dayna Terrazas[2], Katie Snell[5], Diego Leon-Rico[1], Karen Buckland[1], Kimberly Gilmour[1], Satiro De Oliveira[2], Christine Rivat[5], Natalia Izotova[1], Stuart Adams[1], Hilory Ricketts[1], Alejandra Davila[2], Chilenwa Uzowuru[1], Beatriz Campo Fernandez[2], Roger P. Hollis[2], Maritess Coronel[2], Ruixue Zhang[2], Serena Arduini[5], Frances Lynn[5], Mahesh Kudari[5], Andrea Spezzi[5], Marco Zhan[6], Rene Heimke[6], Ivan Labik[6], Kenneth Cornetta[7], Robert Sokolic[4], Michael Hershfield[8], Manfred Schmidt[6], Fabio Candotti[9], Harry L. Malech[10], Adrian J. Thrasher[1], H. Bobby Gaspar[5]

[1]UCL GOSH Institute of Child Health, London, United Kingdom,[2]University of California, Los Angeles, Los Angeles, CA,[3]Great Ormond Street Hospital NHS Trust, London, United Kingdom,[4]National Human Genome Research Institute, Bethesda, MD,[5]Orchard Therapeutics, London, United Kingdom,[6]GeneWerk, Heidelberg, Germany,[7]Indiana University School of Medicine, Indianapolis, IN,[8]Duke University, Durham, NC,[9]Lausanne University Hospital, Lausanne, Switzerland,[10]National Institute of Allergy and Infectious Diseases, Bethesda, MD

Introduction: Severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) is a rare and life-threatening disorder caused by *ADA* gene mutations leading to compromised immune function. Current treatment guidelines suggest enzyme replacement therapy prior to definitive treatment with hematopoietic stem cell transplantation or gene therapy. An investigational gene therapy (GT) consisting of autologous $CD34^+$ hematopoietic stem cell progenitors (HSPCs) transduced *ex vivo* using a self-inactivating lentiviral vector (LV) encoding the human *ADA* cDNA sequence under the control of a shortened human elongation factor 1α gene promoter (EFS-ADA LV) was studied in trials in the US and EU. Methods: Fifty patients with ADA-SCID (30 in the US and 20 in the EU) were treated with lentiviral gene therapy following non-myeloablative busulfan conditioning. An analysis was conducted integrating two US studies (using fresh and cryopreserved formulations of OTL-101) at 24-months' follow-up alongside an EU study (fresh formulation) with 36-months' follow-up. Results: Overall survival was 100% for all analyses up to 24 and 36 months. Event-free survival (in the absence of enzyme replacement therapy reinstitution or rescue allogeneic hematopoietic stem cell transplant) was 96.7% (US studies) and 100% (EU patients) at 12 months, 96.7% and 95%, respectively at 24 months, and 95% (EU patients) at 36 months. Engraftment of genetically modified HSPCs persisted in 29/30 US patients and 19/20 EU patients up to last follow-up. Patients exhibited sustained metabolic detoxification and normalized ADA activity levels. Immune reconstitution was robust with T cell counts reaching or approaching normal ranges at last follow up, including increased naïve T cell numbers. 89.7% of US patients and 100% of EU patients discontinuing immunoglobulin replacement therapy by 24 and 36 months, respectively demonstrating improved B cell function. Most adverse events were mild. No evidence of monoclonal expansion, leukoproliferative complications, or emergence of replication-competent lentivirus was noted and no events of autoimmunity or graft-versus-host disease occurred. Conclusions: Treatment of ADA-SCID with *ex vivo* lentiviral HSPC gene therapy resulted in high rates of overall and event-free survival, with sustained *ADA* gene expression, metabolic correction, and functional immune reconstitution.

41. Efficient Ex-Vivo Selection of Gene Edited Human Hematopoietic Stem/Progenitor Cells

Martina Fiumara*[1,2], Samuele Ferrari*[1], Elisabetta Mercuri[1], Aurelien Jacob[1,3], Luisa Albano[1], Angelo Lombardo[1,2], Pietro Genovese#[1], Luigi Naldini#[1,2]

[1]SR-TIGET, Ospedale San Raffaele, Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]Milano-Bicocca University, Monza, Italy

Hematopoietic stem/progenitor cells (HSPCs) gene editing based on homology directed repair (HDR) prospectively allows the treatment of human hematological diseases by *in situ* gene correction while maintaining physiologic regulation. However, in most hematological diseases, gene corrected HSPC would not gain selective advantage over the unedited counterpart; therefore, current HDR editing efficiencies might be insufficient to establish robust therapeutic benefit. Selection of HDR-edited HSPCs, followed or not by ex vivo expansion, would allow administering higher numbers and/or proportions of corrected cells, improving efficacy of HSC therapy while lowering the conditioning requirement and the toxicity of the procedure. Here, we developed selection strategies that couple gene correction and transient Selector expression by Means of Artificial Transcription activators (SMArT). In one design, we fused the selector (e.g. GFP, ΔNGFR) open-reading frame with the corrective coding sequence of the targeted gene by a

self-cleaving 2A peptide. Since transcription of the edited gene is often low in HSPCs and might not suffice for detectable selector expression, an ArT directed against the targeted gene promoter was co-delivered by mRNA with the editing machinery. Upon editing therapeutically relevant genes in human HSPCs, we achieved transient selector overexpression in all HSPC subpopulations, allowing enrichment of HDR-edited cells up to 90% in the sorted fraction. To avoid selector co-expression in the HSPC progeny physiologically expressing the edited gene, which in some setting may cause toxicity and/or immunogenicity, we improved the SMArT design by introducing in the HDR-template an independent selector cassette regulated by a minimal promoter (minP) that is nearly inactive at basal level. Transient delivery of an ArT binding on the genome flanking the minP but outside the homology region comprised in the HDR-template induced selector expression only in HDR-edited HSPCs. Transactivation, however, was weaker in the more primitive HSPC subpopulations, likely due to the distance between the Art binding site and the transcriptional start site (TSS). To overcome this limitation, we modified the SMArT HDR-template by incorporating multiple tetracycline operator (TetO) repeats close to the minP and transiently co-expressed a tetracycline-regulated TransActivator (tTA) by RNA. Multiplexed ArT binding in close proximity of the TSS allowed robust and transient selector expression in the most primitive HSPC subpopulations. We then tuned tTA activity by an initial pulse of doxycycline administration to suppress transactivation while non-integrated template is still in excess and reached up to 100% HDR editing in the selector-positive fraction. SMArT was portable to several disease relevant loci (*IL2RG*, *AAVS1* and *CD40LG*), compatible with different HSPCs sources and with clinically compliant selectors. On-going experiments are aimed to stringently evaluate the repopulation potential of SMArT-selected HSPCs. Overall, we anticipate that enrichment of HDR-edited HSPCs by SMArT would allow to broaden clinical applicability and tolerability of HSC gene editing.

42. Targeted Genome Editing of Hematopoietic Stem Cells for Treating Recombination Activating Gene 1 (RAG1) Immunodeficiency

Maria Carmina Castiello[1,2], Nicolò Sacchetti[1], Elena Draghici[1], Samuele Ferrari[1], Valentina Vavassori[1], Chiara Brandas[1], Denise Minuta[1], Martina Di Verniere[1], Aurelien Jacob[1], Enrica Calzoni[1,3], Marita Bosticardo[3], Luigi Daniele Notarangelo[3], Luigi Naldini[1], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche (CNR), Milano, Italy,[3]Laboratory of Clinical Immunology and Microbiology, NIAID, NIH, Bethesda, MD

Recombination Activating Genes (*RAG*) are tightly regulated molecules during T and B cell differentiation. Mutations in *RAG* genes result in a broad spectrum of immunological disorders including T-B- SCID and leaky forms. Curative treatment is hematopoietic stem cell transplantation but donor availability is still limited and unsatisfactory outcomes have been described with partially HLA-matched donors. Gene replacement therapy have been studied in pre-clinical models, however safety concerns are related to unregulated *RAG1* expression. Thus, we developed a specific genome editing (GE) strategy, based on the delivery of engineered nucleases and DNA template, to correct *RAG1* mutations and restore gene expression and function. We firstly assessed the minimal therapeutic dose of functional cells by competitive transplant experiments into *Rag1*$^{-/-}$ mice, which showed that low proportion of wild-type or *Rag1*$^{+/-}$ lineage negative bone marrow cells can correct immune defects. We exploited the CRISPR/Cas9 platform combined with the delivery of an AAV6 donor carrying the corrected codon sequence downstream splice acceptor site sequence to use the endogenous *RAG1* promoter. A panel of 12 guides was screened and we selected the best performing one in terms of cutting efficiency assessed by T7 surveyor assay. The selected guide delivered with the corrective AAV6 donor resulted in restoration of RAG1 activity assessed by an in vitro LV-based recombination assay in Rag-1 KO cells. We apply our GE strategy to human CD34+ derived from cord blood or mobilized peripheral blood (MPB) of healthy donors (HD) and we obtained high editing efficiency even in the most primitive hematopoietic stem and progenitor cells (HSPC), assessed as homology directed repair (HDR) efficiency by ddPCR. We exploited an organoid platform to assess the T cell differentiation potential of edited HSPC coupling the GE and the T cell differentiantion protocols optimized for our purposes. Human CD34+ cells edited in *RAG1* locus differentiated in CD3+ TCRαβ+ cells demonstrating that our strategy preserves the T cell differentiation potential. Next, we edited CD34+ cells derived from MPB of HD and two RAG1 patients carrying hypomorpic *RAG1* mutations resulting in a reduced recombination activity and presenting with a combined immunodeficiency with granuloma formation and autoimmunity. Edited cells were transplanted into NSG mice to assess the engraftment capability and kept in culture for phenotypical and molecular analyses. The HPSC phenotype and HDR efficiency (40%) were similar between HD and RAG1-derived edited cells *in vitro.* Importantly, GE did not impact the engraftment and multilineage differentiation of edited HSPC in NSG mice. Moreover, we observed a peripheral selective advantage of edited cells in NSG mice transplanted with edited CD34+ cells derived from RAG1 patients.Overall, our findings suggest that we set up an efficient and promising GE platform for the correction of RAG1 deficiency, that would potentially benefit treatment of RAG1 patients who lack matched donors.

Musculo-skeletal Diseases

43. The Long-Term Efficiency of the scAAV. U7.ACCA Vector in Inducing Dystrophin Expression in Adult Dup2 Mice

Liubov V. Gushchina, Adrienne J. Bradley, Kelly M. Grounds, Aisha Suhaiba, Emma Frair, Calli Bellinger, Tabatha Simmons, Natalie Rohan, Nicolas Wein, Kevin M. Flanigan

Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH

Duplications in exon 2 of the *DMD* gene, encoding the dystrophin protein, account for around 10% of all duplication mutations associated with the X-linked Duchenne muscular dystrophy (DMD). As part of

the preclinical development of a U7snRNA vector currently in a clinical trial, our group has previously evaluated the therapeutic efficacy, absence of off-target splicing effects in AAV9.U7snRNA mediated skipping of exon 2 in a murine Dmd model, and lack of toxicity in non-human primates. Here we report long-term dystrophin expression data following treatment of 3-month-old Dup2 mice with the scAAV.U7.ACCA vector, which contains four copies of U7snRNA targeted to the exon 2 splice acceptor and splice donor sites. Dup2 males received a single intravenous infusion of 3E13 vg/kg, which is the minimal efficacious dose (MED) dose in ongoing first-in-human clinical trial (ClinicalTrials.gov NCT04240314). The age matched Dup2 treated with diluent and C57Bl/6 mice were used as controls. All animals (n=9-11) were sacrificed 18-month post vector administration. The RT-PCR results showed that a single scAAV9.U7.ACCA vector injection resulted in a significant exon 2 skipping in tibialis anterior (TA), diaphragm (Dia) and heart tissues, showing an average of 46%, 32% and 73% total therapeutic transcripts, respectively. To determine the degree of functional rescue, we performed in situ physiology studies on TA and in vitro on Dia muscles. Treated with diluent, both Dia and TA muscles from 21-month-old Dup2 mice exhibited a functional deficit with a significant (45-61%) reduction in specific force output compared with C57Bl/6 (Bl6) mice. The significant force drop was also observed in diluent treated Dup2 mice compared with Bl6 mice following a rigorous fatigue protocol. The single scAAV9.U7.ACCA injection resulted in a dramatic improvement in specific force output, which increased up to 64-76% in Dia and TA muscles, respectively, and better protection of the TA muscle from repeated fatigue, which improved up to 73%. Overall, these data support our previous findings showing that scAAV9.U7.ACCA provides long-term protection by restoring the disrupted dystrophin reading frame in straight muscles from Dup2 mice and functional recovery of TA and Dia muscles 18-month post vector administration.

44. Towards an Off-the-Shelf Cell Therapy for Bone Healing: Use of an Immortalized, Genetically Modified Cell Line as a Proof of Concept

Rodolfo E. De la Vega[1,2], Michael J. Coenen[1], Gresin Hawse[1], Joseph A. Panos[1], Christopher H. Evans[1]

[1]Rehabilitation Medicine Center, Mayo Clinic, Rochester, MN,[2]cBITE, MERLN Institute - Maastricht Unversity, Maastricht, Netherlands

Introduction: Bone morphogenetic protein-2 (BMP-2) is the most potent clinically available osteogenic cytokine. Yet, after almost two decades in the market, it has limited clinically approved use conditions, requires supraphysiological doses, and side effects are a concern. Gene transfer has been suggested as a means to improve and prolong delivery while lowering side effects. *Ex vivo* gene transfer using autologous cells has been studied in this regard but is limited by the high cost associated with harvesting, tissue culture expansion, transduction, and characterization under GMP conditions prior to re-implantation. This project explores an alternative approach by generating and evaluating a stably-transduced, BMP-2 expressing, immortalized cell line to serve as a proof of concept for an eventual off-the-shelf, allogeneic cell therapy product for bone healing in humans. METHODS: We used HEK293 cells because of their high proliferation rate, ease of maintenance and transduction. Cells were transduced with a lentivirus coding for the human BMP-2 gene driven by the CMV promoter. Clones were selected by puromycin, expanded, BMP-2 production characterized by ELISA and cells frozen at either $5x10^5$ (low), $1x10^6$ (medium) or $3x10^6$ (high) cells/vial. Individual vials were thawed and cells encapsulated in fibrin just prior to surgical implantation in a rat, 5-mm, femoral, critical size bone defect model. The high dose of 11 µg BMP-2 bridges the defect, while empty defects fail to bridge, acting as positive and negative controls, respectively (n=10/group). FK506 was used to prevent xenograft rejection. Defect bridging was monitored via weekly radiographs until euthanasia at 12 weeks. A subset of animals receiving $1x10^6$ cells (n=5) was euthanized at 4 weeks for gDNA extraction from the defect site to determine the presence of implanted cells. RESULTS: Two clones were selected and expanded. Clone CL1K showed significantly higher BMP-2 production per cell than CL20K (Fig. 1A). CL1K was expanded and BMP-2 production was confirmed up to passage 50 (Fig. 1B). After 12 weeks (Fig. 1C), defect bridging seemed to be correlated to the number of implanted cells, with 20%, 60% and 70% of animals bridging in the low, medium and high groups, respectively. qPCR was able to detect gDNA from the implanted cell line in 3 out of 5 animal samples, with one of these animals presenting with solid mass at the femoral defect site. DISCUSSION: Although 293 cells cannot differentiate into osteoblasts, the BMP-2 expressing CL1K cells were able to bridge large bone defects. It is widely assumed that *ex vivo* gene delivery for bone healing must use osteoprogenitor cells as vehicles. However, these data show otherwise and suggest a novel approach to bone healing. We know of no other reports where a cell unable to differentiate into osteoblasts has been used to induce bone healing in a bone defect model. 293 cells are not an ideal candidate for *ex vivo* therapies because of the risk of tumorigenesis, but this experiment serves as a proof of concept for the prospect of an allogeneic off-the-shelf cell line for bone healing. CONCLUSIONS: These novel findings provide a basis for the development of a convenient and effective *ex vivo* gene therapy product for bone healing. Future work is needed to optimize the conditions needed for this approach to be successful for clinical translation.

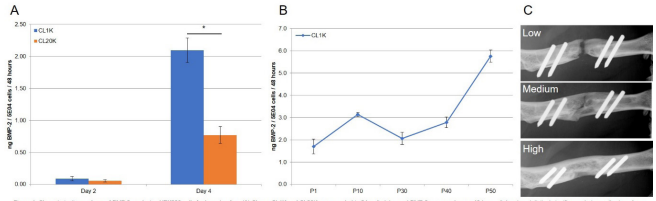


Figure 1. Characterization and use of BMP-2 producing HEK293 cells for bone healing. (A) Clones CL1K and CL20K were seeded in 24-well plates and BMP-2 measured every 48 hours. * denotes statistical significance between the two clones. (B) BMP-2 production of CL1K throughout multiple passages. (C) Radiographs of critical size bone defects treated with ascending doses of CL1K cells after 12 weeks.

45. Correction of Clcn1 Mis-Splicing Reverses Muscle Fiber Type Transition in Mice with Myotonic Dystrophy

Ningyan Hu, Thurman Wheeler

Massachusetts General Hospital, Boston, MA

Background: DM1 is caused by an expanded CTG repeat in the DMPK gene. Characteristics of this multisystem disorder include myotonia (delayed muscle relaxation), progressive weakness, muscle wasting, and cardiac conduction defects. Clinical features of DM1 arise from expression of DMPK transcripts that contain expanded CUG repeats (CUG)exp that accumulate in nuclear inclusions of skeletal muscle

and other affected tissues. This pathogenic RNA readily binds proteins in the muscleblind-like (MBNL) family that are required for normal regulation of alternative splicing, resulting in loss of MBNL protein function. In the HSALR transgenic and Mbnl1 knockout mouse models of DM1, alternative splicing patterns in muscle tissue are very similar. A clinical manifestation of Mbnl1 loss of function includes myotonia, a delayed muscle relaxation caused by mis-splicing of muscle chloride channel Clcn1. In human DM1 muscle, oxidative muscle fibers are upregulated and preferentially atrophic as compared to glycolytic fibers. **Methods**: We crossed HSALR transgenic mice with Mbnl1 knockout mice to create a double homozygous mouse model of DM1. Tibialis anterior muscles were injected with an antisense morpholino oligo that target Clcn1 exon 7a for skipping. The contralateral muscle were injected with the 5' - 3' invert of the active drug. We measured Clcn1 splicing by RT-PCR, transgene expression by droplet digital PCR, and myosin fiber type by immunolabeling and ddPCR. **Results**: In muscles receiving the active treatment, Clcn1 splicing was corrected by 16 days after injection. Type 2B glycolytic muscle fibers were nearly absent in untreated double mutant mice and 50% of the overall total in treated mice. Expression of ACTA1-CUG transcripts and the muscle regeneration marker embryonic myosin (Myh3) was reduced. **Conclusions**: Chronic severe myotonia results in muscle fiber type transition from glycolytic to oxidative in models of DM1. Reversal of Clcn1 mis-splicing is sufficient to rescue muscle fiber type patterns and reduce muscle fiber damage. **Grant support**: Muscular Dystrophy Association.

46. Characterization of Acute Toxicity after High-Dose Systemic Adeno-Associated Virus in Nonhuman Primates, Including Impact of Vector Characteristics

Juliette Hordeaux, Chunjuan Song, Erik Wielechowski, Ali Ramezani, Cecilia Dyer, Elizabeth L. Buza, Jessica Chichester, Peter Bell, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Dose-limiting toxicities have occurred following intravenous administration of high doses of adeno-associated virus (AAV) to target the musculoskeletal or central nervous systems. Acute elevations in liver enzymes and/or reductions in platelets have been observed in most high-dose AAV clinical trials. Although infrequent, severe toxicities have included anemia, renal failure, complement activation, and, in the worst cases, fatal hepatobiliary disease. We previously reported the development of acute thrombocytopenia and transaminitis in most nonhuman primates (NHPs) within days of of receiving high doses of AAV (in the 1x1014 GC/kg range). Some animals recover while some develop a lethal syndrome of coagulopathy, liver failure, hemorrhage, and shock. The acute initial presentation of thrombocytopenia and transaminase elevation is common to NHPs and humans and has not been observed in other species in our experience. Although the progression of the initial acute toxicity may differ in human patients with pre-existing conditions compared to healthy animals, we propose that NHPs can be used as a model to better characterize the acute toxicity of high doses of AAV to reduce risk in clinical trials. We conducted studies aimed at investigating the effect of capsids, prophylactic steroids, and vector purification methods on the incidence and severity of acute toxicity following high-dose AAV systemic administration. We administered ten rhesus macaques (16 months to 4.6 years old with anti-capsid neutralizing titers < 5 and weighing 3-7 kg) with AAV9 or an engineered capsid derived from AAV9 (AAV-PHP.eB) encoding green fluorescent protein at 1 or 2 x 1014 GC/kg. We then monitored for acute toxicity for 2 weeks post-administration. All vectors were produced from transient transfection of adherent HEK293 cells and either purified using affinity column chromatography or an iodixanol gradient. Both purification methods yielded more than 70% of full capsids. We observed acute toxicity in the majority of animals, regardless of the purification method, characterized by thrombocytopenia, liver enzyme elevation with or without hyperbilirubinemia, and increased coagulation times on day 3 post-administration. Most animals recovered from the initial toxicity except for two AAV-PHP.eB-dosed animals that had to be humanely euthanized due to severe coagulopathy. Prophylactic steroids appeared to help with the recovery from the initial toxicity but did not prevent it from occurring. The toxicity was dose dependent, with AAV9 capsid having a narrow safety margin: 1 x 1014 GC/kg was well tolerated whereas a 2-fold increase led to thrombocytopenia in the majority of animals. Importantly, we observed complement activation concurrent with the thrombocytopenia on day 3 with activation of the alternate pathway and elevation of complement Bb fragment and SC5b-9 membrane attack complex, whereas the classical pathway did not appear to be activated (unchanged C4 and C4a levels). Accordingly, there were no detectable IgM antibodies to the capsid on day 3, suggesting that immune complexes were not the cause of toxicity at 3 days post-administration in NHPs. Collectively, our data suggest that the acute toxicity that occurs after high-dose AAV in NHPs causes thrombocytopenia and liver injury on day 3 post-administration and resolves by day 7 in the majority of animals. This toxicity is capsid dependent, dose dependent, unrelated to a vector purification method, and involves transient activation of the alternate complement pathway.

47. Long-Term Hematopoietic Stem Cell Lentiviral Gene Therapy Corrects Neuromuscular Manifestations in Preclinical Study of Pompe Mice

Niek P. van Til[1,2], Yildirim Dogan[1], Cecilia Barese[1], Zeenath Unnisa[1], Swaroopa Guda[1], Rena Schindler[1], John Yoon[1], Mary Jacobs[1], Abhishek Chiyyeadu[1], Daniella Pizzurro[1], Bianling Liu[1], Mirjam Trame[1], Tim Maiwald[1], Christine Oborski[1], Axel Schambach[3], Claudia Harper[1], Richard Pfeifer[1], Chris Mason[1,4]

[1]AVROBIO, Inc, Cambridge, MA,[2]Child Neurology, Emma Children's Hospital, Amsterdam University Medical Centers, Vrije Universiteit and Amsterdam Neuroscience, Amsterdam, Netherlands,[3]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,[4]Advanced Centre for Biochemical Engineering, University College London, London, United Kingdom

Pompe disease is an inherited disorder caused by acid alpha-glucosidase (GAA) deficiency, leading to lysosomal glycogen accumulation in the heart, skeletal muscles, and the central nervous system (CNS). Pompe disease presents with generalized muscle weakness, and, if untreated, the most severely affected patients typically succumb

early in life due to cardiorespiratory failure. The standard of care is enzyme replacement therapy, which requires lifelong treatment, but does not prevent disease progression. Hence, there is a clear unmet medical need to develop treatment options for Pompe disease patients that could provide a lifelong, life-altering benefit. We used a lentiviral vector driven approach to overexpress a chimeric human *GAA* transgene with a glycosylation independent lysosomal targeting (GILT) tag in the hematopoietic system of a Pompe disease mouse model. A novel therapeutic lentiviral vector carrying a clinically proven promoter was selected and tested for long-term efficacy in Pompe mice. Supraphysiological GAA enzyme activity levels were achieved in hematopoietic cells and plasma following transplantation with genetically modified hematopoietic stem and progenitor cells (HSPCs) in pre-conditioned Pompe mice. Urinary hexose tetrasaccharides (Hex4), a breakdown product of glycogen, were reduced in treated Pompe mice. GAA enzyme activity was detected in the murine heart, skeletal muscles, spinal cord, and at lower levels in the brain at eight months after transplantation. Glycogen clearance was complete in the murine heart, diaphragm, brain, spinal cord, and in most of the skeletal muscles tested. Consequently, pathological heart remodeling was reversed. Locomotor function was improved. Average vector copy numbers in leukocytes were below five in all the experimental groups. Importantly, administering the lowest dose median VCN ~2 provided robust glycogen reduction indistinguishable from higher doses (median VCN >3) administered. In addition, glucose levels remained stable during the study and the hematopoietic reconstitution of transplanted gene-modified HSPCs was similar to controls. In summary, our candidate vector was shown, in mouse models, to be a potentially effective therapeutic approach for long-term alleviation of cardiomyopathy, muscle weakness and CNS pathology in Pompe disease. We believe this approach could translate into a clinical application of single-dose therapy for Pompe disease patients.

48. Downregulation of the Genetic Modifier *PITPNA* as Means of Therapy in Duchenne Muscular Dystrophy

Matthias R. Lambert, Yuanfan/Tracy Zhang, Janelle M. Spinazzola, Jeffrey J. Widrick, James R. Conner, Louis M. Kunkel

Genetics and Genomics, Boston Children's Hospital, Boston, MA

Duchenne muscular dystrophy (DMD) is a severe genetic disorder caused by mutations in the *DMD* gene. Absence of dystrophin protein leads to progressive degradation of muscle function and leads to premature death. Although there are several promising strategies under investigation to restore dystrophin protein expression, there is currently no cure for DMD, and the development of dystrophin-independent therapies is essential. The recent advances in whole-genome/exome sequencing and the use of large-scale databases have enabled the identification of several genetic modifiers that influence clinical presentation and represent an unexplored territory for therapy. In our lab, we identified *PITPNA* as a new genetic modifier of DMD in two exceptional mildly affected Golden Retriever Muscular Dystrophy (GRMD) dogs. Downregulation of *PITPNA* allowed dystrophin-deficient dogs to retain functional muscle and normal lifespan. This was confirmed in our dystrophin-deficient zebrafish in which *pitpna* downregulation by morpholino antisense nucleotide resulted in improved muscle structure and function. In the present study, we explored different strategies that both aimed to downregulate *PITPNA* and prevent the muscle pathology. In dystrophin-deficient zebrafish, we developed a straightforward phenotypic drug screening assay that would be inconceivable in mouse model systems. We identified that phosphodiesterase 10A (PDE10A) inhibitors improved muscle integrity and reduced *pitpna* expression. The PDE10A pathway was confirmed with the use of *pde10a* morpholino and we combined different functional assays that showed improvement in locomotion, muscle, and vascular function as well as long-term survival in *dmd* zebrafish. In dystrophin-deficient mice, we are currently evaluating the potential benefit of *Pitpna* downregulation on muscle pathology based on two different strategies: (i) the use of PDE10A inhibitors and (ii) the use of Pitpna shRNA-AAV. Despite recent advances in dystrophin replacement strategies, there is still precedence to pursue pharmacological therapies targeting genetic modifiers that can complement dystrophin-based therapies and are independent of patients' genetic mutations. In different animal models of DMD, downregulation of the genetic modifier *PITPNA* presents several reasons for there to be further studied of it as a potential DMD therapeutic via two strategies that may benefit patients.

49. Non-Genotoxic Conditioning to Increase Gene Therapy Safety in a Rare Bone Disease

Valentina Capo[1,2], Sara Penna[1,3], Ludovica Santi[1], Andrea Cappelleri[4,5], Stefano Mantero[2,6], Elena Fontana[2,6], Eugenio Scanziani[4,5], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Milan Unit, CNR-IRGB, Milan, Italy,[3]Dimet, University of Milano-Bicocca, Monza, Italy,[4]Department of Veterinary Medicine, University of Milan, Lodi, Italy,[5]Mouse and Animal Pathology Lab (MAPLab), Fondazione UniMi, University of Milan, Milan, Italy,[6]Humanitas Clinical and Research Center - IRCCS, Rozzano, Italy

Autosomal recessive osteopetrosis (ARO) is a rare genetic disease, affecting osteoclast differentiation or function. Most ARO patients present mutations in *TCIRG1* gene, encoding the a3 subunit of V-ATPase proton pump, necessary for the acidification of bone resorption lacunae by osteoclasts. Symptoms include dense and brittle bones, limited bone marrow cavity, anaemia and progressive nerve compression, leading to death in the first decade of life. To date, hematopoietic stem cell transplantation (HSCT) is the only therapeutic option, but it is limited by availability of HLA-matched donors, toxicity of conditioning regimens and significant morbidity. We and others proposed gene therapy (GT) as an alternative strategy to overcome donor-related issues. However, the burden of conventional myeloablative conditioning on patients remains a strong unmet clinical need. Thus, we evaluated the use of a novel non-genotoxic conditioning in GT setting of *TCIRG1*-dependent ARO. In particular, we tested antibody-drug conjugates (ADCs), formed by anti-cKit or anti-CD45 antibody conjugated to the saporin toxin, able to make space in bone marrow (BM) and to promote hematopoietic stem and progenitor cell (HSPC) engraftment without off-target toxicity in adult mice. Since ARO symptoms occur very early in life, we evaluated the HSPC depleting potential of ADCs on WT newborn mice, administered via temporal vein at 1 day of life. We sacrificed mice 2 days after treatment

and observed partial HSPC depletion in BM, spleen and peripheral blood. Notably, we did not observe histological lesions or increase in apoptosis (evaluated with Caspase 3 immunohistochemistry) due to organ toxicity in kidney, brain and BM of ADC-treated mice. To evaluate the efficacy of non-genotoxic conditioning in favouring HSPC engraftment early in life, we performed mismatched HSCT in WT ADC-conditioned mice, and compared it to transplantation after sublethal total body irradiation or without conditioning. Twenty weeks after transplant, we observed low but persistent donor chimerism in ADC-treated mice compared to irradiated controls in peripheral blood, BM, spleen and thymus.As reported in literature, very low level of donor cells engraftment is sufficient to restore bone phenotype in osteopetrotic mice. We hypothesized that ADC conditioning could successfully guarantee bone phenotype amelioration and reduction of the conditioning toxicity in the osteopetrotic mouse model. This approach may be even more advantageous in the GT setting, in which autologous HSCT avoids the risk of graft rejection. We plan to apply ADC conditioning on osteopetrotic mice before the transplant of lentiviral vector GT Lin- cells, to test the efficacy of our strategy on this severe bone disease model. *Acknowledgements.* This project has received funding from the European Calcified Tissue Society.

Novel AAV Capsids for Brain, Eye and Muscle Tissues

50. Endothelial-Tropic AAVs for Genetic Access to Whole-Brain Vasculature in Wild-Type Mouse Strains Following Non-Invasive Systemic Delivery

Xinhong Chen, Damien A. Wolfe, Sripriya Ravindra Kumar, Timothy F. Miles, Erin E. Sullivan, Acacia M. Hori, Xiaozhe Ding, Viviana Gradinaru

BBE, Caltech, Pasadena, CA

The neurovascular unit (NVU) is a vital yet understudied component of the nervous system. Malfunction of non-neuronal cell types within the NVU, including endothelial cells, can facilitate the progression of neurological disorders (Yu et al, Frontiers in Neuroscience, 2020), but limited options for cell-type specific transgene delivery hamper its study. Adeno-associated virus (AAV) vectors for gene delivery to the brain are commonly administered via intra-cranial injections, resulting in tissue damage and limited, uneven spatial coverage. Systemic AAV delivery provides a non-invasive, brainwide alternative for genetic access. Having engineered vectors that efficiently cross the blood-brain-barrier (BBB) with broad tropism in rodents (e.g. AAV-PHP.eB), we turned our focus to engineering cell-type-specific vectors that could access vasculature without targeting other components of the NVU. Using M-CREATE directed evolution (Kumar et al, Nature Methods, 2020), we identified a family of endothelial-enriched capsid variants, including one named AAV-CAP.X1. Following intravenous (I.V.) injection, AAV-CAP.X1 targets vasculature with high cell-type specificity and efficiency throughout the body, including the brain. After injecting 3E11 vg total of AAV-CAP.X1 packaging CAG-GFP into adult C57BL/6J mice, 97% (+/- 0.8%) of the GFP+ area in the hippocampus are CD31+ (demonstrating specificity), and 73% (+/- 9.1%) of the CD31+ area in the hippocampus is GFP+ (proving efficiency; note that an increased dosage of 1E12 vg per mouse resulted in even greater CD31+ labeling without losing specificity). As AAV-CAP.X1 vascular infectivity in the periphery may complicate applications that focus on brain-specific endothelial transduction, we introduced point mutations on the AAV-CAP.X1 capsid and incorporated microRNA target sites into the cargo genome that successfully de-target AAV-CAP.X1 from the liver without impairing brain transduction. AAV-CAP.X1 can be used across multiple genetically diverse mouse strains, with efficient labeling of both capillaries and arteries in the brains of C57BL/6J, FVB/NJ, CBA/J, and BALB/cJ mice following I.V. administration. We also observed a significant increase in transduction compared to its parent capsid AAV9 on multiple human-derived cell lines *in vitro*. In its brain-targeted form, AAV-CAP.X1 could be paired with pre-clinical therapeutic cargo both to probe vascular contributions to neurological disease and to inform intervention strategies. More broadly, gene delivery via endothelial-tropic AAV capsids could, in principle, be applied to study diverse pathologies that may benefit from vascular remodeling. Our evolving knowledge regarding vascular pathology in COVID-19 that could underlie generalized organ dysfunction demonstrates the timeliness and potential importance of such vectors.

51. RNA-Driven Evolution of AAV Capsid Libraries Identifies Variants with High Transduction Efficiency in Non-Human Primate Central Nervous System

Mathieu Nonnenmacher, Shaoyong Li, Wei Wang, Matthew A. Child, Amy Z. Ren, Katherine Tyson, Nilesh Pande, Xiaodong Lu, Jiangyu Li, Xiao-Qin Ren, Jianyu Shang, Michael Hefferan, Jay Hou, Omar Khwaja

Voyager Therapeutics, Cambridge, MA

Widespread transduction of the central nervous system (CNS) by viral or non-viral vectors still represents a considerable challenge in gene therapy. Local delivery of Adeno-Associated Virus (AAV) vectors to the CNS by intraparenchymal or intrathecal administration typically results in strong but heterogeneous transduction and is associated with potential risks related to invasive delivery. By contrast, intravascular delivery of engineered AAV vectors capable of crossing the blood-brain barrier should allow a broader CNS transduction, thanks to the high density of the brain vascular network. We applied our previously described RNA-driven biopanning TRACER platform to perform directed evolution of an AAV9 capsid library in cynomolgus monkeys (*Macaca fascicularis*). Following two rounds of intravenous dosing and neuron-specific library selection, a synthetic pool of variants was tested by multiplexed RNA enrichment analysis. This yielded a series of capsid variants with enhanced performance relative to AAV9. Five selected candidates were tested individually by low dose intravenous injection and their tropism for the CNS analyzed by measuring transgene RNA expression, viral DNA biodistribution and immunohistochemistry. All five variants were markedly improved over AAV9, with a subset showing 10-fold or more improvement of transduction in the brain. The highest performing variant displayed more than 1,000-fold higher RNA expression in the brain and 100-fold higher in the spinal cord. Immunohistochemical analysis indicated that this enhanced new

variant displays a predominant neuronal tropism and widespread transduction of multiple brain regions including the cortex, thalamus, putamen and brainstem. Transduction was strikingly high in deep cerebellar nuclei. We propose that this novel capsid variant has potential for use in multiple CNS indications.

52. Expanding the Utility of Intravitreal AAV via a Capsid Variant That Overcomes Neutralization by Anti-AAV2 NAbs in Human Vitreous

Siddhant S. Gupte[1], Sanford L. Boye[1], Wei Li[1], Sergei Zolotukhin[1], Paul Gamlin[2], Siva Iyer[3], Shannon Boye[1]

[1]Pediatrics, University of Florida, Gainesville, FL,[2]Ophthalmology, University of Alabama Birmingham, Birmingham, AL,[3]Ophthalmology, University of Florida, Gainesville, FL

Purpose: Adeno-associated virus (AAV) based gene therapies are approved for treating retinal disorders. While subretinal injection (SRI) leads to efficient gene transfer, intravitreal injection (IVI) is being pursued as a less invasive approach but is complicated by the presence of neutralizing antibodies (NAbs) against AAV in the vitreous. ~70% of the population has pre-existing anti-AAV2 NAbs, which can reduce therapeutic transgene expression to sub-therapeutic levels. As such, the presence of NAbs in the serum is currently used as an exclusion criterion for clinical trials utilizing IVI of AAV. We previously showed that 10% of human vitreous samples screened (31/301) contained high levels of AAV2 NAbs (>95% inhibition of transduction at 1:4 dilution). Here, we evaluated matched vitreous/serum samples (from the same individual) to determine the correlation between respective levels of AAV2 NAbs. We also continued to evaluate the ability of P2-V1, an AAV capsid variant identified through directed evolution and screening in non-human primate (NHP) for the ability to evade neutralization in these samples. Lastly, we characterized the transduction of P2-V1 in macaque following IVI at a clinically relevant dose. The ultimate goal is to identify gene therapy vectors capable of successfully treating the largest proportion of patients. **Approach:** Self-complementary smCBA-mCherry and CBA-GFP vectors were packaged in AAV2, P2-V1 or AAV2.7m8 via plasmid transfection. Vectors used in the NHP experiment also underwent affinity column purification. Vitreous and blood (processed to serum) were collected from patients undergoing vitreoretinal surgeries. They were screened for AAV2 NAbs by infecting HEK293 cells with AAV2-mCherry at MOI of $5x10^3$ in the presence of vitreous or serum at 1:4 dilution followed by 4-fold dilutions ranging from 1:10 to 1:10,240. Expression was quantified via flow cytometry. Samples reducing AAV2 transduction >95% at 1:4 dilution were classified as inhibitory. The NAb50 titer (reciprocal dilution at which 50% inhibition of transduction occurs) for each sample was determined. AAV2, P2-V1 and AAV2.7m8 were similarly compared head-to-head in the presence of inhibitory vitreous samples. 1e11vg of AAV2(benchmark) or P2-V1 CBA-GFP vector was IVI into macaque. Transgene expression was recorded in life at 3 and 6 weeks post injection by fluorescent fundoscopy and by immunohistochemistry after sacrifice in retinal sections. **Results:** In head-to-head comparisons using inhibitory vitreous samples, P2-V1 had a 4-64 fold higher NAb50 value than AAV2 in 20 of 29 samples, and AAV2.7m8 in 16 of 22 samples. P2-V1 displayed higher transduction efficiency following IVI in NHP retina compared to AAV2. Of the 19 matched vitreous and serum samples tested so far, 9 serum and 4 vitreous samples inhibited AAV2. All 4 inhibitory vitreous samples had corresponding neutralizing serum, albeit with 4-64 fold higher NAb50. **Conclusions:** P2-V1 outperformed AAV2 and AAV2.7m8 in the presence of human vitreous samples containing anti-AAV2 NAbs and showed higher transduction than AAV2 in NHP retina. While more samples are being assessed, our results with matched serum and vitreous samples suggest that serum NAb levels are a more sensitive measure of a patient's NAb titer than vitreous. We are exploring whether blood/retinal barrier integrity is predictive of higher vitreous NAbs. The ability of P2-V1 to evade neutralization by the matched samples containing anti-AAV2 NAbs will also be studied. Use of P2-V1 may expand the number of patients that can participate in IVI-based clinical trials.

53. Breaking Thru the Human Blood Brain Barrier: Discovering AAV Vectors Targeting the Central Nervous System Using a Transwell Model

Ren Song[1], Katja Pekrun[1], Themasap A. Khan[2], Feijie Zhang[1], Sergiu Pasca[2], Mark A. Kay[1]

[1]Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA,[2]Stanford University School of Medicine, Stanford, CA

Recombinant adeno-associated virus (AAV) vectors for genetic and acquired central nervous system (CNS) disorders continue to gain popularity exemplified by the FDA approval of Zolgensma[R], an AAV9 vector expressing the SMN1 gene from motor neurons for the treatment spinal muscular atrophy. Unfortunately, extremely high doses of the drug are required, leading to varying degrees of adverse effects, including death. A major limitation in the field is the relatively low penetration of vectors across the human blood-brain barrier (BBB). Once across the BBB, selective transduction of various cell types would also be important depending on the type of disorder being treated. In order to enhance human BBB penetration and select for CNS cell selective AAV capsids, we developed two key techniques. First, we used an *in vitro* transwell BBB system with human endothelial cells and separate layers of human iPS cell neurons and astrocytes derived from 3D organoid cultures. We then tested a pool of 18 known AAV vectors by passaging them through our model system. The pool included AAVs known to cross the BBB more efficiently, such as AAV9 and AAV-rh.10, and those recognized not to cross the BBB (e.g AAV-DJ). The proportion of AAV-9 and AAV-rh10 capsids was enriched in the flowthrough by ~2-fold, while AAV-DJ was reduced by ~5-fold. We created a complex AAV capsid library with ~1 million variants, each containing a unique DNA barcode, using multispecies interbreeding. Three different genetic evolutionary selection schemes were carried out in astrocytes, neurons and the flow thru media. After multiple passages through the humanized BBB transwell system, we used both high-throughput single molecule capsid DNA (2.2kb) sequencing (PacBio) as well as barcode sequencing (Illumina), to identify the capsids that were enriched in the three screens. Multiple enriched AAV variants were made up of complex capsid chimeric sequences that have enhanced ability to pass through the BBB and transduction of astrocytes and/or neurons. We have validated six viral vectors from the screen for astrocytes and all six are able to cross the endothelial cell layer and

transduce astrocytes 37 to 91-times better than AAV9. We also found four viral vectors from the screen for neurons that were 3.6 to 20-times better than AAV9 at crossing both the endothelial and astrocyte cell layers. These four vectors are also 1.6 to 4.0-times better than AAV9 at directly transducing iPS-derived neurons. We evaluated the capsids in mice and found several that are liver de-targeted. Our next plan is to pool the best capsids and determine their BBB penetration in non-human primates. Towards this plan, we have selected 6 of the candidates, pooled them together and established their relative BBB penetration in the transwell system, which was predicted as when each was tested individually. Our work supports the use of a human transwell system for selecting improved capsids for CNS based gene therapeutics.

54. Expanding the AAV Toolbox for Cerebellar Transduction: Identifying and Characterizing Novel Variants in Non-Human Primates and Mice

Megan S. Keiser, Yong Hong Chen, Paul T. Ranum, Xueyuan Liu, Congsheng Cheng, David Leib, Geary Smith, Amy Muehlmatt, Luis Tecedor, Beverly L. Davidson

Children's Hospital of Philadelphia, Philadelphia, PA

Introduction: New tools for gene therapy have opened the door to addressing an almost infinite number of genetic lesions, however delivering these tools to the appropriate anatomical location and cell type remains a significant bottleneck. Spinocerebellar ataxias (SCAs) are a family of hereditary movement coordination disorders broadly characterized by neurodegeneration of the cerebellum and brainstem. Here, we characterize the hindbrain tropism of newly identified capsids from an ongoing directed AAV evolution screen in nonhuman primates (NHPs) for future delivery of SCA gene therapies **Methods**: Directed evolution libraries were comprised of AAV1, AAV2, and AAV9 variants generated by addition of random 7-mer peptides into wild-type capsids. Top hits were identified following three consecutive rounds of enrichment based off pooled results from 14 individual brain regions including cerebellar cortex, deep cerebellar nuclei (DCN) and brainstem. Two capsids with enrichment in the cerebellum and brainstem were generated to express fluorescent reporters for visualization of transduction in vivo. Individual variants were delivered to NHPs or adult mice by ICV injection or direct injection in the DCN. **Results**: Following a single ICV injection, a top AAV9 variant was able to transduce Purkinje cells across multiple lobules of the cerebellum as well as a wide variety of other cerebellar cell types including Bergman glia, granule cells, and dispersed interneurons in NHP brain. Injection of the AAV9 variant ICV to adult mice revealed transduction of Purkinje cells. In addition, a top AAV2 capsid variant showed uniformly high levels of Purkinje cell expression in superficial lobules of the cerebellum. Other areas highly transduced in the adult murine brain included the motor cortex, basal ganglia, thalamic nuclei, and brainstem neuronal populations known to be affected in SCA types 1, 2, and 3. Direct parenchymal injections of our AAV2 variant to the DCN of adult mice revealed nearly complete transduction of Purkinje cells in rostral lobules of the cerebellum with positive transduction of multiple brainstem nuclei. Further testing to characterize the biodistribution profiles following different routes of administration in adult mice and nonhuman primates is ongoing. **Conclusion**: These newly identified variants expand the utilities of AAVs to treat cell types refractory to standard AAV serotypes, and improve targeted delivery following directed or broad administration to treat multiple forms of the spinocerebellar ataxias.

55. Capsid Display of Cell-Penetrating Peptides Yields AAVs with Enhanced Brain Penetration in Both Rodents and Primates

Yizheng Yao[1], Jun Wang[1,2], Yi Liu[1], Yuan Qu[1], Kaikai Wang[1], Yang Zhang[1], Yuxin Chang[1], Zhi Yang[1], Jie Wang[1], Choi-Fong Cho[1], Fengfeng Bei[1]

[1]Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, MA,[2]Renmin Hospital of Wuhan University, Wuhan, China

Adeno-associated viruses (AAVs) have emerged as promising vectors for gene therapy in the central nervous system (CNS). Engineering of AAV capsid has met with varying degrees of success in generating neurotropic AAV variants, as studies of several AAVs with strong CNS tropism in small model animals fail to translate to large animals such as non-human primates (NHPs). Here, we applied a rational design approach of engineering CNS-penetrating AAVs by displaying cell-penetrating peptides (CPPs) on the AAV capsid. Individual CPPs were inserted into the capsid of AAV9, and the resulting variants were screened for brain transduction after intravenous administration. Peptide sequence optimization by multiple rounds of iteration was performed for leading CPP candidates. Such design approach yielded two variants, namely AAV.CPP.16 and AAV.CPP.21, with 6-249 folds of higher efficiency than AAV9 in brain transduction across four mouse strains. Importantly, the advantage of AAV.CPP.16 over AAV9 for CNS gene delivery translates from mice to NHPs, as AAV.CPP.16 is approximately 5-fold more efficient in systemically transducing the brain in NHPs. In the meanwhile, only a modest increase of efficiency over AAV9 was observed for AAV.CPP.21 in NHPs, in contrast to its more notable brain penetration in mice. Further characterization of AAV.CPP.16 revealed the new variant transduces both neuron and astrocytes in the CNS with notable tropism to the motor neurons in particular. No capsid-associated immuno-toxicity was detected in NPHs based on behavioral monitoring and blood assays, and no cellular abnormality was observed in the dorsal root ganglion cells, which could be associated with potential AAV toxicity. Furthermore, no evidence of break-down of the blood-brain barrier (BBB) by AAV.CPP.16 was observed, and neither AAV.CPP.16 or AAV.CPP.21 binds to Ly6a, a previously identified receptor that is capable of mediating BBB crossing for certain AAV variants. Ongoing studies are further looking into the mechanisms of action on AAV.CPP.16's tropism to CNS tissues. Together, our study reports a novel CNS-tropic AAV vector AAV.CPP.16 with potential applications for gene therapy in both rodents and primates.

56. Engineering AAV6-Based Vectors for Improved Ocular Transduction Following Intravitreal and Intracameral Injection

Sean M. Crosson[1], Shreyasi Choudhury[2], James Peterson[1], Antonette Bennett[3], Diego Fajardo[1], Andras Komaromy[4], Mavis Agbandje-McKenna[3], Sanford L. Boye[5], Shannon E. Boye[1]

[1]Pediatrics, Cell and Molecular Therapy, University of Florida, Gainesville, FL,[2]Ophthalmology, University of Florida, Gainesville, FL,[3]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL,[4]Small Animal Clinical Sciences, University of Michigan State, East Lansing, MI,[5]Pediatrics, Powel Gene Therapy Center, University of Florida, Gainesville, FL

Purpose: The transduction and tropism of AAV in the eye is dependent on vector administration route. Intravitreal injection (IVI) is less surgically complex than a subretinal injection but requires AAVs that traverse the inner limiting membrane, a behavior that relies on the capsid's ability to bind heparan sulfate proteoglycan (HSPG). Transduction of trabecular meshwork (TM) in the anterior segment similarly depends on HSPG binding. The purpose of our study was to rationally engineer AAV6-based vectors for improved transduction of retina and TM following intravitreal or intracameral (ICI) injection, respectively. **Methods:** We evaluated transduction of HEK293, 661W, and rMC-1 cells by AAV1 and AAV6 using flow cytometry. We generated a series of AAV6-based mutants containing previously described proteasomal avoidance (PA) mutations to surface-exposed S, T, and/or Y residues [AAV6(S551V+S663V), AAV6(T492V+S663V), and AAV6(T492V+Y705F+Y731F)] and evaluated their performance in rMC-1 cells. WT mice were IVI with 1E9 vg of AAV6(T492V+Y705F+Y731F), 'aka' AAV6(3pMut), AAV6, or benchmark shH10(Y445F), and evaluated at 6 weeks post-injection (p.i.) by fundoscopy and immunohistochemistry (IHC). Additional variants with increased HSPG affinity or PA, AAV6(3pMut)D532N, AAV6(3pMut)D532R, and AAV6(4pMut) were IVI in mice and evaluated as above. Heparin binding assays were performed using heparin-conjugated agarose beads and gravity flow columns. WT rats received ICI injections of AAV6, AAV6(3pMut), or AAV6(3pMut) D532N (2E9 vg) and the TM, lens, and cornea were imaged by IHC 4 weeks p.i. **Results:** AAV6 variants harboring PA mutations transduced rMC-1 cells with significantly higher efficiency than unmodified AAV6, with AAV6(3pMut) having the highest transduction. In IVI mice, AAV6(3pMut) outperformed both AAV6 and shH10(Y445F) while all had similar tropism for Müller glia (MG) and retinal ganglion cells (RGCs). Addition of D532N and D532R mutations to AAV6(3pMut) increased heparin affinity, more so for AAV6(3pMut) D532R; while the addition of a 4th PA mutation [AAV6(4pMut)] slightly decreased heparin affinity. In IVI mice, AAV6(3pMut)D532N transduction was comparable to AAV6, while AAV6(4pMut) had reduced transduction and AAV6(3pMut)D532R did not transduce. All infectious variants had MG and RGC tropism. AAV6(3pMut) D532N transduced TM most efficiently, followed by AAV6, then AAV6(3pMut). **Conclusions:** Incorporation of PA mutations onto AAV6 enhances retina transduction via IVI. However, addition of a 4th PA mutation [AAV6(4pMut)] diminished retinal transduction, indicating PA alone is not the sole determinant of transduction via IVI. This reduction is likely attributed to this capsid's decreased HSPG affinity, which may be influenced by the hydrophobic nature of PA mutations. Conversely, retinal transduction by AAV6(3pMut) D532N was comparable to AAV6 but worse than AAV6(3pMut) via IVI, suggesting that increased HSPG affinity counteracts the effects of PA mutations. In TM, AAV6(3pMut)D532N transduction was higher than AAV6 and AAV6(3pMut) suggesting that increased HSPG affinity is a main a driver of TM transduction, rather than PA. Overall, these results suggest that transduction of ocular tissues by AAV requires a balance between the capsid's HSPG affinity and PA.

Preclinical Gene Therapy for Neurologic Diseases I

57. *ST3GAL5* Gene Replacement in CNS Restores Gangliosides Production and Improves Survival in a Mouse Model of GM3 Synthase Deficiency

Huiya Yang[1], Karlla Brigatt[2], Jia Li[1], Kazuhiro Aoki[3], Michael Tiemeyer[3], Robert H. Brown[4], Dan Wang[a5], Kevin A. Strauss[a2], Guangping Gao[a1]

[1]Horae Gene Therapy Center and Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[2]Clinic for Special Children, Strasburg, PA,[3]Complex Carbohydrate Research Center and The Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA,[4]Department of Neurology, University of Massachusetts Medical School, Worcester, MA,[5]Horae Gene Therapy Center and RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

GM3 synthase (ST3GAL5) deficiency is caused by biallelic mutations of the *ST3GAL5* gene, and results in complete systemic deficiency of GM3 and its downstream a- and b-series cerebral gangliosides (GM1, GD1a, GD1b, and GT1b). The clinical phenotype is characterized by hearing and visual impairment, intractable epileptic encephalopathy, irritability, failure to thrive, progressive microcephaly, developmental stagnation, and reduced survival. GM3 synthase deficiency follows an autosomal recessive pattern with an estimated incidence of approximately 1 per 1,200 births in Old Order Amish communities of North America. No disease-modifying treatment is currently available. Here, we hypothesize that *ST3GAL5* replacement therapy targeted to the central nervous system (CNS) could drive ST3GAL5 expression in neurons and oligodendrocytes, restore the endogenous production and trafficking of cerebral gangliosides, and thereby rescue the severe neurodevelopmental phenotype. We first used lentivirus-mediated gene replacement to transfer human h*ST3GAL5* cDNA *in vitro*, which restored GM3 production in patient-derived fibroblasts. More importantly, h*ST3GAL5* replacement in patient iPSC-derived cortical neurons reconstituted GM3 as well as its major downstream a- and b-series brain gangliosides. These *in vitro* data indicate the strong therapeutic potential of human *ST3GAL5* cDNA gene replacement. Next, to prove our hypothesis *in vivo*, we packaged h*ST3GAL5* constructs into AAV9 capsids. To examine the safety and efficacy of transgene expression, we first delivered AAV vectors via intracerebroventricular (ICV) administration to wild type (WT) neonatal C57BL/6 mice. We observed robust transgene expression in CNS without short-term vector-associated toxicity. Furthermore,

to assess the therapeutic efficacy of *ST3GAL5* gene replacement, we delivered AAV9-h*ST3GAL5* vectors in *St3gal5/B4galnt1* double knock-out (DKO) mice, which are unable to synthesize any cerebral gangliosides and mirror the phenotype of human GM3 synthase deficiency. Direct ICV treatment of neonatal DKO mice restored the production of GD1a, GD1b, and GT1b, improved physical growth, and extended median survival from 20 days to more than 5 months. To our surprise, administration of AAV9-h*ST3GAL5* via systemic injection triggered acute liver damage 2-days post injection that culminated in animal death. At the mRNA level, h*ST3GAL5* expression was 30-fold higher than normal in liver, which may be the cause of the liver damage. We therefore modified our vector design from a ubiquitous to neuron-specific promoter, which alleviated acute liver toxicity. In ongoing studies, we are further improving rAAV-based h*ST3GAL5* replacement vectors and testing their safety and efficacy in *St3gal5-/-* and *St3gal5-B4galnt1* double knockout mice. [a]Co-corresponding authors

58. CRISPR/Cas9 Strategies to Treat Spinocerebellar Ataxia Type 1

Kelly Fagan[1], Megan Keiser[2], Beverly Davidson[2]

[1]University of Pennsylvania, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

Spinocerebellar ataxia type 1 (SCA1) is an autosomal dominant neurodegenerative disease that causes progressive loss of motor coordination, respiratory issues and eventual death. SCA1 is caused by expansion of the polyglutamine repeat region in the *ATXN1* gene. Normal *ATXN1* alleles contain 6-42 CAG trinucleotide repeats with interspersed CAT nucleotides, while disease alleles have an uninterrupted CAG region with 39-100+ repeats. The mechanism of SCA1 pathogenesis is unknown; however, some features of the disease include neuronal degeneration and formation of toxic mutant ATXN1 (mATXN1) nuclear inclusions. Although mATXN1 is expressed ubiquitously, it affects primarily Purkinje cells (PCs). There are currently no treatment options for SCA1. We hypothesize that CRISPR-Cas9 editing of *ATXN1* will reduce mutant ATXN1 and be therapeutically beneficial. We designed two different strategies to reduce ATXN1; the first uses a single guide RNA (gRNA) to target near the exon-exon junction to induce nonsense mediated decay, while the second approach employs a dual guide system to delete the CAG repeat region. gRNAs were optimized in vitro, with each approach significantly reducing *ATXN1* expression. The single guide approach reduced *ATXN1* mRNA levels by 40-45% ($p \leq 0.02$) and protein by approximately 20% ($p \leq 0.01$) and the dual guide approach reduced levels of mRNA and protein levels by 70-75% ($p<0.001$) and 45-65% ($p \leq 0.03$), respectively. For testing in vivo, SCA1 mice were crossed to *sp*Cas9 transgenic mice; SCA1 mice express human *ATXN1* with an expanded 82 CAG repeats in Purkinje cells, and show progressive motor deficits and neuropathology. Recombinant AAVs (rAAVs) expressing the optimized gRNAs (EE2 and EE4) from the exon-exon strategy were delivered directly to the deep cerebellar nuclei of 5-week-old SCA1/*sp*Cas9 mice for transduction of Purkinje cells. The EE2 gRNA reduced protein and mRNA levels by 55% ($p=0.05$) and 50% ($p=0.02$), respectively and EE4 decreased ATNX1 protein and mRNA levels by 10% ($p=0.9$) and 20% ($p=0.07$) compared to saline injected controls. The EE2 gRNA more effectively reduced ATXN1 levels, and studies are in progress to assess its impact on SCA1 mice phenotypes.

59. Rescue of Molecular and Motor Phenotypes in CGG Knock-In Mice with CRISPR Mediated Deletion of the Trinucleotide Repeat

Carolyn M. Yrigollen[1], Laura Ohl[1], Euyn Lim[1], ShuJuan Zheng[2], Kasey Brida[3], Yong Hong Chen[1], Alejandro Mas Monteys[1], Beverly L. Davidson[1]

[1]CCMT, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Temple University, Philadelphia, PA,[3]University of Alabama at Birmingham, Birmingham, AL

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a neurodegenerative disorder that is caused by a premutation allele (55-200 CGG repeats) in the 5' untranslated region of the *FMR1* gene. Gene-editing using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) has previously been shown to delete the CGG repeats of *FMR1 in vitro*, but this has not yet been reported *in vivo,* nor the molecular and phenotypic effects of such a treatment strategy. We evaluated a Cas9 based therapy for its ability to correct *FMR1* trinucleotide repeats in the *Fmr1* CGG knock-in (KI) mouse with approximately 130 CGG repeats. These mice exhibit motor and memory impairments and are used to model FXTAS. Two guideRNAs (gRNAs) were used to target the CGG repeats for deletion upon Cas9 mediated cleavage. Dual AAV vectors containing Cas9 and the gRNAs were injected into mouse striatum and tissues harvested three weeks post injection. Isolated DNA showed complete or partial deletion of the CGG repeats along with deletion of adjacent nucleotides upstream and downstream of the target site. The transcriptional start site and the start codon of *Fmr1*, elements required for functional gene expression, remained intact. Striatal expression of *Fmr1* in untreated KI mice had a 3-fold upregulation compared to WT littermates, consistent with the known upregulation of this transcript with CGG repeat expansion. However, KI mice treated with targeting gRNAs/Cas9 had significantly lower *Fmr1* expression than untreated mice ($p < 0.0001$), with levels similar to WT. Treated and untreated mice from both genotypic groups had Fmrp levels that did not differ significantly from each other, suggesting the gene editing that corrected the regulation of *Fmr1* mRNA levels did not hinder synthesis of the encoded protein in *Fmr1* CGG knock-in mice. To investigate whether rescue of *Fmr1* transcriptional regulation would result in phenotypic rescue, neonatal mouse pups were injected at P0 to P1 bilaterally into the cerebral ventricles with the dual AAV vectors. Motor performance was evaluated at 12-15 weeks, 28-30 weeks and 52-54 weeks of age by accelerating rotarod. Motor impairment occurs in KI mice by 28 weeks, and results in quicker falls from the apparatus than is observed in WT littermates. Mice that were treated with the CRISPR constructs performed better than untreated knock-in mice, $p = 0.0041$ and were not statistically different their WT littermates at 28-30 weeks of age. At the late stage rotarod the *Fmr1* treated mice continued to perform similarly to the WT animals, but statistical differences were no longer observed between treatment groups. Additional analysis of behavioral and molecular data is ongoing.Our study is the first to demonstrate *in vivo* editing of expanded CGG repeats in *Fmr1* using CRISPR. Here we have shown that our guides enable safe excision, removing the expanded repeat, rescuing expression of *Fmr1* mRNA, and maintaining Fmrp synthesis. Importantly, we show *in vivo* CRISPR mediated editing of the *Fmr1* trinucleotide repeat has a therapeutic benefit, rescuing motor deficits present in aged KI mice. These results indicate that

CRISPR-mediated gene editing has the potential to ameliorate the degenerative pathology present in FXTAS and further development of this strategy for treatment of FXTAS and other Fragile X-associated disorders is necessary.

60. Transthyretin Gene Therapy as a Modulator of Alzheimer's Disease Progression

Ana Rita Batista[1], Robert M. King[2], Thais Hernandez[1], Monique Otero[1], Paola Rodriguez[1], Matthew J. Gounis[2], Miguel Sena-Esteves[1]

[1]Dep of Neurology and Horae Gene Therapy Center, University of Massachussets Medical School, Worcester, MA,[2]NECStR, University of Massachussets Medical School, Worcester, MA

Epidemiological studies and work in transgenic animals suggest that transthyretin (TTR) levels in plasma and/or CSF may modulate Alzheimer's disease (AD) presentation and/or progression. AD patients exhibit lower levels of TTR in CSF compared to age-matched healthy individuals. Similarly, genetic reduction of TTR in mouse models of AD is associated with earlier onset and more severe phenotypes. The current study was designed to determine the potential of somatic AAV mediated TTR expression to modulate disease presentation in AD.We designed an AAV9 vector to express TTR specifically in liver and choroid plexus using a TTR promoter to drive transgene expression. Two-month-old male and female 5XFAD mice were injected systemically with 2E12 gc AAV9-TTRp-TTR^{T119M} (n=12M+12F), or PBS as controls (n=12M+12F). At nine months of age animals underwent neurobehavioral tests to assess cognitive function, and 3D isotropic T2-weighted MRI to evaluate differences in brain volume. Post-mortem assessments include histological evaluation of plaque burden, quantification of Abeta levels in different brain structures, quantification of neurofilament light-chain (NfL) in serum, among others still on-going.Cognitive function was assessed through measurement of working and spatial memory using forced and spontaneous alternation tasks in the Y-maze, and novel object recognition tests. We observed behavioral improvements in the females only, namely regarding in the percent time interacting with novel object. General locomotion and anxiety were evaluated using the open field test, where AAV treated females presented higher locomotor activity and increased exploratory behavior. Interestingly, no differences were observed in males. Surprisingly, MRI analysis revealed increased CSF volume in all treated animals compared to controls, with females showing larger differences.Histological analysis of plaque burden using 6E10 antibody staining showed a remarkable difference in the treated females in comparison to controls. Analysis of the males is still ongoing.Our initial results show that systemic delivery of an AAV9 vector that expresses TTR in appropriate tissues improves cognitive function, locomotor activity and anxiety symptoms in 5XFAD mice, but this benefit is more noticeable in females. The unexpected increase in CSF volume may be related to increased TTR production by the choroid plexus of AAV treated animals, and additional experiments are ongoing to determine the cause of this outcome.Overall, this new AAV vector represents a therapeutic platform to study biologically relevant questions about the role of TTR in AD.

61. CRISPR/Cas9-Mediated Excision of ALS/FTD-Causing Hexanucleotide Repeat Expansion in C9ORF72 Rescues Major Disease Mechanisms *In Vivo* and *In Vitro*

Katharina E. Meijboom[1], Abbas Abdallah[1], Nicholas Fordham[1], Hiroko Nagase[1], Tania F. Gendron[2], Gopinath Krishnan[3], Tomás Rodriguez[3], Alireza Edraki[3], Meghan Blackwood[1], Erik Sontheimer[3], Fen-Biao Gao[3], Robert H. Brown[3], Zane Zeier[4], Christian Mueller[1]

[1]Gene Therapy Center, UMASS Medical School, Worcester, MA,[2]Mayo Clinic, Jacksonville, FL,[3]UMASS Medical School, Worcester, MA,[4]University of Miami, Miami, FL

A hexanucleotide repeat expansion (HRE) consisting of $GGGGCC_{24+}$ in the *C9ORF72* gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Both are fatal neurodegenerative diseases with no current approved treatments that significantly slow disease progression or extend life expectancy. Several hypotheses have emerged to explain how this HRE causes neuronal death, including haploinsufficiency, sequestration of RNA-binding proteins in the nucleus, and toxic repeat-associated non-ATG (RAN) dipeptide production. In the present study we used a CRISPR/Cas9 gene-editing approach to remove the HRE from the C9ORF72 genomic locus, designing guide RNAs (gRNAs) flanking the HRE, and delivered Cas9 and gRNAs via adeno-associated virus serotype 9 (AAV9) vectors. Here, we demonstrate successful excision of the HRE in C9ORF72 in primary cortical neurons and in the brains of three mouse models containing the C9ORF72 expanded HRE (ranging from 500-600 repeats) as well as in iPSC motor neurons and brain organoids (450 repeats). This resulted in a reduction of RNA foci and poly-dipeptides and a rescue of haploinsufficiency, the major hallmarks of C9-ALS/FTD. This work is, to our knowledge, the first to demonstrate an *in vivo* therapy that addresses both toxic gains-of-function conferred by to mutant RNAs and polydipeptides, but also haploinsufficiency, making this an extremely attractive therapeutic approach to these diseases.

62. C9ORF72 Variant-Specific RNA Interference Rescues C9-ALS/FTD Molecular Hallmarks *In Vivo* and *In Vitro*

Katharina E. Meijboom, Nicholas Fordham, Meghan Blackwood, Gabriella T. Cabrera, Robert H. Brown, Christian Mueller

UMASS Medical School, Worcester, MA

Amyotrophic lateral sclerosis (ALS) is a terminal neurodegenerative disease that affects upper and lower motor neurons, causing progressive muscle weakening and respiratory failure. A hexanucleotide repeat expansion (HRE) consisting of $GGGGCC_{23+}$ in the intronic region of C9ORF72, contributes to 40% of familial and 10% of total ALS cases. Up to 50% of patients with this expansion also develop frontotemporal dementia (FTD). The major proposed disease mechanisms behind this HRE include haploinsufficiency, RNA binding proteins sequestration in the nucleus, and toxic repeat-associated non-ATG (RAN) dipeptide

production. Both C9ORF72 ALS and FTD are aggressive diseases with no treatments to significantly slow disease progression or extend life expectancy. We are developing two AAV-RNAi gene therapy approaches using artificial microRNAs packaged in AAVrh10 to: (1) specifically target C9ORF72 mRNA variants that contain the HRE (variant (V) 1 and V3) and preserve the most abundant variant, V2, which does not contain the HRE, to avoid haploinsufficiency; (2) specifically target the intron containing the HRE, thereby only targeting incorrectly spliced transcripts. Using approach (1) we significantly lowered levels of V1 in treated primary neurons of C9ORF72 BAC-transgenic mice expressing an expanded repeat (500) compared to untreated groups, while overall C9ORF72 levels remained unaffected. We next treated adult C9ORF72 BAC-transgenic mice through striatal injections and similarly reduced levels of V1 mRNA containing the HRE, but not in V2 in the striatum. This reduction of HRE-containing transcripts by artificial miRNAs resulted in a decrease of GP poly dipeptides and RNA foci. Our preliminary studies using approach (2) have demonstrated a large, significant decrease of intron-containing transcripts. Future experiments will test the efficacy of both therapeutic approaches *in vivo* and in patient-derived motor neurons to eliminate existing - and prevent the formation of - toxic dipeptides and RNA foci formation, to ultimately rescue motor neuron pathology in ALS and FTD.

63. Restoration of *Scn1a* Expression after Symptom Onset in a Novel Model of Dravet Syndrome Rescues Seizures and Behavioral Alterations

Gaia Colasante[1], Nicholas Valassina[1], Simone Brusco[1], Linda Serra[1], Alessia Salamone[1], Gabriele Lignani[2], Vania Broccoli[1]

[1]Division of Neuroscience, Ospedale San Raffaele, Milan, Italy,[2]Division of Neuroscience, UCL, London, United Kingdom

Dravet syndrome is a severe epileptic encephalopathy that begins during the first year of life and leads to severe cognitive and social interaction deficits. It is mostly caused by heterozygous loss-of-function mutations in the SCN1A gene, which encodes for the alpha-subunit of the voltage-gated sodium channel (Nav1.1) and is responsible mainly of GABAergic interneuron excitability. While different therapies based on the upregulation of the healthy allele of the gene are being developed, the dynamics of reversibility of the pathology are still unclear. In fact, whether and to which extent the pathology is reversible after symptom onset and if it is sufficient to ensure physiological levels of Scn1a during a specific critical period of time are open questions in the field and their answers are required for proper development of effective therapies.We generated a novel *Scn1a* conditional knock-in mouse model (Scn1a floxSTOP) in which the endogenous Scn1a gene is silenced by the insertion of a floxed STOP cassette in an intron of Scn1a gene; upon Cre recombinase expression, the STOP cassette is removed, and the mutant allele can be reconstituted as a functional Scn1a allele. In this model we can reactivate the expression of Scn1a exactly in the neuronal subtypes in which it is expressed and at its physiological level. Those aspects are crucial to obtain a final answer on the reversibility of DS after symptom onset. In fact, for all the gene therapy approaches that are being explored in different laboratories, several factors converge on the final therapeutic efficacy: the real ability of the strategy in boosting Scn1a gene expression at single cell level, the targeting of the correct neuronal subtype and the total number of cells reached by the treatment.We exploited this model to demonstrate that global brain re-expression of the Scn1a gene when symptoms are already developed (P30) led to a complete rescue of both spontaneous and thermic inducible seizures and amelioration of behavioral abnormalities characteristic of this model. We also highlighted dramatic gene expression alterations associated with astrogliosis and inflammation that, accordingly, were rescued by Scn1a gene expression normalization at P30. Moreover, employing a conditional knock-out mouse model of DS we reported that ensuring physiological levels of Scn1a during the critical period of symptom appearance (until P30) is not sufficient to prevent the DS, conversely, mice start to die of SUDEP and develop spontaneous seizures. These results offer promising insights in the reversibility of DS and can help to accelerate therapeutic translation, providing important information on the timing for gene therapy delivery to Dravet patients.

AAV Biology, Engineering, Immunology and Animal Modeling

64. A Multi-Mechanistic Anti-Angiogenic AAV Gene Therapy Product Candidate, 4D-150, for the Treatment of Wet Age-Related Macular Degeneration (wAMD) and Diabetic Macular Edema (DME): Intravitreal Biodistribution, Transgene Expression, Safety and Efficacy in Non-Human Primates

Peter J. Francis[1], Peter J. Francis[1], Christian Burns[2], Paul Szymanski[2], Meredith Leong[3], Nima Mohaghegh[4], Christopher Schmitt[5], Austin Klein[5], Roxanne H. Croze[5], Melissa A. Calton[3], Devi Khoday[6], Melissa Kotterman[2], Katherine Barglow[7], David Schaffer[1], David Kirn[1]

[1]4D Molecular Therapeutics, Emeryville, CA,[2]Discovery & Engineering, 4D Molecular Therapeutics, Emeryville, CA,[3]Translational Medicine, 4D Molecular Therapeutics, Emeryville, CA,[4]Bioinformatics, 4D Molecular Therapeutics, Emeryville, CA,[5]HCDM, 4D Molecular Therapeutics, Emeryville, CA,[6]Upstream Process Development, 4D Molecular Therapeutics, Emeryville, CA,[7]Process and Analytical Development, Characterization, 4D Molecular Therapeutics, Emeryville, CA

Introduction Major blinding conditions such as wAMD and DME are characterized by abnormal retinal angiogenesis resulting in leakage, hemorrhage and scarring with consequent vision loss. We have designed an intravitreally-delivered multi-mechanistic anti-angiogenic gene therapy (4D-150) which could prove an attractive alternative to currently-approved biologics by eliminating the need for frequent injections combined with the potential for improved efficacy. 4D-150 is comprised of the retina targeted and evolved intravitreal vector R100 and is engineered to deliver the aflibercept protein sequence (anti-VEGF A&B, Placental growth factor, PlGF) plus a second

sequence encoding ddRNAi targeting the angiogenic factor VEGF C. In our preliminary *in vitro* experiments, we have shown dose-related expression in human iPSC-derived retinal cells and demonstrated functional activity. **Methods** We assessed the in vivo efficacy of R100. anti-VEGF gene therapy (a 4D-150 prototype) in the validated NHP laser-induced choroidal neovascularization (CNV) model of wAMD across a broad range of therapeutically relevant doses. NHP were dosed IVT with the 4D-150 prototype and six weeks post-administration, underwent retinal laser photocoagulation to induce CNV lesions. After two and four weeks, the numbers of clinically relevant grade IV CNV lesions were assessed by fluorescein angiography. Anti-VEGF protein was assessed by ELISA over 12 months by serial in vivo aqueous fluid samples. We subsequently performed a study in nonhuman primates (NHP) to assess the safety of 4D-150 and to measure expression of the aflibercept protein (retina and ocular fluids) and expression of VEGFC RNAi in the retina. **Results** In the NHP CNV model, the 4D-150 prototype resulted in complete suppression of grade IV CNV lesions (0/72) in treated eyes compared to vehicle (19/72) at doses as low as 1E11vg/eye ($p<0.0001$). Measurable and dose-dependent anti-VEGF protein was detected in aqueous fluid as early as 14 days post dosing and sustained through 12 months. There were no ocular or systemic toxicities at any dose tested. Specifically, there was no evidence of chronic intraocular inflammation (uveitis). Single IVT administration of 4D-150 resulted in high levels of ocular and retinal aflibercept protein together with highly robust VEGF C RNAi expression, without evidence of toxicity. **Conclusion** 4D-150 is a multi-mechanistic anti-angiogenic gene therapy that can be delivered by the simple and safe intravitreal route of administration. 4D-150 was designed with the goal of improved efficacy over single mechanism anti-angiogenic approaches by inhibiting multiple VEGF isoforms, as well as PlGF, within the retina. An intravitreal anti-VEGF prototype of 4D-150 resulted in efficacy and safety through 12 months in the NHP CNV model. Intravitreal 4D-150 resulted in sustained and high levels of functional aflibercept and anti-VEGF C RNAi. 4D-150 holds potential for the intravitreal treatment of wAMD and DME.

65. Evolving Synthetic AAV Variants for Genome Editing in Immune Cell Populations

Jonathan Ark[1], William Nyberg[2], Patrick Havlik[3], Angela To[2], Justin Eyquem[2], Aravind Asokan[3]

[1]Molecular Genetics and Microbiology, Duke University, Durham, NC,[2]Parker Institute for Cancer Immunotherapy, UCSF, San Francisco, CA,[3]Surgery, Duke University, Durham, NC

The immune system is an important target for genetic manipulation, with implications for cancer immunotherapy using T or NK cell populations, correction of monogenic blood disorders using hematopoietic stem cell populations or plasma cell-based therapies. A majority of approaches to achieve homology directed repair ex vivo are performed using AAV serotype 6 to deliver the single stranded DNA repair or donor template. However, significant room for improvement with regard to cellular targeting and efficiency of donor template or transgene delivery exists. To achieve such, we adopted our structure-driven evolution approach to engineer AAV capsid libraries that can transduce primary T-cells, B-cells, NK cells, monocytes/macrophages and hematopoietic stem cells of human and mouse origin. Upon cycling, newly evolved AAV variants were significantly enriched by > 1000 fold from the parental library. Highly conserved structural epitopes were mapped onto the capsid surface of lead capsid candidates, but amino acid residues were distinct for different cell lineages and with human vs murine origin as well. To assess the potential for improved transduction, we are evaluating lead AAV capsid variants in different human and mouse immune cell lineages. Notably, a human T cell evolved variant Ark312 and a mouse T cell variant Ark313 displayed 10-20 fold higher transduction efficiency in human T cells and in mouse T cells, 100-1000 fold improved transduction at low multiplicities of infection (MOI) showing a 100-1000 fold increased mean fluorescence index (MFI) with 40-fold increase in % transduced cells. Neither AAV variant infected immune cells from the other species, demonstrating host selectivity. When used to deliver an HDR/donor template, Ark312 significantly increased knock-in frequency compared to AAV6 across a range of incubated viral titers. Remarkably, Ark313 enabled highly efficient HDR in primary mouse T cells with a knockin frequency reaching <25% at an MOI of 3e3 and above 50% at 1e5, whilst wtAAV6 afforded less than 10% at the highest MOI. These synthetic AAV variants and others under evaluation have the potential to enable greater ex vivo knockin efficiencies in a wide spectrum of immune cell-based therapies and ultimately help pave the way for immunoengineering in vivo.

66. Real Time Blood Brain Barrier Disruption in a Multi-Species Model

AR Batista[1], RM King[2], WC Baker[1], MJ Gounis[2], H. Gray-Edwards[1], M. Sena-Esteves[1]

[1]UMASS, Worcester, MA,[2]NECStR, UMASS, Worcester, MA

Central nervous system (CNS) transduction by gene therapy is highly efficient upon local delivery. However, local CNS delivery is invasive and involve complex neurosurgical procedures that carry considerable risk for patients. Systemic delivery is the simplest route to achieve broad distribution of therapeutics in CNS, but they have to cross the blood brain barrier (BBB). Several approaches have been used to enhance the efficiency of AAV CNS gene transfer, including use of mannitol or focused ultrasound to temporarily disrupt the BBB, and the development of new AAV capsids. New AAV capsids developed by in vivo capsid evolution proved remarkably efficient for CNS gene delivery in mice, but the lack of superiority to AAV9 in non-human primates (NHP) raised questions about translatability and stopped further development as a new platform for human trials in neurological diseases. Methods that facilitate a transient opening of the BBB allowing for successful systemic delivery of AAV gene therapy, and the subsequent closing of the BBB, so that its function remains intact, are vital. Here we present the application of a BBB disruptive peptide, K16ApoE, that we show to achieve this transient BBB opening in multiple species.Initial studies were performed in young adult BALB/cJ mice by co-injecting in the tail vein 5E11 vg AAV vector encoding Firefly luciferase (FLuc) with peptide, or injection of K16ApoE alone followed by AAV 30 min later. At 3-weeks post-injection, FLuc activity was measured in CNS and liver. In a second phase, we evaluated the BBB-disrupting properties of K16ApoE in other species by MRI using dynamic gadolinium (Gd) enhancement. Three different species were used: mouse, sheep and NHP, in a paired fashion, where one animal in each pair received a co-injection of 0.1 mmol/Kg Gd and K16ApoE and the other Gd only. The MR sequence was the same for all animals:

baseline 3D T1W MPRAGE for structural imaging, dynamic 2D T1W imaging with a dynamic time of 20 sec for a total of 1 hour, and finally post injection repeat of the 3D T1W MPRAGE. Co-injection of AAV9-FLuc with K16ApoE enhanced FLuc activity in brain by ~100 fold compared to AAV9-FLuc alone, ~1,000 fold with AAVrh10 and ~10 fold with AAV2, a capsid that otherwise would not reach the brain when injected systemically. When we separated the administration of AAV9 and peptide, there were no differences in FLuc activity compared to control animals. For the second phase of the experiment, animals imaged during co-injection of Gd and K16ApoE showed higher levels of maximum signal enhancement within the brain compared to those that only received Gd (Figure 1). The contrast enhancement was observed at 2-3 min post injection and reached maximum after approximately 20 min, whereby the signal remained constant for the remainder of the imaging. In the animals that only received Gd, the signal was seen to increase immediately, consistent with contrast in the vascular system, and then return to baseline levels in less than 3 min. All animals recovered from anesthesia normally with no apparent side effects.K16ApoE peptide is able to transiently open the BBB and allows for transport of different AAV capsids as well as Gd over a span of less than 30 min, when compared to animals that did not receive peptide where there was little to no crossing of the BBB. The use of this peptide was well tolerated by all the species. The combination of systemic AAV gene therapy with a peptide that temporarily disrupts the BBB is a powerful approach to potentiate therapies for neurological diseases.

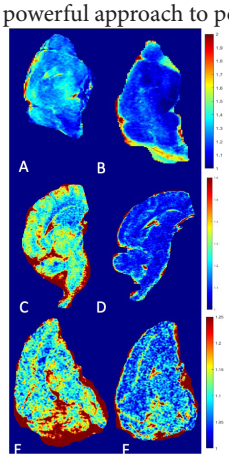


Figure 1:
Maximum enhancement maps for the dynamic T1W MRI. First column (A,C,E): Animals co-injected with gadolinium and K16ApoE. Second column (B,D,F): Animals injected with gadolinium alone. In the top row (A,B) shows mouse brains, the second row (C,D) shows sheep brains, and the final row (E,F) shows NHP brains. In all the species shown, the animals that received injection of gadolinium combined with peptide show greater levels of maximum enhancement over the brain parenchyma. In the animals that received gadolinium alone, only the choroid plexus (*) showed enhancement.

67. AAV2:2.retro-Mediated Delivery of Mutant Huntingtin throughout Cortico-Basal Ganglia Circuitry Leads to the Progressive Development of Motor and Cognitive Decline, along with Microstructural Changes in White and Gray Matter, in a Novel Rhesus Macaque Model of Huntington's Disease

Alison R. Weiss[1], William A. Liguore[1], Kristin Brandon[1], Xiaojie Wang[1,2], Zheng Liu[1,2], Jackie Domire[1], Dana Button[1], Christopher D. Kroenke[1,2,3], Jodi L. McBride[1,3,4]

[1]Division of Neuroscience, Oregon National Primate Research Center/ OHSU, Beaverton, OR,[2]Advanced Imaging Research Center, OHSU, Portland, OR,[3]Department of Behavioral Neuroscience, OHSU, Portland, OR,[4]Neurology, OHSU, Portland, OR

To create a nonhuman primate model of the neurodegenerative brain disorder, Huntington's disease (HD), we injected adult rhesus macaques into the caudate and putamen with a 1:1 mixture of AAV2 and AAV2.retro expressing a fragment of m*HTT* bearing 85 CAG repeats. Previous work by our lab shows that this injection strategy leads to the expression of mutant HTT protein, and the formation of hallmark HTT+ inclusions, throughout the striatum as well as dozens of cortical and subcortical brain structures due to the strong retrograde capability of AAV2.retro. Here, we queried the disruption of cortico-basal ganglia circuitry for 14-months post-delivery of this mHTT construct (HTT85Q, n=6), a control HTT construct bearing 10 CAG repeats (HTT10Q, n=6) or PBS buffer (n=5). We characterized the emergence of motor and cognitive phenotypes to link behavioral changes with disruptions in cortico-basal ganglia circuitry using multi-modal neuroimaging techniques including diffusion tensor imaging (DTI) and T1/T2-weighted magnetic resonance imaging (MRI). To achieve this, we evaluated animals using complex behavioral tasks to assess fine motor coordination (Lifesaver Retrieval Task), gross motor function (NHP-specific neurological rating scale), working memory (Spatial Delayed Response) and object recognition (Delayed Non-Match to Sample) to complement our neuroimaging battery. Compared to buffer and HTT10Q treated controls, animals treated with AAV2:2retro-HTT85Q showed a progressive development of mild orofacial dyskinesia, aberrant forelimb posture, forelimb chorea, incoordination, hindlimb slowness (bradykinesia) and/or tremor, which were exacerbated by the dopamine receptor agonist apomorphine. Compared to baseline measures, control animals also performed better on the Lifesaver Retrieval Task than HTT85Q animals. Moreover, compared to controls, HTT85Q animals exhibited impaired spatial working memory, but preserved object recognition. Voxel-based DTI analysis revealed many white and gray matter regions with alterations in fractional anisotropy in HTT85Q animals, suggesting that mHTT expression resulted in microstructural changes throughout the cortico-basal ganglia circuit. Similar voxel-based approaches are currently being applied to the T1w/T2w images using tensor-based morphometry to address whether HTT85Q leads to localized changes in brain volume. Additional efforts are also underway using positron emission tomography (PET) imaging to explore perturbations in the dopamine system (F18-Fallypride) as well as regional changes in glucose metabolism (F18-FDG). These data

demonstrate the feasibility of generating AAV-based models of HD in nonhuman primates that exhibit the hallmark motor and cognitive behavioral phenotypes, as well as neuropathological manifestations, of HD. Using a combination of AAV2 and AAV2.retro allowed for the expression of mHTT throughout the cortico-basal ganglia circuit, versus just the striatum, leading to the creation a NHP model of this disease that more closely depicts the neuropathology observed in human HD patients. Therefore, our studies also set the stage for developing novel biomarkers of disease manifestation, as well as using this gene delivery approach to test promising therapeutics in our model.

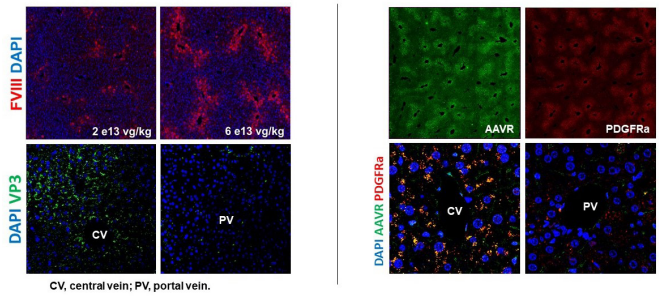
68. Investigating Mechanisms of Variability of AAV5-hFVIII-SQ Expression in Mice

Su Liu, Bridget Yates, Lisa Razon, Britta Handyside, Lening Zhang, Ryan Murphy, Jessica Felix, Catherine Vitelli, Weiming Zhang, Cheng Su, Sherry Bullens, Stuart Bunting, Sylvia Fong

BioMarin Pharmaceutical Inc., Novato, CA

Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) is an investigational gene therapy under development for the treatment of severe hemophilia A. Significant intersubject variability has been observed in AAV5-hFVIII-SQ gene expression outcomes across species and trials. We systematically investigated host factors in mice that may affect transgene expression at transduction, transcription, and protein translation/secretion. Male C57BL6 mice were administered a single vector dose ($1x10^{13}$ to $2x10^{14}$ vg/kg) intravenously. Levels of liver FVIII DNA, RNA, and protein; plasma FVIII protein; and various markers were analyzed. At doses of $1\text{-}3x10^{13}$ vg/kg (producing therapeutic levels), significant correlation was noted between levels of liver vector DNA and FVIII protein ($r = 0.7312$, $P < 0.0001$), suggesting that hepatocyte transduction (from vector uptake, trafficking, genome processing to retention) may be an important contributing factor. At higher doses of vector ($\geq 6x10^{13}$ vg/kg), a strong correlation between levels of liver Grp78, a chaperone protein responsible for folding and secreting proteins, and plasma FVIII protein levels ($r = 0.7613$; $P < 0.0001$) was observed, suggesting individuals who have a greater intrinsic ability to fold FVIII protein may secrete higher levels of mature protein into circulation. While neutralizing AAV5 antibodies can inhibit transduction, other non-antibody soluble factors may also impact transduction. For example, levels of liver vector DNA positively correlated with predosing serum cholesterol ($r = 0.491$; $P = 0.0279$) and progesterone levels ($r = 0.496$; $P = 0.0261$). Next, we determined if abundance and expression patterns of AAV receptor (AAVR) and platelet-derived growth factor receptors (PDGFRa and b), known coreceptors of AAV5, correlated with AAV5 vector transduction. AAVR and PDGFRa expression exhibit a preferential pericentral vein pattern, similar to hepatocellular AAV5 capsid distribution and FVIII expression (**Figure**). There was a significant correlation between FVIII and PDGFRa expression ($r = 0.9546$; $P = 0.0115$) but not PDGFRb ($r = 0.7636$; $P = 0.1330$) or AAVR ($r = 0.1796$; $P = 0.7726$). Levels of DNA repair enzymes that facilitate the transformation of single-stranded vector DNA to circular genomes may also influence AAV vector transduction. The expression of one such exonuclease, Artemis (*DCLER1C*), was significantly correlated with AAV5-FVIII vector DNA levels ($r = 0.6822$; $P = 0.0052$). Next, we identified factors that may contribute to AAV-FVIII transcriptional variability. There was a significant correlation between FVIII transcripts and mRNA levels of RNF121 ($r = 0.7188$; $P = 0.003$), Phf5A ($r = 0.5970$; $P = 0.0154$), and HNF1α (a transcription factor that binds to the promoter of AAV5-FVIII-SQ vector; $r = 0.7158$; $P = 0.0054$). Overall, we demonstrated that AAV5-FVIII-SQ intersubject variability may be driven by multiple contributing host-mediated mechanisms: transduction, transcription, and protein folding/secretion. Additional studies that further investigate the mechanistic drivers of AAV5 gene therapy variability are ongoing and may help identify predictive biomarkers of transgene expression and/or therapeutic approaches to decrease variability and optimize outcomes.

Figure. AAVR and PDGFRa expression in the liver of C57BL6 male mice shares a pericentral bias with FVIII transgene and vector capsid protein expression



CV, central vein; PV, portal vein.

69. Thermoresponsive Polymer-AAV Nanoparticle Vectors Improved Transgene Expression on Immunized Murine Model

Kai Wang[1], Min Zheng[1], Chengwen Li[2], Zongchao Han[1]

[1]Ophthalmology, The University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Gene Therapy Center, The University of North Carolina at Chapel Hill, Chapel Hill, NC

A great hurdle in adeno-associated virus (AAV) mediated gene therapy is pre-existing or re-administration-related humoral/cellular immune responses. Earlier, we developed an elastin-like polypeptide (ELP)-based AAV delivery system and evaluated the performance in vitro in cell cultures. Our results showed that the virions encapsulated in the ELP nanoparticles can be shielded from neutralizing antibodies against AAV capsids. In this study, we have generated more ELP-AAV formulations (ELP $(KV_2F)_{64}$-AAV, ELP V_{60}-AAV, and PNIPAM-AAV) and screened them by neutralizing antibody assay. We found that ELP $(KV_2F)_{64}$ is the most effective polymer to protect AAV virion, and therefore we have used this ELP-AAV vector for further studies. Mass spectroscopy analysis indicated that the polymer was bound to residual K507, R566, and K649 of AAV VP3, and the molecular weight of ELP bonded VP3 was increased by 200 kDa. In vivo studies showed that the ELP-AAV2/9 nanoparticle vectors effectively transduced targeted tissue without tropism change compared to free AAV through subretinal injection, intramuscular injection, and retro-orbital injections. Histological examination indicated that no toxicity was observed on the major organs (kidney, liver, spleen, heart, lung, and brain) of mice injected with ELP-AAV2/9. The ELP-AAV2/9 vectors were also studied on the mice that were immunized with IVIg or prior AAV intramuscular injection via retro-orbital injection 2 hours after IVIg infusion or 2 weeks after intramuscular injection, respectively. Our

results suggested that the ELP-AAV2/9 vectors significantly improved the reporter gene expression compared to free AAV and did not change the AAV tropism. Our ongoing studies are focusing on the delivery of using high-doses of ELP-AAV2/9 and alternative AAV serotypes. If successful, this method will create a novel strategy to potentially solve the immunogenic problem in AAV re-administration in clinic.

70. AAV Vector Dose Dependent Redundant and Non-Redundant Roles of TLR9 and IL1R Signaling in CD8+ T Cell Activation upon Muscle Gene Transfer

Ning Li, Roland Herzog

Department of Pediatrics, IU School of Medicine, Indianapolis, IN

Adeno-associated viral (AAV) vectors are evaluated in multiple clinical trials for the treatment of neuromuscular disorders. However, immune responses to the transgene product remains a concern. Viral vectors are initially sensed by the innate immune system, which shapes subsequent adaptive immune responses. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), while IL-1 receptors (IL-1Rs) intermediate downstream of pathways trigged by PAMPs or by tissue damage. They act as major sensors of pathogens for innate immune responses. Release of IL-1 cytokine serves as a damage signal to active IL-1R pathways upon infection and/or tissue injury. TLR9 is an endosomal DNA receptor that responds most potently to unmethylated CpG motifs as found in bacterial and viral DNA. Myd88 as a universal and essential adaptor is recruited when TLR9 and IL-1R signaling is activated. Our previous studies found that cross-priming of AAV capsid-specific $CD8^+$ T cells depended on TLR9-MyD88 pathway. Similarly, others documented TLR9-dependent $CD8^+$ T cell responses against non-secreted transgene products such as LacZ and hemagglutinin upon muscle-directed AAV gene transfer. In our previous studies, we found that $CD8^+$ T cell responses to a secreted ovalbumin (ova) transgene product were substantially reduced (although not entirely eliminated) upon muscle gene transfer in TLR9-deficient mice [J Innate Immun. 7:302-14]. Here, we performed intramuscular injections with 2 doses of single-stranded ssAAV1-CMV-ova vectors ($2X10^{10}$ and $2X10^{11}$ vg) in wild-type (WT) C57BL/6 and innate sensing knockout (TLR9-/-, MYD88-/- and IL-1R-/-) mice. Using MHC tetramer ($H2\text{-}K^b$ -SIINFEKL), ova-specific $CD8^+$ T cell frequencies were monitored in peripheral blood for up to 6 weeks. As expected, transgene product-specific $CD8^+$ T cell responses were much reduced in MyD88-/- mice, in which 0.2% and 1.7% $tetramer^+$ of CD8 frequencies were found at low and high doses, respectively. To our surprise, TLR9-/- and IL-1R-/- mice only showed a substantially reduced response (1.2% and 0.1% $tetramer^+$ of CD8) at the low dose when compared to WT animals (12% $tetramer^+$ of CD8, $p<0.0001$, n=5/group), whereas $CD8^+$ T cell responses were similar in TLR9-/- and WT mice (16% and 15% $tetramer^+$ of CD8) and only slightly decrease in IL-1R-/- mice (11% $tetramer^+$ of CD8) at the high dose (n=5/group). To further investigate these findings, we prevented activation of IL-1R by pre-treatment with a combination of IL-1α and IL-1β antibodies followed by IM injections of $2X10^{11}$ vg of ssAAV1-CMV-ova vector to TLR9-/- mice. As a result, substantially reduced $CD8^+$ T cell responses (4% $tetramer^+$ of CD8) were observed in these animals compared to TLR9-/- mice that received isotype antibodies (13% $tetramer^+$ of CD8, p=0.003, n=5/group). Our data reveal that sensing of the AAV genome by TLR9 and sensing of IL-1 release by IL-1R are both critical for the $CD8^+$ T cell response to the transgene product at lower vector doses. Thus, absence of one of the pathways dramatically impaired the response. However, TLR9 or IL-1R driven signaling pathways are sufficient to drive the response at higher vector doses, so that elimination of one of these now redundant pathways is not sufficient to blunt the response. Rather, both pathways need to be targeted at higher vector doses. We propose that use of CpG-depleted vectors (or TLR9 inhibitors) combined with IL-1R antagonist will be beneficial to prevent CD8+ T cell responses in AAV muscle gene transfer protocols that require high vector doses.

CAR Modified Cellular Therapies

71. Pre-Selected CAR $T_{N/SCM}$ Outperform CAR T_{BULK} in Driving Tumor Eradication in the Absence of Severe CRS and ICANS

Silvia Arcangeli[1], Claudia Mezzanotte[1], Camilla Bove[1], Barbara Camisa[1], Laura Falcone[1], Francesco Manfredi[2], Rossana Norata[3], Francesca Sanvito[4], Maurilio Ponzoni[4], Beatrice Greco[1], Fabio Ciceri[5], Chiara Bonini[2], Attilio Bondanza[1], Monica Casucci[1]

[1]Innovative Immunotherapies, San Raffaele Hospital, Milan, Italy,[2]Experimental Hematology Unit, San Raffaele Hospital, Milan, Italy,[3]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), San Raffaele Hospital, Milan, Italy,[4]Pathology Unit, San Raffaele Hospital, Milan, Italy,[5]Department of Hematology and Stem Cell Transplantation, San Raffaele Hospital, Milan, Italy

Capability of CAR T cells to expand and persist in patients emerged as a fundamental factor accounting for better outcome and durability of antitumor responses. These features inversely correlate with T-cell differentiation, suggesting that the enhanced T-cell fitness typical of early memory T cells may significantly improve CAR T cell therapeutic potential. Presently, however, whether pre-selecting specific memory T-cell subsets before manipulation would be really beneficial is still an open issue, especially as regard toxicity. Therefore, we deeply investigated the efficacy and safety profiles of CAR T cells generated from isolated naive/stem memory T cells ($T_{N/SCM}$), as compared to those derived from unselected T cells (T_{BULK}). As expected, CAR $T_{N/SCM}$ were less lytic than CAR T_{BULK} and produced lower amounts of pro-inflammatory cytokines when stimulated with CD19+ targets *in vitro*, even though displaying a similar proliferative capacity. When challenged against tumor cells in HSPC-humanized mice, limiting doses of CAR $T_{N/SCM}$ showed superior antitumor activity compared to CAR T_{BULK} and the unique ability to protect mice from leukemia re-challenge, together with higher *in vivo* expansion, persistence and a better CAR T cell fitness. Indeed, as evaluated by BH-SNE algorithm, after leukemia encounter CAR $T_{N/SCM}$ were characterized by prevalence of early memory T-cell subsets, together with the expression of multiple activation markers and a limited enrichment of inhibitory receptors, as opposed to the more exhausted and terminally differentiated phenotype typical of CAR T_{BULK}. Notably, at limiting doses and low tumor burdens no cases of severe Cytokine Release Syndrome (sCRS) were reported. Conversely, when infusing high doses of CAR T cells in mice with

high tumor burdens, sCRS and Immune effector-Cell Associated Neurotoxicity Syndrome (ICANS) were only elicited by CAR T_{BULK}, with more than 50% of mice experiencing a drastic weight loss and increased serum elevation levels of IL-6 and SAA, culminating in the death of the treated mice. Moreover, multifocal brain hemorrhages were only found in the CAR T_{BULK} treated cohort, in contrast to the group infused with CAR $T_{N/SCM}$, in which only one mouse presented with a small hemorrhagic focus. Interestingly, similar results were obtained with CAR $T_{N/SCM}$ harboring a CD28 rather than a 4-1BB co-stimulatory molecule, indicating that CAR $T_{N/SCM}$ are intrinsically less prone than CAR T_{BULK} to trigger detrimental infusional toxicities, independently of the CAR design strategy. In conclusion, $T_{N/SCM}$ pre-selection during CAR T cell manufacturing allows for deeper and more durable anti-tumor responses in the absence of sCRS and ICANS, significantly widening the therapeutic index of current CAR T cell approaches.

72. CD5 CAR T-Cells Avoid Self-Elimination by Continuously Degrading CD5 Protein

Royce Ma[1], Divya Popat[1], Alex Chaumette[1], Alexandre Carisey[2], Mary K. McKenna[1], Madhuwanti Srinivasan[1], Norihiro Watanabe[1], Malcolm K. Brenner[1], Maksim Mamonkin[1]

[1]Center for Cell & Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Pediatrics, Baylor College of Medicine, Houston, TX

Chimeric antigen receptor (CAR) T-cells for T-cell malignancies have been clinically effective. However, CARs specific to T-cell antigens may also result in extensive fratricide of CAR T-cells, precluding their expansion. This constraint does not apply equally to all the T cell antigens potentially targetable by CAR-Ts. Thus, we have shown that T-cells expressing CD5-specific CARs produce limited initial fratricide and then become resistant to self-targeting despite having high CD5 expression prior to CAR transduction. Such minimal fratricide coincides with rapid and complete loss of detectable CD5 expression on the cell surface without affecting CD5 gene transcription, suggesting post-translational downmodulation of CD5 protein. However, the exact mechanisms underpinning antigen removal and the resulting resistance to fratricide in CD5 CAR T-cells remain unclear; an improved understanding of these processes may guide future efforts to target additional T cell antigens that are currently excluded from consideration due to excessive fratricide. Ligation of CD5 with monoclonal antibodies induces its capping and internalization. Using time-lapse microscopy in T-cells freshly transduced with CD5 CAR, we observed rapid aggregation and internalization of surface CD5 from the cell membrane. Western Blot analyses revealed complete removal of the CD5 protein in CD5 CAR T-cells thus ruling out epitope masking or intracellular sequestration of the antigen. These results suggested CAR-mediated ligation of CD5 *in cis* triggers continuous internalization and degradation of CD5 protein in T-cells. Indeed, replacing endogenous CD5 protein with an engineered CD5 variant containing myc-/FLAG-tags on the N-/C-termini resulted in complete loss of both tags in T-cells co-expressing CD5 CAR but not control CD19 CAR indicating the entire CD5 molecule is removed. Neither CAR nor CD5 signaling was required for antigen downmodulation as removal of intracellular signaling portions of each respective molecule did not ablate CD5 downregulation. The process of CD5 removal can be initiated both *in cis* and *in trans*. CD5 CAR T-cells induced rapid *in trans* downmodulation of surface CD5 expression in both resting and activated primary T-cells upon short coculture with concurrent inter-cellular transfer of CD5 CAR molecules to target T-cells. Importantly, *in trans* removal of CD5 was observed in both normal and malignant T-cell lines suggesting this mechanism can limit availability of CD5 on target T-cells leading to resistance to cytotoxicity. Similarly to the *cis*-mechanism, complete removal of CD5 protein *in trans* was observed in target T-cells expressing dual-tagged [N-]myc/[C-]FLAG CD5 co-cultured with CD5 CAR Ts. However, in contrast to the *cis*-downmodulation, we detected robust release of soluble CD5 protein into culture supernatant during coculture of normal CD5+ T-cells with CD5 CAR T-cells, suggesting CD5 protein can also be shed or secreted from target cells upon contact with CD5 CAR T-cells. This study unravels a novel mechanism of fratricide evasion in T-cells expressing a T lineage antigen-specific CAR mediated by continuous removal of target antigen. Furthermore, rapid downmodulation of CD5 on normal and malignant T-cells may contribute to their resistance to CD5-directed cytolysis. These results are supported by clinical observations from an ongoing Phase I clinical study in which CD5 CAR T-cells could produce complete regression of recalcitrant T-cell tumors without fully ablating the endogenous T-cell compartment. Understanding the mechanisms of fratricide resistance can inform design of other T lineage-specific CARs and improve outcomes in patients with T-cell malignancies.

73. Abstract Withdrawn

74. Investigating the Therapeutic Efficacy of Disruption of Cell Intrinsic Checkpoint Regulator CTLA-4 in Chimeric Antigen Receptor T Cells

Sangya Agarwal[1,2], Angela Aznar[1,2], Tong Da[1,2], Weimin Kong[1,2,3], Mercy Gohil[1,2], Megan M. Davis[1,2], Joseph A. Fraietta[1,2,3], Gabriela Plesa[1,2], Regina M. Young[1,2], David L. Porter[4], Carl H. June[1,2]

[1]Center for Cellular Immunotherapies, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[2]Parker Institute of Cancer Immunotherapy, University of Pennsylvania, Philadelphia, PA,[3]Department of Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[4]Division of Hematology/Oncology, Department of Medicine and Abramson Cancer Center, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Cancer immunotherapy is a rapidly growing field that has led to multiple successful treatment strategies including monoclonal antibodies (MAb), inhibitory receptor (IR) blockade, and adoptive cell transfer (ACT). ACT modified to express chimeric antigen receptors (CARs) can redirect T cells to tumor-associated antigens and has shown impressive clinical efficacy in patients with refractory leukemia and lymphoma. 90% of pediatric patients with acute lymphoblastic leukemia (ALL) respond to CD19 CAR T cell therapy (CART19), whereas only 26-35% of patients with chronic lymphocytic leukemia (CLL) show complete responses (CR). It is unclear why responses are less frequent in CLL compared to ALL. The heavy pre-treatment

received by CLL patients likely contributes to lower response rates with T cell therapy, and disease progression is worsened due to profound T cell defects characterized by elevated expression of IRs such as PD-1 and CTLA-4. We hypothesized that disruption of CTLA4 would improve CAR T cell efficacy in CLL based on numerous observations. Fraietta et. al. assessed CLL patient apheresis samples and CART19 products to show that CR was associated with elevated levels of CD27+PD1-CD8+ T memory cells whereas the non-responders (NR) showed an exhausted phenotype with high levels of multiple IRs. We studied 14 patients with advanced, heavily pretreated CLL who received at least one dose of CART19. Patients with CRs exhibited high *in vivo* expansion and persistence of infused CAR T cells, as opposed to NR's. Importantly, at peak levels of *in vivo* CAR expansion, NR's had elevated levels of CTLA-4 expression which correlated with poor CLL patient responses to CART19 therapy. Additionally, multiple studies show CTLA4 is amongst the top 20 differentially upregulated genes in dysfunctional CD8+ T cells from various tumors such as hepatocellular carcinoma, melanoma, non-small cell lung cancer, and pancreatic adenocarcinoma. In summary, these data suggest that eliminating CTLA-4 mediated T cell inhibition can be clinically beneficial. We, therefore, performed disruption of CTLA4 using CRISPR technology in primary human T cells from healthy donors. Our data demonstrate that the knockout (KO) of CTLA-4 leads to maintenance of surface CAR expression and higher tumor clearance in a chronic re-stimulation model using CART19 cells against NALM6 tumor cells. In xenograft models of ALL, KO of CTLA-4 increases the anti-tumor efficacy of CART19 cells. We then performed CTLA4 disruption in CLL patient T cells who had not responded to CAR T cell therapy to determine whether dysfunctional CAR T products from CLL patients can be invigorated by CTLA-4 KO. In the chronic re-stimulation model of NALM6, CTLA-4 KO CAR T cells from CLL patients maintained CAR expression and lower tumor burden, relative to subject-matched unedited CAR T cells. Thus, disruption of CTLA4 in CD19 CAR T cells from NR CLL patient cells endows them with superior anti-tumor efficacy, suggesting that CTLA4 disrupted CAR T cell products may enhance the success rate of CAR T cell therapy for CLL patients. This technology can be feasibly expanded to other tumor indications and increase the overall efficacy of CAR T cells. These IND-enabling studies will support the translation of this therapy to the clinic.

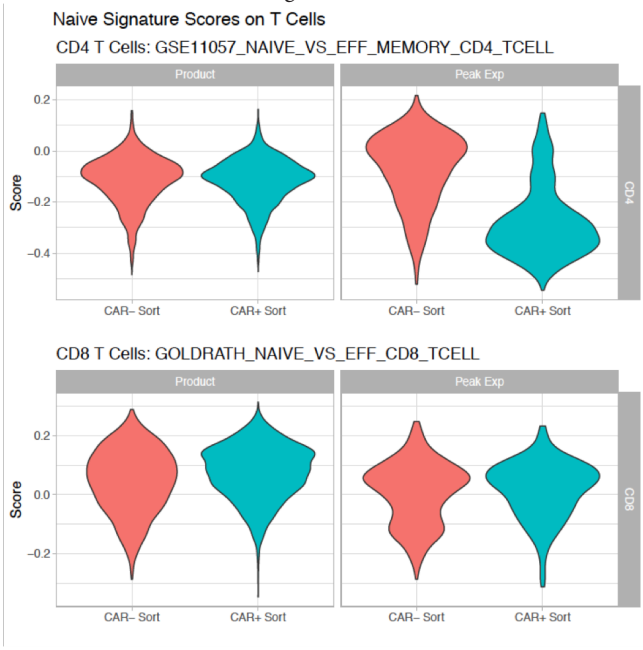
75. Non-Human Primate Derived CD20 CAR T Cells Elicit a Bystander Effect on CD8 but Not CD4 CAR- T Cells

Ulrike Gerdemann[1,2], James Kaminski[3,4], Ryan A. Fleming[3], Emily Ho[5], Victor Tkachev[3], Connor McGuckin[3], Fay Eng[5], Xianliagn Rui[6], Jennifer Lane[3], Michael C. Jensen[7], James Rottman[5], Alex K. Shalek[8], Leslie S. Kean[1,2]

[1]Pediatric Hematology/Oncology, Dana Farber Cancer Institute, Boston, MA,[2]Pediatric Hematoloty/Oncology, Boston Children's Hospital, Boston, MA,[3]Pediatric Hematology/Oncology, Boston Children's Hospital, Boston, MA,[4]Broad Institute of MIT and Harvard, Boston, MA,[5]BluebirdBio, Cambridge, MA,[6]Pediatric Hematology/Oncology, Boston Children' Hospital, Boston, MA,[7]Ben Towne Center for Childhood Cancer Research, Seattle Children's Research Institute, Seattle, WA,[8]Broad Institute of MIT and Harvard, Cambridge, MA

The recent clinical successes of CAR T cell (CAR T) therapy are reshaping the field of hematology-oncology. However, despite the significant response to CD19 CAR T therapy in B-ALL, 10-20% of patients fail to enter remission and, perhaps more importantly, 30-50% of patients who achieve remission relapse within a year. Given these results, it is critical to understand not only the drivers of CAR T effector functions but also the mechanisms by which one can induce a tumoricidal bystander effect in surrounding effector cells. Performing detailed analysis of CD19 CAR Ts in human subjects is challenging mostly due to the limited sample volume available. To address this shortcoming, we have now utilized a clinically relevant Non-Human Primate (NHP) CD20 CAR T model to identify mechanisms of the CAR T specific immune response. For this we adoptively transferred CD20 CAR Ts to lymphodepleted rhesus macaques. CAR T expansion resulted in B cell ablation, associated with clinical symptoms of cytokine release syndrome. CAR T persisted an average of 4 weeks, at which point loss of CAR T was followed by B cell recovery. Flow analysis of the CAR- T population showed only minimal activation status of CD4 CAR- Ts and expansion of CAR Ts was more prominent in CD8 T cells. To further reveal the bystander effect of CAR- Ts we performed single cell sequencing analysis of sorted CAR+ and CAR- Ts from the product and at time of maximum proliferation. As expected, CAR- and CAR+ Ts in the infusion product showed similar transcriptomic profiles, however, state of activation differed significantly between CD4 CAR- and CD4 CAR+ Ts at peak of expansion (Wilcoxon test, p-value <0.001). While after infusion CD4 CAR- Ts reverted to memory- like state, CD4 CAR+ Ts maintained an effector state. In contrast, CD8 CAR+ and CD8 CAR- Ts both displayed a very similar effector pattern in the product and at maximum expansion, suggesting, that CAR- CD8 Ts, in contrast to CAR- CD4 Ts, are subject to a significant bystander effect. This is also reflected in the analysis of differentially expressed genes: at time of peak expansion we detected > 200 differentially expressed genes in between CD4 CAR- and (n=832) CAR+ Ts (n=594). In contrast differential gene expression analysis of CD8 CAR+ (n=163) vs CAR- Ts (n=556) at peak expansion did not demonstrate significant differences. Ingenuity Pathway Analysis comparing differentially expressed genes in CD8 CAR+ and CAR- Ts in peak expansion vs infusion product suggests a role of mir155-

driven pathways in the CD8 CAR- T bystander effect.In summary these data show for the first time that a CAR induced bystander effect is predominantly elicited in CD8 CAR- Ts. Further dataset analyses as TCR based T cell tracking are underway to uncover additional mechanism of the immune regulation of CAR Ts in the NHP model.



The signature score for each cell was calculated using the software package VISION, which treats cell signatures from MSigDB as scores (Upregulated = +1,Downregulated = -1), and multiplies these scores by the normalized expression counts in each cell. Results are displayed as violin plots, separated by CAR+/CAR- status, and timepoint.

76. Enhanced Generation of T-Cell Derived Naïve Pluripotent Cells as a Renewable Cell Source for the Mass Manufacture of Off-the-Shelf CAR T Cell Therapies

Yi-Shin Lai, Hui-Ting Hsu, Mochtar Pribadi, Greg Bonello, Megan Robinson, Pei-Fang Tsai, Bi-Huei Yang, Gloria Hsia, Amanda Yzaguirre, Raedun Clarke, Tom Lee, Ramzey Abujarour, Bahram Valamehr

Fate Therapeutics, San Diego, CA

Induced pluripotent stem cells (iPSCs) are a promising renewable cell source for the mass manufacture of uniform chimeric antigen receptor (CAR) T cell products that can be banked and validated in advance to relieve major manufacturing cost and logistical obstacles. iPSCs can be generated from a variety of somatic cell types (commonly fibroblasts or CD34+ cells) but T cells are an ideal starting material for the derivation and engineering applications related to iPSC-derived T cell therapies. However, a major obstacle toward the use of T cells for the generation of iPSCs is that specialized cells such as T or B lymphocytes are not readily amenable to cellular reprogramming and often fail to dedifferentiate into bona fide pluripotent cells. To develop a robust non-integrating plasmid-based system for T cell reprograming, we applied both genomics and functional screening approaches to identify novel reprogramming factors that could markedly improve reprogramming of terminally differentiated somatic cells, including T cells. To enhance cellular reprogramming and induce naïve pluripotency, we used our previously described stage-specific media supplemented with small molecule drivers of naïve pluripotency and inhibitors of differentiation (MEKi, GSK3i, ROCKi and TGFbi). Using the new set of reprogramming factors that includes the master regulator of pluripotency gene *OCT4* and optimized medium formulations, iPSCs were generated in a highly efficient manner from T cells derived from multiple donors, with the fraction of TiPSCs reaching 7-15% of the population by day 18 post induction of cellular reprogramming and further increasing to 36-50% at day 26, enabling the ability for multiplexed engineering at the iPSC stage. To generate clonal TiPSC clones, we sorted single reprogrammed cells into 96-well plates and expanded multiple TiPSC clones for extensive characterization and selection. Quantitative PCR analysis revealed that all TiPSC clones were free of reprogramming plasmids and no longer had the potential to dedifferentiate. RNA sequencing data confirmed that the TiPSC clones were equivalent to control iPSC lines and pluripotency was further confirmed by the ability of the TiPSC clones to differentiate into representatives of the 3 germ layers in a trilineage differentiation assay. The generated feeder-free and single cell-derived TiPSCs showed enhanced expression of naïve pluripotency markers (e.g. KLF4, DMNT3L, PRDM14) and lower expression of primed pluripotency markers (e.g. THY1, OTX2, ZIC2). Importantly, TiPSC clones differentiated efficiently into homogenous T cell populations and maintained normal karyotype over extended culture duration. Next, we tested if the generated TiPSC clones maintained stable pluripotent profile and genomic integrity following stress-inducing manipulations including multiplexed engineering, subcloning and cryopreservation. Selected cryopreserved TiPSC clones were thawed and engineered by CRISPR-mediated insertion of CAR into the TCR alpha constant locus, re-cloned at the single cell level and re-banked. The engineered CAR+ TiPSC clones continued to maintain a homogenous pluripotent phenotype and normal karyotype, and differentiated into CAR T cells that exerted effective cytotoxicity against target cancer cells. Collectively, our data describe improvements in the generation of footprint-free and single cell-derived naive TiPSCs that are amenable for multiple rounds of multiplexed engineering, subcloning and cryopreservation and facilitate the replacement of donor-derived T cells as the preferred source for a more consistent, homogenous, cost effective and off-the-shelf CAR T cell product.

77. Precise Targeting of AML with First-in-Class OR / NOT Logic-Gated Gene Circuits in CAR-NK Cells

Brian S. Garrison[1], Han Deng[1], Gozde Yucel[1], Nicholas W. Frankel[1], Marcela Ayala Guzman[1], Russell Gordley[1], Michelle Hung[1], Derrick Lee[1], Marcus Gainer[1], Kathryn Loving[1], Jenny Chien[1], Tiffany Pan[1], Wesley Gorman[1], Travis Wood[1], Wilson Wong[2], Philip Lee[1], Tim Lu[1], Gary Lee[1]

[1]Senti Biosciences, South San Francisco, CA,[2]Department of Biomedical Engineering, Boston University, Boston, MA

Background: Given the poor prognosis and long-term survival of relapsed/refractory acute myeloid leukemia (AML) patients, more efficacious therapies are greatly needed. While chimeric antigen receptor (CAR) cell therapies have provided some extraordinary

clinical responses, developing effective CAR cell therapies for AML has been challenging due to: (a) the lack of a single target antigen robustly expressed across both AML leukemic stem cell (LSC) and immature leukemic blast cell subpopulations, and (b) the lack of truly AML-specific target antigens, since current targets are also expressed on healthy tissues and may result in off-tumor toxicity. Using logic-gated gene circuits, we are engineering CAR-NK cells to overcome these longstanding challenges. **Methods:** To maximize clearance of AML tumor cells and minimize toxicities, we used a proprietary bioinformatics pipeline to identify the optimal combinations of AML and healthy tissue target antigens to incorporate into OR and NOT logic-gated CAR gene circuits. These first-in-class CD33 OR FLT3 NOT Endomucin (EMCN) gene circuits enable allogeneic CAR-NK cells to target CD33 and/or FLT3 expressing AML tumor cells (LSCs and blasts) but not healthy FLT3+ hematopoietic stem cells (HSCs). **Results:** First, for the CD33 OR FLT3 activating CAR (aCAR) portion of the logic circuit, we demonstrated that engineered primary human NK cells expressing both CD33 and FLT3 aCARs exhibited up to 75% cytotoxicity and significant cytokine secretion (GrB, IFN-g, and TNF-a) against multiple leukemia cell lines in vitro, including MOLM13, THP1, and SEM. Importantly, these CD33 OR FLT3 CAR-NK cells also significantly reduced tumor burden and improved mouse survival within a MOLM13 xenograft leukemia model. Second, for the NOT gate portion of the logic circuit to protect FLT3+ healthy HSCs, we developed inhibitory CARs (iCARs) (NOT gates) that recognize the HSC-specific EMCN surface antigen, which is expressed on up to 70% of healthy HSCs but not AML cells. We demonstrated that FLT3 aCAR-NK cells engineered with an EMCN-specific iCAR protected over 50% of FLT3+ EMCN+ cells from FLT3 aCAR-mediated cytotoxicity. Next, to more closely replicate a clinical context, we mixed FLT3+ EMCN- (AML-like) and FLT3+ EMCN+ (HSC-like) target cells and demonstrated that FLT3 NOT EMCN CAR-NK cells exhibit preferential killing of FLT3+ EMCN- target cells, demonstrating that our NOT-logic gene circuit controls NK-mediated responses on a cell-by-cell basis. **Conclusion:** This work represents the first time NK cells have been engineered with OR and NOT logic-gated CAR gene circuits, wherein the OR gate provides increased AML LSC/blast tumor clearance (to prevent relapse), and the NOT gate protects healthy HSCs from off-tumor toxicity, which may preserve hematopoiesis and mitigate the need for bone marrow transplant. Beyond AML, logic-gated CAR-NK cell technology has applicability to other cancer-associated antigens limited by potential off-tumor toxicity.

Gene Therapies for Hemoglobinopathies

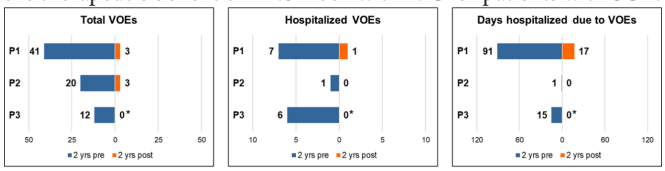
78. Early Results from a Phase 1/2 Study of ARU-1801 Gene Therapy for Sickle Cell Disease (SCD): Safety and Efficacy of a Modified Gamma Globin Lentivirus Vector and Reduced Intensity Conditioning Transplant

Michael Grimley[1], Monika Asnani[2], Archana Shrestha[1], Sydney Felker[1], Carolyn Lutzko[1], Paritha I. Arumugam[1], Scott Witting[1], Jennifer Knight-Madden[2], Omar Niss[1], Charles T. Quinn[1], Christopher Lo[3], Courtney R. Little[3], Joseph W. McIntosh[3], Punam Malik[1]

[1]Cincinnati Children's Hospital Medical Center, Cincinnati, OH,[2]Carribean Institute for Health Research, Kingston, Jamaica,[3]Aruvant Sciences, New York, NY

ARU-1801 is an investigational gene therapy drug product to treat sickle cell disease, consisting of autologous CD34+ hematopoietic stem cells and progenitors (HSPCs) transduced with a lentiviral vector (LV) encoding a modified γ-globin^{G16D} gene. It is being evaluated in patients with SCD in the ongoing Phase 1/2 MOMENTUM study (NCT02186418). Preliminary studies in SCD mice have suggested HbF^{G16D} may have a more potent anti-sickling effect than wild-type HbF. As a high potency anti-sickling globin, HbF^{G16D} is believed to allow ARU-1801 to be effective with reduced intensity conditioning (RIC), resulting in fewer toxicities and lower resource utilization than myeloablative approaches, expanding access to gene therapy to a broader group of SCD patients. Here, we present long-term clinical data on the first two patients (P1 and P2) and 10-month follow-up on patient 3 (P3), the first patient treated with a newer manufacturing process (Process II) to improve engraftment and increase HbF^{G16D} expression. As of December 2021, 3 patients treated with ARU-1801 have follow-up of >9 months. ARU-1801 demonstrated a favorable safety profile with no treatment-related adverse events to date. RIC resulted in neutrophil engraftment within 7-9 days (median 7 days), and platelet engraftment within 6-12 days (median 7 days). Under the initial manufacturing process, P1 has shown steady VCN of 0.2, stable expression of 20% HbF^{G16D} and 31% total anti-sickling globin (ASG, composed of endogenous HbF, HbA2 and ARU-1801-derived HbF^{G16D}), with 64% F-cells at 2 years post ARU-1801 infusion. P2 had a sub-therapeutic exposure to melphalan secondary to renal hyperfiltration and rapid clearance of melphalan, resulting in lower engraftment. Despite lower engraftment (VCN of 0.1) and hence lower HbF^{G16D} levels, P2 maintained stable 22% ASG expression and 36% F-cells at 2 years post ARU-1801 infusion due to sustained increases in endogenous HbF and HbA2. Under the new manufacturing process, P3 has demonstrated a stable VCN of 0.7 (latest measurement at month 9) with 41% ASG expression and 27% HbF^{G16D} at month 10. Furthermore, P3 has 92% F-reticulocytes at month 6, showing near pan-cellular expression of HbF. Treatment with ARU-1801 has resulted in remarkable improvement in clinical outcomes. As shown in Figure 1, patients had 12-41 VOEs (median, 21) in the 24 months prior to treatment with ARU-1801 and were hospitalized for 1-7 of those VOEs (median, 6). Through 24 months post ARU-1801 treatment, Patients 1 and 2 have seen 93% and 85% reductions in the number of VOEs, and Patient 3 has had no VOEs through 10 months of follow-up, a 100% reduction. The corresponding

cumulative days in hospital associated with those VOEs has decreased from 1-91 days (median, 15) to 0-17 (median, 0), representing an average 93.8% reduction. These results are an encouraging sign of the therapeutic benefit of ARU-1801 with RIC for patients with SCD.



*VOE data for patient 3 is 10 months post gene therapy infusion

79. Immunereconstitution in Transfusion Dependent Beta-Thalassemia Patients Treated with Hematopoietic Stem Cell Gene Therapy

S. Scaramuzza[1], S. Marktel[1,2], F. Giglio[2], M. P. Cicalese[1,3], M. R. Lidonnici[1], C. Rossi[1], V. Calbi[1,3], N. Masera[4], E. D'Angelo[5], N. Mirra[5], R. Origa[6], I. Tartaglione[7], S. Perrotta[7], G. Viarengo[8], L. Santoleri[9], R. Milani[9], S. Gattillo[9], A. Calabria[1], E. Montini[1], G. Graziadei[10], L. Naldini[1,11], M. D. Cappellini[10], A. Aiuti[1,3,11], F. Ciceri[1,2,11], G. Ferrari[1,11]

[1]SR-TIGET, Milano, Italy,[2]Haematology and BMT Unit, IRCCS San Raffaele Scientific Institute, Milano, Italy,[3]Pediatric Immunohematology, IRCCS San Raffaele Scientific Institute, Milano, Italy,[4]Pediatric Department University of Milano-Bicocca, San Gerardo Hospital, Monza, Italy,[5]Pediatric Clinic/DH, Fondazione IRCCS Ca' Granda, Milano, Italy,[6]University of Cagliari, Cagliari, Italy,[7]Università degli studi della Campania "Luigi Vanvitelli", Napoli, Italy,[8]Immunohematology and Transfusion Medicine Service, Fondazione IRCCS Policlinico S. Matteo, Pavia, Italy,[9]Blood Transfusion Service, IRCCS San Raffaele Scientific Institute, Milano, Italy,[10]Rare Disease Center, Fondazione IRCCS Ca' Granda, Milano, Italy,[11]University Vita-Salute San Raffaele, Milano, Italy

Transfusion-dependent β-thalassemia (TDT) is a disorder due to mutations in the gene encoding the ß-globin chain causing a reduced or absent production of hemoglobin A leading to severe anemia and lifelong transfusion dependence.Gene therapy has been recently accepted as a possible alternative to the only curative treatment represented by allogeneic bone marrow (BM) transplantation. We developed a gene therapy approach based on autologous mobilized hematopoietic stem cell transduced by LV vector, expressing human ß-globin gene, administered by intrabone injection, following a myeloablative conditioning (NCT02453477). Nine patients with severe TDT with different genotypes have been treated with a drug product with a median cell dose of 19.5x10^6 CD34$^+$ cells/kg, a transduction efficiency from 38 to 77% and a median vector copy number/genome (VCN) in bulk CD34$^+$ cells of 0.9 (range 0.7-1.5). Overall, gene therapy was generally well-tolerated with no adverse events related to the investigational product. No severe infectious-related adverse events were reported, except for those related to neutropenia as expected after conditioning. Insertion site analysis demonstrated highly polyclonal engraftment with no clonal dominance. Clinical outcome showed a reduction of transfusion requirement both in frequency and volume in adult patients up to more than 50%. Among the pediatric patients, 4 out of 6 discontinued transfusions shortly after gene therapy and are transfusion-independent at the last follow-up (up to 60 months). A robust and persistent engraftment was observed in 7 out of 9 patients, with a marking of BM progenitors that, in engrafted patients, ranged between 25.3 and 79.8% and with a median VCN in CD34$^+$ cells of 0.53 (range 0.34-2.21). As a relevant target, BM erythroid cells were stably marked (VCN range 0.3-2.5).Similar values were retrieved in the myeloid compartment and B lymphocytes while a lower VCN (range 0.12-1.48) was observed in CD3$^+$ cells. Focusing on lymphocytes, different rates of cell count increase were reported in B and T cells. In particular, CD4$^+$ T cells remained below the normal range for a prolonged period in all the patients, resulting in an inverted CD4/CD8 ratio, consistent with reported allogeneic bone marrow transplant experience. Flow-cytometry, TREC and KREC evaluation performed at different time-points showing a slow but progressive recovery of immunocompetency. Importantly, despite a slow increase in the number, both innate and adaptive immune cells showed good response to mitogens and pathogens with an adequate antibody titer documented in response to vaccinations. All patients were enrolled in a long-term follow-up study that will provide results on long-term clinical efficacy and safety of this gene therapy.

80. Multiplex Base Editing of Hematopoietic Stem and Progenitor Cells to Enrich Therapeutic Cells Post Engraftment

Olivier Humbert[1], Emily S. Fields[1], Mallory J. Llewellyn[1], Savannah M. Cook[1], Gregory A. Newby[2,3], Jonathan Yen[4], George S. Laszlo[1], Stefan Radtke[1], Roland B. Walter[1,5], Mitch J. Weiss[4], David R. Liu[2,3], Hans-Peter Kiem[1,5]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA,[2]The Broad Institute of Harvard and MIT, Cambridge, MA,[3]Harvard University, Cambridge, MA,[4]St. Jude Children Research Hospital, Memphis, TN,[5]University of Washington, Seattle, WA

A significant obstacle in current hematopoietic stem cell (HSC) gene therapy studies is the inability to consistently achieve sufficiently high engraftment of engineered cells to provide long-term therapeutic efficacy. Here we propose a strategy to increase engraftment of genome-edited cells after transplantation using multiplex base editing. Base editors are ideal for multiplexing since they introduce precise genetic alterations without double-stranded DNA breaks and thus prevent risk for chromosomal translocations. CD34+ cells were simultaneously edited both at a therapeutic gene and at a selection gene for evaluation in transplantation studies. As therapeutic target, we focused on the gamma-globin (HBG) locus for the reactivation of fetal hemoglobin (HbF) to treat hemoglobinopathies. This target has already been validated using CRISPR/Cas9-based approaches in several pre-clinical studies, including by our group. As edit that can be enriched by selection, we investigated the myeloid differentiation antigen CD33. Since CD33 is widely expressed on neoplastic myeloid cells, a wide array of CD33-directed drugs has been developed and tested. Previous findings demonstrated that inactivation of CD33 in HSCs by CRISPR/Cas9-editing does not impact the engraftment and multilineage differentiation potential of these cells. We used the adenine base editor ABE8e as editing platform, which was recently evolved from ABE7.10 for more robust activity. Human CD34+ cells electroporated with ABE8e mRNA targeting both the HBG and CD33 sites were transplanted in the murine model. Editing efficiency

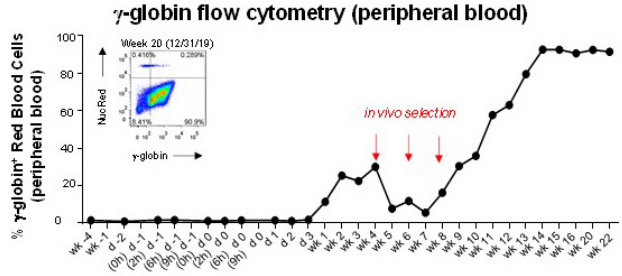
in infused cells averaged 40% for HBG and 80% for CD33. Notably, clonal assays demonstrated that about 75% of treated cells displayed edits at both targets in the same cell. Engraftment was comparable between the multiplex-edited group and the mock electroporated group. Furthermore, editing levels in peripheral blood (PB) remained high for both targets during the course of the experiment. Monocytes generated from engrafted cells displayed considerably reduced CD33 expression. Treatment of the animals with gemtuzumab ozogamicin confirmed resistance of edited cells to this highly potent CD33 antibody-drug conjugate. To further validate our multiplex editing strategy in an autologous transplantation setting, we utilized our nonhuman primate model described previously (Humbert et al., STM 2019). The CD34+CD90+ HSC-enriched cell population was edited with the same ABE8e-based approach as described above, yielding editing efficiencies in infused cells averaging 50% and 70% for HBG and CD33, respectively, with 70% containing edits at both targets. At 3 months post-transplantation, editing measured in total nucleated cells stabilized at about 10% (HBG) and 20% (CD33). CD33 expression measured in PB granulocyte was reduced to 65% as compared to >90% in a control animal, and HbF expression, as determined by F-cell frequency, reached 12% of total red blood cells. We will next use CD33-directed drugs in this animal to determine the in vivo ability to efficiently enrich for CD33- and thus HBG- edited cells as a means to enhance HbF to therapeutic levels for hemoglobinopathies. Together, our results demonstrate efficient engraftment and persistence of multiplex base edited CD34+ or CD34+CD90+ HSC-enriched cells in murine and NHP models. If enrichment is successful, this approach could serve as the basis for a broadly applicable method to treat other genetic blood diseases.

81. In Vivo HSC Gene Therapy for Hemoglobinopathies: A Proof of Concept Evaluation in Rhesus Macaques

Chang Li[1], Hongjie Wang[1], Sucheol Gil[1], Aphrodite Georgakopoulou[1], Stefan Radtke[2], Evangelia Yannaki[3], Thalia Papayannopoulou[4], Hans-Peter Kiem[5], Andre Lieber[1]

[1]Division of Medical Genetics, University of Washington, Seattle, WA,[2]Fred Hutchinson Cancer Research Center, Seattle, WA,[3]G. Papanicolaou Hospital, Thessaloniki, Greece,[4]Division of Hematology, University of Washington, Seattle, WA,[5]Division of Medical Genetics, Fred Hutchinson Cancer Research Center, Seattle, WA

Current gene therapy or genome editing studies for hemoglobinopathies require highly sophisticated medical facilities to perform hematopoietic stem cell (HSC) collections/selections and genetic modifications. In addition, patients receive high-dose chemotherapy to facilitate engraftment of gene-modified cells. Thus, current gene therapy protocols will not be accessible to most patients suffering from hemoglobinopathies. Here we describe a highly portable and scalable approach using in vivo HSC gene therapy to potentially overcome these limitations. The central idea of our in vivo HSC gene therapy approach is to mobilize HSCs from the bone marrow, and while they circulate at high numbers in the periphery, transduce them with an intravenously injected HSC-tropic, helper-dependent adenovirus HDAd5/35++ gene transfer vector system. Transgene integration is either achieved by a Sleeping Beauty transposase (SB100x) in a random pattern or by homology-directed-repair into a safe genomic harbor site. Currently, an in vivo selection system (involving the mgmt^{P140K} gene/low-dose O^{6}BG/BCNU) is employed to achieve >80% marking levels in peripheral blood cells. We demonstrated safety and efficacy of our approach in mouse models for thalassemia intermedia, Sickle Cell Disease, and hemophilia A, where we achieved a phenotypic correction. We now present data in 3 rhesus macaques. We show that treatment with G-CSF/AMD3100 resulted in efficient HSC mobilization into the blood circulation and subsequent intravenous injection of the HDAd5/35++ vector system (total 1-3 x10^{12} vp/kg, in two doses) was well tolerated. The longest follow-up thus far is 24 weeks after in vivo HSC transduction with a human-γ-globin expressing HDAd5/35++ vector. After in vivo selection with O^{6}BG plus low dose of BCNU, γ-globin marking in peripheral red blood cells rose to ~90% and was stable for the duration of the study (see Figure). γ-globin levels in red blood cells were ~18% of adult α1-globin (by HPLC). No abnormalities in genome and transcriptome analyses of animal #1 were found at the time of scheduled necropsy. We show that a new prophylaxis regimen (dexamethasone, IL-6R, IL-1bR antagonists, saline bolus IV) was able to mitigate all side effects associated with intravenous HDAd5/35++ vector administration. Analysis of day 3 bone marrow showed 30% transduced HSCs. Vector DNA biodistribution studies demonstrated very low or absent transduction of most tissues (including testes and CNS). Analysis of bone marrow showed efficient, preferential HSC transduction and re-homing of transduced CD34$^+$CD90$^+$ cells to the bone marrow. At week 4, about 5% of progenitor colony-forming cells demonstrated stable transduction with integrated vector, and this frequency increased after starting the in vivo selection. The level of human mgmt^{P140K} mRNA expression in PBMCs also increased after in vivo selection. This is the first proof-of-concept study that in vivo HSC gene therapy could be feasible in humans without the need of high-dose chemotherapy conditioning and without the need for highly specialized medical facilities.



82. Hematopoietic Reconstitution and Lineage Commitment in HSC Gene Therapy Patients Are Influenced by the Disease Background

Andrea Calabria[1], Giulio Spinozzi[1], Fabrizio Benedicenti[1], Daniela Cesana[1], Luca Del Core[1,2], Serena Scala[1], Samantha Scaramuzza[1], Maria Rosa Lidonnici[1], Alessandra Albertini[1], Simona Esposito[1], Fabiola De Mattia[1], Maryam Omrani[1], Valeria Calbi[1], Francesca Fumagalli[1], Marco Grzegorczyk[2], Ernst Wit[3], Giuliana Ferrari[1,4], Luigi Naldini[1,4], Alessandro Aiuti[1,4], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Bernoulli Institute for Mathematics, Groningen, Netherlands,[3]Universita della Svizzera Italiana, Lugano, Switzerland,[4]Vita Salute San Raffaele University, Milan, Italy

Lentiviral vector (LV) based hematopoietic stem cell (HSC) gene therapy (GT) applications have shown a favorable efficacy and safety profile for the treatment for a variety of genetic diseases. While this is a promising strategy for the treatment of diverse diseases, several factors, including transplantation protocols, patients' age and the underlying genetic disease, may impact on the kinetics of hematopoietic reconstitution, lineage specification, efficacy, and safety. To understand the impact of patient-specific factors and disease background on the hematopoiesis after transplantation, we studied the clonal reconstitution and multilineage potential over time in 48 HSC-GT patients affected by 3 different diseases: 29 with metachromatic leukodystrophy (MLD, a neurodegenerative lysosomal storage disorder), 10 with Wiskott-Aldrich syndrome (WAS, a B and T cell immunodeficiency) and 9 with β-thalassemia (β-Thal, a hemoglobinopathy). As reported previously, these HSC-GT treatments resulted in clinical benefits for most patients. We analyzed the LV genomic Integration Sites (IS), a genetic marker for clonal identity, from the DNA of $CD34^+$ cells as well as myeloid, B, T and erythroid cell lineages purified from blood and bone marrow, harvested at different time points after HSC-GT (longest follow up to 9 years). Integration site analysis yielded >3 million unique IS and showed highly polyclonal reconstitution, multilineage marking and no signs of genotoxicity in all patients.Regardless of disease background, all patients showed a similar pattern of hematopoietic reconstitution over time, characterized by: an early phase up to 9 months after GT, where the myeloid cells are highly polyclonal whilst T and B cells have a less complex repertoire; a second phase of 9 up to 18 months, where the polyclonality of lymphoid cells increases; and a third phase where the complexity of the lineages decreases and stabilizes. During the early phase of hematopoietic reconstitution an average of 80,000 active hematopoietic stem and progenitor cell (HSPCs)/patient contributed to hematopoiesis. However, the active HSPC pool decreased to an average 11,000 after 9 months, suggesting that short lived progenitors have a relevant role in sustaining the early phases of hematopoietic reconstitution. Differences across diseases were found when we analyzed the multilineage potential of individual clones and their commitment towards a specific lineage over time. In MLD patients multilineage clones reached a proportion of 75% on the total engineered clones, decreased over time to 50%, remaining stable thereafter. Myeloid-committed clones increased over time and stabilized at 40%. The decrease in multilineage clones over time was also observed in WAS and β-Thal patients. However, in WAS patients we did not observe a concomitant increase in myeloid committed cells rather an increase of the commitment in the T-cell lineage, as expected by the selective advantage in T cells, whereas in β-Thal patients several clones showed erythroid commitment.These novel data suggest that the disease condition influences the proportion and the type of lineage-committed cells over long periods of time, and that the engrafted HSPC pool respond to the disease-specific physiopathology in a dynamic fashion to restore normal hematopoiesis.

83. Lentiviral Mediated Gene Therapy for Pyruvate Kinase Deficiency: Updated Results of a Global Phase 1 Study for Adult and Pediatric Patients

José Luis López Lorenzo[1,2], Ami J. Shah[3,4,5], Susana Navarro[2,6,7], Julián Sevilla[8], Lucía Llanos[1,2], Begoña Pérez Camino de Gaisse[1,2], Sol Sanchez[1,2], Bertil Glader[5,9], May Chien[5,9], Oscar Quintana-Bustamante[2,6,7], Brian C. Beard[10], Kenneth M. Law[10], Miriam Zeini[10], Grace Choi[10], Eileen Nicoletti[10], Gayatri R. Rao[10], Maria Grazia Roncarolo[3,4,5], Juan A. Bueren[2,6,7], Jonathan D. Schwartz[10], José C. Segovia[2,6,7]

[1]Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain,[2]Unidad Mixta de Terapias Avanzadas, Instituto de Investigación Sanitaria Fundación Jiménez Díaz, Madrid, Spain,[3]Center for Definitive and Curative Medicine, Stanford University, Stanford, CA,[4]Div. of Pediatric Hematology/Oncology/Stem Cell Transplant and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA,[5]Stanford Children's Hospital, Palo Alto, CA,[6]Centro de Investigación Biomédica en Red de Enfermedades Raras, Madrid, Spain,[7]Instituto de Innovación Biomédica, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain,[8]Hospital Infantil Universitario Niño Jesus, Madrid, Spain,[9]Dept. of Pediatrics, Div. of Hematology/Oncology, Stanford University School of Medicine, Stanford, CA,[10]Rocket Pharmaceuticals, Inc., Cranbury, NJ

Introduction: Pyruvate kinase deficiency (PKD) is a rare inherited hemolytic anemia that results from mutations in the *PKLR* gene leading to decreased red cell pyruvate kinase activity and impaired erythrocyte metabolism. Manifestations include anemia, reticulocytosis, splenomegaly and iron overload, and may be life-threatening in severely affected individuals. Current treatments are limited to blood transfusions, iron chelation therapy, and splenectomy which are associated with significant side effects. Preclinical studies in a clinically relevant PKD murine model have demonstrated that infusion of gene-modified Lin^- bone marrow (BM) cells may ameliorate the PKD phenotype. Based on compelling preclinical data, a global Phase 1 clinical trial RP-L301-0119 (NCT04105166) is underway to evaluate the feasibility and safety of lentiviral mediated gene therapy in adults and pediatric subjects with severe PKD. **Methods:** A total of 6 adult and pediatric subjects with severe PKD (defined as severe and/or transfusion-dependent anemia despite prior splenectomy) will be enrolled. Peripheral blood (PB) hematopoietic stem cells (HSCs) are collected on 2 consecutive days via apheresis after mobilization with granulocyte-colony stimulating factor (G-CSF) and plerixafor. $CD34^+$ HSCs are enriched, transduced with PGK-coRPK-

WPRE lentiviral vector, and cryopreserved. Following release testing of the investigational product (IP), RP-L301, myeloablative conditioning with therapeutic drug monitoring guided busulfan is administered over 4 days. RP-L301 is then thawed and infused. Patients are followed for safety and efficacy assessments for 2 years post-infusion. **Results:** As of January 2021, 2 adult subjects have been treated. Patient 1 received 3.9x10^6 CD34$^+$ cells/kg with mean vector copy number (VCN) of 2.73. At 3 months post-infusion hemoglobin has normalized, with PB VCN of 1.55. Patient 2 received 2.4x10^6 CD34$^+$ cells/kg with mean VCN of 2.08. Neutrophil engraftment occurred within 2 weeks for both patients. No adverse events have been attributed to RP-L301. 6- and 3-month post-treatment data will be presented for Patients 1 and 2, respectively. **Conclusions:** Hematopoietic stem cell mobilization using G-CSF and plerixafor appears feasible and effective in adult PKD patients. RP-L301 was successfully manufactured to meet the required specifications for the Phase 1 clinical study and administered without short-term infusion related complications. Preliminary efficacy was evident in Patient 1 during the initial 3 months post-RP-L301 as demonstrated by hemoglobin normalization and PB genetic markings.

84. Base Editing of the -200 Region of the γ-globin Promoters Leads to Fetal Hb Reactivation and Rescues the Sickle Cell Disease Phenotype in Primary Patient Cells

Panagiotis Antoniou[1], Giulia Hardouin[1], Pierre Martinucci[1], Giacomo Frati[1], Megane Brusson[1], Marion Rosello[2], Giulia Maule[3], Filippo Del Bene[2], Anna Cereseto[3], Wassim El Nemer[4], Jean-Paul Concordet[5], Annarita Miccio[1]

[1]IMAGINE Institute, Paris, France,[2]Institut de la Vision, Paris, France,[3]CIBIO, Trento, Italy,[4]Institut National de la Transfusion Sanguine, Paris, France,[5]Museum National d'Histoire Naturelle, Paris, France

β-hemoglobinopathies, β-thalassemia and sickle cell disease (SCD), are caused by mutations affecting the production of the adult hemoglobin (Hb). Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an attractive therapeutic option. The clinical severity of β-hemoglobinopathies is alleviated by the co-inheritance of mutations causing hereditary persistence of fetal Hb (HPFH) in adult life. To reactivate fetal γ-globin expression, nuclease-based editing approaches have been explored. Site-specific nucleases, however, generate double-strand breaks (DSBs) in the genome, raising safety concerns for clinical applications. Base editing (BE) allows the introduction of point mutations (C>T by cytidine base editors, CBEs; A>G by adenine base editors or ABEs) without generating DSBs.HPFH mutations in the promoters of the 2 γ-globin (*HBG1/2*) genes either disrupt the binding sites (BS) of fetal Hb (HbF) repressors or generate BS for HbF activators. In particular, mutations clustering ~200 nucleotides upstream of the *HBG* TSSs either reduce LRF binding (e.g. -197 C>T) or recruit KLF1 (-198 T>C). In this study, we used base editors to recapitulate HPFH mutations in the -200 region in the *HBG* promoters.First, we explored the BE system to introduce C>T mutations in the LRF BS. The absence of the canonical *Spy*Cas9 NGG PAM close to the LRF BS prompted us to test a variety of non-NGG CBEs including CBE-NRCH, CBE-SpG, CBE-SpRY and a novel BE containing NAA PAM Cas9 variant. CBEs edited 7 out of 8 cytidines of the LRF BS in an erythroid cell line (K562) with efficiencies of up to ~60%. These C>T conversions include not only known HPFH mutations but also HPFH-like mutations that can further impair LRF binding.We tested this strategy in HSPCs from SCD patients and achieved BE efficiencies of up to ~45%. A progenitor assay indicated no alteration in the multilineage differentiation of edited HSPCs. HSPCs were differentiated in mature red blood cells (RBCs). The expression of erythroid markers was similar in control and edited samples and the production of mature RBCs was not affected by the BE treatment. We observed a potent γ-globin reactivation with a high frequency of HbF$^+$ cells and a concomitant decrease in the HbS content/cell, as detected by RT-qPCR, HPLC and flow cytometry. Importantly, the pathological RBC sickling phenotype was substantially improved in the edited samples. Finally, we used GUIDE-seq coupled with targeted sequencing to evaluate the potential off-target activity of gRNAs disrupting the LRF BS.We then compared BE strategies targeting the -200 region of the *HBG* promoters that either disrupt the LRF BS or create a *de novo* KLF1 BS in SCD HSPCs. The superior efficiency in generating the KLF1 BS by ABEs was associated with higher levels of HbF in mature RBCs and colonies derived from erythroid progenitors, compared to a strategy merely disrupting the LRF BS. Similarly, the RBC sickling phenotype was further improved in the edited samples carrying the KLF1 BS.In conclusion, we developed efficient BE strategies to disrupt repressor BS or create activator BS in the *HBG* promoters that led to therapeutically relevant HbF levels. Validation of the above-described results in HSPCs *in vivo* will provide sufficient proof of efficacy and safety to enable the clinical development of base-edited HSPCs for the therapy of β-hemoglobinopathies.

Immune Responses to AAV Vectors

85. Declining FVIII Activity Following Hepatic AAV Gene Transfer Because of Translational Shutdown Linked to an Immune Response

John S. S. Butterfield[1], Thaís B. Bertolini[2], Annie R. Piñeros[2], Kentaro Yamada[2], Sandeep R. P. Kumar[2], Jyoti Rana[2], Moanaro Biswas[2], Cox Terhorst[3], Roland W. Herzog[2]

[1]University of Florida, Gainesville, FL,[2]IU School of Medicine, Indianapolis, IN,[3]Harvard Medical School, Boston, MA

Adeno-associated virus (AAV) gene therapy aims to provide sustained replacement in diseases of protein deficiency, such as clotting factor VIII (FVIII) for the X-linked bleeding disorder hemophilia A (HA). Excitingly, liver-directed AAV-FVIII gene transfer led to complete correction of HA in clinical trials. However, these high levels of therapeutic expression were unstable and declined in years 2-4. We identified a sub-strain of BALB/c-HA mice that reliably forms an adaptive immune response against FVIII upon hepatic gene transfer with AAV8 vector expressing codon-optimized human FVIII under a liver-specific promoter. Administering mTOR inhibitor rapamycin for the eight weeks following gene transfer prevented formation of antibodies to FVIII and capsid, allowing vector readministration. Elimination of antibody formation resulted in initial FVIII activity

levels of 20-60% of normal while the mice were on immune suppression. However, in multiple experiments, average FVIII levels consistently declined to less than 5% by week 16 (with 50-100% of animals having undetectable levels, depending on the experiment). Therefore, this model allowed us to uncouple loss of FVIII expression from humoral immune responses. To counter potential cellular immune responses, we included monoclonal antibodies (mAb) in the rapamycin regimen. Combining rapamycin with a mAb to IL-15, a cytokine critical for NK and memory CD8 T cells, modestly preserved FVIII activity. We confirmed the effectiveness of blocking IL-15 signaling, and in fact improved upon this approach, by combining rapamycin with a Fc-silent mAb to the shared IL-2/IL-15 receptor beta chain (CD122). Eight weeks after immune modulation was stopped, livers of mice given anti-CD122 were still NK-depleted. In addition, Kupffer cells had lower expression of inflammatory molecules, suggesting that the improvement in FVIII activity levels by IL-15 blockade was due to suppression of an inflammatory immune response. Furthermore, we found CD8 T cell depletion with anti-CD8 after rapamycin treatment reduced the number of mice that entirely lost FVIII activity similarly to anti-IL-15 and anti-CD122 treated cohorts, indicating a contribution of CD8 T cells. However, the remaining FVIII activity was less than 5%. Together, the results suggest that multiple cell types contribute to the immune response, including CD8 T and NK cells. IHC stains revealed that rapamycin-treated animals that lost FVIII activity had no FVIII protein expression in the liver, while those that had circulating levels due to IL-15 blockade showed FVIII expression in hepatocytes. Among animals that received immune suppression, mRNA levels were similar regardless of FVIII protein expression, and CD8 T cell depletion only modestly increased vector copy numbers compared to other rapamycin-treated animals. Interestingly, all transduced mice retained AAV copies that positively correlated with hFVIII mRNA levels but not FVIII protein expression. Mice that received vector without immune suppression—thus formed antibodies against FVIII—had 2-3 times higher gene copy numbers and mRNA levels but showed only few FVIII expressing hepatocytes. Therefore, expression was lost primarily because of a translational shutdown that could in part be prevented by dampening cellular immune responses. We are now investigating whether an unfolded protein response to FVIII contributed to the shutdown and the inflammatory response. A model of gradual loss of FVIII expression largely due to translational shutdown with a lesser contribution of hepatocellular injury/cytotoxicity would explain the observations in clinical AAV-FVIII gene therapy.

86. Requirements for Cross-Presenting Dendritic Cells and CpG Motifs in CD8$^+$ T Cell Response to AAV Gene Transfer

Thais B. Bertolini[1], Jamie L. Shirley[2], Sandeep Kumar[1], Xin Li[1], Irene Zolotukhin[3], Roland Herzog[1]

[1]Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN,[2]Department of Medicine, University of Colorado, Aurora, CO,[3]Department of Pediatrics, University of Florida, Gainesville, FL

Gene therapy using adeno-associated virus (AAV) has emerged as one of the most promising treatments for various genetic diseases. However, the development of immune response to the viral vector or the transgene product might compromise the outcome for long-term success. Immune stimulatory CpG motifs, which are unmethylated in viral DNA, trigger innate immune receptors and induce AAV capsid specific CD8$^+$ T cells. TLR9 sensing of CpG motifs in the AAV vector genome has been implicated in the literature to serve as a signal for activation of CD8$^+$ T cells. For instance, TLR9 signaling in plasmacytoid dendritic cells (DCs) leads to IFN I production, which in turn licenses conventional DCs to prime anti-capsid CD8$^+$ T cells. XCR1$^+$ DCs are most efficient in cross-presenting antigen to CD8$^+$ T cells and are comprised of organ-resident CD8α$^+$ DCs and migratory CD103$^+$ DCs. We quantified the capsid-specific CD8$^+$ T cell response by tetramer stain upon intramuscular (IM) injection of AAV2 vector (1x10^{11} vg/mouse) containing an ovalbumin-derived surrogate epitope in C57BL/6 mice. To determine the putative role of these DCs, we used XCR1-DTR mice to deplete XCR1$^+$ DC by administration of diphtheria toxin (DT). Alternatively, we treated WT C57BL/6 mice with neutralizing anti-CD103. C57BL/6 mice treated with anti-CD103 showed a significantly lower frequency of capsid-specific CD8$^+$ T cell response compared with controls. Depletion of XCR1$^+$ DCs gave a more dramatic outcome and resulted in nearly complete elimination of the response. Next, we evaluated the role of CpG motifs in the immune response. For that, a vector was constructed based on an entirely CpG-free expression cassette. It contained a CpG-free edited sequence of the coding region for human coagulation factor IX (FIX) and a synthetic intron under transcriptional control of a CMV enhancer/EF1α promoter combination and SV40 polyA signal. This cassette was inserted in between AAV2 ITRs and packaged into the AAV2- SIINFEKL capsid. C57BL/6 mice received IM injections (1x10^{11} vg/mouse) of AAV2-SIINFEKL depleted of CpG motifs (AAV2-SIINFEKL-CpG$^-$) or CpG rich control vector (AAV2-SIINFEKL-CpG$^+$; containing native, not CpG depleted sequences). AAV-SIINFEKL-CpG$^-$ administration markedly reduced anti-capsid CD8$^+$ T cell response as observed by the decrease of % tetramer$^+$ CD8$^+$ T cells. In contrast, we did not observe differences in capsid-specific antibody formation between groups. Next, we evaluated the immune response to FIX in male hemophilia B (C3H/HeJ F9$^{-/Y}$) mice injected IM with AAV1-CpG$^+$ or AAV1-CpG$^-$ vector (1x10^{11} vg/mouse, n=5/group). AAV1-CpG$^-$ induced a substantially reduced (~8 fold), but not completely absent, CD8$^+$ T cell infiltration and ~2 fold higher numbers of hFIX expressing muscle fibers, as quantitated by image analysis of immunofluorescent stained sections of skeletal muscle. We conclude that XCR1$^+$ DCs are the critical subset of conventional DCs that presents AAV capsid antigen to CD8$^+$ T cells, which in part occurs through XCR1$^+$CD103$^+$ DCs. Furthermore, CpG motifs are a major activation signal for CD8$^+$ T cell responses against capsid and transgene product in AAV muscle gene transfer but are not a major factor for antibody formation.

87. Defining and Overcoming Preexisting T-Cell Adaptive Immunity to SaCas9 CRISPR-Cas Genome Editors

Andrea T. Lee

Hematology, St. Jude Children's Research Hospital, Memphis, TN

In vivo CRISPR-Cas genome editing has the potential to transform human medicine by directly correcting disease-causing mutations in affected cells in the body. Cas9 from *Staphylococcus aureus* (*S. aureus;* SaCas9) was the first orthologue discovered with high activity in mammalian cells that is small enough to be encoded in an AAV vector and has shown great clinical potential in preliminary *in vivo* studies.

One major concern regarding the durability of promising *in vivo* genome editing therapeutic strategies using Cas9 is the potential for immune rejection of Cas9-expressing edited cells. Pre-existing adaptive T cell immunity to the Cas9 variants derived from *Streptococcus Pyogenes* (SpCas9) and S.*aureus*, common human pathogen, have been reported. While SaCas9-specific T cells were identified, the adaptive T cell immune response to SaCas9 has not been fully characterized. Here we screened peripheral blood mononuclear cells (PBMCs) from healthy HLA-typed donors using an IFN-γbased enzyme-linked immune absorbent spot (ELISPOT) assay using a peptide library consisting of 15 amino acid long peptides with an 11 amino acid overlap that covered the entire coding region of SaCas9 to sensitively measure the SaCas9-specific T cell response. We found that 24% (8 of 33) of donors screened exhibited a positive T cell response to SaCas9 that was CD4-restricted in 2 of 2 analyzed positive donors. Next, we performed an unbiased epitope screen to map immunodominant epitopes using our SaCas9 peptide library for two donors. For one donor, we defined the minimal 13 amino acid epitope that was recognized by SaCas9-specific CD4 T cells. Next, we performed alanine scanning of the minimal epitope and identified 7 positions that significantly reduced T cell recognition. We engineered corresponding alanine mutations into SaCas9 to determine whether these SaCas9 variants would retain high genome editing activity. Six of 7 SaCas9 alanine variants-maintained genome editing activity at levels comparable to wild-type SaCas9. In conclusion, our results illuminate the prevalence and type of T cell-mediated immunity towards SaCas9 and offer a proof-of-principle solution of engineering immune silent variants to overcome the challenge of preexisting adaptive T cell immunity to CRISPR-Cas genome editors.

88. Clinical Outcomes in Patients with and without Pre-Existing Neutralizing Antibodies to the Vector: 6 Month Data from the Phase 3 HOPE-B Gene Therapy Trial of Etranacogene Dezaparvovec

Michael Recht[1], Frank W.G. Leebeek[2], Wolfgang Miesbach[3], Nigel S. Key[4], Susan Lattimore[1], Giancarlo Castaman[5], Eileen K. Sawyer[6], David Cooper[6], Valerie Sier-Ferreira[7], Steven W. Pipe[8]

[1]The Hemophilia Center at Oregon Health & Science University, Portland, OR,[2]Erasmus MC, University Medical Center, Rotterdam, Netherlands,[3]University Hospital Frankfurt, Frankfurt, Germany,[4]University of North Carolina, Chapel Hill, NC,[5]Center for Bleeding Disorders and Coagulation, Careggi University Hospital, Florence, Italy,[6]uniQure Inc., Lexington, MA,[7]uniQure BV, Amsterdam, Netherlands,[8]University of Michigan, Ann Arbor, MI

Introduction: Etranacogene dezaparvovec is an investigational gene therapy for hemophilia B (HB) comprising an adeno-associated virus serotype 5 (AAV5) vector containing a codon-optimized Padua variant human factor IX (FIX) gene with a liver specific promoter. Although most gene therapy clinical studies exclude participants (pts) with pre-existing neutralizing antibodies (NAb) to the capsid serotype, early clinical studies and nonhuman primate data suggest that generally prevalent titers of anti-AAV5 NAbs may not preclude successful transduction with etranacogene dezaparvovec. **Aims and methods:** A Phase 3, Health Outcomes with Padua gene; Evaluation in Hemophilia B (HOPE-B; NCT03569891) was established to further assess efficacy and safety of etranacogene dezaparvovec in adults with HB. Adult males with severe or moderate-severe HB (FIX≤2%) on prior routine FIX prophylaxis were enrolled into this open-label, single-dose, single-arm, multinational trial. Pre-existing NAbs to AAV5 were assessed but not exclusionary. Participants entered a ≥6 month lead-in period, then received a single dose of etranacogene dezaparvovec ($2x10^{13}$gc/kg) without prophylactic immunosuppression. The co-primary endpoints are change in FIX activity at 26 and 52wks and 52wk annualized bleeding rate compared to lead in. Here, outcomes at 26 weeks in participants with and without pre-existing NAbs to AAV5 are analyzed using descriptive statistics and a correlation analysis. **Results:** 54 participants were dosed and completed 26 wks of follow-up, 31 (57.4%) had no AAV5 NAbs. Of the 23 (42.6%) with AAV5 Nabs at baseline (BL), the median titer was 56.9 (1^{st}-3^{rd} quartile 23.3-282.5) with a distribution representative of the general population. The max NAb titer was 3212. One participant with a NAb titer of 198 received a partial dose and was excluded from the assessment of NAb impact on efficacy. A single participant with a NAb titer of 3212 did not respond and remained on prophylaxis. All other participants (n=52) discontinued prophylaxis and remain prophylaxis-free at 26 weeks. No correlation of pre-existing NAbs with FIX activity was observed up to a titer of 678 (n=52, r=-0.28 [95% CI -0.51, 0.00], R^2=0.078). Mean FIX activity at 26 weeks was 32.7 IU/dl (min <2, max 90.4, 1^{st}-3^{rd} quartile 16.3-42.6, n=22) in participants with NAbs versus 41.3 IU/dl (min 8.4, max 97.1, 1^{st}-3^{rd} quartile 31.3-52.7, n=31) in those without. Most common treatment-related AEs were transient transaminitis requiring corticosteroids (2/23 pts with NAbs; 7/31 pts without), infusion-related reactions (5/23 pts with NAbs; 2/31 pts without), headache (2/23 pts with NAbs; 5/31 pts without) and influenza-like illness (4/23 pts with NAbs; 3/31 pts without). No deaths and no inhibitors to FIX were reported. **Conclusions:** FIX activity was similar in participants without and with pre-existing NAbs to AAV5 up to a titer of 678; there were insufficient data to assess a relationship with higher titer NAbs. No relationship between AAV5 NAbs and safety was observed. This study demonstrates for the first time successful treatment of patients with pre-existing NABs at generally prevalent levels with an AAV5 construct, supporting broad eligibility for AAV5-based therapies.

89. Abstract Withdrawn

90. IL-1a and IL-1b Are Essential for Inflammasome Independent $CD8^+$ T Cell Responses to Hepatic AAV Gene Transfer

Sandeep Kumar[1], Moanaro Biswas[1], Annie R. Piñeros[1], Ype P. De Jong[2], Roland W. Herzog[1]

[1]Pediatrics, Indiana University, Indianapolis, IN,[2]Gastroenterology and Hepatology, Weill Cornell Medicine, New York, NY

We previously demonstrated that liver directed gene therapy with low dose adeno-associated virus serotype 8 (AAV8) vector elicited a $CD8^+$ T cell response to the transgene product, resulting in the loss of expression in murine liver. Further, we showed that these $CD8^+$ T cell response were independent of TLR9 sensing, instead relying on the IL-1R1/MyD88 pathway and $CD4^+$ T cell help. Using proliferation of adoptively transferred, CellTrace violet labeled transgene specific $CD8^+$ T cells as a readout, we demonstrated that cross presenting

$CD11c^+XCR1^+$ DCs are critical for MHC I antigen presentation. In the present study we sought to delineate the role of IL-1α, IL-1β and the role of inflammasomes in mediating cellular responses to the AAV encoded transgene. Further, we studied kinetics of $CD4^+$ and $CD8^+$ T cell activation following AAV mediated hepatic gene delivery. Since IL-1R1 is a cognate receptor for both IL-1α and IL-1β, we used anti-IL-1α and anti-IL-1β to block IL-1 signaling. Wildtype (WT) C57BL/6 mice (n=8/group) were treated with anti-IL-1α, anti-IL-1β or both antibodies one day prior to intravenous (IV) injection with AAV8-OVA ($1x10^9$ vg/mouse). Treatment was continued 2x/week for four weeks. Control WT-C57BL/6 mice (n=8) received only AAV8-OVA. PBMC were screened for OVA-specific $CD8^+$ T cells using class I MHC tetramer after 4 weeks. In order to study the role of inflammasomes, knockouts mice of specific inflammasomes (NLRP1, NLRP3, AIM-2 and Caspase-1) on C57BL/6 background (n=8) were injected with AAV8-OVA and OVA-specific $CD8^+$ T cells were quantified. To understand the activation kinetics of $CD4^+$ and $CD8^+$ T cells, AAV8-OVA injected C57BL/6-WT mice were euthanized on days 3, 7, 10 and 14 (n=5). $CD4^+$ and $CD8^+$ T cells from liver, hepatic lymph nodes and spleen were further analyzed for expression of activation markers (CD44, CD69 and CD107a). Naive C57BL/6-WT mice (n=5) were used to obtain baseline status of $CD4^+$ and $CD8^+$ T cells. Consistent with earlier findings, 75% of control WT mice developed a $CD8^+$ T cell response to OVA. Blocking of either IL-1α or IL-1β reduced the number of mice (33% and 50% respectively) that developed a $CD8^+$ T cell response. Moreover, only 18% of mice developed a $CD8^+$ T cell response when both IL-1α and IL-1β were blocked. These results indicate that both IL-1α and IL-1β play a critical and in part redundant role in mediating $CD8^+$ T cell response to an AAV encoded transgene during hepatic gene delivery. Since inflammasomes are an important mediator of IL-1β driven immune responses, we tested if any of the inflammasomes were required. Similar to WT mice, knockout mice for different inflammasome developed OVA specific $CD8^+$ T cell response indicating that these cellular responses were independent of inflammasome machinery. Following AAV8-OVA administration, increased expression of CD44, CD69 and CD107a was observed on $CD4^+$ and $CD8^+$ T cells isolated from liver as compared to hepatic lymph nodes or spleen. Activated $CD4^+$ and $CD8^+$ T cells were observed in the liver as early as day 3 post AAV administration. In summary our results suggest that during AAV mediated liver gene transfer, both IL-1α and IL-1β mediates $CD8^+$ T cell activation to AAV encoded transgene, which is independent of any inflammasome machinery. Further, our results imply that these cellular responses are initiated in the liver.

91. Novel miRNA-Binding Sites That Recruit miR-652 and miR-223 in AAV Vector Designs Boost Transgene Levels and Synergistically Suppress Cell-Mediated Immunity

Manish Muhuri[1,2,3], Wei Zhan[1,2,3], Yukiko Maeda[1,3,4], Jia Li[1,2], Anoushka Lotun[1], Jennifer Chen[1], Phillip W. L. Tai[a1,2,3], Guangping Gao[a1,2,5]

[1]University of Massachusetts Medical School, Horae Gene Therapy Center, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[3]VIDE Program, University of Massachusetts Medical School, Worcester, MA,[4]Department of Medicine, University of Massachusetts Medical School, Worcester, MA,[5]Li Weibo Institute for Rare Diseases Research, University of Massachusetts Medical School, Worcester, MA

Recombinant adeno-associated virus (AAV) vector gene therapy offers tremendous promise for the treatment for a variety of genetic diseases. Regulatory approvals for the treatment of two genetic diseases have already been received and clinical development for many more are on the horizon. Exciting advancements have been made in the gene delivery technologies, from the identification of novel AAV serotypes, to the development of novel vector delivery techniques. One of the challenges for long-term success of gene therapy is the development of immune response to the transgene product. This effect is attributed to the undesirable transduction of antigen presenting cells (APCs), which in turn triggers host immunity towards rAAV-expressed transgene products. miRNA-mediated regulation to detarget transgene expression from APCs has shown promise for reducing immunogenicity. Skeletal muscle has been considered a viable target for AAV vector-mediated gene transfer to achieve sustained production of secreted therapeutic proteins. We have previously shown that miR-142 mediated detargeting allows continued expression of transgene in myofibers, represses cytotoxic T cell response and blunts the activation of co-stimulatory signals. However, the combinatorial effect of more than one miRNA binding site in the 3'-UTR of the transgene on anti-transgene immunity has not been reported previously. In this study, we performed *in vitro* screening of binding sites for 26 miRNAs that were selected based on their high expression levels in APCs, such as dendritic cells (DCs) and macrophage cell lines, but low in myoblasts. We identified two novel miRNA binding sites, miR-652BS and miR-223BS, that are efficient at APC detargeting *in vitro*, either individually or in combination with miR-142BS. Intramuscular administration of rAAV1 vectors containing either miR-142+652BS or miR-142+223BS demonstrate higher transgene expression in skeletal myoblasts as compared to previously published detargeting constructs, with negligible anti-OVA IgG production. Immunophenotyping of cells isolated from liver, spleen and muscle tissues revealed suppression of DC and co-stimulatory signals, and macrophage activation. In addition, there was a marked reduction in OVA specific CD8+ T cell response in those tissues accompanied by a reduction in the production of inflammatory cytokines, TNFα and IFNγ. Moreover, we present evidence that miR-142-, miR-652-, and miR-223-mediated detargeting also leads to significant repression of Th17 response *in vivo*. Transgene detargeting mediated by the combination of miR-142BS and miR-652BS within the same vector cassette proves to be the most efficient at muting transgene specific immunity. Our approach, thus, advances the efficiency of

miRNA-mediated detargeting to achieving synergistic reduction of transgene-specific immune responses and the development of safer and more efficient delivery vehicles for gene therapy.

Novel Factors in AAV Transduction and AAV Genomes

92. Chemical Mediated Recruitment of Epigenetic Modifiers Regulate Adeno-Associated Virus Episomal Transgene Expression

Jessica Umana[1], Liujiang Song[2], Xufen Yu[3], Jian Jin[3], Matthew L. Hirsch[2], Nathaniel Hathaway[1]

[1]Chemical Biology and Medicinal Chemistry, University of North Carolina - Chapel Hill, Chapel Hill, NC,[2]Gene Therapy Center, University of North Carolina - Chapel Hill, Chapel Hill, NC,[3]Icahn School of Medicine, Mount Sinai, New York, NY

Optimistic clinical data using AAV gene therapy has been observed for disorders of the muscle, blood, brain, and those affecting vision. In all these applications, the AAV vectors administered to humans have one thing in common: they are uncontrollable at the level of transgene expression. Following transduction, AAV vector genomes form circular concatemers and limited studies have demonstrated these episomes associate with histones, as well as transcriptional activators and repressors. These observations suggest a formal possibility that AAV episomes are, in part, restricted for transgene expression, alluding to the ability to modulate their epigenetic composition to enhance and/or repress the transcriptional activity. Based on these observations, it was hypothesized that AAV transgene expression can be controlled at the episomal level through targeted recruitment of epigenetic regulators of gene expression. We have developed a technology, known as chemical epigenetic modifiers (CEMs), that utilizes chemically induced proximity and endogenous epigenetic proteins to regulate specific chromosomal gene expression. The CEM technology was adapted to AAV vectors (termed AAV-CEMtrol) to investigate transgenic episome regulation post-transduction. Following AAV2 or AAV8-CEMtrol transduction in vitro, the exogenous addition of different CEMs that recruit distinct transcriptional activators (CBP/p300 or BRD4) to the recombinant AAV episome demonstrated dose-dependent and highly specific rAAV-borne reporter induction. These novel observations in well controlled and rigorous experiments demonstrate unambiguously, that AAV episomes are naturally restricted for expression in human cells and allude to the ability to enhance and potentially repress therapeutic transgene expression at a fixed AAV vector dose following in vivo administration. Towards that end, pharmacokinetic studies following a single intraperitoneal injection demonstrated the safety and bioavailability of the leading CEM in multiple tissues. Future experiments include mechanistic ChIP analyses to elucidate the exploited biology as well as performance investigations of AAV-CEMtrol regulation in vivo in response to CEM administration. These data demonstrate the ability to significantly induce AAV transgene expression using particular CEMs that can recruit epigenetic regulatory machinery to transgenic episomes. This novel approach may provide a novel approach to activate and potential repress AAV transgene expression towards controllable, and thus safer, AAV gene therapeutics.

93. The Human Silencing Hub (HUSH Complex) Is a Potent Regulator of AAV Transgene Silencing

Madhuvanthi Vijayan, Anshuman Das, Grace Stafford, Zachary Elmore, Eric Walton, Aravind Asokan

Duke University, Durham, NC

Therapeutic transgenes delivered by recombinant AAV (rAAV) vectors are known to be subject to transgene silencing as observed in several clinical trials. This poses a challenge towards achieving efficient gene expression, often requiring high vector doses. However, the latter has shown the potential to cause dose-dependent liver toxicity amongst other adverse effects. Importantly, the underlying silencing mechanisms impacting rAAV transgene expression are not well understood. In order to identify potential cellular pathways involved in rAAV transgene silencing/expression, we screened a panel of host factors known to be involved in epigenetic regulation of foreign DNA. This targeted screen revealed novel restriction factors of rAAV transduction, particularly components of the HUSH (HUman Silencing Hub) complex. The HUSH complex is known to be associated with H3K9me3-dense genomic regions and functions to repress cellular genes. The HUSH complex has also been shown to transcriptionally silence unintegrated retroviral genomes by recruiting NP220 to the viral DNA followed by methyltransferases and deacetylases. Here, we show here that CRISPR KO of individual HUSH complex members such as MPP8, PPHLN1, TASOR, SETDB1 and NP220 leads to a robust increase in AAV mediated transduction, both at the level of mRNA transcripts and translated protein. This observation was independent of AAV capsid serotype, promoter elements and observed in case of both single stranded and self-complementary rAAV vectors. Interestingly, rAAV transduction leads to increased expression of HUSH complex members like TASOR and PPHLN1, implying a role for host sensing mechanisms. Interaction of NP220 with the rAAV genome and how this changes the epigenetic landscape is currently under investigation. Lastly, we show that pharmacological inhibition of deactylases enhances rAAV transgene expression. Our approach underscores the importance of understanding host cell mechanisms that can silence rAAV transgenes. Moreover, these findings provide a roadmap towards exploring druggable aspects of rAAV genome expression and engineering the rAAV genome to potentially circumvent silencing.

94. Effects of Sexual Dimorphism and Genetic Background on AAV Tissue Transduction in Mice Following Intravenous Administration of a Diverse Capsid Pool

Elad Firnberg, Jenny M. Egley, Chunping Qiao, Samantha A. Yost, April R. Giles, William M. Henry, Karolina J. Janczura, Justin Glenn, Devin S. McDougald, Kirk Elliot, Randolph Qian, Subha Karumuthil-Melethil, Ye Liu, Olivier Danos, Andrew C. Mercer
Gene Transfer Technologies, REGENXBIO, Inc., Rockville, MD

Introduction: The field of AAV gene therapy is quickly growing from treating small numbers of patients with rare diseases to more common clinical indications that affect larger populations. However, the literature on the effects of sexual dimorphism and genetic background on AAV transduction is limited to a small number of serotypes. In this study, we sought to characterize the tissue transduction properties of 118 pooled AAV capsids in male and female mice as well as male *mdx* mice (C57BL/10ScSn-*DMD*^{mdx}/J), a common model for Duchenne muscular dystrophy. **Results**: The AAV capsid pool consisted of 118 vectors, comprising both naturally occurring serotypes and engineered variants, with a barcoded CAG-GFP transgene. The AAV pool was administered by intravenous injection at a dose of 1.77 x 10^{13} GC/kg to female C57BL/6 (n=5), male C57BL/6 (n=5), and male *mdx* (n=5) mice, all six to eight weeks old. Vector genome copies (GC) per diploid cell and transcripts per µg RNA were quantified by digital PCR with primers for GFP and mouse glucagon as an endogenous genome control. Next generation sequencing was used to assess the relative transduction of all 118 capsids. Our results indicate that transduction differences by sex are tissue-specific and similar trends were observed across all AAV capsids and clades. In muscle tissues, we saw a 9-fold increase in GC/cell in the heart and 2-fold increase in gastrocnemius in female compared to male mice. By contrast, in liver we found a 2-fold increase in GC/cell in male compared to female mice. Male C57BL/6 mice had higher GC/cell in muscle tissues, including gastrocnemius, tibialis anterior, triceps, and diaphragm, compared to *mdx* mice. Surprisingly, this trend was highly reversed at the RNA expression level with a 24-fold increase in transcripts/µg RNA in *mdx* tibialis anterior, 8-fold in *mdx* gastrocnemius, and 2-fold in *mdx* heart compared to C57BL/6 male mice. In liver, both male C57BL/6 and *mdx* mice had similar GC/cell; however, C57BL/6 male mice had 3-fold higher transcripts/µg RNA compared to *mdx* mice. **Conclusions**: Differences in AAV gene transfer efficiency by sex or genetic background were found to be tissue dependent. Furthermore, trends in vector genome quantity and RNA expression level were not always concordant. Thus, both sex and genetic background of animal models should be considered in the development of AAV gene therapy.

95. High Throughput Screening of Diverse Mini-Promoter Libraries within AAV via Expression Linked Promoter Selection (ELiPS)

Kazuomori K. Lewis[1], Joost van Haasteren[1], David V. Schaffer[2]
[1]Bioengineering, UC Berkeley, Berkeley, CA,[2]Chemical and Biomolecular Engineering, Bioengineering, and Neuroscience, UC Berkeley, Berkeley, CA

The limited carrying capacity of the AAV genome (4.7 kb) necessitates the careful design of transgenes to maximize gene therapy efficacy and safety. A major design component of the transgene is the promoter, which dictates when, where, and how much of a therapeutic payload is expressed. Some applications require strong, ubiquitous expression, yet the common constitutive promoters - such as CMV (>750 bp) and CAG (>1600 bp) - are moderately sized and thus place limitations on the rest of the vector genome (e.g. Cas9). Truncated versions of these promoters are typically significantly weaker. Other applications call for cell or tissue specific expression, yet available specific promoters tend to be large and weaker in expression than constitutive promoters. We have developed a platform for the efficient generation of large (>10^7) libraries of synthetic promoters that can be functionally screened using AAV vectors for the high throughput selection of promoters based on their expression properties in cells or tissues of interest. We initially focused on engineering small (~200 bp), strong ubiquitous promoters for use in AAV-mediated gene delivery. To date, the largest screen of synthetic promoters in mammalian cells has been a microprinted array of 52,000 library members. We have developed a method termed ELiPS (Expression Linked Promoter Selection) in which synthetic promoters are built sequentially from small motifs in coordinated steps, allowing precise control of promoter size. ELiPS enables the construction of synthetic promoter libraries in which a barcode in the 3` UTR of the mRNA transcript is directly linked to the identity of the promoter that drove its expression, a design that is amenable to next generation sequencing analysis of promoter strength. Based on bioinformatic analysis of gene expression databases, we engineered two large libraries containing over 5x10^7 short (~200 bp) synthetic promoters intended for ubiquitous, AAV-mediated gene expression. Through just a single round of selection in an initial experiment in HEK 293T cells, we have identified a synthetic promoter of 218 bp termed ELiPS-L2-293T.1 (EL2T.1) that has 76% of the activity of the CAG promoter (1664 bp) and 82% of the activity of the CMV promoter (808 bp) - via flow cytometry, MFI_{CAG} 8570 ± 611, MFI_{CMV} 7985 ± 1128, $MFI_{EL2T.1}$ 6583 ± 1118. The expression level of EL2T is also not statistically significantly different from that of CMV ($p = 0.159$, two-tailed Student's t-test, unequal variance). This approach is currently being applied to screen for small, strong, constitutive promoters in human tissue models. Our ELiPS platform is thus highly effective in the generation and high throughput screening of large synthetic promoter libraries. We are now in the process of applying our platform technology to identify strong, short promoters in human *in vitro* culture models and furthermore anticipate its translation *in vivo* to derive tissue and cell-specific promoters.

96. GMEB2 is a Conserved Cellular AAV Restriction Factor That Inhibits Transduction of Human Stem Cells

Amanda M. Dudek, Nicole M. Johnston, Sriram Vaidyanathan, Sridhar Selvaraj, Matthew H. Porteus
Pediatrics, Stanford University, Palo Alto, CA

Cellular factors that inhibit AAV transduction are of great interest to identify areas of improvement for AAV-based gene therapies for both episomal AAV expression as well as AAV-mediated genome editing. Here, we have identified the P79 component of the parvovirus initiation factor (PIF) complex, GMEB2, as a highly conserved AAV restriction

factor. Knock-out of GMEB2 increases transduction in cell lines and primary cells across all tested AAV capsids including AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV9, and AAVDJ and at both high and low genome copy number, demonstrated by an up to 10-fold increase in GFP positive cells. At high transduction levels (above 90% GFP+ in WT cells), MFI is drastically increased demonstrating a potential of GMEB2 modulation to increase not only overall number of transduced cells, but also expression level within the cell. Increased transduction in GMEB2 KO cells is observed for constructs with a variety of promoters, such as CMV and UBC demonstrating that this restriction is specific to the AAV sequence. GMEB2 is highly expressed in primary cell types that are inefficiently transduced by AAV including human induced pluripotent stem cells and human airway stem cells. In contrast, CD4+ T cells and hematopoietic stem and progenitor cells (HSPCs) which are more efficiently transduced by AAV express little or no detectible GMEB2. Using a highly efficient multiplexed guide RNA/Cas9 strategy for knock-out, we demonstrate that loss of GMEB2 increases transduction in clinically relevant primary human airway bronchial stem cells which we have previously used for AAV-mediated genome editing as a corrective therapy for Cystic Fibrosis. Subcellular fractionation and western blot experiments demonstrate that although GMEB2 is reported to be a DNA-binding protein, restrictive cell types express high amounts of cytoplasmic and/or membrane associated GMEB2 rather than nuclear-localized GMEB2. Droplet digital PCR from knock-out cell fractions transduced with AAV demonstrate an increase in overall genome number in the lysates, while maintaining the overall genome distribution in cytoplasmic vs. membrane vs. nuclear fractions, suggesting that the restrictive effect of GMEB2 occurs at an early stage in the entry pathway. Additionally, in the presence of nucleofection the beneficial effect of GMEB2 KO is lost, suggesting that nucleofection at the time of AAV transduction may bypass the restrictive activity of GMEB2. These studies demonstrate a highly conserved AAV restriction factor which is capsid independent and influences the transduction efficiency of primary human stem cells.

97. Rationally Designed Inverted Terminal Repeats Improve AAV Vector Production

Liujiang Song[1,2], Zhenwei Song[1], R. Jude Samulski[1,3], Matthew L. Hirsch[1,2]

[1]Gene Therapy Center, University of North Carolina, Chapel Hill, NC,[2]Department of Ophthalmology, University of North Carolina, Chapel Hill, NC,[3]Department of Pharmacology, University of North Carolina, Chapel Hill, NC

Gene delivery approaches using Adeno-associated virus vector (AAV) are currently the leading method of human gene therapy. In all clinical AAV gene therapy applications, the only conserved viral DNA sequences in recombinant AAV (rAAV) are based on the inverted terminal repeats of serotype 2 (ITR2), which are required for multiple aspects of the AAV life cycle such as genome replication and encapsidation. Our laboratory has engineered a panel of functional synthetic ITRs (synITRs) via rational nucleotide substitutions and deletions of putative host factor binding sites. To assess the influence of ITR sequence variations, transgenic genomes flanked by ITR2 or synITRs were evaluated for rAAV production. Additionally, several biological characteristics were analyzed including: 1) the impact of the ITR sequences on rAAV genome replication, 2) the packaging efficiency and genomic integrity, and 3) overall rAAV titers. This study also considered the impact of the synITR sequences on inherent ITR transcriptional activity and exogenous promoter function following rAAV transduction. Derived data demonstrated a phenomenon of ITR sequence-dependency on rAAV yield, as well as the capsid:genome packaging ratio. Notably, vectors exhibiting synITR257, an ITR2 size-matched sequence in which selected guanine-cytosine rich regions were substituted with adenine-thymine nucleotides, showed a significant three-fold increase in vector production compared to ITR2-based rAAV as determined by probe-based qPCR, supported by alkaline gel electrophoresis, and capsid packaged ITR sequencing. The hypothesis of increased transgenic genome replication to account for the increased rAAV yield was refuted as results indicate that vector genomes employing synITR257 replicate less efficiently than ITR2-flanked genomes in production cells. This is the first evidence showing that transgenic genome replication is not a rate-limiting step for rAAV production using 293 triple transfection production methodology. Interestingly, synITR257 significantly enhanced the encapsidation efficiency of rAAV vectors determined by the capsid:genome ratio compared to ITR2. Furthermore, preliminary results suggest that the synITRs and ITR2 may have different inherent transcriptional activity following transduction with a promoter-less luciferase transgene cassette. However, in the context of a strong exogenous promoter, reporter activity was not significantly different between synITR257 and ITR2 following transduction of the evaluated cell lines. The collective data demonstrate that rAAV transgenic genome replication is not rate-limiting for rAAV production and that modulation of ITR sequences influence rAAV yield, potency, and thus represents another optimization step for enhanced and potentially safer AAV-based gene therapy drugs.

98. Characterization of AAV Inverted Terminal Repeats by Atomic Force Microscopy

Marianne Laugel*[1], Yeraldinne Carrasco-Salas*[2], Blandine Simon[2], Mathieu Mevel[1], Oumeya Adjali[1], Cendrine Moskalenko#[2], Magalie Penaud-Budloo#[1]

[1]INSERM UMR 1089, University of Nantes, Nantes, France,[2]UMR 5672, ENS de Lyon, Lyon, France

The rational design of a recombinant adeno-associated vector (rAAV) consists of removing the three open reading frames of the wild-type AAV and cloning a sequence of interest between the two inverted terminal repeats (ITR). Hence, ITRs are the only viral elements kept in the AAV vector genome. AAV ITRs are 143-167 nucleotides in length. These viral telomeres are composed of imperfect palindromic regions folding into a particular T-shaped structure. Essential for vector production, they contain the minimal elements required *in cis* for rAAV genome replication and encapsidation. With the numerous successes of gene therapy clinical trials and the commercialization of AAV-based drugs, there is a need for better understanding and characterizing these vectors. In particular, ITRs have been shown to be involved in cell-specific transcription, leading to renew interest in these fascinating sequences. In this work, we have developed a protocol allowing the visualization of AAV vector genomes by Atomic Force Microscopy (AFM), and specifically the assessment of ITR secondary structures. Single-stranded genomes flanked by wild-type AAV2 ITR were extracted from AAV vectors, incubated with Mg2+ and absorbed on a Mica surface for AFM imaging in air and in Peak Force mode. Length

and height of observed molecules were calculated using Matlab and Gwyddion programs. A majority of intact full-length rAAV genomes of approximatively 1.5 μm (4.5kb) were observed, validating our DNA extraction method. The presence of folded ITRs at the extremities of the vector genome was confirmed by height measurement. Moreover, three types of ITR secondary structures were found: a T-shape structure consistent with the most energetically stable structure predicted by RNAfold, a linear form consistent with inter-molecular annealing of unfolded ITRs and an intermediate folding conformation. Our study confirms that ITRs are dynamic structures that can adopt diverse folding structures. Controlling experimental parameters, our method could give new insights into the secondary structures of rAAV DNA, and particularly of ITRs, in different cellular compartments. Further investigations will be necessary to determine how these secondary structures could impact the rAAV molecular fate and DNA-triggered cellular responses.

Preclinical Gene Therapy for Neurologic Disorders II

99. A Novel Exon Specific U1 snRNA Therapeutic Strategy to Prevent Retinal Degeneration in Familial Dysautonomia

Anil Chekuri[1,2], Elisabetta Morini[3], Emily Logan[1], Aram Krauson[1], Monica Salani[1], Giulia Romano[4], Federico Riccardi[4], Franco Pagani[4], Luk H. Vandenberghe[4], Susan A. Slaugenhaupt[3]

[1]Center for Genomic Medicine, Massachusetts General Hospital Research Institute, Boston, MA,[2]Department of Neurology, Harvard Medical School, Boston, MA,[3]Department of Neurology, Harvard Medical School, Center for Genomic Medicine, Massachusetts General Hospital Research Institute, Boston, MA,[4]Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Familial dysautonomia (FD) is an autosomal recessive neurodegenerative disorder caused by a splice mutation in the gene encoding Elongator complex protein 1 (*ELP1*, also known as *IKBKAP*). A T-to-C base change in the 5' splice site of *ELP1* exon 20 results in exon 20 skipping with tissue specific reduction of ELP1 protein predominantly in central and peripheral nervous system. In addition to complex neurological phenotype, FD patients also exhibit progressive retinal degeneration severely affecting their quality of life. To test novel splicing-targeted therapeutic approaches, we developed a phenotypic mouse model of FD, *TgFD9; Ikbkap*$^{20/flox}$ which exhibits most of clinical features of the disease while displaying the same tissue specific mis-splicing observed in patients. Here, we report a thorough characterization of the retinas of our FD mouse using SD-OCT and immunohistochemical assays during disease progression. Our findings showed a significant decrease in the thickness of the retinal nerve fiber layer (RNFL) and the ganglion cell layer (GCL) starting from 3 months of age. Retinal whole-mount analysis showed reduction of RGC cell counts from 6 months of age. Histopathological analysis of the optic nerve from FD mice using neurofilament (NF) staining indicated diffuse degeneration of axonic bundles demonstrating that our mouse model correctly recapitulates the retinal degeneration observed in patients. To restore correct *ELP1* splicing defect and rescue retinal degeneration, we have designed a novel splice targeted therapy using modified version of the spliceosomal U1 snRNAs (ExSpeU1s) that permit targeted binding to intronic sequences downstream of the mutant 5' splice site enabling efficient recruitment of spliceosomal machinery. We have analyzed the efficiency of splicing correction of the ExSpeU1s through *in vivo* delivery using adeno associated vectors (AAV). Our findings suggest that our novel FD mouse model exhibit most of retinal degeneration pathology observed in FD patients. Our *in vivo* preliminary data demonstrate the valuable therapeutic potential of ExSpeU1 RNA delivery to treat retinal degeneration in FD.

100. Novel RNA-Targeting Gene Therapy Approach for Usher's Syndrome Type II Retinitis Pigmentosa

Daniel Gibbs, Rea Lardelli, Greg Nacthrab, Daniela M. Roth, Shawn Lee, Claire Geddes, Alistair Wilson, Nandini Narayan, Dimitrios Zisoulis, Ranjan Batra

Locanabio, Inc., San Diego, CA

Mutations in the USH2A gene are the most common cause of Usher Syndrome type II (USH2) and non-syndromic autosomal recessive retinitis pigmentosa (10-15% of ARRP). Mutations in exon 13 of USH2A gene are the most common mutations in USH2A gene (~16,000 patients in the North America and EU) and lead to loss of function of the very large (600KD) Usherin (USH2A) protein. USH2A isoforms lacking exon 13 lead to the production of a slightly truncated functional protein. Recently, genome-editing and antisense oligonucleotide (ASO)-based approaches have been described to target USH2A exon 13 at the DNA and RNA levels, respectively. Antisense oligonucleotides require recurrent delivery in the intravitreal space and DNA targeting entails the risk of permanent DNA-level off-target effects. Here we describe an RNA-targeting CRISPR/Cas13d gene therapy approach for skipping USH2A exon 13. Our *in vitro* data demonstrates that our candidate shows >98% efficacy in skipping exon 13 (alternative splicing) leading to production of the slightly shortened but functional Usherin protein. Furthermore, we packaged this candidate in a single AAV gene therapy delivery vector for one-time treatment. We screened various AAV serotypes including but not limited to AAV2, AAV5, AAV7, AAV8 and AAVrh10 to optimize delivery to the photoreceptors in wild-type mice. We also constructed another version of our potential therapeutic to skip mouse exon 12 (equivalent to the human exon 13) that requires two guide RNAs along with dCas13d, which shows dose-dependent and highly efficient exon 12 skipping *in vitro*. Finally, we show photoreceptor transduction, safety (4 and 8 weeks), and molecular efficacy (exon 12 skipping) of our AAV-packaged USH2A therapeutic candidate post sub-retinal delivery *in vivo* in mice. These findings support the development of RNA-targeting gene therapies using highly efficacious CRISPR/Cas13d exon skipping in USH2A for patients with exon 13 mutations.

101. Evolution of Modified AAV Vectors in Rhesus Macaque Cochlea

Paul T. Ranum[1], Stephen R. Chorney[2], Yong Hong Chen[1], Megan S. Keiser[1], Xueyuan Liu[1], Congsheng Chen[1], Geary Smith[1], Amy Muehlmatt[1], Luis Tecedor[1], Robert C. O'Reilly[2], Beverly L. Davidson[1,3]

[1]Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA,[2]Division of Pediatric Otolaryngology, The Children's Hospital of Philadelphia, Philadelphia, PA,[3]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Introduction: Gene therapy is a powerful tool to combat hearing loss and deafness, but its clinical implementation is impeded by the temporal bone anatomy, a high diversity of genetic lesions, and myriad cochlear sensory- and supporting-cell types. The identification of adeno-associated virus (AAV) capsids capable of efficient hair-cell transduction, including Anc80L65 and AAV2, has brought cochlear gene therapy closer to a clinical reality for some forms of genetic deafness. However, for genetic lesions impacting hearing at the level of cochlear supporting cells, additional vectors with varying tropism characteristics are needed. To meet this need we implemented a directed AAV evolution screen in the nonhuman primate (NHP) cochlea to identify a library of AAV capsid variants with diverse transduction properties capable of satisfying the requirements of current and future cochlear gene therapy designs. **Methods:** Peptide modified AAV libraries were first generated by insertion of random peptides into AAV1, AAV2, and AAV9. In vivo selection was performed in two successive enrichment steps in which our AAV capsid library was delivered to tissues of interest. Tissues were then recovered and capsid variants were extracted and used as the basis for the subsequent round of in vivo enrichment. At each round of enrichment NGS amplicon sequencing libraries were generated and sequencing data was used to track enrichment of each capsid configuration. Initially, our AAV capsid library was delivered to the NHP brain by Intracerebroventricular (ICV) injection. In this experiment we observed striking and unexpected transduction of NHP cochlear hair-cells and supporting cells by AAV9 derived capsid variant. This unexpected transduction of cochlear cells following ICV injection may occur via the fluid connection of CSF to the perilymph bathing the cochlear aqueduct. This finding prompted us to test direct injection of the AAV9 variant by round window membrane injection with lateral canal fenestration (RWM+LCF) and to undertake a separate cochlea-specific AAV enrichment study in which we delivered our AAV capsid library directly to the cochlea via the same RWM+LCF method. One cochlea received direct injection of 1999 and four cochlea (two NHPs) received injections of the AAV capsid library over the course of two rounds of in vivo enrichment. **Results:** Here we report the successful delivery and selection of modified AAV vectors in the cochlea of Rhesus macaque. Using a fluorescent reporter, we observed that the AAV9 variant transduces cochlear inner hair cells effectively, though incompletely, as well as an assortment of supporting cells including cells of the stria-vascularis and spiral ganglion neurons. Sequencing results from the directed cochlear AAV evolution strategy reveal an assortment of enriched capsid variants for which small-library- and fluorescence-based validation is ongoing. Given the remarkable similarity with the human cochlea, the Rhesus macaque animal model provides a tremendous opportunity to refine both delivery methods and identify AAV capsid variants with high potential for clinical applicability.

102. A Novel Retinal Gene Therapy Strategy for Batten Disease and Beyond

Maura Schwartz[1], Archana Jalligampala[2], Alex Campbell[3], Isabella Palazzo[3], Shibi Likhite[1], Andrew Fischer[3], Maureen McCall[2], Kathrin Meyer[1]

[1]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Kentucky Lions Eye Institute, Louisville, KY,[3]The Ohio State University, Columbus, OH

Batten Disease is a fatal, lysosomal storage disorder characterized by cognitive and motor deficits, vision impairments, and seizures. Loss of vision is a characteristic in 10 of the 13 Batten Disease subtypes. Our group has pioneered AAV9 gene therapy treatments that achieve widespread transduction of the brain and spinal cord. However, based on non-human primate data, AAV9 transduction of the retina after CSF delivery might be suboptimal. Thus, strategies to further improve retinal targeting using the same AAV9 gene therapy vector already in clinical trial could further improve Batten Disease gene therapy. Similar to most genetic ocular diseases, photoreceptor degeneration is the most commonly cited pathology in patients. However, recent studies suggest that in some subtypes of Batten Disease such as CLN3 and CLN6, expression must be rescued within cells of the inner nuclear layer (INL) of the retina to prevent vision loss. However, a full visual rescue was not achieved in mouse studies with INL targeting alone. We performed single-cell RNA sequencing in non-human primates to confirm CLN gene expression and found a generally ubiquitous, low expression in almost all cell-types of the retina. This suggests the need for a wide-ranging retina-tropic AAV that efficiently targets the INL, among other cell-types, which currently does not exist. We have recently discovered that administration of neuraminidase (NA), a sialidase enzyme, prior to or in combination with AAV9. GFP, drastically increases transduction through all the retinal layers in the murine and porcine retina. In mice, we delivered 11 mU of Neuraminidase prior to or in combination with $2e^{10}$vg AAV9.GFP. This manipulation resulted in an increase of GFP+ cells in the murine inner retina. Hand counts of GFP+ cells co-localized with cell-specific counterstains confirmed a 40% increase in AAV9 transduction in Müller glia, a 15% increase in amacrine cells, a 30% increase in bipolar cells, and a 78% increase in horizontal cells. Moreover, we translated this promising treatment paradigm to a WT porcine model. Intravitreal injections delivered 844mU of Neuraminidase prior to or in combination with $2e^{12}$ vg AAV9.GFP. Confocal imaging of retinal sections shows GFP expression in almost all, if not all, retinal cell types. This suggests that it may be possible to target every cell-type of the retina with a single AAV vector. This is especially important with respect to the treatment of Batten Disease and has many additional implications. Also, this strategy would be highly useful in the field of optogenetics, where there is a need for an efficient method to express light-sensitive opsins in cells of the INL to restore sight in individuals that have already lost their photoreceptors.

103. Reprogramming to Recover Youthful Epigenetic Information and Restore Vision

Yuancheng Lu[1], Benedikt Brommer[1], Xiao Tian[1], Anitha Krishnan[1], Margarita Meer[1], Chen Wang[1], Noah Davidsohn[1], George Church[1], Konrad Hochedlinger[1], Vadim Gladyshev[1], Steve Horvath[2], Morgan Levine[3], Meredith Gregory-Ksander[1], Bruce Ksander[1], Zhigang He[1], David Sinclair[1]

[1]Harvard Medical School, Boston, MA,[2]Department of Human Genetics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA,[3]Department of Pathology, Yale School of Medicine, New Haven, CT

Aging is a degenerative process that leads to tissue dysfunction and death. A proposed cause of aging is the accumulation of epigenetic noise that disrupts gene expression patterns, leading to decreases in tissue function and regenerative capacity. Changes to DNA methylation patterns over time form the basis of aging clocks, but whether older individuals retain the information needed to restore these patterns—and, if so, whether this could improve tissue function—is not known. Over time, the central nervous system (CNS) loses function and regenerative capacity. Using the eye as a model CNS tissue, here we show that ectopic expression of Oct4 (also known as Pou5f1), Sox2 and Klf4 genes (OSK) through AAV2 in mouse retinal ganglion cells restores youthful DNA methylation patterns and transcriptomes, promotes axon regeneration after injury, and reverses vision loss in a mouse model of glaucoma and in aged mice. The beneficial effects of OSK-induced reprogramming in axon regeneration and vision require the DNA demethylases TET1 and TET2. These data indicate that mammalian tissues retain a record of youthful epigenetic information—encoded in part by DNA methylation—that can be accessed to improve tissue function and promote regeneration in vivo.

104. Efficacious, Safe, and Stable Inhibition of Corneal Neovascularization with rAAV-*KH902* in a Mouse Model of Corneal Alkali injury

Wenqi Su#[1,2], Shuo Sun#[1,3], Bo Tian#[1], Phillip W. L. Tai[4], Jihye Ko[4], Yongwen Luo[4], Xiao Ke[5], Qiang Zheng[5], Hua Yan*[2], Guangping Gao*[4], Haijiang Lin*[1]

[1]Department of Ophthalmology & Visual Sciences, University of Massachusetts Medical School, Worcester, MA,[2]Ophthalmology, Tianjin Medical University General Hospital, Tianjin, China,[3]Tianjin Key Laboratory of Retinal Fuctions and Diseases, Tianjin International Joint Research and Development Centre of Ophthalmology and Vision Science, Eye Institute and School of Optometry, Tianjin, China,[4]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[5]Chengdu Kanghong Pharmaceuticals Group, Chengdu, China

Introduction: Corneal avascularity is an essential prerequisite of corneal transparency. Pathological conditions such as inflammation, hypoxia, and limbal barrier dysfunction disrupt the balance of pro- and anti-angiogenic factors. Under these conditions, corneal neovascularization (CoNV) can occur, causing a decrease in corneal transparency and visual impairment. CoNV develops in over 1.4 million patients in the United States per year. Among the well-studied anti-VEGF drugs in clinical testing, periodic injections of KH902 (trade name, conbercept) can inhibit ocular neovascularization. However, more efficacious and long-lasting treatment for patients with clinical CoNV is an urgent need. To this end, we investigated the long-acting anti-angiogenesis effects and safety of rAAV-delivered *KH902* in a mouse corneal injury model. **Methods:** In this study, we packaged the *KH902* transgene into rAAV vectors, and delivered them by single intrastromal or subconjunctival injections. The transduction efficiency of vectored *KH902* and its cell tropism in the cornea were determined by immunostaining. Droplet Digital PCR was used to quantitate *KH902* mRNA expression. Potential side effects were evaluated by measuring the central corneal thickness (CCT) at various time points by using optical coherence tomography (OCT). Immunostaining of corneal sections with CD11b and F4/80 was performed to assess immune responses two weeks post rAAV-*KH902* injection. Progression of CoNV in an alkali burn-induced model was tracked and quantified at 5 and 10 days, and 2, 3, 4, 8, and 12 weeks post-administration of rAAV-*KH902* by live animal imaging microscopy. Activation of DLL4/Notch signaling and ERK, downstream of effectors of VEGF signaling, were assessed by immunostaining and Western blot. **Results:** We demonstrate that rAAVs can successfully mediate KH902 expression in corneal keratocytes after intrastromal administration, while subconjunctival administration resulted in poorer transduction of keratocytes. *KH902* mRNA expression in the cornea reached peak levels at 1-2 weeks and then gradually declined. Nevertheless, *KH902* was still detectable at three months post intrastromal delivery. In addressing the safety of rAAV-*KH902* in the cornea, we found that high doses (1.6×10^{10} vg/eye) of rAAV-*KH902* triggered an intense immune response, while lower doses (8×10^{8} vg/eye) caused a minimal response. Based on this finding, we adopted the lower dose for our therapeutic studies in the corneal alkali-burn model for CoNV. We observed that rAAV-*KH902* conferred a dramatic suppression of CoNV for at least three months post-alkali burn without adverse events. In contrast, we observed that the inhibition of CoNV conferred by a single administration of the manufactured conbercept drug lasted only for 14 days post injection. Furthermore, the therapeutic outcomes produced by rAAV-*KH902* treatment were concomitant with the downregulation of DLL4/Notch signaling and ERK activation in the treated tissues. **Conclusion:** Our study demonstrates the potential and relative safety of rAAV-based, long-term anti-angiogenesis therapy with *KH902* in the treatment of CoNV. # The authors contributed equally to this work. * Corresponding authors.

105. Efficacy of a Vectorized Anti-Tau Antibody Using Systemic Dosing of a Blood Brain Barrier Penetrant AAV Capsid in Mouse Models of Tauopathies

Wencheng Liu[1], Jerrah Holth[1], Maneesha Paranjpe[1], Xiao-Qin Ren[2], Yanqun Shu[2], Giridhar Murlidharan[1], Charlotte Chung[1], Alex Powers[1], Emalee Peterson[1], Abigail Ecker[1], Usman Hameedi[3], Kyle Grant[3], Vinodh Kurella[2], Dillon Kavanagh[2], Omar Khwaja[1], Jay Hou[2], Steve Paul[1], Kelly Bales[1], Todd Carter[1]

[1]Neuroscience, Voyager Therapeutics, Cambridge, MA,[2]Vector Engineering, Voyager Therapeutics, Cambridge, MA,[3]Vector Production, Voyager Therapeutics, Cambridge, MA

Anti-tau immunotherapy is a promising therapy for tauopathies, including Alzheimer's disease (AD), frontotemporal dementia (FTD) and progressive supranuclear palsy (PSP). While passive immunization with anti-tau monoclonal antibodies has been shown by several laboratories to reduce age-dependent tau pathology and neurodegeneration in mouse tauopathy models, these studies have typically used repeated high weekly doses of antibody and reported only moderate reduction of tau pathology. We have previously demonstrated broad distribution and expression of a vectorized anti-tau antibody in the mouse brain using a blood-brain barrier (BBB) penetrant AAV capsid administered intravenously (IV). This gene therapy-based approach has potential advantages, including continuous expression of antibody in the central nervous system (CNS) after a single administration of vector, increased CNS levels of tau antibody relative to passive immunotherapy, and the potential to target intracellular tau aggregates. Here we describe studies characterizing the onset of transduction following dosing with a vectorized tau antibody and its efficacy in mouse models of tauopathy. AAV vectors comprising a novel capsid and a transgene encoding an anti-tau monoclonal antibody were administered by IV bolus to wild type mice. Biodistribution and cellular tropism in the CNS were evaluated by ELISA and (or) immunostaining, and vector genome levels were quantified using digital PCR, resulting in the detection of antibody expression soon after dosing. We then investigated efficacy in reduction of tau pathology in mouse tauopathy models. Treatment with our vectorized antibody resulted in durable antibody expression in the CNS and a corresponding reduction in CNS insoluble pathological tau and neurofibrillary tangles. Our results indicate that systemic dosing of a vectorized anti-tau antibody using a BBB-penetrant AAV capsid results in reduced tau pathology and may represent a new single-dose therapeutic strategy for treating various tauopathie

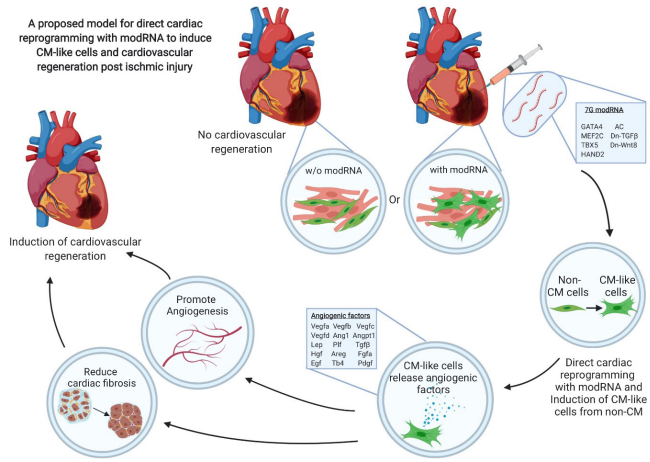
Synthetic/Molecular Conjugates and Physical Methods for Delivery

106. Combinatorial Modified mRNA Induces Cardiovascular Regeneration Post Muscle Ischemic Injury

Keerat Kaur[1], Asharee Mahmood[1], Hanna Girard[1], Ann Anu Kurian[1], Magdalena Zak[1], Maria Paola Santini[1], Elena Chepurko[1], Vadim Chepurko[1], Djamel Lebeche[1], Jason C. Kovacic[1], Roger J. Hajjar[2], Shahin Rafii[3], Lior Zangi[1]

[1]Cardiology, Icahn School of Medicine, Mount Sinai, New York, NY,[2]Phospholamban Foundation, Amsterdam, Netherlands,[3]Cardiology, Weill Cornell Medicine, New York, NY

Despite advances in the preventative medicine, ischemic heart disease remains a major cause of morbidity and mortality in the industrialized world, causing significant societal and economic burden. Post myocardial infarction (MI), the adult mammalian heart has limited regenerative capacity to recover from the loss of cardiomyocytes (CM). Thus, reprogramming non-cardiomyocytes (non-CMs) into cardiomyocyte (CM)-like cells in vivo is a promising strategy for cardiac regeneration. However, the current viral-based gene transfer delivery methods have low and erratic transduction efficiency that precludes these technologies from being translated to the clinic. Modified mRNA (modRNA)-based gene delivery features transient but potent protein translation and low immunogenicity, with minimal risk of insertional mutagenesis. Here we used a modRNA gene delivery platform to deliver different stoichiometry of cardiac-reprogramming genes (Gata4, Mef2c, Tbx5 and Hand2) together with reprogramming helper genes (Dominant Negative (DN)-TGFβ, DN- Wnt8a and Acid ceramidase (AC)), named 7G, to induce direct cardiac reprogramming post MI. In this study, we found that a combination of 7G modRNA cocktail can play a vital role in cardiac reprogramming. Compared to previously established Gata4, Mef2C and Tbx5 (GMT) reprogramming cocktail, 7G modRNA reprogrammed twice the number of non-CMs to CMs (from 28% to 57%) in vitro. Importantly, repeated 7G modRNA transfection results in beating CMs and complete cardiac reprograming *in vitro*. Using our lineage tracing model, we showed that one-time delivery of the 7G-modRNA cocktail at the time of MI partially reprogrammed ~25% of the non-CMs in the scar area to CM- like cells. Furthermore, 7G modRNA treated mice showed significantly improved cardiac function, longer survival, reduced scar size and greater capillary density than control mice 28 days post-MI. We attributed the improvement in heart function post modRNA delivery of 7G or 7G with increased Hand2 ratio (7G-GMT Hx2) to significant upregulation of 15 key angiogenic factors without any signs of angioma or edema. Intriguingly, 7G modRNA cocktail was also able to induce to neovascularization in mouse hindlimb ischemia model, indicating that 7G-modRNA cocktail administration promotes vascular regeneration in cardiac and skeletal muscle post ischemic injury. Thus, combinatorial, first of its kind 7G modRNA cocktails presents a safe and high efficiency gene delivery approach with therapeutic potential to treat ischemic diseases.



107. Sustained Episomal Transgene Expression *In Vivo* Driven by Non-Viral DNA Delivery to Rodent Liver

Stoil Dimitrov[1], Joe Sarkis[2], Kevin Smith[3], Ben Geilich[2], Sean Severt[2], Kevin Davis[4], Edward Acosta[4], Karen Olson[4], Molly Bell[4], Kristine Burke[4], Shan Naidu[4], Eric Jacquinet[4], Carla Leite[2], Andrew Auerbach[2], Nicholas Amato[2], Erik Owen[2], Jordan Santana[2], Tatiana Ketova[2], Brian Fritz[2], Christopher Tunkey[2], Bhargav Tilak[2], Jeffrey Pimentel[1], Mychael Nguyen[1], Susanna Canali[1], Jared Iacovelli[1], Eric Huang[1]

[1]New Venture Labs, Moderna, Cambridge, MA,[2]Platform Research, Moderna, Cambridge, MA,[3]Process Development, Moderna, Cambridge, MA,[4]Non-Clinical Sciences, Moderna, Cambridge, MA

Efficient non-viral delivery of exogenous DNA vectors *in vivo* has the advantage of decreased immunogenicity in comparison to current virally-mediated delivery approaches and largely increased payload capacity. The increased maximal size of the delivered exogenous DNA allows for the introduction of complex transcription units containing the gene of interest together with endogenous or tissue-specific promoter and enhancers, as well as additional mammalian transcription or stability control elements. Here we describe a successful non-viral, Lipid nanoparticles (LNP)-mediated delivery of non-integrating plasmid DNA vectors *in vivo* that drive hepatocytes-specific expression in rodents and allow for long-term stable episomal expression of a transgene. Plasmid DNA-mediated delivery of Firefly Luciferase gene, under control of the liver-specific Alpha-1 Antitrypsin (AAT) promoter, resulted in stable, steady-state Luc expression in mouse hepatocytes for six weeks until study termination. LNP-mediated delivery of plasmid DNA encoding a secreted Fc fragment of human IgG1, driven by the liver-specific AAT promoter, resulted in sustained, dose-dependent expression of the secreted hIgG-Fc protein that lasted for six months in Sprague-Dawley rats. Importantly, plasmid DNA-mediated liver-specific transgene expression resulted in mild, transient elevation of ALT and AST six hours after dosing, which resolved by 48h post-delivery. No other changes in the comprehensive blood biochemistry profile of the test subjects were detected. We have observed transient, dose-dependent activation of Type I interferon response after plasmid DNA delivery *in vivo*. In both mouse and rat serum, levels of IFN-alpha, IP-10, MCP-1, MCP-3 and RANTES were transiently elevated at 6h post-delivery and decreased 24h to 48h post dosing. In conclusion, we have demonstrated that LNP-mediated delivery of DNA vectors *in vivo* can lead to long-term, stable tissue-specific expression of both intracellular or secreted proteins in mouse and rat hepatocytes. Expression of transgenes can last for at least 24 weeks. LNP-mediated DNA delivery resulted in mild and transient elevation of liver enzymes and serum inflammatory cytokines.

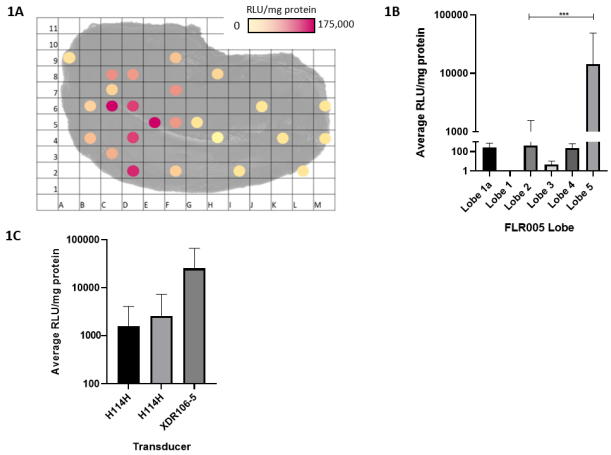
108. Optimization of Transcutaneous Ultrasound Mediated Gene Delivery into Large Animals

Megan A. Manson[1], Feng Zhang[1], Carley M. Campbell[1], Peter Kim[1], Kyle P. Morrison[2], Maura Parker[3], Keith R. Loeb[3], Dominic Tran[1], Masaki Kajimoto[4], Rainier Storb[3], Carol Miao[1]

[1]Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]Sonic Concepts, Inc., Bothell, WA,[3]Fred Hutchinson Cancer Research Center, Seattle, WA,[4]Seattle Children's Research Institute, Seattle, WA

Hemophilia A is an X-linked clotting disorder characterized by a deficiency of functional factor VIII (FVIII) protein. In previous experiments, we performed transcutaneous ultrasound mediated gene delivery (UMGD) of a FVIII plasmid into normal canines with success using the H114H focused transducer. In order to increase the treatment area, we have recently developed a new transducer, XDR106-5. This transducer is a 5-element, unfocused transducer that leverages both PNP output to circumvent tissue attenuation, as well as a larger treatment area for effective gene delivery. The unique 5-element configuration allows the user to easily scan the transducer to cover a greater treatment area as opposed to the H114 focused transducer, which has a small area of focus. In preparation for future experiments in hemophilia A canines, we aimed to optimize the surgical protocol and ultrasound parameters and tested an improved transducer design. A pGL-4 plasmid encoding the luciferase reporter gene was injected in combination with RN18 microbubbles (MBs) into the canine liver lobes through a fluoroscopy-guided balloon catheter (either a 12 mm x 20 mm or a 14 mm x 20 mm balloon) inserted into the hepatic vein via jugular vein access. Simultaneously, the transcutaneous therapeutic ultrasound, either H114H or XDR106-5, was applied to induce cavitation in the liver using a pulsed US treatment. Blood was collected at 24 hours and assessed for alanine and aspartate aminotransferase levels. Animals were sacrificed on day one, and liver tissue was harvested and divided into 1 cm x 1 cm sections and subjected to luciferase and Bradford assays. Representative sections were selected from each liver lobe and subjected to H&E staining and reviewed by a certified pathologist. Hemophilia A dogs tend to be larger and therefore have larger blood vessels, so we tested a new balloon catheter in a normal canine of comparable size (22.5 kg). The 12 mm x 20 mm balloon catheter was used in lobe 2, while the 14 mm x 20 mm balloon catheter was used in lobe 5. Lobe 5 demonstrated high expression (Figure 1A) and had significantly higher luciferase expression compared to lobe 2 (Figure 1B). Liver histology showed little change in the liver on day one following the procedure, with no significant injury noted. ALT and AST values were elevated, but at a comparable level to canines from previous experiments, and no veterinary concern was noted. Preliminary data from pig experiments showed that the H114H and XDR106-5 transducers induced similar levels of luciferase expression (RLU/mg protein) in individual sections. However, the XDR106-5 transducer allowed a much larger area of the lobe to be treated. This led to a tenfold increase in average RLU/mg protein per lobe: 2089 RLU/mg on average when treated with H114H and 25486 RLU/mg when treated with XDR106-5 (Figure 1C). We are able to successfully optimize the transcutaneous UMGD procedure in

canines by adjusting the surgical procedure for larger canines, and by incorporating a new ultrasound transducer which effectively treats a larger proportion of a targeted lobe.



109. Assembling Several mRNA Strands for Facilitating mRNA Delivery with and without Using Carriers

Satoshi Uchida[1,2], Naoto Yoshinaga[3], Kyoko Koji[3], Horacio Cabral[3]

[1]Kyoto Prefectural University of Medicine, Kyoto, Japan,[2]Innovation Center of NanoMedicine (iCONM), Kawasaki Institute of Industrial Promotion, Kawasaki, Japan,[3]The University of Tokyo, Tokyo, Japan

For developing messenger RNA (mRNA) delivery carriers, it is still challenging to prevent enzymatic degradation before reaching target cells. Although complicated design of polymers or lipids has contributed to solve this issue to some extent, use of such novel materials requires tremendous processes before clinical approval. Herein, we propose a simple strategy of modulating mRNA steric structure that improves nuclease resistance and delivery efficiency of naked mRNA and existing mRNA carriers. To prevent nuclease attack to mRNA by steric hindrance, we structured mRNA by preparing nanoassemblies (NAs) from several mRNA strands, which were crosslinked with each other using RNA crosslinkers hybridizing to mRNA. NAs thus prepared had about 8 mRNA strands and diameter below 100 nm on average. NAs showed around 100-fold increase in nuclease stability compared to naïve mRNA in their naked form. Further mechanistic studies of NAs indicated the contribution of their high-order structure, as well as secondary structure, on the improvement of nuclease stability. Interestingly, NAs retained their translational activity despite their high-order structure, which prevented nuclease attack. Förster resonance energy transfer-based observation of NA structure in intracellular environment indicates that NAs were dissociated selectively in intracellular environment through endogenous RNA unwinding mechanisms coupled with 5′ cap dependent translation. Ultimately, NAs improved mRNA introduction efficiency in their naked form after transfection to culture cells and to mouse brain. Next, we studied the potential of NAs to improve functionality of an existing mRNA carrier. Polymeric micelles (PMs) from poly(ethylene glycol) (PEG)-polylysine with a core-shell structure of PEG shell and mRNA-containing core were selected because polylysine is widely used for complexing nucleic acids. Furthermore, PMs loading plasmid DNA were used in a clinical trial. Both PMs loading naïve mRNA and NAs showed average size below 100 nm, with almost neutral ζ-potential. Further physicochemical evaluations including transmission electron microscopic observation and analytical ultracentrifuge revealed that PMs loading NAs possessed a shell with denser PEG chains and a core with more tightly packaged mRNA compared to PMs loading naïve mRNA. As a result, use of NAs in PMs boosted mRNA nuclease stability in serum and in blood circulation after intravenous injection to mice compared to PMs loading naïve mRNA. Ultimately, PMs loading NAs yielded efficient mRNA introduction after transfection to culture cells and to mouse brain. Importantly, PMs loading NAs were stably distributed to whole brain after injection to cerebrospinal fluid, leading to efficient protein expression in wide area of mouse brain. Notably, the utility of NA formulation in naked and PM-loaded forms was demonstrated using several types of mRNA. Conclusively, our strategy effectively improves the functionalities of naked mRNA and existing mRNA carriers, just by changing mRNA structure without the use of additional materials, providing a versatile platform for future clinical applications.

110. Development of Hydrodynamics-Based Gene Therapy for Liver Cancer

Kenya Kamimura[1], Takeshi Yokoo[1], Hiroyuki Abe[1], Masato Ohtsuka[2], Hiromi Miura[2], Hiroshi Nishina[3], Shuji Terai[1]

[1]Niigata University, Niigata, Japan,[2]Tokai University, Isehara, Japan,[3]Tokyo Medical and Dental University, Tokyo, Japan

Liver cancer is one of the leading causes of cancer-related deaths worldwide. The primary liver cancer is mainly hepatocellular carcinoma (HCC) and currently available therapeutic options, including embolization and chemotherapy, are not effective against advanced-stage liver cancer due to its heterogeneity, high risk of recurrence, and drug resistance. Although recent developments in the field of molecular-based strategies shed light on chemotherapy for HCC using molecular targeting agents, these agents also have limitations due to the heterogeneity of HCC. Therefore, it is essential to develop a novel therapy, and in this context, we evaluated the gene therapy for HCC using a diphtheria toxin fragment A (DTA) gene-expressing plasmid using a nonviral hydrodynamics-based procedure in this study. The complementary DNA of DTA was inserted into the pIRES2 plasmid vector containing an internal ribosome entry site. The expression of DTA was regulated by a chicken β-actin promoter and a cytomegalovirus enhancer (pCAG-DTA) or the human alpha-fetoprotein (AFP) promoter (pAFP-DTA). The antitumor effect of DTA expression in HCC cell lines including HLE, HLF, and Huh7 and AFP promoter selectivity was evaluated *in vitro* by examining HCC cell growth. We then examined the *in vivo* effect and safety of the AFP promoter-selective DTA expression using an HCC mouse model established by the hydrodynamic gene delivery of the yes-associated protein (YAP)-expressing plasmid. Mice with the YAP-expressing plasmid demonstrated an HCC incidence of >80% and expression of AFP protein. First, we confirmed the effect of DTA on the inhibition of protein synthesis by the cells based on the disappearance of tdTomato protein expression and GFP protein expression cotransfected upon the delivery of the DTA plasmid. The proliferation of HCC cell lines was

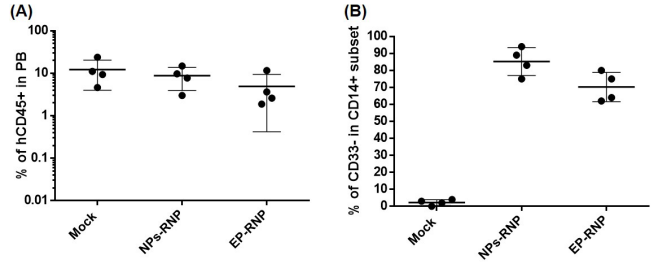
significantly inhibited to <20% upon DTA expression in HCC cells in an AFP promoter-selective manner. In addition, concentration of AFP in the cell culture medium was significantly inhibited by pAFP-DTA transfection. Moreover, a significant inhibition of HCC incidence of 80% to 10%; and a suppression of the AFP tumor marker from 13,448.2 ± 8787.2 to 193.5 ± 129.5 ng/mL and des-gamma-carboxy prothrombin from 590.6 ± 306.7 to 70.4 ± 37.7 ng/mL were evidenced in mice treated with hydrodynamic gene delivery of DTA within 2 months after YAP gene delivery. Serum biochemical factors revealed improvement of hepatobiliary enzymes after the DTA gene therapy due to the shrinkage of the tumor, and no adverse events were observed. These results indicate, for the first time, the effect and safety of hydrodynamics-based gene delivery of DTA for HCC in an *in vivo* mouse model.

111. Hematopoietic Stem and Progenitor Cells-Targeted Polymeric Nanoparticles for In Vivo Gene Therapy

Rkia El Kharrag[1], Kurt Berckmueller[1], Margaret Cui[1], Ravishankar Madhu[1], Anai Perez[1], Olivier Humbert[1], Hans-Peter Kiem[1,2], Stefan Radtke[1]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA,[2]Department of Medicine, University of Washington School of Medicine, Seattle, WA

Autologous hematopoietic stem and progenitor cell (HSPC) gene therapy has the potential to permanently cure millions of patients suffering from hematological diseases and disorders. However, current approaches depend on expensive specialized cleanrooms for the manipulation of HSPCs *ex vivo* limiting the feasibility and routine clinical application. Consequently, HSPC gene therapy remains inaccessible to many patients especially in infrastructure poor regions. HSPCs-targeted *in vivo* delivery of gene therapy agents could overcome this bottleneck enhancing the portability of gene therapy by avoiding expensive infrastructure. With the aim to perform HSPCs-targeted gene therapy *in vivo*, we adapted a biodegradable poly (β-amino ester) (PBAE)-based nanoparticle (NP) formulation with targeting ability for the modification of HSPCs. We benchmarked the NP-based delivery approach against our existing electroporation (EP)-mediated CRISPR/Cas9 gene-editing strategy to knockout CD33 on HSPCs and protect them from anti-CD33 targeted acute myeloid leukemia immunotherapy. NPs were generated with Cas9 ribonucleoprotein (RNP) complexes and loaded NPs evaluated for size and charge to correlate physiochemical properties with the outcome, and establish quality control standards. NPs and EP gene-edited $CD34^+$ cells were evaluated phenotypically by flow cytometry and functionally in colony-forming cell (CFC) assays, and in NSG xenograft model. The optimal characteristics for RNP-loaded NPs were determined at 150-250 nm and 25-35 mV. Physiochemical assessment of RNP-loaded NP formations provided an upfront quality control of RNP components detecting degraded components significantly and enhancing the predictability and success of editing. NPs achieved more than 85% CD33 knockout using 3-fold lower dose of CRISPR nucleases compared to EP. No impact on erythromyeloid differentiation potential of gene-edited cells in CFC assays was observed. Most importantly, NP-modified CD34+ cells showed efficient and sustained gene editing in *vivo* with improved long-term multilineage engraftment potential in peripheral blood (PB) and bone marrow stem cells in comparison to EP-edited cells **(Fig. A,B)**. Here, we show that PBAE-NPs enable efficient CRISPR/Cas9 gene editing of human CD34+ cells without compromising the viability and long-term multilineage engraftment of human HSPC *in vivo*. Most importantly, we defined physiochemical properties of PBAE-NPs that enable us to not only determine the integrity of our gene-editing agents, but also predict the efficiency on long-term engrafting HSPCs. The requirement of 3-fold less reagents compared to EP, the ability to lyophilize quality-controlled and ready to administer gene therapy reagents, and the opportunity to engineer the surface of PBAE-NPs with HSPC targeting molecules are the technological foundation for feasible, portable, and safe *in vivo* HSPC gene therapy. Ease of production and versatility of cargo options will hopefully enable the field of *in vivo* gene therapy to make a significant step towards a more accessible and portable gene therapy platform. **Figure. A)** Human CD45+ engraftment and **B)** CD33 knockout percentage in CD14+ subset in PB of mice transplanted with CD33 gene-edited CD34+ cells at week 14 post transplantation.



112. Delivery of CRISPR/Cas9 for Recovering the Expression of the Endogenous FVIII in Hemophilia A Mice

Chun-Yu Chen[1], Xiaohe Cai[1], Carol H. Miao[1,2]

[1]Immunity & Immunotherapies, Seattle Children's Hospital Research Institute, Seattle, WA,[2]Department of Pediatrics, University of Washington, Seattle, WA

Hemophilia A (HemA) is a bleeding disorder resulting from a deficiency of the X-linked factor VIII (FVIII) gene. Currently HemA patients is routinely infused with FVIII protein 3-4 times per week as prophylactic treatment, which is costly and inconvenient. Gene therapy represents a very promising alternative method to treat HemA patients. Advancement of biocompatible nanoparticle (NP) technology enabled delivery of nucleic acids safely into the targeted organs. In combination of CRISPR/Cas9 technique, *in vivo* gene edition to correct the mutant FVIII and regain the expression of FVIII is feasible. We examined the *in vitro* gene delivery efficiency of DNA encapsulated NPs and *in vivo* FVIII gene editing, respectively. Since FVIII protein is mainly and naturally made in liver sinusoidal endothelial cells, we synthesized NPs that can selectively target endothelial cells. DNA encapsulated NPs was synthesized using nanoprecipitation by the combination of an organic phase containing chondroitin sulfate and sorbitan ester and an aqueous phase containing p2X-GFP plasmid DNA. DNA encapsulation efficiency was examined by DNA electrophoresis. By titrating different DNA:NPs ratios, our results showed that NPs can carry plasmid DNA efficiently at saturating concentrations.

Next, we used HUVEC cells as endothelial cell model to evaluate the transfection efficiency of DNA encapsulated NPs. The transfected cells were analyzed by flow cytometry after transfection using NPs carrying p2X-GFP plasmid. GFP expression was detected in ~26% of the cells, indicating NPs/DNA can successfully transfect HUVEC cells. Next, we evaluated the efficacy of different sgRNAs for *in vivo* gene editing of mutant FVIII gene by hydrodynamic injection of Cas9/sgRNA plasmid into HemA mice. An immunodeficient hemophilia A (NSG HA) mice that contained premature stop codon in exon 1 of FVIII were used as the HemA mouse model. Two different sgRNAs that can edit wild type FVIII sequence (mF8sgRNA) or mutant FVIII exon 1 sequence (NSGHAsgRNA) were designed, respectively. Efficiency of gene editing was estimated *in vitro* by T7E1 assay using mouse embryonic fibroblast NIH3T3 cells. The results showed that mF8sgRNA can specifically induce double-strand breaks in wild type FVIII gene of NIH3T3 cells. The Cas9/sgRNA plasmids were hydrodynamically injected to NSG HA mice and the FVIII expression was examined by aPTT assay after one week. Both FVIII-targeting sgRNAs can promote the recovery of FVIII expression in NSG HA mice, suggesting the successful gene editing in mutant FVIII. After injection of Cas9/NSGHA sgRNA expression plasmid, FVIII activities maintained at least one month after treatment. Our data showed high transfection efficiency of DNA encapsulated NPs in HUVEC cells. Furthermore, we investigated *in vivo* gene editing using CRISPR/Cas9 technology to correct the mutated FVIII gene and regain the expression of FVIII protein in NSG HA mice. Our future goal is to use NPs that carry Cas9/sgRNA plasmid to correct the mutant FVIII gene in NSG HA mice.

Targeted Gene and Cell Therapy for Cancer

113. Gene-Based Immune Reprogramming Overcomes the Immunosuppressive Microenvironment of Liver Metastases and Enables Protective T Cell Responses

Thomas Kerzel[1], Federica Pedica[2], Stefano Beretta[1], Eloise Scarmadella[1], Tamara Canu[3], Rossana Norata[2], Lucia Sergi Sergi[1], Marco Genua[1], Renato Ostuni[1], Anna Kajaste-Rudnitski[1], Antonio Esposito[3], Masanobu Oshima[4], Francesca Sanvito[1], Mario Leonardo Squadrito[1], Luigi Naldini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]IRCCS San Raffaele Hospital, Milano, Italy,[3]Experimental Imaging Center, San Raffaele Scientific Institute, Milano, Italy,[4]Division of Genetics, Cancer Research Institute, Kanazawa University, Kanazawa, Japan

The liver hosts an immune suppressive environment, which favors metastatic seeding and proliferation of cancer cells. Current pharmacological treatments, including most recent immunotherapies, fail in the presence of liver metastases (LMS). Therefore, identifying new interventional tools to break tumor tolerance and unleash immune responses in patients with LMS is of pivotal importance. Our group has shown that vesicular stomatitis virus G protein-pseudotyped lentiviral vectors (LVs) delivered systemically to mice and non-human primates efficiently target and transduce liver cells, including liver resident macrophages termed Kupffer cells (KCs). Building on these findings, we developed a novel LV-based platform, termed KC-LV, to selectively engineer KCs in vivo with the goal of delivering therapeutic molecules specifically to LMS. To this aim, the KC-LV design exploits a reconstituted mannose receptor c type 1 (MRC1) promoter, active in macrophages, including KCs. To further fine-tune KC specificity, the KC-LV also includes microRNA target (miRT) sequences that prohibit LV transgene expression in liver sinusoidal endothelial cells and hepatocytes. We observed that systemic delivery to mice of a KC-LV driving the fluorescent marker GFP resulted in transgene product expression in KCs, but not in other liver cell types or organs, such as brain, gut, lung, lymph nodes and bone marrow. In mice bearing LMS, GFP expression was enriched in the tissue rim surrounding LMS. To leverage KC-LV as a therapeutic platform we included a sequence encoding for interferon a (IFNα), a cytokine with pleotropic immune function. Long term analysis of mice treated systemically with IFNα KC-LV showed a rapidly established, vector dose-dependent and sustained IFNα expression, with no signs of hepatotoxicity, neutropenia or macroscopic skin reactions. IFNα KC-LV systemically delivered to mice challenged with colorectal cancer LMS, either obtained from cancer cell lines or organoids, significantly delayed tumor growth and achieved, in some mice, a complete response. Furthermore, mice that completely cleared LMS were refractory to rechallenge with matched cancer cells indicating persisting adaptive immune protection. Single cell RNA sequencing analysis of LMS revealed upregulation of IFN-responsive genes and altered activation/polarization profile in tumor infiltrating host cells in mice treated with IFNα KC-LV indicating robust immune reprogramming of the LMS microenvironment. In particular, IFNα KC-LV promoted macrophage skewing from a protumoral (M2-like) to an antitumoral (M1-like) polarization state and expansion of LMS specific CD8 T cells. In summary, we have developed an innovative gene-based platform that upon a single well-tolerated intravenous LV infusion can rapidly establish a protective response against mouse LMS through promotion of macrophage reprograming and adaptive immune activation.

114. Inducible Tumor-Targeted Interferon-α Gene Therapy Inhibits Glioblastoma Multiforme in Mouse Model without Adverse Systemic Effects

Filippo Birocchi[1,2], Melania Cusimano[1], Federico Rossari[1,2], Stefano Beretta[1], Anna Ranghetti[1], Marco Genua[1], Renato Ostuni[1,2], Stefano Colombo[1], Ivan Merelli[1], Paola M. V. Rancoita[1,2], Barbara Costa[3], Angel Peter[3], Nadia Coltella[1], Luigi Naldini[1,2]

[1]SR-TIGET San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]Vita-Salute San Raffaele University, Milano, Italy,[3]German Cancer Research Center (DKFZ), Heidelberg, Germany

Background: The pleiotropic immunostimulatory effects of cytokines such as interferon alpha (IFN-α) have been extensively investigated in cancer. However, systemic administration leads to sharp oscillations in cytokine plasma levels and OFF-target toxic effects, which strongly

limit clinical application. The possibility to specifically target cytokines to the tumor establishing a local source that maintains physiological-range concentrations may thus open up new therapeutic perspectives. To this aim, our group has exploited a population of tumor-associated TIE2 expressing macrophages (TEM) to deliver IFN-α specifically to tumors through a cell and gene therapy approach. Transplantation of hematopoietic stem cells (HSCs) transduced with lentiviral vector (LV) expressing *Ifna1* under the control of the *Tie2* promoter induces TEM-specific release of the cytokine in the tumor microenvironment (TME), reprogramming it towards immunological activation.Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor. The unsuccessful results of current therapies make GBM treatment an unmet clinical need. Its strongly immunosuppressive TME and the poor drug delivery across the blood brain barrier, make GBM a suitable candidate for testing our cell-based gene therapy. **Methods:** Mice were transplanted with HSCs transduced with *Tie2-Ifna1* LV. To further improve our platform, we developed an inducible strategy based on fusing destabilizing domains (DD) to the cytokine, thus allowing control of the timing and amount of IFN-α secreted in the TME. Both strategies were tested in a new syngeneic mouse model of GBM, called mGB2, which closely recapitulates several features of the human pathology. **Results:**Upon GBM challenge, mice transplanted with *Tie2-Ifna1* LV transduced HSCs showed remarkable tumor inhibition and improved survival over controls. Notably, a fraction of mice cleared the tumor and survived long term suggesting induction of protective immunity. Similar results were obtained with the inducible platform. The tumor-targeted loco-temporal regulated release of IFN-α induced anti-cancer effect in stringent therapeutic settings, when it was switched ON in already established tumors, and was associated with more effective, durable and safer profile compared to systemic treatment with recombinant IFN-α. Single-cell RNA sequencing and FACS analysis of the tumor infiltrate revealed substantial activation of IFN responsive genes in monocyte/macrophages, B cells and dendritic cells, accompanied by decreased exhaustion of T cells. Moreover, IFN-α gene therapy treatment polarized tumor-associated macrophages (TAMs) towards a pro-inflammatory phenotype, while impairing the recruitment and/or induction of an immunosuppressive subset of TAM, whose gene signature was found to have negative prognostic value in the human disease. **Conclusions:** These results indicate that IFN-α gene therapy can reprogram the GBM TME and stimulate anti-cancer immune response. The promising pre-clinical results obtained so far from our group led to the registration of the first drug based on this strategy, named Temferon®, which is currently in phase I/II clinical trial in our Institute for the treatment of patients with GBM. Our data also demonstrate the feasibility of inducible and intra-tumoral targeted delivery of an immune activating cytokine, thus opening the way to broader application to other cytokines and tumor types.

115. Design and Demonstration of Potent In Vitro and In Vivo Activity for CART-ddBCMA, a BCMA-Targeted CAR-T Cell Therapy Incorporating a Non-scFv Binding Domain

Janine M. Buonato, Justin Edwards, Liubov Zaritskaya, Ankit Gupta, Laura Richman, David Hilbert, David LaFleur, David Tice

Arcellx, Giathersburg, MD, MD

Chimeric Antigen Receptor (CAR) T cell therapies directed against B-cell maturation antigen (BCMA) have demonstrated compelling clinical activity and manageable safety in subjects with relapsed and refractory Multiple Myeloma (RRMM). Prior reported CAR-T cells have encoded humanized or murine scFvs, or camelid heavy chain antibody fragments with 41BB or CD28 co-stimulatory domains. Herein, we describe the generation and preclinical evaluation of CART-ddBCMA, an anti-BCMA CAR-T cell therapy encoding a non-scFv, synthetic binding domain targeting BCMA with a 4-1BB costimulatory motif and CD3-zeta T cell activation domain. The BCMA binding domain was discovered from a phage display library of D domains based on a ~8 kD highly stable scaffold and engineered for reduced immunogenicity. A human tissue cross-reactivity study using the BCMA binding domain as the test article demonstrated that the binding domain was specific for BCMA. Preclinical in vitro studies of CART-ddBCMA cocultured with BCMA-positive cell lines demonstrated highly potent, dose-dependent measures of cytotoxicity, cytokine production, T cell degranulation, and T cell proliferation. Furthermore, in each assay CART-ddBCMA performed as well as T cells incorporating the BCMA-directed scFv-based C11D5.3 CAR. CART-ddBCMA demonstrated in vivo efficacy in a disseminated BCMA-expressing tumor model in NSG™ immunocompromised mice. Mice engrafted with 5×10^6 U266 cells and grown for 37 days rapidly cleared tumor burden within 7 days following injection of 4.5×10^6, 1.5×10^6, or 0.5×10^6 CART-ddBCMA cells, and tumor clearance was sustained throughout the duration of the experiment. The level of CART-ddBCMA cells detected in circulation 14 days post-T cell transfer was dependent on the initial dose, and at each dose CAR expression was maintained in 90+% of hCD3+ T cells. Based on these promising preclinical data CART-ddBCMA is being studied in a first-in-human phase I clinical study to assess the safety, pharmacokinetics, immunogenicity, efficacy, and duration of effect for patients with RRMM (NCT04155749).

116. A SOX2 Engineered Epigenetic Silencer Factor Represses the Cancer Genetic Program and Eradicate Glioblastoma Development

Vania Broccoli[1], Alessandro Sessa[1], Federica Banfi[1], Valerio Benedetti[1], Mattia Zaghi[1], Luca Massimino[2], Simone Bido[1], Laura Argelich[1], Serena Giannelli[1], Antonio Niro[1]

[1]San Raffaele Scientific Institute, Milano, Italy,[2]Humanitas University, Milano, Italy

Glioblastoma multiforme (GBM) is the most common and lethal brain cancer in adults (1-5 cases per 100,000 people per year, 12-15 months of median survival). This is due, in addition to the aggressiveness of the disease, to the inefficacy of current therapies to substantially

increase the overall survival. Patients usually undergo tumor resection followed by adjuvant radio- and chemo-therapies that can't prevent recurrences. It has been proposed that some stem cell-like cancer cells (CSCs) with tumor-initiating potential remain in preserved tissue, resist the adjuvant treatments, leading to the re-appearance of the disease. By rational engineering of the SOX2 cancer-associated transcription factor, we generated a synthetic epigenetic repressor named as SOX2 Epigenetic Silencer (SES) that maintains the ability to bind to a large group of its original targets inducing their long-term silencing. By deleting the C-terminal domain of SOX2 and fusing two epigenetic repressor domains, SES is able to effectively inhibit the SOX2 tumorigenic molecular network (rather than activating it as the original TF), including genes crucial for tumor maintenance and growth. By doing this, SES kills both glioma cell lines and patient-derived CSCs both *in vitro* and *in vivo*. Following *in situ*viral transduction of GBM xenografts in mice, we have demonstrated that SES induced a strong growth inhibition of pre-formed tumor of human origin. Remarkably, we have also proved that SES is not harmful to neurons and glia and does not trigger significant transcriptional changes in these cells. Nevertheless, we equipped the vector for our anti-cancer therapy with either a cell-cycle specific promoter or a micro-RNA binding sequence which restricts SES expression exclusively in cancer cells. These data suggest that in situ viral delivery of SES can target the residual cancer cells spread in the tissues and inhibit their growth and survival while leaving unaffected brain parenchyma cells. This approach enables the development of synthetic epigenetic silencers by engineering oncogenic transcription factors that can repress entire tumorigenic transcriptional pathways that are critical for supporting cancer development. Our strategy paves the way to an unprecedented therapeutic opportunity for the treatment of aggressive brain tumors endowed with high levels of efficacy and safety.

117. Oncolytic Adeno-Immunotherapy Expressing IL-12p70 and Immune Checkpoint Blockade PD-L1 Minibody Modulates the Host Immune System to Enable HER2.CAR T-Cells to Cure Pancreatic Tumors

Amanda Rosewell Shaw, Caroline Porter, Tiffany Yip, Way Champ Mah, Katie McKenna, Matthew Dysthe, Robin Parihar, Malcolm Brenner, Masataka Suzuki

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Oncolytic Adenoviral vectors (OAds) encoding immunomodulatory transgenes ("Armed"OAds) have shown promise as cancer immunotherapy agents. To overcome the limited transgene capacity of OAds, we have combined OAd with a Helper-dependent Ad (HDAd) (CAd system) to locally provide both oncolysis and multiple immunomodulatory molecules encoded by an HDAd. While intratumoral administration of CAd is insufficient to cure metastasized tumors, we have previously demonstrated that combination of local CAd injection with systemic human epidermal growth factor receptor 2 (HER2) specific chimeric antigen receptor T-cells (HER2.CART) provides significant anti-tumor activity in various solid tumor xenograft models including orthotopic metastatic model. Based on these encouraging preclinical studies, we proposed this combination immunotherapy with CAd producing IL-12p70, PD-L1 blocking antibody, and an HSVtk safety switch (CAd*Trio*) to treat patients with HER2 positive solid malignancies, including pancreatic adenocarcinoma (PDAC) because high expression levels of HER2 have been associated with poor prognosis in PDAC patients. Since treatment with single immunotherapies (checkpoint inhibitor, oncolytic virus, CAR T-cell) showed limited anti-tumor efficacy in PDAC patients, in this study we evaluated whether our combination immunotherapy enables control of PDAC growth in multiple PDAC models including humanized mice. Combinatorial treatment with CAd*Trio* and HER2.CART cures PDAC tumors within 25 days without tumor recurrence in xenograft models. HER2.CART is the dominant contributor of our combination therapy to the anti-tumor activity in xenograft PDAC models. However, in one tumor model, CAd*Trio* enhanced early HER2.CART infiltration/expansion at the tumor site, leading to better overall anti-tumor activity than treatment with HER2.CART alone. To address how PDAC tumor microenvironment contributes to our immunotherapy, we evaluated our immunotherapies in humanized mouse models. We found that intratumoral CAd*Trio* treatment induced type I IFN responses, including chemotaxis, to enable HER2.CART migration to the tumor site resulting in significant tumor control, and long-term complete responses. In contrast to xenograft models, HER2.CART treatment alone could not cure PDAC tumors in humanized mice. Additionally, using an advanced PDAC tumor model in humanized mice, we found that local CAd*Trio* treatment repolarized distant tumor microenvironments, leading to improved HER2.CART anti-tumor activity at a metastatic tumor site. Elucidation of immune gene signatures indicate CAd*Trio*-dependent stimulation of NK cells, together with *in vitro* data showing CAd*Trio* components and type I IFN induced by CAd*Trio* enhance NK cell anti-PDAC activity, suggest that host immune cells activated through our immunotherapy contribute to the overall anti-tumor effect of our combinatorial treatment. Our data demonstrate that CAd*Trio* and HER2.CART provide complementary activities to eliminate PDAC tumors and may represent a promising therapy for PDAC patients. We have initiated a Phase I clinical trial at BCM (NCT03740256) that has recruited its first patients to test CAd*Trio* with HER2.CART in patients with HER2-positive solid tumors including PDAC.

118. UCARTCS1A, an Allogeneic CAR T-Cell Therapy Targeting CS1 in Patients with Relapsed/Refractory Multiple Myeloma (RRMM): Preliminary Translational Results from a First-in-Human Phase I Trial (MELANI-01)

Krina Patel[1], Mini Bharathan[2], Francisco J. Esteva[2], David Siegel[3], Adriana Rossi[4], Mark G. Frattini[2], Julianne Smith[5], Carrie Brownstein[2]

[1]Lymphoma Myeloma, UT MD Anderson Cancer Center, Houston, TX,[2]Cellectis, Inc., New York, NY,[3]John Theurer Cancer Center, Hackensack, NJ,[4]Weill Cornell Medical College, New York, NY,[5]Cellectis, S.A., Paris, France

MELANI-01 (NCT04142619) is a phase I dose-escalation trial of UCARTCS1A, an allogeneic chimeric antigen receptor (CAR) T-cell therapy targeting the CD2 subset-1 (CS1) antigen in patients (pts) with RRMM. UCARTCS1A production includes knockout of the T-cell receptor (to minimize the risk of GVHD) and CS1 (to avoid fratricide

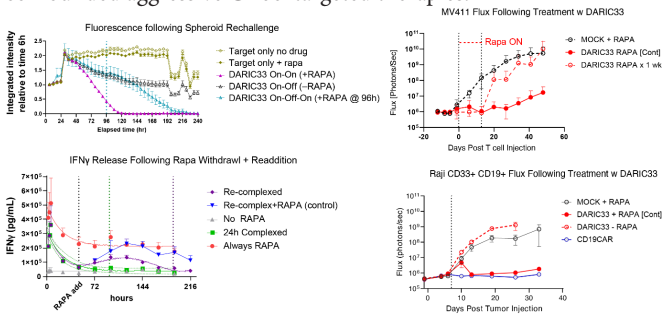
during manufacturing). After lymphodepletion with fludarabine/cyclophosphamide, 5 pts received UCARTCS1A: 4 pts in dose level 1 (DL1) (1×10^6 cells/kg) and 1 pt in DL2 (3×10^6 cells/kg). Clinical response per IMWG criteria and exploratory correlates including CS1 expression on MM cells, UCARTCS1A expansion and persistence, changes in serum biomarkers (cytokines, ferritin, liver enzymes), and host immune cell reconstitution were assessed. In all pts, CS1 was expressed in >97% of detected plasma cells. After administration, UCARTCS1A cells were detected in 3/5 pts, with expansion observed in 1 DL1 pt and 1 DL2 pt. In the DL1 pt (4 prior lines of MM therapy), peak UCARTCS1A level in whole blood by flow cytometry was 78 cells/μL and vector copy number (VCN) was 27,385 copies/μg DNA at day (d) 28. This pt achieved an MRD-negative partial response (PR) at d28 and a very good PR (VGPR) by month (mo) 3. Serum lambda light chains (LC) decreased from 503.2 to <1.3 mg/L and M protein from 2.9 to 0.1 g/dL between d0 and mo 3, with LC detectable only by immunofixation at mo 3. Notable clinical findings included grade (G) 2 cytokine release syndrome (CRS), CAR-related hemophagocytic lymphohistiocytosis (HLH), and elevated serum biomarkers including IL-6, IL-8, and IFNγ. This pt died on d109 from organizing pneumonia in the context of prolonged severe lymphopenia in the absence of myeloma progression. In the DL2 pt (13 prior lines of MM therapy), peak UCARTCS1A level was 42 cells/μL (d11) and VCN was 141,090 copies/μg DNA on d9. Serum M protein and kappa LC decreased from 0.9 to 0.4 g/dL and from 15.4 to 1.4 mg/L, respectively, from d0 to d14. The pt achieved a PR at d14. Notable clinical findings included G4 CRS. This pt died on d25 as a result of G5 hemorrhagic pancreatitis in the context of CRS, CAR-related HLH, disseminated mucormycosis, and pseudomonal pneumonia. Among the 3 nonresponding pts in DL1 (11 to 15 prior lines of MM therapy), 1 pt maintained stable disease and 2 pts experienced disease progression. UCARTCS1A cell expansion was associated with meaningful clinical responses, though with notable toxicity including G2-G4 CRS and G5 events. After a brief clinical hold, the study has resumed with protocol updates to address the potential risk of prolonged lymphopenia and severe CRS. These preliminary results support further investigation of UCARTCS1A in RRMM.

119. A Drug-Regulated Anti-CD33 Chimeric Antigen Receptor with Potent Anti-AML Activity and a Reversible On-Off Switch

Jacob S. Appelbaum[1,2], Unja Martin[3], Joy Zhang[3], Kaori Oda[1], Mark Pogson[3], Giacomo Tampella[1], Dong Xia[3], Anne-Rachel Krostag[3], April Price[3], Pauline So[3], Wei-Hang Leung[3], Alexander Astrakhan[3], Jordan Jarjour[3], Joshua Gustafson[1], Michael Jensen[1]

[1]Seattle Children's Therapeutics, Seattle, WA,[2]Hematology, University of Washington, Seattle, WA,[3]bluebird, bio, Seattle, WA

Background: Acute myeloid leukemia (AML) antigens are expressed on normal hematopoietic precursors. Regulated CAR T cells are an attractive strategy for achieving (1) therapeutic efficacy, (2) hematopoietic recovery, and (3) exhaustion avoidance by temporally constraining antigen-dependent activation. We previously described dimerizing agent regulated immunoreceptor complex targeting CD33 (DARIC33), wherein antigen binding and T cell signaling domains of a chimeric antigen receptor are spatially separated and fused to the rapamycin (RAPA)-dependent dimerization domains FRB* and FKBP12. RAPA heterodimerizes antigen binding and signaling receptors, reconstituting CD33-dependent T cell activation. Here, we probe pharmacologic control of DARIC33 T cells using systems for pulsatile RAPA dosing. We find that DARIC33 T cells show nearly complete RAPA-dependent anti-CD33 activity and in vivo efficacy comparable to a clinically validated CD19 CAR. **Results:** DARIC33 T cells preactivated with RAPA for 24hr were washed and cultured in RAPA-free media for different periods prior to stimulation with CD33+ target cells in vitro. Pre-activated DARIC33 T cells showed high levels of cytokine release followed by a progressive decline to baseline levels. Re-addition of RAPA restored antigen-dependent cytokine release to levels of DARIC33 T cells maintained in RAPA continuously. We also found DARIC33 T cells efficiently eliminate CD33+ spheroids in the presence of RAPA. Following spheroid challenge, DARIC33 T cells were isolated and cultured in RAPA-free media for 48 hours, followed by rechallenge with a second spheroid target. DARIC33 T cells exhibited significantly reduced cytotoxicity after RAPA wash out. However, re-addition of RAPA rescued T cell cytotoxicity, leading to complete spheroid clearance. Thus two in vitro systems show DARIC33 T cell activation by RAPA and reduced cytokine release or cytotoxicity following RAPA withdrawal. In both systems, depressed effector function of DARIC33 T cells following RAPA withdrawal can be restored through re-addition of RAPA. To assess RAPA modulation of DARIC33 activity in vivo, NSG mice engrafted with CD33+ MV4-11 AML cells were treated with DARIC33 T cells + various durations of RAPA. Mice treated continuously with RAPA cleared tumors, whereas mice treated for only 1 week showed tumor regression followed by relapse, confirming RAPA withdrawal deactivates DARIC33 T cells. Next, Raji lymphoma cells, engineered to express CD33 at levels similar to CD19, were injected into NSG mice. The engrafted CD33+CD19+ Raji tumors were then treated with DARIC33 T cells or control CD19 CAR T cells in the presence or absence of RAPA. Mice treated with 10e6 CD19 CAR T cells or 30e6 DARIC33 T cells + RAPA controlled Raji tumor growth. Anti-tumor activity was not observed in mice treated with 30e6 DARIC33 T cells in the absence of RAPA. **Conclusions:** These data demonstrate that DARIC33 T cells exert potent anti-AML activity that can be modulated with RAPA. The resulting temporal control of T cell activation may enable optimization of durable effector function, and avoid long-term aplasia that has confounded aggressive CD33-targeted therapies.



Upstream Process Development for AAV Vector Production

120. Co-Identification and Characterization of Host and Viral Protein Interactomes during AAV Production by Two Different Proximity Labeling Methods

Ji Sun Lee, Guangping Gao*, Jun Xie*
Horae Gene Therapy Center, The University of Massachusetts Medical School, Worcester, MA

Scalable manufacturing of high titer and high potency vectors is a challenge for translating recombinant Adeno-Associated Virus (rAAV)-based gene therapeutics into clinical applications. Efficiencies in AAV genome replication, capsid assembly and genome encapsidation are the keys to the success of vector production. Understanding the interactomes in producer cells during AAV vector production will provide insights into developing more efficient production platforms. The proximity-dependent biotin identification method allows detecting protein-protein interactions with a promiscuous biotin ligase fused to a bait protein. These biotinylated proteins can be selectively isolated by biotin-streptavidin capture and identified by mass spectrometry. We grafted two types of biotin ligase, BioID2 or TurboID onto the surface of AAV2 capsid. BioID2 is a smaller biotin ligase and the major disadvantage is its slow kinetics, which necessitates labeling with biotin for 18-24 hours. By contrast, TurboID is greater in size but enable the labeling in ten minutes. Both engineered AAV2 capsids can biotinylate un-biotinylated substrates just like a purified biotin ligase. During AAV vector production by using 293 cell transient transfection method, biotin and ATP were added to the culture medium. Seventy-two hours post transfection, proteins were then selectively isolated by streptavidin-coated beads for mass spectrometry analysis. Rep proteins were found biotinylated regardless of the presence of packageable genomes, suggesting the formation of Rep-Capsid complex before genome encapsidation. This pre-encapsidation complexes were also previously detected by coimmunoprecipitation, co-sedimentation, and yeast two-hybrid analyses (Ralf Dubielzig, et al., 1999). In addition, a total of 61 cellular proteins were co-identified by both BioID2 and TurboID technologies as direct or indirect interactors with viral proteins possibly involved in vector genome replication, capsid assembly, and genome encapsidation. The main predicted biological functions of the 61 cellular coding genes are associated with cell cycle, immune response, and cell growth by GO term analysis. Ongoing studies aim to further validate contributions of those cellular genes to AAV production process. Our findings may shed some lights to developing strategies to improve vector empty/full particle ratios and virus genome titer. *Co-corresponding authors.

121. The Effects of ITR Structure and Plasmid Backbone on Plasmid Stability and Yield

Ruofan Wang, Shaw Camphire, David Ho, Christopher Porzondek, Bo Li, Guo-an Wang
Vigene Biosciences, Rockville, MD

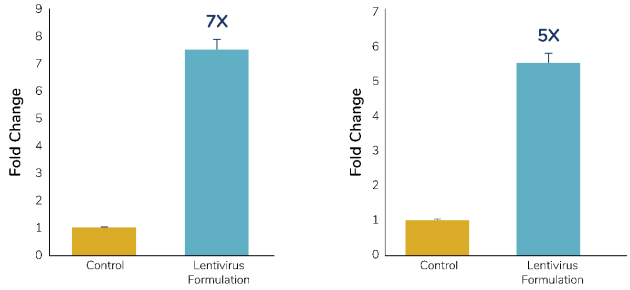
In viral vector production, plasmid integrity is of paramount importance. Plasmid yield is the number one KPI in plasmid manufacturing, affecting process design, product quality and overall cost. It is common knowledge that AAV ITR-carrying plasmids tend to be unstable, resulting in plasmid variants, extra DNA fragments, and low production yield. Here we present a comprehensive study to document these changes and to understand the causes of ITR instability by means of NGS and restriction analyses. We found that both ITR structure and plasmid backbone contributed to such instability. Our data showed that modification of either ITR structure or plasmid backbone would reduce, and even eliminate, deletions in ITR and result in an increase of plasmid yield from 30% to several folds.

122. Increasing Gene Therapy Vector Production Using Viral Sensitizer Molecules

Jean-Simon Diallo
Virica Biotech, Inc., Ottawa, ON, Canada

Inefficient manufacturing processes can have several important implications during product development. The first and most obvious is cost of goods (COGs), which is driven upwards when GMP compliant material is required. Related are the practical considerations of manufacturing virus using an inefficient process, which means longer times to produce target amounts of virus, more substantial needs in personnel, physical space, and investments in infrastructure. Lastly, there is the issue of maximum feasible dose, where the maximum dose of a manufactured virus given to a patient is limited by the amount of virus that can be produced. Viral Sensitizers (VSETMs) encompass a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently overcoming cellular antiviral defenses. VSEs can be used in a range of applications such as improving virus manufacturing yield, improving tumor infection by oncolytic viruses, or transduction of cells by common gene therapy vectors like AAV, adenovirus, and lentivirus. Owing to different molecular mechanisms through which they operate, VSEs can be combined, adapted, and formulated for specific uses. To improve lentivirus production, we employed high throughput screening and DOE methodology to develop a VSE formulation that could improve 3rd generation lentivirus production in adherent HEK293T cells (VSE-LentiTM). We designed a high-throughput method to identify single then multi-VSE compound formulations that enhance lentivirus production. Different transfection reagents, and production scales as well as commonly used lentivirus production enhancers were tested. We found VSE-LentiTM to be compatible with several transfection reagents (PEI Pro, lipofectamine, TransIT lenti). VSE-LentiTM was able to enhance lentivirus production whether added at the time of transfection or up to 24h prior to transfection. Enhancements in lentivirus production were observed over 72 hours, without a requirement to resupply compounds. Improvements in yield observed using VSE-LentiTM were condition-dependent but in pre-optimized conditions lentivirus production conditions, VSE-LentiTM led to >5X increase in TU/ml.

Figure 1. VSE-Lenti™ molecules were added to adherent HEK293T cells at the time of transfection in combination with PEI transfection reagent. Following transfection with luciferase-expressing 3rd generation lentivirus plasmids, a 5-7-fold increase in luciferase activity was observed.)



123. Transcriptional Response of HEK293 cells to Clinical-Scale Recombinant Adeno Associated Virus Production by Transient Transfection

Cheng-Han Chung[1], Christopher Murphy[1], Douglas McCarty[2], Jeffrey Pavlicek[1], Erik Barton[1]

[1]Pharmaceutical Sciences, Bioprocess Research and Development, Gene Therapy Process Development, Pfizer Inc., Morrisville, NC,[2]Rare Disease Research Unit, Pfizer Inc., Morrisville, NC

The Pfizer gene therapy platform relies on transient transfection of HEK293 cells with three plasmids encoding the viral genes required for rAAV replication and assembly. Improving rAAV production is hampered by a lack of fundamental understanding of host cell cofactors and cellular responses to AAV production. We hypothesized that rAAV productivity is constrained by cellular stress or antiviral responses. To investigate potential bottlenecks to productivity, we defined the kinetic transcriptional response of HEK293 cells to rAAV production in a clinical manufacturing-relevant process. We found ~2000 genes differentially expressed after transient transfection and during rAAV production compared with pre-transfection control. Pathways involved in detection of external stimulus, defense response to virus and inflammatory response were regulated at different times post transfection. This indicates that multiplexed cellular responses were triggered by rAAV production, which may negatively impact rAAV productivity. Systematic analyses of the cellular transcriptional response to rAAV production may illuminate genes limiting rAAV yields, thereby enabling the rational design of a next-generation rAAV manufacturing platforms.

124. Vector Engineering of pRep-Cap and pHelper Enhanced AAV Productivity by Triple Transfection in Suspension HEK293 Cells

Caitlin Tripp[1], Jennifer Wang[1], Tsai-Yu Chen[2], Wenling Dong[2], Senthil Ramaswamy[1], Bingnan Gu[1]

[1]R&D, Lonza Houston Inc, Houston, TX,[2]Process Development, Lonza Houston Inc, Houston, TX

As a leading viral vector for *in vivo* gene therapy, AAV is non-pathogenic and can effectively infect a wide range of human tissues. Currently, most AAV-based gene therapy pipelines rely on AAV manufacturing via transient triple plasmids transfection in human HEK293 cells. However, the productivity of the platform needs to be improved in order to meet the growing demand of recombinant AAV for clinical development and commercialization. Previously, we isolated a highly productive clonal cell 5B8 in suspension HEK293, and established a robust cGMP AAV manufacturing platform that was based on triple transfection and supported production in single-use bioreactor up to 250L scale for multiple AAV serotypes. To continue to improve our platform, we designed and evaluated a panel of pRep-Cap and pHelper plasmid vectors for AAV production. For pRep-Cap vector, we focused on rebalancing expression of Rep and Cap. Through engineering of their native viral promoters, we identified the best combination of Rep-Cap expression levels in pLHI_Rep-Cap [a] that could double the AAV titer across multiple serotypes, e.g. AAV2, AAV5, AAV9, and Anc80L65. The improvement was confirmed with different AAV transgenes in multiple runs in 250L scale. For pHelper vector, we screened five additional candidate helper genes and/or isoforms. Remarkably, we found that the additional co-transfection of one candidate, but not others, with standard triple plasmids could increase the AAV titer 50%-100% over just standard triple transfection method. Consistently, the enhancement was dose-dependent, as more plasmids co-transfection resulted in higher AAV titer up to 100% more. Furthermore, we subcloned the overexpression cassette into the current state-of-the-art vector to create a new generation of pHelper plasmid, named pLHI_Helper [b]. The pLHI_Helper plasmid could be manufactured at the same cost as regular pHelpler but would provide more than 50% productivity compared to the state-of-the-art vector. Taken together, our study suggested that optimization of vector design in AAV manufacturing plasmids is feasible and can significantly improve the AAV productivity in large scale by triple transfection in suspension HEK293 cells. [a, b] Patent pending.

125. High Titer rAAV Production upon Upstream Process Development of Stable Helper-Virus Free ELEVECTA® Producer Cells

Juliana Coronel, Aishwarya Patil, Ahmad Al-Dali, Tom Braß, Helmut Kewes, Christian Niehus, Jens Wölfel, Kerstin Hein, Nicole Faust, Silke Wissing

Cevec Pharmaceuticals GmbH, Cologne, Germany

In recent years, the number of gene therapy products in the biopharma clinical pipeline have increased, as well as the need for scalable manufacturing processes for viral vector production. Recombinant adeno-associated virus (rAAV) is widely used as viral gene therapy vector, however the delivery of the required amounts of AAV-vector particles is still a challenge. We have recently developed a stable helper-virus free rAAV production platform named ELEVECTA®. This AAV production platform consists of mammalian suspension cells which have stably integrated all components necessary to produce AAV, namely the adenovirus helper functions E2A, E4ORF6, VA RNA, as well as AAV replicase, AAV capsid and the gene of interest (GOI) flanked with the AAV ITRs. Production in this system is initiated by induction via doxycycline. Stable rAAV production using the ELEVECTA platform has been proven with different serotypes as well as different GOIs. To further develop the production process using the ELEVECTA® production platform, upstream process optimization was performed with one of CEVEC's AAV8 proof of concept single cell clones. The optimized upstream process, developed in the ambr® 15 was first scaled to 10 L, then in a collaboration with Pall Corporation it

was successfully scaled-up to 200 L using the Allegro™ single-use stirred tank bioreactor. A major advantage of fully stable AAV producer cell lines is that this allows for thorough upstream process development. In order to deploy an intensified large-scale process for rAAV production, we applied ATF perfusion technology. The ATF-based perfusion set-up at lab-scale consisted of a stirred tank bioreactor connected to an ATF unit. The cells were inoculated in chemically defined medium free of animal-derived components and cultivated in standard conditions. After reaching the target viable cell density, rAAV production was induced. The harvest was done from the whole cell suspension 4 to 5 days post-induction. Noteworthy, this ATF perfusion process resulted in very high titers (E15 vg/L) and high percentage of full particles (35-40 %).

126. Genome-Wide CRISPR Activation Screen Reveals That SKA2 and ITPRIP Increase AAV Manufacturing via Cell Cycle Modulation

Hyuncheol Lee[1], Christopher Barnes[2], David Ojala[2], David Schaffer[1,2]

[1]California Institute for Quantitative Biosciences (QB3), University of California, Berkeley, Berkeley, CA,[2]Chemical & Biomolecular Engineering, University of California, Berkeley, Berkeley, CA

Introduction and Methods Recombinant adeno-associated viruses (rAAV) are a leading gene delivery platform; however, current rAAV manufacturing methods cannot meet the demands of the field, which will increasingly be a barrier for the clinical development and commercialization of gene therapies. To address this issue, we developed an AAV-specific genome-wide screening strategy to identify gene targets whose upregulation promotes rAAV packaging. Specifically, an AAV vector library carrying Synergistic Activation Mediator (SAM) guide RNA libraries was generated, and HEK293T cells expressing SAM machinery (a dCas9-VP64 fusion with MS2-P65-HSF1) were infected with the AAV library for 48 hours to provide time for modulation of target host factor expression. The cells were then transfected with rAAV packaging genes (pAAV2) and pHelper for another 72 hours to package the rAAV with each vector genome. After the iterative rounds of selection, Next Generation Sequencing was used to analyze the AAV genomes and thus the guide RNAs targeting host factors that increased rAAV packaging. **Results** The most enriched guide RNA targeted the spindle and kinetochore associated complex subunit 2 (SKA2) transcript variant 2 and inositol 1, 4, 5-trisphosphate receptor interacting protein (ITPRIP). In the SKA2 or ITPRIP expressing stable cell line, AAV packaging was increased by 2.2-fold and 3.3-fold, respectively. A cell line expressing both SKA2 and ITPRIP increased AAV titer by 3.8-fold. We analyzed multiple mechanistic steps where target gene expression could impact AAV vector production, including transfection, viral gene expression, the cell cycle, and others. We found that AAV vector genome replication in SKA2 or ITPRIP expressing cell line was higher than that in WT cells. In addition, during AAV production ITPRIP expression increases the proportion of cells in the S-phase, when AAV vector genome replication is known to occur. Moreover, EdU incorporation in SKA2 expressing cells showed elevated S-phase synthesis, suggesting SKA2 may influence cell cycle kinetics during rAAV packaging. Consistent with this finding, culturing in high confluency to arrest in the G0 phase antagonized the effect of SKA2 and ITPRIP, indicating the effect of SKA2 and ITPRIP in rAAV packaging is mediated by cell cycle modulation. Finally, we performed capsid ELISA and immunoblotting using crude lysate samples normalized by same copy number or same protein concentration and showed expression of SKA2 and ITPRIP increased the AAV full/empty capsid ratio, which suggests increased vector genome replication promoted genome loading into virions. **Conclusions** This broad screening strategy offers a new approach to improve rAAV producing cell lines as well as to unveil the host factors relating to virus packaging. Taken together, our genome wide activation screens revealed host factors, SKA2 and ITPRIP, that increased vector genome replication, the full capsid ratio, and consequently AAV production. **Disclosure** C.B., D.S.O., and D.V.S. are inventors on patents related to cell lines for increased production of AAV. D.V.S is a co-founder of 4D Molecular Therapeutics, which develops novel rAAV therapeutic vectors for clinical use.

Presidential Symposium and Top Abstracts

127. Base Editing Rescues Sickle Cell Disease in Human Hematopoietic Stem Cells and in Mice

Jonathan S. Yen[1], Gregory A. Newby[2,3], Kaitly J. Woodard[1], Thiyagaraj Mayuranathan[1], Cicera Lazzarotto[1], Yichao Li[1], Heather Sheppard-Tillman[4], Shaina N. Porter[5], Yu Yao[1], Kalin Mayberry[1], Kelcee A. Everette[2,3], Yoonjeong Jang[3], Christopher J. Podracky[3], Elizabeth Thaman[6], Christopher Lechauve[1], Akshay Sharma[7], Jordana M. Henderson[8], Michelle F. Richter[2,3], Kevin T. Zhao[2,3], Shannon M. Miller[2,3], Tina Wang[2,3], Luke W. Koblan[2,3], Anton P. McCaffrey[8], John F. Tisdale[9], Theodosia A. Kalfa[6,10], Shondra M. Pruett-Miller[5], Shengdar Q. Tsai[1], Mitchell J. Weiss[1], David R. Liu[2,3]

[1]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN,[2]Merkin Institute of Transformative Technologies in Healthcare, Broad Institute of Harvard and MIT, Cambridge, MA,[3]Department of Chemistry and Chemical Biology and Howard Hughes Medical Institute, Harvard University, Cambridge, MA,[4]Department of Pathology, St. Jude Children's Research Hospital, Memphis, TN,[5]Cell and Molecular Biology, St. Jude Children's Research Hospital, Memphis, TN,[6]Division of Hematology, Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH,[7]Bone Marrow Transplantation & Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN,[8]TriLink BioTechnologies, San Diego, CA,[9]Molecular and Clinical Hematology Branch, NHLBI/NIDDK/NIH, Bethesda, MD,[10]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

Sickle cell disease (SCD) is a catastrophic disorder caused by a mutation in the *HBB* gene encoding the β-globin subunit of adult hemoglobin. We used a bespoke adenine base editor (ABE8e-NRCH) to convert the SCD allele (HBB^S) to hemoglobin Makassar (HBB^G), a naturally occurring non-pathogenic allele. Delivery of mRNA encoding ABE8e-NRCH and an HBB^S-targeting single guide RNA (sgRNA) into $CD34^+$

hematopoietic stem and progenitor cells (HSPCs) from patients with SCD using a clinical electroporation method resulted in 80% conversion of *HBB*^{S} to *HBB*^{G} with no perturbation of erythropoiesis. Reticulocytes from edited CD34$^+$ cells showed an 80% shift of total β-like globin protein from sickle (β^S) to Makassar (β^G), and a 3-fold decrease in hypoxia-induced sickling. Sixteen weeks after transfer of edited human CD34$^+$ cells into adult immunodeficient mice, the *HBB*^{S}-to-*HBB*^{G} editing frequency in engrafted cells was 68%, indicating durable modification of HSCs. Reticulocytes from the bone marrow of engrafted mice showed a shift of 63% of total β-like protein from β^S to β^G, and a 5-fold decrease in hypoxia-induced sickling. Since human red blood cells (RBCs) do not survive in mouse peripheral blood, we also examined the physiological effects of *HBB*^{S} base editing by electroporating ABE8e-NRCH and sgRNA ribonucleoprotein into HSPCs from a mouse harboring human SCD alleles, followed by transplantation into irradiated host mice. Sixteen weeks after transplantation, 56% of *HBB*^{S} alleles were converted to *HBB*^{G} in bone marrow-repopulating donor HSPCs. Circulating RBCs showed a shift of 84% of total β-like protein from β^S to β^G, and a 3-fold decrease in hypoxia-induced sickling. Mice that received base-edited HSPCs showed rescue of hematologic parameters to near-normal levels and reduced splenic pathology compared to mice transplanted with unmodified HSPCs. Secondary transplantation of edited bone marrow confirmed durable phenotypic rescue of long-term hematopoietic stem cells and when performed with different ratios of edited and unedited HSPCs revealed that ≥20% *HBB*^{S}-to-*HBB*^{G} editing is required for phenotypic rescue, confirming that base editing treatment substantially exceeds this threshold. Human HSPCs showed evidence of p53 activation and large DNA deletions or rearrangements following treatment with Cas9 nuclease targeting *BCL11A*, but not following treatment with ABE8e-NRCH targeting *HBB*^{S}. These findings suggest a potential one-time autologous treatment for individuals with SCD that eliminates pathogenic *HBB*^{S}, generates benign *HBB*^{G}, and minimizes undesired consequences of making double-strand DNA breaks.

128. Safety and Efficacy Results with a Single Dose of Autologous CRISPR-Cas9-Modified CD34$^+$ Hematopoietic Stem and Progenitor Cells (HSPCs) in Transfusion-Dependent β-Thalassemia (TDT) and Sickle Cell Disease (SCD)

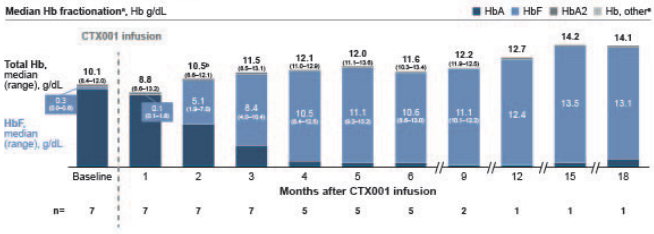
T.W. Ho[1], H. Frangoul[2], Y. Bobruff[1], M.D. Cappellini[3], S. Corbacioglu[4], C.M. Fernandez[1], J. de la Fuente[5], S. Grupp[6], R. Handgretinger[7], S. Imren[8], A. Kattamis[9], J. Lekstrom-Himes[8], F. Locatelli[10], Y. Lu[8], M. Mapara[11], S. Mulcahey[8], M. de Montalembert[12], D. Rondelli[13], N. Shanbhag[8], S. Sheth[14], S. Soni[1], M.H. Steinberg[15], M. Weinstein[1], J. Wu[16], D. Wall[17]

[1]CRISPR Therapeutics, Cambridge, MA,[2]The Children's Hospital, Nashville, TN,[3]University of Milan, Milan, Italy,[4]University of Regensburg, Regensburg, Germany,[5]St Mary's Hospital, London, United Kingdom,[6]University of Pennsylvania, Pennsylvania, PA,[7]University of Tübingen, Tübingen, Germany,[8]Vertex Pharmaceuticals Inc, Boston, MA,[9]University of Athens, Athens, Greece,[10]University of Rome, Rome, Italy,[11]Columbia University, New York, NY,[12]University of Paris, Paris, France,[13]University of Illinois at Chicago, Chicago, IL,[14]Cornell University, New York, NY,[15]Boston University, Boston, MA,[16]University of British Columbia, Vancouver, BC, Canada,[17]University of Toronto, Toronto, ON, Canada

BCL11A is a key transcription factor that suppresses the production of fetal hemoglobin (HbF) in red blood cells (RBCs). In TDT and SCD, elevated HbF is associated with fewer transfusions and clinical complications. To reactivate HbF in RBCs we used the CRISPR-Cas9 platform to edit the erythroid enhancer region of *BCL11A* in HSPCs ex vivo (CTX001™). We present safety and efficacy results from patients (pts) infused with CTX001 with ≥3 months (mo) of follow-up (f/u). CLIMB THAL-111 (TDT, NCT03655678) and CLIMB SCD-121 (SCD, NCT03745287) are multicenter, first-in-human studies of CTX001. Pts aged 12-35 years (ys) with TDT (all genotypes) receiving ≥10 units/y of packed RBC transfusions in the prior 2 ys, and those with severe SCD (≥2 vaso-occlusive crises (VOCs)/y requiring medical care in the prior 2 ys) were eligible. We collected peripheral CD34$^+$ HSPCs by apheresis after mobilization with G-CSF and plerixafor (TDT) or plerixafor alone (SCD). We edited the erythroid enhancer region of *BCL11A* in the enriched CD34$^+$ cells using a specific CRISPR guide-RNA and Cas9 nuclease. Pts received myeloablative busulfan before infusion. We monitored engraftment, AEs, total Hb, HbF, hemolysis, F-cells, RBC transfusions (TDT), and VOCs (SCD). 7 TDT pts (median f/u 8.9 mo, range 3.8-21.5) and 3 SCD pts (median f/u 7.8 mo, 3.8-16.6) received CTX001. Median neutrophil and platelet engraftment were achieved on Day 32 (20-39) and 37 (29-52) respectively in TDT pts, and on Day 22 (17-30) and 30 (30-33) in SCD pts. The safety profile after infusion was generally consistent with myeloablative conditioning and autologous bone marrow transplant. 4 serious AEs (SAEs) related or possibly related to CTX001 occurred in 1 TDT pt in the context of HLH: HLH, headache, ARDS, and IPS. All resolved at time of analysis. The other 9 pts reported no CTX001-related SAEs. Pts received their last transfusion within 2 mo after infusion and showed increases in Hb and HbF over time (Figure). The first pts have been transfusion-free for >20.5 (TDT) and >16.0 (SCD) mo. No SCD pts have

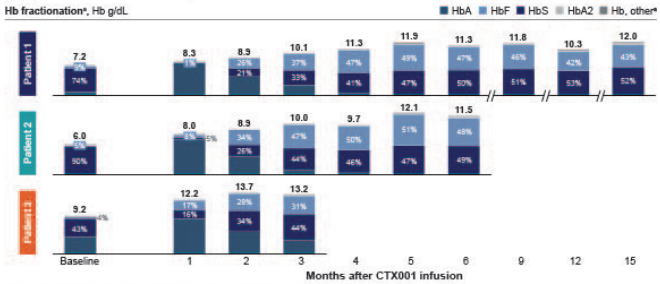
had a VOC since infusion. The first SCD pt has been VOC-free for >16.6 mo. CTX001 led to increases in HbF and total Hb in all treated pts. Its post-infusion safety profile is generally consistent with myeloablation. All 7 TDT pts have been transfusion-free for ~2 mo and the 3 SCD pts have had no VOCs. These early data demonstrate CTX001 is a potential functional cure for treatment of TDT and SCD.

A. Median Hb fractionation and total Hb in patients with TDT (N=7). HbF (g/dL) is indicated in light blue and total Hb (g/dL) appears at the top of each bar (Frangoul et al. ASH 2020. Abstract 004)



Hb: hemoglobin; HbA: adult hemoglobin; HbF: fetal hemoglobin; TDT: transfusion-dependent β-thalassemia
*Hb adducts and other variants; †With respect to Patient 2, total Hb from local laboratory and Hb fraction from central laboratory

B. Hb fractionation and total Hb in patients with SCD (N=3). HbF (%) is indicated in light blue and total Hb (g/dL) appears at the top of each bar (Frangoul et al. ASH 2020. Abstract 004)



Hb: hemoglobin; HbA: adult hemoglobin; HbF: fetal hemoglobin; HbS: sickle hemoglobin; SCD: sickle cell disease
*Hb adducts and other variants

129. Immunostimulatory Bacterial Antigen-Armed Oncolytic Measles Virotherapy Significantly Increases the Potency of Anti-PD1 Checkpoint Therapy

Eleni Panagioti[1,2], Cheyne Kurokawa[1], Kimberly Viker[1], Arun Amayappan[1], Sotiris Sotiriou[3], Kyriakos Chatzopoulos[3], Katayoun Ayasoufi[4], Aaron Johnson[4], Ianko Iankov[1], Evanthia Galanis[1]

[1]Molecular Medicine, Mayo Clinic, Rochester, MN,[2]Neurosurgery, Brigham and Womens Hospital, Harvard Medical School, Boston, MA,[3]Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN,[4]Immunology, Mayo Clinic, Rochester, MN

Clinical immunotherapy approaches are lacking efficacy in the treatment of glioblastoma (GBM). In this study, we sought to reverse local and systemic GBM-induced immunosuppression using the Helicobacter pylori neutrophil-activating protein (NAP), a potent TLR-2 agonist as a novel immunostimulatory transgene expressed in an oncolytic measles virus (MV) platform, retargeted to allow viral entry through the urokinase-type plasminogen activator receptor (uPAR). While single agent murine anti-PD1 treatment or repeat *in situ* immunization with MV-s-NAP-uPA provided modest survival benefit in MV resistant models, the combination treatment led to synergy with a cure rate of 80% in mice bearing intracranial GL261 (*P= 0.047)*and 72% in mice with CT-2A tumors (*P= 0.007*). Combination NAP-immunovirotherapy induced massive influx of lymphoid cells in mouse brain, with $CD8^+$ T cell predominance; therapeutic efficacy was $CD8^+$T cell dependent. Inhibition of the IFN response pathway using the JAK1/JAK2 inhibitor ruxolitinib significantly decreased PD-L1 expression on myeloid-derived suppressor cells (MDSCs) in the brain and potentiated the therapeutic effect of MV-s-NAP-uPA and anti-PD1. Our findings support that measles virus strains armed with bacterial immunostimulatory antigens represent an effective strategy to overcome the limited efficacy of immune checkpoint inhibitor based therapies in GBM creating a novel and promising translational strategy for this lethal brain tumor.

130. In-Vivo Engineered B Cells Retain Memory and Secrete High Titers of Anti-HIV Antibodies in Mice

Alessio David Nahmad[1], Cicera Lazzarotto[2], Natalie Zelikson[1], Mary Tenuta[3], Talia Kustin[1], Inbal Reuveni[1], Miri Horovitz-Fried[1], Iris Dotan[1], Rina Rosin-Arbesfeld[1], Adi Stern[1], James Voss[3], Shengdar Tsai[2], Adi Barzel[1]

[1]Tel Aviv University, Tel Aviv, Israel,[2]St Jude Children's Research Hospital, Memphis, TN,[3]The Scripps Research Institute, La Jolla, CA

Eliciting a potent and neutralizing antibody response to diverse and rapidly mutating viruses is a long-standing clinical challenge. HIV specific broadly neutralizing antibodies (bNAbs) can suppress viremia, as demonstrated recently by combination therapy. However, the mean elimination half-life of the bNAbs in patients is shorter than a month, requiring constant administration to prevent the virus from rebounding. Moreover, individuals with prior HIV resistance to the antibodies are excluded from trials and resistance to one antibody occurs when the concentration of the second diminishes. Persistence may be addressed by constitutive expression, however anti-drug antibodies may develop, possibly due to improper glycosylation. B cell engineering provides an opportunity to express a bNAb for adaptive immunity. Both mucosal protection and systemic clearance may be achieved by Class Switch Recombination (CSR). Somatic Hypermutation (SHM) followed by affinity maturation may allow for counteracting viral escape and memory retention allows for increased titers upon viral resurgence. We recently demonstrated that, upon adoptive transfer, bNAb engineered B cells undergo differentiation, memory retention, CSR, SHM and clonal expansion. However, extensive, and expensive *ex-vivo* manipulations hinder clinical potential of this approach. Furthermore, allogeneic B cell therapy necessitates MHC-II compatibility to receive T cell help. To overcome these limitations, we engineer B cells *in-vivo*. In particular, we demonstrate that an injection of two AAV-DJ vectors, one coding for CRISPR/Cas9 and another coding for a bNAb donor cassette, allows for site specific integration in B cells. Following immunizations, we show memory retention and bNAb secretion at high titers. Antibodies secreted by the engineered B cells were found to be of multiple isotypes and IgGs could neutralize autologous and heterologous pseudoviruses. The engineered antibody coding genes underwent somatic hypermutation and clonal selection. Detected engineered cells by flow cytometry included B cells in the blood, plasmablasts and germinal center B cells in the spleen, indicating B lineage differentiation. Biodistribution of the donor AAV over time indicated CRISPR-dependent expansion of engineered B cells only in lymphatic tissues. We further assessed the

possible off target effects of our *in vivo* B-cell engineering approach and found limited CRISPR/Cas9 off-target cleavage, using unbiased, highly sensitive, CHANGE-Seq analysis. Finally, we reduced on-target cleavage at undesired tissues by expressing Cas9 from a B cell specific promoter and by coding the gRNA in the donor vector. In summary, we demonstrate that B cells can be safely engineered *in-vivo*. We propose that *in-vivo* B cell engineering should be considered for novel future applications, to address other persistent infections or to treat autoimmune diseases, genetic disorders, and cancer.

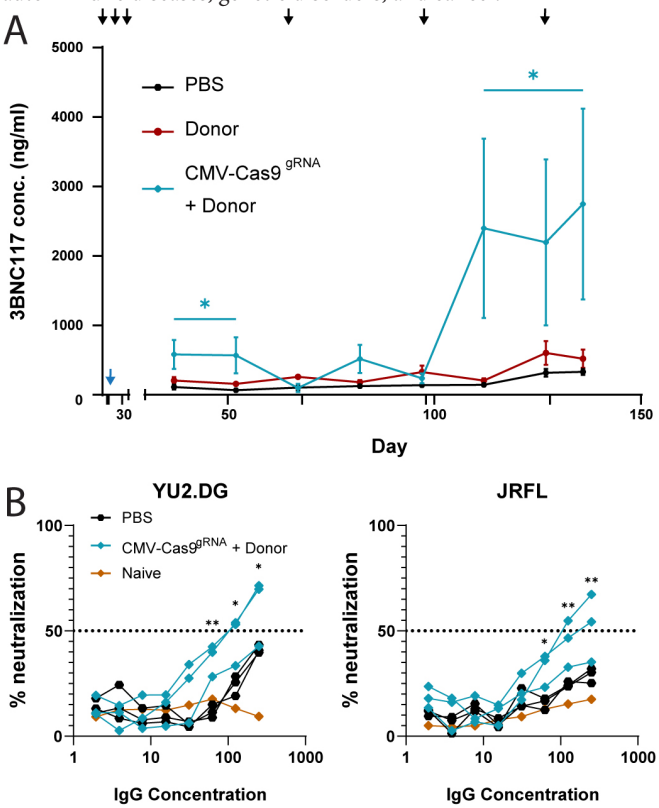


Figure: in-vivo engineered B cells retain memory and secrete high titers of anti-HIV antibodies in mice. A. Serum titers of the antibody integrated in the cells (3BNC117). Black arrows indicate immunizations, blue arrow indicate AAV injection. B. Engineered B cells secrete functional bNAbs. Neutralization of autologous (YU2.DG, left) and heterologous (JRFL, right) pseudoviruses using purified IgGs from last time point as in A.

AAV Therapies for Neurological and Sensory Diseases

131. AAV-Mediated GJB2 Gene Therapy Rescues Hearing Loss and Cochlear Damage in a Mouse Model of Congenital Hearing Loss Caused by Conditional Connexin26 Knockout

Pranav D. Mathur[1], Phillip Uribe[1], Stephanie Szobota[1], Jeremy Barden[1], Sairey Siegel[1], Christopher Bartolome[2], Xiaobo Wang[1], David Jaramillo[2], Anne Harrop-Jones[1], Rayne Fernandez[1], James Vestal[1], Rodrigo Pastenes[1], Bonnie Jacques[1], Steven Pennock[2], Adrian Timmers[2], Fabrice Piu[1], Mark Shearman[2], Alan C Foster[1]

[1]Otonomy Inc., San Diego, CA,[2]AGTC, Cambridge, MA

According to the NIDCD, 2-3 out of every 1,000 children in the United States are born with some degree of hearing loss, with more than half due to genetic factors. Mutations in the *GJB2* gene which encodes the gap junction protein Connexin 26 (CX26) are the most common forms of non-syndromic deafness, responsible for approx. 50% of cases. While in most subjects the onset of hearing loss is prelingual and moderate to severe, in some subjects, hearing loss due to loss of CX26 is mild and progressive. In the inner ear, expression of CX26 is vital for the function of various non-sensory cell types including support cells and fibrocytes. Results from mouse and human studies have revealed that mutations in *Cx26* ultimately lead to near total degeneration of cochlear hair cells. Since the constitutive homozygous *Cx26* knockout is embryonic lethal, we utilized conditional knockouts to study the effect of losing CX26 protein in the cells of the inner ear. We utilized two different conditional knockout strains (*Cx26* cKO) generated by crossing $Cx26^{loxp/loxp}$ mice with either an inducible *cre* mouse line or with a constitutive *cre* mouse line. Using the inducible *cre* line, we knocked out *Cx26* with temporal control and observed varying degrees of hearing loss and cochlear defects dependent on the time of *cre* induction. Early postnatal *cre* induction caused severe to profound hearing loss in the *Cx26* cKO mice when assessed at postnatal day 30, whereas later induction of *cre* resulted in mild to moderate hearing loss that was progressive in nature. Constitutive *cre Cx26* cKO animals, by virtue of embryonic *cre* expression in the inner ear tissues, displayed severe to profound hearing loss across all frequencies tested. The availability of these various mouse models enabled us to evaluate AAV-mediated *GJB2* gene therapy across a spectrum of hearing loss severity that mimics known human phenotypes. We previously reported the identification of novel adeno-associated viral (AAV) vectors that produce efficient expression of a gene of interest in cochlear support cells in rodents and non-human primates. We designed an AAV vector with an optimized capsid, promoter and human *GJB2* gene elements (OTO-825) that provides excellent expression of CX26 in cochlear support cells and fibrocytes. We also generated an identical AAV vector that expresses CX26 with a FLAG-tag to allow identification of virally expressed CX26 (OTO-825-FLAG). In cell-based assays, utilizing HeLa cells that do not normally express CX26, both OTO-825 and OTO-825-FLAG induced expression of CX26 that was correctly trafficked to the cell membrane. Injection of OTO-825-FLAG into the cochleae of mice provided near total expression of CX26-FLAG in our cells of interest throughout the

cochlea (from base to apex). Compared with vehicle, intracochlear administration of OTO-825 to postnatal *Cx26* cKO mice substantially restored CX26 expression and provided a marked improvement in hearing across multiple frequencies. In addition, OTO-825-injected *Cx26* cKO mice had greatly improved cochlear morphology relative to those injected with vehicle. Sub-cellular localization of the CX26 protein in rescued animals was normal and apparent in support cells throughout the cochlea, and these animals showed increased numbers of surviving hair cells. These preclinical results with AAV-mediated GJB2 gene therapy support the use of OTO-825 as a clinical candidate to treat congenital hearing loss caused by GJB2 deficiency.

132. AAV9 Mediated Delivery of PUF RNA Targeting System Corrects Molecular and Functional Defects in Amyotonic Dystrophy Type 1 Mouse Model

Ranjan Batra[1], Daniela M. Roth[2], Claire Geddes[1], Haydee Gutierrez[1], Nandini Narayan[1], Aaron Berlin[3], Greg Nachtrab[3], Dan Gibbs[3]

[1]R&D, Locanabio, Inc., San Diego, CA,[2]Molecular Biology, Locanabio, Inc., San Diego, CA,[3]Vector Development, Locanabio, Inc., San Diego, CA

Myotonic dystrophy type I (DM1) is a multisystemic autosomal-dominant inherited disorder caused by CUG microsatellite repeat expansions (MREs) in the 3' untranslated region (UTR) of the DMPK mRNA. Previously, we showed that a CRISPR-based RNA-targeting gene therapy has the potential to eliminate toxic RNAs expressed from repetitive tracts in DM1 in primary patient cells and a DM1 mouse model. We engineered a novel PUF RNA-binding protein system derived from the naturally-occurring human PUM1 protein linked with an RNA endonuclease (E17) derived from human ZC3H112A to target and cleave expanded DM1-related CUG repeats. AAV9-packaged PUF-E17 was delivered via intramuscular (IM) and intravenous (IV) injections to adult (8-weeks old) HSALR DM1 mice. We report sustained PUF-E17 expression at 4- and 12-weeks post injection. Consequently, we observed ~70% decrease in CUG nuclear foci by RNA-FISH, ~50% decrease in the levels of CUG-containing RNA, and efficient reversal of DM1-related mis-splicing to near wildtype levels 4 weeks post injection. We also compared RNA-targeting Cas9, Cas13d, and PUF-E17 systems in vivo and show comparable efficacy in elimination of toxic CUG repeats. Finally, we show that PUF-E17 treatment is safe in HSALR mice giving us multiple safe treatment options for this neuromuscular disorder.

133. Intracorneal and Sequential Contralateral Dosing of AAV-opt-ARSB Reverses MPS VI Corneal Clouding

Matthew L. Hirsch[1], Jacklyn H. Salmon[2], Liujiang Song[1], Darby Roberts[2], Jacquelyn J. Bower[1], Prabhakar Bastola[1], Brian C. Gilger[2]

[1]Ophthalmology, University of North Carolina, Chapel Hill, NC,[2]Clinical Sciences, North Carolina State University, Raleigh, NC

Mucopolysaccharidosis VI (MPS VI) is a rare, autosomal recessive lysosomal storage disease caused by mutations in ARSB which encodes arylsulfatase B. Corneal blindness in MPS VI patients compromises life quality and there is no effective treatment. The purpose of these studies was to validate an AAV gene therapy for MPS VI corneal clouding. Initially, the human ARSB ORF was compared to codon optimized cDNA sequences to identify the variant most productive for arysulfatase B activity in vitro (termed opt-ARSB). MPS VI felines, homozygous for a null ARSB mutation, presented diffuse mild corneal clouding and peripheral corneal vascularization OU with no retinal dysfunction. At 21 weeks of age, $ARSB^{-/-}$ felines were unilaterally administered AAV8-opt-ARSB ($1e^9$ viral genomes (vg)) via intracorneal injection. Clinical ophthalmic examinations (slit lamp biomicroscopy, tonometry, pachymetry, ophthalmoscopy, corneal OCT) were performed throughout the study and corneal confocal microscopy was employed at the experimental conclusion. Following the AAV8-opt-ARSB injection, the pre-existing corneal clouding cleared in the central 85-95% of the cornea within 3 weeks. In contrast, the severity of corneal disease progressed in the contralateral vehicle treated cornea. Eight weeks following the initial vector injection, the vehicle treated $ARSB^{-/-}$ cornea was administered AAV8-opt-ARSB ($1e^9$vg). Within 2 weeks of this sequential injection clearance of the storage disease was observed. All AAV8-opt-ARSB injections to the corneal stroma were well tolerated and all vector treated $ARSB^{-/-}$ corneas exhibited >85% clarity until the humane endpoint (90 days following the sequential AAV8-opt-ARSB administration). Vector biodistribution analyses, vector derived cDNA abundance, histological analyses, as well as serum AAV8 neutralizing antibody titers will also be presented. The collective data following intracorneal injection of AAV8-opt-ARSB in MPS VI felines, in conjunction with the large data set of a similar approach for MPS I corneal disease, suggest that intracorneal AAV gene therapy is safe and effective to reverse corneal clouding observed in several lysosomal storage diseases. Importantly, this is the first study to demonstrate in any disease model that sequential AAV vector dosing of the cornea, is safe and remarkably therapeutic with no immunological or adverse consequences.

134. Efficacy and Biodistribution of Anc80-RK-hRPGRIP1 Gene Therapy in a Mouse Model of Rpgrip1 Deficiency and in Non-Human Primate

Binit Kumar, Lambert Edelmann, Greg Voronin, Anna Mollin, Eun-Hee Park, Eun-Hee Park, Mehdi Doroudchi, Steve DeMarco, Stephen Jung, Hur Dolunay Kanal, Marla Weetall

PTC Therapeutics Inc, South Plainfield, NJ

Introduction: Mutations in the *RPGRIP1* (retinitis pigmentosa GTPase regulator interacting protein 1) gene are a cause of LCA6 (Leber congenital amaurosis 6) and account for ~3-5% of total autosomal recessive blindness. RPGRIP1 plays a vital role in the development and maintenance of photoreceptors due to its role in the trafficking of proteins along the connecting cilium. A null mutation in the *RPGRIP1* gene leads to progressive and irreversible blindness typically during childhood. Other mutations are associated with a later onset of disease. We are currently investigating RPGRIP 1 gene therapy in a *Rpgrip1* knockout mouse model. Here, we describe biodistribution studies in wildtype (WT) mice and non-human primates (NHP) and efficacy studies in *Rpgrip1* knockout mice. **Methods:** An initial biodistribution and dose response study was conducted using five doses (between 6.76

x 10^9 - 8.02 x 10^{11} vg/eyetotal dose) in 4-6 week-old WT C57BL/6J mice and analyzed for vector biodistribution using qPCR and protein expression using an ECL (Enhanced Chemiluminescence) assay. The extent of Anc80-hRK-RPGRIP1 transduction (at doses 4.5 x 10^{10} and 4.5 x 10^{11} vg/eye in NHP and 2e9 vg/eye in mouse) was determined using *in situ* hybridization (ISH) probes against RPGRIP1 DNA and mRNA in mouse and NHP. To assess efficacy of RPGRIP1 gene replacement therapy in PND21 *Rpgrip1*$^{-/-}$ mice, an Anc80 AAV vector containing a human rhodopsin kinase promotor driving the expression of RPGRIP1 was injected subretinally into one eye of each *Rpgrip1*$^{-/-}$ mouse at doses of 7.36 x 10^{10}, 2.43 x 10^{11} or 8.02 x 10^{11} vg/eye. Each contralateral eye was injected with vehicle as a control. At 12- and 24-weeks post-subretinal injections, the full field electroretinograms (ERGs) and optical coherence tomography (OCT) were recorded for measuring retinal function and structure, respectively. *Rpgrip1*$^{-/-}$ mice injected subretinally with Anc80-hRK-RPGRIP1 were analyzed for RPGRIP1 protein localization using immunohistochemistry along with other markers, such as acetylated tubulin and rootletin. **Results:** Subretinal delivery of Anc80-hRK-RPGRIP1 in WT C57BL/6J resulted in a dose-dependent increase in AAV vector distribution and human RPGRIP1 protein expression. Similarly, NHP retinas showed a dose dependent increase in Anc80-hRK-RPGRIP1 transduction and mRNA expression analyzed using ISH assay. ERG and OCT analysis showed improved preservation of photoreceptor/retinal function and improved photoreceptor survival in the treated eyes compared to untreated eyes 12 weeks after subretinal injection in *Rpgrip1*$^{-/-}$ mice. Subretinal delivery of the Anc80-hRK-RPGRIP1 in *Rpgrip1*$^{-/-}$ mice confirmed human RPGRIP1 protein was expressed specifically in photoreceptors and localized correctly in the connecting cilia. **Conclusions:** The present study demonstrates improvement in photoreceptor survival and function after subretinal delivery of Anc80-hRK-RPGRIP1. Efficacy was associated with the proper localization of human RPGRIP1 protein at the connecting cilium.

135. Bicistronic AAV Gene Therapy for Tay-Sachs and Sandhoff Diseases

Toloo Taghian[1], Deborah Fernau[1], Kalajan L. Mercado[2], Lauren Ellis[2], Elise Diffie[2], Amanda Gross[2], Anne S. Maguire[2], Ana Rita Batista[1], Stephanie Bertrand[3], Monique Otero[1], Rachel Prestigiacomo[3], Rachael Gately[3], Jillian Gallagher[1], Hannah Lahey[1], Amanda Taylor[2], Jey Koehler[2], Douglas R. Martin[2], Miguel Sena-Esteves[1], Heather L. Gray-Edwards[1]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Auburn University, Auburn, AL,[3]Tufts Cummings School of Veterinary Medicine, Grafton, MA

Tay-Sachs and Sandhoff diseases (TSD, SD) are neurodegenerative diseases, with similar clinical manifestations, caused by mutations in alpha or beta subunit of enzyme Hexosaminidase (Hex) respectively. Deficiency of Hex results in GM2 ganglioside storage and neuronal death. The Hex isozyme (Hex A) that degrades GM2 in humans is a heterodimer of alpha and beta subunits; therefore, co-expression of both subunits is necessary for restoration of Hex function. Here we describe the therapeutic efficacy of a single, bicistronic AAV9 vector construct that delivers both Hex subunits simultaneously in a naturally occurring sheep model of TSD. TSD sheep were injected with 1E14 vg total intravenously (IV, n=5, 5 days of age) or via the cerebrospinal fluid (CSF, n=5, 3-6 weeks of age). Sheep treated intravenously survived to 18±5 months of age and CSF treated sheep are all ongoing with the oldest now more that 3 years of age. Untreated TSD sheep reach the humane endpoint at ~ 9 month of age. GM2 ganglioside levels in CSF of 1- and 2-year-old animals treated by CSF administration were completely normalized (P<0.0009 vs TSD; P<0.88 vs normal), however, the GM2 ganglioside levels in the IV cohort were between that in untreated TSD and normal sheep. Both IV and CSF AAV treated TSD sheep exhibit marked attenuation of neurologic disease as well as normal cognition as measured by maze testing. Magnetic resonance spectroscopy (MRS) of treated sheep thalamus at ~2 years of age showed normalization of markers of neuronal health, myelination and metabolism in the CSF cohort and a slight improvement in neuronal health in the IV cohort. Diffusion tensor imaging (DTI) analysis demonstrates the decrease in microstructural integrity and increase in water diffusion in white matter of TSD brain which is a consistent finding in neurodegenerative diseases. IV treatment partially corrected these changes in some brain regions. IV treatment resulted in Hex A activity in spinal cord at ~ 70% and 100% of normal level in cervical and lumbar sections, ~ 20% in cerebellum and brainstem and lower levels in other brain regions. GM2 content in caudal aspect of the brain correlates (>80%) with Hex A activity. HexA level in skeletal muscle was ~90% of normal, but levels were lower in skeletal and other peripheral organs and tissues. Further studies are ongoing with both cohorts. Non-invasive imaging and GM2 content analysis on CSF have been routinely performed on long term CSF cohort (~ 3 years of age) to monitor the therapeutic efficacy using non-invasive biomarkers of the disease and provide data to further aid clinical translation. To enable early assessments of biomarkers and biochemistry in postmortem tissues after CSF administration, a short-term CSF treatment cohort has been initiated (n=4, endpoint ~6 months of age). These data show promise for a minimally invasive treatment for TSD and SD using this bicistronic vector construct which will provide the basis for submission of a pre-IND application.

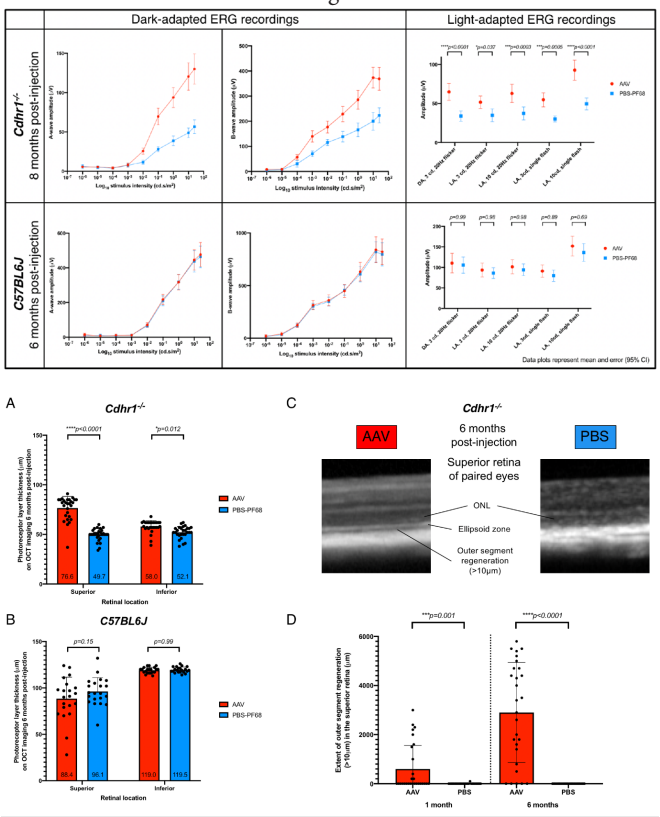
136. Gene Therapy Rescues Cone and Rod Function in a Pre-Clinical Model of *CDHR1*-Associated Retinal Degeneration through Restoration of Photoreceptor Outer Segments

Imran H. Yusuf, Michelle E. McClements, Robert E. MacLaren, Peter Charbel Issa

Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, United Kingdom

Purpose To evaluate the efficacy and safety of retinal gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration - an as yet untreatable, blinding disorder characterised by progressive cone and rod photoreceptor degeneration. **Methods** *Cdhr1*$^{-/-}$ *(n=28)* and *C57BL6J* control mice *(n=23)* underwent paired sub-retinal injections of AAV8.GRK1.CDHR1 ($1.5x10^8$) and PBS vehicle control in the fellow eye at 4 weeks of age. Dark- and light-adapted electroretinography (ERG) was undertaken at 2, 4, 6 and 8 months post-injection. Photoreceptor layer thickness measurements were compared using optical coherence

tomography (OCT) imaging at 1 and 6 months post-injection. **Results** In *Cdhr1*$^{-/-}$ mice, sub-retinal delivery of AAV8.GRK1.CDHR1 rescued A-wave amplitudes ($p<0.0001$ at all time points) and B-wave amplitudes ($p<0.0001$ from 6 months) on the dark-adapted ERG luminance series when compared with PBS-injected control eyes (**Fig.1**). Light-adapted flicker ERG amplitudes were greater in AAV-treated eyes at 8-months post-injection ($p<0.0001$). Sub-retinal injection of AAV8.GRK1.CDHR1 preserved photoreceptor layer thickness in the superior retina versus PBS-injected control eyes at 6-months post-injection (mean: 76.6µm versus 49.7µm; $p<0.0001$; **Fig.2**). OCT changes consistent with photoreceptor outer segment regeneration and restoration of the ellipsoid zone were only identified in AAV-treated eyes, with therapeutic effect seen as early as 1-month post-injection ($p=0.001$; **Fig.2**). In *C57BL6J* mice, there was no difference in ERG assessments (A-wave, $p=0.65$; B-wave, $p=0.47$; Cone responses, $p=0.09$; **Fig.1**) or photoreceptor thickness measurements at 6 months between AAV and PBS-injected eyes ($p=0.19$; **Fig.2**). **Conclusion** These data provide proof-of-principle of the efficacy and safety of *CDHR1* gene therapy in a pre-clinical model of *CDHR1*-associated retinal degeneration. Rod and cone rescue occur through prevention of photoreceptor cell death and regeneration of photoreceptor outer segments. A follow-on clinical trial in patients with *CDHR1*-associated retinal degeneration is warranted.



137. scAAV9 Gene Replacement Therapy for Epileptic SLC13A5 Deficiency

Rachel Marion Bailey, Lauren Bailey, Morgan Schackmuth, Irvin Garza

Center for Alzheimer's and Neurodegenerative Diseases, University of Texas Southwestern Medical Center, Dallas, TX

SLC13A5 Deficiency is an autosomal recessive disorder. This severe and rare form of epileptic encephalopathy is due to mutations in the *SLC13A5* gene, which codes for a plasma membrane sodium-dependent citrate transporter. To date all tested mutations result in no or a much reduced amount of the citrate transported inside the cells. Affected children present with seizures beginning within a few days of birth that persist throughout life. They show difficulty with speech production, limited and slow motor progress and many never achieving independent walking. Currently there are no treatments for SLC13A5 deficiency that target the underlying cause of disease and patients require constant supervision and care. Anti-epileptic drugs have varying success even amongst siblings, and children can succumb to complications of the seizures. Gene replacement therapy represents a therapeutic option for SLC13A5 Deficiency. We developed a self-complementary vector encoding a codon-optimized human SLC13A5 gene (scAAV9/SLC13A5), the unaltered design of which could be appropriate for human use. This study aims to compare safety and efficacy of scAAV9/SLC13A5 delivered intrathecally-lumbar puncture (IT) or intra-cisterna magna (ICM) in SLC13A5 knockout (KO) mice and wild type (WT) littermates. KO mice do not have an overt phenotype; however, similarly to patients, they have increased extracellular citrate and abnormalities in TCA intermediates. KO mice have low, but increased epileptic activity as compared to WT mice and continuous EEG recordings can detect seizure types ranging from myoclonic and focal and generalized convulsive, like those seen in patients. KO mice and WT littermates were treated with scAAV9/SLC13A5 via IT delivery or ICM delivery at 10-12 weeks of age. Mice were monitored for weight and survival. Blood was collected at baseline and then monthly after injection and ~5 months after treatment, mice received telemetry implants to record EEG and EMG activity over two 60 hour recording sessions. These mice were then tested for susceptibility to seizure induction by pentylenetetrazol (PTZ). In agreement with previous studies, at baseline, KO mice had increased plasma citrate levels as compared to WT littermates. At one-month post-treatment and beyond, KO mice treated with scAAV9/SLC13A5 had significantly decreased plasma citrate levels while KO mice treated with vehicle had sustained, high citrate levels. EEG assessments showed that vehicle treated KO mice had increased spike train activity and seizure frequency compared to their WT littermates and, importantly, that vector treatment reduced this epileptic activity with greater rescue achieved with ICM delivery than IT delivery. Using the Racine scale, SLC13A5 KO mice also had increased PTZ-induced seizure susceptibility, which was rescued to WT levels with ICM delivery of scAAV9/SLC13A5 and to a lesser extent with IT delivery. Additionally, the safety profile of the SLC13A5 vector was excellent with no adverse effects on weight, general activity or survival in KO or WT mice with either IT or ICM delivery. Overall, our preclinical results suggest that gene replacement therapy with scAAV9/SLC13A5 could provide a meaningful benefit to SLC13A5 patients.

Advances in Cellular and Immunotherapies?

138. Dissecting the Transcriptional and Epigenetic Landscape of hiPSC-Derived Neural Stem and Progenitor Cells: Implications for Cell Therapy Approaches

Vasco Meneghini[1], Marco Luciani[1], Chiara Garsia[1], Luca Petiti[2], Ingrid Cifola[3], Stefano Beretta[1], Ivan Merelli[1,3], Clelia Peano[4,5], Annarita Miccio[6], Angela Gritti[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Computational and Chemical Biology, Fondazione Istituto Italiano di Tecnologia, Genova, Italy,[3]Institute for Biomedical Technologies (ITB), National Research Council (CNR), Segrate, Italy,[4]Institute of Genetic and Biomedical Research, UoS of Milan, National Research Council (CNR), Rozzano, Italy,[5]Genomic Unit, IRCCS Humanitas Clinical and Research Center, Rozzano, Italy,[6]Laboratory of Chromatin and Gene Regulation during Development, INSERM UMR1163, Imagine Institute, Paris, France

Human induced pluripotent stem cell (hiPSC)-derived neural stem/progenitor cells (hiPS-NSPCs) are a promising cell source for cell therapy approaches and for the development of novel *ex vivo* gene therapy strategies to target neurodegenerative disorders with unmet clinical need. We have shown that intracerebral transplantation of genetically modified hiPS-NSPCs provides full rescue of the enzymatic deficit in the murine model of metachromatic leukodystrophy (MLD), a fatal demyelinating disease caused by genetic mutations of the arylsulfatase A (ARSA) enzyme. The optimization of hiPS-NSPC production (purity, homogeneity) and a comprehensive safety assessment are mandatory in view of prospective clinical application. In this study, we evaluated the transcriptional and epigenetic mechanisms underlying the hiPSC to neural commitment and complemented these data with phenotypic and functional studies in order to define a comprehensive hiPS-NSPC signature and safety profile. Computational integration of RNA-seq and ChIP-seq data revealed a strong downregulation of pathways regulating pluripotency, cell cycle, and cancer-related processes with the concomitant appearance of a distinct "neural signature" in hiPS-NSPCs. Interestingly, we highlighted a dramatic change in the usage of cell-specific enhancers and super-enhancers during hiPSC neural commitment, suggesting its major role in the generation and maintenance of hiPS-NSPCs. Differences in the transcriptomic and epigenetic profiles between hiPS-NSPCs and human fetal NSCs (hfNSC, used as reference) could be ascribed to culture conditions, regionalization pattern, and differentiation potential, with no major signs of abnormal differentiation and activation/misregulation of cancer-related pathways attributable to a pluripotent "memory". Single cell RNA-seq analyses confirmed that hiPS-NSPCs are a heterogeneous cell population mainly composed by radial glial stem cells and committed neuronal and glia progenitors, and do not include pluripotent cells. Indeed, upon intraventricular transplantation in neonatal immunodeficient mice hiPS-NSPCs engrafted and widely dispersed in the brain parenchyma, migrating along the rostro-causal axis and differentiating in mature neurons and glial cells, with no sign of abnormal cell proliferation up to 12 months after treatment.This study contributes to the development of strategies for increasing safety and efficiency of hiPS-NSPC transplantation approaches for the treatment of neurodegenerative and demyelinating disorders.

139. Receptor Engineered TRuC Tregs Maintain a Regulatory Phenotype and Are Suppressive in a Murine Model of Hemophilia A

Jyoti Rana, Sandeep R. P. Kumar, Maite Munoz, Moanaro Biswas

School of Medicine, IUPUI, Indianapolis, IN

The most serious complication to clotting factor VIII (FVIII) replacement therapy in hemophilia remains the development of inhibitory antibodies (inhibitors), which occurs in a significant proportion of patients with severe disease. Tolerance to exogenously administered FVIII is strongly dependent on regulatory T cells (Tregs) and it is expected that "redirecting" antigen specificity by engineering Tregs with synthetic receptors will overcome low inherent precursor frequencies and simultaneously augment suppressive functions in Treg cellular therapy. Our preliminary data indicate that employing a high affinity chimeric antigen receptor (CAR) molecule paradoxically results in heightened signaling and loss of suppressive activity in transduced Tregs. Therefore, we applied an alternative approach, which is to complex a FVIII antibody based single-chain variable fragment (scFv) to the N-terminus of murine CD3ε subunit of TCR complex. The resulting TCR fusion construct (TRuC) Treg is able to recapitulate TCR based signaling in an MHC-independent fashion. We initially confirmed TCR dependent surface expression of TRuCs by flow cytometry using a FVIII binding assay, where co-transduction of FVIII-TRuC with TCR and CD3 components resulted in transportation of the engineered receptor to the plasma membrane surface in $5KC\alpha^{-}\beta^{-}$ cells (which lack TCRα and β) and in human HEK-293 cells (which lack TCR and CD3). FVIII stimulation of TRuC-Tregs *in vitro* led to upregulation of CD69, Ki67, CD28, FoxP3, and a 5-fold increase in CTLA4 expression (p=0.0001, 1-way ANOVA). TRuC Tregs secreted significantly lower levels of cytokines IL-2, IL-4, IL-17, IL-10 and IFNγ as compared to CAR Tregs. This was confirmed by intracellular cytokine staining. Phospho-flow cytometry and western blotting confirmed dampened expression of critical signaling proteins pAKT S473, pERK and pS6 in TRuC Tregs, which was similar to levels observed on triggering the endogenous TCR in Tregs with anti-CD3/28 microbeads. These results confirm that FVIII stimulated TRuC Tregs maintain a Treg phenotype. Importantly, FVIII stimulation and TRuC signaling did not result in a loss of lineage stability in transduced Tregs, as TRuC Tregs from FoxP3-GFP mice retained FoxP3 expression in vivo (92.75±0.3% GFP^{+} cells). We next investigated whether TRuC Treg functional responses was sufficient to maintain a suppressive phenotype *in vitro* and *in vivo*. FVIII TRuC Tregs were able to suppress the *in vitro* proliferation of TRuC Tconv responders when stimulated with low dose (0.1 IU/mL) BDD-FVIII, without any requirement for antigen presentation. *In vivo,* naïve BALB/c *F8*e16$^{-/-}$ recipient mice were infused with $5x10^5$ sorted TRuC Tregs or polyclonal thymic derived (t)Tregs ($5x10^5$ or $1x10^6$) followed by 8 weekly IV injections of 1.5 IU BDD-FVIII. FVIII TRuC Tregs were more effective at suppressing inhibitor formation as compared to polyclonal tTregs. 7 out of 8 animals in the

TRuC Treg group did not develop detectable inhibitors (0.23±0.23 BU/mL) at 4 weeks compared 8 out of 8 in the control group (69.42±33.99 BU/mL). At 8 weeks, control mice had a mean inhibitor titer of 151.4±48.6 BU/mL compared to 15.4±10.4 BU/mL in the TRuC Treg group. αFVIII IgG1 titers were also significantly lower in the TRuC Treg group (5238±3862 ng/mL) compared to the $5x10^5$ (28429±3862 ng/mL, p=0.0042) or $1x10^6$ (21821±8020 ng/mL, p=0.0438) tTreg groups at 8 weeks, suggesting a more sustained tolerogenic effect. In conclusion, this study suggests that adoptive cellular therapy with antigen specific engineered TRuC Tregs is able to suppress antibody formation against the soluble therapeutic protein FVIII in an MHC-independent manner, in spite of persisting only transiently in vivo (~7 days). More studies are required on regulating activation thresholds to maintain optimal suppressive function in engineered Tregs.

140. Functional Benefit of Mitochondrially Augmented HSPCs: Improved Engraftment and Alterations in Immune Cell Differentiation

Noa Sher[1], Elad Jacoby[2,3], Moriya Ben Yakir-Blumkin[1], Shiri Blumenfeld-Kan[1], Yehuda Brody[1], Amilia Meir[2], Ayelet Shabtay-Orbach[1], Gat Pozner[1], Tina Napso[1], Natalie Yivgi-Ohana[1], Amos Toren[2,3]

[1]Minovia Therapeutics Ltd, Tirat Hakarmel, Israel,[2]Pediatric Hematology and Oncology, Cell Therapy Center, The Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Tel Hashomer, Israel,[3]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

mtDNA associated disorders (either deletions, mutations or depletion) lead to multisystemic disease, often severe at a young age, with no disease-modifying therapies. In mitochondrial augmentation therapy (MAT), hematopoietic stem and progenitor cells (HSPCs) are enriched with healthy donor mitochondria ex vivo. This process allows cells to import mitochondria harboring full length mtDNA, resulting in increased mitochondrial content and improved function. HSPCs were chosen as recipient cells for MAT due to their demonstrated ability to alleviate systemic (hematological and non-hematological) pathologies. We demonstrated that mitochondrial augmentation of HSPCs is dose-dependent and confers functional benefit in both healthy donor and patient-derived HSPCs. We showed that HSPCs can be enriched with up to 34.9% exogenous mtDNA, resulting in increased oxygen consumption rate. To assess the potential effects of mitochondrial augmentation on human HSPCs, we used a humanized NSGS mouse transplanted with cord-blood derived CD34+ cells from a patient with Pearson Syndrome (PS), a mtDNA deletion syndrome. In this non-conditioned model, mitochondrially augmented CD34+ cells had improved long-term engraftment in the NSGS mice, as confirmed by flow cytometry and dPCR. Multi-lineage hematopoietic potential of engrafted cells was demonstrated, and animals which received mitochondrially enriched cells had significantly higher percentages of human CD3+ T cells. To investigate persistence in an immunocompetent animal, we developed a mouse model in which all nuclei were labelled with red fluorescence (Tomato) and all mitochondria were labelled with green fluorescence (Dendra). Recipient Polg mice had a point mutation in the mitochondrial DNA polymerase gene, leading to accumulation of mtDNA mutations. We were able to show long term persistence of daughter cells without preconditioning treatment. Continuous in vivo transfer of exogenous mitochondria to recipient host hematopoietic cells was observed and persisted up to 4.5 months post transplantation, the last time point tested. Cells of both myeloid and lymphoid lienage were recipients of mitochondria in the peripheral blood. Taken together, these data provide evidence supporting feasibility of augmentation of human HPSCs and for the potential of MAT as a therapeutic modality for the treatment of mitochondrial disorders.

141. Memory Enriched Epstein-Barr Virus {EBV} Specific T-Cells with Broader Target Antigen Repertoire for the Treatment of EBV+ Malignancies

Sandhya Sharma[1,2], Naren Mehta[1], Kathan Parikh[1], Ayea El-Ghazali[1], Mae Woods[1], Tim Sauer[1], Huimin Zhang[1], Birju Mehta[1], Vicky Torrano[1], Bambi Grilley[1], Helen Heslop[1,3,4], Cliona Rooney[1]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Graduate School of Biomedical Sciences, Baylor College of Medicine, Houston, TX,[3]Houston Methodist, Houston, TX,[4]Texas Childrens Hospital, Houston, TX

Almost 40 % Hodgkin's and Non-Hodgkin Lymphoma {HL/NHL} patients carry the EBV genome in a type 2 latency state in which 4 viral type-2 latency antigens {T2-Ag} are expressed. T2-Ag-specific T-cells from lymphoma patients are difficult to expand, likely because they are rendered anergic in the tumor microenvironment, and circulate with low frequency. In a clinical trial-NCT01555892 using patient-derived, EBV-specific T cells {EBVSTs} to target these tumors, ~25% of EBVST lines failed our manufacturing criteria due to lack of antigen specificity or failure to grow. Even in successfully expanded lines, over 50% recognized only 1 or 2 antigens, which could lead to tumor escape by antigen modulation. We ***hypothesized*** that low antigen specificity could result from the expansion of non-specific bystander cells in our cultures, and that enrichment of memory T-cells prior to culture as well as stimulation with additional viral tumor antigens could improve their potency. To enrich for memory T-cells, we **depleted the CD45RA+ fraction** of PBMCs that contains naïve T-cells, T-regs and NK-cells prior to EBVSTs generation. To broaden the tumor-specific T-cell repertoire, we evaluated EBV lytic cycle antigen expression in HL biopsies and combined EBV lytic antigen and T2-Ag peptide libraries to generate **broad repertoire {BR}-EBVSTs** with specificity to both in γ-IFN ELIspot assays. Depletion of CD45RA+ cells prior to EBVSTs generation increased the frequency of T2-Ag specific T-cells by 2-10 fold, and enhanced proliferation and cytotoxicity compared to EBVSTs generated from unfractionated PBMCs. Most importantly, we restored the ability of patient EBVSTs to respond to EBV T2-Ags. This unexpected finding suggested that CD45RA+ cells are capable of inhibiting the reactivation and expansion of T2-Ag specific T-cells. In support of our proposal to target both T2- and lytic antigens, we identified both T2-latent and lytic cycle transcripts in HL biopsy samples and demonstrated that BR-EBVSTs could be generated by stimulation with a combination of T2- and lytic-cycle antigens. Following adoptive transfer into an EBV+ murine xenograft model, autologous BR-EBVSTs cleared tumor more rapidly {average by

day 15} than T2-EBVSTs {average by day 30} and better limited metastatic spread. Notably human GM-CSF and IFN-γ serum levels were 2-fold or higher in mice treated with BR-EBVSTs, which should create a more pro-inflammatory tumor milieu leading to increased tumor killing and epitope spreading in lymphoma patients. **BR-EBVSTs from memory enriched CD45RA+ subset depleted PBMCs** are now under evaluation in our amended clinical trial. We generated EBVSTs with high antigen specificity from 7/7 lymphoma patients and treated five, including the first patient whose EBVSTs had failed manufacturing using the previous protocol {Fig 1.}

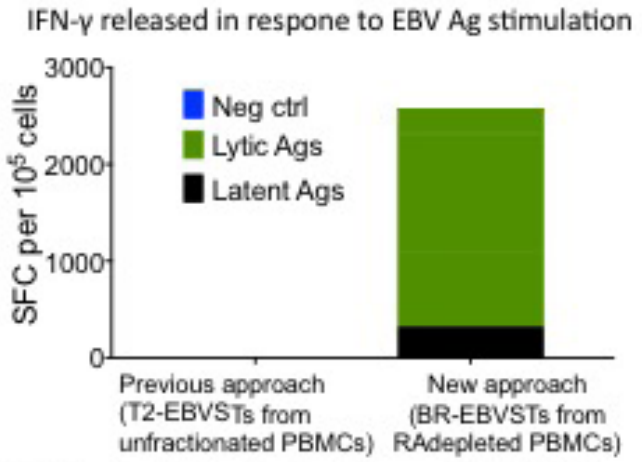


Fig 1.) Bar Graph illustrating the antigen specificity of EBVST lines generated for patient 1 by two different approaches. Y-axis represents the Spot Forming Cells (SFC) as a measure of number of cells that secreted IFN-γ in response to EBV antigen stimulation.

We have observed an increase in the frequency of T-cells recognizing both T2- and lytic antigens in patient PBMCs post-infusion for up to 3 months. Long-term follow-up and comparison with patients who received T2-EBVSTs from unfractionated PBMCs will determine if these changes indeed produced a more potent clinical product.

142. Non-Viral Engineering of CAR-NK Cells Using the *TC Buster* Transposon System™

Emily J. Pomeroy, Walker S. Lahr, Beau R. Webber, Branden S. Moriarity

Pediatrics, University of Minnesota, Minneapolis, MN

Immunotherapy with T cells and NK cells modified with viral vectors to express a chimeric antigen receptor (CAR) has shown remarkable efficacy in clinical trials. However, viral vectors are limited in their cargo size, carry the risk of insertional mutagenesis, and large-scale manufacturing for clinical use can be cost-prohibitive. Thus, CAR delivery via DNA transposon engineering has been pursued as an alternative due to convenient and cost-effective production and a better safety profile. Engineering via transposition is accomplished using a two-component system: a plasmid containing a gene expression cassette flanked by inverted terminal repeats (ITRs) and a transposase enzyme that binds to the ITRs and integrates the transposon into the genome. Here, we sought to use the newly developed *TC Buster*™ (Bio-Techne) transposon system to deliver a transposon containing a CD19-CAR-DHFR-EGFP expression cassette (3.7 kb transposon, figure 1A) to primary human peripheral blood (PB) NK cells. However, the use of transposons in NK cells has been very limited due to DNA toxicity and induction of a type I interferon response. Thus, we optimized methods to avoid this, including using DNase in recovery media and delivery via Nanoplasmid vectors which have a small (<500 bp) backbone, high supercoiling, and are regulatory compliant. We optimized activation, electroporation, recovery, and expansion conditions to achieve 49.6% (±4.64%) integration efficiency using an evolved hyperactive *TC Buster*™ transposase (Hyp-TCB) (Figure 1B). Our cargo also contained a mutant dihydrofolate reductase (DHFR) which allowed us to select for stable transposon integration using methotrexate (MTX). Our optimized protocol achieves manufacturing in 20 days and results in 99.2% (±0.5%) CAR+ NK cells expanded 837.9-fold (±88.6) from input (Figure 1C, 1D). We tested CAR-NK cells in functional assays against CD19-expressing Raji cell lines. CAR-NK cells produced significantly more IFNγ and TNFα than CAR-negative NK cells when co-cultured with Raji (Figure 2A, 2B). CAR-NK cells also expressed significantly more CD107a on their surface, a marker of degranulation (Figure 2C). In killing assays, CD19-CAR-NK cells killed over 90% of Raji cells in 24 hours at effector-to-target ratios as low as 1:3 (Figure 2D). Our work provides a platform for robust delivery of multicistronic, large cargo via transposition to primary human PB NK cells. We are currently using this platform to deliver larger cargo of interest that far exceeds the carrying capacity of viral vectors. We demonstrate that CAR-NK cells can be enriched using MTX selection, while maintaining high viability and function, and they can be expanded to clinically relevant numbers in a matter of weeks. In recent experiments we have demonstrated successful delivery of *TC Buster*™ transposons for large cargo integration with CRISPR/Cas9 for targeted gene knockout in a single electroporation event. This non-viral approach for multiplex editing of NK cells represents a versatile, safer, and more cost-effective option for the manufacture of CAR-NK cells compared to viral delivery.

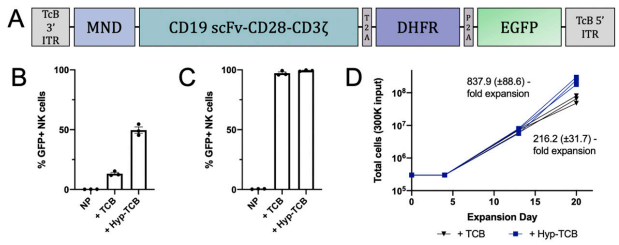


Figure 1. Delivery of a CD19-CAR-DHFR-GFP transposon using the evolved hyperactive TC Buster Transposon System. (A) The 3.7 kb transposon flanked by TC Buster ITRs, containing an MND promoter, CD19 CAR, mutant DHFR selection gene, and enhanced GFP. Elements are separated by 2A self-cleaving peptides. Transposon was delivered in a nanoplasmid (NP) backbone. (B) Primary human peripheral blood NK cells (n=3 human donors) were electroporated with the nanoplasmid (NP) alone or in combination with mRNA encoding either TC Buster (TCB) or the evolved hyperactive mutant TC Buster (Hyp-TCB). Two days after electroporation, NK cells were expanded with mbIL21-expressing feeder cells for 1 week. After expansion, GFP expression was measured by flow cytometry. (C) NK cells were expanded again with mbIL21-expressing feeder cells for one week in media containing 250 nM methotrexate (MTX) and then GFP expression was measured by flow cytometry. (D) Fold expansion was calculated over the course of the experiment.

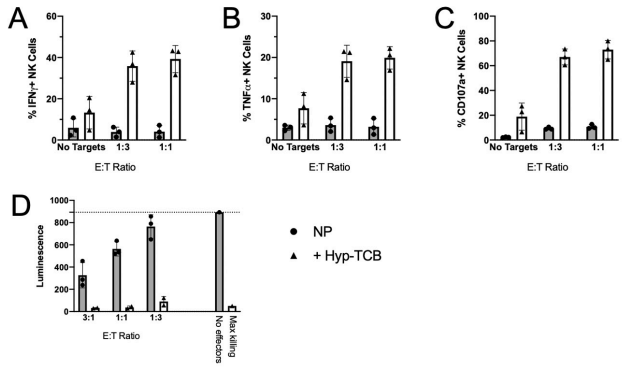


Figure 2. CD19-CAR-NK cells engineered via transposition show enhanced activity and killing against CD19+ target cells. MTX-selected NK cells (n=3 human donors) expressing CD19-CAR were co-cultured with luciferase-expressing, CD19-positive Raji target cells. (A-C) After co-culture, NK cells were analyzed by flow cytometry for expression of IFNγ, TNFα, and CD107a. (D) Target cell killing was measured by luciferase expression after co-culture.

143. Adoptively Transferred, *In Vitro*-Generated Alloantigen-Specific Type 1 Regulatory T (Tr1) Cells Persist Long-Term *In Vivo*

Alma-Martina Cepika[1], Pauline P. Chen[1], Rajni Agarwal[1], Gopin Saini[1], David M. Louis[2], Laura C. Amaya-Hernandez[3], Liwen Xu[4], Parveen Shiraz[4], Keri M. Tate[5], Dana Margittai[5], Neehar Bhatia[5,6], Everett Meyer[4], Alice Bertaina[1], Mark M. Davis[2,7,8], Rosa Bacchetta[1,6], Maria Grazia Roncarolo[1,3,6]

[1]Pediatrics, Stanford University School of Medicine, Stanford, CA,[2]Institute for Immunity, Transplantation and Infection, Stanford University School of Medicine, Stanford, CA,[3]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA,[4]Medicine, Stanford University School of Medicine, Stanford, CA,[5]Laboratory for Cell and Gene Medicine, Stanford University School of Medicine, Stanford, CA,[6]Center for Definitive and Curative Medicine, Stanford University School of Medicine, Stanford, CA,[7]Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA,[8]Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA

Allogeneic, HLA-mismatched hematopoietic stem cell transplantation (allo-HSCT) can be a life-saving treatment for many high-risk hematological malignancies, as well as for certain non-malignant disorders. However, the largest obstacle to wide-spread use of allo-HSCT is its frequent and life-threatening side-effect, graft-vs-host disease (GvHD). GvHD is caused by HSCT donor-derived effector T (Teff) cells that attack patient's HLA-mismatched healthy tissues. GvHD can be treated, but all standard-of-care treatments also induce generalized immunosuppression, increasing the risk of relapse, infection, and death. To alleviate GvHD without causing generalized immunosuppression, we aim to leverage type 1 regulatory T (Tr1) cells. Tr1 cells are inducible, IL-10+FOXP3- subset of regulatory T cells that was shown to correlate with tolerance in allo-HSCT patients, and prevent GvHD in mice. Tr1 cells suppress the activity of Teff cells, which cause GvHD *in vivo*, through soluble IL-10 and co-inhibitory surface receptors. We have generated alloantigen-specific Tr1 cells by co-culturing HSC donor-derived CD4 T cells with patient-derived tolerogenic dendritic cells, which present patient alloantigens and induce differentiation of CD4 T cells into Tr1 cells. During the differentiation, Tr1 cells clonally expand, developing a highly restricted TCR repertoire. The final Tr1-enriched cell product, called T-allo10, is anergic and suppresses Teff cell proliferation specifically in response to alloantigens, but not irrelevant antigens (Cepika AM, Chen PP et al, J ImmunoTher Cancer 2020; Vol. 8, S3:146). T-allo10 cells are being tested for safety in a phase I clinical trial in children and young adults with hematological malignancies undergoing allo-HSCT (ClinicalTrial.gov ID: NCT03198234). In this trial, T-allo10 cells are adoptively transferred one day before allo-HSCT, and patients are immunomonitored in 19 time points from day 0 to 360. One year-post treatment, the first three patients are alive, have met the safety criteria, and are GvHD and cancer-free (Agarwal R et al, Biol Blood Marrow Transplant 2020; Vol. 26 (3): S272-S273). To demonstrate the long-term survival of adoptively transferred Tr1 cells in these first three patients, we FACS-sorted the highly purified Tr1 cells from patient's T-allo10 cell product, and compared their TCR repertoire to non-Tr1 cells from the same product, parental CD4 T cells, and control Teff cells. We determined the top 20 most highly enriched TCR clonotypes of Tr1 cells, and then compared this data to the patient whole blood TCR repertoire in all 19 time-points. Using this method as complementary to peripheral Tr1 cell detection by flow cytometry, we demonstrate that Tr1 cells persist in patients for up to 1-year post adoptive transfer. This implies that Tr1 cells represent a durable cell therapy option to modulate antigen-specific tolerance.

144. Endothelial Progenitor Cells Engineered to Overexpress Endothelial NO-Synthase May Improve Infarct Healing: Results from the Enhanced Angiogenic Cell Therapy - Acute Myocardial Infarction (ENACT-AMI) Trial

Duncan Stewart[1], Michael Kutryk[2], Hung Q. Ly[3], Christopher A. Glover[4], Alexander Dick[5], Kim Connelly[2], Shaun G. Goodman[2], Howard Leong-Poi[2], Leslie Carlin[1], Rose Gaudet[1], Monica Taljaard[6], David Courtman[1]

[1]Sinclair Centre for Regenerative Medicine, Ottawa Hospital Research Institute, Ottawa, ON, Canada,[2]St. Michael's Hospital, Toronto, ON, Canada,[3]Cardiology, Montreal Heart Institute, Montreal, QC, Canada,[4]Cardiology, University of Ottawa Heart Institute, Ottawa, ON, Canada,[5]University of Ottawa Heart Institute, Ottawa, ON, Canada,[6]Ottawa Methods Centre, Ottawa Hospital Research Institute, Ottawa, ON, Canada

Introduction: Despite modern reperfusion therapies for acute myocardial infarction (AMI), many patients are left with a significant area of injury leading to scar formation that contributes to a dysfunctional myocardium and the development of heart failure. The goal of the Enhanced Angiogenic Cell Therapy-AMI (ENACT-AMI) trial was to determine whether intracoronary delivery of early outgrowth endothelial progenitor cells (EPCs) engineered to overexpress endothelial NO-synthase (eNOS) would improve global left ventricular ejection fraction (LVEF) (primary outcome) and infarct size as assessed by cardiac MRI (CMR) in patients with LAD territory ST elevation myocardial infarction treated with evidence-based therapies. **Methods**: ENACT-AMI (NCT00936819) was a double-blind placebo-controlled trial in which participants were randomized to one of 3 arms:

1) saline placebo; 2) EPCs; or 3) eNOS-transfected EPCs. EPCs were derived from circulating mononuclear cells by 7-8 days of differential culture on fibronectin in EGM supplemented with endothelial growth factors using fully sourced GMP-compatible reagents. Transfection was performed using a plasmid DNA vector (pVax) containing the human eNOS sequence combined with JetPEI (Polyplus). Target sample size was 100 participants. Groups were compared using analysis of covariance (ANCOVA). **Results**: The trial was terminated due to slow recruitment after 47 patients were enrolled at three Canadian sites over six years (n=18 placebo, n=15 EPCs; n=14 eNOS-transfected EPCs). The groups were comparable with respect to demographic variables including age (56.1±9.7 years), cardiac risk factors, pre-existing cardiac disease, peak CK and troponin values and baseline LVEF by cardiac magnetic resonance imaging (40.7±9.3). Intracoronary cell delivery (20M cells; 20±5 days post-AMI) was well tolerated. At 6 months, there were no significant differences in the primary endpoint of LVEF between groups (mean difference between the average of the two EPC groups vs. placebo: 0.5% [95% Confidence Interval (CI) 2.9% to 3.9%). The secondary outcome of left ventricular infarct mass indexed to LV size at 6 months demonstrated no significant difference between the average of the two EPC groups versus placebo (p=0.72); however, a significant difference was seen in those receiving eNOS-transfected EPCs compared to EPCs (-6.6; CI -12.0 to -1.1, p=0.02). Only four major cardiovascular events were observed over an average follow up of 4.1±1.6 years, and these were equally distributed across groups. **Conclusions**: While there were no significant differences in LVEF, the results of the ENACT-AMI trial suggest that intracoronary delivery of gene-enhanced EPCs reduced infarct size and LV diastolic diameter in patients with large anterior wall AMI consistent with improved scar healing. These findings have important implications for remodelling and require confirmation in larger clinical trials.

CAR-Based Cancer Gene Therapy

145. Chimeric Antigen Receptor Macrophages (CAR-M) Induce Anti-Tumor Immunity and Synergize with Immune Checkpoint Inhibitors in Pre-Clinical Solid Tumor Models

Stefano Pierini[1], Rashid Gabbasov[1], Alison Worth[1], Linara Gabitova[1], Daniel Blumenthal[1], Yumi Ohtani[1], Olga Shestova[2], Maksim Shestov[2], Saar Gill[2], Sascha Abramson[1], Thomas Condamine[1], Michael Klichinsky[1]

[1]Carisma Therapeutics, Philadelphia, PA,[2]Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA

Despite the remarkable efficacy achieved by CAR-T cell therapy in hematologic malignancies, translating these results for solid tumors remains challenging. We previously developed human CAR-M and demonstrated that adoptive cell transfer of CAR-M into xenograft models of human cancer controls tumor progression and improves overall survival[1]. Given that CAR-M are professional antigen presenting cells, we established a fully immunocompetent syngeneic mouse model to evaluate the interaction of CAR-M with the tumor microenvironment (TME) and the potential for induction of a systemic anti-tumor immune response. Murine bone marrow-derived CAR-expressing macrophages (muCAR-M) were efficiently engineered to express an anti-huHER2 CAR using the chimeric adenoviral vector Ad5f35. In addition to efficient gene delivery, Ad5f35 transduction promoted a pro-inflammatory (M1) phenotype in murine macrophages. MuCAR-M, but not control untransduced (UTD) macrophages, specifically phagocytosed HER2+ target cancer cell lines and killed HER2-expressing murine CT26 colorectal and human AU-565 breast cancer cells in a dose-dependent manner. Moreover, CAR-M induced MHC-I and MHC-II expression on tumor cells and cross-presented tumor-associated antigens (TAA) resulting in CD8+ T cell activation. To evaluate muCAR-M in an immunocompetent *in vivo* setting, BALB/c mice were engrafted with subcutaneous CT26-HER2+ tumors and treated with HER2-CAR or UTD macrophages. CAR-M treated mice showed significant tumor control and improved survival compared to control groups. Analysis of the TME showed increased intratumoral immune infiltration as well as an increase in T cells reactive to the CT26 MHC-I antigen gp70, indicating enhanced epitope spreading. Mice that achieved complete responses (CRs) after CAR-M therapy were protected against antigen-negative relapse in a HER2- CT26 (CT26-Wt) rechallenge model. This anti-tumor immune response was CD3+ T cell-mediated and suggested induction of long-term memory against TAA. To evaluate the systemic anti-tumor immune response, we simultaneously engrafted BALB/c mice with CT26-HER2+ and CT26-Wt tumors on opposite flanks and treated mice with local administration of CAR-M to the HER2+ tumors. After CAR-M treatment, 75% of mice cleared their CT26-HER2+ tumors and the growth rate of the contralateral CT26-Wt tumors was significantly reduced, demonstrating an abscopal effect. Given the impact of CAR-M on the endogenous adaptive immune system, we evaluated the combination of CAR-M with PD1 checkpoint inhibitor therapy in the CT26-HER2 model, which is resistant to anti-PD1 monotherapy, and found that the combination further improved tumor control and overall survival. These results demonstrate that CAR-M reprogram the TME, induce epitope spreading, and orchestrate a systemic immune response against solid tumors. Moreover, our findings provide rationale for the combination of CAR-M with immune checkpoint inhibitors. The anti-HER2 CAR-M, CT-0508, is under evaluation in a phase I clinical trial for patients with HER2 overexpressing solid tumors (NCT04660929).

1. Klichinsky M, Ruella M, Shestova O, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. Nat Biotechnol. 2020;38(8):947-953.

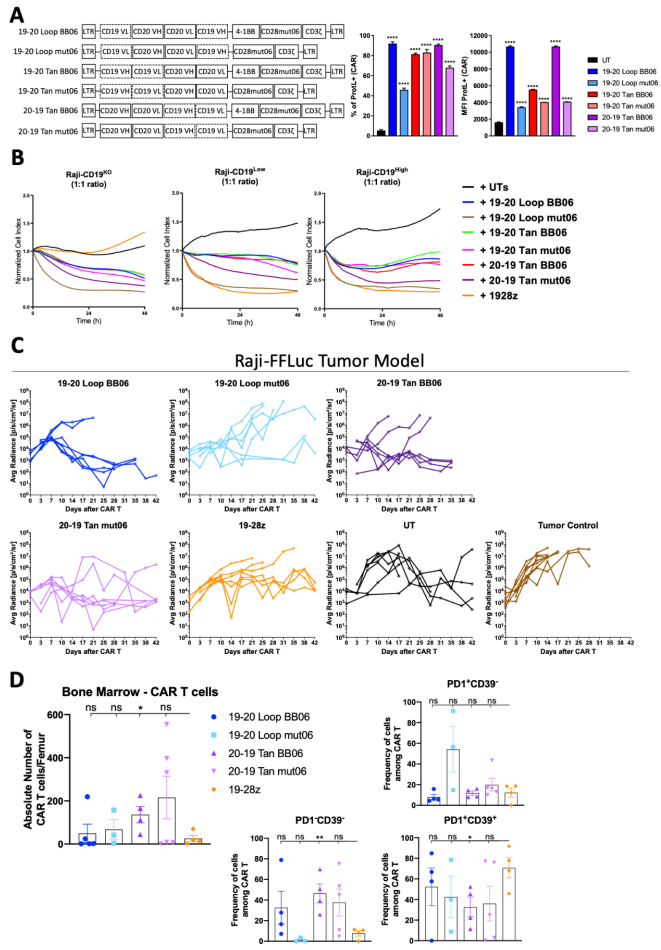
146. Bivalent CD19/CD20-Specific CAR T Cells with 4-1BB and Mutated CD28 Co-Stimulatory Domains Show Enhanced Function

Emiliano Roselli, Gongbo Li, Kristen Spitler, Justin Boucher, Kayla M. Reid, Sae Bom Lee, Nhan Tu, Marco L. Davila

Immunology, Moffitt Cancer Center, Tampa, FL

Despite the high response rates of CD19 CAR T cell therapy, the loss or downregulation of CD19 has been reported as a common mechanism of tumor resistance. Current strategies focused on overcoming antigen escape include the use of CAR T cells able to recognize more than one target antigen. Additionally, the choice of co-stimulatory domains

impact CAR T cell expansion, persistence and antitumor function. Our recent study found that mutating the CD28 endodomain to retain signaling only through the PYAP motif (mut06) rendered enhanced antitumor activity to CAR T cells, which was in part due to resistance to T cell exhaustion (Boucher et al. 2020). For this work we developed bi-specific constructs encoding for single bivalent CAR molecules able to recognize both human CD19 (FMC63) and CD20 (Leu16) in a loop or tandem configuration. To take advantage of the bi-specificity and the enhanced co-stimulatory domain we developed second generation bivalent CARs with mutated CD28 (mut06) and third generation CARs containing both 4-1BB and mut06 (BB06). We determined efficient surface CAR expression in all constructs and increased central memory phenotype in third generation BB06 CARs (Figure 1A). We detected secretion of significant levels of IFNγ, IL-2, TNFα, and minimal levels of IL-6 upon engagement to either CD19 or CD20 with the highest levels observed when both antigens were present. We determined in vitro cytotoxicity by a real-time assay (RTCA) against either antigen alone or both and observed significant lytic effect of all constructs with second generations (mut06) CARs showing a faster response (Figure 1B). We then analyzed in vivo antitumor function of these bivalent CAR T cells against a xenograft model of Raji-FFLuc. Third generation CAR T cells with mutated CD28 (BB06) showed a more effective tumor growth control compared to mono-specific anti-CD19 with unmutated CD28 co-stimulation (1928z)(Figure 1C). Importantly, these BB06 CAR T cells showed increased persistence in the mice at day 45 post-inoculation compared to 1928z. Furthermore, BB06 CAR T cells displayed significantly lower frequency of cells expressing the exhaustion-associated markers PD1, TIM3 and CD39 compared to 1928z and second-generation mut06 cells (Figure 1D). In a subsequent in vivo experiment using Raji cells only expressing CD20 (Raji-CD19KO) we were able to validate our previous results showing 20-19 tandem BB06 as the most effective construct against this CD19KO model. To date our results demonstrate that these bi-specific CAR T cells are able to recognize and trigger an effector function upon engagement of CD19 and/or CD20 and, most importantly that the combination of 4-1BB and this new mutated form of CD28 as co-stimulation enhance the antitumor function of cells. This work supports the concept that CAR T cell therapies can be improved by optimizing their design, including this dual antigen-binding domain and modified co-stimulatory motifs.



147. Combining IAP Inhibitors with CAR T Cell Therapy to Treat Glioblastoma

Edward Z. Song[1], Benjamin I. Philipson[1], Radhika Thokala[1,2], Zev A. Binder[2], Donald M. O'Rourke[2], Michael C. Milone[1]

[1]Department of Pathology and Laboratory Medicine and Center for Cellular Immunotherapies, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Antigen heterogeneity is one of the major obstacles to successful chimeric antigen receptor (CAR) T cell treatment in solid tumors including glioblastoma, as antigen-negative tumor cells could escape from the CAR-T cells. To address this issue and improve the efficacy of CAR-T cell therapy for glioblastoma, we are developing an approach that combines IAP inhibitors, a new class of small molecules, with CAR-T cells to treat glioblastoma. IAP inhibitors can induce the degradation of inhibitor of apoptosis (IAP) proteins. By antagonizing IAP proteins, IAP inhibitors could sensitize glioblastoma cells, including antigen-negative cells, to apoptosis induced by CAR-T cell-derived tumor necrosis factor (TNF). Meanwhile, IAP inhibitors may promote CAR-T cell survival, proliferation and cytokine production including TNF via NF-kB inducing kinase (NIK)-mediated NF-κB pathways. We demonstrate that the IAP inhibitor Birinapant, currently in early phase clinical

studies, induces the degradation of cellular IAP1 (c-IAP1) in the human glioblastoma cell line M059K, and exogenous TNF and Birinapant synergistically kill M059K via apoptosis. We further show that while CAR-T cells targeting glioblastoma antigen epidermal growth factor receptor variant III (EGFRvIII) do not directly kill M059K parental cells lacking EGFRvIII expression, Birinapant enhances the "by-stander" cell death of M059K parental cells when co-cultured with M059K cells transduced with EGFRvIII and the anti-EGFRvIII CAR-T cells, which express TNF when stimulated. Furthermore, our data show that conditioned medium from activated CAR-T cells mediates M059K cell death in the presence of Birinapant, and this death is partially blocked by TNF neutralization by infliximab, supporting the role of TNF in the mechanism of by-stander killing. In addition, we investigated how the IAP inhibitor Birinapant affects CAR-T cell functions. Exploiting an *ex vivo* repetitive stimulation assay, we show that Birinipant promotes CAR-T cell proliferation as measured by a higher fraction of cells in S-phase and also enhances TNF production by the CAR-T cells with prolonged antigen stimulation. These effects are associated with the activation of both the canonical and non-canonical NF-kB pathways. Glioblastoma xenograft models with heterogeneous tumor antigen expression are currently being developed to evaluate the efficacy of combining CAR-T cells with Birinipant *in vivo* in order to prevent escape due to antigen heterogeneity. In conclusion, our results support the potential for combining IAP inhibitors such as Birinipant as a novel approach to addressing antigen heterogeneity and tumor escape that represent a major hurdle to CAR-T cell-based immunotherapies.

148. B-CLL-Mediated Insufficient Activation Is CAR-Independent

McKensie A. Collins, Weimin Kong, In-Young Jung, Stefan Lundh, Joseph A. Fraietta, J. Joseph Melenhorst
University of Pennsylvania, Philadelphia, PA

Background: Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in the western world, accounting for nearly 1/3rd of adult leukemia diagnoses. While standard-of-care chemo-immunotherapies are initially successful in treating this disease, the lack of curative therapeutic options means that most patients will ultimately succumb to their disease. Chimeric Antigen Receptor (CAR) T cell therapy has proven effective, but only in a small subset of patients. Improving response rates to this therapy will provide much-needed curative options for this patient population. **Findings:** We have previously shown that the inability of CLL cells to activate CAR T cells drives this lack of response. In a serial re-stimulation model, we observed defects in both CD19- and ROR1-directed CAR T cell proliferation, cytokine production, and cytotoxicity. Flow cytometry showed that CLL-stimulated CAR T cells maintained an un-activated profile, suggesting that CLL cells fail to stimulate CAR T cells rather than rendering them non-functional. We further showed that these defects were non-permanent and could be rescued by stimulating CAR T cells with an artificial antigen presenting cell (aAPC) with or without the presence of CLL cells. Immunophenotyping of our B-CLL biobank showed that the majority of CLL cells (18/20 patients, 90%) express the IL-2 receptor alpha chain (CD25) at a high level. We hypothesized that CD25 may be part of an active IL-2R signaling complex, allowing CLL cells to outcompete the CAR T cells for IL-2, limiting their activation and explaining the observed phenotype. We supplemented IL-2 into CLL/CAR T cell co-cultures which rescued the proliferative capacity of the CAR T cells and partially rescued cytokine production. Further, we performed intracellular cytokine staining of CAR T cells stimulated with aAPCs and CLL cells. Interestingly, we found that CLL cells fail to stimulate IL-2 production after either a 6- or 12-hour incubation, disproving the IL-2 sinking hypothesis. However, we did observe low-level CD107a acquisition, suggesting that the CAR T cells retain some cytotoxic function. We next proposed that low-level expression of co-stimulatory or adhesion molecules on CLL cells may impair CAR T cell activation. Immunophenotyping of the CLL cells showed low level expression of molecules such as CD54, CD80, and CD86. We hypothesized that up-regulation of these molecules could improve CAR T cell targeting of CLL cells. We activated CLL cells via CD40L and IL-4 which resulted in subsequent up-regulation of CD54, CD58, CD80, and CD86 as well as other molecules. Stimulation of CAR T cells with these activated CLLs enhanced CAR T cell proliferation and cell-conjugate formation, indicating stronger cell-to-cell interactions. Therefore, improving CLL stimulatory capacity can rescue T cell dysfunction. To assess whether IL-2 addition and CD40 ligation were synergistic, we combined the two assays; however, we saw no additional improvement over IL-2 addition alone. We showed that rescue via either IL-2 addition or CD40 ligation was not CAR-specific, as we observed similar defects and rescue with both a ROR1-targeting CAR and the gold standard CD19-targeting CAR. **Conclusions:**These data indicate that CAR T cell dysfunction in CLL is mediated by insufficient activation of CAR T cells rather than true defects in cell function. Improving the stimulatory capacity of CLL cells via IL-2 addition or CD40 activation may enable better clinical responses. Additionally, the requirement for enhanced co-stimulation and adhesion even in a second-generation CAR T cell containing a co-stimulatory signaling domain is a unique finding. Further, the conserved effect between CD19- and ROR1-targeting CARs suggests that these results may be broadly applicable to CAR T cell therapies, and may be relevant in other indications beyond CLL.

149. CAR Design and Expression Determine Hyper-Proliferative States in TET2 Deficient T Cells

Nayan Jain[1], Zeguo Zhao[1], Archana Iyer[1], Michael Lopez[1], Judith Feucht[2], Richard Koche[1], Julie Yang[1], Yingqian Zhan[1], Michel Sadelain[1]
[1]Memorial Sloan Kettering Cancer Center, New York, NY,[2]University of Tübingen, Tübingen, Germany

TET2 disruption through a chance integration of a 4-1BB chimeric antigen receptor (CAR) lentiviral vector led to the emergence of a dominant CAR T cell clone, which coincided with tumor clearance in a chronic lymphocytic leukemia (CLL) patient (Fraietta et. al. Nature 2018). The enhanced proliferative ability ascribed to the *TET2* disruption opened the possibility of treating patients with lower doses of TET2 deficient CAR T cells than are currently required. In a pre-clinical murine model of human acute lymphoblastic leukemia (ALL), we studied the effect of CAR design in determining the effect of *TET2* editing on T cell phenotype and anti-tumor efficacy. To assess the effect of *TET2* disruption on CAR T cell therapeutic efficacy, we treated immune deficient mice bearing the human ALL cell line, NALM6, with limiting doses of either *TET2* edited (CRISPR/Cas9) or

unedited CAR T cells. We investigated the two most widely used second generation CD19-specific CARs encompassing the costimulatory domain of either CD28 (Rv-1928z) or 4-1BB (Rv-19BBz). *TET2* editing enhanced the anti-tumor efficacy of Rv-19BBz CAR T cells and promoted the acquisition of a central memory phenotype. In contrast, *TET2* editing did not alter the anti-tumor efficacy of Rv-1928z CAR T cells or their differentiation state. As Rv-1928z CAR T cells have a stronger induction of effector differentiation than Rv-19BBz CAR T cells, this divergence led us to hypothesize that *TET2* editing acts in concert with CAR signaling to remodel the T cell phenotype. To test this hypothesis, we edited *TET2* in two additional CAR designs that have been shown to limit T cell effector differentiation over retrovirally encoded CD28 costimulated CARs: 1928z driven by the *TRAC* promoter (*TRAC*-1928z) and Rv-1928z co-expressing the 4-1BB ligand (Rv-1928z-41BBL). Indeed, disruption of *TET2* enhanced the anti-tumor efficacy of both these CAR T cells and increased their early central memory phenotype. However, *TET2* edited CAR T cells, over time, attain a hyper-proliferative phenotype with a near total loss of effector function. This state was consistently associated with biallelic *TET2* editing. The frequency with which *TET2* edited T cells achieved hyper-proliferation depended on the signaling properties of the CAR receptor. This hyper-proliferative state is associated with sustained upregulation of cell cycle factors and shares features with some T cell leukemia/lymphoma. Exome analysis identified point mutations and chromosomal aberrations in hyper-proliferative cells, but they were not conserved across different populations, suggesting an alternate mechanism as a likely candidate for driving the hyper-proliferation. Chromatin accessibility analysis revealed that loss of *TET2* sets an epigenetic state that allows for sustained elevated levels of BATF3, which in turn drives a MYC-dependent proliferative program in *TET2* deficient CAR T cells. Our study shows that disruption of *TET2* may enhance the proliferation and persistence of T cells, depending on their CAR receptor, but that *TET2* deficient CAR T cells eventually uncouple their proliferative program from effector function thus impairing their therapeutic potency.

150. Tumor-Responsive, Multifunctional CAR-NK Cells Cooperate with Impaired Autophagy to Infiltrate and Target Glioblastoma

Jiao Wang[1], Sandro Matosevic[1,2]

[1]Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN,[2]Center for Cancer Research, Purdue University, West Lafayette, IN

Background: Tumor antigen heterogeneity, a severely immunosuppressive tumor microenvironment (TME), and lymphopenia resulting in inadequate immune intratumoral trafficking all contribute to glioblastoma (GBM) being highly resistant to therapy [1]. Though intratumoral presence of NK cells is beneficial to GBM patients, current GBM immunotherapies have struggled to overcome these challenges and demonstrate sustained clinical improvements in patient overall survival (OS). As a result, new immunotherapeutic approaches for this deadly cancer that are able to address all three of these critical hurdles are needed. **Methods:** We generated multifunctional human NK (CD73.mCAR-pNK) cells that express a dual-specific CAR redirected against ligands for NKG2D and GBM-associated GD2 receptors, and a third functional moiety that can be activated in the GBM TME to address immunometabolic suppression of NK cell function: tumor-associated proteases (TAP) mediated site-specific release of anti-CD73 scFv which can inhibit the activity of CD73 independently of CAR signaling and decrease the local concentration of adenosine (**Fig.1A**-**C**). To address insufficient homing of NK cells into the tumor bed, we combined these cells with chloroquine (CQ) as an adjuvant to inhibit autophagy in GBM and evaluated these cells against patient-derived intracranial GBM models in vivo (**Fig.1D**). **Results:** When administered with CQ, CD73.mCAR-pNK cells could effectively target orthotopic patient-derived GBM xenografts, demonstrating effective anti-tumor responses (**Fig.1E** and **F**), potent inhibition of CD73 expression (**Fig.1G** and **H**) and remarkable suppression of adenosine production in the local tumor (**Fig.1I** and **J**). In addition, we also unveiled a complex reorganization of the immunological profile of GBM induced by inhibiting autophagy. In particular, pharmacologic impairment of the autophagic process promoted a significant secretion of chemokines, including CCL5 and CXCL10, which were favorable to NK cell infiltration (**Fig.1K**). In response to combination therapy with CD73.mCAR-pNK cells and CQ, we detected significantly elevated presence of NK cells in GBM tumors in brains of treated mice, which correlated to higher chemokine levels (**Fig.1L** and **M**). **Conclusions:** We built a novel, sophisticated combinatorial platform for GBM: a multifunctional immunotherapy based on genetically-engineered, human NK cells bearing multiple anti-tumor functions, including local tumor responsiveness, that addresses, for the first time, key drivers of GBM resistance to therapy: antigen escape, immunometabolic reprogramming of immune responses, and poor immune cell homing. **References** [1] Pombo Antunes AR, Scheyltjens I, Duerinck J, Neyns B, Movahedi K, Van Ginderachter JA. Understanding the glioblastoma immune microenvironment as basis for the development of new immunotherapeutic strategies. Elife. 2020;9:e52176.

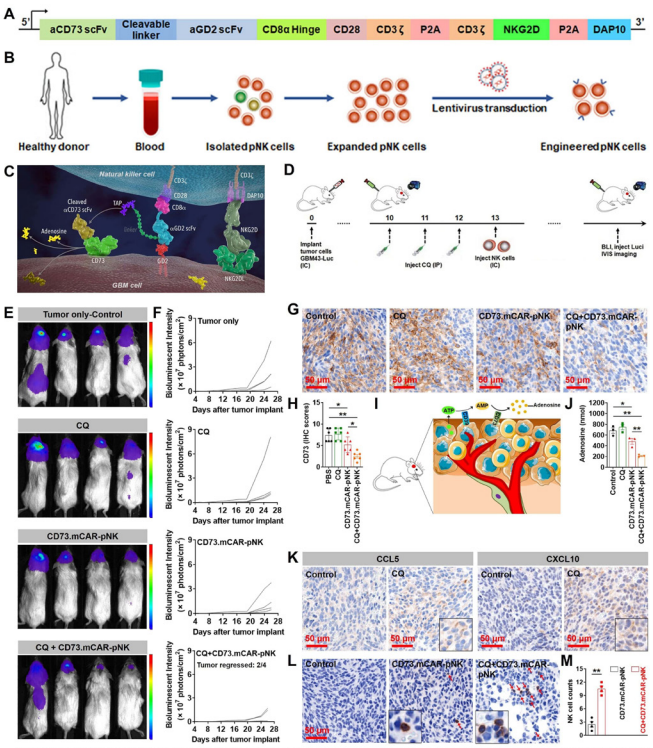


Fig. 1. (**A**) Schematic representation of transgene representing the complete multi-functional construct: tumor-responsive anti-CD73 scFv-secreting dual-specific CAR targeting NKG2DL and GD2. (**B**) Schematic illustration showing the process of viral engineering human primary NK (pNK) cells to generate CD73.mCAR-pNK cells. (**C**) Illustration showing the multifunctional, tumor-responsive engineered NK cells and their working mechanisms against GBM. (**D**) Schematic diagram showing the in vivo treatment program through an orthotopic patient-derived GBM xenograft model. (**E**) Bioluminescence imaging of mice in each treatment groups on day 26 after tumor implantation. (**F**) Tumor growth is shown for individual mice from each group over time monitored using bioluminescent imaging. (**G**) Immunohistochemical (IHC) staining for CD73 performed on tumor sections from indicated treatment groups using anti-CD73 antibody. Scale bar = 50 μm; 200× magnification. (**H**) IHC scores of CD73 expression performed on tumor sections from different treatment groups. (**I**) Schematic diagram showing extracellular adenosine production in the GBM tumor microenvironment. (**J**) Adenosine concentration in local brain tissues of mice in each treatment group (n = 3). (**K**) Immunohistochemical (IHC) staining of CCL5 (left two panels) and CXCL10 (right two panels) performed on indicated tumor sections in different treatment groups using anti-CCL5 and anti-CXCL10 antibodies, respectively. Scale bar = 50 μm; 200× magnification. (**L**) Immunohistochemical (IHC) staining for NK cells performed on tumor sections from indicated treatment groups using anti-NKp46 antibody. Scale bar = 50 μm; 200× magnification. (**M**) Quantification of NK cell infiltration into intracranial tumors treated with CD73.mCAR-pNK cells or CQ + CD73.mCAR-pNK cells. Here, cell counts were recorded in 4 consecutive high-power fields (HPFs) at 200× magnification. Data were measured from independent samples. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$. p values in (M) were determined using the two-tailed Student's t-test, and in (H and J) using one-way ANOVA analysis.

151. ADCLEC.syn1 Is a Novel Combinatorial CAR Platform for Enhanced Therapeutic Index in AML

Sascha Haubner, Jorge Mansilla-Soto, Sarah Nataraj, Jae Park, Xiuyan Wang, Isabelle Rivière, Michel Sadelain

Center for Cell Engineering and Immunology Program, Memorial Sloan Kettering Cancer Center, New York, NY

Relapsed/refractory (r/r) AML is associated with very poor prognosis. The only curative option to date is allogeneic stem cell transplantation which is limited due to high treatment-related toxicity and therapeutic failure, creating a high medical need for potent yet tolerable novel therapies. CAR therapy has high potential for successful application beyond CD19-positive B cell malignancies, however suitable CAR targets in AML still need to be identified. Integrating proteomic and transcriptomic target expression data we have previously discovered n=24 CAR candidate targets with a favorable profile in AML and normal tissues and provided a rationale for several combinatorial CAR approaches in AML (Perna et al. Cancer Cell 2017). We have further validated favorable target pairs among our 24 candidates, based on a multimodal in-depth analysis including multiparameter spectral flow cytometry of primary r/r AML and normal bone marrow samples, along with normal tissue immunohistochemistry studies and mass-spectrometry. We focus here on ADGRE2 and CLEC12A, two cell surface molecules highly co-expressed in AML but with largely non-overlapping expression profiles in normal tissues. We hypothesized that rational combinatorial CAR design targeting both ADGRE2 and CLEC12A enhances anti-leukemic efficacy without cumulating potential on-target/off-tumor toxicity. Using a bicistronic gamma-retroviral vector, we screened different combinatorial CAR formats targeting ADGRE2 and CLEC12A. Specific to the combined target expression profile in malignant versus normal cells, we fine-tuned both scFv affinities considering total avidity, eventually achieving thresholds for mediating cytolysis in the context of optimized CD3 zeta signaling. In addition, we further optimized the chimeric receptor combination by evaluating different hinge/transmembrane and costimulatory domains. To provide a platform for identification of the ideal combinatorial CAR design, we established in-vitro and in-vivo models based on a human AML cell line with up- or down-regulated antigen levels of ADGRE2 and CLEC12A to mimic both AML antigen-low escape and toxicity to normal cells. Ultimately, using therapeutically relevant T cell doses we were able to identify a combinatorial CAR format that allowed complete and durable AML remission across relevant target levels while sparing cell line clones representing normal cells.

Cardiovascular and Pulmonary Gene Therapy

152. Systemic *Hps1* Gene Augmentation Prevents Pulmonary Manifestations in a Mouse Model of Hermansky-Pudlak Syndrome

Shachar Abudi[1,2], Marina Zieger[3], John D. Burke[1], Lisa J. Garrett[4], Yair Anikster[2,5], Bernadette R. Gochuico[1], William A. Gahl[1,6], Christian Mueller[3], May C. Malicdan[1,6]

[1]Human Biochemical Genetics Section, NHGRI, NIH, Bethesda, MD,[2]Tel Aviv University, Tel Aviv, Israel,[3]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[4]Transgenic Mouse Core, NHGRI, NIH, Bethesda, MD,[5]Sheba Medical Center, Ramat Gan, Israel,[6]NIH UDP, NHGRI, NIH, Bethesda, MD

Mutations in *HPS1* cause Hermansky-Pudlak syndrome type 1 (HPS-1), an autosomal recessive disorder manifesting with oculocutaneous albinism, a bleeding diathesis, and a highly penetrant and lethal form of pulmonary fibrosis (HPSPF). A prominent finding in the lungs of HPSPF patients is the presence of enlarged and foamy type II alveolar cells (AECII), which are thought to have critical roles in alveolar homeostasis. Alteration in the function of AECII cells have been shown to promote dysregulated repair and pathogenic activation of fibroblasts, ultimately leading to fibrosis. There is no FDA-approved treatment for HPSPF; the identification of effective therapy has been hindered by the lack of preclinical models that represent the human HPS genotype and phenotype. Because *HPS1* mutations are loss-of-function, we hypothesized that introduction of a normal copy of *HPS1* could be a strategy for treatment. With the goal to establish gene therapy for HPS-1, we systemically administered adeno-associated virus (AAV) harboring the open reading frame of the murine *Hps1* to a novel *Hps1* knockout mouse that we generated.We deleted *Hps1* in mice by using CRISPR-Cas9 ($Hps1^{delta/delta}$). These mice recapitulated human HPS-1 phenotypes, and presented with enlarged and foamy

AECII, increased susceptibility to bleomycin-induced PF, reduced pigmentation, and a bleeding diathesis. Molecularly these mice show no *Hps1* mRNA expression in their lungs and other tissues. To determine which AAV serotype is suited to target AECII, we injected AAV5 or AAV8 harboring GFP to one-day old via the facial vein. We performed *in situ* hybridization of RNA, immunohistochemistry of GFP protein and molecular quantification. Our results reveal that AAV5 and AAV8-GFP are highly expressed in lung cells, including AECII, after 1 month of augmentation.For *in vivo* gene augmentation, we injected systemically AAV5 or AAV8 harboring murine *Hps1* to one-day old mice. Phenotypic characterization included molecular analysis, examination of lung pathology, and measurement of lung physiology. Six months after gene augmentation mice given AAV5-Hps1 or AAV8-Hps1 revealed an increase of about 80% in *Hps1* mRNA in their lungs compared to lungs of untreated mice that did not express *Hps1* mRNA. More importantly, histological analysis showed improvement of the lung phenotype including reduction in the size and number of enlarged and foamy AECII (Figure 1), which correlated with higher *Hps1* expression. Taken altogether, our results show that gene augmentation of AAV can prevent the pulmonary manifestations in our *Hps1* knockout mouse and can be used in designing therapeutic options for HPSPF.

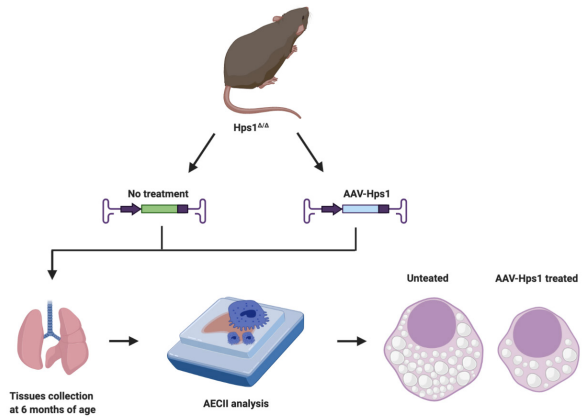


Figure 1. Schematic presentation of approach to gene augmentation. AAV5 or AAV8 encoding *Hps1* were injected into Hps1$^{delta/delta}$ mice. Tissues were collected and analyzed after 6 months. Lung histology of Hps1$^{delta/delta}$ untreated mice reveals enlarged and foamy type II alveolar epithelial cells (AECII). Mice that were transduced with AAV5 or AAV8 *Hps1* had smaller and less foamy AECII.

153. Generation of a Human 3D Lung Model for Therapeutic Gene Editing in Surfactant Protein B Deficiency

Helena C. M. Meyer-Berg, Stephen C. Hyde, Deborah R. Gill

Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom

Surfactant protein B (SP-B) deficiency is a rare autosomal recessive disease of the lung leading to severe respiratory distress that is fatal within the first months of life. Conventional therapies such as steroids or surfactant replacement therapy are ineffective in these individuals. Gene therapy has the potential to treat SP-B deficiency by restoring surfactant homeostasis. Cross-species variation can hamper gene therapy development, because of variable expression of viral entry receptors crucial for (e.g. rAAV) vector entry. Moreover, genomic differences complicate testing of targeted gene editing tools in non-human models. We established a human model of SP-B deficiency to address these complications, choosing a human 3D-lung model generated from the human embryonic stem cell line RUES2. These lung bud organoids (LBOs), described by Chen and colleagues [Nat Cell Biol, (2017) 19: 542], exhibit a bias towards generation of lung parenchyma, especially surfactant-producing ATII cells. We previously used this model to screen rAAV serotypes suitable for transduction of the human parenchyma and identified rAAV6.2 as a promising candidate, concluding that LBOs are a suitable lung gene therapy model [Stem Cell Res Ther 2020 11: 448]. To grow SP-B deficient LBOs, RUES2 embryonic stem cells were gene edited to achieve the 121ins2 (C > GAA) mutation in SFTPB - the most common mutation in SP-B deficiency (ca. 66% of cases). HDR gene editing was performed by electroporation of Cas9 RNP (Synthego) and ssDNA donor. Because of the low efficiency of HDR editing, electroporation was first optimised. The highest levels of HDR gene editing were achieved using the CA137 electroporation programme (Lonza), with up to 89 % indel formation and 59 % knock-in achieved as determined by ICE analysis. After cloning and screening of 58 clones, 53 % were identified as 121ins2 edited via restriction fragment length polymorphism analysis. Next, 14 RUES2 121ins2-edited clonal lines were confirmed by Sanger sequencing reactions, twice after isolation and twice during clonal expansion. Two clones were taken forward for further characterisation. A normal karyotype was confirmed for both via KaryoStat microarray and the retention of stem cell status was examined for both lines by immunocytochemistry for markers SOX2, OCT4, TRA-1-60 and SSEA4. LBOs were grown from the parental line and from one of the 121ins2 edited clones. Firstly, endoderm was induced and induction efficiency determined by flow cytometry. The parental cell line and the edited clone were 90.2 % and 90.5 % double positive for the endoderm markers CXCR4 and c-Kit, respectively. During anterior foregut endoderm induction, expression of FOXA2 was confirmed at similar levels in both cell lines with immunocytochemistry. Finally, lung progenitor organoids were placed in Matrigel for maturation and branching. The morphology was documented over the entire organoid generation and at no point were differences between cell lines observed. Buds developed within 2 weeks, with similar budding efficiency observed. On day 77 of maturation, Western blot analysis indicated expression of SP-B in the wild-type organoids and its absence in the 121ins2 edited LBOs. In summary, we have generated a human embryonic stem cell line to model the SP-B deficient lung, with opportunity to screen for gene therapies or therapeutic gene editing strategies, in an effort to accelerate translational research.

154. Vectored Immunoprophylaxis for COVID-19 (COVIP)

Yue Du[1], Kamran Miah[1], Habib Omar[1], Helena Meyer-Berg[1], YanQun Wang[2], JinCun Zhao[2], Stephen Hyde[1], Deborah Deborah[1]

[1]Nuffield Department of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom,[2]State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Health, the First Affiliated Hospital, Guangzhou, China

A quest for effective therapeutics and prophylactic strategies for COVID-19 pandemic is still an active pursuit. Protein-based therapeutics, such as monoclonal antibodies (mAbs) that target epitopes on the SARS-CoV-2 Spike (S) protein represent a novel approach, but such therapeutics typically have short half-lives, necessitating repeated delivery. A more promising strategy against SARS-CoV-2 infections is to use vector-mediated immunoprophylaxis (VIP) by directing the sustained production of neutralising mAb into serum or in a localized fashion. Among multiple recombinant viral vectors, our preferred vector is based on a third-generation, self-inactivating simian immunodeficiency virus (rSIV) pseudotyped with the Fusion and Haemaglutinin-Neuraminidase surface glycoproteins from Sendai virus (rSIV.F/HN), which directed a sustained expression of broadly neutralising mAb to confer completely protection against supra-lethal influenza infection (Tan 2020 Thorax 2020;75:1112). As a comparison, we also included recombinant Adeno-associated virus (rAAV) vector 9 and 8 serotypes, which are gold standard to offer VIP against pathogens of importance.Since the laboratory BALB/c mice are not naturally susceptible to Coronavirus, we provided human ACE2 (hACE2) in trans via rAAV9 vector to facilitate SARS-CoV-2 pseudovirus (encoding luciferases; S-LV.luciferase) entry in lungs. Intranasal (IN) delivery of S-LV to the hACE2-expressing mice resulted in dose-dependent luciferase expression, which peaked at peaked at 7 days post-delivery (8.72E4±4.14E4 p/s/cm2/sr, Average Activity Radiance). Ultimately, we utilised hACE2-expressing murine model and S-LV as a means to investigate a novel VIP for COVID-19 (COVIP) strategy.The sequence for anti-SARS-CoV-2 monoclonal antibody (mAb) NC0321, isolated from the PBMCs of a COVID-19 convalescent patient, was inserted into the rSIV.F/HN.hCEF, rAAV.hCEFI and rAAV.CASI vectors. To test prophylaxis, 1e11 GC AAV9 or 5e8 TU rSIV.F/HN mAb vector were co-administrated in a single IN administration with 1e11 GC rAAV9.hACE2; 1e11 GC of rAAV9 mAb vector was delivered by intramuscular injection and followed by an IN delivery of rAAV9.hACE2 under the same anaesthesia conditions. The NC0321 IgG expression level was increased from day 7 to day 14, reaching a plateau at day 14 onwards. At day 28 post-delivery, IgG expression level was about 0.5,1 and 5 μg/ml for rSIV.F/HN, rAAV9, and rAAV8 vectors expressing NC0321, respectively. In all three cases, NC0321 IgG expression in sera was significantly higher than that in naïve mice (****, *p<0.001*). At 28 days post-delivery, the IgG expression level in the mouse epithelial lining fluid was about 5, 50 and 20 μg/ml for rSIV.F/HN, rAAV9, and rAAV8 vectors expressing NC0321, respectively. Encouragingly, on maximal challenge with S-LV.luc to hACE2-expressing mice, luciferase expression was significantly reduced in mice intranasally dosed with rSIV.F/HN and rAAV9 vector expressing NC0321 compared with an isotype control (**, *p=0011* or *0.0052*, respectively; AUC of the average luminescence). Interestingly, rAAV8-mediated expression of NC0321 conferred no significant protection.Here we compare different vector platforms and delivery routes for SARS-CoV-2 specific antibody gene transfer based on a hACE2 murine model. Our study indicates that vector delivery of mAb by direct lung inhalation route is efficient to confer protection against SARS-CoV-2 mimic. This COVIP strategy may offer protective immunity for vulnerable individuals unable to mount an effective immunological response for COVID-19.

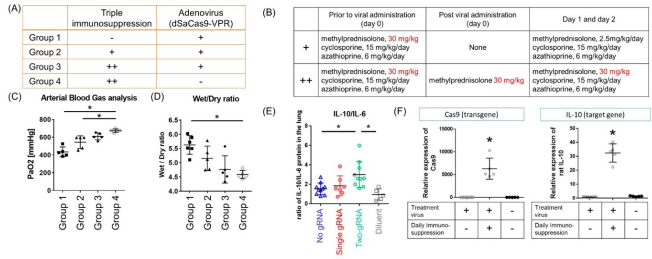
155. Impact of Transplant Immunosuppression on In Vivo Lung-Selective CRISPR/Cas9 Therapeutics for Lung Transplantation

Kumi Mesaki, Stephen Juvet, Zehong Guan, Jim Hu, Alan Davidson, Marcelo Cypel, Mingyao Liu, Shaf Keshavjee

University Health Network, Toronto, ON, Canada

Background: Donor lung gene modification using CRISPR/Cas9 holds promise in lung transplantation (LTx). Immunologic enhancement of donor lungs by epigenetic or genetic modification using CRISPR/Cas9 could address the shortage of donor organs, improve outcomes, and eventually eliminate the need for life-long systemic immunosuppression. For CRISPR/Cas9 therapeutics to modulate a whole donor organ, efficient delivery is critical to achieve the therapeutic effects. Adenoviral vectors provide high efficacy with rapid expression and have sufficient capacity to deliver the Cas9 and guide RNAs (gRNAs) in a single package. Nonetheless, adenoviruses are known to have significant immunogenicity, particularly in the lung - an organ vulnerable to inflammation and injury. Consequently, expression of transgenes after adenoviral delivery is transient. Further, Cas9 is itself immunogenic, thus creating an additional potential barrier to clinical translation of CRISPR/Cas9 therapeutics with adenoviral vectors. Anti-rejection immunosuppression required in LTx can be utilized to minimize vector-related inflammation. Furthermore, viral delivery to the organ ex vivo, prior to host exposure to the graft further facilitates this process. This has made the possibility of gene therapy more promising. We therefore hypothesized that the use of transplant immunosuppression could permit effective CRISPR/Cas9 therapeutics in vivo in the lung.
Methods: Adenovirus expressing a Cas9 activator (dSaCas9-VPR) was trans-bronchially delivered to Lewis rats at a high dose (2.5E8 PFU/rat). The effect of standard triple immunosuppression (methylprednisolone, cyclosporine, and azathioprine) with or without additional methylprednisolone on vector-related inflammation was assessed in four groups (Fig A, B) after 72 hours. Next, to elucidate the impact of immunosuppression on the persistence of epigenome editing, adenovirus expressing Cas9 activator and gRNAs was delivered to activate the IL-10 gene at low dose (1E8 PFU/rat), and assessed after day 14 with or without daily immunosuppression.
Results: Delivery of high dose adenovirus expressing Cas9 activator without immunosuppression (Group 1) worsened oxygenation and lung edema: PaO2 (435±55 vs 674±20, p<0.0001), wet/dry ratio (5.63±0.33 vs 4.59±0.14, p=0.0013) compared to the diluent group (Group 4, Fig C, D). Triple immunosuppression (Group 2) improved oxygenation compared to the no immunosuppression group (Group 1). Additional steroid administration (Group 3) further improved the condition of the lung leading to PaO2 (610±38, p=0.35) and wet/dry (4.77±0.48,

p>0.99) ratios comparable to the diluent (control) group (Group 4). Adenovirus expressing two gRNAs induced IL-10 gene expression with minimal inflammation at low dose, as shown by a significantly increased IL-10/IL-6 protein ratio in the lung (Fig E). Daily immunosuppression sustained IL-10 gene activation until day 14 (relative expression; 32.3±6.5 vs 0.67±0.14, p<0.0001, Fig F) as well as Cas9-expression and viral DNA, suggesting immunosuppression inhibited elimination of the transduced cells. **Conclusion**: Transplant immunosuppression ameliorates inflammation and prolongs the effect of CRISPR/Cas9 epigenome editing with adenovirus in the lung. This study illustrates a strategy for effective gene editing by managing vector-related inflammation in an in vivo CRISPR/Cas9 therapeutics.



156. First Proof-of-Concept of miQURE™ Based Gene Targeting in the Liver: Lipid Lowering and Atherosclerosis Suppression by AAV-miQURE™-Mediated ANGPTL3 Targeting

Vanessa Zancanella[1], Astrid Valles-Sanchez[1], Carlos Vendrell Tornero[1], Maroeska Oudshoorn-Dickmann[1], Hendrina Wattimury[1], Kristel van Rooijen[1], Mark van Veen[1], Monika Golinska[1], Elsbet J. Pieterman[2], Nanda Keijzer[2], Hans M. G. Princen[2], Geurt Stokman[2], Martin de Haan[1], Ying Poi Liu[1]

[1]uniQure Biopharma B.V., Amsterdam, Netherlands,[2]TNO Metabolic Health Research, Leiden, Netherlands

We have developed a proprietary, next-generation miQURE™ technology, where a transcript encoding artificial microRNAs (miRNAs) is packaged into adeno-associated viral (AAV) vectors for mRNA silencing. Our AAV5-miQURE™ targeting mutant huntingtin in the brain is currently being tested in a phase 1/2a clinical study in Huntington Disease patients. In the current study, we have used Dyslipidemia as indication to investigate the potency of AAV-based miQURE™ to lower Angiopoietin-like 3 (ANGPTL3) in the liver. ANGPTL3 loss-of-function genetic variants have been associated with low levels of plasma triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol, and a decreased risk of cardiovascular diseases. Pharmacological inhibition of ANGPTL3 mimics the phenotype of individuals carrying mutations impairing ANGPTL3 and lowers plasma lipids in mice, monkeys and humans. After *in vitro* testing, the most promising miQURE™ candidates were tested for their efficacy to lower ANGPTL3 mRNA and ANGPTL3 plasma levels in wild-type and transgenic mice with a humanized lipoprotein metabolism (APOE*3-Leiden.CETP). The lead candidate resulted in wild-type mice in a dose-dependent decrease in ANGPTL3 mRNA expression (up to ~77%) and a subsequent up to ~90% reduction in ANGPTL3 plasma levels. APOE*3-Leiden.CETP mice received AAV-miANGPTL3 treatment with or without the standard of care treatment, statin (atorvastatin). AAV5-miANGPTL3 delivery to the liver resulted in a significant and sustained reduction in triglycerides and plasma total cholesterol, whereas the levels in the vehicle and control scrambled miRNA groups remained unchanged. Furthermore, a significant decrease in total atherosclerotic lesion area could be observed in the AAV-miANGPTL3 treated group (58% compared to scrambled miRNA). Treatment with AAV-miANGPTL3 was associated with maintenance of an non-diseased plaque phenotype and reduction in the lesion severity. A further reduction in total cholesterol and triglycerides exposure and in the total atherosclerotic lesion area was observed in the combination treatment with atorvastatin. To provide proof of concept of liver directed miANGPTL3 expression, a non-human primates study was conducted. In this study, the animals received a high caloric diet to induce hyperlipidemia. Similar to the mouse study, the main goal was to assess miRNA, mRNA and relevant plasma lipid markers. The AAV-miANGTPL3 treatment was well-tolerated in all animals during the in-life phase of 22 weeks. However, the individual animal response on the high calory diet varied greatly, resulting in variable data. Nevertheless, preliminary results suggest that the AAV5-miANGTPL3 group showed a slight lowering in total cholesterol and triglycerides. In conclusion, we here report the first proof-of-concept of a miQURE™-based approach in the liver. The combination of tolerability of miQURE™ expressed in the liver of non-human primates and proof-of-concept in mice supports the continuation of its development for liver-directed indications and translation in the clinic.

157. Abstract Withdrawn

158. Electroporation Mediated Gene Transfer of MRCKα to the Lungs of Mice Effectively Treats Pre-Existing Acute Lung Injury

Jing N. Liu[1], Michael Barravecchia[2], David A. Dean[2]
[1]The Cellular and Molecular Pharmacology and Physiology, University of Rochester School of Medicine and Dentistry, Rochester, NY,[2]Pediatrics, University of Rochester School of Medicine and Dentistry, Rochester, NY

Acute Lung Injury (ALI) and its more severe form Acute Respiratory Distress Syndrome (ARDS) are severe conditions characterized by alveolar fluid accumulation and insufficient gas exchange, ultimately leading to acute respiratory failure. Both impaired alveolar fluid clearance (AFC) and a disrupted alveolar-capillary barrier contribute to the pathogenesis of ALI/ARDS. Most attempts at therapy in the past have focused on enhancing AFC, but repairing the alveolar-capillary barrier is likely also needed for effective treatment. We previously have shown that overexpression of the β1 subunit of the Na^+, K^+-ATPase (β1- Na^+, K^+-ATPase) increases AFC in lungs of multiple species. We also have found that electroporation-mediated gene delivery of the β1- Na^+, K^+-ATPase rescues lipopolysaccharide (LPS) induced ALI by upregulating tight junction (TJ) proteins and pulmonary barrier function, demonstrated by decreased lung permeability, total protein and cellularity in bronchoalveolar lavage (BAL) fluid, and improved overall outcome of lung injury. While studying how the β1- Na^+, K^+-ATPase increases lung barrier, we identified MRCKα (CDC42 binding protein kinase alpha) as an interacting partner of the β1 subunit. Previous data from our lab indicate that MRCKα mediated β1's upregulation of TJ proteins and epithelial barrier integrity in cultured cells. However, it is unknown whether MRCKα can upregulate pulmonary barrier function and treat LPS induced ALI *in vivo*. Understanding this question would help to determine the therapeutic potential of MRCKα for ARDS. Plasmids expressing MRCKα or β1- Na^+, K^+-ATPase were delivered individually or in combination by aspiration and transthoracic electroporation to mice which had been pre-injured by LPS intratracheal administration 24 hours earlier. Two days after gene delivery, various endpoint assays were performed to evaluate lung edema, permeability, inflammation and histological injury. We found that overexpression of MRCKα alone attenuated LPS-increased edema, lung leakage, BAL cellularity and protein concentration, restored TJ protein expression, and improved overall outcome of lung injury, similar to gene transfer of the β1- Na^+, K^+-ATPase. However, we found that unlike β1- Na^+, K^+-ATPase, gene transfer of MRCKα alone did not enhance AFC. These results indicate that MRCKα could benefit the pre-injured lungs by reducing pulmonary edema, restoring lung barrier function and reducing inflammation. Moreover, they also suggest that improving barrier function alone may be of equal or even more benefit than improving AFC in order to treat ALI/ARDS. (Supported by: NIH grants HL120521, HL131143, and HL138538 and an AHA predoctoral fellowship (JL))

Clinical Trials and Advanced Preclinical Studies for Neurologic Diseases

159. Gene Therapy Candidate for Metachromatic Leukodystrophy (MLD): Summary of Preclinical *In Vivo* Data Following an Intravenous Delivery of HMI-202

Jacinthe Gingras, Thia St-Martin, Katie Gall, Tania A. Seabrook, Jason Lotterhand, Israel Rivas, Nancy Avila, Michael Mercaldi, Jennifer Newman, Shiva Krupa, Teresa Wright, Omar Francone, Albert Seymour
Homology Medicines Inc, Bedford, MA

Metachromatic leukodystrophy (MLD) is an inherited autosomal recessive lysosomal storage disorder (LSD) with a great unmet medical need. This fatal neurodegenerative LSD occurs in three forms: late infantile (prevalence of 1 in 40,000), juvenile, and adult. The late infantile and juvenile forms represent the majority of the MLD patients where mortality at 5 years is estimated at 75% and 30%, respectively. MLD is most commonly caused by mutations in the *ARSA* gene and patients suffering from the disease are deficient in arylsulfatase-A (ARSA) enzyme activity. The disease is characterized by accumulation of sulfatides to supraphysiologic and toxic levels in the peripheral organs and nervous system. In the brain, excess sulfatides lead to the destruction of myelin, a key protective layer of the nerve fibers that enhances propagation of action potentials. Herein, we report preclinical gene therapy data where a single intravenous (IV) dose of HMI-202 (AAVHSC15-human-*ARSA* (h*ARSA*)) crosses the blood-nerve- and blood-brain-barriers (BNB and BBB) in juvenile non-human primates (NHP) and in the *Arsa* KO murine model of MLD. In the HMI-202-treated adult *Arsa* KO mice, hARSA expression patterns are nearly identical to that of murine Arsa (mArsa) distribution in the nervous system of wild type age-matched littermates, in both neuronal and glial cellular profiles. In HMI-202-treated adult *Arsa* KO mice, we show a dose-response relationship in hARSA enzyme activity, transcript, and vector genomes in the central nervous system (CNS). As early as 1 week following administration (earliest time-point of collection), near-normal human adult levels of hARSA activity are detected in the CNS of HMI-202-treated adult *Arsa* KO mice, and levels are sustained at or above normal adult human brain levels throughout the study (52 weeks post-dose). Similarly, hARSA enzyme activity is detected 1 week post-dose in the CNS of *Arsa* KO neonates and is sustained out to 12 weeks post-dose (end of study). Furthermore, we demonstrate modulatation of key biochemical markers in the CNS, including murine neuronal sulfatides, myelin and lymphocyte (MAL) transcript, lysosomal-associated membrane protein-1 (LAMP-1), and glial fibrillary acidic protein (GFAP) levels in HMI-202-treated *Arsa* KO mice. Lastly, using the rotarod assay, we demonstrate a functional motor benefit in HMI-202-treated *Arsa* KO mice dosed prior to the

detectable accumulation of CNS neuronal sulfatides (~2 months of age). In summary, a single-IV dose of HMI-202 crossed the BNB and BBB in lower (mice) and higher (NHP) species. In addition, the ability to achieve hARSA enzyme activity levels at or above normal human adult brain levels, rapid onset of expression, durability, broad biodistribution, modulation of key biomarkers, and functional motor benefit in a murine MLD disease model was demonstrated. These preclinical data from IND-enabling studies continue to support the further optimization and development of HMI-202 as a gene therapy for the treatment of MLD.

160. Gene Replacement Therapy for Angelman Syndrome

Justin Percival, Kasturi Sengupta, Long Le, Khalid Arhzaouy, Heather Born, Elizabeth Buza, Cecilia Dyer, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Angelman syndrome (AS) is a rare neurodevelopmental disorder affecting approximately half a million individuals worldwide. AS is characterized by intellectual and physical disability, seizures, impaired sleep, and gut dysfunction. Many of these deficits result from a loss of the maternally inherited ubiquitin protein ligase E3A (*UBE3A*) allele. At present, therapeutic options for AS are limited. Adeno-associated virus (AAV)-based gene replacement therapy represents a promising strategy for restoring *UBE3A* isoform expression and mitigating AS severity. However, the *UBE3A* gene encodes three isoforms, and it is currently unclear which *UBE3A* isoform is the most effective candidate. We addressed this uncertainty by comparing the efficacy of AAV vectors delivering codon-optimized human *UBE3A* isoforms in a mouse model of AS. Western blotting and immunohistochemical analyses indicated that intracerebroventricular injections of AAV-UBE3A human isoform 1 and 2 vectors into neonatal control and AS mice resulted in robust protein expression. Isoform 1 replacement significantly improved gait, nest-building ability, and motor coordination in a dose-dependent manner in AS mice. In contrast to isoform 1, isoform 2 further impaired nest-building ability and motor coordination in AS mice. Ongoing toxicology studies in nonhuman primates suggest that a high dose of AAV-UBE3A isoform 1 vector injected into the cisterna magna has no significant adverse effects. Taken together, these data indicate that the AAV-UBE3A isoform 1 vector is the most therapeutically effective option with a promising safety profile. These preclinical findings represent an important step forward in the development of gene replacement therapy for AS.

161. An AAV-miRNA for Androgen Receptor Knockdown in Spinal and Bulbar Muscular Atrophy

Eileen Workman, Julia Johansson, Mariya Kostiv, Christian Hinderer, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Introduction: Spinal and bulbar muscular atrophy (SBMA) is an X-linked, slowly progressive motor neuron disease caused by a polyglutamine (CAG) expansion tract within exon 1 of the androgen receptor (AR). The expansion results in the nuclear aggregation of the AR protein, which then causes motor neuron degeneration almost exclusively in males due to androgen-mediated activation of toxicity. To date, no effective treatment has been approved for SBMA. Since knockdown of the androgen receptor in neurons does not appear to cause adverse effects, lowering AR levels in SBMA is an attractive strategy for treating the disease. Our gene therapy approach involves delivering an AAV vector expressing an miRNA targeting the AR to motor neurons. **Results:** We screened for artificial miRNAs targeting the AR in cell culture and identified a candidate miRNA. Artificial miRNAs were cloned into a miR-155 backbone with a cytomegalovirus promoter and transfected into HEK293 cells. Using qPCR and Western blot analysis, we identified an AR-targeting candidate miRNA, GTP-miR001, which shares homology in mouse, human, and rhesus macaque. GTP-miR001 was able to knock down up to 60% of AR expression in HEK293 cells. The candidate miRNA was packaged into an AAV.PHP.eB vector for *in vivo* testing in wild-type C57BL/6J mice. It was determined that IV injection of AAV.PHP.eB.GTP-miR001 at 3e11 was sufficient to achieve greater than 50% knockdown of the androgen receptor in the mouse brain and 70% knockdown in the spinal cord. To evaluate the potential efficacy of GTP-miR001 for the treatment of SBMA, we tested an AAV serotype hu68 vector expressing the miRNA in AR97Q SBMA transgenic mice. These mice express high levels of the human AR with a pathogenic repeat expansion and display a very severe SBMA phenotype with progressive hindlimb weakness that ultimately results in death. Males have a median survival of 91 days and females have a median survival of 184 days. Intravenous administration of the AAVhu68.GTP-miR001 vector in 3- or 5-week-old mice resulted in efficient knockdown of the AR, with modest improvements in survival and motor phenotype that correlated with the degree of AR suppression. Treating AR97Q mice as neonates yielded robust improvements in weight gain, motor phenotype, and survival, indicating the necessity of early intervention in this rapidly progressive mouse model. **Conclusions:** We have identified a candidate AAV vector expressing an artificial miRNA for the treatment of SBMA. This vector was capable of knocking down AR protein expression *in vitro* and *in vivo*. Further, we were able to achieve improvement of the phenotype and survival of AR97Q SBMA transgenic mice when subjects were injected at the neonatal stage. Ongoing studies include the determination of vector toxicity and knockdown of AR protein in nonhuman primates.

162. AXO-AAV-GM1 for the Treatment of GM1 Gangliosidosis: Preliminary Results from a Phase I-II Trial

Cynthia J. Tifft[1], Precilla D'Souza[1], Jean Johnston[1], Maria Acosta[1], Caroline Rothermel[1], Audrey Thurm[2], Ajith Karunakara[3], Benjamin Thorp[3], Peter Ross[3], John Jameson[3], Toby Vaughn[3], Donna Valencia[3], Erika De Boever[3], Gavin Corcoran[3]

[1]Office of the Clinical Director, NHGRI, National Institutes of Health, Bethesda, MD,[2]National Institute of Mental Health, National Institutes of Health, Bethesda, MD,[3]Sio Gene Therapies, New York, NY

GM1 gangliosidosis is a rare, inherited neurodegenerative disorder caused by mutations in the *GLB1* gene which encodes the lysosomal hydrolase β-galactosidase (β-gal). The resulting enzyme deficiency leads to a toxic accumulation of GM1 ganglioside, predominantly in the

central nervous system (CNS) where its rate of synthesis is the highest, but also in peripheral tissues. GM1 gangliosidosis is uniformly fatal, and there are no disease-modifying treatments currently available. As this is a monogenic disorder, it is an ideal target for gene therapy to deliver β-gal to the CNS and periphery, with resulting potential to halt further neurodegeneration, restore function and ameliorate symptoms. This is a preliminary analysis from an ongoing open-label, single-arm, Phase I-II trial (ClinTrials.gov, NCT03952637) in which subjects with a confirmed genetic and biochemical diagnosis of GM1 gangliosidosis were treated with AXO-AAV-GM1 (AAV9-GLB1), an investigational gene therapy utilizing an adeno-associated virus (AAV9) vector to deliver a functional copy of the *GLB1* gene. All subjects received 1.5 $x10^{13}$ vg/kg of AXO-AAV-GM1 administered via intravenous infusion and immune modulation with rituximab, sirolimus and glucocorticoids. The primary endpoint of the trial is safety/tolerability, secondary efficacy endpoints include Vineland-3, brain MRI, motor function and disease severity. Biomarkers of disease progression or stabilization (βgal activity and GM1 ganglioside in serum and CSF) are also assessed. Six-month follow-up data are presented from 4 subjects with the late infantile (Type IIa) form and one with juvenile (Type IIb) disease. AXO-AAV-GM1 was generally safe and well-tolerated and there have been no serious adverse events (SAEs) related to gene therapy. One SAE was described: a single subject experienced bacterial sepsis due to a PICC line infection, which was considered to be unrelated to the investigational product, and which resolved following line removal and administration of IV antibiotics. The most common adverse events were considered mild to moderate. Four subjects had AST elevations that were considered to be adverse events. None required clinical intervention or had associated clinical sequelae. There were no other adverse events indicative of impaired liver function including serum bilirubin, GGT, and ALT. Serum β-gal enzyme activity was sampled at 9 distinct post-baseline timepoints between Day 7 and Month 6. The mean increase from baseline ranged from 71-138% across the 9 time points. At Month 6, serum enzyme activity was sustained with an increase from baseline ranging from 33%-128% across the 5 subjects. Subjects were assessed by multiple measures of neurodevelopment including the Vineland Adaptive Behavior Scales 3rd Edition (VABS-3), Upright and Floor Mobility Score, and Clinical Global Impression (CGI). All 5 subjects demonstrated disease stability at 6 months post-treatment as assessed by VABS-3 Growth Scale Value scores, Upright and Floor Mobility Score, and CGI relative to baseline values. Per protocol, brain MRIs were not collected for the 6-month follow-up analysis but will be completed at later timepoints. We will continue to follow-up these subjects and are now evaluating additional subjects receiving 4.5 $x10^{13}$ vg/kg dose of AXO-AAV-GM1.

163. AXO-Lenti-PD Gene Therapy for Parkinson's Disease: Efficacy, Safety, and Tolerability Data from the Second Cohort in Open-Label Dose Evaluation Study SUNRISE-PD at 6 Months Post Administration

Gavin Corcoran[1], Ajith Karunakara[1], Ben Vaughn[2], Elimor Brand-Schieber[1], Thomas Foltynie[3], Roger A. Barker[4], Stéphane Palfi[5]

[1]Sio Gene Therapies Inc., New York, NY,[2]Rho Inc., Durham, NC,[3]Department of Clinical and Movement Neurosciences, UCL Institute of Neurology, London, United Kingdom,[4]John van Geest Centre for Brain Repair, Department of Clinical Neuroscience, Addenbrooke's Hospital, Cambridge, United Kingdom,[5]AP-HP, Groupe Hospitalier Henri-Mondor, DMU CARE, Neurochirurgie, Créteil, France

AXO-Lenti-PD (ALPD, previously known as OXB-102), a novel gene therapy for Parkinson's disease (PD), uses a lentiviral vector to deliver the three genes required for endogenous dopamine synthesis (TH, CH1 and AADC), into the putamen with the goal of improving motor fluctuations and long term quality of life. In the Phase I/II study OXB-102-01 (SUNRISE-PD; NCT03720418), six subjects were dosed with ALPD: Cohort1 (n=2; Low dose, 4.2E+6 Transducing Units [TU]) and Cohort2 (n=4; Mid dose, 1.4E+7 TU). Efficacy data reported here are from Cohort1 and Cohort2 6-month visits (6M). Given the small sample size, only descriptive statistics were planned. Two of four subjects did not have UPDRS "OFF" 6M data recorded: one due to COVID-19 site limitations and the other declined the OFF assessment. The two evaluable subjects in Cohort 2 had 20.5-point (40%) improvement from baseline (BL) in UPDRS III (Motor Examination) 'OFF'. UPDRS Part II (Activities of Daily Living) "OFF" had 13.5-point improvement (71%). Subjects used the Hauser home diary for up to 3 consecutive days within the week prior to the study visit to log their motor function (either Asleep, ON without dyskinesia, ON with non-troublesome dyskinesia, ON with troublesome dyskinesia, or OFF) at 30 minute intervals normalized to 16 waking hours. Good ON time (the sum of ON without dyskinesia and ON with non-troublesome dyskinesia) increased from BL by 2.2 hours (h) across the four Cohort2 subjects at 6M, while OFF time decreased by 2.3h. Levodopa equivalent daily dose (LEDD) decreased 271.0 mg, 13% lower than at BL. Table 1 summarizes individual and mean changes.

Table 1 Change from Baseline					
Subject	OFF Time (h)	Good ON Time (h)	LEDD (mg)	UPDRS II OFF	UPDRS III OFF
Cohort 1					
1001	+2.8	-2.4	-98	-26	-20
1003	-0.5	+2	-150	-13	-14
Mean	**+1.2**	**-0.2**	**-124 (11%)**	**-19.5 (65%)**	**-17.0 (29%)**
Cohort 2					
2002	-0.6	+0.6	0	-12	-22
2004	-0.2	+0.1	-700	-	-
2006	-3.9	+3.5	-466	-	-
2007	-4.6	+4.5	+81	-15	-19
Mean	**-2.3**	**+2.2**	**-271 (13%)**	**-13.5 (71%)**	**-20.5 (40%)**

A total of four serious adverse events (SAEs), all unrelated to ALPD and resolved, have been reported: Parkinson's disease (ie, worsening of non-motor OFF related to anxiety) and Major depression in a Cohort1 subject; Confusional state and Wound infection in a Cohort2 subject. No hypersensitivity, immune or endotoxicity related adverse events have been reported. No serious unexpected suspected adverse reactions (SUSARs) have been reported. No subject died or discontinued from the study. The 6M data from the first two cohorts in SUNRISE-PD trial showed that ALPD gene therapy was generally well tolerated, and the initial efficacy data suggest the potential for a clinically relevant effect. Further evaluation of ALPD is planned using a higher dose/volume open label cohort followed by sham-controlled study.

164. Safety Evaluation of IV-Administered BBP-812, an AAV9-Based Gene Therapy for the Treatment of Canavan Disease, in Mice and Juvenile Cynomolgus Macaques

David W. Scott, Jeremy Rouse, Kirsten Romero, Rachel Eclov, Mayank Kapadia, Daniel McCoy, Clayton W. Beard

Aspa Therapeutics, Raleigh, NC

Canavan Disease (CD) is a rare pediatric leukodystrophy caused by aspartoacylase deficiency. The disease is characterized by elevated levels of the aspartoacylase substrate N-acetylaspartic acid and patients present with a lack of psychomotor development with an average lifespan of less than ten years. We are developing BBP-812, an AAV9-based gene therapy containing a codon modified human *Aspa* transgene, to introduce functional aspartoacylase into CD patients. Proof of concept studies in Aspa -/- mice demonstrated that intravenous (IV) dosing of BBP-812 could reverse signs and symptoms of CD. To better understand the safety of IV delivered BBP-812, we conducted studies in non-human primates (NHP; juvenile cynomolgus macaques) and a GLP-toxicology study in C57Bl/6 mice. The NHP study included IV doses of $3.14x10^{13}$, $1.05x10^{14}$, and $3.14x10^{14}$ vg/kg with animals sacrificed three and eight weeks after dosing. In the mouse study, animals were dosed at $1.0x10^{14}$, and $3.0x10^{14}$ vg/kg and were sacrificed four, twelve, and twenty-four weeks after dosing. Safety readouts for both studies included clinical chemistry and hematology, immunology, and microscopic evaluation of major organs systems. For the NHP study, the microscopic evaluation of the nervous system included four spinal cord regions and eight dorsal root ganglia (DRG) per animal. Key findings in the NHP study were a transient increase in ALT and AST in the highest dose IV treatment group at day 3 which returned to normal without intervention by day 8. No other changes in hematology or clinical chemistry occurred during the study. Immunological analysis revealed that all treated animals developed antibodies to AAV9 and the majority of animals developed antibodies to Aspa after dosing. There were no adverse test article-related microscopic changes in the study at any dose level at either necropsy timepoint. Test article-related changes were observed only in the liver (portal infiltrates and/or increased cellularity) in some of the $3.14x10^{14}$ vg/kg treated animals. These findings were considered minor, did not impact the clinical health of the animals, and were not associated with any tissues damage. Evaluation of the spinal cord and DRG revealed sporadic minimal findings that were also present in control animals. Importantly, there was no evidence of axonopathy in the spinal cord or DRGs as has been reported with other AAV9-based therapies. In the GLP-toxicology study in mice there were no adverse test article related changes in any clinical chemistry or hematology during the study. Creatine kinase and lactate dehydrogenase were decreased at both dose levels at all timepoints but this was not considered adverse. Immunological analysis revealed that all BBP-812 treated animals generated antibodies to AAV9 while no animals generated antibodies against Aspa. T-cells were monitored for reactivity to AAV9 and Aspa. There was a minor response in some animals to AAV9 with no response detected towards Aspa. There were no microscopic changes in the study in any group at any time point. Based on these findings, the NOAEL from the NHP study was determined to be $3.14x10^{14}$ vg/kg and from the GLP toxicology study in mice was determined to be $3.0x10^{14}$ vg/kg of IV-administered BBP-812. These studies support the continued development of BBP-812 for the treatment of CD.

165. Gene Replacement Therapy for *SURF1*-Related Leigh Syndrome Using AAV9

Qinglan Ling, Matthew Rioux, Steven Gray

UTSW, Dallas, TX

SURF1(surfeit locus protein 1)-related Leigh syndrome (LS) (also known as Charcot Marie Tooth disease type 4K) is an early onset neurodegenerative disorder characterized by reduction in the assembly factor of complex IV, resulting in disrupted mitochondrial function. Here, we hypothesized that functional gene replacement strategy could restore mitochondrial functions in LS caused by *SURF1* loss-of-function mutations. A codon-optimized version of the human *SURF1* (h*SURF1*) packaged within self-complementary adeno-associated virus serotype 9 (AAV9) viral vectors (AAV9/h*SURF1*), which have been shown to induce robust expression in central nervous system (CNS), was designed and generated as the treatment entity. Our and other studies indicated that the knockout (KO) of *SURF1* in mice reduced complex IV/cytochrome *c* oxidase (COX) activity and MT-CO1 (mitochondrially encoded cytochrome *c* oxidase I) protein expression in multiple organs, as well as induced lactic acidosis. Thus, we treated juvenile *SURF1* KO mice with AAV9/h*SURF1* through broad CNS-

directed delivery (intrathecal (IT) administration, or a combination of intrathecal and intravenous administration), in a manner that is compatible with human translation. Our data indicate that 4 weeks after intrathecal administration into juvenile mice, the COX activity was partially and significantly rescued in all tissues tested, including liver, brain and muscle. Furthermore, our histology study suggest AAV9/h*SURF1*-treated mice showed dose-dependently increased h*SURF1* mRNA expression and restoration of MT-CO1 protein expression in the brain, which further supported our findings in COX activity. Additionally, we tested endurance capacity and lactic acidosis 4 weeks and 9 months after the treatment in a separate group of mice. Our data suggest that the gene replacement treatment also mitigated the lactic acidosis upon exhaustive exercise at mid-age. However, doubling the total amount of virus by administering through both intravenous and intrathecal route did not confer any significant improvement compared to an intrathecal route alone in any of the parameters tested above, except the COX activity in liver. This suggests a single dose IT administration of AAV9/h*SURF1* is effective and sufficient in improving SURF1 deficiency-related dysfunctions. The toxicity of the vectors was evaluated through intrathecal administrating into wildtype (WT) mice at a maximum feasible dose. There were no toxicities observed in either the in-life portion of the study or after microscopic examination of major tissues up to a year following gene transfer. Taken together, we propose gene replacement therapy through IT administration of AAV9/h*SURF1* as a potential treatment worthy of further development for *SURF1*-related LS patients. Further, this general approach might be amenable as a potential treatment for patients with other related forms of LS or other mitochondrial disorders.

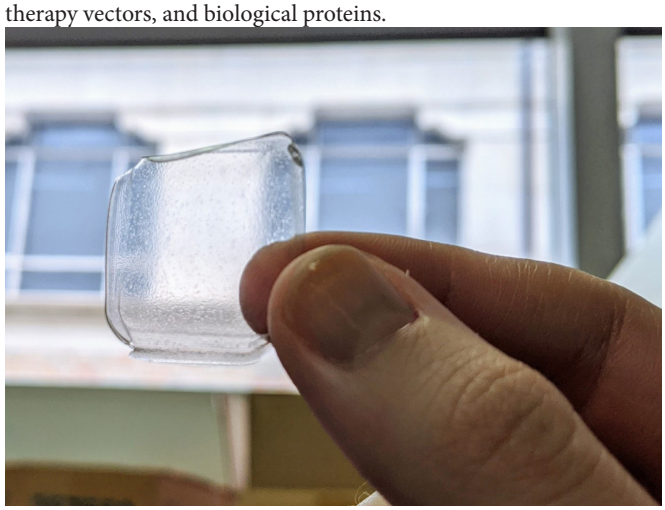
Downstream Process of Vector Manufacturing

166. Novel Platform for Transport and Delivery of Recombinant Adeno-Associated Virus without Need for Cold Storage during Transit

Maria A. Croyle[1,2,3], Trang Doan[1], Matthew D. Le[1], Irnela Bajrovic[1,4], Lorne Celentano[4], Charles Krause[4], Haley G. Balyan[4], Abbie Svancarek[4], Lakmini Wasala[4], Angela Mote[4], Anna Tretiakova[4], R. Jude Samulski[3,4]

[1]College of Pharmacy, University of Texas at Austin, Austin, TX,[2]John R. LaMontagne Center for Infectious Disease, Austin, TX,[3]Jurata Thin Film, Chapel Hill, NC,[4]AskBio, Research Triangle Park, NC

Within the past 30 years, significant progress has been made in the development of gene vectors with several therapies receiving approval by the European Medicines Agency (EMA) and the U.S. Food and Drug Administration (FDA). Despite this progress, these products are stored and shipped at ≤ -70°C, similar to current COVID19 vaccines. This poses significant logistic and economic issues with respect to global distribution and access to life-saving medicines. In an effort to address these important issues, we developed a novel method for stabilizing live viruses in a peelable thin film matrix that can be stored at ambient temperature (see figure). In these studies, an AAV9 vector (AAV9-CBA-Luc) was mixed with film base formulation, poured into 1 ml molds and dried under aseptic conditions. Films were peeled and packaged in individual particle free-bags with foil overlays and stored at room temperature under controlled humidity. A sublot of films were shipped from Texas to North Carolina via overnight courier in an envelope without ice or cold blocks for in vivo testing. Control preparations were stored in a commercial liquid formulation at room temperature and 4 and -80 °C for comparison. Over 95% of the original infectious dose of AAV was recovered after drying as determined by *in vitro* transduction assays measuring transgene expression and internalized viral genome copies. In contrast, infectious virus particles could not be detected in the liquid formulation after 30 days at room temperature with large aggregates detected by dynamic light scattering at 14 days transitioning to subviral particulates at day 30. Stability profiles of the same preparation stored at 4 °C for 30 days were not statistically different from vector stored in film at room temperature at the same time point. Films taken from room temperature storage and placed at 40 °C with 20% relative humidity for 3 days demonstrated a loss of less than 5% of infectious titer. Initial mechanistic studies suggest that elements of the film matrix directly bind to capsid proteins to stabilize them and shield them against environmental stressors. *In vivo* studies in mice demonstrated that vector biodistribution and transgene expression profiles of vector dried within the film matrix were similar to those of vector stored frozen in the commerical formulation. Taken together, these results suggest that storage of AAV in our novel matrix facilitates easy transport of vector to remote sites without compromising in vivo performance. Companion studies using enveloped and non-enveloped viruses (Ad, influenza) further suggest that this approach could be a universal technology for room temperature storage of vaccines, gene therapy vectors, and biological proteins.



167. Exposing the Content of Different AAV Fractions after Ultracentrifugation

David Dobnik, Mojca Janc, Maja Štalekar, Nejc Košir, Tjaša Jakomin, Rebecca Vollmeier Kovačić, Nika Savodnik, Polona Kogovšek

National Institute of Biology, Ljubljana, Slovenia

Recent developments in the biomedical fields have enabled that viruses can also be used to help us fight against numerous diseases. Even though the production of recombinant viral vectors began 40 year ago, the established technologies still haven't reached the optimal level.

Different product and process related impurities affect the efficiency of the production. To improve the overall production process, advanced analytical techniques are needed to accurately follow presence of desired product and possible impurities in various production steps. A variety of methods have been developed and utilised to characterize viral vectors, but only a limited number of comparative studies have been done to demonstrate correlations between them. Non-related analytical techniques usually also produce non-comparable results. We have used different analytical techniques (Quantitative real-time PCR (qPCR), digital droplet PCR (ddPCR), high throughput sequencing (HTS), enzyme-linked immunosorbent assay (ELISA) and transmission electron microscopy (TEM)) to thoroughly characterize four fractions of AAVs (empty, intermediate, full and heavy particles) coming from CsCl gradient purification. The aim of the study was to compare those different fractions in terms of vector and impurities content and at the same time evaluate the correlations between different analytical methods. The results have shown that the fractions were relatively similar in terms of relative content of vectors and impurities, with exception of heavy fraction. There was no clear correlation between the results of different methods that would hold for all of the fractions. Nevertheless, the results offered an interesting insight into the presence of impurities, vector genomes and viral particles in different fractions. Those results will be presented and discussed in view of content of partially filled vectors, comparability of analytical methods and importance for creating a wider picture of the virus vector sample.

168. Optimization of Affinity Purification for Adeno-Associated Viral Vectors

Huiren Zhao, Songli Wang, W. Hans Meisen
Genome Analysis Unit, Amgen Inc., South San Francisco, CA

Purification of recombinant AAV (rAAV) via affinity chromatography with Poros™ CaptureSelect™ (PCS) AAVX, AAV8 or AAV9 affinity resins achieves good purity and high vector concentration with minimal vector loss. Additionally, this purification process is highly scalable. Despite the advantages of affinity purification, we and others have observed that significant host cell proteins (HCPs) and other impurities such as endotoxins are co-eluted with rAAV. The goal of this study was to optimize the washing step during affinity chromatography to remove contaminants and increase rAAV purity. In this study, we systematically evaluated a variety of washing reagents including 1M NaCl, 0.2 M $MgCl_2$, low pH (pH4 and pH5), 0.5 M arginine, 0.1% Tween 20 and 1-2% OTG (octyl β-D-1-thioglucopyranoside) for their ability to improve rAAV8-EGFP affinity purification using PCS AAVX. In these experiments purity was evaluated by Coomassie blue stain of SDS-PAGE gels, titer by the CyQuant method, and endotoxin by the Limulus Amebocyte Lysate (LAL) method. A large batch of rAAV8-EGFP was generated and used as the starting material for all rAAV8 affinity purifications. Gradients of wash buffers were applied in some purifications to identify the best concentration of the reagent. In these studies, we observed that some of the wash buffers tested resulted in the loss of rAAV8 by capsid protein immunoblot including 0.1% Tween 20, 50 mM Citrate buffer (pH 4.0) and 0.5 M arginine. These findings highlight the challenge of identifying washing buffers that can remove impurities while retaining the association of the rAAV and the affinity ligand. rAAV8 vector was not washed off by 1M NaCl, 0.2 M $MgCl_2$, 50 mM Citrate buffer (pH 5.0), or 2% OTG as determined by immunoblot. Compared to PBS wash alone, these reagents reduced the amount of contaminating proteins and endotoxins (about 66-100%) from the vector preparation. Among the reagents evaluated, 2% OTG resulted in the greatest reduction in contaminating proteins and endotoxins (<LoD) compared to PBS wash alone. Using this protocol we achieved yields approximately 88% of PBS wash alone suggesting OTG wash results in minimal vector loss. Our preliminary data also showed that OTG wash can be used to purify rAAV1 (1% OTG wash), rAAV2 (1% OTG wash), and rAAV5 (2% OTG wash) with PCS AAVX resin. A 2% OTG can also be used to successfully purify rAAV9 with PCS AAV9 resin. We observed approximately half of rAAV9 was lost after 2% OTG wash using PCS AAVX resin suggesting differences in how rAAV9 interacts with the two resins. Currently, other serotypes are being tested with OTG wash. Our group previously published an optimized rAAV production protocol in suspension HEK 293T cells with triple-transfection (Zhao, et al. Mol Ther Clin Dev & Methods, 2020). When this OTG wash was combined with a modified protocol that eliminates sodium butyrate, we were able to produce about $5\text{-}6\times10^{14}$ VG of purified rAAV8-Ef1α-EGFP from one liter of suspension HEK 293T cell culture. The yield achieved via this suspension HEK 293T cell platform and our affinity purification scheme offers high scalability and a comparable yield to *sf9* cells (Kurasawa et al. Mol Ther Clin Dev & Methods, 2020) making it an appealing option for rAAV generation.

169. Characterization of rAAV Key Quality Attributes Generated from a Highly Optimized, Hela 3.0 Producer Cell Line (PCL) Production Platform

Nicholas Richards[1], Caroline Adams[2], Kevin Larpenteur[2], Justin Tedeschi[2], Samuel Wadsworth[2], K. Reed Clark[2], Matthew Fuller[2]
[1]Ultragenyx Pharmaceuticals, Inc, Cambridge, MA,[2]Ultragenyx Gene Therapy, Cambridge, MA

A key challenge facing the gene therapy field today is ensuring that manufacturing capabilities surpass current standards to ensure accessibility and affordability for all eligible patients, as well as facilitating treatments for diseases that require higher doses for efficacy, such as Duchenne muscular dystrophy. To address these needs, we have developed a HeLa-based rAAV manufacturing platform that has demonstrated scalable 2000L production in an industrial setting to support ongoing Phase I/II clinical trials. Recently, we have further optimized our HeLa Producer Cell Line (PCL) platform to HeLa 3.0 by genetically modifying existing, highly productive monoclonal PCLs. Utilizing an RNA-seq directed screening method, we identified specific genes that modulate rAAV production and knocked out those genes *via* CRISPR/Cas9-mediated genome editing, which increased productivity up to 5 fold. While titer improvements are important, ensuring the quality and fidelity of both the production platform and manufactured vector is vital. To this end, two HeLa 3.0 producer cell lines, developed for separate clinical programs, were interrogated to characterize attributes previously identified as essential for generation of a robust, stable cell line including rAAV genome amplification, integration site analysis, kinetic analysis of rAAV production and overall fitness. In addition, viral products generated from both HeLa 3.0 PCLs were analyzed for key quality attributes, such as genome integrity,

capsid protein ratio, and empty: full ratio to establish comparability with existing, efficacious vectors. Importantly, the newly optimized HeLa 3.0 production system demonstrated the robustness required to facilitate production of high quality rAAV vector product.

170. AAVX Resin Binding Site Identification via Library Screening Analysis on Novel AAV Vectors

Stewart Craig[1], Zachary Thorpe[1], Rebecca McDonnell[1], Kimberly Le[1], Allegra Fieldsend[1], Deepak Grover[1], Sri Siripurapu[1], Stephanie Malyszka[1], Laura K. Richman[1], Luk H. Vandenberghe[1,2], Christopher Tipper[1]

[1]Affinia Therapeutics, Waltham, MA,[2]Grousbeck Gene Therapy Center, Mass Eye and Ear and Harvard Medical School, Boston, MA

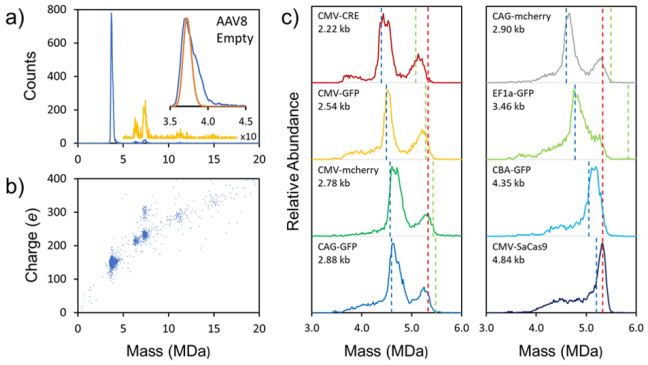
A major effort by AAV-based gene therapy manufacturers is focused on improving yield and reducing COGs independent of AAV serotype to enable the potential treatment of rare and non-rare diseases with a one-time dose of gene therapy. Key among these efforts is improved affinity capture. Affinity capture is a scalable method that effectively removes host impurities from AAV vector preparations. As with any receptor/ligand complex, the stringency of capture of the AAV particle is a function of the dissociation constant (Kd) of the interaction. Identifying and understanding the specific chemistries of the interaction may allow engineering and modulation of the Kd. Similar to AVB, AAVX is an affinity resin that utilizes a camelid antibody to capture AAV vector particles. Camelid antibodies are single chain and exhibit extended CDRs, making them ideal for reaching confined epitopes and conjugation to porous substrates. The development of the resin by ThermoFisher has been recognized as a major improvement to downstream processes. AAVX resin can bind most tested serotypes with an affinity high enough to withstand the stringent wash conditions required for therapeutic development. Here, we investigate the potential AAV epitope responsible for binding to AAVX resin. The use of AAV vector libraries to address manufacturability concerns has not been widely reported. The rich datasets produced using diverse yet informatically manageable vector libraries engineered via ancestral sequence reconstruction provide an opportunity to answer questions of manufacturability at multiple process steps. Exemplifying this utility, we have preliminarily identified the region of the AAV vector where the AAVX epitope resides. Processing the Anc80 vector library via AVB and AAVX affinity resins revealed differential efficiency of vector capture. By informatic analysis, the single residue responsible for this observation was identified. Interestingly, this binding site is located within the 3-fold axis, which is distinct from the AVB binding site near the 5-fold axis. Further studies to definitively identify critical primary-to-tertiary elements of the AAVX epitope are ongoing.

171. Analysis of Gene Therapy Products by Charge Detection Mass Spectrometry

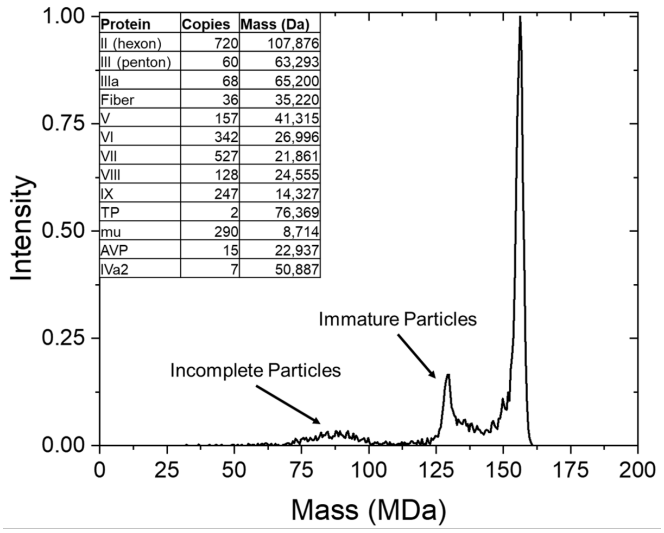
Benjamin E. Draper[1], Lauren F. Barnes[2], Martin F. Jarrold[1,2]

[1]Megadalton Solutions, Bloomington, IN,[2]Chemistry, Indiana University, Bloomington, IN

New analytical tools in cell and gene therapy will improve patient outcomes. In general, commercial mass spectrometers have an effective upper mass limit around a megadalton (MDa), a limit that arises because of the loss of charge state resolution in the mass-to-charge (m/z) spectrum. Cell and gene therapy platforms fall beyond this range, but a relatively new technique called charge detection mass spectrometry (CDMS) can circumvent this limitation and bring the precision of mass spectrometry to cell and gene therapy. CDMS simultaneously measures the m/z and charge (z) of individual particles allowing for a direct calculation of mass. This extends accurate mass measurements into the MDa to gigadalton (GDa) regime. Accurate mass measurements obtained from CDMS can be used to assess a range of critical quality attributes for gene therapy products. This work will highlight how CDMS has been demonstrated for viral vector platforms and genomic material. Adeno-associated virus (AAV) vectors have a mass in the 3-5 MDa range depending on the packaging of genomic material. Direct mass measurement allows for the packaging to be assessed and the gene of interest to be identified for intact capsids. We show that the differences between the masses of empty particles and particles with the genome of interest (GOI) are correlated with the expected genome mass. In addition, CDMS provides a robust orthogonal technique to quantify empty, partial, and full particles as well as aggregates that form through both capsid degradation and capsid adherence. Adenovirus vectors have a well-established use in cancer treatments and hold great promise in vaccine research. Here we show the first high resolution mass measurement for a virus with a mass over 100 MDa. Using CDMS, we correlate the measured mass for a range of packaged genome lengths to the amount of co-packaged counterions and proteins important for genome stability. In addition, we use the accurate charge measurement to assess structure and identify incomplete, defective, and empty particles of adenovirus. Due to the importance of genomic material in gene therapies, we show how CDMS can assess the purity and mass of plasmids. Building off the mass measurements of plasmids we also able to directly detect and measure the genomic material from disassembled AAV and adenovirus capsids. In the case of AAV both ssDNA and dsDNA corresponding to the GOI were detected and used to identify the purity of the packaged contents. All spectra and results showed above use a single CDMS platform that is robust and highly sensitive. A few benchmarks include: AAV analysis low titer samples (1e10 vp/mL) in heterogeneous matrices, mass measurements with low volumes (10-20 µL), and analysis times of less than 1 hour.



(Figure 1)



(Figure 2)

172. Use of SPTFF in Continuous Downstream Manufacturing of Adeno-Associate Viruses

Rajeshwar Chinnawar, Shawn Tansey, Nicholas Marchand

R&D, Pall Biotech, Westborough, MA

In recent years, pre-clinical and clinical development in the gene therapy industry has been rapidly growing. To meet the industry's requirement for large quantities of GMP-compliant therapeutic viral vector, there's a need for high-efficiency equipment and consumables. A typical downstream process for AAV manufacturing involves a combination of unit operations including clarification of crude harvest, chromatography, concentration, diafiltration and sterile filtration. A few challenges in AAV processing include processing-time and shear sensitivity of the product, and safety concerns of the product. Adding TFF membranes into a process can reduce working volumes, and application of single-use consumables can mitigate safety concerns. Replacing traditional recirculating TFF with newer Single-Pass TFF (SPTFF) technologies has the potential to reduce shear exposure, reduce processing time by integrating with unit operations before and/or after, and improve process yields. In this work we implemented Palls innovative Cadence SPTFF technology for in-line concentration to optimize an AAV downstream process. An SPTFF device was connected to an upstream depth filtration assembly with a small break tank. The post-SPTFF-concentrated viral vector stream was continuously pumped through a sterile filter. This work demonstrates the use of an integrated, continuous SPTFF operation in an AAV process that achieves 40% reduction in processing time while maintaining a 96% Yield.

Immunotherapy and Vaccines

173. A Humanized EBV Mouse Model to Evaluate the Safety/Impact of Human T_{reg} Cell Therapy on Antiviral Immune Responses

Swati Singh, Stefan Lachkar, Noelle Dahl, Christina Lopez, Yuchi Honaker, Claire Stoffers, Anna Zielinska-Kwiatkowska, Iram Khan, Karen Sommer, David J. Rawlings

Seattle Children's Research Institute, Seattle, WA

Regulatory T cells (Treg) suppress immune cell activation and thus have key roles in preventing excessive immune responses and autoimmunity. Adoptive cell therapy with Treg is currently being tested clinically to provide tolerance for graft vs. host disease, organ transplant and autoimmune diseases. Due to potential limitations of Treg frequency, stability and ex vivo expansion, we have utilized gene editing to enforce FOXP3 expression in peripheral blood $CD4^+$ cells, driving them to adopt Treg-like phenotypes and functions. We refer to these cells as engineered Tregs (Eng Tregs). While Eng Tregs show robust suppression of pathological immune responses in mouse models, it is theoretically possible that adoptively transferred Treg could have undesirable off-target effects such as suppressing immune responses against pathogens, either during novel exposures or the re-activation of latent viruses such as Epstein-Barr virus (EBV). While most humans (95%) will have been infected with EBV by adulthood, infection is usually well-controlled by the immune system. However, a reservoir of latently infected B cells persists for decades, kept in check by immunological surveillance; the latent EBV can become re-activated when the immune system is suppressed. The goal of our study was to evaluate the safety/impact of engineered Treg (Eng Tregs) on viral responses. Since EBV does not infect mouse cells, we tested this in the following humanized mouse model. From a G-CSF-mobilized human donor, we purified autologous $CD34^+$ peripheral blood stem cells (PBSC), $CD4^+$ T cells, and thymic Treg (tTreg). The $CD34^+$ PBSCs were transplanted into busulfan-conditioned NOD-*scid*-IL2Rg^{NULL} (NSG) mice. After stable engraftment (12-13-weeks post-transplant), humanized NSG were infected with EBV, then subsequently treated with either autologous tTreg or Eng Tregs ($CD4^+$ T cells edited to stably express FOXP3 utilizing HDR-based CRISPR/Cas9 targeted integration of FOXP3 cDNA with an MND promoter into *FOXP3)*. Eight weeks later, we quantified circulating EBV loads and the immunophenotype of human lymphocytes in the spleen, blood, and bone marrow. These experiments included control mice that were treated with T cell-depleting antibodies in place of Treg; these mice rapidly developed B cell proliferative disease associated with EBV infection/re-activation and had high EBV loads vs. mice receiving EBV alone. This control demonstrated the role of the human $CD4^+$ and $CD8^+$ T cells in keeping the EBV infection in check in this model. Importantly, the viral loads, % human B cell and % activated human $CD8^+$ T cells in response to EBV infection were equivalent between mice that received Eng Tregs, tTreg, or EBV alone. Further, we were able to detect $LNGFR^+$ $FOXP3^+$ cells in the Eng Tregs cohort at his time point, suggesting the stability of Eng Tregs in this pro-inflammatory setting. These combined data demonstrate that Eng Tregs do not impact the immune responses required to control EBV infection in this EBV-infected humanized

mouse model; demonstrating an effective in vivo system to evaluate whether human Treg-based cell therapies can preserve desired anti-viral immune responses.

174. Candidate Selection in BALB/c Mice towards a Single Dose AAV-Based COVID19 Prophylactic Vaccine

Wenlong Dai, Nerea Zabaleta, Urja Bhatt, Reynette Estelien, Dan Li, Dawid Maciorowski, Julio Sanmiguel, Ruchi Chauhan, Allison Cucalon, Cheikh Diop, Maya Kim, Abigail Sheridan, Luk H. Vandenberghe

Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

The public health crisis of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) exceeds 100 million cases worldwide. While several protective vaccines are becoming available, several challenges to attain levels of population immunity remain. We hypothesized that, based on prior developments, an AAV-based preventative vaccine could address some of the biological and logistical limitations to an effective vaccination campaign at a global scale, importantly for example single dose efficacy. In the present study, we designed 9 AAV2/rh32.33 vector based COVID-19 genetic vaccine candidates (AAVCOVID) expressing the wild type full length spike (S) protein, stabilized S derivatives and several soluble S-based antigen, namely S ectodomain, S1 and receptor binding domain (RBD), the latter both in ssAAV and scAAV context. Wuhan S and D614G variant strains were used as the basis for antigen design. AAVCOVID candidates were evaluated for immunogenicity following single dose intramuscular (IM) injection in BALB/c mice at either 10^{10} gc/mouse or 10^{11} gc/mouse for a period of 3 months post injection. From these studies, a candidate selection was performed for further development. Results demonstrate variable levels, yet overall rapid and robust antigen specific binding and neutralizing antibody responses. Interestingly, serum antibody isotype profiles differed qualitatively between membrane anchored antigens and soluble antigens, suggesting a more pronounce Th2 phenotype for the soluble antigen vaccine candidates. However, all candidates induced robust IFN-γ, but not IL4, T cell response by ELISpot assay at a 3-month time point, indicative of a Th1 biased response for all AAVCOVID candidates. For the duration of the immune response, the serum antibody titer reached the peak at month 2 and started to decrease at month 3. Remarkably, T cell responses in the low dose group mice were equivalent to modestly higher than those in high dose group. Taken together, these results indicate that the AAVCOVID platform induces high level neutralizing responses that are complemented with robust T-cell immunogenicity. Based on these data and other strategic parameters, AAVCOVID-1 (membrane anchored pre-fusion stable S) and AAVCOVID-3 (secreted S1) progressed to further studies toward consideration of one candidate for clinical development.

175. AAV Specific CAR Regulatory T Cells Mitigate Immune Responses against AAV Gene Therapy

Motahareh Arjomandnejad[1], Thomas Nixon[1], Qiushi Tang[1], Meghan Blackwood[1], Katelyn Sylvia[1], Alisha M. Gruntman[1,2,3], Guangping Gao[1], Terrence Flotte[1,2], Allison M. Keeler[1,2]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Department of Pediatrics, University of Massachusetts Medical School, Worcester, MA,[3]Department of Clinical Sciences, Department of Clinical Sciences, Cummings School of Veterinary Medicine at Tufts University, Grafton, MA

Despite the success of AAV vectors as powerful gene therapy tools, the immune responses to AAV capsids have limited their therapeutic applications. As observed in clinical trials, systemic delivery of AAV leads to capsid specific immune responses and clearance of the transgene by CD8 T cells while intramuscular delivery of AAV, leads to long-term transgene expression despite an immune response. Differences in transgene expression is thought to be due to T regulatory cell (Treg) infiltration in muscles creating tolerance to AAV capsids. Hence, we examined the ability of Tregs to induce local and specific immunosuppression rather than steroid mediated non-specific immune suppression. We previously generated AAV specific chimeric antigen receptor T cells (AAV-CAR), and AAV specific regulatory T cells (CAR-Treg) which recognize major AAV capsid variants. AAV-CARs are cytotoxic to AAV infected target cells and this killing is suppressed by AAV-CAR-Tregs. In murine models, AAV-CARs cleared AAV infected cells. Herein, we confirmed the cytotoxicity of AAV-CARs and the suppressive ability of AAV-CAR-Tregs against the AAV capsid variants- AAV1, AAV2, AAV3b, AAV 5, AAV6, AAV8, AAV9 and rh32.33. Further, AAV-CAR-Tregs inhibited the proliferation and interferon gamma (IFN-γ) production of AAV-CAR T cells when stimulated with AAV capsid. Next, we utilized AAV-rh32.33 capsid to elicit a robust immune response in mice. Animals were injected with AAV-rh32.33 expressing alpha 1 anti-trypsin (AAT) followed by intravenous delivery of AAV-CAR-Tregs, non-specific expanded T-regs, or saline. AAT expression was stable for 25 weeks in both Treg and AAV-CAR-Treg groups, despite high level of anti-capsid antibody with no detectable expression 3 weeks post AAV injection in the saline group. Histology of injected muscles revealed detectable AAT staining in AAV-CAR-Treg, or non-specific Tregs groups and not in the untreated group. Tregs can induce tolerance in an antigen independent pathway via the bystander effect, where antigen activated Tregs create local suppressive environment which can suppress other antigens. To determine if AAV-CAR-Tregs can function similar to endogenous Tregs, we tested if they AAV capsid specific AAV-CAR-Tregs could block immune responses to transgene. We designed a cytotoxicity assay with mixed antigens of CAR T cells and CAR-Tregs which revealed that AAV-CAR-Tregs could suppress CAR T cells specific to other antigens. To test this principle in vivo, we delivered AAV1 expressing the immunogenic OVA (AAV-OVA) transgene followed by delivery of either AAV-CAR-Tregs, expanded non-specific T-regs, or saline. Stable transgene expression was observed in the animals receiving AAV-CAR-Tregs, or non-specific T-regs, and was reduced 2 weeks after AAV-OVA in the saline group. Interestingly, all groups produced anti-OVA antibodies despite treatment. Moreover, histology of injected

muscles revealed severe focal, and diffuse myositis in untreated animals, with significantly reduced inflammation observed in the muscles of the animals treated with AAV-CAR-Tregs, despite a robust OVA expression. Together, this data demonstrates that AAV-CAR-Tregs are powerful tools to model the cellular immune response against AAV capsid. Therapeutically, AAV-CAR-Tregs potentially can modulate immune responses directed against AAV capsids and immunogenic transgenes without the detriments of systemic immunosuppression.

176. Engineered Protein M Analogs Enhance the Ability to Suppress Vector Neutralizing Antibodies and Generate a Window for Successful Gene Delivery

Charles Askew, David Thieker, Zheng Chai, Brian Kuhlman, Chengwen Li

University of North Carolina, Chapel Hill, NC

Patient derived neutralizing antibodies (NAbs) to viral vectors are a major hurdle to using these advanced biologics as traditional drugs. NAbs block therapeutic gene delivery in those with pre-existing vector immunity, and prevent vector dose titration after the first treatment. Of the strategies in development to reduce the effect of pre-existing NAbs or blunt the generation of antibodies during treatment, most suffer from limited efficacy to bring NAb titers below a level that permits vector administration, and none demonstrate instantaneous NAb suppression. Previously we demonstrated that a soluble fragment of mycoplasma Protein M transiently blocks NAbs to adeno-associated virus (AAV), which enabled successful in vivo systemic gene delivery over a 1,000-fold concentration range of anti-AAV NAbs as compared to controls without Protein M where the same AAV dose was neutralized. Protein M binds to the Fab region of all antibody classes at a conserved site common to humans, non-human primates, rodents, and most mammalian species. Protein M binding temporarily blocks all antigen recognition through steric hinderance of the complementarity-determining regions, and generates a transient gene delivery window whereby any vector is universally protected from neutralization. Additionally, we demonstrate that use of Protein M with a variety of vectors instantaneously blocks NAbs for successful gene delivery, enabling robust gene expression in the presence of neutralizing serum. While generating feasibility data we quickly discovered that Protein M is highly unstable at body temperature, posing a significant challenge for in vivo use and scalable protein manufacturing. Instantaneous Protein M unfolding occurs at 41°C, resulting in precipitation and aggregation. Incubating a truncated naturally occurring Protein M sequence at 37°C caused protein unfolding after only 15 minutes and loss of antibody blocking function. Therefore, we used structural and computational modeling to design 850+ Protein M analogs with enhanced properties. We produced a library of stabilized mutants using *in silico* site saturation mutagenesis and free energy prediction, and then screened single and combinatorial mutants. Stabilizing mutations resulted in a distribution of analogs with increased melting temperatures, from 42°C degrees to 65°C and greater. Improvements in thermostability were accompanied by significantly decreased aggregation, increased solubility, and ability to highly concentrate the protein. Stabilized Protein M variants improved in vitro function, whereby several analogs retained antibody blocking ability after more than 72hrs at 37°C. Using an engineered analog, PM129, we now demonstrate successful readministration of AAV in mice immunized by AAV one-month before secondary administration. PM129 blocks NAbs after systemic intravenous dosing shortly before AAV administration, as well as when PM129 is admixed with AAV and administered in a single intramuscular injection. While PM129 is a novel foreign protein with the capacity to elicit inhibitory antibodies to itself, we are able to successfully redose PM129 and AAV even when prior doses of PM129 have been administered. This suggests PM129 is capable of outcompeting self-neutralizing antibodies, which likely represent only a small fraction of the total serum antibody pool. Finally, safety assessments of PM129 were conducted in mice demonstrating no observed adverse reactions in over 40 mice included in the high dose cohort. Minimal B cell or T cell proliferation was observed after in vivo administration and collected serum samples failed to activate complement C5 or demonstrate immune complex formation.

177. *In Vivo* HSC Gene Therapy with High-Level, Erythroid-Specific Expression of a Secreted SARS-CoV-2 Decoy Receptor

Hongjie Wang[1], Sucheol Gil[1], Chang Li[1], Christopher Doering[2], Philip Ng[3], Steve Roffler[4], Hans-Peter Kiem[5], Andre L. Lieber[1]

[1]University of Washington, University of Washington, Seattle, WA,[2]Emory University, Atlanta, GA,[3]Baylor College of Medicine, Houston, TX,[4]Academia Sinica, Taipei, Taiwan,[5]FHCRC, Seattle, WA

We developed a technically simple and portable *in vivo* hematopoietic stem cell (HSC) transduction approach that involves HSC mobilization from the bone marrow into the peripheral blood stream and the intravenous injection of an integrating, helper-dependent adenovirus (HDAd5/35++) vector system. HDAd5/35++ vectors target human CD46, a receptor that is abundantly expressed on primitive HSCs. Transgene integration is achieved by a hyperactive Sleeping Beauty transposase (SB100x) and transgene marking in peripheral blood cells can be increased by *in vivo* selection. Here we directed transgene expression to HSC-derived erythroid cells using beta-globin regulatory elements. We hypothesized that the abundance and systemic distribution of erythroid cells can be harnessed for high-level production of secreted therapeutic proteins. We first demonstrated that our approach allowed for sustained, erythroid-lineage expression of a bioengineered human factor VIII, termed ET3, leading to phenotypic correction of the bleeding defect in $hCD46^{+/+}/F8^{-/-}$ hemophilia A mice as measured by chromogenic assay, aPTT assays and tail bleeding assays (PMID: 31585952). We then used this approach for continuous expression of a SARS-CoV-2 decoy receptor, i.e. a secreted, human ACE2 extracellular domain fused to human constant IgG1 domains to form an antibody-like structure (sACE2-Ig) capable of mediating virus opsonization. We performed *in vivo* HSC transduction of CD46-transgenic mice with a HDAd5/35++-sACE2-Ig vector. Serum sACE2-Ig levels reached 500-1300 ng/ml after *in vivo* selection. The sACE2-Ig protein in the serum from these animals was active in blocking the infection of $293\text{-}ACE2^{+}$ cells with a CoV2-Spike protein-pseudotyped lentivirus vector. Lineage-negative bone marrow cells from HDAd5/35++-sACE2-Ig-transduced CD46-transgenic mice will be transplanted into lethally irradiated K18-hACE2 transgenic

mice. The resulting mice will express sACE2-Ig and will be susceptible to SARS-CoV-2 infection. Furthermore, we performed *in vivo* HSC transduction with the integrating HDAd5/35++sACE2-Ig vector in a rhesus macaque. HSC mobilization by subcutaneous administration of G-CSF/plerixafor and intravenous HDAd injection at dose of $3.2x10^{12}$ vp/kg was well tolerated after appropriate cytokine prophylaxis (dexamethasone, tocilizumab, anakinra). The HSC transduction efficacy and serum sACE2-Ig levels after *in vivo* selection will be reported. Ultimately, we plan to test whether continuous sACE2-Ig expression could provide protection against challenges with CoV-2 variants in the mouse and NHP models.

178. A Platform for Genome Editing of Human B Cells to Produce Single-Chain Antibody-Like Molecules That Recapitulate Antibody Functionality

Geoffrey L. Rogers, Xiaoli Huang, Chun Huang, Atishay Mathur, Paula M. Cannon

Molecular Microbiology and Immunology, University of Southern California, Los Angeles, CA

Genome editing of B cells offers opportunities to create cells that express antibodies with characteristics that cannot be elicited by vaccination. However, the complex regulatory pathways governing antibody function, which include alternative splicing, class switch recombination, and somatic hypermutation, suggest that editing the endogenous immunoglobulin (Ig) locus may be required to fully reconstitute such functions in an engineered antibody. Towards that goal we have developed a strategy to engineer the human Ig locus based on the design of single-domain antibodies (sdAbs) - heavy-chain only antibodies found in camelids that comprise an antigen-binding VHH domain and heavy chain Fc region. By inserting a promoter-VHH-splice donor cassette within an intron of the human IgG1 gene (*IGHG1*), a spliced mRNA is produced that fuses the VHH to constant domains of IgG1 and results in both a recombinant membrane-bound B cell receptor (BCR) and the matched secreted antibody. As a proof of principle, we used CRISPR/Cas9 to engineer human B cell lines with HIV Env-specific VHH domains. We observed anti-HIV BCR cell surface expression, signaling after engagement with the HIV Env antigen, and secretion of functional sdAbs capable of neutralizing HIV. Long-term culture of the cells resulted in signatures of somatic hypermutation, with the sequence changes strongly localized to AID hotspot motifs, and consequential alterations in antibody functionality. Such an ability may provide advantages against a highly mutable target such as HIV. Expanding the approach to primary human B cells, we combined CRISPR/Cas9 with AAV6 homology donors to insert anti-HIV VHH cassettes at *IGHG1*. Over 20% of cells expressed HIV-specific human IgG as measured by flow cytometry, and the engineered B cells could be expanded >50-fold over 11 days using an animal-component free system. After *in vitro* differentiation that skewed the B cells towards an antibody-secreting cell phenotype, we observed a shift in splicing of the chimeric antibody mRNA towards the secreted isoform and increased anti-HIV sdAb secretion. Importantly, anti-HIV sdAb secretion tracked with total IgG secretion by unmodified cells on a per cell basis (R^2=0.99), indicating that alternative splicing of the transgene was co-regulated with endogenous antibody splicing by the differentiation state of the B cell. Finally, supernatants from engineered B cells were able to neutralize HIV infection with an IC50 equivalent to the recombinant VHH-Fc protein, indicating that sdAbs generated by this strategy are properly produced and secreted despite the presence of other endogenous IgGs in this system. In conclusion, we have established a versatile platform to reprogram human B cells to express engineered single-chain antibodies. It can be used with a variety of antigen-recognizing domains including VHH domains, scFvs and soluble receptor derivatives, expanding the range of antibody-like molecules that can be produced from the Ig locus. Multiplexed cassettes, comprising for example multiple VHH domains linked together by flexible linkers, can also be inserted and used to produce molecules with enhanced or multivalent functionalities. We believe the technology is broadly applicable and could be considered for any antibody-based therapy that would benefit from long-term *in vivo* production, including targets in cancer, autoimmune diseases, or other infectious diseases.

179. Immune Modulation Preceding AAV9-GLB1 Gene Therapy Preserves the Possibility for Re-Dosing in Children with GM1 Gangliosidosis

Precill D'Souza[1], Kirsten E. Coleman[2], Caroline E. Rothermel[1], Manuela Corti[2], Cynthia J. Tifft[1], Barry J. Byrne[2]

[1]Office of the Clinical Director, NHGRI, National Institutes of Health, Bethesda, MD,[2]Powell Gene Therapy Center, University of Florida College of Medicine, Gainesville, FL

GM1 gangliosidosis is a rare, autosomal recessive, multisystem lysosomal storage disorder with relentless neurodegeneration and no effective therapy. AAV9 has broad distribution with intravenous administration and demonstrated tropism to the CNS. However, preexisting immunity to AAV9 decreases the effectiveness of transgene delivery and precludes the re-dosing of children as they grow. Here we present results of 6 Type II GM1 patients (ages 2.5 to 9 years) who received 1.5 x 10^{15} vg/kg AAV9-*GLB1* intravenously following a novel immune modulation pre-conditioning regimen to deplete CD19+ B cells and inactivate CD3+ T cells. All 6 patients received 4 intravenous doses of rituximab (375 mg/m^2) beginning 21 days prior to gene transfer and oral rapamycin (0.5-1 mg/m^2/day) to maintain a trough level of 5-10 ng/mL beginning 21 days prior to gene transfer and continuing 180 days following gene delivery. Methylprednisolone (1 mg/kg IV) was administered 60 minutes prior to gene transfer and prednisone (0.5mg/kg/d) was given orally for 3 days following dosing. CD19+ B cells were depleted following 2 does of rituximab in all 6 subjects with recovery beginning 3-6 months following gene delivery. There were no serious infections in any subject with the exception of one patient who developed bacterial sepsis from PICC line malfunction at 2 weeks following gene transfer. Quantitative immunoglobulins remained within normal ranges for all subjects through 6 months following gene transfer. Side effects of mucositis (4 patients) and mild neutropenia (3 patients) resolved without sequalae. IgM and IgG antibody titers to AAV9 did not change in 4 of 6 subjects. One subject, thought to be possibly pre-immune to AAV, had a delayed IgG response beginning at 14 days following vector administration that remained elevated through

180 days. A second subject had delayed IgM response to viral capsid that peaked at 30 days followed by a modest IgG response beginning at 30 days and persisted through 180 days. The pharmacokinetics of vector genome copies in serum for these 2 subjects were not different than the remaining 4 subjects who did not show a response. Following gene transfer, liver functions including alanine aminotransferase (ALT) and gamma-glutamyl-transferase (GGT) were within normal limits. Baseline aspartate aminotransferase (AST) was mildly elevated in all subjects consistent with our observations in a cohort of GM1 patients (mean=61.35 U/L, N=38). No subject had AST elevations greater than 3X their baseline value. C3 and C4 remained within the normal range and 3 subjects had mild thrombocytopenia (lowest value of 88 K/mcL). Immune modulation was safe and well tolerated in our cohort and there were no complications in the days immediately following gene delivery. Two patients developed delayed antibody to the viral capsid, one of whom was likely pre-immune to AAV. Four patients showed no rise in antibody titer following gene transfer leaving open the possibility for re-dosing with the same AAV9 vector in the future.

New Gene Editing Technologies and Applications

180. Modulation of DNA Repair Pathways by HDR-CRISPR Promotes Seamless Genome Editing in Primary Human Hematopoietic Cells

Antonio Carusillo, Raul Schäfer, Manuel Rhiel, Daniel Türk, Tatjana I. Cornu, Toni Cathomen, Claudio Mussolino

Transfusion Medicine and Gene Therapy, Universitätsklinikum Freiburg, Freiburg Im Breisgau, Germany

The CRISPR-Cas system is a robust platform for genome editing. The introduction of a DNA double-strand break (DSB) at precise gene locations can be exploited to achieve targeted gene knockout by harnessing the error-prone non-homologous end-joining (NHEJ) pathway. However, using CRISPR-Cas technology for precise genome editing via homology-directed repair (HDR) remains challenging, with HDR frequencies below the threshold required for clinical translation. Common strategies to increase HDR-mediated DSB repair include the use of chemicals either to inhibit NHEJ or to arrest the cells in those cell cycle phases when HDR is most active. However, the global effects of these drugs pose serious safety concerns if applied in clinically relevant settings. To address this issue, we devised a strategy to recruit HDR-promoting factors or NHEJ-inhibiting proteins to the DSB site. This is achieved via the direct fusion of particular protein-protein interaction domains to the Cas9 nuclease. We generated 16 different Cas9-fusion proteins (referred to as HDR-CRISPR) and extensively investigated their impact on DNA repair pathway choice by using two reporter systems, the traffic light reporter (TLR) and the BFP-to-GFP (B2G) assay. These two assays allowed us to investigate the outcome of DNA repair mediated by a DNA donor supplied either as a plasmid or oligodeoxynucleotides (ODN) respectively. Our results indicate that HDR-CRISPRs enhanced HDR frequency 3-fold over baseline levels. The simultaneous reduction of NHEJ-mediated repair resulted in a 5-fold increase in the HDR:NHEJ ratio when using our best performing HDR-CRISPR. Next, we assessed the capability of HDR-CRISPR to precisely integrate a large GFP expression cassette into the endogenous *AAVS1* safe harbor locus of K562 and Jurkat cell lines. Independently of the cell type, the use of HDR-CRISPR resulted in a 2.5-fold increase in targeted integration as compared to samples receiving the unmodified Cas9. The most efficient HDR-CRISPR fusion was then tested for its ability to promote HDR-mediated repair in clinically relevant primary human cells. HDR-CRISPR was delivered to T lymphocytes and hematopoietic stem cells (HSCs) in the form of RNA. Using an appropriate ODN as a repair template, we aimed at introducing a stop codon within exon 3 of the *CCR5* gene to generate immune cells resistant to HIV infection. HDR-CRISPR led to a 2-fold increase in precise genome editing events as compared to the use of an unmodified Cas9. In conclusion, our data support the hypothesis that DSB repair choice can be altered through the local recruitment of key DNA repair factors capable of either promoting HDR or inhibit NHEJ, also in clinically relevant cells.

181. Capturing and Characterizing Single Cell Allelic Heterogeneity of CRISPR-Cas9 Gene Editing *In Vivo*

Jesse A. Weber, Jonathan F. Lang, Alejandro M. Monteys, Beverly L. Davidson

Children's Hospital of Philadelphia, and University of Pennsylvania, Philadelphia, PA

Translating CRISPR/Cas9 technology into *in vivo* therapeutic applications necessitates a strong understanding of the potential sites of delivery and the nature of subsequent gene editing events. Editing-dependent reporter mouse strains (e.g. Ai6 and Ai14 (both containing lox-stop-lox-fluorescent protein at the *ROSA26* locus)) in combination with adeno-associated virus (AAV) delivered CRISPR/Cas9 offers a sensitive way to address some of these delivery and editing questions. In some occurrences, a deletion affecting both alleles of a gene may be desired. To assess the frequency and fidelity of biallelic editing, we generated compound Ai14/Ai6 heterozygote mice. Injection of AAVs encoding Cas9 protein and gRNAs targeted to the transgenic *ROSA26* locus will edit the stop cassette and activate tdTomato and/or ZsGreen from the Ai14 and Ai6 reporter alleles, respectively. Interestingly, after AAVgRNA delivery, the most frequent outcome was that only one of the two reporter alleles was activated in transduced hepatocytes, suggesting allelic heterogeneity within single cells following *in vivo* editing. The experiment was repeated *in vitro* using Ai14/Ai6 mouse embryonic fibroblasts and SpCas9/gRNAs- or SaCas9/gRNAs-expressing plasmids. Following transfection, we again observed heterogeneity in reporter activation. Next, we used flow to separate cells into double positive (tdTomato+/ZsGreen+), single positive (tdTomato+/ZsGreen- or tdTomato-/ZsGreen+), or double negative (tdTomato-/ZsGreen) populations and sequenced DNA from tdTomato-/ZsGreen+ single positive cells at the Ai14 (non-expressing) and Ai6 (expressing) alleles. As expected, the Ai6 allele contained the desired CRISPR-mediated deletion of the stop cassette. However, at the non-expressing Ai14 allele, the most common editing outcome was indels at both gRNA cuts sites without deletion of the stop cassette. Contrary to our expectation, the apparent reason for non-activation at the Ai14 allele was not lack of editing, but rather, undesired editing. The NHEJ DNA-repair inhibitor,

Ku57788, increases CRISPR deletion efficiency *in vitro*. To test its effects on biallelic deletions, Ai14 /Ai6 mouse embryonic fibroblasts were treated with Ku57788 and transfected with SpCas9/gRNAs- or SaCas9/gRNAs-expressing plasmids. Flow-sorted cells exhibited a marked increase in single positive cells of both reporter alleles, but most importantly exhibited a near 2-fold increase in the number of cells with a successful biallelic deletion. This suggests that efforts to transiently inhibit DNA repair may increase biallelic CRISPR deletion efficiency.

182. Dual-HDR Editing Strategies for the Development of Islet-Specific Regulatory T Cells (EngTregs) for Restoration of Immune Tolerance in Type 1 Diabetes

Ahmad Boukhris[1], Peter Cook[1], Soo Jung Yang[2], Iram Khan[1], Jane Buckner[2], David Rawlings[1]

[1]CIIT, Seattle Children's Research Institute, Seattle, WA,[2]Benaroya Research Institute, Seattle, WA

Adoptive transfer of engineered regulatory T cells (EngTregs) represents an emerging therapeutic approach to promote immune tolerance in the setting of stem cell or solid organ transplantation, and in autoimmune diseases including type 1 diabetes (T1D). We previously developed robust methods to generate polyclonal EngTregs from CD4+ T cells by homology-directed-repair (HDR)-based gene editing. This approach introduces a ubiquitous promoter (MND) into the *FOXP3* locus downstream of the Treg-specific demethylated region (TSDR), resulting in high level FOXP3 expression and stable Treg-like phenotype and function. Polyclonal EngTregs cells generated by this approach are beneficial for systemic autoimmune conditions (including IPEX and GvHD) but lack the fine-specificity required to control tissue-specific autoimmune diseases driven by locally-expressed self-antigens. In this study, we describe a robust dual-HDR approach to generate and enrich antigen-specific EngTregs cells. Knock-out of the endogenous T cell receptor alpha constant (*TRAC*) gene and replacement with an islet-specific TCR is predicted to generate EngTregs capable of localizing to APCs expressing islet antigens in the pancreatic lymph nodes and/or pancreas. We evaluated three strategies for introducing an islet-specific TCR into the *TRAC* locus in parallel with HDR-editing of the *FOXP3* locus. To overcome the anticipated lower efficiencies of dual-HDR editing, we generated HDR donor cassettes designed to simultaneously introduce a heterodimeric, chemically-induced signaling complex (CISC) that mimics IL-2 signaling in response to an exogenous dimerizer, rapamycin. For our dual-HDR approach, the dimerizing components of the CISC cassette were introduced via two separate locus-specific editing events (in *TRAC* and *FOXP3*), thereby enabling specific selection and enrichment of only the dual-edited, islet-specific EngTregs. We first attempted a *TRAC* knock-in cassette incorporating an MND promoter, the full islet-TCR coding domain, and half of the CISC cassette. This strategy produced dual-edited cells that enriched in response to CISC dimerization. However, due to the large size of the TRAC-targeting repair template, the initial editing rates were low. To reduce the size of the HDR cassette, we pursued a promoter capture strategy, introducing the islet TCR downstream of the endogenous TRAC promoter. Notably, split-CISC test constructs using fluorophore markers showed that the these dual-edited cells failed to enrich, implying that CISC functionality requires high surface density of the synthetic receptor. Taking these findings into account, we designed an in-frame knock-in construct with an MND promoter driving CISC expression followed by the partial islet-TCR sequence (bearing the TCR beta chain and the alpha chain variable region) hijacking the endogenous alpha constant gene. Using this approach, we observed strong CISC-based enrichment of dual positive, antigen specific, EngTreg cells. After culturing cells in dimerizer, edited cells enriched from initial dual HDR-editing rates of 5-10% to >80% within 7 days, and these populations expressed high-levels of FOXP3 and relevant Treg surface markers. Our findings demonstrate that dual-HDR editing using a 'split IL-2 CISC' is a viable strategy for generating antigen-specific EngTreg cells. This approach has the capacity to deliver targeted immune tolerizing therapy to treat or prevent T1D and is likely to be easily adapted for use with alternative TCRs (or CARs) designed to target tissue-specific autoimmune or inflammatory diseases.

183. A Dimeric, Luminescent Biosensor for Imaging Unique DNA Sequences in Individual Cells

Nicholas G. Heath[1], David J. Segal[1], Henriette O'Geen[1], Nicole B. Coggins[1], Jacob Corn[2]

[1]Genome Center, UC Davis, Davis, CA,[2]Institute of Molecular Health Sciences, ETH, Zurich, Switzerland

One of the most prominent bottlenecks in the gene editing process is the ability to identify and isolate individual cells with desired edits within a population of treated cells. Current approaches typically require time-consuming and labor-intensive single cell isolation and population expansion followed by destruction of some portion of an expanded cell population for downstream *in vitro* analysis of DNA sequence content. Cell types that exhibit low efficiencies in transfection, editing, single cell isolation, or population expansion can be particularly challenging. To compound this problem, homology directed repair (HDR) can exhibit extremely low efficiency in certain cell types. A promising alternative to these and other destructive DNA detection assays could be the direct biosensing of edited DNA sequences in living cells. In recent years, an extensive arsenal of biosensing tools has been developed based on the clustered regularly interspaced short palindromic repeat (CRISPR) platform, including those that detect the presence of specific DNA sequences both *in vitro* and in live cells. In fact, the CRISPR/Cas gene editing system has been extensively modified for imaging endogenous genomic loci, but the vast majority of current approaches utilize monomeric fluorescent reporter-based biosensors, such as dCas9-GFP. A major drawback to these systems is that each monomeric sensor produces a signal whether bound to its target DNA or not, resulting in a high fluorescent background that negatively impacts the signal-to-noise ratio. For this reason, such "always-on" sensors must rely on obtaining a high local concentration of probes to distinguish signal from noise, limiting their use to highly repetitive elements that can be targeted by one gRNA or to unique sequences targeted by 50 or more gRNAs. In contrast, dimeric "turn-on" DNA biosensors offer the possibility of achieving signal production solely upon binding of two subunits to the target DNA and reassembly of a bright reporter. Furthermore, luminescent reporters are an attractive alternative to fluorescent reporters in biosensing experiments as cellular background luminescent signal is essentially nonexistent. This is due to the different nature of light production in

luminescent reporters where a catalytic reaction of an enzyme with its substrate produces light, eliminating the need for exogenous excitation light.Taking these points into consideration, we have developed a luminescence-based, dimeric DNA sequence biosensor that provides a sensitive readout for DNA sequences through proximity-mediated reassembly of two independently optimized fragments of NanoLuc luciferase (NLuc), a small, bright reporter. Reconstitution of NLuc becomes more favorable upon binding of two guide RNAs (gRNAs) to two DNA target sites with a defined orientation and spacing. Using this "turn-on" probe, we demonstrate rapid and sensitive detection of as low as 190 amol transfected target DNA, presenting a reliable approach for DNA biosensing. Across several cell-based delivery approaches, we were able to achieve approximately 2.5 - 20-fold increase in signal in live populations of cells transfected with the dimeric biosensor and various target DNA scaffolds compared to populations transfected with the dimeric biosensor but no target DNA. The future goals for this system include detection of single-copy genomic sequences at endogenous loci and validation of gene sequence changes after genome editing experiments in live cells.

184. Efficient CRISPR-Cas9-Mediated Gene Knockout and Interallelic Gene Conversion in Human Induced Pluripotent Stem Cells Using Non-Integrative Bacteriophage-Chimeric Retrovirus-Like Particles

John De Vos[1], Joffrey Mianné[1], Chloé Nguyen Van[1], Chloé Bourguignon[1], Mathieu Fieldès[1], Engi Ahmed[1], Christine Duthoit[2], Nicolas Martin[3], Alexandra Iché[3], Régis Gayon[3], Florine Samain[3], Lucile Lamouroux[3], Pascale Bouillé[3], Arnaud Bourdin[1], Said Assou[1]

[1]U1183, INSERM, Montpellier, France,[2]U1183, Flash Therapeutics, Toulouse, France,[3]Flash Therapeutics, Toulouse, France

The combination of CRISPR/Cas9 technology with human induced pluripotent stem cells (hiPSC) has tremendous potential for basic research and cell-based gene therapy. However, fulfilling these promises relies on our capacity to efficiently deliver exogenous nucleic acids into these cells and harness the repair mechanisms induced by the nuclease activity.Since gene editing systems require low and short-term expression in order to avoid off-target effects, RNA delivery is favored over DNA delivery. RNA delivery presents major advantages compared to DNA delivery, being that it is completely safe and devoid of any recombination events in the host genome. It is actually the most versatile, flexible, and safe mean for human therapy.Here, we investigated the potential of bacteriophage-chimeric retrovirus-like particles for the non-integrative delivery of RNA molecules in hiPSC. We found that these particles efficiently convey RNA molecules for transient expression in hiPSC, with minimal toxicity and without affecting cell pluripotency and subsequent differentiation. We then used this system to transiently deliver the CRISPR-Cas9 components (Cas9 mRNA and sgRNA) to generate a gene knockout with a high indel level (up to 85%) at several loci into hiPSC. Strikingly, when using an allele-specific sgRNA at a locus harboring compound heterozygous mutations, the targeted allele was not altered by NHEJ/MMEJ, but was repaired at high frequency using the homologous wild type allele, suggesting interallelic gene conversion.Our results highlight the potential of bacteriophage-chimeric retrovirus-like particles to efficiently deliver RNA molecules in hiPSC, and describe for the first time genome engineering by gene conversion in hiPSC. Harnessing this DNA repair mechanism could facilitate the therapeutic correction of human genetic disorders in hiPSC.

185. Triggering P53 Activation and Trapping of Transcriptionally Active Recombinant AAV Sequences Are Inadvertent Consequences of HSC Genome Editing

Samuele Ferrari*[1], Aurelien Jacob*[1,2], Marianne Laugel[3], Véronique Blouin[3], Stefano Beretta[1], Valentina Vavassori[1], Luisa Albano[1], Ivan Merelli[4], Eduard Ayuso[5], Oumeya Adjali[3], Magalie Penaud-Budloo[3], Pietro Genovese[1], Luigi Naldini[1,6]

[1]San Raffaele Telethon Institute for Gene Therapy, Ospedale San Raffaele, Milan, Italy,[2]University of Milano-Bicocca, Monza, Italy,[3]INSERM UMR 1089, University of Nantes, Nantes, France,[4]National Research Counsil, Segrate (MI), Italy,[5]INSERM UMR 1089, University of Nantes, Milan, France,[6]Vita-Salute San Raffaele University, Milan, Italy

Gene editing by homology-directed repair (HDR) in human hematopoietic stem cells (HSC) holds therapeutic potential for the treatment of hematological diseases by *in situ* correction of pathogenic mutations or site-specific integration of a transgene expression cassette. Induction of DNA double strand breaks by nucleases (e.g. CRISPR/Cas ribonucleoprotein) and cell transduction with single-stranded Adeno-Associated Vector serotype 6 (ssAAV6) to deliver the donor template for HDR cumulativelytrigger a robust p53-dependent DNA damage response (DDR), which delays HSC proliferation, lower the yield of edited cells and ultimately results in oligoclonal reconstitution after xenotransplantation. Transient p53 inhibition released cell cycle block and improved size and clonal composition of the human graft. Although ssAAV6 is the major contributor to p53 pathway activation, the elements responsible for such DDR signaling as well as their persistency in edited HSC have not been fully characterized. We did not observe significant differences in kinetics or extent of p53 pathway activation due to targeted loci, transgene cassette or ssAAV6 purification process, suggesting a shared viral component as DDR trigger. However, HSC edited and transduced with genome-free ("empty") AAV6 did not promote p53 response over nuclease-only treated cells and robustly engrafted in immunodeficient mice, thus ruling out capsid-dependent effect and confirming that ssAAV genomes can activate the p53 pathway. Delivery of the HDR template by self-complementary (sc)AAV6 triggered a more prolonged p53 transcriptional response and further reduced repopulation capacity of edited cells compared to ssAAV6-edited ones. Since recombinant (r) AAV genomic elements may trigger detrimental cellular responses in cultured HSC, we evaluated persistence of vector sequences in edited cells by deep sequencing the edited allele. A low fraction of HSCs unexpectedly carried on-target trapping of rAAV DNA fragments despite the transient delivery of editing components in these actively proliferating target cells. This finding was consistent across different editing loci and transgene cassettes and was confirmed in other human primary hematopoietic cells. Edited HSC with on-target trapping of rAAV DNA fragments engrafted in immunodeficient mice and

persisted long-term after transplant (median allele frequency < 0.5%). Sequence alignment of trapped fragments to the parental rAAV genome showed prevalence of the inverted terminal repeats (ITR) including the Rep binding element and the terminal resolution site. Of note, hematopoietic cells transduced with ssAAV6 harboring a promoter-less reporter cassette showed low but detectable ITR-dependent transcriptional activity. We are currently investigating the underlying mechanism of such rAAV fragment trapping and whether ITR-driven transcription may contribute to the adverse impact of AAV on HSC. This work will help to design strategies in order to overcome these inadvertent consequences of HDR template delivery and improve the efficiency and predicted safety of gene editing.

186. Accurate Quantification of CRISPR/Cas9 Induced Large Deletions, Insertions and Chromosomal Rearrangements Using SMRT Sequencing with Unique Molecular Identifiers

So Hyun Park, Mingming Cao, Gang Bao

Bioengineering, Rice University, Houston, TX

CRISPR/Cas9 induced double-stranded breaks (DSBs) can result in complex gene editing outcomes, including small insertions and deletions (INDELs), large INDELs, and complex chromosomal rearrangements, which may have pathogenic consequences. The current gold standard for quantifying Cas9 activity is short-range PCR amplification followed by next-generation sequencing (NGS). This method relies on amplifying short sequences around the cut site, thus significantly limits the accuracy in quantifying alleles with large INDELs or chromosomal rearrangement or loss. We previously developed the LongAmp-Seq (Long-range PCR Amplification based Sequencing) assay, a combination of long-range PCR and NGS. LongAmp-seq utilizes the widespread availability of Illumina NGS platform and provides high throughput discovery of large deletions as well as small INDELs at the Cas9 cut-site. However, we found that small amplicons carrying large deletion events were falsely overrepresented during long-range PCR, making accurately quantifying large deletions difficult. Here, we used *S. pyogenes* (Spy) CRISPR gRNA/Cas9 (RNP) to target beta-globin gene (*HBB*) in hematopoietic stem and progenitor cells (HSPCs) from patients with sickle cell disease, adapted the dual-unique molecular identifiers (UMI) tagging for long-read amplicon sequencing, and analyzed the induced mutagenesis. The 5.5 kb *HBB* region around the Cas9 cut-site was dual-UMI tagged using two PCR cycles, followed by a second and third PCR to enrich the dual UMI-tagged template molecules. The SMRT-bell library composed of RNP-treated and untreated samples was sequenced on a PacBio Sequel II 8M flowcell in circular consensus sequencing (CCS) mode. The PacBio subreads were converted to HiFi reads and subjected to UMI-based de-duplication and variant calling. The raw PacBio CCS reads without UMI clustering generated 300k reads with full-length coverage and dual UMIs of the correct length and pattern. Reads were binned based on the concatenated UMI pair. In RNP treated sample, we detected diverse large deletions of up to 4 kb and insertions up to 600 bp, and complex local rearrangement events. An overrepresentation of large deletion fragments was demonstrated by higher UMI coverage. After UMI-based de-duplication, the frequency of deletions larger than 100bp decreased from 40% to 31%, and the frequency of deletions larger than 1kb decreased from 15% to 11%. We quantified a diverse array of HBB gene editing outcomes including large deletions/insertions and complex rearrangements using the dual-UMI long-read amplicon sequencing. Our results showed that the current assessments of gene editing rates using short-range PCR based NGS could miss a substantial proportion of Cas9-induced mutations, leading to a large error in gene editing frequencies. With the rapid development of CRISPR-based therapeutic applications, there is an unmet need to develop a simple, accurate and reliable method to determine the complex gene editing outcomes and study the potentially detrimental consequences of unexpected mutations.

Novel AAV Biology and Platform Technologies

187. Effect of pH and Temperature on AAV2 Capsid Structure and Stability

Joshua Alexander Hull[1], Justin Kurian[1], Antonette Bennett[1], Balasubramanian Venkatakrishnan[1], Mario Mietzsch[1], Paul Chipman[1], Chen Xu[2], Duncan Sousa[3], Mandy Janssen[4], Timothy Baker[4], Robert McKenna[1], Mavis Agbandje-McKenna[1]

[1]Biochemistry and Molecular Biology, University of Florida, Gainesville, FL,[2]Medical School Cryo-EM Core Facility, University of Massachusetts, Worcester, MA,[3]Department of Biological Sciences, Florida State University, Tallahassee, FL,[4]Department of Chemistry and Biochemistry and Division of Biological Sciences, University of California San Diego, San Diego, CA

Adeno-associated viruses (AAV) and recombinant AAVs (rAAVs) must enter the host cell by endo-lysosomal trafficking, where the capsid is exposed to an increasingly acidic (pH 7.4-5.5) environment[1]. During this process, a viral phospholipase A_2 (PLA_2) enzyme, within the capsid viral protein 1 (VP1), functions to allow the capsid to escape the endo-lysosomal pathway to traffic to the nucleus for uncoating and genome replication[1,2]. For this function, the PLA_2 undergoes a switch from the capsid interior to exterior, necessitating proposed capsid rearrangements which are currently poorly understood[3,5,6]. This process can be induced with heat (~60 ^oC)[3,5,6] as a surrogate. Towards understanding the necessary capsid rearrangements associated with PLA_2 externalization, we present the structure of AAV2 virus-like particles determined under 10 different conditions to 2.7-4.0 Å resolution, including pre-incubation at varying temperatures (4, RT, 55, and 60 ^oC) and pH (7.4, 6.0, 5.5, and 4.0), by single particle reconstruction cryo-electron microscopy. At RT pre-vitrification, capsid rearrangements are observed at the 2-fold axes in previously described variable regions (VR) III and IX at pH 5.5 and 4.0. In addition, unmodeled density, previously interpreted as the unique region of VP1 (VP1u) containing the PLA_2[4,5], was observed at room temperature irrespective of pH. At neutral pH with 60 ^oC pre-vitrification, the rearrangement of the 2-fold VR IX can be partially reproduced. We demonstrate that low pH conditions, and heat as a surrogate, create capsid dynamics at the 2-fold axis suggesting this region as well as the 5-fold pore are essential for PLA_2 externalization

and function. [1]Florian Sonntag *et al.* 2006 [2]Anne Girod *et al.* 2002 [3]Svenja Bleker *et al.* 2005 [4]Stephanie Kronenberg *et al.* 2001 [5]Stephanie Kronenberg *et al.* 2005 [6]Joshua Grieger *et al.* 2007

188. Development of a Split Rep/Cap System to Improve AAV Capsid Production

Derek Carbaugh[1], Audry Fernandez[1], Lester Suarez[1], Richard J. Samulski[1,2]

[1]R&D, AskBio, Durham, NC,[2]Gene Therapy Center, University of North Carolina, Chapel Hill, NC

Adeno-associated virus (AAV)-based gene therapy is rapidly becoming established as a clinical therapy for genetic diseases. Despite their great promise, one of the largest limitations to their widespread use is the inability to manufacture enough high quality AAV vectors quickly and cost effectively. The wild-type AAV genome comprises inverted terminal repeats that flank two open reading frames (ORFs): *rep* and *cap*. The *rep* ORF includes four overlapping genes encoding the proteins required for AAV replication and packaging, whereas the *cap* ORF contains overlapping sequences encoding viral proteins 1, 2, and 3 (VP1-VP3), which interact to form a capsid. Recombinant AAV (rAAV) is constructed by replacing the *rep* and *cap* genes with a therapeutic gene; consequently, an adequate source of *rep* and *cap* proteins must be provided *in trans* for the rAAV lifecycle to proceed. Under normal conditions, large Rep78 and small Rep52 are derived from two distinct promoters, p5 and p19, respectively. The transcribed mRNAs then undergo splicing to form Rep68 and Rep40. WT AAV expresses VP1-3 from its p40 promoter, which overlaps with the *rep* ORF. These VPs, which only differ in their N-terminus, are produced by alternative splicing and leaky scanning from one reading frame to achieve a molar ratio of VP1:VP2:VP3 = 1:1:10. However, inefficient splicing may result in inefficient *cap* production and the best VP ratio for the most efficient assembly of correct viral capsids is still uncertain. To overcome the problem of inefficient splicing and attempt to optimize VP ratios we have split the *rep* and *cap* ORFs by cloning *cap* upstream of *rep*, driven by a pCMV promoter. Additionally, since Rep78 and Rep52 have been shown to be sufficient for replication, we modified *rep* to produce a codon optimized *rep* (coRep), ablating donor and acceptor sites for Rep68 and Rep40 splicing, as to only produce Rep78 and Rep52. Our split coRep/Cap system will allow us to optimize the expression level of both the *rep* and the *cap* proteins through codon optimization, modification of Kozak sequences, promoters, transcription start sites, post-translational modifications, etc, while avoiding altering protein splicing, to improve production of AAV capsids for use in gene therapy.

189. Intravenous Administration of a Barcoded and Pooled AAV Library for the Comprehensive Characterization and Comparison of Capsid Tropisms

April R. Giles[1], Samantha A. Yost[1], Elad Firnberg[1], Jenny M. Egley[1], Karolina J. Janczura[2], William M. Henry[2], Ye Liu[1], Subha Karumuthil-Melethil[2], Andrew C. Mercer[1]

[1]Gene Transfer Technologies, REGENXBIO, Rockville, MD,[2]Target Discovery, REGENXBIO, Rockville, MD

As the breadth and quantity of potential applications for adeno-associated viral (AAV) vector-mediated gene therapy expands, so does the demand for capsids with unique properties tailored to address each indication. REGENXBIO's NAV® Technology Platform is comprised of over 100 proprietary AAV vectors. We sought to thoroughly evaluate the potential of these AAVs to perform efficiently as gene therapy vectors. The productivity of each vector was evaluated by triple plasmid transfection into suspension HEK293 cells, screening primarily for supernatant yield and secondarily on cell pellet yield. Based on these criteria, 56 NAV® Platform vectors were selected for inclusion in the study; an additional 49 engineered AAV variants and 13 commonly used AAVs were also included. Each AAV was produced individually, packaging a CAG-GFP transgene cassette with a unique barcode, and pooled in approximately equal concentrations to generate a library of 118 barcoded AAVs. This library was administered intravenously to both mice (C57BL/6) and non-human primates (NHPs, cynomolgus macaques) at a dose of 1.77e13 GC/kg. Next-generation sequencing (NGS) was used to determine the relative abundance of barcoded genomes and transcripts present in a number of target tissues, allowing for comprehensive characterization of each capsid in the pool, direct comparison between capsids of interest, evaluation of clade-based transduction patterns, and translation of findings between species. Three, six, and twenty-four hours after dosing, we sampled NHP blood to determine the rate of vector clearance. AAV6 was the most rapidly cleared vector, with no genome copies detected at any time point post administration. Clade E vectors (e.g. AAV8, rh.10) trended towards quicker blood clearance compared to members of other clades, while AAV variants designed to de-target the liver, as well as AAV4, remained in circulation longer. In the livers of both species, vectors belonging to clade E were the most enriched relative to vectors from other clades. Clade C vectors were the least enriched in the livers of both species. Clade A members transduced mouse liver poorly but efficiently transduced NHP liver, largely driven by AAV6. We identified a previously uncharacterized clade E AAV that was as efficient as AAV8 in transducing both mouse and NHP liver but transduces the peripheral organs from both species 8-38-fold less efficiently than AAV8; this AAV was significantly less tropic for the central and peripheral nervous systems than AAV8 in both species. Heart transduction was dominated by clade F vectors (e.g. AAV9) in both species. Clade E vectors were enriched in mouse heart, while clade A, B, and C vectors transduced this tissue poorly, but these trends did not translate to NHP heart. Additionally, we identified a clade C AAV that transduced NHP heart with similar efficiency to AAV9 but, unlike AAV9, failed to efficiently transduce the liver (over 20-fold reduction) and other peripheral tissues; however, this vector did not efficiently transduce mouse heart. We also discovered that a single fringe AAV (not assigned to an existing clade) accounted for over 35% of all AAV genomes and transcripts in the mouse lung, while this property did not translate to the NHP lung. Taken together, these findings highlight the importance of direct, simultaneous comparison of the performance of these AAVs to several commonly used AAV vectors in multiple species and across tissues to fully characterize a given variant. From this study, we have identified capsids uniquely suited to address specific indications while also identifying trends to steer future capsid engineering efforts.

190. AAV Capsid Property Estimation Is Improved by Combining Single-Molecule ID Tags and Hierarchical Bayesian Modeling of Experimental Processes

Kathy Lin, Jakub Otwinowski, Brian Tobin, Sam Wolock, Jamie Kwasnieski, Cem Sengel, Roza Ogurlu, Eryney Marrogi, Megan Cramer, Sylvain Lapan, Anna Wec, Chris Reardon, Nishith Nagabhushana, Helene Kuchwara, Heikki Turunen, Shireen Abestesh, Ilke Akartuna, Alexander Brown, Farhan Damani, Jorma Gorns, Jeff Jones, Elina Locane, Stephen Malina, Hanna Mendes Levitin, Patrick McDonel, Stephen Northup, James Oswald, Amir Shanehsazzadeh, Sam Sinai, Michael Stiffler, Flaviu Vadan, Adrian Veres, Lauren Wheelock, Justin Yan, Eric Kelsic, Jeff Gerold

Dyno Therapeutics, Cambridge, MA

AAV capsid engineering has been accelerated by the application of high-throughput synthesis and sequencing technologies to measure the properties of many barcoded capsid variants in parallel. While these techniques significantly increase the number of capsid variants that can be screened, they also introduce errors that decouple barcodes from designed capsids and obscure the measurement signal. These errors include cross-packaging, template switching, and errors in DNA synthesis. To address these issues, we developed a paired library construction and Bayesian modeling strategy that identifies sets of sequences which are likely to contain errors. Incorporation of additional random DNA IDs subdivides the barcoded sequence populations. Each barcode-ID can then be tracked through the stages of plasmid production, capsid assembly, and transduction. We probabilistically model the measurement process and sources of decoupling between barcode-IDs and designed capsids and infer parameters describing the different production, transduction, and error rates, with Bayesian methods. In particular, we leverage probabilistic programming and stochastic variational inference to infer complex models of these mechanisms on large data. To combine information from multiple experiments in a principled manner, we model variation between samples and jointly model production and transduction rates. Notably, we infer for each barcode-ID whether it is correctly linked with its designed capsid sequence, which we verify experimentally with independent measurements. Thanks to this identification of errors, inferred production and transduction rates are significantly less noisy, as shown by the distribution of wild-type and stop codon controls. For an in vitro transduction assay, our analysis reduced the false positive transduction rate among VP1 stops and narrowed the set of high-performing variants without impacting wild-type controls. We verify the accuracy of our uncertainty estimates with replicate data and low-throughput validation data. ID tracking also enables estimation of new quantities of interest, such as the numbers of transfected and transduced cells per capsid variant. In addition, accurate uncertainty quantification is critical for identifying promising variants for further study and can be utilized for improving models that predict capsid properties from their sequence. We explore the utility of uncertainty estimates by training a simple sequence-function model jointly with our experimental process model. The resulting model learns the effect of mutations on capsid properties by integrating information from multiple variants, which may be necessary when measurements are noisy. Overall, our approach can be simply incorporated into many barcode-based assays of capsid function and significantly improves estimation of packaging and transduction in large, barcoded AAV libraries.

191. Hydroxylation of *N*-acetylneuraminic Acid Influences the *In Vivo* Tropism of *N*-linked Sialic Acid-Binding Adeno-Associated Viruses AAV1, AAV5 and AAV6

Estrella Lopez-Gordo, Alejandro Orlowski, Arthur Wang, Thomas Weber

Cardiovascular Research Center (CVRC), Icahn School of Medicine at Mount Sinai, New York, NY

Adeno-associated virus (AAV) vectors are promising candidates for gene therapy. However, a number of recent preclinical large animal studies failed to translate into the clinic. This illustrates the formidable challenge of choosing the animal models that promise the best chance of a successful translation into the clinic. Several of the most common AAV serotypes use sialic acid (SIA) as their primary receptor. However, in contrast to most mammals, humans lack the enzyme CMAH, which hydroxylates Neu5Ac into Neu5Gc. Here, we investigate the tropism of SIA-binding AAV1, 5, and 6 in wild-type (WT) and CMAH knock-out ($CMAH^{-/-}$) mice. All three serotypes showed significant differences in tropism in $CMAH^{-/-}$ when compared to WT mice. Transduction of heart was 5.8-fold, 3-fold and 5.4-fold lower in $CMAH^{-/-}$ mice for AAV1, 5 and 6, respectively. Importantly, the most dramatic difference in transduction was observed for AAV5 in the skeletal muscle, where transduction was 30-fold higher ($p<0.001$) in $CMAH^{-/-}$ mice. Expression in the brain, kidney and lung was overall much lower than that observed in heart, liver and skeletal muscle for all the AAV serotypes tested, and only AAV1 showed 1.4-fold lower transduction of $CMAH^{-/-}$ mice lung. The staining pattern and abundance of α2-3/6 *N*-linked SIA-containing glycoproteins that serve as AAV1, 5 and 6 receptors showed no dramatic differences in tissue between $CMAH^{-/-}$ and WT mice. Together, these data suggest that tropism of AAV1, 5 and 6 may not only depend on the type of linkage (e.g: α2-3 or α2-6) between the glycans that form *N*-linked SIA, as described in the literature, but it also may be influenced by particular modifications on these glycans such as the hydroxyl group that is present on Neu5Gc but missing on Neu5Ac. Thus, the relative abundance of Neu5Ac and Neu5Gc in tissue may play a role in AAV tropism. These findings are important for a deeper understanding of the mechanisms dictating AAV tropism and are fundamental for the identification of translationally relevant animal models for AAV research.

192. High-Throughput Screening of AAV Productivity to Enable Rapid Capsid Characterization

Jenny M. Egley, April R. Giles, Samantha A. Yost, Elad Firnberg, Chunping Qiao, Kirk Elliott, Randolph Qian, Devin S. McDougald, Ye Liu, Olivier Danos, Andrew Mercer

REGENXBIO Inc., Rockville, MD

Adeno-associated virus (AAV) mediated gene therapy is a rapidly growing field with immense potential to deliver curative treatments to patients with unmet needs. Hundreds of unique, naturally occurring AAV capsid sequences, as well as innumerable engineered capsids, have been identified; yet, outside of the commonly used capsid serotypes (AAV1-12), there is a general lack of characterization of these capsids for overall production, packaging efficiency, and other properties impacting their implementation. One challenge for screening large numbers of AAV capsids is the low throughput of triple plasmid transfection production systems. Here we describe a high-throughput, 96-well platform that allows us to screen hundreds of capsids simultaneously to define potential capsids of interest more rapidly. This method produces AAV vectors by triple plasmid transfection of a suspension HEK293-based system in 1 mL culture volume using 96-well deep well plates sealed with gas-permeable membranes. The plates are incubated in an INFORS Multitron shaking incubator modified to a 3 mm orbital throw at 1000 rpm to achieve the optimal oxygen and CO_2 levels. Initially, we screened approximately 150 naturally occurring capsids packaging CAG-firefly luciferase (total genome length of 4.0 kb) in duplicate and found minimal well-to-well or plate-to-plate variation, demonstrating this system's robustness. Post-production harvest and in-plate cell lysis allowed the evaluation of total and secreted AAV titer concurrently. The overall productivity in the supernatant and lysed cell pellet of these vectors was comparable to that of those produced in cultures of larger volume grown in a shaker flask under the same triple transfection conditions after determining viral genome titer by quantitative polymerase chain reaction (qPCR), further validating this method. Next, we screened the same set of capsids for their ability to package a larger transgene, with a total genome length of 4.7 kb, to determine the impact of genome length on vector yield. We found several highly homologous capsids within the same clade demonstrated varying levels of secretion and overall production, suggesting the size of the transgene cassette has a significant impact on the yield of vector in a capsid-specific fashion. This screening platform can be further employed to identify residues critical for AAV structure-function relationships, such as secretion, via residue swapping or alanine scanning experiments when such approaches would not be reasonably feasible in a lower throughput method. Under these described conditions, several hundred AAV vectors can be screened rapidly in parallel with reduced hands-on time and potential for automation, demonstrating that this system is a valuable tool for accelerating capsid engineering efforts to develop the next-generation AAV vectors for gene therapy.

193. AAV-GPseq Analysis of Vectors from HEK293 and BEV/Sf9 Production Platforms Reveals Differential Genome Heterogeneity and Enrichment of Potential Innate Immune DNA Epitopes in Empty Capsids

Ngoc Tam Tran[1,2], Kristina Weber[3], Suk Namkung[1,2], Eduard Ayuso[4], Oumeya Adjali[4], Cécile Robin[4], Eric Devine[4], Veronique Blouin[4], Magalie Penaud-Budloo*[4], Guangping Gao*[1,2], Phillip W. L. Tai*[1,2]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[3]Pacific Biosciences Inc., Menlo Park, CA,[4]INSERM UMR 1089, University of Nantes, Nantes, France

The use of adeno-associated virus (AAV) vectors in gene therapy has shown promise for treating a wide-range of diseases, especially for rare diseases. As a consequence, AAV vector production has been pushed to meet the high standards needed to deliver safe and effective doses to patients. In the past two decades, vector manufacturing has made remarkable advancements to meet large-scale production demands for clinical trials and approved treatments in today's markets. AAV vectors have been extensively studied to assess their safety and efficacy. The presence of empty AAV capsids and particles containing "inaccurate" vector genomes in preparations have been a subject of concern for nearly 20 years. Whether they are considered as contaminants or can act as immune decoys to improve therapy depends on the criteria for assessing vector potency and efficacy. Nonetheless, the current trend for AAV vector manufacturing is to obtain the highest purity of full particles as possible. Several methods are employed to separate empty capsids from full particles; but thus far, no single technique can guarantee empty or intermediate (genome truncation) capsid-free preparations. Therefore, these non-full particles remain critical concerns. Unfortunately, the exact vector genome compositions of full, intermediate, and empty capsids, remain largely unknown. In this work, we use AAV-Genome Population Sequencing (AAV-GPseq) to explore the compositions of DNase-resistant, encapsidated vector genomes produced by two common production pipelines: triple-transfection in human embryonic kidney cells (HEK293) and baculovirus expression vectors in *Spodoptera frugiperda* insect cells (BEV/Sf9). As we have found previously, "full particles" can contain truncated genomes irrespective of the production and purification methods that generate them; and as expected, intermediate capsids are predominately composed of inaccurate or chimeric genomes. We also demonstrate that empty particles purified by CsCl gradient ultracentrifugation are not truly empty, but are instead packaged with genomes comprised of a single truncated and/or unresolved inverted terminal repeat (ITR). Our findings suggest that the frequency of these "mutated" ITRs correlate with the abundance of inaccurate genomes, which may yield a higher degree of empty capsids. For example, trident-shaped mutant ITRs are carried by unresolved species and form self-complementary AAV genomes. Intriguingly, our results show that vectors manufactured by HEK293 production show a high degree of homogeneity, while the BEV/Sf9 system produces a high degree of truncated and unresolved species. Our work shows that fragments bearing a single ITRs found in the empty capsids may impact drug safety and efficacy. The mechanisms

underpinning how these species are formed is not fully understood, but our findings may lead to further study in vector design and capsid extraction methods to improve AAV vector production. *equal co-corresponding authors

Clinical Trials Spotlight Symposium

194. Systemic AAV Delivery Activates the Classical Complement Pathway Leading to Thrombotic Microangiopathy

Stephanie M. Salabarria[1], Samantha L. Norman[1], Julie A. Berthy[1], Melissa E. Elder[1], Kirsten E. Coleman[1], Carmen E. Leon-Astudillo[1], Lawrence R. Shoemaker[1], Precilla D'Souza[2], Cynthia J. Tifft[2], Manuela Corti[1], Barry J. Byrne[1]

[1]Pediatrics, University of Florida, Gainesville, FL,[2]Pediatric Undiagnosed Diseases Program, National Human Genome Research Institute, Bethesda, MD

Gene replacement therapy by *in vivo* delivery of adeno-associated virus (AAV) is attractive as a potential treatment for a variety of genetic disorders. A critical challenge for the success of AAV-mediated gene therapy is the host immune response, which poses ongoing concern about patient safety as well as efficacy of gene transfer and expression. AAV therapy induces humoral and cellular responses to both capsid and transgene. After antigen presentation, T cells stimulate reactive B cells, which transform into viral capsid antibody-producing plasma cells. The humoral response promotes classical-pathway activation of the complement system, triggered by capsid antigen-IgM complexes (Ag:Ab complex). Typically, the Ag:Ab complex leads to C3 convertase assembly, resulting in membrane attack complex (sC5b-9) formation. Leukocyte activation, from convertase-generated C3a fragments and from sC5b-9, propagates cellular injury by release of inflammatory mediators. Here we present data on 16 subjects who each received an AAV9 gene transfer product and underwent daily immunosurveillance following systemic administration of the AAV vector. These subjects are compared to six subjects who also received AAV9 in conjunction with a targeted immune modulation regimen with limited use of glucocorticoids (Abstract by D'Souza P). Our objective is to understand the mechanism of the innate and adaptive humoral immune responses following systemic AAV9 gene transfer. Subjects ranged in size 3-43.0 kg and were treated with an AAV9 vector by IV infusion. Two dosing cohorts were evaluated, with three subjects in the first cohort (5×10^{13} vg/kg) and thirteen in the high dose cohort (2×10^{14} vg/kg) across two indications. Patients tolerated the infusion very well for the initial 48 hours, however at 72 hours, nausea, vomiting and malaise developed, despite pre-medication with IV or PO glucocorticoids. Decline in platelet count began at 24 hours post-infusion, independent of other early markers of immune activation. On post-infusion day 4 - 5, the platelet count declined more rapidly, along with an increase of D-dimer and other indicators of endothelial activation. Coincident with the first appearance of IgM directed against the vector capsid, C3 and C4 levels both fell, consistent with classical complement activation. Ba, a marker of alternative complement activation, did not increase. By Day 7, both C3 and C4 levels were in many cases below lower limit of detection and sC5b-9 levels had peaked in all patients. Review of the peripheral blood smear demonstrated schistocytes and LDH was elevated in some cases, confirming hemolysis and microangiopathy. These findings of immune activation are in contrast to an independent cohort of six patients who received an AAV9 vector following pre-treatment with an immune modulation regimen that prevents humoral adaptive response. In this cohort, no compliment activation was observed in the absence of anti-capsid Ab. **Conclusion:** Therapeutic use of high-dose AAV is associated with activation of both adaptive and innate immune responses. These responses significantly impact safety. Importantly, current clinical findings were not anticipated from pre-clinical rodent or NHP studies which impacted the ability to respond to these findings in the clinical setting. Interventions to inhibit adverse effects of complement activation following AAV therapy have been utilized to ensure patient safety while optimizing efficiency of AAV gene transfer and expression.

195. Activity of Banked (Off-the-Shelf) CD30.CAR-Modified Epstein-Barr Virus-Specific T Cells in Patients with CD30-Positive Lymphoma

David H. Quach, Carlos A. Ramos, Premal D. Lulla, Sandhya Sharma, Haran R. Ganesh, Yezan F. Hadidi, Sachin Thakkar, Luis Becerra-Dominguez, Birju Mehta, Silvana Perconti, Eric Anderson, Emily M. Hsieh, Olga Dakhova, Huimin Zhang, Bambi J. Grilley, Malcolm K. Brenner, Helen E. Heslop, Rayne H. Rouce, Natalia Lapteva, Cliona M. Rooney

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

The manufacturing of individual patient-derived CAR T-cells is frequently unsuccessful, can be too time consuming to benefit acutely ill patients, and is difficult to economically scale up to treat large numbers of patients. "Off-the-shelf" T cell products that are banked from healthy donors would improve accessibility, allow more rapid treatment of patients, and reduce costs. Major obstacles to successful treatment with allogeneic T cells include graft-versus-host disease (GVHD) and graft rejection, mediated by host and recipient alloreactive T cells, respectively. To address GVHD, we are using banked allogeneic Epstein-Barr Virus-specific T cells (EBVSTs) that have been safely used without producing GVHD in more than 300 recipients. To prevent graft rejection we have expressed a chimeric antigen receptor for CD30 (CD30.CAR) in EBVSTs. CD30 is upregulated by alloactivated T cells, which then become targets for CD30.CAR-EBVSTs. The CD30.CAR also targets CD30-positive lymphoma and has proved safe and effective in prior clinical trials (NCT02917083) using autologous CAR-T cells, while EBVSTs can target CD30-negative, EBV-positive malignancies via their native TCRs. Hence, off-the-shelf CD30.CAR EBVSTs should eliminate the alloreactive T cells they elicit in allogeneic hosts, and therefore persist for sufficient time to eliminate CD30 or EBV-positive lymphoma without causing GVHD. We have initiated a Phase I clinical trial using banked off-the-shelf CD30.CAR EBVSTs to treat patients with relapsed or refractory CD30-positive lymphomas. The trial evaluates three fixed doses, escalating from 4×10^7 to $1 \times$

10^8 to 4 x 10^8 of CD30.CAR EBVSTs infused after lymphodepleting chemotherapy. Although CD30.CAR killing is not HLA restricted, selection of the CD30.CAR EBVST product for each recipient is based on the best HLA class I and class II match. This allows endogenous EBV to boost the *in vivo* activity of CD30.CAR EBVSTs via their native TCRs, and will provide additional reactivity for patients with EBV-positive tumors. We have currently treated three patients on the first dose level and no dose-limiting toxicities have been observed; in particular no cytokine release syndrome (CRS) or GVHD of any grade. The first two patients have received PET/CT scans 6 weeks after infusion. The first patient showed complete resolution in all but one site, which had low level persistent activity (Graded PR). The second patient had marked reduction of several areas of disease although he also had enlargement of cutaneous lesions so the overall assessment was a mixed response (MR). Despite these objective clinical responses, we detected no expansion or persistence of CD30.CAR EBVSTs in peripheral blood. Although CD30.CAR EBVSTs were detected in blood 3-4 hours after infusion by qPCR, their numbers had declined by the week 1 time point. We will continue to assess the safety and efficacy of CD30.CAR EBVSTs at higher doses. In the future, we will evaluate whether CD30.CAR EBVSTs can serve as a platform for other CAR/TCRs to target other malignancies. In conclusion, we have shown that banked CD30.CAR EBVSTs are an immediately available off-the-shelf cell therapy that even at the lowest dose level have activity against CD30+ lymphoma without evident toxicity.

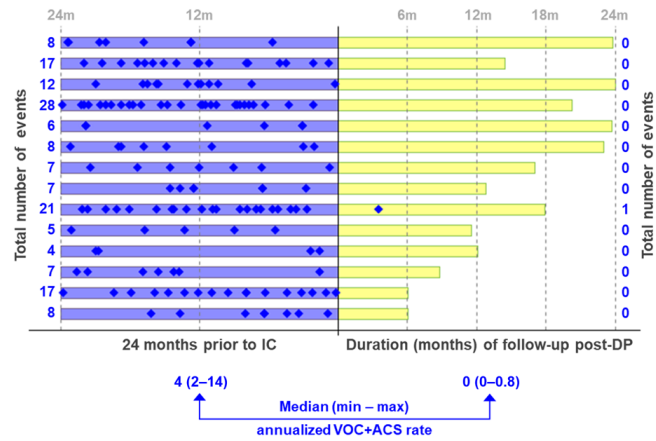
196. Updated Results from HGB-206 LentiGlobin for Sickle Cell Disease Gene Therapy Study: Group C Data and Group A AML Case Investigation

John F. Tisdale[1], Alexis A. Thompson[2,3], Janet L. Kwiatkowski[4,5], Markus Y. Mapara[6], Lakshmanan Krishnamurti[7], Banu Aygun[8], Kimberly A. Kasow[9], Stacey Rifkin-Zenenberg[10], Manfred Schmidt[11], Mauris Nnamani[12], Alex Miller[12], Ren Chen[12], Dennis Kim[12], Sunita Goyal[12], Jennifer Jaroscak[15], Julie Kanter[13], Mark C. Walters[14]

[1]Cellular and Molecular Therapeutics Branch NHLBI, National Institutes of Health, Bethesda, MD,[2]Northwestern University Feinberg School of Medicine, Chicago, IL,[3]Ann & Robert H. Lurie Children's Hospital of Chicago, Chicago, IL,[4]Children's Hospital of Philadelphia, Philadelphia, PA,[5]U Penn Perelman School of Medicine, Philadelphia, PA,[6]Columbia University Medical Center, New York, NY,[7]Children's Healthcare of Atlanta, Emory University, Atlanta, GA,[8]Cohen Children's Medical Center, Queens, NY,[9]UNC, Chapel Hill, NC,[10]Hackensack University Medical Center, Hackensack, NJ,[11]GeneWerk GmbH, Heidelberg, Germany,[12]bluebird bio, Inc., Cambridge, MA,[13]University of Alabama Birmingham, Birmingham, AL,[14]UCSF Benioff Children's Hospital, Oakland, CA, [15]Medical University of South Carolina, Charleston, SC

The phase 1/2 HGB-206 study (NCT02140554) evaluates efficacy and safety of LentiGlobin for SCD (bb1111) gene therapy (GT) using a modified human β-globin gene that produces GT-derived anti-sickling hemoglobin HbA^{T87Q}. As of February 2021, following the report of two Suspected Unexpected Serious Adverse Reactions (SUSARs), HGB-206 is on clinical hold. Patients (pts; ≥12–≤50 yrs) with SCD and recurrent severe vaso-occlusive events, including acute episodes of pain and acute chest syndrome (ACS), were enrolled and infused with LentiGlobin, CD34+ cells collected by plerixafor mobilization/apheresis and transduced with BB305 LVV, following myeloablative busulfan conditioning. Laboratory evaluations, SCD-related outcomes, and AEs were monitored. Data are median [min-max] unless otherwise stated. As of 3 March 2020, 40 Group C pts (23.5 [12–38] yrs) began cell collection; 25/40 were treated with LentiGlobin and followed for 12.1 (2.8–24.8) months (mos). All pts stopped RBC transfusions by 90 days post-treatment (tx). In 16 evaluable pts with ≥6 mos follow-up, median total Hb at last visit was 11.5 (9.6–16.2) g/dL, with HbA^{T87Q} contribution of 5.2 (2.7–9.4) g/dL, HbS of 6.1 (4.9–7.8) g/dL, and median HbS ≤60% of total Hb. In 14 pts with ≥6 mos of follow-up and a history of vaso-occlusive crisis (VOC) or ACS, the annualized VOC+ACS rate was 4.0 (2.0–14.0) in the 2 yrs before tx. No ACS/serious VOCs were observed post-tx. One nonserious Grade 2 VOC occurred ~3.5 mos post-tx, resulting in a 99.5% (95% confidence interval, 92.4%–100%) mean reduction in the annualized VOC+ACS rate post-tx (**Fig 1**). The most common nonhematological Grade ≥3 AEs post-tx were stomatitis (n=15) and febrile neutropenia (n=11). SAEs reported in ≥2 pts post-tx were nausea, opioid withdrawal syndrome, and vomiting (all n=2). Three pts had LentiGlobin-related nonserious Grade ≤2 AEs. One death, unlikely related to LentiGlobin, occurred >18 mos post-tx in a pt with significant baseline SCD burden. Beyond the reported data-cut, two SUSARs were reported. One Group C pt had persistent anemia 6 mos after transplant that is being investigated. In Group A (overall data not reported here), one pt treated >5 yrs ago had acute myeloid leukemia; data from recent investigations on this case will be presented. LentiGlobin for SCD GT results in near pancellular $β^{A-T87Q}$ expression, reduced HbS, and increased total Hb. Complete resolution of VOC/ACS was observed in nearly all pts. The safety profile post-LentiGlobin remains generally consistent with risk of autologous stem cell transplant, myeloablative conditioning, and underlying SCD.

Figure 1. VOC+ACS Post-LentiGlobin Treatment



Mean reduction of annualized VOC + ACS rate post-LentiGlobin treatment is 99.5% (95% CI, 92.4%-100%)

Investigator-reported AEs of VOC or ACS are shown; Patients with ≥ 4 VOC/ACS at baseline before IC and with ≥ 6 months of follow-up post-DP infusion are included.
ACS, acute chest syndrome; AE, adverse event; CI, confidence interval; DP, drug product; IC, informed consent; VOC, vaso-occlusive crisis.

197. ADVM-022 Intravitreal Gene Therapy for Neovascular AMD - Results from the Phase 1 OPTIC Study

Szilárd Kiss[1], Carol Hoang Roller[2], Adam Turpcu[2], Carol Chung[2], Aaron Osborne[2]

[1]Weill Cornell Medicine, New York, NY,[2]Adverum Biotechnologies, Redwood City, CA

Introduction: Intravitreal gene therapy has the potential to significantly reduce treatment burden and improve vision outcomes in patients with neovascular AMD (nAMD). OPTIC (NCT03748784) is a phase 1 study designed to assess the safety, tolerability and efficacy of a single intravitreal injection of ADVM-022 (AAV.7m8-aflibercept gene therapy) in patients with nAMD. The results presented here are from Cohort 1-4. **Methods:** Patients were administered a single intravitreal injection of ADVM-022 at 6x10^11 vg/eye (Cohort 1: n=6, Cohort 4: n=9) or at 2x10^11 vg/eye (Cohort 2: n=6, Cohort 3: n=9). Incidence and severity of adverse events, change in visual acuity (BCVA), change in central retinal thickness (CST), and the need for and number of supplemental aflibercept injections were evaluated. **Results:** As of October 15, 2020, median follow-up was 86 weeks (Cohort 1), 64 weeks (Cohort 2), 48 weeks (Cohort 3) and 16 weeks (Cohort 4). Patients in all 4 cohorts previously received frequent anti-VEGF injections (mean 7.1-9.2 injections in the prior 12 months) to maintain relatively good baseline BCVA (mean 65.0-65.9 ETDRS letters) prior to receiving ADVM-022. ADVM-022 continues to be well tolerated with a favorable safety profile. All ADVM-022-related ocular adverse events were mild (78%) to moderate (22%). Ocular inflammation predominantly affecting the anterior segment, when observed, has been responsive to steroid eye drops. No cases of retinal involvement or vasculitis were reported. A significant reduction in anti-VEGF injection burden to patients was observed with both doses; 14/15 (high dose) and 10/15 (low dose) patients remained supplemental anti-VEGF injection free. Mean annualized anti-VEGF injection frequency was reduced by 99% (high dose) and 85% (low dose) after ADVM-022. For Cohorts 1-3, data pending for cohort 4, BCVA was maintained with a mean change of -2.5 to +0.2 ETDRS letters, and CST improved with a mean change of -19.7 to -132.7 µm. **Conclusions:** ADVM-022 is designed to provide continuous expression of aflibercept following a single intravitreal injection. Over 80% of patients with nAMD in OPTIC have not needed any supplemental anti-VEGF injections up to 92 weeks follow-up, and ADVM-022 has been well tolerated. Both doses of ADVM-022 showed potential to reduce treatment burden and improve patient vision outcomes.

Figure 1. Substantial reduction in anti-VEGF treatments following a single IVT injection of ADVM-022 in patients that require frequent treatments to maintain vision.

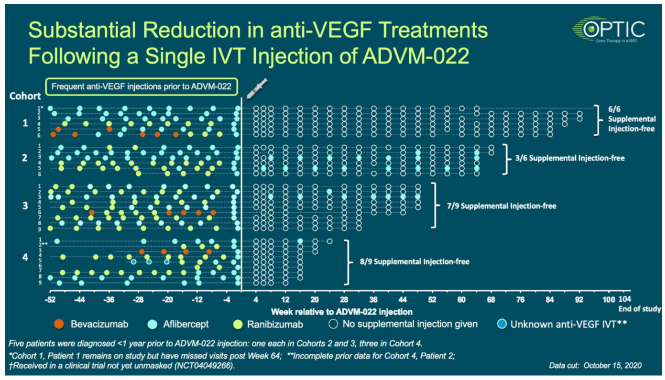
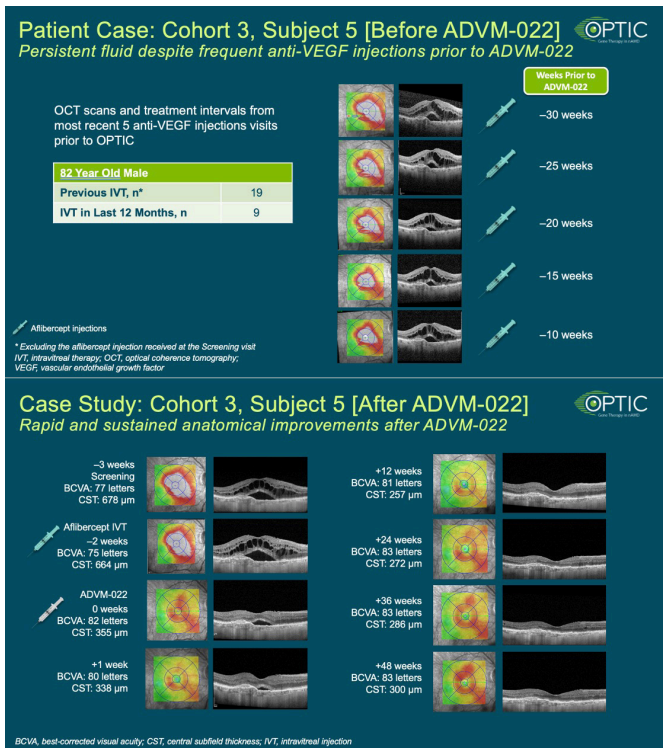


Figure 2. Patient case of before and after ADVM-022 treatment show rapid and sustained anatomical improvements.



198. Natural Killer T Cells Expressing a GD2-CAR and IL-15 Are Safe and Can Induce Complete Remission in Children with Relapsed Neuroblastoma - A First-in-Human, Phase 1 Trial

Andras Heczey[1], Amy Courtney[1], Ka Liu[1], Migmei Li[1], Nisha Ghatwai[1], Olga Dakhova[1], Xin Xu[1], Ho Ngai[1], Erica Di Piero[1], Andrew Sher[1], Barbara Savoldo[2], Gianpietro Dotti[2], Leonid Metelitsa[1]

[1]Baylor College of Medicine, Houston, TX,[2]University of North Carolina, Chapel Hill, NC

Background: Despite remarkable progress in B cell malignancies, T cells expressing chimeric antigen receptors (CARs) remain largely ineffective in solid tumors in part because T cells do not traffic

effectively to or survive in tumor tissues. Due to their natural anti-tumor properties and ability to localize to the neuroblastoma (NB) tumor site, Vα24-invariant natural killer T cells (iNKTs) are promising immune effectors for CAR-based immunotherapies targeting NB and other solid tumors. In pre-clinical studies, iNKTs expressing an optimized GD2-CAR with a CD28 costimulatory endodomain and IL-15 demonstrated superior anti-tumor activity, providing rationale to evaluate CAR-iNKTs in NB patients. **Methods**: Following validation of current good manufacturing practice (cGMP) protocols for generation and clinical-scale expansion of CAR-iNKTs, we initiated a phase 1 clinical trial to assess the safety, persistence, and efficacy of autologous CAR-iNKTs in patients with relapsed/refractory NB (NCT03294954) using a 3+3 dose-escalation design. Eleven patients have enrolled to date and have received infusions on four dose levels (DL; CAR+ iNKTs/m^2): DL1 (3x10^6), DL2 (1x10^7), DL3 (3x10^7), and DL4 (1x10^8) following lymphodepletion. **Results**: Median age of patients was 7 years (range 2-12 years), all had stage 4 relapsed or refractory high-risk NB. Patient-derived iNKTs were successfully expanded to produce ≥10^9 CAR-iNKTs, resulting in 93.5% median iNKT purity (range: 74.1-97.2%) and 54.4% median CAR expression (range: 20.2-87.7%). Lymphodepletion-induced toxicities were detected as expected, and patients did not experience grade 2 or higher toxicities related to CAR-iNKTs. All 11 treated patients showed evidence of CAR-iNKT expansion peaking at two-to-four weeks post-infusion, with a median of 1,144 NKTs (range: 46-4,903) and 40 CAR+ iNKTs (range: 4-697) detected per ml of peripheral blood. Analysis of tumor biopsies showed trafficking of CAR-iNKTs to metastatic lesions on all DL. Antitumor activity was quantified using Curie scores and the International Neuroblastoma Response Criteria. Five patients progressed, four had stable disease (including one with 30% reduction in Curie score and another with clearance of bone marrow metastases), one had a partial response, and one achieved complete remission (**Fig 1**). The frequency of CD62L+ iNKTs in the infused products correlated with the total number of CAR-iNKTs detected in peripheral blood for four weeks post-infusion (AUC, r=0.65, p=0.029). Moreover, CAR-iNKT AUC normalized to disease burden (AUC/Curie score) was associated with response to therapy (**Fig 2**). **Conclusions**: The results indicate that adoptively transferred autologous CAR iNKTs are safe, expand *in vivo*, localize to the tumor site, and can induce complete regression of metastatic lesions in NB patients.

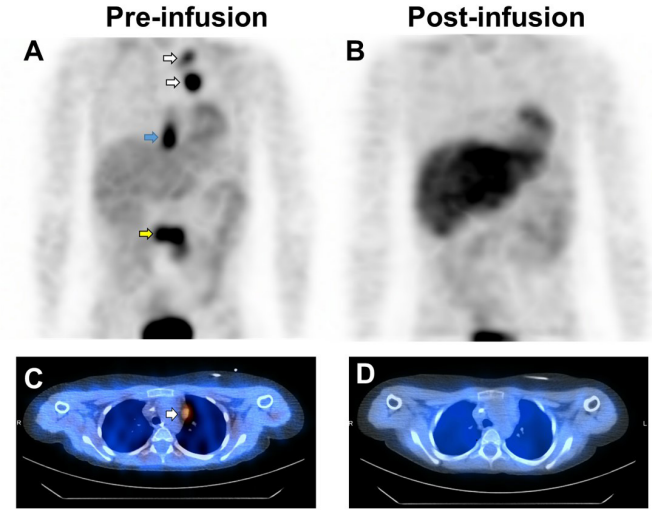


Fig 1. CAR-iNKTs induce complete remission in a child with relapsed / refractory high-risk neuroblastoma: Pre- and post-infusion MIBG scans are shown. Panels A-B: Maximum intensity projection SPECT images of the chest, abdomen and pelvis; panels C-D: Axial SPECT image through the chest at the level of the great vessels. Arrows demonstrate MIBG avid paratracheal and prevascular (white), mediastinal (blue), and retroperitoneal lymph nodes (yellow) pre-infusion with complete resolution of uptake following treatment. Note that the heart muscle, liver and bladder show physiologic high MIBG uptake, which is not related to neuroblastoma.

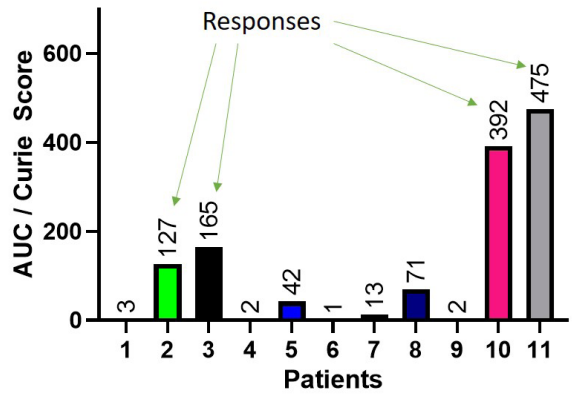


Fig 2. CAR-iNKT expansion and persistence is associated with antitumor activity: The concentration of CAR-iNKTs was measured pre-infusion and weekly post-infusion to calculate area under the curve (AUC). Curie score was measured by standard MIBG scans pre- and post-infusion. AUC / Curie score ratio is shown for each infused patient to demonstrate antitumor activity in relationship to total in vivo CAR-iNKT dose and tumor burden.

199. Long Term Follow Up for the Development of Subsequent Malignancies in Patients Treated with Genetically Modified Immune Effectors

David Steffin[1], Ibrahim Muhsen[2], Nabil Ahmed[1], Meenakshi Hegde[1], Olga Dakhova[1], Tao Wang[3], Mengfen Jessie Wu[3], Stephen Gottschalk[4], Sarah Whittle[1], Premal Lulla[5], Max Mamonkin[5], Bilal Omer[1], Rayne Rouce[1], Andras Heczey[1], Leonid Metelitsa[1], Laquisa Hill[5], Carlos Ramos[5], Cliona Rooney[5], Malcolm Brenner[5], Helen Heslop[1]

[1]Pediatric Hematology/Oncology, Baylor College of Medicine, Houston, TX,[2]Hematology/Oncology, Houston Methodist Hospital, Houston, TX,[3]Dan L. Duncan Cancer Center, Baylor College of Medicine, Houston, TX,[4]Bone Marrow Transplantation and Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN,[5]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Background: Subsequent malignancies (SMs) are a well-documented complication in patients receiving chemotherapy, with a cumulative incidence of 3-4% over 15-20 years. Treatment with genetically modified immune effectors (IEs) is an effective therapy for patients with many hematological malignancies and is being evaluated in solid tumors; however, little is known about their long-term effects. Insertional mutagenesis after retroviral genetic modification remains a concern, given reports of patients developing SMs after gene therapy using retrovirus-transduced hematopoietic stem cells for inherited diseases. To date, there have been no reported SMs related to IEs, though minimal literature exists. **Methods:** We reviewed data from 349 patients treated across 26 pediatric and adult investigator-initiated cell therapy trials at the Center for Cell and Gene Therapy at Texas Children's Hospital and Houston Methodist Hospital. All patients received genetically modified IEs using a retroviral vector to treat relapsed and/or refractory hematologic malignancies and solid tumors. All studies included, we collected over 24 years of data. **Results:** Of 349 patients reviewed, 13 (3.7%) developed SMs with four patients having two malignancies each for a total of 16 SMs. There was no correlation between the type of SM and IE infused. Five patients developed hematologic malignancies (three with myelodysplastic syndrome; one with acute myeloid leukemia, and one with T-cell lymphoblastic lymphoma [T-LBL]). 11 patients developed solid tumors (neural sheath tumor, thyroid adenoma, basal cell carcinoma, renal cell carcinoma, meningioma, squamous cell carcinoma of the penis, urothelial carcinoma, basal cell carcinoma, squamous cell carcinoma, ductal cancer, spindle cell carcinoma). PCR analysis was used to detect the cell therapy transgene in 10/13 tumor biopsies; no copies were detected. Replication complement retroviral (RCR) testing was performed on peripheral blood in 13 patients with all results negative. The patient that developed T-LBL also had a medical history significant for Constitutional Mismatch Repair Deficiency Syndrome (CMMR-D), which alone greatly increases cancer risk. PCR testing for IE transgene on the lymphoma biopsy was negative;since lymphoma is one of the most common malignancies associated with CMMR-D, his malignancy was thought to be unrelated to IE therapy. As an additional safety parameter, we evaluated the vector copy number of IEs prior to cell therapy infusion. We assessed 93 patients to date, with an average copy number per transduced cell of 4.48 (range 0.42-9.40). None of these patients have developed SMs to date although the follow up duration is shorter. **Conclusions:** To our knowledge, this is the largest follow up of SMs in patients treated with IEs. Rates of SM were very low and consistent with patients receiving standard chemotherapy. In the SMs detected, we did not see evidence of insertional mutagenesis, nor was RCR testing positive in any patients. These preliminary results suggest that IEs do not appear to increase the risk for SMs. Ongoing work by our group seeks to compare these data to a disease-matched control group who did not receive genetically modified cellular therapy.

200. Updated Results of Transpher A, a Multicenter, Single-Dose, Phase 1/2 Clinical Trial of ABO-102 Gene Therapy for Sanfilippo Syndrome Type A (Mucopolysaccharidosis IIIA)

Kevin M. Flanigan[1], Nicholas JC Smith[2], Maria L. Couce[3], Maria Escolar[4], Kristen V. Truxal[1], Kim L. McBride[1], Maria J. de Castro[3], Maria Fuller[5], Astrid Pañeda[6], Juan Ruiz[6]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Paediatric and Adult Neurometabolic Diseases, Women's and Children's Hospital, Adelaide, Australia,[3]Metabolic Unit, Department of Pediatrics, Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain,[4]Department of Pediatrics, University of Pittsburgh Medical Center, Pittsburgh, PA,[5]SA Pathology, Women's and Children's Hospital, Adelaide, Australia,[6]Abeona Therapeutics Inc, Madrid, Spain

Transpher A is a Phase 1/2 clinical trial assessing the safety and efficacy of a single intravenous administration of ABO-102, a self-complementary AAV9-based vector encoding human *SGSH*, for treating mucopolysaccharidosis IIIA (MPS-IIIA). MPS IIIA is a neurodegenerative lysosomal storage disorder manifesting early in childhood for which there is no clinically approved treatment. Eighteen patients have been enrolled across three dose cohorts: Cohort 1, $5x10^{12}$ vg/kg, n=3; Cohort 2, $1x10^{13}$ vg/kg, n=3; Cohort 3, $3x10^{13}$ vg/kg, n=12. Cohorts 1 and 2, and 8 of 12 patients in Cohort 3, have completed 24-month follow-up. After 24 months, patients are transferred to a Long-Term Follow-Up study where they are monitored for safety and efficacy for 3 subsequent years. Primary endpoints for Transpher A are safety and neurocognitive development (compared to natural history studies), and secondary endpoints include biomarker determination or brain and liver volume, among others. Intravenous administration of ABO-102 was well tolerated, with no serious drug-related adverse events (Follow-up Cohort 1: 51.8-55 months; Cohort 2: 43.8-46.4 months; Cohort 3: 3-40.3 months). Cohort 3 (the highest dose) was associated with rapid, dose-dependent, and statistically significant reductions of CSF heparan sulfate (HS) at all time points, including at 24-months. Statistically significant reductions for systemic biomarkers (plasma and urine HS and total urine glycosaminoglycans) and liver volumes were observed in Cohort 3 for the duration of follow-up. Neurocognitive evaluation showed continuous gain of cognitive skills in younger patients (≤30 months, DQ>60) in Cohort 3, with a follow-up of 18-36 months, well beyond the point where natural history reports relentless neurodegeneration. Overall, intravenous administration of ABO-102 in MPS-IIIA patients showed a favorable long-term safety profile and led to statistically significant reductions

in CNS and systemic biomarkers, with clear indications of meaningful neurocognitive benefit in the youngest patients treated with $3x10^{13}$ vg/kg, before advanced neurodegeneration.

Base Editing and Gene Editing Approaches

201. Intracellular RNASE Activity Limits the Efficiency of mRNA-Based Gene Editing in Nonhuman Primate Hematopoietic Stem and Progenitor Cells

Christopher W. Peterson[1,2], Rasika Venkataraman[1], Sowmya S. Reddy[1], Teresa Einhaus[1], Stefan Radtke[1], Olivier Humbert[1], Hans-Peter Kiem[1,2]

[1]Fred Hutch, Seattle, WA,[2]University of Washington, Seattle, WA

Introduction

Gene editing of hematopoietic stem and progenitor cells (HSPCs) is a promising approach for numerous human pathologies, including cancer, hemoglobinopathies and HIV-1. In the nonhuman primate (NHP) model, we previously showed that CRISPR-Cas9 ribonucleoprotein complexes (RNPs) led to more efficient editing than mRNA-encoded ZFNs, although notably, these studies targeted separate loci involved in hemoglobinopathies (HBG), and HIV-1 (CCR5). Here, we compared CCR5 CRISPR-Cas9 RNPs and CCR5 ZFN mRNA head-to-head in NHP HSPC and identified mechanisms that underlay the differential editing efficiency and engraftment in our previous NHP studies. We hypothesized that HSPC culture conditions and intracellular RNases were the primary factors that hampered ZFNs. To address whether CCR5 inactivation itself negatively impacted the engraftment of autologous HSPCs, we applied an optimized editing protocol that overcame these barriers, and compared the engraftment of CCR5- and HBG-edited cells in the same autologous NHP hosts.

Methods

$CD34^+$ HSPCs were isolated from mobilized NHP and edited with CRISPR-Cas9 RNPs or mRNA-encoded ZFNs targeting the 5' coding region of NHP CCR5. ZFN mRNA experiments compared HSPC culture conditions with or without fetal bovine serum (FBS) and co-delivered RNase inhibitors. In order to rapidly assess the engraftment potential of each CCR5-edited NHP HSPC product, a novel NHP-immunodeficient mouse model was constructed and applied. To address whether locus-specific differences impacted engraftment of NHP HSPCs, cells were edited separately with CCR5 or HBG CRISPR-Cas9 RNPs, infused into a cohort of 3 autologous hosts, and tracked for up to 18 months *in vivo*.

Results

We found that FBS-free media nearly doubled CCR5 ZFN editing efficiency in true hematopoietic stem cells, though editing in this subset remained lower than in early and committed progenitor populations. Consistent with our previous NHP studies, CCR5 ZFN-edited HSPCs in FBS-containing media demonstrated <5% engraftment in immunodeficient mice, whereas cells cultured in FBS-free media displayed up to 66.45% engraftment. Co-delivery of RNase inhibitors led to a >6-fold mean increase in ZFN editing efficiency. CCR5 CRISPR Cas9 RNPs consistently outperformed CCR5 ZFN mRNA with or without RNase inhibitors, displaying editing efficiencies of up to 91.46%. Direct comparison of CCR5 and HBG-edited HSPC in the same NHP hosts revealed no impairment in engraftment of CCR5-edited HSPC, relative to HBG-edited HSPC.

Conclusions

Intracellular RNases are a likely mechanism underlying differential efficiency of ZFN mRNA- vs. CRISPR-Cas9 RNP-based gene editing. FBS-containing media negatively impacted the engraftment of ZFN-edited HSPC, reinforcing the use of serum-free media formulations in pre-clinical and clinical studies. Importantly, our new NHP data clearly show that decreased engraftment of CCR5 ZFN-edited NHP in historical studies is more likely due to one or both of these factors, rather than CCR5 inactivation *per se*. Our optimized HSPC gene editing platform and powerful large animal model position us to address critical questions in the field, including approaches to enhance the engraftment of homology directed repair (HDR)-edited HSPCs, and the therapeutic impact of HDR-edited HSPCs in numerous disease settings.

202. Enhancing the RNA Base-Editing Activity, Functionality and Specificity of the ADAR2 Deaminase Domain

Dhruva Katrekar[1], Nathan Palmer[1], Yichen Xiang[1], Anushka Saha[1], Dario Meluzzi[2], Prashant Mali[1]

[1]Bioengineering, University of California, San Diego, La Jolla, CA,[2]Department of Medicine, University of California, San Diego, La Jolla, CA

Adenosine deaminases acting on RNA (ADARs) can be repurposed to enable programmable RNA editing, however their exogenous delivery leads to transcriptome-wide off-targeting, and additionally, enzymatic activity on certain RNA motifs, especially those flanked by a 5' guanosine is very low thus limiting their utility as a transcriptome engineering toolset. To address these issues, we explored comprehensive ADAR2 protein engineering via three approaches: First, we performed a novel deep mutational scan of the deaminase domain that enabled direct coupling of variants to corresponding RNA editing activity. Experimentally measuring the impact of every amino acid substitution across 261 residues, i.e. ~5000 variants, on RNA editing, revealed intrinsic domain properties, and also several mutations that greatly enhanced RNA editing. Second, we performed a domain-wide mutagenesis screen to identify variants that increased activity at 5'-GA-3' motifs, and discovered novel mutants that enabled robust RNA editing. Third, we engineered the domain at the fragment level to create split deaminases. Notably, compared to full-length deaminase overexpression, split-deaminases resulted in >1000 fold more specific RNA editing. Taken together, we anticipate this comprehensive deaminase engineering will enable broader utility of the ADAR toolset for RNA biotechnology and therapeutic applications.

203. Novel CRISPR-Associated Transposase Systems for Targeted DNA Integration

Daniela S. A. Goltsman, Jason Liu, Audra E. Devoto, Cristina N. Butterfield, Brian C. Thomas, Christopher T. Brown

Metagenomi, Emeryville, CA

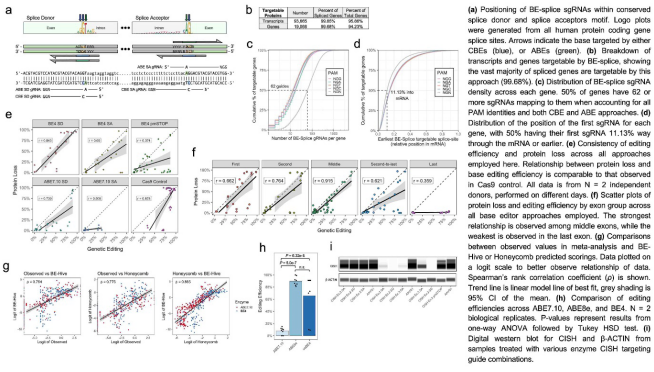
CRISPR nucleases are promising systems for genome editing due to their programmable nature. These nucleases can readily be applied for gene disruption, wherein genome repair pathways produce small insertions and deletions at a target site. However, targeted DNA integration remains a challenge due to the dependence on, and low efficiency of the homology directed repair machinery of the cell. Transposases are found in all domains of life, and are known for their efficient integration of large DNA cargo. Although some transposases are targeted (i.e they recognize specific DNA motifs), the fact that most enzymes cannot easily be re-programmed makes them challenging systems for genome engineering and therapeutic applications. CRISPR associated transposases (CAST) are rare, newly discovered mobile elements that can be reprogrammed to integrate at novel genomic sites using guide RNAs. Here, we mined extensive metagenomic datasets to identify novel CAST systems and tested their programmable targeted integration capabilities. From analysis of an extensive metagenomic database spanning the equivalence of hundreds of thousands of microbial genomes, we identified a novel CAST encoding a Type V-K nuclease-defective effector within a Tn7-like transposon. Targeted integration was tested in vitro using a PAM plasmid library as a target, and confirmed via PCR amplification and sequencing of the integration products. The native CAST uses a dual guide RNA. However, we engineered a reduced single guide RNA and show that the system preferentially directs DNA integration in one orientation, along with producing a 3-5 bp target site duplication. Extensive engineering of the guide RNA, transposon terminal inverted repeats, and protein fusions minimized the size of the system while maintaining targeted integration. Unlike systems that require endogenous repair pathways for integration, we do not expect that CAST will have significant limitations on the size of the DNA template that can be integrated. The broad applicability of programmable, predictable DNA integrases make novel CAST systems a highly desirable addition to the genome editing toolbox.

204. CRISPR-Cas9 Cytidine and Adenosine Base Editing of Splice-Sites Mediates Highly-Efficient Disruption of Proteins in Primary and Immortalized Cells

Mitchell G. Kluesner, Walker S. Lahr, Cara-Lin Lonetree, Nicholas J. Slipek, Xiaohong Qiu, Branden A. Smeester, Patricia N. Claudio Vázquez, Samuel Pitzen, Emily J. Pomeroy, Madison J. Vignes, Samuel P. Bingea, Aneesha A. Andrew, Beau R. Webber, Branden S. Moriarity

Pediatrics, University of Minnesota, Minneapolis, MN

Background. CRISPR systems afford an unprecedented ability for targeted gene editing. These applications are of particular interest for cell based immunotherapies, where the multiplexed disruption of immunosuppressive genes such as *PDCD1, CTLA4, or CISH*, in tandem with the introduction of specific tumor antigen receptors yields promise in the development of therapies to solid tumors. While CRISPR nucleases work well for single gene editing, double stranded break (DSB) induction during multiplex editing raises concerns of large scale genomic rearrangements, reduced fitness, and oncogenesis. An alternative tool to edit genes without the need for DSBs are CRISPR-Cas9 base editors. Despite the array of papers demonstrating the various ways base editors can be used to disrupt genes and modulate splicing (BE-splice approach), there are two main gaps in the field, namely 1) a comprehensive tool for designing both cytidine base editors (CBEs) and adenine base editors (ABEs) sgRNAs to target both splice donor (SD) and splice acceptor (SA) sites, and 2) a head-to-head comparison of the methods of base editing mediated disruption encompassed across, ABEs, CBEs, BE-splice methods, and pmSTOP induction. **Results.** Here we develop an easy-to-use webtool SpliceR (z.umn.edu/splicer), for the design of base editing sgRNAs to target splice-sites of any Ensembl annotated organism. Analysis of the human genome showed that 95.86% of all protein coding gene transcripts, and 99.85% of all transcripts from genes that undergo splicing are targetable. We assessed these predictions, and compared BE-splice to the induction of premature stop codons in a mid-throughput screen of sgRNAs targeting genes in the TCR-CD3 MHC Class I immune synapse. Among the genes in our screen we found three main trends; 1) 4th generation CBE mediated disruption was higher and more reliable than 7th generation ABE mediated disruption, 2) Across CBE and ABE, SD sgRNAs produced more robust knockouts, and 3) BE-splice sgRNAs produced more frequent knockouts than pmSTOPs sgRNAs. Following the development of 8th generation ABEs, we designed a panel of sgRNAs targeting the immunohibitory gene *CISH*. We found that both ABE and CBE can disrupt proteins at the transcriptional level, and employing ABE8e eliminated the disparities between rAPOBEC1-BE4 and ABE7.10. To further assess the context dependencies of rAPOBEC1-BE4 and ABE7.10, we performed a meta-analysis of the base editing literature. Using these findings in tandem with teachings from the machine learning algorithm BE-Hive, we developed a simplified algorithm for scoring sgRNAs we term "Honeycomb". We found that Honeycomb performed comparably to BE-Hive in the ability to rank sgRNAs for BE-splice, while being >10,000x faster. Ultimately the learnings from this work have been integrated into the scoring algorithm of SpliceR. **Conclusion.** Here, we demonstrate a robust method for base editor mediated gene disruption, accompanied by a modular design tool that will be of use to basic and translational researchers alike, which holds particular promise in the sensitive landscape of cell-based therapies.



(a) Positioning of BE-splice sgRNAs within conserved splice donor and splice acceptors motif. Logo plots were generated from all human protein coding gene splice sites. Arrows indicate the base targeted by either CBEs (blue), or ABEs (green). **(b)** Breakdown of transcripts and genes targetable by BE-splice, showing the vast majority of spliced genes are targetable by this approach (99.68%). **(c)** Distribution of BE-splice sgRNA density across each gene. 50% of genes have 62 or more sgRNAs mapping to them when accounting for all PAM identities and both CBE and ABE approaches. **(d)** Distribution of the position of the first sgRNA for each gene, with 50% having their first sgRNA 11.13% way through the mRNA or earlier. **(e)** Consistency of editing efficiency and protein loss across all approaches employed here. Relationship between protein loss and base editing efficiency is comparable to that observed in Cas9 control. All data is from N = 2 independent donors, performed on different days. **(f)** Scatter plots of protein loss and editing efficiency by exon group across all base editor approaches employed. The strongest relationship is observed among middle exons, while the weakest is observed in the last exon. **(g)** Comparisons between observed values in meta-analysis and BE-Hive or Honeycomb predicted scorings. Data plotted on a logit scale to better observe relationship of data. Spearman's rank correlation coefficient (ρ) is shown. Trend line is linear model line of best fit, grey shading is 95% CI of the mean. **(h)** Comparison of editing efficiencies across ABE7.10, ABE8e, and BE4. N = 2 biological replicates. P-values represent results from one-way ANOVA followed by Tukey HSD test. **(i)** Digital western blot for CISH and β-ACTIN from samples treated with various enzyme CISH targeting guide combinations.

205. Cleavage-Free dCas9 Knock-In Gene-Editing Tool Leveraging RNA-Guided Targeting of Recombineering Proteins

Le Cong, Chengkun Wang, Jason K. W. Cheng, Yuanhao Qu, Qianhe Zhang
Stanford University, Stanford, CA

Exemplified by CRISPR-Cas9 systems, gene-editing technology is a powerful collection of tools for probing the mechanisms of human health and diseases. Recently, limitations of the initial CRISPR methods have stimulated new modalities of editing. However, existing tools that use Cas9 cutting or nicking causes DNA damage at on- and off-target sites, leading to potential cellular stress, unwanted mutations, and enrichment of p53-mutant cells. Additionally, most gene-editing tools rely on endogenous DNA repair mechanisms, particularly for knock-in of large transgenes. This results in significant levels of indels and non-homologous events. Here, we developed a cleavage-free knock-in gene-editing tool using catalytically-dead dCas9 and microbial single-strand annealing proteins (SSAPs), termed **SSAP-assisted Flanking-homology-directed dCas9 editor (SAFE-dCas9)**. SAFE-dCas9 generated near-zero indels at target loci while achieving comparable knock-in efficiencies as wild-type Cas9 in human cell line and stem cells. Using chemical perturbations, we showed that SAFE-dCas9 was less dependent on endogenous mammalian repair pathways, and our data supported the proposed model that SSAPs may promote DNA strand exchange when genomic DNA becomes transiently accessible via dCas9 unwinding and R-loop formation. We demonstrate that this new editing tool is compatible with human stem cell engineering and also could be delivered via AAV-based vector. Overall, this tool could open new opportunities towards safer genome engineering in mammalian cells with minimal DNA damage.

Highlights of this work are:

1) Engineer SAFE-dCas9 editor using dCas9 and recombineering single-stranded annealing protein (SSAP) with near-zero DNA damage (Figure 1A);

2) Metagenomic screening of phage SSAPs to identify the most efficient homolog for dCas9-based gene-editing in human cells (Figure 1B);

3) Chemical perturbation of endogenous DNA repair pathways (MRN complex, RAD51) to demonstrate that SAFE-dCas9 is significantly less dependent on key mammalian DNA repair steps;

4) Propose a mechanistic model based on the ATP-independent SSAP activity with dCas9 unwinding of genome targets, supported by inhibitor and cell synchronization assays;

5) apply SAFE-dCas9 to perform knock-in editing in human embryonic stem cells (hESC) with comparable efficiencies as Cas9 approach (Figure 1C);

6) develop a compact miniSAFE-dCas9 tools with 50% overall size reduction, compatible with SaCas9 and AAV delivery.

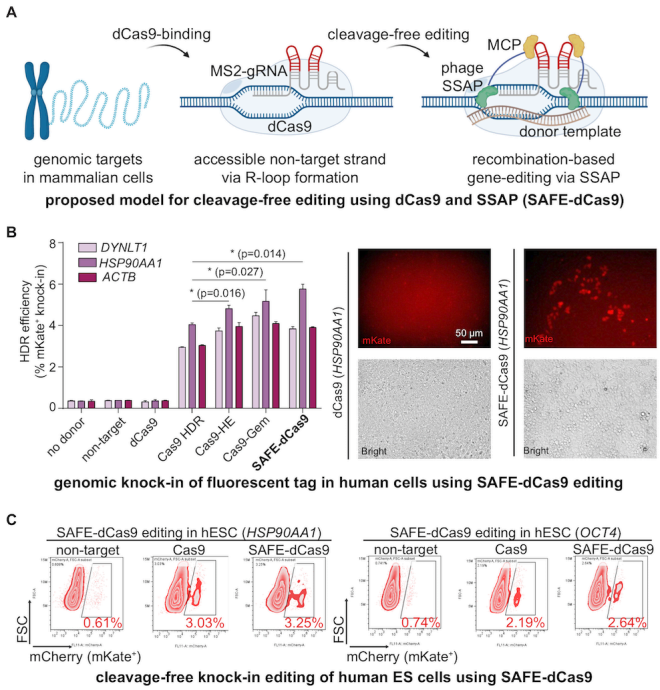


Figure 1. Development and demonstration of SAFE-dCas9 editor. (A) model of cleavage-free editing using dCas9 and microbial SSAP protein. (B) genomic knock-in of fluorescent tag (mKate) in human cells using SAFE-dCas9 editor compared with existing methods such as Cas9 HDR. Cas9-HE and Cas9-GEM are enhanced version of Cas9 HDR published previously. (C) Flow cytometry assay showing SAFE-dCas9 knock-in editing in human embryonic stem cell (hESC) with comparable efficiency as Cas9 HDR at two endogenous loci (HSP90 and OCT4).

206. Novel CRISPR Associated Gene Editing Systems Discovered in Metagenomic Samples Enable Efficient and Specific Genome Engineering for Cell Therapy Development

Gregory J. Cost, Rebecca C. Lamothe, Meghan D. Storlie, Andres Rivas, Diego A. Espinosa, Daniela S. A. Goltsman, Lisa Alexander, Cristina N. Butterfield, Christopher T. Brown, Brian C. Thomas
Metagenomi, Emeryville, CA

Gene-editing technology has revolutionized molecular therapeutics, enabling DNA-engineering-based approaches to treat disease. Despite this, development of medicines using gene editing has been hampered by technological, immunological, and legal limitations. We described previously the discovery of novel type II and type V gene-editing systems from metagenomic data, the characterization of these systems in vitro, and a demonstration of their activity in immortalized cell lines. Here, we substantially advance this work with three separate, novel gene-editing systems, demonstrating their utility for cell therapy development. We express and purify the nuclease components of both type II and type V effectors and concentrate them to ~90 and ~300 uM, respectively. We show that all three systems are capable of reproducible, high-frequency gene editing in primary human

T cells, disrupting the T cell receptor (TCR) alpha-chain constant region in up to 95% of cells and both copies of the TCR beta-chain constant region in up to ~90% of cells. Neither treatment had an effect on T cell viability. Such robust editing activity at the TCR loci will permit efficient engineering and manufacture of allogeneic chimeric antigen receptor- (CAR) and TCR-based cell therapies. We then demonstrate multiplexed gene editing in T cells with the simultaneous knock-out of a second locus relevant to cell therapy development. These edits were achieved using three novel gene-editing systems that we have developed, both using a single system or multiple systems. Further, we use our novel gene-editing systems to exploit homology-dependent DNA repair to integrate a CAR construct into the TCR alpha-chain locus (in up to ~60% of T cells), and demonstrate high-level CAR expression and antigen-dependent CAR-T cytotoxicity. Cas9 is notorious for tolerating mismatches and bulges in its target, resulting in high levels of unwanted DNA double-strand break formation. In contrast, interrogation of our gene editing systems using GUIDE-seq reveals exclusively on-target activity - zero off-target cleavage sites - even at doses greatly in excess of those required for high-frequency gene editing. Finally, as our systems are taken from microbes found in environmental samples rather than from human pathogens, we expect pre-existing immunity to our nucleases will be quite rare. In all, we show that three new gene-editing systems have the activity, specificity, and translatability necessary for use in cell therapy development.

207. Base Editing Approaches for the Treatment of β-hemoglobinopathies through Disruption of the Erythroid-Specific *BCL11A* Enhancer to Reactivate Fetal Hb

Panagiotis Antoniou[1], Giulia Hardouin[1], Pierre Martinucci[1], Megane Brusson[1], Giacomo Frati[1], Jean-Paul Concordet[2], Annarita Miccio[1]

[1]IMAGINE Institute, Paris, France,[2]Museum National d'Histoire Naturelle, Paris, France

β-hemoglobinopathies, β-thalassemia and sickle cell disease (SCD), are caused by mutations affecting the synthesis or the structure of the adult hemoglobin (Hb). Transplantation of autologous, genetically modified hematopoietic stem cells (HSCs) is an attractive therapeutic option for patients lacking a suitable HSC donor. The clinical severity of β-hemoglobinopathies is alleviated by fetal γ-globin reactivation, which can be achieved by down-regulating BCL11A, a master repressor of fetal Hb (HbF) synthesis. In particular, to reduce BCL11A levels, genome editing approaches based on site-specific nucleases that disrupt either the GATA1 or ATF4 activator binding site (BS) within the erythroid-specific *BCL11A* enhancers (+58Kb and +55Kb region respectively) have been explored. CRISPR/Cas9 nuclease-mediated disruption of the GATA1 or the ATF4 BS is associated with potent BCL11A downregulation and γ-globin upregulation. Site-specific nucleases, however, generate double-strand breaks (DSBs) in the genome and raise safety concerns for clinical applications, particularly when used in DSB-sensitive HSCs. Base editing (BE) is a CRISPR-Cas9-based genome editing technology that allows the introduction of point mutations (C>T by cytidine deaminase or CBE and A>G by adenine deaminase or ABE) in the DNA without generating DSBs.In this study, we used BE to target the GATA1 or the ATF4 BS within the erythroid-specific *BCL11A* enhancer, modulate BCL11A expression and reactivate HbF. Firstly, we performed a sgRNA screening in an erythroid cell line (K562) and identified the most efficient sgRNAs. Each sgRNA led to the editing of different nucleotides within the GATA motif (TGATAAA). The overall BE efficiency reached values of 32%. In the majority of cases, we detected no insertions or deletions in base-edited samples. Then, we tested our BE strategy in a human adult erythroid progenitor cell line (HUDEP2) expressing mainly β-globin. Even though BE efficiency was higher than in K562 cells and reached values of 72%, no significant increase of HbF was observed, as evaluated by RT-qPCR, HPLC and flow cytometry in differentiated erythroid cells. These results suggest that GATA1 binding might not play an essential role in *BCL11A* gene activation in this widely used erythroid cell line model.Hence, we tested our BE strategy in hematopoietic stem/progenitor cells (HSPCs) obtained from SCD patients with novel enzymes that edited each one of the nucleotides of the GATA motif in a more various way. HbF levels in colonies derived from edited erythroid progenitors were different between samples carrying different mutations within the GATA motif, implying that some nucleotides are more essential for the GATA1 binding capacity and the consequent *BCL11A* gene activation. Similarly, disruption of the ATF4 BS in SCD HSPCs by different mutations within the ATF4 motif, led to variable levels of HbF in erythroid colonies.In conclusion, we developed an efficient BE strategy to disrupt the GATA1 BS in the erythroid-specific *BCL11A* enhancer in SCD HSPCs that led to therapeutically relevant HbF levels. It is worth noting that our DSB-free approach could be simultaneously combined with BE strategies aiming to reactivate HbF by introducing mutations in the *HBG1/2* promoters associated with hereditary persistence of fetal hemoglobin (HPFH) in adulthood, with the final goal of achieving higher levels of HbF expression. The above-described results are currently being validated in patient HSPCs, *in vitro* and *in vivo*, to provide sufficient proof of efficacy and safety to enable the clinical development of base-edited HSCs for the therapy of β-hemoglobinopathies.

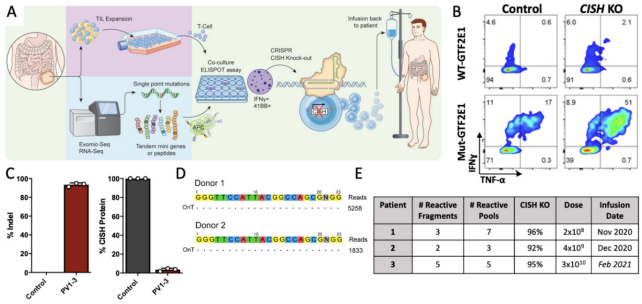
Cancer Immunotherapy

208. Clinical Application of CRISPR Edited Tumor Infiltrating Lymphocytes in Gastrointestinal Cancer

Beau R. Webber[1], Matthew J. Johnson[1], Walker S. Lahr[1], Miechaleen D. Diers[1], Nicholas J. Slipek[1], David H. McKenna[2], Darin Sumstad[3], R. Scott McIvor[4], Modassir Choudhry[5], Timothy K. Starr[6], Emil Lou[7], Branden S. Moriarity[1]

[1]Pediatrics, University of Minnesota, Minneapolis, MN,[2]Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN,[3]Molecular and Cellular Therapeutics, University of Minnesota, Minneapolis, MN,[4]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN,[5]Intima Bioscience, New York, NY,[6]Obstetrics, Gynecology, and Women's Health, University of Minnesota, Minneapolis, MN,[7]Hematology and Oncology, University of Minnesota, Minneapolis, MN

Neoantigen-specific tumor infiltrating lymphocytes (TIL) have shown promise clinically but fail to consistently elicit durable tumor regression. The intracellular checkpoint CISH negatively regulates T cell receptor (TCR) signalling and adoptive transfer of Cish-/- T cells achieves pronounced and durable tumor regression in vivo in mice. We identified that CISH is preferentially expressed in human TIL compared to T cells from peripheral blood, and at the single-cell transcriptome level CISH expression is inversely correlated with markers of T cell activation; suggesting that CISH is a key regulator of TIL response to neoantigens. To further characterize CISH function in human T cells and determine whether TIL neoantigen reactivity could be enhanced by CISH inhibition, we developed a CRISPR/Cas9-based strategy to knockout (KO) CISH in human T cells with high-efficiency (>90%) and without detectable off-target editing as measured by amplicon sequencing of computationally predicted off-target loci and unbiased GUIDE-Seq in human T cells. In peripheral blood T cells, CISH KO enhanced proliferation, cytokine polyfunctionality, and cytotoxicity in vitro. To determine if CISH KO similarly enhanced TIL function, we developed a clinical-scale, cGMP-compliant manufacturing process for CISH disruption in primary human TIL. In cGMP process validation runs we achieved highly efficient CISH KO at the genomic (93.4±1.9%) and protein (96.6±1.6%) level without detectable off-target editing while maintaining high viability and expansion (1071±393-fold). Compared to WT controls, CISH KO TIL exhibited increased cytokine-dependent proliferation and enhanced TCR avidity and neoantigen recognition. Strikingly, we identified examples where neoantigen reactivity was lost during rapid expansion (REP) in control cultures but retained in the CISH KO TIL product. Similarly, we found that CISH KO was capable of unmasking reactivity against common TP53 mutations. Intriguingly, hyperactivation in CISH KO TIL did not increase differentiation, suggesting that CISH KO may uncouple activation and differentiation pathways. Based on these preclinical studies, we recently initiated a human clinical trial at the University of Minnesota evaluating CISH edited neoantigen-reactive TIL in patients with treatment refractory metastatic gastrointestinal cancer (NCT04426669). Cellular manufacturing met release criteria for 3/3 patients to date across a dose range of 2e8-3e10 with an average CISH KO efficiency of 94.3 ± 2.1%. Currently, two patients have received TIL infusion without evidence of acute adverse toxicity related to the cellular product. Clinical updates will be presented.



Patient	# Reactive Fragments	# Reactive Pools	CISH KO	Dose	Infusion Date
1	3	7	96%	$2x10^8$	Nov 2020
2	2	3	92%	$4x10^9$	Dec 2020
3	5	5	95%	$3x10^{10}$	*Feb 2021*

Figure 1. Production and characterization of CISH KO TIL. (**A**) Clinical pipeline and manufacturing process. (**B**) *CISH* KO TIL exhibit enhanced reactivity against mutated neoantigen presented by autologous antigen presenting cells. (**C**) Efficiency of *CISH* KO at the genetic (left) and protein (right) level across three independent cGMP process validation (PV) runs. (**D**) Off-target analysis in *CISH* edited TIL using genome-wide unbiased GUIDE-seq. (**E**) Table summarizing the first three clinical TIL products including the number of neoantigen reactive TIL subcultures (fragments) pooled for infusion and the total number neoantigen peptide pools eliciting reactivity from selected fragments.

209. Single-Cell Sequencing Approach for the Discovery of Mutant IDH1 Reactive T Cell Receptors from a Glioma Vaccine Trial and an MHC-Humanized Mouse Model

Khwab Sanghvi[1,2], Lukas Bunse[1,2], Ching Leng Tan[1], Edward Green[1], Katharina Lindner[1], Matthias Bozza[3], Isabel Poschke[4], Wolfgang Wick[5,6], Michael Platten[1,2]

[1]CCU Neuroimmunology and Brain Tumor Immunology, German Cancer Research Center (DKFZ), Heidelberg, Germany,[2]Department of Neurology, University Hospital Mannheim, Mannheim, Germany,[3]DNA Vector Research Laboratory, German Cancer Research Center (DKFZ), Heidelberg, Germany,[4]Immune Monitoring Unit, National Center for Tumor Diseases (NCT), Heidelberg, Germany,[5]CCU Neurooncology, German Cancer Research Center (DKFZ), Heidelberg, Germany,[6]Department of Neurology, University Hospital Heidelberg, Heidelberg, Germany

Discovery of exploitable tumor-specific antigens is central to the development of clinically relevant immunotherapeutic strategies for cancer. Serving this purpose is the mutation in gene encoding isocitrate dehydrogenase 1 (IDH1) which is amongst the most frequent in diffuse gliomas. We have previously shown the therapeutic efficacy of a peptide vaccine encoding the IDH1R132H (mIDH1) in an MHC-humanized A2.DR1 mouse tumor model. The immunogenicity of this peptide vaccine was, in turn, successfully tested in a multicenter, first-in-man clinical trial (NOA-16; NCT02454634). To identify mIDH1 specific T cell receptors (TCRs), we investigated the lesion infiltrating leukocyte (LIL) repertoire of a patient with pseudoprogression, indicative for anti-cancer inflammation, using single-cell RNA and VDJ sequencing. Patient PBMCs subjected to antigen-specific *in vitro* T cell expansion were also sequenced to compare peripheral and LIL repertoire. In turn, to facilitate testing of TCRs in an autologous, HLA-agnostic setting with limited sample availability, we developed a protocol for the rapid expansion of patient PBMCs. Candidate TCRs were delivered into target cells using a safe, novel S/MAR based gene therapy vector and validated using NFAT luciferase-based T cell activation assays designed for testing TCRs against antigens presented on MHC class II complexes. We identified a unique transcriptional signature for the mIDH1 reactive TCR indicative of it being an orchestrator of inflammation. In a similar approach, we identified reactive TCRs from an antigen-specific T cell line generated from vaccinated A2.DR1 mice. The proof of principle identification of mIDH1 reactive TCRs demonstrates the feasibility of exploiting immune responses against CD4-restricted neo-epitopes as a first step in developing an adoptive TCR-transgenic T cell therapy for glioma patients.

210. Epigenome Editing Enables PD1 Silencing in Primary T Cells

Maria Silvia Roman Azcona, Toni Cathomen, Claudio Mussolino

Institute for Transfusion Medicine and Gene Therapy, Medical Center - University of Freiburg, Freiburg im Breisgau, Germany

The programmed cell death protein 1 (PD1) is an inhibitory receptor whose expression is associated with the exhaustion-mediated immunosuppression of Chimeric Antigen Receptor (CAR) T cells. The exhaustion state typically leads to functional unresponsiveness

and proliferative impairment, thus profoundly affecting the ability of the CAR T cells to battle against malignant cells. Interestingly, disrupting the interaction of PD1 with its ligand programmed cell death 1 ligand 1 (PDL1) through checkpoint inhibitors has shown to reverse the exhaustion state and reinvigorate the immune response. As a consequence, PD1 blockade in CAR T cells is currently under scrutiny for enhancing the immune response against some types of tumors. However, since the use of PD1-blocking antibodies triggers systemic immune-unleashing effects, potential fatal side effects, such as acute anaphylaxis and systemic inflammatory response syndrome are major concerns. Strategies to interrupt the PD1-PDL1 interaction in a more specific and safer manner are of outmost importance. To impair this interaction, we devised an approach to silence the expression of the PD1 coding (PDCD1) gene in primary T cells via epigenome editing. We generated six Designer Epigenome Modifiers (DEMs) targeting the PDCD1 promoter at different positions. Upon binding mediated by their customizable TALE-based DNA binding domain, deposition of de novo cytosine methylation at the target site drives gene silencing. Initial functional screening in a reporter cell line identified four DEMs capable of long-term silencing of the reporter gene. Delivery of the best-performing DEM in primary T cells led to sustained silencing of the endogenous PDCD1 gene with 5-fold and 3-fold reduction of transcript and protein levels, respectively. Bisulfite sequencing of the targeted promoter confirmed that silencing was associated with increased deposition of DNA methylation. Current experiments aim at inactivating the PDCD1 gene in CAR T cells directed against hematological malignancies. Taken together, our approach represents a novel strategy to modulate the PD1 inhibitory pathway through epigenome editing. A thorough assessment of DEM-associated on-target and off-target effects on CAR T cell activity and physiology will allow us to explore the full potential of this novel technology in future cancer immunotherapy applications as a novel and potentially safer approach to overcome tumor-associated immunosuppression.

211. mRNA Delivery of an Evolved Bispecific Single Domain Antibody to Synergize the Immune Checkpoint Blockade Therapy for Liver Malignancies

Rihe Liu

Eshelman School of Pharmacy, UNC Chapel Hill, Chapel Hill, NC

mRNA-based gene-therapy is considered as a revolutionary approach that could "disrupt the drug industry", as exemplified by the recent success in developing mRNA vaccines against SARS-CoV-2. Application of mRNA therapeutics in treating human malignancies has great potential. One of the urgent unmet need in cancer treatment is how to expand the immune checkpoint blockade (ICB) to a broader range of cancer types, while minimizing the immune-related adverse events (irAES) that frequently occur in the cancer immunotherapy. Liver malignancies are among the tumor types that are resistant to ICB therapy due to their high enrichment of tumor associated macrophages (TAMs) with little T cell infiltration in the tumor microenvironment (TME). Conceptually, mRNA therapeutics promise to turn the liver of patients into immunotherapeutic-making factories and can be used to address numerous mechanistic and translational questions that are challenging in the field. To identify critical targets that are involved the immunosuppression in the liver malignancies, we performed correlation gene signature analysis and IHC studies in human HCC patients' samples and found that CCL2 and CCL5 are two major chemokines responsible for attracting TAM infiltration and inducing their polarization towards cancer-promoting M2-phenotype. To reverse this immunosuppressive process, we applied directed molecular evolution to develop a humanized single-domain antibody that bispecifically binds and neutralizes CCL2 and CCL5 (BisCCL2/5i) with high potency and specificity. One major challenge in cancer immunotherapy is the lack of an effective strategy that allows preferential delivery of a therapeutic agent to the disease organs while minimizing its systemic side toxicity. Taking advantage of the very small size of this innovative BisCCL2/5i (~13 kDa), we encapsulated the mRNA encoding BisCCL2/5i in a clinically approved lipid nanoparticle (LNP) platform, resulting in a liver-homing biomaterial that allows transient yet efficient expression of BisCCL2/5i in the diseased organ in a multiple dosage manner (Figure 1). This BisCCL2/5i mRNA nanoplatform significantly induces the polarization of TAMs toward the antitumoral M1 phenotype and reduces immunosuppression in the TME, with a superior antitumor efficacy than simultaneous administration of anti-CCL2 and anti-CCL5 antibodies. The combination of BisCCL2/5i with ICB therapy produced a robust immune response and significantly improved long-term survival in mouse models of primary liver cancer and liver metastasis of pancreatic and colorectal cancers (Figure 1). Our work provides an effective bispecific targeting strategy that could broaden the ICB therapy to include multiple types of malignancies in the human liver.

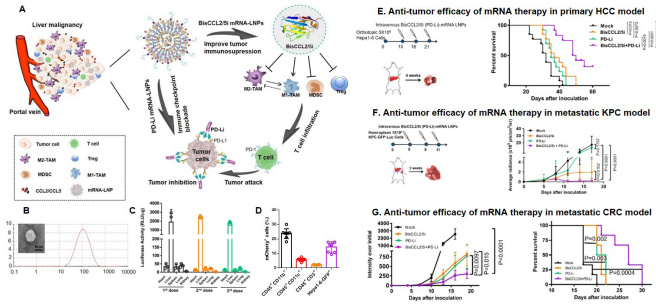


Figure 1. A. Schematic overview of BisCCL2/5i-mediated regulation of the tumor microenvironment and synergy of the ICB immunotherapy. **B.** Size distribution of mRNA-loaded LNPs. **C.** Quantification of luciferase activity in different organs after repeated administration of Luc mRNA-LNPs, demonstrating that mRNA-LNPs were almost exclusively delivered to the liver. **D.** mRNA-LNPs were mainly internalized by tumor cells (GFP+) and monocytes (CD11b+) in the liver. **E-G:** Anti-tumor immunotherapeutic efficacy of mRNA therapeutics in treating liver malignancies in mouse models of the primary HCC hepa1-6 liver cancer (**E**), liver metastasis of KPC pancreatic cancer (**F**), and liver metastasis of CT26 colorectal cancer (**G**). Syngeneic tumor-bearing mice were treated intravenously with various formulations, including BisCCL2/5i mRNA-LNPs, PD-Li mRNA-LNPs, and BisCCL2/5i mRNA-LNPs + PD-Li mRNA-LNPs, respectively, versus the HcRed mRNA-LNPs as the mock control.

212. Exhausted, Tumor-Specific T Cells Can Be Exploited to Generate a Library of T-Cell Receptors for Adoptive T Cell Therapy in Blood Malignancies

Francesco Manfredi, Lorena Stasi, Silvia Buonanno, Fabio Ciceri, Ruggiero Eliana, Chiara Bonini

VIta-Salute San Raffaele University, Milan, Italy

Aggressive blood malignancies can benefit from the immune pressure imposed by allogeneic Hematopoietic Stem Cell Transplantation (allo-HSCT), able to eradicate cancer cells surviving chemo-radiotherapy. However, allo-HSCT may also mediate allo-reactions with potentially fatal outcomes. In order to boost HTSC efficacy while reducing toxicities, adoptive T cell therapy with genetically engineered T cells redirected against tumor antigens (ACT) is an appealing therapeutic candidate. However, ACT applicability in blood tumors is limited by

the low number of known tumor-reactive T-Cell Receptors (TCRs). In order to concur to tackle this issue, tumor-specific T cells circulating in blood cancer patients who underwent allo-HSCT were purified and then in vitro expanded by means of dextramers, tetramer-like molecules reacting against immunodominant peptides from MAGE-A2, WT1, Survivin and hTERT tumor-associated antigens, restricted for HLA-A0201 and HLA-A2402 alleles. With this protocol, markedly oligoclonal tumor-specific T-cell cultures were isolated from N=13/20 patients. To assess their functionality, T-cell cultures were challenged with a cell line pulsed with the appropriate tumor-specific peptide. Despite documented in vitro enrichment and expansion, cell cultures failed to lyse target cell lines, suggesting that T cells were hypo-functional. To circumvent hypo-functionality, the dominant TCRs from N=6 dextramer-derived T-cell cultures were reconstructed and transferred into donor T cells which endogenous TCR have been previously knocked-down by means of CRISPR/Cas9 system. Newly engineered T cells proved able to specifically recognize and eliminate peptide-pulsed cell lines in in vitro killing assays. A high-dimensional flow cytometry analysis performed on N=33 patients strengthened the hypothesis that tutor-specific T cells are hypo functional ex vivo. Indeed, tumor-specific, but not viral-specific T cells preferentially expressed multiple inhibitory receptors and showed an impaired bipartite differentiation program, with accumulation of stem cell-like memory T cells and terminal effectors. Interestingly, such phenotype was accentuated in those patients deemed to relapse. In order to take advantage of the unique exhaustion signature of tumour-specific T cells, we also adopted a reverse approach for the isolation of tumor-specific TCRs, exploiting inhibitory receptors to segregate from three Acute Myeloid Leukemia patients a T cell fraction positive for multiple inhibitory receptors (IR+) and putatively enriched with tumor specificities. We then serially stimulated with autologous Leukemic-Antigen Presenting Cells (L-APCs) the IR+ and the control IR- fraction. In all three patients, L-APCs promoted the occurrence of a dominant TCR clonotype in the IR+ but not in the IR- group. The dominant TCR inserted into TCR-knocked-down donor T cells led to the generation of engineered T cells that efficiently lysed autologous leukemia in vitro while sparing healthy bone marrow cells. In summary, our data suggest the presence of circulating, though exhausted, tumor-specific T cells in patients after allo-HSCT. Here we show that by exploiting dextramers or the IR signature of cancer-specific T cells, it is feasible to isolate novel tumor-specific TCRs with different specificity, affinity and peptide sensitivity to be employed in ACT.

213. Changes in the Tumor Microenvironment in Patients with Glioblastoma Multiforme Treated with IFN-a Immune Cell & Gene Therapy (TEM-GBM_001 Study)

Bernhard Gentner[1], Gaetano Finocchiaro[2], Francesca Farina[3], Alessia Capotondo[4], Marica Eoli[5], Elena Anghileri[5], Maya Ganzetti[3], Matteo Carabba[3], Valeria Cuccarini[6], Francesco Di Meco[7], Federico Legnani[7], Bianca Pollo[8], Maria Grazia Bruzzone[6], Marco Saini[7], Paolo Ferroli[7], Roberto Pallini[9], Alessandro Olivi[9], Rosina Paterra[8], Mariagrazia Garramone[4], Stefania Mazzoleni[10], Valentina Brambilla[10], Tiziana Magnani[10], Gabriele Antonarelli[3], Matteo Naldini[1], Matteo Barcella[1], Nadia Coltella[1], Carlo Russo[10], Luigi Naldini[1], Fabio Ciceri[3]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Neuro-Oncology Unit - San Raffaele Hospital, Milan, Italy,[3]Hematology and Bone Marrow Transplantation Unit - San Raffaele Hospital, Milan, Italy,[4]San Raffaele Telethon Institute for Gene Therapy Clinical Lab, Milan, Italy,[5]Neuro-Oncology Unit - Istituto Nazionale Neurologico Carlo Besta, Milan, Italy,[6]Neuroradiology Unit - Istituto Nazionale Neurologico Carlo Besta, Milan, Italy,[7]Neurosurgery Unit - Istituto Nazionale Neurologico Carlo Besta, Milan, Italy,[8]Neuropathology Unit - Istituto Nazionale Neurologico Carlo Besta, Milan, Italy,[9]Neurosurgery Unit - Policlinico Gemelli Hospital, Rome, Italy,[10]Genenta Science, Milan, Italy

Genetically modified cell-based therapies are of great relevance in immuno-oncology due to their potential for tumor specificity & potential durability. We have a genetically modified cell-based platform, for ex-vivo transduction of autologous HSPCs with a lentiviral vector expressing the IFNa transgene (Temferon) & delivery by autologous stem cell transplantation (ASCT). Specific control mechanisms selectively target transgene expression to Tie-2 expressing macrophages (TEMs) by combining Tie-2 promoter & post-transcriptional regulation exerted by miRNA target sequences. TEM-GBM is an open-label, Phase I/IIa dose-escalation study evaluating safety & efficacy of Temferon in 21 newly diagnosed patients with glioblastoma & unmethylated MGMT promoter. By 4th January 2021, 15 patients had enrolled; 9 received Temferon (D+0) with follow up at 48-546 days. One patient died from sepsis & respiratory failure at D+60 & 1 patient died from progressive disease (PD) at D+403. PD occurred in 7 patients 27-239 days from D+0, within expectations for this tumor type. Temferon was well tolerated without dose-limiting toxicity. Median neutrophil & platelet engraftment occurred at D+13 & D+12, respectively. SAEs include infections, venous thromboembolism, brain abscess, hemiparesis, GGT elevation & poor performance status compatible with ASCT, concomitant medications & PD. Temferon-derived differentiated cells, as determined by the presence of vector genomes in peripheral blood & bone marrow, were evident at D+30 (median VCN in CD33+ BM cells 11%, reflecting variability in input cell dose) & persisted at lower levels up to 18 months. The built-in transgene expression control mechanism was effective, evidenced by very low plasma IFNa concentrations (median 5pg/ml at D+30). Concomitantly with PD, IFNa was detectable in the cerebrospinal fluid of 2 patients at 3 & 6 months, respectively. Four patients underwent second surgery. Tumors had gene-marked cells & increased expression of IFN-responsive gene

signatures compared to diagnosis, indicative of local IFNa release by TEMs. Biopsies of a stable as compared to a progressing lesion in 1 patient (as defined by MRI) had a higher proportion of T cells & TEMs within the myeloid infiltrate & an increased IFN-response signature. The T-cell immune repertoire changed with evidence for expansion of tumor-associated clones. Tumor microenvironment characterization by scRNA & TCR sequencing is ongoing. The results support the safety of Temferon treatment & provide early evidence of its potential to modulate the TME of GBM patients as predicted by preclinical efficacy studies.

214. Small Molecule-Regulated Gene Circuit for Controlling Cytokine Expression in Cell Therapies

Michelle E. Hung[1], Divya Israni[2], Huishan Li[2], Yin Yin Chong[1], Poornima Ramkumar[1], Allison Drain[2], Allison Quach[1], Mengxi Tian[1], Rishi Savur[1], Niran Almudhfar[1], Chen-Ting Lee[1], Abla Bakir[1], Edwin Cruz[1], Carmina Blanco[1], Travis Wood[1], Mario Lorente[1], Brandon Lee[1], Brett Kiedaisch[1], Russell Gordley[1], Marcela Guzman Ayala[1], Gary Lee[1], Tim Lu[1], Ahmad Khalil[2]

[1]Senti Bio, South San Francisco, CA,[2]Boston University, Boston, MA

Background: The efficacy of CAR-T and CAR-NK cell adoptive immunotherapies in solid tumors is hampered by poor persistence and limited effector functions in the immunosuppressive tumor microenvironment. Pro-inflammatory cytokines, such as IL-12, have been shown in preclinical studies to enhance CAR-T cell activity and stimulate the innate immune antitumor response. However, systemic administration of recombinant IL-12 or poorly regulated secretion by armed CAR-T cells triggers severe toxicity in humans, limiting the clinical application of potent immunomodulatory payloads. A technology for titratable small-molecule regulation of cytokine production by armed CAR cells would enhance antitumor efficacy, precision and safety in patients. Methods: We designed a gene circuit to regulate gene expression via FDA-approved, orally dosed NS3 inhibitors. The circuit consists of a synthetic transcription factor and a cognate synthetic promoter consisting of zinc finger binding sites and a minimal promoter. Synthetic transcription factors comprise a genome-orthogonal zinc finger DNA binding domain linked to transcriptional activation domains via the NS3 protease and NS3 protease-cleavable linkers. At baseline, NS3 cleaves the transcription factor, inactivating it. NS3 inhibitors stabilize the transcription factor so that transcription occurs from the synthetic promoter. Results: Promoters were optimized in immune cells to minimize basal expression, resulting in limited production of IL-12 in the absence of the NS3 inhibitor grazoprevir (GZR) (~140pg/1e6 cells in 48h), while maintaining high GZR induced expression (~20,000pg/1e6 cells in 48h). Production of IL-12 returned to baseline after 48h without GZR. The transcription factor was optimized to maximize GZR sensitivity, resulting in induction at concentrations as low as 10nM, well below the Cmax in humans of 230nM. This sensitivity should enable fine-tuned control of induction at tolerable concentrations of GZR. The gene circuit was then tested in vivo - NSG mice were dosed with 75mg/kg GZR intraperitoneally on days 1-4, and received 20e6 engineered cells on day 2. IL-12 was detected at 1,642 pg/mL on day 4 in plasma of mice treated with the maximum dose of GZR, compared to 19 pg/mL in vehicle- treated mice (groups were statistically different, $p<0.01$). On day 8 (4 days after final GZR dose), differences in IL-12 levels were not statistically significant between GZR- and vehicle-treated mice and were between 20-30 pg/mL for all groups. The >100 fold upregulation in IL-12 serum levels between GZR and vehicle treated mice indicates that this gene circuit provides a wide dynamic range of control of cytokine level that will enable precise tuning through small-molecule dosing. Conclusion: We optimized small-molecule sensitivity and basal gene expression of a synthetic transcription factor/promoter gene circuit for drug-inducible regulation of gene expression. The circuit was used to regulate IL-12 production in primary immune cells and demonstrated >100 fold dynamic range of control of IL-12 levels in vivo. Further experiments can further optimize cell and small-molecule dosing to generate ideal therapeutic levels of IL-12 in armed CAR cells. This platform technology may also be applied to regulation of other cytokines and immune effectors to enhance efficacy of CAR immune cells.

Gene Therapy for Lysosomal Storage Disorders

215. RGX-121 Gene Therapy for the Treatment of Severe Mucopolysaccharidosis Type II: Interim Analysis of the First in Human Study

Roberto Giugliani[1], Maria Escolar[2], Can Ficicioglu[3], Paul Harmatz[4], Marie-Laure Nevoret[5], Yoonjin Cho[6], Dawn Phillips[5], Paulo Falabella[5]

[1]Department of Genetics, UFRGS, Medical Genetics Service, HCPA, Porto Alegre, Brazil,[2]University of Pittsburgh, Pittsburgh, PA,[3]Children's Hospital of Philadelphia, Philadelphia, PA,[4]UCSF Benioff Children's Hospital Oakland, Oakland, CA,[5]Clinical Development, REGENXBIO, Rockville, MD,[6]Biostatistics, REGENXBIO, Rockville, MD

Mucopolysaccharidosis type II (MPS II) or Hunter syndrome is an x-linked lysosomal storage disease caused by a deficiency of iduronate-2-sulfatase (I2S) leading to an accumulation of glycosaminoglycans (GAG) in tissues. Severe MPS II results in irreversible neurocognitive decline and behavioral symptoms that are not addressed by intravenously administered enzyme replacement therapy (ERT) with recombinant I2S. RGX-121 is a recombinant adeno-associated virus serotype 9 capsid (AAV9) containing a human iduronate-2-sulfatase expression cassette (AAV9.CB7.hIDS). In an MPS II murine model, AAV-mediated transfer of the iduronate-2-sulfatase gene into the cerebrospinal fluid demonstrated increased I2S activity in the brain, corrected biochemical changes due to I2S deficiency, and improved neurobehavioral function. Vector distribution and reduced levels of GAGs were also observed in peripheral organs, as well as normalization of liver size and weight. Delivery of the IDS gene directly to the central nervous system (CNS) could provide a permanent source of secreted I2S, allowing for long-term cross correction of cells throughout the brain. In addition, transduction of RGX-121 into peripheral organs could provide correction of MPS II systemic disease. In this phase

1/2, first-in-human, multicenter, open-label, dose escalation trial (NCT03566043), RGX-121 is administered as a one-time, image-guided injection into the cisterna magna or lateral cerebral ventricle of participants with severe MPS II ages 4 months to 5 years. Participants are evaluated for safety, tolerability, and efficacy for 104 weeks after administration. Assessments include biomarkers in the CSF, plasma and urine; neurodevelopmental scales of cognition, language, motor, and adaptive behavior; audiometry; and imaging of the brain, liver and spleen. Eight participants have been enrolled in 2 dose cohorts (Cohort 1: $1.3x10^{10}$ and Cohort 2: $6.5x10^{10}$ genome copies/gram brain); 2 participants have never received ERT. As of January 4, 2021, RGX-121 is reported to be well-tolerated in all eight patients with no administration or drug-related serious adverse events. In all participants, CSF levels of heparan sulfate, a biomarker of neuronopathic disease in MPS II, showed consistent reductions measured up to 104 weeks. CSF I2S enzyme concentration was not present at baseline but was measurable in all Cohort 2 participants after RGX-121 administration. Interim neurodevelopmental testing demonstrated ongoing skill acquisition in cognition, language and/or motor domains in all participants with at least 6 months of follow-up. Additionally, there is emerging evidence of systemic RGX-121 efficacy indicated by plasma I2S enzyme expression, total urine GAGs, and abdominal ultrasound imaging.

216. AVR-RD-01, an Investigational Lentiviral Gene Therapy for Fabry Disease: Clinical Data Trends from Phase 1 and Phase 2 Studies up to 3.5 Years

Mark Thomas[1], Kathy Nichols[2], Ben Carnley[1], Mirjam Trame[3], Chris Mason[3,4], Fatemeh Tavakkoli[3]

[1]Royal Perth Hospital, Perth, Australia,[2]Royal Melbourne, Parkville, Australia,[3]AVROBIO, Cambridge, MA,[4]Advanced Centre for Biochemical Engineering, University College London, London, United Kingdom

Fabry disease (FD) is a monogenic lysosomal disorder associated with pathological accumulation of substrates and metabolites, including globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3), which may lead to chronic kidney and cardiac disease. AVR-RD-01 is an investigational lentiviral gene therapy that involves infusion of autologous stem cells genetically modified with a lentiviral vector which inserts into the human genome a complementary deoxyribonucleic acid sequence that encodes for functional human AGA enzyme deficient in FD. In a Phase 1 safety study of AVR-RD-01, 5 adult male patients, on enzyme replacement therapy, received a single infusion of AVR-RD-01. Three out of 5 patients have discontinued ERT after receiving AVR-RD-01. In an ongoing Phase 2 study evaluating the safety, tolerability and efficacy of AVR-RD-01, four adult treatment-naïve classic Fabry disease male patients received a single infusion of AVR-RD-01. Across both studies, all participants demonstrated increases in plasma and leukocyte AGA activity and decreases in Gb3 and lyso-Gb3 in plasma, that are now sustained up to 42 months in the Phase 1 study and up to 30 months in the Phase 2 study. Estimated glomerular filtration rates (eGFR) for all participants show positive trends, indicating stable kidney function up to 32 months post AVR-RD-01 treatment. Kidney biopsy at 48 weeks post AVR-RD-01 treatment for the first participant in the Phase 2 study demonstrated an 87% reduction in renal peritubular capillary Gb3 inclusions compared to baseline. Across all 4 patients enrolled to-date in Phase 2, cardiac mass and function remain stable at 1 year post AVR-RD-01 treatment. Overall, the therapy is well tolerated, and adverse events were consistent with the conditioning regimen, underlying FD and pre-existing conditions, and none were related to AVR-RD-01 drug product. The latest results from the ongoing Phase 1 and Phase 2 studies will be shared, as well as the results from the second evaluable kidney biopsy at 48 weeks post AVR-RD-01 treatment in the Phase 2 study.

217. Updated Results of Transpher B, a Multicenter, Single-Dose, Phase 1/2 Clinical Trial of ABO-101 Gene Therapy for Sanfilippo Syndrome Type B (Mucopolysaccharidosis IIIB)

Maria J. de Castro[1], Kevin M. Flanigan[2], Bénédicte Héron[3], Nicole Muschol[4], Maria L. Couce[1], Kristen V. Truxal[2], Kim L. McBride[2], Claudia Ravelli[3], Luise Ammer[4], Mona Lindschau[4], Maria Fuller[5], Ana B. del Campo[6], Juan Ruiz[6]

[1]Metabolic Unit, Department of Pediatrics, Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain,[2]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[3]Centre de Référence des Maladies Lysosomales, Hôpital Trousseau, Paris, France,[4]Department of Pediatrics, University Clinic Hamburg-Eppendorf, Hamburg, Germany,[5]SA Pathology, Women's and Children's Hospital, Adelaide, Australia,[6]Abeona Therapeutics, Madrid, Spain

Transpher B is a Phase 1/2 clinical trial assessing the safety and efficacy of a single intravenous administration of ABO-101, a single-stranded AAV9-based vector encoding human *NAGLU*, for treating Mucopolysaccharidosis IIIB (MPS-IIIB). MPS-IIIB is a neurodegenerative lysosomal storage disorder manifesting early in childhood. Twelve patients have been enrolled in three cohorts, including two pairs of siblings: Cohort 1, $2x10^{13}$ vg/kg, n=2, follow-up 22-36 months; Cohort 2, $5x10^{13}$ vg/kg, n=5, follow-up 12.1-19 months; Cohort 3, $1x10^{14}$ vg/kg, n=4, follow-up 1.6-9.8 months. One patient from Cohort 1 rolled-over to a Long-Term Follow-Up study to be followed for safety and efficacy for 3 additional years. Intravenous administration of ABO-101 was well tolerated, with no infusion adverse events and only one serious drug-related adverse event (one patient in Cohort 3, prolonged hospitalization to monitor vomiting and fever following treatment). ABO-101 administration normalized plasma enzyme activity by day 7, remaining within normal limits for up to 3 months in Cohort 1 and 6 months in Cohorts 2 and 3. ABO-101 also induced a rapid reduction in CSF heparan sulfate (HS), as early as day 30 which was sustained at later timepoints, including month 24 in the only patient yet to reach that time-point in Cohort 1. Likewise, systemic biomarkers (HS in plasma and urine and total urinary glycosaminoglycans) showed a rapid decrease, which was sustained during follow-up in Cohorts 2 and 3. Liver volume decreased in all patients. Limited follow-up duration currently precludes adequate assessment of neurological outcomes. In summary, intravenous administration of ABO-101 in children with MPS-IIIB was well tolerated and showed a clear biochemical response, including normalization of enzyme activity in plasma up

to 6 months, improvements in CNS and systemic biomarkers, and reductions in liver volume. Longer follow-up is required to evaluate neurodevelopmental changes.

218. Safety and Efficacy of Liver-Directed Gene Therapy in Patients with Mucopolysaccharidosis Type VI

Nicola Brunetti-Pierri[1,2], Rita Ferla[1,2], Virginia M. Ginocchio[1], Alessandro Rossi[2], Simona Fecarotta[3], Roberta Romano[2], Giancarlo Parenti[1,2], Yilmaz Yildiz[4], Stefano Zancan[5], Valentina Pecorella[1], Mafalda Graziano[1], Margherita Dell'Anno[1,2], Marialuisa Alliegro[1], Francesca Santamaria[2], Raffaella Brunetti-Pierri[6], Francesca Simonelli[6], Vincenzo Nigro[1,7], Maria Vargas[8], Giuseppe Servillo[8], Francesco Borgia[9], Ernesto Soscia[10], Olivier Danos[11], Jean-Brice Marteau[12], Stefania Galimberti[13], Mariagrazia Valsecchi[13], Philippe Veron[14], Federico Mingozzi[14], Francesca Fallarino[15], Giancarlo La Marca[16], Alberto Auricchio[1,9]

[1]Telethon Institute of Genetics and Medicine, Pozzuoli (NA), Italy,[2]Department of Science Translational Medicine, Federico II University, Naples, Italy,[3]DAI Materno-Infantile, Federico II University, Naples, Italy,[4]Children's Hospital, Hacettepe University, Ankara, Turkey,[5]Fondazione Telethon, Roma, Italy,[6]Multidisciplinary Department of Medical, Surgical and Dental Sciences, Università degli Studi della Campania "Luigi Vanvitelli", Naples, Italy,[7]Department of Biophysics and Pathology, Università degli Studi della Campania "Luigi Vanvitelli", Naples, Italy,[8]Department of Neurosciences, Reproductive and Odontostomatological Sciences, Federico II University, Naples, Italy,[9]Department of Advanced Biomedical Sciences, Federico II University, Naples, Italy,[10]Department of Clinical and Experimental Medicine, Federico II University, Naples, Italy,[11]REGENXBIO Inc., Rockville, MD,[12]Genosafe SAS, Evry, France,[13]Center of Biostatistics for Clinical Epidemiology, Department of Medicine and Surgery, Milano-Bicocca University, Monza, Italy,[14]Genethon, Evry, France,[15]Department of Medicine and Surgery, University of Perugia, Perugia, Italy,[16]Department of Paediatrics A. Meyer Children's Hospital, University of Florence, Florence, Italy

Mucopolysaccharidosis type VI (MPS VI) is a multi-system and severe lysosomal storage disorder caused by deficiency of arylsulfatase B (ARSB) that results in widespread accumulation and urinary excretion of glycosaminoglycans (GAG). Enzyme replacement therapy (ERT) is currently available for treatment of MPS VI but has several limitations including the requirement of multiple and costly intravenous administrations, risks related to intravenous infusions, and limited efficacy on some disease manifestations.In a phase I/II open label, dose escalation clinical trial (NCT03173521) nine MPS VI subjects of 4 years of age or older discontinued ERT at least one week prior to the intravenous injection of an AAV2/8 vector expressing human *ARSB* under the control of a liver-specific promoter (AAV2/8.TBG.h*ARSB*). Enrolled subjects received a low dose of $6x10^{11}$ genome copies (GC)/kg (n=3), an intermediate dose of $2x10^{12}$ GC/kg (n=2), and a high dose of $6x10^{12}$ (GC)/kg (n=4). In contrast to the low and intermediate dose, subjects of the high dose cohort received prophylactic treatment with steroids. Vector infusion was well tolerated in all subjects without severe adverse events related to the vector. Subjects in the low and intermediate dose cohorts showed serum ARSB activity levels of approximately 20% of normal range that were sustained over time but not sufficient to maintain urinary GAG at the same levels achieved by ERT. Subjects of the high dose cohort had ARSB activities ranging from 30% to 100% of normal levels and their urinary GAG showed moderate or no increase compared to the ERT levels. Moreover, subjects of the high dose cohort remained without ERT for up to 12 months post-gene therapy with no evidence of worsening of their clinical manifestations. Although further clinical follow up is needed, the data of this first-in-human clinical trial show that AAV8-mediated liver-directed gene therapy for MPS VI is safe and results in sustained ARSB expression with preliminary evidence of biochemical and clinical improvements.

219. Ex Vivo Hematopoietic Stem Cell Gene Therapy for Mucopolysaccharidosis Type IH (Hurler Syndrome): An Interim Analysis with a Median Follow Up of 19 Months

Bernhard Gentner[1], Maria Ester Bernardo[1], Francesca Tucci[1], Francesca Fumagalli[2], Silvia Pontesilli[2], Paolo Silvani[2], Erika Zonari[1], Simona Miglietta[1], Eugenio Montini[1], Andrea Calabria[1], Fabio Ciceri[2], Stefania Galimberti[3], Giancarlo La Marca[4], Rossella Parini[5], Luigi Naldini[1], Alessandro Aiuti[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]San Raffaele Hospital, Milano, Italy,[3]Milan-Bicocca University, Monza, Italy,[4]Meyer Hospital, Firenze, Italy,[5]San Gerardo Hospital, Monza, Italy

Transplantation of genetically-corrected autologous hematopoietic stem and progenitor cells (HSPC) represents a promising treatment approach for patients with lysosomal storage disorders. We conducted a phase I/II gene therapy (GT) study in children affected by Hurler syndrome. Eight patients were infused with autologous HSPC transduced with an alpha-L-iduronidase (IDUA)-encoding lentiviral vector following conditioning with busulfan and fludarabine. Seven of 8 patients had previous enzyme replacement therapy (ERT), which was discontinued several weeks before GT. Median transduction efficiency of the products was above 80%, with a median of 2.2 vector copies (VCN) per cell. All patients had rapid hematologic recovery, with median neutrophil engraftment on day +20, rapid resolution of neutropenic fever (median duration: 1.5 days in the 6 patients that experienced it) and short thrombocytopenia, limited to grade 3 in 5 out of 8 patients and median platelet engraftment occurring at day+12 in the 3 patients that experienced grade 4 thrombocytopenia. GT did not induce IgG immune responses to IDUA, and the 5 patients that tested positive for antibodies while on ERT before GT cleared them within 3 months. With a median follow up of 19 months (range: 11-28) as of Nov 27, 2020, all patients are alive and well and show evidence for highly polyclonal, stable long-term gene marking in mononuclear cells with a median VCN of 1.0 (range: 0.2-4.0) at 9-12 months after GT. Median blood IDUA activity at 1 year was 108 micromol/L/h (range: 30-139), five-fold higher than the average of normal subjects (range: 3.8-35). Most patients excreted normal glycosaminoglycan (GAG) levels in the urine at 9-12 months after GT. Importantly, IDUA activity was detectable in the cerebrospinal fluid (CSF) suggesting central enzyme expression, hypothesized to be provided by local engraftment

of microglia-like cells derived from transduced HSPC. Heparan sulfate levels in the CSF decreased up to 20-fold post-GT suggesting central metabolic correction. All patients demonstrate a stable cognitive score, stable motor function, reduced joint stiffness, improved findings on brain and spine MRI and normal growth. Our preliminary results show encouraging safety and efficacy data, highlighting the potential of genetic engineering of HSPC for the treatment of MPSIH.

220. A Targeted AAV Gene Therapy Product Candidate, 4D-310, for the Treatment of Fabry Disease: Intravenous Biodistribution, Transgene Expression and Safety in Non-Human Primates

Kevin Whittlesey[1], Gabriel Brooks[1], Paul Szymanski[1], Ghezal Beliakoff[1], Julie Nye[1], Jessica Sepulveda[1], Roxanne Croze[1], Chris Schmitt[1], Janelle Bickta[1], Katy Barglow[1], Melissa Kotterman[1], Peter Francis[1], David Schaffer[1,2], David Kirn[1,2]

[1]4D Molecular Therapeutics, Emeryville, CA,[2]University of California, Berkeley, Berkeley, CA

RATIONALE: Fabry disease (FD) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, which encodes α-galactosidase A (AGA). Mutations in *GLA* that diminish AGA activity result in intracellular accumulation of its substrate globotriaosylceramide (Gb3) and associated disease. While AGA enzyme replacement therapy reduces Gb3 levels in plasma and in endothelial cells, AGA uptake into critical diseased tissues such as the heart and blood vessel walls is highly inefficient. Cardiovascular disease, the leading cause of death in FD, thereby remains a high unmet medical need. We designed the AAV gene therapy product candidate 4D-310 to treat FD systemically, including both through AGA expression directly within target organs such as the heart, and through stable secreted AGA levels in the plasma after a single intravenous (IV) administration. 4D-310 is comprised of the heart targeted and evolved intravenous vector C102, and is engineered to deliver a codon-optimized human *GLA* transgene driven by a ubiquitous promoter. In the mouse FD knockout model, IV 4D-310 resulted in dose-related AGA expression within the heart and plasma and was associated with significant reductions in Gb3 substrate isoforms. Prior to initiation of clinical trials in humans, we carried out a study of IV 4D-310 in NHP. **METHODS:** A dose-ranging study of 4D-310 was performed by IV administration in cynomolgus macaque NHPs ($3x10^{12}$-$5x10^{13}$ vg/kg). Termination was at 8 weeks. Parameters examined included *in vivo* safety measures, hematology, serum chemistries, vector biodistribution, transgene expression, plasma and tissue AGA transgene activity and protein levels. **RESULTS:** 4D-310 was well-tolerated in NHPs up to 5x1013 vg/kg (the highest dose tested). There were no significant test article-related clinical findings or effects on hematology, chemistry or coagulation tests. Supraphysiologic dose-dependent plasma AGA activity plateaued by Day 15 after dosing (the earliest time post-dose examined) and was sustained through the end of the study; mean plasma AGA activity at study termination ranged from 420% to as high as 6,700% of baseline levels ($P<0.05$). 4D-310 vector DNA was detected in the heart and liver in a dose-dependent manner and mirrored GLA transgene expression. We also evaluated AGA protein in tissues by immunohistochemistry. Highest expression of AGA was seen in liver, heart and carotid artery. In regard to the heart, the highest levels of expression were noted in the left ventricle and interventricular septum. **CONCLUSIONS:** 4D-310 demonstrated safety and secreted high-level and stable AGA activity in plasma and throughout the heart and blood vessel walls. These data in a primate model demonstrate the potential of 4D-310 for a dual mechanism-of-action in Fabry disease by leveraging cell-autonomous production of AGA within the heart plus high plasma enzyme levels. 4D-310 has the potential to address the unmet medical need in FD patients, including AGA expression within cardiovascular tissues.

221. Efficacy of Intracerebroventricular Adeno-Associated Virus Encoding Iduronidase with Dorsal Root Ganglia-Detargeting Sequences in a Mouse Model of Mucopolysaccharidosis Type I

Yanchun Li, Juliette Hordeaux, Brianne Jeffrey, Yanqing Zhu, Peter Bell, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Mucopolysaccharidosis type I (MPSI) is a recessive lysosomal storage disorder caused by a mutation in the α-L-iduronidase (*IDUA*) gene. IDUA enzyme deficiency results in the accumulation of glycosaminoglycans in the central nervous system (CNS) and peripheral organs, causing a devastating neurodegenerative and systemic disease. Administering adeno-associated virus (AAV) encoding IDUA into the cisterna magna is remarkably efficacious in large animal models and is currently being assessed in humans. However, nonclinical studies on nonhuman primates demonstrate that delivering AAV vectors into the CNS is associated with dorsal root ganglion (DRG) toxicity. Our team recently developed a new approach to ameliorate this safety concern by introducing sequence targets for miR183 into the untranslated region of the transgene. This optimization specifically downregulates transgene expression in DRG neurons and the corresponding toxicity without affecting transduction in the CNS of nonhuman primates. In this study, we further investigated the efficacy of this new approach in an MPSI mouse model. We administered AAVhu68 constructs encoding a new codon-optimized IDUA, with or without target sites for miR183, to homozygous *IDUA*-knockout (KO) mice via intracerebroventricular injection. We evaluated the efficacy and immunogenicity of both vectors 1-3 months post-administration. We found that both vectors significantly restored IDUA activity in the CNS and peripheral organs. Interestingly, we observed higher enzymatic activity for vectors expressing IDUA-miR183 compared with IDUA alone in the brain, spinal cord, liver, kidney, and serum. Importantly, both constructs showed similar efficacy in reducing lysosome-associated membrane protein 1 immunostaining in the CNS of *IDUA*-KO mice, demonstrating a correction in lysosomal storage. Both constructs elicited similar levels of humoral response to the transgene and were equally well tolerated in the mice. This proof-of-concept study demonstrates that DRG-detargeting sequences do not

modify the efficacy of AAVhu68 encoding IDUA and may represent a viable approach for improving the safety profile of a CNS-directed AAV therapy for MPSI.

Lentiviral Vector Manufacturing

222. Stabilization of Lentiviral Vector Genomic RNA during Production Using a Novel RNA Chaperone Leads to Increased Yields

Jordan Wright[1], Cristina Nogueira[1], Zsofia Miskolczi[1], Catarina R. Vieira[1], Lucy Barnes[1], Beihui Liu[1], Amy R. Barker[1], Emma Burton[1], Emily R. Hilbourne[1], Lisa Cooper[2], Dogan Gur[2], Jessica M. Bourne[2], Rui C. Sanches[2], Kirstie Pemberton[2], Ciaran M. Lamont[2], Oliver Goodyear[2], Thomas Evans[1], Charles Moore-Kelly[1], R. A. Raposo[1], Laura Moyce[3], Daniel Blount[3], Amy L. Keating[4], Gayathri Devarajan[4], Tiziana Coradin[4], Sara Ferluga[1], Hannah J. Stewart[1], Kyriacos A. Mitrophanous[1], Nicholas G. Clarkson[1], Dan C. Farley[1]

[1]Platform Research, Oxford BioMedica, Oxford, United Kingdom,[2]Process R&D, Oxford BioMedica, Oxford, United Kingdom,[3]Translational Science Group, Oxford BioMedica, Oxford, United Kingdom,[4]Early Development Group, Oxford BioMedica, Oxford, United Kingdom

The use of lentiviral vectors (LVs) for gene delivery will increase substantially over the coming years, and there is considerable work being undertaken to improve the quality and quantity of their manufacture. The two broad areas of the manufacturing process are 'Upstream' and 'Downstream' phases, wherein the biological and physical properties of these vectors are the subject of intense research. In addition, LV transgene payloads will become larger and more complex as greater functionality and control of transgene expression are required. A key aspect of LV particle assembly is the amount of steady-state vector genome RNA (vgRNA) in the production cell, which generally correlates strongly with vgRNA length, but can vary according to the specific transgene sequences. Here we report on improvements that can be employed during the Upstream phase in enhancing the activity of lentiviral vector particles produced from manufacturing cells, such that these gains are retained to final product, irrespectively of the steps taken during Downstream phase. We have developed a new technology based on modified U1 snRNAs to increase lentiviral vector out-put titres when exogenously co-expressed with vector components. We replaced the splice donor annealing sequence of the U1 snRNA to various heterologous 'targeting' sequences that are complementary to sites within the vgRNA. By targeting the modified U1 snRNA to the packaging sequence (Psi) of the LV genome we could enhance the steady-state levels of vgRNA in the cytoplasm, and observed a concomitant increase in vector harvest titres. These Psi-targeted U1 snRNAs are able to effectively chaperone LV genomic RNA, possibly by avoiding nuclear decay mechanisms. Wild type HIV-1 has been shown to employ a polyadenylation suppression mechanism by recruiting endogenous U1 snRNA at its major splice donor in order to suppress premature polyadenylation of the upstream polyA site in the 5'LTR, prior to proviral transcription induction by tat. However, in providing an example of how HIV-1 biology and 3rd generation LV vectorology can substantially differ, we show that premature polyadenylation at the 5'LTR of LV genome cassettes does not occur to any great extent, and that Psi-targeted U1 snRNA is able to enhance the LV titres of genomes with a functionally mutated 5'polyA site. This indicates that Psi-targeted U1 snRNA operates in a novel manner over those previously described for other uses of modified U1 snRNA. We will present data regarding optimisation of the targeting U1 snRNA and how these new class of enhancers have been used to increase titres of some LV-CARs by >8-fold in 5L bioreactors. Finally, we demonstrate that residual Psi-targeted U1 snRNA is cleared to very low levels during Downstream processing, and show production of functional CAR-T cells generated using this approach. We are now transitioning this new technology into large scale GMP manufacturing.

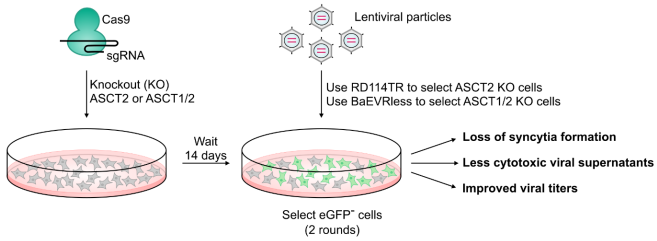
223. Knockout of Entry Receptors in Virus Producer Cells for Improved Titer and Quality BaEVRless-Pseudotyped Retroviral Particles

Denise Klatt[1], Kayla E. Wright[1], Susanne Wolf[2], Axel Schambach[2,3], Els Verhoeyen[4,5], David A. Williams[1,3], Christian Brendel[1,3]

[1]Gene Therapy Program, Dana Farber/Boston Children's Cancer and Blood Disorders Center, Harvard Medical School, Boston, MA,[2]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,[3]Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, MA,[4]Centre International de Recherche en Infectiologie (CIRI), Université Lyon, Université Claude Bernard Lyon 1, INSERM, U1111, CNRS, UMR 5308, Ecole Normale Supérieure de Lyon, Lyon, France,[5]Université Côte d'Azur, INSERM U1065, Centre Méditerranéen de Médecine Moléculaire, Nice, France

The viral envelope forms the outer layer of a retroviral particle and largely determines its tropism and biophysical properties. Baboon endogenous retrovirus (BaEV) and RD114 glycoproteins efficiently pseudotype retroviral vectors and are superior to the commonly used VSV-G envelope for gene transfer into hematopoietic cell types such as HSC or NK-cells. BaEV and RD114 glycoproteins and their engineered derivatives (BaEVRless, RD114TR) bind to the neutral amino acid transporter ASCT2 for cell entry, and BaEV-derived envelopes additionally use the ASCT1 receptor, which increases their tropism for HSC. The production of high-quality viral particles is crucial in all viral-based gene therapy approaches but is expensive and difficult to scale for clinical applications. The production of BaEV-enveloped vectors is hampered by strong cytopathic effects due to fusogenicity, syncytia formation and cell death during vector production. Here, we used CRISPR-Cas9 to knockout the entry receptors ASCT1 and/or ASCT2 in HEK293T cells to prevent super-infection and syncytia formation during production of alpha-retroviral particles. Both knockout cell lines proliferate analogous to wild type (WT) HEK293T cells (doubling time of 0.9-1.2 days) indicating that the ASCT1/2 deficiency is tolerated. The loss of ASCT2 is sufficient to abrogate the infectivity of BaEV-pseudotyped viral particles in HEK293T cells (<1% GFP^+ cells) suggesting that ASCT2 is the main entry receptor in this cell type. Knockout (KO) of ASCT2 or ASCT1/2 completely eliminates syncytia formation during virus production, which is associated

with reduced cell death and lower accumulation of toxic debris in concentrated viral supernatants as shown by a 3-fold reduction of apoptotic cells after transduction of K562 cells with ASCT2 or ASCT1/2 KO compared to WT-derived viral supernatants. Improved cell fitness and reduced sequestration of viral particles due to elimination of self-infection during virus production led to a 2.2-fold increase in viral titers using ASCT2 KO producer cells, which increased up to 3.6-fold during a larger scale production. The benefit was less pronounced for RD114TR-pseudotyped viral particles (1.5-fold increase in titer) indicating that the excessive syncytia formation observed only with BaEV-pseudotyped viral particles rather than sequestration of viral particles through self-infection during vector production is the main cause of reduced titers. In summary, we demonstrate that knockout of BaEV and RD114 entry receptors eliminates syncytia formation, reduces cytotoxicity of concentrated viral supernatants and improves viral titers. Thus, ASCT2 KO HEK293T cells are a suitable starting point for the generation of a stable packaging cell line to produce BaEVRless-pseudotyped viral particles for future clinical applications.



224. Development of pEMBR™- An Improved Adenovirus Helper Plasmid for AAV Production

Ashley Craddick, Linsey Vanderberry, Mason Bonitz, Corben Davis, Steven Wesel, Michael Swenor, Brooklyn Eagan, Blake Gursky, Adam Davis, David Dismuke

Technology Development, Forge Biologics, Grove City, OH

Treatments for genetic diseases are becoming a reality as gene therapies continue to demonstrate promising results in clinical trials and move through regulatory approvals. For this promise to become realized, safe and effective manufacturing methods need to be employed. One concern for the production of recombinant adeno-associated virus (rAAV) vectors is the size and composition of the plasmid that provides the adenovirus helper genes in transient transfection platforms. Historically, forms of this plasmid have been derived by cloning portions of the adenovirus genome into plasmid backbones using convenient restriction enzyme sites. While this has generated functional adenovirus helper plasmids used by the field, they often contain unnecessary adenoviral genes that could be expressed and might induce a heighten immune response following rAAV administration. Additionally, the common adenovirus helper plasmids are often large, increasing the difficulty and cost of manufacturing as a starting material for GMP production of rAAV. To circumvent these potential issues we designed the pEMBR™ adenovirus-helper gene plasmid. The pEMBR plasmid was configured to deliver the subset of the adenoviral genes required for robust rAAV production in a smaller (12kb) and theoretically safer design. The pEMBR plasmid was synthesized de novo, sequence verified, and scaled up for use in large-scale rAAV manufacturing. Production of rAAV studies were performed to compare vector yields when using the pEMBR plasmid versus other commercially available adenovirus helper plasmids. Vector quality and activity were also assessed from rAAV produced with the different adenovirus helper plasmids to confirm that rAAV produced with pEMBR is equivalent, if not superior. Taken together, these results demonstrate that the pEMBR adenovirus helper plasmid generates rAAV of high yield and quality, in a potentially safer and easier to produce design.

225. Genetically Engineering Packaging Cells to Enhance Titer and Infectivity of Lentiviral Vectors

Jiaying Han[1], Kevin Tam[2], Curtis Tam[2], Roger P. Hollis[2], Donald B. Kohn[2]

[1]Molecular and Medical Pharmacology, UCLA, Los Angeles, CA,[2]Microbiology, Immunology & Molecular Genetics, UCLA, Los Angeles, CA

Lengthy and complex lentiviral vectors (LVs), such as the β-globin LVs commonly used for the treatment of hemoglobinopathies, often have low titers and sub-optimal gene transfer efficiency for human hematopoietic stem and progenitor cells (HSPCs), hindering clinical translation and commercialization for *ex vivo* gene therapy. We elucidated the mechanisms of titer and gene transfer reduction in complex vectors and developed strategies to improve vector RNA and particle production. These studies resulted in a multiply modified packaging cell line and protocol, which creates more effective LVs for gene therapy. Previously, we observed that a high percentage of Lenti-βAS3FB globin LV viral genomic RNAs were incomplete toward the 3′ end in packaging cells and in released vector particles. The incomplete vector genomes impeded reverse transcription in target cells, limiting stable gene transfer to HSPCs. The vector production was further hindered by host antiviral factors in 293T cells, such as protein kinase R (PKR), which inhibits vector protein translation for reverse-oriented LVs. To improve vector RNA and particle production, we explored several strategies: shortening the β-globin vector length from 9 kb to 5.3 kb; packaging with HIV-1 Tat protein; and knocking out *PKR* in 293T cells. In this current study, we have further improved our packaging process by conducting a targeted CRISPR-Cas9-mediated knockout screen and we identified five host antiviral factors that were impeding LV production. Knocking out each these antiviral factors led to a 2~6-fold increase in viral titer. In addition, overexpressing transcription elongation factors improved the production of complete RNA, thereby increasing titer by 2~3 fold. The versatile packaging cell line we created may be used to improve the production of many LVs. As these approaches not only improve titer but also enhance gene delivery, this may facilitate clinical applications by increasing the number of patient doses produced in each batch of LV.

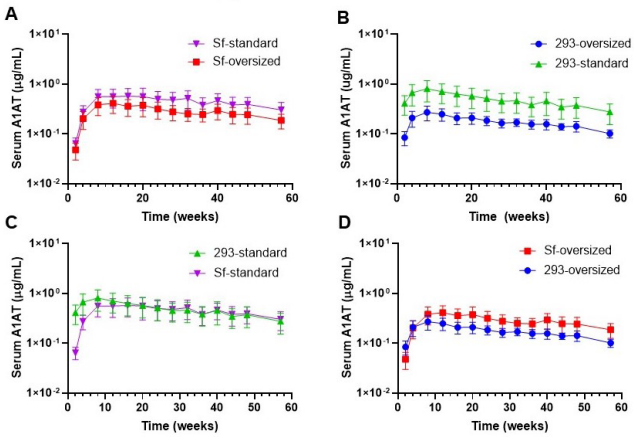
226. Long-term Expression Comparison of Adeno-Associated Virus (AAV) Vector Produced in HEK293 vs Sf Cell Lines

Britta Handyside, Lening Zhang, Bridget Yates, Lin Xie, Choong-Ryoul Sihn, Ryan Murphy, Taren Bouwman, Katina Ngo, Brian Kaplowitz, Natalie Fredette, Olivia Gorostiza, Brian Baridon, Vishal Agrawal, Christa Cortesio, Hassib Akeefe, Jill Woloszynek, Cheng Su, Weiming Zhang, Peter Colosi, Sherry Bullens, Gabor Veres, Stuart Bunting, Sylvia Fong

BioMarin Pharmaceutical Inc., Novato, CA

While production of recombinant adeno-associated virus (rAAV) vectors has predominately occurred using HEK293 cells, growth limitations in this adherent cell line provide a challenge in producing sufficient yields of rAAV for widespread commercial use. *Spodoptera frugiperda* (Sf)-based cell lines offer a more efficient alternative to producing sufficient amounts of standard-sized rAAVs, though questions remain regarding their capacity to efficiently produce larger rAAVs. We compared "oversized" (4970 bp) and "standard-sized" (4600 bp) rAAV5 human A1-antitrypsin (rAAV5-hA1AT) vectors manufactured in HEK293 or Sf cells and assessed the long-term durability of hA1AT protein expression in mice. Mice were treated with $6x10^{13}$ vg/kg of either vector at 8 weeks of age. Blood was collected serially through week 57 for the assessment of circulating hA1AT. Mice treated with Sf-produced standard-sized vector had 65% higher serum hA1AT levels at week 57 than mice treated with Sf-produced oversized vector (**Figure 1A**, $P = 0.0232$). A similar pattern was observed in mice treated with HEK293-produced vectors, with 209% higher serum hA1AT levels noted at week 57 in mice treated with HEK293-produced standard-sized vectors when compared to treatment with HEK293-produced oversized vectors (**Figure 1B**, $P = 0.0015$). Up to 8 weeks post treatment, serum hA1AT levels were higher in mice treated with HEK293-produced standard-sized vector; by week 12 through week 57, there was no difference between HEK293- vs Sf-produced groups (**Figure 1C**). Serum hA1AT levels were comparable in mice treated with Sf- or HEK293-produced oversized vectors for up to 8 weeks post treatment (**Figure 1D**). By week 12 through week 57, mice dosed with Sf-produced oversized vector had slightly higher circulating hA1AT levels compared with the HEK293 group, with 77% greater circulating hA1AT levels noted in mice treated with Sf-produced oversized vector by week 57 when compared to mice treated with HEK293-produced oversized vector. For all groups, levels of circulating hA1AT protein reached steady state by weeks 8-12, and dropped 47%-63% by week 57. Overall, long-term durability of rAAVs produced by either Sf or HEK293 was comparable, albeit with slightly different kinetics in the early phase of expression. Oversized vector produced in either cell line resulted in lower expression levels. Additionally, Sf cells produced oversized vectors in sufficient quantities for use in studies and may represent a viable alternative to HEK293 cells for production of larger rAAV products.

Figure 1. Serum hA1AT levels after AAV5-hA1AT transduction in mice. **A)** Serum hA1AT mice treated with vectors produced in Sf cells, **B)** serum hA1AT in mice treated with vectors produced in HEK293 cells, **C)** serum hA1AT in mice treated with standard-sized vector, **D)** serum hA1AT in mice treated with oversized vector.



AV5-hA1AT, AAV human Factor VIII A1AT; HEK, human embryonic kidney; Sf, *Spodoptera frugiperda*.

227. Processing of Lentiviral Vectors Pseudotypes Using Anion Exchange and Affinity Chromatography

Yuefei Huang, Michael P. Marino, Jakob Reiser

Division of Cellular and Gene Therapies, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Silver Spring, MD

In order to simplify the processing of large volumes of lentiviral vector (LVV) supernatants involving LVVs pseudotyped with VSV-G, the measles virus (MV) hemagglutinin (H), or the Tupaia Paramyxovirus (TPMV) H glycoprotein, we previously implemented a concentration and purification strategy based on anion exchange (AEX) membrane chromatography. LVV supernatants processed in this way showed enhanced purity and reduced toxicity compared to vectors concentrated using standard ultracentrifugation protocols. To improve vector purity, we are currently exploring strategies based on immobilized metal ion affinity chromatography (IMAC) to concentrate LVV pseudotypes bearing hexahistidine (6 x His)-tagged envelope (Env) glycoproteins. Initial experiments focused on LVVs pseudotyped with a 6 x His-tagged, receptor-blind MV H glycoprotein displaying IL-13. Such pseudotypes were captured on HiTrap IMAC HP columns using the AKTA Pure L1 chromatography system and batch eluted using buffers containing imidazole. The recoveries observed were up to 37% of the input samples. We also tested the ability of the IMAC strategy to capture lentiviral vectors pseudotyped with the commonly used VSV-G Env glycoprotein bearing a 6 x His tag at position 8 of the mature VSV-G glycoprotein. The recoveries observed were up to 7% of the input vector sample. The reduced recovery observed using VSV-G pseudotypes compared to MV H pseudotypes is possibly related to limited accessibility of the 6 x His tag present on VSV-G. To improve exposure of the 6 x His tag, we investigated additional sites within the VSV-G ectodomain that can tolerate 6 x His tags while retaining the function of the VSV-G protein. To do this we designed variants with 6 x His tags inserted at positions 2 and 352 of the mature VSV-G glycoprotein. Additionally, we explored double-6 x His tags inserted at positions 2, 8 and 352 of the mature VSV-G glycoprotein. The data obtained showed that the presence of single or double-6 x His tags inserted at position 2 resulted in vector titers comparable to those

obtained using unmodified VSV-G. Vector titers involving VSV-G chimeras with 6 x His tags inserted at positions 8 or 352 resulted in lower titers. Studies aimed at optimizing the conditions for IMAC purification of LVVs bearing 6 x His tagged Env glycoproteins and improving their recovery and stability are ongoing.

228. Separation of Empty Capsids from Full Capsids for AAV Gene Therapy Using a Flow through and Step Elution Approach

Abhiram Arunkumar, Nripen Singh

Technical Operations, Voyager Therapeutics, Cambridge, MA

Adeno-associated viral vectors (AAVs) have emerged as a preferred delivery vector for gene therapies, particularly for the treatment of several particular genetic diseases. During the production of AAVs, the infection process results in the production of both "full" capsids that contain the transgene of interest, and "empty" capsids which do not contain the transgene of interest. Recent studies have indicated that empty capsids can reduce the effective dosage of the vector, and also potentially reduce the efficacy of the AAV therapy by stimulating unwanted immunogenic side effects. Enriching full capsids from empty capsids is thus an important step in the production of AAV gene therapies. While several studies have reported using ion-exchange chromatography to separate empty capsids from full capsids, most of these methods require the use of shallow salt gradients, extremely sensitive flow-rate control (±1% control tolerance) and provide low productivity. This presentation discusses Voyager's efforts to identify effective empty/full separation resins (ligand chemistry, ligand density and particle size) using displacement chromatography to maximize selectivity. Gradient elution experiments are first conducted to identify and screen a range of binding conditions for both the empty and full AAV capsids. Based on the information collected from gradient elution experiments, appropriate conditions have been identified to enrich full AAV capsids. The outcomes of this work indicate that high yield (>70%) and high purity (>80% full) can be achieved in manufacturing-friendly schemes by selectively modulating buffer salts and ligand density.

Metabolic and Muscle Diseases, Tissue and Immunological Engineering

229. Immunogenicity of An AAV-Based, Room-Temperature Stable, Single Dose COVID-19 Vaccine in Mouse and NHP

Nerea Zabaleta[1], Wenlong Dai[1], Urja Bhatt[1], Jessica A. Chichester[2], Julio Sanmiguel[1], Reynette Estelien[1], Kristofer T. Michalson[2], Cheikh Diop[1], Dawid Maciorowski[1], Wenbin Qin[3], Elissa Hudspeth[3], Allison Cucalon[1], Cecila D. Dyer[2], M. Betina Pampena[2], James J. Knox[2], Dan Li[1], Maya Kim[1], Abigail Sheridan[1], Nadia Storm[4], Rebecca I. Johnson[4], Aisling Ryan[1], Ruchi Chauhan[1], Marion McGlynn[2], Brian Price[5], Anna Honko[4], Anthony Griffiths[4], Sam Yaghmour[3], Robert Hodge[6], Michael R. Betts[2], Mason W. Freeman[7], James W. Wilson[2], Luk H. Vandenberghe[1]

[1]Grousbeck Gene Therapy Center, Schepens Eye Research Institute, Mass Eye and Ear, Boston, MA,[2]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Novartis Gene Therapies, San Diego, CA,[4]Department of Microbiology and NEIDL, Boston University School of Medicine, Boston, MA,[5]Albamunity, Boston, MA,[6]Novartis Gene Therapies, Libertyville, IL,[7]Center for Computational & Integrative Biology, Massachusetts General Hospital, Boston, MA

The utility of AAV as a preventative vaccine platform has previously been shown in preclinical and clinical studies. AAV encoding a foreign transgene induces functional B and T cell transgene-specific response. Generally, however, AAVs have a limited pro-inflammatory and even tolerogenic potential, which are both helpful for gene therapy, but undesirable for gene-based vaccines. AAVrh32.33 is a chimera of two natural isolates from rhesus macaques that is uniquely capable of inducing functional anti-viral immunity upon IM injection in mouse and NHP in a vaccine context. The goal of this study was to develop AAVrh32.33-based vaccines against SARS-CoV-2. We have designed two clinically relevant vaccine candidates that consist of AAVrh32.33 carrying the stabilized full-length SARS-CoV-2 Spike (S) or the secreted S1 subunit, named AAVCOVID19-1 (AC1) and AAVCOVID19-3 (AC3), respectively. A single prime IM vaccination elicited high binding and neutralizing antibody responses in two mouse strains, BALB/c and C57BL/6, which were followed for 24 weeks. Robust binding and neutralizing antibody responses against the transgene were developed in all animals treated at high dose ($1x10^{11}$ gc/mouse), while modest reduction was observed in low dose groups ($1x10^{11}$ gc/mouse). AC1 showed a better response in terms of magnitude and neutralizing/binding ratios. Low dose-treated BALB/c animals were boosted with an AAV1 expressing the same antigen as AC1 on week 24 showing a boost in antibody titers. T cell responses were robust by IFN-γ ELISpot, while no response was detected in the IL-4 ELISpot. A pilot NHP study (n=2/candidate), IM dosed with $1x10^{12}$ gc/NHP, revealed a kinetic difference between both candidates. AC3-injected animals reached antibody plateau on week 5 and AC1 had detectable antibodies on week 5 and a continued increase afterwards. Both maintained stable titers (neutralizing 1:640 -1:10,240) until at least 20 weeks post-vaccination.

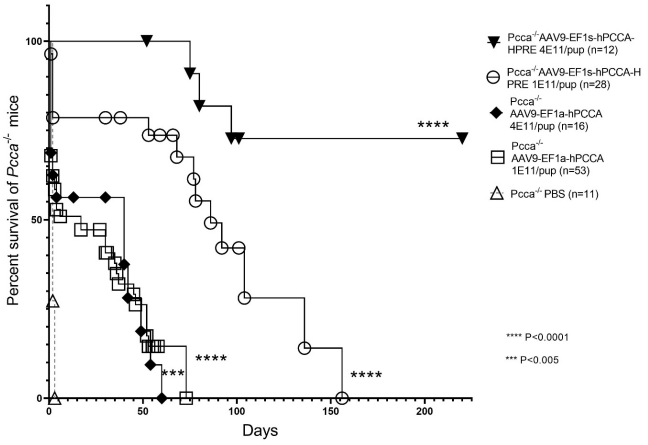
Live virus and pseudovirus neutralizing antibodies followed the same trend as the binding antibodies. T cell responses were slightly higher in AC3-treated animals compared to AC1 animals. Responses against the capsid revealed slow kinetics of neutralizing antibodies that reached plateau around week 16 with low titers (≤1:320). Importantly, no cross-reactivity was detected against AAV serotypes 1,2,5,8 and 9. Currently, a SARS-CoV-2 challenge study in 24 cynomolgus macaques is being performed, in which protection of a lower dose ($1x10^{11}$ gc/NHP) in a single dose will be analyzed. Finally, we have shown in various studies that AAVrh32.33 has low seroprevalence among the population and that AAVCOVID candidates are stable for at least 1 month at 4°C and room temperature. In conclusion, AC1 and AC3 vaccines elicit high antibody responses in various preclinical models and showed quantitative and kinetic differences. Ongoing challenge studies will reveal if lower doses protect NHP from SARS-CoV-2 infection.

230. Systemic AAV9 Gene Therapy Rescues Propionic Acidemia (PA) Mice from Neonatal Lethality and Provides Sustained Therapeutic Benefits

Lina Li[1], Eun-Young Choi[1], Pam S. Head[1], Sam Myung[1], Stephanie Smith[1], Erik Wagner[2], Amy Wang[2], Catherine Chen[2], Xin Xu[2], Elizabeth Ottinger[2], Donald Lo[2], Philip J. Brooks[2], Randy J. Chandler[1], Charles P. Venditti[1]

[1]NHGRI/NIH, Bethesda, MD,[2]NCATS/NIH, Rockville, MD

Background: Propionic acidemia (PA) is a severe autosomal recessive organic acid metabolic disorder caused by propionyl-CoA carboxylase (PCC) deficiency. PCC is a mitochondrial enzyme catalyzes the conversion of propionyl-CoA to D-methylmalonyl-CoA in the pathway of propionyl-CoA oxidation. It is composed of α- and β-subunits encoded by *PCCA* and *PCCB* genes respectively. Mutations in either the *PCCA* or *PCCB* genes can cause PA. In this report, we studied AAV9 gene delivery for PA caused by PCCA deficiency in two murine models of PA. Methods: Two new Pcca alleles - an early nonsense mutation ($Pcca^{p.Q133LfsX41}$) and a hypomorphic missense mutation ($Pcca^{p.A134T}$) - were generated using genome editing and characterized. $Pcca^{p.Q133LfsX41/p.Q133LfsX41}$ mice totally lack PCC activity and manifest uniform lethality by the second day of life, recapitulating the severe neonatal lethal phenotype of PA , while $Pcca^{p.Q133LfsX41/p.A134T}$ mice are viable as adults, yet display markedly reduced PCC activity and mildly elevated metabolic biomarkers of PA. These newly created murine models of PA were then employed to study the efficacy of AAV9 gene therapy. Two AAV9 vectors, AAV9-EF1a-hPCCA and AAV9-EF1s-hPCCA-HPRE, were prepared and used to treat the respective PA mice, either as neonates or young adults. AAV9-EF1a-hPCCA and AAV9-EF1s-hPCCA-HPRE vectors were delivered at low dose (1E11 VG/pup or 8.3E12 VG/kg) or high dose (4E11 VG/pup or 3.3 VG/kg) via retroorbital injection to newborn $Pcca^{p.Q133LfsX41/p.Q133LfsX41}$ mice. Although the untreated $Pcca^{p.Q133LfsX41/p.Q133LfsX41}$ mice experience 100% lethality by DOL 2, both AAV9 vectors provided effective rescue from neonatal lethality that was accompanied by improvements in clinical (prolonged survival, weight gain), metabolic (reduced plasma 2- methycitrate levels), and enzymatic (increased hepatic and cardiac PCC enzymatic activity) parameters. The AAV9-EF1s-hPCCA-HPRE vector appeared more efficacious and was selected for further study (See below Figure). Separate studies used adult (2-5 month) hypomorphic $Pcca^{p.Q133LfsX41/p.A134T}$ mice. One month after systemic delivery via the retroorbital plexus of AAV9-EF1s-hPCCA-HPRE at a vector dose of 5E12 VG/kg , the treated hypomorphic PA mice had AAV9 transgene expression in the liver and heart as measured by RT-qPCR, RNA in situ hybridization, Western blotting, and increased PCC enzyme activity, with vector genomes detected in the same organs. Conclusions: Systemic gene therapy using newly developed mouse models has enabled facile vector testing across the clinical spectrum of propionic acidemia and provides the preclinical foundation to support AAV9 gene delivery for translation to human clinical trials for PCCA deficiency.



231. A Randomized, Double-Blind, Placebo-Controlled, Gene-Delivery Clinical Trial of rAAVrh74.MHCK7.micro-dystrophin for Duchenne Muscular Dystrophy

Jerry R. Mendell[1,2], Perry B. Shieh[3], Zarife Sahenk[1], Kelly Lehman[1], Linda P. Lowes[1], Natalie F. Reash[1], Megan Iammarino[1], Lindsay N. Alfano[1], Jeremy D. Woods[3], Christy L. Skura[3], Howard C. Mao[3], Loretta A. Staudt[3], Rachael A. Potter[1,4], Danielle Griffin[1,4], Sarah Lewis[1,4], Larry Hu[4], Sameer Upadhyay[4], Teji Singh[4], Louise R. Rodino-Klapac[4]

[1]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics and Neurology, The Ohio State University, Columbus, OH,[3]Ronald Reagan UCLA Medical Center, Los Angeles, CA,[4]Sarepta Therapeutics, Inc., Cambridge, MA

Background: Adeno-associated virus (AAV)-mediated gene transfer therapy has shown potential as a treatment for Duchenne muscular dystrophy (DMD). Earlier findings, including those from preclinical studies and a Phase 1b/2 clinical trial, warrant further investigation of gene transfer therapy in DMD. We designed an AAV vector (rAAVrh74) containing a codon-optimized human micro-dystrophin transgene driven by a muscle-specific promoter (MHCK7) with a cardiac enhancer; this gene therapy, named SRP-9001, was tested in a 3-part multicenter Phase 2 clinical trial (NCT03769116, "Study 102"). Part 1 of the trial is a 48-week randomized, double-blind, placebo-controlled period. Part 2 is an ongoing 48-week period in which placebo patients from Part 1 received SRP-9001 in a crossover blinded fashion. Part 3 is

a planned open-label follow-up period that will last up to 212 weeks. **Objective:** The purpose of Study 102 is to evaluate the safety and efficacy of intravenous rAAVrh74.MHCK7.micro-dystrophin (SRP-9001) in patients with DMD. **Methods:** Key eligibility criteria included ambulatory boys aged ≥4 to <8 years with a confirmed *DMD* gene mutation (exons 18-58), an established clinical diagnosis, and stable steroid dosing (≥12 week). The trial randomization was stratified by age group (4-5 years [≥4 to <6], and 6-7 years [≥6 to <8]). In Part 1 of Study 102, 20 patients received SRP-9001 and 21 received placebo. The intended target treatment dose in Study 102 was $1.33x10^{14}$ vg/kg (linear qPCR, supercoiled plasmid standard equivalent of $2x10^{14}$ vg/kg), which is the same dose previously used in Study 101 (NCT03375164). The primary biological endpoint was change in micro-dystrophin expression (measured by western blot; baseline to Week 12). The primary functional endpoint was change in North Star Ambulatory Assessment (NSAA) (baseline to Week 48). Safety endpoints included serious adverse events and treatment-emergent adverse events. **Results:** The primary biological endpoint of micro-dystrophin expression at 12 weeks post treatment was met. Although treated patients showed a positive separation from placebo patients on the NSAA at all timepoints, the trial did not achieve statistical significance on the primary functional endpoint of change in NSAA total score at Week 48 in treatment versus placebo arms (P=0.37). Pre-specified subgroup analysis of the 4- to 5-year-old group showed a statistically significant (P=0.0172) difference between treatment (+4.3) and placebo (+1.9) at Week 48. Although the baseline NSAA score of the 4- to 5-year-old group was well balanced across treatment and placebo arms, it was significantly imbalanced (P=0.0046) in the 6- to 7-year-old group, which may have contributed to the lack of statistical significance on the overall functional endpoint. Treatment-related adverse events were transient and manageable, with no clinically relevant complement activation observed. **Conclusions:** These findings further support the hypothesis that SRP-9001 has a robust biological and physiological effect, which may be clinically relevant in people with DMD. The results of Part 1 of Study 102 reinforce a favorable benefit-risk profile. These data will provide important information for our ongoing clinical development program.

232. Unprecedented Low Dose of AAV-Mediated Gene Transfer Corrects the Pathology in a Model for Fukutin-Related-Protein Deficiencies

Evelyne Gicquel[1], Marine Faivre[1], Susan Brown[2], Laurine Buscara[1], Nathalie Daniele[1], Emmanuel Thevenot[1], Isabelle Richard[1]

[1]Genethon, Evry, France,[2]Comparative Biomedical Sciences, Royal Veterinary College, London, United Kingdom

Mutations in the gene coding for Fukutin related protein (FKRP) can lead to different diseases, ranging from very severe multi-organ syndromes like Walker Warburg Syndrome (WWS) and Muscle Eye Brain disease (MEB), to muscle defects as Congenital Muscle Dystrophy (CMD) and the mildest but more frequent form Limb Girdle Muscle Dystrophy type 2i (LGMD2i or LGMD-R9 FKRP-related). These diseases, all part of the dystroglycanopathies group, are characterized by the defective glycosylation of alpha-dystroglycan (aDG), a membrane glycoprotein involved in the cell/matrix anchoring of muscle fibers. FKRP is one of the multiple proteins involved in the aDG glycosylation process, acting with its partner Fukutin as a ribitol-transferase. We previously published a proof-of-principle of the efficiency of AAV-mediated transfer of the FKRP gene to correct the myopathology in a KI mouse model reproducing the most frequent mutation (L276I) in LGMD-R9. Since the phenotype of this model is relatively modest, we developed a muscle specific FKRP knock-out mouse model, based on Cre-lox recombination technology. This new mouse model, named *HSA-FKRPdel*, has a normal life span but present a severe dystrophic process in the skeletal muscle. Defects of glycosylation of aDG and of its binding to laminin were observed, as well as progressive histological dystrophic signs as centronucleation, inflammation, necrosis and fibrosis. Functional evaluation also revealed a reduced force of *HSA-FKRPdel* mice muscles. Seric and in situ biomarkers of muscle dystrophy pathways were assessed and highlight activation of pathways relative of regeneration, inflammation and fibrosis. The *HSA-FKRPdel* mouse model was then used for a dose effect study of an AAV9 expressing FKRP under the expression of a muscle promoter. The results of the study indicate that a dose of 5e12 vg/kg corrects to the wild-type level the myopathology at the histological and functional levels. No adverse events or signs of toxicity was observed even at 20x the efficient dose. These preclinical studies demonstrated efficacy and safety in AAV-mediated transfer of FKRP and are relevant for defining the doses to be used in clinical trials for FKRP deficiencies.

233. AAV Mediated Apelin Transduction Facilitates Cell Cycle Entry of cTnT-Positive Cells in the Heart

Andrew Park[1], Travis McMurphy[1], Su Mi Choi[1], Weike Bao[2], Alex Alfaro[3], Qing-Dong Wang[2], Herren Wu[1], Yasuhiro Ikeda[1]

[1]Antibody Discovery & Protein Engineering, AstraZeneca, Gaithersburg, MD,[2]Early Cardiovascular Renal and Metabolism, AstraZeneca, Gaithersburg, MD,[3]Clinical Pharmacology & Safety Sciences, AstraZeneca, Gaithersburg, MD

Aims Terminally differentiated cardiomyocytes do not undergo mitotic division, resulting in minimal cardiac regenerative capacity upon injury. Previous studies have reported that apelin-13 can enhance cardiac repair via enhanced proliferation of myocardial stem cells and/or induction of neovascularization in the injured heart. Here our aim is to test the impact of AAV-mediated apelin gene delivery on the proliferation of cardiac cells in vivo. **Methods** AAV9 vectors encoding a full length pro-apelin was produced using triple plasmid transfection and purified through iodixanol gradient. 2E11 genome copies of AAV vectors were intravenously administered into C57bl/6 mice (4-8 weeks old). After 5 days of post AAV injection, mice were put under drinking water with BrdU (0.8mg/ml) for 10 days. At 15 days of post AAV injection, mouse hearts were harvested for FACS analysis, immunohistochemistry (IHC) and transcriptomic analysis. Serum samples were also harvested to measure circulating levels of apelin-13 by ELISA. **Results** AAV administration resulted in a 5-fold increase in apelin-13 levels in vivo when compared to the control group. IHC analysis found increased number of Ki67 (cell proliferation marker)-positive cells in the heart sections. When cardiac cells were dissociated and characterized for BrdU uptake and expression of a cardiomyocyte

marker cTnT by FACS, we found BrdU-positive cells were mostly cTnT-positive and increased 5-fold in the AAV-apelin-treated group. RNAseq analysis identified activation of cell cycle and cell division pathways, with marked upregulation of CDK1, KIF14, BUB1B, PLK1, RGCC, TTK, and KNTC1 genes, upon AAV-apelin treatment. **Conclusions** AAV-mediated pro-apelin gene delivery achieved sustained apelin-13 overexpression in mice, which induced cell cycle entry of cTnT-positive cells in the heart. This strategy may offer a cardiac regenerative therapy for the injured heart.

234. Vectorized SARS-CoV-2 Human IgG Expression in Mice and Ovine Animal Models Is Feasible and Well Tolerated

Amira D. Rghei[1], Laura P. van Lieshout[1], Benjamin McLeod[1], Yanlong Pei[1], Hugues Faust[2], Gary P. Kobinger[2], Brad Thompson[3], Byram W. Bridle[1], Leonardo Susta[1], Khalil Karimi[1], Sarah K. Wootton[1]

[1]Pathobiology, University of Guelph, Guelph, ON, Canada,[2]Département de microbiologie-infectiologie et d'immunologie, Université Laval, Quebec City, QC, Canada,[3]Avamab Pharma Inc., Calgary, AB, Canada

The novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged from China in late 2019. Though two SARS-CoV-2 vaccines have received regulatory approval for emergency use in North America, reactogenic mRNA-based vaccines have been contraindicated in some frail elderly individuals. The elderly and immunocompromised have a lessened ability to respond robustly to classical antigen-based vaccines. Given the 2019 novel coronavirus pandemic is disproportionately affecting the elderly, it is important to have a prophylactic approach that would avoid dependence on the endogenous immune system.Monoclonal antibody (mAb) therapy is a proven therapeutic modality for preventing and treating infectious diseases. While there has been a deluge of well-characterized, potent mAbs providing protection against SARS-CoV-2, production of clinical grade mAbs for passive immunization is labour-intensive, technically challenging, and results in short-lived immunity. One strategy to circumvent the problems associated with passive immunization is to use the host as a "bioreactor" to produce neutralizing mAbs *in vivo* by employing a method called vectored immunoprophylaxis (VIP). VIP consists of a single intramuscular injection of an adeno-associated viral (AAV) vector encoding pathogen-specific broadly neutralizing mAbs that get secreted into the bloodstream.In this study, we vectorized three human mAbs (31C2, 15A7 and CR3022) that bind the SARS-CoV-2 spike protein, two of which were isolated from convalescent patients (15A7 and 31C2) and characterized their expression and safety profiles in mice. AAV-mAb genomes were packaged into the AAV6.2FF capsid and injected intramuscularly (IM) into eight BALB/c mice at 1e11 vector genomes (vg). AAV6.2FF-31C2 expressed human (h)IgG in the serum with a peak average plasma concentration of 175.1 ug/mL and an average of 276.5 ug/mL hIgG in bronchoalveolar lavage fluid. AAV6.2FF-15A7 and -CR3022 expressed lower peak average plasma concentrations of 22.7 μg/mL and 30.1 μg/mL, respectively. To investigate potential toxicity or pathology associated with AAV-mAb expression in mice, groups of male and female BALB/c mice were administered AAV6.2FF-31C2 IM at doses of 1e11 vg, 2e11 vg and 6e11 vg, with cohorts of each dose evaluated at endpoints of 7, 28 and 56 days post AAV administration. A dose dependent increase in 31C2-hIgG in murine plasma was observed between the low, mid and high doses, with averages of 67.9 μg/mL, 127.7 μg/mL and 137.8 μg/mL, respectively. Biodistribution of AAV in various tissues revealed dose dependent increase in vg copy number in the kidney, liver and brain was observed between cohorts, whereas no difference was observed in the lungs, heart and spleen. Serum biochemical parameters and hematological histology indices were all normal.A second animal study utilizing lambs was conducted to establish the feasibility of AAV-mAb expression in a large animal model. Three two-week-old male lambs were administered 1e13 vg/kg of AAV6.2FF-31C2 IM in the rump. hIgG expression increased steadily throughout the 28-day study, resulting in day 28 concentrations of 21.4- 46.7 μg/mL, indicating practical scale up from rodent to large animal models. Blood samples were collected weekly and serum biochemical parameters and hematological indices were all normal. Histopathology on injection site, lungs, liver, heart, spleen, kidney, pancreas, testes, and brain and did not reveal any signs of toxicity. This data demonstrates the tolerability and feasibility of AAV-VIP in a large animal model.

235. Intravenous AAV5 Gene Therapy with Human CYP21A1 Corrects Phenotypic Deficiencies of the 21-Hydroxylase Kockout Mouse Model and Demonstrates Durability and Safety in Non-Human Primates and Mice

Rachel Eclov[1], Sophie Le Fur[1,2], David Scott[1], Christine Dos Santos[3], Marie-Pierre Belot[1,2], Mayank Kapadia[1], Terra Lewis[1], Jeremy Rouse[1], Kirsten Romero[1], Alexandre Stella[4], Cyndie Goumeaux[1,2], Philippe Liere[2], Philippe Hantraye[5], François Amalric[4], Clayton Beard[1], Pierre Bougnères[1,2]

[1]Adrenas Therapeutics, BridgeBio, Raleigh, NC,[2]Therapy Design Consulting, Vincennes, France,[3]INSERM U1195, Paris, France,[4]Proteomics Unit, CNRS, Institut de Pharmacologie et de Biologie Structurale, Toulouse, France,[5]Molecular Imaging Research Center, Neuratris, DRF-CEA, Frontenay-aux-Roses, France

Classic 21-OH-deficiency (21OHD) due to defects in the *CYP21A2* gene leads to deficiencies in cortisol and aldosterone, resulting in 5-fold higher mortality, adrenal crises, short stature, virilization, subfertility, obesity, and cardiovascular disease. BBP631, an AAV5 gene therapy expressing human *CYP21A2,* is being developed for systemic delivery to patients with 21OHD. BBP-631 was administered IV to $Cyp^{-/-}$ 15-week old mice, and disease phenotypes compared with untreated $Cyp^{-/-}$ mice for 10 weeks. Vector genome copies (VGC) of human *CYP21A2* transgene and mRNA normalized to mouse GAPDH were measured in adrenals and other organs. Adrenal 21-OH protein expression was evaluated using customized nano-LCMSMS proteomics to detect a peptide specific to human 21-OH, normalized to GAPDH peptides. Treated mice demonstrated a dose dependent weight gain that correlated with adrenal VGC, transgene mRNA level, and 21-OH protein expression. The elevated plus maze test showed improvement in the response of $Cyp^{-/-}$ mice to stress in proportion to the vector dosage. Accumulation of progesterone, the only substrate of 21-OH in mice, was detected using stable isotope dilution mass spectrometry and was reduced in BBP-631 treated mice. Gene therapy with BBP-631 of

Cyp$^{-/-}$ mice was robust and dose dependent at the molecular level and for the correction of disease phenotypes. A biodistribution and safety study was performed in 2-2.5-year old NHPs (cynomolgus macaques). Following IV administration BBP-631 no adverse safety signals for clinical observations, body or organ weights, urinalysis, hematology, serum chemistry, gross pathology, histopathology (including thoracic spinal cord), immunohistochemistry (no CD4lymphocyte infiltrates), or anti-drug responses (cellmediated immune responses to AAV5 capsid or human 21-OH) across all doses. Dosedependent VGC and stable mRNA transcript expression were detected in adrenals through Week 24. Proteomic methods showed a dose-dependent and persistent expression of human 21-OH protein up to 9 to 24% of endogenous 21-OH levels. Overall, the results indicate that delivery of BBP631 is well tolerated with persistent mRNA and protein expression in adrenals of NHPs for a duration of at least 24 weeks. In WT mice, a GLPcompliant toxicology study evaluated safety (clinical observations, pathology, hematology, clinical chemistry, histopathology, and immune response) and biodistribution at Weeks 4, 12 and 24. Vector genome and transgene expression were detected in adrenal gland and other tissues. No test-article related toxicity was observed at any study timepoint or dose tested following a single IV administration of BBP-631. This study demonstrates that BBP-631 is welltolerated with a No Adverse Event Level (NOAEL) of 3.0×10^{14} vg/kg, and has durable transgene transfer and expression in adrenal glands. Non-clinical findings support BBP631 therapeutic potential in a first-in-human clinical trial.

New Technologies Advancing Gene Therapy for Neurologic Diseases

236. Combined Transgene and Intron-Derived miRNA Therapy Reverses Motor Phenotypes in SCA1 Mice

Ellie M. Carrell[1], Megan S. Keiser[1], Beverly L. Davidson[1,2]

[1]Raymond G. Perelman Center for Cellular and Molecular Therapeutics, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Spinocerebellar ataxia type I (SCA1) is an autosomal dominant neurodegenerative disease caused by a (CAG) expansion within the coding region of the *ATXN1* gene. Native ATXN1 incorporates into transcriptional repression complexes, and aberrant incorporation of polyglutamine-expanded ATXN1 has recently been shown to be the primary driver of neurotoxicity in the cerebellum. As a therapeutic strategy, we have previously demonstrated that virally expressed RNA interference (RNAi) molecules targeting *ATXN1* can prevent and reverse disease phenotypes in SCA1 mice. We have also shown that overexpression of the ATXN1 homolog, ATXN1-like (ATXN1L) can improve disease readouts when delivered pre-symptomatically, presumably through displacement of mutant ATXN1. Given the efficacy of both approaches in isolation, we developed a dual expression transgene that incorporates an ATXN1-targeting miRNA (miS1) into an upstream intron of a human ATXN1L (hATXN1L) minigene (AAV.hATXN1L int2 miS1). Here we tested whether this approach could effectively reverse disease phenotypes in SCA1 mice. Varying doses (6.48e7 to 8e9 vg) of vector were delivered directly to the deep cerebellar nuclei of SCA1 mice at 12 weeks of age following motor symptom onset as measured by rotarod performance. Motor phenotypes were again assessed by rotarod at 22 weeks, prior to tissue harvest. Ongoing experiments show that delivery of our dual expression transgene can arrest or improve motor deficits in B05 mice following symptom onset. While motor performance of animals injected with saline deteriorated over the course of the experiment, all vector-treated groups showed significant increases in latency to fall over saline treated animals at 22 weeks. Molecular analyses further indicate tolerability in mouse brain supporting development of our vector for therapeutic use.

237. Combining MALDI-Based Metabolic Imaging and Molecular Analysis for Effective and Informative Assessment of Therapeutic Efficacy in Sub-Anatomical Regions of the CNS after rAAV Gene Therapy

Dominic J. Gessler, Anoushka Lotun, Guangping Gao

Gene Therapy Center, UMass, Worcester, MA

Gene and cell therapies have introduced new therapeutic avenues that are reshaping the medical landscape, especially for previously untreatable conditions. Therapeutic efforts for central nervous system (CNS) diseases are posed with particular challenges due to the brain's cellular heterogeneity and degree of subanatomical specialization. Previous work indicates that systemically delivered recombinant adeno-associated virus (rAAV) transduces different brain regions in a serotype-dependent manner; however, the implications of this transduction heterogeneity on therapeutic efficiency are poorly understood. To address this question, we hypothesized that different brain regions might require different amounts of rAAV transduction and thus transgene expression to achieve therapeutic effect. We used a mouse model lacking expression of the aspartoacylase (Aspa$^{-/-}$) gene, which leads to pathognomonic differences in N-acetylaspartate (NAA) distribution across the brain, thus serving as a readout for transgene activity. These Aspa$^{-/-}$ brains display spongiform degeneration and myelin loss, particularly in subcortical regions such as the thalamus, cerebellum, and brainstem. Using Matrix-Assisted Laser Desorption/Ionization (MALDI)-based metabolic imaging, we found NAA levels in wildtype (Wt) brains were similar across different brain regions. In contrast, Aspa$^{-/-}$ brains showed elevated NAA levels in regions corresponding with the most prominent myelin loss and degeneration. Juvenile Aspa$^{-/-}$ mice were then injected intravenously with rAAV expressing a wildtype (treatment group) or a mutated, enzymatically non-functional, Aspa gene (control group) and sacrificed at either 1 or 4 weeks after treatment. In the treatment group, we found that NAA was homogenously reduced to the levels similar to Wt brains, contrasting that of the control group which instead resembled Aspa$^{-/-}$ mice. We also analyzed other critical metabolites, including myelin lipids, and found a time-dependent change between the two treatment time points. Interestingly, this time-dependency was associated with changes in certain subanatomical regions. Given that serotype-dependent rAAVs variably transduces different brain regions, this suggests that the therapeutic effect might be dependent on the transgene levels. To test this hypothesis, we used digital PCR to quantify rAAV genomes

and transgene mRNA in the same brain regions selected for metabolic imaging. Surprisingly, brain regions, e.g., cerebellum, demonstrated low transgene expression but high therapeutic effect as seen by histopathology and MALDI imaging. This was particularly interesting because it indicates that the therapeutic gene dose requirements differ between brain regions and per metabolite, a finding which may be informative for other pathologic conditions and thus might guide further fine tuning of the gene therapy design. In conclusion, combining metabolic imaging and molecular analysis allow for the characterization of pathological hotspots and spatial-temporal high-resolution molecular quantification of gene therapy efficacy and thus can foster the understanding of gene therapy application for disease processes.

238. Evolution of Modified AAV in Rhesus Macaque Brain

Yong Hong Chen[1], Paul T. Ranum[1], Megan S. Keiser[1], Xueyuan Liu[1], Congsheng Cheng[1], David Leib[1], Geary Smith[1], Amy Muehlmatt[1], Luis Tecedor[1], Beverly L. Davidson[1,2]

[1]Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Molecular gene therapy tools have become capable of addressing almost infinite combinations of genetic lesions impacting innumerable cell types. Delivering these tools to the anatomical location and cell types required by each therapy remains an important bottleneck. Here, we report an ongoing directed AAV evolution screen in nonhuman primates (NHPs) to identify a library of AAVs with diverse transduction properties capable of meeting the needs of current and future gene therapy designs. We first generated peptide-modified AAV libraries by addition of random peptides into AAV1, AAV2, and AAV9, followed by two rounds of *in vivo* enrichment via delivery into the cerebrospinal fluid (CSF). The input library, and viral DNA recovered from 14 brain regions collected after each round were subjected to NGS, along with RNA from the round 2 tissues. Our NGS data show successful enrichment of barcodes from AAV1, AAV2, and AAV9 libraries yielding different candidates enriched for each serotype. We observed that each of the AAV1, AAV2, and AAV9 libraries had unique enrichment characteristics, with AAV1 and AAV2 yielding more total capsid variations. AAV1 and AAV2 top hits were variable across tissues and represented a lower percentage of observed capsid variants. In comparison, AAV9 had a lower number of total capsid variants detected and a top hit representing >40% of capsid variants detected in some tissues. From the AAV9 derived capsid library, the top-ranking variant "1999," was consistently the most enriched across all tissues tested in both DNA and RNA, constituting a hit with very broad transduction capacity. Our screen also yielded examples of vectors with highly specific tropism, for example, the AAV1-derived capsid variant "1382" was found to be highly enriched in both round2 DNA and RNA hits from Ependyma, and was largely absent from other tissues. In-vivo fluorescent reporter validation of capsid variants "1999" and "1382" in both Mouse and NHPs confirmed broad and highly specific transduction patterns respectively. These results confirm the capacity of our pipeline to produce AAV capsid variants with diverse traits enabling gene therapy approaches with distinct delivery requirements. By combining the rich capsid enrichment datasets with our purpose-built analytical pipeline, we have created a NHP AAV-capsid database from which users can identify candidate AAV capsids with user-defined properties.

239. Real-Time MR Tracking of AAV Gene Therapy with Enzyme-Activated MR Probes

Toloo Taghian[1], Ana Rita Batista[1], Sarah Kamper[2], Michael Caldwell[2], Laura Lilley[2], Paola Rodriguez[1], Katerina Mesa[1], Shaokuan Zheng[3], Robert M. King[3,4], Matthew J. Gounis[3], Sophia Todeasa[1], Anne S. Maguire[5], Douglas R. Martin[5], Miguel Sena-Esteves[1], Thomas J. Meade[2], Heather L. Gray-Edwards[1]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Northwestern University, Evanston, IL,[3]University of Massachusetts Medical School, Worcester, MA,[4]Worcester Polytechnic Institute, Worcester, MA,[5]Auburn University, Auburn, AL

Transformative results of adeno-associated virus (AAV) gene therapy in patients have demonstrated unequivocally the potential of *in vivo* gene transfer to treat monogenic diseases. However, the field lacks a non-invasive way to assess expression and duration of gene expression. Here we describe a new MR imaging technology for real-time reporting of enzyme-based therapeutics in any organ. For the first-time, we show reliable tracking of enzyme expression after AAV gene therapy encoding lysosomal acid β -galactosidase (β-gal) using a self-immolative β-gal responsive probe. This gadolinium-based agent has an exposed arm that is the enzymatic target of the therapeutic, which, upon cleavage, results in MR signal enhancement. We have previously shown that GM1 mice (β-gal$^{-/-}$) treated with AAV encoding β-gal (GLB-1), results in global distribution, with highest β-gal levels in the CNS after intracranial delivery and in the liver after intravenous (IV) administration. Similarly, intrathecal (IT) administration of β-gal-responsive contrast agent, 1 month after administration of AAV (10^{10} vg, thalamus, unilateral, n=4), resulted in strong MR signal enhancement in CSF and parenchyma. Enhancement of CSF occurred rapidly after intrathecal administration (< 20 minutes) with an increasing ratio of parenchymal signal / CSF signal that indicates penetration of contrast agent from the CSF into the brain tissue. Distribution of the probe within the brain was greatest on the ventral aspect with a gradient projecting dorsally. IT administration of the agent in wild type mouse (n=2) resulted in partial enhancement, however; the enhancement was absent in the untreated β-gal$^{-/-}$ mouse (n=2). The detection range was linear and extended to at least 3-logs with a strong correlation between enhancement and β-gal enzyme activity within the brain (R^2=0.81). Intraperitoneal administration of the contrast agent, 1 month after AAV9 treatment of β-gal$^{-/-}$ mouse (IV, $3X10^{11}$ vg, n=4), resulted in sharp rise of signal over the first 40-50 minutes after which it plateaued for the duration of the MRI (> 2 hours). Enzymatic activity of AAV-IV treated, WT (n=2) and β-gal$^{-/-}$ mouse (n=2) correlated with enhancement (R^2 = 0.94). This class of contrast agents can be modified to interact with the active sites of other enzymes and could be extended to other gene therapies, enzyme replacement

therapy and/or CRISPR based technologies. With >1800 gene therapies in phase I/II clinical trials, development of non-invasive methods to assess gene expression is crucial to the future of the gene therapy field.

240. Inclusion of a Degron Reduces Levels of Undesired Inteins after AAV-Mediated Protein *Trans*-Splicing in the Retina

Patrizia Tornabene[1], Ivana Trapani[1], Miriam Centrulo[1], Elena Marrocco[1], Renato Minopoli[1], Mariangela Lupo[1], Carolina Iodice[1], Carlo Gesualdo[2], Francesca Simonelli[2], Enrico Maria Surace[3], Alberto Auricchio[1]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy,[2]University of Campania L. Vanvitelli, Naples, Italy,[3]Federico II University, Naples, Italy

Split intein-mediated protein *trans*-splicing expands AAV gene transfer, thus overcoming AAV limited cargo capacity. However, non-mammalian inteins persist as *trans*-splicing by-products, and this could raise safety concerns for AAV intein clinical applications. In this study, we tested the ability of several degrons to selectively decrease levels of inteins after protein *trans*-splicing, and found that a version of *E. coli* Dihydrofolate reductase, that we have shortened to better fit into the AAV vector, is the most effective. We show that subretinal administration of AAV intein armed with this short degron is both safe and effective in a mouse model of Stargardt disease (STGD1) which is the most common form of inherited macular degeneration in humans. This supports the use of optimized AAV intein for gene therapy of both STGD1 and other conditions which require transfer of large genes.

241. Drug-Regulated Splicing Switch for Gene Expression Control

Alex Mas Monteys, Amiel A. Hundley, Paul T. Ranum, Euyn Lim, Luis Tecedor, Amy Muehlmatt, Beverly L. Davidson

University of Pennsylvania/CHOP, Philadelphia, PA

To date, gene therapies for human application rely on engineered promoters that cannot be finely controlled. Here, we report a universal switch element that allows precise control for gene silencing or gene replacement after exposure to a small molecule. Importantly, these small molecule inducers are in human use, are orally bioavailable when given to animals or humans, and can reach both peripheral tissues and the brain. Moreover, the switch system (Xon) does not require the co-expression of any regulatory proteins. Using Xon, translation of desired elements for gene knockdown or gene replacement occurs after a single oral dose, and expression levels can be controlled by drug dose or in waves with repeat drug intake. This universal switch can provide temporal control of gene editing machinery and gene addition cassettes that can be adapted to cell biology applications and animal studies. Additionally, due to the oral bioavailability and safety of the drugs employed, the Xon switch provides an unprecedented opportunity to refine gene therapies for more appropriate human application.

242. Targeted Gene Therapy with Engineered Systemic AAVs for the Central and Peripheral Nervous Systems Prevents Motor Coordination Phenotypes in a Mouse Model of Friedreich's Ataxia

Acacia Hori, Anastasiya Grebin, J. Elliott Robinson, Xinhong Chen, Nick Flytzanis, Nick Goeden, Ken Chan, Ben Deverman, Viviana Gradinaru

Biology and Biological Engineering, California Institute of Technology, Pasadena, CA

Friedreich's Ataxia (FA) is a genetic disease characterized by progressive ataxia and cardiomyopathy. In FA, insufficient expression of frataxin (FXN) increases cell susceptibility to damage from oxidative stress. Neuronal degeneration in the deep cerebellar nuclei and dorsal root ganglia (DRGs) cause loss of motor coordination, while cardiomyopathy in FA contributes to early death. Systemically delivered AAVs can non-invasively target genetic cargo to diverse sites throughout the body, with some serotypes able to reach the CNS. However, at high doses, these vectors can cause severe toxicity, emphasizing the need for targeted, efficient gene delivery vectors. In this study, we used engineered AAVs to target FXN to cell types of pathophysiologic relevance to FA, while de-targeting the liver and cell types typically spared in human disease. A DNA plasmid containing FXN and its putative gene regulatory elements and control constructs were packaged into AAV.CAP-B10[1] and AAV-PHP.PNS2, two next generation systemic AAVs that have demonstrated transduction biases toward CNS and PNS respectively. A cocktail of both capsids packaging either the FXN or control cargo were delivered intravenously to a pilot cohort of young adult FA model mice (inducible shRNA-based FXN knockdown mice (FXNiKD)[2], n=7 per group) 12 weeks prior to doxycycline-induction of the disease phenotype. Motor, sensory and cardiac function was assessed using the beam traversal test, gait analysis, weightlifting and electrocardiography before quantitative tissue analysis was performed to assess FXN levels and pathologic hallmarks. Exogenous FXN expression mimicked the endogenous patterns of non-diseased mice in the CNS and DRGs, with reduced liver expression. Importantly, prophylactic AAV-FXN expression prevented induction of motor and coordination deficits as measured by foot slips and time to traverse the narrowing beam, compared to controls, with performance resembling wildtype littermates. We are investigating whether it is also possible to reverse existing motor deficits and pathology in mice using targeted AAV-FXN intervention after induction of the disease phenotype. These findings demonstrate the utility of engineered AAVs for pre-clinical research to test precision gene therapies in neurodegenerative disease models.[1] Flytzanis, N.C., Goeden, N., Goertsen, D., Cummins, A., Pickel, J., Gradinaru, V. Broad gene expression throughout the mouse and marmoset brain after intravenous delivery of engineered AAV capsids. bioRxiv 2020.06.16.152975; doi: https://doi.org/10.1101/2020.06.16.152975 [2] Chandran V, Gao K, Swarup V, Versano R, Dong H, Jordan MC, Geschwind DH. Inducible and reversible phenotypes in a novel mouse model of Friedreich's Ataxia. Elife. 2017 Dec 19;6:e30054. doi: 10.7554/eLife.30054. PMID: 29257745; PMCID: PMC5736353.

Oligonucleotide Therapeutics

243. Robust RNA Editing via Recruitment of Endogenous ADARs Using Circular Guide RNAs

Dhruva Katrekar[1], James Yen[1], Yichen Xiang[1], Anushka Saha[1], Dario Meluzzi[2], Yiannis Savva[3], Prashant Mali[1]

[1]Bioengineering, University of California, San Diego, La Jolla, CA,[2]Department of Medicine, University of California, San Diego, La Jolla, CA,[3]Shape Therapeutics, Seattle, WA

Akin to short-hairpin RNAs and antisense oligonucleotides which efficaciously recruit endogenous cellular machinery such as Argonaute and RNase H to enable targeted RNA knockdown, simple long antisense guide RNAs can recruit endogenous adenosine deaminases acting on RNA (ADARs) to enable programmable A-to-I RNA editing, without requiring co-delivery of any exogenous proteins. This approach is highly specific, however the efficiency is typically lower than seen with enzyme overexpression. Conjecturing this was due in part to the short half-life and residence times of guide RNAs, here we engineer highly stable circular ADAR recruiting guide RNAs (cadRNAs), which can be delivered not only by genetically encoding on DNA vectors, but also transfection of RNA molecules transcribed *in vitro*. Using these cadRNAs, we observed robust RNA editing across multiple sites and cell lines, in both untranslated and coding regions of mRNAs, vastly improved efficiency and durability of RNA editing, and high transcriptome-wide specificity. High transcript-level specificity was achieved by further engineering to reduce bystander editing. Additionally, *in vivo* delivery of cadRNAs via adeno-associated viruses (AAVs) enabled robust 38% RNA editing of the mPCSK9 transcript in C57BL/6J mice livers, and 12% UAG-to-UGG RNA correction of the amber nonsense mutation in the IDUA-W392X mouse model of mucopolysaccharidosis type I-Hurler (MPS I-H) syndrome. Taken together, cadRNAs enable efficacious programmable RNA editing with application across diverse protein modulation and gene therapeutic settings.

244. *In Vivo* Delivery of AAV.U7 Induce Efficient Exon Skipping for a Mutational Hotspot of the *DMD* Gene Results in Protein Restoration & Force Improvement in Skeletal Muscles, Heart & Diaphragm

Dhanarajan Rajakumar[1], Ding Li[1], Daniel Lesman[1], Caleb Gaffney[1], Yacidzohara Rodriguez[1], Courtney Young[2,3], Melissa J. Spencer[2,3], Kevin M. Flanigan[1,4], Nicolas Wein[1,4]

[1]Center for Gene Therapy, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH,[2]Molecular Biology Institute, University of California, Los Angeles, CA,[3]Department of Neurology, University of California, Los Angeles, CA,[4]Departments of Pediatrics, The Ohio State University, Columbus, OH

Two clinical phenotypes are caused by mutations of the *DMD* gene. Duchenne Muscular dystrophy (DMD), a severe and common muscular dystrophy is caused by absence of dystrophin. Milder Becker muscular dystrophy (BMD) associated with truncated but functional dystrophin. Mutations which do not affect the reading frame of the transcript leads into milder phenotype. Exon skipping is based on restoring the reading frame of the transcript by masking exon/intron definition sites by antisense oligonucleotides. As a result, a truncated but functional dystrophin is being produced similarly as what is observed in BMD. Traditionally used antisense oligonucleotides (AON) have two limitations 1. stability - requires repeated injections; 2. tropism - limited entry in major affected organs (e.g. heart) upon systemic administration. To overcome these disadvantages, we and others demonstrated an alternative approach i.e., AAV.U7 mediated antisense delivery. An antisense sequence is carried by a small nuclear RNA (in this study, U7snRNA) to increase the stability and using a viral vector such as adeno-associated virus (AAV) to improve tropism. Based on our promising results for mice and patients carrying an exon 2 duplication, we decided to utilize a similar approach for another mutational hot spot in the DMD gene: the exon 44. Skipping of exon 44 could be beneficial to ~6-8% of patients. We initially evaluated the efficiency of our candidates *in vitro* using patients derived skin biopsy converted into transdifferentiated myoblasts. Our objective in this study was to evaluate the efficiency of our lead candidate *in vivo* via intramuscular and systemic deliveries. We used hDMD/mdx del45 mice in this study, a new humanized DMD mice model (with mdx background) that is missing human exon 45. This model express neither mouse nor human dystrophins and presents a dystrophic muscle pathology. We evaluated exon skipping at the mRNA level using RT-PCR and protein restoration using western blot and immunostaining. Muscle function was assessed using muscle physiology apparatus. *In vivo,* 1- and 3-months post intramuscular injections, exon 44 skipping was efficient (≥80%), which led to expression of a truncated dystrophin (at least ≥50%) and associated proteins. Muscle force was tested by measuring tibialis anterior specific force and eccentric contraction. Both demonstrated muscle improvement (by ~50% compared to untreated mouse). Based on our previous success with a different AAV.U7 program for DMD, we did systemic minimal efficacious dose (MED) studies. Three months post systemic MED data shows about 60% of dystrophin restoration in heart muscle and improvement in TA /diaphragm force generation with the highest dose. To explore systemic efficiency using another lead candidate, we performed an additional study using similar high dose and evaluated it for 3 months post injection. This vector restored dystrophin (≥50%) in different muscles (TA, diaphragm and heart), significantly improved muscle eccentric contraction (≥50%) in TA and muscle specific force in TA and diaphragm. To conclude, our lead candidate can induce efficient *DMD* exon 44 skipping, resulting into dystrophin production and muscle strength improvement in major muscle groups affected in DMD. This AAV.U7-exon 44 skipping vector represent a promising candidate that could help ~6-8% of DMD patients.

245. A Versatile Platform for ADAR-Mediated RNA Editing In Vivo in Preclinical Models

Prashant Monian

Wave Life Sciences, Cambridge, MA

The ADAR (adenosine deaminases acting on RNA) family of enzymes catalyze adenine (A) to inosine (I) changes in RNA, which are read as guanine (G) by the translational machinery. Recruiting endogenous

ADAR enzymes using chemically modified oligonucleotides to direct specific editing holds great promise for treating human disease. PRISM™, Wave Life Sciences' proprietary discovery and drug development platform, enables us to develop stereopure oligonucleotides—those in which the chiral configuration of backbone linkages (*R*p or *S*p) are precisely controlled at each position—that direct sequence-specific RNA editing using endogenous ADAR enzymes. We will present preclinical *in vivo* data in mouse and non-human primates (NHPs) showing that stereopure oligonucleotides are effective when delivered as naked oligonucleotides or GalNAc-conjugates in the absence of any delivery vehicle via systemic, intracerebroventricular or intrathecal administration. We show editing in myriad tissues, including liver, multiple regions of the CNS, kidney and lung. The most promising oligonucleotides support up to 50% editing *in vivo*. These preclinical investigations lay the foundation for development of RNA-editing therapeutics with potential to treat human genetic disease.

246. Human miRNA mir-675 Inhibits DUX4 Expression and May Be Exploited as a Potential Treatment for Facioscapulohumeral Muscular Dystrophy

Nizar Y. Saad[1], Mustafa Al-Kharsan[2], Sara E. Garwick-Coppens[1], Gholamhossein Amini Chermahini[1], Madison A. Harper[1], Andrew Palo[1], Ryan L. Boudreau[3], Scott Q. Harper[1]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Department of Neurology, UNM School of Medicine, Alburquerque, NM,[3]Department of Internal Medicine, University of Iowa, Iowa City, IA

Double homeobox protein 4 (DUX4) emerged as an important gene because of its linkage to Facioscapulohumeral muscular dystrophy (FSHD), a devastating disease affecting up to 870,000 people worldwide. The DUX4 locus is normally repressed by heterochromatin in adult muscle, but silencing mechanisms are absent or reduced in FSHD muscle, thereby permitting DUX4 expression. When present in muscle, the DUX4 gene product, a transcription factor, activates genes involved in cell death and dysfunction, leading to muscular dystrophy. The most direct route to FSHD therapy will likely involve inhibiting DUX4 in muscle, but currently there are no approved DUX4-targeted treatments that slow disease progression or improve muscle weakness. In prior work, we designed artificial microRNAs targeting DUX4 mRNA for degradation through the RNAi pathway and demonstrated that RNAi-based gene therapy could improve FSHD-associated phenotypes in FSHD mice and human myotubes. Here we pursued an alternative and completely novel strategy to treat FSHD with RNAi therapy, using a natural microRNA mir-675. Accordingly, we provided the first evidence that a natural human microRNA, mir-675, could silence DUX4 expression via RNAi and counteract DUX4-associated phenotypes. We then developed two novel FSHD therapies relying upon mir-675 upregulation with gene therapy and using small molecules. Both strategies resulted in decreased expression of DUX4 and DUX4-associated outcomes in FSHD mice and human FSHD myotubes. To our knowledge, this is the first study demonstrating the use of small molecules to suppress a dominant disease gene using an RNAi mechanism.

247. The FORCE™ Platform Achieves Robust Knock Down of Toxic Human Nuclear *DMPK* RNA and Foci Reduction in DM1 Cells and in Newly Developed hTfR1/DMSXL Mouse Model

Stefano Zanotti, Cody Desjardins, Nelson Hsia, Timothy Weeden, Ryan J. Russo, Lydia Schlaefke, Monica Yao, Aiyun Wen, Scott Hildebrand, John Najim, Qifeng Qiu, Brendan Quinn, Kim Tang, Mo Qatanani, Romesh Subramanian, Oxana Beskrovnaya

Research, Dyne Therapeutics, Waltham, MA

Myotonic dystrophy type I (DM1) is a rare, life-threatening neuromuscular disease caused by the toxic expansion of CUG repeats in the *DMPK* mRNA. The toxic mutant *DMPK* transcript is trapped in the cell nucleus where it forms hairpin-loop structures that sequester MBNL splicing factors, form nuclear foci, and consequently lead to splicing defects that drive disease progression in skeletal, cardiac, and smooth muscle. Therapies with antisense oligonucleotides (ASO) designed to reduce *DMPK* mRNA have shown promise as potential DM1 treatments; however, their translation to the clinic has been hampered by insufficient delivery to muscle. To overcome the limitations of ASO delivery to cardiac, skeletal, and smooth muscle, we developed the FORCE™ platform by conjugating a fragment antibody (Fab) against the human transferrin receptor (hTfR)1, which is highly expressed on the surface of muscle cells, with ASOs targeting human *DMPK* mRNA. To determine the potential of FORCE conjugates to correct the DM1 phenotype *in vitro*, we tested their ability to knock down the human *DMPK* transcript, reduce nuclear foci, and correct splicing defects in myoblast cultures from DM1 patients with different numbers of CUG repeats. We report that the FORCE conjugate selected as our lead candidate for DM1 caused over 50% *DMPK* mRNA knock down in cells with either ~380 or ~2600 *DMPK* CUG repeats, leading to a reduction in nuclear foci and correction of splicing defects measured by *BIN1* exon 11 inclusion. To enable efficacy analysis of our lead candidate against toxic human *DMPK* mRNA *in vivo*, we generated an innovative hTfR1/DMSXL mouse model that expresses the *hTfR1* transgene and carries a human *DMPK* gene with more than 1,000 CTG repeats, which is representative of a severe DM1 phenotype. In agreement with the results in DM1 patient-derived cells, intravenous injections of our lead candidate in hTfR1/DMSXL mice resulted in a 39 - 60% reduction of the toxic human nuclear *DMPK* mRNA in the gastrocnemius, tibialis anterior, diaphragm, and heart. In these mice, our lead candidate reduced levels of human *DMPK* foci in the heart, confirming the findings from the mRNA expression analysis. In conclusion, studies with a fully human FORCE conjugate targeting human *DMPK* mRNA produced encouraging data regarding the potential of the FORCE platform to be an effective therapy for patients afflicted with DM1.

248. *In Vivo* Delivery of Suppressor tRNA Overcomes a Pathogenic Nonsense Mutation in Mice

Jiaming Wang*[1,2], Yue Zhang*[1,2], Craig A. Mendonca*[1], Lingzhi Ren[1], Jialing Liang[1], Chen Zhou[1], Jia Li[1], Guangping Gao[a1,2,3], Dan Wang[a1,4]

[1]Gene Therapy Center, University of Massachusetts, Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[3]Li Weibo Institute for Rare Diseases Research, University of Massachusetts Medical School, Worcester, MA,[4]RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

A nonsense mutation introduces a premature termination codon (PTC) in mRNA that prevents full-length protein synthesis, and accounts for ~11% of human pathogenic mutations. Engineered tRNAs that can decode a termination codon and readthrough a PTC are known as suppressor tRNAs (stRNAs), and have long been considered as potential therapeutic agents targeting PTCs. However, their *in vivo* therapeutic efficacy and safety have been understudied compared to other readthrough agents such as aminoglycosides. In this study, we aim to develop an *in vivo* stRNA therapy delivered by AAV vectors as platform therapeutics for nonsense mutations, such as the *IDUA-W402X* mutation found in the majority of Hurler syndrome patients. We first screened a panel of stRNAs for readthrough activity in HEK293 cells and Hurler syndrome patient fibroblasts that carry homozygous *IDUA-W402X* (TGG>TAG) nonsense mutation; this mutation abrogates the lysosomal enzyme iduronidase (IDUA) activity. One stRNA was able to restore IDUA enzymatic activity above the targeted therapeutic threshold of 0.5% of normal level. This stRNA gene was packaged as recombinant AAV9 (rAAV9) and systemically delivered to a mouse model of Hurler syndrome harboring the *Idua-W392X* mutation (TGG>TAG) analogous to the human mutation. At 10 weeks post-treatment, serum IDUA activity was restored up to 6% of wildtype (WT) level, accompanied by 70% reduction in urine glycosaminoglycans (GAGs), the primary substrates accumulated due to IDUA deficiency. Furthermore, IDUA activity was restored to 9% and 27% of WT levels in the liver and heart, respectively, which led to significant decrease in tissue GAGs (95% and 66% reduction in the liver and heart, respectively). Elevation of other lysosomal enzymes triggered by GAG accumulation, a hallmark of several lysosomal disorders including Hurler syndrome, was almost completely normalized. Local but not systemic rAAV9.stRNA delivery to the brain and skeletal muscle achieved significant IDUA activity restoration, which correlates with stRNA gene delivery efficiency. These results demonstrate that rAAV9.tRNA could alleviate whole-body disease burden and tissue pathology caused by the *Idua-W392X* nonsense mutation. Ribosome profiling of patient fibroblasts revealed that, whereas G418 (the benchmark readthrough aminoglycoside) induced high global readthrough at normal stop codons and strong perturbation in translation elongation, stRNA had a much milder impact and only acted on the UAG stop codon. Similarly, the rAAV9-stRNA-treated mouse liver exhibited UAG-restricted global readthrough and unperturbed translation elongation, indicating a better safety profile than aminoglycoside. We did not observe gross abnormalities nor any changes of comprehensive serum clinical chemistry in the treated mice. tRNA sequencing is ongoing to assess endogenous tRNA homeostasis following stRNA treatment. The functional stRNAs identified in this study could be potentially used to treat a range of diseases caused by a UAG PTC. Compared to gene replacement and CRISPR-based gene editing, the small gene size of stRNA is highly amenable to AAV vector delivery, and lack of foreign protein expression has a favorable immunological profile. *These authors contributed equally to this work. [a]Co-corresponding authors.

249. The 3´tsRNAS Are Aminoacylated Further Implicating Their Role in Ribosome Biogenesis during Tissue Homeostasis and Cancer

Ziwei Liu[1], Hak Kyun Kim[2], JIngpeng Xu[3], Mark A. Kay[3]

[1]Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA,[2]Chung-Ang University, Seoul, Korea, Republic of,[3]Stanford University School of Medicine, Stanford, CA

Emerging evidence indicates that tRNA-derived small RNAs (tsRNAs) are involved in fine-tuning gene expression and become dysregulated in various cancers. There are at least 6 major types of tsRNAs derived from the mature or precursor tRNA genes in the genome. We recently showed that the 22nt LeuCAG tsRNA from 3´ end of the mature $tRNA^{Leu}$ is required for efficient translation of two ribosomal protein mRNAs and ribosome biogenesis. Inactivation of this tsRNA induced apoptosis in rapidly dividing cells and in patient-derived orthotopic hepatocellular carcinomas implanted in mouse liver (Kim et al., Nature 2017). Here we used bioinformatic algorithms to establish the expression profiles of the more than 150, 3'-tsRNAs present in human normal versus tumor paired tissues. We found a number of 3'tsRNAs that are up or down regulated in nine human cancers evaluated to date suggesting they may play a role in the oncogenic process perhaps by regulating the cell's protein synthetic capacity. The mechanism involved in the generation of the 3´-tsRNAs has remained elusive and it is unclear if the 3´-ends of 3´-tsRNAs are aminoacylated. Here we report an enzymatic method utilizing exonuclease T to determine the 3´charging status of tRNAs and tsRNAs. Our results showed that the LeuCAG 3´-tsRNA is fully charged and originated solely from the charged mature tRNA. When the leucyl-tRNA synthetase was knocked down, less tsRNA was generated while the mature tRNA was not. Because the aminoacyl-tRNA synthetases form a complex on actively translating ribosomes, our results support a model where the tsRNAs are generated during translation and finely tune ribosomal protein synthesis, and thus regulate ribosome biogenesis during homeostasis and altered growth conditions including cancer. These small RNAs represent novel targets for treating cancer.

Pharmacology/Toxicology Studies or Assay Development

250. Liquid-Biopsy-Integration-Site-Sequencing Allows Safety Studies and Longitudinal Monitoring of Vector Integration Sites in LV and AAV-Based In-Vivo GT Applications

Daniela Cesana[1], Andrea Calabria[1], Laura Rudilosso[1], Pierangela Gallina[1], Fabrizio Benedicenti[1], Giulio Spinozzi[1], Monica Volpin[1], Giulia Schiroli[1], Alessandra Magnani[2], Marie Pouzolles[3], Francesca Fumagalli[1], Valeria Calbi[1], Maximilian Witzel[4], Frederic D. Bushman[5], Alessio Cantore[1], Pietro Genovese[1], Naomi Taylor[3], Valérie S. Zimmermann[3], Christoph Klein[4], Alain Fischer[2], Marina Cavazzana[2], Emmanuelle Six[2], Alessandro Aiuti[1], Luigi Naldini[1], Eugenio Montini[1]

[1]Division of Regenerative Medicine, SR-TIGET, Milan, Italy,[2]Laboratory of Human Lympho-Hematopoiesis, INSERM, Paris, France,[3]Institut de Génétique Moléculaire de Montpellier, CNRS, Montpellier, France,[4]Dr. von Hauner Children's Hospital, LMU, Munich, Germany,[5]Department of Microbiology, UPeNN, Philadelphia, PA

Clonal tracking techniques based on vector integration enable monitoring of the fate of engineered cells in the blood of gene therapy (GT) patients and allow assessment of the safety and efficacy of these procedures. However, due to the limited number of cells that can be tested and the impracticality of studying cells residing in peripheral organs without performing invasive biopsies, this approach provides only a partial snapshot of the clonal repertoire and dynamics of genetically modified cells and reduces the predictive power as a safety readout.We have developed Liquid Biopsy Integration Site sequencing (LiBIS-seq), a PCR technique optimized to quantitatively retrieve vector integration sites (IS) from cell-free DNA (cfDNA) released into the bloodstream by dying cells residing in different tissues. We applied LiBIS-seq to cfDNA purified from plasma samples harvested at different time points post-transplant from 7 patients of the lentiviral (LV)-based hematopoietic stem cell (HSC) GT trial for metachromatic leukodystrophy and retrieved >10,900 IS. The IS analyses on cfDNA confirmed that all patients had a polyclonal repertoire and no signs of genotoxicity as reported previously. Furthermore, LiBIS-seq enabled the early detection of a T-cell lymphoma expanding in thymus and triggered by insertional activation of LMO2 in a patient of a γ-retroviral-based clinical trial for X-linked severe combined immunodeficiency. Of note, the aberrant expansion was not identified by conventional IS analyses carried on circulating cells. We next evaluated whether LiBIS-seq can detect aberrant clonal expansions in the context of in vivo liver-directed GT applications. To reach this goal, we systemically injected a genotoxic LVs in newborn mice (N=14) to experimentally induce hepatocellular carcinoma and histiocytic sarcoma by insertional mutagenesis. IS retrieved from the DNA of tumor-bearing tissues and cfDNA collected overtime demonstrated that the malignant and highly dominant clones can be efficiently identified also by LiBIS-seq in up to 75% of treated-mice. These data demonstrate that LiBIS-Seq can overcome the need of solid tissue biopsies for safety studies through IS analyses in in vivo GT applications. Finally, we challenged the ability of LiBIS-seq in retrieving AAV IS from the cfDNA of ZAP70 knock-out mice that were intrathymically injected by an AAV8 expressing the therapeutic transgene (N=8). By using a specific multiplexed PCR protocol, we retrieved 829 AAV IS from lymph-node DNA and, more importantly, 207 AAV IS from the cfDNA collected over time. Both IS datasets showed an enrichment of AAV IS within the TCR-a chain gene, likely due to the high level of RAG-mediated somatic recombination occurring in this locus during TCR maturation which favors AAV integration. In conclusion, LiBIS-Seq can interrogate the clonal repertoire of genetically modified cells residing in solid organs in a dynamic fashion and without the need to perform invasive solid tissue biopsies and allows to perform predictive safety studies in LV and AAV-based in-vivo GT applications.

251. Physiologically Based Pharmacokinetic Modeling for the Biodistribution of Adeno-Associated Virus Serotype 8 after Intravenous Administration in Mice and Non-Human Primates

Kefeng Sun[1], Zhiwei Zhang[2], Glen Ko[2], Erica L. Bradshaw[3]

[1]Shire Human Genetic Therapies, A Takeda Company, Cambridge, MA,[2]RES Group Inc., Needham, MA,[3]Quantitative Solutions, Takeda California Inc., San Diego, CA

Introduction: For recombinant adeno-associated virus (AAV) gene therapies, biodistribution studies in nonclinical species are typically conducted prior to clinical trials. Such studies are generally time consuming, require large numbers of animals (especially when multiple time points are included), and incur high cost for the sponsor. Physiologically based pharmacokinetic (PBPK) modeling has been widely used to predict whole body distribution of therapeutic modalities such as small molecules and proteins. Given the fact that the tissue tropism of an AAV vector is mostly determined by its serotype, a PBPK model could thus be utilized to describe and to project the biodistribution time course of recombinant AAVs with the same serotype regardless of the DNA cassette sequence within. **Methods**: Using a middle-out modeling approach, an ordinary differential equation (ODE) based, whole body PBPK model for intravenously (IV) administered AAV serotype 8 (AAV8) was constructed utilizing in vitro and in vivo vector genome (VG) data from mouse and non-human primates (NHP) in the public domain, and qualified with a separate set of in vivo VG data. The tissues and matrices included in the model were plasma, liver, spleen, heart, kidney, lymph nodes, skeletal muscle, brain, gastrointestinal tract, pancreas and bone marrow. Important physiological processes of the AAV captured by the model included organ blood flow, tissue permeation, uptake and pre-uptake elimination, intracellular trafficking, and formation and loss of episomal DNA. **Results**: The PBPK model was able to accurately capture the short-term (0-21 days post-dose) and long-term (21 days to several years) durability of AAV vector genomes in somatic tissues (in VG per diploid genome or VG per µg DNA), as well as the time course of AAV concentration in circulation (in VG per mL), in both mouse and NHP. Key findings include: (1) Uptake of AAV8 via Kupffer cells

and the spleen macro-phagocytic system and other systemic routes eliminated up to 50% of all injected AAV dose prior to tissue uptake; (2) Mouse and NHP displayed quantitatively similar rates of uptake for the same somatic tissues and overall biodistribution pattern of AAV8; (3) While liver accounted for 80%-90% of all AAV8 that was taken up into tissues, 90% to 95% of all AAV8 internalized by hepatocytes was degraded by proteasomes and non-specific mechanisms in the nucleus, eventually yielding episomal DNA at around 1% (range: 0.3% to 3%) of the total equivalent AAV dose by 3 weeks post-dose; and (4) Long-term rates of loss of episomal DNA in tissues could be partially explained the tissue cell lifespan. **Conclusion**: The novel PBPK model for IV-administered AAV8 can provide *a priori* knowledge of durability of the presence of vector genomes in multiple organs, thereby informing nonclinical and clinical study designs in AAV dose, matrix collection and sampling time points. The model also establishes a foundation for projecting biodistribution for other AAV serotypes after recalibration with serotype-specific in vivo data.

252. LC-MS Confirmation of Single Amino Acid Correction by Base Editing

Bo Yan, Valerie J. Winton, S. Haihua Chu, Michael S. Packer, Calvin Lee, Sarah Smith, Adam Hartigan, Carlo Zambonelli, Francine M. Gregoire, Manmohan Singh, Giuseppe Ciaramella

Beam Therapeutics, Cambridge, MA

Base editing is emerging as a powerful next generation editing technology. Base editing provides precise single base editing without introducing cutting of DNA or RNA strands. It has enabled diverse therapeutic strategies since 2016. There are multiple analytical assays needed to measure the base editing efficacy. First, DNA editing efficiency (A to G or C to T editing) can be quantified by next generation sequencing (NGS) as a straightforward readout. However, genomic level information by itself does not account for potential post-transcriptional regulation and post-translational modifications that can occur with gene expression. Thus, detection of single amino acid mutations expressed at the protein level is a key component to confirm the mechanism of action (MoA) and efficacy of base editing. Developing adequate analytical assays to confirm precise base editing at the protein level is critical and challenging. For example, the traditional ELISA method relies on generation of antibodies that specifically recognize point mutations on proteins. The generation and screening of antibodies may not always be feasible, and this process adds a significant amount of effort and cost to the assay development. Here, we describe applications using liquid chromatography mass spectrometry (LC-MS) for multiple analytical assays to confirm the precise correction at the amino acid level by base editing. We discuss assay development strategy and unique challenges. We will also present case studies of assay development for ex vivo and in vivo base editing therapies, such as in sickle cell disease and alpha-1 antitrypsin deficiency. In summary, LC-MS provides unique solutions for confirmation and quantitation of single amino acid corrections after base editing.

253. Lack of Germline Transmission in Male Mice Following Administration of AAV5-hFVIII-SQ, an Investigational Gene Therapy for Hemophilia A

Carlos Fonck, Cheng Su, Jeremy Arens, Elli Koziol, Jaydeep Srimani, Joshua Henshaw, Andrea Van Tuyl, Sundeep Chandra, Christian Vettermann, Charles A. O'Neill

BioMarin Pharmaceutical Inc., Novato, CA

Introduction. Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) is an adeno-associated virus serotype 5-mediated gene therapy encoding a B-domain deleted human factor VIII gene controlled by a hepatocyte-specific protomer currently under investigation as a treatment for hemophilia A. In this study, we assessed the potential for germline transmission of the hFVIII-SQ transgene from treated male mice to their offspring following a single intravenous injection. A novel 2-stage model was used to estimate statistical power, defined as the probability of detecting germline transmission to ≥1 F1 offspring. **Methods.** B6.129S6-*Rag2*^{tm1Fwa} N12 male mice received a single intravenous injection of vehicle solution (Group 1, n = 14) or $6x10^{13}$ vector genomes (vg)/kg of AAV5-hFVIII-SQ (Group 2, n = 16; Group 3, n = 34). Group 1 and 2 males were mated with naïve females on day 4 post-dose, when vg concentration in semen was expected to peak, and Group 3 males were mated with naïve females on day 37 post-dose, when 1 full spermatogenesis cycle was expected to complete. The presence of transgene DNA in liver and testes from the F0 males and liver from the F1 offspring was evaluated using quantitative polymerase chain reaction. In addition, FVIII protein levels were measured in F0 males using an enzyme-linked immunoabsorbent assay to confirm that AAV5-hFVIII-SQ administration resulted in successful cell transduction and transgene protein expression. The probability of germline transmission of the hFVIII-SQ transgene was estimated using a novel 2-stage statistical method that estimated a confidence range based on the number of F0 males dosed with AAV5-hFVIII-SQ and the number of F1 offspring sired by those males (**Figure 1**). **Results.** Following AAV5-hFVIII-SQ administration, transgene DNA was detected in the liver and testes of all F0 male mice in Groups 2 and 3, confirming that successful gene transfer occurred. In evaluated Group 3 F0 males, 28/33 had detectable hFVIII protein in their plasma. In total, 77, 59, and 80 F1 offspring were assessed from Groups 1, 2, and 3, respectively. No F1 mice sired by F0 males dosed with vehicle were positive for transgene DNA. Similarly, no transgene DNA was detected in the livers of F1 offspring sired by F0 males dosed with AAV5-hFVIII-SQ ($6x10^{13}$ vg/kg; Groups 2 and 3; **Figure 2**). Based on power calculations using the 22 F0 males that had offspring in Groups 2 and 3 and their combined 139 F1 pups, there is 99.2% confidence that the risk of germline transmission is <5%. No adverse clinical observations or other findings in F0 males or F1 offspring were attributed to AAV5-hFVIII-SQ. **Conclusion.** There was no evidence of transmission of the hFVIII-SQ transgene through the germline from male mice who received a single $6x10^{13}$ vg/kg dose to their offspring, following mating at 2 different time points. Novel statistical methods to estimate the probability of detecting ≥1 germline transmission event following gene transfer confirmed that it is highly unlikely.

Figure 1. Study power calculation

$$\text{Probability (detecting transgene DNA in ≥1 F1 offspring)} = 1 - \prod_{i=1}^{N}[1 - P_1 + P_1 * (1 - P_2)^{m_i}]$$

Where

P_1 is the probability an F0 male acquires and transmits transgene DNA to the F1 offspring.

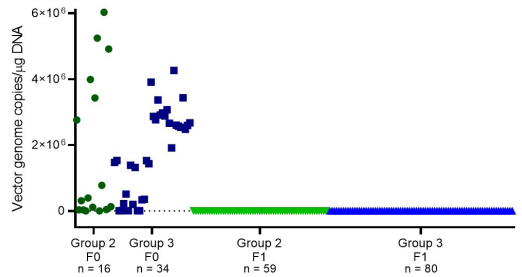
P_2 is the probability an F1 offspring inherits and retains the transgene DNA.

$P_1 * P_2$ is the total probability of germline transmission.

N denotes the number of F0 males with successful mating.

m_i denotes the number of F1 offspring from the i^{th} F0 male.

Figure 2. Vector genome copy number in individual liver tissue samples



F0 males and F1 males and females are shown. No vector genome DNA was detected in Group 1 F0 or F1 samples.

254. DNA Sequence Analysis of Recombinant Adeno-Associated Viral Integrations Events Recovered from Hepatocellular Carcinomas in Mice Reveals Enhancer Insertion as the Mechanism of Vector Genotoxicity

Randy J. Chandler[1], James C. Mullikin[2], NISC Comparative Sequencing Program[2], Charles P. Venditti[1]

[1]MGMMGB, National Institutes of Health, Bethesda, MD,[2]NISC Comparative Sequencing Program, National Institutes of Health, Bethesda, MD

Background: Donsante et al. first noted the development of hepatocellular carcinoma (HCC) in mice after recombinant adeno-associated virus (rAAV) exposure in 2001, and in 2007, documented that rAAV insertions in the *Rian* locus widely dysregulated expression of *cis* genes around the integration. Over the past decade, numerous other groups, working with varied mouse models, have reported similar findings, specifically that rAAV exposure causes mice to develop HCC in later life. We previously studied a large number of AAV HCCs that formed after the treatment of neonatal mice, and noted that many, but not all, of the integration events mapped into a tiny 92 base pair microRNA locus (*Mir341*) that is only present in mice and rats (Chandler et al 2016). Furthermore, an influence of the vector enhancer and promoter on genotoxic risk was documented. Motivated by the report of pathogenic wildtype AAV integrations in human HCCs, the documentation of clonal rAAV expansions in canine models of hemophilia, and now, the very recent description of HCC in hemophilia patient following rAAV gene therapy, we have undertaken studies to fully characterize the genomic features of pathogenic AAV integrations to understand the mechanism(s) underlying vector toxicity.Methods: Using a combination of whole genome sequencing (10X genomics) and targeted PCR amplification, we completely sequenced 5, and partially sequenced 6, rAAV integrations from 11 unique HCCs, each arising in a different mouse.Results: The full length rAAV integration events contained a truncated vector genome that ranged in size from 428 to 1,216 base pairs, and lacked any portion of the therapeutic transgene. Common features included a truncated 5' ITR, the endogenous AAV enhancer element, and the enhancer element from the vector transgene. The structure of the insertions varied, with some integrations harboring an additional internal truncated 5' ITR juxtaposed to a vector enhancer and promoter, suggesting that the event derived from a tail to head concatemer. Of note, one rAAV integration additionally contained the origin of replication (*ori*) from the plasmid used to package the vector. Conclusions: The presence of both AAV and transgene enhancers as minimally conserved vector elements in all HCC integrations indicates that enhancer insertion is the mechanism of rAAV genotoxicity, and tumorigenesis ensues in part, due to the upregulation of *cis* genes. Furthermore, the lack of a vector encoded cDNA in any of the recovered rAAV integrations likely explains why the therapeutic transgene was not detected at high copy numbers in AAV HCCs in previous studies. The fact that contiguous plasmid sequences were also detected in one AAV HCC integration raises an additional concern regarding potential vector toxicity, and highlights the need to implement sequence based quality control measures in AAV manufacturing. As rAAV gene therapy becomes widely translated, HCC, a common GI malignancy, may continue to be observed in treated patients, and it will then become even more critical to understand rAAV integration and to incorporate observations from animal models to aid in the design of inherently less genotoxic, and safer, gene therapy vectors.

255. Towards a Clinical Trial of Gene Therapy for Retinitis Pigmentosa Associated with Usher Syndrome Type IB

Rita Ferla[1,2], Fabio Dell' Aquila[1], Monica Doria[1], Ivana Trapani[1], Maria Ferraiuolo[3], Alessia Noto[3], Fabiana Grazioli[3], Virginia Ammendola[3], Stefano Colloca[3], Alberto Auricchio[1]

[1]Telethon Institute of Genetics and Medicine, Pozzuoli, Italy,[2]Department of Science Translational Medicine, Federico II University, Naples, Italy,[3]Reithera srl, Castel Romano, Italy

Usher syndrome type IB (USHIB) due to biallelic mutations in the *MYO7A* gene, is the most severe form of Usher syndrome, which is the most common form of retinitis pigmentosa associated with hearing loss.To transfer the large MYO7A gene to the retina, we have developed dual hybrid adeno-associated viral vector serotype 8 (dual AAV8.h*MYO7A*) which reconstitute full-length MYO7A protein to therapeutic levels in the Shaker-1 (sh1) mouse model of USHIB. In view of a gene therapy clinical trial for USHIB retinitis pigmentosa, we performed a GLP-compliant non-clinical study to assess the safety of dual AAV8.h*MYO7A*.A 13-weeks study was conducted in non-human primates (NHPs) using a GMP-like lot of dual AAV8.h*MYO7A*. NHPs received a single sub-retinal injection in the right eye of either the formulation buffer as control (CTR group) or the dual AAV8.h*MYO7A* at one of the following doses: a low dose of $1.37x10^{12}$ (LD group) and a high dose of $3.75x10^{12}$ total genome copies (GC)/eye (HD group), which correspond to 1.6X and 4.3X the highest dose proposed for

the clinical trial, respectively.Sub-retinal injection of formulation buffer was well-tolerated. Eyes treated with dual AAV8.h*MYO7A* showed microscopic alterations that were dose-dependent in terms of severity, and improved at the end of the study compared to the interim evaluation (6-9 weeks post-injection), suggesting recovery over time. Optical coherence tomography (OCT) supported the histopathological findings with areas of retinal degeneration and presence of hyper-reflective sub-retinal material away from the injection site, as well as alteration of the IS/OS line in the macula, exclusively in the HD group. Consistent with this, electroretinography (ERG) showed a dose-dependent reduction of retinal function, which improved over time with some eyes in the LD-group falling in the range of normality at the end of the study.In conclusion, our data showed that sub-retinal administration of dual AAV8.h*MYO7A* at the low dose results in a mild toxicity which improved over time. Importantly, some of the observed alterations were similar to those previously reported for AAV8 administered in NHPs at similar doses and then safely used in humans. Based on this, we believe that this safety study, once completed by biodistribution, expression and immunology data, will pave the way for a gene therapy clinical trial for USHIB retinitis pigmentosa.

256. A GLP Safety and Biodistribution Study of AXO-Lenti-PD Manufactured via Two Processes Delivered at a Higher Volume and Flow Rate

Thomas Pack[1], Peter Ross[1], Barak Gunter[2], Mark Johnson[3], Michelle Kelleher[4]

[1]Early Development Group, Sio Gene Therapies, Durham, NC,[2]Surgical Safety Assessment Group, Charles River Laboratories, Mattawan, MI,[3]Safety Assessment Group, Northern Biomedical Research, Norton Shores, MI,[4]Translational Sciences Group, Oxford Biomedica, Oxford, United Kingdom

AXO-Lenti-PD is an EIAV-based lentiviral gene therapy being developed for Parkinson's disease that delivers the 3 enzymes needed to convert striatal neurons into dopamine factories. In this GLP study, we evaluated the safety, toxicology, biodistribution, and immunogenic potential of $4x10^6$ TU/hemisphere AXO-Lenti-PD when delivered bilaterally via a SmartFlow™ cannula into the putamen regions of the brains of NHPs. For this safety study, we investigated a higher volume (250 µL/hemisphere) and flow rate (up to 5 µL/min) than previously used (100 µL/hemisphere delivered at up to 3 µL/min) as well as the effects of AXO-Lenti-PD produced with a new suspension-based manufacturing process. The study groups were: 28- and 90-day vehicle groups (n=2 per group), 28- and 90-day LacZ (surrogate vector) groups (n=2 per group), 28- and 90-day adherent process manufactured AXO-Lenti-PD groups (n=4 per group), and a 90-day suspension process manufactured AXO-Lenti-PD group (n=3). Safety and toxicology were assessed by monitoring the clinical and neurological status of the animals and by pathological analysis of CNS and non-CNS tissues. Biodistribution and shedding were assessed by qPCR and RT-qPCR analysis, respectively, with extensive tissue sampling of the brain, peripheral organs, and shedding matrices (serum, urine, saliva, surgical site swabs, and CSF). Immune responses were evaluated via Western blots against viral and transgene components. In-life observations did not show any effects of AXO-Lenti-PD (adherent or suspension) or LacZ on clinical observations, body weight, ophthalmoscopic examination, functional/neurological behavior, and cardiac examination. There were no effects of LacZ or AXO-Lenti-PD (adherent or suspension) on peripheral organ pathology. In the brain, neuropathological review found no adverse microscopic findings associated with LacZ or either AXO-Lenti-PD (adherent or suspension) treatment. Any microscopic findings in the brain were non-adverse and limited to minimal to slight, mononuclear cell infiltrates in and near infusion sites with no evidence of neuroinflammation or neurodegeneration. In the day 90 groups, residual inflammatory responses in the brain were consistent between vehicle-, LacZ- and AXO-Lenti-PD (adherent and suspension)-administered groups and tissues exhibited the expected healing responses to the trauma associated with cannulation and infusion into the brain. Biodistribution of AXO-Lenti-PD (adherent or suspension) was localized primarily to the injection target brain area. AXO-Lenti-PD expression in distal brain region (eg. cerebellum) or peripheral organ samples (including PBMCs) was undetectable with no constant expression. In all groups, there was no evidence of viral shedding at any time point, consistent with primary localization of the virus to the injection target region. Immune responses were limited to the viral components in both AXO-Lenti-PD-adherent (7/8 animals - VSV-G viral envelope; 2/8 animals - EIAV p26 capsid) and AXO-Lenti-PD-suspension (1/3 animals - VSV-G; 1/3 animals - EIAV p26 capsid) groups. There were no responses against the transgene components in any AXO-Lenti-PD-treated animal. Overall, the results of the study did not identify any LacZ- or AXO-Lenti-PD-related adverse effects associated with a higher volume and flow rate of infusion or any differences in the safety profile of adherent- or suspension-manufactured AXO-Lenti-PD material.

RNA Virus Vectors

257. Combinatorial Relief of Multiple Innate Immune Blocks Allows Efficient Gene Engineering of Quiescent Human Hematopoietic Stem Cells

Erika Valeri[1,2], Giulia Unali[1,2], Francesco Piras[1], Ivan Cuccovillo[1], Nathaniel R. Landau[3], Anna Kajaste-Rudnitski[1]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Vita-Salute San Raffaele University, School of Medicine, Milan, Italy,[3]Department of Microbiology, NYU School of Medicine, New York, NY

Quiescent human hematopoietic stem and progenitor cells (HSPC) remain highly refractory to genetic manipulation potentially due to the expression of high levels of antiviral innate immune factors. The HIV-1 restriction factor SAMHD1 interferes with reverse transcription (RT) by decreasing the nucleotide pools, in non-dividing, quiescent cells such as macrophages but its role in HSPC has been poorly investigated. To address the role of SAMHD1 in human HSPC, we used lentiviral vectors (LV) that directly incorporate into vector particles the SIV accessory protein Vpx, which degrades SAMHD1. While Vpx-incorporation gave only a modest benefit in transduction efficiency in HSPC pre-stimulated with growth-promoting cytokines, a significant

advantage was observed when transducing freshly isolated, quiescent HSPC. Vpx was additive with Cyclosporin H (CsH), a compound we have shown to overcome an innate block to vector entry in HSPC that acts prior to the SAMHD1-imposed block to RT. However, the most striking benefit was observed when CsH was combined with addition of deoxynucleosides (dNs) during transduction of quiescent HSPC, yielding up to 60% of transduction at the moderate multiplicity of infection (MOI) of 25. Importantly, this benefit was particularly strong in the most primitive $CD34^+CD133^+CD90^+$ fraction of HSPC from either cord blood or the clinically relevant mobilized peripheral blood source resulting in an average 8-fold increase in gene transfer efficacy over control cells. Of note, similarly to Vpx, adding dNs did not benefit transduction in pre-stimulated HSPC even in combination with CsH, suggesting that nucleotide pools are not a limiting factor in less quiescent cells. Experiments to assess engraftment and transduction levels of long-term HSC *in vivo* are on-going. Because shorter *ex vivo* culture of HSPC preserves their engraftment capacity *in vivo*, we hope to achieve an ideal combination of high engraftment and efficient transduction using the combination of CsH and dNs in unstimulated HSPC. Overall, our results indicate that multiple innate immune barriers restrict gene transfer into quiescent HSPC and pave the way for the development of genetic engineering strategies directly in unstimulated HSPC.

258. Administration during Liver Growth Improves the Efficiency of Lentiviral Vector Based *In Vivo* Gene Therapy in Mice

Francesco Starinieri[1,2], Michela Milani[1], Cesare Canepari[1,2], Chiara Simoni[1], Mauro Biffi[1], Fabio Russo[1], Tiziana Plati[1], Eugenia Cammarota[3], Luigi Naldini[1,2], Alessio Cantore[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]Vita Salute San Raffaele University, Milano, Italy,[3]IRCCS San Raffaele Scientific Institute, Milano, Italy

The liver is one of the most studied target organs for *in vivo* gene therapy, due to its involvement in several metabolic and coagulation diseases. Adeno-associated viral (AAV) vectors are widely adopted for liver-directed gene therapy, however their episomal state challenges their use in pediatric patients, because proliferating hepatocytes may dilute the AAV genomes, leading to progressive decrease in therapeutic efficacy with liver growth. In contrast, lentiviral vectors (LV) efficiently integrate into the target cell chromatin and are maintained as cells divide. We have previously developed LV that achieve stable transgene expression in the hepatocytes of adult mice, dogs and non-human primates after intravenous (i.v.) delivery. Here, we set out to investigate the stability of liver-directed LV gene therapy in mice during post-natal growth and homeostasis. We first compared the efficiency of gene transfer in mice treated at different ages, 1-day old (newborn), 2-week-old (juvenile) or 8-week-old (adult) by i.v. administration of weight-matched LV expressing FIX. We observed the highest transgene output at steady state in juvenile, followed by newborn and the lowest in adult treated mice. To assess if the differences in FIX output were due to different transduction efficiency, we measured the percentage of transgene-positive liver area. We did not detect differences between newborn and juvenile treated mice, while the transduced area in adults was significantly lower. Hepatocytes transduced at multiple LV copies may cause higher transgene output even with similar transgene-positive area, thus, to evaluate this possibility, we treated mice by i.v. administration of a mix of 3 LV expressing different fluorescent proteins. We observed cells expressing 2 or 3 markers in mice treated at all ages, indicating that multiple transduction events occur, but the percentage of multi-LV copy hepatocytes was similar between ages. Moreover, adult-treated mice showed a preferential transduction of the periportal area, even more evident for multiple-transduced cells. LV transduction was more homogenous throughout the liver lobule in newborns, while the periportal bias started to appear in juvenile treated mice, suggesting that the biological process underlying this outcome is established during growth. We then investigated the impact of hepatocyte proliferation on transgene output. We evaluated clonal proliferation of LV-transduced and untransduced hepatocytes in Alb-Cre/Rosa26-Confetti mice during growth and homeostasis up to 1 year of age and showed that only a fraction of cells generates continuously growing clusters, while most hepatocytes remain quiescent. Proliferation rate was highest in the first week of life, then progressively decreased. There were no differences in the proportion or extent of growth between transduced and untransduced hepatocytes, excluding that higher transgene output in juvenile treated mice was due to selective expansion of transduced cells. These data show that timing of LV administration strongly impacts on gene therapy efficacy, with more efficient and homogenous gene transfer in younger compared to adult mice and the highest transgene output observed in juvenile treated mice, for reasons that are currently under investigation and may be related to polyploidization of transduced hepatocytes. Overall, our work will inform about the mechanisms underlying optimal output and long-term stability of LV-mediated liver gene transfer in young mice and establish the rationale for its application to the gene therapy of pediatric patients.

259. MicroRNA Detargeting Proves Superior to Genetic Attenuation for Balancing Safety and Efficacy of Oncolytic Mengovirus in Immunodeficient Glioblastoma Mouse Model

Yogesh R. Suryawanshi, Rebecca A. Nace, Stephen J. Russell, Autumn J. Schulze

Molecular Medicine, Mayo Clinic, Rochester, MN

Oncolytic Mengovirus $vMC_{24}NC$, detargeted from cardiac and nervous tissue, exhibited significant efficacy in an immunocompetent syngeneic murine plasmacytoma model. Mengovirus is an attractive oncolytic virus candidate for treating a variety of cancers, including GBM due to its natural tropism for neuronal tissue and ability to reach central nervous system when injected systemically. However, the efficiency, specificity, and stability of $vMC_{24}NC$ microRNA response elements has not been tested in immunocompromised models. In this study, we compared the safety and efficacy of microRNA detargeted $vMC_{24}NC$ against $vMC_{24}\Delta L$, a Mengovirus that does not express Leader protein, in an immune deficient human glioblastoma xenograft model (U87-MG). The Leader protein is a known virulence factor that has been implicated as an inhibitor of nucleo-cytoplasmic transport and IFN-α/β antagonist. Although $vMC_{24}\Delta L$ exhibited reduced toxicity in murine glial cells likely due to suboptimal replication, *in vitro*,

the virus replicated in nervous and cardiac tissues *in vivo,* causing a significant level of toxicity in athymic nude mouse when injected directly into subcutaneous human glioblastoma (U87-MG) tumor xenografts or intracranially. In contrast, the microRNA response elements in $vMC_{24}NC$ prevented virus replication in nervous and cardiac tissues diminishing virus toxicity while effectively reducing U87-MG xenografts and improving overall survival. Safety of $vMC_{24}NC$ is dependent on the stability of microRNA targets that are prone to mutations/deletions, hence further optimization of $vMC_{24}NC$ design is needed to improve the stability of microRNA targets to bolster the safety of virus. In summary, microRNA detargeting is a superior and effective strategy to eliminate Mengovirus toxicity and warrants further development for clinical application in patients with variable immune status.

260. CTCF-Based Chromatin Insulators and Enhancers in Lentiviral Vectors Impact Genome Topology and Vector Safety

Monica Volpin[1], Ivan Merelli[2], Davide Cittaro[3], Mei Chee Lim[4], Roberta Alfieri[2], Leonardo Ormoli[1], Andrea Calabria[1], Daniela Cesana[1], Fabrizio Benedicenti[1], Giulio Spinozzi[1], Pierangela Gallina[1], Melissa Fullwood[4], George Stamatoyannopoulos[5], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), Milan, Italy,[2]Institute of Biomedical Technologies (CNR), Milan, Italy,[3]Center for Translational Genomics and Bioinformatics, Milan, Italy,[4]Cancer Science Institute Singapore, Singapore, Singapore,[5]University of Washington, Seattle, WA

Self-Inactivating (SIN) Lentiviral Vectors (LVs) have demonstrated great efficacy and safety in preclinical models and clinical trials. Still, SIN LV integrations are not entirely neutral to the cell genome and can interfere with physiological gene expression by various genotoxicity mechanisms. In this work, we study the impact of LV insertions on chromatin architecture in 10 different K562 cell clones harboring from 20 to 60 SIN LV integrations carrying CTCF-based chromatin insulators (CI) and/or strong enhancers. Such features are able to interact with distant genomic loci but their effects on the 3D genome conformation in the context of LV integration is poorly explored. At first, we tailored a lentiviral-vector specific chromatin conformation capture method (LV-4C) able to retrieve the integrated LV together with the genomic sequence flanking the vector insertion locus fused to the long-range interacting site. We observed that the majority of the inserted features interact hundreds of Kb from the vector insertion site. Interestingly, while the LV without CI interacts with genomic loci upstream and downstream the IS, inducing gene deregulation at such sites, the vector harboring the CI fully redirects long-range interactions towards genomic loci upstream the LV IS that are enriched for CTCF-motives in convergent orientation to the ones present in the LV ($p<0.01$). At these interaction-sites, the genes upstream the IS were not deregulated, while for genes downstream the IS, where no chromatin-loops are formed, we observe vector-mediated aberrant transcription of host genes. We next expanded our study to address global genome topology by HiC, that allows to capture genomic interactions between all regions of the genome, extending our analysis beyond the vector-captured interactions. We examined the composition of topologically associated domains (TADs) and observed differences in TADs compositions for all tested vectors vs. controls. Consistently with the LV4C results, we observed that the insulators present in the LV interact with genomic CTCF-sites in convergent orientation ultimately redirecting the genomic interactions towards these sites. Finally, we performed an *in vivo* genotoxicity study to assess the safety of these vectors in a tumor prone mouse model based on *Cdkn2a* full and heterozygous KO. We observed that mice injected with insulated vectors harboring the CTCF-site in specific orientations showed improved survival kinetics compared to the non-insulated LV. Integration sites analysis in tumor infiltrated tissue indicate that non-insulated LVs or LVs with CTCF-insulators in specific orientation impact the mechanism of genotoxicity by oncogene activation. On the other hand LVs with CTCF insulators in other configurations resulted to be safe. Taken together our findings show that CTCF-sites within LVs can affect host chromatin architecture depending on their orientation within the vector backbone. Importantly, the directionality of CTCF-mediated interactions has also a profound impact in terms of safety of LV insertion *in vivo*.

AAV Vectors - Clinical Studies

261. AAV8-Mediated Liver-Directed Gene Therapy as a Potential Therapeutic Option in Adults with Glycogen Storage Disease Type Ia (GSDIa): Updated Phase 1/2 Clinical Trial Results

David F. Rodriguez-Buritica[1], Ayesha Ahmad[2], Mari Luz Couce Pico[3], Terry G. Derks[4], John Mitchell[5], Rebecca Riba-Wolman[6], Jiani Mou[7], Allen Poma[7], Vassili Valayannopoulos[7], Eric Crombez[7]

[1]University of Texas McGovern Medical School, Houston, TX,[2]University of Michigan, Ann Arbor, MI,[3]Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain,[4]University of Groningen, Groningen, Netherlands,[5]Montreal Children's Hospital, Montreal, QC, Canada,[6]University of Connecticut, Farmington, CT,[7]Ultragenyx Gene Therapy, Cambridge, MA

Introduction: Glycogen storage disease type Ia (GSDIa) results from a deficiency of the enzyme glucose 6-phosphatase (G6Pase) which is essential for glycogenolysis and gluconeogenesis. Decreased endogenous glucose production causes severe hypoglycemia during fasting. DTX401 is an AAV8 vector that expresses the human glucose-6-phosphatase catalytic subunit (*G6PC*) gene under transcriptional control of the normal G6Pase promoter. **Methods:** The GSDIa phase 1/2 gene therapy study (NCT03517085) is a global, open-label dose escalation trial evaluating the safety, tolerability, and efficacy of a single DTX401 intravenous infusion in adults with GSDIa. Cohorts of subjects were treated with DTX401 at doses of 2.0×10^{12} Genome Copies (GC)/kg and 6.0×10^{12} GC/kg. **Results:** Three subjects in Cohort 1 received DTX401 at 2.0×10^{12} GC/kg; three subjects each in Cohorts 2, 3, and 4 received 6.0×10^{12} GC/kg. The three subjects in Cohort 4 received a prophylactic steroid regimen starting at Day 0 under an amended protocol designed to optimize the steroid approach to the

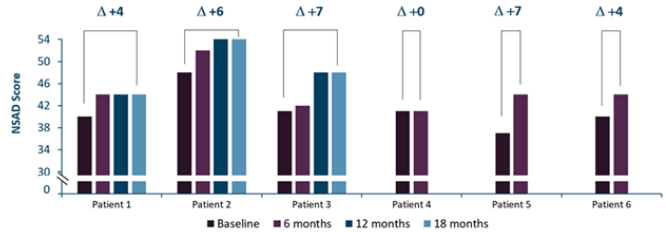
vectorinduced immune response; these subjects are still completing followup and their results are not included in this abstract. DTX401 was generally well-tolerated. No infusion-related or treatment-related serious adverse events (AEs) were reported. All AEs were mild or moderate in severity. Most subjects had transient asymptomatic elevations in ALT that responded well to steroid administration, similar to previous observations with AAVbased gene therapies. All nine subjects eliminated at least one dose of cornstarch per day by their most recent assessment. The mean reduction in frequency of cornstarch use was 4.0 doses/day for Cohort 1, 1.3 doses/day for Cohort 2, and 3.0 doses/day for Cohort 3 at the last onstudy visit. In the nine subjects from Cohorts 1 through 3, least squares mean (95% CI) reduction in total daily cornstarch intake from baseline to Week 52 was 77% (63% to 91%; [$p < 0.0001$]). In Cohort 3, where continuous glucose monitoring was implemented, an average 69% reduction in cornstarch intake from baseline to most recent completed onstudy visit (Week 45 to Week 48) was associated with a mean 11% increase (73% to 84%) in the percentage of time spent in euglycemia, defined as blood glucose levels in the normal range (60 mg/dL to 120 mg/dL). Seven of nine subjects had decreases in body weight following DTX401 treatment, with a mean decrease of 12% from mean baseline weight in these seven subjects. Subject interviews were performed after week 24 and/or Week 52 visits. At these timepoints, subjects reported improved physical/mental health, increased energy/strength, normalization of daily activities, weight loss, greater mental acuity, and reduced stress. **Conclusions:** DTX401 had an acceptable safety profile and sustained improvement in biological G6Pase activity to Week 52 in the nine subjects from Cohorts 1 through 3, with a significant reduction in total daily cornstarch intake. Percentage of time spent in euglycemia increased, and in most subjects, body weight decreased. Subjects also reported increased quality of life across a range of measures. A minimum of 12 weeks of efficacy and safety data from all subjects in Cohort 4, as well as additional follow-up for subjects in Cohorts 1, 2, and 3, will be available at the time of the meeting.

262. Safety, β-Sarcoglycan Expression, and Functional Outcomes from Systemic Gene Transfer of rAAVrh74.MHCK7.SGCB in Limb Girdle Muscular Dystrophy Type 2E/R4

Louise R. Rodino-Klapac[1], Eric R. Pozsgai[1,2], Sarah Lewis[1,2], Danielle A. Griffin[1,2], Aaron S. Meadows[1,2], Kelly J. Lehman[2], Kathleen Church[2], Natalie F. Miller[2], Megan A. Iammarino[2], Linda P. Lowes[2], Jerry R. Mendell[1,3]

[1]Sarepta Therapeutics, Inc., Cambridge, MA,[2]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH,[3]Department of Pediatrics and Neurology, The Ohio State University, Columbus, OH

Introduction: Limb-girdle muscular dystrophy type 2E/R4 (LGMD2E/R4) is caused by mutations in the β-sarcoglycan (*SGCB*) gene that result in loss of functional protein, affecting other structural components of the dystrophin-associated protein complex. Clinically, LGMD2E/R4 usually manifests with progressive hip/shoulder muscle weakness and often includes cardiac involvement and elevated creatine kinase (CK). We designed a self-complementary rAAVrh74.MHCK7.hSGCB construct (SRP-9003) to restore functional SGCB to muscles. Here we report initial findings of a Phase 1/2 clinical gene transfer trial of SRP-9003. **Methods:** In this first-in-human, single-center, open-label, systemic gene delivery, Phase 1/2 study (NCT03652259), 6 patients with LGMD2E/R4 received SRP-9003 in escalating doses. Eligible patients were age 4–15 years, with *SGCB* gene mutation (both alleles), negative for rAAVrh74 antibodies, and >40% on 100-meter walk/run (MWR). Cohort 1 (n=3) received a single IV infusion of $1.85x10^{13}$ vg/kg (linear qPCR, supercoiled plasmid standard equivalent of $5x10^{13}$ vg/kg); Cohort 2 (n=3) received a single IV infusion of $7.41x10^{13}$ vg/kg (linear qPCR, supercoiled plasmid standard equivalent of $2x10^{14}$ vg/kg). For all patients, prednisone 1 mg/kg/day was initiated 1 day before treatment (tapering after 30-60 days). Primary endpoint was safety. Secondary endpoint was change in SGCB expression from baseline to week 8. Other endpoints included CK decrease, North Star Assessment of Limb-girdle Muscular Dystrophies (NSAD), and timed functional tests (100MWR, 10MWR, 4-stair climb, and time to rise). **Results:** Initial results show systemic administration of SRP-9003 is well tolerated with no unexpected immunologic responses observed. Efficient transduction and robust SGCB protein expression were observed in all patients post-infusion. Relative to normal controls, immunofluorescence data for Cohorts 1 and 2 showed 51% and 72% of fibers positive for SGCB, respectively, and staining intensity was 47% and 73%. Protein levels detected by Western blot were 36.1% and 62.1% of normal controls. SGCB expression led to reconstitution of the sarcoglycan complex. CK at day 90 was reduced by 83.4% and 89.1% from baseline in Cohorts 1 and 2, respectively. Six months after SRP-9003 infusion, patients from both cohorts experienced improvements in functional measures compared with baseline (Figure). For Cohort 1, data up to 18 months post-infusion are available and demonstrate improvements in NSAD and timed tests maintained over baseline. **Conclusion:** These data suggest the long-term efficacy of SRP-9003 gene transfer therapy, supporting advancement of the clinical development program.



263. IGNITE-DMD Phase I/II Study of SGT-001 Microdystrophin Gene Therapy for Duchenne Muscular Dystrophy

Carl A. Morris, Cathryn M. Clary, Susan Redican, Sidney Spector, Courtney Shanks, Kristy J. Brown, Joel S. Schneider, J. Patrick Gonzalez

Solid Biosciences, Cambridge, MA

IGNITE-DMD is an open-label Phase I/II ascending dose study investigating the safety and efficacy of a single intravenous infusion of SGT-001, an AAV9 microdystrophin gene therapy for Duchenne muscular dystrophy (DMD). SGT-001 is designed to deliver a functional surrogate of the dystrophin protein absent in DMD. This

unique microdystrophin construct includes the neuronal nitric oxide synthase (nNOS) binding domain, which has been shown to protect muscles from ischemic damage in preclinical studies. Primary outcomes in IGNITE-DMD include safety evaluations and measurement of microdystrophin expression by Western blot. Evaluations of motor function, pulmonary function, disease biomarkers, and quality of life are also performed as secondary endpoints. Dose escalation to the current level of 2E14 vg/kg occurred following assessment of microdystrophin expression that was below the level of quantification of the Western blot assay in the 3 subjects administered the initial dose of 5E13 vg/kg. At Day 90 following administration of SGT-001 at 2E14 vg/kg, all subjects showed microdystrophin expression with approximately 5-17.5% of normal dystrophin by Western blot and 20-70% of positive muscle fibers by immunofluorescence. Molecular function of the microdystrophin protein was further demonstrated by the restored sarcolemmal localization of the critical associated proteins β-sarcoglycan and nNOS in microdystrophin positive muscle fibers. In addition, levels of serum creatine kinase (CK), a biomarker for membrane stability, were substantially decreased in these subjects at 1 year post-treatment. Acute treatment-related serious adverse events that have all fully resolved were previously communicated and included complement-mediated inflammatory response, thrombocytopenia, and transient increases in hepatic transaminases. Prophylactic administration of complement inhibitors and increased glucocorticoid dose have since been implemented in the clinical protocol for subsequent patients. In addition to protein expression and biomarker results, positive results were observed in functional and quality of life assessments at 1 year post-treatment and will be described. These data in totality continue to support the ongoing investigation of SGT-001 as a transformative therapy for DMD.

RNA Virus Vectors

264. Automation-Assisted Workflow for High-Throughput Lentiviral Library Construction

Richard Parker-Manuel[1], Laurent Houzet[2], Marina Perez-Cerezuela[2], Marek Kucej[2], Kadri Maal[2], Vaughan Robert Leydon[2], Claire Greenwood[3], Matt Burridge[3], Simon Pollack[3], Qian Liu[4], Ryan Cawood[5]

[1]Library and Vector Development, OXGENE, Oxford, United Kingdom,[2]Antibody & Viral Vector Manufacture, OXGENE, Oxford, United Kingdom,[3]Laboratory Automation, OXGENE, Oxford, United Kingdom,[4]Gene Therapy, OXGENE, Oxford, United Kingdom,[5]OXGENE, Oxford, United Kingdom

Library screening has become a powerful tool for the identification of genetic sequences endowing a specific phenotype. To deliver these genetic libraries into host cells, lentiviral vectors are used extensively as they allow long-term, heritable, genetic modification of a wide range of cell types. To enable more efficient and robust lentiviral library construction, we developed an automation-assisted workflow consisting of high-throughput DNA/gRNA library design and plasmid construction, 96-well format lentivirus production, and automated lentivirus titration assays. To design gRNA libraries, we use either published sequences or a newly developed gRNA design tool, GRNADE. Library cloning is achieved by preparing oligonucleotides with compatible overhangs into a CRISPR lentiviral transfer plasmid (LTP). The oligos are inserted into the LTPs and plasmid DNAs are purified with aid of liquid handlers, matched to a standard concentration and then quality checked by Sanger sequencing. Samples of the validated gRNA LTPs are then prepared in 96-well plates for high-throughput lentiviral production. This workflow enables us to construct arrayed libraries of up to 1000 guides, and the processes are adaptable to develop other library types such as promoter and scFv libraries. The high-throughput lentiviral construction pipeline was coupled with an optimised packaging plasmid system in adherent and suspension HEK293 cell lines in 96 well plate format. The critical process parameters such as cell density before and at seeding, DNA quantity, plasmid ratio, post-transfection supplements, and time of harvest were optimised, achieving an average lentiviral titre of 1-5E7 TU/mL. We demonstrate that by configuration of Hamilton STAR line liquid handling platforms with other integrated devices, the process of HEK293 cell seeding, transfection and titration can be fully automated. Through sequential optimisation of this platform, we achieve lentiviral yields closely comparable to large viral production formats.

265. Lentiviral Vector Use in Pre-Clinical Work for Gene and Cell Therapy Application - Method and Feasibility

Romain Genard, Selly Hung, Lauriane Padet, Renée Riffon, Simon Authier

Immunology, CRL, Laval, QC, Canada

Gene and cell therapy are currently used as treatment in a broad range of diseases for which no effective treatment exists or conventional therapy failed. Use of lentiviral vector (LVV) has been demonstrated to be a strong and safe tool to rely on in order to deliver a specific gene to a pre-determined target cell which will result in a therapeutic effect. We developed a robust methodology, in a GLP environment, designed to conduct proof of concept as well as toxicology study in mouse model in order to support the growing needs in gene and cell therapy field such as enzyme replacement therapy. Briefly, bone marrow cells were harvested from disease mouse models followed by Hematopoietic Stem Cell (HSC) isolation. HSC were transduced with LVV then dosed to diseased mice. The HSC enrichment and LVV expression from cell culture was evaluated by flow cytometry. All those steps were designed and carried out with multiple checkpoints (such as flow cytometry check for HSC enrichment, LVV expression evaluation in cultured cells at various timepoint by flow cytometry and CFU monitoring) and using characterized assay, in order to standardize the process and ensure the repeatability of the methods. Results from two different studies that followed this process showed expected level of transduced cell's engraftment. Work is also on-going to perform the LVV transduction on cells from larger animal models, such as non-human primates, in order to bring to the gene and cell therapy field, combined with the drug discovery field, as much support and available tools as possible. The benefice of this process can be multiplied and transposed to a various type of cells/disease models within GLP environment requirements.

266. Recombinant Avian Orthoavulavirus-1 Engineered to Express Variants of the SARS-CoV-2 Spike Protein Induces Mucosal and Systemic Immune Responses

Jacob Yates[1], Robert C. Mould[1], Lily Chan[1], Jason P. Knapp[1], Yeganeh Mehrani[1], Yanlong Pei[1], Phuc H. Pham[1], Alexander Leacy[1], Lisa A. Santry[1], Betty-Anne McBey[1], Pierre P. Major[2], Byram W. Bridle[1], Leonardo Susta[1], Sarah K. Wootton[1]

[1]Pathobiology, University of Guelph, Guelph, ON, Canada,[2]Juravinski Cancer Centre, Hamilton, ON, Canada

In response to the severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) global pandemic a plethora of vaccine candidates have been developed. The main neutralization and protective antigen of SARS-CoV-2, the spike (S) protein, is a homo-trimeric type I transmembrane fusion protein consisting of two subunits, S1, mediating receptor recognition and S2, responsible for fusion of the virus with the cell membrane. The S protein exists in two conformations, a "pre-fusion" state where the S1 subunit is exposed and a "fusion" conformation where the S1 subunit hinges to expose the S2 subunit to promote fusion. Previous research on SARS-CoV-1 and Middle East respiratory syndrome vaccines demonstrated more robust immune responses when using prefusion-stabilized versions of S. It also emphasized the importance of inducing a T-helper cell type 1-biased immune response rather than one focused on type 2 T-helper cells, as the latter has been associated with the development of eosinophilic interstitial pneumonia and enhanced disease upon infection. Avian orthoavulavirus-1 (AOaV-1) is a non-segmented, negative-sense single-stranded RNA virus belonging to the *Paramyxoviridae* family. In mammalian species this virus has a strong safety profile, derived in part by the virus' inability to counteract the mammalian interferon system. Additionally, as an acute cytoplasmic virus with an encapsulated genome, AOaV-1 does not establish chronic infection or integrate in the host genome. In mammals, AOaV-1 has a natural tropism for lung epithelial cells and when administered intranasally (IN), induces effective mucosal and systemic immune responses. Furthermore, as AOaV-1 is antigenically distinct from human paramyxoviruses, its ability to act as a vaccine platform would not be compromised by pre-existing immunity. Finally, AOaV-1 can be lyophilized and stored at 4°C with minimal reduction in titer, without the need of ultra-low temperature freezers. Given these promising characteristics, we have developed recombinant AOaV-1s (rAOaV-1s) expressing multiple versions of the S protein, with the goal of producing AOaV-1-vectored vaccines against SARS-CoV-2. The first-generation vaccine vector, AOaV-1-SARS-CoV2-S expresses the wild-type S protein and the second-generation vector, AOaV-1-SARS-CoV2-PFS, expresses a pre-fusion stabilized (PFS) S protein variant. Administration of the first-generation vector IN and intramuscularly in C57BL/6 and Balb/c mice in a two-dose vaccination regimen induced systemic S-specific T cell and antibody responses. Spike-specific T cells were quantified by flow cytometric detection of intracellular cytokines induced by stimulation with a S protein-derived peptide pool. Serum IgG and bronchoalveolar lavage fluid IgA antibody responses against S were quantified using an enzyme-linked immunosorbent assay. Virus neutralizing titers were determined using a S-pseutodyped lentivirus vector. In collaboration with the National Research Council, efficacy of the first- and second-generation vaccines is currently being compared in the hamster challenge model. Additionally, each vector has been engineered to co-express murine interleukin-12 to further promote a Th1-biased immune response and enhance clearance of SARS-CoV-2-infected cells, preventing eosinophilic immunopathology and will be evaluated in the K18-hACE2 challenge model.

267. *In Vivo* Gene Delivery to T cells and Hepatocytes Achieved through a Re-Targetable Fusogen Platform

Kyle Trudeau, Patty Cruite, Matteo Stoppato, Akinola Emmanuel, Jess Elman, Allyse Mazzarelli, Shirisha Amatya, Caspian Harding, James Kaczmarek, Sonya Iverson, Hanane Ennajdaoui, Katie Sullivan, Samantha Crocker, Anna Liang, Kelan Hlavaty, Jason Rodriguez, Lauren Broom, Justine Cunningham, Kutlu Elpek, Suvi Jain, Trevor McGill, Terry Fry, Donna Dambach, Michael Laska, Albert Ruzo, Lauren Pepper, Jagesh V. Shah

Sana Biotechnology, Cambridge, MA

Effectively addressing cell-specific delivery would markedly improve the therapeutic potential of gene therapy. Leveraging a system based on paramyxoviral fusogens, we have developed a platform for tailoring targeted fusogens to cellular receptors of choice, thereby enabling a much greater degree of cell-specific delivery in a modular fashion across cell types. Here, we demonstrate our platform's ability to generate engineered, targeted fusogens against multiple receptors for lentiviral vector (LVV) gene delivery in both T cells and hepatocytes. Fusogen engineering begins with the identification of specific surface proteins unique to, or selectively enriched on, the target cell of choice. We then generate single polypeptide chain affinity binders recognizing these receptors, combine these with a receptor-blinded fusogen and test these engineered fusogens for specificity and transduction efficiency *in vitro*, and then *in viv*o. Protein optimization methods on high performing candidates can increase transduction efficiency and have been demonstrated across several receptor and binder modalities, suggesting they are generalizable. For T cells, we have generated multiple fusogens against CD8 and CD4 receptors. Our CD8-targeted fusogens have been generated from both VHH and scFv modalities (Fusogen #1 and Fusogen #2, respectively, in figures). When pseudotyped on an LVV carrying a GFP transgene, CD8-targeted fusogens showed selective and efficient *in vitro* CD8 T cell transduction in human PBMCs (Figure 1, upper panel). Low transduction on a CD8-negative cell panel showed further selectivity, particularly when compared to VSV-G, a fusogen with low cell-type selectivity (Figure 1, lower panel). *In vivo* delivery in an adoptive transfer model demonstrated efficient and selective CD8 T cell transduction (Figure 2). These CD8-targeted fusogens have also delivered an anti-CD19-CAR (FMC63-BBz) transgene selectively to CD8-positive T cells and demonstrated dose-dependent *in vitro* tumor cell killing and *in vivo* tumor eradication. For hepatocytes, targeted fusogen LVVs showed on-receptor

specificity in engineered cell lines and primary human hepatocytes (PHH). Our protein optimization methods increased PHH transduction efficiencies of hepatocyte-targeted fusogen LVVs to levels reported for VSV-G LVVs and with greater specificity. The ability to build targeted fusogens against a pre-defined receptor supports rapid and intentional design of cell-selective interventions with applicability to a wide range cell types. These experimental results demonstrate the potential of fusogen-targeted LVVs for *in vivo* cell-specific gene delivery to address a diverse set of diseases, including cancer and hereditary metabolic disease.

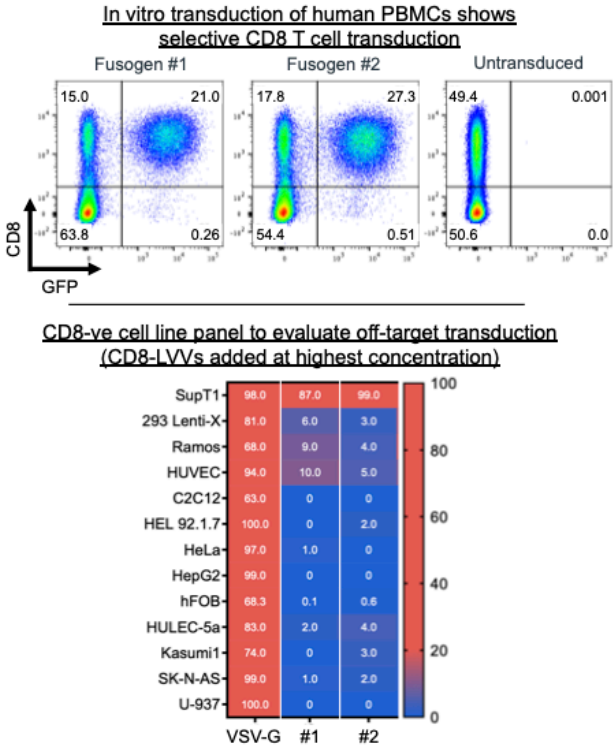


Figure 1

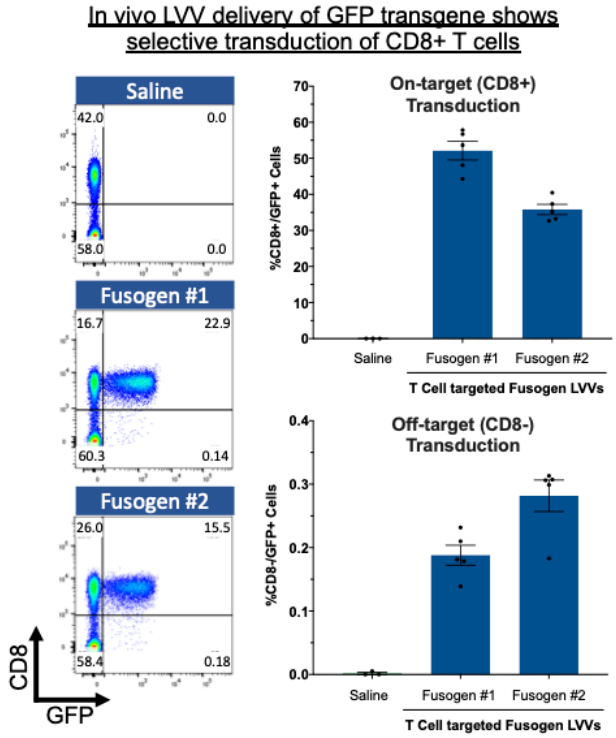


Figure 2

268. Assessment of F/HN Pseudotyped Lentiviral Vector Following Intravenous Delivery to Mice

Robyn V. Bell, Nikhil Faulkner, Eric W. F. W. Alton, Uta Griesenbach

National Heart and Lung Institute, Imperial College London, London, United Kingdom

The UK Respiratory Gene Therapy Consortium, has developed a lentivirus pseudotyped with Sendai virus envelope proteins F and HN (rSIV.F/HN) for efficient pulmonary gene transfer. Contrary to other viral vectors, rSIV.F/HN generates prolonged and stable gene expression after a single dose (approximately 2 years in mice), retaining efficiency after repeated administration. Importantly the vector may hold promise for broader disease indications. More recently, rSIV.F/HN delivered topically to murine lungs successfully achieved therapeutic plasma protein levels of secreted proteins, Factor VIII and alpha-1-antitrypsin. To investigate further the suitability of rSIV.F/HN vector for treatment of systemic diseases, intravenous administration to wildtype mice was characterised and compared to topical administration. Vector encoding gaussia luciferase (rSIV.F/HN-gLux) was utilised to compare the lungs and the liver as factories for secretion of protein into the blood. Mice were treated with increasing doses of vector, intravenously or topically to the lungs (n=6/group), and blood sampled via tail vein puncture at regular intervals post-dosing. Serum levels of gaussia luciferase demonstrated that intravenous delivery of rSIV.F/HN led to persistent (at least 6 months) transgene expression in mice, roughly one log higher than a matching dose of vector delivered topically. To characterise

biodistribution, vector encoding firefly luciferase (rSIV.F/HN-Lux) was delivered intravenously (n=8) and bioluminescent imaging performed 28 days post-dosing. Transgene expression was observed in the spleen, liver, lungs, gonads and kidneys following intravenous delivery, in contrast to highly localised respiratory tract expression following topical administration. Finally, repeat administration was investigated by comparing transduction efficiency between mice receiving one dose of rSIV.F/HN-Lux and mice delivered two doses of control vector followed by a final dose of rSIV.F/HN-Lux at monthly intervals (n=6/group). Mean transgene expression in the group of mice dosed repeatedly through intravenous administrations was 77% lower compared to the mice treated with one dose intravenously. We have shown previously that efficacy is retained following three doses administered by topical delivery to the respiratory tract, suggesting the intravenous route may provoke a differential anti-vector immune response. In summary, persistent levels (at least 6 months) of secreted protein were observed systemically following intravenous delivery of rSIV.F/HN vector, with higher transduction efficiency observed compared to topical administration. However, reduced efficacy following repeated vector administration may impact intravenous delivery for diseases that require life-long protein expression.

269. Towards Enhancing Signal Peptides for Respiratory Gene Therapy with Secreted Proteins

Jack W. Hickmott, Possawee Prasertsuk, Robyn V. Bell, Mario Chan, Uta Griesenbach, Eric W. F. W. Alton

National Heart and Lung Institute, Imperial College London, London, United Kingdom

Introduction: Using a recombinant SIV lentiviral vector pseudotyped with Sendia virus F and H/N proteins (rSIV.F/HN) allows for robust and sustained transduction of the airways and lungs in mice. The success of this vector is driving a cystic fibrosis gene therapy program towards clinical trials. Additionally, we have previously shown that stable transduction of the murine lung with rSIV.F/HN can be used to secrete clinically relevant levels of alpha-1 antitrypsin. However, similar transduction of the human lung will require larger amounts of virus and technologies that improve protein secretion could decrease the necessary dose. One approach to improving protein secretion is by employing a strong signal peptide. Signal peptides are heterogenous sequences at the N-terminus of secreted proteins. During translation, signal peptides signal for translocation of the nascent protein to endoplasmic reticulum and subsequent secretion. Previously it has been shown that a synthetic signal peptide, Secrecon, can boost the secretion of reporter genes such as secreted alkaline phosphatase. Here we explore if signal peptides are a viable strategy for boosting the secretion of therapeutically relevant proteins such as alpha-1 antitrypsin from a lentiviral expression plasmid. **Methods:** Plasmids encoding AAT with the endogenous AAT signal peptide or with the Secrecon signal peptide were transfected into HEK293T or A549 cells (n=8) and AAT production was measured by ELISA. **Results:** In HEK293T cells, exchanging the endogenous AAT signal peptide for the Secrecon signal peptide doubled the amount of AAT secreted into the media (mean = $3.911x10^{-4}$ ng AAT/cell and $8.603x10^{-4}$ ng AAT/cell, respectively). However, in A549 cells, the AAT signal peptide lead to the secretion of more than double the amount of AAT as Secrecon did (mean = $1.843x10^{-4}$ ng AAT/cell and $0.709x10^{-4}$ ng AAT/cell, respectively), suggesting a possible tissue-specific difference. Therefore, endogenous signal peptides from proteins expressed in the target respiratory tissue, could offer superior secretion and be less likely to trigger an immune response than the synthetic Secrecon. To identify candidate lung signal peptides, a bioinformatic analysis of proteins secreted from the lungs into the blood stream or lung epithelial lining fluid was performed. Five candidate proteins were identified: Fibronectin (CLEC3B) Cartilage Acidic Protein 1 (CRTAC1), Alpha-2-macroglobulin (A2M), Uteroglobin (SCGB1A1), and Pulmonary surfactant associated protein A (SFTPA2). Studies are underway to assess the ability of these signal peptides to enhance secretion. **Conclusions:** Exchanging the AAT signal peptide for Secrecon boosts AAT secretion in HEK293T cells. Further evaluation of Secrecon, and lung signal peptide candidates, in models that better represent the lungs such as air-liquid interface culture and mice, could identify an optimal signal peptide for respiratory gene therapy.

270. Lentiviral Gene Therapy of Chronic Granulomatous Disease (CGD) - Expression and Functional Comparison of Universal and Tissue-Specific Promoters

Haokun Yuan[1], Hongwei Liu[1], Yuchen Liu[2], Rui Zhang[2], Lung-Ji Chang[1,2]

[1]Medicine, UESTC, Chengdu, China,[2]Shenzhen Geno-Immune Medical Institute, Shenzhen, China

Chronic granulomatous disease (CGD) is a congenital immunodeficiency characterized by severe life-threatening infections. CGD patients lack reactive oxygen species (ROS) in phagocytic leukocytes and often develop widespread tissue granulomas. The current treatment for CGD is lifelong antibiotic prophylaxis or hematopoietic stem cell transplantation (HSCT). However, most patients cannot find matched donors for HSCT. Gene therapy (GT) has become the most promising treatment for CGD. GT can restore ROS production in HSCs. The ROS produced in HSCs are involved in the balance of quiescence, self-renewal, proliferation and differentiation processes. GT based on ectopic expression of NADPH oxidase using non-tissue specific promoters can over-produce ROS in HSCs, which could promote apoptosis and induce differentiation of resting HSCs, thus reducing the self-renewal ability and resulting in significant exhaustion of stem cell pool. In this study, we developed lentiviral vectors (LVs) carrying a universal promoter (EF1a) or tissue-specific promoters driving the expression of green fluorescent protein (GFP) or CYBB (cytochrome B-245 beta chain, or p91-phox). The tissue specific promoters included myeloid-specific (miR223, CD68) and endothelial specific (VEC) promoters. *In vitro* assays using EA-hy926 cells, a human endothelial cell line, and HeLa cells revealed that the lowest GFP expression was detected with miR223-GFP as compared with EF1a-, CD68-, or VEC-GFP under similar vector copy number. We further examined myeloid-specific expression using mouse HSCs (mHSCs). Myeloid differentiation was induced using mouse G-CSF, and GFP expression was compared in both differentiated (diff) and undifferentiated (undiff) cells. The results showed expression of diff vs. undiff cells as following: EF1a 80 vs. 79%, miR223 92 vs. 65%, CD68 51 vs. 48% and VEC 31 vs. 34%, indicating myeloid specificity of the

miR223 promoter. To investigate LV-CYBB gene activities, we utilized a CGD mouse model (X-CGD; B6.129S-Cybb^{tm1Din}/J). Functional analysis based on dihydrorhodamine (DHR)123 assay using the CGD mHSCs revealed restored therapeutic range of ROS after LV-CYBB gene transduction at the following levels: EF1a (~37%), miR223 (~31%), and CD68 (~29%) as compared with WT (~57%). The packaging efficiencies of the different LV-CYBB vectors were compared and the results showed that LV-miR223-CYBB was packaged over 10^9 IU/ml at levels 19-fold greater than LV-CD68-CYBB and 5-fold greater than LV-EF1a-CYBB. Phagocytosis of GFP+ *E. coli* by granulocytes using differentiated CGD mHSCs was also examined, and we detected similar activities as following: wild type (GFP+ 87%), EF1a-CYBB (77%), miR223-CYBB (84%) and CD68-CYBB (82%), indicating that LV transduction *per se* did not affect the phagocytosis function of the granulocytes. Our study illustrated that the LV-miR223 transgene displayed high vector packaging and transgene delivery efficiencies with myeloid-specificity. Translational potential of these tissue specific promoters in human HSCs and in CGD mice is under investigation to support future clinical application.

AAV Vectors - Virology and Vectorology

271. Tyrosine Chemically Modified AAV Vectors for Gene Delivery in Retina

Mathieu Mevel[1], Aurélien Leray[2], Mohammed Bouzelha[1], Sébastien Gouin[2], Joanna Demilly[1], Magalie Penaud-Budloo[1], Oumeya Adjali[1], Eduard Ayuso[1], David Deniaud[2], Thérèse Cronin[1]

[1]INSERM UMR1089, CHU de Nantes, University of Nantes, Nantes, France,[2]CORAIL team, CEISAM UMR 6230, University of Nantes, Nantes, France

Recombinant Adeno-Associated-Virus-derived vectors (rAAV) provide a relevant clinical platform for gene transfer. Despite an increasing number of clinical trials and the commercialization of the first AAV-based products, there are still some critical limitations to address particularly when the vector is administered systemically: (i) high doses are usually required to achieve a therapeutic effect; (ii) the transduction of non-target tissues is generally observed; (iii) an absence of efficacy in case of pre-existing viral neutralizing antibodies (NAb). Our team has already reported chemically modified AAV vectors at amine residues with improved properties.[1] These modifications significantly reduced interactions between rAAV capsids and NAb and increased selectivity towards targeted cells. Here, we describe a new chemical modification of rAAV capsids at tyrosine residues.[2] Tyrosine to phenylalanine mutations of rAAV capsid have already been shown to increase cell transduction efficiency. Thus, we hypothesized that chemical modification of tyrosine residues with molecules of interest would have multiple benefits in term of rAAV vector efficacy. The molecule comprises a ligand for a selective tissue tropism, a linker and a reactive function specific to tyrosine residue to achieve bioconjugation on rAAV particles. Several molecules have been synthesized and efficiently bioconjugated on rAAV serotype 2 capsids. We have demonstrated the formation of a covalent bond between these molecules and the tyrosine residues of the capsid by dot blot and Western blot. Finally, tyrosine-modified rAAV2 particles were more efficient in transducing murine retina than non-modified rAAV2. The characterization of chemical and biological properties of these tyrosine-modified rAAV vectors will be discussed. This new rAAV technological platform could help to improve AAV selectivity and gene transduction efficiency, which are still long standing issues for successful clinical translation of rAAV products. [1] M. Mével, et al., *Chem. Sci.* **2020**, 10.1039.C9SC04189C. [2] Patent No. 19185879.4

272. Categorical Thinking Applied to the World of AAV Engineering: A Rationally Designed AAV-LK03 Variant with Improved Liver Biodistribution

Marti Cabanes Creus[1], Renina Navarro[1], Erhua Zhu[2], Grober Baltazar[1], Sophia Liao[1], Matthieu Drouyer[1], Ian E. Alexander[2,3], Leszek Lisowski[1,4]

[1]Translational Vectorology Unit, Children's Medical Research Institute, Westmead, Australia,[2]Gene Therapy Research Unit, Children's Medical Research Institute, Westmead, Australia,[3]Discipline of Child and Adolescent Health, The University of Sydney, Sydney, Australia,[4]Laboratory of Molecular Oncology and Innovative Therapies, Military Institute of Medicine, Warsaw, Poland

Recent successes in gene therapy clinical trials have intensified the interest in utilising adeno-associated viruses (AAV) as vectors. AAV-LK03, a bioengineered variant, has recently reached clinic in the context of liver-directed therapy. AAV-LK03 was developed using directed evolution in a xenograft mouse model of human liver. In order to organise the knowledge around this xenograft model, and with the aim to simplify its inherent complexity, we have applied the concept of *categorical thinking* to study AAV mediated liver transduction. Acknowledging that the observed transduction variability among AAV variants ranges along a continuous spectrum, rather than falls within fixed categories, we nevertheless found the categorization to be a valuable process when applied to the world of AAV bioengineering. As a proof of concept, we focused on the first key step in AAV transduction, the glycan-based cell attachment. While AAV-LK03 is composed of fragments of multiple parental AAVs, it shares the key amino acids responsible for attachment to heparan sulfate proteoglycan (HSPG) with AAV3b. Using targeted mutagenesis, we generated a set of AAV-LK03 mutants displaying either higher or lower affinity for HSPG and divided them into four categories based on the observed *in vivo* transduction profile as follows. Stronger HSPG attachment significantly hampered liver transduction, whilst a similar liver-detargeting phenotype - although probably arising via an opposite mechanism - was observed for HSPG-binding ablated vectors. AAV-LK03, which presents moderate HSPG-attachment belongs to a third category, characterized by vectors that can transduce the liver but that present a marked periportal transduction profile in xenograft models and in larger animals. Based on these results, we rationally designed an AAV-LK03 variant harbouring two point mutations (R594E and D598H, designated as AAV-LK03-REDH) that lower, but maintain, HSPG attachment. AAV-LK03-REDH would thus be a representative of a fourth category of vectors that on the HSPG-binding spectrum lies between AAV-LK03 and HSPG-binding ablated variants. Using barcoded vectors encoding for different fluorescent markers, we

developed a strategy that allows for simultaneous testing of vector potency using next generation sequencing and immunofluorescence imaging. We compared the biodistribution of AAV-LK03 and AAV-LK03-REDH within the same hepatic lobules. In animals presenting a humanization index of 20%, AAV-LK03-REDH was markedly superior at reaching and transducing the clusters of human hepatocytes, whereas AAV-LK03 remained periportal. With highly humanized livers, AAV-LK03-REDH displayed an overall 3-fold increase in vector genomes and transgene RNA, as measured by next generation sequencing. More importantly, AAV-LK03-REDH was able to infiltrate deeper into the hepatic lobule towards the central vein, leading to transduction of hepatocytes in additional hepatic zones. In summary, we found *categorical thinking* as a powerful tool to rationally design the next generation of AAV variants, such as AAV-LK03-REDH. The superior transduction of this variant observed in highly repopulated mice warrant further analyses in larger animals.

273. Use of Transmission Electron Microscopy and Deep Learning for Classification of AAV Capsids

Cynthia Swanson, Michael Staup, Jeffrey Crews, Alfred Inman, Danielle Brown

Charles River, Durham, NC

Adeno-associated virus (AAV) is commonly used as the delivery system for gene therapies. A major challenge in manufacturing these AAV vectors is the presence of impurities (i.e., empty or partial capsids), which may affect the efficacy and safety of the vector product (i.e., full capsid). It is essential to determine the ratio of these impurities with that of the vector product, since this is a critical quality requirement for the AAV production process. Traditionally, analytical techniques have been utilized to quantify empty, full, and partially full capsids, including analytical ultracentrifugation (AUC), capillary isoelectric focusing (cIEF), and ion exchange chromatography (IEC). However, none of these techniques allow visualization of the capsids. Transmission electron microscopy (TEM) has emerged as a novel method for visualization of AAV capsid integrity and morphology. Electron micrographs can be used to characterize the capsids as empty, full, and partially full. Other attributes such as aggregation and purity can also be assessed. Analysis of TEM images has traditionally been performed manually. However, technological advances in image analysis and the introduction of deep learning have allowed automated image analysis to be performed on high resolution gray-scale digital images. We introduce a novel artificial intelligence-based image analysis algorithm for automated classification of negatively stained AAV capsids into full, empty, and partially full, with rapid quantification of capsid ratios (Figure 1). These images can be assessed by pathologists and image analysis scientists for accuracy, and images with labels applied can be archived indefinitely. This creates a robust quality control and study re-creation process that fulfills regulatory requirements. The results generated by the algorithm were verified against a manual analysis performed by a trained observer blinded to the automated output, and a high correlation was observed between methods. This method should be considered the new gold standard for AAV capsid ratio determination.

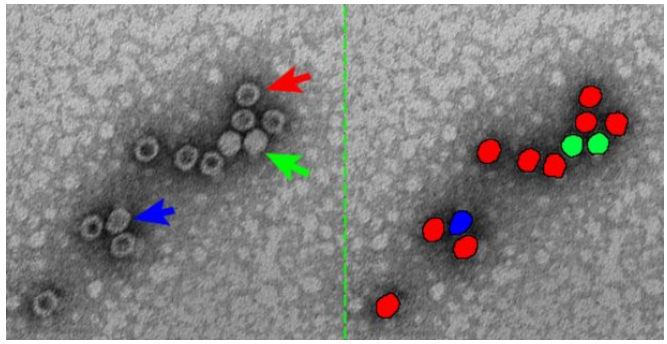


Figure 1. Classification of AAV capsids into full (green), empty (red), and partially full (blue) through artificial intelligence on transmission electron micrographs.

274. Evaluation of Methods to Enable Multicistronic Transgene Expression in an AAV Vector System

Bruce Schnepp, Emilia Bora, Ilya Slabodkin, Weiran Shen, Nachi Gupta, Zhu Pirot, Melissa Rhodes, Brian Furmanski

Kriya Therapeutics, Redwood City, CA

Given the genome size limitations of adeno-associated viruses (AAVs), expression of multicistronic vectors - multiple genes or open reading frames for expression of large proteins - remains a challenge in the field of AAV gene therapy. These challenges must be addressed to enable the efficient and consistent AAV-mediated expression of large therapeutic proteins, such as monoclonal antibodies. Monoclonal antibodies have been developed over decades to address a range of disease areas, including in oncology, immunology, neurology, and infectious disease. They are large molecules whose characteristic structure is defined by two identical light and heavy chains joined by disulfide bonds. One-time delivery of an AAV vector containing the genes coding for therapeutic monoclonal antibodies may address traditionally difficult-to-treat diseases by enabling durable and focal production. Importantly, a single therapeutic administration of a vectorized monoclonal antibody could eliminate the need for lifelong repeat injections and substantially reduce the burden associated with conventional monoclonal antibody therapy. Here, we describe the construction of a modular AAV-mediated monoclonal antibody expression cassette to assess different configurations of heavy and light chains as well as incorporation of linkers, including furin 2A (F2A) peptides, internal ribosome entry sites (IRES), and dual promoter systems. We evaluated in vitro transfection of plasmids in HEK293 cells. The construct containing the F2A in a heavy chain then light chain configuration was the highest expressing plasmid. Plasmids utilizing an IRES produced an unequal ratio of heavy and light chains. The lowest concentrations were observed with a dual promoter system, and as with the IRES system unequal ratios of heavy and light chains were expressed in the dual promoter system. We further evaluated the different plasmid constructs through hydrodynamic tail vein injection in mice to compare expression levels and serum levels of the monoclonal antibodies in vivo. The pattern of expression levels in mice was generally similar to the pattern observed in vitro, with the F2A heavy followed by light chain resulting in the highest level of expression. These results provide insight into modular expression cassette designs for AAV-mediated delivery of monoclonal antibodies and

multicistronic transgenes, and should be considered alongside other factors to guide the assembly of an optimal vectorized monoclonal antibody expression cassette.

275. Development of Universal, Strong Mini-Promoters for Recombinant Adeno-Associated Viral (rAAV) Vectors

Sunghee Chai

Pediatrics, Oregon Health & Science University, Portland, OR

rAAVs have emerged as an efficient gene delivery tool. Discovery of various natural serotypes and recent development of recombinant capsids significantly advanced the transduction efficiency of rAAVs in a variety of cells and tissues. On the other hand, much less effort has been made for maximizing expression of the rAAV cargo DNA, since current AAV vectors mainly rely on well-established promoters for gene expression. Among those, CMV and CAG promoters belong to the most frequently used strong promoters providing universal activity. The capacity of DNA packaging in rAAV capsids is limited (4.7 kb). Hence, the large size of the existing strong promoters is a drawback in delivering genes and gene editing tools of large sizes, reaching the limits of the viral packaging capacity. To improve AAV as a gene therapy tool, discovery of small but strong promoters is a crucial step. Here we report two new strong mini promoters, called INS84 and GCG110, with universal activity in rAAV expression vectors. These promoters are only 84 and 135 base pairs in size, respectively. They showed strong expression of a reporter transgene from rAAV in human and mouse cells and tissues, including human hepatocytes in primary cultures, humanized mice in vivo and human pancreatic islet cells. Expression levels in these tissues were comparable to those obtained with the much larger CAG promoter. Until now, viral vectors utilized (or 'borrowed') promoters that are characterized in the context of plasmid expression vectors or germ-line transgenes. Our strong mini-promoters for rAAV expression suggest a new direction for developing promoters for viral vectors, specifically that the large size of promoters required for expression in the context of plasmid vectors is often not necessary for strong expression in an rAAV vector.

276. Deep Tropism Profiling of Barcoded AAV Capsid and Cargo Pools in Intact Tissue Using High-Throughput Ultrasensitive Sequential FISH

Min Jee Jang, Gerard M. Coughlin, Yameng Zhang, Noushin Koulena, Simone Schindler, Long Cai, Viviana Gradinaru

Biology and Biological Engineering, Caltech, Pasadena, CA

Genetic access to specific cell types through minimally invasive routes is of particular interest in basic research and clinical applications. Extensive efforts have been made in engineering gene delivery vectors, such as recombinant adeno-associated viruses (rAAVs), and gene regulatory elements to achieve this goal. Despite many interesting candidates, revealed for example from directed evolution via M-CREATE (Sripriya Ravindra Kumar *et al.*, *Nature Methods*, 2020), histology-based characterization presents a bottleneck due to the limited number of variants and/or cell types that can be investigated at once. To address this, we have developed ultrasensitive sequential FISH (useqFISH) for multiplexed detection of both endogenous and barcoded transgene transcripts in intact tissue with single-molecule resolution. By combining two amplification strategies (rolling circle amplification, RCA, and hybridization chain reaction, HCR), we achieved a 2.7- or 6.7-fold increased signal-to-background ratio of useqFISH in comparison to one with RCA or HCR only amplification, respectively. UseqFISH allowed us to detect endogenous genes with a single probe pair (20-nucleotide (nt) for each) and, in transfected cell cultures, to distinguish capsid variants with genomes differing by only 7-mer peptide modification. We further improved useqFISH by establishing an automated single-molecule imaging and microfluidic solution exchange system and an analytical pipeline for 3D imaging data. To demonstrate the applicability of useqFISH for in vivo AAV profiling, we employed this method to further characterize a pool of 6 AAV capsid variants that we found to be highly efficient for brain-wide and/or cell-type biased transduction in the mouse brain following systemic delivery. We designed unique nucleic acid barcodes (160-nt) in the 3'UTR of each viral genome and retro-orbitally injected the pooled AAVs into 2 C57BL6/J mice at a dose of 5e10 viral genomes (vg) per variant (total 3e11 vg/mouse). For transcript detection, 11 canonical cell-type markers (e.g., Slc17a7, Gad1, Pvalb, SST, VIP, etc) were used together with probes against the viral genome barcodes, to characterize the cell-type tropisms of each variant. Next, we designed a pool of 103 barcoded AAV genomes carrying 4 tandem repeats of a unique miRNA target site. We packaged these genomes into AAV-PHP.eB and delivered to 3 C57BL6/J mice at a dose of 1e10 vg/variant (total ~1e12 vg/mouse). Using useqFISH, we were able to assess the ability of each miRNA target site to dampen transgene expression in different cell types, thereby revealing useful intersectional strategies to refine cell-type-specific transgene expression with capsid/cargo combinations. These results demonstrate that useqFISH allows for high-throughput characterization of pooled genetic variants of viral capsids and gene regulatory elements in intact tissue and thus enables comprehensive profiling of genetic toolkits for precise access to targets of interest.

277. Simultaneous Detection of AAV Genome, Transcript, and Protein Localization in Intact Cells and Tissues at High Resolution

Máté Borsos, Xinhong Chen, Viviana Gradinaru

California Institute of Technology, Pasadena, CA

Recent years have seen significant progress in AAV capsid engineering for gene delivery with increased efficiency and desired cell-type specificity to match the needs of pre-clinical research and gene therapy. The cellular uptake of AAVs, however, can stop short of the ultimate goal of cargo protein production - due to AAV silencing, insufficient nuclear transport, inefficient uncoating, failed second-strand synthesis, or other still to be discovered mechanisms. Defining the relationship between AAV genome uptake, transcription and cargo protein synthesis efficiencies in different cell types and tissues can help bypass key bottlenecks in gene delivery and guide effective AAV engineering. We adapted a recently published in-situ-transcription-based signal amplification method, the "Zombie technique" (*1*), to detect AAV genomes in a variety of fixed cells and tissues. Zombie involves producing 20 to 380 base-pair long barcode transcripts by exogenously-supplied T7 phage polymerase, which are then visualized at high

resolution in intact cells through fluorescent in situ hybridization (FISH) by hybridization chain reaction (HCR). We demonstrate that Zombie enables simultaneous detection of AAV genomes and AAV transcripts by FISH and cargo proteins by immunofluorescence. Consequently, we can determine the rates of AAV genome nuclear uptake, cargo transcription and cargo translation at different time points post-infection. Combining the Zombie method with cell-type-specific markers (protein immunolabelling or mRNA FISH) can give insights into subcellular AAV processing in complex tissues and help pinpoint to limiting factors in the overall transduction process. Interestingly, we find that after direct brain injection of AAV6, although cargo genomes do enter the nuclei of microglia, these genomes do not result in functional cargo protein production in this cell type. Furthermore, we analyze the pattern of AAV genome nuclear uptake, transcription and translation in mice after systemic delivery of AAV9 and engineered AAV capsids (AAV.PHP.eB, AAV.CAP-B10) (*2, 3*) across brain, liver and spleen. In conclusion, the Zombie technique adapted to AAV vectors enables simultaneous cargo genome, mRNA and protein visualization and thus can help differentiate cellular and subcellular processing steps of engineered gene delivery vectors, facilitating their refinement for basic and translational research.

1. A. Askary *et al.*, *Nat. Biotechnol.* **38**, 66-75 (2020).
2. K. Y. Chan *et al.*, *Nat. Neurosci.* **20**, 1172-1179 (2017).
3. N. Flytzanis *et al.*, bioRxiv doi.org/10.1101/2020.06.16.152975 (2020).

278. Building a Stable Transgene Expression Cassette Plasmid to Enhance the Quality and Potency of AAV Gene Therapy Vectors

Ting-Wen Cheng[1], David Souza[1], Annette Sievers[2], Ilya Tikh[2], Steven Kumpf[3], Jessie Qian[3], Tom Lanz[3], Clark Pan[1], Robert D. Bell[1]

[1]Rare Disease Research Unit, Pfizer Inc, Cambridge, MA,[2]BioMedicine Design, Pfizer Inc, Cambridge, MA,[3]Drug Safety Research & Development, Pfizer Inc, Groton, CT

The use of adeno-associated virus (AAV) for gene therapy has been accelerated in recent years. Large-scale AAV vector production with consistent purity and potency remains a challenging hurdle to overcome. DNA deletions and mutations within the inverted terminal repeats (ITR) of the transgene plasmid can occur during bacterial plasmid amplification and AAV production processes thereby hampering vector quality. Here we describe engineered ITR transgene plasmids containing both stable and unstable ITR sequences and further evaluated AAV packaging quality and potency. The occurrence of ITR mutations and deletions within the stable ITR plasmid was confirmed to be significantly reduced during large-scale plasmid production and purification. Packaged ssDNA content in AAV vectors that were generated using various ITR plasmids was also examined by next-generation sequencing to identify transgene sequences and non-transgene DNA fragments. Moreover, we evaluated AAV vector potency in vitro and in vivo by measuring the expression and activity of a reporter gene under the control of various regulatory elements. Our results indicate that AAV vector production and potency benefits from the use of a stable ITR transgene plasmids.

279. Abstract Withdrawn

280. Abstract Withdrawn

281. Abstract Withdrawn

282. Abstract Withdrawn

283. Satellite Sub-Genomic Particles Are Key Regulators of Adeno-Associated Virus Life Cycle

Junping Zhang[1], Jenni Firrman[2], Matthew Chrzanowski[3], Richard J Samulski[4], Weidong Xiao[1]
[1]The Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN,[2]Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA,[3]Lewis Katz School of Medicine, Temple University, Philadelphia, PA,[4]Department of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC

Historically, AAV defective interfering particles (DI) were known as abnormal virions arising from natural replication and encapsidation errors. Through single virion genome analysis, we revealed that a major category of DI particles contains a double stranded DNA genome in a "snapback" configuration (SBG). The 5'-SBGs include the P5 promoters and partial rep gene 5 sequences. The 3'-SBGs contains the capsid region. The molecular configuration of 5'-SBGs allowed double stranded RNA transcription in their dimer configuration, which in turn regulate AAV rep expression and may improve AAV packaging. In contrast, 3'-SBGs at its dimer configuration increased levels of cap protein. The generation and accumulation of 5'-SBGs and 3'-SBGs appears to be coordinated to balance the viral gene expression level. Therefore, the 10 functions of 5'-SBGs and 3'-SBGs may help maximize the yield of AAV progenies. We postulate that AAV virus population behaved as a colony and utilizes its subgenomic particles to overcome the size limit of viral genome and encodes additional essential functions.

284. Examining the Fidelity of Direct Nanopore Sequencing across ssAAV and scAAV ITRs: Revealing the Benefits and Limitations for Fast Proofing the Integrity of Intact Vector Genomes

Suk Namkung, Ngoc Tam Tran, Alexander Brown, Dan Wang, Jun Xie, Guangping Gao*, Phillip W. L. Tai*
Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Quality control assessment of adeno-associated virus (AAV) gene therapy vectors is essential to ensure safety and efficiency of the therapy; yet, gauging vector genome integrity and composition remains a challenge. Development of third-generation sequencing methods, including nanopore sequencing, have enabled the profiling of vector heterogeneity with varying degrees of accuracy. In an effort to find alternatives to obtaining single-genome sequencing resolution of recombinant (r)AAV vectors that can be achieved by AAV Genome-Population sequencing (GPseq), we have evaluated the capacity of long-read sequencing by Oxford Nanopore Technologies MinION sequencer to capture the full-length viral genome, from inverted terminal repeat (ITR)-to-ITR. We sequenced single-stranded (ss) and self-complementary (sc)AAV vector genomes, as well as the parental plasmids used to produce these vectors by nanopore to, 1) evaluate differences in sequencing the two forms of rAAV genomes, 2) validate whether the ssAAV and scAAV reads accurately reflect the actual vector genome population

in preparations, and 3) assess the processivity and accuracy of nanopore sequencing through the inverted terminal repeats (ITRs). Our analysis was also able to specifically identify truncation hotspots, indicating that nanopore sequencing can guide the engineering of optimal rAAV genomes. Sequencing of the parental plasmids also confirmed that these truncation hotspots are indeed found in vector preparations and not an artifact of the sequencing platform. In addition, we observe reads that support the notion that particles are packaged with vector genomes that arise by rolling hairpin replication. We find that although nanopore sequencing is limited in studying specific aspects of AAV vectorology due to its inability to resolve homopolymers, it excels in detecting vector heterogeneity and serves as a rapid and lower cost alternative for proofing vectors. *Co-corresponding authors

285. Network Analysis of Complex Novel AAV Vector Library Datasets for De-Risking Gene Therapy Candidate Selection

Deepak Grover[1], Sri Siripurapu[1], Stephanie Malyszka[1], Dharmendra Goswami[1], Lisa Stanek[1], Eric Zinn[2], Eva Andres-Mateos[2], Allegra Fieldsend[1], Julio Sanmiguel[2], Urja Bhatt[2], Erin Merkel[2], Chiekh Diop[2], Christopher Tipper[1], Luk H. Vandenberghe[1,2], Laura K. Richman[1]

[1]Affinia Therapeutics, Waltham, MA,[2]Grousbeck Gene Therapy Center, Mass Eye and Ear and Harvard Medical School, Boston, MA

Adeno-associated virus (AAV) vectors have emerged as the main gene delivery vehicles for the treatment of a wide variety of human diseases. Engineering novel AAV vectors is an exciting emerging area to broaden its reach by addressing the limitations of tissue and cell transduction profile of conventional serotypes. Previous work utilized evolutionary intermediates of viral vectors using ancestral sequence reconstruction (ASR) and developed methods to functionally evaluate ASR vector libraries by high throughput next generation sequencing in vivo. This approach has led to the identification of novel vectors with highly differentiated tissue tropism compared to naturally occurring serotypes currently in development. The generation of large datasets (for ~38,000 vectors) during NGS screens faces the challenge of data analysis to answer relevant questions such as vector distribution within tissues and cell types of interest. These datasets may suffer from bias such as 1) technical inconsistencies and variability in tissue sampling, DNA/RNA isolation and NGS process itself (2) differential vector manufacturability of library members, resulting in screening data resembling a negative binomial distribution, and a mixture of many Poisson distributions with different means, and (3) inherent structural and functional characteristics of the vectors that add to the overall complexity. In the absence of suitable corrections, these challenges could result in both false positive and false negative findings. Algorithms that consider the network structure in NGS data have recently emerged as powerful genomic tools. To address these potential sources of bias, we extrapolated one such weighted correlation network approach for our use case. In this approach, we incorporated the network topology in datasets to detect nonlinear association and decipher the collective dynamics of AAV variant tropism in tissues of interest. We applied this algorithm to an AAV library screen conducted in two mature female rhesus macaques that were injected intracisternally with a pooled library preparation plus spike-in controls. The animals were euthanized 7 days post injection, and seventeen CNS tissues including those from the cortex, deep brain, brainstem, and spinal cord regions were collected. Frozen tissues were processed for DNA isolation. Barcodes indicating the presence of vector DNA were recovered and counted by computational analysis of resulting NGS datasets. First and foremost, using this approach we were able to identify new tissue transduction patterns for these novel capsid variants in the CNS. We further identified subnetworks of novel capsid variants that were observed in specific brain regions. Network analysis conducted for this NHP screen revealed several AAV variants with distinct CNS tropism, potency and specificity profiles. Novel AAV variants were identified ranging from very narrow to broad anatomic CNS transduction patterns. In summary, we describe analytical methods that enable the high-resolution identification of AAV variants with unique transduction profiles, where conventional analysis has fallen short due to various sources of noise. These findings allow de-risking via more predictive AAV candidate selection tailored to the indication profile and pave the way for future structure-function modeling and higher analytics such as Machine Learning.

286. AAV^{mod2}, an AAV Capsid Engineered to Independently Detarget the Liver and Enhance Gene Delivery to Skeletal Muscle

Kevin Olivieri[1], Zachary Thorpe[1], Stewart Craig[1], Doug Sanders[1], Levy Te[1], Luk H. Vandenberghe[1,2], Laura K. Richman[1], Christopher Tipper[1]

[1]Affinia Therapeutics, Waltham, MA,[2]Grousbeck Gene Therapy Center, Mass Eye and Ear and Harvard Medical School, Boston, MA

Engineering novel adeno-associated virus (AAV) vectors is an exciting emerging area in gene therapy to broaden its reach by addressing the limiting clinical characteristics of conventional serotypes. As has been previously reported, AAV alteration leading to a liver-detargeting phenotype was discovered within the Anc80 library, one of nine libraries derived by ancestral sequence reconstruction. The residues necessary and sufficient for this phenotype were identified and confirmed in vivo. Importantly, the ability to detarget the liver has been successfully transferred to naturally occurring serotypes, and AAV^{mod1} has emerged as a potential lead vector with favorable transduction profile for muscle disorders while detargeting the liver. Demonstration of specific, possibly synergistic, overlay of independent structure/function phenotypes is critical to advance our understanding of vector modification and resulting alteration in tissue tropism. The use of peptide insertion to enhance or alter AAV targeting is well known. Here, we describe a peptide insertion that improved muscle tropism in mice. Furthermore, the peptide insertion combined with AAV^{mod1} further enhanced the overall muscle transduction profile. C57BL/6 mice were injected with four CAG.GFP variants: AAV9, AAV^{mod1}, AAV$^{mod1.5}$ (peptide insertion), and AAV^{mod2} (mod1+peptide insertion), at doses of $1e^{13}$ and $5e^{13}$ vg/kg. Upon sacrifice 28 days later, tissues were flash frozen for biodistribution and fixed for immunohistochemistry (IHC). IHC showed characteristic staining of the liver and muscle with AAV9. As suggested by earlier work, AAV^{mod1} showed reduced liver staining and AAV$^{mod1.5}$ enhanced muscle staining. Interestingly, the combination of modifications in AAV^{mod2} showed uncompromised

features of both engineered vectors, and perhaps qualitatively enhanced muscle delivery. Biodistribution by ddPCR confirmed that the peptide insertion alone had improved targeting of skeletal muscle, 5x better by gc/diploid genome versus AAV9, with a 50x improved gene expression by eGFP mRNA versus the housekeeping gene RPP30. $AAV^{mod1.5}$ is otherwise akin to its parental serotype in terms of robust hepatocyte transduction. AAV^{mod1} had a >10x reduction in liver gc/diploid genome and a >1000x reduction in liver expression. As observed by IHC, AAV^{mod2} liver-detargeting was unaffected by the muscle enhancement. Expression in the muscle by ddRT-PCR was equivalent between $AAV^{mod1.5}$ and AAV^{mod2}. Quantitative measure of GFP signal by IHC is ongoing. Future studies will test the transduction profile of AAV^{mod2} in BALB/c mice and NHPs.

287. AAV Capsid Development in Primary Human Retinal Explants

Adrian Westhaus[1], Steven Eamegdool[2], Leszek Lisowski[1], Anai Gonzalez-Cordero[3], Robyn Jamieson[2], Milan Fernando[3], Grady C. Smith[3], Giorgia Santilli[4], Adrian J. Thrasher[4]

[1]Translational Vectorology, Children's Medical Research Institute, Westmead, Australia,[2]Eye Genetics, Children's Medical Research Institute, Westmead, Australia,[3]Stem Cell Medicine, Children's Medical Research Institute, Westmead, Australia,[4]Molecular and Cellular Immunology, University College London, London, United Kingdom

Gene therapy interventions for degenerative diseases affecting the eye are quickly becoming the new reality for treating - and potentially curing - blindness. However, ineffective delivery of the therapeutic transgene to photoreceptors and other cell types of the human retina are hampering the wide-spread success to date. To address this technical limitation, we applied our proprietary Functional Transduction (FT) selection platform, which selects for novel capsids based on their expression strength in target cells to perform AAV library selection on primary human retinal explants. After performing two rounds of RNA-based library selection in polarized retinal explants, we compared the top 20 selected variants using a next-generation sequencing (NGS)-based strategy in preclinical models of the human retina such as primary human explants and iPSC-derived retinal organoids. Furthermore, the most functional variants identified in the NGS screen were individually validated using an immunofluorescence (IF)-based readout. Based on the data collected we were able to divide the selected variants into two distinct functional groups. One group of capsids showed a broad profile of transduction, labelling various cell types of the human retina, including photoreceptors. The second group consisted of capsids that showed an extremely strong and specific transduction of Müller glia cells in human tissue, which is something that, to our knowledge, has not been reported to date. Lastly, testing the vector performance in primary human and iPSC-derived retinal pigment epithelium (RPE), we identified very strong and promising candidates that significantly outperform AAV2, the FDA approved serotype used in the voretigene neparvovec gene therapy, for transduction of RPE.

288. Disorder in the C-terminus of VP Dictates AAP Requirement for AAV Capsid Assembly

Swapna Kollu, Helen Rappe Baggett, Anusha Sairavi, Hiroyuki Nakai

Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR

Assembly-activating protein (AAP) is a non-structural viral protein coded in a frame-shifted manner within the *cap* gene of adeno-associated virus (AAV). Although AAP supports capsid assembly in AAV serotypes 1-12; AAV serotypes 4,5, 11 rh32.33 are capable of assembling capsids even in the absence of AAP. It remains unclear why certain AAV serotypes are capable of AAP-independent capsid assembly. Here we demonstrate that AAV variants with enhanced intrinsic disorder in regions of the C-terminus of VP are capable of AAP-independent capsid assembly. Bovine AAV (BAAV) and goat AAV (AAV-Go.1) have sequences similar to those of AAV4 and 5, respectively, and recapitulate their phenotype of AAP-independent capsid assembly. However, this is not the case for AAV11 and 12, the close phylogenetic neighbors; presenting an ideal case for a study to uncover mechanisms dictating AAP requirement. In the current study, we propose a model that explains why certain AAV variants are capable of AAP-independent capsid assembly. Upon mutational analysis, we found that a single amino acid in the C-terminus of VP3 could alter AAP requirement in AAV 4, 11 and 12. In AAV4 and AAV11, a P-to-L mutation at the corresponding positions 722 and 721 respectively, can convert AAV4 and 11 from being AAP-independent to AAP-dependent. Conversely, in AAV12, a L-to-P mutation at the corresponding position 730 can convert AAV12 from being AAP-dependent to AAP-independent. We designated this position (722 in AAV4, 721 in AAV11 and 730 in AAV12) as the "critical position" or CP. The CP lies in the C-terminus of VP, close to the VR-IX loop that is proximal to the 2-fold axis of VP. In the AAV5 VP context, we have found that replacing 12 amino acids (dodecapeptide) of AAV5 at position 648 and 650 with each corresponding dodecapeptide from AAV2 VP leads to loss of AAV5's AAP independency. In AAV5, position 648 and 650 are in the HI loop, close to the 5-fold axis. Thus, for AAV4, 5, 11 and 12; we have variants that are either AAP-dependent or independent. For each of these serotypes and variants, we determined the degree of VP protein disorder using the Predictor of Natural Disordered Regions (PONDR) and compared the PONDR values between VPs showing distinct degrees of AAP dependency. We found that the disorder near the mutated region in the AAP-independent VPs always tends to be higher than that in the AAP-dependent VPs . The mutated regions are either near the 2-fold axis (for AAV4,11 and 12) or the 5-fold axis (for AAV5). Thus, AAP-independent VPs tend to have a greater degree of protein disorder than those that are AAP-dependent in these two regions. In the AAV icosahedron, interdigitation of VP subunits at the 2-fold and 5-fold axes is poor compared to that at the 3-fold axis. Enhanced flexibility at the 2-fold and 5-fold axes likely promotes VP subunit interdigitations at VP-VP interfaces, leading to more stable VP oligomerization and subsequent capsid assembly. Conversely, VPs with reduced flexibility at the 2-fold or 5-fold axis do not autonomously oligomerize and are more prone to degradation, necessitating non-VP factors for promoting VP oligomerization via the 2-fold or 5-fold axis; and therefore, they are dependent on AAP for VP oligomerization and subsequent capsid

assembly. Taken together, our data suggest that enhanced disorder in the C-terminus of VP that promotes structural flexibility in the 2-fold and 5-fold axis regions plays a critical role in conferring the ability to undergo AAP-independent capsid assembly.

289. Ly6a Receptor Affinity Impacts Blood-Brain Barrier Penetration and the Tissue Targeting Properties of AAV9-PHP.B Vectors after Systemic Delivery

R. Alexander Martino, Joshua Sims, Qiang Wang, Gui Hu, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

AAV9-PHP.B—a gene therapy vector based on adeno-associated virus 9 (AAV9)—crosses the blood-brain barrier (BBB) in C57BL/6 mice with unprecedented efficiency. AAV9-PHP.B achieves this brain-transduction phenotype through its interactions with Ly6a, a cellular receptor expressed on the surface of microvascular endothelial cells in the murine brain. Here, we explored how the intrinsic affinity of the PHP.B insert peptide for Ly6a and the valency of its presentation on the AAV9 capsid surface impact receptor engagement and BBB crossing. First, we developed a series of PHP.B peptide mutations that modulate the intrinsic affinity of AAV9 insert vectors for Ly6a. Surprisingly, AAV9-PHP.B insert variants with both increased and decreased receptor affinity localized to the brain with equal efficiency. However, BBB crossing and brain transduction were attenuated for variants with both weaker *and* tighter Ly6a binding, indicating that the original PHP.B peptide is near an affinity optimum for BBB function, which is defined by more than BBB localization alone. Because the valency of PHP.B peptide presentation also influences receptor binding *in vitro*, we explored the valency requirements for BBB function by generating a series of mosaic AAV9:AAV9-PHP.B capsids and using these capsids to define activity thresholds. We discuss the role of affinity and valency in targeted vector design to overcome delivery obstacles in humans.

290. A Novel AAV Library Design Yields Capsids That Cross the Blood-Brain-Barrier in Mice

Joshua Sims, Yuan Yuan, Shiva Shrestha, Jacqueline B. Murphy, Sharon Lian, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

The adeno-associated virus (AAV) capsid can safely deliver gene correction to many tissues following intravenous (IV) delivery. However, even so-called "neurotropic" natural serotypes show inefficient gene transfer to the central nervous system due to the blood-brain barrier (BBB). Here we present a novel AAV library strategy to discover peptides capable of directing AAV9 across the BBB. We first identified hundreds of candidate peptides that had the potential to interact with proteins residing on brain capillaries. We then produced a library of AAV9 variants displaying these peptides on the capsid surface in a variety of structural contexts. Next-generation sequencing (NGS) analysis of capsid genes from brain tissue revealed remarkable brain-transduction activity of a variant—YGY—that harbors a 13 amino-acid insertion at HVR8. We deployed an NGS-based optimization pipeline to simultaneously improve vector production yield and BBB-crossing in AAV9-YGY. Optimized AAV9-YGY variants show superior brain transduction from IV delivery compared to a benchmark engineered capsid—AAV9-PHP.B—in C57BL/6J mice. Unlike AAV9-PHP.B, however, YGY variants also function in strains that are not permissive to AAV9-PHP.B, such as Balbc. We discuss the implications of this work for targeting the AAV capsid to tissue-specific receptors and contrast it to more typical, unbiased, large-library screening approaches, which often prove ineffective in large animal models.

291. The Nucleolar-Enriched Deubiquitinating Enzyme USP36 Regulates AAP Ubiquitination

Swapna Kollu, Anusha Sairavi, Mushui Dai, Hiroyuki Nakai

Molecular and Medical Genetics, Oregon Health & Science University, Portland, OR

Recombinant adeno-associated virus (AAV) is an FDA-approved gene delivery vector that is increasingly finding applications for therapy of various diseases. Discovering new ways to enhance vector production by understanding the AAV capsid assembly process can significantly reduce the cost of AAV vector-based gene therapy. Capsid assembly is regulated by a non-structural viral protein called assembly-activating protein (AAP) and host cellular factors. To identify the host cellular proteins that interact with AAP and are involved in the capsid assembly process, we conducted a BioID proximity-based proteomics study and identified USP36, a nucleolar-enriched deubiquitinating enzyme (DUB), as a cellular protein that potentially interacts with AAP2 (AAV2 AAP) . We have previously shown that USP36 plays roles in effective AAV2 virion formation and maintaining steady-state levels of AAP2, having led us to propose a hypothesis that USP36 regulates AAP2 by altering its ubiquitin status. Here we provide definitive evidence showing that USP36 interacts with AAP2 and regulates poly-ubiquitination of AAP2 through its conserved catalytic triad (Cys-His-Asn/Asp). To detect ubiquitination on AAV viral proteins in HEK293 cells, we first expressed His-tagged ubiquitin with AAV2 VP3 and/or FLAG-tagged AAP2 (FLAG-AAV2) and performed a nickel pull-down experiment. Despite clear demonstration of poly-ubiquitinated VP3, poly-ubiquitination of FLAG-AAP2 could be barely detected by the standard immunoblotting procedure. Subsequently, we explored various conditions for optimizing the AAP2 immunoblotting procedures and successfully established a very sensitive assay by which we can unambiguously assess poly-ubiquitination status of AAP2. The new assay uses an HA-tagged AAP2 (HA-AAP2) that, unlike FLAG-AAP2, does not carry amino acids Lys, Cys, Arg, Ser or Thr within the tag, ruling out any surreptitious ubiquitination of the tag itself. With this new robust assay, we investigated how USP36 might regulate poly-ubiquitination of AAP2 when USP36 is overexpressed in HEK293 cells. To this end, we created a USP36 mutant (USP36mt) carrying amino acid mutations within the conserved catalytic triad. USP36 belongs to the USP family of deubiquitinating enzymes that are cysteine-dependent proteases containing the Cys-His-Asn/Asp catalytic triad. This triad has been shown to be important for deubiquitinating enzyme (DUB) activity in other DUBs belonging to the USP family; therefore, we expected that USP36mt is devoid of the DUB activity. The results showed that the poly-ubiquitin tags of AAP2 were reduced by over-expressing the wild-type USP36 but were unchanged with USP36mt. This demonstrated that USP36 regulates

AAP2 ubiquitination, USP36mt is incapable of deubiquitinating AAP2, and the catalytic triad in USP36 indeed mediates the DUB catalytic activity and is responsible for deubiquitination of AAP2. To investigate interaction of USP36 and AAP2, we co-expressed FLAG-USP36 and HA-AAP2 in HEK293 cells, followed by FLAG immunoprecipitation and subsequent probing with an HA antibody. We found that FLAG-USP36 pulled down HA-AAP2, providing evidence that USP36 and AAP2 can interact in HEK293 cells. To our knowledge, this is the first study of regulation of post-translational modifications on AAP by cellular proteins and how the regulation relates to the AAP stability.

292. Identification of a Kidney Specific AAV Following Insertion of Tissue Homing Peptides in Variable Region IV

Samantha A. Yost, Elad Firnberg, Justin D. Glenn, April R. Giles, Jenny M. Egley, Karolina J. Janczura, William M. Henry, Subha Karumuthil-Melethil, Jared B. Smith, Ye Liu, Andrew C. Mercer

Research and Early Development, REGENXBIO Inc., Rockville, MD

The use of adeno-associated viruses (AAV) as gene delivery vectors is an area of accelerated growth and development, offering promising treatments for many severe, unmet medical needs. Early capsid engineering studies produced variants with enhanced properties for gene transfer including increased potency, escape from immune barriers, cell type and tissue specificity, and manufacturability. For instance, the variable region eight (VR-VIII) capsid loop was among the first determined to be amenable to peptide insertion, and insertions at this location have been used extensively to confer enhanced utility to the capsid. In order to provide additional opportunities for peptide randomization or insertion of purification tags and homing peptides, we used available high-resolution structures to identify variable region IV (VR-IV) as an area of high flexibility and low structural similarity between serotypes. Positions within VR-IV were previously probed with FLAG peptides and effects on AAV packaging efficiency, potency, and surface exposure of the peptide were evaluated (Yost *et al*. ASGCT 2019). The most promising insertion site (immediately after AAV9 residue S454) was then selected and used as a template for insertion of previously identified homing peptides to test the potential of retargeting AAV to tissues of interest. Individual capsids containing homing peptides were tested previously *in vitro* in several cell lines (Lec2, HT-22, hCMEC/D3, and C2C12) and combined as a DNA-barcoded pool in C57/BL6 mice through intravenous (IV) administration of $1x10^{13}$ GC/kg. Tissue transduction and transgene expression were analyzed by next generation sequencing. Capsids containing highly charged peptides (FLAG or 8-Asp) severely impaired transduction of all tissues. Insertion of a kidney homing peptide (CLPVASC), originally identified by Pasqualini and Ruoslahti in 1996, significantly reduced transduction in the liver and moderately reduced transduction in muscle tissues while maintaining kidney transduction levels compared to the parental AAV9. Pooled vector results were confirmed through a single vector administration mouse study. In a nonhuman primate study of IV administered, barcoded vector pool ($2x10^{13}$ GC/kg), these trends were replicated. Our data suggest this kidney homing peptide insertion at AAV9 VR-IV has the promising feature of liver and muscle de-targeting while maintaining kidney transduction efficiency in rodent and primate. Further capsid engineering combined with optimized route of administration are warranted for efficient gene transfer in kidney.

293. Optimization of Adeno-Associated Viral Vector-Mediated Transduction of the Retina: Comparison of Five Short Promoters

Bart Nieuwenhuis[1,2], Elise Laperrousaz[1], Joost Verhaagen[2,3], James W. Fawcett[1,4], Keith R. Martin[1,5,6], Andrew Osborne[1]

[1]University of Cambridge, Cambridge, United Kingdom,[2]Netherlands Institute for Neuroscience, Amsterdam, Netherlands,[3]Vrije Universiteit Amsterdam, Amsterdam, Netherlands,[4]Institute of Experimental Medicine, Prague, Czech Republic,[5]Centre for Eye Research Australia, Melbourne, Australia,[6]University of Melbourne, Melbourne, Australia

Recombinant adeno-associated viral vectors (AAVs) are the vectors of choice for gene delivery in the retina. In particular, AAV serotype 2 became apparent as vector with outstanding transduction of retinal ganglion cells. The AAV serotype is however not the only factor contributing to the effectiveness of gene therapies. DNA regulator units classified as promoters influence the strength and cell-selectivity of transgene expression. This study is a side-by-side comparison between five short promoters designed to maximise AAV2 cargo space for gene delivery: chicken β-actin (CBA), cytomegalovirus (CMV), short CMV early enhancer/chicken β-actin/short β-globulin intron (sCAG), mouse phosphoglycerate kinase (PGK) and human synapsin (SYN) promoter. AAV2 harbouring these promoters and the DNA coding sequence for eGFP were intravitreally injected into the eyes of adult C57BL/6J mice and tissues of the visual system were examined after 4 weeks. eGFP expression was strongest in the retina, optic nerves and brain when driven by the SYN or CAG promoters. The other three promoters had relatively weak expression by comparison. The SYN promoter also expressed almost exclusively in retinal ganglion cells compared to CBA, CMV and CAG, which directed eGFP expression in a variety of cell types across multiple retinal layers. The PGK promoter had predominant expression in retinal ganglion cells and AII amacrine cells but was weaker than SYN. Taken together, this promoter comparison study contributes to optimising AAV-mediated transduction in the retina, and could be valuable for research in ocular disorders, particularly those with large or complex genetic cargos.

294. Purification of Extracellular Vesicle Enveloped AAV Vectors Increases Resistance to Antibody Neutralization and Allows Biochemical Characterization

Ming Cheng[1], Laura Dietz[2], Yi Gong[3], Florian S. Eichler[3], Josette Nammour[3], Carrie Ng[3], Dirk Grimm[2], Casey A. Maguire[1]

[1]Massachusetts General Hospital, Charlestown, MA,[2]University of Heidelberg, Heidelberg, Germany,[3]Neurology, Massachusetts General Hospital, Charlestown, MA

Adeno-associated virus (AAV) is a non-enveloped DNA virus. However, in conditioned media of packaging cells producing recombinant AAV vector, AAV capsid can associate with the interior and surface of

extracellular vesicles (EVs), sometimes referred to as exosomes. Since then we and others have demonstrated that exosome-enveloped AAV, exo-AAV, can enhance transduction *in vivo,* as well as evade neutralizing antibodies. While promising, these data were generated with differential centrifugation to isolate the exo-AAV. This method results in a heterogeneous mixture of exo-AAV, co-precipitating proteins, as well as free AAV capsids. To accurately define the properties of exo-AAV, here we used a density gradient method to purify exo-AAV which separates it from denser free capsids. We next performed head to head comparisons of standard AAV, differential centrifuged exo-AAV, and gradient purified exo-AAV for antibody evasion and transgene expression in the murine brain. We found purified exo-AAV to be more resistant to neutralizing antibodies than the other AAV preparations. Direct intracranial injection of purified exo-AAV into mice resulted in robust transduction, which trended higher than the other preparations of AAV. We also identified the recently described membrane-associated accessory protein (MAAP) by mass spectrometry of purified exo-AAV preparations. Together, these data suggest that higher purity exo-AAV will have beneficial characteristics for in vivo gene delivery and also may lead to mechanistic insights into the incorporation of AAV into EVs.

295. CpG-Depleted AAV Vectors Result in Durable Transgene Expression in a Novel Mouse Model That Mimics the Transgene-Limiting Inflammatory Response Observed in Human Clinical Trials

Susan Faust, Joseph E. Rabinowitz
NxGEN Vector Solutions, Washington, DC

In clinical settings, activation of de novo and pre-existing cellular and humoral immunoreactivity toward AAV viral capsid and encoded transgene antigens has compromised safety and long-term gene replacement success. This was not observed in the preclinical studies. We established a mouse model of hepatic AAV gene transfer that mimics the inflammatory immune responses that limit transgene durability in human clinical trials of liver gene transfer without requiring a "model antigen" such as OVA, which can add an unnecessary variable to the experiment and does not closely model the conditions in the clinic. We used a uniquely immunogenic AAV vector, AAVRh32.33, that mimics in mice the vibrant inflammatory response observed in clinical trials. C57BL/6 mice were I.V. injected with 1E11 GC of AAV8 or AAVRh32.33, expressing a LacZ reporter gene. Liver tissue was recovered on days 7 and 28 post vector administration and histochemistry was performed to assess B-gal expression in the liver. AAV8 injected mice exhibited stable transgene expression while AAVRh32.33 gene transfer resulted in an almost complete loss of B-gal expressing cells by day 28. As hepatotoxicity indicated by brief elevation in liver transaminases can be associated with an immune response toward the vector or transgene in human clinical trials, we assayed for this in our experimental model. Serum was collected and AST and ALT levels were quantified at various time points. The loss of transgene expression correlated with a transient elevation of ALT levels similar to those observed in FIX human clinical trials. To investigate the relationship between the loss of transgene expression with accompanying elevation in ALT levels and immunoreactivity, MHC I tetramer stain and ELISPOT were performed to quantify transgene reactive CD8+ T cells and primed transgene responsive IFN-g-producing cells. Mice that received the AAVRh32.33 vector exhibited a significant increase in the percentage of LacZ-responsive CD8+ T cells compared with mice that received the AAV8 vector. A significant increase of primed transgene antigen-reactive IFN-g ELISPOT responses were also observed in mice that received the AAVRh32.33 but not the AAV8 vector. Using this novel mouse model, we next assessed the effect of CpG-depletion of the immunogenic AAVRh32.33 vector on induction of innate and adaptive immune responses following hepatic gene transfer by comparing the performance of two vectors expressing a β-gal reporter gene that differed only by the abundance of CpG motifs: 324 CpG motifs in the CpG-unmodified AAVRh32.33 vector versus 16 CpG motifs in the CpG-depleted AAVRh.32.33 vector. Following I.V. administration of 1E11 GC of the modified and unmodified AAVRh32.33 vectors, CpG-unmodified AAVRh32.33 vector transgene expression was rapidly eliminated from mouse hepatocytes while CpG-depleted AAVRh32.33 vectors achieved sustained transgene product expression. The CpG-unmodified AAVRh32.33 vectors also induced maturation of conventional and plasmacytoid dendritic cells in spleens of injected mice, which was linked to stimulation of transgene product-specific T cell responses. Both activation of innate and adaptive immune responses were strongly attenuated upon CpG reduction of the AAV vector, and only the CpG-reduced AAVRh32.33 vectors resulted in durable transgene expression. These results establish a clinically relevant mouse model of AAV hepatic gene transfer that truly mimics the initial expression and subsequent transgene loss that has been observed in the clinical setting and demonstrates that CpG depletion of AAV vector genomes provide an essential strategy to improve the clinical outcome of AAV-mediated gene transfer.

296. Identification of New Adeno-Associated Virus 9 (AAV9) Capsid Variants Targeting the BALB/c Mouse Brain through Directed Evolution

Serena G. Giannelli[1], Mirko Luoni[1], Eleonora Conti[1], Antonio Niro[1], Martin Trepel[2], Jakob Körbelin[3], Vania Broccoli[1,4]
[1]Neuroscience, San Raffaele Scientific Institute, Milan, Italy,[2]Department of Hematology and Medical Oncology,, University Medical Center Augsburg, Augsburg, Germany,[3]ENDomics Lab, Department of Oncology, Hematology and Bone Marrow Transplantation, University Medical Cente Hamburg-Eppendorf, Hamburg, Germany,[4]Neuroscience, National Research Concilium (CNR), Milan, Italy

The intravenous route is considered a safe route of vector administration employed in gene therapy. Such a strategy nonetheless requires vectors displaying high transduction efficiency but also high specificity, in order to achieve optimal delivery of the target tissue and avoid possible detrimental effects. Systemic delivery of therapeutic vectors into the central nervous system (CNS) bears even more obstacles, since the blood-brain barrier restricts the passage of molecules, including AAV capsid. Cerebral endothelial cells play a key role in BBB integrity and function. Therefore, a recombinant AAV specifically targeting these cells could be helpful for the development of new therapeutic

approaches for various CNS diseases. Nonetheless, in the latest years interest has increased for vectors that, not only target brain endothelial cells, but even cross them, thus reaching the cerebral parenchyma and infecting neurons and glia cells. The clinical relevance of such a tool would be invaluable and could bear innumerable applications in CNS pathologies. In this perspective capsid directed evolution focused on the natural serotypes AAV9 since its ability to cross the BBB - although restricted - was very well known. Using a peptide display approach based on AAV9 Deverman *et al*., discovered various capsids with the ability to cross the BBB, among which PHP.eB is the most efficacious in mice. Systemic administration of these vectors in non-human primates (NHP) revealed a limited capacity to cross the BBB. Species-specific brain transduction was found to be based on a small protein, called Ly6a present in the murine genome but not in human and primates, thus lessening the translability of these vectors. Starting from these premises we executed an in vivo screening approach using a AAV9 display peptide library to select novel brain targeting capsid variants. The peptide library was inserted in the AAV9 capsid with additional W503A mutation which is known to erase the AAV9 natural biding on galactose thus facilitating new interactions.In order to avoid Ly6a binding the selection was carried out using BALB/c mice that present mutations in the Ly6a locus that significantly decrement its expression. Three enriched capsid variants were identified after four consecutive rounds of selection. All of them displayed prevalent endothelial cell transduction that we identified and characterized on transduced mouse brains after tail vein injections. Brain cell transduction and neuronal targeting was also observed with interesting differences between brain regions with two capsid variants and equally conserved in BALB/c mice.

297. The Key Amino Acids Play a Critical Role in the Production of the Haploid Adeno-Associated Virus

Zheng Chai[1], Amaury Pupo[1], Scott Anglin[1], Lester Suarez[1], R. Jude Samulski[1,2]

[1]Research & Development, Asklepios BioPharmaceutical, Inc., Durham, NC,[2]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Adeno-associated virus (AAV) has been successfully applied for human gene therapy as a delivery vector. At present, two AAV vector-based gene therapy products have been approved by FDA. However, vector engineering which could enhance transduction efficiency and avoid immune response is still an important strategy for novel vector development. Previously, haploid vectors have been reported for their high transduction efficiency and neutralizing antibody evasion. It also showed that the VP1 and VP2 from one serotype and the VP3 from another serotype might change the tropism of the AAV vectors. However, the ratio of two different plasmids, containing VP1/2 and VP3 of the haploid vectors, also needed to be optimize. For future application in AAV vector manufacturing, we established the all-in-one constructs including VP1/2 from AAV8, VP3 from either AAV rh10 or AAV rh74 within one plasmid. The haploid AAV8.8_rh10, AAV8.8_rh74 vectors, the parental AAV8 and AAV rh10 were produced by three plasmid co-transfection into Pro-10 cells. The results showed that the virus titer of the haploid AAV8.8_rh10 was similar to those of the parental vectors AAV8 and AAV rh10. However, the virus titer of the haploid AAV8.8_rh74 was much lower than those of the parental vectors. Next, we analyzed protein sequences of VP3s of AAV rh10 and rh74 and found that there were only 5 amino acid differences. Therefore, we generated 5 mutants on AAV8.8_rh10 background with the rh74 substitutions and evaluated the vector production. We found that the virus titers of AAV8.8_rh10 V378 Del and AAV8.8_rh10 V379W (VP3 numbering) were much lower than that of AAV8.8_rh10 but were similar with that of AAV8.8_rh74. The other three mutant haploid vectors achieved the same amount of the virus vectors as that of AAV8.8_rh10. To confirm that these two amino acids play an important role in the virus production, we reversely generated 4 mutants on AAV8.8_rh74 background, incorporating the rh10 amino acids. Since the positions 378 and 379 are very close, we combined them together and named the haploid vector as AAV8.8_rh74_VV. After the vectors were produced, the results showed that the virus titer of the AAV8.8_rh74_VV was similar with that of AAV8.8_rh10, while the other 3 mutant vectors were similar with AAV8.8_rh74. The result firmly proved that these two amino acids, at position 378 and 379 of VP3 play an important role in vector production. In summary, the data indicates that the all-in-one construct could achieve a similar amount of haploid AAV vectors as the parental vectors. Moreover, some key amino acids could affect the production of the AAV vectors, which may need additional attention during manufacturing process.

AAV Vectors - Virology and Vectorology

298. A CRISPRa Screen Identifies Transcription Factors That Can Silence or Activate rAAV Genomes

Eric M. Walton, Joshua B. Black, Charles A. Gersbach, Aravind Asokan

Duke University, Durham, NC

Gene therapies based on recombinant AAV vectors have been consistently plagued by relatively low transgene expression and eventual transgene silencing over time, despite the persistence of viral genomes within the target tissue. These deficiencies have necessitated the use of very high viral titers, leading to toxic side effects in clinical trials. Using a high-throughput pooled CRISPR activation (CRISPRa) screen, we have identified multiple host transcription factors (TFs) that are capable of influencing rAAV transgene expression. Specifically, we utilized deactivated Cas9 (dCas9)-based gene activation to overexpress ~1,500 putative TFs in the human genome and assess the impact of such on transgene expression from cassettes incorporating different promoters in a liver cell culture model. Along with putative effectors that emerged as activating for specific promoters, we have identified TFs that can enhance rAAV transgene expression in a promoter independent manner. Interestingly, some of these factors, when overexpressed, were transcriptionally activating despite the presence of a KRAB domain, which is canonically involved in transcriptional silencing. Additionally, we identified a different set of TFs that are capable of potently silencing rAAV transgenes carrying different promoters. Interrogation of predicted binding sites within different elements in the rAAV vector genome and deeper analysis of host pathways yielded additional insight into the putative mechanisms by which these factors may modulate rAAV transgene expression. These data provide a roadmap for improvements to vector genome design

by leveraging (or circumventing) the activity of host TFs with a goal to potentially reduce the vector dose and improve the persistence of rAAV-mediated gene therapies.

299. Subgenomic Flaviviral RNA Elements Enhance rAAV Vector mRNA Stability and Transduction

Rita M. Meganck[1,2], Sven Moller-Tank[3], Jiacheng Liu[4], Longping V. Tse[2], Heather A. Vincent[1], William F. Marzluff[2], Aravind Asokan[1]

[1]Duke University, Durham, NC,[2]UNC Chapel Hill, Chapel Hill, NC,[3]Regeneron Pharmaceuticals, Tarrytown, NY,[4]Sloan Kettering Institute, New York, NY

Suboptimal transgene expression using recombinant AAV vectors is a significant challenge facing gene therapy. This has necessitated the use of high viral doses, leading to toxic side effects in clinical trials. One potential approach to improve AAV vector efficacy is to enhance rAAV genome derived mRNA persistence to achieve higher transgene expression. Here, we leverage naturally occurring viral RNA elements that function through diverse mechanisms to promote mRNA translation, enhance mRNA stability, and subvert host antiviral responses. Specifically, we exploit subgenomic flaviviral RNAs (sfRNAs), which antagonize the exoribonuclease XRN1, resulting in increased stability of a subset of host mRNAs, and inhibit the host interferon (IFN) response. To determine if AAV transcripts are subject to XRN1-mediated decay, we first transduced XRN1 KO cells with several AAV serotypes and observed increased transduction compared to scrambled controls. We then engineered AAV genomes by incorporating sfRNA elements from different flaviviruses [Dengue virus (DENV), Zika virus (ZIKV), Japanese Encephalitis virus (JEV), Yellow Fever virus (YFV), Murray Valley Encephalitis virus (MVEV) and West Nile virus (WNV)] and observed increased transduction efficiency in vitro. This effect was context-dependent; increased transduction efficiency was only observed when the sfRNA element was incorporated in cis as a 3' untranslated region (UTR) of the luciferase transgene mRNA. Further molecular dissection revealed that while the sfRNA stem loop regions were dispensable, two distinct dumbbell (DB) structures within the DENV2 sfRNA were necessary and sufficient to confer improved AAV transduction. Surprisingly, the ability of the DENV2 sfRNA and DB RNA elements to enhance AAV transduction appeared to be XRN1-independent. Rather, we find that sfRNA elements likely function through stabilization of AAV transgene-derived mRNA, increasing overall transduction. These proof-of-concept studies demonstrate that engineering novel RNA elements into AAV genomes can enhance AAV mRNA stability and transduction efficiency, and potentially reduce AAV vector doses needed to achieve effective therapeutic gene expression.

300. Evaluating Partner Fitness by Interfaces Interaction in Potential AAV Haploids

Amaury Pupo[1], Zheng Chai[1], Scott Anglin[1], Lester Suarez[1], Richard Jude Samulski[1,2]

[1]R&D, Asklepios BioPharmaceutical, Inc., Research Triangle, NC,[2]Gene Therapy Center, University of North Carolina, Chapel Hill, NC

Transcapsidation approaches consisting of the transfection of combinations of AAV serotype helper plasmids to produce mosaic recombinant AAV capsid has been established (Rabinowitz *et al.* (2004), J. Virol. 78: 4421-4432). We have introduced the term Polyploid (when utilizing more than two parental AAV helpers) or Haploids (when only using two) to better describe this approach. For example, AAV capsids made from VP1/VP2 of one serotype and VP3 donated from a unique serotype, or combinations thereof, would represent the generation of novel Haploid AAV capsid Vectors. Haploid AAVs have the potential to uniquely combine structural advantages of parental AAVs and have successfully demonstrated 1) synergistic effects in transduction, 2) unexpected new tropisms, and 3) the ability to escape Nab (Chai *et. al.* (2019), Viruses 11: 1138). We have performed this study to identify good/better partners to make a haploid. We go beyond sequence identity analysis to predict the feasibility of new AAV haploids by calculating the interaction energies of the new interfaces that appear in the haploids with the original interfaces in the parent AAVs. Based on this approach, specific mutations can be predicted to improve the interaction energies in the haploid. We applied this analysis to two new haploids: AAV88rh.10 and AAV88rh.74 and correlated the results with production capsid titers of the haploids and mutants, demonstrating the advantages of computer guided mosaic virus assembly and the potential to design novel AAV vectors going forward *in silico*.

301. A CRISPR/Cas9 Genome Wide Screen and Cryo-EM Structure Reveal Receptor Switching by a Synthetic AAV Variant

Anshuman Das[1], Patrick Havlik[1], Jonathan Ark[1], Mario Mietzsch[2], Mavis Agbandje-McKenna[2], Aravind Asokan[1]

[1]Duke University, Durham, NC,[2]University of Florida, Gainesville, FL

Gene therapy in the past decade has greatly benefitted from the use of naturally occurring Adeno-associated viruses (AAV), as recombinant vectors for gene delivery to different cells and tissues. Several groups including ours have developed capsid engineering approaches to impart selective tissue tropism, improve transduction efficiency and evade neutralizing antibodies with an overall goal to improve safety and efficacy of gene therapy modalities. However, the biology of evolved AAV variants and how such may differ from that of natural AAV strains is often overlooked. Using an iterative, structure-guided evolution approach, we previously identified a novel human hepatotropic variant of AAV8 called AAV Hum.8 (Havlik et al., *JVI*, 2020). In contrast to the naturally occurring AAV8 serotype, Hum.8 showed robust transduction of human hepatocytes in culture as well as in a human liver xenograft mouse model. Here, we performed a genome wide CRISPR/Cas9 screen in human hepatoma Huh7 cells and identify Integrin Beta 1 (ITGB1) as a novel, essential host factor for Hum.8 transduction. Cell surface binding and more significantly, the uptake of Hum.8 requires ITGB1, but is independent of AAVR, the putative receptor for AAV8 and other serotypes. Using a high resolution cryo-EM reconstructed 3D structure of Hum.8, we map the newly evolved ITGB1 binding epitope to variable region IV (VR-IV) on the capsid and structurally annotate the differences between AAVR and ITGB1 footprints. Taken together, our results provide structure-function correlates for a newly evolved, synthetic AAV variant.

Further, our approach underscores the potential for structure-guided evolution to improve AAV transduction through receptor switching and exploitation of cognate cell entry pathways independent of those employed by natural AAV serotypes.

302. The 2.5Å Structure of AAV5 Bound to Its Cellular Receptor AAVR - Implications for Antibody Neutralization Mechanisms

Mark A. Silveria, Edward E. Large, Grant M. Zane, Tommi A. White, Michael S. Chapman
Biochemistry, University of Missouri, Columbia, MO

Adeno-Associated virus (AAV) is gathering momentum as the leading vector for human gene therapy capable of delivering transgenes of up to 4-5 kilobases. Despite its success, our molecular understanding of host interactions remains limiting in improving cellular targeting, immune evasion, and efficiency. Using a 2 domain construct of the primary AAV entry receptor, AAVR, a 2.5 Ångström resolution *cryo*-EM structure of AAV5 bound to AAVR was obtained. This structure, along with a 2.1 Å AAV5 native structure, allow analysis of atomic interactions between AAV5 and AAVR at amino acid level. AAV5 binds to the first PKD domain of AAVR, PKD1, which is in contrast to AAV2, which primarily binds to the second PKD domain. Furthermore, the binding sites for AAV5 antibodies ADK5a, ADK5b, and 3C5 overlap with that of AAVR. This finding, combined with *in vitro* experiments revealing competition in binding between antibodies and receptor, provide a new perspective on AAV neutralization mechanisms that have previously been thought to be independent of receptor-mediated cell entry. Our findings add to the constraints that will need to be satisfied in the design of vectors resistant to immune neutralization.

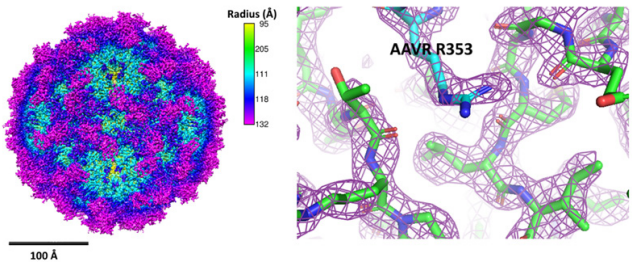


Figure: AAV5 bound to its cellular receptor AAVR. Left - AAV5 bound to AAVR colorized by distance from the center of the virus particle. Right - The interacting residues in the binding site of the AAV5-AAVR complex with the carbon atoms colored cyan in AAVR and green in AAV5.

303. AAVid™: A Platform for Intelligently Engineering AAV Capsids from Massively Diverse Libraries

Thomas A. Packard, Erica Cates, Lan Guo, Kevin Stein, Jeremy Bazinet, Lauriel Earley, Duan Kun Lee, Adam Dingens, Robert Loyd, Thomas Long, Adrian Briggs, David Huss, Francois Vigneault
Shape Therapeutics, Seattle, WA

Current adeno-associated virus (AAV)-based gene therapies lack tissue-targeted tropism, reducing therapeutic efficacy and increasing risk of off-target toxicities. Engineering of the viral capsid through variant libraries is a common strategy to modify AAV biodistribution. However, low diversity of capsid libraries and translational relevance of the biological filters limit current approaches to engineering AAV tropism. To overcome these limitations, we developed the AAVid™ capsid discovery platform that generates and screens massively diverse combinatorial variant libraries of assembly-competent AAV capsids in non-human primates (NHP). Each AAVid™ campaign begins by identifying the target cell-binding region of the AAV capsid and creating a targeted combinatorial library of defined variants by silicon-based synthesis. For our AAV5-based campaign we selected capsid VP1 positions 581-589, a primarily solvent-exposed region involved in the natural tropism of wildtype AAV5. From a starting sequence pool of 100 billion amino acid variants, we produced an assembly-competent virus library of ~1.7 billion unique virions. The library virus was administered intravenously to NHPs and 4 weeks later the animals were euthanized for tissue harvest and capsid variant recovery. Our results demonstrate broad distribution of AAVid™ variants in all NHP tissues sampled, including multiple regions of the central nervous system. Importantly, our variant recovery pipeline appends a unique-molecular identifier (UMI) that enables high resolution of variant frequency. Following sequencing, we featurize the variant regions and use machine learning (ML) to model likely deterministic capsid features defining tropism. This enables selection of candidate variants with high target-tissue specificity that are absent/low in other tissues, representing a fundamental leap forward from iterative positive enrichment, directed-evolution techniques. In summary, the AAVid™ capsid discovery platform enables identification of novel, tissue-specific AAV variants by using massively diverse capsid libraries, direct-to-NHP biological filtering and ML-guided AAVid™ variant selection.

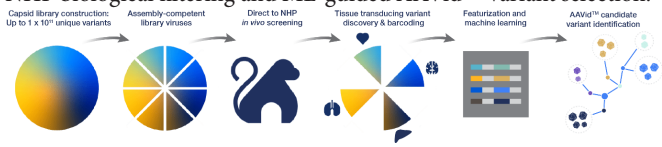


Figure 1: Overview of the AAVid™ capsid discovery platform. Massively diverse targeted combinatorial variant libraries are assembled into viruses and directly selected in NHP. Tissue transducing variants are recovered, and UMIs/barcodes appended. The recovered variant sequences are featurized and ML is used to predict deterministic characteristics identifying candidate variants for validation studies.

304. Functional Characterization of AAVHSC Compared to AAV Serotypes: Activation of Cellular Pathways In Vitro and In Vivo Transduction Properties

Omar L. Francone
Homology Medicines, Inc, Bedford, MA

Functional Characterization of AAVHSCs Compared to AAV serotypes: Activation of Cellular Pathways *In vitro* and *In Vivo* Transduction Properties
Duong K, Boyd M, Smith S, Behmoiras L, Smith L, Fasano J, Lehnert B, Chittoda M, Avila N, Faulkner D, Lotterhand J, Sengooba A, Tzianabos A, Seymour A and Francone O.L.
Adeno-associated virus (AAV) has been successfully used in the clinic

to deliver functional copies of genes to treat various diseases. Efforts to gain further insights about their tropism, cellular trafficking, and mode of actions are critical to identify and select safe and efficacious capsids to be used in humans. A family of AAVs isolated from human hematopoietic stem cells (HSCs) called AAVHSCs has broad tissue tropism across multiple cell types in mice and non-human primates. We examined and compared functional properties of AAVHSCs and AAV9 (AAVs of the same Clade F) and AAVs of other clades including AAVs 1-8 capsids containing a self-complementary CBA-GFP transgene in human iPSC. AAV1/2/3/6/8 induced apoptosis, with AAV2/3/6 being the most potent in eliciting cell death in iPSCs when compared to other AAVs. This finding was driven through a p53-dependent pathway including its downstream targets p21, caspase-3, and PARP. By contrast, no or little upregulation of p53 was detected in iPSC transduced with AAVHSCs. Considering that changes in p53, p21, CHEK2 and cleaved Parp were similar amongst AAV1/2/3/6, we selected AAV2 as representative for AAV1, AAV3 and AAV6. AAV2 impacted cell proliferation as demonstrated by cell cycle arrest at G2/M phase with a concomitant decrease in the proportion of cells in G0/G1, S and G2/M phases while no impact was observed in iPSC, primary human fibroblasts or skeletal myoblasts/myotubes transduced with AAVHSC15. Upregulation of p53 and cell death were not related to the amount of virus present in the cells but rather intrinsic differences in the intracellular transport to the nucleus between AAV2 and AAVHSCs in iPSC. To begin assessing the *in vivo* translation of these findings, a biodistribution study was performed in mice using CBA-Luc packaged in AAV2/5/6/8 and AAVHSC capsids as well as an editing mF8-Luc vector packaged in AAV6 and AAVHSC15. AAVHSC7/15/17 were determined to be the most efficient liver transducers among capsids tested, as determined by luciferase expression and vector genomes. These data provide further insights into the mechanisms of AAVHSC transduction and expression that are being applied in the selection of AAVHSC capsids in the development of genetic medicine therapeutics.

305. Distinct Functional Roles for AAV Capsid Surface Epitopes Revealed through Structure-Guided Evolution

Lawrence Patrick Havlik[1,2], Daniel K. Oh[1], Anshuman Das[1], Ruth M. Castellanos Rivera[2], Aravind Asokan[1]

[1]Duke University, Durham, NC,[2]UNC Chapel Hill, Chapel Hill, NC

The AAV capsid has distinct surface features that dictate receptor binding and antibody neutralization. We utilize structure-guided evolution to demonstrate that the different epitopes that constitute these surface footprints can be engineered to impart distinct biological properties. Two novel AAV8 variants evolved by infectious cycling, dubbed AAV840 and AAV880, with engineered variable regions IV and VIII, respectively, show 1-2 orders of magnitude higher transduction than AAV8 vectors. These variants are functionally distinct; while AAV880 shows improved cell surface binding, the more potent AAV840 variant shows increased cell surface binding as well as cellular uptake. A rationally engineered AAV840/880 combo variant shows decreased transduction implying that the newly evolved infectious pathways are distinct and not synergistic. Upon further exploration, we discover that AAV880 shows a switch in cell surface glycan recognition, while AAV840 has acquired the ability to utilize a novel receptor. Competitive inhibition, glycan cleavage and CRISPR-based knockout assays demonstrate AAV880 exploits sulfated glycosaminoglycans for cell attachment, while AAV840 utilizes Integrin β1 (ITGB1) for cellular uptake. Moreover, AAV840 shows drastically reduced dependence on KIAA0319L, the cognate AAV Receptor (AAVR), for efficient transduction. Finally, grafting the integrin binding epitope evolved on AAV840 onto variable region IV in other capsid serotypes (AAV6/9/rh.10) phenocopies improved transduction efficiency. Our studies suggest that different surface footprints on AAV capsids can be engineered to exploit distinct mechanisms of viral cell entry.

306. Mechanistic Elucidation of Adenovirus Mediated Enhancement of Recombinant Adeno-Associated Virus (rAAV) towards Efficient Low Dose Gene Therapy

Prabhakar Bastola[1,2], Liujiang Song[1,2], Matthew L. Hirsch[1,2]

[1]Gene Therapy Center, University of North Carolina at Chapel Hill, CHAPEL HILL, NC,[2]Ophthalmology, University of North Carolina, Chapel Hill, NC

Multiple recombinant adeno-associated virus (rAAV) clinical trials have observed immunological and tumorigenic concerns related to high dose rAAV, resulting in decreased clinical efficacy and safety. Such reports highlight the importance of investigating novel approaches towards achieving optimal therapeutic efficacy using low dose rAAV administration, while also decreasing high titer vector costs and associated production concerns. Current rAAV optimization largely overlooks the natural biology of AAV infection in target cells. Upon cellular entry of rAAV, successful transgene expression is facilitated through several cellular pathways, while reports demonstrate restriction by others. Identification and specific modulation of the inhibitory cellular pathways exploit a natural approach towards improving the efficacy of low dose rAAV therapy. Natural AAV biology has perhaps already overcome these transduction efficiency challenges observed in the clinics. For instance, multiple studies have shown that adenovirus coinfection results in substantial enhancement of rAAV transduction in vitro and in vivo; however, the precise mechanism associated with the enhancement remains elusive. Data from our recent studies confirmed the dramatic enhancement of rAAV transduction upon adenovirus serotype 5 (Ad5) coinfection in multiple cell lines in vitro (>50-fold). Successful rAAV transduction is reliant on rAAV infection steps that include viral binding to the cell surface, entry, endosomal trafficking and escape, nuclear import, capsid uncoating, second strand genome synthesis, and episomal persistence. Data thus far have eliminated viral entry and second-strand synthesis as the discrete steps in Ad5-mediated rAAV transduction enhancement. To delineate the Ad5 protein(s) involved in rAAV transduction enhancement, we utilized transient plasmid transfection of Ad5 helper plasmid (pXX680), as well as multiple key Ad5 protein expression plasmids to show that concurrent expression of two Ad5 proteins, E1B-55K and E4ORF6, mediate rAAV transduction enhancement (10-20 fold) in vitro. E1B-55K and E4ORF6 have been shown to display a well-established E3-Cul5 ligase-mediated proteasomal degradation of cellular substrates such as p53, Mre11, integrin α3, and DNA ligase IV. To investigate the role of the Ad5 infection and subsequent E3-Cul5 ubiquitin ligase-mediated degradation of cellular substrates towards rAAV transduction enhancement, we created *Tp53* CRISPR-Cas9

knockout cell lines, and subsequently investigated the role of p53 towards rAAV transduction enhancement. Preliminary results indicate that isogenic *Tp53* knockout in HEK293 cells mediate similar rAAV transduction enhancement upon Ad5 coinfection. Furthermore, rAAV harboring a synthetic inverted terminal repeat (ITR), unable to bind p53 in vitro, elicit Ad5-mediated rAAV transduction enhancement similar to rAAV harboring wildtype ITRs, suggesting that p53 does not play a significant role towards Ad5-mediated rAAV transduction enhancement. Data regarding Ad5-mediated rAAV enhancement in multiple *Tp53* knockout cell lines and, separately, using various p53 functional inhibitors will also be presented. In parallel, cellular substrates such as Cul5 and Mre11 will be investigated in knockout cell lines as well as using clinically relevant pharmacological inhibitors. The collective data derived from these studies contribute to the understanding of the influences of Ad on the enhancement of rAAV transduction and may find relevance towards safer and more effective low dose rAAV clinical gene therapy.

307. Abstract Withdrawn

308. Subgenomic Satellite Particle Generation in Recombinant AAV Vectors Results from DNA Lesion/Breakage and Non-Homologous End Joining

Junping Zhang[1], Ping Guo[2], Xiangping Yu[2], Jenni Firrman[3], Nianli Sang Sang[4], Roland Herzog[1], Weidong Xiao[1]

[1]The Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN,[2]School of Biomedical Science, Huaqiao University, Xiamen, China,[3]United States Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA,[4]Department of Biology, College of Arts and Sciences, Drexel University, Philadelphia, PA

Recombinant AAV (rAAV) vectors have been developed for therapeutic treatment of genetic diseases. Nevertheless, current rAAV vectors administered to patients often contain non-vector related DNA contaminants. Here, we present a thorough molecular analysis of the configuration of non-standard AAV genomes generated during rAAV production. In addition to the sub-vector genomic size particles containing incomplete AAV genomes, our results found that rAAV preparations were contaminated with multiple categories of subgenomic particles with either snapback genomes or vector genomes with deletions in the mid regions. Through CRISPR and restriction enzyme-based in vivo and in vitro modeling, we identified that the main mechanism leading to the formation of non-canonical genome particles occurred through nonhomologous end joining of fragmented vector genomes caused by genome lesions or DNA breaks that were generated by the host cell/environment. The results of this study advance our understanding of AAV vectors and provide new clues on improving vector efficiency and safety profile for use in human gene therapy.

309. Machine Learning-Accelerated AAV Engineering through the Generation of Production-Fit Capsid Libraries

Fatma-Elzahraa Eid, Ken Chan, Albert T. Chen, Isabelle G. Tobey, Qin Huang, Qingxia Zheng, Yujia Alina Chan, Ben Deverman

Stanley Center, Broad Institute, Cambridge, MA

Recombinant AAV vectors are now used in two approved gene therapies and are under preclinical and clinical evaluation for an array of indications. To broaden the application of gene therapies for currently intractable indications and enable the cost-effective scaling of AAV-based gene therapy manufacturing, there have been growing efforts to use protein engineering and directed evolution to engineer AAVs with both enhanced transduction characteristics and high production yields. The latter in particular has been a considerable obstacle to deriving engineered AAVs that can be cost-effectively and practically harnessed for gene therapy. Indeed, many groups have screened high diversity AAV capsid libraries and identified candidates with enhanced

functions; however, these do not necessarily lead to the development of capsids that are ultimately suitable for clinical translation due to low production yield or low capsid stability. To address this limitation, we built and validated machine learning (ML)-designed libraries enriched for high production yield, peptide-modified AAV capsid variants. First, we designed AAV9 7-mer modified capsid libraries that uniformly sample from the sequence space with the objective of training an ML model to accurately predict capsid fitness. A long short-term memory neural network was trained on the fitness of capsid variants and resulted in a model that accurately predicted the fitness of unseen variants (r = 0.94 predicted versus measured fitness). The model was then harnessed to generate a novel library composed of a defined set of fit capsid variants. The resulting "Fit4Fxn" library, which comprises hundreds of thousands of unique sequences, uniformly samples the production-fit sequence space and enables the generation of highly reproducible experimental data from downstream functional assays. This new approach of screening production-fit capsid libraries for functions of interest has the potential to accelerate the development of advanced AAV vectors for human gene therapy.

310. Insulin Resistance Negatively Impacts Early rAAV Transduction Events

Sean Carrig, Ashley T. Martino

Pharmaceutical Science, St. John's University, Jamaica, NY

Insulin resistance has the potential to effect virus uptake. We have previously established an acute insulin augmentation protocol - giving an acute administration of insulin during viral uptake yields a significant (2-3-fold) increase in gene transfer both *in vivo* and *in vitro*. When insulin receptors (INSR) are bound and endocytosed, receptors proximate to the INSR will be taken in as well. Our data supports that an increase in insulin receptor endocytosis causes an increase in viral uptake and therefore gene expression. Firstly, we utilized an *in vitro* insulin resistance model using HepG2 treated with 4.5g/L glucose media with 500mM recombinant human insulin and/or sodium palmitate. Insulin resistance was confirmed through the uptake of FITC conjugated insulin through flow cytometry, AKT/phospho-AKT protein levels measured through Western blot, and insulin receptor gene expression measured through qPCR. After insulin resistance was established, 10,000 MOI AAV2-CMV-sc-eGFP was administered to both normal and insulin resistant HepG2 for 6 hours and incubated for 3 days. After 3 days live cells were analyzed through flow cytometry. These cells showed reduced gene expression in both the palmate induced and long-term insulin induced insulin resistance. These results are significant, yielding a 1.5-fold decrease in GFP+ cells when compared to non-insulin resistant HepG2. Cells that underwent acute insulin augmentation did not achieve the typically seen 2-3-fold increase, rather only a 5-10% increase was observed. Additionally, we previously reported on the inhibition of Phosphoinositide 3-kinase (PI3K) and Glycogen synthase kinase 3 (GSK3) in human hepatoma cells (Hep3B and HepG2). Inhibition of PI3K leads to a reduced efficacy of the insulin augmentation protocol, while inhibition of GSK3 leads to reduced levels of gene expression in Hep3B cells. Hep3B cells have a dysregulation in the insulin signaling pathway stemming from a deletion of p53 while HepG2 cells have a closer to physiological insulin signaling pathway. The inhibition of GSK3 in cultured HepG2 cells leads to a 1.5-2-fold increase in GFP expression, suggesting that portions of the insulin signaling pathway is crucial for rAAV uptake. Inhibition of GSK3 has also been shown to improve insulins effects in hepatocytes and skeletal muscle. To generate an insulin resistant model *In vivo*, C57BL/6J mice were implanted subcutaneously with a Linbit sustained release 28-day insulin pellet and fed high carbohydrate chow for the duration of the insulin pellets release. Insulin resistance was confirmed via glucose challenge and relative insulin receptor tissue levels in qPCR. Normal and Insulin resistant mice were then injected either intramuscularly with AAV1-CMV-sc-hFIX, or intrasplenic with AAV8-CMV-sc-hFIX with or without an insulin augmentation protocol. Normal mice that received an insulin augmentation protocol had an approx. 2-3-fold increase in serum hFIX over normal mice which did not receive insulin - typical of the insulin augmentation protocol. Whereas insulin resistant mice had a reduced level of serum hFIX when compared to normal mice. Overall, this data suggests that insulin resistance plays a significant in AAV uptake and gene transfer. A portion of the increased gene expression may be caused during the insulin receptors endocytosis. Several receptors and co-receptors for AAV are either located proximally to the insulin receptor or have crosstalk between similar receptor tyrosine kinases. Insulin resistant cells and mice have a reduced quantity of insulin receptors and therefor have reduced uptake. Another potential factor for increased gene expression lies within the insulin receptor signaling cascade as shown by the potent effects of inhibiting GSK3.

311. AAV Selection: Expressing the Future of Directed Evolution

Adrian Westhaus[1], Marti Cabanes-Creus[1], Timo Jonker[1], Erwan Sallard[1], Erhua Zhu[1], Scott Lee[2], Milan Fernando[2], Grady C. Smith[2], Renina G. Navarro[1], Giorgia Santilli[3], Adrian J. Thrasher[3], Anai Gonzalez-Cordero[2], Ian E. Alexander[4], Leszek Lisowski[1]

[1]Translational Vectorology, Children's Medical Research Institute, Westmead, Australia,[2]Stem Cell Medicine, Children's Medical Research Institute, Westmead, Australia,[3]Molecular and Cellular Immunology, University College London, London, United Kingdom,[4]Gene Therapy Research, Children's Medical Research Institute, Westmead, Australia

The power of AAV Directed Evolution for identifying novel vector variants with improved properties is well established, as evidenced by numerous publications reporting bioengineered AAV variants over the last decade. However, most capsid variants have been identified using either replication-competent (RC) selection platforms or PCR-based capsid DNA recovery methods, which can bias the selection towards efficient virus replication or unproductive vector trafficking, respectively, both properties that would lower functionality of the therapeutic vector. Therefore, the need for more advanced AAV capsid evolution selection methods is apparent. The focus of this study was to develop and validate Functional Transduction (FT)-based methods for rapidly identifying novel AAV variants based on transgene expression in target cells. We engineered a novel AAV selection platform that supports AAV recovery from library-encoded RNA expressed from the native AAV-p40 promoter and comprehensively compared it to existing capsid replication and DNA recovery strategies. Following these validations, we performed a side-by-side comparison by conducting *in vivo* library selection in humanized FRG mice. Specifically, the same

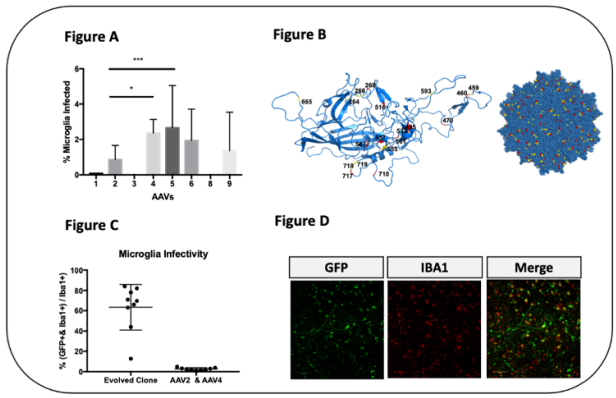
capsid library was cloned into the conventional RC and our novel RNA-based FT selection platforms. Based on the selection kinetics and function of novel capsids identified in this xenograft model of the human liver, we identified mRNA-based FT selection as the most optimal AAV selection method. Driven by the encouraging results from selection in human hepatocytes we wanted to expand the use of the FT superior technology to more complex tissues of the CNS using iPSC-derived human cortical brain and retina as exemplars. To this end we fine-tuned the promoter element to restrict capsid library expression to specific cell types within a larger population of cells. Specifically, the p40 promoter was linked to a number of transcriptionally activating or silencing elements which allowed us to develop a library selection platform for bioengineering of neuronal and photoreceptor-specific novel AAV capsids.

312. Directed Evolution of Adeno-Associated Virus for Enhanced Gene Delivery in Human Microglia Cells

Danqing Zhu[1], Galina Schmunk[2], Tomasz Nowakowski[2], David V. Schaffer[3]

[1]Quantitative Biosciences, University of California - Berkeley, Berkeley, CA,[2]University of California - San Francisco, San Francisco, CA,[3]University of California - Berkeley, Berkeley, CA

Introduction: Microglia, the brain's resident macrophages, are a crucial component of central nervous system function. Dysfunction of microglia likely contributes to the development of several major brain disorders, including Alzheimer's, glioblastoma, neuropathic pain and others. Genetically manipulating endogenous microglial populations *in situ* is thus a promising therapeutic approach to counteract disease pathology. However, such strategies remain challenging due to difficulties in efficient and targeted delivery of therapeutics to this relatively rare cell population. Adeno-associated virus (AAV) vectors are highly promising gene delivery vehicles for an increasing range of clinical applications given their favorable safety profile. Furthermore, AAV capsid proteins are encoded by a single *cap* gene, which can be modified to engineer the virus's tropism and other infectious properties. Here we apply directed evolution for developing efficient AAV vectors that target and transduce microglia in primary human brain tissue, which can serve as basic biological tools to investigate neuro-immune interactions as well as therapeutic delivery vehicles to treat a range of neurological disorders. **Methods:** Directed evolution can achieve improvements in AAV vector performance without pre-existing knowledge of virus-cell interactions. To optimize our library design, we applied multiple techniques to genetically diversify the *cap* gene and develop variants that overcome current delivery barriers. Specifically, we transfected nine different libraries separately into packaging cell lines to produce viral particles. Purified capsid libraries were then administered using an equimolar mixture of AAV libraries to live fetal brain slices (age 19- 23 weeks), and functional selective pressure was imposed by harvesting only the microglia population using magnetic-activated cell sorting 72 hours after infection. Using PCR-based recovery of *cap* variants, we performed three iterative rounds of phenotypic selection, which successfully drive convergence toward the fittest clones that transduce human microglia cells. **Results:** Most natural AAV serotypes transduce microglia cells poorly (**Fig A**). Here we showed less than 5% of GFP expression co-localized with microglia marker (Iba1+) in natural AAV serotypes. After three rounds of selection, Sanger sequencing analysis of 30 clones revealed convergence on one variant from ancestral library represented ~54% of the clones recovered (**Fig B**). To determine whether the evolved clone can transduce microglia cells more efficiently and specifically, we packaged recombinant GFP virus and administrated it onto brain slices. Our results demonstrated an up to 6-fold increase of total microglial transduction (**Fig C**) compare to existing vectors. Interestingly and surprisingly, the variant was also highly selective for microglia and transduced this target at a percentage that was 7-fold higher than non-microglial cells. Representative images are also shown (**Fig D**). In summary, we have developed an efficient and targeted AAV variant to overcome current challenges for microglia delivery. The novel variant will enable future investigation of molecular mechanisms underlying neural disorders and provide potential therapeutic strategies for disease treatment.



313. Isolating Natural Adeno-Associated Viruses from Porcine Tissues

Qiang Wang, Gui Hu, Xiaobin Liu, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

As adeno-associated virus (AAV) mediated gene therapy expands to cover more diseases, the demand is rising for new AAV capsids that can optimize gene delivery. There are three main strategies to obtain new AAV capsids: tapping the natural diversity of AAV, directed evolution, rational design, or a combination thereof. We screened for new AAVs in porcine tissues with the high-fidelity polymerase chain reaction (PCR) protocol we previously reported. Among the porcine tissues we tested, the small intestine samples showed a PCR-positive rate (targeting a small and relatively diverse region flanked by conserved regions for primer binding) much higher than samples of liver, heart, lung, and spleen. We then ran 5'-RACE and 3'-RACE to find good PCR primer binding sites that covered the capsid genes and successfully recovered more than 20 new porcine AAV capsid genes, AAVpoG001-AAVpoG025. The close hits in Genbank of those new porcine AAV isolates are AAVpo1, AAVpo2.1, AAVpo4, and AAVpo5. The AAVpo2.1-like and AAVpo4-like new AAV isolates showed good transduction in Huh7 cells. AAVpoG013 and AAVpoG015 had the best Huh7 cell transduction and were selected for further analysis. Vectors with both capsids had very good yields (n=1 for each capsid) and showed mouse liver transduction through intravenous injection with an efficiency comparable to AAV8 (i.e., the benchmark of mouse

liver gene delivery). We also evaluated AAVpoG015 for its serological profile. Preliminary results showed that AAVpoG015's neutralizing antibody titer was only one-fold lower than AAV8's titer when tested with two monkey sera from AAV8 vector-injected animals even though the two capsids are distant (the protein sequence similarity between AAVpoG015 and AAV8 is only 79%). Our results indicate that 1) natural AAV diversity is rich; 2) the small intestine is rich in natural AAVs among porcine samples (unlike human samples we previously investigated); and 3) AAVpoG013 and AAVpoG015 could be good capsid candidates for liver gene delivery.

314. Novel AAV Capsids for Enhanced Gene Transfer to the Cerebellum, Spinal Cord, and Schwann Cells

Xin Chen[1], Thomas Dong[1], Widler Casy[1], Yuhui Hu[1], Daphne Chen[2], Thomas McCown[2], Steven Gray[1]

[1]Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX,[2]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

A capsid DNA shuffling and directed evolution process was pursued to generate new AAV variants for nervous system gene transfer. Forty-eight novel AAV capsid variants were recovered after biopanning our library in mice following three rounds of lumbar intrathecal (IT) administration. To evaluate the biodistribution and cellular tropism of the recovered capsids, the vector variants were generated with a self-complementary GFP transgene. They were then individually administered into wild type (WT) adult mice via tail intravenous (IV 2E11 vg/mouse in 200 μL bonus, n=4) or lumbar intrathecal (IT 1E10 vg/mouse in 5 μL bonus, n=4) injection. Biodistribution analysis was preformed ten days post-injection by qPCR on the brain, spinal cord, dorsal root ganglia (DRG), sciatic nerve, and major peripheral organs. In the IV cohort, all variants did not traverse the blood-brain-barrier to any extent relative to AAV9. Interestingly, some of the capsids showed peripheral organ tropisms that we were not selecting for, such as enhanced kidney or spleen biodistribution compared to AAV9, which are under further investigation. In the IT cohort, more than fifteen variants that had biodistribution values at least ten times greater than AAV9 in the brain and spinal cord, combine with reduced biodistribution to the liver. Three top variants C304, C101, and B108 were chosen for further studies based on their biodistribution pattern from the brain, spinal cord, and sciatic nerve. To directly evaluate transduction patterns, the variants C304, C101, and B108 were administered into WT adult mice via lumbar IT injection (2E11 or 5E10 vg/mouse in 5 μL bonus, n=3). Histological analysis was preformed twenty-one days post-injection by immunohistochemistry (IHC) with anti-GFP antibody. Quantitative analysis of scanned images with HALO™ Image Analysis Platform confirmed that both C304 and C101 expressed in the brain stem (~2 and ~2-fold), cerebellum (~2 and ~5-fold), lumbar spinal cord (~10 and ~5-fold), and sciatic nerve (~5 and ~4-fold) higher than AAV9. In addition, B108 expressed ~5 fold higher than AAV9 across brain regions including cortex, subcortex, brain stem, and cerebellum. Unfortunately, neither of the capsids transduced deeper brain structures like the striatum equivalently to AAV9. To further explore the cell tropism, IHC with anti-Calbindin or anti-S100 antibody was performed on cerebellum or sciatic nerve to label Purkinje or Schwann cells, respectively. Our preliminary results indicated that both C304 and C101 transduced and expressed more in Purkinje and Schwann cells, while B108 transduced and expressed more in Purkinje cells as compared to AAV9. These results demonstrated that C304, C101, and B108 may be appropriate to treat cerebellar diseases with Purkinje cell involvement, while C304 and C101 may be appropriate to treat many peripheral demyelinating neuropathies with Schwann cell involvement. Ongoing studies include assessing these candidate vectors in non-human primates.

315. Expanding the Vector Toolkit: Investigating Transduction Efficiencies of AAV Vectors

Luke Fennell, Jennifer Lyles

Biology, Francis Marion University, Florence, SC

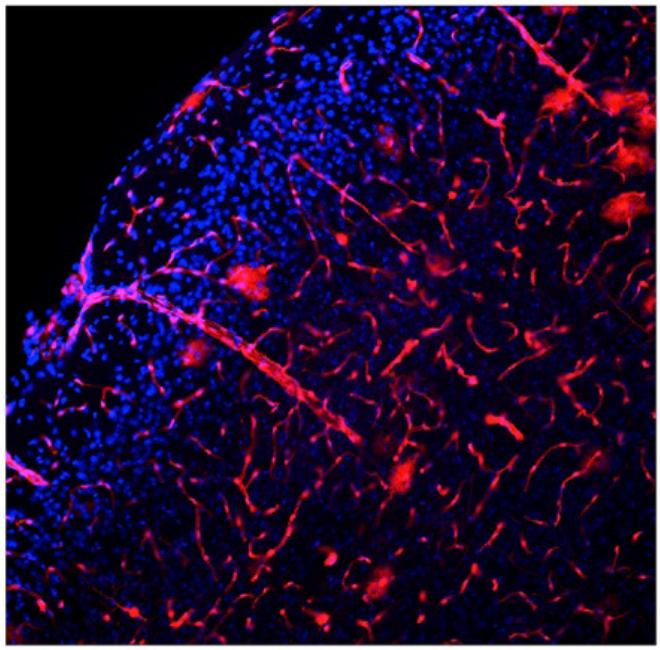
Adeno-associated viral (AAV) vectors are known for their long-term persistence following a single administration of the vector—a property that is critical to the success of the therapy. A potential barrier to long-term persistence is the initial entry of the vector into the host cell. It is known that different serotypes of AAV have different affinities for various cell types. As a result, the field has shifted towards the use of alternative AAV serotypes, including newly engineered hybrid serotypes, depending on the target tissue. Additionally, AAV vectors may contain either single-stranded (ss) or self-complementary (sc) genomes. The configuration of the vector genome upon nuclear entry has also been shown to have an effect on transduction efficiency, with scAAV vectors typically resulting in greater gene expression. Continuing to characterize the transduction efficiencies of all AAV serotypes, both naturally occurring and newly engineered, in common laboratory cell lines will aid in expanding the vector "toolkit" for researchers and clinicians to ensure that the most suitable vector is being used for the appropriate target tissue or cell line. In this study, the transduction efficiencies of ssAAV-DJ and scAAV-DJ - an engineered serotype derived from the shuffling of eight wild-type viruses - were investigated in HEK293 and HeLa cells using fluorescence microscopy and qPCR. In a side-by-side comparison with ssAAV-2 and scAAV-2, AAV-DJ demonstrated greater gene expression, regardless of genome configuration or cell type. Unexpectedly, both serotypes packaging single-stranded (ss) vector genomes resulted in greater expression as compared to those packaging self-complementary (sc) vector genomes in HEK293 cells. This may be due to a discrepancy in availability of cellular factors required for second-strand synthesis of ssAAV genomes in HEK293 cells and HeLa cells. Interestingly, there were no significant differences in the concentration of intracellular vector genomes, regardless of serotype, genome status, or cell type; however, substantial differences in the level of gene expression were observed. It is possible that a subset of vector genomes may be silenced upon nuclear entry, dependent upon serotype, genome status, and cell type. Further research is required to elucidate underlying molecular mechanisms responsible for these observations.

316. An AAV Capsid Mediates Highly Selective Transduction of Brain Endothelium and Pericytes after Systemic Delivery in Mice

Siobhan McCarthy[1], Killian S. Hanlon[1], Eloise Hudry[2], Josette Nammour[1], Patricia L. Musolino[1], Casey A. Maguire[1]

[1]Massachusetts General Hospital, Charlestown, MA,[2]Novartis, Cambridge, MA

Neurodegeneration and cerebrovascular disease share an underlying microvascular dysfunction and selective targeting of brain endothelium and pericytes enables gene-targeted therapeutic interventions. The brain vasculature can also serve as a source of secreted therapeutic proteins to neurons and glial cells due to its high density throughout the central nervous system. We performed two rounds of *in vivo* selection with an AAV9 capsid scaffold displaying a heptamer peptide library to isolated capsids that traffic to brain after intravenous (iv) delivery. Next generation sequencing was performed to identify the peptide sequence profile and subsequently, we separately produced candidate capsids packaging a single-stranded AAV-CBA-Cre cassette and injected them iv into Ai9 mice which have a CBA-Floxed-STOP-tdTomato reporter. One capsid, termed AAV-PR, mediated striking transduction of the vasculature as visualized by tdTomato fluorescence. To observe whether the new capsid could enable relevant levels of transgene expression in brain vasculature, we injected AAV-PR packaging a self-complementary AAV-CBA-GFP genome in adult C57BL/6 mice and sacrificed at days 5 and 28 post injection. Immunofluorescence analysis of GFP expression co-localized with endothelium and pericytes markers but not with smooth muscle, neuronal, glial or microglial/monocyte markers. The high tropism without the need for tissue-specific promoters, suggests that this capsid is physically targeted to the vasculature. In all, our data suggests that AAV-PR may be a useful capsid for genetic modification of vasculature for different applications in mice. Future experiments will determine whether AAV-PR's properties translates to larger species.



317. Identification of Transcriptional Start Sites in a Tandem Promoter by 5'-RACE and NGS

Kirk W. Elliott, Chunping Qiao, Devin S. McDougald, Joseph T. Bruder, Olivier Danos, Ye Liu

Research and Early Development, Regenxbio Inc., Rockville, MD

Introduction A key imperative of many gene therapy approaches is the ability to achieve stable therapeutic levels of transgene expression in a tissue-specific manner, concomitant with transgene immune tolerance. It has been proposed that tandem promoters, which are capable of driving transgene expression in a tissue of choice as well as in the liver, may help achieve long-term expression following gene transfer. Liver expression is believed to result in an immunological state of tolerance towards the transgene product, while the tissue-specific transcriptional elements drive high-level, long-term expression. We and others have previously reported using such tandem promoters driving therapeutic gene expression of, *e.g.*, therapeutic antibody or secreted lysosomal enzyme. However, important questions on the impact of the tandem promoter configuration on transcription remain unanswered. This study seeks to understand the activity of individual *vs* tandem promoters in their intended tissues, as well as the influence of the tandem configuration on transcription start sites (TSS), mRNA processing and translation. **Results** We employed Rapid Amplification of cDNA Ends (RACE) and next-generation sequencing (NGS) to identify TSS in a tandem promoter. Green fluorescence protein reporter constructs driven by muscle-specific (CK8), liver-specific (hAAT), or tandem promoter LMTP6 (CK8-hAAT) were transfected into muscle (C2C12) and liver (Huh7) cell lines. Tandem configurations were engineered to deplete any start codon (ATG) present in the 3' promoter sequence. After C2C12 differentiation and confirmation of transgene expression, the transfected cells were harvested and mRNA was subjected to cDNA synthesis, tailing and PCR amplification. The purified RACE PCR products were subjected to NGS analysis with MiSeq. Our results revealed that: The CK8 promoter only had a unique cluster of TSS in both muscle and liver cell lines. The hAAT promoter had strong expression in liver cells with one unique cluster of TSS and weak expression in muscle cells with one major cluster and one minor cluster of TSS. The tandem LMTP6 promoter had three TSS clusters in both liver and muscle cell lines. The major TSS of LMTP6 in liver and muscle cells were identical to the TSS of individual hAAT and CK8 promoters, respectively. **Conclusions** RACE plus NGS is a valuable method to identify TSS and alternative splicing sites on promoters. Our results indicate that the contributing liver or muscle promoters in a tandem design can maintain their individual tissue specificity and TSS in cell lines. Depletion of start codon ATG from the 3' promoter sequence is necessary to ensure only downstream transgene start codon will be used for translation initiation from both promoters. Future work includes analyzing cDNA from mouse liver and muscle tissues after administration of AAV containing the tandem promoter to confirm the current finding from cell lines.

318. Abstract Withdrawn

319. Continued *In Vitro* and *In Vivo* Characterization of Novel AAV Vectors Engineered for Muscle Gene Delivery

Jennifer Green[1], Tiffany Willacy[1], Jessica Boehler[1], Alina Dhruva[1], Hui Meng[2], Margaret Beatka[2], Emily Ott[2], Mariah Prom[2], Michael W. Lawlor[2], Peter Pechan[1], Carl Morris[1]

[1]Solid Biosciences, Cambridge, MA,[2]Department of Pathology and Neuroscience Research Center, Medical College of Wisconsin, Milwaukee, WI

Recombinant adeno-associated viral vectors (rAAV) demonstrate great promise as the leading platform for clinical gene delivery. A variety of AAV serotypes currently exist and enable transduction of multiple tissues for the treatment of many genetic and other complex diseases. For Duchenne Muscular Dystrophy (DMD), gene therapy is under investigation to replace the absent dystrophin with a smaller, functional micro-dystrophin (uDys). Since muscle constitutes a large proportion of body mass, a high dose of vector administered systemically is necessary. Identification and development of more muscle-tropic vectors should improve safety and efficacy of DMD gene therapy. Previously, we described AAV-SLB101, a rationally designed novel AAV capsid identified as part of an internal screening program. AAV-SLB101 resulted in a greater than 8-fold increase in uDys protein expression when compared to the natural serotype AAV9 *in vitro* in both mouse and human DMD skeletal muscle cells. In addition, two independent DMD^{mdx} mouse studies confirmed significant increases in biodistribution (4- to 7-fold) and uDys expression (1.5- to 2.5-fold) in heart and quadriceps, respectively, after 14 days. Based on these data, we have further explored the use of AAV-SLB101 in a dose response and time dependent manner in DMD^{mdx} mice compared to AAV9. Additional *in vitro* assays were also performed to understand transduction increases observed with AAV-SLB101 over AAV9. More information on the capacity and specificity of engineered rAAVs in DMD muscle and other related cells will enhance our understanding of muscle tropism of these novel capsids, and ultimately may lead to the design of more efficacious muscle-tropic vectors.

320. Optimization of AAV Loading into Extracellular Vesicles as a Method to Avoid Neutralizing Antibodies

Bhargavee Gnanasambandam, Madeleine Youniss, Ke Xu, Palak Shah, Aaron Noyes, Russell McConnell, Jonathan D. Finn

Codiak Biosciences, Cambridge, MA

Adeno-associated virus (AAV) is a commonly used gene therapy vector due to its favorable safety profile, stability, and duration of transgene expression. One major limitation of current AAV vectors are neutralizing antibodies (nAb), which are present in many potential patients (preventing treatment) and develop in all patients following an initial dose (preventing re-administration). Encapsulation within exosomes (exoAAV) is a potential strategy to protect AAV from antibody-mediated neutralization, allowing efficient transduction even in the presence of high nAb titers. We have developed a method for generation of high purity exoAAV that, compared to free AAV, have demonstrated increased *in vitro* potency and resistance to nAb. exoAAV was functional when administered intravitreally to rats. In an effort to increase exoAAV yields, we compared several production factors to increase exoAAV yield. Combining optimized transfection conditions, cell type, and harvest conditions generated markedly (multi-log) higher exoAAV yields. exoAAV were resistant to nAb found in human IVIg, which completely blocked transduction by free, un-encapsulated AAV. Our results show that production of functional, nAb-resistant exoAAV can be significantly increased through optimized production procedures.

321. Development of an AAV Vector Screening Assay in Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes

Ajay Kumar, Justin Boyd, Arpana Khatri, Suryanarayan Somnathan, Jane Owens, Nicolas Christoforou

Pfizer Inc, Cambridge, MA

Adeno Associated Viruses (AAVs) have emerged as effective vectors for nucleic acid delivery into target tissues for the treatment of inherited monogenetic diseases. Several AAV capsids, including AAV9, can efficiently and selectively, transduce heart tissue making them vectors of choice for delivering genes to the heart. Developing or testing novel expression cassettes containing regulatory elements that confer cardiac specificity or interrogating batch-to-batch variability necessitates a testing platform that is relevant to the human heart and allows for a high-throughput screen of a range of variables. Our goal was to develop and optimize an in vitro AAV vector screening assay using human induced pluripotent stem cell-derived cardiomyocytes (HiPSC-CMs). We tested a range of parameters including: AAV capsids, promoters, viral titer (MOI), co-transduction with wild type adenovirus, and timing of delivery or readout. We were successful in identifying a set of parameters allowing efficient HiPSC-CM transduction, transgene expression, and dose-dependent signal detection and quantification. Ultimately, we aim to incorporate this platform within specific programs with a goal of assessing AAV potency for expression of disease-relevant payloads.

322. Development of Capsid-Modified Next Generation AAVrh74 Vectors with Increased Transduction Efficiency in Primary Human Skeletal Muscle Cells: Implications in Gene Therapy of Muscular Dystrophies

Keyun Qing, Jakob Shoti, Barry J. Byrne, Arun Srivastava

Pediatrics, University of Florida, Gainesville, FL

It has become increasingly clear that the host immune response correlates directly with the AAV vector dose. For example, whereas a dose of up to $1x10^{14}$ vgs/kg of AAV8 vectors was safe, a dose of $3x10^{14}$ vgs/kg has been shown to be associated with the death of 3 patients in a gene therapy trial of X-linked myotubular myopathy (*Hum. Gene Ther.*, 31: 787, 2020). Although a dose of $2x10^{14}$ vgs/kg of AAVrh74 vectors has been shown to be well-tolerated in patients with Duchenne muscular dystrophy (*JAMA Neurol.*, 77: 1122-1131, 2020), it would be desirable to achieve clinical efficacy at a significantly lower vector dose. We have previously reported that site-directed mutagenesis of

surface-exposed specific tyrosine (Y) to phenylalanine (F) residues results in the next generation ("NextGen") AAV2 vectors that are significantly more efficient at reduced doses (*Proc. Natl. Acad. Sci. USA*, 105: 7827-7832, 2008; *Mol. Ther.*, 18: 2048-2056, 2010), and are less immunogenic (*Blood*, 8: 121: 2224-2233, 2013). Since most, if not all, of surface-exposed Y residues are also conserved in AAVrh74, we generated the following corresponding Y-F mutants of AAVrh74 vectors: Y733F single-mutant (SM), and Y733+447F double-mutant (DM). The transduction efficiency of these vectors expressing the EGFP reporter gene was respectively up to ~12-fold and ~16-fold higher than that of the conventional AAVrh74 vectors in HeLa cells (**Figure 1A**). The Y-F mutant vectors were also significantly more efficient in transducing an immortalized mouse myoblast cell-line, C2C12 (**Figure 1B**). Sincewe have previously reported that inclusion of site-directed mutagenesis of surface-exposed threonine (T) to valine (V) residues further augments the transduction efficiency of AAV2 vectors (*PLoS One*, 8: e59142, 2013), we next generated a Y733+Y447F+T494 triple-mutant (TM) ssAAVrh74 vector, which was up to ~5-fold more efficient than the first generation ssAAVrh74 vector in primary human skeletal muscle cells (**Figure 1C**). Studies are currently underway to evaluate the efficacy of the SM, DM, and TM ssAAVrh74 vectors in skeletal muscle in a murine model *in vivo*. Taken together, these studies suggest that the use of NextGen AAVrh74 vectors may lead to the potentially safe and effective gene therapy of human muscular dystrophies at reduced doses, without the need for immune-suppression.

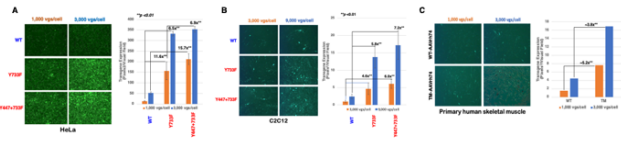


Figure 1: Transduction efficiency of WT and Y-F mutant ssAAVrh74 vectors in human HeLa (**A**), mouse C2C12 (**B**), and primary human skeletal muscle cells (**C**). Cells were transduced with each vector at the indicated vector genome copy numbers vgs/cell (vgs)/cell at 37ºC for 2 hrs, and transgene expression was visualized under a fluorescence microscope 72 hrs post-transduction. Data were quantitated using the ImageJ software.

This research was supported by a sponsored research grant from Sarepta Therapeutics.

323. Use of CRISPR/Cas9-Mediated Disruption of CNS Cell Type Genes to Profile Transduction of AAV by Neonatal Intracerebroventricular Delivery in Mice

Tess Torregrosa, Sydney Lehman, Sam Hana, Galina Marsh, Shanqin Xu, Kathryn Koszka, Nicole Mastrangelo, Alexander McCampbell, Christopher Henderson, Shih-Ching Lo

Biogen Inc., Cambridge, MA

Adeno-associated virus (AAV) transduction efficiency and tropism are conventionally determined by high expression of a fluorescent reporter gene. Emerging data has suggested that such conventional methods may underestimate AAV transduction for cells in which reporter expression from AAV vectors is undetectable. To explore an alternative method that captures AAV transduction in cells in which low expression of a cargo is sufficient for the intended activity, we sought after CRISPR/Cas9-mediated gene disruption. In this study, we use AAV to deliver CRISPR/guide RNA designed to abolish the genes NeuN, GFAP or MOG expressed specifically in neurons, astrocytes, or oligodendrocytes respectively in the central nervous system (CNS) of mice. Abrogated expression of these cell-type-specific genes can be measured biochemically in CNS subregions and provide a quantitative assessment of AAV transduction in these CNS cell types. By using this method, we compared CNS transduction of AAV9, AAV-PHP.B, and AAV-PHP.eB delivered via intracerebroventricular injection (ICV) in neonatal mice. We found both AAV-PHP.B and AAV-PHP.eB resulted in a marked disruption of the NeuN gene by CRISPR/Cas9, significantly greater than AAV9 in several brain regions and spinal cord. In contrast, only modest disruption of the GFAP gene and the MOG gene was observed by all three AAV variants. Since the procedure of ICV circumvents the blood-brain barrier, our data suggests that, independent of their ability to cross the blood-brain barrier, AAV-PHP.B variants also exhibit remarkably improved neuronal transduction in the CNS. We anticipate this approach will facilitate the profiling of AAV cellular tropism in murine CNS.

AAV Vectors - Preclinical and Proof-of-Concept Studies

324. Novel Genome Editing Therapy Improves Disease Phenotype and Survival of a Mouse Model of Methylmalonic Acidemia

Amy Bastille, Xiaohan Zhang, Nikhil Ramesh, Jenisha Vora, Elizabeth McCarthy, Dylan Frank, Chih-Wei Ko, Carmen Wu, Noel Walsh, Jing Liao, Susana Gordo, Lauren Drouin, Matthias Hebben, Kyle Chiang, Nelson Chau, Shengwen Zhang

LogicBio Therapeutics, Lexington, MA

Methylmalonic acidemia (MMA) is a rare genetic disorder most commonly caused by mutations in the mitochondrial methylmalonyl-CoA mutase (MMUT). MMA patients suffer from frequent episodes of metabolic instability, which can lead to developmental delay and other neurological abnormalities and are potentially lethal. There is no cure for MMA; the current standard of care mainly consists of a highly restricted diet. However, even under strict dietary management, many patients remain metabolically unstable, and liver transplantation becomes the only therapeutic alternative for these pediatric patients. The rAAV vector mLB-001 is a mouse-specific surrogate of a nuclease-free, promoterless GeneRide™ vector designed to precisely insert a functional *MMUT* open reading frame (ORF) into the *Albumin* locus via homologous recombination. The MCK-Mut murine model of MMA, a *Mmut* knockout expressing Mmut in muscle, was used to investigate the therapeutic efficacy of mLB-001. A single intravenous administration of mLB-001 at doses ranging $3x10^{13}$ and $1x10^{14}$ vg/kg to neonates (postnatal day 0-1) or adults (2 months old) successfully transduced the liver tissues and edited the hepatocytes to express MMUT protein in a dose-dependent manner. Immunohistochemistry analyses indicated that the proportion of corrected hepatocytes expanded over time, suggesting a survival advantage of MMUT-

expressing hepatocytes. Site-specific genomic integration of *MMUT* ORF resulted in dose- and time-dependent increase in Albumin-2A, a circulating biomarker for precise gene editing, which was accompanied by an increase in MMUT mRNA and protein expression in MMA mice. A high protein diet challenge mimicking metabolic crisis led to significant body weight loss in the vehicle-treated animals while the body weight of the mLB-001-treated animals was maintained. Furthermore, in response to diet challenge, MMA mice treated with mLB-001 at all dosing levels showed >80% animal survival, compared to 35% survival in vehicle-treated animals at 2 months post-challenge. In summary, by mimicking diet management of MMA patients with a low protein diet, we were able to significantly extend the life of the MMA mice and demonstrate the therapeutic benefit of a single intravenous administration of mLB-001 on biochemical and clinical outcomes. These findings support the development of GeneRide™ technology-based gene therapy for children with this devastating disease.

325. Comparative Genomic Analyses of rAAV Integrations

Tyra G. Wolfsberg[1], Stephanie N. Smith[1], Anh-Dao Nguyen[1], Randy J. Chandler[1], Karl-Dimiter Bissig[2], Charles P. Venditti[1]

[1]NIH/NHGRI, Bethesda, MD,[2]Department of Pediatrics, Duke University, Durham, NC

BACKGROUND: In a wide variety of murine models, recombinant AAV (rAAV) has been documented to cause hepatocellular carcinoma (HCC) after integration, and in hemophiliac canines, clonal proliferation of hepatocytes after rAAV gene therapy has been recently described. However, neither the mechanism(s) nor the site preferences for rAAV integration are fully understood. We have therefore initiated an effort to use comparative genomics to examine a subset of predicted human orthologs of integration sites (IS) that have been associated with HCC formation in murine models, and augmented our analysis through an examination of rAAV integrations independently captured and assessed in a humanized mouse model. **METHODS:** We first compiled a subset of 58 HCC-associated rAAV integration sites that map to the mouse Rian locus from previously published studies, and used a lift-over to assign these sites to the better characterized human genome. Of the 58 mouse sites, which spanned 70 kb at the Rian locus, upstream of Rtl1, 29 mapped successfully to a 48.5 kb region of the human genome that overlaps RTL1 and MEG8. In parallel, we analyzed a representative dataset of 878 rAAV IS in the human genome along with 1743 from the mouse. These IS were captured from the chimeric livers of an rAAV-infected mouse model in which the mouse livers were repopulated with human hepatocytes. In order to remove any potential bias due to the experimental capture protocol, we first discarded all sequencing reads that contained vector sequence and subsequently removed all IS that mapped to any integration site that we had previously identified. To explore integration site characteristics in both mouse and human, we created 10,000 simulated datasets, each with the same number of mock integration sites as were in the experimental datasets. We determined the genomic context of both the experimental and the simulated IS, which enabled us to assign statistical significance to the rAAV integrations in and around genes. **RESULTS:** The 29 Rian integration sites that successfully mapped to the human genome clustered into three groups, with many mapping to regions with enhancer-like signatures and transcription factor binding sites. Of the 29 IS that failed to lift over to the human genome, 19 were contained in the mouse Mir341 locus. The supramolecular organization of the region surrounding the mapped IS appears to respect two long range chromatin domains, one predicted to form between the 3' end of RTL1 and a distant non-coding enhancer located >200 kb upstream, and another domain, contained within the first, defined by the MEG8 promoter and a downstream enhancer. A more global assessment of 878 integration sites in the human genome showed that integrations are more likely to map within and around 5 kb of genes ($p < .0001$), specifically in exons, first exons, and 5 kb upstream (all with $p < .0001$). The 1743 rAAV integrations into the mouse genome also tend to occur within 5 kb of genes ($p \leq .02$) and in first exons ($p \leq .04$). **CONCLUSIONS:** Comparative genomic studies using rAAV IS derived from a well-established hotspot show clustering in a putative chromatin domain formed around RTL1 that is densely populated with transcription factor binding sites and regulatory elements. Similar regions within and upstream of genes emerged as preferred sites for rAAV integration in our global IS analysis. In aggregate, the rAAV IS patterns we describe are reminiscent of integration preferences noted with retroviral vectors, where LTR enhancer insertion into promoters mediates genotoxic sequelae after gene therapy. rAAV integrations in preclinical models should be aggregated and further assessed using comparative genomics, which might inform the design of safer vectors.

326. Abstract Withdrawn

327. rAAV-Mediated Inhibition of Vascular Endothelial Growth Factor for the Treatment of Retinal Vascular Pathologies without Causing Vasculitis

Shun-Yun Cheng[1], Yongwen Luo Luo[2], Anneliese Malachi[1], Jihye Ko[3], Qin Su[3], Jun Xie[3], Bo Tian[1], Haijiang Lin[1], Xiao Ke[4], Qiang Zheng[4], Philip W. L. Tai[3], Guangping Gao[3], Claudio Punzo[1]

[1]Ophthalmology, University of Massachusetts School of Medicine, Worcester, MA,[2]College of Veterinary Medicine, Guangzhou, China,[3]Horae Gene Therapy Center, University of Massachusetts School of Medicine, Worcester, MA,[4]Chengdu Kanghong Pharmaceutical Group, Chengdu, China

The wet form of age-related macular degeneration (AMD) is characterized by neovascular pathologies that can result in retinal edemas and subsequent vision loss if left untreated. Inhibition of vascular endothelial growth factor (VEGF) has been used to successfully treat neovascular pathologies of the eye. Nonetheless, some patients require frequent intraocular anti-VEGF injections, raising the risk of complications from the procedure and the burden to both, doctors and patients. rAAV-mediated expression of anti-VEGF proteins is an attractive alternative to reduce the risk to affected patients. However, controversy remains as to the safety of sustained VEGF inhibition in the eye. Here, we test the safety and efficacy of delivering the anti-VEGF drug Conbercept (KH902) with rAAV vectors in two mouse models of vascular pathology. We used the oxygen-induced retinopathy model, as well as the laser damage-induced model of choroidal neovascularization. To assess the efficacy of rAAV-*KH902*, we

quantified the number of aneurysms in the oxygen-induced retinopathy model and the number of edemas, as well as the size of the neovascular lesions in the laser damage model. We found that intravitreal delivery of rAAVs expressing KH902 can successfully reduce the number of aneurysms, edemas, as well as the growth of the choroidal neovascular lesions. Serotypes with high transduction efficiencies were found to induce, in a dose dependent manner, a vascular sheathing pathology that is characterized by immune cell infiltrates, reminiscent of vasculitis. This pathology was accompanied by increased expression in vascular cells adhesion molecule 1, which promotes extravasation of immune cells from the vasculature. Importantly, low viral doses were still able to reduce edemas and lesion size without causing any vascular sheathing pathology. The data suggest that rAAV-mediated expression of anti-VEGF drugs can be employed safely for the treatment of neovascular eye pathologies.

328. Targeted Correction of Exons 23-26 of the Factor VIII Gene to Correct the I22I Inversion in Hemophilia A by AAVHSC-Mediated Genome Editing

Lakshmi V. Bugga, Ka Ming Pang, Szu-Chi Su, Saswati Chatterjee

Department of Surgery, City of Hope, Duarte, CA

Approximately 45% of hemophilia A patients carry the I22I inversion in which exons 1-22 of the Factor VIII gene (F8) are inverted relative to exons 23-26. This inversion splits the full length FVIII protein into two truncated proteins which are transcribed in opposite directions. To correct the I22 inversion and restore the full length FVIII protein, we evaluated AAVHSC-mediated targeted insertion of the cDNA of FVIII exons 23-26 at the end of exon22 of the FVIII gene. AAVHSC editing vectors mediate homologous recombination-based genome editing without the need for prior nuclease-mediated cleavage of genomic DNA. We optimized the design of FVIII I22I editing vectors for the insertion of promoterless reporters downstream of exon 22 of the F8 gene. Flow cytometry and sequence analysis showed that AAVHSC editing vectors successfully inserted the promoterless GFP open reading frame (ORF) at specified locations within the FVIII gene in both cell lines as well as primary human and canine liver sinusoidal endothelial cells (LSEC) in vitro. This formed the basis for the design of correction vectors to edit the I22I inversion. Editing vectors for FVIII gene correction were designed to insert codon optimized exon 23 to 26 cDNA and a reporter gene coding region. The homology arms were designed to insert the correction cassette at the end of exon 22 of the FVIII gene, such that successful correction of the I22 inversion would be dependent upon transcription from the chromosomal FVIII promoter. Specific mCherry expression in AAVHSC edited primary human LSEC indicated successful insertion of the correction sequence in the FVIII gene. The frequency of editing ranged from 7% to > 30% in primary human LSEC, and was dependent upon homology arm configurations. Editing was confirmed at the molecular level by targeted integration assays employing amplification of junction sequences using mCherry-specific and chromosome-specific primers. Sequence analysis revealed precise and seamless insertion of the exon 23-26 cassette into the FVIII gene and the absence of insertion/deletion mutations. Preliminary data confirmed expression of the correction cassette in primary human and canine endothelial cells. Studies are underway to evaluate the restoration of expression of the full-length FVIII protein and correction of the I22I inversion.

329. FLT201, a Novel Investigational AAV-Mediated Gene Therapy Candidate for Gaucher Disease

Carlos Miranda[1], Fabrizio Comper[1], Petya Kalcheva[1], Elisa Chisari[1], Clement Cocita[1], Samantha Correia[1], Jalpa Pandya[1], Benjamin Liou[2], Miriam Canavese[1], Natalie Northcott[1], Emmaline Stotter[1], Erald Shehu[1], I-Mei Yu[1], Jaminder Khinder[1], Rose Sheridan[1], Ying Sun[2], Amit Nathwani[3], Romuald Corbau[1]

[1]Freeline, Stevenage, United Kingdom,[2]Cincinnati Children's Hospital Medical Center, Cincinnati, OH,[3]University College London, London, United Kingdom

Gaucher disease (GD) is one of the most common lysosomal storage disorders. Mutations in the *GBA1* gene attenuate or abrogate the activity of the lysosomal acting enzyme glucocerebrosidase (GCase). Enzyme replacement therapy (ERT) and substrate reduction therapy are currently standard of care for the treatment of non-neuronopathic, GD type 1 (GD1). However, significant unmet needs remain. Intravenous (IV) infusion every 2 weeks is required because of the short half-life of ERTs. This produces a high burden of treatment and consumes significant healthcare resources. Also, response to treatment can be variable and incomplete, leading to significant life-limiting symptoms and associated reduced quality of life. AAV-mediated liver-directed gene therapy may address unmet needs for GD1 patients by providing sustained, endogenous production of GCase following a single IV infusion. FLT201, an investigational gene therapy for the treatment of GD1, is a novel, potent, engineered AAV capsid (AAVS3) containing an expression cassette that encodes for a GCase variant 85 ($GCase_{var85}$). The $GCase_{var85}$ contains two novel amino acid substitutions to the mature human GCase, which results in a 20-fold increase in GCase half-life at lysosomal pH conditions and a 10-fold increase in half-life in mouse serum. $GCase_{var85}$ catalytic parameters (K_M) are comparable to wild-type GCase and ERTs used to treat GD1. The potency and functionality of $GCase_{var85}$ when secreted by the liver following treatment with AAV8 pseudotyped FLT201 [AAV8-FLT201] were evaluated in GCase-deficient mice ($Gba^{9v/null}$) and compared to liver-secreted wild-type GCase following treatment with AAV8 pseudotyped FLT200 [AAV8-FLT200] and velaglucerase alfa (an ERT used to treat GD). $Gba^{9v/null}$ mice (8 weeks of age) received a single injection of either AAV8-FLT200 or AAV8-FLT201 at $2x10^{10}$, $2x10^{11}$, or $2x10^{12}$ vg/kg. Velaglucerase alfa was administered every 2 weeks at a dose of 60 U/kg. Treatment-naïve wild-type and $Gba^{9v/null}$ mice were included as comparators. Animals were followed for 12-weeks to allow the GD disease hallmarks to develop in the $Gba^{9v/null}$ mouse model. AAV8-FLT201 treatment resulted in a rapid and sustained dose-dependent increase in circulating enzyme levels that were 42-fold higher than observed with AAV8-FLT200 at 12 weeks post-injection. AAV8-FLT201 fully restored GCase activity in plasma, white blood cells and all tissues analyzed (liver, spleen, bone marrow and lung). In contrast, AAV8-FLT200 failed to restore tissue GCase activity in $Gba^{9v/null}$ mice, particularly in bone marrow and lung where levels stayed below 50% of those observed in wild-

type mice. Importantly, restoration of tissue GCase activity achieved with AAV8-FLT201 resulted in clearance of lipid storage cells and normalization of lyso-Gb1 biomarker levels in all tissues examined. Based on the data collected in *Gba*$^{9v/null}$ mice and clinical data for our AAVS3 platform, the use of FLT201 compared to FLT200 should allow reduction of the predicted efficacious clinical dose by at least 10-fold. Pre-clinical data show that AAV8 pseudotyped FLT201 genome is well tolerated and results in a significant increase in potency in GCase deficient mice, including hard-to-reach tissues such as bone marrow and lung. Therefore, FLT201 has the potential to address areas of current unmet medical need in GD1 patients.

330. Improving the *In Vivo* Gene Targeting Efficiency of Liver-Directed rAAV Vector Using the Nucleotide Analog Class of Ribonucleotide Reductase Inhibitors

Shinnosuke Tsuji[1], Calvin J. Stephens[1], Giulia Bortolussi[2], Katja Pekrun[1], Feijie Zhang[1], Gustavo de Alencastro[1], Gabriele Baj[3], Andres F. Muro[2], Mark A. Kay[1]

[1]Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA,[2]International Centre for Genetic Engineering and Biotechnology, Trieste, Italy,[3]Life Sciences, University of Trieste, Trieste, Italy

While the liver is one of the most attractive target organs for gene therapy using recombinant adeno-associated viral (rAAV) vectors, their use in neonatal or pediatric patients presents several challenges. These include loss of episomal vector genomes resulting from normal or disease related liver growth. Second, the potential for insertional mutagenesis and vector-promoter activation of a proto-oncogene induced hepatocellular carcinoma remains a concern. In order to expand the clinical application of rAAV, our group previously reported a strategy using a rAAV vector for homologous recombination (AAV-HR) without the use of nucleases (Barzel, et al, Nature, 2015) in which we can specifically target a therapeutic coding sequence onto the end of an endogenous coding gene before the translation termination signal. Thus, a chimeric mRNA is produced that makes both the endogenous and therapeutic protein. This technology has been used to treat mouse models of hemophilia B, Crigler-Najjar, ZZ alpha-1-antitrypsin deficiency and methylmalonic acidemia.In current and prior studies, we and others found that the efficiency of AAV-HR in mouse liver occurs at best in ~1% of hepatocytes. This limits the clinical application of the technology for some disorders. Here, we tested a series of small molecule compounds with various different mechanisms of action in the context of AAV-HR and identified that ribonucleotide reductase (RNR) inhibitors significantly enhanced the AAV-HR efficiency in mouse and human liver cell lines by 2 to 5 fold. Because these drugs may have multiple modes of action, we used RNAi knockdown to confirm reduction of RNR activity contributed to the increased HR efficiency. Furthermore, short term administration of an FDA-approved nucleotide analog type of RNR inhibitor, fludarabine, increased the *in vivo* efficiency of AAV-HR by 4-5 fold in murine liver at a dose that did not cause serious toxicity. In contrast, hydroxyurea failed to show *in vivo*efficacy possibly related to its mechanism of action as well as its low accumulation in the liver. In addition, we were able to show that fludarabine administration induced transient DNA damage signaling in both proliferating and quiescent hepatocytes. Surprisingly, *in vivo* BrdU labeling implicated that the majority of AAV-HR events occurred in non-proliferating hepatocytes in both the fludarabine and no drug treatment animals. These data suggest that induction of transient DNA repair signaling in non-dividing hepatocytes is responsible for enhancing the efficiency of AAV-HR in mice treated with fludarabine. We also demonstrated that fludarabine treatment significantly increased the *in vivo* CRISPR/Cas9-mediated hepatic gene editing efficiencies. In total, we show that the transient use of RNR inhibitors at the time of AAV vector administration can act on quiescent cells and provide a safe strategy to enhance genome editing events for therapeutic purposes.

331. Development of AAV-Based CRISPR/Cas9 Therapies for Correcting Duchenne Muscular Dystrophy by Targeted Genomic Integration

Adrian Pickar-Oliver[1], Christopher Nelson[1], Joel Bohning[1], Veronica Gough[1], Robin M. Perelli[2], Andrew P. Landstrom[2], Charles A. Gersbach[1]

[1]Biomedical Engineering, Duke University, Durham, NC,[2]Cell Biology, Duke University, Durham, NC

Genome engineering technologies are the foundation of exciting potential therapies for correcting genetic diseases. Specifically, Duchenne muscular dystrophy (DMD) is one of the most prevalent lethal heritable childhood diseases characterized by progressive muscle degeneration and weakness resulting from mutations in the dystrophin gene. In most cases (~60%), the mutations consist of deletions in one or more of the 79 exons that disrupt the reading frame of the dystrophin transcript. Previous therapeutic strategies typically aim to generate expression of a truncated but functional dystrophin protein that restores the dystrophin associated protein complex (DAPC) and demonstrates functional restoration in animal models of DMD. For example, several groups have adapted the CRISPR/Cas9 technology for gene editing in cultured human DMD cells and the mdx mouse model of DMD to restore the dystrophin reading frame by removing specific exons. However, there remains a need to develop gene editing strategies to evaluate the restoration of complete and functional dystrophin protein. This could be accomplished by the targeted insertion of the exonic regions that are lost from the patient's genome. Previously, AAV delivery of CRISPR/Cas9 for homology-independent targeted integration (HITI) was developed for genome editing of neurons *in vivo*. Here, we demonstrate AAV-based HITI-mediated gene editing therapies for correcting the dystrophin gene. Specifically, we adapt the CRISPR/Cas9 gene editing technology to direct the targeted insertion of missing exons into the dystrophin gene. As a therapeutically relevant target, we optimize HITI-mediated genome editing strategies in a humanized mouse model of DMD in which exon 52 has been removed in mice carrying the full-length human dystrophin gene (*hDMDΔ52/mdx* mice). To achieve targeted integration, an AAV vector containing the deleted genome sequence including exon 52 was co-delivered with AAV encoding Cas9/gRNA expression cassettes. Following local injection in the tibialis anterior muscle, we have confirmed targeted exon 52 genomic integration, splicing of exon 52 in dystrophin transcripts, and dystrophin protein restoration in our *hDMDΔ52/mdx* mouse model. Additionally, to address a larger

patient population, we are evaluating insertion of a single "superexon" that encodes the complete dystrophin cDNA sequence downstream of exon 51 in cultured cells and in our *hDMDΔ52/mdx* mouse model. Current studies include assessment of HITI-mediated editing following systemic injection. Combined with AAV delivery, the development of HITI-mediated strategies for targeted insertion of missing exons provides a method to restore full-length dystrophin, which could potentially lead to improved functional outcomes.

332. Increased Transgene Expression in Rat Retinal Ganglion Cells by Using Human Mini-Promoters Compatible with rAAV

Victor Guedes de Araujo[1], Mariana Dias[1], Thaís Gonçalo[1], Taliane Vasconcelos[1], Gabriel dos Santos[1], William Hauswirth[2], Rafael Linden[1], Hilda Petrs-Silva[1]

[1]Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil,[2]Department of Ophthalmology, University of Florida, Gainesville, FL

Introduction: As retinal gene therapy field matures, it became crucial to improve experimental protocols to facilitate its translation to human trials. Some clinical trials use ubiquitous promoters such as CBA/CMV, which promote high expression level but can cause side effects since they are not cell specific. To limit such effects, specific promoters have successfully been used to target a variety of retinal cell types. However, between the promoters designated for retinal ganglion cells (RGCs), there are some restrictions: too large, not specific, and not strong in promoting expression. Thus, an important step for gene therapies under development for RGC-related disorders is the availability of small promoters, capable of promoting specific gene expression at suitable levels of expression for the therapy. **Aim:** In this work, we assess the capacity of transgene expression in RGCs of six mini-promoters (MiniPs) designed by bioinformatics tools. **Methods:** The MiniPs sequences were packaged in rAAV vectors driving the direct reporter green fluorescent protein (GFP). Adult *Lister Hooded* rats had intravitreal injection of rAAV vectors and their retinas were immunolabeled 4 weeks post-injection and analyzed by confocal microscopy. To quantify the number of GFP-expressing RGCs, we used the RCG marker, Brn3a. Moreover, Image J software was used to determine GFP intensity in individual GFP-expressing Brn3a+ cells. Statistics were assessed using one-way analysis of variance with Dunnett's post hoc test in GraphPad Prism 7.00 software. $p < 0.05$. All animal procedures were approved by the Ethics Committee on the Use of Animals in Scientific Experimentation from The Health Sciences Center, Federal University of Rio de Janeiro (#083/17). **Results:** Our results demonstrate that different retinal cell types can be efficiently target using the rAAV-MiniPs, especially RGCs. Indeed, three MiniPs, Ple25 2kb (36.43%±5.49; n=5); Ple25 0.75kb (37.45%±5.79; n=5); and Ple53 (43.36%±3.52; n=5), have potentially increased the number of RGCs expressing the transgene compared to ubiquitous promoter CBA/CMV (11.25%±3.88; n=5). Furthermore, quantification of GFP fluorescence intensity in individual Brn3a-positive RGCs showed that MiniPs Ple53 drove strong GFP expression in RGCs with significantly higher intensity levels at 1.31-fold increase. **Conclusion:** Thus, this study identified a functional set of human mini-promoters suitable for AAV-mediated ocular gene delivery applicable in basic and preclinical research, making them potential tools for gene therapies where preferential transgene expression in RGCs is desired.

333. When Size Matters: FVIII Construct Optimization Leveraging ceDNA, a Non-Viral Gene Therapy Platform

Russell Monds, Luke Hamm, Nicholas Parsonnet, Liz Nelson, Ashley Penvose, Zhong Zhong, Deb Klatte

Generation Bio, Cambridge, MA

Development of successful gene therapies requires strategies for optimization of transgene expression that navigate the vast combinatorial complexity of sequence design space. Strategies that assume functional independence of genetic elements are attractive as they allow for independent screening of distinct genetic element modules, followed by combinatorial screening of only the best performers from each module. However, violation of this assumption increases the risk that either improved combinations are not identified or that unanticipated interactions between components limit the effectiveness of modular combinations. Additional constraints on the exploration of sequence design space are encountered by viral gene therapies, such as AAV, due to intrinsic limitations on the size of genetic elements that can be encoded. Meeting these challenges will likely require both new platform technologies and construct optimization strategies. We have developed a non-viral gene therapy platform to deliver and durably express therapeutic proteins systemically *in vivo*. It is comprised of ceDNA, an engineered, double-stranded, linear, covalently closed-ended DNA construct, formulated in a cell-targeted lipid nanoparticle delivery system, ctLNP. This platform permits transgene optimization largely free from the size limitation of viral platforms. We have taken advantage of this feature to perform a semi-combinatorial optimization of FVIII expressing ceDNA for the treatment of Hemophilia A. Here we report identification of increases in construct potency across a range of genetic elements, including codon optimization, promoters, introns, secretion signals, 5' and 3' UTRs. Together, these optimizations resulted in marked improvements in FVIII peak expression *in vitro*, which translated to improved potency of ctLNP-ceDNA in mouse models of Hemophilia A. We also discovered numerous instances of sequence context dependence that impacted performance of individual genetic elements, highlighting the limitations of modularity as a framework for construct optimization. For instance, the performance of 5' UTR sequences was shown to be highly dependent on the codon optimization of the open reading frame, which was not anticipated based on current mechanistic understanding of translation initiation. Uncovering important interaction effects between genetic elements also offers the chance to develop design rules that allow focusing of optimization efforts without sacrificing design complexity. In this regard, we uncovered important design principles governing the function and impact of intronic sequences on transgene performance. Optimization of constructs for gene therapy applications will need to meet new challenges as the field develops, such as incorporation of more complex regulation to enable better response of gene therapies to individual disease presentation. Towards that end, we have shown how relaxed constraints on the size of cis-regulatory elements can open the opportunity for different dimensions of optimization in the future.

334. Increasing AAV-CRISPR Gene Insertion Rates by Modulating DNA Repair Mechanisms

Sylvia P. Thomas[1], Wei Zhang[2], Bernard Thebaud[3], Byram Bridle[1], Sarah K. Wootton[1]

[1]Department of Pathobiology, University of Guelph, Guelph, ON, Canada,[2]Department of Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada,[3]The Ottawa Hospital Research Institute, Ottawa, ON, Canada

With the revolutionary advancement presented by CRISPR-Cas9 technology, gene editing has become a tool applicable across several sectors. Medical applications of this machinery can be employed to correct monogenic diseases and administration of this tool *in vivo* is attainable through delivery by viral vectors, in particular adeno-associated viral vectors (AAV). AAV-CRISPR can deliver and integrate a gene of interest into a specific locus by utilizing DNA repair pathways after its expressed nuclease performs a double stranded break (DSB). This DSB will initiate the DNA repair mechanism inherent in the cell, which consists of two distinct pathways - non-homologous end joining (NHEJ) and homology directed repair (HDR). In mammalian cells, NHEJ is the favoured pathway for DNA repair. This preference is a detriment to current AAV-CRISPR approaches that depend on HDR. However, there are several unique proteins at play in both repair pathways that can be modified to shift the repair mechanisms in favour of HDR. Changes to the activity of these pathway components can occur through the application of DNA repair pathway modulators that work to increase HDR through NHEJ inhibition or HDR enhancement. In our study, the application of these modifiers to increase HDR is investigated *in vitro* and *in vivo* with a dual vector AAV-CRISPR platform. AAV6.2FF, a recombinant viral vector that has demonstrated strong lung tropism, will deliver two separate cassettes, one with the repair template carrying a reporter gene and the other with the CRISPR machinery. We have previously delivered this AAV-CRISPR platform carrying a GFP expressing template into murine models and analyzed this expression (Figure 1 and 2). This baseline insertion rate will be compared to the levels of HDR when this AAV-CRISPR platform is delivered in combination with HDR enhancers that modulate the DNA repair pathway. This AAV-CRISPR combinatorial platform is tested *in vitro* by transducing mouse lung epithelial cells (MLE-12 cells) and analyzing reporter gene expression 5 days post transduction. Gene expression is initially examined by with a multi-detection plate reader and with a fluorescence microscope. This expression is then further quantified through flow cytometry to determine the efficiency of HDR. After analyzing the variance between the different treatment groups, the ideal modulators will be selected and investigated *in vivo* in mice. Lungs will be harvested 3 weeks post vector administration and the number of edited cells quantified by flow cytometry. This study will help provide a comparison between these DNA repair pathway modulators when applied to a dual vector AAV-CRISPR platform.

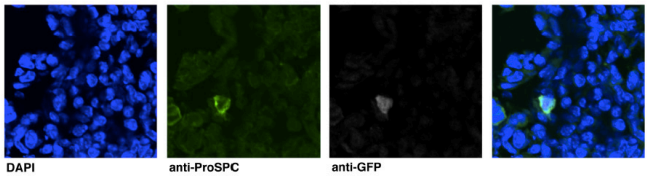


Figure 1. Immunofluorescence analysis of AAV6.2FF-CRISPR-GFP expression.

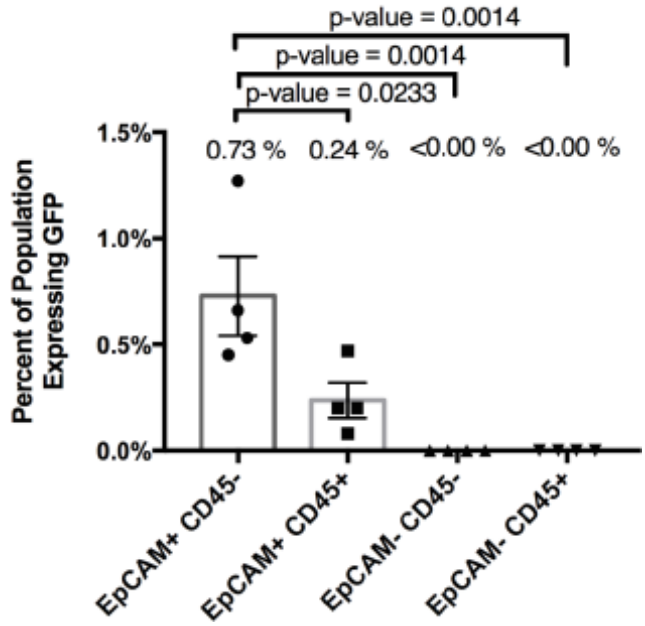


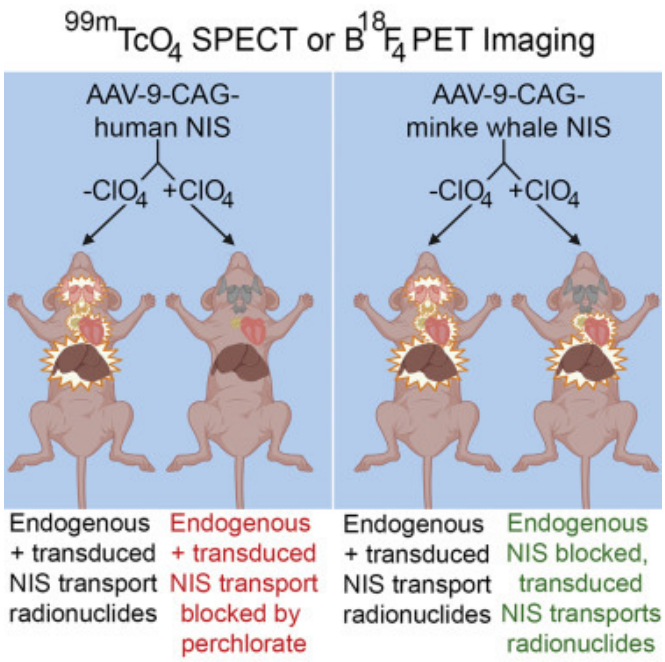
Figure 2. Delivery of AAV6.2FF-CRISPR-GFP platform and analysis by flow cytometry.

335. Superior *In Vivo* Tracking of AAV-9 Gene Therapy with the Sodium Iodide Symporter (NIS) from Minke Whale and Systemic Perchlorate Inhibition

Susanna C. Concilio[1], Lukkana Suksanpaisan[2], Linh Pham[1], Kah-Whye Peng[1], Stephen J. Russell[1]

[1]Department of Molecular Medicine, Mayo Clinic, Rochester, MN,[2]Imanis Life Sciences, Rochester, MN

The sodium iodide symporter (NIS) is used as a reporter gene to noninvasively follow the distribution and longitude of viral vector-mediated gene expression using single-photon emission computed tomography (SPECT) and positron-emission tomography (PET). However, endogenously expressed NIS limits the resolution and specificity of NIS imaging. This study examines whether the perchlorate-resistant NIS protein from minke whale (*Balaenoptera acutorostrata scammoni*) combined with pretreatment with the NIS inhibitor perchlorate can block endogenous uptake while maintaining transport *in vitro* and *in vivo*. SPECT and PET imaging studies were performed in nude mice treated with adeno-associated virus (AAV)-9 vectors encoding either human NIS or minke whale NIS, with and without subcutaneous perchlorate pretreatment prior to intravenous radioisotope administration. These experiments revealed that minke whale NIS continued to act as a reliable reporter gene in the presence of perchlorate, even when radiotracer transport by human NIS or endogenous mouse NIS was ablated.



336. Transducing the Liver as an Antibody Factory Using AAVHSCs

Yogeshwar Sharma, Hillard Rubin, Nancy Avila, Meghan Scarpitti, Jason Lotterhand, April Hayes, David Rappoli, Minglun Wang, Jose Israel Rivas, Benjamin Lehnert, Marissa Stanvick, Diane Golebiowski, Andrew Pla, Serena Dollive, Wei Wang, Gustavo Cerqueira, Matt Elliott, Gabe Cohn, Miranda Rubin, Laura Van Lieshout, MiJeong Kim, Michael Blum, Lindsay Hyde, Shiva Krupa, Tim Kelly, Carmen M. Barnes, Omar Francone, Albert Seymour

Homology Medicines, Inc, Bedford, MA

Chronic dosing of therapeutic antibodies is used as a treatment for many diseases, including autoimmune disorders, complement-related diseases, inflammatory disorders, wet AMD and others. Repeat dosing is associated with complications, increased risk for the patient, discomfort, and lack of compliance. AAV-mediated gene therapies offer the potential to change the paradigm of antibody delivery. A single dose of a vectorized antibody may be able to overcome the peaks and troughs characteristic of antibody pharmacokinetics with persistent and durable antibody levels, alleviating the need for chronic dosing and its associated risks. Taking advantage of the natural tropism of AAVHSCs (AAV capsids isolated from human hematopoietic stem cells (HSCs)), we designed AAVHSC vectors to produce functional antibodies *in vivo* using liver-specific promoters to preferentially express to this tissue. As a proof of concept, we focused on complement-related disorders and used anti-complement protein 5 antibody as payload. Antibody expression was demonstrated *in vitro* in transformed hepatoma cell lines (Hepa1-6, Alexander, HuH7, HepG2), primary hepatocytes (murine and human), and *in vivo* in mouse and human hepatocytes. In both *in vitro* and *in vivo* studies, the fully assembled antibodies were highly expressed (human IgG ELISAs and WB) and were functional (competitive ELISAs and *ex vivo* hemolysis assay). Stable and robust IgG expression *in vivo* was successfully demonstrated in NOD-SCID mice for the duration of the study (up to 26 weeks). *In vivo* expression was dose-dependent and steadily increased during the first 5 weeks, reaching a plateau by ~7 weeks post dose. Analysis of the cellular lysates at the end of study demonstrated the antibody was efficiently secreted and that there were no protein aggregates in the liver even at the highest doses examined, with serum IgG levels >20mg/mL. IgG levels were dependent on vector design and capsid, and capsid-ranking differed in mouse vs human hepatocytes. In summary, we showcase the potential for AAV-mediated gene transfer in transforming a liver to an antibody-producing factory. By utilizing AAVHSCs, which have broad tissue tropism across different cell types and species, we may be able to deliver an appropriate antibody payload to the liver and maintain durable levels of IgG production and therapeutic expression to meet the needs of patients with a variety of diseases currently managed with chronic antibody dosing.

337. Preclinical Considerations for Novel AAV Gene Therapies

Kimberley Hoefen

Consulting Services, Azzur Group, Lakewood, CO

The field of gene therapy (GT) offers new possibilities for treating and even curing many unmet medical needs. GT is a therapeutic intervention that genetically alters or modifies cells by gene delivery most commonly using viral vectors, with adeno-associated virus (AVV) leading the field for many novel GT therapeutics. The ability to generate recombinant virus lacking viral genes while allowing for long-term transgene expression and very low immunogenicity renders AVV one of the safest and most common tools for GT. Preclinical study design needs to mimic the proposed clinical trial design in the target population and the route of administration (ROA) as well as the demonstration of any AAV's association with unregulated expression of the transgene, immunogenicity, expression duration, off-target distribution, any germline transmission and environmental shedding. Preclinical safety and efficacy programs for GT are generally case-by-case with regulatory agencies encouraging early interaction and innovative risk-based approaches. The FDA has recently created a new process called Initial Targeted Engagement for Regulatory Advice on CBER products Meetings (INTERACT) that is intended to facilitate early interactions on innovative programs. This presentation will discuss key factors to consider in the preclinical development of a novel AAV GT and early regulatory interactions with the FDA.

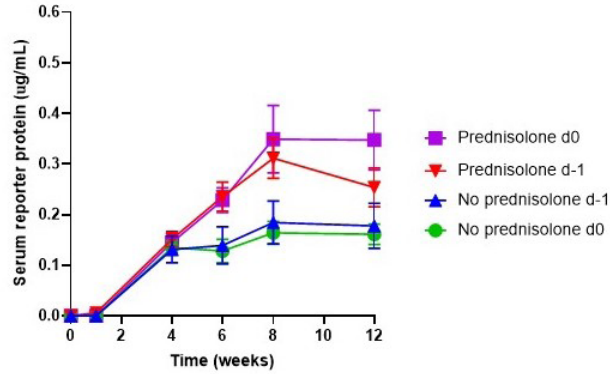
338. The Effect of Prophylactic Corticosteroid Treatment on Adeno-Associated Virus (AAV)-Mediated Gene Expression

Britta Handyside, Lening Zhang, Bridget Yates, Lin Xie, Brian Kaplowitz, Olivia Gorostiza, Ryan Murphy, Natalie Fredette, Brian Baridon, Katina Ngo, Silvia Siso, Vishal Agrawal, Christa Cortesio, Hassib Akeefe, Jill Woloszynek, Weiming Zhang, Cheng Su, Peter Colosi, Sherry Bullens, Gabor Veres, Stuart Bunting, Sylvia Fong

BioMarin Pharmaceutical Inc., Novato, CA

Administration of adeno-associated virus (AAV)-based gene therapy may elicit immune responses to the viral capsid, the viral genome, or the transgenic protein product. Preclinical studies suggest that corticosteroid treatment may modulate immune responses to AAV vectors, leading to long-lasting alterations in AAV-mediated expression. In earlier work, prednisolone treatment initiated one week after valoctocogene roxaparvovec (AAV5-hFVIII-SQ) administration did not alter FVIII expression in mice. Here, we explored whether administration of corticosteroids prior to gene therapy might block early immune responses and promote transgene expression in mice. Daily prednisolone (2 mg/kg) was orally gavaged to mice for 4 weeks starting either 1 day or 2 hours prior to treatment with an AAV5 vector ($6x10^{13}$ vg/kg). After the first dose of prednisolone, mice were treated with an AAV5 vector delivering human a1-antitrypsin transgene driven by a liver-specific promoter (AAV5-HLP-hA1AT). For controls, mice were gavaged with water instead of prednisolone for 4 weeks starting either 1 day or 2 hours prior to treatment with an AAV5 vector. Starting 6 weeks post-AAV dosing and through 12 weeks when transgene expression reached steady state, mice in the prednisolone-treated groups (1 day or 2 hours prior to AAV administration) showed significantly higher levels (1.5- to 2.2-fold) of serum hA1AT protein than non-prednisolone-treated groups (**Figure 1**). In prednisolone-treated groups, the number of hepatocytes transduced with vector DNA and full-length repaired vector genomes was higher than non-prednisolone-treated groups. Full-length vector genome DNA levels significantly correlated with liver hA1AT RNA and serum hA1AT protein levels (r = 0.68; *P* <0.0001). Additionally, there was a trend toward lower interindividual variability in the prednisolone-treated groups (**Figure 2**). Overall, corticosteroid use prior to AAV treatment increased liver-directed AAV expression that is associated with higher levels of full-length vector genomes in mice and may represent a potential strategy for the reduction of interindividual variability in AAV-mediated gene therapy.

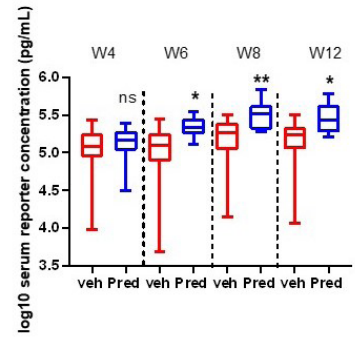
Figure 1. Serum hA1AT protein levels over 12 weeks



Data are mean ± standard deviation.

d, day; hA1AT, human a1-antitrypsin.

Figure 2. Serum hA1AT concentration over time in mice treated with AAV5-hA1AT



**P <0.01; *P <0.05; ns (P ≥0.1).

Whiskers represent minimum and maximum values.

hA1AT, human a1-antitrypsin; ns, not significant; Pred, prednisolone; veh, vehicle; W, week.

339. Second Generation APOE2^{+}APOE4^{-} AAV-Mediated Gene Therapy for APOE Homozygotes at Risk for Alzheimer's Disease

Rachel Montel, Esther Frenk, Ronald G. Crystal, Katie M. Stiles

Genetic Medicine, Weill Cornell Medical College, New York, NY

Alzheimer's disease (AD) is a progressive degenerative neurological disorder associated with a strong genetic risk in polymorphisms of the apolipoprotein E (*APOE*) allele. Inheritance patterns of *APOE* alleles demonstrate that *APOE4/4* homozygotes have a 14.5-fold increased risk of developing AD, while *APOE2/2* homozygotes are protected against developing AD. The knowledge that the presence of *APOE2* in *APOE2/4* heterozygotes markedly reduces the *APOE4* risk led to the development of a 1st generation preventative AD gene therapy delivering *APOE2* to the brain of *APOE4* homozygotes. In the present study, we hypothesized that a 2nd generation gene therapy using an adeno-associated virus (AAV) expressing therapeutic *APOE2* with artificial microRNAs (miRNA) targeting endogenous *APOE4*

may further mitigate the risk and limit AD development of *APOE4* homozygotes. To test this hypothesis, we evaluated silencing of *APOE4* expression using a series of siRNAs targeting *APOE4* in the U-87 human glioblastoma cell line. Of these siRNAs, one sequence (siRNA2) significantly silenced *APOE* expression and was selected for generating novel *APOE*-targeting miRNAs that were incorporated into an artificial miRNA cassette for expression in the AAV vector. These miRNAs were cloned into the intron in the CAG promoter 5' of the transgene or in the 3' untranslated region (UTR) of the pAAV expression cassette. To determine the efficiency of silencing by the miRNAs, the *APOE4* target site was cloned into the pmirGLO-luciferase plasmid and co-transfected into 293T cells with pAAV expression plasmids containing *APOE*-targeting miRNAs. There was a >40% reduction in luciferase activity (representing *APOE4* expression) in the presence of *APOE*-targeting miRNA compared to the control scrambled miRNA. In parallel, an *APOE2* expression cassette was designed that was resistant to silencing by the miRNAs. To test expression of the modified *APOE2* cDNA, 293T cells were transfected, followed by analysis of APOE2 expression by quantitative PCR and Western analysis. The mRNA and protein expression levels from the miRNA resistant *APOE2* cDNA were similar to unmodified *APOE2*. These observations demonstrate that *APOE4* can be targeted for silencing by miRNA incorporation into an AAV expression cassette with a therapeutic *APOE2* as a 2nd generation gene therapy for the *APOE2*$^{+}$*APOE4*$^{-}$ treatment of *APOE4* homozygous individuals at high risk for the development of Alzheimer's disease.

340. Optimization of Human FIX Expression Cassettes for Superior Liver-Targeted Gene Therapy of Hemophilia B

Xiaojun Liu[1], Kentaro Yamada[2], David M. Markusic[2], Roland W. Herzog[2], Pengcheng Zhou[1], Jason Zhang[1]

[1]GeneLeap Biotechnology LLC, Luye Boston Research & Development LLC, Woburn, MA,[2]Department of Pediatrics, Gene and Cell Therapy Program, Herman B. Wells Center for Pediatric Resear, Indiana University, Indianapolis, IN

Gene therapy offers a potential cure for hemophilia B, and significant progress has been achieved in liver-directed gene transfer mediated by adeno-associated vectors (AAV). Recent clinical trials with AAV vectors containing hFIX expression cassettes showed encouraging efficacy in patients, while long-term safety and efficacy related to high vector doses remain significant concerns. Thus, further optimization of AAV vectors and human FIX expression cassettes may benefit hemophilia B gene therapy's ultimate success. In this report, we constructed more than 70 human FIX expression vectors using different combinations of regulatory elements, codon-optimized and mutated FIX nucleotide sequences, and Toll-like receptor 9 (TLR9) inhibitory elements. All FIX expression constructs were packaged with an AAV8 capsid. The potency of engineered AAV constructs in FIX expression were evaluated in cultured human liver cells, wild type C57BL/6, and FIX-deficient hemophilia B (C3H/HeJ-F9$^{-/Y}$) mice via intravenous injection. Our systemic screening identified that the construct pXLLY027 are > 25-fold superior to the benchmark construct in cultured HepG2 liver cells in FIX expression as measured with a human FIX-specific ELISA kit and the potency evaluated by measuring plasma FIX activity with a chromogenic assay. Our in vivo studies demonstrated that AAV8.pXLLY027 vectors express significantly higher FIX expression and FIX activity than benchmark vector AAV8.scAAV-LP1-cohFIX-Padua at different time points after dosing by intravenous injection of packaged AAV8 vectors into wild type C57BL/6 and in C3H/HeJ-F9-/Y on day 28 and 56 post-injection. In addition, interesting effects of TLR9 sequences on FIX expression were identified in vivo. Together, a superior candidate AAV8.pXLLY027 with improved FIX expression and potency, over the benchmark, was successfully generated through systemic optimization of molecular structure and composite regulatory elements.

341. Evaluation of AAV9 and AAVrh.10 mCherry Vectors in Adult Cynomolgus Macaques after Intracisterna Magna Administration

Shen Shen, Branka Grubor, Edward Plowey, Pete Clarner, Kalyani Nambiar, Melissa Kirkland, Stefan Hamann, Patrick Trapa, Eric Tien, Dale Morris, Junghae Suh

Biogen, Cambridge, MA

Recombinant AAV vectors are a promising delivery platform for gene therapy. Despite recent clinical successes of AAV gene therapy in a few CNS indications, higher transduction efficiency in the CNS is imperative to develop gene therapies for a broad spectrum of neurological and neuromuscular diseases. AAV9 and AAVrh.10 have been the leading serotypes for CNS in clinical and preclinical studies. However, limited data have been reported to compare these two serotypes with high dose levels in large animals. Therefore, the objective of this study was to evaluate the biodistribution, transduction efficiency, and toxicity profile of AAV9 and AAVrh.10 delivered at high dose levels via intracisterna magna (ICM) administration in adult Cynomolgus Macaques. AAV9 and AAVrh.10 vectors encoding mCherry driven by the CAGGS promoter were produced at UMASS Medical School Viral Vector Core (Worcester, MA). The vectors were purified by ultracentrifugation and quantified by transgene-specific ddPCR. Vectors were administered via ICM injection at high dose levels (AAV9: 1.5e14 vg and 3.0e14 vg; AAVrh.10: 1.5e14 vg) in a high dose volume (2.5 ml). 12-14 days post dosing, animals were euthanized due to adverse clinical signs in a subset of cohorts. CNS and peripheral tissues were collected to evaluate vector DNA biodistribution, transgene mRNA expression, spatial transduction efficiency at single-cell level, and histopathology. Biodistribution analysis demonstrated high viral load in the spinal cord, dorsal root ganglia (DRG), and liver. In the brain, AAV vector genomes were also detected in subcortical regions, while cortical tissues showed higher viral load. Transgene mRNA expression analysis by RT-qPCR showed high transgene expression in spinal cord and a few DRG samples for both AAV9 and AAVrh.10 dosed at 1.5e14 vg. Interestingly, transgene mRNA expression was significantly lower in subcortical structures, whereas the expression in cortical regions were still moderate. In comparison with animals dosed with AAV9 at 1.5e14 vg, animals dosed with AAV9 at 3.0e14 vg showed higher biodistribution and transgene expression across sampled tissues. Spatial transduction analysis using RNAscope and AI-based single-cell segmentation and detection algorithms demonstrated 64% to 91% transduction among sampled spinal cord ventral horn motor neurons. Mean transduction percentages ranging from 40 - 81% were observed in sampled DRG neurons. In cortical regions, neuronal transduction was limited (2-9%) and in vasculocentric clusters, consistent with

perivascular flow of AAV from cerebrospinal fluid (CSF) to interstitial fluid (ISF). In subcortical and basal ganglia regions, sparse transduction was detected. Histopathology analysis showed minimal to moderate axonal degeneration in dorsal funiculi and minimal to mild gliosis and mononuclear cell infiltration in spinal cord. Consistent with previous publications, minimal to moderate neuronal degeneration/necrosis accompanied by minimal to marked mononuclear cell infiltration were also detected in DRG from these animals. Minimal to moderate neuronal degeneration/necrosis with gliosis, mononuclear cell infiltration and axonal degeneration was also observed in the brain. In summary, we report a preclinical study with AAV9 and AAVrh.10 mCherry vectors delivered via the ICM route in adult cynomolgus monkeys at high dose levels. These results demonstrate superior spinal cord transduction, limited brain transduction, and adverse histopathology findings in the spinal cord, DRG, brain, and liver. Contributing factors could include high dose levels, high dose volume in CSF, and toxicity of the mCherry transgene.

342. PR001 Gene Therapy Increased GCase Activity and Improved Gaucher Disease Type 1 Phenotypes in Animal Models

Patty Sheehan, Laura D. Heckman, Li Chin Wong, Zhaohui Yang, Hsuan-Ni Lin, Sid Kamalakaran, Swetha Garimalla, Jason Politi, Yong Dai, Franz Hefti, Asa Abeliovich

Prevail Therapeutics, New York, NY

Gaucher disease (GD) is caused by biallelic mutations in the glucocerebrosidase (*GBA1*) gene, with severe mutations leading to neurological manifestations (neuronopathic Gaucher disease; nGD) and more moderate mutations leading to a primarily peripheral disease (Gaucher disease type 1; GD1). The *GBA1* encoded enzyme glucocerebrosidase (GCase) is a key lysosomal enzyme required for the normal metabolism of glycolipids. GCase deficiency causes the accumulation of glycolipid substrates, resulting in lysosomal dysfunction that leads to inflammation and other pathological changes. With our gene therapy product candidate, PR001, we aim to increase GCase activity in GD1 patients in order to ameliorate lysosomal dysfunction and manage the disease manifestations with a one-time treatment. Using mouse models of GD, we evaluated the therapeutic effects of PR001 administered via intravenous (IV) injection. Post-treatment, animals were assessed for GD-like phenotypes in the liver, lung, and spleen. PR001 delivery via IV injection resulted in broad biodistribution and dose-dependent increases in GCase activity, reductions in glycolipid substrate accumulation, and reductions in inflammation-associated endpoints. Long-term studies evaluating the persistence of PR001 and its therapeutic effects are ongoing. Overall, PR001 demonstrated efficacy in two independent mouse models of GD supporting further development of PR001 for patients with GD1.

343. AAV Intein-Mediated Factor VIII *Trans*-Splicing for Gene Therapy of Haemophilia A

Federica Esposito[1], Hristiana Lyubenova[1], Patrizia Tornabene[1], Stefano Auricchio[1], Edoardo Nusco[1], Cristina Olgasi[2], Antonia Follenzi[2], Alberto Auricchio[1]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli (Naples), Italy,[2]Department of Health Sciences University of Piemonte Orientale "Amedeo Avogadro", Novara, Italy

Haemophilia A (HemA) is a common X-linked bleeding disorder (1:5000 males), caused by deficiency of human clotting factor VIII (*FVIII*). The current management of HemA involves administration of either recombinant *FVIII*, or of a recently approved bispecific antibody, Emicizumab. However, lifelong intravenous infusions of either of the two are still required.Liver gene therapy with adeno-associated viral (AAV) vectors holds great promise to provide long-term FVIII expression after a single administration. However, HemA poses a great challenge to AAV-delivered gene therapy due to the size of the *FVIII* coding sequence (~7 kb) that exceeds the canonical AAV cargo capacity (~ 4.7 kb). Some of the B-domain deleted (B-dd) *FVIII* variants tested in gene therapy clinical trials also are at the limit of AAV packaging capacity.To overcome this limitation, we developed a protein *trans*-splicing (PTS) strategy using two AAVs, each encoding one half of the large and highly active B-domain deleted (B-DD) N6-*FVIII* variant (~5 kb) flanked by split inteins. We show that AAV intein successfully reconstitute full-length N6-*FVIII* both *in vitro* and in mouse liver and achieve supraphysiological levels of FVIII plasma activity in haemophilic mice in the absence of *FVIII* neutralising antibodies. Additionally, a codon optimized (codop) version of these constructs allows delivery of therapeutic doses of AAV in the range of (~150 IU/dl). Therefore, systyemic administration of AAV intein N6-*FVIII* is a potential therapeutic option for HemA.

344. Discovery of Novel AAV Capsids for Osteoarthritis Gene Therapy

Yukiko Maeda, Guangping Gao #, Phillip W. L. Tai #

Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Arthritis is the leading cause of disability among adults in the United States. 54 million adult Americans have doctor-diagnosed arthritis. Osteoarthritis (OA) is the most common type of arthritis. Risk factors include excess weight, family history, age, and previous injury. When the cartilage wears away, bone rubs against bone, causing pain, swelling, and stiffness. Over time, joints can lose strength and pain may become chronic. Currently, there are no disease-modifying drugs for OA. Gene therapy represents a new treatment strategy that can promote cartilage repair, especially for OA. Recombinant adeno-associated viruses (rAAVs) have emerged as the gene therapy vector of choice since they can transduce non-dividing cells, confer long-term transgene expression, are non-pathogenic, and have relatively low immunogenicity profiles. Its relatively small size (~20 nm) enables rAAVs to penetrate the dense cartilage matrix. Numerous rAAV serotypes have been tested for their capacity to treat arthritis via gene therapy. Among them, AAV2 confers the most efficient transduction of mouse arthritic chondrocytes. Unfortunately, preexisting neutralizing antibodies to AAV2 may limit patient access. It is therefore important

to expand the diversity of capsid variants for rAAV gene therapy vectors. To discover novel AAV capsids that can efficiently transduce the arthritic knee, our laboratory previously identified a large library of AAV proviral capsid sequences from human tissues by single-molecule real-time (SMRT) sequencing and bioinformatics pipelines. In this current work, we tested 42 novel capsid variants that are related to AAV2 for their capacity to transduce OA knee joints. We examined the transduction efficacy of the variants in an OA mouse model generated by surgical destabilization of the medial meniscus (DMM). DMM is known to mimic clinical meniscal injury, a known cause for OA development; and more importantly, allowed us to screen for high-performance capsids that can transduce tissues undergoing structural and biological changes concurrent with OA progression. Two weeks after the DMM surgery, disorganized articular surface and cell death are observed in articular cartilage. AAV variant vectors packaging barcoded transgenes were injected as a mixture into mouse OA knee joints two weeks after DMM surgery and in untreated knees. Two weeks after the injection, OA and intact knee joints, which encompass articular cartilage, synovium, and ligament around the joint space, were dissected. RNA and DNA were subsequently extracted. Following library construction and deep sequencing analysis, we identified three novel capsids that presented higher transduction levels of the barcoded transgene than AAV2 in both intact and OA knee respectively. Interestingly, four variants were found to exclusively transduce either healthy or injured knee joints. These novel AAV2 variants may improve gene therapy vectors for OA and other joint diseases. # Co-corresponding authors

345. Enhanced Repair of Human Osteochondral Defects upon Implantation of Human Bone Marrow Aspirates Modified by rAAV Gene Transfer and Overexpression of *sox9* and TGF-b via pNaSS-Grafted Poly(e-caprolactone) Film-Guided Delivery

Cedric Marmillon[1], Jagadeesh K. Venkatesan[1], Céline Falentin-Daudré[2], Amélie Leroux[2], Veronique Migonney[2], Magali Cucchiarini[1]

[1]Center of Experimental Orthopadie, Saarland University, Homburg, Germany,[2]Université Paris 13-UMR CNRS 7244-CSPBAT-LBPS-UFR SMBH, Bobigny, Paris, France

INTRODUCTION: Gene transfer using recombinant adeno-associated viral (rAAV) vectors is a powerful tool to enhance cartilage repair. Here, we tested the delivering therapeutic (chondrogenic *sox9* and TGF-β) rAAV vectors in reparative human bone marrow aspirates by vector delivery using poly(sodium styrene sulfonate) (pNaSS)-grafted poly(ε-caprolactone) (PCL) films for implantation in a human osteochondral defect model as a potential scaffold-guided gene therapy option. **Methods**:The PCL films were fabricated using a spin-coating method. The following conditions were tested: absence of pNaSS grafting (PCL conditions) *versus* pNaSS grafting (1.3 x 10^{-5} mol/g pNaSS; pNaSS-PCL conditions). rAAV-*lacZ* carries the *E. coli* β-galactosidase (*lacZ*) reporter gene, rAAV-FLAG-h*sox9* a human *sox9* sequence, and rAAV-hTGF-β a human transforming growth factor beta 1 sequence, all controlled by the CMV-IE promoter/enhancer. Immobilization of rAAV on the films was performed by adding the vectors (40 µl) with 0.002% poly-L-lysine. 150 µl of human bone marrow aspirates (hBMAs) were placed on the films with fibrinogen/thrombin and implanted in 4-mm diameter of human osteochondral defects (hOCDs) and kept in 750 µl chondrogenic medium for up to 21 days. The proteoglycan contents were assessed by DMMB assay, the DNA contents by Hoechst 33258 assay, and the total protein contents by BCA Protein assay. Total RNA was extracted cDNA amplification was performed by real-time RT-PCR with GAPDH as control for normalization. A t-test was employed with $P \leq 0.05$ considered statistically significant. **RESULTS:** Treatment with rAAV *sox9* and TGF-β enhanced type-II collagen deposition in hBMAs via PCL film-guided gene transfer via implantation in hOCDs (**Fig. 1** and **Tables 1** and **2**). Similar results were noted when measuring the normalized type-II collagen contents and increased the normalized proteoglycan contents but only via pNaSS-grafted films ((**Tables 1** and **2**). These results were corroborated by real-time RT-PCR of the profiles for COL2A1 and ACAN expression (**Fig. 2**). Interestingly, reduced premature expression of COL1A1 and COL10A1 (**Fig. 2**). Overall, the effects reported with the pNaSS-grafted PCL films were more potent than when using ungrafted films. **Conclusions:** These results show the potential of pNaSS-grafted PCL film-guided therapeutic rAAV gene transfer in hBMAs as a novel healing platform for implantation in articular cartilage defects.

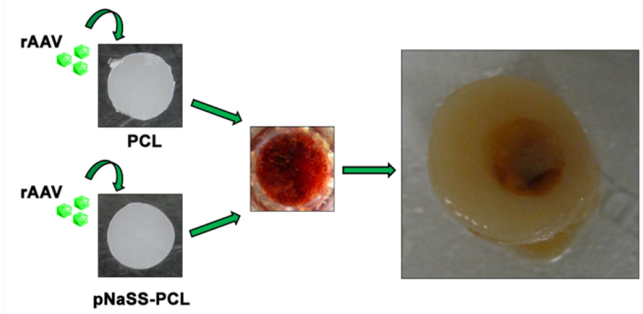


Fig. 1. PCL film-guided rAAV-mediated gene transfer in hBMAs for implantation in hOCDs.

Table 1. Biochemical analyses in hOCDs treated with hBMAs modified by application of rAAV-coated PCL films (day 21)

Parameters	-/PCL	*lacZ*/PCL	*sox9*/PCL	TGF-β/PCL
Type-II collagen (ng/ml)	0.83 (0.40)	0.90 (0.23)	3.72 (0.10)[a,b]	4.34 (0.50)[a,b]
Proteoglycans (µg/µg total proteins)	0.06 (0.20)	0.07 (0.30)	0.09 (0.40)	0.08 (0.10)
DNA (ng/µg total proteins)	0.19 (0.10)	0.20 (0.30)	0.25 (0.20)[a,b]	0.24 (0.20)[a,b]
Type-II collagen/DNA (ng/ng)	0.07 (0.20)	0.06 (0.20)	0.12 (0.20)[a,b]	0.13 (0.10)[a,b]
Proteoglycans/DNA (µg/ng)	0.32 (0.30)	0.34 (0.20)	0.35 (0.10)	0.35 (0.40)

Data are given as means (SD). Statistically significant versus a-/PCL and blacZ/PCL.

Table 2. Biochemical analyses in hOCDs treated with hBMAs modified by application of rAAV-coated pNaSS-grafted PCL films (day 21)

Parameters	-/pNaSS-PCL	*lacZ*/pNaSS-PCL	*sox9*/pNaSS-PCL	TGF-β/pNaSS-PCL
Type-II collagen (ng/ml)	0.97 (0.60)	0.99 (0.40)	4.72 (0.30)[a,b]	5.38 (0.40)[a,b]
Proteoglycans (µg/µg total proteins)	0.05 (0.10)	0.08 (0.30)	0.10 (0.20)	0.09 (0.10)
DNA (ng/µg total proteins)	0.19 (0.30)	0.21 (0.30)	0.26 (0.10)[a,b]	0.25 (0.10)[a,b]
Type-II collagen/DNA (ng/ng)	0.03 (0.30)	0.07 (0.40)	0.16 (0.10)[a,b]	0.18 (0.30)[a,b]
Proteoglycans/DNA (µg/ng)	0.27 (0.30)	0.28 (0.20)	0.40 (0.50)[a,b]	0.39 (0.10)[a,b]

Data are given as means (SD). Statistically significant *versus* [a]-/pNaSS-PCL and [b]*lacZ*/pNaSS-PCL.

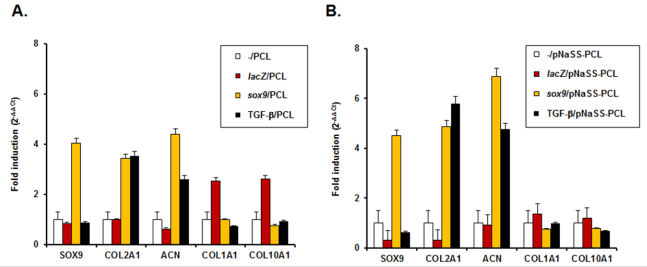


Fig. 2. Real-time RT-PCR analysis in hOCDs treated with hBMAs modified by application of (**A**) rAAV-coated PCL films and (**B**) rAAV-coated pNaSS-grafted PCL films (day 21).

346. Long-Term Safety Studies of AAV Vectors Delivered to Liver or Skeletal Muscle in Tumor-Prone p53 Model Show No Increase of Oncogenicity

Joerg Schuettrumpf[1,2], Benjamin J. Samelson-Jones[1,3], Ralf Buente[4], Valder R. Arruda[1,4]

[1]The Children's Hospital of Philadelphia, Philadelphia, PA,[2]Goethe University, Frankfurt a.M., Germany,[3]University of Pennsylvania, Philadephia, PA,[4]University of Pennsylvania, Philadelphia, PA

Introduction: Recombinant AAV (rAAV) is the first vector for in vivo delivery to receive regulatory approval. Though rAAV genomes remain predominantly non-integrating, rare integrations have been observed in preclinical in vivo models. However, it is not known to what degree these integration events are oncogenic. The assessment of the oncogenic risk of rAAV is essential to understanding the safety of rAAV. The recent report of the development of hepatocellular carcinoma (HCC) after liver-directed rAAV gene therapy trial for hemophilia B spotlights the issues. Here, we report the risk of tumor formation following rAAV administration into p53 heterozygous (p53 (+/-)) deficient mice, a provocative mouse model for the risk of oncogenesis that develop spontaneous tumor in an age-dependent manner. ***Results:*** To evaluate the oncogenesis of rAAV, we delivered rAAV serotype 2 vectors to the liver or skeletal muscle of mouse models p53 (+/-) and p53 wild-type (p53-WT) littermate C57/Bl6 controls. The liver-directed cohort received 4 x 10^{12} vg/kg rAAV with a liver-specific promoter encoding FIX or vehicle control; the muscle-directed cohort received intramuscular (IM) injection of AAV-CMV-null vector at 4 x 10^{12} vg/kg and vehicle control in the contralateral limb. Tumor development was quantified at 12 months or subsequently at time of death. Histological analysis was blinded to treatment and mouse genotype. The rate of tumor formation for the p53 (+/-) model was 0.41 lesions per mouse after liver-gene therapy (n=17) and 0.48 in the vehicle control group (n=23) (p=0.7). As expected, the rate of tumor formation in the p53-WT controls was lower than the p53 (+/-), but also did not differ between animals that received liver-directed gene therapy and vehicle controls. Only 1 vehicle control p53 (+/-) animal developed HCC, though several animals developed other liver cancers and metastases. In p53 (+/-) that received IM AAV and vehicle evaluated at 12 months (n=24), 1 osteosarcoma (OS) was identified in the limb that received AAV and 1 OS was identified in the limb that received vehicle. Four additional OS were found in the muscle-directed cohort that were followed up to 600 days (n=30) with 2 being in the AAV-administered limb and 2 being in the vehicle limb. The OS in the AAV-administered limb had low vector copy numbers (0 - 0.95 copies/cell) compared to healthy muscle tissue (3.4-14.4 copies/cell); likewise, liver-tumors in the mice that received systemic AAV also had lower copy number than the surrounding healthy tissue. Integration analysis of the muscle tumors did not demonstrate clonal expansion with vector integrants and analysis of liver tumors only showed a limited number of expansion without integration into specific oncogenes. Lastly, we carried out a long-term survival study after vector administration. As expected, the cancer predisposition in the p53 (+/-) mice increased their mortality; however, there was no difference in mortality between that received muscle-directed AAV (n=73), liver-directed AAV (n=53), and vehicle controls (n=211) for the 600 days of follow up. Likewise, there was no difference in the mortality of p53-WT that received muscle-directed AAV (n=53), liver-directed AAV (n=27), and vehicle controls (n=168). ***Conclusions:*** In this provocative study, we observe no increase in tumor formation either after systemic AAV vector administration or local IM administration. Cumulatively, this data support the low risk of oncogenesis after AAV administration and the overall safety of AAV based gene therapy.

347. A Non-Human Primate Biodistribution Study Comparing Multiple Routes of Administration of AAV9

Jennifer L. Daily[1], Suzanne R. Burstein[2], Nicholas W. Keiser[1], Stuart R. Cobb[1]

[1]Neurogene Inc., New York, NY,[2]Neurogene, New York, NY

Introduction: Conditions such as Batten disease are associated with widespread neurodegeneration that affects all divisions of the nervous system including central and peripheral nerves, as well as sensory systems such as the retina. In such indications, use of a single gene therapy product would be an ideal approach if it were feasible to broadly target the nervous system, peripheral nerves, and retina. We have conducted a well-controlled study to evaluate the transduction efficiency of a single AAV9 product across these different nervous system domains. Using non-human primates, which exhibit nervous system and eye morphology and immunophysiology similar to that of humans, we assessed a range of intra-CSF, systemic, and ocular targeted routes of administration for efficient targeting of the nervous system and retina with AAV9. **Methods:** To compare distribution efficiency in both the central and peripheral nervous systems, animals (n=3/group) were treated with 1.1e13 vg of an AAV9 vector containing a human CLN5 transgene (AAV9-CLN5) in a 1ml administration volume via intra-CSF delivery using intracisternal magna (ICM), unilateral intracerebroventricular (ICV), or intrathecal lumbar (IT-L) injection. In one group of ICV-treated animals, the same dose was administered (1.1e13 vg) using a higher volume (4X) to assess injection volume effects on biodistribution; an additional cohort was administered 1.1e13 vg via IV injection. One month following treatment, brain, spinal cord, and peripheral nerve tissues were processed for qPCR measurement of vector biodistribution from all animals receiving CNS-directed treatments using an independently qualified assay. To compare ocular routes of administration, animals (n=2/group) were treated with 2.8e11 vg of AAV9-CLN5 via bilateral intravitreal (IVT), suprachoroidal (SC), or subretinal (SR) injections. Eye tissues from ocular-treated animals were collected one-month post-treatment and assessed for biodistribution using RNAScope. **Results:** The AAV9-CLN5 vector was

well-tolerated in all treatment groups. The biodistribution results of this study indicate that route of administration is an important variable in targeting specific tissues underlying neurological and retinal disease. Both ICM and ICV administration generated broad biodistribution throughout the brain and spinal cord; the four-fold increase in ICV injection volume did not impact vector biodistribution. Transduction efficiency with IT-L was comparable to ICM and ICV in the posterior brain regions and lumbar spinal cord, but lower than ICM and ICV in the anterior brain regions and the cervical and thoracic spinal cord. All intra-CSF delivery routes targeted the peripheral nerves with similar efficiency. RNAScope analysis of eye tissues revealed regional differences in biodistribution with IVT, SC, and SR injection routes. SR administration was most efficient in targeting the retina and photoreceptors. **Conclusions:** These results, together with the technical feasibility and safety profile of each delivery route, will help inform the selection of the route(s) of administration for AAV9 gene therapy products targeting complex neurological disease affecting multiple domains of the nervous system. Given the differences in biodistribution with each route of administration, the regional specificity of disease pathology should be considered for each specific indication. For indications with both neurological and retinal manifestations, such as Batten disease, a combination of delivery routes may be required to effectively treat key target tissues.

348. Adenoviral Transduction of Dickkopf-1 Mitigates Silica-Induced Silicosis in Lungs of Mice

Qian Cai[1], Jia Ma[1], Jing Wang[2], Jieda Cui[3], Dandan Yang[1], Jiali Yang[1], Jing Xue[1], Juan Chen[3], Xiaoming Liu[1]

[1]Department of Life Science, Ningxia University, Ningxia, China,[2]Department of Pathology, General Hospital of Ningxia Medical University, Ningxia, China,[3]Department of Pulmonary and Critical Care Medicine, General Hospital of Ningxia Medical University, Ningxia, China

Silicosis is an occupational disease caused by inhalation of crystalline silica dust, which is hallmarked by a progressive *pulmonary fibrosis*. To date, there is not a conventional drug available for silicosis treatment, therefore it is an unmet medical need in developing novel therapeutic approach for this dreadful disease. Aberrant Wnt/β-catenin signaling has been recognized as a key driver in the development of pulmonary fibrosis, and a therapeutic target for treatment of fibrotic diseases. Dickkopf-1 (Dkk1), an inhibitor of Wnt/β-catenin signaling is a biomarker in many diseases related to infection, autoimmunity,and fibrosis. In light of above findings, we thus hypothesis that DKK1 may be a rational target for silicosis lung disease. In test this hypothesis, we first examined the concentrations of Wnt signaling ligand WNT3A and inhibitor DKK1 in plasmas of patients with pneumoconiosis including silicosis. Notably, an increased circulating WNT3A and DKK1 proteins were observed in patients with pneumoconiosis including silicosis compared to healthy subjects. Consistently, the experimental studies in mice also revealed a reactivated Wnt/β-catenin signaling, along with an increased Wnt3a and Dkk1 proteins in lungs of silica-induced silicosis mice, as compared to controls. Given the fact of the great promise of viral gene therapy for treatments of many chronic lung diseases, such as cystic fibrosis, and the pulmonary fibrosis, implying it may also represent an effective mean for treatment of silicosis. To test this concept, the alterations of pathology and Wnt/β-catenin signaling activity were evaluated in lungs of silica-challenged mice that infected with an adenoviral vector expressing Wnt3a or Dkk1 in this study. As expected, the intratracheal delivery of an adenoviral vector expressing murine Dkk1 (AdDkk1) led to a dramatical inhibition of Wnt/β-catenin signaling activity, coupled with a significantly mitigation of silicotic pathogenesis, as accessed by the reduction of epithelial-mesenchymal transition (EMT) markers and extracellular matrix (ECM) deposition in silica-challenged mouse lungs, in comparison with the "null" control vector AdBglII. Mechanistic analysis further demonstrated that the AdDkk1-mediated amelioration of silicosis was in part by inhibiting the silica-induced TGF-β/Smad signaling, which was further confirmed by in vitro studies in human airway epithelial cells. In addition, the augmented Dkk1 expression was able to inhibit silica-induced epithelial cell proliferation in polarized human bronchial epithelial cells. As expected, the adenoviral vector expressing murine Wnt3a (AdWnt3a) exerted an opposite effect to AdDkk1. This study provides an insight of underlying mechanism of Wnt/β-catenin signaling in the pathogenesis of silicosis, and an evidence of proof-of-concept that the promise of viral vector-mediated Dkk1 gene transduction in treatment of silicosis lung disease.

AAV Vectors - Preclinical and Proof-of-Concept Studies

349. Carbon Dot-Guided Delivery of Therapeutic (sox9, TGF-β) rAAV Vectors to Enhance the Biological Activity of Human Articular Chondrocytes

Weikun Meng[1], Jagadeesh K Venkatesan[1], Ana Rey-Rico[2], Gertrud Schmitt[1], Susanne Speicher-Mentges[1], Françoise Pons[3], Henning Madry[1], Luc Lebeau[3], Magali Cucchiarini[1]

[1]Saarland University, Homburg/Saar, Germany,[2]Universidade da Coruña, Coruña, Spain,[3]CNRS-University of Strasbourg, Illkirch, France

INTRODUCTION: Scaffold-assisted gene therapy is a highly promising tool to treat cartilage injuries, including osteoarthritis (OA). In our previous observations showing the potential of carbon dot (CD)-guided rAAV gene transfer in human bone marrow-derived mesenchymal stromal cells (hMSCs) , we examined here the ability of an optimized CD (MC148) to deliver chondroreparative *sox9* and TGF-β rAAV vectors to human articular chondrocytes, to treat OA. **METHODS:**rAAV-*lacZ* carries the *E. coli* β-galactosidase (*lacZ*) reporter gene, rAAV-FLAG-h*sox9* a human *sox9* sequence, and rAAV-hTGF-β a 1.2-kb human transforming growth factor beta 1 sequence, all controlled by the CMV-IE promoter/enhancer . Human OA chondrocytes were seeded in 48-well plates (5,000 cells/well), maintained in DMEM medium and incubated at 37°C during 12 h before addition of the CDs. The CDs (MC148) were generated through pyrolysis of citric acid in the presence of *N,N*-dimethylethylenediamine (DMEDA) and next mixed with rAAV-*lacZ,* rAAV-FLAG-h*sox9*, or rAAV-hTGF-β in equal parts and applied to the cells for up to 21 days . Histological and immunohistochemical analyses were performed

on monolayer culture (anti-sox9, anti-TGF-β and by ELISA (R&D Systems), anti-type-I, anti-II, and anti-X collagen). Alcian blue staining (matrix proteoglycans) was was quantified by overnight solubilisation in 6 M guanidine hydrochloride to monitor absorbance at 595 nm. Cell viability was monitored by Cell Proliferation reagent WST-1 (Roche Applied Science). Each condition was performed in duplicate in three independent experiments. **RESULTS AND CONCLUSIONS**Successfully therapeutic gene delivery of rAAV *sox9* and TGF-β was achieved in human OA chondrocytes via MC148 for at least 21 days (**Fig. 1A**). compared with all other conditions (**Fig. 1B**). Overexpression of *sox9* and TGF-β via *sox9*/MC148 and TGF-β/MC148 delivery, respectively, increased the deposition of type-II collagen and matrix proteoglycans in human OA chondrocytes over time (21 days) while further decreasing type-I collagen and type-X expression *versus* control conditions (**Fig. 2**). In addition, both *sox9*/MC148 and TGF-β/MC148 treatments allowed to trigger cell proliferation after 21 days relative to control conditions (**Fig. 3**). **CONCLUSIONS**: Delivery of rAAV *sox9* and rAAV TGF-β via MC148 CDs is capable of activating the biological activities in human OA chondrocytes .These results showing CDs as a novel, safe and effective platforms to treat human OA.

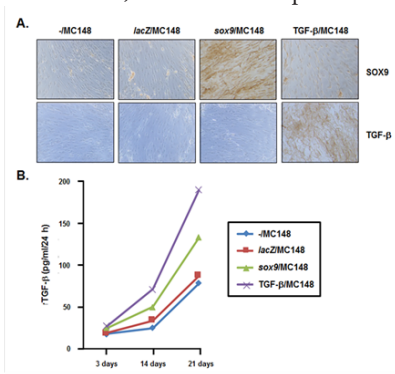


Fig. 1. Detection of transgene (SOX9, TGF-β) expression in human OA chondrocytes following CD-guided rAAV gene transfer. **(A)** Evaluation of SOX9 and TGF-β expression by immunocytochemistry after 21 days of treatment with the therapeutic systems (rAAV-FLAG-h*sox9*/MC148, i.e. *sox9*/MC148; rAAV-hTGF-β/MC148, i.e. TGF-β/MC148). **(B)** Analysis of TGF-β expression by ELISA at the denoted time points. Control conditions included CDs carrying rAAV-*lacZ* (*lacZ*/MC148) or lacking vector treatment (-/MC148).

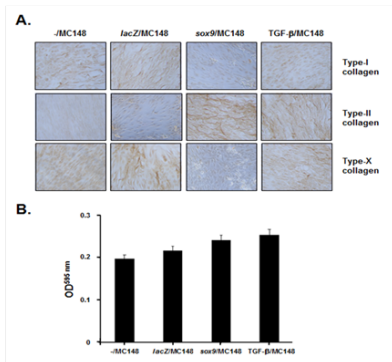


Fig. 2. Evaluation of matrix deposition in human OA chondrocytes following CD-guided rAAV gene transfer. (A) Analysis of type-I, -II, and -X collagen deposition by immunocytochemistry and (B) evaluation of alcian blue staining (B) (matrix proteoglycans) by spectrophotometry (OD595 nm) after 21 days of treatment with the therapeutic systems (C) (sox9/MC148 or TGF-β/MC148) versus control conditions (lacZ/MC148 and -/MC148).

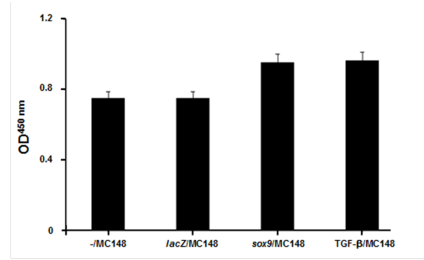


Fig. 3. Analysis of cell proliferation in human OA chondrocytes following CD-guided rAAV gene transfer. The indices of cell proliferation were monitored using the Cell Proliferation reagent WST-1 (OD^{450nm}) after 21 days of treatment with the therapeutic systems (*sox9*/MC148 or TGF-β/MC148) *versus* control conditions (*lacZ*/MC148 and -/MC148).

350. Benefits of Photopolymerizable Hydrogels for the Effective Delivery of rAAV Vectors in Human Bone Marrow Derived Mesenchymal Stem Cells

Weikun Meng[1], Jagadeesh K Venkatesan[1], Riccardo Beninatto[2], Devis Galesso[2], Gertrud Schmitt[1], Susanne Speicher-Mentges[1], Henning Madry[1], Magali Cucchiarini[1]

[1]Saarland University, Homburg/Saar, Germany,[2]Fidia Farmaceutici SpA, Padua, Italy

Injured adult articular cartilage has a limited ability for self-healing. Scaffold-guided rAAV gene transfer has the potential to overcome neutralizing antibodies against by protecting the vectors from neutralization while increasing their gene transfer efficacy by controlling their spatial and temporal delivery. Here, we examined the feasibility of delivering rAAV vectors in human MSCs (hMSCs) via photopolymerizable hydrogel systems for cartilage repair. **METHODS:** rAAV-*lacZ* carries the E. coli β-galactosidase (*lacZ*) reporter gene and rAAV-RFP the Discosoma sp. red fluorescent protein gene (RFP). hMSCs were (passage 1) were seeded in 48-well plates (5,000 cells/well). The hydrogel systems (FID119 F3 and FID119 F4) were prepared by overnight dissolution of the compounds (300 mg of FID119 F3, i.e. F3; 200 mg of FID119 F4, i.e. F4) 10 ml NaCl 0.9% (w/v). The F3 and F4 hydrogel systems were mixed with rAAV-*lacZ* or rAAV-RFP in equal parts using a BTC® Medical Europe UV-Lamp (5 min) and directly applied to the cultures for up to 21 days. Release of rAAV from the hydrogels was evaluated by ELISA.Transgene (RFP) expression was detected by live fluorescence imaging. Cell viability was monitored using the Cell Proliferation reagent WST-1. **RESULTS AND CONCLUSIONS** The F3 and F4 hydrogels were both capable of encapsulating rAAV as noted by effective detection of Cy3 vector labeling relative to control conditions (**Fig. 1**). The rAAV release profiles (**Fig. 1C**) showed that both the F3 and F4 hydrogels effectively released rAAV over a prolonged period of time (21 days) compared with control conditions. Specifically, the F4 hydrogel system was the most potent to support a controlled release of rAAV over time (**Fig. 1C**). Transgene (RFP) expression upon direct contact of the hydrogels with hMSCs over time revealed that rAAV-RFP released from the F3 and F4 hydrogels promoted an effective overexpression of the RFP reporter gene in the cells for at least 21 days *versus* the various control conditions tested (**Fig. 2**). Equally important, cell proliferation indices in the cells showed no deleterious effects of the F3 or F4 hydrogel systems carrying rAAV relative to the various control conditions tested (**Fig.**

3). **CONCLUSION:** Combining rAAV gene transfer with the use of photopolymerizable (FID119) hydrogels may provide novel, promising tools to enhance cartilage repair by implanting MSCs modified to overexpress therapeutic (chondroregenerative) genes via controlled delivery of rAAV using in particular the F4 hydrogel.

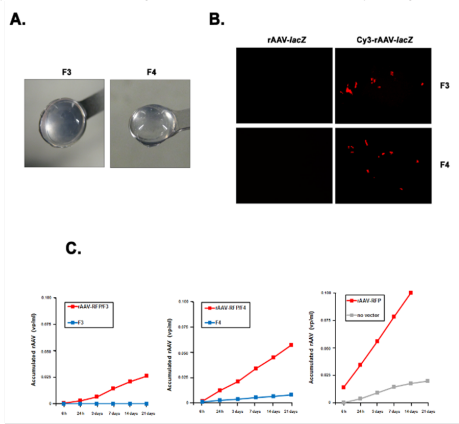


Fig. 1. Characterization of the FID119 hydrogel systems employed to deliver rAAV vectors. (**A**) Macroscopic views of the F3 and F4 hydrogels. (**B**) Detection of Cy3-labeled rAAV vectors encapsulated in the F3 and F4 hydrogels after 24 h. (**C**) rAAV release profiles from the F3 and F4 hydrogels over time relative to hydrogels lacking rAAV vectors, to hydrogel-free rAAV release, and to a condition lacking both the hydrogel and rAAV.

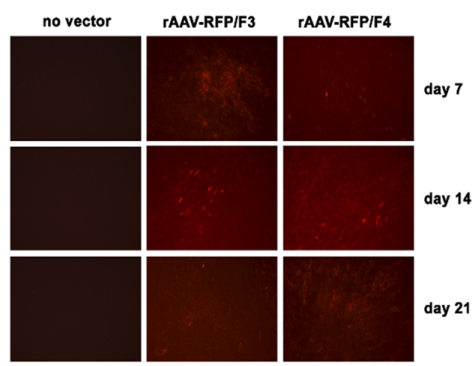


Fig. 2. Detection of transgene (RFP) expression in hMSCs via FID119 hydrogel-guided rAAV gene transfer. RFP expression was monitored by live fluorescence imaging at the denoted time points in the presence of rAAV-RFP/F3 and rAAV-RFP/F4 *versus* a no vector control treatment.

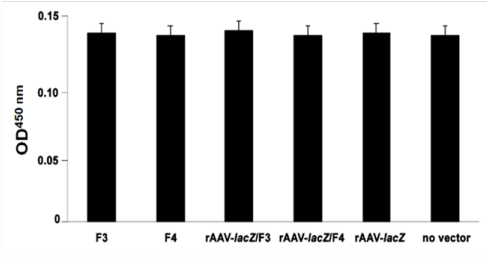


Fig. 3. Analysis of cell proliferation in hMSCs upon FID119 hydrogel-guided rAAV gene transfer. The indices of cell proliferation were monitored after 21 days using the Cell Proliferation reagent WST-1 (OD^{450nm}) in the presence of rAAV-*lacZ*/F3 and F4/rAAV-*lacZ*/F4 *versus* conditions of hydrogels (F3 or F4) without rAAV, hydrogel-free rAAV-*lacZ*, and no vector treatment.

351. Expression of Desmoplakin (*DSP*) Using a Split AAV Vector System

Kyle Chamberlain, Thomas Weber

Cardiology, Icahn School of Medicine at Mount Sinai, New York, NY

Recombinant adeno-associated virus vectors (rAAVs) for the treatment of various inherited and acquired disorders are currently in numerous stages of development. One limitation of rAAVs is the limited cargo capacity of ~5 kb. For most therapies this is not a concern, as the median human protein size is ~375 amino acids, and easily allows for the incorporation of a promoter, poly A, and other regulatory elements into the expression cassette. However, for certain diseases the therapeutic payload vastly exceeds the packaging capacity of a single AAV vector. Thus, approaches to overcome this packaging constraint have been an active area of research for AAV gene therapy, for example, in the treatment of Duchenne muscular dystrophy. Arrhythmogenic right ventricular cardiomyopathy (ARVC) is an inherited heart disease with most known causes associated with pathogenic variants in genes encoding desmosomes, a specialized cell-to-cell adhesion complex. Desmoplakin (DSP) is a structural protein that forms part of the desmosome. Missense or nonsense mutations in one copy of the DSP gene can cause a haplo-insufficiency of protein, leading to decreased desmosome integrity. DSP deficiency is a potential target for rAAV therapy. However, the DSP coding sequence is 8.6kb long, which - coupled with a promoter and other regulatory elements - vastly exceeds the packaging capacity of a single AAV vector. Here, we have utilized a dual AAV vector approach to split the therapeutic payload across two vectors. In addition to the split vector approach, we also utilized minimized CMV and polyA sequences to reduce further the genome size. An HA tag was included at the 3' terminus to enable antibody labelling of full-length transgenic vs endogenous DSP. Dual AAV vectors were manufactured and tested in vivo in adult and neonatal C75BL6 mice. Although a Western Blot of extracted proteins from mouse hearts 1-month p.i. showed that transgenic DSP was detectable, further analysis of mRNA levels revealed that transgenic levels of DSP were only about 5% that of endogenous DSP. Ultimately, because DSP-dependent ARVC is caused by a haplo-insufficiency of DSP protein, expression of transgenic DSP at ~5% of endogenous DSP levels will likely not prove sufficient to compensate for the reduction in DSP expression caused by mutations in the DSP gene. Therefore, while this work shows that minimized regulatory elements in context of a split AAV vector system are likely insufficient to provide a therapeutic benefit in patients with DSP mutations, it does show that there may be useful applications of minimized CMV and polyA sequences in genetic diseases where low amounts of protein provide a therapeutic effect.

352. Characterization of New Pfizer AAV Vectors

Charles Shyng[1], Michael Michalec[1], Kimberly Zaraspe[1], Ting-wen Cheng[2], Ricardos Tabet[2], LouJin Song[3], JinCheng Pang[3], Yuhang Liu[4], RDRU Production Group Pfizer[1], Allison Lewis[1], Jeffrey Morin[5], Katherine Hales[3], Seungil Han[4], Robert Bell[2], Joseph Rabinowitz[1], Clark Pan[2]

[1]Rare Disease Research Unit, Pfizer, Inc, Morrisville, NC,[2]Rare Disease Research Unit, Pfizer, Inc, Cambridge, MA,[3]Internal Medicine Research Unit, Pfizer, Inc, Cambridge, MA,[4]Medicinal Sciences, Pfizer, Inc, Groton, CT,[5]Pfizer, Inc, Cambridge, MA

Naturally occurring viral serotypes typically exhibit broad tissue tropisms, relatively high neutralizing antibodies in the general population, as well as difficulty with manufacturing at scale that may complicate targeted delivery of genetic payloads and limit patient

access. Preferably, newly engineered constructs should possess targeted tropism for the tissue of interest, evade neutralizing antibodies, and support manufacturability. In addition to previous efforts to insert peptides on externalized domains to confer tissue tropism, we have generated new AAV vectors harboring between 35 and 120 non-linear amino acid differences by making large domain exchanges between different viral serotypes (comprising over 30% of the serotype sequence including VRIV through VRVIII). Cryo-electron microscopical analysis of the new vectors indicated a seamless integration of the parental foundational sequences and the swapped domain. Two variants, PFAAV-1 and PFAAV-2, were studied in mice using a bidirectional CMV driven GFP/Firefly luciferase as the transgene: PFAAV-1 and PFAAV-2 utilized AAV6 and AAV7, respectively, for the domain swap into AAV9. PFAAV-1 showed equivalent cardiac and muscle tropism compared to AAV9 but significantly greater de-targeting of the liver. PFAAV-2 showed targeted liver transduction when compared to AAV9. Immunohistochemical staining for GFP demonstrated an equivalent area of transduction between AAV9 and PFAAV-1 in the ventricle and atria of the heart, while staining of the liver showed a significant reduction in GFP positivity. Conversely, PFAAV-2 showed an equivalent area of transduction compared to AAV9 in the liver but significantly decreased transduction in the heart. The immunohistological data reflected the *in vivo* and *ex vivo* bioluminescence data. PFAAV-1 showed minimal to no GFP positivity throughout the liver which was confirmed by both ddPCR (for viral genomes) and RNA expression studies. These vectors also demonstrated characteristics that are supportive of manufacturability at the 4L scale using HEK293 cells in suspension cultures. Both variants have similar vg/cell production compared to the parental AAV9 serotype, and can utilize the same affinity purification methodology as the parental AAV9 serotype. These data demonstrate the feasibility of transferring tropism features between serotypes while retaining key characteristics of manufacturing.

353. AAV-Mediated Aldehyde Dehydrogenase 2 (ALDH2) Gene Therapy to Treat Osteoporosis in Ethanol Consuming ALDH2 Deficient Mice

Anna Camilleri[1], Michelle Cung[2], Odelya E. Pagovich[1], Matthew B. Greenblatt[2], Ronald G. Crystal[1], Katie M. Stiles[1]

[1]Genetic Medicine, Weill Cornell Medical College, New York, NY,[2]Pathology and Laboratory Medicine, Weill Cornell Medical College, New York, NY

Aldehyde dehydrogenase 2 (ALDH2) deficiency affects 8% of the world population and 35 to 40% of East Asian populations. The ALDH2*2 allele, the most common genetic variant, is the result of a glutamic acid-to-lysine substitution at position 487 (E487K). ALDH2 is a key enzyme in ethanol metabolism; with alcohol consumption, the lower oxidizing ability of the ALDH2*2 variant results in systemic accumulation of toxic acetaldehyde. Acetaldehyde interferes with bone metabolism by suppressing early osteoblast progenitor formation leading to decreased bone formation. There are definitive epidemiologic links between ALDH2 deficiency and increased risk for osteoporosis and hip fracture that are further increased with alcohol consumption. In mouse models of ALDH2 deficiency, chronic ethanol consumption results in severe osteopenia. Our previous studies in *Aldh2* E487K knock-in homozygous ($Aldh2^{E487K+/+}$) mice demonstrated that a one-time administration of an adeno-associated virus serotype rh.10 gene transfer vector expressing the coding sequence of human ALDH2 (AAVrh.10hALDH2) corrected the ALDH2 deficiency and prevented bone loss resulting from subsequent chronic ethanol consumption. We hypothesized that administration of AAVrh.10hALDH2 would also treat prior bone loss associated with ALDH2 deficiency and chronic ethanol ingestion. To assess this hypothesis, AAVrh.10hALDH2 was administered intravenously [10^{11} genome copies (gc)] to male and female $Aldh2^{E487K+/+}$ mice (n=6/group) 6 wk after commencing chronic ethanol ingestion (10% ethanol in drinking water). Mice were assessed at intervals for body weight and rotarod performance. Mouse femurs were evaluated by micro computed tomography (μCT) of the trabecular and cortical bone. Compared to water drinking littermates 12 wk after vector administration, untreated $Aldh2^{E487K+/+}$ mice had decreased body weight and performed poorly on the rotarod. In contrast, AAVrh.10hALDH2-treated $Aldh2^{E487K+/+}$ chronic ethanol drinking mice had increased body weight (males $p<0.0004$; females $p<0.02$) and enhanced rotarod performance (males $p<0.02$; females $p<0.04$). In the femur μCT analysis, untreated $Aldh2^{E487K+/+}$ mice showed significant bone loss in both the trabecular bone and midshaft cortex compared to control (water) littermates. Strikingly, with administration of AAVrh.10hALDH2, there was enhancement of trabecular bone volume and significant recovery of cortex thickness. In summary, in the $Aldh2^{E487K+/+}$ murine model with chronic ethanol ingestion, AAVrh.10hALDH2 therapy can mediate recovery of bone mass for treatment of osteoporosis associated with chronic ethanol consumption and ALDH2 deficiency.

354. AAV-Based MCOLN1 Gene Transfer as a Therapeutic Strategy for Mucolipidosis IV

Yulia Grishchuk[1], Samantha DeRosa[2], Madison Sangster[1], Victoria Miller-Browne[2]

[1]Center for Genomic Medicine and Department of Neurology, MGH, Boston, MA,[2]Center for Genomic Medicine, MGH, Boston, MA

Mucolipidosis IV (MLIV) is an orphan disease leading to debilitating psychomotor deficits and vision loss. It is caused by loss-of-function mutations in the *MCOLN1* gene that encodes the lysosomal transient receptor potential channel mucolipin1, or TRPML1. With no existing therapy, the unmet need in this disease is very high. Here we showed that AAV-mediated CNS-targeted gene transfer of the human *MCOLN1* gene rescued motor function and alleviated brain pathology in the MLIV mouse model. Using the AAV-PHP.b vector in symptomatic mice, we showed long-term reversal of declined motor function and significant delay of paralysis. Next, using self-complimentary AAV9 clinical vector, we showed that its intracerebroventricular administration in post-natal day 1 mice significantly improved motor function, myelination and reduced lysosomal storage load in the MLIV mouse brain. Based on our data and general advancements in the gene therapy field, we propose scAAV9-mediated CSF-targeted *MCOLN1* gene transfer as a therapeutic strategy in MLIV.

355. *In Vitro* and *In Vivo* Analyses of Dual Vector Otoferlin Expression to Support the Clinical Development of AK-OTOF (AAVAnc80-hOTOF Vector)

Eva Andres-Mateos, Francesc Puig-Basagoiti, Danielle R. Lenz, Yukako Asai, Ivy K. Hughes, Hao Chiang, Richard M. Churchill Jr., Shimon P. Francis, Yuanzhao Darcy, Michelle D. Valero, Robert Ng, Michael J. McKenna, Emmanuel J. Simons, Gregory S. Robinson, Jennifer A. Wellman

Akouos, Inc., Boston, MA

Millions of people worldwide have hereditary hearing loss because one of their genes generates an incorrect version of a specific protein the ear requires for hearing. Gene therapy using adeno-associated viral (AAV) vectors is a promising therapeutic modality for such inner ear conditions. The *OTOF* gene encodes otoferlin, a protein that plays a critical role in the priming, fusion, and replenishing of synaptic vesicles at the inner hair cell synapse during sound encoding. The majority of patients with biallelic mutations in *OTOF* have a congenital, Severe to Profound sensorineural hearing loss. Because the approximately 6-kilobase length of the *OTOF* complementary DNA exceeds the packaging capacity of a single AAV vector, a dual vector approach is utilized. AK-OTOF (AAVAnc80-hOTOF) is a dual vector consisting of component AAVAnc80 vectors encoding the upstream, or 5', and the downstream, or 3', sequences of human otoferlin; the preclinical-stage product candidate is intended for the treatment of patients with *OTOF*-mediated hearing loss. In dual AAV vector approaches, two transgenes, each of which contain a portion of the full-length transcript, are packaged separately; the two resulting vectors are mixed and provided together to a target cell population. A target cell needs to receive copies of both the upstream and the downstream transgenes and, based on the dual vector design, these transgene products will recombine and generate a full-length mRNA transcript. In order to support the clinical development of AK-OTOF, several different analyses were performed to show that the dual vector otoferlin gene therapy expresses full-length human otoferlin mRNA in a human cell line and expresses full-length human otoferlin protein both in a human cell line and in NHP inner hair cells of the cochlea. *In vitro* studies with several ratios of the 5' and 3' hOTOF AAVAnc80 vectors were conducted to determine the optimal ratio(s) for efficient reconstitution of full-length human otoferlin protein. A comprehensive evaluation of the mRNA transcripts formed as a result of dual vector transduction showed minimal undesired transcripts. Protein expression analysis clearly identified only the full-length protein product, with no detectable truncated proteins. To evaluate full-length otoferlin expression *in vivo*, cynomolgus macaques received intracochlear administration of dual vector AAVAnc80 encoding a 3'-FLAG-tagged human otoferlin protein. Following a one-month in-life duration, cochlear histology revealed FLAG staining in the IHCs of NHPs, indicating expression of full-length human otoferlin. These complementary IND-enabling nonclinical studies, demonstrating consistent recombination of the component vector transgene products and generation of full-length otoferlin protein, provide support for future clinical development of AK-OTOF for the treatment of *OTOF*-mediated hearing loss.

356. Novel Adeno Associated Vector-Based Gene Therapy for the Autosomal Recessive Non-syndromic Deafness (DFNB9)

Jérôme Nevoux[1], Ghizlene Lalhou[1], Baptiste Plion[1], Lauranne Alciato[1], Marie Giorgi[1], Charlotte Calvet[1], Danica Ciric[1], Patrice Vidal[2], Laurent Desire[2], Marie-Jose Lecomte[1], Christine Petit[1], Saaid Safieddine[1]

[1]Genetics and Physiology of Hearing Laboratory, Institut de l'Audition/Pasteur, Paris, France,[2]Sensorion, Montpellier, France

The autosomal recessive deafness (DFNB9) is affecting 2 to 8% of all cases of congenital genetic deafness. It is due to mutations in the gene encoding otoferlin (*OTOF*). Otoferlin is highly expressed in the inner hair cells (IHC) wherein is critical for the synaptic vesicles fusion with the presynaptic plasma membrane. *OTOF* gene defect leads to the failure synaptic transmission between inner hair cells (IHC) and auditory nerve resulting in a profound deafness. There are no approved curative therapies for otoferlin deficiency and cochlear implantation is the only option proposed to young patients (<18 months old). Even though this solution improves the quality of life and language acquisition, hearing recovery is limited and thus a more targeted treatment for DFNB9 is an unmet medical need. Due to its recessive genetic origin DFNB9 is an ideal target for gene therapy. Adeno-associated virus (AAV) is the vector of choice for *in vivo* gene therapy. A major limitation to the development of gene therapy for DFNB9 is the size of coding sequence of *OTOF* largely exceeding AAV packaging capacity. To overcome this limitation, we adopted a dual vector strategy combining trans-splicing and overlapping vectors. Under the control of a constitutive promoter, Otof de novo expression in IHC showed efficacy to reverse deafness of DFNB9 mouse model. Beyond this proof of concept, IHC-restricted expression of otoferlin may be advantageous for long-term expression of the therapeutic transgene. Thus, we aimed to develop a therapeutic recombinant vector candidate using cell-specific components (capsid and promoter). In a first set of experiments, using a constitutive promoter and the GFP as a reporter gene, we demonstrate that the AAV serotype selected from mice studies is effective in non-human primate, allowing to efficiently target IHCs. Then, using the IHC-restricted promoter in DFNB9 mice and non-human primates, we investigated specificity and quantified GFP expression in IHC of both species and evaluated the potential side effects of our surgical approach in non-human primates. Our non-human primate data showed an effective transduction rate of the relevant target IHC at levels compatible with therapeutic intervention of Otoferlin deficiency and a complete recovery after surgery, which constitutes a major step toward future clinical trials in DFNB9 patients using our therapeutic recombinant vector.

357. Novel Allele Independent Treatment of an ADRP Model by RAAV-CRISPR/CAS9 Mediated HITI

Brian Rossmiller[1], Takeshi Iwata[2], Phillip W. L. Tai[1], Guangping Gao[1]

[1]Horae Gene Therapy Center, The University of Massachusetts, Worcester, MA,[2]National, National Hospital Organization, Tokyo, Japan

Retinitis pigmentosa is the leading cause of hereditary blindness with 30-40% of cases attributable to autosomal dominant retinitis pigmentosa (ADRP). ADRP is caused by mutations in at least 24 known genes, with 30% arising in the rhodopsin gene (Rho). There are currently no effective long-lasting treatments. Fortunately, advances in gene therapy, such as RNAi and ribozyme-based knock-down and replacement have proven useful. However, these approaches only slow the rate of degeneration. In our novel work, we have created a long-term allele-independent gene correction modality using CRISPR/Cas9. We demonstrated, in wild-type mice, that subretinal injection of rAAV gene-editing vectors can achieve allele-independent replacement of Rho through cDNA insertion with homology-independent transgene integration (HITI). One-month post-treatment, we observed a 60% reduction of endogenous Rho, without compromising retinal function. Furthermore, we achieved a six-fold increase in ERG a-wave response in a mouse model of ADRP (I307N Rho heterozygous mice), one-month post-treatment. The a-wave remained significantly higher out to six months. This was coupled with an increase in the expression of several photoreceptor-specific genes, such as Rho, Arrestin, GNAT1, etc. Finally, deep sequencing confirmed that treatment of retinas resulted in a 5.8% insertion rate and 88.1% indels events. In conclusion, we demonstrate the feasibility of allele-independent treatment of ADRP with the aid of CRISPR/Cas9. The insertion of Rho cDNA from a self-complementary AAV allowed for allele-independent gene correction at therapeutic levels that maintained significant retinal function and structure. This work will serve as a model for future allele independent research in the treatment of retinal degenerative diseases.

358. Demonstration of Tolerability of a Novel Delivery Approach and Secreted Protein Expression Following Intracochlear Delivery of AK-antiVEGF (AAVAnc80-antiVEGF Vector) in Non-Human Primates

John Connelly[1], Ivy K. Hughes[1], Ann E. Hickox[1], Shimon P. Francis[1], Christopher A. Shera[2], Alec Salt[2], Eric Horowitz[1], Kathleen Lennon[1], Jean Phillips[1], Jenna Soper[1], Michelle D. Valero[1], Jennifer A. Wellman[1], Greg Robinson[1], Emmanuel J. Simons[1], William F. Sewell[3], Michael J. McKenna[1], Eva Andres-Mateos[1]

[1]Akouos, Inc., Boston, MA,[2]Consultant to Akouos, Inc., Boston, MA,[3]Co-Founder and Consultant to Akouos, Inc., Boston, MA

AK-antiVEGF is a gene therapy vector designed for the potential treatment of patients with vestibular schwannomas (VS), or benign tumors that originate from the cells surrounding the vestibulocochlear nerve within the internal auditory canal. Common symptoms associated with VS include hearing loss, tinnitus, and dizziness; as tumors continue to grow, they can compress the brainstem, representing a concern for more serious morbidity and, in very rare cases, mortality. Therapy with inhibitors of vascular endothelial growth factor (VEGF) offers a potential opportunity to attenuate VS tumors, rather than using invasive alternatives such as surgical resection and/or radiation therapy. These current standard of care options often have significant morbidity (e.g., facial paralysis and hearing loss). Systemic Avastin® (bevacizumab) has been shown to decrease vestibular tumor size and improve hearing in neurofibromatosis type 2 (NF2) patients; however, long-term systemic administration of VEGF inhibitors can be associated with significant safety concerns. AK-antiVEGF (AAVAnc80-antiVEGF) is a preclinical development product candidate intended to treat individuals with VS by gene transfer to the inner ear to promote localized expression and secretion of an anti-VEGF protein; the objective is to provide local exposure of VEGF inhibitor at the VS site, thereby limiting systemic exposure and minimizing the potential for the adverse effects associated with systemic administration. In the planned approach, cochlear and vestibular cells of the inner ear transduced by AK-antiVEGF secrete anti-VEGF protein into perilymph, an inner ear fluid that is in diffusional continuity with the interstitial and perineural spaces of the vestibulocochlear nerve where VS tumors are located. We evaluated the tolerability and exposure of AK-antiVEGF, and resulting anti-VEGF protein expression, following one-time bilateral administration at two different doses in cynomolgus macaques followed for either 2 months or 6 months. The data generated in this preliminary, non-GLP tolerability and exposure study show no systematic differences in tolerability, as measured using both physiologic and histologic assessments, between vector-dosed animals and vehicle-control animals in both the 2-month and 6-month follow-up groups. Additionally, anti-VEGF protein was detected at biologically active levels in perilymph fluid. Finally, we performed computational modeling of anti-VEGF diffusion as a function of distance from the inner ear to the location in the internal auditory canal where VS tumors typically originate. Based on this computational model, the anti-VEGF protein concentrations measured in perilymph, once diffused to the tumor site, are expected to remain within a predicted therapeutically relevant range, supporting the feasibility of the intended clinical route of administration for AK-antiVEGF. Taken together, these data demonstrate that the long-term expression of anti-VEGF protein following intracochlear delivery of AK-antiVEGF is robust and well tolerated, supporting the future clinical development of AK-antiVEGF for the treatment of vestibular schwannomas.

359. Investigational Liver Gene Transfer of C1-INH for the Treatment of Hereditary Angioedema

Christopher R. Riling, Frank Pankowicz, Hayley Hanby, Michelle Scriver, Steve Ioele, Valeriya Posternak-Ball, William J. Quinn, Daniel M. Cohen, George M. Preston, Marco Crosariol, Sean M. Armour, Federico Mingozzi

Liver Research & Development, Spark Therapeutics, Inc, Philadelphia, PA

Hereditary angioedema (HAE) is an autosomal dominant genetic disorder characterized by episodic attacks of acute angioedema that can have severe and sometimes fatal consequences. HAE is caused predominantly by deficiency in the first component of complement inhibitor (C1-INH) that leads to aberrant production of bradykinin, driving painful and unpredictable episodes of subcutaneous or submucosal swelling. Treatment with C1-INH-replacement therapy increases survival, reduces attack frequency and severity, and is a current standard of care for HAE disease patients. However, current treatment modalities have several significant drawbacks such as the potential for breakthrough attacks, safety/tolerability, high patient burden, and potential for limited compliance, highlighting the need

for new treatment options. Gene transfer for hereditary angioedema offers the potential for a one-time therapeutic intervention with the potential to overcome some of these drawbacks. Here we show that a novel investigational liver-directed adeno-associated viral (AAV) gene therapy with an optimized C1-INH encoding transgene results in sustained, dose-dependent, circulating levels of C1-INH in mice. Additionally, we show that AAV-mediated C1-INH expression in C1-INH null mice both reduces bradykinin, the mediator of HAE attacks, and stabilizes levels of circulating C4, a diagnostic marker of HAE. The observed sustained levels of circulating C1-INH and concomitant reduction of bradykinin are promising preclinical results supporting the further evaluation of liver gene transfer with AAV-C1-INH in clinical trials.

360. Synthetic AAV Vectors for the Potential Gene Therapy of Hemophilia in Children

Jakob Shoti, Keyun Qing, Arun Srivastava

Pediatrics, University of Florida, Gainesville, FL

Recombinant AAV serotype vectors and their variants have been, or are currently being used for gene therapy for hemophilia in several Phases I/II/III clinical trials in adult patients. However, none of these trials thus far has included children with hemophilia since the traditional liver-directed AAV gene therapy will not work in these patients because of the following reasons: (i) Up until age 10-12, the liver is still growing and dividing, and with every cell division, the AAV vector genomes will be diluted out due to their episomal nature; and (ii) Repeated gene delivery will be needed, but repeat dosing, even with an ideal AAV vector is not an option because of pre-existing antibodies to AAV vectors following the first administration. We hypothesized that the use of a synthetic AAV vector, devoid of AAV capsid proteins, and capable of repeat dosing, could be developed. To this end, we further improved a novel No-End (NE) AAV DNA, a previously described hybrid genome that consists of a gene cassette covalently flanked by AAV inverted terminal repeats (ITRs) with no open ends (*Gene*, 119: 265-272, 1992). Although this linear, NE-DNA is exonuclease-resistant, and can be used to express a transgene following delivery into mammalian cells, the stability of NE-DNA and kinetics of transgene expression have not been studied in detail. In addition to optimizing the NE-DNA production protocol, multiple truncations of the AAV-ITR were generated and assessed for transgene expression and longevity from an EGFP expression cassette under the control of the human α-fetoprotein (AFP) promoter (**Figure 1A**). An optimal NE-DNA construct (NE-TR75) was found to mediate transgene expression in human hepatocellular carcinoma cell line, Huh7, up to 4-weeks post-transfection (**Figure 1B**). The transgene expression half-life of NE-TR75 DNA was observed to be ~1.6-fold longer (~9.8 days compared with ~6.1 days with linear DNA) and ~1.8-fold longer than circular plasmid (~5.3 days). The longevity of transgene expression also correlated well with the stability of the NE-TR75 DNA construct containing the EGFP expression cassette under the control of the human liver cell-specific trans-thyretin (TTR) promoter up to 35 days post-transfection as determined by PCR analysis of extrachromosomal DNA (**Figure 1C**). We have also generated the NE-TR75-human clotting factors IX (hF.IX) and VIII (hF.VIII) DNAs under the control of the TTR promoter, and studies are currently underway to evaluate to efficacy and longevity of hF.IX and hF.VIII expression in human hepatic cells. In future studies, encapsidation of the NE-TR75-TTR-hF.IX and NE-TR75-TTR-hF.VIII DNAs in liver-targeted synthetic liposomes may allow repeat dosing and sustained transgene expression from these synthetic AAV vectors. Furthermore, encapsidation of NE-TR75-TTR-hF.IX and NE-TR75-TTR-hF.VIII DNA in exosomes may also provide a viable approach for the potential gene therapy for hemophilia in children since exosome-associated AAV-F.IX vectors have been shown by others to achieve robust delivery to the liver, evading pre-existing humoral immunity to AAV capsids, leading to a phenotypic correction in hemophilia B mice (*Blood Adv,* 1: 2019-2031, 2017).

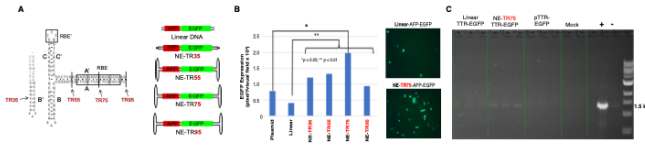


Figure 1: (**A**) schematic structures of the AAV inverted terminal repeat (ITR) in its hairpin configuration and linear and No-End EGFP expression cassettes under the control of the human α-fetoprotein promoter (AFP) flanked by AAV-ITRs of various indicated lengths. (**B**) Comparative analyses of transgene expression from linear and NE-DNAs with ±1SD error, and representative images of transgene expression 4-weeks post-transfection. (**C**) Agarose gel electrophoresis of PCR-amplified fragments from low mol. wt. DNAs from transfected cells 35-days post-transfection. The expected 1.5 kb DNA fragments and positive [+] and negative [-] controls are shown.

361. Preclinical Safety Evaluation of the *Silence & Replace* AAV Technology for Alpha-1 Antitrypsin Deficiency in Non-Human Primates

Carmen Unzu[1], Erin Merkel[1], Hongbin Xu[1], Amany Awad[1], Katie Black[1], Valeria Berlfein[1], Scott Loiler[1], Christian Mueller[2], Florie Borel[1]

[1]Apic Bio, Cambridge, MA,[2]University of Massachusetts Medical School, Worcester, MA

Alpha-1 antitrypsin (AAT) deficiency is an autosomal codominant disease affecting an estimated 100,000 individuals in the United States alone. It is the most common genetic cause of emphysema, which results from low levels of active AAT. However, the common Z mutation is also associated with liver disease, which typically develops later in life and can affect both hetero- and homozygotic patients. To address AAT deficiency, we have adopted a *Silence & Replace* strategy using a dual-function AAV3B vector (THRIVE™), that simultaneously silences endogenous AAT using an artificial miRNA and expresses an active AAT protein as replacement. The strategy demonstrated dose-dependent efficacy in proof-of-concept experiments on lung disease in AAT knockout mice as well as on liver disease in PiZ transgenic mice. In this study, we dosed non-human primates with $5x10^{13}$ gc/kg of the dual-function AAV3B vector and evaluated AAV, miRNA and human AAT immunogenicity upon systemic administration. One group of animals were treated with an immunosuppression (IS) regimen throughout the study to explore the potential of a drug combination to control the immune response against the vector or its elements. Two weeks upon administration, a mild, subclinical rise in liver transaminases that resolved in days was observed. In addition, there was an increase in CD20 and CD19 cell populations in peripheral blood three weeks upon injection that was completely controlled by the IS regimen. Histopathology analysis of the target tissue, the liver, and

dorsal root ganglia (DRG) did not show vector or transgene-related toxicity at the given dose. Moreover, vector biodistribution analysis by ddPCR and dual DNA/RNAscope confirmed AAV3B capsid tropism to the liver. AAV3B and human AAT ELISpot results were performed to complement histopathology data. Finally, human and non-human AAT expression in serum was quantified by LC-MS/MS. In conclusion, the *Silence & Replace* AAV Technology showed promising safety results for the treatment of Alpha-1 Antitrypsin Deficiency at the target dose in non-human primates. Furthermore, our data support the use of an immunosuppression regimen for long-term AAT expression.

362. CSF Delivery of Anc80L65 in Nonhuman Primates Results in More Widespread Gene Transfer Throughout the Central Nervous System Compared to AAV9

Lisa M. Stanek[1], Roberto Calcedo[1], Bryan Mastis[1], Doug Sanders[1], Robert May[1], Luke Lewis[1], Matt Edwards[1], Christopher Tipper[1], Stewart Craig[1], Deepak Grover[1], Hongxing Wang[1], Levy Te[1], Luk H. Vandenberghe[1,2], Laura K. Richman[1]

[1]Affinia Therapeutics, Waltham, MA,[2]Grousbeck Gene Therapy Center, Mass Eye and Ear and Harvard Medical School, Boston, MA

Adeno-associated virus (AAV) gene transfer is a powerful therapeutic modality for treating a number of neurologic disorders. Engineering novel AAV vectors is an exciting emerging area to broaden its reach by addressing the poor distribution throughout the CNS and inefficient transduction of target cell populations using conventional serotypes. The novel AAV vector Anc80L65 was designed based on ancestral sequence reconstruction with sequence information derived from known extant AAVs. Anc80L65 is 101 residues (more than 10%) divergent from AAV9 and was shown to be superior to AAV9 at targeting the brain and spinal cord after intravenous injection in mice and distributed more broadly throughout the CNS. Recent process optimization has significantly improved manufacturing yields of Anc80L65. These data warranted further testing of Anc80L65 in larger animals. Here, we evaluated distribution of single-stranded AAV9 and Anc80L65 encoding the EGFP reporter 14 days following injection by either lumbar puncture (LP) injection into the lumbar cistern (approximately L3-L4) or intracisterna magna (ICM) injection ($4x10^{13}$ gc/animal; $2x10^{13}$ vg/ml) in adult cynomolgus macaques. We demonstrate that a single injection of Anc80L65 into the CSF of adult cynomolgus monkeys led to the efficient transduction of broad regions of the CNS. Following ICM injection, Anc80L65 distributes more broadly throughout the cortex and into deep brain nuclei compared to AAV9. Following LP injection, Anc80L65 distribution throughout the cortex was on par with ICM delivery and superior to that seen with AAV9 via ICM delivery. AAV9 showed limited transduction in the cortex following LP delivery. AAV9 and Anc80L65 efficiently transduced spinal cord ventral horn motor neurons with both routes of administration. Quantification of transduction across regions as well as tropism in neurons vs. glial cells are currently being evaluated. This work demonstrates the ability of Anc80L65, a rationally designed novel AAV, to target widespread regions of the CNS following CSF routes of delivery and outperforms the distribution of AAV9 in targeting cortical and deep brain regions. The ability of Anc80L65 to mediate efficient gene transfer and expression in neurons and astrocytes throughout the brain and spinal cord of NHPs supports further investigation in a wide range of neurologic disorders.

363. Treatment of Feline GM1 Gangliosidosis by Cerebrospinal Fluid Delivery of AAV9 or AAVrh10

Courtney Garrett[1], Amanda L. Gross[1,2], Heather Gray-Edwards[3], Miguel Sena-Esteves[4,5], Douglas R. Martin[1,2]

[1]Scott Ritchey Research Center, Auburn University College of Veterinary Medicine, Auburn, AL,[2]Department of Anatomy, Physiology & Pharmacology, Auburn University College of Veterinary Medicine, Auburn, AL,[3]Department of Radiology, University of Massachusetts Medical School, Worcester, MA,[4]Department of Neurology, University of Massachusetts Medical School, Worcester, MA,[5]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Feline GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency of lysosomal β-galactosidase (β-gal). The feline disease faithfully emulates the pathology of GM1 in humans, making cats an ideal animal model for studying this disease. Adeno-associated viral (AAV) gene therapy for GM1 aims to restore β-gal activity and minimize disease progression. Choosing the appropriate AAV serotype and delivery route is critical for GM1 therapy since the vector must display tropism for cells that can achieve therapeutic effect and reach the areas most affected. Intracranial injection of AAV into the thalamus and deep cerebellar nuclei resulted in a 7.5-fold increase in the lifespan of GM1 cats, but the procedure is invasive and requires a craniotomy for delivery to the brain parenchyma. Injection into the CSF via the cisterna magna (CM) is less invasive and could potentially improve biodistribution to certain parts of the CNS and peripheral organs. This study compares the effects of AAV serotype on survival, β-gal distribution, viral distribution, and disease progression in GM1 cats following CM injection using serotypes AAV9 and AAVrh10. Each cohort received 1.5e13 vector genomes/kg body weight at 2.2 ± 0.3 months of age (symptom onset occurs at ~4 months). The two vectors were compared by size and zeta potential, and atomic force microscopy and electron microscopy were each used to compare structural differences. β-gal activity in the GM1+AAV9 cohort for the cerebellum was restored to 0.8 ± 0.2 -fold normal and 0.8 ± 0.3-fold normal in the spinal cord; however, for the GM1+AAVrh10 cohort cerebellar β-gal activity was 0.6 ± 0.1-fold normal and 0.7 ± 0.3-fold normal in the spinal cord. In addition, activity remained minimal in the cerebrum for both cohorts (untreated animals exhibit little to no β-gal activity in the brain and spinal cord). Moreover, both cohorts displayed similarly improved β-gal activity in peripheral tissues. For example, β-gal activity in the heart increased 2.5 ± 2.1-fold normal for GM1+AAV9 cats and approximately 2.8 ± 1.6-fold normal for GM1+AAVrh10 cats. In the liver, β-gal activity was limited to 0.26 ± 0.14-fold normal for GM1+AAV9 cats and approximately 0.25 ± 0.14-fold normal for GM1+AAVrh10 cats, which is improved activity compared to the untreated cohort. 7T MRI documented delayed neurodegeneration in both cohorts, which correlated with delayed clinical symptoms such as inability to stand. These symptoms are reached at approximately 8 months in untreated animals, but were delayed by 2+ months in both treatment cohorts . Glycerophosphocholine and phosphoscholine (GPC+PCh), an MRS biomarker that increases with loss of myelin

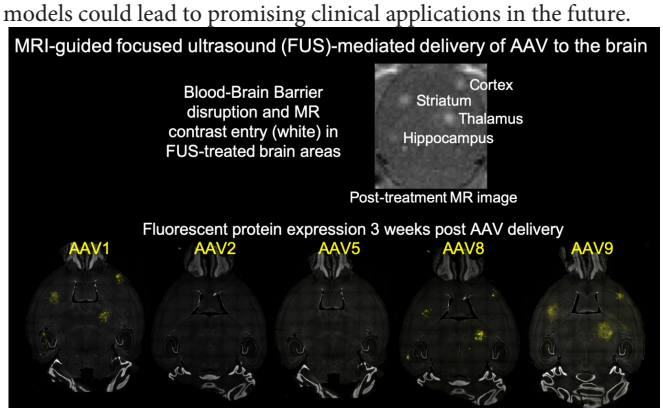
integrity, showed no preservation in the GM1+AAVrh10 cohort, and partial preservation in the cerebellum of the GM1+AAV9 CM cohort. Ultimately, untreated GM1 cats (n=9) survived 7.9 ± 0.3 months, GM1+AAV9 (n=3) cats survived 13.9 ± 1.9 months, and GM1+AAVrh10 (n=3) cats survived 11.9 ± 0.9 months. Statistical significance was found between the survival of untreated GM1 cats and GM1+AAV9 treated cats (p=0.0089) as well as untreated cats and GM1+AAVrh10 treated cats (p=0.0089) but there was no statistical significance found between AAV9 and AAVrh10 treated cats (p=0.1966). This study indicates efficacy of AAV therapy in both treatment cohorts and suggests that CM-injected AAV9 delays pathology compared to AAVrh10 for the feline model.

364. The Impact of Serotypes and Anatomical Regions on the Efficacy of Focused Ultrasound-Mediated AAV Delivery to the Brain

Rikke H. Kofoed[1], Kate Noseworthy[1], Chinaza Dibia[1], Nathalie Vacaresse[1], Kullervo Hynynen[2], Isabelle Aubert[1]

[1]Biological Sciences, Sunnybrook Health Sciences Centre, Toronto, ON, Canada,[2]Physical Sciences, Sunnybrook Health Sciences Centre, Toronto, ON, Canada

Introduction The blood-brain barrier (BBB) is a major obstacle for gene therapies aiming to deliver adeno-associated virus (AAV) to the central nervous system (CNS). New AAV serotypes are being developed to decrease the intravenous dose of AAV required to cross the BBB and provide widespread gene delivery to the CNS. In certain conditions, however, targeting specific regions for gene transfer may be ideal. We are developing novel strategies to overcome the BBB, non-invasively, and deliver AAVs in one treatment to a single or multiple specific brain region(s). Here, we combined transcranial focused ultrasound (FUS) with microbubbles circulating in the bloodstream to transiently increase the permeability of the BBB, enabling the entry of intravenously administered AAVs in FUS-targeted brain areas. The goal of this study is to establish standards of efficient FUS-mediated delivery of AAVs from the blood to the brain. **Study design** We compared the gene delivery efficacy of 5 commonly used AAV serotypes encoding fluorescent proteins (FP), injected intravenously at $3.3x10^{12}$ GC/kg and delivered to specific brain areas using FUS (n=4-5). We counted the number of neurons and astrocytes expressing FP relative to the total number of neurons and astrocytes in the FUS-targeted region of the cortex, striatum, thalamus, and hippocampus. We also measured the size of the transduced areas and the distribution of vector genome copies in 13 peripheral organs. **Highlighted results** AAV9 led to greater (*p<0.05) percentages of FP+ neurons in the FUS spots of the striatum and thalamus (4.0±2.6% and 5.5±1.5%) compared to AAV1, 2, 5, and 8. In the cortex and hippocampus, significantly more FP+ neurons were found with AAV9 (1.2±0.6% and 1.0±0.6%) than with AAV2 and AAV5, but not compared to AAV1 and AAV8. The percentages of FP+ astrocytes in the striatum and thalamus were higher with AAV9 (13.2±5.5% and 12.4±3.6%) compared to AAV1, 2, 5, and 8. In the hippocampus, no significant difference was found in the percentages of FP+ astrocytes (ranging from 0.05 to 3.6%) transduced by AAV1, 2, 4, 8 and 9. In the cortex, however, AAV9 (5.4±2.4%) and AAV8 (4.6±2.1%) gave significantly higher percentage FP+ astrocytes compared to AAV2 and AAV5, but not compared to AAV1. In contrast to other brain regions evaluated, the surface area of FP expression by AAV9 in the thalamus was significantly higher (1.2±0.5 mm^2) compared to AAV1, 2, 5, and 8. This suggests that the distribution of AAVs from the site of entry depends both on the serotype, and the properties of the brain area targeted with FUS. The genome copies/cell of AAV5 was several folds higher (*p<0.05) in the liver, spleen, and lung compared to other serotypes, suggesting that the low level of FUS-delivered AAV5 to the brain may be caused by a preferential peripheral uptake. **Conclusion** This study provides a comprehensive analysis of the efficacy of FUS-mediated AAV delivery and transgene expression to the brain. Optimizing FUS-mediated gene delivery to the brain in preclinical models could lead to promising clinical applications in the future.



365. siRNA Regulation of Erythropoietin Gene Therapy

Fiona M. Hart, Katie M. Stiles, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

The ability to regulate gene expression initiated by adeno-associated virus (AAV) gene therapy vectors post-administration would extend the safety and scope of conditions that could be treated by gene therapy. Erythropoietin (EPO), a hormone produced in the kidney, mediates production of bone marrow-derived red blood cells. Anemia, a common complication of chronic kidney disease, is currently treated with intermittent parenteral administration of recombinant human EPO. AAV-mediated EPO gene therapy has been explored, but there is a risk that persistent EPO transgene expression levels cannot be controlled and, if too high, will lead to polycythemia. We hypothesized that building an "off-switch" in the AAV expression cassette would enable EPO transgene expression to be temporarily shut down if the red blood cell level became too high. To test this hypothesis, we inserted two target sites for unique small interfering RNAs (siRNA) into the 3' untranslated region (UTR) of the pmirGLO-luciferase dual reporter system. Transfection of the luciferase reporter into 293T cells followed by transfection of the cognate siRNAs resulted in 80-93% (p<0.04) silencing of luciferase expression compared with control siRNAs. Both target sites were cloned into the 3'UTR after the human EPO cDNA with a hemagglutinin tag (hEPO-HA). After co-transfection in 293T cells, siRNA1 (0.5-5 pmol) silenced hEPO-HA expression from the pAAV expression cassette in a dose-dependent manner (59 to 90%). This *in vitro* data suggests that incorporation of unique siRNA target sites into the 3' UTR of pAAV expression cassettes could be an effective strategy to temporarily reduce the expression of the transgene from

AAV gene therapy vectors mediated by exogenous administration of cognate siRNAs. The ability to control expression is independent of the transgene or promoter and opens up new areas for gene therapy where the ability to regulate therapeutic gene expression would add to the effectiveness of the therapy.

366. A Gene Therapy Approach to Preventing COVID19: AAV-Delivery of Potent, Engineered Ace2 Decoys to the Airway

Joshua Sims, Christian Hinderer, Makoto Horiuchi, Sharon Lian, Kristofer Michalson, Cecilia Dyer, Kevin B. Turner, Hailey Shankle, Jenny A. Greig, Rosemary Meggersee, Shiva Shrestha, Henry Hoff, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

The novel human pathogen SARS-CoV2 has infected tens of millions of people with Coronavirus Disease 2019 (COVID-19) and resulted in more than 1 million deaths worldwide. To establish infection, the spike protein of SARS-CoV-2 binds to the receptor angiotensin-converting enzyme 2 (Ace2) on human airway cells. Here we demonstrate the fast-acting, intranasal AAV-mediated delivery of Ace2 decoy receptor genes directly to the airway of mice and non-human primates. Soluble Ace2 decoy protein secreted into the airway surface liquid is capable of binding to the SARS-CoV2 spike protein and neutralizing the pseudotyped CoV-2 reporter virus. We improved the potency of the decoys 1000-fold by screening large libraries of Ace2 variants and optimizing their fusion partners. We discuss the advantages of a decoy-based prophylaxis relative to vaccination and neutralizing antibody-based approaches.

367. Dual AAV Vector Strategy for Expression of Large Genes Targeted for Stargardt Disease Gene Therapy Development

Brianna Barrett, Scott Kerns, Brian Kevany, Linas Padegimas

Abeona Therapeutics, Cleveland, OH

Purpose: Stargardt disease is an inherited, chronic, and progressive retinal dystrophy caused by mutations in the *ABCA4* gene resulting in the most common form of juvenile macular degeneration, for which there are currently no therapeutic options. The ABCA4 coding sequence is 6,822 nucleotides in length and therefore exceeds the packaging capacity of a typical AAV capsid. To enable delivery of large genes, several dual AAV vector strategies have been employed, including homologous recombination, trans-splicing, and intein-mediated protein splicing, but each method has advantages and limitations. To overcome these hurdles, we have created a dual AAV approach that allows efficient recombination of N-terminal and C-terminal ABCA4 fragments by exploiting Cre recombinase. **Methods**: The first AAV vector encodes the N-terminal region of the ABCA4 gene and also expresses Cre recombinase via a self-cleaving T2A peptide. The second AAV vector encodes the C-terminal region of the ABCA4 gene. Cre recombinase recognizes LoxP sites inserted into each ABCA4 construct and combines the N- and C-terminal fragments resulting in a full-size ABCA4 expression cassette that will exist episomally within the cell. During the same recombination process, the Cre recombinase gene loses its promoter and inactivates itself. **Results:** We have demonstrated that infection in tissue culture with a 1:1 ratio of AAV.ABCA_N and AAV.ABCA_C vectors results in efficient ABCA4 coding sequence reconstitution and full-length ABCA4 protein production within 48 hours. Tissue culture cells infected with ABCA_N and ABCA_C vectors lacking Cre recombinase show no detectable full-length ABCA4 protein after up to 120 hours. **Conclusions:** Full-length ABCA4 protein can be efficiently reconstituted from two independent AAV vectors by utilizing Cre recombinase. Future studies exploring Cre-mediated ABCA4 reconstitution *in vivo* and the ability of dual AAV-delivered ABCA4 to clear lipofuscin build-up will be essential milestones to move this early-stage program towards the clinic.

368. High Affinity Cardiac Gene Transfer Mediated by Systemic Delivery of AAVrh.10 Vectors

Bishnu P. De[1], Jonathan B. Rosenberg[1], Clarisse Jose[1], Alvin Chen[1], Edward Fung[2], John Babich[2], Douglas J. Ballon[2], Mehdi Gasmi[3], Brahim Belbellaa[3], Dolan Sondhi[1], Stephen M. Kaminsky[1], Ronald G. Crystal[1]

[1]Genetic Medicine, Weill Cornell Medical College, New York, NY,[2]Radiology, Weill Cornell Medical College, New York, NY,[3]Adverum Biotechnologies Inc, Redwood City, CA

One of the challenges of effective adeno-associated virus (AAV)-mediated gene therapy for cardiac disorders is the choice of delivery of the AAV vector to the myocardium. Several methods have been evaluated including open thoracotomy direct intramyocardial, coronary artery, retrograde coronary sinus and cardiac bypass recirculating delivery. While all are effective to varying degrees, all of these methods are invasive, time consuming and costly. Based on preliminary studies with intravenous (IV) administration to Balb/c mice of AAVrh.10 expressing the mCherry marker gene driven by the CAG constitutive promoter at $5x10^{12}$ gc/kg resulting in diffuse high level expression of mCherry throughout the myocardium, we explored the hypothesis that, based on the high affinity of AAVrh.10 for the myocardium, IV administration of AAVrh.10 vectors would provide high levels of myocardial gene transfer. Three murine and two nonhuman primate (NHP) studies were assessed, all using the CAG highly active constitutive promoter. IV administration of AAVrh.10hFXN coding for human frataxin to Balb/c mice ($2.5x10^{13}$ gc/kg, assessed at 2 wk) resulted in 42±6% vector genomes in the heart compared to liver, resulting in similar levels of human FXN protein (1800±280 ng/mg protein) in heart and liver ($p>0.6$). IV administration of AAVrh.10AAT coding for human α1-antitrypsin to C57Bl/6 mice ($5x10^{12}$ gc/kg, assessed at 3 months) resulted in 61±30% AAT mRNA levels in heart *vs* liver. IV administration of AAVrh.10luc coding for the luciferase reporter gene to C57Bl/6 mice ($5x10^{12}$ gc/kg, assessed at 6 wk) resulted in 60±14% luciferase light units in heart *vs* liver. The high affinity of AAVrh.10 for the myocardium was also demonstrated in NHP studies. IV administration of $5\text{-}7x10^{12}$ gc/kg of AAVrh.10hFXN to NHP assessed at 21 days delivered 4-5 vector genome copies/diploid genome resulting in 25 to 40 ng human FXN/mg protein, a level at or above normal cardiac endogenous FXN levels. Finally, evaluation of

cardiac *vs* liver distribution of the AAVrh.10 compared to the AAV9 capsid was assessed by positron emission tomography of the I-124-labeled 24 hr following IV administration demonstrated the heart to liver ratio of I-124 AAVrh.10 biodistribution was 61% compared to only 13% for I-124 labeled AAV9. In summary, based on the high affinity of AAVrh.10 for the myocardium, intravenous administration of AAVrh.10 vectors, an inexpensive, minimally invasive outpatient procedure, is an effective delivery strategy for effective AAV-mediated gene therapy.

369. Successful Transduction of Pancreatic Acinar and Islet Cells by Intra-Pancreatic Duct Injection of AAV9 Vector in Non-Human Primates

Kei Adachi[1], Masahiro Horikawa[1], Helen R. Baggett[1], Gregory A. Dissen[2], Theodore Hobbs[2], Paul Kievit[2], Diana Takahashi[2], Marcela Brissova[3], Alvin C. Powers[3], Charles T. Roberts[2], Markus Grompe[1], Hiroyuki Nakai[1,2]

[1]Oregon Health & Science University, Portland, OR,[2]Oregon National Primate Research Center, Beaverton, OR,[3]Vanderbilt University, Nashville, TN

Recent advances in various technologies for cell fate conversion offer an innovative approach to treat various human diseases by reprograming one cell type into another. Of relevance to the development of novel gene therapy approaches to treat type 1 diabetes (T1D) is the demonstration of successful reprogramming of non-beta cells into insulin-producing beta cell-like cells in rodent models by vector-mediated delivery of key transcription factors to target non-beta cells from which beta cell-like cells will regenerate. In order to translate this approach to the clinic, it is critical to establish a clinically relevant effective and safe approach to deliver an AAV vector to the pancreas and show successful pancreatic gene transfer in large animals including non-human primates (NHPs). Here we report successful establishment of a real-time image-guided retrograde pancreatic duct (PD) injection method to deliver AAV vectors to pancreatic cells and demonstrate that the method is safe and can successfully transduce both pancreatic acinar cells and islet endocrine cells in rhesus macaques using an AAV9 vector. We also report pharmacokinetic profiles of AAV9 vector injected via the PD route in the macaques. In the study, for the purpose of establishing proof-of-concept of the PD approach, we sought to establish a surgical approach using NHPs as an alternative to ERCP (endoscopic retrograde cholangiopancreatography)-based drug delivery to the pancreas because an endoscopic approach in NHPs weighing several kilograms is unfeasible due to the small animal size. Using two rhesus macaques negative for anti-AAV9 neutralizing antibodies, we performed laparotomy, made a small incision of the duodenum wall, directly visualized the ampulla of Vater, and inserted under real-time fluoroscopy a catheter into PD with the catheter tip wedged at the proximal portion of the pancreatic body. Backflow of PD-injected agents from PD to the duodenum could be prevented by selecting an appropriate size of the catheter. We then injected 1.4×10^{13} vg of AAV9-CAG-tdTomato over a period of 2 min following a 20 min dwelling time period. Blood samples were collected multiple times pre- and post-injection to assess safety and determine pharmacokinetic profiles of PD-injected AAV9 vector. The animals were euthanized 4 weeks post-injection and multiple tissues including the pancreas and the liver were harvested for downstream molecular and histological analyses. Although serum amylase and lipase levels were elevated following the injection, the levels went down to the normal range within 3 days post-injection and no clinical signs suggesting post-procedure pancreatitis were observed. The AAV9 vector leaked into the systemic circulation with the vector concentrations in blood reaching a peak at 8 h post-injection, resulting in vector genome dissemination to non-pancreatic organs to some degree. In the pancreas, the AAV9 vector transduced many acinar cells and some islets with a few showing good endocrine cell transduction. In summary, our study demonstrated that the PD injection is safe and transduces both acinar and islet cells with AAV9 vector. However, this local approach with AAV9 still allows vector spillover in other non-target organs. We are currently searching AAV capsids that can transduce pancreatic cells much more effectively than AAV9 via PD in NHPs while minimizing off-target vector dissemination, using our AAV Barcode-Seq.

370. Assessment of Whole Body Anti-Capsid Neutralizing Immunity Using Quantitative I-124 Whole-Body Functional Positron Emission Tomography Imaging of Adeno-Associated Viral Vector Biodistribution in Nonhuman Primates

Jonathan B. Rosenberg[1], Edward Fung[2], Bishnu P. De[1], Alvin Chen[1], Dolan Sondhi[1], Stephen M. Kaminsky[1], John Babich[2], Ronald G. Crystal[1], Douglas J. Ballon[2]

[1]Genetic Medicine, Weill Cornell Medical College, New York, NY,[2]Radiology, Weill Cornell Medical College, New York, NY

For *in vivo* adeno-associated virus (AAV) gene therapy to be successful, it is critical that AAV capsid biodistribution following administration is sufficient to mediate effective therapy to the target organ(s). A major barrier to effective capsid biodistribution is pre-existing neutralizing antibodies directed against the AAV capsid. Other than setting an arbitrary serum anti-capsid neutralizing antibody titer for effective therapy, there is no parameter to assess in humans whether preexisting anti-capsid immunity will hinder the effectiveness of AAV *in vivo* gene therapy. With the goal of developing an *in vivo* assessment of effective delivery of a therapeutic AAV vector, we have developed I-124 labeled AAV capsid methodology that permits rapid (1 to 72 hr) quantitative positron emission tomography (PET) imaging of capsid biodistribution following *in vivo* I-124-AAV vector administration. Assessment of real-time biodistribution of I-124 radioiodinated AAV capsids (AAVrh.10, 9, and 5) following intravenous (IV) or intracisternal (IC) delivery was carried out in capsid immune-naive and capsid-immune African Green nonhuman primates (n=7, *chlorocebus aethiops*, NHP), whose adult average head and body length approximate small children, in a human Siemens PET/CT system for rapid whole-body imaging. PET scans were performed at 1, 24, 48, and 72 hr, at an effective spatial resolution of approximately 5.0 x 5.0 x 5.0 mm^3. In capsid immune-naive NHP, total body clearance of all serotypes, whether administered by the IV or IC routes, had a biological half-life of 54.1 to 84.5 hr. In marked contrast, total body clearance of all serotypes and routes in capsid-immune NHP had a half-life of 24.1 to 25.6 hr ($p<0.0007$). The % I-124 AAV in the spleen in capsid-immune NHP was 6.1 to 8.6% compared to 1.3 to 1.9% in capsid naive animals ($p<0.00009$). Importantly, as a

measure of whole body anti-capsid immunity, analysis of % capsid in spleen compared to serum anti-capsid neutralizing titer demonstrated a high correlation (r^2=0.95), i.e., the I-124 PET quantification of capsid in the spleen provides has a high correlation with anti-capsid neutralizing titer. These observations support the concept that I-124-AAV capsid PET scanning may be a useful biomarker to assess functional anti-capsid immunity in humans prior to gene therapy.

371. AAV Gene Therapy: A Novel Treatment for Food Allergy

Miguel Gonzalez Visiedo, David M. Markusic
Pediatrics, Indiana University, Indianapolis, IN

Introduction: Food allergy (FA) is a health problem with an increased incidence, with children being the most affected population. Sensitization to food allergens in humans can occur in the gut, respiratory tract or even the skin and the most common food triggers are milk, eggs, peanut and tree nuts. FA results from an abnormal response in gut immune tolerance mediated by activated dendritic cells that present antigens to Th2 cells that lead to activation of B cells and immunoglobulin E (IgE) class switching. Previous studies have linked a loss in regulatory T cell number and/or function to the development of FA therefore, we hypothesized that we could induce allergen specific regulatory T cells using adeno-associated vectors (AAV) liver gene therapy to prevent and treat allergen sensitization and reduce the frequency of severe allergic reactions. **Methods:** 6-week-old heterozygous flaky tail ($FT^{+/-}$) female mice were injected with 10^{11} vg of AAV8-OVA either with a liver-specific (TTR) or ubiquitous promoter (EF1α) or an AAV8 vector expressing FIX protein as control prior to or after sensitization with OVA. Mice were sensitized by epicutaneous administration of *Alternaria alternata* protein extract follow by OVA protein. Then, mice were challenged by IP OVA injection and the core body temperature (CBT) and symptom score (SS) were recorded every 15 minutes. One week after the challenge, splenocytes were collected and restimulated with OVA for flow cytometry studies. Mice were bled at different timepoints to measure basophil activation (an IgE mediated allergy marker), OVA-specific IgE and IgG1 and OVA expression in plasma. **Results:** AAV8-OVA gene therapy provided protection from allergen sensitization CBT TTR (-0.64 ± 0.815) and EF1α (0.73 ± 0.17), while a significant decrease in CBT (-4.71 C ± 1.456) and higher SS (3.28 ± 0.6442) was observed in the control group during challenge. However, the TTR-OVA treated mice had lower SS (0.5 ± 0.3504) compared with the EF1α treated group (1.125 ± 0.3504), even though the OVA levels in plasma were almost 10 times lower than EF1α-OVA group, suggesting that the protective effect was dependent on hepatocyte restricted expression. As expected, AAV8-OVA treated groups did not show any significant increase in CD200R expression, a marker for basophil activation, as well as lower levels of both OVA specific IgG1 and IgE compared to the control group. OVA restimulated splenocytes from the TTR and EF1α-OVA treated groups showed significantly lower Th2 ($p<0.0001$ for both) and T follicular helper cell ($p<0.0001$ for both), and higher T follicular regulatory cell (TFR) ($p<0.0001$ for both) frequencies compared to the control group. Next, we investigated if we could treat pre-existing OVA FA with this therapy. Mice were sensitized and challenged with OVA and then injected with $1x10^{12}$ vg of the TTR-OVA or $1x10^{11}$ vg with EF1a-OVA or FIX AAV8 vectors and challenged four weeks later. We selected a higher dose for TTR-OVA based on earlier studies in BALB/c mice and hemophilia B mice. In this pilot study we obtained similar results compared with the prevention study above. Mice treated with either AAV8-OVA vector had a significant reduction in hypothermia and lower SS during the challenge compared to control. However, in this study EF1α-OVA treated mice had reduced CBT changes (-0.82 C ± 0.6434) compared to TTR-OVA group (-2.68 C ± 1.196). These data suggest that higher OVA levels are needed for the reversal of established allergy. **Conclusions:** Our data show that AAV gene therapy can be an effective treatment for severe OVA allergy reactions in a skin sensitized FA model. The protection from FA appears to be mediated by an increase in TFR frequency that leads to a reduction in OVA-specific antibody formation. We were also able to significantly ameliorate the severe allergic symptoms produced upon exposure with OVA in prior sensitized mice, highlighting the potential to translate this therapy into FA patients.

372. Optimization of GeneRide-Encoding UGT1A1 Vector Improved Efficacy in a Mouse Model of Crigler-Najjar Syndrome

Chih-Wei Ko[1], Amy Bastille[1], Shengwen Zhang[1], Elizabeth McCarthy[1], Susana Gordo[1], Jenisha Vora[1], Dylan Frank[1], Nikhil Ramesh[1], Jing Liao[1], Lauren Drouin[1], Matthias Hebben[1], Kyle Chiang[1], Roshan Padmashali[2], Ankit Gupta[2], Vivian Choi[2], Nelson Chau[1], Qiang Xiong[1]
[1]LogicBio Therapeutics, Lexington, MA,[2]Takeda Pharmaceuticals, Cambridge, MA

Crigler-Najjar (CN) Syndrome Type 1 is an ultra-rare, neonatal onset monogenetic disease caused by complete loss of function of a liver-specific enzyme, UDP-glucuronosyltransferase family 1 member A1 (UGT1A1). These patients show neonatal hyperbilirubinemia leading to irreversible CNS damage and mortality if left untreated. Standard of care consists of blue light therapy for >10 hours per day. Liver transplant is the only curative option due to reduced blue light therapy efficacy as patients grow. GeneRide™ is an AAV-based, promoterless, nuclease-free, genome editing technology that leverages the natural process of homologous recombination to insert, site-specifically, a copy of a therapeutic transgene into the genome (*Barzel A, et al. 2015 Nature*). Daughter cells of the corrected cells would also carry the transgene. Thus, GeneRide™ is especially attractive in treating pediatric patients compared to conventional liver-targeted AAV-based gene therapy which may gradually lose efficacy as the liver grows. Treating newborn *Ugt1a1*$^{-/-}$ mice with a tool GeneRide™ vector encoding UGT1A1, we have demonstrated the full rescue of neonatal lethality (*Ko C., et al. 2020 ASGCT*). As neonatal mouse liver has a dramatically different immunity and metabolic profile that more represents pre-term babies in human (*Nakagaki B., et al. 2018 J Hepato*), we further evaluated the therapeutic effect when vectors were administered at pediatric ages to support translation into the clinic. Based on the liver growth curve comparison between mouse and human, post-natal day (PND) 14 to 28 in mice mimics pediatric human ranging from 0.5~5 years old (*Dutta S., et al. 2015 Life Sciences*). PND14 and PND28 dosing in mice showed significant increase in GeneRide™ activities compared to neonatal dosing.

The GeneRide™ vectors were further optimized to improve gene editing. Compared to the benchmark tool vector, the optimized vector showed greater than 4-fold increase in genome editing frequency which was accompanied with a proportional increase in UGT1A1 expression and the biomarker for site-specific integration, Albumin-2A in circulation. When the optimized GeneRide™ vector was dosed in *Ugt1a1*$^{-/-}$ mice at the equivalent of pediatric ages, we observed substantial lowering of total bilirubin, the key biomarker of efficacy. Taken together, we have demonstrated the therapeutic potential of an optimized GeneRideTM vector encoding UGT1A1 in treating pediatric patients with Crigler-Najjar Syndrome.

373. Pilot Study of M002, a Novel AAV9-Mediated Gene Therapy for Treatment of Mucolipidosis Type II (MLII) Using a Modified Phosphotransferase (S1S3)

Lin Liu[1], Jennifer Srnak[1], Yicheng Zhao[1], Steven Le[2], Alexander Sorensen[2], Balraj Doray[3], Robert Gotschall[1], Patricia Dickson[2], Stuart Kornfeld[3]

[1]M6P Therapeutics, St. Louis, MO,[2]Division of Genetics and Genomic Medicine, Washington University School of Medicine in St.Louis, St. Louis, MO,[3]Divisions of Hematology, Washington University School of Medicine in St.Louis, St. Louis, MO

The GlcNAc-1-phosphotransferase: lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (PTase), localized in the early Golgi, is the key enzyme for the generation of the mannose 6-phosphate (M6P) tag on acid hydrolases to enable binding to mannose 6-phosphate receptors (M6PRs) in the late Golgi and subsequent trafficking of the tagged enzymes to lysosomes. The hexameric PTse enzyme (α2β2γ2) is encoded by two genes, *GNPTAB* and *GNPTG* gene. Mucolipidodsis type II (MLII), also called inclusion cell (I-cell) disease, is a rare genetic lysosomal storage disorder caused by mutations in the *GNPTAB* gene. Deficiency of PTase results in impaired lysosomal trafficking of acid hydrolases due to their inability to bind the MPRs, with concomitant hypersecretion of the enzymes into the plasma of patients. This results in premature death by the age of 10 due to the massive accumulation of undegraded substrates in the lysosome. Currently, there is no treatment for MLII. We recently reported the generation of a modified truncated form of phosphotransferase (S1S3) which show high enzyme activity in cells. Compared to the wild type *GNPTAB* cDNA at 3.7 Kb, the S1S3 cDNA is around 1.7 Kb which makes it more feasible for AAV insertion. Here, we describe the development of a novel AAV9 mediated gene therapy - M002, using the modified S1S3 PTase under control of the hybrid CAG promoter (chicken beta-actin promoter fused by cytomegalovirus enhancer). *In vitro* transduction experiments with M002 using HEK293 and/or HepG2 cells show that S1S3 expression and binding of endogenous lysosomal enzymes to the cation-independent mannose 6-phosphate receptor (CI-MPR) occur in an M002 dose-dependent manner. To investigate the efficacy of the gene therapy *in vivo*, a *GNPTAB* knockout (*GNPTAB*$^{-/-}$) mouse model was utilized (Gelfman, Vogel et al. 2007). Heterozygous (*GNPTAB*$^{+/-}$) mice were maintained and bred for homozygous knockout animals (*GNPTAB*$^{-/-}$). At week 4, the *GNPTAB*$^{-/-}$ mice were injected with M002 at 2E11 vg/mouse intravenously. Serum samples were collected before injection (day 0), and after injection periodically, and assayed for lysosomal enzyme activity and CI-MPR binding. Our preliminary data show that a single dose administration of M002 partially reduces the level of lysosomal enzymes secreted into the serum at 2 weeks post-treatment. Further, we present evidence that in the M002 treated animal, the serum lysosomal enzymes we tested exhibited 3-5 times more binding to the CI-MPR than observed with WT serum. Serum enzymes from the untreated mice had no detectable binding to the CI-MPR. The circulating M6P tagged lysosomal enzymes could potentially increase the cross-correction by allowing the enzymes to be internalized by other tissues/cells. Biochemical and histological analysis will be scheduled at 12 weeks post-treatment. In conclusion, our pilot proof-of-concept data demonstrates that M002, an AAV9 mediated gene therapy for treatment of MLII mice decreases serum lysosomal enzymes and restore the M6P content of acid hydrolases.

374. Development of Genome-Modified Generation X Single-Stranded AAVrh74 Vectors with Improved Transgene Expression in Primary Human Skeletal Muscle Cells

Jakob Shoti, Keyun Qing, Barry J. Byrne, Arun Srivastava

Pediatrics, University of Florida, Gainesville, FL

The naturally occurring AAV contains a single-stranded DNA genome, and expresses the viral genes poorly, because ssDNA is transcriptionally-inactive, and there is no RNA polymerase that can transcribe a ssDNA. Similarly, transgene expression levels from recombinant ssAAV vectors are also negatively impacted. We have previously reported that the D-sequence in the AAV inverted terminal repeat (ITR) at the 3'-end of the vector genome plays a significant role in limiting transgene expression from ssAAV vectors (*Proc. Natl. Acad. Sci., USA*, 94: 10879-10884, 1997). We identified a binding site for the NF-κB negative regulatory factor (NRF), known to suppress transcription, in the D-sequence in the AAV-ITR. Substitution of the D-sequence in the left ITR (LC1), or the right ITR (LC2) resulted in generation X ("GenX") ssAAV vectors, which mediated up to 8-fold improved transgene expression (*J. Virol*., 89: 952-961, 2015). In the present study, we evaluated whether encapsidation of these modified ssAAV genomes in AAVrh74 capsids would also lead to increased transgene expression. HeLa cells were transduced with WT, LC1, and LC2 vectors expressing the hrGFP reporter gene at 1,000, 3,000, and 10,000 vgs/cell and hrGFP fluorescence was quantitated 72 hrs post-transduction. These results, shown in **Figure 1A**, document ~5 and ~2.5-fold increase in transgene expression mediated by LC1 and LC2 vectors, respectively (p <0.01). The observed increase in transgene expression was not due to increased entry of LC1 and LC2 vectors, as documented by approximately similar numbers of the vector genomes quantitated by qPCR analyses of low mol. wt. DNA samples isolated from transduced cells with each of these vectors (**Figure 1B**). The extent of the transgene expression from these vectors was also evaluated in primary human skeletal muscle cells transduced at 1,000, 3,000, and 10,000 vgs/cell of each of these vectors. Quantitation of fluorescence images indicated that ssLC1-AAVrh74 vectors averaged ~13-fold increase, and ssLC2-AAVrh74 vectors averaged ~5-fold increase in transgene expression compared with that from the conventional ssAArh74 vectors (**Figure 1C**). Based on our previously published studies with NextGen AAV2 and AAV3 serotype vectors (*Hum. Gene*

Ther. Meth., 27: 143-149, 2016), we anticipate that encapsidation of LC1 and LC2 GenX AAV genomes into NextGen AAVrh74 capsids, it would be feasible to achieve significantly higher levels of transgene expression in a murine model *in vivo*. These observations have significant implications in the potential use of GenX AAVrh74 vectors at further reduced doses in gene therapy of muscular dystrophies.

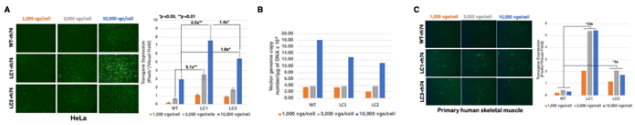


Figure 1: (**A**) Transduction efficiency of WT, LC1, and LC2 ssAAVrh74 vectors in human HeLa cells. (**B**) Vector genome copy numbers in HeLa cells transduced with WT, LC1, and LC2 ssAAVrh74 vectors. (**C**) Transgene expression mediated by WT, LC1, and LC2 ssAAVrh74 vectors in primary human skeletal muscle cells. Cells were transduced with each vector at the indicated vector genome copy numbers/cell at 37°C for 2 hrs, and transgene expression was visualized under a fluorescence microscope 72 hrs post-transduction. Data were quantitated using the ImageJ software.

This research was supported by a sponsored research grant from Sarepta Therapeutics.

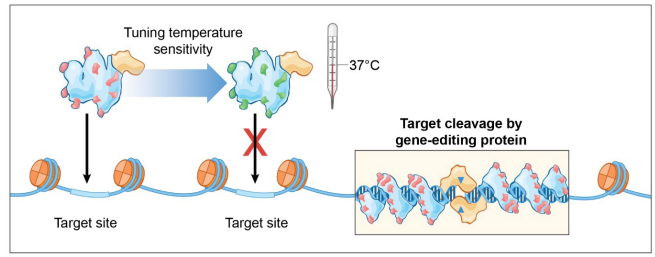
Gene Targeting and Gene Correction

375. DNA Binding Domains Containing Novel Repeat Sequences Enable Temperature-Tunable Gene Editing in Primary Human Cells

Yemi Osayame[1], Franklin Kostas[2], Mitchell R. Kopacz[2], Mackenzie Parmenter[1], Christopher B. Rohde[2], Matthew Angel[2]

[1]Novellus, Inc., Cambridge, MA,[2]Factor Bioscience, Cambridge, MA

Sequence-specific gene-editing endonucleases, such as zinc finger nucleases (ZFNs), clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9), and transcription activator-like effector nucleases (TALENs) are being used in the development of many gene and cell therapies. However, these gene-editing endonucleases have seen limited *in vivo* application due to concerns about effects on genomic loci outside the intended cut site. TALENs consist of a DNA binding domain containing repeat-variable diresidues (RVDs) that confer site specificity fused to a nuclease catalytic domain. The RVDs contact individual bases in the target DNA sequence, and are connected by two alpha-helices linked by a short, disordered loop. We hypothesized that engineered gene-editing endonucleases comprising DNA-binding domains containing novel loops could exhibit different on-target cutting activity as well as altered specificity, due to the potential of this linkage to determine the degree of conformational disorder of the DNA-binding domain. We tested the ability of gene-editing endonucleases containing novel linkages to edit genes in primary cells, and discovered a striking temperature-dependence of gene-editing with certain linkages. We identified engineered endonucleases that efficiently edit only at sub-physiological temperatures, as well as endonucleases capable of high-efficiency editing in primary cells at 37 °C. We observed high-efficiency gene editing with the novel endonucleases in primary human dermal fibroblasts, primary epidermal keratinocytes, induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs). Additionally, we measured the specificity of the engineered endonucleases at various editing temperatures in cultured cells using LAM-PCR based sequencing. We show that engineering the RVD linkage of gene-editing endonucleases has the potential to improve on-target activity as well as sequence specificity in gene therapies.



376. Expanding PAM Recognition of CRISPR-Associated Endonucleases by Domain Engineering

Cristina N. Butterfield, Aaron Lin, Daniela S. A. Goltsman, Lisa Alexander, Morayma Temoche-Diaz, Alan R. Brooks, Christopher T. Brown, Brian C. Thomas

Metagenomi, Emeryville, CA

RNA guided CRISPR-associated (Cas) endonucleases have been widely used for a variety of genome-editing applications. However, genome targetability is often constrained by intrinsic sequence specificities of protospacer adjacent motifs (PAMs). Here, we develop a strategy to expand PAM recognition of highly active, naturally-occurring Cas endonucleases by leveraging domain diversity discovered from mining extensive metagenomic datasets. Guided by predicted structural model information we engineered functional protein variants recognizing non-canonical PAMs by swapping domains from closely, as well as distantly related enzymes into highly-active Cas endonucleases. In vitro biochemical results suggest that the engineered variants maintain high levels of activity while converting to new PAM specificities. Notably, we converted a GC rich PAM to an A rich PAM and show that this variant retains the mammalian gene editing activity levels of the catalytic nuclease used as backbone. This system successfully targets Albumin intron 1 in Hepa1-6 cells with up to 50% NHEJ by mRNA transfection. These results demonstrate the ability to tune natural enzymes for new targets without sacrificing efficiency through structure-based protein engineering. The modularity and diversity of PAM specificity provides an ease-of-use approach to rapidly engineer CRISPR nucleases with additional target compatibility.

377. Improved Tissue Engraftment of Human Genome-Edited Hematopoietic Stem Cells with Busulfan-Based Myeloablation

Edina Poletto[1], Pasqualina Colella[2], Shaukat Khan[3], Shunji Tomatsu[3], Natalia Gomez-Ospina[2]

[1]Gene Therapy Center, Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil,[2]Pediatrics, Stanford, Stanford, CA,[3]Nemours/ Alfred I. duPont Hospital for Children, Wilmington, DE

Autologous hematopoietic stem cell transplantation using genetically modified cells holds immense promise for the one-off treatment of hematological and non-hematological disorders. Accordingly, genome editing tools and protocols for the efficient and safe editing of hematopoietic stem and progenitor cells (HSPCs) are being heavily pursued. While approaches that rely on non-homologous end-joining have advanced rapidly into the clinic, approaches based on homology-directed repair have been slowed by the decreased engraftment potential often observed in HSPCs that have undergone repair. We previously described a highly efficient *ex vivo* genome editing platform in human HSPCs to express therapeutic proteins and applied it to preclinical models of two of the most common lysosomal storage disorders: Mucopolysaccharidosis type 1 (MSPI) and Gaucher. For MPSI, human HSPCs that underwent targeted addition of iduronidase expression cassettes into the *CCR5* locus demonstrated sustained enzyme expression, maintain long-term repopulation, and multi-lineage differentiation capacity in vivo. However, cells that underwent homology-directed repair had ~5-fold lower long-term engraftment capacity than cells that did not. Also, enzyme reconstitution and biochemical storage in the CNS was low. Despite this, transplantation of the edited HSPCs improved biochemical and neurobehavioral abnormalities in an immunocompromised mouse model of the disease (MPSI-NSG). In these studies, we aimed to determine if the conditioning regimen prior to transplantation impacts the engraftment and therapeutic efficacy of edited HSPCs. We compared the standard irradiation protocol used in NSG mice (sub-lethal, 2.1 Gy) with full Busulfan-based myeloablation (68 mg/kg over four days) in MPSI-NSG mice in transplantation experiments using unselected preparations of human HSPCs that had undergone targeted addition of PGK-Iduronidase expression cassette. We measured human cell chimerism and engraftment of the edited cells as well as metabolic correction in hematopoietic and non-hematopoietic organs. While both protocols resulted in a similar level of human cell chimerism in the bone marrow, spleen, and peripheral blood (Median (min, max) bone marrow: Bu 73 % (25, 91) and TBI 45 % (2.1, 97)), Busulfan-based myeloablation resulted in three-fold higher engraftment of the edited cells (20 % ± 13 of edited CCR5 alleles in the bone marrow compared to 5.7 % ± 4.1 in the irradiation group ($p = 0.0009$)). Because the pre-transplant cell product had an edited allele fraction of 30 %, this meant an allele drop of 5.2-fold for irradiation compared to 1.5-fold for Busulfan. Busulfan conditioning also resulted in higher enzyme reconstitution levels in all organs tested: ~3-fold in serum, 15-fold in the liver, 25-fold in the spleen, and 2.5-fold in the CNS. Increased iduronidase expression with Busulfan conditioning also resulted in improved metabolic correction, as measured by reduced urinary GAG excretion and tissue GAG storage in transplanted mice. In the CNS, Busulfan conditioning led to a significant reduction in GAG accumulation in the brain ($p = 0.0118$), while no reduction was observed in irradiated mice. ***These data suggest that conditioning regimens can impact the engraftment of edited HSPCs. Busulfan-based myeloablation is a better protocol for testing the long-term repopulation capacity of edited HSPCs. Furthermore, transplantation of HPSCs in Busulfan-conditioned recipients can achieve better expression of therapeutic proteins in target organs, particularly in the CNS, thereby constituting a better conditioning approach for non-hematological and neurological diseases.***

378. GUIDE-seq-2: A Simplified, Scalable, and Accurate Improved Method for Defining Genome-Wide Activity of CRISPR-Cas Nucleases in Living Cells

GaHyun Lee, Cicera R. Lazzarotto, Yichao Li, Shengdar Q. Tsai

Hematology, St. Jude Children's Research Hospital, Memphis, TN

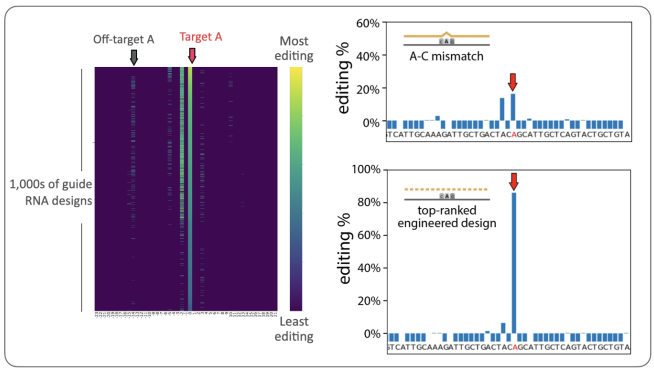
CRISPR-Cas genome editing nucleases are a powerful, programmable gene editing technology that enable targeted modification in the genomes of living cells. Towards the development of safe and effective genome-edited based cell therapies, it is important to first experimentally define the genome-wide activity of the editing nucleases to minimize the risk of unexpected off-target mutations with potentially adverse biological consequences. Genome-wide unbiased identification of double stranded breaks enabled by sequencing (GUIDE-seq) is a sensitive, unbiased method of defining the genome-wide activity of genome editors in living cells. It is based on the integration of an end-protected double-stranded oligodeoxynucleotide tag into the sites of nuclease-induced double stranded breaks. The tag-containing genomic DNA (gDNA) is then amplified for high-throughput sequencing. Advantages of the original GUIDE-seq method are high sensitivity compared to other cell-based methods for defining genome-wide activity. Some disadvantages are that it is challenging to scale to large number of samples and it is not possible to directly distinguish potential misamplification artifacts. To address these limitations, we developed an improved high-throughput version of GUIDE-seq called GUIDE-seq-2. GUIDE-seq-2 is a simplified and scalable version of GUIDE-seq that utilizes Tn5-transposon-based tagmentation and a single PCR step for library preparation. Tn5 transposase is an enzyme widely used in genomic assays for simultaneous *in vitro* DNA transposition and fragmentation (tagmentation), that simplifies the library preparation process. By introducing tagmentation into the GUIDE-seq workflow, we were able to eliminate three library preparation steps (random gDNA shearing, end repair/A-tailing and adapter ligation steps), as well as the associated required DNA purification steps, as Tn5 simultaneously fragments the DNA and add the required adapters for sequencing. We simplified the series of GUIDE-seq PCRs by generating tag-specific primers combined with the i7 barcode primers, reducing the number of PCR reactions required for library preparation. Additionally, the use of a single PCR step for library preparation enabled a primer design capable of distinguishing mispriming artifacts without changing the length of GUIDE-seq tag and affecting tag integration efficiencies. Overall, GUIDE-seq-2 reduces the gDNA input requirement by 4-fold, eliminates the need for specialized equipment for DNA shearing, reduces processing steps, time and costs, and enables filtering for bona fide tag GUIDE-seq tag integration events. We compared the sensitivity of GUIDE-seq and GUIDE-seq-2 in 10 CRISPR-Cas9 sgRNA target sites across 6 therapeutically relevant loci in human primary T cells (*CCR5*, TRAC, *PDCD1*, *CTLA4*, AAVS1 and *CXCR4*), previously characterized by GUIDE-seq and CHANGE-seq, with variable numbers of known off-targets. We found a strong correlation between the read counts from GUIDE-seq and GUIDE-seq-2 at 9 out of 10 sites ($R^2>0.9$), with high overlap with original

GUIDE-seq at most sites. Overall, GUIDE-seq-2 is a simplified, highly scalable method with comparable level of sensitivity to the original GUIDE-seq for defining genome wide off-target activity in living cells.

379. Deep Screening of Guide RNAs Enables Therapeutic RNA Editing with Endogenous ADAR

Brian J. Booth, Yiannis Savva, Susan Byrne, Rick Sullivan, Stephen Burleigh, Adrian W. Briggs
Shape Therapeutics, Seattle, WA

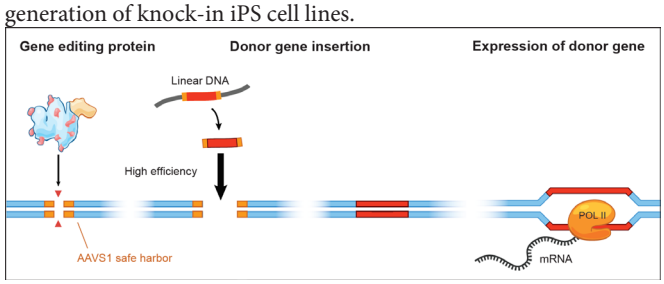
A promising approach for gene therapy engages the natural RNA-editing enzyme ADAR to correct pathogenic G->A mutations, control RNA splicing, or modulate protein expression and function. ADAR-based editing uses a short guide RNA (gRNA) to redirect ADAR's deaminase activity and convert a target adenosine into inosine, which is read by cellular machinery as guanosine.An ideal ADAR-based RNA-editing therapy would function with the natural levels of ADAR present in cells and be deliverable as a gene-encoded vector to maximize treatment durability for serious diseases. However, all ADAR gRNA designs published to date either a) fail to enable efficient editing by endogenous ADAR, b) show poor specificity for the target adenosine, or c) require chemical modification of the gRNA, precluding gene-encoded delivery.To solve this significant challenge, we built the RNAfix™ high-throughput screening platform that tests up to 500,000 structurally unique gRNA designs against a desired target adenosine. This method rapidly identifies gRNA sequences that create unique, highly efficient and specific ADAR substrates when bound to target mRNA. We will present the power of the RNAfix™ platform to discover novel gRNAs that enable, for the first time, highly efficient and specific editing of disease-relevant targets with gene-encoded gRNAs and endogenous ADAR. ***For example, as shown in Figure 1 below, by screening alibrary of thousands of different gRNA sequences against a clinical targetadenosine in the LRRK2 gene, we discovered gRNAs that enable ADAR to editthe target base with far greater efficiency and specificity than gRNAsconstructed according to currently published design rules. We will demonstratethe applicability of this screening method to this and other targets, as wellas validation experiments in preclinical disease models.***



380. Knock-In iPS Cell Line Generation Using End-Modified Linear DNA Donors

Aidan M. Simpson[1], Christopher B. Rohde[2], Mitchell R. Kopacz[2], Jasmine K. Harris[2], Franklin Kostas[2], Caglar I. Tanrikulu[3], Matthew Angel[2]
[1]Biological Engineering, Massachusetts institute of Technology, Cambridge, MA,[2]Factor Bioscience, Inc., Cambridge, MA,[3]Novellus, Inc., Cambridge, MA

To produce a targeted knock-in, a gene-editing endonuclease is used to create a double strand break (DSB) at a target site in the genome, and a plasmid donor containing a transgene as well as homology arms is inserted at the target site. However, rates of on-target integration using plasmid donors are very low, especially in primary cells and induced pluripotent stem cells (iPSCs). We hypothesized that the use of end-modified linear DNA donors could result in higher insertion rates and increased target specificity when compared with traditional plasmid donors. We synthesized end-modified linear donors using PCR with standard primers, 5'-biotinylated primers, and primers containing a 5′ polyethylene glycol (PEG) linker connected to a random 21-nucleotide single-stranded DNA sequence. We hypothesized that these donors could exhibit lower cytotoxicity, increased persistence in cells, and improved rates of on-target integration. To examine these characteristics, we compared the three donors in knock-in experiments using primary human fibroblasts and iPSCs. End-modified linear donors encoding green fluorescent protein (GFP) and a puromycin resistance gene were synthesized and electroporated into fibroblasts and iPSCs together with mRNA encoding NoveSlice gene-editing proteins targeting a sequence within the AAVS1 locus. We optimized the electroporation parameters and cell culture conditions for simultaneous delivery of gene-editing mRNA and donor DNA to iPSCs. Using GFP expression levels as a marker, the standard PCR donor resulted in the highest insertion rate, followed by the 5′ ssPEG donor and the biotinylated donor. All three donors resulted in higher integration rates than a plasmid containing the same sequence. Transfected iPSCs formed colonies of cells with uniform GFP expression that were isolated and propagated as stable knock-in lines. We show that end-modified linear donors integrate at higher rates than plasmid donors. Use of these donors may therefore represent a preferred approach for the generation of knock-in iPS cell lines.



381. Using Novel Technologies to Improve upon Gene-Editing Therapeutics for Alzheimer's Disease

Sahil Malhotra
Duke University, Durham, NC

Numerous approaches—both correlational and mechanistic—have identified genes that influence the development of Alzheimer's Disease

(AD). Particular mutations, especially the E4 isoform of APOE, have been shown to increase the probability of developing AD, as well as triggering an earlier onset. Correcting these deleterious mutations via gene editing with CRISPR-Cas9 is a promising route to developing therapeutics for AD. The standard approach to gene editing is to encode the editing complex, plus promoters and any other necessary genetic elements, as a plasmid within a viral vector. However, the viral vector approach has a number of limitations. Plasmid size must be small, on the order of 10 kb or less, depending on the vector type. Other issues include ensuring the efficacy and accuracy of the gene-editing complex to prevent potentially harmful off-target editing activity. Establishing control over when and in which tissues the editing complex is active is also important. A number of developments in gene editing have opened several avenues to address these limitations. Cell-cycle-dependent CRISPR models aim to limit CRISPR-Cas9 activity only to stages of the cell cycle in which homology-directed repair (HDR) of DNA damage would allow for the successful introduction of desired mutations using a provided template while minimizing the risk of collateral DNA damage. Another type of gene editor, cytosine base editors (CBEs), only catalyzes cytosine to thymine (C → T) transitions; CBEs are relatively small and may be well-suited to correcting the pathogenic E4 APOE isoform, which can be corrected with a single C → T transition. Further improvements to spatial and temporal control over editor expression may be reversibly achieved using an operon system, most notably the tetracycline-dependent tet-on/tet-off system. These advances would help create a robust, safe, and more effective gene-editing system. In the context of AD, they may increase the likelihood of realistically creating a viable gene-editing therapeutic.

382. AsCas12a Ultra Nuclease Facilitates the Rapid Generation of Therapeutic Cell Medicines

Liyang Zhang[1], John Zuris[2], Ramya Viswanathan[2], Jasmine Edelstein[2], Rolf Turk[1], Bernice Thommandru[1], H. Tomas Rube[3], Steve Glenn[1], Michael Collingwood[1], Nicole Bode[1], Sarah Beaudoin[1], Swarali Lele[2], Sean Scott[2], Kevin Wasko[2], Christopher Borges[2], Mollie Schubert[1], Gavin Kurgan[1], Cecilia Fernandez[2], Vic Myer[2], Richard Morgan[2], Mark Behlke[1], Christopher Vakulskas[1]

[1]Integrated DNA Technologies, Coralville, IA,[2]Editas Medicine Inc, Cambridge, MA,[3]University of California - Merced, Merced, CA

Though AsCas12a fills a crucial gap in the current genome editing toolbox, it exhibits relatively poor editing efficiency, restricting its overall utility. We isolated a novel engineered variant, "AsCas12a Ultra", that dramatically increased the on-target editing efficiency by 40-fold over wild-type (WT) protein. Nearly 100% editing efficiency at all sites examined in HSCs, iPSCs, T cells, and NK cells were achieved when Ultra was delivered as ribonucleoprotein (RNP) at a relevant dose, which virtually eliminated the guide selection process required by WT RNP. We show that the AsCas12a Ultra maintains high on-target specificity thereby mitigating the risk for off-target editing, and making it ideal for complex therapeutic genome editing applications. Comparing to the current state-of-art Cas12a variants (enAsCas12a), Ultra exhibits greater on-target efficiency and better specificity. In primary T cells, we simultaneous targeted three clinically relevant genes at >90% efficiency, and demonstrated up to 60% and 40% efficiencies for single- and double-transgene knock-in. With Ultra, we achieved site-specific knock-in of a chimeric antigen receptor (CAR) in NK cells up to 50% efficiency, which afforded enhanced anti-tumor NK cell recognition, and potentially enabling the next generation of allogeneic cell-based therapies in oncology. Overall, AsCas12a Ultra is an advanced CRISPR nuclease with significant advantages in both basic research and in the production of gene edited cell medicines.

383. Long-Lasting Analgesia via Targeted *In Situ* Repression of Nav1.7

Ana Moreno[1], Fernando Aleman[1], Glaucilene Catroli[2], Matthew Hunt[2], Michael Hu[3], Amir Dailamy[3], Andrew Pla[3], Sarah Woller[2], Nathan Palmer[4], Udit Parekh[5], Daniella McDonald[3], Amanda Roberts[6], Tony Yaksh[2], Prashant Mali[3]

[1]Navega Therapeutics, San Diego, CA,[2]Department of Anesthesiology, University of California San Diego, San Diego, CA,[3]Department of Bioengineering, University of California San Diego, San Diego, CA,[4]Division of Biological Sciences, University of California San Diego, San Diego, CA,[5]Department of Electrical Engineering, University of California San Diego, San Diego, CA,[6]Animal Models Core, Scripps Research Institute, La Jolla, CA

Current treatments for chronic pain rely largely on opioids despite their substantial side effects and risk of addiction. Genetic studies have identified in humans key targets pivotal to nociceptive processing. In particular, a hereditary loss-of-function mutation in Nav1.7, a sodium channel protein associated with signaling in nociceptive sensory afferents, leads to insensitivity to pain without other neurodevelopmental alterations. However, the high sequence and structural similarity between Nav subtypes has frustrated efforts to develop selective inhibitors. Here, we investigated targeted epigenetic repression of Nav1.7 in primary afferents via epigenome engineering approaches based on clustered regularly interspaced short palindromic repeats (CRISPR)-dCas9 and zinc finger proteins at the spinal level as a potential treatment for chronic pain. Towards this end, we first optimized the efficiency of Nav1.7 repression in vitro in Neuro2A cells, and then by the lumbar intrathecal route delivered both epigenome-engineering platforms via adeno-associated viruses (AAVs) to assess their effects in three mouse models of pain: carrageenaninduced inflammatory pain, paclitaxel-induced neuropathic pain and BzATP-induced pain. Our results show effective repression of Nav1.7 in lumbar dorsal root ganglia, reduced thermal hyperalgesia in the inflammatory state, decreased tactile allodynia in the neuropathic state; and no changes in normal motor function in mice. We anticipate this Long-lasting Analgesia via Targeted in vivo Epigenetic Repression of Nav1.7, a methodology we dub LATER, might have therapeutic potential in management of persistent pain states.

384. A Longitudinal Study of Juvenile Methylmalonic Acidemia (MMA) Mice Treated by Target Integration of *MMUT* into *Albumin* with a Promoterless AAV Vector

Leah E. Venturoni[1], Randy J. Chandler[1], Nelson Chau[2], Jing Liao[2], Susan Gordo[2], Mark Kay[3], Adi Barzel[2], Charles Venditti[1]

[1]NHGRI, NIH, Bethesda, MD,[2]LogicBio Therapeutics, Cambridge, MA,[3]Departments of Pediatrics and Genetics, Stanford University, Stanford, CA

MMA is a rare and heterogenous inborn error of metabolism most commonly caused by a deficiency of methylmalonyl-CoA mutase (MMUT). Patients suffer from frequent episodes of metabolic instability, severe morbidity, and early mortality. Although elective liver transplantation has emerged as a treatment option for severely affected patients and can eliminate the potentially lethal metabolic instability associated with this disorder, gene therapy has been explored in MMA mouse models as an alternative therapy to liver transplantation. Conventional adeno-associated viral (AAV) mediated gene delivery was highly effective in the treatment of neonatal mice with severe MMA. However, as has been reported in many other mouse models, hepatocellular carcinoma was observed in aged MMA mice, as well as control littermates, that were treated with conventional AAV in the neonatal period. To minimize the potential of vector-related insertional mutagenesis and preserve MMUT expression after therapeutic gene delivery, we designed a promoterless AAV vector that utilizes homologous recombination to achieve site-specific gene addition of human *MUT* into the mouse albumin (*Alb*) locus, Alb-2A-MMUT (GeneRide™). Previously, we reported that after a latency period of several months, MMA mice treated as neonates displayed continuous enhancement of MMUT expression accompanied by markers of improved clinical condition such as weight gain, reduction of disease associated plasma biomarkers and increased 1-C-13 propionate oxidation capacity. Aged MMA mice, treated as neonates with very high (2.5e12 VG/pup) doses of vector, have not developed HCC (30 mice followed 13 to 26 months after treatment). We have now investigated the efficacy of the treatment of juvenile animals with MMA. A time course study has revealed increasing levels of liver MMUT expression, a reduction of plasma methylmalonic acid, and increasing levels of a treatment plasma biomarker, Alb-2A, over time in the treated animals. Additionally, mutants treated as juveniles have similar Alb-MMUT integration rate , a treatment biomarker, as seen in the treated neonates at the same point post treatment (at 2 months post treatment an average of 0.041±0.025 for 4 juvenile treated animals vs. 0.096±0.034 for 12 neonatally treated animals; P=0.39). As with neonatal treatment, plasma Alb-2A levels correlated with Alb-MMUT integration rates and hepatic MMUT protein expression in juvenile MMA mice, further emphasizing the use of this circulating protein biomarker as a means to track correction and expansion of the modified cells longitudinally. It is likely that the increased hepatic turnover associated with the underlying disease creates an environment where therapeutic integration events into the *Alb* locus can be enriched in the absence of physiologic liver growth, which if translatable to humans, would expand the therapeutic window for genome editing into this locus as a treatment for MMA.

385. Targeting Weakly Expressed Loci with Self-Cleaving Guide RNAs

Amita Tiyaboonchai[1], Catherine L. May[2], Klaus H. Kaestner[2], Markus Grompe[1]

[1]Department of Pediatrics, Oregon Health and Science University, Portland, OR,[2]Department of Genetics, University of Pennsylvania, Philadelphia, PA

Promoterless GeneRide rAAV vectors are typically designed to integrate into the highly expressed Albumin (Alb) locus resulting in supraphysiological expression levels of the therapeutic gene. We have developed a method based on self-cleaving guide RNA (scgRNA) to introduce a gene disruption cassette into the GeneRide vector in cis. The scgRNA consists of a gRNA flanked by two self-cleaving ribozymes. The ribozymes permit the proper processing of an active gRNA from a polymerase 2 transcript. To determine if functional scgRNA GeneRide is also produced from lower expressing loci, vectors containing the scgRNA were designed to integrate into a series of genes with decreasing strength of expression relative Alb including transthyretin (Ttr), phenylalanine hydroxylase (Pah) and factor IX (F9). To test these loci, the Ai9-tdTomato reporter mouse system was utilized. In this system tdTomato gene expression is activated by CRISPR/cas9 mediated disruption of a stop cassette upstream of the reporter. The gRNA required for CRISPR/cas9 mediated cutting can be expressed only after precise, homologous integration of the promoterless GeneRide vector. Hence, the frequency of integration can be determined by the frequency of tdTomato positive hepatocytes. The various targeting rAAV scgRNA-GeneRide vectors and a negative control vector were delivered into both neonatal and adult mice. Hepatocytes from neonatally injected mice were analyzed at 8 to 12 weeks of age, while adult mice were analyzed 2 weeks after the rAAV delivery via flow cytometry and histology. Both neonatal and adult mice had tdTomoato positive hepatocytes from all vectors. In the control vector, there were low levels of tdTomato expression in the liver indicating slight leakiness within the system, most likely due to random 5'UTR integration of the rAAV vector. In both neonates and adults tdTomato expression from the Alb, Ttr and Pah loci were significantly higher than the controls suggesting that scgRNA can successfully be expressed from polymerase 2 promoters that are up to 100x weaker than Alb. Having demonstrated that functional scgRNA can be expressed from lower expressing endogenous promoters, we wanted to determine if the scgRNA could be utilized for genetic lineage tracing of weakly expressed genes. As a proof of concept, the Keratin 14 (Krt14) gene was chosen as its expression in the mouse is well characterized. A transgenic mouse was generated containing a scgRNA targeting the stop codon in Ai9-tdTomato downstream of the terminal coding exon of Krt14. This mouse was bred to animals which constitutively express spCas9 and Ai9-tdTomato. Tissues from the mouse were examined and tdTomato was found to be expressed from the tongue, salivary glands, skin and thymus corresponding to previous reports of Krt14 expression patterns. As expected, the kidney, liver, spleen, bladder, pancreas and testis were found to be tdTomato negative. Together these experiments demonstrate that functional scgRNA can be expressed from the promoters of even weakly expressed genes and mediate CRISPR/Cas cutting at distant loci. Our methodology has the potential for the use

of scgRNA in selection of gene targeted hepatocytes in gene therapy and also for lineage tracing of genes through a wider dynamic range than previously described methods.

386. Rescuing the Haploinsufficiency in 22Q11.2 Deletion Syndrome

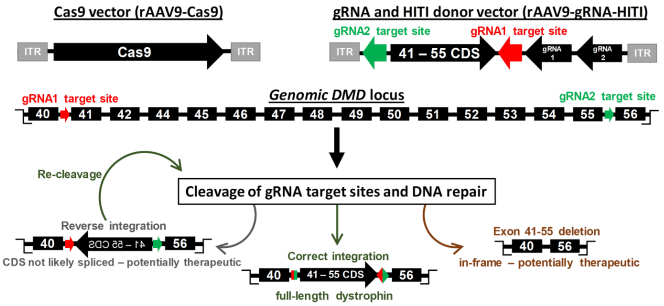
Niraj Rajiv Punjya
Department of Biochemistry and Molecular Medicine, UC Davis, Davis, CA

22q11.2 deletion syndrome (22q DS) is a disorder characterized by a spontaneous chromosomal microdeletion on the order of three million base pairs, containing approximately forty protein coding genes. The disorder occurs in about 1/4000 live births and is considered the second most common cause of intellectual disability in the world. Patients with the deletion exhibit wide ranging symptoms, including neurological and behavioral symptoms such as attention deficit hyperactive disorder, anxiety, and schizophrenia. Here, we describe a strategy to upregulate a subset of these forty protein coding genes to rescue the haploinsufficiency using a dead Cas12a (dCas12a) protein fused to transcriptional activators. We will be using dCas12a instead of the more widely used dCas9 because Cas12a is more suited for multiplex activation of multiple genes due to being a smaller protein with a smaller overall guide length. To upregulate multiple genes, we will employ a multiplex approach designing a single plasmid that contains up to 25 guide RNAs. The prevailing theory regarding the gene-phenotype relationship with 22q is that there is a wide variety of complex interactions with many shared molecular targets between the genes involved in the deletion. A multiplexed dCas12a approach could therefore be both a critical tool for mapping the genetic causes of the 22Q DS phenotypes, as well as for developing multi-gene therapies.

387. Homology-Independent Targeted Integration for Correction of DMD Distal Hotspot Mutations

Anthony A. Stephenson, Julian R. Havens, Stefan Nicolau, Tatyana Vetter, Kevin M. Flanigan
Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH

Duchenne muscular dystrophy (DMD) is a progressive muscle disease which manifests in childhood as difficulty walking, progresses to loss of ambulation by the teens, and leads to death in early adulthood. DMD is caused by loss of the muscle-specific structural protein dystrophin resulting in poor muscle cell integrity. Chronic cycles of muscle degeneration in individuals with DMD causes a decline in muscle mass, strength, cardiac performance, and ventilation over time. Adeno-associated virus (AAV) vectorized gene editing with CRISPR-Cas9 has recently emerged and shown great potential for restoring dystrophin protein expression in animal models of DMD. However, these approaches often generate mutant dystrophin isoforms with unknown clinical benefits. Importantly, as most patients exhibit single or multiple exon deletions within a mutation hotspot in the distal region of the *DMD* gene (exons 45 - 55), knock-in gene editing to restore full-length dystrophin expression is highly desirable. Progress in knock-in gene editing for DMD is stifled by the low efficiency of homology-mediated knock-in within non-dividing cells. However, a recently described CRISPR-Cas9-based technology called homology-independent targeted integration (HITI) allows knock-in with the ubiquitous and efficient non-homologous end joining DNA repair pathway. Here, we designed a HITI gene editing approach to replace exons 41 - 55 with a single "mega-exon" of ~2.5 kb comprised of the coding sequence of exons 41 - 55 without the intervening introns. We designed CRISPR guide RNAs (gRNAs) that target introns 40 and 55 and screened their gene editing activity in HEK293 cells. Using the most active gRNAs, we tested HITI replacement of exons 41 - 55 in HEK293 cells and found that we could induce knock-in of the ~2.5 kb mega-exon in place of the natural ~175 kb exon 41 - 55 locus. Next, we packaged the HITI gene editing system into AAV and tested it in patient-derived myogenic cells and via systemic delivery into a humanized mouse model of DMD exon 45 deletion. This work provides proof of concept data in support of a HITI gene editing approach to replace exons 41 - 55 that could benefit ~37% of DMD patients with mutations that lie between intron 40 and 55.



388. A Non-Viral Approach for B-Cell Engineering

Kanut Laoharawee, Matthew J. Johnson, Evan W. Kleinboehl, Joseph J. Peterson, Kenta Yamamoto, Walker S. Lahr, Emily J. Pomeroy, Beau R. Webber, Branden S. Moriarity
Pediatrics, University of Minnesota, Minneapolis, MN

B cells are a subgroup of adaptive immune cells that have the unique ability to differentiate into long-lived plasma cells, producing a large quantity of protein (aka antibody) for many years or even decades. B cells make up approximately 2%-8% of leukocytes and can be easily isolated from peripheral blood. These characteristics have generated great interest in the use of B cells as a novel cell-based therapy for protein delivery to patients with enzymopathies. To this end, we previously developed and optimized a robust protocol for engineering B cells using CRISPR/Cas9 and recombinant AAV (rAAV) (PMID: 30108345 and 33226023). Moving forward, we tested this viral-based approach for engineering B cells to express a therapeutic enzyme to treat enzymopathies. We chose to investigate mucopolysaccharidosis type I (MPS I) as a disease model. MPS I is a genetic disease caused by mutations in the gene responsible for producing the a-L-iduronidase (IDUA) enzyme. We constructed a cassette comprising a therapeutic cDNA of *IDUA* that is co-expressed with RQR8 driven by MND promoter. The RQR8 is an extracellular epitope of CD34 and CD20, which is not normally expressed on B cells. Thus, RQR8 is used as an engineered-cell marker that can be detected by flow cytometry. Due to the large size of this gene cassette, the homology arms (HAs)

were 250 base pairs (bp) to meet rAAV capacity (4.7 kb). The 250bp homology arms in this viral vector produced low knock-in (< 5%) frequency when compared to 1kb HAs (> 50%). Our finding emphasizes a major drawback associated with viral-based approaches. In addition, rAAV transduction has been reported to activate an immune response, leading to rapid clearance of the rAAV-transduced cells (PMID:21321191). Furthermore, production is costly and turn-around time is much longer when compared to plasmid DNA. These bottlenecks prompted us to explore alternative approaches. We chose a non-viral strategy using a plasmid donor for HDR, which is not limited by cargo size capacity and may also eliminate the issue of immunity associated with rAAV-based methods. To test this non-viral approach, we introduced pMax *GFP* plasmid in B cells, resulting in 60% GFP-positive B cells. Next, we tested CRISPR/Cas9 mediated HDR using a nanoplasmid™ vector as a DNA donor molecule. Nanoplasmids™ are a 500 bp circular DNA without traditional bacterial and antibiotic resistance genes. We constructed an HDR template consisting of a promoterless *EGFP* plasmid (*SA-EGFP*) flanked on both sides with 1kb HAs to the AAVS1 locus. Using this method, we observed up to 28% EGFP-positive B cells. Homology mediated end-joining (HMEJ) has been reported to integrate transgenes with higher efficiencies than HDR (PMID:32412410). In order for HMEJ to occur, the donor template must be delivered in the form of linearized double stranded DNA. To compare HDR to HMEJ, we developed an HMEJ template by inserting a synthetic CRISPR/Cas9 target site that does not exist on human genome (hereafter termed universal target site) upstream of the left HA and downstream of the right HA of the *SA-EGFP*. We delivered Cas9, AAVS1 gRNA, and universal gRNA along with either the HDR or HMEJ nanoplasmid™. We observed similar rates of EGFP-positive B cells in both HDR and HMEJ, although the HMEJ approach was harder on B-cell viability (< 30%) when compared to HDR (40%). However, B cells from both methods started to rebound within 4 days of engineering. Moving forward, we used HDR for our subsequent experiments. Next, we cloned the *IDUA-RQR8* cassette with 250-bp HAs targeting the IgH locus into a nanoplasmid™. Surprisingly, we observed up to 19% engineering efficiency using the non-viral engineering approach, which was higher than the viral-based approach (5%). This data suggests that our non-viral approach can be used for better engineering efficiency in B cells.

389. Allele-Specific Knockdown of Mutant HTT Protein via Editing at Coding Region SNP Heterozygosities

Sarah Oikemus[1], Edith L. Pfister[2], Ellen Sapp[3], Kathryn Chase[2], Lori A. Kennington[2], Edward Hudgens[1], Rachael Miller[2], Lihua J. Zhu[1], Akanksh Chaudhary[1], Eric O. Mick[4], Miguel Sena-Esteves[5], Scot A. Wolfe[1,6], Marian DiFiglia[3], Neil Aronin[2,7], Michael H. Brodsky[1]

[1]Department of Molecular Cell and Cancer Biology, University of Massachusetts, Medical School, Worcester, MA,[2]Department of Medicine, University of Massachusetts, Medical School, Worcester, MA,[3]Department of Neurology, Harvard Medical School and MassGeneral Institute for Neurodegenerative Disease, Charlestown, MA,[4]Department of Population and Quantitative Health Sciences, University of Massachusetts, Medical School, Worcester, MA,[5]Horae Gene Therapy Center, University of Massachusetts, Medical School, Worcester, MA,[6]Biochemistry and Molecular Pharmacology, University of Massachusetts, Medical School, Worcester, MA,[7]RNA Therapeutics Institute, University of Massachusetts, Medical School, Worcester, MA

Huntington's disease (HD) is an autosomal dominant disease caused by an expansion of the CAG repeat region in the huntingtin (HTT) gene. CAG repeat expansion results in the production of a toxic mutant protein with an extended polyglutamine tract. Reduction or elimination of the mutant mRNA and protein is expected to provide therapeutic benefit. While partial silencing of HTT in the brain is tolerated in mice and large animals, wild-type huntingtin has important neuronal functions and the consequence of long-term inactivation of total huntingtin are not established. One strategy for specific targeting of the mutant allele is targeting of heterozygous single nucleotide polymorphisms (SNPs) in the huntingtin gene. CRISPR-Cas9 based gene editing provides a potential method to permanently prevent expression of the huntingtin mRNA and protein. We have used CRISPR-Cas9 nuclease to target sites in the coding region of the human huntingtin that are frequently heterozygous. Expression of a single CRISPR-Cas9 nuclease targeting a coding SNP results in a high frequency of insertion/deletion mutations in the targeted HD allele *in vitro* and *in vivo* and causes a reduction in mutant huntingtin protein without the production of truncated protein products. Therefore, allele-specific targeting of coding SNPs using CRISPR-Cas9 is a feasible approach to inactivate autosomal dominant mutations that cause genetic disease.

390. DNA Methylation Editing of X-linked Intellectual Disability Genes via Split dCas9 AAV9

Julian Alexander Nandor Matthias Halmai, Fiona Buchanan, Casiana Gonzalez, Peter Deng, Jennifer Waldo, Jasmine Carter, David Cameron, Kyle Fink

Department of Neurology, UC Davis, Sacramento, CA

There are more than 35 known X-linked intellectual disability genes present on the X chromosome that have a severe clinical manifestation in females. Due to random X chromosome inactivation in females, X-linked dominant Mendelian diseases can result in a mosaic of cells expressing the mutant and wild-type alleles. Strikingly more than 15% of genes naturally escape from the epigenetic X chromosome

inactivation dosage compensation mechanism. These escape genes demonstrate promoter DNA hypomethylation. One potential gene correction strategy previously demonstrated by our group is to reactivate the silenced wild type allele in cells expressing a loss-of-function mutant allele by DNA demethylation editing of the gene promoter. Here, we demonstrate reactivation of X-linked disability genes in patient-derived cell models. Targeting of a CRISPR/dCas9 DNA demethylase reduces DNA methylation levels from the promoter, resulting in de-repression of the silenced allele as assessed by ultradeep sequencing. We show that this one-fits all toolbox is specific for the intended target sites. In addition, we demonstrate pre-clinical feasibility of our approach via the utilization of intein-mediated trans-splicing of large CRISPR/Cas9 effector fusion proteins in vivo, allowing for central nervous system delivery via AAV9. This epigenome editing platform will help expand the list of clinically actionable genetic diseases in the future.

391. A Novel Bimodal Gene Construct for Multiplex Cell Engineering and the Development of Allogeneic Chimeric Antigen Receptor T-Cells

Audrey Roussel-Gervais[1], Antonija Šakić[1], Sten Ilmjärv[1], Ophelie Cherpin[2], Patrick Salmon[2], Karl-Heinz Krause[2,3], Marco Alessandrini[1,2]

[1]Antion Biosciences, Geneva, Switzerland,[2]Pathology and Immunology, University of Geneva, Geneva, Switzerland,[3]University Hospitals of Geneva, Geneva, Switzerland

Chimeric antigen receptor (CAR) T-cell therapy is an established form of treatment for B-cell malignancies, and regulatory approval of similar treatments against other cancer indications is expected in the near future. The field is progressing rapidly as researchers look to improve numerous aspects relating to treatment safety, efficacy and feasibility. Key features in this regard include the design of multivalent CARs to target diverse tumors, suppression of inhibitory receptors to prevent CAR T-cell exhaustion, expression of stimulatory cytokines to improve CAR T-cell fitness, and facilitating off-the-shelf administration through disruption of the T-cell receptor (TCR) and HLA molecules. Gene modifying T-cells to achieve these additional layers of complexity depends heavily on the use of safe and efficient molecular strategy. Our aim was to develop a single, bicistronic gene construct for co-expression of a CAR and a gene silencing microRNA cassette (miCAR). The latter is based on a substantially optimized gene architecture previously reported by our group, which allows for expression of numerous microRNAs and the ability to achieve multiplex gene silencing. For development of miCAR constructs, we cloned a second generation anti-CD19 CAR into optimized multi-hairpin gene constructs, driven by its own promoter such that it is expressed independently from the multiplex microRNA cassette. All constructs were packaged into lentiviral vectors and delivered to primary T-cells to assess gene silencing, CAR expression and activity of our miCAR T-cells. By screening for up to 10 target sequences per gene, we demonstrate gene silencing efficiencies of 75-95% against relevant cell surface receptors, namely TCR, HLA class I molecules and the inhibitory receptor PD1. Following transduction of primary T-cells with a miCAR gene construct, we are able to show simultaneous multiplex gene silencing and expression of the CAR and a selection marker (RQR8). Notably, we achieved over 50% transduction of primary T-cells, all of which uniformly express both the CAR and microRNA cassette. Our allogeneic miCAR19 T-cells also maintained specific cytotoxicity of CD19 expressing cells. We demonstrate proof of principle for efficient delivery of a genetic payload with multiplex cell engineering capabilities, including the expression of both a CAR and selection marker, as well as expression silencing of three relevant genes. We believe miCAR T-cells offer a promising immunotherapeutic solution for application to numerous disease indications.

392. Novel CRISPR-Cas13d System to Target Highly Conserved Sequences of SARS-CoV-2 and Other Human Coronaviruses

Mouraya Hussein, Zaria Andrade dos Ramos, Monique Vink, Ben Berkhout, Elena Herrera-Carrillo

Medical Microbiology, Amsterdam UMC, Amsterdam, Netherlands

The current SARS-CoV-2 pandemic is a major global health burden. Although protective vaccines have rapidly become available since the beginning of 2021, concerns remain as new variants of the virus continue to appear worldwide. Gene editing-based antiviral approaches may offer an attractive alternative as this method can be rapidly adjusted to new viral sequences. Sequence-specific gene editing tools such as CRISPR-Cas enable the inactivation of viral genomes. Specifically, the novel CRISPR-Cas13d system that specifically cleaves RNA makes it ideal for targeting the SARS-CoV-2 RNA genome. In this study, we aimed at using CRISPR-Cas13 to target viral RNA sequences that are phylogenetically conserved, not only among different SARS-CoV-2 variants, but also among the other human coronaviruses, thus preparing for a future zoonotic outbreak of a novel coronavirus. 3282 complete genome sequences of SARS-CoV-2 variants were aligned and analyzed in order to identify conserved sequences. Moreover, SARS-CoV-2 sequences were aligned with those of other human coronaviruses (SARS-CoV, MERS-CoV, HCoV-229E, HCoV-NL63, HCoV-OC43 and HCoV-HKU1) to identify broadly conserved sequences. Based on a panel of selected target sequences, 23-nucleotide crRNAs were designed and their efficacy was predicted using a computational approach. Besides targeting the positive-sense RNA genome, also crRNAs that target the negative-sense RNA replication intermediate were included. The knock-down efficiency of the crRNAs was tested *in vitro* in transfected cells with a designed multi-target luciferase reporter construct. The antiviral activity was subsequently validated by scoring the crRNA knock-down efficiency in cells transfected with the SARS-CoV-GFP replicon based on the SARS-CoV-1 pathogen. Transfection of susceptible cells with this replicon and replication of the viral RNA results in the expression of GFP, which thus reflects the extent of viral replication. We used this reporter system to monitor the suppression of viral replication by crRNAs. We identified a panel of crRNAs targeting highly conserved SARS-CoV-2 genome sequences with a high predicted activity. A strong reduction of the luciferase signal was induced by the majority of these crRNAs compared to a negative control construct. Selected crRNAs also demonstrated efficient targeting of the SARS-CoV-GFP replicon, confirming that the designed crRNAs exhibit broader anti-coronavirus activity in a biologically relevant setting. This study demonstrates

potent targeting of highly conserved SARS-CoV-2 sequences by the novel CRISPR-Cas13 system in cell culture. The results emphasize the importance of rational design of gene-editing tools to develop antiviral strategies in order to prepare for future zoonotic outbreaks of novel pathogenic coronaviruses.

393. Novel Families of CRISPR Systems Enriched in Small Effectors with Genome Editing Capability

Daniela S. A. Goltsman, Lisa Alexander, Jason Liu, Cindy J. Castelle, Audra E. Devoto, Morayma M. Temoche-Diaz, Alan R. Brooks, Rebecca C. Lamothe, Gregory J. Cost, Cristina N. Butterfield, Brian C. Thomas, Christopher T. Brown

Metagenomi, Emeryville, CA

CRISPR nucleases have begun to revolutionize gene and cell therapy due to their versatility and programmability. However, many in vivo approaches are not currently possible owing to the size, and potential off-target or immunogenic effects associated with many of the systems currently under development. We mined an extensive genome-resolved metagenomics database and identified small CRISPR effectors from natural environments to develop into genome editing tools with the potential to overcome these limitations. Our analysis encompassed hundreds of thousands of microbial genomes and uncovered a vast diversity of novel CRISPR effectors, which we grouped into families based on phylogenetic analysis. We identified families composed of highly-novel sequences that were enriched in proteins smaller than 1,200 amino acids, including one family with several active and diverse representatives that is enriched in exceptionally small effectors (≤1,000 aa). Enzymes from these families had predicted catalytic and binding domains with expected functional residues. For selected representatives, we predicted tracrRNA sequences, designed single guide RNAs, and demonstrated cleavage activity for members of >15 families. Notably, these systems exhibited novel guide RNA structures compared with each other and with previously characterized systems, and have various PAM motif requirements. Several systems showed levels of editing activity in E. coli comparable to or significantly in excess of spCas9, and exhibited promising levels of genome editing in immortalized cell lines when tested across a variety of genomic targets. The nucleases described here are particularly interesting systems given their potential to enable more flexible delivery options using, for example, adeno-associated viruses. Importantly, some of these systems exhibit low levels of off target editing based on GuideSeq analysis, making them especially suitable for in vivo applications. Furthermore, the small size and single guide designs have the potential to improve production and developability of these systems as required for therapeutic applications. These enzymes represent next generation tools with the potential to cure currently untreatable diseases.

394. Iterative Screen Identifies Amphiphilic Peptides That Confer Enhanced Delivery of CRISPR Associated Nucleases and Adenine Base Editors to Airway Epithelia

Soumba Traore[1], Sateesh Krishnamurthy[1], Christine Wohlford-Lenane[1], Katarina Kulhankova[1], Bernice Thommandru[2], Garrett Rettig[2], Mark Behlke[2], Gregory A. Newby[3], Stéphanie Hallée[4], Vicky Caron[4], Coraline Lauvaux[4], Xavier Barbeau[4], Mario Harvey[4], Joannie Roberge[4], David R. Liu[3], David Guay[4], Paul B. McCray[1]

[1]Pediatrics, University of Iowa, Iowa City, IA,[2]Integrated DNA Technologies, Coralville, IA,[3]Merkin Institute of Transformative Technologies in Healthcare, Broad Institute of Harvard and MIT, Cambridge, MA,[4]Feldan Therapeutics, Quebec City, QC, Canada

Disease-causing mutations in *CFTR* gene lead to the dysfunction of airway epithelial cells, contributing to the onset and progression of cystic fibrosis (CF). Therapeutic proteins or genome-editing reagents can modify these disease-associated mutations in the respiratory epithelium; however, the lack of an appropriate delivery mechanism in vivo remains a challenge. We developed amphiphilic peptides with properties that facilitate the effective delivery of CRISPR-associated (Cas) nuclease and adenine base editor (ABE) ribonucleoproteins (RNPs) to well-differentiated primary cultures of human airway and nasal epithelia in vitro and mouse large and small airway epithelia in vivo. To further optimize delivery, we iteratively screened rationally designed amphiphilic peptides to deliver SpCas9 and AsCas12a RNPs in primary cultured human airway epithelial cells at an air-liquid interface. The targeted genomic locus was PCR amplified and subsequently sequenced using next generation sequencing (NGS) to quantify the occurrence of imprecise non-homologous end joining (NHEJ) repair as a proxy for delivery and editing efficiency. This approach successfully identified peptides with improved delivery properties. These candidate peptides were then used in further iterative screening. Peptides with the best delivery properties were investigated in vivo for SpCas9 and AsCas12a RNP editing in transgenic Rosa $^{mT/mG}$ mice (tdTomato mouse). To assess precise genome editing and functional correction of the *CFTR* gene, ongoing studies will evaluate ABE-RNP delivery with amphiphilic peptides in human nasal and airway epithelial cells with *CFTR* mutations using NGS and CFTR function endpoints. In summary, this screening strategy facilitated identification of novel peptides that improve the delivery of Cas nucleases and ABE RNPs to airway and nasal epithelia. These peptides provide new resources for therapeutic protein delivery to respiratory epithelial cells.

395. Extinction of All Infectious HIV in Cell Culture by the CRISPR-Cas12a System

Minghui Fan, Zongliang Gao, Elena Herrera-Carrillo, Ben Berkhout

Amsterdam UMC, Amsterdam, Netherlands

Extinction of all infectious HIV in cell culture by the CRISPR-Cas12a systemThe CRISPR-Cas9 system has been used successfully for genome editing in various organisms. We previously reported inhibition of the human immunodeficiency virus (HIV) in cell culture infections and subsequent viral escape when a single guide RNA (gRNA) was

used, but also complete inactivation of all infectious HIV with certain combinations of two gRNAs. The recently described RNA-guided endonuclease system CRISPR-Cas12b (formerly called Cpf1) may provide an even more promising tool for genome engineering with increased activity and specificity. We observed inhibition of HIV in cell culture infections when a single guide RNA (called crRNA) was used, but also witnessed viral escape in some cultures. Most importantly, one of these crRNAs was able to achieve full HIV inactivation (sterilizing CURE), albeit not in all test cultures. We subsequently tried to further improve the CURE activity by testing dual crRNA combinations in a spreading HIV infection. We demonstrate that dual crRNA combinations exhibit more robust antiviral activity than a single crRNA attack. For instance, dual-crRNA therapy can prevent virus escape in long-term cultures. We analyzed the target sequences in the HIV proviral genomes in escape cultures but also in CURE cultures and compared them to the original Cas9 system for inactivation of the integrated HIV DNA genome. We disclose that DNA cleavage by the Cas12a endonuclease and subsequent DNA repair cause mutations with a sequence profile that is distinct from that of Cas9. Both CRISPR systems can induce the typical small deletions around the site of DNA cleavage, but Cas12a does not induce the pure DNA insertions that are routinely observed for Cas9. Surprisingly, we did not detect excision of the HIV sequences between the two Cas12a cleavage sites, which was proposed as the HIV-inactivation mechanism in a dual Cas9 CURE strategy. Instead, we observed efficient HIV inactivation by means of "hypermutation", that is the generation of virus-inactivating indel mutations at both DNA cleavage sites as a result of error-prone DNA repair. We propose that the different architecture of the Cas9 versus Cas12a endonuclease can explain this effect.

396. Cure Sickle Cell Initiative Standardized Data Forms for Genetic Therapy Studies

Sophie Lanzkron[1], Alexis Thompson[2], Traci Mondoro[3], Patrick Carroll[4], Michael DeBaun[5], Julie Kanter[6], Punam Malik[7], Deepa Manwani[8], John Pierciey[9], Mark Walters[10], Traci Clemons[11], Victoria Coleman-Cowger[11], Sherita Alai[11]

[1]Johns Hopkins School of Medicine, Baltimore, MD,[2]Northwestern University, Chicago, IL,[3]Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute, Bethesda, MD,[4]Johns Hopkins Medicine, Baltimore, MD,[5]Vanderbilt University Medical Center, Nashville, TN,[6]Medical University of South Carolina, Rockville, MD,[7]Cincinnati Children's Hospital, Cincinnati, OH,[8]Montefiore Medical Center, Bronx, NY,[9]bluebird bio, Cambridge, MA,[10]University of California, San Francisco, Oakland, CA,[11]Emmes, Rockville, MD

The Cure Sickle Cell Initiative (CureSCi) was created in 2017 to accelerate the development of safe and promising genetic therapies to improve the lives of individuals with sickle cell disease (SCD). The CureSCi has worked to actively engage the SCD community of patients, family members, caregivers and advocates to work together on a path to finding genetic-based cures while also encouraging collaboration among researchers, industry, non-profit organizations, and policy-making agencies. As a component of CureSCi, genetic research-specific common data elements (CDEs) for SCD were curated and developed in order to: accelerate clinical research development; build upon consensus data elements; and facilitate start-up of multi-center clinical research efforts. In early 2020, the CureSCi CDE Working Groups (WGs) comprised of clinicians, researchers, patient advocates and industry members from 35+ institutions/organizations/companies began to review current study forms and recommendations in SCD clinical studies from American Society of Hematology (ASH), US Food and Drug Administration (FDA), consensus measures for Phenotypes and eXposures (PhenX) catalog, Center for International Blood and Marrow Transplant Research (CIBMTR), and various other research entities. CDEs were identified, developed, and vetted by experts in the scientific community through a process that was transparent and inclusive, including a 6-week public review period. The five (5) WGs: 1) Genetics and Assays; 2) Physical Examination/ Medical History; 3) Cardiopulmonary, Renal and Cerebrovascular; 4) Outcomes; and, 5) Monitoring for Side Effects; compiled this library of recommended CDEs to represent the current state of science and annotated them in a hierarchical fashion. Core data elements represent the essential/required information applicable to any SCD genetics study; Supplemental-Highly Recommended data elements are essential for specific domain but not required in all SCD genetics studies. Supplemental data elements are those commonly collected in clinical research studies where use depends upon the study design, protocol or type of research involved; and Exploratory data elements require further validation but fill current gaps and are reasonable to use with the understanding that there is limited validation in the target group. These CDEs are posted on the Curesickle.org website and on the National Library of Medicine (NLM) website (https://cde.nlm.nih.gov). The NHLBI is strongly encouraging use of the recommended CDEs by the clinical research community to expedite study start-up, standardize data collection and allow for future data sharing. A standardized set of clinical research recommendations will increase efficiency and effectiveness of SCD clinical research studies, increase data quality and help educate new clinical investigators in the active field of SCD genetic research. This first iteration of the CDE recommendations will be updated on an annual basis to ensure incorporation advances in gene therapy and gene editing as well as other developed standards for SCD.

397. RNA-Seq Reveals Allele-Specific Expression of Somatic Mutations in Neuroblastoma

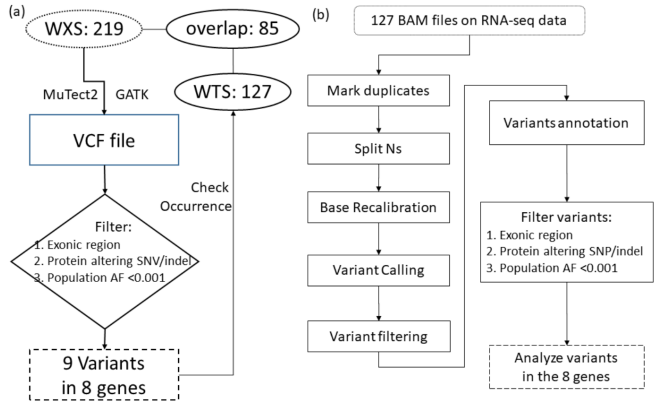
Lan Sun[1,2], Qian Liu[3], Lingli Tu[1], Xiaoqing Li[2,4], Jingyi Wang[2,4], Kai Wang[3], Jiang F Zhong[1]

[1]Department of Otolaryngology, Keck School of Medicine, University of Southern California, Los angeles, CA,[2]Department of Oncology, the People's Hospital of Bishan District, Chongqing, China,[3]Children's Hospital of Philadelphia, Philadelphia, PA,[4]Chongqing Medical University, Chongqing, China

Neuroblastoma (NB) is one of the most common solid tumors in children, accounting for approximately 8% of all pediatric malignancies and 15% of childhood cancer deaths. Somatic mutations in several genes such as ALK have been associated with neuroblastoma progression and can facilitate the discovery of novel therapeutic strategies. However, differential expression of mutated and wild-type alleles on the transcriptome level is not well studied. In this study, we analyzed 219 whole-exome sequencing datasets (with somatic mutations detected by MuTect from paired normal and tumor samples)

and prioritized mutations in eight candidate genes as potential driver mutations. Meanwhile, we analyzed 127 RNA-seq samples (of which 85 also had DNA-seq data available) for allele-specific expression levels of each mutation. Our integrated analysis of somatic mutations and allele-specific expression levels confirmed the presence of allele-specific expression of somatic mutations in neuroblastoma including MYCN, ALK and PTPN22. The allele-specific expression of mutations suggests that the same somatic mutation may have different effects on clinical outcomes of tumors. Our study also suggests possible involvement of ZNF44 as a candidate driver gene for neuroblastoma. In summary, this study demonstrates the value of examining allele-specific expression levels of somatic mutations through the analysis of RNA-Seq data to assess the effects of somatic mutations in different patients. Improved understanding of allele-specific expression of somatic mutations can facilitate development of personalized treatment for neuroblastoma in precision medicine.

Gene	Chr	Position	Ref	Alt	Patients number
RIMS4	20	44,758,168	C	G	19
RUSC2	9	35,560,530	A	G	8
ALK	2	29,209,798	C	T	7
ALK	2	29,220,829	G	T	6
MYCN	2	15,942,195	C	T	3
PTPN11	12	112,450,394	G	A	2
ALOX12B	17	8,072,868	T	G	2
ZNF44	19	12,273,632	CA	C	2
CNGB1	16	57,962,594	G	GAGCTAGGGGAAGTTGAGGGC	2



398. Engineered Extracellular Vesicles as Versatile Ribonucleoprotein Delivery Vehicles for Efficient and Safe CRISPR Genome Editing

Baisong Lu[1], Xingang Yao[2]

[1]Institute for Regenerative Medicine, Wake Forest Institute for Reg. Med., Winston Salem, NC,[2]School of Pharmaceutical Sciences, Southern Medical University, Guangzhou, China

Transient delivery of CRISPR-based genome editing effectors is important to reduce off-target effects and immune responses. Recently extracellular vesicles (EVs) have been explored for Cas9 ribonucleoprotein (RNP) delivery. However, lack of mechanisms to enrich RNPs into EVs limited the efficiency of EVs as a RNP delivery vehicle. Here we describe a mechanism to actively enrich RNPs into EVs. We used the specific interaction between RNA aptamer and aptamer-binding protein (ABP) to enrich RNPs into EVs. We inserted RNA aptamer com into single guide RNA (sgRNA), and fused com-binding ABP Com to both termini of tetraspan protein CD63 that is abundant in exosomes. We found that the Com/com interaction enriched Cas9 and adenine base editor (ABE) RNPs into EVs, via forming a three-component complex including CD63-Com fusion protein, com-modified sgRNA, and Cas9 or ABE. The RNP enriched EVs are efficient in genome editing and transiently expressed. The system is capable of delivering RNPs targeting multiple loci for multiplex genome editing. In addition, Cas9 from different species can be used together. The EV-delivered RNPs are active *in vivo*. The data show that the aptamer and ABP interactions can be utilized to actively enrich RNPs into EVs for improved genome editing efficiency and safety.

399. Targeted Correction of Severe Combined Immunodeficiency (SCID) of Athabascan-Speaking Native Population

Patricia N. Claudio Vázquez[1,2,3], Walker S. Lahr[1,3,4], Emily J. Pomeroy[1,3,4], Mitchell G. Kluesner[1,3,4], Beau R. Webber[1,3,4], R. S. McIvor[1,2,3,4], Branden S. Moriarity[1,2,3,4]

[1]Department of Pediatrics, University of Minnesota, Minneapolis, MN,[2]Department of Genetics, Cell Biology & Development, University of Minnesota, Minneapolis, MN,[3]Center for Genome Engineering, University of Minnesota, Minneapolis, MN,[4]Masonic Cancer Center, University of Minnesota, Minneapolis, MN

Severe combined immunodeficiency of Athabascan-speaking Natives (SCID-A) is a primary immunodeficiency disease (PID) resulting from deficiency of Artemis protein, which is required for hairpin-opening during nonhomologous end-joining (NHEJ) in V(D)J gene rearrangement. Thus, in SCID-A, the lack of Artemis causes absence or very low abundance of mature T and B lymphocytes and hypersensitivity to double-stranded breaks (DSB), leading to severe infections. Current therapy for SCID-A is allogeneic hematopoietic stem cell transplant (HSCT), for which there is a significant risk of morbidity/mortality. *Ex vivo* lentiviral complementation of Artemis in SCID-A hematopoietic stem cells (HSCs) for HSCT is currently in clinical trials. However, lentiviral gene transfer has shown suboptimal gene expression and adverse genotoxic reactions. As a superior genetic therapy to repair Artemis and avoid genotoxicity, we propose site-directed correction of the founder premature stop codon (pmSTOP) mutation in the *Artemis* gene, testing two gene-editing approaches: homologous recombination (HR) and conservative base editor (BE) substitution.For HR-mediated correction, we utilized Cas9 nickase to avoid genotoxic DSB inductions and delivered a template for DNA repair using recombinant adeno-associated virus (rAAV), achieving >50% correction in K562SCID-A cells of the pmSTOP codon that leads to Artemis deficiency. For BE-mediated conversion, we utilized ABE8.20m-NG and ABE8e-NG to target the mutation. ABE8.20m-NG was unable to convert both adenosine targets while ABE8e-NG was

able to achieve >20% editing (Figure 1) at A1 and A2, respectively, converting the pmSTOP codon to a tryptophan, resulting in a conservative substitution, and restoring the Artemis open reading frame. This conservative substitution was functionally validated using a previously established hairpin-opening assay (Figure 2). We plan to correct Artemis in SCID-A patient HSCs with subsequent *in vitro* and *in vivo* functional testing and analogous genomic analyses to generate proof-of-concept results supporting clinical testing for immune restoration in SCID-A patients. Results of these genetic correction studies will be applicable to the treatment of other inherited diseases by introduction of conservative amino acid substitutions.

Figure 1

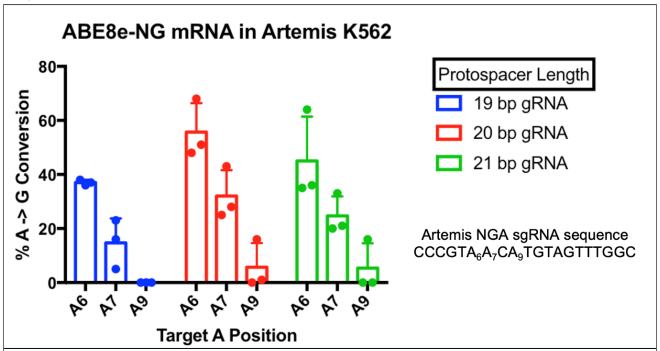


Figure 1. Efficiency attained by Sanger sequencing in Artemis K562s with ABE8e-NG system using 19, 20 and 21 bp gRNAs.

Figure 2

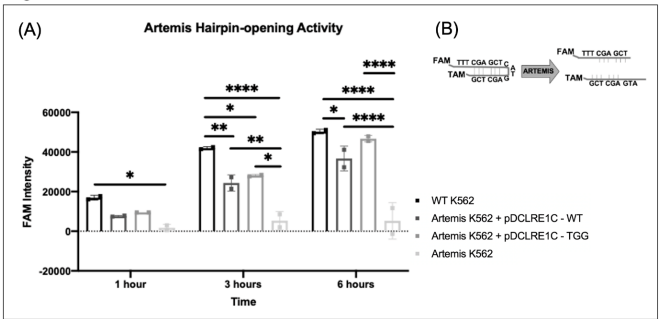


Figure 2. Artemis hairpin-opening activity. **A,** Enzymatic activity of WT K562s, SCID-A K562s and SCID-A K562s transfected with WT plasmid and TGG measured over time. **B,** synthetic hairpin with FAM fluorophore at 5' and TAMRA quencher at 3' cleaved by Artemis.

400. PRIME Editing Permits to Introduce High Frequency Specific Point Mutations in the Dystrophin Gene

Cedric Happi Mbakam, Joel Rousseau, Guillaume Tremblay, Jacques-P Tremblay

Molecular Medicine, CHUL de Québec Research Center, Laval University, Quebec City, QC, Canada

Mutations in the dystrophin gene lead to neuromuscular disorders such as Duchenne Muscular Dystrophy which is a lethal X-linked hereditary disease with the prevalence of 19.8 per 100 000 males' birth. Currently available clinical therapies with corticosteroids or with morpholino antisense oligomer injections provide limited phenotypic improvement. Our study aimed to measure the PRIME editing technology efficiency. This technology uses a PRIME editor plasmid (PE2 or PE3) coding for a Moloney murine leukaemia virus reverse transcriptase fused with the Cas9 H840A nickase, and a plasmid coding for a pegRNA containing a primer binding sites (PBS) and a reverse transcriptase template (RTT). It permits specific nucleotide substitutions, deletions or insertions in the genome. We designed different pegRNAs targeting several hDMD exons (9, 20, 35,43, 52, 55, and 61) to introduce a STOP codon by modifying a single nucleotide. HEK293T cells were harvested from DMEM culture media three days after being simultaneously transfected with the PE2 and pegRNA. Exons were PCR amplified and sequenced using the Sanger method. Results were analysed using the EditR program to estimate the editing percentage. We confirmed that PRIME editing permits the specific C to T, G to A, A to T, and G to T substitutions in the DMD gene with an editing efficiency between 6 to 11 % (PE2) and 23% (PE3). Repeated transfections 6 days after the first one showed up to 15 % (PE2) edition in exons 9 and 35. An additional mutation in NGG PAM sequence (exon 35) improved a PE2 result to 40% for a single transfection. Thus, PRIME editing permits the specific substitutions in the DMD gene and might be used to correct point mutations in the DMD gene to lead to dystrophin expression.

401. Novel Small Molecule Combinations That Favour CRISPR/Cas9-Mediated Homology-Directed Repair

Abhishek Srivastava[1], Suzanne Snellenberg[1], Richard Parker-Manuel[1], Tom Payne[1], Leonard Seymour[2], Ryan Cawood[1]

[1]Discovery, OXGENE, Oxford, United Kingdom,[2]Department of Oncology, University of Oxford, Oxford, United Kingdom

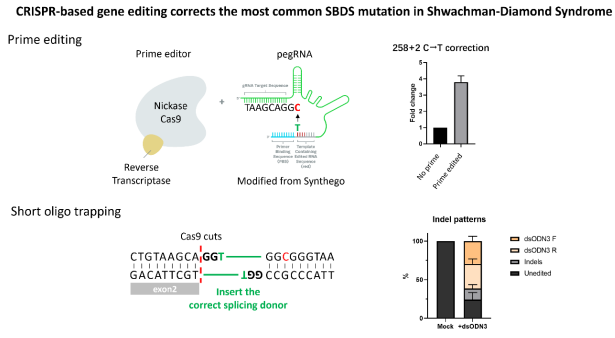
Correction of genetic disorders by gene editing technologies is useful in genome research and shows great therapeutic potential. CRISPR/Cas9 technology can be used for both gene knock-out and knock-in to reverse the effect of a mutated gene. DNA double strand breaks (DSBs) are generated by CRISPR/Cas9, which are typically repaired by the competing repair pathways, non-homologous end joining (NHEJ) or homology-directed repair (HDR). Precise genome editing is facilitated by HDR; however, its efficiency is low and the NHEJ pathway is the preferred choice for repair. Here, we screened small molecules against an in-house generated reporter HEK293 cell line to identify molecules that enhance HDR following editing with wild-type (wt) and nickase Cas9 (D10A). We found four compounds that increased HDR efficiency using wtCas9, and seven increased HDR efficiency using paired nicking. By combining these compounds into cocktails, HDR frequencies were further increased (over 350%) for both wt and nickase Cas9. Altogether, we identified two sets of unique HDR enhancers, one set that works specifically with wtCas9 and a second set that works with paired nickases. The small molecules are easy to use, have minimal effect on cell viability and are compatible with a variety of cell lines and type II and type V CRISPR effector nucleases Cas9 and Cas12a (Cpf1).

402. CRISPR-Based Gene Editing Corrects the Most Common SBDS Mutation in Shwachman-Diamond Syndrome

Chi-Yuan Zhang

Dana-Farber Cancer Institute, Boston, MA

Shwachman-Diamond syndrome (SDS) is an autosomal-recessive disease characterized by multisystem disorders, including hematopoietic dysfunction and leukemia predisposition. Approximately 90% of patients harbor mutations in the Shwachman-Bodian-Diamond syndrome (SBDS) gene on chromosome 7. Here, we compared two CRISPR-based gene editing methods for their ability to correct the by far most frequent mutation, the 258+2T to C splice site mutation in intron 2 of the SBDS gene, and to restore functional splicing. We established an SBDS-mCherry reporter cell line carrying 258+2T to C mutation to facilitate screening for successful editing after gene editing. The first method is Prime editing, which uses an elongated guide RNA to directly copy a 14 nucleotides (nt) wildtype sequence template encoded by the guide RNA into the 258+2 position without inducing double-strand breaks into the genome. Alternatively, we tested the efficacy of using a short oligo trapping approach to reconstitute a functional splice site. A stabilized 30 nt repair oligo was co-delivered with spCas9 and is inserted into the site of DNA double-strand break induced by Cas9 upstream of position 258+2 via non-homologous end joining repair (NHEJ). Both approaches led to ~11% of C to T correction of transfected cells in the SBDS-mCherry reporter line, as assessed by an increase in mCherry fluorescence by flow cytometry and confirmed by sequencing. Since in quiescent hematopoietic stem cells (HSCs) the NHEJ repair pathway dominates, the short oligo trapping strategy was tested in both SDS patient-derived fibroblasts (FHCRC32) and mobilized healthy donor CD34 cells. Within FHCRC32, we reproducibly achieved a correction rate of more than 60%, which recovered the correct mRNA splicing and SBDS protein expression to approximately 90% of the wildtype. Within CD34 cells, we targeted the pseudogene of SBDS1, called SBDSP1, since it also harbors the 258+2T to C mutation and is readily available using healthy donor HSC rather than rare SDS patient HSCs. Furthermore, in SBDSP1, the repair outcomes are not influenced by potential selection processes as is expected to be the case for editing of the SBDS1 locus. In this setting, we were able to achieve a correction rate of more than 40%. Our findings indicate that the most common 258+2T to C mutation in SBDS is amenable to gene editing based correction using two different CRISPR-based methods. Ongoing experiments aim at further improvement of repair rates, assessment of the risks, benefits, and threshold correction rates for each methodology.



Gene Targeting and Gene Correction

403. Investigating the Impact of Donor Co-Localization on Homology-Directed Repair with a Cas9-Monomeric Streptavidin Fusion and Biotinylated Donors

Grace Ellen McAuley[1], Ralph Valentine Crisostomo[2], Zulema Romero[1], Donald B. Kohn[3]

[1]Microbiology, Immunology & Molecular Genetics, University of California, Los Angeles, Los Angeles, CA,[2]Microbiology, Immunology & Molecular Genetics; Molecular Biology Interdepartmental Program, University of California, Los Angeles, Los Angeles, CA,[3]Microbiology, Immunology & Molecular Genetics; Molecular and Medical Pharmacology; Pediatrics, University of California, Los Angeles, Los Angeles, CA

Genome editing techniques involving CRISPR/Cas9 associated systems have revolutionized the field of biology by utilizing the overlapping pathways of DNA repair to construct permanent genetic changes. The two main pathways of double-stranded DNA repair in eukaryotic cells are known as homology-directed repair (HDR) and non-homologous end joining (NHEJ). The error-prone NHEJ pathway generates various insertions or deletions at the Cas9-induced double-stranded break (DSB) sites. Alternatively, HDR utilizes homologous donor DNA to yield precise modifications of the DSB. HDR is of particular interest to genetic editing for its ability to encode precise and specific edits. However, increasing the ratio of HDR to NHEJ is complex due to HDR's short active span in the cell cycle and competition with NHEJ in the S and G2 phases. Another major limitation of HDR is the time needed to search for a homologous donor template. Donor co-localization to the DSB has been shown to increase HDR events up to 90% in experiments utilizing a cationic polymer, polyethylenimine, to deliver Cas9-monomeric streptavidin (Cas9-mSA) ribonucleoprotein (RNP)-biotinylated donor complexes into HEK293 cells (Roche et al, 2018). Additionally, studies using Cas9-mSA mRNA and biotinylated donor DNA microinjected in murine embryos at the HDR-permissive two-cell stage produced up to 95% knock-in efficiency (Gu et al, 2018). Previous reports utilizing the high affinity avidin-biotin binding approach for donor co-localization fail to describe the impact of the technique through electroporation delivery unaccompanied by additional HDR-enhancing factors. To address this gap, we co-electroporated Cas9-mSA with biotinylated single-stranded oligo donors (ssODNs) to determine if donor co-localization alone can eliminate the cellular search for a homologous donor, and push DNA repair towards HDR. Here, we examine the effect of HDR-mediated gene correction using an spCas9-mSA (Gu et al, 2018) alongside a variant including a flexible GSG (Gly-Ser-Gly) linker (spCas9-GSG-mSA). Using the Lonza nucleofector, we co-electroporated the Cas9 plasmid fusions in a BFP+ K562 reporter cell line with 5'-biotinylated GFP ssODNs complementary to the upper or lower strand of the BFP gene. In parallel, we co-electroporated in-vitro transcribed (IVT) Cas9-mSA mRNA in the HL60 and Jurkat cell lines with 5'-biotinylated ssODNs targeted to the *HBB* gene to knock-in two base changes. Flow cytometry was used to analyze editing outcomes in the BFP+ K562 cells, with green fluorescence representing HDR, while Tracking Indels by Decomposition (TIDE) and Inference of CRISPR Edits (ICE) analysis were used to interpret editing results from experiments in HL60 and Jurkat cells. Preliminary data show

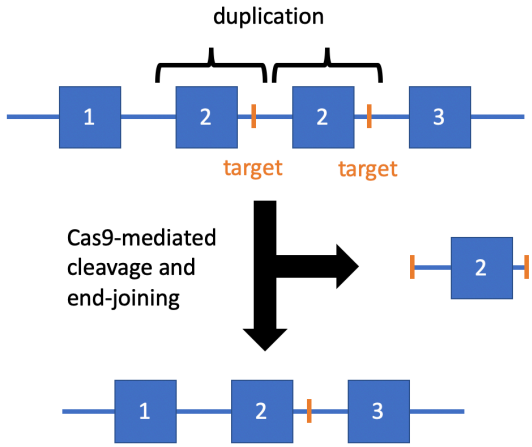
nonsignificant improvements in the HDR/NHEJ ratio in both the K562 BFP to GFP model and studies targeted to the HBB locus. However, comparisons of spCas9-mSA and spCas9-GSG-mSA suggest a flexible linker can enhance overall nuclease activity. We demonstrate that donor co-localization alone is not sufficient to significantly improve the HDR/NHEJ ratio through electroporation of editing reagents. Further studies will focus on editing outcomes in human peripheral blood stem cells (hPBSCs) to validate our results. Studies will also be done to test additional factors affecting the avidin-biotin binding approach such as, steric hindrance at the DSB alongside the kinetics of nuclease activity and avidin-biotin binding and release.

404. CRISPR/Cas9 Correction of Single and Multi-Exon Duplications in Duchenne Muscular Dystrophy

Stefan Nicolau[1], Anthony A. Stephenson[1], Tatyana A. Vetter[1], Julian Havens[1], Liubov V. Gushchina[1], Kevin M. Flanigan[1,2]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Departments of Neurology and Pediatrics, Ohio State University, Columnus, OH

Duchenne muscular dystrophy (DMD) is an X-linked disorder presenting with muscle weakness, elevated creatine kinase levels, and cardiomyopathy. The disorder is caused by mutations in the *DMD* gene, most commonly single or multiple exon deletions or duplications. The latter account for 10-15% of all DMD patients and are most commonly located in the 5' region of the gene. Exon 2 is the most frequently duplicated exon. In these patients, genome editing via the CRISPR/Cas9 system has the potential to restore full-length dystrophin expression. We devised a genome editing strategy employing a single guide RNA (gRNA) targeting the duplicated region, which comprises exon 2 and part of the adjacent introns. This approach would allow correction of isolated duplications of exon 2, as well as multi-exon duplications spanning exon 2 (figure). Potential gRNAs targeting human *DMD* were screened in vitro in HEK293 cells and further evaluated in patient-derived myogenic cell lines. The most active gRNA achieved ~65% correction of exon 2 duplication and ~45% correction of exon 2-6 duplication on the transcript level in patients' cells. This demonstrated the feasibility of a single gRNA approach to delete large (up to ~200 kb) regions of genomic DNA and correct single or multi-exon duplications. Mouse-targeting gRNAs were screened in vivo by electroporation in a mouse model of exon 2 duplication. The most active mouse-targeting gRNA was packaged into an AAV vector and used for further in vivo studies. Intramuscular injection was performed in the tibialis anterior of 12-week-old mice. Quantitative immunofluorescence analysis performed 4 weeks post-injection showed restoration of dystrophin expression in 15% of muscle fibres. These studies lay the groundwork for further translational evaluation of exon duplication correction for DMD using the CRISPR/Cas9 system.



405. *Investigational Genetic Medicine Approaches for Phenylketonuria (PKU)*

Diana Lamppu, Jason Wright, Ludovic Benard, Eric Faulkner, Nancy Avila, Omar Francone, Albert Seymour

Homology Medicines, Inc, Bedford, MA

PKU is an autosomal recessive monogenic disorder. >98% of cases are due to mutations in the phenylalanine hydroxylase (*PAH)* gene, resulting in deficient PAH activity, a hepatic enzyme that catalyzes the formation of tyrosine from phenylalanine (Phe). Untreated PKU results in progressive, irreversible neurological impairment during infancy and early childhood (Lichter-Konecki, 2019). Restricting protein and Phe intake is standard of care for most PKU patients. Relaxing of dietary restrictions results in loss of metabolic control and wide fluctuations in Phe that are associated with progressive neurological damage (Christ, 2010; Enns, 2010; Janzen, 2010). Current U.S. treatment guidelines (Vockley, 2014) indicate that blood Phe ≤360 µM does not require therapy or dietary modification. Gene therapy has the potential to deliver a functional copy of human *PAH* (hPAH) to the liver, restoring PAH enzyme activity and normal Phe levels thus potentially eliminating dietary restrictions. A gene transfer (GT) approach, with episomal gene expression, is feasible in a fully developed liver. Because the human liver undergoes rapid growth during the first 10-15 years, (Pryce, 2014; Shankle, 1983; Chouker, 2004), a gene editing (GE) approach with integration of the *PAH* gene into the target locus would allow maintained expression throughout the period of growth as the cells divide. GT and GE vectors expressing hPAH were packaged in AAVHSC15. The GE vector transgene has locus- and species-specific homology arms flanking the *hPAH* sequence that are designed to guide the DNA to the target *PAH* locus and integrate through non-nuclease-based, AAV-mediated homologous recombination. Mouse surrogate vectors were used in a murine model of PKU (Pah^{enu2}), while a human-targeted GE vector was used in a humanized-liver murine model. The vectors were administered as a single intravenous (IV) injection. The Pah^{enu2} mouse has baseline blood Phe >1000µM. These mice were fed normal chow throughout the studies for consistent Phe intake. The xenograft mouse has a liver comprised of >90% human hepatocytes, following gradual repopulation of the liver compartment

with human hepatocytes (Azuma, 2007). The xenograft model does not have *Pah/PAH* gene mutations. Phe was measured by mass spectrometry. Livers were processed to measure vector genomes and mRNA by ddPCR, and integration of the GE vector transgene in the target locus by next generation sequencing (NGS). Data from Pah^{enu2} mice were used to establish a dose-response relationship between hPAH mRNA expression and Phe levels. Results from the humanized-liver murine model were compared to results from Pah^{enu2} mice, providing the basis for dose extrapolation from the mouse surrogate to the human-specific construct. Blood Phe normalization (<120 μM) in Pah^{enu2} mice was achieved within 1-2 weeks post-dose and maintained for the duration of the studies with both GT and GE vectors. Similar hPAH mRNA levels were observed across species with the mouse- and human-specific constructs and were consistent with levels needed for Phe reduction in Pah^{enu2} mice. Gene insertion at the target locus was confirmed by NGS analysis. Integration rates were similar in Pah^{enu2} mice and human-liver xenograft mice. These data demonstrated that a single IV dose of AAVHSC15-based GT or GE gene therapy resulted in a sustained reduction of Phe in Pah^{enu2} mice on a normal chow diet. The human-targeted GE vector demonstrated human-specific editing. The option of a GT or GE approach has the potential to deliver the hPAH gene to hepatocytes resulting in sustained expression in a fully developed liver or a growing liver, respectively.

406. Leveraging CHANGE-seq to Define the Genome-Wide Activity of Cas12a

Cicera R. Lazzarotto, Yichao Li, Varun Katta, Shengdar Q. Tsai

St Jude Children's Research Hospital, Memphis, TN

CRISPR-Cas systems, first discovered in prokaryotes as essential for bacterial adaptative immunity, have become broadly adopted as an easily programmable genome editing technology. Novel genome-editing based therapies based on Cas9, the first CRISPR-Cas nuclease widely used for genome editing, are advancing to various stages of human clinical trials. Cas12a nucleases, derived from a type V CRISPR system, is an alternate genome editing tool with substantial differences from Cas9: (1) Cas12a is a single-RNA-guided nuclease that does not require a tracrRNA; (2) Cas12a cleavage activity produces staggered double-stranded breaks (DSBs); (3) Cas12a recognizes thymidine-rich DNA sequences as the PAM at the 5' end (5'-TTTV-3') of target sequences. The alternate PAM requirement of Cas12a offers a unique opportunity to target sites in the genome that lack the 3'-NGG PAM sites required by SpCas9. Despite broad adoption of CRISPR-Cas systems for genetic engineering, CRISPR-Cas genome editors lack perfect specificity for their target sites and unintended 'off-target' sites may be affected. We recently developed CHANGE-seq (Circularization for High-throughput Analysis of Nuclease Genome-wide Effects by Sequencing), a fast, streamlined, Tn5 tagmentation-based assay for measuring the genome-wide activity of Cas9 *in vitro* that is easily scalable to many targets and samples, allowing us to understand fundamental principles that determine engineered nucleases cellular specificity. To understand factors that contribute to Cas12a specificity, we optimized CHANGE-seq for Cas12a. Due to differences in DNA ends resulting from Cas9 and Cas12a cleavage activity, adjustments in the CHANGE-seq workflow were required to achieve proper enrichment and identification of Cas12a on- and off-target cleavage sites genome-wide. We assessed the genome-wide activity of AsCas12a (AsCas12a Ultra - IDT) in two crRNA target sites (*DNMT1* site 3 and Matched site 6) previously evaluated by GUIDE-seq using CHANGE-seq, and all sites identified in cells by GUIDE-seq were identified *in vitro* by CHANGE-seq. Additionally, we leveraged CHANGE-seq to define the genome-wide off-target activity of four engineered AsCas12a variants (enCas12a, enCas12a RR, enCas12a RVR, enCas12a-HF1), in the two crRNA target sites mentioned above. As expected, we found that the specificity of Cas12a-HF1 *in vitro* was higher than the other variants for both of the target sites evaluated. Currently, we are applying CHANGE-seq to perform high-throughput characterization of therapeutically relevant Cas12a targets for genome editing of human primary T cells. CHANGE-seq enables the rapid characterization of Cas12a genome-wide activity at a scale not previously achievable by other methods and represents a simple and rapid method to routinely define global genome editing activity of CRISPR-Cas12a.

407. Abstract Withdrawn

408. Determinants of Persistence in Epigenetic Editing

Henriette O'Geen, Marketa Tomkova, David J. Segal

Genome Center UC Davis, Davis, CA

Precise regulation of gene expression is critical for development and cell identity. Misregulation of this tightly controlled process can lead to disease such as cancer or neurological disorders. Distinct epigenetic marks (DNA methylation and post-translational histone modifications) have been associated with expressed or silenced genes as well as with regulatory elements in the genome. Traditionally, epigenetic drugs are used to induce changes in an untargeted manner and thus can alter epigenome signatures throughout the genome. With the RNA-guided Cas9/CRISPR complex, we now have a tool that can easily and precisely target a 20-bp sequence in the genome. Targeted epigenetic regulators (epi-dCas9) are based on fusions of epigenetic effector domains to the catalytically inactive dCas9. Epi-dCas9 can regulate transcription in a targeted manner without altering genetic information. Longevity and heritability of induced epigenetic changes and associated expression changes have not been comprehensively studied. We and others have shown that targeting dCas9 fused to a single effector domain is rarely sufficient to induce a long-term repressed state, but a combination of dCas9-fusions to transcriptional repressors (KRAB-dCas9) and DNA methyltransferases (DNMT3A-dCas9 + DNMT3L) is required for engineering robust and stable silencing. Although combinatorial epi-dCas9 treatment induces the epigenetic switch and change in gene expression, endogenous cellular processes are required to maintain this altered epigenetic state. So far studies of so called hit-and-run engineering of heritable silencing have focused on a handful of genes with success that is largely gene and cell-type specific. Clearly, a better understanding is needed of the targetable epigenome that is amenable to persistent gene silencing and an understanding of the pathway(s) to accommodate persistent gene silencing. Here we investigate the impact of pre-existing chromatin environment that make a target locus amenable to persistent targeted epigenome editing. Correlating the persistence of gene silencing with ENCODE RNA expression, epigenome, and 3D interaction data offered a glimpse of critical chromatin features in one cell type. In addition, the ability to engineer

persistent epigenetic silencing will depend on the available cellular factors in the target cell. We therefore combined epi-dCas9 treatment with a orthogonal CRISPR-based knock out screen to identify cellular genes involved in epigenetic persistence. Not only will this information advance the capabilities of us and others to create targeted persistent epigenetic changes for the study and treatment of disease, it will also provide fundamental insights into the mechanistic steps required to transition from one epigenetic state to another.

409. A Novel CRISPR Associated Type V Editing System Derived from Metagenomic Samples with Potent Activity in Liver Cells

Morayma M. Temoche-Diaz, Janet Mei, Cristina N. Butterfield, Andres Perez Rivas, Daniela S. A. Goltsman, Christopher T. Brown, Brian C. Thomas, Alan R. Brooks

Metagenomi, Emeryville, CA

CRISPR-Cas based genome editing systems are being widely used to make specific changes to mammalian genomes with the goal of treating a wide range of human diseases. The liver is an attractive target organ for in vivo genome editing therapies because of the availability of delivery technologies including AAV and lipid nanoparticles that can efficiently deliver nucleic acids. However, attempts to edit the liver in vivo have relied almost exclusively on the Cas9 nucleases derived from Streptococcus pyogenes or Staphylococcus aureus. Recent publications demonstrating that humans have pre-existing immunity to both these proteins raises concerns about their clinical application. We have harnessed metagenomics to create a large database of previously unidentified CRISPR systems. In vitro screening followed by validation in mammalian cells in culture identified several active systems including MG29-1, a member of the Type V CRISPR family. MG29-1 is 1280 amino acids in length, exhibits only 41 % amino acid identity to Francisella tularensis Cas12a/cpf1, and recognizes a KTTN PAM (estimated frequency 1 in 16 bp). It utilizes a single guide RNA (sgRNA) comprised of a 22 nt constant region and a 20 to 25 nt spacer. MG29-1 was identified from an unclassified bacterial species found in a deep-sea hydrothermal vent and therefore it is unlikely that humans have pre-existing immunity to this nuclease. Previously, we reported that MG29-1 was active in HEK293 cells (Aliaga Goltsman et al: The CRISPR Journal, 3, 454-461). To evaluate the potential of MG29-1 to edit genes in the liver, 19 sgRNAs targeting mouse serum albumin were evaluated in the mouse liver cell line Hepa1-6. Editing was tested by nucleofection of ribonuclear proteins (RNP), and by co-transfection of sgRNA and mRNA encoding MG29-1. Eight of the sgRNAs generated INDELS at a frequency of 50% or greater by one or both transfection methods, and six guides generated greater than 80% INDELS. One of the most potent MG29-1 guides exhibited similar or superior activity as commercial spCas9 over a range of RNP doses from 1 to 20 pmoles per 2e5 cells. In order to identify highly modified guides with increased resistance to nucleases we tested chemical modifications (2' O-methyl, 2' Fluoro and phosphorothioate) at different positions in the sgRNA. The editing efficiency of the chemically modified guides was evaluated in Hepa1-6 cells. Guides with chemical modifications on up to 60% of the bases retained full activity. The potent editing activity, small sgRNA, and a protein size compatible with delivery of the nuclease and guide in a single AAV, makes MG29-1 an attractive candidate for in vivo editing applications targeting the liver.

410. Epigenome-Base Strategies for Efficient and Safe Editing of APOEε4 Allele: Implications for Alzheimer's Disease

Ornit Chiba-Falek[1], Ashley Kilgore[1], Gabriella MacDougall[1], Joseph Rittiner[2], Boris Kantor[2]

[1]Neurology, Duke University, Durham, NC,[2]Neurobiology, Duke University, Durham, NC

Carrying the APOEε4 variant significantly increases lifetime risk for Late Onset of Alzheimer's disease (LOAD), and the presence of two copies is associated with further increased risk for earlier disease onset. We and other groups have suggested that alteration in the expression of APOEε4 isoform may play important role in the etiology of LOAD. Here, we report the development of novel epigenome-editing approach utilizing CRISPR/Cas- tools to achieve an efficient and specific augmentation of APOE/ε4 expression. The developed system based on CRISPR-deactivated Cas9 (dCas9) variant VRER fused with catalytic domain of DNA-methyltransferase 3A and 3L (DNMT3A & 3L). Applying the system to human induced pluripotent stem cell (hiPSC)-derived mature neurons carrying ApoEε4 alleles resulted in fine downregulation of APOE mRNA and protein, such providing a groundwork and *proof-of-concept* for the development of new gene therapy-based approach for LOAD. Our epigenome therapy strategy for intervention with *APOE* expression based on dCas9 technology is *translational* toward the development of 'smart drug' for LOAD.

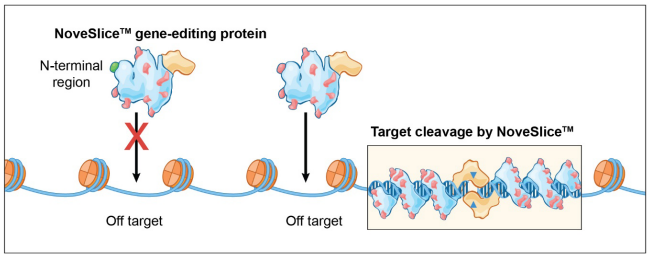
411. Removing T_0 Constraint Reveals Differences in Specificity of Engineered Gene-Editing Proteins

Mackenzie Parmenter, Mitchell R. Kopacz, Christopher B. Rohde, Matthew Angel

Factor Bioscience Inc., Cambridge, MA

Gene editing proteins offer an efficient means of knocking out, inserting and repairing nucleic-acid sequences in living cells. However, while gene-editing proteins can efficiently target pre-determined sequences, they can also cleave similar sequences throughout the genome, albeit with lower efficiency (so-called "off-target" editing). Many gene editing technologies include design constraints that limit the sequences that can be targeted, for example CRISPR-Cas9 requires a PAM sequence and TALENs require a thymine (T) in the zero position of the target site ("T_0"). A recently described temperature-sensitive gene-editing protein with flexible linkers, NoveSlice, shares the T_0 requirement, limiting the available target sites in regions of low sequence complexity. We sought to remove the T_0 requirement of TALEN and NoveSlice through amino-acid substitution of key residues in the N-terminal region of the DNA-binding domain. We designed and synthesized constructs encoding NoveSlice and TALEN proteins comprising various N-terminal regions using site directed mutagenesis. We tested the gene editing efficiency of these novel proteins by mRNA transfection into primary human keratinocytes. After 48 hours we amplified the target site, a region near the COL7A1 exon 73 splice acceptor site, and assessed editing using

T7 endonuclease I. From this initial screen, we identified the most promising N-terminal region that had the highest editing efficiency in a target site with an N_0. Surprisingly, when measuring cutting of an N_0-containing target site, we observed "off-target" editing by TALENs while there was no editing by NoveSlice under these same conditions. Thus, the NoveSlice gene-editing protein showed a higher degree of specificity when compared to TALENs for this clinically relevant target site. These data suggest that the NoveSlice gene-editing protein can yield higher specificity than TALENs targeting the same site in primary human cells, and thus could offer an advantage in the development of both *ex-vivo* and *in-vivo* gene editing therapies.



412. In Situ Gene Editing of Retinal Pigment Epithelium <RPE>by TAGE, Novel Programmable Ribonucleoproteins

Rina Mepani

Spotlight Therapeutics, Hayward, CA

Despite remarkable advances, delivery remains the most significant hurdle to realizing the full therapeutic potential of genome editing. To enable *in vivo* cell-selective delivery of gene editing molecules safely and efficiently, we developed a new class of biologics called Targeted Active Gene Editors (TAGE). TAGE are modular, programmable ribonucleoproteins that target selected cell types and edit specific genes. Here, we demonstrate preclinical proof of principle with *in vivo* retinal pigment epithelium (RPE) cell editing following a single subretinal TAGE administration. Using the same ocular-targeted scaffold with a base editor payload, we evaluated whether the TAGE could mediate a functional effect in an RPE disease model. These studies, bypassing the complexities and safety concerns associated with viral and nanoparticle delivery vehicles, demonstrate the broad potential of *in vivo* TAGE delivery for gene editing.

413. Abstract Withdrawn

414. MiniABCA4 Gene Replacement Therapy: A Single Vector-Based and Mutation-Independent Approach to Treat ABCA4-Associated Retinal Disease

Bhubanananda Sahu[1], Jennifer Lecouter[2], Abraham Scaria[2], Kourous Rezaei[2], Guangping Gao[3], Hemant Khanna[1]

[1]Ophthalmology and Visual Science, University of Massachusetts Medical School, Worcester, MA,[2]Iveric Bio, Iveric Bio, New York, NY,[3]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA

Mutations in the ATP-binding cassette-A4 (ABCA4) lead to autosomal recessive forms of Stargardt disease (STGD1) and is a frequent cause of early onset retinal degeneration (~1/8,000-10,000 individuals). Adeno-associated viral (AAV) vectors are currently the most efficient vectors for gene delivery to the retina. However, the development of a gene therapy for ABCA4 has been challenging because the size of the *ABCA4* cDNA (~6.8 kb) is too large to be packaged into conventional AAV vectors. Although dual vector approaches have been previously reported to show some promise in animal models, our goal is to design a mutation-independent gene therapy delivered with a single AAV vector to treat STGD1. We designed five shorter versions of ABCA4 (miniABCA4) that can be delivered into the subretinal space using single AAV vectors. The AAV-miniABCA4 transgenes were packaged into AAV capsids and delivered subretinally into 4-6 weeks old *Abca4*^{ko} mice. We show that three out of five minigenes can reduce, in an age-dependent manner, the autofluorescence in the retinal pigmented epithelium, which is caused by the absence of ABCA4 in the retina. In addition, diminished autofluorescence is well correlated with immunofluorescent detection of miniABCA4 expression in the restored photoreceptor outer segments, which are lost in the late stage of Stargardt disease. Our preliminary results indicate that the miniABCA4 approach with conventional AAV vectors may have the potential for mutation-independent gene therapy STGD1 patients.

415. Exploring the Potential of GSTM1 Gene Augmentation in a Cellular Model of Dry-AMD

Manas R. Biswal, Riddhi Vichare
Pharmaceutical Sciences, Taneja College of Pharmacy, University of South Florida, Tampa, FL

Purpose: Loss of Retinal Pigment Epithelial (RPE) cells and photoreceptors leads to Age-related macular degeneration (AMD), one of the leading causes of blindness in the elderly worldwide. Oxidative stress-mediated damage is one of the factors that impairs the survival of RPE and photoreceptors in dry-AMD. We sought to determine the role of human Glutathione S-Transferase Mu-1 (GSTM1) in protecting RPE cells from oxidative stress-induced changes. **Method**: We cloned human GSTM1 cDNA with P2A tag into a lenti-plasmid vector and later made a lentivector to express GSTM1. After that, we infected human retinal pigment epithelial cells (ARPE-19), screened stable ARPE-19 cells expressing GSTM1 with supplementation of Puromycin antibiotic in the media, and performed western blotting to check GSTM1 expression in stable cells. To mimic cellular model of Dry-AMD, H_2O_2 was used to induce oxidative stress on stable and control ARPE-19 cells. We employed MTT assay and flow cytometry to study cell viability. Real time PCR (RT-PCR) was used to measure other antioxidant genes' expression in response to GSTM1 gene augmentation. For immunocytochemical analysis, we stained differentiated RPE cells with ZO1 junctional protein and fluorescence images were analyzed to evaluate cellular integrity. We also cloned GSTM1 cDNA into an AAV vector and tested in cell culture to validate protein expression. **Results**: The western blotting with GSTM1 antibody confirmed exogenous GSTM-1 protein expression and further affirmed P2A antibody. The MTT (n=6) and flow cytometry assay (n=3) demonstrated significant cell viability compared to the control cells ($p< 0.05$) in response to H2O2 (200μM and 500μM) induced oxidative stress. The RPE junctional integrity was maintained in the GSTM1 expressing differentiated cells as compared to the controls. RT-PCR analysis is in progress to check the induction of antioxidant genes. **Conclusion**: GSTM1 gene augmentation in RPE cells can protect against oxidative stress-mediated changes. Our research opens an avenue for a potential gene therapy strategy using AAV to reverse oxidative stress-mediated changes, thus preventing RPE cell death associated with progressive vision loss in Dry-AMD.

416. Optimization of Long-Read Sequencing Protocol to Assess the Efficacy of Gene Editing for Duchenne Muscular Dystrophy

Landon A. Burcham, Christopher E. Nelson
Biomedical Engineering, University of Arkansas, Fayetteville, AR

Duchenne Muscular Dystrophy (DMD) is an X-linked recessive monogenic disorder, which makes it an ideal candidate for *in vivo* gene editing. It affects 1 in 5000 males and the life expectancy is 26 years of age. The principal symptom associated with DMD is severe muscle weakness, which ultimately leads to cardiomyopathy and compromised respiratory function. Previous work has shown that CRISPR-mediated genome editing is sustained for over one year in mouse models of DMD and muscle function has been restored in large animal models of DMD. Important safety considerations remain unanswered including the immune response to CRISPR and the creation of unintended genome modifications. The aim of this work was to optimize a long-read sequencing protocol for unbiased assessment of genome-editing outcomes in the genomic DNA and transcript. A reliable method for assessing unintended on-target outcomes is crucial as numerous effects have been observed, such as large deletions, inversions, and vector integration in viral delivery applications. Aberrant splicing events have also been observed by targeted RNA sequencing as well as multi-exon skipping. CRISPR gene editing is known to induce alternative splicing of mRNAs, which could lead to multiple RNA isoforms that result from a single genome editing approach. The diverse RNA isoforms are difficult to detect with short-read sequencing, and thus cDNA analysis using a reliable long-read sequencing workflow will give insight into unexpected editing outcomes. In this study, we used dual guide RNAs for targeted deletion of exon 23 to restore dystrophin expression in mice. We compared on-target modifications with multiple delivery approaches, including viral vector delivery. Several enrichment approaches were explored to optimize for read depth, including PCR amplification, hybridization capture, and CRISPR digestion. For PCR amplification, primers were optimized for amplification of ~12kb of the gDNA or cDNA. The hybridization-based capture approach involves optimizing conditions for DNA fragmentation and hybridization to single-stranded oligonucleotide probes. The CRISPR-based enrichment involves the cleavage and isolation of target regions using the dual CRISPR gRNAs. Initial optimizations were performed on DNA that was extracted from NIH3T3 cells. Mouse cells were transfected with a pair of single guide RNAs that excised exon 23 and sequenced using long-read sequencing (Oxford Nanopore) and validated by short-read sequencing (Illumina). After optimizing the enrichment and sequencing protocols in cell culture, mouse samples that had been treated with CRISPR-Cas9 were used for further study and optimization. The next stage of this work is to sequence samples that utilize viral vector delivery to assess vector integration during gene editing.

417. Nonviral Gene Delivery of CRISPR Epigenome Editing System to Human Mesenchymal Stem Cells

Andrew Hamann, Kelly Broad, Tyler Kozisek, Angela K. Pannier
Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE

Introduction: Human mesenchymal stem cell (hMSC) applications could be advanced by safe and efficient gene delivery. Nonviral systems, which usually deliver plasmid DNA (pDNA) complexed with cationic reagents, are safer and more flexible than viral vectors, but are less efficient, especially in hMSCs. In this work, we optimize a transfection protocol to enable, for the first time nonvirally in hMSCs, CRISPR epigenome editing to upregulate expression of target genes with dCas9-p300 and promoter-targeting guide RNAs (gRNAs), a technology first demonstrated in the easily transfected HEK293T cells [1]. **Methods:** HEK293Ts or adipose hMSCs (hAMSCs) were seeded in 96-well plates and transfected with three commonly used reagents (Turbofect™, Lipofectamine 3000™, or 25 kDa branched polyethylenimine), complexed with pDNA encoding for reporter proteins to optimize pDNA and reagent dose. In subsequent experiments using optimized doses, pDNA encoding dCas9-EGFP or dCas9-p300 was co-delivered with a gRNA targeting the IL1RN

promoter [1], either from a separate pDNA which also encoded for the dsRed fluorescent reporter, or as synthetic RNA oligos. Reporter expression was quantified with imaging after 48 hrs, and target IL1RN mRNA upregulation was quantified by qRT-PCR. **Results:** Optimized EGFP transfection of HEK293Ts and hMSCs with pDNA (120 ng/well) complexed with Turbofect at a 1:3 ratio (µg:µL), resulted in about 70% and 20% transfection efficiency, respectively, and was significantly higher than with optimized LF3K and PEI complexes. Using these optimized doses, dCas9-EGFP transfection efficiency was about 85% and 10% in HEK293Ts and hAMSCs, respectively (**Fig 1A**), while transfection efficiency of the co-delivered gRNA pDNA reporter (dsRed) was about 65% and 5% in HEK293Ts and hAMSCs, respectively (**Fig 1B**). Consistent with seminal work [1], dCas9-p300 co-delivered with gRNA pDNA to HEK293Ts resulted in IL1RN mRNA upregulation of over 3000-fold, relative to controls with no gRNA delivery. In comparison to HEK293Ts, identically treated hAMSCs displayed little to no IL1RN mRNA upregulation over controls, presumably due to low transfection efficiency. To achieve higher gRNA delivery than when delivered on separate pDNA in hAMSCs, we used synthetic gRNA oligos co-delivered with dCas9-p300 pDNA, which achieved up to 50-fold target gene IL1RN mRNA upregulation over controls in some hAMSC donors, but synthetic gRNA toxicity also resulted in significantly decreased cell viability.

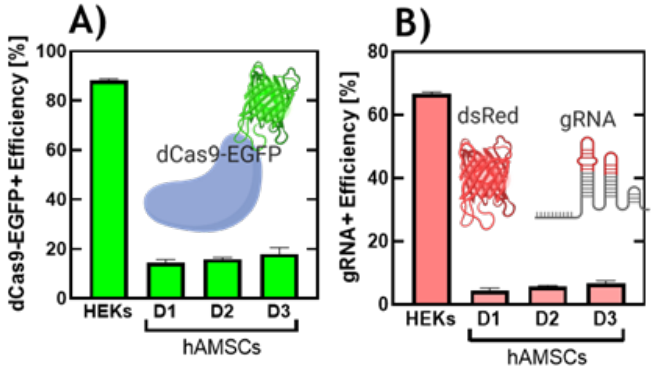


Figure 1: **A)** dCas9-EGFP transfection efficiency in HEK293T cells (HEKs) and hAMSCs derived from multiple donors (denoted as D#) after optimized co-delivery of pDNA expressing dCas9-EGFP (60 ng/well) and pDNA expressing dsRed reporter with gRNA (60 ng/well) **B)** Corresponding gRNA transfection efficiency from experiment described in **A**. Figure created with BioRender.com. **Conclusion:** These results demonstrate the significant challenge of translating technologies first described in easily transfected cell lines to therapeutically relevant primary cells using safe gene delivery methods. Future work aims to increase transfection efficiency of CRISPR systems to hMSCs, in part by delivering both gRNA and dCas9 within the same pDNA. **References:** [1] Hilton et al, *Nat Biotechnol*, 2015, 33, 510.

418. The New Frontier in the Development of LOAD Therapies Targeting APOE

Anna Yang[1,2], Boris Kantor[3,4,5], Ornit Chiba-Falek[6,7]

[1]Duke University, Durham, NC,[2]Division of Translational Brain Sciences, Department of Neurology, Duke University Medical Center, Durham, NC,[3]Viral Vector Core, Duke University, Durham, NC,[4]Department of Neurobiology, Duke University Medical Center, Durham, NC,[5]Duke Center for Advanced Genomic Technologies, Duke University, Durham, NC,[6]Department of Neurobiology, Duke University, Durham, NC,[7]Center for Genomic and Computational Biology, Duke University Medical Center, Durham, NC

Alzheimer's disease (AD) has a critical unmet medical need. The consensus around the amyloid cascade hypothesis has been guiding pre-clinical and clinical research to focus mainly on targeting beta-amyloid for treating AD. Nevertheless, the vast majority of the clinical trials have repeatedly failed, prompting the urgent need to refocus on other targets and shifting the paradigm of AD drug development towards precision medicine. One such emerging target is apolipoprotein E (APOE), identified nearly 30 years ago as one of the strongest and most reproduceable genetic risk factors for late-onset Alzheimer's disease (LOAD). Here we conducted a comprehensive literature search to explore APOE as a new therapeutic target for AD treatments. We specifically review three avenues of gene therapy strategies for targeting apoE including antisense oligonucleotides, gene editing, and base editing. Carrying the APOE e4 variant significantly increases the lifetime risk for LOAD, while APOE e2 confers a protective effect. Gene therapy approaches have the potential to facilitate two mainstream strategies for LOAD treatment: reduce the risk allele - e4 and generate the protective allele - e2. Three approaches have showed promising results in targeting APOE e4: (1) An ASO that led to an APOE-mRNA reduction of about 50% and showed a decrease in Aβ deposition and plaque-associated neuritic dystrophy in a mouse model of amyloidosis. (2) CRISPR-Cas9 gene editing by knock in repair of the e4 allele showed promising results in a hiPSC derived model in which conversion from e4 to e3 ameliorated various AD-related phenotypes including increased tau phosphorylation, Aβ production, and GABAergic neuron degeneration. (3) Base editing through the conversion of a single-base point mutation of C to T using a modified deactivated Cas9 showed greater efficiency in the correction of the e4 allele to e3 compared to typical gene editing methods while also reducing the number of non-specific events. Two approaches have also showed promising results mediated by APOE e2: (1)Harnessing the protective effect of apoE2 through viral-mediated overexpression and (2) gene/base editing conversion of apoE4 to apoE2 as promising treatment methods for AD. The literature search confirmed both the suitability and applicability of APOE as a potential target moving closer towards the development of a novel precision medicine gene-therapy for AD.
Keywords: APOE, Alzheimer's Disease, Beta-Amyloid, Gene Therapies, Antisense Oligonucleotides, Gene Editing

419. VDJ Targeting - Nuclease-Free B Cells Engineering Using VDJ Recombination

Daniel Nataf, Iris Dotan, Alessio D. Nahmad, Tal Akriv, Miriam Horovitz-Fried, Adi Barzel

School of Neurobiology, Biochemistry & Biophysics, Tel Aviv University, Tel Aviv, Israel

B cell engineering has remarkably evolved is the last few years, harnessing the potential of B cells to produce therapeutic antibodies and to provide long-term protein-replacement solutions. We and others have demonstrated that engineered B cells not only migrate to germinal centers and secret antibodies at protective levels against HIV-1 and respiratory syncytial virus (RSV) infections, but also differentiate into memory and long-lived plasma cells. Most of current B cell engineering approaches use CRISPR-Cas9 nucleases to target the integration of an antibody coding gene into the immunoglobulin heavy locus of *mature* B cells. However, engineering of mature B cells requires their pre-activation, leading to the formation of DSBs in the process of class switch recombination (CSR) in addition to the CRISPR/Cas9 breaks. While the erroneous repair of such CSR induced DSBs might lead in rare cases to chromosomal translocations and the formation of B cell lymphomas, introducing an additional DSB using CRISPR-Cas9 may severely aggravates such genotoxic risk.Here we describe the engineering of primary developing B cells using a novel nuclease-free approach called VDJ targeting. A recombinant adeno-associated viral vector (rAVV), coding for an Ab gene, is inserted into the IgH locus by the endogenous recombination activating gene (RAG) complex during V(D)J recombination, without use of exogenous nucleases and the risks they carry. We previously described the integration of Ab genes into immortalized and inducibly-differentiating B cells. Here we demonstrate for the first time, not only high transduction rates (>40%) of actively developing mouse precursor B cells using AAV-DJ, but also the integration and expression of a broadly neutralizing antibody against HIV-1. The rAAV cassette codes for a single-chain antibody, terminated with a splice donor and a recombination signal sequence (RSS), compatible to recombine with endogenous RSS in the IgH locus. The integrated sequence can then undergo splicing with the endogenous IgH constant segment to be expressed as a fully functional B cell receptor (BCR). In addition, we successfully devised an *in-vitro* differentiation protocol of the precursor B cells into *immature* and *mature* B cells. Approximately 43% out of the CD19+ population displayed an immature phenotype and an additional 40% displayed a mature phenotype. These cells will be further capable of undergoing activation and differentiation into memory and plasma cells in future experiments. On top of avoiding the genotoxic risks of using exogenous nucleases, targeting developing B cells may hold additional advantages in the field of autoimmune diseases. Korniotis *et al.* have shown that the adoptive transfer of TLR9-stimulated precursor B cells facilitated their maturation into regulatory B cells, leading to protection against the onset of type-1 diabetes and experimental autoimmune encephalomyelitis in relevant mouse models. Combining VDJ targeting in precursor B cells with TLR9 stimulation may thus pave the way to antigen-specific regulatory B cells as a promising cell therapy for autoimmune diseases.

420. Use of a Split AAV Delivery Platform for the Evaluation of dxCas9 for Allele-Specific Silencing in Huntington's Disease

Jennifer J. Waldo, Julian Halmai, Peter Deng, David Cameron, Jasmine Carter, Casiana Gonzalez, Isaac Villegas, Jan Nolta, Kyle Fink

Department of Neurology, Stem Cell Program and Institute for Regenerative Cures, MIND Institute, UC Davis, Sacramento, CA

Huntington's disease (HD) is a rare, autosomal dominant neurodegenerative disorder caused by a trinucleotide expansion in exon 1 of the Huntingtin gene (HTT), which leads to neuronal dysfunction and cell death. Healthy *HTT* is implicated in axonal trafficking and trophic factor regulation, making targeted allele-specific reduction important. This study aimed to target single nucleotide polymorphisms (SNPs) in HD patient-derived cells to reduce expression of mutant *HTT* using CRISPR epigenome editing.Heterozygous SNPs near regulatory regions of the *HTT* promoter were confirmed in HD patient cells, allowing for the design of allele-specific gRNAs. Our novel vector expressing dxiCas9 containing repressive effector domains allowed for broader PAM site coverage and higher binding specificity compared to SpdCas9. CRISPR constructs were introduced to an HD patient fibroblast line and knockdown of total *HTT* transcript levels was assessed to screen for gRNA selection. Knockdown was assessed at multiple loci in the *HTT* gene and significant downregulation was achieved using several of our gRNAs that were designed to be allele-specific. Targeting of rs762855 or rs3856973 resulted in significant downregulation compared to a non-treated control. Interestingly, synergy between multiplexed gRNAs was not observed. A gRNA targeting rs762855 also showed significant downregulation in primary cortical neurons derived from YAC128 mice.The delivery of large proteins into the CNS continues to be a barrier to success when using these gene-editing tools. By using a split AAV system, large proteins such as Cas9 can be split into two domains fused to inteins that are able to recombine and create a full-length Cas9 in target cells. Previously established gRNAs targeting *HTT* were cloned into the split AAV vector system containing dxCas9 fused to KRAB. These AAV were injected into the striatum of YAC128 mice containing the full-length human *HTT* locus. Knockdown of *HTT* was assessed via qPCR, western blot, and immunohistochemistry (IHC). These studies support the potential of targeted allele-specific strategies paired with split AAV delivery for CNS disorders.

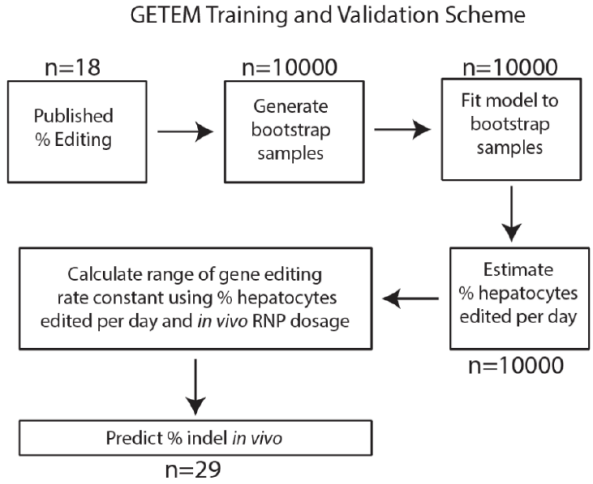
421. Design of Efficacious Somatic Cell Genome Editing Strategies for Recessive and Polygenic Diseases

Amritava Das[1], Jared Carlson-Stevermer[2], Amr Abdeen[2], David Fiflis[2], Benjamin Grindel[2], Shivani Saxena[2], Tugce Akcan[2], Tausif Alam[2], Heidi Kletzien[2], Lucille Kohlenberg[2], Madelyn Goedland[2], Micah Dombroe[2], Krishanu Saha[2]

[1]Bioethics, Morgridge Institutes for Research, Madison, WI,[2]University of Wisconsin - Madison, Madison, WI

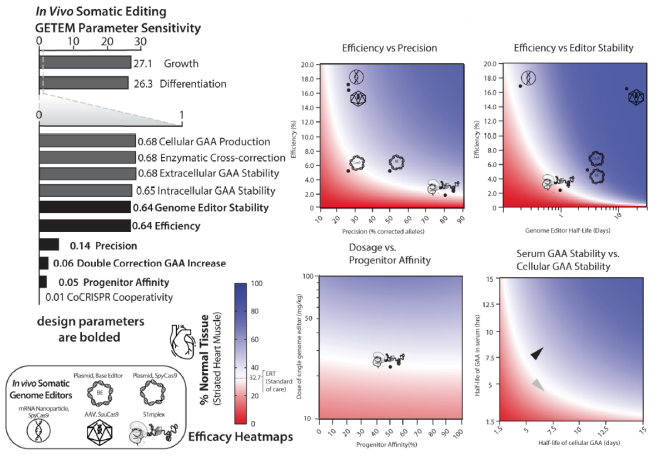
Compound heterozygous recessive or polygenic diseases could be addressed through gene correction of multiple alleles. However, targeting of multiple alleles using genome editors could lead to mixed genotypes and adverse events that amplify during tissue morphogenesis. Here we demonstrate that Cas9-ribonucleoprotein-based genome editors can correct two distinct mutant alleles within a single human cell precisely. Gene-corrected cells in an induced pluripotent stem cell model of Pompe disease expressed the corrected transcript from both corrected alleles, leading to enzymatic cross-correction of diseased cells. We developed a quantitative in silico model for the in vivo delivery of genome editors validated using data from 47 mice across 9 studies

using 6 different types of editors. Simulated data showed concordance with experimental results from 6 editors (SpyCas9, SauCas9, RA6.3, Split ABE, MMEJ and NmeCas9) delivered via plasmid, mRNA LNP, RNP or AAV.



Validation scheme for mathematical modeling

We simulate genome editor injection into the developing human infant liver, we identify progenitor targeting, delivery efficiencies, and suppression of imprecise editing outcomes at the on-target site as key design parameters that control the efficacy of various therapeutic strategies. This work establishes that precise gene editing to correct multiple distinct gene variants could be highly efficacious if designed appropriately.



Model findings indicating key trade-offs and sensitivity of various gene editing parameters. Methods to increase therapeutic outcome include treating with an enzyme stabilizing adjuvant, or using a more stable genome editor delivery system.

422. Digital Correction of Fanconi Anemia Patient Mutations for Gene Therapy

Christopher J. Sipe[1,2,3,4], Mitchel G. Kluesner[1,2,3,4], Sam P. Bingea[1], Aneesha A. Andrew[1], Samantha P. Graham[5], Emily J. Pomeroy[1,2,3,4], Walker S. Lahr[1,2,3,4], Margaret L. Macmillan[1,6], John E. Wagner[1,6], R. Scott McIvor[1,2,3,4,5], Beau R. Webber[1,2,3,4], Branden S. Moriarity[1,2,3,4]

[1]Pediatrics, University of Minnesota, Minneapolis, MN,[2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN,[3]Center for Genome Engineering, University of Minnesota, Minneapolis, MN,[4]Stem Cell Institute, University of Minnesota, Minneapolis, MN,[5]Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN,[6]Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN

Fanconi anemia (FA) is a rare genetic disease characterized by bone marrow failure and cancer predisposition. Mutations can affect any of the 22 FA DNA repair pathway proteins that are essential to inter-strand crosslink (ICL) repair and the repair of double strand breaks (DSBs) through homologous recombination (HR)[1], making traditional nuclease mediated HR genetic correction virtually impossible. Current therapy for FA is hematopoietic cell transplant which is met with complications, such as immune rejection, graft versus host disease and increased cancer risk. To improve upon this allogeneic approach, gene therapy efforts have focused on lentiviral transduction of FA genes or CRISPR-Cas9 homology directed repair approaches to correct FA mutations, but these have not achieved significant levels of phenotypic improvement. To that end, we deployed the Cas9 base editors (BEs), cutting-edge 'digital' genome editing technologies that allow for mutation correction without a DSB or a HR DNA donor molecule. As *FANCA* is a key member of the FA core complex and accounts for 60% of all FA patient cases, we focused on *FANCA* mutations initially. To first determine if base editing is operational in FA cells, we deployed the cytosine deaminase (CBE) to correct a *FANCA* c.3934+2T >C mutation in patient cells. We delivered CBE mRNA and our gRNA by electroporation and found low level initial base editing (>5%). We then optimized gRNAs by extending the editing 'window' within the gRNA binding site by three nucleotides and achieved >20% editing (**Figure 1**). Next, we targeted the most common *FANCA* founder mutation seen in Spanish Romani ancestry[2]. The mutant nonsense codon (TAG) resulting from a *FANCA* c.295 T >C mutation was converted to tryptophan (TGG) using the adenosine base editor (ABE) with an efficiency of 70% editing (**Figure 2**). Tryptophan is conserved and is similar to the WT glutamine (CAG). For both mutations targeted, corrected cells had a growth advantage and became resistant to mitomycin C (MMC), which demonstrates phenotypic rescue (**Figure 2**). Further, base editing efficiencies using ABE were much higher than indel induction using Cas9 nuclease with the same gRNAs, highlighting base editing as an ideal approach for FA gene therapy without the need for DSBs. This work constitutes an important advance in a personalized medicine pipeline for optimizing reagents prior to the editing of precious CD34+ and bone marrow samples in autologous *ex vivo* genetic therapy for any base edit targetable FA mutation.

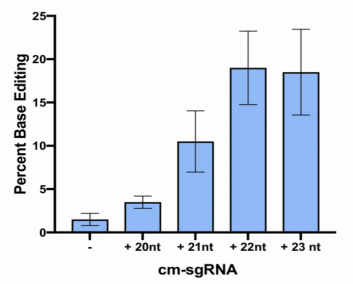


Figure 1. Targeting *FANCA* c.3934+2T>C in patient fibroblasts with CBE using extended gRNAs improves editing efficiency. Sanger sequencing five days post electroporation, n=2 biological replicates.

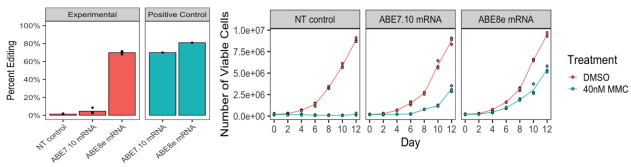


Figure 2. Positive control *B2M*. Comparison of ABE7.10 and ABE8e targeting *FANCA* c.295 T>C in patient derived LCL. Initial base editing (left) five days post electroporation using Sanger sequencing. MMC outgrowth (right), n=2 biological replicates.

423. The rhAmpSeq™ CRISPR Analysis System Enables up to 95% Sensitivity and Specificity in Detection of 0.5% Allele Frequency Variants

Garrett R. Rettig

Integrated DNA Technologies, Coralville, IA

Targeted amplification with the rhAmpSeq™ CRISPR system facilitates straight-forward preparation of targeted next generation sequencing (NGS) libraries, and it simplifies amplicon sequencing, which is the gold-standard method for characterizing the efficiency and specificity of genome editing with CRISPR systems. Amplicon sequencing provides the most detailed description of on- and off-target editing events. We have previously presented rhAmpSeq sequencing library preparation coupled with CRISPR editing analysis with CRISPRAltRations, which is a well-validated data analysis tool informed by a logical understanding of CRISPR editing events. Here, we investigate the sensitivity and specificity afforded by coupling these tools by in-depth evaluation of 20 genomic targets, in which a particular emphasis is placed on low frequency editing events by including only samples that have an INDEL rate of ≤3.5%; furthermore, the comparison includes a matched set of unedited control samples. When applying the requirements of >5,000x read coverage depth, a background INDEL frequency of ≤0.4% in unedited, matched, negative control samples, and a true INDEL signal of >0.5%, our investigation suggests a sensitivity and specificity in the evaluation of CRISPR editing can approach 95%.

424. Tailor-Made OMNI50 CRISPR-Associated Nuclease Facilitates Precise ELANE Mono Allelic Knockout to Promote Neutrophil Differentiation in Severe Congenital Neutropenia

Yosef Dicken[1], Liat Rockah[1], Vahagn Makaryan[2], Nadav Marbach Bar[1], Peter Sabo[2], Lital Povodovski[1], Matan Gabay[1], Tzlil Bar[1], Tanoya Poulsen[2], Asael Herman[1], Lior Izhar[1], David C. Dale[2], Rafi Emmanuel[1]

[1]Emendo Biotherapeutics, Ness Ziona, Israel,[2]Medicine, University of Washington, Seattle, WA

Heterozygous mutations are the leading cause of the vast majority of genetic disorders. Potential treatment strategies for these disorders include complete biallelic knockout or gene correction via Homology Directed Repair (HDR), Prime editing or Base editing. Such strategies, however, may prove problematic due to the potential loss of the essential function of the normal allele product and limited coverage of diverse monogenic mutations within the patient population, respectively. Severe Congenital Neutropenia (SCN) is an example of such disorders, wherein more than 130 heterozygous mutations in the ELANE gene result in misfolding of neutrophil elastase, leading to the death of myeloid cells and block in neutrophil differentiation. Here, we present a CRISPR-based mono allelic knock out strategy targeting heterozygous sites of single nucleotide polymorphisms (SNPs) that are associated with the majority of ELANE mutated alleles (Figure 1). We used directed-evolution and protein engineering capabilities to optimize the CRISPR nuclease (OMNI-50) and design compact guide RNAs that effectively and precisely cleave at the SNPs of SCN patient CD34+ bone marrow cells. We demonstrated that while unedited patient cells presented significant abnormalities in proliferation and differentiation, consistent with the hematopoietic defect in SCN patients, a single allele ELANE knockout fundamentally improved these cellular abnormalities and resulted in the maturation of the edited cells into active neutrophils. Thus, these results manifest the therapeutic potential of our tailor-made optimized gene editing approach that could be available to a broad population of patients with a variety of genetic disorders.

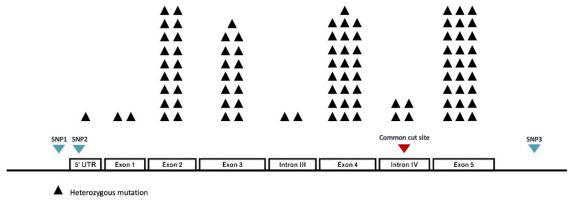


Figure 1: Representative heterozygous mutations in ELANE gene associated with Severe Congenital Neutropenia that can be linked with adjacent SNPs.

425. Single Base Resolution - Methodologies and Bioinformatic Tools to Treat Point Mutations Using CRISPR-Cas Systems

Roy Rabinowitz, Dani Offen

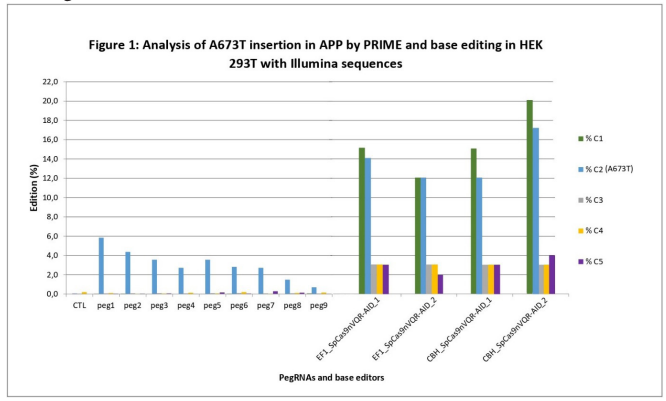
Molecular Genetics and Biochemistry, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

The CRISPR-Cas system holds a great promise in the treatment of diseases caused by genetic variations. The specificity of the system enables researchers to target a particular locus within the genome. However, the ability of the CRISPR-Cas system to distinguish between two sequences differing in a single nucleotide is limited. Therefore, Cas9 is prone to both genome-wide and allelic off-targeting. We performed a meta-analysis of previously reported data to characterize the specificity profile of the CRISPR-Cas system. Our analysis implies the existence of a sub-region within the guide-RNA (gRNA) seed sequence that confers an increased degree of specificity compared to other positions. A thorough investigation of the seed sequence implies a lack of single-nucleotide specificity. Several approaches to increase the specificity of the genome-editing system were previously suggested. Part of the work presented here is focused on two of which, namely SNP-derived PAM to specifically target an allele of interest and base editing that allows precise correction of point mutations with no involvement of DNA double strand breaks. We established bioinformatic tools to assist in designing gRNAs for treating point mutations by the two distinct approaches. **CrisPam** identifies SNP-derived PAMs to specifically target a single nucleotide variant in an allele-specific manner. The PAM sequence is essential for successful DNA binding and cleavage by the Cas enzyme. While there may be several PAM sequences for some Cas enzymes, the PAM requirement is stringent. Thus, by identifying novel PAM sequences that were generated by the variant nucleotide, CrisPam suggests potential Cas enzymes and their corresponding gRNAs to perform allele-specific targeting. Base editing allows precise conversion of a target nucleotide without involving DNA double strand breaks. The editing occurs within a region called the activity window in which all target nucleotides will undergo transition. The editing may result in bystander editing and cause additional unintended mutations. However, in some special cases termed synonymous corrections, bystander editing results in the proper amino acids sequence. **BE-FF** predicts the outcome of base editing and identifies precise corrections of the target nucleotide, as well as synonymous corrections which broaden the gene editing scope of base editors. These tools are designed to serve experimental researchers and assist in the process of gRNA design and identification of suitable CRISPR systems. Both CrisPam and BE-FF are freely available at https://www.danioffenlab.com/web-tools.

426. Comparison of Prime Editing and Base Editing for the Insertion of a Mutation in the APP Gene

Guillaume Tremblay, Joël Rousseau, Cedric Happi-Mbakam, Antoine Guyon, Jacques P. Tremblay
Molecular Medicine, Laval University, Quebec, QC, Canada

Introduction: The field of genetic engineering is developing rapidly, allowing researchers to cure diseases which were once considered incurable using gene therapy. Several biological tools that generate a DNA correction have been developed since the discovery of CRISPR. However, problems arise in the selection of these tools as "off-target events" occur during editing, causing unwanted and potentially detrimental changes. The objective of this study was to compare prime and base editing for the insertion of the A673T mutation in APP gene. **Methods**: Cas9 nickases (Cas9n) were paired with a Target-AID to create cytosine deaminase base editors. The SpCas9 nickase used was mutated into Cas9nVQR with an NGAN protospacer adjacent motif (PAM) to better insert the A673T mutation. Several sgRNA lengths, from 17 bp to 22 bp, were used to optimize the deamination window. Prime editing guide RNAs (pegRNA) were designed by varying the length of the primer binding site (PBS) and the reverse transcriptase template (RTT) sequence. For the editing, one guide was first used on the target gene (PE2); afterwards, additional guides were used to favor DNA editing (PE3, PE3b). The correction was introduced in HEK 293T cells and deep sequencing was used to determine the percentage of edition. Results were estimated by BAM file extraction and verified with CRISPResso2. **Results:** Successful base editing occurred at a maximum rate of 17% for the A673T mutation alongside notable off-target mutations mainly at the level of the C1 nucleotide where a rate of 20% was observed. Prime editing demonstrated precise editing in up to 6% of cells for the A673T mutation with no off-target mutations within the target window (Figure 1). **Conclusions:** Additional methods will be needed to optimize prime editing. Prime editing offers a better precision for the edition of a single nucleotide when compared to base editing.



427. Enhancing SaCas9 Target Specificity by Rational Directed Mutagenesis

Hind Ghezraoui
OXGENE, Medawar Centre, Oxford, United Kingdom

Continued development of the CRISPR/Cas9 system is making therapeutic gene editing a viable biomedical tool. However, delivery of *Streptococcus pyogenes* (SpCas9) by adeno-associated viral vectors (AAV) is challenging due to the small packaging capacity of AAV (~4.7 kb). This can be overcome by using the minimal SpCas9 ortholog *Staphylococcus aureus* Cas9 (SaCas9). Unfortunately, while many high-fidelity SpCas9 variants have been reported, far fewer SaCas9 variants have eliminated off-target editing, leaving off-target cleavage of unintended genomic sites a critical issue to be resolved for this ortholog. We developed a reporter plasmid that can be used to examine on- and off-target editing efficiency and used it to screen rationally engineered SaCas9 variants to identify those SaCas9 variants with enhanced specificity that could effectively discriminate a single base mismatch. We identified SaCas9 variants that dramatically reduced off-target effects, whilst maintaining robust on-target activity comparable to wild-type SaCas9. We confirmed editing at three genomic loci by CRITIC (CRISPR EdiTing InterferenCes) analysis using Sanger trace sequences. Thus, our variants could be used for genome editing applications where high fidelity is required.

428. Advancing Epigenetic Approaches in CRISPR-dCAS9 Systems

Mohanapriya Cumaran
Biomedical Engineering, Duke University, Durham, NC

Late-onset Alzheimer's disease (LOAD) is a type of neurodegenerative disease that affects more than 5.8 million Americans and causes major neuronal degeneration and dementia.[1] The hallmarks of this disease include toxic plaques of amyloid beta protein (AB), and neurofibrillary tangles made out of misfolded tau protein.[1] Apolipoprotein E is a protein that is encoded by the *APOE* gene and its E4 isoform has been shown to increase an individual's risk for LOAD.[2] Due to the overall aging population, Alzheimer's places a socioeconomic burden on family, healthcare workers and on the US healthcare system. Since conventional pharmacological treatments have not been successful, gene therapy is being utilized to develop a safe and reliable treatment for Alzheimer's.CRISPR-Cas9 is a tool utilized in genetic engineering to modify genes. While this tool is an immune system mechanism in bacteria, it is being utilized in various base and prime editing techniques to repair damaged DNA.[4] The CRISPR identifies the sequence of interest to be modified while the Cas9 protein catalytically degrades this sequence. In addition to these repair mechanisms, CRISPR is used in epigenetic editing where epigenetic modifier proteins such as DNMT3a, KRAB-MeCP2, MBD proteins, HP1A and HP1B are complexed with CRISPR-dCas9 constructs to modify expression of genes rather than changing the genetic sequence. Unlike traditional CRISPR-based systems that cleave DNA sequences, this project uses dCas9, dead Cas9, which removes the catalytic component of CRISPR and allows for the epigenetic modification of DNA instead.[5,6] These constructs are delivered to cells using viral vectors such as adeno-associated viruses (AAV) and lentiviruses. The overall goal of developing CRISPR dCas-9 constructs is to develop a reliable, safe therapeutic technique that uses an epigenetic effector complexed with CRISPR-dCas9 to epigenetically repress the expression of the *APOE* gene. CRISPR-Cas9 does offer a prospective therapeutic approach to genome editing, but obstacles include the size constraint of large CRISPR-Cas9 complexes with regard to packaging into smaller AAV viruses, as well as off-target mutagenesis due to lack of specificity.[12,13]

429. CRISPR/Cas13b Site-Directed RNA Editing of Pathogenic Adenosine Nucleotides Associated with Neurodevelopmental Disorders

Jasmine L. Carter, Julian A. N. M. Halmai, Casiana E. Gonzalez, Chloe J. Welch, Jennifer J. Waldo, Peter Deng, Nicole Bellini, Whitney Cary, Jan A. Nolta, Kyle D. Fink
Stem Cell Program and Institute for Regenerative Cures, University of California, Davis, Sacramento, CA

Pathogenic G-to-A mutations that manifest as neurological disorders have traditionally been challenging to edit in neuronal cells as current strategies require host-cell DNA repair pathways and incorporation of a template sequence. ADAR2 is a brain-enriched protein which post-transcriptionally modifies double-stranded RNA (dsRNA) by deaminating adenosine nucleotides to inosine, thereby enabling translational machinery to create a functional adenosine to guanosine edit within the transcript. The CRISPR associated protein 13 (dCas13b) is a catalytically inactive RNA binding protein which achieves site directed RNA editing when fused to the ADAR2 deaminase domain (ADAR2DD). A guide RNA (gRNA) sequence forms a dsRNA substrate for dCas13b-ADARDD2 binding at the complementary target while enabling RNA editing at a single base. However, the CRISPR Cas13 system has yet to be employed for RNA editing of G-to-A mutations in neuronal cells or multiple pathogenic adenosine variants within the same transcript. Our lab created induced pluripotent stem cell (iPSC) from fibroblasts of individuals with pathogenic G-to-A mutations in exon 5 (E198K) and exon 12 (E420K) of protein phosphatase 2, regulatory subunit B', delta (*PPP2R5D)* which are causative for the neurodevelopmental disorder Jordan's Syndrome. Here, we have differentiated isogenic and variant iPSCs to neural stem cells (NSCs) to evaluate RNA editing efficiency and efficacy.Towards this goal, we have designed and screened exon 5 (E198K) and exon 12 (E420K) gRNA to identify lead gRNA which have high on-target editing and low-moderate off-target editing in the *PPP2R5D* transcript. We demonstrate gRNA structure is readily amenable to fit sequence constraints within the target transcript, thus improving on-target editing efficiency. We have also used our *in vitro* neurodevelopmental model to understand the consequence of G-to-A mutations on *PPP2R5D* targets at the phosphoproteomic and transcriptomic levels. To our knowledge we are the first to employ the CRISPR RNA editor system to correct G-to-A mutations in a patient-specific neuronal model. These studies support the potential for CRISPR/Cas13 systems to selectively edit mutant transcripts harboring G-to-A mutations while providing an alternative editing technology for neurodevelopmental disorders.

Oligonucleotide Therapeutics

430. Dual SELEX - Parallel Selections against Two Chemokine Receptor Targets

Chen Li, John C. Burnett
Center for Gene Therapy, City of Hope Beckman Research Institute, Duarte, CA

There are over 20 Chemokine receptors, which are cell surface-expressed receptors that respond to chemokines. While normally found on healthy tissues, these receptors are also aberrantly expressed on cancer cells. CXCR4 (C-X-C chemokine Receptor type 4) is a chemokine receptor that is expressed in various cancers, among them are breast and prostate cancers. High expression level of CXCR4 in breast cancer is an indicator for metastatic potential and poor prognosis. CXCR4 is also a receptor required for HIV (Human Immunodeficiency Virus) entry. Another chemokine receptor, CXCR7, is found in prostate cancers as well. Furthermore, it has been found that CXCR4 and CXCR7 can form heterodimers that can enhance cell migration in vitro. Notably, CXCR4 and CXCR7 share command chemokine ligand, CXCL12 and artificial ligand AMD3100. This feature makes it challenging to develop aptamers that are highly specific to CXCR4 but not CXCR7, and vice versa. Aptamers are short oligonucleotides with high affinity for their targets; they also possess the ability to transport therapeutic RNA into cells - thus making them ideal candidates for developing targeted therapies for chemokine receptor-overexpressing cancers. We have

performed two parallel SELEXs (Systemic Evolution of Ligands by Exponential enrichment) to select RNA aptamers against CXCR4 and CXCR7. Specifically, we are selecting for aptamers that will bind with high specificity to either CXCR4 or CXCR7. We also saved RNA pools to allow future selection for apatmers that are specific to CXCR4/CXCR7 heterodimers. We performed five rounds of selection to enrich for aptamers that are specific to CXCR4, CXCR7 or the CXCR4/CXCR7 heterodimers. Afterwards, we proceed to two separate selections to further enrich aptamers towards CXCR4 alone and CXCR7 alone. Throughout the selection process, we performed high throughput sequencing and employed bioinformatics approaches to identify aptamer candidates for affinity testing. Apart from selecting aptamers with high binding affinity, we are also exploring the functional properties of our aptamers. To the best of our knowledge, there is little study about their potential roles in signaling transduction, so we are testing our aptamer candidates with a particular focus on this aspect of their functions. We are using two platforms for this purpose: (1) the Presto-Tango assay, for measuring β-arrestin recruitment and (2) the Wound Healing assay, for measuring stimulation/retardation in cell movements. We are also using HIV Portection assay to measure the ability of aptamer to protect CXCR4-expressing cells from HIV entry. With the functional characterizations mentioned above, we hope to deepen the therapeutic potentials of aptamers.

431. INTASYL™ PH-762 Self-Delivering RNAi Targeting PD-1 Enhances the Therapeutic Efficacy of Systemically Administered HER2-Targeted CAR-T Cells in a SKOV3 Model of Human Ovarian Adenocarcinoma in NCG Mice

Benjamin Cuiffo, Melissa Maxwell, Dingxue Yan, Brianna Rivest, James Cardia, Simon P. Fricker

R&D, Phio Pharmaceuticals, Marlborough, MA

Targeted chimeric antigen receptor engineered T cells (CAR-T) therapies have proven clinically successful in treating hematological malignancies. However, the therapeutic potential of CAR-T in treating solid tumors remains limited, due in part to intrinsic mechanisms of T cell exhaustion triggered by the immunosuppressive tumor microenvironment of solid tumors, including tumor-mediated upregulation of T cell inhibitory receptors such as PD-1. To overcome this hurdle, strategies to functionally silence PD-1 and/or other mediators of T cell exhaustion are under development. Gene editing strategies to permanently delete PD-1 have been demonstrated to enhance CAR-T efficacy against solid tumors in preclinical models and in initial clinical studies. However, the clinical outlook for gene edited CAR-T therapies may be hampered by safety considerations deriving from the permanent deletion of T cell suppressive mechanisms and off-target impacts of gene editing to the CAR-T product, as well as technical challenges including relatively low editing efficiencies and integrating into clinical protocols of autologous CAR-T production. The INTASYL™ platform is a self-delivering RNAi technology that imparts small molecule-like properties to interfering RNAs, providing efficient delivery to target cells without need for specialized formulations or drug delivery systems. As such INTASYL can be easily incorporated into CAR-T manufacturing protocols. INTASYL provides robust and highly specific on-target gene silencing. While durable, the effects of INTASYL-mediated silencing are transient, potentially mitigating safety considerations arising from permanent deletion of either T cell suppressors such as PD-1 or off target genes. Here we assessed the potential of the PD-1 targeted INTASYL PH-762 to enhance the therapeutic efficacy of HER2-targeted CAR-T cells (HER2CART; ProMab) in treatment of a subcutaneous HER2-expressing SKOV3 model of human ovarian cancer in NCG mice. On-target silencing of PD-1 was demonstrated in HER2CART cells *in vitro*. For the *in vivo* study, 5 μM PH-762 was administered to the HER2CART cell product under simultaneous activation with human CD3/CD28-coated beads (simulating a CAR-T manufacturing expansion phase); control HER2CART cells were similarly bead-activated but remained untreated. Twenty-four hours-post INTASYL treatment / activation, equal numbers (5e05 cells) of viable INTASYL-treated or untreated CAR-T cells were administered intravenously to mice bearing SKOV3 tumors of ~150 mm^3 (N = 4 / group); a control group was administered vehicle only. Tumor volumes and body weights were recorded. Compared to vehicle, HER2CART therapy initially inhibited mean tumor volume growth over the first half of the study however, tumor volumes of untreated HER2CART therapy groups caught up to those of the vehicle control group by study conclusion (Day 15). In contrast, INTASYL PH-762-treated inoculum of HER2CART cells (PH-762 HER2CART) exerted a statistically significant, robust and durable inhibition of tumor growth. Tumors were collected at endpoint, dissociated, and stained with human-specific antibody panels to uncover PH-762 mediated phenotypic changes to the tumor resident CAR-T cells. Taken together, these data using a HER2-targeted CAR-T cell product against a HER2-expressing ovarian cancer xenograft provide proof of concept for the application of PD-1 checkpoint inhibition with INTASYL in CAR-T cells prior to adoptive transfer to enhance the therapeutic efficacy of CAR-T cell therapy.

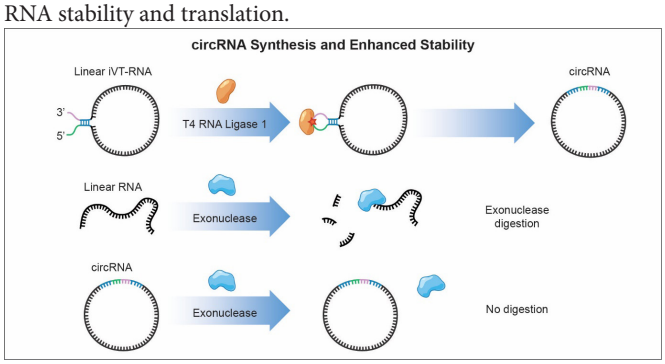
432. Splint and Ribozyme-Free Enzymatic Synthesis and Purification of Long Circular RNA for In Vitro Translation in Human Cells

Aisha Svihla[1], Christopher Rohde[2], Matthew Angel[2]

[1]Northeastern University, Boston, MA,[2]Factor Bioscience Inc., Cambridge, MA

Messenger RNA (mRNA) has many applications in biological research and therapeutic development. However, its relatively short half-life limits the use of mRNA in applications where long-term protein translation is desired. Circular RNAs (circRNAs) are a novel class of RNA molecules with enhanced stability that exist in many organisms, including *C. elegans*, *Drosophila melanogaster*, mice, and humans. Engineered circRNAs have been used for a variety of applications including microRNA sponges and protein expression. The covalently closed structure of circRNAs makes them resistant to degradation by exonucleases, leading to extended protein translation in cells. Methods for synthesizing circRNAs include the use of a splint molecule to bring the 5' and 3' ends of linear RNA in close proximity for ligation and the use of ribozymatic methods in conjunction with self-splicing introns to covalently link the 5' and 3' ends of an *in vitro*-transcribed RNA molecule. However, these methods suffer from low efficiency or result in cytotoxic byproducts that must be removed using high-performance liquid chromatography (HPLC). We show that circRNA can be efficiently synthesized without cytotoxic byproducts using

T4 RNA Ligase 1 and rationally designed short RNA sequences that form secondary structures designed to enhance ligation efficiency. Specifically, we show that RNA sequences that form a hairpin loop in close proximity to the 5' and 3' ends better support intramolecular ligation than RNA sequences without such a hairpin loop. We demonstrate that this method of ligation is applicable to a variety of RNA sequences, including long RNAs. CircRNA containing an internal ribosome entry site (IRES) 5' to a sequence encoding green fluorescent protein is readily translated in primary human fibroblasts with minimal cytotoxicity. In summary, we present a simple, cost effective method of producing protein-encoding circRNA sequences that removes the need for complex purification methods, which may find use in biomedical applications that can benefit from extended RNA stability and translation.

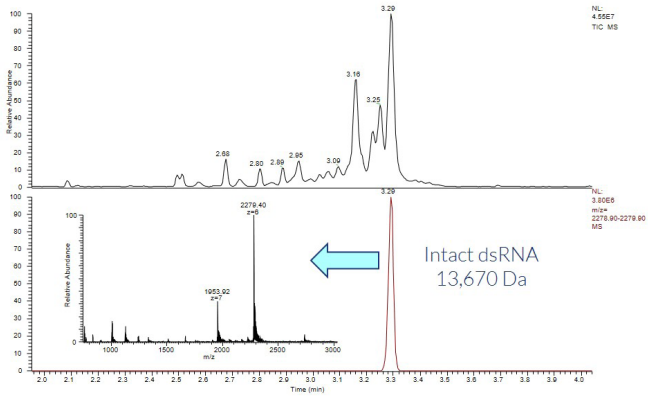


433. Characterization of Single-Stranded and Double-Stranded Oligonucleotides by ZipChip Microchip Electrophoresis-Mass Spectrometry

Scott Mellors

908 Devices, Boston, MA

The recent explosion of gene and cell therapies has drawn attention to the relative lack of analytical tools available for the detailed characterization of nucleic acid based drugs. The methods available for separation and mass spectrometry analysis can be counted on one hand, and do not currently offer the level of sophistication that has been achieved in the protein analysis world. While chromatographic methods exist and are under continued development, electrophoresis holds some particular advantages for nucleic acid analysis, as the human genome project clearly demonstrated. Here we demonstrate a new method that performs capillary zone electrophoresis with integrated electrospray ionization mass spectrometry on the microfluidic platform known as ZipChip (908 Devices). ZipChip analysis of single-stranded oligonucleotides was first reported last year. The method uses microfluidic chips with negatively charged surface chemistry to both repel the negatively charged oligonucleotides and provide a strong electroosmotic flow to drive the analytes through the separation channel. The separations are performed with a background electrolyte based on an ammonium acetate buffer, pH adjusted near pH 9. Positive electrospray is performed directly off of the corner of the microfluidic device, yielding surprisingly high sensitivity and improved robustness versus negative ESI. An orbitrap mass spectrometer (Thermo Scientific) is being used to provide high resolution and accurate mass detection. The method described here builds on that initial work by analyzing a double-stranded RNA oligonucleotide (21-mer). It was found that the double-stranded structure remains intact during both the separation and the gentle ESI conditions of the method, allowing the double-stranded structure to be studied directly. It was further found that simple dilution of the sample with DMSO chemically denatures the molecule to allow analysis of the single strands. Because the chemical denaturing only requires a different dilution solvent for the sample, one can perform back to back analyses focusing on the double strand in one analysis, then the single strands immediately following. And because the ZipChip method only takes about 5 minutes per run, both analyses can be complete in about 10 minutes. Current work is focused on defining the limits of the separation power of this method. In general, variations to molecular structure which yield a change in charge or shape are more easily separated by CE than they are by LC methods, while size and polarity differences are easier to separate by LC. The most up to date results will be presented.



434. Functional Characterization of CD3-Specific DNA Aptamers

Anna Miodek, Anais Boutserin, Cecile Bauche, Renaud Vaillant, Frederic Mourlane

Ixaka, Villejuif, France

A number of therapeutic strategies that modulate T cell immunity by targeting CD3, the signaling component of the T-cell receptor, are widely used in clinics as immunosuppressive agents in transplantation (OKT3), autoimmune type I diabetes and psoriasis (teplizumab and otelixizumab). More recently, bispecific therapies retargeting the cytotoxic activity of effector T cells by binding to CD3 to tumors expressing tumor-associated antigen have demonstrated striking activity in patients across different cancers (blinatumomab). Ixaka has been focusing on the development of CD3-specific aptamers to generate new targeting agents to specifically deliver anti-cancer therapeutics to T lymphocytes. We have screened and selected G-quadruplex-based DNA aptamers that bind to CD3 ε/γ or CD3 ε/δ protein complexes expressed on human T lymphocytes. By combining biophysical and on-cell binding methods, CD3-specific interactions with nanomolar range affinities were reported with the best candidates. Sequence-optimized derivatives have been engineered with abasic sites, base substitutions or N-terminal functionalization that resulted in improved affinity, specificity and serum stability. They exhibit remarkable cross-specificity with human, mouse and cynomolgus isoforms and extended serum stability. Isolated aptamers recognized an epitope on CD3 which is different from the epitopes covered by OKT3 and other T-cell activating antibodies. Accordingly, these aptamers did

not induce any activation of primary human T lymphocytes nor the internalization of CD3 receptors upon binding, which is of particular interest for the development of inert targeting agents without any immunomodulating properties. Preliminary *in vivo* biodistribution data will also be presented. All together these lead CD3 aptamers exhibit unique properties and qualify for further preclinical and clinical development of CD3-targeting therapeutics.

435. Engineering and Functional Characterization of Anti-PSMAxCD3 Bispecific Aptamers

Anna Miodek, Anais Boutserin, Cecile Bauche, Renaud Vaillant, Frederic Mourlane

Ixaka, Villejuif, France

Bispecific therapies redirecting cytotoxic effector T cells to malignant cells by simultaneous binding to CD3, the signaling component of the T-cell receptor, and tumor-associated antigens have demonstrated striking activity in patients across different cancers (blinatumomab and catumaxomab). Currently over 80 bispecific antibody fragments/derivatives are under active clinical developments in oncology, half of which involves CD3 recognition. Ixaka has been developing CD3-specific aptamers as new targeting agents to specifically deliver *in vivo* anti-cancer therapeutics to T lymphocytes. These G-quadruplex-based binders with nanomolar affinities recognize epitopes on the CD3 receptor that are different from OKT3 and other T-cell activating antibodies. They exhibit remarkable cross specificity with human, mouse and cynomolgus isoforms and extended serum stability. In order to further explore the therapeutic potential of these leads, we engineered PSMAxCD3 bispecific aptamers to develop new anticancer agents able to recruit cytotoxic T cells to induce killing of prostate-specific membrane antigen (PSMA)-positive tumor cells. Hexynyl-modified anti-CD3 moieties carrying hexaethylene glycol linkers at their 5'ends were dimerized by click chemistry with the model anti-PSMA RNA A10 aptamer functionalized with an azide group and TEG-biotin/Cy5 tag at its 3' end. By combining biophysical and on-cell binding methods, we demonstrated that dimerization did not alter the specificity and affinity of PSMA and CD3 monomers due to steric hindrance. The serum stability of the PSMAxCD3 heterodimers was not any different from that of individual monomers despite the HEG linker and triazole inter-nucleotide dimerization. In vitro cytotoxicity assays revealed specific PSMA-positive cell killing with PSMAxCD3 aptamers when control monomer A10 lacking the CD3 binding moiety did not induce any cytotoxicity. Engineered bispecific aptamers are thus able to recruit effector T lymphocytes to target cells to redirect their cytolytic machinery and eliminate a particular cell population. Based on these promising properties, PSMAxCD3 aptamers have been selected for further preclinical and clinical development as prostate cancer therapeutics.

436. Identifying Molecular Mechanisms of Compounds That Prime Nonviral Gene Delivery to Human Mesenchymal Stem Cells

Tyler Kozisek, Andrew Hamann, Angela K. Pannier

Dept. of Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE

Human mesenchymal stem cells (hMSCs) are under intense research for applications in cell therapeutics due to unique properties, however, the intrinsic therapeutic properties of hMSCs could be enhanced by gene delivery. Viral gene delivery is efficient but suffers from safety concerns. Conversely, nonviral gene delivery, while safer than viral methods, suffers from inefficiency, especially in hMSCs. To address the shortcomings of nonviral gene delivery to hMSCs, our group has demonstrated that pharmacological 'priming', the addition of compounds to the culture media to modulate transfection, can increase transfection compared to a vehicle control (VC). Specifically, we have shown that the glucocorticoid dexamethasone can increase transfection outcomes while decreasing transfection-induced cytotoxicity compared to a VC. Furthermore, we expanded our priming library by screening the NIH Clinical Collection, which includes 700+ clinically approved drugs, and identified new classes of hMSC priming compounds, such as antibiotics, antihypertensives, and flavonoids. However, the mechanism by which these newly identified compounds modulate transfection in hMSCs has yet to be investigated. Therefore, in this work, our objective was to identify the priming mechanisms of the newly identified drug classes, as well as further our understanding of the glucocorticoid priming mechanism in hMSCs, by identifying possible genes that these priming compounds may be modulating. Drug Set Enrichment Analysis (DSEA) was used to identify possible genes that may be modulated by antibiotics, antihypertensives, and glucocorticoids. Using compounds from each drug class that produced the largest overall fold change in transfection compared to a VC, specifically rifabutin and rolitetracycline for antibiotics, nicardipine for antihypertensives, and clobetasol, dexamethasone, fluorometholone, and fluticasone for glucocorticoids, DSEA identified five genes potentially modulated by positive priming compounds (i.e., compounds that increase transfection compared to a VC; Figure 1A) and five genes potentially modulated by negative priming compounds (i.e., compounds that decrease transfection compared to a VC; Figure 1A). Next, we experimentally validated the DSEA results by priming hMSCs with the above compounds and assaying for gene expression levels of the predicted genes, relative to a VC, 24 hours after transfection using RT-PCR. Dexamethasone increased endogenous expression of yes-associated protein 1 (YAP1) by 5-fold compared to the VC (Figure 1B), while all negative priming compounds decreased endogenous expression of YAP1 compared to the VC (Figure 1B), which agreed with the DSEA results (Figure 1A). Furthermore, perturbing YAP1 with verteporfin (VP), a YAP1 inhibitor, significantly decreased transgenic luciferase expression in hMSCs primed with glucocorticoids compared to hMSCs that were primed with glucocorticoids but did not receive VP (Figure 1C), suggesting that YAP1 may play a key role in hMSC transfection. Together, these data show that hMSC transfection priming compounds can modulate transfection outcomes through regulation of specific genes, such as YAP1, and suggests that further investigation into specific genes that are modulated by both positive and negative priming compounds could lead to a better understanding of the biology of hMSC transfection.

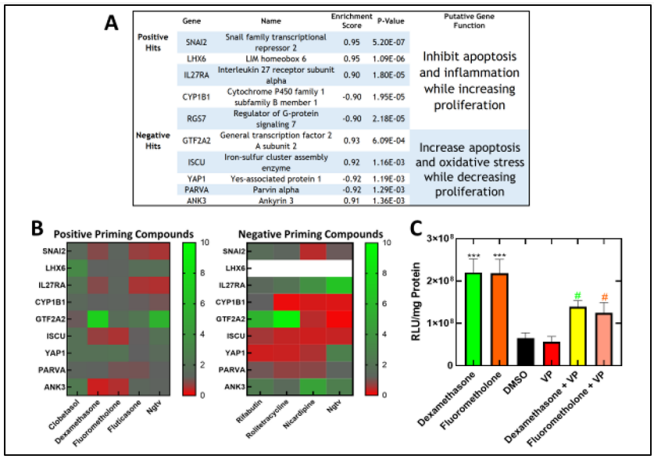


Figure 1: Potential endogenous gene regulation by bMSC priming compounds and the effects these priming compounds have on hMSC transfection. A) List of ten potential endogenous genes modulated by positive and negative priming compounds as predicted by DSEA. Enrichment score indicates the direction of modulation by the group of priming compounds and the P-value asses how the priming compounds consistently modulate the indicated gene in the direction of the enrichment score. B) RT-PCR analysis of endogenous gene expression for DSEA predicated genes for both positive and negative priming compounds relative to a vehicle control (DMSO). C) Transgenic luciferase expression in hMSCs following priming and YAP1 inhibition with verteporfin (VP). * indicate significance relative to DMSO. # indicate significance relative to the same priming compound condition without inhibition of YAP1 with VP. Significance accepted at p<0.05.

437. Comparison of Peptide-Linked PMO Sequences for Inducing Exon 2 Skipping in the Dup2 Mouse Model of Duchenne Muscular Dystrophy

Liubov V. Gushchina[1], Adrienne J. Bradley[1], Aisha Suhaiba[1], Kelly M. Grounds[1], Tatyana Vetter[1], Emma Frair[1], Calli Bellinger[1], Frederick J. Schnell[2], Gunnar Hanson[2], Tabatha Simmons[1], Kevin M. Flanigan[1]

[1]Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Sarepta Therapeutics, Cambridge, MA

Duchenne muscular dystrophy (DMD) is caused by mutations that disrupt the reading frame of the *DMD* gene, leading to lack of dystrophin protein. Exon skipping therapies are designed to exclude a specific exon from the mRNA and restore the reading frame. Two such antisense phosphorodiamidate morpholino oligomers (PMO) have been approved for therapeutic use. These target exon 51 or exon 53 to result in expression of an internally-trunctated but functional dystrophin protein; in contrast, skipping of one copy of an exon in a patient with a single exon duplication may restore an entirely wild-type *DMD* transcript and dystrophin protein. In the case of exon 2 duplications—the most common duplication mutation in DMD—there is a wide therapeutic window: complete exon exclusion will result in expression from a downstream internal ribosome entry site (IRES) of an N-deleted protein with nearly normal function, as patient who express it walk into the eight decade. Here we compare the efficacy of several cell-penetrating peptide-conjugated PMOs, each targeting the exon 2 splice acceptor site. In a double-blind fashion, we compared the efficacy of one previously-characterized and 4 novel PPMO sequences delivered intravenously to 12-week-old mice in a single intravenous injection of 40 mg/kg; saline-treated Dup2 and C57Bl/6 mice served as controls (n=4-7 in each cohort). Mice were sacrificed 15 days post-injection for assessment of exon skipping efficiency by RT-PCR, and evaluation of dystrophin expression by immunofluorescent analysis and western blot. Exon skipping was the primary endpoint, and therapeutic transcripts lacking one (WT) or both (Del2) copies of exon 2 were detectable with all PPMOs in all skeletal muscles. Two sequences resulted in significantly greater and generally comparable skipping, but the efficiency of exon skipping was most pronounced in cohort 5, which showed the highest mean therapeutic transcripts of 13.3% (gastrocnemius), 7.9% (tibialis anterior) and 17.1% (quadriceps). The rank order of these sequences was confirmed by dystrophin expression studies. These results suggest lead candidates for future clinical development of exon 2 skipping PPMOs, including multiple-dose studies in the Dup2 mouse.

438. Ethical Issues in Individualized Antisense Oligonucleotide Therapies

Lisa Kearns

Division of Medical Ethics, NYU Grossman School of Medicine, New York, NY

The U.S. FDA approved the first antisense oligonucleotide (ASO) therapy in 1998. Two decades later, in 2019, the first individualized ASO therapy was developed, for a single patient. In fact, that year saw the development of two custom-made ASO therapies, both for ultrarare neurological disorders: milasen, named for a girl with a unique genetic mutation associated with Batten disease, and jacifusen, named for a young woman with an extremely rare mutation associated with early onset ALS. The development and administration of such "bespoke" therapies raise critical ethical issues. Because no clinical trials were conducted—the ASOs had never been tested in humans before they were administered to the patients, and were only briefly tested in rodents—there were no data on either efficacy or safety. Such data forms the foundation for the informed consent process because they relate the risks and benefits that patients (or families/surrogates) must weigh before consenting to treatment. Safety and efficacy data are also an integral part of the treatment protocols that regulatory agencies and ethics review boards/IRBs evaluate as part of their oversight duties. Although data are scarce in traditional Phase 1 trials, for these individualized ASO therapies scant preclinical testing was coupled with the hope that the therapies would be curative. This is generally not the case in clinical development. The enormous resources, human and financial, necessary to develop and administer these ASOs raise troubling questions about justice. As a policy position, it is difficult to argue that such considerable expenditures of person-power and money would not be better spent on therapies that would benefit more than one patient. It is morally indefensible to argue that first-in-class therapies for fatal or life-limiting diseases should be available only to those who can afford them. Research costs are typically covered by developers, who intend to bring products to market and thus recoup their investments, but there is no market incentive to develop individualized therapies. If insurers or governments do not cover experimental therapies, as is often the case, the cost falls to patients. Their lives depend on funding the development of exorbitantly expensive therapies (the success of which is hardly assured). In the field of individualized genetic interventions, researchers are also physicians and patients are also research participants, raising concerns about conflicts of interest. Suspicions of conflict may be heightened when one considers the potential financial rewards for institutions sponsoring the development of the interventions. These ethical obstacles may seem insurmountable, but

they can be mitigated. After Boston Children's Hospital created milasen, it launched the Oversight Committee on Personalized Experimental Therapeutics as a dedicated review board for future similar cases. This effort could serve as a model for other ASO or gene therapy developers. As custom ASO therapies proliferate, FDA reviewers will become more experienced in both classes of therapies and the patients they target, as will ethics review committees. Dedicated, experienced reviewers should prove to be the best guarantee of a sound informed consent process for unique investigational therapies. The justice questions will be much harder to manage, given the enduring inequities in access to healthcare in the US and globally. Educating all stakeholders in individualized ASOs about the attendant ethical considerations will go a long way toward establishing policies and practices to protect the safety of and ensure justice for patients.

439. A MicroRNA-Based Cell Selective Therapy to Treat Coronary Artery Disease by Inhibiting Restenosis and Accelerating Vessel Healing

Ishani Wickramage, Richa Banerjee, Isabella Hetherington, Teresa Darcey, Hana Totary-Jain
Molecular Pharmacology and Physiology, University of South Florida, Tampa, FL

Atherosclerotic cardiovascular disease (CVD) is one of the leading causes of morbidity and mortality worldwide. Percutaneous coronary intervention has vastly improved patient outcomes. Yet, high rates of in stent restenosis are associated with bare-metal stents (**Fig A**). Despite reducing the occurrence of in stent restenosis by inhibiting the proliferation of vascular smooth muscle cells (VSMCs), drug-eluting stents increase risk of thrombosis and neoatherosclerosis due to the non-selective drug eluted from the stents inhibiting reendothelialization as well (**Fig B**). To overcome these obstacles, we previously performed a proof-of-principle study in which we uniquely leveraged the endothelial cell (EC)-specific miRNA, miR-126, to design an adenoviral vector containing target sequences complementary to the mature miR-126-3p strand at the 3'-end of cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Ad-p27-126TS). This configuration enables exogenous p27 overexpression and inhibition of VSMCs proliferation, while selectively protecting ECs. This novel approach achieved striking therapeutic results in a rat carotid artery balloon injury model, selectively inhibiting neointimal hyperplasia and inflammation while promoting vessel reendothelialization, reducing hypercoagulability and restoring the endothelium-dependent vasodilatory response to levels indistinguishable from uninjured controls (PMID: 25133430). To enhance the translational potential of this miRNA switch and circumvent the safety concerns associated with recombinant virus-based therapy, we utilized a synthetic p27-encoding mRNA switch that contains one miR-126 target site at its 5'UTR, which was delivered using a cationic amphipathic cell penetrating peptide (p5RHH) that forms a compact, self-assembled, endonuclease-resistance nanoparticle of < 200 nM in size and possessed an intrinsic endosomolytic activity upon endosomal acidification. When administered in atherosclerotic or femoral artery wire injury mouse models, the nanoparticles delivered their payload specifically to the atherosclerotic regions or to the endothelial denuded regions, respectively, and not to other organs. Repeated retro-orbital venous administration of the p27-switch nanoparticle to a femoral artery wire injury mouse model for two weeks resulted in significant reduction of restenosis and enabled vessel reendothelialization (**Fig C,** in press Molecular Therapy). To further advance this therapeutic approach and accelerate vessel healing by promoting endothelial repair, we are currently optimizing the concurrent delivery of mRNA encoding vascular endothelial growth factor A (VEGF-A), a powerful mediator of angiogenesis together with the p27 miRNA switch (**Fig D**). Our work demonstrates the potential of this mRNA nanotherapeutic platform to selectively target atherosclerotic plaques or neointimal hyperplasia following angioplasty while protecting and accelerating endothelial cell growth and vessel healing, which will significantly advance the fight against atherosclerotic CVD.

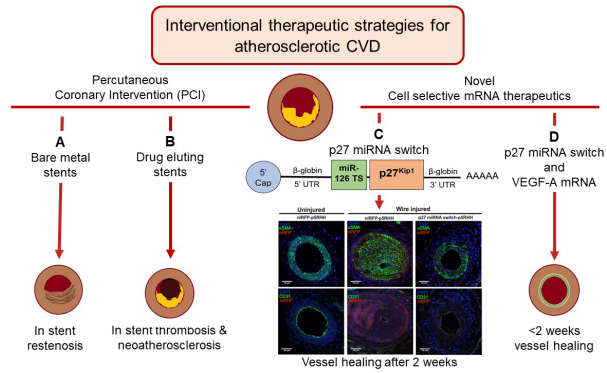


Fig A-D: Outline of CVD therapeutics and immunofluorescence confocal images of α smooth muscle cell actin (αSMA) and endothelial cell coverage (CD31) of uninjured and wire injured femoral arteries of mice treated with control niRFP mRNA (n=5) or p27-miRNA switch nanoparticles (n=6) every 72 hours for 2 weeks after injury.

440. RNA Therapeutics for the Treatment of Brain Cancer

Martin Joseph Hicks, Flobater Gawargi
Biology, Monmouth University, West Long Branch, NJ

Individuals diagnosed with glioblastoma multiforme (GBM) have a short life expectancy of 12-15 months. Current strategies are often limited by the blood-brain barrier. This project is to develop therapies to bypass challenges to effective and continuous drug delivery to the brain, targeting cancer-driving genes. Tumor blood vessel formation depends on vascular endothelial growth factor receptor 2 (VEGFR2), while tumor cell proliferation is stimulated by epidermal growth factor receptor (EGFR). Both are important for tumor cell survival. In our lab, we are developing an innovative therapy that can bypass the blood brain barrier by developing RNA therapies to alter the splicing mechanism of the VEGFR2 and EGFR genes to reduce or block their activation, thus stop tumor cell angiogenesis and growth. Our approach uses an adeno-associated virus gene transfer vector encoding RNA therapeutics targeting critical elements of the EGFR and VEGFR2 pre-mRNA transcript. The 'pre-mRNA structurome' can be used to uncover and determine the accessibility of targetable regions. Our approach has the potential to deliver one single dose of gene therapy directly to the GBM tumor environment and block the production of EGFR and VEGFR2 and activate the expression of a stable therapeutic isoform of the two genes. To advance our therapeutic strategy, we have analyzed

the secondary structure of the two genes using selective 2' hydroxyl acylation and primer extension followed by mutational profiling (SHAPE-MaP). SHAPE-MaP reactivity profiles were generated revealing the structure of splicing and cryptic polyadenylation signal (PAS) elements within the targeted region. We identified enhancer binding motifs surrounding the 5' splice site and hidden elements of the cryptic PAS. Based on these structural profiles, we generated RNA therapies to unravel the hidden PAS to activate expression of the short therapeutic isoform.

441. Characterization and Treatment of a Novel Mouse Model of Duchenne Muscular Dystrophy Using U7snRNA to Mediate Exon Skipping for the Second Calponin Homology Domain of Dystrophin

Daniel N. Lesman[1], Ding Li[1], Dhanarajan Rajakumar[1], Hayat Al-Tabosh[1], Rachel Rafferty[1], Yacidzohara Rodriguez[1], Nicolas Wein[1,2]

[1]Nationwide Children's Hospital, Dublin, OH,[2]Department of Pediatrics, The Ohio State University, Columbus, OH

Duchenne Muscular Dystrophy (DMD) is a severe, progressive, genetic neuromuscular disease caused by mutations in the *DMD* gene encoding dystrophin and affects ~1:5,000 males. Since the *DMD* gene is too large for traditional gene-replacement therapies using Adeno-Associated Virus (AAV), alternative approaches such as exon skipping have been explored. The concept relies on removing a selected exon to restore an open reading frame, leading to the production of a truncated, but functional protein. While exon skipping has been attempted in the past with limited success in dystrophin restoration, our group and others have explored a vectorized exon-skipping approach (VES; a combination of AAV.U7snRNA-exon skipping) to overcome this low level of dystrophin restoration. Promising preclinical results using VES for patients carrying an exon 2 duplication are now in clinical trial. In this study, we focus on another mutational hot spot: exons 6-8, which if skipped should lead to the production of a functional, internally truncated protein. Here we tested several VES constructs to mediate exon 6-8 skipping. This could benefit 3-4% patients with DMD. We chose to evaluate VES targeting the human *DMD* gene. We used in vitro cell models derived from human skin biopsies carrying various mutations at the targeted locus and a new humanized mouse model. The mouse model does not express murine dystrophin due to a nonsense mutation in its *DMD* gene. In addition, this mouse contains the humanized *DMD* gene with a nonsense mutation in exon 7 introduced using CRISPR/Cas9 (hDMDm7). Following the creation of the hDMDm7 mouse, we evaluated 3-month and 6-month old hDMD and hDMDm7 mice. Several VES constructs were designed and tested *in vitro* and *in vivo*. We screened several VES *in vitro* in HEK293. Most of the VES constructs were able to induce skipping of the three exons. Following this pre-screening, we chose four lead candidates. These four lead VES candidates were then evaluated in the hDMDm7. We have previously characterized this hDMDm7 model through several tests. The hDMDm7 mouse presents a reduction of muscle integrity with >90% centronucleation at 3- and 6-months of age as determined by H&E staining. Little to no dystrophin expression was determined by western blot. Additionally, in terms of muscle force, the average percent drop over 10 consecutive eccentric contractions was 59% in hDMDm7 vs 9% in hDMD. These results are in-line with previously established DMD mouse models. Intramuscular injections using our 4 VES lead candidates were performed into the tibialis anterior of hDMDm7 mice at 6- and 7- weeks of age. Exon skipping and dystrophin expression were measured in mice injected at 6-weeks at 12-weeks of age. Muscle force was measured at 3-months post-injection in 7-week injected mice. This analysis is ongoing. In conclusion, we identified several VES vectors able to efficiently skip three exons at least in vitro. Although promising, these results are currently being confirmed in the new hDMDm7 mouse model. This VES for exons 6-8 could be beneficial for 3-4% of DMD patients.

442. Exon Skipping Targeting Neurofibromin Exon 17 as a Therapeutic for Neurofibromatosis Type I

Deeann Wallis[1], Andre Leier[1], Marc Moore[2], Michael Daniel[1], Hui Liu[1], Ludwine Messiaen[1], Bruce Korf[1], Alex Hyde[1], Laura Lambert[1], Robert A. Kesterson[1], George Dickson[2], Linda Popplewell[2]

[1]Genetics, UAB, Birmingham, AL,[2]Department of Biological Sciences, Royal Holloway - University of London, London, United Kingdom

Exon skipping involves the modification of RNA splicing using specific antisense oligonucleotides (ASOs) and allows "skipping" of one or more exons carrying pathogenic DNA sequence variants. We investigated the feasibility of utilizing an exon skipping approach as a genotype-dependent therapeutic for neurofibromatosis type 1 (NF1) by determining which *NF1* exons might be skipped while maintaining neurofibromin function. Human neurofibromin is well-known as a GTPase activating protein (GAP), but outside of its GAP-related domain (GRD), it is unclear how critical other regions are for function. Subsequent *in silico* analysis predicted exons that can be skipped with minimal loss of neurofibromin function. Utilizing a novel *Nf1* cDNA system, we performed a functional screen to determine the effects of exon skipping on *in vitro* neurofibromin expression and GRD function. Loss of single exons 12, 17, 25, 41, 47, or 52 maintained significant GRD function in at least two Ras activity assays. Exons 18/19, 20 and 28 are critical for GRD function; deletion of exons 20, 41, or 47 lead to significantly lower levels of neurofibromin. Skipping of exons 17 or 52 results in both the highest neurofibromin levels and the greatest suppression of Ras activity. We designed antisense phosphorodiamidate morpholino oligos (PMOs) to skip exons 17, 47, and 52 and evaluated them in human cell lines that we generated via CRISPR/Cas9 with mutations in these exons. While all oligos efficiently caused skipping of the desired exons to restore NF1 expression, only oligos targeting exons 17 and 52 restored both NF1 expression and function. Assessment of NF1 patient databases indicates that mutations resulting in deletion or skipping of exons 17 and 52 has not been reported; and truncating pathogenic variants in each exon account for ~0.91 and 0.25% of unrelated NF1 cases respectively. Further, homozygous deletion of exon 17 in a novel mouse model is compatible with viable and grossly healthy animals, providing proof-of-concept that exon 17 may not be essential for murine neurofibromin function. Hence, exon skipping might be used for intragenic exon 17 mutations affecting a significant portion of individuals.

443. Antisense Oligonucleotide Mediated Increase in OPA1 Improves Mitochondrial Function in Fibroblasts Derived from Patients with Autosomal Dominant Optic Atrophy (ADOA)

Aditya Venkatesh[1], Syed Ali[1], Raymond S. Oh[1], Donna Sonntag[1], Zhiyu Li[2], Taylor McKenty[1], Brittany A. Slipp[1], Robert B. Hufnagel[2], Jeffrey H. Hoger[1], Gene Liau[1]

[1]Stoke Therapeutics, Bedford, MA,[2]National Institutes of Health, Bethesda, MD

ADOA is the most common inherited neuro-ophthalmic disorder which starts in the first decade of life and leads to progressive, irreversible vision loss from degeneration of retinal ganglion cells (RGCs). More than 400 mutations have been reported in ADOA and most patients harbor mutations in the *OPA1* gene that lead to haploinsufficiency. Reduced OPA1 levels are associated with impaired mitochondrial function in RGCs leading to apoptosis. Currently, there is no approved disease-modifying treatment for ADOA. Targeted Augmentation of Nuclear Gene Output (TANGO) uses antisense oligonucleotides (ASOs) to reduce inclusion of a non-productive, alternatively spliced exon in *OPA1*. We previously demonstrated that TANGO ASOs can be utilized to increase OPA1 expression in cultured human cells and in the rabbit retina. In this study, we use fibroblasts from patients with ADOA as an *in vitro* model to evaluate efficacy of our approach. Skin fibroblasts were obtained from 3 unrelated patients with ADOA, each with different heterozygous *OPA1* mutations. ASOs were delivered by lipid-based transfection. RNA and protein were isolated to assess changes in *OPA1* mRNA splicing and OPA1 protein levels. Mitochondrial respiration was evaluated by measuring the oxygen consumption rate (OCR) using the XFe96 Extracellular Flux Analyzer from Seahorse Biosciences. Flow cytometry with DCFDA dye was used to detect intracellular reactive oxygen species (ROS). All 3 fibroblast lines showed decreased expression of *OPA1* mRNA and OPA1 protein when compared to wild-type fibroblasts. Patient fibroblasts exhibited impairment in mitochondrial respiration as seen by decreased basal OCR, ATP-linked respiration, and spare respiratory capacity. Intracellular ROS levels also increased. TANGO ASOs reduced the non-productive exon inclusion, increased total OPA1 protein levels, and improved mitochondrial function in patient fibroblasts. We validated the use of patient fibroblasts as an *in vitro* model of ADOA pathophysiology. Improvement in mitochondrial function suggests that ASO-mediated increase in OPA1 can potentially modify disease progression in ADOA in a mutation-independent manner.

444. Engineered Exosomes Efficiently Deliver STAT6 Antisense Oligonucleotides to Tumor Associated Macrophages (TAMs) Resulting in Potent Local and Systemic Anti-Tumor Activity

Sushrut Kamerkar, Charan Leng, Olga Burenkova, Su Chul Jang, Christine McCoy, Kelvin Zhang, William Dahlberg, Kyriakos Economides, Timothy Soos, Dalia Burzyn, Sriram Sathyanarayanan

Codiak BioSciences, Cambridge, MA

Reprogramming anti-inflammatory, 'M2' TAMs to a proinflammatory 'M1' phenotype by selectively silencing transcription factors that drive the M2 phenotype is an attractive strategy to relieve TAM-induced immunosuppression and induce anti-tumoral immune responses. Exosomes are natural intercellular messengers composed of 30-200 nm (~100 nm average) membrane delimited particles. Exosomes can transfer RNA and protein cargoes to recipient cells and mediate cell-cell communication locally and between organs. These observations have led to the development of engineered exosomes for directing the delivery of diverse therapeutic payloads, including short interfering RNAs, antisense oligonucleotides, cytokines and immune modulators to desired target cells (Dooley et al 2021). Exosome surface glycoproteins may enhance exosome uptake by TAMs, thus allowing preferential delivery of therapeutic molecules to these cells within the tumor microenvironment (TME) to manipulate cell-specific biology. By combining the inherent property of exosomes as a natural intercellular communication system and RNA silencing technology, we have developed a novel, engineered exosome therapeutic candidate loaded with antisense oligonucleotides (ASO) targeting STAT6 (exoASO-STAT6), a key transcription factor that controls the tumor-induced M2 phenotype in TAMs.Compared to free ASO, exosome-mediated ASO delivery was found to both enhance ASO drug uptake and silence STAT6 expression by 2x in human M2 polarized macrophages in vitro. Target gene knockdown induced by exoASO-STAT6 was persistent for up to 10 days in cultured mouse, cynomolgus monkey and human M2 macrophages. Importantly, the exoASO-STAT6 treatment induced macrophage M2 to M1 reprogramming in vitro, as demonstrated by a 7x decrease in M2 marker CD163 and a 25x increase in pro-inflammatory cytokines such as IL-12 or TNFα. With IV in vivo dosing, exosome-associated ASO showed an 11x increase in drug uptake in blood monocytes and MDSCs, and a 5.8-fold increase in TAMs and MDSCs in the tumor compared to free ASO. Efficacy studies in the CT26 tumor model (IT dosing) showed potent dose-dependent single agent activity of exoASO-STAT6 with 94% tumor growth inhibition and 80% complete responses at the efficacious dose. exoASO-STAT6 anti-tumoral activity was impaired by prior CD8 T-cell depletion and no tumor growth was observed after tumor cell re-challenge of responder animals, demonstrating the involvement of T-cells and induction of immunological memory. Systemic IV administration of exoASO-STAT6 also showed significant single agent activity in an orthotopic model of HCC unresponsive to other immunotherapies (61% reduction in tumor mass and complete elimination of tumor lesions in 50% of treated mice). Gene expression analysis showed a decrease in M2 markers (e.g., Tgfb1 and Ccl17) and an increase in M1 markers such as IL1b induced by exoASO-STAT6 treatment. A significant increase in interferon and cytotoxic T-cell gene signatures was also observed, as well as a decrease in pathways involved in tumor progression such as angiogenesis and cell proliferation. In summary, exoASO-STAT6 is a novel exosome therapeutic for selectively silencing STAT6 in TAMs, resulting in effective reprogramming of immunosuppressive macrophages to a pro-inflammatory phenotype, which in turn translates into potent single agent anti-tumor activity in multiple checkpoint refractory tumor models.

445. Primary Cultured Cryopreserved Human Hepatocytes for the Evaluation of Efficacy of Gene Therapy Drug Candidates

Albert P. Li, Qian Yang
In Vitro ADMET Laboratories, Columbia, MD

Primary cultured human hepatocytes (PCHH) are considered the "gold standard" in vitro experimental system for the evaluation of human drug properties which, due to species differences, may not be readily assessed using preclinical animal models. A recent advancement in our laboratory is the optimization of human hepatocyte cryopreservation, resulting in PCHH with high viability, plating efficiency, and prolonged culture duration (>4 weeks) as near confluent monolayer cultures with stable gene expression. For gene therapy modalities to be developed to combat liver diseases where hepatocytes are the intended target cells, PCHH can be applied towards the selection of modality candidates during early development based on efficacy, potency, as well as in vitro hepatotoxicity. A comparison of results with PCHH and primary cultured animal hepatocytes will provide useful information guiding the selection of an appropriate animal model for the prediction of human in vivo properties. PCHH can also be used in the IdMOC™ microphysiological experiment system for the evaluation of specificity and toxicity of gene therapy modalities. IdMOC™ (patents allowed in US, Korea, EU, and China) allows the co-culturing of multiple cell types as physically separated (discrete) but interconnected (integrated) by a common overlying medium. An IdMOC culture containing the intended target and nontarget cells allows the evaluation of specificity of the efficacy of gene therapy drug candidates via their on-target and off-target effects. An example of an IdMOC system is a co-culture of cardiomyocytes, hepatocytes, neuronal cells, vascular endothelial cells, kidney epithelial cells, and pulmonary epithelial cells for the evaluation of a gene therapy drug candidate targeting the hepatocytes using gene expression for the evaluation of the intended effects on the target (hepatocytes) as well as the unintended off-target (nonhepatocyte) effects. Toxicological potential of the gene therapy drug candidates per se can be assessed in the two experimental systems using cytotoxicity endpoints such as mitochondrial metabolism (e.g. WST-1) and apoptosis (e.g. caspase 3/7 activation). We propose that primary cultured cryopreserved human hepatocytes and the Integrated Discrete Multiple Organ Co-culture (IdMOC™) represent efficient species-relevant in vitro experimental models that can be applied routinely as in vitro experimental systems to advance the development of gene therapy modalities, providing human-specific data on efficacy, specificity, and toxic potential with substantially lower test material requirements than that required for in vivo animal studies.

446. Lowering the Pathogenic Exon 1 HTT Fragment by AAV5-miHTT Gene Therapy

Marina Sogorb-Gonzalez[1], Fanny Mariet[1], Christian Landles[2], Steffi Jonk[1], Pavlina Konstantinova[1], Sander van Deventer[1], Gillian P. Bates[2], Melvin M. Evers[1], Astrid Valles[1]
[1]uniQure Biopharma B.V., Amsterdam, Netherlands,[2]Department Neurodegenerative Disease, Queen Square Institute of Neurology, UCL, London, United Kingdom

Huntington disease (HD) is a genetic neurodegenerative disease caused by a trinucleotide repeat in the huntingtin gene (HTT). Despite its monogenic cause, HD pathology is highly complex and targeting the leading pathogenic mechanisms is relevant for developing disease-modifying therapies. Increasing evidence indicates that, besides the mutant full-length HTT protein, exon 1 HTT fragments generated by aberrant splicing are very prone to aggregate and contribute to HD pathology. These findings suggest that reducing the expression of the pathogenic exon 1 HTT protein might achieve a greater therapeutic benefit than targeting only full-length mutant HTT protein. We have developed an engineered microRNA which targets exon 1 and full-length HTT (miHTT), delivered via adeno-associated serotype 5 virus (AAV5). AAV5-miHTT is currently being tested in HD patients in a Phase I/II clinical study. Preclinical studies with AAV5-miHTT demonstrated HTT lowering in several rodent and large animal models by reducing full-length HTT mRNA and protein and rescuing HD phenotypes. In the current study, we evaluated the ability of AAV5-miHTT to reduce the exon 1 HTT mRNA and exon 1 HTT protein in HD mice (homozygous and heterozygous Q175 knock-in mice). Polyadenylated exon1 HTT mRNA was successfully detected in striatum and cortex by 3'RACE and qPCR. To detect soluble HTT full length and soluble exon 1 fragments, specific antibodies were used in the HTRF (homogeneous time-resolved fluorescence) platform. After intrastriatal administration of AAV5-miHTT, dose-dependent lowering of both full-length HTT and mis-spliced exon 1 HTT mRNA was observed in striatum and cortex in Q175FDN mice. In accordance, total soluble mutant HTT and exon 1 HTT were also reduced in a dose-dependent manner. These results present the first evidence that AAV5-miHTT reduces both full length and exon 1 HTT levels and support the potential therapeutic value of the first RNAi-based gene therapy for HD to enter clinical trials.

447. Development of *In Vitro* Transcribed mRNA Therapeutics for Cystic Fibrosis

Ruhina Maeshima[1], Martin G. Attwood[1], Amy J. Walker[1], Aristides D. Tagalakis[1], Robin J. McAnulty[2], Christopher O' Callaghan[1], Stephen L. Hart[1]
[1]Great Ormond Street Institute of Child Health, University College London, London, United Kingdom,[2]Respiratory Centre for Inflammation and Tissue Repair, University College London, London, United Kingdom

Cystic fibrosis (CF) is a recessive disease that affects approximately 10,000 people in the UK. The disease is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Absent/non-functional CFTR leads to an imbalance of sodium, chloride and bicarbonate ion transport, and production of thick, sticky mucus in the lung which results in chronic bacterial infection and inflammation. Gene replacement therapy with viral/non-viral vectors has been explored in the last 25 years but all failed to show significant clinical efficacy. *In vitro* transcribed (IVT) mRNA has emerged in the last few years as a new approach for protein replacement. IVT mRNA offers potential advantages of greater efficiency of expression as mRNA delivery is only required to the cytoplasm rather than the nucleus, and safety as there is no risk of genomic integration. We are developing CFTR IVT mRNA therapy for CF to replace to wild type CFTR protein using receptor-targeted nanocomplex (RTN) formulations that consist of liposomes, and specific receptor targeting

peptides and nucleic acid. We have optimised the ability of RTNs to transfect primary cystic fibrosis bronchial epithelial (CFBE) cells at submerged culture, air-liquid interface (ALI) culture and mouse lung using reporter IVT mRNAs. CFTR mRNA was also delivered and the transfection efficiency was assessed by immunoblotting in CFBE cells at submerged culture and ALI culture. We first optimised the RTN formulation, comparing combinations of three different cationic liposomes and five peptides. As a result, we identified a novel formulation for mRNA delivery that achieved almost 100% cellular uptake efficiency, and 90% transfection efficiency, compared to a maximum of approximately 20% with a plasmid reporter. The same formulation was able to deliver the mRNAs in ALI cultured cells and mouse lungs. CFTR mRNA was successfully delivered to a lung epithelial cell line, 16HBE14o-, primary normal epithelial cells (NHBE) and CF epithelial (CFBE) cells. In addition, we found co-delivery of the commercial drug, corrector of CFTR: Lumacaftor (VX-809) improved the expression or stability of CFTR protein in CFBE cells in submerged culture. Moreover, CFTR protein expression was shown to be upregulated in ALI culture of CFTR cells transfected with RTN CFTR mRNA by Western blot. Luciferase IVT mRNA transfection to mouse lung has also successfully shown to induce Luciferase protein expression that is 200-fold higher than a plasmid DNA coding the gene. IVT mRNA of CFTR delivered by RTNs is a promising novel therapeutic for cystic fibrosis. In addition, the flexibility of lipoplex allows co-delivery of CFTR mRNA with Lumacaftor, which is a promising significantly improved CFTR expression.

448. Photoresponsive miR-34a/Nanoshell Conjugates Enable Light-Triggered Gene Regulation to Impair the Function of Triple-Negative Breast Cancer Cells

Megan N. Dang[1], Carolina Gomez Casas[1], Emily S. Day[1,2,3]

[1]Biomedical Engineering, University of Delaware, Newark, DE,[2]Materials Science and Engineering, University of Delaware, Newark, DE,[3]Helen Graham Cancer Center & Research Institute, Newark, DE

Introduction Triple-negative breast cancer (TNBC) is an aggressive disease that requires new interventions. A promising therapy is to introduce tumor suppressive miR-34a into TNBC cells. Unfortunately, naked miR-34a is not effective, while nanocarriers designed to increase miR-34a stability and cellular uptake lack specificity and potency. To overcome these limitations, we conjugated miR-34a to photoresponsive gold nanoshells (NS) to be released upon excitation with continuous wave (CW) or nanosecond (ns) pulsed near-infrared light for on-demand gene regulation. We show ns pulsed light releases miRNA more effectively than CW light, and that miR-34a/NS can regulate downstream gene targets following irradiation to reduce TNBC cell viability, proliferation, and migration. These findings signify miR-34a/NS as promising tools for controlled gene regulation of TNBC. **Methods** *Nanoparticle (NP) Synthesis:* NS were coated with miRNA duplexes containing a 3'-thiol and passivated with mPEG-SH. The size and surface charge of bare NS and miRNA/NS were measured by dynamic light scattering (DLS) and zeta potential. miRNA loading was measured using an Oligreen assay. *Release Studies:* NS were exposed to CW or ns pulsed 810 nm light for 10 min at 0.1, 0.2, or 0.4 W/cm^2 and monitored for bulk temperature changes. After irradiation, released RNA was quantified to determine the number of duplexes remaining bound to NS and the RNA structure was evaluated by gel electrophoresis. *In vitro Studies*: To confirm released miR-34a is delivered into the cytoplasm and regulates downstream target genes, MDA-MB-231 cells were incubated with miR-34a/NS or miR-co/NS at equivalent RNA doses of 100 nM, irradiated at 0.1 W/cm^2 for 10 min, then incubated for 1 or 24 hr before miR-34a, mRNA, and protein levels were analyzed. To assess the functional impact of miR-34a delivery, cells were treated as before and analyzed for metabolic activity, proliferation, and migration at various timepoints. **Results** DLS and TEM revealed NS were monodisperse and spherical with increases in diameter and charge observed upon miRNA conjugation (**Fig 1A**). Oligreen quantification showed relatively equal loading of miR-34a and miR-co, with 7300 and 5500 RNA/NS. Thermal measurements showed that NS temperatures increase during irradiation at levels corresponding to power density. Excitingly, ns pulsed irradiation induced more RNA release from NS than CW light at all power densities. Further, gel electrophoresis confirmed that released miRNA retains its double-stranded structure following irradiation (**Fig 1B**). At 1 hr post irradiation, miR-34a expression was ~1.2X higher in cells exposed to miR-34a/NS + ns pulsed light than cells exposed to CW light. At 24 hr post irradiation, the mRNA and protein expression of miR-34a target genes decreased in cells treated with miR-34a/NS + ns pulsed light. Correspondingly, cells exposed to miR-34a/NS + ns pulsed light had significantly reduced metabolic activity and proliferation 72-hr post irradiation, and cell migration was inhibited by ~65% at 48-hr post irradiation. **Conclusion** These data demonstrate that miR-34a/NS can facilitate on-demand gene regulation in TNBC cells to reduce cell proliferation and migration. This approach offers unprecedented spatiotemporal control over miRNA delivery.

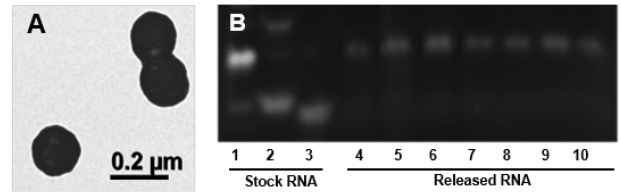


Figure 1. (A) miRNA Conjugate. Transmission electron micrograph of miR-co/NS conjugates. **(B) miRNA Release.** Gel electrophoresis of stock miRNA (1-3) duplex, sense, and antisense compared to miRNA released from NS upon ns-pulsed irradiation at varying power densities of (4-10) 0.02, 0.04, 0.06, 0.15, 0.32, and 0.46 W/cm^2.

449. Turning off AAV-Delivered Transgenes Using Artificial microRNAs

Youjun Wu, Katie M. Stiles, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

Once administered *in vivo* to a non-proliferating cell population, adeno-associated virus (AAV) gene transfer vectors mediate persistent expression. While persistent expression following a single administration is ideal for treating hereditary disorders, current technology does not provide a means of turning off expression should there be adverse effects from the transgene product. With the goal of developing an "off-switch" that would halt transgene expression if needed following *in vivo* AAV-mediated gene therapy, we hypothesized that incorporation of unique artificial microRNA (amiRNA) target

sequences into the AAV expression cassette would enable suppression of transgene expression with subsequent delivery of a different serotype AAV vector coding for the amiRNA cognate to the target sequence in the previously administered expression cassette. To test this hypothesis, we introduced 22 nt unique sequences not present in the human and mouse transcriptome into the 3' untranslated region (UTR) of the transgene of a therapeutic AAV expression cassette. To mediate efficient transgene knockdown, two tandem copies of the amiRNAs targeting the unique sequences were embedded in the miR-155 enhanced SIBR (eSIBR) scaffold and cloned into the 3'UTR of the reporter gene mCherry in another AAV expression cassette. Three different target sites and two amiRNAs targeting each site were evaluated. A dual luciferase reporter assay identified 4 out of 6 amiRNAs that significantly inhibited luciferase activity by interaction with their target sites. The "off-switch" system was further evaluated *in vitro* by testing its ability to repress the expression of AAV transgene anti-IgE, a recombinant DNA-derived humanized IgG1κ monoclonal antibody that binds to human immunoglobulin E (IgE). AAV-mediated delivery of anti-IgE was designed to provide long-term expression of anti-IgE to protect against allergen-specific-induced allergic reactions such as peanut allergy. Co-transfection of HEK293T cells with both anti-IgE and targeting amiRNA pAAV expression plasmids significantly reduced the secreted anti-IgE in culture medium compared with control amiRNA. Additionally, anti-IgE protein expression was effectively inhibited in both HEK293T cells and mouse hepatocyte H2.35 cells by immunofluorescence staining. Anti-IgE transgene mRNA was markedly reduced after exposure to amiRNAs, indicating amiRNA-induced transgene mRNA degradation. The two amiRNAs demonstrating the most pronounced transgene inhibition showed the highest level of primary amiRNA (pri-amiRNA) expression, suggesting that the amount of pri-amiRNA correlates with the level of transgene repression. In summary, this data provides a proof-of-concept for the use of amiRNAs to switch off transgene expression, a strategy that may improve the safety of AAV-mediated gene therapies.

450. Novel Lentiviral Vectors for Gene Therapy of Sickle Cell Disease Combining Gene Addition and Gene Silencing Strategies

Mégane Brusson[1], Anne Chalumeau[1], Pierre Martinucci[1], Fulvio Mavilio[2], Marina Cavazzana[1], Mario Amendola[3], Annarita Miccio[1]

[1]Imagine Institute, INSERM UMR1163, Paris, France,[2]Department of Life Sciences, Modena University, Modena, Italy,[3]Genethon, INSERM UMR951, Evry, France

Sickle cell disease (SCD) is due to a mutation in the β-globin (*HBB*) gene causing the production of the sickle β^S-globin chain. The sickle Hb (HbS, $\alpha_2\beta^S_2$) polymerizes, leading to the formation of sickle-shaped red blood cells that cause vaso-occlusions and organ damage. Transplantation of autologous hematopoietic stem/progenitor cells (HSPCs) transduced with lentiviral vectors (LVs) expressing an anti-sickling β-globin transgene (βAS LV) is a promising curative treatment; however, it is partially effective in SCD patients, who still present elevated HbS levels. Here, we aim to improve LVs to boost therapeutic β-like globin levels without increasing the mutagenic vector load in HSPCs. We developed 2 novel LVs expressing βAS together with an artificial microRNA (amiR) targeting either the fetal Hb (HbF) repressor BCL11A (βAS/amiRBCL11A) or the β^S-globin (βAS/amiRHBB). By downregulating BCL11A, amiRBCL11A re-activates the expression of the endogenous anti-sickling fetal γ-globin, which, together with βAS, should improve the clinical course of SCD; β^S-globin downregulation should favor βAS incorporation in Hb tetramers, increase therapeutic Hb levels and ameliorate the SCD phenotype. First, we developed βAS/amiRBCL11A LV by inserting the amiR in the βAS intron 2 under the control of *HBB* promoter/enhancers to limit BCL11A downregulation to the erythroid lineage and reduce potential amiR-related cellular toxicity and off-target effects. We showed that amiR insertion did not affect LV titer nor βAS expression in a human erythroid cell line (HUDEP2). BCL11A downregulation in HUDEP2 led to γ-globin gene de-repression and a high proportion of HbF^+ cells (RTqPCR, HPLC, flow cytometry). Importantly, the total amount of therapeutic β-like globins was substantially higher in βAS/amiRBCL11A LV- than in βAS LV-transduced cells, with no impairment in cell viability or erythroid differentiation. In parallel, we designed 17 amiRs targeting *HBB* and generated the corresponding βAS/amiRHBB LVs. We tested these LVs in HUDEP2 and selected 2 efficient amiRs able to downregulate β-globin at mRNA and protein levels (RT-qPCR and Western Blot). Finally, we tested βAS/amiRBCL11A and βAS/amiRHBB LVs in HSPCs from SCD patients. HSPC-derived erythroid cells transduced with βAS/amiRBCL11A LV showed increased HbF levels. To further reduce β^S-globin levels, we targeted the β^S-globin mRNA using the βAS/amiRHBB LV. Of note, we modified the βAS transgene by inserting silent mutations that prevent its recognition by the amiR (βASm). Efficient HSPC transduction by βASm/amiRHBB LV led to a substantial decrease of β^S-globin transcripts in HSPC-derived erythroid cells compared to the βAS LV-transduced cells (RTqPCR) at a VCN/cell of 2. Notably, the amiR specifically down-regulated β^S-globin, without affecting βAS expression. HPLC analysis showed that β^S-globin downregulation led to a ~2-fold decrease of HbS in βASm/amiRHBB- *vs* βAS LV-transduced cells (25% and 46% of the total Hb, respectively). This was associated with an increase of the therapeutic Hb in βASm/amiRHBB LV- *vs* βAS LV-transduced erythroid cells (57% and 35% of the total Hb, respectively). Finally, a clonal assay of hematopoietic progenitors showed no impairment in HSPC viability and differentiation towards the erythroid and myeloid lineages upon transduction with bifunctional LVs. In conclusion, we created a LV able to concomitantly silence the β^S-globin and express βAS, achieving clinically relevant levels of therapeutic Hb. Therefore, the combination of gene addition and gene silencing strategies can improve the efficacy of current therapeutic approaches, representing a novel treatment for SCD.

451. Investigation of the Impact of miR-758 in Breast Cancer

Clodagh P. O'Neill[1], Katie Znidericz[1], Alanna Stanley[2], Róisín M. Dwyer[1,3]

[1]Discipline of Surgery, National University of Ireland Galway, Galway, Ireland,[2]Anatomy, National University of Ireland Galway, Galway, Ireland,[3]Science Foundation Ireland (SFI) Research Centre for Medical Devices (CÚRAM), National University of Ireland Galway, Ireland

Improved understanding of microRNA expression and function in cancer has revealed a range of microRNAs that negatively regulate many

oncogenic pathways, thus representing potent tumour suppressors. Therapeutic delivery of these microRNAs to the site of tumours and metastases provides a promising avenue for cancer therapy. The miR-379 microRNA family is thought to have strong anti-cancer synergism. Previous research by this group has proven the tumour suppressive role of miR-379 in the breast cancer setting. The current research focuses on two more members of this microRNA family: miR-411 and miR-758. Two invasive breast cancer cell lines (MDA-MB-231, HCC-19954) were transfected with miR-411, miR-758 or a scramble miRNA sequence using Lipofectamine® RNAiMAX. Transfection success was confirmed by qPCR. The impact of elevated miRNA expression on cell proliferation, cytoskeleton remodelling, migration and capacity to stimulate angiogenesis was determined. Cell proliferation was analysed using CellTiter 96® Aqueous One Solution Assay. Changes in cell cytoskeleton were determined through F-actin, vinculin and paxillin staining. Corning Transwell Inserts with 8μm pores were used to quantify migration. Cell conditioned media (CCM) was harvested from transfected cells and the ability to stimulate tubule formation determined using Human Umbilical Vein Endothelial Cells (HUVECs). Significant elevation of miR-758 or miR-411 was confirmed in both HCC-1954 and MDA-MB-231 cell lines after transfection with miRNA mimics. In the presence of elevated miR-411 or miR-758 there was no change in cell viability observed in either cell population. Interestingly, cytoskeleton staining revealed a change in intracellular distribution of F-actin. While control cells maintained the wild-type elongated morphology with well-polarized actin bundles, miR-758 transfected cells displayed increased volume and loss of polarity. Consequently, a significant decrease in migration of MDA-MB-231 cells expressing miR-758 was observed. HUVECs incubated with CCM from cells transfected with miR-758 demonstrated a reduced capacity to form tubules compared to those incubated with CCM from control cells. This exciting preliminary data highlights potential for miR-758 as a potent tumour suppressor in breast cancer, with potential for synergistic effects with miR-379. Studies are ongoing to determine the specific mRNA targets of miR-758 that mediate the effects observed.

452. Purification Process Development of Plasmid DNA for Gene Therapy

Hassan Sakhtah[1], Tao Xiang[1], Nida Zubairy[1], Jared Kaufman[1], Chiming Yu[1], Roy Lin[1], Jaime de Souza[2]

[1]Boston Institute of Biotech, Southborough, MA,[2]Milliporesigma, Burlington, MA

Plasmids are small double stranded DNA molecules that are separate from the chromosome. Plasmids are present naturally in bacterial cells and are commonly used as raw materials in mRNA and gene therapy vaccines. These plasmids can be used as the gene template for mRNA vaccines, or to provide the necessary genes, such as rep/cap/gene of interest, in adeno-associated virus (AAV) processes. The current work outlines Millipore's collaboration with Boston Institute of Biotechnology, LLC, Southborough MA, a biotechnology contract development and manufacturing organization (CDMO), to develop unit operations for the purification of a plasmid DNA used for AAV gene therapy application. Currently the purification of pDNA in a pure form ready for therapeutic use still presents various challenges starting from the lysis step. It also requires development of highly reproducible and scalable processing methods that meet regulatory manufacturing standards. The development here focuses on purification of the supercoiled isoform of pDNA and the use of scalable technologies on process steps of lysate clarification, ultrafiltration/diafiltration, capture using membrane ion exchange chromatography, and sterile filtration.

453. Tumor-Targeted Delivery of Exosome-Encapsulated Antisense Oligonucleotides Using Neural Stem Cells

Tomasz Adamus, Chunsong Yu, Chia-Yang Hung, Mohamed Hammad, Linda Flores, Kokilah Muthaiyah, Piotr Swiderski, Sergey Nechaev, Qifang Zhang Zhang, Karen Aboody, Marcin Kortylewski

Beckman Research Institute, Duarte, CA

Hypoxia-driven tumor tropism of neural stem cells (NSCs) creates an opportunity for the drug delivery. Here, we demonstrate that NSCs can effectively transport oligonucleotide therapeutics (ONTs), such as antisense oligonucleotides (ASO) into brain tumor microenvironment. We used previously described conjugates of STAT3ASO with Toll-like receptor-9 (TLR9) agonists, CpG oligodeoxynucleotides tor gymnotic loading of NSCs. Following endocytosis, CpG-STAT3ASO was released into cytosol and colocalized with $CD63^+$ vesicles. Over 3 days post-uptake, NSCs released extracellular vesicles, mainly $CD63^+CD81^+$ exosomes, loaded up to 80% with fluorescently-labeled CpG-$STAT3ASO^{Cy3}$. The CpG-STAT3ASO-loaded exosomes showed potent immunostimulatory properties, compared to native exosomes, inducing immune activation, NF-κB signaling and IL-12 production by human dendritic cells or mouse macrophages. Next, we verified antitumor efficacy of the NSC-mediated CpG-STAT3ASO delivery against subcutaneous GL261 glioma in mice. Peritumoral injections of 5×10^5 NSCs loaded *ex vivo* with CpG-STAT3ASO inhibited tumor growth similarly as the equivalent amount of CpG-STAT3ASO alone. Finally, using orthotopic GL261 tumors, we demonstrated that NSC-mediated delivery improves oligonucleotide transfer into the glioma microenvironment compared to injection of naked oligonucleotides. Correspondingly, we observed the enhanced activation of glioma-associated microglia by the NSC-delivered CpG-STAT3ASO. We anticipate that further optimization of NSC-mediated delivery can benefit application of oligonucleotides to glioma therapy.

Synthetic/Molecular Conjugates and Physical Methods for Delivery

454. Albumin-Decorated Exosomes Demonstrate Extended Circulation Time In Vivo

Xiuming Liang[1], Dhanu Gupta[1], Valentina Galli[2], Wenyi Zheng[1], Christopher Davies[2], Justin Hean[2], Eleni Kyriakopoulou[2], Xiuna Yang[2], Jorge de Andres[2], Doste R. Mamand[1], Risul Amin[1], Zheyu Niu[1], Rim Jawad[1], Oscar Wiklander[1], Antje Zickler[1], Manuela Gustafsson[1], Andre Gorgens[1], Joel Z. Nordin[1], Samir EL Andaloussi[1]

[1]Karolinska Institutet, Stockholm, Sweden,[2]Evox Therapeutics Limited, London, United Kingdom

Exosomes have shownpromise as potential therapeutics for the treatment of diseases. However, theirrapid clearance after administration could be a limitation in certaintherapeutic settings. Here we design and study an exosome engineering strategywhich dramatically extends the circulating half-life of exosomes in vivo, byintroducing albumin onto the surface of the exosome through surface engineeringof albumin binding domains (ABDs). ABDs were added to the large extracellularloops of select tetraspanins (CD63, CD9 and CD81) and these engineered exosomesexerted robust binding capacity to mouse and human albumins. By coating thesurface of the exosome with albumin we show that the circulating concentrationsof exosomes increased more than 10-fold after both intravenous andintraperitoneal injection. Moreover, these engineered exosomes also showed considerablelymph node (LN) and solid tumor accumulation, which may have utility when usingexosomes for immunomodulation and immunotherapy. The increased circulationhalf-life of exosomes may also be important when combined with tissue-specifictargeting ligands. In conclusion, by introducing ABDs onto exosomes, we havesignificantly extended the circulation half-life of exosomes, which couldprovide significant benefit for their therapeutic use in a variety of diseaseindications.

455. Electro-Mechanical Transfection of T Cells at Manufacturing Scale

John Zhao, Jessica Sido, James Hemphill, Bethany Grant, Cullen Buie, Paulo Garcia

Kytopen, Cambridge, MA

Adoptive cell therapies are dependent on reliable, repeatable, reproduceable methods of cell engineering to deliver the payload of interest to cells in a manufacturing environment. Of the non-viral solutions available, batch electroporation has gained traction; but this method limits throughput and has had challenges maintaining high viability and overall yield, particularly as it is scaled to manufacturing volumes. Our novel solution addresses these limitations by utilizing electro-mechanical transfection: continuous fluid flow combined with a low-energy electric field. This technique boasts high efficiency, high viability, and higher overall yield while easily scaling to manufacturing volumes. This novel electro-mechanical transfection technique, delivered via the *Flowfect™* System developed by Kytopen Corporation (Cambridge, MA), results in fast and efficient payload delivery. Electro-mechanical transfection utilizes the mechanical energy inherent in a high Reynolds number flow combined with a precise electric field delivered to an in-line flow cell. The result is a transfection process that can be implemented equally at small scale (e.g. 100 uL) and large scale (e.g. 5mL or more). Additional benefits of continuous fluid flow include integration with an automated liquid handling system, for high throughput transfection optimization, and delivery in a closed system, for engineered cell-based product manufacturing. A demonstration of this technology at low volume for rapid optimization and scale up to larger volume is shown in the delivery of GFP reporter mRNA to primary T cells. We also demonstrate optimization of electro-mechanical transfection parameters for specific cell type and payload combinations, the goal being to obtain high efficiency while maintaining high viability. This optimization was performed using a small volume high throughput application of the *Flowfect™* technology, utilizing a 96-well plate to identify settings producing optimal output. Optimal electro-mechanical transfection parameters were also confirmed in multiple donors. The optimal settings were then transferred to a large volume application of the *Flowfect™* technology to produce therapeutically relevant quantities of engineered cells. This compelling case-study, shown at multiple scales, supports the claim that electro-mechanical transfection can be used for primary cell editing, and that the *Flowfect™* technology will enable rapid translation of therapeutic innovations from the bench to the clinic.

456. Development of Lentiviral Vector Respiratory Tract Dosing Methods in Anticipation of Rodent GLP Toxicology Assessment

Anthony Sinadinos[1], Ana Sergijenko[1], Cuixiang Meng[1], Toby Gamlin[2], Stephen Hyde[2], Deborah R. Gill[2], Uta Griesenbach[1], Eric W. F. W. Alton[1]

[1]National Heart and Lung Institute, Imperial College London, London, United Kingdom,[2]Radcliffe Department of Medicine, University of Oxford, Oxford, United Kingdom

Introduction: We have developed a Simian Immunodeficiency Virus (SIV)-based lentiviral vector pseudotyped with the Sendai-virus envelope glycoproteins (F/HN) (rSIV.F/HN) that is effective at transducing pulmonary epithelium in vivo. To prepare for a first-in-man clinical trial, we have developed protocols that can be used in a mouse GLP-toxicology study to efficiently transduce the nose or lungs. In addition, we have used a radiopharmaceutical as a surrogate for the viral vector, to quantify dose deposition in target organs. **Mouse nose transduction protocol:** We aimed to efficiently target gene transfer to the murine nose with minimisation of vector loss to non-target organs, including the lung. We compared our standard 100 µL bolus administration ("nasal sniffing") to pipetting of small volume doses (2 x 5 µL). All animals were treated with 1e7 TU/mouse of rSIV.F/HN expressing luciferase (n=5-6/group). 10-12 days after transduction, both methods resulted in non-significantly different effective transduction of the nasal cavity. However, in contrast to administration via nasal sniffing, pipetting of small volumes prevented gene expression in the lungs. Technetium radiotracer (5MBq of

99mTc-DTPA/mouse) showed that 98.1±1.2% of the pipetted dose was retained in the head. In contrast, nasal sniffing only retained 36.1±12.0% in the head, with the rest of the radioactivity dispersing into lungs (34.0±15.6%) and the rest of the body (29.9±4.5%). To increase vector delivery to the nasal epithelium further, we used up to 10 x 5 μL doses (at 5 min intervals) to a maximum of 2.4e8 TU/mouse, n=5/group. We observed a dose-related increase in gene expression ($p<0.0001$, $r2=0.87$). Importantly, even after 10 x 5 μL doses the majority of the radio-tracer (90.0±2.8%) was retained in the head. **Mouse lung transduction protocol:** We aimed to maximize transgene delivery to the lung, comparing nasal sniffing to an oropharyngeal (OP) delivery method (1e7 TU/50 μL dose for both techniques). There was no significant difference in gene expression within the lung with either technique. We further compared volume distribution using technetium as a radiotracer (5MBq of 99mTc-DTPA/mouse, n=5-6/group). Mice were culled 10 min after administration and radioactivity was compared in the lungs, head and rest of the body. No significant differences in quantities of radioactivity were detectable in the lungs using either delivery method (sniffing: 40.7±18.4%, OP: 37.0±10.9%). However, nasal sniffing is able to deliver double the volume of that for OP. **Conclusions:** We have developed protocols suitable for GLP toxicology studies in the murine nose and lung to optimise vector delivery to the target organ. By defining dose distribution with a radiotracer we are better able to calculate effective viral titres and overages compared to proposed clinical doses in future toxicology studies.

457. Non-Viral Polymer Nanoparticle Delivery of mRNA to the Brain after Intrathecal Lumbar Injection

Brian P. Mead, Angeleica Zabronsky, Julia Suffoletta, Audrey Hollingsworth, Franco Duarte, Santanu Maity, Timothy C. Fong, Kunwoo Lee

GenEdit, Inc., South San Francisco, CA

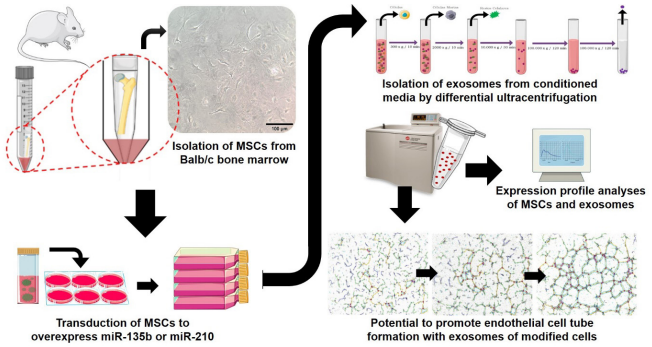
Clinical translation of CNS-directed gene therapies demonstrates the potential to treat and cure a broad array of diseases. However, the lack of efficient and safe targeted delivery systems still limits use in therapeutic applications. Even the most clinically advanced adeno associated viruses (AAVs) are hampered by inefficient or off-target delivery, pre-existing immunity, genome integration, and costly manufacturing. Non-viral delivery systems are an alternative to address these issues. We have developed chemically synthesized co-block polymers that can efficiently encapsulate RNA, DNA, proteins, or CRISPR-Cas9 ribonucleoprotein and deliver these cargos to specific organs and tissues after intravenous (IV) or intrathecal (IT). Our proprietary polymer nanoparticles (PNP) have the potential to deliver to targeted regions of the brain after IT administration into the lumbar cerebrospinal fluid. Previously, we have shown that one candidate PNP formulation, GEN-150, can deliver a mRNA cargo to the brain after IT lumbar injection in mice. Delivery of the cargo was observed in cells adjacent to the ventricles. Further engineering of the polymer nanoparticle was conducted to understand properties that are important for targeted delivery and penetration in the parenchyma. Indeed, the GEN-190 series of nanoparticles, encapsulating a firefly luciferase reporter mRNA, were administered IT and mediated efficient and broadly distributed bioluminescence in the brain. Tissue distribution of GEN-190 PNPs was further assessed via histological assessment using GEN-190 encapsulating Cre mRNA in the Ai9 TdTomato transgenic mouse model. Our results showed delivery to the hindbrain with deeper penetration into the parenchyma and transfection of both neurons and astrocytes. These data suggest that the polymer nanoparticles can deliver gene therapy cargos via IT administration having a potential for gene therapeutic approaches to treat neurological diseases affecting the target brain regions.

458. Effect of Overexpression of miR-135b and miR-210 on Mesenchymal Stromal Cells on the Production of Extracellular Vesicles with Angiogenic Factors

Juliana M. F. Vieira[1], Laura N. Zamproni[2], Rafael S. Lindoso[3], Sang W. Han[1]

[1]Department of Biophysics, Federal University of São Paulo (UNIFESP), Sao Paulo, Brazil,[2]Department of Biochemistry, Federal University of São Paulo (UNIFESP), Sao Paulo, Brazil,[3]Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

Critical Limb Ischemia (CLI) is characterized by narrowing of the arteries of the lower limbs. The available treatments for CLI are limited due to the comorbidities and advanced age of patients, motivating the development of new treatments. A promising approach is the use of microRNAs for angiogenic therapy. The miR-135b and miR-210 microRNAs are angiogenic regulators that have an important impact on signaling pathways in response to hypoxia. In addition, extracellular vesicles (EVs) of mesenchymal stromal cells (MSCs) are known to support tissue regeneration after ischemic injuries and are potential vehicles for the delivery of microRNAs. - In this study, we aimed to investigate whether overexpression of miR-135b or miR-210 in MSCs can produce EVs enriched with these miRNAs and improve their angiogenic activity. For this purpose, bone marrow MSCs from Balb/C mice were transduced with a lentiviral vector containing a gene expression cassette for miR-135b or miR-210, and expression profiles of angiogenic genes and microRNAs were evaluated by RT-qPCR. EVs from MSCs were isolated and purified by ultracentrifugation and characterized by flow cytometry and NTA (Nanoparticle Tracking Analyzer). The concentrations of angiogenic microRNAs in EVs were measured by RT-qPCR and these EVs were used to assess pro-angiogenic activity on HUVECs. As result, we observed that the modification of MSCs with miR-210 or miR-135b promoted overexpression of respective microRNAs. The MSCs overexpressing miR-135b presented an upregulation of miR-210-5p and follistatin, a glycoprotein associated with angiogenesis and vasculogenesis. MSCs overexpressing miR-210 led to negative regulation of miR-221 and increase of miR-296. Moreover, EVs derived from the modified MSCs were enriched with both target miRNAs and promoted further formation of tubular structures in HUVEC. Collectively, our results reveal that EVs can be enriched with a specific angiomiR by modifying of MSCs with a lentiviral vector, and these EVs showed increased angiogenic activities, indicating an interesting strategy for EV-based therapy for ischemic diseases.



459. Using Novel Intein Technology to Deliver Exosome-Based Therapeutics

Justin Hean[1], Claire Pearce[1], Pijus Brazauskas[1], Charlie de Havilland[1], Charlotte Stainsby[1], Maria Jacintha Victoria[1], Thaine Mayes[1], Zoe Rees[1], Xiuna Yang[1], Jorge Salazar[1], Trang Mai[1], Vrinda Sreekumar[1], Gianmaria Vincenzi[1], Luke Haslett[1], Stefania Fedele[1], Yi Yang[1], James Veale[1], Daria Farcas[1], Andrew Christie[1], Jacqueline Vieira[1], Olusegun Abiola[1], Tania Selas-Castineiras[1], Sonam Gurung[2], Julien Baruteau[2], Sonya Montgomery[1]

[1]Evox Therapeutics, Oxford, United Kingdom,[2]University College London, London, United Kingdom

Exosomes are nonimmunogenic extracellular vesicles, secreted by most cell types, that play a critical role in cell-to-cell communication by proteins and nucleic acids transfer. We aim to leverage the natural delivery capabilities of exosomes to deliver therapeutics to treat life-threatening rare diseases and develop a new medicine modality. We utilise an engineered mini-intein (pH sensing, cis-cleaving) based technology that, when combined with an exosome targeting fusion, enables the luminal loading of soluble therapeutic protein cargo. Subsequently, we have generated luminally-loaded exosomes capable of delivering drug cargos destined for the cell cytoplasm, without impacting the inherent activity of the drug itself. Here we describe steps taken in the transformation of this technology from proof-of-concept through translation into preclinical development and advancement towards the clinic. One of our main focusses is developing a treatment for Argininosuccinic aciduria (ASA), a rare, life-limiting autosomal recessive urea cycle disorder caused by a mutation in the arginosuccinate lyase (ASL) gene. This therapeutic candidate delivers exosome mediated soluble ASL protein to hepatocytes. Here we describe targeted extracellular vesicle (EV) loading of entirely soluble ASL when expressed as a cleavable ASL fusion protein, compared to wild-type ASL which is exclusively intracellular. In addition, we show *in vitro* confirmation of the ability of complex engineered proteins to fold correctly and retain activity comparable to wild-type human ASL enzyme. Finally, we present data to demonstrate functionality of exosome-delivered soluble ASL protein through *in vivo* modification of biomarkers of disease in a hypomorphic knock-in $Asl^{Neo/Neo}$ murine model. Lastly, we also demonstrated proof of concept of the modularity of this intein-based design through successful loading of protein cargos for arginosuccinate synthetase deficiency (ASSD), a second urea cycle disorder caused by a mutation in the ASS1 gene leading to a deficiency in hepatic enzyme arginosuccinate synthetase (ASS) that results in high levels of citrulline and ammonia.

460. Cationic Diblock Copolymer Nanoparticles Increase pDNA Payload and Improve pDNA Cell Delivery

Kenneth R. Sims Jr.[1], Danielle Huk[2], Cherry Gupta[1], Stephanie Kute[3], Anthony Duong[1]

[1]Advanced Materials and Energetics, Battelle Memorial Institute, Columbus, OH,[2]Health Outcomes & Biotechnology Solutions, Battelle Memorial Institute, Columbus, OH,[3]Medical Devices and Health Analytics, Battelle Memorial Institute, Columbus, OH

Delivery vehicles constructed using synthetic chemistry (*i.e.*, polymers) have the potential to solve the greatest challenge for new pharmaceuticals, particularly emerging genetic medicines - safe and effective delivery of large payloads. Gene editing technology based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a rapidly growing subset of genetic medicine that has the potential to increase the efficacy and accessibility of treatments for genetic disorders. Rather than delivering short gene segments to replace proteins or modulating protein translation via silencing, CRISPR enzymes directly edit patient cell genomes, permanently correcting genetic mutations. Whether the editing is done with plasmid deoxyribonucleic acid (pDNA) encoding the CRISPR ribonucleoprotein (RNP) or by administering the RNP itself, the main obstacle for gene therapy continues to be the ability to deliver the payload to the correct cells while avoiding adverse immune responses. Virus vectors have dominated recent clinical research as delivery vehicles for genetic medicines, but they are too small and too immunogenic to effectively deliver CRISPR gene editors to cells, so the development of new delivery vehicles is needed. We designed and constructed polymer delivery vehicles, which are reconfigurable, affordable, and scalable, to solve the challenge of delivering large CRISPR pDNAs and RNPs. Here, we used a cationic diblock, copolymer nanoparticle (NP) containing 2-(dimethylamino) ethyl methacrylate (DMAEMA), butyl methacrylate (BMA), and 2-propylacrylic acid (PAA) synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization (Fig 1A) to demonstrate the ability to form complexes with pDNA up to 48.5 kb (Fig 1B) and to deliver pDNA cargo encoding a Cas9-GFP fusion protein and a non-targeting gRNA to HEK293 cells (Fig 1C). Doses of pDNA (1.5 µg pDNA/well; *i.e.*, 5 pg/cell, ~9.5×10^{-6} pmol/cell) coupled with a NP concentration (57 nM; *i.e.*, 3.8×10^{-5} pmol/cell for a 4:1 NP to pDNA molar ratio) demonstrated the highest level of transfection efficiency - significantly greater than the commercial benchmark Lipofectamine 3000 as indicated by fluorescence intensity (Fig 1C). Upon evaluation of cytotoxicity, we identified a direct correlation between pDNA dose and cell viability for the NP concentration of 57 nM (Fig 1D). The 1.5 µg DNA dose with 57 nM NP was significantly more cytotoxic than Lipofectamine, but doubling the DNA dose negated the cytotoxic effect (Fig 1D). These results demonstrate a proof-of-concept for the use of RAFT diblock copolymers for use in

delivering CRISPR-Cas9 pDNA to cells. We are working to further optimize the delivery system to maximize cell transfection while minimizing cell cytotoxicity.

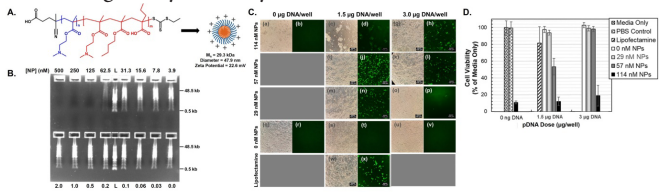


Figure 1. A. Scheme showing the diblock copolymer composition and a cartoon of the self-assembled cationic micelle NP. NP characteristics: Overall M_n=29.3 kDa, Diameter=47.9 nm, Zeta Potential=22.6 mV. **B.** Photo of electrophoresis gel showing the NP's ability to load a pDNA ladder (up to 48.5 kb) at NP concentrations between 62.5 nM and 500 nM. **C.** Brightfield and fluorescence images of HEK293 cells treated with a range of pDNA concentrations (0, 1.5, or 3 µg/well; 0 – 1.9 x 10^{-5} pmol/cell) with and without NPs (0, 29, 57, or 114 nM) or Lipofectamine (positive control). **D.** Cell viability results shown as a % of the positive media only control for Lipofectamine and varied NP concentrations (0, 29, 57, or 114 nM) loaded with different amounts of pDNA (0, 1.5, or 3 µg/well; 0 – 1.9 x 10^{-5} pmol/cell).

461. Targeted Gene Delivery to Cervical Cancer Cells In Vitro Using Green Synthesized Copper Oxide Nanoparticles

Keelan Jagaran, Moganavelli Singh

Biochemistry, University of KwaZulu-Natal, Durban, South Africa

Cervical cancer is responsible for 90% of female deaths in low- to middle- income countries according to the World Health Organisation. This cancer type has been identified as the fourth most frequent cancer affecting women and is a global health crisis resulting in thousands of deaths, worldwide. The sector of clinical oncology is riddled with the devasting outcomes of patients presenting with various cancer types, on a daily basis. Current treatment approaches although effective cause deleterious effects to healthy organs as a result of non-target specificity and their inability to evade efflux pumps. Hence, this treatment strategy is rendered "short-term". Recent technological advancements have provided a means of enhancing patient diagnostics, therapeutics and monitoring abilities. A breakthrough in traditional medicine has emerged in the amalgamated form of medicine and nanotechnology, known as nanomedicine, and is revolutionizing the sector, by overcoming the challenges experienced by traditional treatments. The current study employs' the use of nanomedicine, utilizing a novel copper oxide nanoparticle with the aim of enhancing diagnostic and therapeutic strategies against cervical cancer. These nanoparticles were biologically synthesised, using the *Azadirachta indica* leaf extract, functionalized with chitosan and poly(ethylene glycol) (PEG), and conjugated to the targeting ligand, folate. This approach vaunts a vast array of beneficial properties, aimed at formulating a nanocomplex with cell specificity, lowered toxicity, good biocompatibility, antibacterial activity and low cost. Nanoparticles and nanocomplexes were characterized by Fourier transform infrared (ATR-FTIR) and UV-vis spectroscopy, transmission electron microscopy (TEM) and nanoparticle tracking analysis (NTA). Copper oxide nanoparticles with an average particle size of 78.2 nm were successfully synthesized. Electrophoretic mobility shift assays confirmed pCMV-*Luc*-DNA binding, while nuclease digestion assays revealed the good protective ability of these nanoparticles towards the bound DNA. *In vitro* cytotoxicity using the 3-[(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay revealed cell viability >70% in the human embryonic kidney (HEK293), cervical carcinoma (HeLa) and breast adenocarcinoma (MCF-7) cells, with significant transgene expression, obtained using the luciferase reporter gene assay. Overall, these nanocomplexes displayed good cell viability and cellular uptake, accentuating their role in gene delivery, warranting future optimisations and *in vivo* studies to be undertaken.

462. Targeted Nano-Particles (TNP) Technology as a Universal and Versatile Platform for Gene Therapy

Rachel Pacherie, Laurence Leclere, Marion Lhuaire, Eva Maunichy, Charles Duchene, Maxime Girardon, Ozgul Tezgel, Frederic Mourlane, Renaud Vaillant, Cecile Bauche

Ixaka, Villejuif, France

Intracellular delivery of nucleic acids to mammalian cells using non-viral gene delivery remains a challenge both *in vitro* and *in vivo*, with transfections often suffering from variable efficacy and limited stability. Ixaka's TNP platform is a unique technology that combines the versatility of negative and positive oligopeptide-modified poly(beta-amino ester)s (OM-PBAEs) as biodegradable and safe transfection material together with the gene transfer efficiency of lentiviral vectors (LV). Here we report on the microfluidics-based formulation of LV particles lacking the VSV-G protein ("bald") encapsulated with OM-PBAE polymers. Biophysical methods (NTA, DLS, Videodrop) have been implemented and are used in routine to monitor nano-particles size, concentration and aggregates. The nano-particles (NP) showed a remarkable stability at room temperature as confirmed by transduction efficiency and biophysical methods. *In vitro*, targeted NP efficiently transduced a wide range of mammalian cells including human and mouse cell lines and primary immune cells, without pronounced cell toxicity. Importantly, *in vitro* and *in vivo* transduction efficiency was achieved without the need of CD3/CD28 activation and cytokines addition normally required with VSV-G pseudotyped LV for immune cells transduction. Cell reprogramming was achieved when intracellularly expressed reporter system (GFP) and surface receptor (Chimeric Antigen Receptor) were used as transgenes. Targeted NP efficiently transduced human neurons and hepatocytes and favorably performed compared to VSV-G pseudotyped LV vectors. Thus, Ixaka's versatile platform offers the potential to target and reprogram a broad range of therapeutic cells not limited to the immuno-oncology field but also various genetic disorders, neurological and ocular diseases.

463. Toward In Vivo Clinical Epigenome Rejuvenation Mediated by Lentiviral/Non-Viral Nanoparticle Delivery of mRNA Encoding Oct4, Sox2 and Klf4 Transcription Factors as a Potential Genome-Safe Transient Epigenetic Gene Therapy Approach for Age-Related Disorders and Degenerative Diseases

Roger Bertolotti

Gene Therapy and Regulation, Faculty of Medicine, University of Nice - Sophia Antipolis, Nice, France

Based on hit-and-run protocols, transient epigenetic gene therapy is aimed at long-term transcriptional gene silencing/activation (down/up regulation) through the transient action of short-lived epigenetic/

epigenomic effectors. Initially based on promoter/enhancer-specific siRNAs or short sense/antisense RNAs/oligonucleotides, it involves DNA methylation/demethylation and post-translational histone modifications. Direct epigenome editing culminates now with double-mutant nuclease-null CRISPR/dCas9 and dCas12a as breakthrough programmable single RNA-guided DNA-binding modules for targeted delivery of a variety of fused epigenetic effector domains such as DNA demethylases/methyltransferases, histone lysine-acetyltransferases/deacetylases, histone lysine methyltransferases/demethylases, and various recruiters of endogenous transcriptional activators/repressors. Such a flexible and cost-effective dCas9/dCas12a-based targeted epigenome editing technology is amenable to multiplex engineering, thereby easing true epigenetic editing achievements that are stable over cell divisions. Stable epigenome remodeling is thus involved in many therapeutic approaches and is a driving force for our focus on age-related epigenetic dysregulations, a major aging concern. Importantly enough, hot-off-the-press in vivo epigenetic youthful reprogramming induced by transient ectopic expression of three of the Yamanaka factors (Oct4, Sox2 and Klf4: OSK) stands as a true breakthrough for age-related pathologies and regenerative Medicine. Unlike the full set of Yamanaka factors (OSKM, M: cMyc), OSK does not erase cellular identity, thereby excluding the genesis of induced pluripotent stem cells (iPS cells) and of teratomas. Although obtained by others using AAV delivery of polycistronic OSK, we believe that the restoration of a youthful transcriptome and DNA methylation pattern in retinal ganglion cells of aged mice without erasing cell identity could straightforwardly translate into a variety of tissue-specific clinical trials mediated by OSK mRNA (polycistronic or optimized cocktail) delivered by lentiviral capsid-based bionanoparticles. The lentiviral capsids secure efficient cell entry and, as shown by others, can readily accommodate and deliver therapeutic mRNA tagged by a 3'end aptamer when a relevant aptamer-binding protein is fused to the viral nucleocapsid protein. Although less fitted to tissue precision Medicine, nucleoside-modified mRNA formulated in non-viral nanoparticles is another attractive option. Therefore, in our therapeutic approach for age-related disorders and degenerative diseases, we are now shifting from targeted epigenome editing (e.g. targeted reactivation of down-regulated genes involved in cellular NAD+ homeostasis) to OSK mRNA-mediated global epigenetic rejuvenation, thereby relying on the reactivation and down-regulation of relevant genes upon restoration of healthy youthful transcriptional landscapes without loss of original cell identity. Our approach based on genome-safe delivery of mRNA or nucleoside-modified mRNA is discussed in light of 1) the four arms of our proposed universal stem cell gene therapy platform and their synergistic combinations, 2) published Cas9 mRNA and Cas9:sgRNA RNP delivery mediated by lentiviral capsid-based bionanoparticles, 3) induced pluripotent stem cell genesis and in vivo teratoma induction, 4) potential synergistic use of senolytics and/or dietary complements and 5) epigenetic memory and epigenetic information theory of aging.

464. Using Base Editing and LNP Delivery to Correct Disease-Causing Mutations Underlying Genetic Liver Diseases

Delai Chen, Shailendra Sane, Raymond Yang, Robert Dorkin, Yi Eric Zhang, Dongyu Chen, Xiao Luo, Yvonne Aratyn-Schaus, Brian Cafferty, James Tam, Krishna Ramanan, Jeffrey Marshall, Aaron Larsen, Michael Packer, Sarah Smith, Carlo Zambonelli, Francine Gregoire, Giuseppe Ciaramella, Manmohan Singh

Beam Therapeutics, Cambridge, MA

Base editing enables programmable single-base genomic mutations and has the potential to permanently cure serious genetic diseases. Realizing its therapeutic potential requires development of (1) new base editors with appropriate PAM specificity, editing window, and efficacy; (2) sgRNA sequence and chemistry for optimal potency and target specificity; (3) safe and effective methods for mRNA and sgRNA delivery to target organs and intracellular compartments. Here we share some of our progress in developing base editing therapeutics for liver-originated genetic diseases using lipid nanoparticle (LNP) as the in vivo delivery system. To treat Alpha-1 Antitrypsin (A1AT) Deficiency, we engineered novel adenine base editor (ABE) variants and chemically modified guide RNAs to efficiently correct the PiZ mutation in patient-derived cells. Using lipid nanoparticles, mRNA encoding ABE and guide RNA were delivered to PiZ transgenic mice, resulting in durable gene correction, improved liver pathology, and increased serum A1AT level. To treat Glycogen Storage Disease Type Ia (GSDIa), novel ABE variants were engineered to correct R83C and Q347X, the two most prevalent mutations associated with the disease. LNP delivery of the sgRNA and ABE-encoded mRNA in transgenic mice resulted in correction of the R83C mutation at a level surpassing those expected to restore glucose homeostasis. Here we report efficient editing in the liver of non-human primates using an LNP delivery system containing ABE and reporter sgRNA. These results indicate that base editors in combination with LNP delivery have the potential to treat genetic liver diseases. Further efforts including optimization of the delivery system are ongoing.

465. A Transgenic System for Active Loading of miRNAs into Exosomes Using Aptamers

Andrew Hamann, Kelly Broad, Angela K. Pannier

Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE

Introduction: Exosomes are extracellular vesicles that are under research for therapeutic applications due to their ability to deliver biomolecules to recipient cells with high efficiency and modulate many pathophysiologic processes. Recent studies suggest that many therapeutic effects of mesenchymal stem cells (MSCs), a cell type that has been investigated in >1000 clinical trials, are actually mediated by exosomes secreted by MSCs, which make MSC exosomes attractive candidates for off-the-shelf therapies that could avoid manufacturing and safety challenges associated with whole cells. Additionally, many therapeutic effects of exosomes are associated with microRNAs (miRNAs) contained within the vesicles. This work aimed to engineer a system to actively load miRNAs of choice into exosomes using

cellular machinery, to produce exosomes with enhanced therapeutic properties. The system uses expression of a minimal scaffold of the vesicular stomatitis virus glycoprotein (mVSVG) [1], a transmembrane protein that inserts into exosomes. mVSVG was fused at the C-terminus to RNA aptamer-binding domains to localize these fusions inside exosomes, and facilitate loading of transgenic precursor miRNA (premiR) containing RNA aptamers. **Methods:** The components of the transgenic miRNA loading system were expressed from plasmids (**Figure 1**).

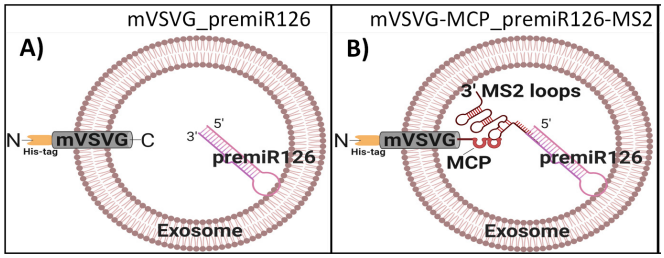


Figure 1: **A)** *mVSVG_premiR126* expresses mVSVG with no C-terminal fusion and premiR126 with no 3' aptamer **B)** *mVSVG-MCP_premiR126-MS2* expresses mVSVG with C-terminal fusion to MS2 coat protein (MCP) and premiR126 with MS2 loop aptamer. Figure created with BioRender.com. HEK293T cells were transfected in 96-well plates with plasmids expressing the described system, using Turbofect reagent. After 48 hrs, exosomes were isolated from media and lysed to extract RNA. Exosomal miRNA was reverse transcribed and amplified with TaqMan Advanced miRNA cDNA Synthesis Kit prior to quantification of relative mature miR126-3p and -5p by qRT-PCR, normalized to mature miR100-5p, using TaqMan Advanced miRNA Assays. All experiments were performed in triplicate (n=3) and on duplicate days. **Results:** Transfection with *mVSVG_premiR126*, resulted in about 10-fold more mature miR126-3p and -5p in exosomes than EGFP control, indicating that increased transcription of a precursor miRNA can increase the amount of the mature miRNA in secreted HEK293T exosomes passively. However, transfection of HEK293Ts with *mVSVG-MCP_premiR126-MS2* constructs (with MS2 loop aptamers linked to either the 3' or 5' end of premiR126) resulted in about 500-fold increases in mature miR126-3p and -5p contained in exosomes, relative to passive loading with *mVSVG_premiR126*. The location of the MS2 aptamers on premiR126 (i.e. 3' or 5' linked) did not bias active loading of mature miR126-3p or -5p into exosomes. In contrast, if MS2 aptamers were linked to both the 3' and 5' ends of premiR126, little to no mature miR126-3p and -5p was loading into exosomes, relative to passive loading with *mVSVG_premiR126*. **Conclusion:** The described system is able to actively load a miRNA of interest into exosomes of transfected cells, while also allowing normal processing to yield high levels of the mature -3p and -5p miRNA species within exosomes, suggesting the system could be used to produce exosomes with enhanced therapeutic properties. Future work aims to translate this technology for engineering of exosomes produced from therapeutically relevant MSCs. **References:** [1] Meyer C, et al. *Int J Nanomedicine*. 2017.

466. Non-Viral Delivery and Expression of Human GCase in Wild-Type Mice

Luke Hamm, Russell Monds, Philip Samayoa, Matt Chiocco, Doug Kerr, Tracy Zimmermann, Haiyuan Sun, Deb Klatte

Generation Bio, Cambridge, MA

Gaucher disease, type I (GD1) is a rare autosomal recessive lysosomal storage disorder caused by loss of function mutations to the *GBA1* gene encoding glucocerebrosidase (GCase). GCase is a lysosomal enzyme catalyzing the metabolism of glucosylceramide (GlcCer). In GD1, progressive accumulation of GlcCer in macrophages particularly of the bone marrow, liver, and spleen causes inflammation leading to hepatomegaly and splenomegaly, anemia, thrombocytopenia, and bone disease. GD1 is the most common variant of Gaucher disease with a prevalence of 1-2 per 100,000. Unlike Gaucher disease types 2 and 3, GD1 does not usually affect the central nervous system. The current standard of care for the management of GD1 is biweekly intravenous enzyme replacement therapy (ERT) with GCase targeted to macrophages by glycosylation modifications to improve mannose 6-phosphate receptor mediated uptake. Even with regular ERT not all patients achieve a complete therapeutic response. Recently, adeno-associated viral (AAV) gene therapy has been considered as a potential treatment for GD1. McEachern and co-workers (McEachern, et al. *J Gene Med*. 2006) demonstrated the feasibility of GBA1 gene therapy in the D409V/null mouse model of GD1 by showing that hepatically expressed GCase was secreted into systemic circulation and reduced the accumulation of GlcCer in liver, lung, and spleen. Despite these successes, there remain significant limitations to AAV gene therapy attributable to the viral capsid. These include development of anti-capsid neutralizing antibodies which limit dosing to a single administration, emerging concerns around safety at higher doses, and costly, scale-limited manufacturing processes. Lipid nanoparticle (LNP) delivery of a non-viral gene therapy could potentially address these limitations. To determine the feasibility of achieving secretion and uptake of human GCase using cell-targeted LNP (ctLNP) delivery of a non-viral gene therapy, transgene constructs expressing human *GBA1* (hGBA) under a liver specific promoter were screened *in vivo* first by i.v. hydrodynamic injection. Wild-type CD-1 mice that received human GBA expressing constructs exhibited dose dependent plasma GCase activity at 3- and 7-days post-dose. Plasma GCase activity was undetectable in vehicle treated animals. The presence of human GCase in splenic macrophages was determined *via* immunofluorescent imaging of tissue sections. Human GBA mRNA expression was evaluated in liver and spleen samples to determine the relative restriction of expression. A follow-up study was conducted to evaluate expression and activity following delivery of a human GBA expressing construct via ctLNP *in vivo*. Analysis of hepatic expression, systemic activity, and splenic macrophage uptake of human GCase from these studies will be presented.

467. Mechanisms of Adoptive T cell Micropharmacies

Christopher M. Bourne[1], Megan M. Dacek[2], Mamadou A. Bah[1], Jesus Romero-Pichardo[3], Thomas J. Gardner[4], Peter Lee[5], Derek Tan[6], David A. Scheinberg[4]

[1]Immunology and Microbial Pathogenesis, Weill Cornell, New York, NY,[2]Pharmacology, Weill Cornell, New York, NY,[3]Industrial Microbiology, University of Puerto Rico, Mayaguez, Mayaguez, NY,[4]Pharmacology, Sloan-Kettering Institute, New York, NY,[5]Chemical Biology, Weill Cornell Medicine, New York, NY,[6]Chemical Biology, Sloan-Kettering Institute, New York, NY

Adoptive T cell therapy reprograms endogenous T cells, ex-vivo, to kill antigen positive cancer cells. Adoptive cell therapy has shown promise for treating B cell malignancies and melanoma, but many barriers limit their efficacy, such as antigen heterogeneity and an immunosuppressive tumor microenvironment. We hypothesized that adoptive T-cells can be used to deliver small cytotoxic molecules to solid tumors to overcome these barriers. Thus, we coupled enzyme prodrug therapies with adoptive T cell therapy, generating SEAKER (Synthetic Enzyme Armed KillER) cells. SEAKER cells can be engineered to secrete a variety of enzymes that are capable of cleaving cognate, engineered prodrugs. Once cleaved, these non-toxic prodrugs are converted into highly toxic chemotherapeutics. As SEAKER cells localize and accumulate in the tumor microenvironment, systemically administered prodrug will be unmasked at the tumor site, mitigating systemic toxicities. Here, we characterized the SEAKER platform using syngeneic, solid tumor mouse models. We demonstrated that the SEAKER platform is feasible and broadly applicable to a wide range of adoptive cell therapies. Our SEAKER platform demonstrated functional enzyme delivery to tumors and improved anti-tumor efficacy in syngeneic tumor mouse models. We characterized the kinetics and biodistribution of SEAKER cells for rapid translation into clinical settings. Coupling enzyme-prodrug therapies to adoptive T cell therapy is efficacious including in fully immunocompetent settings.

468. Microfluidic Droplet Squeezing for Genome Editing in Human Primary T Cells

Byeongju Joo[1], Jeongsoo Hur[1], Gi-Beom Kim[1,2], Seung-Gyu Yun[3], Aram Chung[1,2]

[1]School of Biomedical Engineering, Korea University, Seoul, Korea, Republic of,[2]Interdisciplinary Program in Precision Public Health, Korea University, Seoul, Korea, Republic of,[3]Department of Laboratory Medicine, College of Medicine, Korea University, Seoul, Korea, Republic of

Recently, cell-based therapies have shown compelling clinical evidence for effective cancer treatment. One representative example of whole cell-based therapy is the chimeric antigen receptor-engineered T cell (CAR-T) therapy, which involves genetically modified T lymphocytes selective activity against tumors. For cells to be equipped with therapeutic functions, their genomic alteration should be accompanied, which can be achieved by delivering external genetic nanomaterials into cells. Traditionally, viral transduction and electroporation are the most popular approaches to engineer cells. However, these methods are limited by their time-consuming processes, safety concerns, low scalability, and/or loss of cell phenotypes and functions, inhibiting clinical efficacy. To address these limitations, microfluidic approaches have emerged, demonstrating unprecedented potential for cell-based therapy. Most reported microfluidic delivery platforms deliver external biomolecules by passing cells through narrow constrictions, mechanically opening the cell membranes. Nevertheless, these approaches are susceptible to clogging, substantially lowering their practicality. Furthermore, the approach intrinsically suffers from high reagent consumption and low plasmid DNA transfection. To overcome these drawbacks, we present a novel intracellular delivery approach, namely "droplet squeezing," leveraging droplet microfluidics with cell mechanoporation. Briefly, cells and target genomic nanomaterials are co-encapsulated within droplets that pass through a series of narrow constrictions for cell membrane permeabilization, enabling a highly efficient internalization of external genes into cells. This approach inherently reduces the external biomolecule consumption substantially and allows near-zero clogging operations. Using the presented droplet squeezing method, mRNA, plasmid DNA, and CRISPR-Cas9 ribonucleoprotein (RNP) were internalized into various cell types, and highly effective hard-to-transfect primary T lymphocyte genomic editing was realized with minimal cell perturbation, taking the delivery performance to the next level.

469. Non-Viral Delivery of Therapeutic mRNA Using Tumor-Tropic Liposomes for the Treatment of Solid Tumors

Shoji Saito[1], Ikumi Nakashima[1], Eiichi Akahoshi[2], Mitsuko Ishihara-Sugano[2], Shigeki Yagyu[1], Yozo Nakazawa[1]

[1]Pediatrics, Shinshu University School of Medicine, Matsumoto, Japan,[2]Corporate Research & Development Center, Toshiba Corporation, Kawasaki, Japan

Introduction: The prognosis of relapsed/refractory cancers remains poor, and the development of new therapies is important. Although chimeric antigen receptor (CAR)-T therapy has shown considerable potential in hematological malignancies, those for solid tumors remain challenging. We have recently developed tumor-tropic liposomes that can specifically deliver therapeutic genes for T-cell leukemia. Using the originally developed lipids and artificial intelligence-optimized lipid compositions, liposomes that can specifically deliver therapeutic genes to leukemia cells but not to normal hematopoietic cells have been developed successfully. Furthermore, the anti-tumor activity of tumor-tropic liposomes loaded with suicide gene system has been demonstrated both, *in vitro and in vivo* against T-cell malignancies. Thus, the present study aimed to determine whether these tumor-tropic liposomes loaded with suicide gene system can be used for treating solid tumors. **Materials and Methods:** Based on the previous study, two types of liposomes (No. 43 and No. 79) were selected as candidates in the present study. To investigate the selective delivery and translation of the encapsulated mRNA, the cancer cells or normal peripheral blood mononuclear cells were incubated with rhodamine-labeled liposomes loaded with eGFP mRNA and examined for rhodamine and GFP expression using flow cytometry. Further, the cells were treated with liposomes loaded with inducible caspase 9 (iCas9) mRNA, with or without a chemical inducer of dimerization (CID) for an assessment of the *in vitro* anti-tumor effects. To investigate the *in vivo* anti-tumor effects, NSG mice were subcutaneously inoculated with MDA-MB-231 cells. The mice were then intratumorally treated with either liposomes loaded with iCas9 mRNA or vehicles on days 7 and 14 after tumor

inoculation. Every 24 h after the liposome infusion, the mice were intratumorally treated with CID. Tumor volumes were calculated from digital caliper measurement data using the formula (length × width^2). **Results:** Flow cytometry analysis showed the efficient uptake (81.6%-98.8%) of rhodamine-labeled liposomes by neuroblastoma (SK-N-SH), glioblastoma (TG98), and breast cancer cells (MDA-MB-231). When these liposomes were loaded with GFP mRNA, SK-N-SH, TG98, and MDA-MB-231 cells highly expressed GFP (80.3-%-97.4%) 24 h after the co-culture (Figure 1A). Furthermore, liposomes loaded with iC9 mRNA exerted anti-tumor effects against SK-N-SH, SK-TG98, and MDA-MB-231 cells in the presence of CID, with increased percentages (43.2%-76.5%) of apoptotic or dead cells (Figure 1B). These cytotoxic effects were partly inhibited by pan caspase inhibitor Z-VAD-FMK. Furthermore, the liposome (No.43) loaded with iC9 in combination with CID significantly suppressed tumor growth in MDA-MB-231-bearing NSG mice (Figure 1C, D). **Conclusion:** Tumor-tropic liposomes can efficiently deliver therapeutic mRNA to neuroblastoma, glioblastoma, and breast cancer cells. Furthermore, tumor-tropic liposomes loaded with suicide gene exhibited anti-tumor effects against these malignant cells. Thus, tumor-tropic liposomes can be used for treating solid tumors.

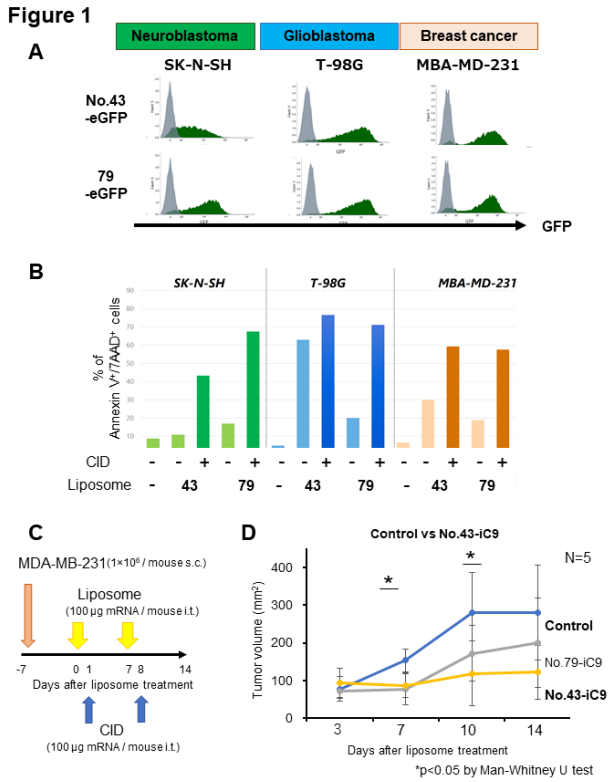


Figure 1. Tumor-tropic liposome exerted anti-tumor effects against solid tumors. (A) GFP expression in tumor cells analyzed by flow cytometry 24h after the co-culture of eGFP mRNA-loaded liposomes. (B) In vitro anti-tumor effects of iC9 mRNA-loaded liposomes against SK-N-SH, T-98G, and MDA-MB-231 in the presence of CID. (C) Experimental plan of the MDA-MB-231 xenograft model (D) Delayed tumor growth in the mice treated with No.43 liposome loaded with iC9 mRNA. CID, a chemical inducer of dimerization; iC9, inducible caspase 9.

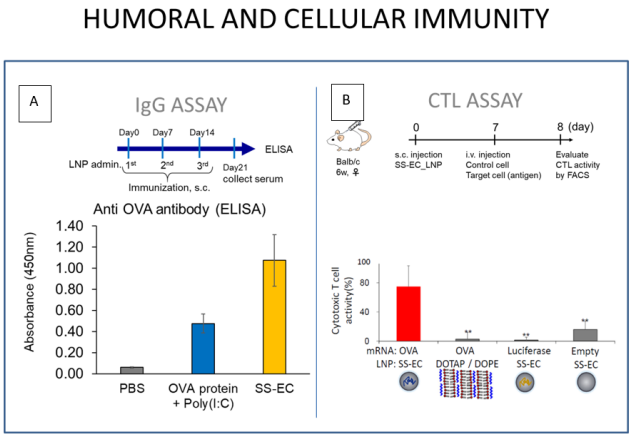
470. Self-Adjuvating Lipid Nanoparticle mRNA Vaccine Elicits T-Cell Responses and Protects against Immune Challenge

Syed M. Reza[1], Shinya Tamagawa[2], Yuta Nakai[2], Kota Tange[2], Hiroki Yoshioka[2]

[1]R&D, NOF Corporation, White Plains, NY,[2]DDS Research Laboratory, NOF Corporation, Tokyo, Japan

Background: Nucleic acid vaccines, DNA and messenger RNA (mRNA), delivered by Lipid Nanoparticles have emerged as promising modalities for infectious disease and for cancer immunotherapy. Currently, there is limited understanding of the mechanisms of antigen presentation and induction of specific T-Cell responses critical to long-term immunity. Further improvements in LNP carriers require a reduction in the 1) systemic toxicity, 2) improved endosomal escape, 3) potent and T-cell specific adjuvating function and 4) targeting to specific antigen presenting cells. **Results:** Previously we have reported that lipid nanoparticles composed of a novel ionizable, biodegradable lipid ("SS-Lipid") can deliver pDNA or mRNA in mice to liver, solid tumors, and other organs via the IV route and achieve high levels of expression. We also evaluated the safety of the lipids in mice where total lipid doses of up to 175 mg/kg were well tolerated. We next evaluated the delivery of DNA and RNA vaccines via the SC route. Multiple SS-Lipid derivatives were synthesized. SS-Lipid LNP encapsulating p-luc DNA were injected SC into BALB/c mice and evaluated by in vivo luciferase assay. The activity of SS-Lipid derivatives with the following lipid tails was 0.4 (SS-M/myristic acid), 1.8 (SS-A/vitamin A), and 6.0 (SS-E/vitamin E) fold higher compared to naked pDNA. Next, the antibody generation response was assessed in BALB/c mice with SS-EC 1.5x μg OVA mRNA injected 3X at weekly intervals and compared with immunization with OVA protein+polyI:C. SS-EC LNP produced a 2.2X higher titer compared with OVA-polyI:C control (Figure 1A). To assess induction of cytotoxic t-cell activity, SS-EC-LNP carrying OVA-encoding mRNA or Luciferase-encoding mRNA were administered at a dose of 0.05 μg of mRNA. The CTL percent lysis activity at 1 week was 75% (SS-EC_OVA-mRNA) of pulsed antigen cells. Sufficient CTL activity was not obtained with other DOTAP liposome, SS-EC_Luc-mRNA and empty LNP. The LNP's were tested for protection against Toxoplasmosis gondii infection in mice immunized with 10 μg of gPF pDNA. Mice immunized with SS-E-LNP had survival of 90% compared with 30% of those immunized with control samples. We then evaluated if robust CTL responses could demonstrate activity against solid tumors. Mice were injected with OVA expressing E.G7 cells as a model for disseminated malignant disease, and treated with SS-EC LNP OVA-mRNA (0.2 μg) after 10 days or SS-EC Luc mRNA as negative control. Tumor growth was significantly repressed in the test animals with negligible growth by day 25. **Conclusion:** We here demonstrate that a novel ionizable lipid SS-E and SS-EC specifically activates macrophages in vivo and this response is correlated with antibody expression and Cytotoxic T-Cell activity. Further efforts are needed to optimize the dosage and formulation towards achieving a single dose vaccine product. These findings suggest LNP composed of SS-E and SS-EC can be effective delivery systems for DNA and RNA vaccines.

FIGURE 1:



471. Design and Optimisation of Synovial-Targeted Non-Viral Lipid Nanoparticles for Systemic Delivery in Rheumatoid Arthritis

Emily R. Young[1], Valeria Nikolaenko[1], Wendy Heywood[1], David J. Gould[2], Stephen L. Hart[1]

[1]Institute of Child Health, UCL, London, United Kingdom,[2]Centre for Biochemical Pharmacology, Queen Mary University of London, London, United Kingdom

Rheumatoid arthritis (RA) is an autoimmune disorder of the joints in which fibroblast-like synoviocytes (FLS) become extremely aggressive, supporting inflammation and cartilage degradation. Current treatment with disease modifying anti-rheumatic drugs increases the risk of serious infection and some patients do not fully respond, or respond at all. Hence, we aim to develop a gene therapy using receptor-targeted nanoparticles (RTN) that selectively delivers therapeutic genes to the inflamed synovium, thereby modulating RA disease without life-threatening immunosuppression. The RTN comprises the cationic lipid DOTMA and the neutral lipid DOPE at a 1:1 ratio, as well as a targeting peptide containing a cationic, 16-lysine DNA packaging domain and an FLS targeting ligand, separated by an endosomally cleavable or a hydrophobic linker to alter RTN stability (Figure 1).The PEGylated cationic lipid ME42 was also investigated, working towards systemic delivery of the gene therapy *in vivo*. Rabbit FLS (HIG-82) were transfected with luciferase or GFP reporter plasmids with RTNs containing different peptides. Chondrocytes (C28/I2) or hepatocytes (HepG2) were used in transfections assessing cell type specificity. Luciferase or GFP expression was measured after 24 or 48-hours, respectively. Mass spectrometry was used to verify cleavage of peptide linkers by the endosomal enzymes furin and cathepsin B. Nanoparticle stability was determined by measuring nanoparticle size over time with a Malvern Zetasizer. RTNs with FLS targeted peptides produced superior transfection efficiency in HIG-82 cells compared to positive control integrin and epithelial targeted peptides. Conversely, when hepatocytes and chondrocytes were transfected, FLS targeted RTNs yielded much poorer transfection efficiency than the positive controls, which transfected all cell types, indicating a targeting specificity for FLS. In all cell types, RTNs with cleavable peptides transfected more efficiently than hydrophobic. Mass spectrometry analysis of peptides following incubation with furin or cathepsin B confirmed the appearance of a cleaved targeting sequence in cleavable, but not hydrophobic peptides. This cleavage following uptake is the suggested reason for the superior transfection seen with this linker. Replacing DOTMA with a similar a triethylene glycol lipid, ME42, demonstrated enhanced transfection efficiency in HIG-82 cells when used with the integrin targeting peptide. However, ME42 RTNs with FLS targeted, or FLS negative control peptides showed extremely low efficiency, presumably due to steric hinderance between PEG and the targeting sequence. To resolve this, nanoparticles with varying ratios of DOTMA:ME42 within the cationic lipid component were made, keeping the neutral lipid component constant. Reducing the ME42 component in FLS targeted RTNs increased transfection efficiency, but even at a ratio of 1:1 DOTMA:ME42, luciferase expression was only half that of DOTMA-only nanoparticles and this came with a sacrifice to stability. ME42-only nanoparticles showed optimal stability in PBS, with no increase in size over 60 minutes. Stability reduced as the proportion of ME42 relative to DOTMA increased. This work provides the basis of a nanoparticle formulation for a synovium targeted RA gene therapy via systemic injection.

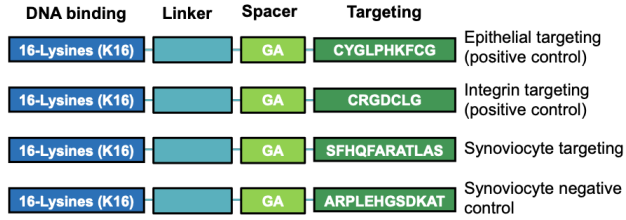


Figure 1: Schematic of peptides, including sequences. The linker region was either cleavable RVRR or hydrophobic XSX, where X is epsilon amino hexanoic acid.

472. Enabling Targeted Cargo Delivery to Hematopoietic Stem and Progenitor Cells with Biomimetic Nanoparticles

Jenna C. Harris[1], Samik Das[2], E. Terry Papoutsakis[2], Emily S. Day[3]

[1]Materials Science and Engineering, University of Delaware, Newark, DE,[2]Chemical and Biomolecular Engineering, University of Delaware, Newark, DE,[3]Biomedical Engineering, University of Delaware, Newark, DE

Introduction: Enabling effective delivery of therapeutic cargo to hematopoietic stem and progenitor cells (HSPCs) is a challenging problem whose solution would transform the treatment of a wide variety of diseases. As HSPCs give rise to all blood cell types in the body, HSPC dysfunction is implicated in many diseases. Current treatment of HSPC disorders involves removal of diseased HSPCs followed by transplant of healthy HSPCs. While transplant results are promising, these procedures are extremely complicated and carry risk of immune reaction. Here, we report the development of biomimetic nanoparticles (NPs) that can deliver therapeutic cargo specifically to HSPCs *in vitro* and *in vivo,* indicating potential to overcome the limitations of HSPC transplants. We show that poly(lactic-co-glycolic acid) (PLGA) NPs wrapped with megakaryocyte (Mk)-derived membranes and loaded with model cargo (DiD fluorophores or gene silencing siRNA) can preferentially bind and enter HSPCs *in vitro*. Preliminary *in vivo* studies show these Mk membrane-wrapped NPs (MkNPs) can target

bone marrow, and specifically, HSPCs, at higher levels than their unwrapped counterparts. **Methods:** DiD-loaded NPs and siRNA-loaded NPs were prepared by single- and double- emulsion solvent evaporation processes, respectively. Membranes were collected from Mk cells and co-extruded with bare NPs to wrap. Successful wrapping was confirmed by transmission electron microscopy (Fig. 1A,B,C), size changes measured by nanoparticle tracking analysis (Fig. 1D) and zeta potential measurements (Fig. 1E). Across several batches, these analyses confirmed MkNP synthesis is highly reproducible. *In vitro* preferential interaction of DiD MkNPs with HSPCs as compared to non-targeted cells was evaluated by flow cytometry and confocal microscopy during a 24 hr incubation period. For gene regulation, siCD34 and siNeg were used as cargo to allow for measurable silencing of CD34 in HSPCs. For *in vivo* studies, BALB/c mice were injected with wrapped or control NPs and the particles allowed to circulate for 16-18 hr. At this point, the mice were sacrificed and their organs and femurs removed. Fluorescence measurements of the tissues were recorded and cells collected from flushed bone marrow were examined by flow cytometry and confocal microscopy.

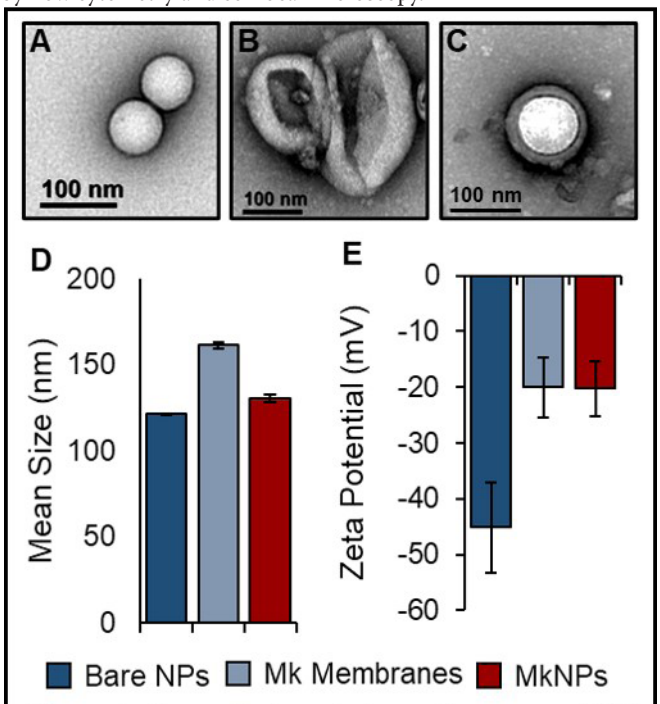


Figure 1. Transmission electron microscopy of (a) bare NPs, (b) Mk membranes, and (c) MkNP where the membrane coating can be seen surrounding the NP. (d) Nanoparticle tracking analysis shows a mean size increase of approximately 10 nm between bare NPs and MkNPs, indicating successful wrapping. (e) Changes in zeta potential charge after wrapping indicate Mk membranes have successfully coated bare NPs to form MkNPs.

Results: HSPCs internalized DiD MkNPs within 24 hr of incubation at a much higher rate than than the two control cell types, confirming the Mk membrane coating facilitates HSPC-specific binding. MkNPs containing siCD34 significantly decreased CD34 expression in HSPCs over 96 hrs by 16%, while MkNPs loaded with siNeg had no impact on CD34 expression. Pilot *in vivo* studies showed that DiD MkNPs exhibit preferential targeting to bone marrow, as both IVIS images of whole tissue and plate reader analysis of homogenized tissue showed greater fluorescence from MkNPs than bare NPs. Further, flow cytometry and confocal microscopy of flushed bone marrow showed that MkNPs specifically interact with HSPCs over other cell types in the bone marrow. In conclusion, MkNPs can be successfully loaded with, and deliver, siRNA cargo to HSPCs *in vitro*, eliciting gene knockdown, and preferentially target HSPCs *in vivo*. These exciting advances pave the way for further optimization of the system and its gene regulation capabilities *in vivo*.

473. Production of Superior RNA CAR T Cells Using a Novel Lipid Nanoparticle Delivery Platform

Reka Geczy, Aruna Balgi, Rita Zhao, Stella Park, Miranda Fujisawa, Andrew Brown, Rebecca DeSouza, Nikita Jain, Andy Geall, Anitha Thomas, Samuel Clark
Research & Development, Precision Nanosystems Inc, Vancouver, BC, Canada

Recent success with CAR T therapies for certain hematologic cancers has revolutionized personalized gene-based intervention, generating substantial interests in employing a similar strategy against solid tumors. To overcome limitations associated with viral transduction, messenger RNA, which allows transient CAR expression, has significant safety and manufacturing advantages. However, the delivery of RNA into cells by electroporation has some challenges. Electroporation often requires a trade-off between transfection efficiency and cell viability, can disrupt normal gene expression, and requires extensive manipulation steps that complicate production of the drug product. In this work, we investigate the generation of RNA CAR T cells using lipid nanoparticle (LNP) delivery (Fig. 1A). LNPs encapsulate RNA within a lipid carrier for delivery to the cell cytoplasm via receptor-mediated endocytosis. We designed an RNA encoding for a second-generation CAR construct with an anti-CD19 scFv binding domain, CD3ζ/4-1BB co-stimulatory domains, and a green florescent protein (GFP) reporter. We optimized a novel LNP reagent using chemically-defined lipid components. Microliter volumes of RNA LNPs were rapidly produced in less than 5 minutes using a scalable microfluidics platform, yielding consistent particles with a diameter of 85 ± 4 nm and an RNA encapsulation efficiency of 98.0 ± 0.4%. Human primary T cells from healthy donors were obtained from commercial sources and activated by CD3/CD28/CD2 stimulation for 72 hours. An aliquot of the RNA LNPs was added to activated T cells at a dose of 100 ng RNA/50,000 cells. Functionality and expression of the CAR construct on the cell surface was confirmed by flow cytometry using soluble CD19 extracellular domain (ECD) ligand and GFP detection. At 24 hours post treatment, homogeneous expression of the CAR was observed in over 50% of cells, with cell viabilities above 90%. Finally, we compared the workflow and performance of the LNPs with electroporation (Fig. 1B). The RNA LNPs were directly added to cells with no further processing, whereas electroporation required centrifugation and resuspension of cells. Compared to electroporation, at the equivalent RNA dose, the LNPs generated a more homogenous level of CAR expression while maintaining higher cell viability. Our findings demonstrate the advantages of the novel LNP reagent and microfluidic platform for

production of RNA CAR T cells, providing a strong case for the utility of LNP-based technology in the development of cell therapies for solid tumor applications. RNA LNPs not only provide a safety mechanism to limit toxicities, but are also fully synthetic and can be rapidly manufactured using microfluidics, enabling small-scale screening of libraries against new targets and subsequent seamless scale-up of lead candidates for clinical translation.

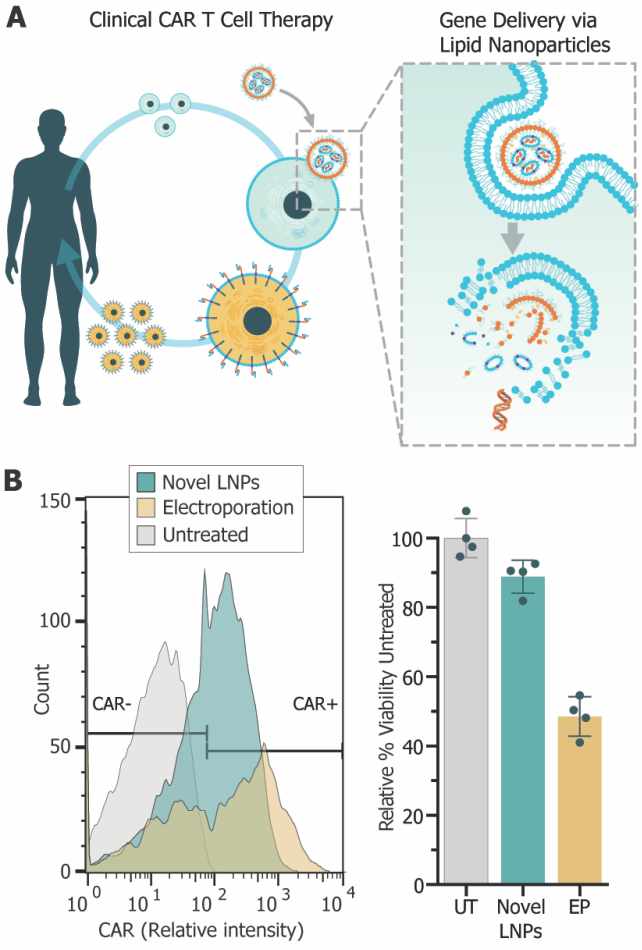


Fig 1. Production of RNA CAR T cells via LNP delivery. A) Workflow and mechanism for LNP delivery. B) Compared to electroporation, LNPs show more homogenous CAR protein expression with 50% transfection efficiency. LNP treated cells maintain > 90% viability, compared to < 50% for electroporation.

474. Vector Optimization for Non-Viral Antibody Gene Therapy and Expression of Human Monoclonal Antibodies in Mice

Nathaniel Silver, Sid Jindal, Nicholas Parsonnet, Luke Hamm, Henry Sun, Debra Klatte, Phillip Samayoa, Doug Kerr

Generation Bio, Cambridge, MA

Since their initial approval over 30 years ago, monoclonal antibodies (mAbs) have seen great success as a therapeutic class. However, their high cost of production as well as the need for frequent administration has limited their widespread use in areas outside of oncology and autoimmune diseases. Antibody gene therapy (AGT) provides an alternative means of delivering specific mAbs, wherein an antibody is vectorized and produced *in vivo*. This enables patients to produce their own biotherapeutic for an extended period. AAV based delivery of vectorized antibodies has demonstrated a capacity to produce efficacious levels of antibodies in a variety of pre-clinical, disease models. However, there remain significant limitations to AAV that have limited its use for AGT in the clinic. In particular, its genetic cargo capacity of ~4.7kb limits encoding of multiple polypeptides, immunogenicity of the viral capsid restricts dosing to a single administration, and costly, scale-limited manufacturing processes prevent use in wide-spread diseases like HIV, Influenza, or COVID19, particularly in the prophylactic setting where they might be the most impactful. Non-viral gene therapy is an attractive solution to address these limitations and enable prophylactic use of AGT on a much broader scale than is possible with passive administration of recombinant mAb or AAV based delivery. We have developed a non-viral gene therapy platform to deliver and durably express therapeutic proteins systemically *in vivo*. It is comprised of ceDNA, an engineered, double-stranded, linear, covalently closed-ended DNA construct, formulated in a lipid nanoparticle delivery system, ctLNP. The ctLNP delivery system has been designed for hepatocyte-specific delivery by using a biological targeting ligand and does not contain any component of the viral capsid, allowing repeated administration. Studies in immunocompetent mice have demonstrated that systemic administration of a single dose of ctLNP formulated ceDNA results in delivery to hepatocytes and durable transgene expression. Administration of a second dose, further increased expression. Here we present the use of our non-viral gene therapy platform to express vectorized monoclonal antibodies systemically *in vivo* and our vector optimization approach to maximize expression. We compare bicistronic, furin/2A-peptide based expression cassettes to paired heavy and light chain vectors as well as multi-promoter, scarless designs. While larger in size, we find that the latter two vector formats, in particular bidirectional promoter cassettes, significantly improve expression of antibodies *in vivo* relative to the bicistronic designs commonly used in AAV vectors to encode multiple peptides. Further optimization of regulatory as well as heavy and light chain sequences enhanced ceDNA based expression, achieving therapeutically relevant levels of systemic mAb expression in mice. These data demonstrate early capabilities of our non-viral AGT platform and provide a path for non-viral delivery of antibodies for disease prevention and treatment.

475. RGD-Conjugated Peptide-Based Polyplexes Combined with Magnetic Nanoparticles as a Delivery System for Herpes Thymidine Kinase Gene to Uterine Fibroids Cells

Sofia Shtykalova[1,2], Anna Egorova[1], Alexander Selutin[1], Marianna Maretina[1], Sergey Selkov[1], Vladislav Baranov[1,2], Anton Kiselev[1]

[1]D. O. Ott Research Institute of Obstetrics, Gynecology, and Reproductology, Saint-Petersburg, Russian Federation,[2]Saint-Petersburg State University, Saint-Petersburg, Russian Federation

Uterine fibroids (UF) occupy the 2nd place in the structure of gynecological pathology and are often the cause of infertility. The possibility of precise ultrasound localization makes the tumors a perfect target for suicidal gene therapy using direct herpes thymidine kinase (HSV1-TK) gene transfer. However, excessive extracellular matrix of UF appears a formidable barrier to deliver therapeutic agents. Non-viral carrier systems are promising for gene delivery being non-toxic and biodegradable, and their application is not limited by severe immunogenicity and inflammation problems. Magnetic nanoparticles (MNPs) are applied in targeted gene delivery by an influence of external magnetic field. Magnetofection can decrease the amount of DNA injected, shorten the time necessary to reach the desired target cells and therefore improve significantly transfection efficiency. Previously we developed a novel αvβ3-integrin-targeted peptide-based carrier for plasmid DNA delivery. Here, for successful penetration into the inner layers of the UF, MNPs were non-covalently bound to DNA-peptide complexes. RGD ligand-conjugated arginine-rich peptides were synthesized and their complexes with pDNA and MNPs were formed. Physicochemical properties of DNA-complexes were studied. The amount of MNPs and incubation time required for successful transfection were found. Significant difference in transfection efficacy with and without magnet was shown. The specificity of DNA delivery was demonstrated by ligand competitive transfections in αvβ3-overexpressed PANC-1 cells. Suicidal gene therapy with HSV1-TK gene delivery and subsequent ganciclovir treatment was held for primary UF cells obtained after myomectomy. Non-covalent conjugation of MNPs does not lead to the destruction of DNA-peptide complexes and does not affect protection of DNA from nuclease degradation. Addition of c(RGDfK) ligand during transfection of PANC-1 cells blocked ligand-conjugated complexes transport significantly. Cellular proliferation AlamarBlue assay showed a decline of proliferative activity in 27% primary UF cells transfected with HSV1-TK gene in comparison with control lacZ gene-transfected cells. Addition of MNPs to DNA-peptide complexes significantly reduces the incubation time required for efficient transfection. The developed DNA-complexes with MNP demonstrated high specificity and transfection efficiency of primary UF cells with subsequent successful suicide gene therapy, which makes them promising for the development of UF gene therapy. The research was supported by RSF grant 19-15-00108.

476. Complex Engineering of Primary Human T Cells Using Non-Viral Intracellular Delivery with the Solupore Platform

Susan Dunne, Darren Martin, Heather Kavanagh, Jessica Schwaber, Shirley O'Dea
Avectas, Maynooth, Ireland

Background: Next-generation immune cell therapy products will require complex modifications using engineering technologies that can maintain high levels of cell functionality. Non-viral engineering methods have the potential to address limitations associated with viral vectors but while electroporation is the most widely used non-viral modality, concerns about its effects on cell functionality have led to the exploration of alternative approaches. We have previously reported the development of our SOLUPORE® technology for non-viral engineering of primary human immune cells. The technology enables development and manufacture of cell therapies, using reversible permeabilization to achieve rapid intracellular delivery of cargos. Here, we have examined the suitability of the SOLUPORE technology for complex engineering of primary human T cells by transfecting T cells that had been already virally transduced and by carrying out sequential gene editing of T cells. **Methods:** T cells were transduced with lentiviral vector to express a CD19 chimeric antigen receptor (LV-CAR) and were then transfected with GFP mRNA using the SOLUPORE technology. Expression and cell viability were measured by flow cytometry. CRISPR-Cas9 protein-gRNA ribonucleoproteins (RNPs) targeting the *TRAC* and *CD7* genes were delivered to T cells individually and sequentially and knock down efficiency and cell viability were measured by flow cytometry. **Results:** In the lentiviral study, T cells that were virally transduced with LV-CAR and then transfected with GFP mRNA were shown to express 65 % GFP in the CAR^+ population and cell viability was >80 %. In the gene editing study, when RNP complexes were delivered individually, CD3 and CD7 expression in the treated populations was reduced to approximately 25 % in both cases. When the *TRAC* RNP was delivered and was followed two days later by the CD7 RNP, approximately 5 % of the treated population retained a $CD3^+/CD7^+$ phenotype and viability remained >90 % when examined 4 days post-delivery of the CD7 RNP. **Conclusion:** These studies demonstrate that the SOLUPORE technology enables efficient complex engineering of T cells, including virally transduced cells, while retaining high levels of cell viability. The ability to carry out multiple modifications in cells while maintaining cell functionality will be critical for the success of next-generation cell therapy products.

477. Accelerating the Production of Self-Amplifying mRNA (saRNA) Vaccines Using Microfluidics

Srinivas Abbina[1], Helena Son[1], Sijo Chemmannur[1], Pierrot Harvie[1], Ariel Zhang[1], Nikita Jain[1], Suraj Abraham[1], Anna Blakney[2], Sitalakshmi Thampatty[1], R. Higgins[1], Aruna Balgi[1], Magnus Cronin[1], G. Darwish[1], Lloyd Jeffs[1], Anitha Thomas[1], R. Shattock[2], Andy Geall[1]
[1]Research & Development, Precision Nanosystems Inc, Vancouver, BC, Canada,[2]Imperial College London, London, United Kingdom

The COVID-19 pandemic has unveiled the potential of messenger RNA (mRNA) based vaccines as the ideal platform for pandemic response. One of the first vaccine to start clinical trials was a non-replicating mRNA vaccine from Moderna (mRNA-1273), the first patient was vaccinated on March 16th at the same time as a Chinese clinical trial was initiated with an adenovirus type-5 (Ad5) vector. Since this time several other SARS-CoV-2 mRNA or saRNA based vaccines have entered clinical trials. Furthermore, the BioNTech/Pfizer vaccine, BNT162b2, was the first COVID-19 vaccine to receive approval, first in the United Kingdom and then Canada, with an impressive 95% efficacy. RNA vaccines provide flexibility in the design and expression of vaccine antigens that mimic antigen structure and expression seen during a natural infection without any possibility of causing the disease. saRNAs have the potential for antigen sparring since lower doses are required for effective immune response compared to non-replicating mRNA-based vaccines. However, saRNAs are inherently more fragile than mRNA and prone to degradation. While lipid nanoparticles and the appropriate buffer composition can protect the saRNA from

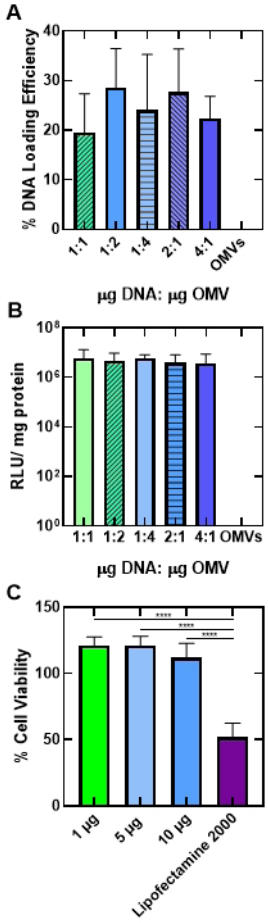
degradation, typical high shear manufacturing methods can affect the potency of the saRNA. Herein, we showcased the utility of microfluidics (NxGen™ platform) to enable low shear, rapid screening of preclinical candidates and the swift advancement to GMP-enabling studies. Accelerated clinical and commercial development of COVID-19 vaccine candidates are possible by reducing the number of engineering batches via seamless transfer of manufacturing process from bench to clinical scale volume, and by eliminating cleaning validation using a fully disposable single use fluid path. The NxGen™ microfluidic platform supports all stages of clinical development through a modular continuous flow capable of producing volumes of 200 mL to > 100 L at outputs up to 12 L/h while retaining the Critical Quality Attributes (CQA) of the drug product. Here, we demonstrate that by using the NxGen™ platform, we were able to rapidly screen preclinical saRNA/LNP vaccine candidates and seamlessly transition into clinical development, scale-up the formulation process and produce material for suitable for human use.

478. Optimization of Loading Outer Membrane Vesicles with Plasmid DNA

Kari Heck[1], Amanda E. Ramer-Tait[2], Angela K. Pannier[1]

[1]Biological Systems Engineering, University of Nebraska- Lincoln, Lincoln, NE,[2]Food Science and Technology, University of Nebraska- Lincoln, Lincoln, NE

Introduction: Gene delivery via the oral route is desirable due to the high rate of patient compliance, ease of administration and large cellular surface area conditions within the gastrointestinal tract[2]. To overcome challenges associated with oral gene delivery, we are developing a novel, biological-based delivery system by loading outer membrane vesicles (OMVs) derived from commensal gut bacteria with plasmid DNA to create DNA-loaded OMV nanocarriers (DNA-OMV NCs). Current work on this project is focused on the optimization of loading methods, transfection of cells *in vitro* and assessing the cytotoxicity of our DNA-OMV NCs. **Methods:** For initial loading studies, OMVs were isolated from *Escherichia coli* DH5-α. Bacteria were grown as described[3] and OMVs were isolated from the bacterial culture using a Vivaspin centrifugal concentrator. Total protein content of the OMVs was quantified using a BCA assay. Plasmid DNA (pEGFP-LUC) encoding for an enhanced green fluorescent and a luciferase fusion protein was loaded into isolated OMV via electroporation using a BioRad Gene Pulser XCell with optimized electroporation parameters. The µg DNA: µg OMV protein ratio was varied between 1:1 - 1:4 while holding electroporation parameters constant to determine the impact of the ratio on DNA loading efficiency. Loading efficiency of the DNA-OMV NCs was determined by applying DNase treatment after electroporation for one hour at 37 ^{0}C. DNA within the DNA-OMV NCs was quantified using Hoechst 33258 dye. Loading efficiency of the DNA-OMV NCs was calculated by dividing the amount of encapsulated DNA in DNA-OMV NCs after DNase treatment by the initial amount of DNA in the DNA-OMV NC preparation. After calculating DNA loading efficiency, DNA-OMV NCs were used to transfect the human embryonic kidney cell line HEK 293T with equal amounts of DNA delivered to cells across all treatments. Non-loaded OMVs were used as a negative control for transfection. Transfection results were quantified by measuring transgenic luciferase activity and normalizing to total cellular protein. Cytotoxicity of DNA-OMV NCs (4:1 DNA:OMV) on Caco2 intestinal epithelial cells was assessed using a WST-8 assay. **Results:** Electroporation voltage and DNA: OMV ratio influenced DNA loading efficiency in DNA-OMV NCs. The highest DNA loading at 31.8 ± 10.9 % was achieved using a 1:2 DNA: OMV ratio **(Fig. 1 A)**. DNA-OMV NC were able to mediate transfection within HEK 293T cells; however, the different DNA:OMV ratios tested did not significantly impact DNA-OMV NC transfection **(Fig. 1 B)**. DNA-OMV NCs showed low cytotoxicity when applied to Caco2 cells **(Fig. 1 C)**. In addition, it was determined that the loaded DNA was associated with the outer surface of the OMV as well as encapsulated inside the OMV (data not shown). **Conclusions:** We have successfully loaded bacterial OMVs with plasmid DNA using electroporation to create DNA-OMV NCs. DNA-OMV NCs can facilitate transfection *in vitro*, albeit at low levels, and show low cytotoxicity to cells. Additionally, varying the DNA:OMV ratio did not impact DNA loading efficiency or transfection efficiency of the DNA-OMV NCs. Together, these data suggest that DNA-OMV NCs created using electroporation are a promising new delivery platform for oral gene therapy. **References:** 1. Kriegel C. *et al.*, Adv Drug Deliv Rev 2013 2. Farris E. *et al,* Curr Opin Biomed Eng 2018 3. Kittana H. *et al.,* Front Immunol 2018



479. Lipid Nanoparticle Library for Non-Viral Delivery of mRNA and saRNA towards Vaccine and Cell & Gene Therapy Applications

Nikita Jain[1], A. Brown[1], Suraj Abraham[1], Srinivas Abbina[1], Helena Son[1], Sijo Chemmannur[1], R. DeSouza[1], R. Sabaten[1], T. Park[1], A. Bernardo[1], Samuel Clarke[1], Anna Blakney[2], R. Shattock[2], Anitha Thomas[1], Andy Geall[1]

[1]Research & Development, Precision Nanosystems Inc, Vancouver, BC, Canada,[2]Imperial College London, London, United Kingdom

Recent FDA approval of ONPATTRO® by Alnylam, emergency use authorization of BioNTech/Pfizer and Moderna mRNA vaccine and the various clinical trials with mRNA-based drugs or vaccines have provided momentum to further develop LNP-based genetic medicine candidates. Ionizable amino-lipid is a major constituent of the lipid nanoparticles for delivering nucleic acid therapeutics. Most of these lipids have been designed for liver-targeted applications. Limited progress has been made in the development of lipids for vaccine applications. More effective ionizable lipids are needed that are non-toxic, can effectively encapsulate nucleic acids, and can release the nucleic acids from the early endosomes of the target cell. Scarcity of lipids that are suitable for vaccination, cell therapy and protein replacement therapies continues to be a problem in advancing many potential therapeutic/vaccine candidates to the clinic. Herein, we describe the development of novel ionizable lipids with apparent experimental pKa in the range of 5.4-7.4 to be used as effective vehicles for nucleic acid therapeutics. Lipid nanoparticles of size 70-100 nm with high encapsulation efficiencies were produced by microfluidic mixing. We studied the transfection efficiency (TE) of LNP-based mRNA formulations of these ionizable lipid candidates in primary human T cells to showcase the effective translation of several protein-encoded mRNAs. The protein expression levels, and the TE were analyzed by ELISA or by Flow Cytometry. The eGFP, rhEPO, and CAR expression in human T cells were found to be significantly higher in comparison to clinically-validated DLin-MC3-DMA, used in ONPATTRO®. We have evaluated various ionizable lipid candidates for protein replacement applications by administering human Erythropoietin (hEPO) encoded mRNA LNPs intravenously at a dose of 0.5 mg/kg in C57BL6 mice. Ionizable lipid candidates were further evaluated using self-amplifying RNA for COVID-19 and Influenza vaccine applications encoding pre-fusion stabilized SARS-CoV-2 full length spike protein and H1N1 Influenza antigen, respectively. Results obtained after antibody characterization using ELISA indicated high titre values. We were able to show that physico-chemical profiles of ionizable lipids are going to be different for cell therapy, protein replacement and vaccine applications. We believe that these studies will pave the path to the advancement in development of cell-based therapy, RNA vaccines, and targeted delivery of other nucleic acids for finding a cure for many rare diseases, where treatment options rarely exist.

480. Slippery Liquid-Infused Porous Surfaces (SLIPS) for Cell Deformation Enabling Intracellular Cargo Delivery

Isaura M. Frost[1], Alexandra Mendoza[2], Tzu-Ting Chiou[3], Natcha Wattanatorn[2], Chuanzhen Zhao[2], Qing Yang[2], Philseok Kim[4], Joanna Aizenberg[5], Satiro De Oliveira[3], Paul S. Weiss[2], Steven J. Jonas[3]

[1]Bioengineering, UCLA, Los Angeles, CA,[2]Chemistry & Biochemistry, UCLA, Los Angeles, CA,[3]Pediatrics, UCLA, Los Angeles, CA,[4]Adaptive Surface Technologies, Inc., Cambridge, MA,[5]School of Engineering and Applied Science, Harvard University, Cambridge, MA

Intracellular delivery technologies that are scalable, cost-effective, and efficient are required to process large populations of cells more effectively into gene and cellular therapy products for patient care. Existing microfluidic approaches that leverage cellular membrane deformation to render cells transiently porous as cells pass through narrow microchannels require specialized equipment and are often hindered by issues with clogging that leads to device failure within minutes. We are developing and testing a rapid deformation technique that consists of passing cells through the pores of slippery liquid-infused porous surface (SLIPS)-modified polyethylene terephthalate (PET) cell culture inserts by application of negative pressure. Delivery of green fluorescent protein (GFP) expression plasmids is demonstrated in Jurkat cells with transfection efficiencies of up to 40% and >80% viability. Fluorescently labeled dextran delivery is demonstrated in both immortalized and primary cell types. These bioinspired omniphobic coatings reduce biofouling and circumvent issues that have precluded existing technologies to enable rapid and sustainable transport of biomolecular payloads to target cell populations. Importantly, these devices can be scaled to process millions of cells in a short time while maintaining costs to a minimum (<$10 per experiment). These good manufacturing practice (GMP)-compatible, table-top approaches do not require specialized equipment, viral vectors, or reagents, offering a versatile and straightforward solution for manufacturing future stem cell-based gene and cellular therapies.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

481. Glycemic Control in a Streptozotocin-Induced Diabetic Mouse Model with AAV-Mediated Expression of Insulin and Glucokinase in Skeletal Muscle

Michele Stone[1], Wallace Harrington[1], Chari Smith[1], Dawn Fellner[1], Jiewu Liu[1], Melissa Rhodes[1], Weiran Shen[1], Zhu Pirot[1], Yunchao Gai[1], Veronica Jimenez[2,3], Xavier Leon[2,3], Maria Molas[2,3], Fatima Bosch[2,3]

[1]Kriya Therapeutics, Redwood City, CA,[2]Center of Animal Biotechnology and Gene Therapy and Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Spain,[3]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain

Diabetes affects about 463 million people worldwide, with type 1 diabetes (T1D) representing 5-10% of all cases. While landmark clinical trials have demonstrated that tight blood glucose control reduces the risk of microvascular and macrovascular complications, fewer than 50% of patients consistently achieve target blood glucose levels. In T1D, lifelong insulin therapy is required to manage hyperglycemia; however, insulin regimens can be highly complex, resulting in poor adherence and frequent, potentially life-threatening hypoglycemic and hyperglycemic events. Skeletal muscle is a major utilizer of glucose in the body, and responsible for the disposal of approximately 70% of circulating glucose after meals. The uptake and utilization of glucose in skeletal muscle is controlled by insulin-stimulated glucose transport through the glucose transporter type 4 (GLUT4) and subsequent phosphorylation to glucose-6-phosphate by hexokinase II. In diabetic muscle, the lack of endogenous insulin results in decreased activity of GLUT4 and hexokinase II. Therefore, by supplying the muscle with a means to sense and remove glucose and process high glucose levels after meals, restoration of glucose homeostasis can be achieved. A one-time administration of an adeno-associated virus (AAV) vector-mediated gene therapy approach expressing insulin and glucokinase has the potential to transform the lives of patients with T1D by genetically engineering skeletal muscle to counteract hyperglycemia. Importantly, this approach requires only tonic levels of insulin to be expressed. Moreover, glucokinase has a low affinity for glucose and thus is only functionally active in phosphorylating glucose at high levels. As such, this gene therapy approach has an inherently low risk of causing hypoglycemia. Previously published studies have demonstrated the efficacy of AAV-mediated delivery of insulin and glucokinase in relevant rodent and dog models of diabetes, with durability established for up to 8 years following administration in diabetic dogs. In this experiment, we confirmed the efficacy of this gene therapy approach in a diabetic mouse model. Mice were first treated with 40 mg of Streptozotocin (STZ) for five consecutive days to deplete beta cells in the pancreas, thereby eliminating production of native mouse insulin and resulting in blood glucose levels reaching ~600 mg/dL. Animals were then given an equal mixture of AAV1-insulin and AAV1-glucokinase in three hindlimb muscles (quadricep, gastrocnemius, tibialis cranialis). In a separate group, animals were administered the same total dose of AAV1-insulin and AAV1-glucokinase to two hindlimb muscles (quadricep and gastrocnemius) in a lower total volume. These animals were compared to STZ-vehicle-treated and non-diabetic vehicle-treated animals (n=10-11/group). STZ-treated diabetic mice that received AAV1-insulin and AAV1-glucokinase restored and maintained normoglycemia and HbA1C levels in fed and fasted conditions, as observed in earlier studies. Therapeutic effects were observed as early as one week following dosing, with significant reductions in hyperglycemia observed by two weeks in the range of untreated control animals. In summary, the joint action of AAV-mediated basal insulin production and glucokinase activity may generate a "glucose sensor" in skeletal muscle that enables tight glycemic control in diabetic animals.

482. Modification of AAV-Insulin and AAV-Glucokinase Vectors in Preparation for Clinical Translation in Patients with Type 1 Diabetes Mellitus

Jason Mallory[1], Miguel Garcia[2,3], Maria Molas[2,3], Xavier Leon[2,3], Veronica Jimenez[2,3], Nachi Gupta[1], Zhu Pirot[1], Weiran Shen[1], Melissa Rhodes[1], Fatima Bosch[2,3]

[1]Kriya Therapeutics, Redwood City, CA,[2]Center of Animal Biotechnology and Gene Therapy and Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra, Spain,[3]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Madrid, Spain

Over the past two decades, gene therapy has evolved from a futuristic concept to tangible, transformative medicines for many patients impacted by debilitating inherited diseases. The recent clinical success with adeno-associated virus (AAV) -mediated gene therapies in rare genetic diseases has paved the way to utilize this innovative new modality for the treatment of more prevalent conditions. Diabetes affects approximately 463 million people worldwide, with type 1 diabetes mellitus (T1D) representing 5-10% of all cases. While landmark clinical trials have demonstrated that tight blood glucose control reduces the risk of microvascular and macrovascular complications, fewer than 50% of patients consistently achieve target glucose levels. A one-time intramuscular administration of AAV -mediated gene therapy co-expressing insulin and glucokinase has the potential to improve the lives of patients with T1D by genetically engineering skeletal muscle to counteract hyperglycemia. Skeletal muscle is responsible for the disposal of approximately 70% of circulating glucose after a meal, but only approximately 20-25% during the fasted state. In preclinical studies, AAV-mediated co-expression of human insulin and rat glucokinase within skeletal muscle ameliorated the diabetic phenotype of streptozotocin-induced diabetic mice and streptozotocin + alloxan-induced diabetic dogs. Eight-year follow-up in dogs demonstrated persistent normoglycemia, complete exogenous insulin independence, maintenance of fasting insulin levels and body weight within the range of healthy dogs, and normalization of other metabolic markers of disease. To support the advancement of insulin + glucokinase gene therapy into clinical application, further vector modifications and improvements have been implemented. AAV vector constructs containing human insulin and human glucokinase were modified to reduce the presence of immunostimulatory features. These

improved constructs were then subcloned into a plasmid containing a CMV promoter and AAV2 inverted terminal repeats. Following plasmid production and purification, constructs were transfected into HEK293 cells and assessed for mRNA expression and intracellular and extracellular protein expression. After comparative analysis in HEK293 cells, selected plasmid constructs were advanced for AAV vector production. After characterization, 2v6.11 cells were transduced by the AAV constructs and assessed for vector infectivity and potency (including mRNA expression, intracellular and extracellular protein expression, and biologic activity) at 48 hours post-transduction. Three independent in vitro infectivity and potency assays for AAV human insulin and human glucokinase vectors were performed. Overall, potency assays with the minimally immunogenic expression cassettes showed similar protein expression and activity in comparison to control vectors. These experiments suggest that modifications of insulin and glucokinase vectors can be implemented without changing the transgene protein expression or activity profiles. These results will be confirmed in a relevant animal model of T1D prior to completing additional IND-enabling studies.

483. ImmTOR Nanoparticles Promote Survival and Enable Repeat Gene Therapy of MMUT-Deficient Mice with Maternally-Transferred Anti-AAV Antibodies

Petr Ilyinskii[1], Alicia Michaud[1], Gina Rizzo[1], Julie LePrevost[1], Christopher Roy[1], Stephanie Elkins[1], Teresa Capela[1], Aparajita Chowdhury[1], Luk Vandenberghe[2], Charles Venditti[3], Sheldon Leung[1], Takashi K. Kishimoto[1]

[1]Selecta Biosciences, Watertown, MA,[2]Massachusetts Eye and Ear Infirmary, Boston, MA,[3]National Human Genome Research Institute, Bethesda, MD

The safety of AAV gene therapy is a key concern, particularly in metabolically fragile pediatric patients. Pre-existing anti-AAV antibodies (Ab) can compromise transduction and may exacerbate inflammatory reactions resulting in adverse events. We have developed tolerogenic ImmTOR nanoparticles encapsulating rapamycin that have been shown to mitigate AAV immunogenicity, allowing for vector re-dosing in mice and NHP (Meliani, Nature Commun, 2018). Moreover, co-administration of AAV vectors and ImmTOR augments liver-directed transgene expression even after the 1st dose (Ilyinskii, 2021, Science Adv, in press). We have previously shown that ImmTOR combined with an AAV-Anc80 vector expressing methylmalonyl-CoA mutase under the control of a liver specific promoter (Anc80-MMUT) mitigated the formation of anti-Anc80 Ab and enabled vector redosing in a mouse model of methylmalonic acidemia (MMA). Here we evaluated the ability of Anc80-MMUT + ImmTOR to rescue juvenile MMA mice with maternally transferred anti-Anc80 Ab. Homozygous male and female $MMUT^{-/-}$;$Tg^{INS\text{-}MCK\text{-}Mmut}$ mice (Manoli, JCI Insight, 2018) were treated with Anc80-MMUT in the absence of ImmTOR prior to breeding. All pups were found to have high levels of maternally transferred anti-Anc80 IgG Ab prior to treatment. Pups were left untreated or treated with 5e12 vg/kg Anc80-MMUT alone, ImmTOR alone, or Anc80-MMUT + ImmTOR at 28 days of age and then retreated 21 and ~100 days later. Treatment with Anc80-MMUT not only failed to rescue the MMA mice, due to the presence of maternally transferred anti-Anc80 Ab, but resulted in increased mortality. Five of 6 animals treated with Anc80-MMUT died in the first 4 weeks after initiation of treatment compared to 2 of 6 untreated mice in the same time period. Interestingly, treatment with ImmTOR alone appeared to provide a survival benefit, with 100% survival during the first 75 days, although the benefit was lost after the last ImmTOR dose, with only 1 of 7 mice surviving until 1 year. In contrast, mice treated with repeated doses of Anc80-MMUT + 300µg ImmTOR showed enhanced survival, with 9 of 10 animals still alive at 1-year post-treatment compared to only 2 of 6 untreated mice. Mice treated with the Anc80-MMUT + ImmTOR showed little or no initial change in plasma methylmalonic acid (pMMA), indicating that the maternally transferred Ab inhibited vector transduction. However, the survival benefit conferred by ImmTOR appeared to enable mice to overcome the increased mortality observed in the vector alone treated group, and the mitigation of de novo anti-Anc80 Ab formation enabled mice to receive therapeutic benefit from the 2nd or 3rd dose after the levels of maternally transferred Ab declined sufficiently to enable transduction. Treatment with ImmTOR alone did not affect pMMA levels, indicating that ImmTOR does not directly correct the underlying genetic defect, but resulted in an immediate drop in plasma FGF21, a biomarker of hepatic damage, suggesting alleviation of MMA-associated pathology. Recently it has been shown that MMA is associated with defects in autophagy that result in the accumulation of dysfunctional mitochondria which trigger cell stress. Rapamycin, the active component of ImmTOR, is a potent inducer of autophagy. Collectively, the combination of ImmTOR and AAV gene therapy vector is a promising approach to mitigate the detrimental impact of maternally-transferred Ab on gene therapy for MMA by promoting survival through stabilization of metabolic dysfunction and enabling repeat dosing through inhibition of de novo formed anti-capsid Ab.

484. AAV-Mediated Gene Therapy Using a Novel Dual Promoter Prevents Pullulanase-Induced Cytotoxic T Lymphocyte Response and Corrects Major Affected Tissues in GSD IIIa Mice

Jeong-A Lim, Priya S. Kishnani, Baodong Sun

Pediatrics Medical Genetics, Duke University, Durham, NC

Background: To overcome the limitation of small AAV packaging capacity, we recently reported a gene therapy approach using a small bacterial glycogen debranching enzyme (Pullulanase) in a mouse model of glycogen storage type IIIa (GSD IIIa). Intravenous injection of an AAV9 vector containing a 2.2-kb codon-optimized Pullulanase cDNA driven by the ubiquitous CMV enhancer/chicken β-actin (CB) promoter (AAV-CB-Pull) into infant GSD IIIa mice significantly decreased (75-80%) glycogen contents in both cardiac and skeletal muscles. However, treatment with the same AAV vector in adult GSD IIIa mice provoked strong transgene-induced cytotoxic T lymphocytes (CTL) response, resulting in only transient Pullulanase expression. In this study, we tested the ability of a novel dual promoter to prevent CTL response against Pullulanase and express the therapeutic transgene in all affected tissues. **Methods**: Three Pullulanase-expressing vectors, including the AAV-CB-Pull, AAV-LSP-Pull containing an immunotolerant liver-specific promoter (LSP), and AAV-Dual-Pull

containing the LSP-CB dual promoter (Dual), were packaged into AAV9 and then intravenously injected into 10-week-old GSD IIIa mice at the same dose of $5x10^{12}$ vector genome (vg)/kg. Mice were sacrificed after two or ten weeks to collect tissues and blood. AAV vector biodistribution, Pullulanase activity and glycogen content were analyzed in tissues. Muscle functional improvement was assessed by treadmill test during the AAV treatment period. **Results**: After two weeks of AAV treatment, immunohistochemical staining of liver sections with an anti-CD4 or CD8α antibody showed that infiltrations of CD4+ and CD8+ positive lymphocytes were abundant in the AAV-CB-Pull treated liver (arrows) but barely detectable in the AAV-LSP-Pull and AAV-Dual-Pull treated livers (arrowheads) (**Fig. 1A**). These data suggest that both the LSP and Dual promoters effectively prevented CTL responses in adult GSD IIIa mice. After ten weeks of treatment, the AAV-Dual-Pull treatment significantly reduced glycogen contents and cleared glycogen accumulations in the liver, heart, and skeletal muscle (quadriceps) (**Fig. 1B**), accompanied by the increased AAV genome copy numbers and elevated Pullulanase activities in these tissues, compared with the untreated GSD IIIa mice. The AAV-Dual-Pull treatment also significantly reduced ALT, AST, and CK levels in plasma, lowered the liver size, reversed hepatic fibrosis, and improved muscle function. In contract, the AAV-LSP-Pull treatment only corrected liver abnormalities and the AAV-CB-Pull treatment had no effect on any of those tissues. **Summary:** Our data demonstrated that the LSP-CB dual promoter retained the ability of the LSP to induce immunotolerance to Pullulanase in adult GSD IIIa mice and drove the expression of the therapeutic enzyme in all major affected tissues, suggesting an effective treatment for patients with GSD IIIa. This treatment approach can also be broadly used for treating other conditions that affect multiple tissues.

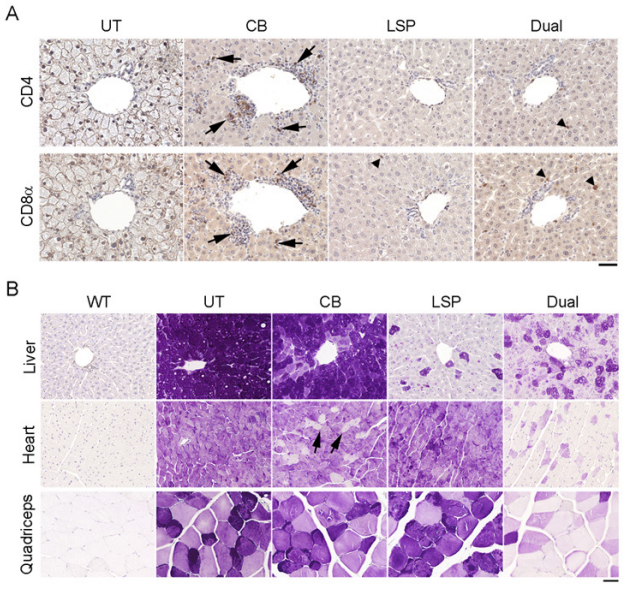


Figure1. The LSP-CB dual promoter prevented Pullulanase-induced cytotoxic T cell responses and reduced glycogen accumulation in major affected tissues of GSD IIIa mice.
A. Paraffin-embedded liver sections two weeks after AAV injection were stained with an anti-CD4 or CD8α antibody to detect cytotoxic T cell responses. Infiltrations of CD4 or CD8α positive lymphocytes (arrows) were abundant in the AAV-CB-Pull (CB) treated livers but only occasionally seen in the AAV-LSP-Pull (LSP) and AAV-Dual-Pull (Dual) treated livers (arrowheads). **B.** Periodic acid–Schiff (PAS) staining of tissue sections was performed to detect glycogen accumulation. Ten weeks after AAV injection, the LSP and Dual treated livers showed light staining of glycogen (purple) while the CB treated livers showed heavily stained glycogen accumulation similar to that in livers of the untreated (UT) GSD IIIa mice. Only the Dual treatment also remarkably cleared glycogen accumulation in the heart and quadriceps. The arrows pointed out the glycogen-free cardiac cells occasionally seen in the CB treated heart. No glycogen accumulation was observed in any tissues of the age-matched wild-type (WT) mice. The images represent at least three mice in each group. Bar=50 μm.

485. Specific Gene Correction and Direct Hepatic Cell Reprogramming as a New Therapeutic Approach for the Treatment of Primary Hiperoxaluria Type 1

Virginia Nieto-Romero[1,2], Aida Garcia-Torralba[1,2], Andrea Molinos-Vicente[1,2], Ramón Garcia-Escudero[3,4], Eduardo Salido[5], Jose-Carlos Segovia[1,2], Maria Garcia-Bravo[1,2]

[1]Biomedical Innovation Unit, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[2]Unidad Mixta de Terapias Avanzadas, Instituto de Investigación Sanitaria Fundación Jiménez (IIS-FJD, UAM), Madrid, Spain,[3]Biomedical Innovation Unit, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Cancer (CIBERONC), Madrid, Spain,[4]Biomedical Research Institute i+12, University, Hospital 12 de Octubre, Madrid, Spain,[5]Pathology Department, Hospital Universitario de Canarias, Universidad La Laguna. Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Tenerife, Spain

Nowadays the only definitive curative treatment for many inherited metabolic liver disorders is organ transplantation. This is the case of Primary Hyperoxaluria Type 1 (PH1), a rare genetic disorder caused by alanine:glyoxylate aminotransferase (AGT) deficiency, a hepatic enzyme involved in the glyoxylate metabolism. Due to the shortage of liver donors, to develop new therapeutic approaches for the treatment of these diseases is essential. Liver cell replacement therapy appears as a promising alternative to liver transplant. However, the use of primary human hepatocytes is hampered by the difficulty to expand and maintain their mature phenotype. An alternative is the use of hepatocyte like cells generated from iPSCs, but it is also limited by the difficulty to generate completely functional mature hepatocytes, in addition to safety issues. To face this challenge we propose the combination of site-specific gene correction and direct cell reprogramming for the generation of autologous phenotypically healthy induced hepatocytes (iHeps) from PH1 patient skin-derived fibroblasts. Following this strategy it would be possible to obtain high amounts of corrected hepatocyte-like cells, avoiding the potentially tumorigenic steps of iPSC generation. We have generated *AGXT* gene corrected cells using two different CRISPR/Cas9 based strategies. In a first strategy, accurate specific point mutation correction (c.853T-C) has been achieved by homology-directed repair (HDR) with ssODN harboring the wild-type sequence. In the second strategy, an enhanced version of *AGXT* cDNA has been inserted near the transcription start codon of the endogenous gene, constituting an almost universal correction strategy for PH1 mutations. Direct reprogramming of fibroblasts into iHeps was conducted by lentiviral overexpression of hepatic transcription factors and *in vitro* culture in defined media. Transcriptome analysis of iHeps generated from healthy donors, as well as from PH1 and gene corrected patient-derived fibroblasts has demonstrated the hepatic identity of the resulting hepatocyte-like cells. Demonstration of *in vitro* and *in vivo* functionality and disease phenotype reversion of gene corrected-PH1 iHeps is ongoing. Patient-derived iHeps would be a useful tool for disease modeling, for studying rare inheritance liver disorders and testing new therapeutic approaches. Moreover, the

development of these advanced therapies set up an alternative cellular source to replace endogenous deficient hepatocytes with autologous functional corrected cells for genetic liver disorders.

486. Developing a Gene Therapy Approach for Guanidinoacetate Methyltransferase Deficiency, a Creatine Deficiency Disorder

Suhail Khoja[1], Matthew Nitzahn[2], Jenna Lambert[2], Adam Eliav[2], Eram Nasser[1], Gerald S. Lipshutz[1]

[1]Department of Surgery, University of California Los Angeles, Los Angeles, CA,[2]University of California Los Angeles, Los Angeles, CA

Creatine deficiency disorders are inborn errors of metabolism of creatine, a high-energy molecule. Creatine is synthesized from arginine and glycine through an intermediate, guanidinoacetate (GAA), and by the sequential enzymatic activities of arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT). Typical characteristics of GAMT deficiency include features of autism, self-mutilation, intellectual disability and seizures; approximately 40% will also have a disorder of movement. GAA toxicity in part has been implicated in the pathophysiology of the disorder. Present day therapy is life-long and two-fold: oral creatine to help replenish cerebral creatine, and supplementation of ornithine with restriction of dietary arginine and protein to control GAA production, which is not always effective. We sought to develop a gene therapy approach for this poorly understood disorder. Our group developed an AAV8 vector expressing human codon-optimized GAMT under a liver-specific promoter and delivered this to 8-12 week old adult GAMT mice after performing dose-finding studies. Following mice for 11 months, mice early on gained weight and nearly matched that of their wild type littermate controls. Monthly serial collection of blood demonstrated a marked early and sustained reduction of guanidinoacetic acid (GAA, neurotoxin associated with brain injury) and essentially a normalization of plasma creatine levels. Urine GAA levels also markedly declined. Behavioral studies demonstrated resolution of abnormalities in grip strength and learning abnormalities detected in the Barnes maze. Neurometabolic phenotype detected by in vivo electrophysiology is currently being conducted to examine if this abnormality is resolved with gene therapy. Examination of the liver by in situ hybridization demonstrated marked expression of GAMT mRNA in the murine liver and also demonstrated panhepatic expression by immunostaining. In conclusion, we have developed a gene therapy approach that normalizes the marked elevation of GAA (with reduced creatine in guanidinoacetate methyltransferase deficiency and at the same time resolves the behavioral phenotype. These findings have important implications for the development of a new therapy for this disorder of creatine metabolism.

487. Gene Therapy for Methylmalonic and Propionic Acidemia Using the Novel Adeno-Associated Viral Vector 44.9

Randy J. Chandler[1], Giovanni DiPasquale[2], Eun-Young Choi[1], Jay A. Chiorini[2], Charles P. Venditti[1]

[1]MGMMGB, National Institutes of Health, Bethesda, MD,[2]NIDCR, National Institutes of Health, Bethesda, MD

Methylmalonic acidemia (MMA) and propionic acidemia (PA) are severe and rare autosomal recessive metabolic disorders caused by defects in the mitochondrial catabolism of propionyl-CoA to succinyl-CoA. Both conditions can result from biallelic mutations in any one of a number of different genes. In the case of MMA, mutations in the methylmalonyl-CoA mutase (*MMUT*) gene are the most common, whereas in PA, mutations in either the propionyl-CoA carboxylase alpha subunit (*PCCA*) *or* subunit beta (*PCCB*) genes occur at equal frequencies. MMA and PA have acute and chronic medical complications, and can be fatal despite management with dietary restriction and co-factor supplementation. The poor outcomes observed in the patients has led to the pursuit of adeno-associated viral (AAV) gene therapy as a potential new treatment option for these disorders. We have previously studied the efficacy of AAV 2, 8, 9 and Anc80 gene therapy vectors in murine models of MMA and PA. Here, we assayed phenotypic and biochemical correction in murine models of MMA and PA after treatment with vectors pseudoserotyped with the novel simian AAV capsid, 44.9, which falls between clade D and E. Gene delivery of *MMUT* using AAV44.9 to either neonatal lethal ($Mmut^{-/-}$) or juvenile MMA mice ($Mmut^{-/-}$ *MCK-*$Mmut^{+}$) could rescue $Mmut^{-/-}$ mice from lethality and reduce plasma methylmalonic acid levels in $Mmut^{-/-}$ *MCK-*$Mmut^{+}$ mice. Similarly, gene delivery of *PCCA* with a AAV44.9 vector to $Pcca^{-/-}$ mice could rescue them from uniform lethality and reduce plasma 2- methylcitrate. Tissue biodistribution and transgene expression studies in treated MMA and PA mice showed high levels of transduction and transgene expression in the liver and heart after gene therapy. Our studies establish that AAV44.9 serotyped vectors are highly potent in mouse models of the common organic acidemias, MMA and PA, and highlight the pronounced hepato-cardiac trophism of this capsid following systemic delivery. Because patients with PA can develop a potentially fatal cardiomyopathy, the use of an AAV44.9 vector might be especially suited for the treatment of this disorder, and by extension, other severe metabolic diseases where hepatic and cardiac targeting is needed, such as glycogen storage disorders and fatty acid oxidation defects.

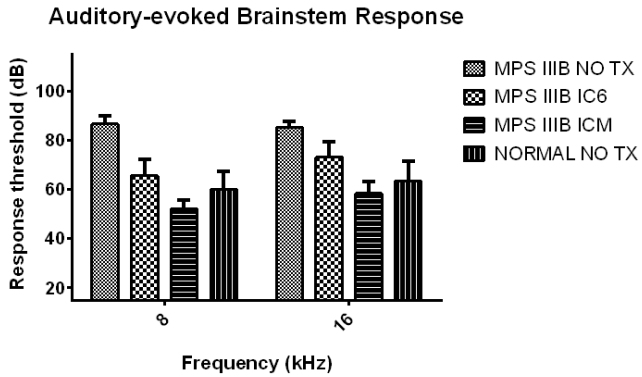
488. Mucopolysaccharidosis Type IIIB Disease Correction by Central Nervous System Delivery of a Modified AAV8 Capsid Expressing Codon Optimized NAGLU in a Mouse Model

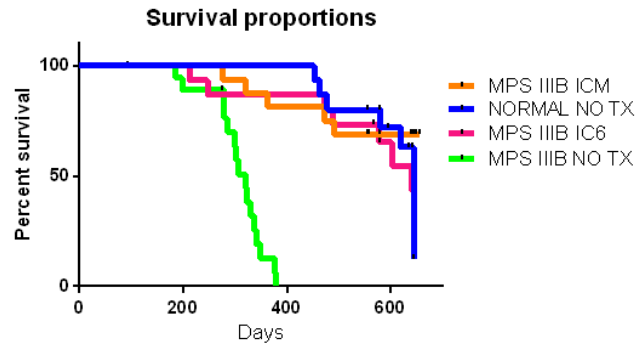
Coy Heldermon[1], Courtney Rouse[1], Kimberly Hawkins[1], Elizabeth Butterworth-Hosaka[2], Mi-Jung Kim[1], Isabella Dinelli[1], Aishwarya Kunta[1], Erinn Rosenkrantz[1], Nadia Kabbej[1], Justin Dalugdug[1], Darin Falk[2]

[1]UF, Gainesville, FL,[2]Lacerta Therapeutics Inc, Alachua, FL

Mucopolysaccharidoses IIIB (MPSIIIB) is an autosomal recessive lysosomal storage disease caused by mutations in the gene N-acetyl-glucosaminidase (NAGLU). Defective NAGLU activity results in aberrant retention of heparan sulfate within lysosomes and progressive central nervous system (CNS) degeneration. Intravenous treatment options are limited by the need to overcome the blood-brain barrier and gain successful entry to the CNS. We previously demonstrated effective disease improvement using AAV5-NAGLU intraparenchymal injection at 6 sites. Additionally, we demonstrated AAV8 provides a broader

transduction area in the MPS IIIB mouse brain compared to AAV5, 9 or rh10. A triple-capsid mutant (tcm) modification of AAV8 further enhanced expression and distribution. Using the MPSIIIB mouse model, we performed a study using either intracranial six site (IC6) injections or cisternal injection of AAVtcm8-coNAGLU compared to no treatment controls to determine disease correction as estimated by the effects on enzyme activity, CNS immune activation, coordination, activity level, hearing, and survival. Histology and enzymatic assay both show each injection method results in supranormal levels of NAGLU expression in the brain. We will show the analysis of running wheel, rotarod, auditory evoked brainstem response, survival, biochemical activity and histologic distribution of NAGLU and immune activation of these cohorts. We have shown complete correction of lifespan, brain NAGLU activity, auditory defects, and lysosomal storage levels in the murine model of MPSIIIB. Acknowledgements: Sanfilippo Children's Research Foundation, Cure Sanfilippo, Lacerta Therapeutics and NIH/NINDS R01NS102624





489. Liver-Directed AAV Gene Transfer for Glycogen Storage Disease Type IA

Louisa Jauze[1], Alexane Cannella Miliano[2], Marine Silva[2], Carine Zitoun[2], Gilles Mithieux[2], Giuseppe Ronzitti[1], Fabienne Rajas[2]

[1]Genethon, Evry, France,[2]INSERM U1213, Lyon, France

Glycogen storage disease type Ia (GSDIa) is a rare monogenetic disease caused by mutations in the glucose-6-phosphatase catalytic subunit 1 (*G6pc1*) gene encoding for the enzyme glucose-6-phosphatase (G6Pase). G6Pase is responsible for the hydrolysis of glucose-6-phosphate into glucose. Mutations reducing G6Pase activity result in severe hypoglycemia, hepatomegaly, and hepatocellular adenomas (HCA) development. Previous studies in mice and dogs showed the potential of gene therapy using AAV expressing *G6PC1* under the control of the human endogenous promoter of the gene (hGPE) to control blood glucose. However, the efficiency of liver gene therapy to prevent hepatic tumor development has not been conclusively demonstrated. Here, we tested the long-term efficacy of AAV9 vectors expressing *G6PC1* under the control of a strong liver-specific promoter (LSP), the hGPE promoter or a promoter derived from the mouse GPE promoter (mGPE) in mice with a liver-specific *G6pc1* deletion (L.G6pc$^{-/-}$ mice). L.G6pc$^{-/-}$ mice were injected with AAV-G6PC1 vectors at a dose of 8x10^{12} vg/kg. Under a standard diet, the three vectors allowed to maintain glycemia in 6h-fasted L.G6pc$^{-/-}$ mice and prevent glycogen accumulation in the liver 6 months post-treatment. In L.G6pc$^{-/-}$ mice fed a high fat/high sucrose (HF/HS) diet, a tumor-promoting diet, higher G6Pase activity was observed after administration of the AAV9-LSP-G6PC1 vector and was associated with reduced tumor formation and rescue of the liver phenotype. This study provides important insight into the strategic choices to make for the translation of liver-targeted AAV gene therapy strategies to the clinic and supports the use of a strong liver-specific promoter for gene therapy in GSDIa.

490. Comparative Cardiac, Neurologic, and Skeletal Effectiveness of Intravenous and Intrathecal AAV9-IDUA Delivered Individually or Combined in a Murine Model of Mucopolysaccharidosis Type I

Lalitha Belur[1], Elizabeth Braunlin[2], Troy Lund[2], Avery Huber[1], Hillary Mantone[1], Andrea Karlen[1], Miles Smith[1], Li Ou[2], Chester B. Whitley[2], Nicholas Buss[3], R. Scott McIvor[1]

[1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN,[2]Pediatrics, University of Minnesota, Minneapolis, MN,[3]REGENXBIO Inc., Rockville, MD

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive storage disease caused by deficiency of α-L-iduronidase (IDUA), resulting in accumulation of heparan and dermatan sulfate glycosaminoglycans (GAGs). Current treatments include allogeneic hematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). However, ERT is ineffective against CNS disease due to the inability of lysosomal enzymes to traverse the blood-brain barrier, and while there is neurologic benefit to HSCT, the level of correction is variable, and the procedure is associated with morbidity and mortality. Preclinical studies of IDUA gene therapy using AAV vectors have provided encouraging results for the treatment of MPS I. In this study, we compare the relative efficacy of intrathecal (IT), intravenous (IV), and combined routes (IT+IV) of AAV9-IDUA vector administration on cardiac, neurologic, and skeletal manifestations of disease. AAV9-IDUA at a dose of 1 x 10^{10}vg was administered either IT, IV, or IT+IV to 2-month old MPS I mice. Control mice included normal heterozygotes and untreated MPS I mice. A gender related effect was observed for IDUA activity in plasma, with levels 100- to 1000-fold higher (in females and males respectively) than normal in all 3 groups. Cardiac valve function analysis by high resolution ultrasound biomicroscopy

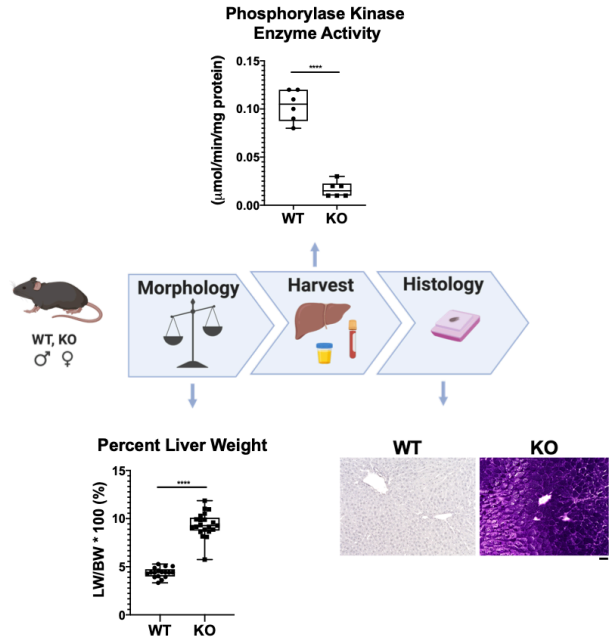
showed aortic insufficiency (AI) in most untreated MPS I mice, while the IT+IV group showed no aortic insufficiency, and the IT and IV groups had only 1 mouse in each group with AI. The ascending aortic diameter was normalized in the IV (all mice) and the IT/IT+IV groups (1 mouse had increased diameter), compared to untreated MPS I mice. IV, IT or IV + IT administration of AAV9-IDUA prevented the emergence of neurocognitive deficit exhibited in MPS I mice, with no significant difference between the treatment groups, as evaluated by Barnes maze and fear conditioning cognitive tests. Skeletal analysis of vector-treated mice by microCT showed normalization of skull width, zygomatic arch diameter, and kyphosis in male mice. Cross sectional moment of inertia was also normalized in both male and female treated mice. Animals were euthanized at 6 months post-treatment, and tissues were harvested and assayed for IDUA activity and GAG levels. IDUA levels in brain and liver showed a gender-related effect, with a 10-fold higher level seen in males. Enzyme activities in the brain were highest after IT administration (10-fold higher than normal), with lowest levels seen after IV administration. Supraphysiological levels of IDUA in liver were seen for all three groups (100-1000-fold higher than normal in females and males, respectively). The three test groups yielded similar levels of enzyme activity in all other organs, and GAG levels were normalized or reduced in all groups. Our results show that AAV9-IDUA vector, administered IV, IT or both resulted in high levels of enzyme activity in major organs, and all three treatments appeared to prevent neurocognitive deficit, cardiac valve dysfunction and skeletal dysplasias in MPS I mice as a model for genetic therapy of human MPS I.

491. Characterization of Liver Pathology in a Novel GSD IX γ2 Mouse Model

Rebecca Gibson, Jeong-A Lim, Su Jin Choi, Leticia Flores, Lani Clinton, Deeksha Bali, Sarah Young, Aravind Asokan, Baodong Sun, Priya Kishnani

Duke University, Durham, NC

Liver Glycogen Storage Disease type IX (GSD IX) accounts for 25% of all GSD cases with an overall estimated prevalence of 1 in 100,000 individuals. GSD IX is defined by a deficiency in PhK, a complex hetero-tetrameric enzyme comprised of four subunits, each encoded by different genes that are associated with different liver GSD IX disease subtypes. Based on a recent literature review, there is mounting evidence that the subtype GSD IX γ2 is associated with a severe progressive liver phenotype. Of published case reports, 95.8% of patients with GSD IX γ2 reported features of liver fibrosis and/or cirrhosis, placing individuals with GSD IX γ2 at higher risk of liver failure, hepatocellular carcinoma, and death. Here, we describe the first known mouse model for liver GSD IX γ2. A *Phkg2*$^{-/-}$ mouse model was generated via targeted removal of the *Phkg2* gene. Male and female knockout (*Phkg2*$^{-/-}$, KO) and wild type (*Phkg2*$^{+/+}$, WT) mice up to 3 months of age were compared for indications of liver pathology. When compared to WT mice, KO mice demonstrated significantly decreased liver PhK enzyme activity, increased liver: body weight ratio, and increased liver glycogen, with no glycogen accumulation observed in the brain, quadricep, kidney, and heart. KO mice demonstrated elevated liver blood markers as well as elevated urine Glc4, a commonly used biomarker for glycogen storage disease. KO mice also demonstrated features of liver structural damage. Hematoxylin & Eosin and Masson's Trichrome stained liver histology slides from KO mice revealed characteristic GSD hepatocyte architectural changes and early liver fibrosis, as have been reported in liver GSD patients. This study provides characterization of the first mouse model for GSD IX γ2. This model will provide a platform for better understanding liver disease progression as well as for the evaluation of novel therapeutics.



492. Combined Efficacy of Insulin and Anti-Inflammatory Gene Therapies for Type 1 Diabetes Mellitus

Fulya Erendor[1], Yunus Emre Eksi[1], Elif Ozgecan Sahin[1], Mustafa Kemal Balci[2], Thomas S. Griffith[3], Salih Sanlioglu[1]

[1]Department of Gene and Cell Therapy, Faculty of Medicine, Akdeniz University, Antalya, Turkey,[2]Department of Internal Medicine, Division of Endocrinology and Metabolism, Akdeniz University, Antalya, Turkey,[3]Department of Urology, School of Medicine, Minnesota University, Minneapolis, MN

Recent studies of insulin gene therapy have mainly focused on the transfer of functional insulin genes to non-pancreatic cells to avoid the autoimmune attack, but insulin secreted by non-beta cells were ineffective to properly manage postprandial hyperglycemia. Compared to other cell types, pancreatic beta cells possess exquisite features in terms of controlled transcription and translation of proinsulin, the presence of prohormone convertase expression, and glucose-sensing machinery, in addition to a regulated secretory pathway. To take advantage of the unique properties of pancreatic beta cells, we constructed 3rd generation lentiviral vectors carrying insulin promoter connected to the insulin gene (LentiINS) targeting pancreatic beta cells. Although intraperitoneal injection of LentiINS to streptozotocin (STZ)-induced diabetic Wistar rats decreased fasting plasma glucose, improved glucose tolerance, and prevented weight loss, it was insufficient in lowering postprandial blood glucose levels. Only, the combination of LentiINS and the anti-inflammatory

vasoactive intestinal peptide (LentiVIP) gene deliveries successfully lowered postprandial blood glucose and suppressed diabetes-related inflammation in diabetic rats. Our results suggested that insulin gene therapy combined with anti-inflammatory/insulinotropic/regenerative gene delivery was more effective than their individual application. TUBITAK:215S820

493. Development of AAV Gene Replacement Therapy for Lysosomal Acid Lipase Deficiency

Patricia Lam[1], Anna Ashbrook[1], Deborah Zygmunt[1], Cong Yang[2], Hong Du[2], Paul T. Martin[1,3]

[1]Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH,[2]Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, IN,[3]Department of Pediatrics, The Ohio State University, Columbus, OH

Lysosomal acid lipase (LAL) is a lysosomal enzyme that hydrolyzes triglycerides (TG) to free fatty acids and cholesteryl esters (CE) to cholesterol. Loss of function mutations in the *LIPA* gene, which encodes LAL, can give rise to Lysosomal Acid Lipase Deficiency (LAL-D). LAL-D presents as one of two rare autosomal recessive diseases: Wolman disease (WD), a severe disorder presenting in infancy characterized by absent or very low LAL activity, and Cholesterol Ester Storage Disease (CESD), a less severe, later onset disease that can manifest in children or adults. Major clinical features of WD include hepatosplenomegaly, liver disease, dyslipidemia, failure to thrive, and premature death. Patients with CESD can present with liver fibrosis, cirrhosis, or failure and can be at increased risk of cardiovascular disease and stroke due to accelerated atherosclerosis. Current treatment for WD and CESD involves enzyme replacement therapy, which, while therapeutic, is not curative. With the goal of providing a more effective treatment for LAL-D, we have developed a recombinant self-complementary (rsc) gene replacement therapy, rscAAVrh74.mCMV.LIPA, using adeno-associated virus (AAV) serotype rhesus 74 (rh74) and a minimal cytomegalovirus (mCMV) promoter to deliver the wild-type human *LIPA* gene to cells and tissues. We tested the therapeutic efficacy of rscAAVrh74.mCMV.LIPA in Lipa knock out mice ($Lipa^{-/-}$). $Lipa^{-/-}$ mice appear normal at birth, but develop enlarged livers, spleens and mesenteric lymph nodes due to accumulation of lipids beginning in the postnatal period, which is coincident with liver damage that can be followed by serum Aspartate transaminase (AST) and Alanine transaminase (ALT) levels. Neonatal (P2 age) and 2-month-old $Lipa^{-/-}$ mice were intravenously injected with 8.4×10^{13} vg/kg of rscAAV.rh74.mCMV.LIPA. At 2, 4, and 6 months post-injection, mice were assessed for AAV biodistribution, LIPA gene and protein expression, serum AST and ALT levels, organ cholesterol and TG levels, and organ LAL enzyme activity. Both injection regimens resulted in a reduction in liver, spleen, and mesenteric lymph node size and weight compared to age-matched mock-treated controls. Injections at 2 months, however, were more efficacious than injections at P2, likely due to robust hepatocyte cell division in the first month of life. Injections at P2 resulted in AAV biodistribution primarily in the heart and lungs, whereas injections at 2 months resulted in greater AAV transduction in the liver and spleen. Both treatments dramatically lowered liver weight and fat content, but mice injected at 2 months showed greater reduction in liver TG and cholesterol levels compared to mice injected at P2. Additionally, LAL enzyme activity in the liver and spleen was restored to normal levels when injected at 2 months, as were elevations in serum AST and ALT. These results demonstrate that AAV gene replacement therapy may be a viable option to treat patients with WD and CESD. This research was supported by grants from LAL-D Aware (USA) and AE LALD (Spain) to the Nationwide Children's Hospital Foundation

494. Developing and Characterizing a Novel Arylsulfatase A-Deficient Mouse Model of Metachromatic Leukodystrophy to Evaluate the Efficacy of Gene Therapy

Gourav Roy Choudhury[1], Juliette Hordeaux[1], Christopher Petucci[2], Brianne Jeffrey[1], Laura Ballentine[1], Melissa White[1], Hongwei Yu[1], Peter Bell[1], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]Metabolomics Core, University of Pennsylvania, Philadelphia, PA

Arylsulfatase-A (ARSA) is a lysosomal enzyme involved in degrading sulfatides, the most common sphingolipids in myelin. Metachromatic leukodystrophy (MLD) is a rare inherited autosomal-recessive disorder characterized by the deficiency of ARSA, resulting in the systemic accumulation of undegraded sulfatides. The toxic accumulation of sulfatides in the central and peripheral nervous systems causes progressive neurodegeneration and demyelination. Clinically, MLD patients present with gait abnormalities, ataxia, convulsions, and difficulties in breathing, leading to a rapid decline in quality of life. The majority of children with the late infantile form of the disease die before the age of 5 years. The only currently approved treatment for MLD, *ex vivo* gene therapy, benefits only pre-symptomatic patients due to the competing kinetics of hematopoietic engraftment in the CNS and disease progression. Other approaches that are used to substitute the enzyme (such as enzyme replacement therapy or hematopoietic stem cell transplantation) do not halt the progression of the disease. Therefore, there is an urgent unmet need for the development of a novel therapeutic approach for MLD. One of the major caveats in the development of MLD treatments is the limited availability of mouse models to test therapies and the paucity of phenotypes in the existing models. Here, we report the development and characterization of a novel ARSA-deficient mouse model to evaluate the efficacy of adeno-associated virus (AAV)-mediated gene therapy for MLD. We collaborated with the Jackson Laboratory to produce a new MLD mouse model using CRISPR-Cas9 gene-editing technology to target the deletion of the ARSA gene in C57BL/6 mice. Three-month-old ARSA knockout (KO) mice and wild-type (WT) littermates were enrolled in a natural history study and monitored for either 9 months (short term) or 15 months (long term) for MLD phenotypes. Parameters included periodic body-weight measurement, neurobehavioral scoring, motor coordination, and gait analysis. At the end of each study, we euthanized the animals and collected tissue samples for sulfatide and immunohistochemistry analyses to identify biomarkers for gene therapy. We showed that ARSA KO mice developed significant neurobehavioral deficits compared to WT controls at around 10 months of age. The deficits in the KO mice manifested as an increase in generalized tremors, hind-limb clasping, and ataxia, which gradually worsened with time. Results from the CATWALK gait analysis revealed

a widening of the base of the hind limbs and gait-pattern abnormalities that manifested as increased irregularity in the step sequence in the ARSA KO mice. Liquid chromatography-mass spectrometry analysis of tissue samples showed a significant increase in C16:0 (14-fold) and C18:0 (9-fold) sulfatide species in the brain and kidney of the ARSA KO mice. Immunohistochemical analysis revealed a significant increase in the lysosomal marker LAMP-1 and astrocyte marker GFAP in the brain and spinal cord of ARSA KO mice compared to WT mice. Collectively, our results demonstrate that our novel ARSA-deficient mouse model shows MLD-like phenotypes and could be a useful tool for testing MLD treatments. Indeed, since demonstrating the utility of this novel MLD mouse model, we have initiated a pilot study to investigate the efficacy of intracerebroventricular AAVhu68 encoding a human codon optimized ARSA transgene at three doses. We are monitoring animals in a long-term study and will present preliminary results from the in-life phase.

495. AAV-Mediated Gene Therapy Rescues GALT Activity and Reduces ER Stress in Classic Galactosemia

Megan L. Brophy, Ting-Wen Cheng, Kevin Le, Ricardos Tabet, Youngwook Ahn, John Murphy, Robert Bell

Rare Disease Research Unit, Pfizer, Cambridge, MA

Classic galactosemia, a recessive autosomal disease, is caused by point mutations in the *GALT* gene that encodes the galactose-1-phosphate uridyltransferase enzyme (GALT), which is essential in galactose metabolism. Without dietary intervention, newborns present with liver inflammation, cataracts, sepsis, and premature death. Long term consequences with dietary intervention include ataxia, speech and intellectual problems, and subfertility. Recombinant adeno-associated virus (AAV)-mediated gene therapy to restore GALT activity offers the potential to address the unmet medical needs of galactosemia patients. We are utilizing fibroblasts derived from classic galactosemia patients and developing a novel mouse model of disease to better understand the pathophysiological consequences resulting from loss of GALT activity. We established a GALT enzyme activity assay and showed fibroblasts derived from galactosemic patients exhibited significant reductions in enzyme activity compared to control fibroblasts. Further analysis revealed that this reduction was likely due to a decrease in GALT protein levels as opposed to mRNA levels. We also confirmed that high galactose conditions induce ER stress in patient fibroblasts. Transduction of classic galactosemia patient fibroblasts with a recombinant AAV vector encoding GALT reconstituted GALT activity and reduced galactose-induced ER stress. We have also developed a novel mouse model. Initial phenotypic characterization of this mouse demonstrated a reduction in GALT protein and activity in the liver, and increased levels of galactose metabolites, both of which are reversed with AAV-mediated gene replacement therapy for GALT. Additional studies will determine if these mice have cerebellar defects, ataxia, and/or reduced fertility and if these aberrant characteristics could be rescued via gene replacement therapy. These cellular and mouse models of galactosemia will advance our understanding of the underlying pathological mechanisms associated with classic galactosemia. AAV-GALT gene therapy holds promise in addressing the unmet medical needs in classic galactosemia patients.

496. Lipid Nanoparticle Encapsulated mRNA Therapy Corrects Serum Total Bilirubin Levels and Extends Survival in a Mouse Model of Crigler-Najjar Syndrome

Jenny A. Greig[1], Joanna Chorazeczewksi[1], Melanie K. Smith[1], Kimberly A. Coughlan[2], Paolo G. V. Martini[2], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]Moderna, Inc., Cambridge, MA

Crigler-Najjar syndrome type 1 (CN1) is an ultra-rare disorder of bilirubin metabolism caused by mutations in the uridine diphosphate glucuronosyl transferase 1A1 (UGT1A1) gene. This disorder is characterized by hyperbilirubinemia and jaundice. Transit of these abnormal bilirubin levels across the blood-brain barrier into brain tissue can cause kernicterus, resulting in irreversible neurological damage. Currently, no cure exists for CN1 and clinical management consists predominantly of phototherapy, which becomes less effective over time. Other illnesses can lead to severe bilirubin excursions, with multiple clinical strategies deployed to reverse the crisis and prevent kernicterus. Liver transplantation is also a treatment option. These limited treatment options highlight the benefit of developing a novel therapeutic approach for this unmet clinical need. Previously, we evaluated the therapeutic potential of a lipid nanoparticle (LNP)-encapsulated mRNA therapy for expression of UGT1A1 in both adult and neonatal CN1 mice. Within 24 hours of intravenous administration (0.2 mg/kg) in adult CN1 mice previously rescued from lethal postnatal hyperbilirubinemia by phototherapy treatment, serum total bilirubin levels rapidly decreased to less than 0.5 mg/dl, which is slightly above wild-type mouse levels. These reductions in total bilirubin levels were sustained for two weeks. Repeated administrations of the mRNA/LNP also resulted in longer-term reductions in total bilirubin levels. We have also previously shown that repeated administrations of mRNA/LNP extended survival of neonatal CN1 mice, which do not survive past the first week of life due to severe hyperbilirubinemia without intervention. All evaluated doses of LNP extended survival of the CN1 mice. After redesigning the mRNA to increase stability, we performed additional single-dose studies in both adult and neonatal CN1 mice. In adult mice, the redesigned mRNA further reduced the serum total bilirubin levels and increased the duration of the effect compared to the previous mRNA. In addition, a single intravenous administration of the redesigned mRNA/LNP within 24 hours of birth extended the median survival of CN1 mice to 17 days. Therefore, we can conclude that LNP mRNA therapy may be suitable for both the prolonged treatment of CN1 and for managing metabolic crises in this disease.

497. Translation of an AAV-Delivered Gene Editing Approach for Transthyretin Amyloidosis from Mice to Nonhuman Primates

Jenny A. Greig[1], Cassandra Gorsuch[2], Joanna K. Chorazeczewski[1], Melanie K. Smith[1], Thomas Furmanak[1], Alexa N. Avitto[1], Scott N. Ashley[1], Wendy Sharer[2], Hui Li[2], Jeff Smith[2], Peter Clark[1], Camilo Breton[1], Derek Jantz[2], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]Precision BioSciences, Inc., Durham, NC

Transthyretin amyloidosis (ATTR) is a rare disease caused by the progressive accumulation of abnormal, misfolded TTR protein into amyloid fibrils which disrupt organ function. There are two primary manifestations of ATTR characterized by the location of the amyloid deposits; polyneuropathy and cardiomyopathy. There are approved treatment approaches for ATTR, including TTR stabilizers and siRNA, but these require repeated administration. A gene-editing approach delivered by adeno-associated vectors (AAV) could provide an effective one-time treatment. We have previously utilized ARCUS meganucleases expressed from AAV vectors for *in vivo* gene editing approaches. For the treatment of ATTR, we have shown up to 59% on-target editing in mice by the TTR5-6 meganuclease expressed from an AAV8 vector. However, this efficacy did not translate well to nonhuman primates (NHPs). We have subsequently repeated these studies with an improved meganuclease, TTR15-16, which targets a different 22 bp sequence in the TTR gene that is conserved in rhesus macaques and humans, but this target site is not present in the mouse genome. To evaluate the ability of this nuclease to edit in mice prior to NHP studies, immunodeficient mice were administered with AAV8.TBG.hTTR(V30M) to supply the human TTR sequence. Two weeks later, we administered mice with one of three doses of an AAV vector expressing the TTR15-16 meganuclease from a liver-specific promoter. There was a dose-dependent significant decrease in human TTR levels of 94.5-99.9% following IV administration at doses of $3x10^{11}$ - $3x10^{13}$ GC/kg. This drop in serum TTR levels correlated with 66% insertions and deletions (indels) in the target sequence located in the hTTR transgene and 92% indels in the transgene RNA. The TTR15-16 nuclease was evaluated in rhesus macaques to determine the translatability of gene editing efficacy from mice to NHPs. At day 18 post-vector administration, liver biopsies were performed and the on-target efficacy of this gene-editing approach was evaluated. In the liver, there were up to 46% indels in the TTR genomic DNA locus and 80% indels in TTR RNA following a dose of $3x10^{13}$ GC/kg. This editing efficacy was reflected in serum TTR levels where there was a concomitant reduction of greater than 95% by day 21 post-vector administration. When the off-target editing activity of the TTR15-16 meganuclease was evaluated, the number of off-target events for this nuclease was reduced compared to other previously described meganucleases with different target genes. In conclusion, the significant reduction in serum TTR levels following genomic editing of the TTR gene by the TTR15-16 meganuclease expressed from an AAV vector indicates that this approach could be effective for the treatment of ATTR.

498. Elucidating Brain White Matter Abnormalities in the Mouse Model of Phenylketonuria

Rachna Manek[1], Cathleen S. Cornell[1], Yinyin Huang[2], Jennifer Johnson[3], Robert Fogle[4], Dan Yu[1], Sue Ryan[3], Xiaoyou Ying[4], Dinesh Kumar[2], Dinesh Bangari[3], Sirkka Kyostio-Moore[1]

[1]Genomic Medicine Unit, Sanofi, Framingham, MA,[2]Translational Science, Sanofi, Framingham, MA,[3]Global Discovery Pathology, Sanofi, Framingham, MA,[4]Global Bioimaging, Sanofi, Framingham, MA

Phenylketonuria (PKU) is a genetic defect of liver enzyme phenylalanine hydroxylase (PAH) that catalyzes the conversion of phenylalanine (Phe) to tyrosine (Tyr). Mutations in the PAH gene lead to partial or complete loss of PAH activity resulting in Phe elevation in blood and consequently in the brain causing various neurotoxic effects. These include white matter deficits observed by MRI in the brains of the early treated PKU patients. Current hypotheses for these deficits are effect of high Phe on myelin biosynthesis by oligodendrocytes, reduction in cholesterol levels and the presence of high oxidative stress in the brains of PKU patients. The goal of our study was to 1) investigate the white matter deficits in a PKU mouse model (Pah^{enu2} mice), 2) understand the causative aspects and 3) to observe the reversibility of these deficits post liver directed AAV-mPAH gene therapy. In our study, adult Pah^{enu2} mice were treated with AAV-mPAH at 1e11 vg/mouse for 3.5 months which normalized the blood Phe levels. The white matter deficit was demonstrated by brain MRI and showed a significant reduction in the volume of corpus callosum (CC) of Pah^{enu2} mice compared to normal heterozygous mice. Similarly, immunostaining for myelin basic protein and Nitroblue staining was reduced in the CC of naive Pah^{enu2} mice. Treatment with AAV-mPAH mice showed improvement for all of these endpoints though the CC volume was not completely normalized. To understand the cause of myelination defects with an unbiased high throughput approach, we performed single nuclei sequencing of the CC region. Our data shows subtle changes in the number of different oligodendrocytes subtypes in the brains of Pah^{enu2} mice and their uneven distribution in a lineage trajectory analysis as compared to the heterozygous Pah^{enu2} mice. We also observed a marked increase in transthyretin (TTR) gene expression in various brain cell types. This protein is mainly involved in transport of thyroid hormone but was recently also shown to play a role in oligodendrocyte development. Treatment with AAV-mPAH was able correct the oligodendrocyte lineage and gene expression changes comparable to heterozygous Pah^{enu2} mice. Ongoing analyses are focused on analyzing the expression profiles and pathways affected in various cell types in the brain to decipher their sensitivity to high Phe levels. In summary, our results showed white matter abnormalities in the corpus callosum of Pah^{enu2} mice and that correction of blood Phe levels can partially correct and prevent these abnormalities. Furthermore, we have demonstrated significant transcriptome and cell population changes in the brains of PKU mice which are likely to contribute to the white matter deficits observed in the PKU brain pathology.

499. The UAB Center for Precision Animal Modeling (C-PAM)

Deeann Wallis[1], The UAB CPAM Team[1], Matthew Might[2], Bradley Yoder[3]

[1]Genetics, UAB, Birmingham, AL,[2]Precision Medicine Institute, UAB, Birmingham, AL,[3]Cell, Developmental and Integrative Biology, UAB, Birmingham, AL

Background: Many persons with rare genetic disorders go without a diagnosis in spite of years of standard medical evaluations. Although genome sequencing now helps to identify the underlying cause for many, sequencing studies often end with the identification of a "variant of unknown significance" that leaves the diagnosis unresolved. Even those who do receive a specific diagnosis may be frustrated by lack of therapeutic options. Disease models: including those based on cells, worms, zebrafish, frogs, mice, or rats that reflect the molecular characteristics of a condition, can not only aid in better understanding of a disorder, but can also be used to identify potential treatments. Until recently, the technology to create small and precise changes to the genome was not readily available. Earlier methods resulted in many models with large changes that completely disrupted a gene and thus did not accurately replicate the molecular pathogenesis of the disease nor the phenotype. New technologies now make it possible to create models with the identical genetic change as the human counterpart. Methods: With this in mind, the University of Alabama at Birmingham, through funding provided by the National Institutes of Health, has developed a Center for Precision Animal Modeling (C-PAM). The C-PAM team consists of scientists and clinicians with expertise in computational and data sciences, human genetics, clinical diagnostics, cellular and molecular biology, animal model generation, and therapeutic modeling and selection. The mission of C-PAM is to provide a national service to generate precision disease models for researchers and physicians working to diagnose, treat, and study genetic diseases. Through coordination with existing UAB centers and laboratories these models can be phenotyped and analysed for functional validation and development of therapeutic screening strategies. Results: Our C-PAM outreach team works closely with the submitting clinician or scientist to coordinate initial evaluation of the model for relevance to the patient phenotype. Generated models are made available to the submitting researcher and to the research community at large. We will discuss processes and methods in place to support prioritization, review, analysis, interpretation, generation, and validation of models. Specifics on the nomination process and how interested individuals can engage with C-PAM and submit variants for consideration will also be provided. Conclusion: C-PAM will leverage existing expertise to fulfill our vision as a national resource for efficient and cost-effective analysis of patient-derived gene variants with production and validation of informative animal models. Development of these models supports generation of knowledge on disease mechanisms and candidate therapeutics. Distribution and further study can further elucidate the specific mechanism by which these genetic changes affect the body. This can finally provide answers as to why these individuals are sick and help find novel treatments for them. The team has established the pipeline and methods and solicits nominations for variants from the community.

500. Hematopoietic Stem Cell Gene Therapy for Cystinosis: Updated Results from a Phase I/II Clinical Trial

Stephanie Cherqui[1], Bruce Barshop[1], Edward Ball[2], Magdalene Dohil[2], Rajan Dohil[2], Donald Kohn[3], Natalie Afshari[2]

[1]Pediatrics, University of California, San Diego, La Jolla, CA,[2]University of California, San Diego, La Jolla, CA,[3]University of California, Los Angeles, Los Angeles, CA

Cystinosis is a lysosomal storage disorder characterized by the accumulation of cystine within the lysosomes of all organs and caused by mutations in the *CTNS* gene encoding the transmembrane lysosomal cystine transporter, cystinosin. Major complications of cystinosis include early renal Fanconi syndrome, kidney failure requiring transplantation, eye defects that can lead to blindness, hypothyroidism, and neuromuscular problem causing swallowing dysfunction and fatal pulmonary aspiration. Cysteamine, the FDA-approved drug to treat cystinosis, only delay the progression of the disease. Preclinical studies showed that transplantation of wild-type hematopoietic stem and progenitor cells (HSPCs) in $Ctns^{-/-}$ mice leads to cystine content decrease in every tissue tested, as well as functional and structural improvement in kidney, eye and thyroid. The mechanism of action involves lysosomal cross-correction via tunneling nanotubes from graft-derived macrophages. We developed an autologous transplantation approach of HSPCs modified ex vivo using a self-inactivating-lentiviral vector to introduce a functional version of the *CTNS* cDNA, pCCL-CTNS (drug product name: CTNS-RD-04). We performed pharmacology and toxicology studies, and manufacturing development for this strategy, and received FDA-approval for a phase I/II clinical trial on December 19, 2018. In July 2019, we started the Phase 1/2 clinical trial at UC San Diego to evaluate the safety and efficacy of CTNS-RD-04 that will include six patients. The manufacturing of the products is performed in the UCLA GMP facility under the leadership of Dr. Donald Kohn. Three patients have been infused so far. White blood cell cystine was decreased in the treated patients with substantial peripheral blood vector copy number (VCN) values. Tissue cystine crystals were shown to be decreased in the skin, eyes and rectal mucosa of patient 1. At the time of the conference, updated data will be presented for the three patients infused to date.

501. Development of Cell-Based Therapies for the Urea Cycle Disorder CPS1 Deficiency

Matthew Nitzahn, Brian Truong, Suhail Khoja, Gerald Lipshutz

UCLA, Los Angeles, CA

Mammalian nitrogen homeostasis is carried out principally in the liver via the urea cycle, where waste ammonia is incorporated into urea for excretion. The first and rate-limiting enzyme in this process is carbamoyl phosphate synthetase 1 (CPS1), which condenses ammonia with bicarbonate to generate carbamoyl phosphate. Functional disruptions of CPS1 cause CPS1 deficiency, a rare inborn error of metabolism, and typically lead to severe neonatal hyperammonemia, causing lethargy, vomiting, coma, and death if untreated. Even with rapid diagnosis and aggressive treatment, current therapies provide

no guarantee of survival and are incapable of preventing recurrent hyperammonemic crises, which cause irreversible neurological injury. The clear need for novel therapeutics immediately suggests a classical gene therapy approach to simply replace the dysfunctional CPS1; indeed, we recently published a proof-of-concept study describing a split AAV-mediated gene replacement strategy that prolongs lifespan and restores ureagenesis in Cps1-deficient mice. Though successful, AAV-based viral therapy for CPS1 deficiency faces multiple challenges, including large cDNA size, cost-prohibitive high viral dosing, potential for carcinogenesis, and episomal loss. One way to address these issues is to use a cell-based therapy, which can be generated *in vitro* and validated prior to implantation; our lab has previously demonstrated that this is a viable strategy for treating Arginase 1 deficiency, another urea cycle disorder. To pursue this approach, we generated 3 CPS1 deficient patient-specific induced pluripotent stem cell (iPSC) lines. Validated iPSCs were then subjected to CRISPR/Cas9 genome editing to introduce a transgenic cassette into the euchromatic *AAVS1* safe harbor. This cassette contains a human codon optimized CPS1 cDNA (hcoCPS1) driven by the human *EF1α* promoter, as well as an in-frame puromycin resistance gene to facilitate selection. By using a constitutively active promoter and gene addition strategy, as opposed to a gene editing strategy, this workflow may be applied to any case of CPS1 deficiency regardless of mutation. Universal applicability is essential because the vast majority of patients have unique mutations that may be spread across the entire length of the gene. Edited clones were subsequently established, and the faithful insertion of the transgenic cassette was confirmed by sequencing. These cells were then differentiated to hepatocyte-like cells (HLCs) and treated with exogenous NH_4Cl to determine their capacity to metabolize ammonia. Surprisingly, edited hcoCPS1-expressing HLCs had higher levels of ammonia in cell culture supernatants than their unedited counterparts, indicating a counterintuitive decrease in ammonia consumption. Reduced ammonia metabolism is not explained by a lack of the essential CPS1 coactivator NAGS, nor by dramatic differences in other urea cycle enzyme expression levels. Interestingly, the glutamine synthetase gene *GLUL* (which condenses glutamate with ammonia) does show a not statistically significant increase in expression in edited cells. This subtle change may suggest that aberrant global amino acid metabolism causes a net increase in ammonia production despite increased CPS1 expression to remove ammonia. The complex and interrelated biochemical homeostasis of ammonia and amino acids may represent an important and underappreciated aspect of hepatocyte biology that hinders gene therapies and merits further investigation.

Metabolic, Storage, Endocrine, Liver and Gastrointestinal Diseases

502. Chimeric Lysosomal Enzymes with Improved Bioavailability to Advance Gene Therapy Strategies for Globoid Cell Leukodystrophy

Federica Cascino[1], Alessandra Ricca[1], Francesco Morena[2], Sabata Martino[2], Angela Gritti[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy

Globoid Cell Leukodystrophy (GLD) is a lysosomal storage disorder (LSD) caused by inherited defects of the β-galactosylceramidase (GALC) gene. The rapid disease progression of the infantile forms and the severe dysfunction of the central and peripheral nervous system (CNS, PNS) pose major issues for the development of effective treatments. Successful gene therapy (GT) approaches for GLD require a safe and pervasive delivery of the functional GALC enzyme to the CNS and periphery. Chimeric lysosomal enzymes with increased secretion and enhanced transport across the blood-brain barrier (BBB) that boost the efficacy of GT approaches in pre-clinical models of similar neurodegenerative LSDs may likewise benefit GLD. To test this hypothesis, we generated novel lentiviral vectors driving the expression of chimeric murine (m)GALC enzymes engineered to express an alternative signal peptide (iduronate-2-sulfatase -IDSsp) and the low-density lipoprotein receptor (LDLr)-binding domain from the Apolipoprotein E II (ApoE II). We evaluated the secretion, bioavailability, safety, and modality of action of chimeric mGALC constructs in GLD murine neural and hematopoietic stem/progenitor cells and progeny, which are relevant cells types in the context of *in vivo* and *ex vivo* GT platforms. We showed that the lentiviral vector-mediated expression of the chimeric GALC enzymes is safe and leads to supranormal enzymatic activity in both neural and hematopoietic cells. Also, we highlighted an advantage of IDSsp.mGALC in comparison to the unmodified GALC in terms of expression and secretion. The transgenic chimeric GALC enzymes reduced the intracellular galactosylceramide (GalCer) storage that is present in murine GLD neurons and glial cells. Importantly, the chimeric GALC enzymes were secreted, recaptured, and delivered to the lysosomes of GALC-deficient neural cells, which were metabolically cross-corrected. Interestingly, the expression of LDLr and LDLr-related proteins in GLD neurons and glial cells reinforces the use of this system to enhance the GALC supply in affected CNS cells and tissues. These results support the rationale of testing the safety and efficacy of these chimeric GALC enzymes in *ex vivo* and *in vivo* GT approaches in GLD animal models, with the final goal of developing novel and more effective GT strategies for this currently untreatable disease.

503. AAV-Based Gene Therapy for PFIC3 (VTX-803) Shows Further Promise with Durable Efficacy in PFIC3 Mice at Different Disease Stages

Nicholas D. Weber[1], Leticia Odriozola[2], Céline Bouquet[3], Blanche Tamarit[3], Anne Douar[3], Cristian Smerdou[2,4], Gloria González-Aseguinolaza[1,2,4]

[1]Vivet Therapeutics S.L., Pamplona, Spain,[2]Division of Gene Therapy and Regulation of Gene Expression, Cima Universidad de Navarra, Pamplona, Spain,[3]Vivet Therapeutics S.A.S., Paris, France,[4]Instituto de Investigación Sanitaria de Navarra (IdISNA), Pamplona, Spain

Gene addition based on liver-directed AAV delivery is emerging as a successful option for treating rare disorders caused by inborn genetic errors. Progressive familial intrahepatic cholestasis type 3 (PFIC3) is a rare metabolic disease caused by inherited mutations in *ABCB4*, a gene encoding for multidrug resistance protein 3 (MDR3), a membrane-bound phosphatidylcholine (PC) floppase involved in the regulation of bile composition. In the absence of fully functional MDR3, insufficient PC is transported into the bile, which results in reduced mixed micelle formation and free bile acids that cause toxicity to hepatocytes and cholangiocytes, eventually leading to cholestasis, cirrhosis and end-stage liver disease requiring transplantation. We have developed an AAV vector delivering a codon optimized MDR3 transgene under the control of a liver specific promoter (VTX-803), and tested it with an AAV8 serotype in a preclinical mouse model of PFIC3. Preliminary studies showed therapeutic efficacy when intravenously administered to two-week-old (pre-fibrotic liver) or five-week-old (fibrotic liver) *Abcb4*$^{-/-}$ mice as seen in the normalization of serum biomarkers of PFIC3, hepatosplenomegaly and liver fibrosis. Further pharmacology studies have expanded the findings and showed a therapeutic effect in older animals (16 weeks) presenting severely fibrotic/cirrhotic livers, without an increase in dose. Long-term studies have shown the vector to be safe and the effect to be durable at least up to 6 months and comparable between 5-week-old and 16-week-old mice. Additionally, a dose range finding study with reduced dose increments has allowed for the more precise establishment of the therapeutic dose in *Abcb4*$^{-/-}$ mice. Finally, it was found that the diseased state of the PFIC3 mouse liver affects transduction efficiency since wildtype mice of the same background were more efficiently transduced with the vector at equivalent doses. These results confirm the strong potential of VTX-803 as a candidate therapy for this devastating childhood disease.

504. Gene Disruption in Humanized Mice Creates a Model of Human Apolipoprotein B Deficiency

Amita Tiyaboonchai[1], Jeffrey Posey[1], Helen Chen[2], Xiping Cheng[2], Markus Grompe[1]

[1]Oregon Health and Science University, Portland, OR,[2]Regeneron Pharmaceuticals, Tarrytown, NY

Better preclinical models are needed to evaluate gene therapy treatments and gain an understanding of metabolic liver diseases. Here, we demonstrate proof-of-principle creation of a humanized mouse model of familial hypobetalipoproteinemia by disruption of the Apolipoprotein B (*ApoB*) gene in human hepatocytes. ApoB is an important structural component of very low-density lipoprotein, low-density lipoprotein (LDL) and chylomicrons. Deficiency in ApoB causes an impairment of lipid export from hepatocytes and leads to decreased levels of circulating ApoB, cholesterol and LDL. It also is a genetic driver of nonalcoholic fatty liver disease. To knock out ApoB, primary human hepatocytes were treated ex vivo with CRISPR/Cas9 ribonuclear proteins (RNPs) consisting of a combination of three guide RNAs (gRNAs) targeting human ApoB and spCas9. The RNP-treated hepatocytes and untransfected control hepatocytes were transplanted into immune deficient Fah$^{-/-}$ (FRGN) mice and allowed to repopulate. Prior to transplantation the insertion and deletion (indel) frequency in human ApoB in the ApoB RNP-treated hepatocytes was approximately 95% ± 8%. Upon repopulation blood lipid analysis was performed. Human ApoB levels were 1.4 mg/dL ± 1.5 mg/dL in group that received ApoB RNP-treated hepatocytes, significantly lower than in mice that received control hepatocytes (18.6 mg/dL ± 13.6 mg/dL), suggesting a phenotypic effect of ApoB deficiency. While there were no differences in cholesterol levels on a normal diet, there were significantly decreased levels of human LDL and human triglycerides in circulation. To determine if a loss of ApoB under the presence of a high fat diet would induce a stronger phenotype, mice were placed on a high fat diet for 9 weeks. Blood lipid analysis was performed at week 2, week 6 and week 9. At the 6-week and 9-week timepoints, compared to the control group, a significantly lower amount of circulating cholesterol was observed in the ApoB RNP-treated group, as well as decreased LDL and triglyceride levels. We hypothesized that this decrease was due to the loss of ApoB limiting cholesterol secretion from cells into circulation. This was verified by analyzing intrahepatic lipid content at terminal harvest. ApoB RNP-treated mice displayed significantly increased levels of cholesterol in hepatocytes and significantly decreased levels of triglycerides compared to the control group. At terminal harvest, human ApoB indel frequency of ApoB RNP treated mice was 97%, unchanged from pre-transplantation levels (p=0.18). Here, we have demonstrated that ex-vivo RNP treatment of human hepatocytes was highly efficient and can be used to generate knockout humanized liver mice. While we have explored the loss of ApoB in this study, in future additional humanized liver knockout mouse models can be created to study various genetic causes of dyslipidemia and other monogenic metabolic diseases.

505. New Gene Therapy Approach for the Treatment of Primary Hyperoxaluria Type 1 (PH1) Patients. Proof of Concept in a Mouse Model of PH1

Andrea Molinos-Vicente[1,2], Aida García-Torralba[1,2], Virginia Nieto-Romero[1,2], Saray Rodríguez-Díaz[3], Alessio Cantore[4,5], Juan Roberto Rodríguez-Madoz[3], Eduardo Salido[6], Jose Carlos Segovia[1,2], María García-Bravo[1,2]

[1]Biomedical Innovation Unit, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[2]Unidad Mixta de Terapias Avanzadas. Instituto de Investigación Sanitaria Fundación Jiménez. (IIS-FJD, UAM), Madrid, Spain,[3]Programa de Medicina Regenerativa, CIMA Universidad de Navarra. Instituto de Investigación Sanitaria de Navarra, IdiSNA, Pamplona, Spain,[4]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy,[5]"Vita-Salute San Raffaele" University, Milan, Italy,[6]Pathology Department, Hospital Universitario de Canarias, Universidad La Laguna. Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Tenerife, Spain

Primary Hyperoxaluria Type 1 (PH1) is a rare genetic disorder caused by alanine:glyoxylate aminotransferase deficiency, an enzyme encoded by the *AGXT* gene, which is specifically expressed in hepatic peroxisomes. These patients suffer from oxalate overproduction in liver that should be excreted by urine. High concentration of oxalate in urine produces renal damage and can result in end-stage renal disease, oxalosis and death. Thirty percent of these patients develop clinical signs in childhood. Currently, the only curative permanent treatment is double kidney and liver transplant. Therefore, new therapeutic strategies are needed. Gene therapy using integrating vectors, such as lentiviral-based ones, is a promising strategy for monogenic diseases such as PH1. Lentiviral vectors (LV), due to their integrating capacity and the low pre-existing immunity in human population, would be promising candidates for the treatment of pediatric patients. Although they are widely used for *ex vivo* approaches, it is very difficult to perform this strategy in the liver due to the low capacity of hepatocytes of maintaining their characteristics *in vitro* and its consequent low engraftment *in vivo*. For this reason, we consider an *in vivo* lentiviral gene therapy protocol as a suitable approach for the treatment of PH1 patients. A preclinical study has been conducted in a mouse model of PH1 to evaluate the therapeutic potential of this approach. Adult *Agxt1* KO mice were injected with different doses of a hepatocyte-specific LV expressing *AGXT* cDNA. Treated mice showed a significant reduction in urine oxalate overproduction, prevention of weight loss and no signs of nephrocalcinosis, all three hallmarks of PH1 disease in *Agxt1* KO model. Due to the cell-autonomous character of this disease, the percentage of corrected cells required to compensate the oxalate production is an important parameter. We are currently working on defining and optimizing the percentage of transduced cells with the therapeutic LV to get a complete reversion of the pathological phenotype. Overall, this study opens the possibility of using LV for the treatment of PH1 and lays the foundations for future therapeutic approaches.

506. Therapeutic Advantage of Combined Gene and Cell Therapy Strategies in a Murine Model of GM2 Gangliosidosis

Davide Sala[1], Francesca Ornaghi[1], Francesco Morena[2], Martina Bazzucchi[2], Manuela Valsecchi[3], Valeria Alberizzi[4], Alessandra Bolino[4], Massimo Aureli[3], Sabata Martino[2], Angela Gritti[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy,[3]Department of Medical Biotechnology and Translational Medicine, University of Milano, Milan, Italy,[4]Department of Neuroscience, IRCCS San Raffaele Scientific Institute, Milan, Italy

Sandhoff disease (SD) is a Lysosomal Storage Disease (LSD) caused by genetic deficiency of the β-N-acetylhexosaminidase (Hex) enzyme. The resulting accumulation of GM2 ganglioside preferentially in neurons has devastating effects on the central nervous system (CNS). The combination of intracerebral gene therapy and hematopoietic stem/progenitor cell (HSPC)-based approaches provides variable benefit in other LSDs. However, the impact of similar therapeutic strategies in halting SD pathology has been poorly investigated. Here, we show the therapeutic advantage of coupling neonatal intracerebral injection of lentiviral vectors (LV) coding for the functional Hex enzyme (intracerebral gene therapy, IC GT) and bone marrow transplant (BMT; performed at 2 months of age) in SD mice. In addition, we clarify the respective contribution of treatments to disease correction. We show that BMT alone delays the onset of symptoms and moderately increase the survival of SD mice (5 months, as compared to 4 months of untreated controls), counteracting neuroinflammation but failing to clear GM2 storage. In contrast, SD mice treated with the combined treatment show a significant increase of lifespan (8 months; study endpoint) and normalization of the pathological phenotype. This benefit correlates with increased Hex activity and remarkable reduction of GM2 storage in neural tissues. Our results suggest that the early enzymatic supply provided by IC GT is crucial for the proficient activity of HSPC progeny, which reduce neuroinflammation and function as a stable reservoir of functional enzyme, delaying the progression of the disease. Overall, our results provide proof of concept of efficacy and tolerability of combining LV-mediated IC GT and BMT in SD mice. We anticipate that this study will boost the development of refined gene/cell-based approaches for treating the brain disease in SD and possibly other LSDs.

507. Long-Term Expression of HMI-203: Investigational Gene Therapy Candidate for Mucopolysaccharidosis Type II (MPS II), or Hunter Syndrome

Laura Smith, Kruti Patel, Jacinthe Gingras, Alec Tzianabos, Lindsay Schulman, Victor Zhivich, Monicah Kivaa, Deiby Faulkner, Arnold Sengooba, Liana Behmoiras, Tania Seabrook, Serena Dollive, Nancy Avila, Jingyan Zhao, Yvonne White, Jenn Newman, Steve Woodcock, Sean Smith, Omar Francone, Albert Seymour

Homology Medicines Inc, Bedford, MA

Mucopolysaccharidosis Type II (MPS II), or Hunter syndrome, is a rare X-linked lysosomal storage disorder (LSD) caused by mutations in the iduronate-2-sulfatase (*IDS)* gene, resulting in loss of I2S enzyme activity leading to subsequent systemic (peripheral organs and central nervous system (CNS)) toxic lysosomal accumulation of glycosaminoglycans (GAGs), which are large polysaccharides made of repeating disaccharide units responsible for providing structure and hydration to the cell. The disease results in skeletal dysplasia, joint stiffness, hepatosplenomegaly and airway obstruction and in severe cases, neurocognitive deficits. Hunter syndrome occurs in approximately 1 in 100,000 to 1 in 170,000 males, and the severe form leads to life expectancy of 10 to 20 years. Herein, we report preclinical gene therapy data where a single intravenous (I.V.) dose of an investigational gene therapy candidate (HMI-203) delivering human *IDS* (hIDS) in the MPS II murine model resulted in significant levels of functionally active hI2S protein in the serum out to 44 weeks post-dose (study on-going). Circulating hI2S protein collected from the serum demonstrated cross-correction activity using an *in vitro* cross-correction assay. Robust *IDS* tissue expression from HMI-203 was demonstrated in the brain, liver, heart, spleen, lung and kidney when compared to age-matched vehicle-treated MPS II mice out to 24 weeks post-dose (last time point evaluated) demonstrating systemic and CNS transduction. Lastly, sustained levels of hI2S demonstrated amelioration of phenotypic symptoms associated with joint and digit deformities in the MPS II murine model starting at onset of the phenotype (between 20 and 28 weeks of age) and was maintained out to 45 weeks following administration. Based on this IND-enabling data, HMI-203 IND-enabling studies are ongoing to support the development of HMI-203 as a gene therapy for the treatment of MPS II.

508. Rapid Production of On-Demand Gene Edited Pigs and Biomedical Reporter Pigs to Accelerate IND-Enabling Studies

Jarryd M. Campbell[1], Kanut Laoharawee[2,3,4,5], Dennis A. Webster[1], Karl J. Clark[6], Adrienne L. Watson[1], Branden S. Moriarity[2,3,4,5], Daniel F. Carlson[1]

[1]Recombinetics, Inc., Eagan, MN,[2]Department of Pediatrics, University of Minnesota, Minneapolis, MN,[3]Masonic Cancer Center, University of Minnesota, Minneapolis, MN,[4]Center for Genome Engineering, University of Minnesota, Minneapolis, MN,[5]Stem Cell Institute, University of Minnesota, Minneapolis, MN,[6]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

Translating promising gene editing technologies like CRISPR-Cas and base editor from basic research tools to therapeutics that cure genetic diseases requires relevant preclinical testing to ensure the safety and efficacy. Preclinical studies in large animal models will provide critical data on dosing and safety of both the editor and the delivery vehicle prior to human use. Genetically engineered swine are beneficial as a preclinical model due to their similar size, anatomy, and physiology to humans as well as their short gestation period (<4 months), large litter size (>6). Technology to produce gene edited swine has rapidly advanced recently, and custom-engineered swine that harbor the targeted therapeutic site from humans in their genome can now be made and delivered in less than 7 months. These animals are particularly useful for late stage IND-enabling studies like dose-escalation, pharmacokinetics and pharmacodynamics analyses to streamline preclinical evaluation for gene editing-based therapies. In addition, these engineered animals will elucidate which organs, tissues, and cells will be targeted by the delivery vehicle in a large animal and allow for toxicity studies to be performed. One way of measuring the specificity of in vivo gene editing in specific organs, tissues, or cells is by using reporter lines, like those pioneered by the rodent community, including the traffic light reporter and the Ai9 reporter. We have developed a suite of biomedical research pig lines with reporters at the *ROSA26* safe-harbor locus in pigs as part of the NIH Somatic Cell Genome Editing consortium. Various lines are being developed that can detect double-strand break activity (Ai9), detect both non-homologous end joining and homology directed repair (traffic light reporter), or detect adenine base editor activity (base editor reporter). The desired editing outcome will activate a fluorescent protein, so analysis can be done on organs, tissues, and cells using immunohistochemistry or in single cells using fluorescence activated cell sorting, or by sequencing. We are also exploring the use of sodium iodide symporter (NIS) co-expression that will allow reporter activation to be detected non-invasively by PET or SPECT imaging. We anticipate these animals will be available with three-month lead time starting Q2 of 2021.

509. GLA Uptake and Metabolic Cross Correction in Fabry Disease Relevant Cell Lines: A Rationale for Liver-Directed AAV Gene Therapy

Jey Jeyakumar[1], Lawrence Tam[1], Azadeh Kia[1], Rose Sheridan[1], Amit Nathwani[2], Romuald Corbau[1]

[1]Freeline Therapeutics, Stevenage, United Kingdom,[2]University College London, London, United Kingdom

Fabry disease (FD) is an X-linked disorder caused by deleterious mutations in the *GLA* gene, which codes for the lysosomal enzyme alpha-galactosidase A (GLA). The resultant accumulation of globotriaosylceramide (Gb3) in many different cell types is thought to be responsible for the pathology observed in FD. A liver-directed adeno-associated virus (AAV)-mediated gene transfer approach may be effective in delivering therapeutic enzymes into Fabry affected cells and restoring enzyme activity. This study was conducted to determine whether hepatocyte-derived GLA enzyme (following AAV gene transfer) can be taken up by disease-relevant cells of human origin *in vitro* and lead to cellular phenotypic correction and storage reversal. Fabry patient-derived fibroblasts and endothelial cells were used as an *in vitro* disease model. CRISPR/Cas9 mediated *GLA* gene knock down cells from podocytes, kidney epithelial cells, and cardiomyocytes, were also generated, which resulted in enzyme-impaired cells similar to those found in Fabry patients. Cellular pharmacokinetics of GLA were investigated for dose, exposure time and intracellular biodistribution. Enzyme uptake studies were carried out using a trans-well co-culturing system, with a human hepatocellular carcinoma (Huh7) cell line as host cell system to be transduced with AAV for transgene overexpression and GLA secretion. Effective uptake of GLA in the recipient cells was evaluated by measuring total cellular protein, cellular enzyme activity and co-localization to lysosomes using microscopy. Following co-culturing with AAV transduced Huh7 liver cells or stable liver cells over- expressing GLA, positive uptake and elevated levels of GLA protein and enzyme activity were detected in key cell lines of Fabry disease with a significant portion targeted to lysosomal compartments. AAV transduction of Huh7 liver cells cultured apically led to a dose-dependent increase in recombinant GLA protein secretion into culture media, and exposure of co-cultured cells to the recombinant enzyme for 48 hours showed a concomitant dose-dependent increase in total cellular GLA protein. Furthermore, enzyme kinetic studies showed that the level of cellular enzyme uptake was sensitive to the level of secreted protein present in the culture media. The level of enzyme activity was also found to be significantly increased in enzyme-deficient Fabry fibroblasts and endothelial cells after co-culturing. We observed clearance of Gb3 storage in Fabry fibroblasts when examined by tandem mass spectrometry (LC-MS/MS). This research demonstrates the ability of hepatocyte-derived GLA (via AAV transduction or stable expression from HuH7 cells) to be taken up by key target cell lines of Fabry disease, and that a significant proportion of the enzyme is effectively delivered to lysosomal compartments where it exerts its degradative function. Metabolic cross correction was due to secretion of the functional enzyme from one cell line followed by intracellular uptake by the deficient cells. These results support the rationale for development of a liver-directed AAV gene therapy for the treatment of Fabry disease.

510. Tunable Delivery of Parathyroid Hormone with the Shielded Living Therapeutics™ Platform Provides a New Modality for Treatment of Hypoparathyroidism

Divya Israni[1], Suyan Li[2], Takako Moriguchi[2], Huishan Li[1], Jared Sewell[2,3], Jie Li[2], Janet Huang[2], Wilson W. Wong[1], Ahmad Khalil[1,4], Hozefa Bandukwala[2], David Peritt[2]

[1]Department of Biomedical Engineering and Biological Design Center, Boston University, Boston, MA,[2]Sigilon Therapeutics, Cambridge, MA,[3]Currently at Catamaran Bio, Inc., Cambridge, MA,[4]Wyss Institute for Biologically Inspired Engineer, Harvard University, Cambridge, MA

Hypoparathyroidism is a rare endocrine disorder with an estimated 60-115,000 patients diagnosed with chronic hypoparathyroidism in the United States each year. Hypoparathyroidism results in low or minimal parathyroid hormone (PTH) production. The most common cause of chronic hypoparathyroidism is surgical damage or removal of the parathyroid glands. Chronic hypoparathyroidism can also be caused by autoimmunity, unknown etiology, in association with underlying disorders or in rare cases, due to genetic disorder. PTH is a critical hormone for regulating calcium homeostasis. Normal plasma levels are tightly maintained in the range of 10-60 pg/mL. While replacement therapy with synthetic PTH hormone is a currently approved option for the treatment of hypoparathyroidism, a key challenge is maintaining therapeutic levels within the physiological range. Overdosing of PTH can lead to the hypercalcemia with significant adverse effects. Thus, modalities that are able to deliver sustained and tunable levels of PTH are needed. Allogeneic cell-based therapy is an attractive potential option. One of the major challenges of allogeneic cell-based therapies is protection from the host's immune system. While many biomaterials (e.g., hydrogels) can provide a physical barrier to allogeneic cells, they themselves can elicit an immune response which results in build-up of pericapsular fibrotic overgrowth (PFO). PFO effectively renders the physical shield impenetrable by the nutrients leading to cell death and making the longevity of such therapy inadequate. Sigilon utilized a library of proprietary small molecules that avoid PFO when conjugated to alginate biomaterials (Bochenek Nat Biomed Eng 2018) to create a modular, cell-based platform with potential for utilization across a range of chronic diseases. The platform consists of genetically modified allogeneic human cells engineered to produce the therapeutic protein of interest, encapsulated in a two-compartment sphere which supports the function of cells (inner compartment) and shields the cells from the host's immune system and PFO (outer layer) (Barney ASGCT 2020). We hypothesized that this platform can be used to deliver sustained and tunable delivery of human PTH. We developed a synthetic gene expression circuit which could be regulated using a small molecule inhibitor of the HCV NS3 protease. PTH production was efficiently induced from cells engineered with this circuit in a dose-regulatable manner. Reciprocally, withdrawal of the inducer led to complete attenuation of PTH production. Dose-responsive PTH production was maintained after encapsulation of engineered cells into the two-compartment spheres. Finally, we demonstrated the functionality of the encapsulated cells *in vivo* with induction of human PTH at physiological levels in recipient animals by oral administration of the small-molecule inducer. Collectively, our data demonstrate

a novel modality utilizing an innovative cell-based platform for the treatment of hypoparathyroidism which provides durable and tunable replacement of PTH, potentially overcoming limitations associated with current therapies.

511. FGF19 Gene Therapy Reduces Steatosis but Not Inflammation and Fibrosis in Two Mouse Models of Non-Alcoholic Steatohepatitis

Martin Borch Jensen, Christopher Carrico, Linda Chio, Ian Driver, Daniel Fuentes, Akela Kuwahara, Francisco LePort, Christopher Towne

Gordian Biotechnology, San Francisco, CA

Gene therapy has had recent clinical success in treating rare, monogenic disorders, through replacement of a missing or non-functional gene by adeno-associated virus (AAV)-mediated expression. Translating this success to diseases without a clear genetic cause is more difficult, however, as the optimal therapeutic payload is often unknown. To address this unmet need, we have developed a platform that allows thousands of candidate gene therapies to be screened simultaneously in rodent and large animal models of complex disease. In parallel to this effort, we have tested existing gene therapy strategies in order to set a benchmark for the novel hits from this screening platform. Non-alcoholic steatohepatitis (NASH) is a severe form of nonalcoholic fatty liver disease with hepatitis and fibrosis that can lead to cirrhosis and liver cancer. The mechanism underlying NASH is poorly understood and there are no approved treatments. Fibroblast growth factor 19 (FGF19) regulates bile acid, carbohydrate, protein, and energy homeostasis and a variant of the recombinant protein is in phase 3 clinical trials for NASH. As AAV expression of FGF19 has been reported to reduce steatosis (fat retention), inflammation and fibrosis in mouse models of NASH, we chose this gene therapy strategy to benchmark our platform. AAV serotype 8 (AAV8) expressing the FGF19 variant, M70, or green fluorescent protein (GFP) was administered intravenously to both the AMLN and FAT mouse models of NASH either before or after onset of inflammation and fibrosis. Greater than 100 ng/mL plasma FGF19 was measured by ELISA one week following delivery of the therapeutic vector; similar to what was reported in previous studies. Interestingly, expression of transgenes (FGF19 or GFP) were lower in animals with increased fibrosis. As FGF19 expression resulted in rapid weight loss in both mouse models, that was not observed in control groups injected with saline or an AAV8 expressing GFP. Plasma and tissue were analyzed two months after vector delivery. As expected, liver steatosis was markedly reduced by FGF19 in both NASH models as measured by histology. Reductions in cholesterol and glucose levels were also observed, further supporting a restoration of lipid metabolism and energy homeostasis. Liver enzyme alanine aminotransferase levels were reduced to normal levels, suggesting a reduction in liver damage. Despite these beneficial effects, however, animals treated with FGF19 had similar levels of infiltrates and collagen deposition compared to control groups as measured by histology. Whereas previous reports had observed amelioration of steatosis, inflammation and fibrosis, only reductions in steatosis were measured in the present study. One hypothesis for this discrepancy is the duration of the gene therapy, which was two months in our study versus six to eight months in other reports. This suggests that the therapeutic effects of FGF19 on inflammation and fibrosis take more than several months to be observed. An additional difference across the experiments was choice of serotype. Earlier studies reporting reductions in inflammation and fibrosis utilized AAV serotype 9, a vector with broader tropism than the AAV8 used in this experiment that has primary tropism for hepatocytes. Future studies will examine these questions and compare effects on disease amelioration with novel candidates identified by our in vivo, high throughput, screening platform.

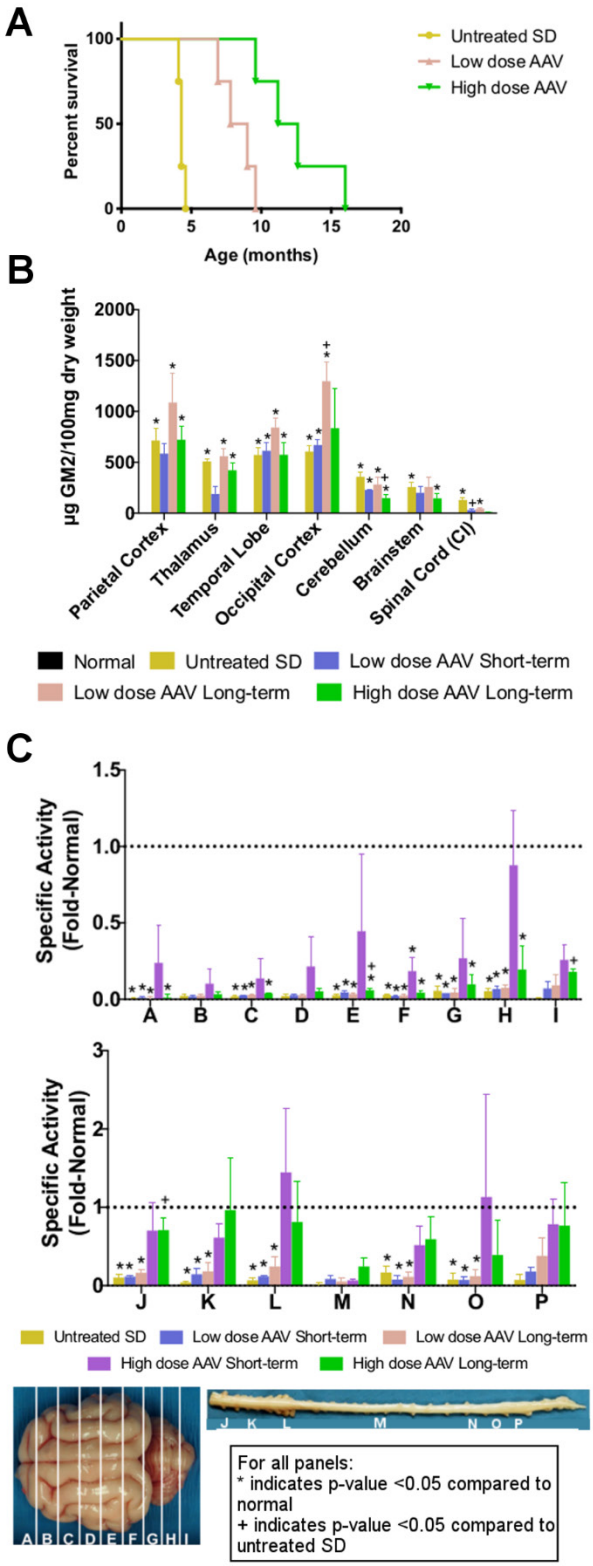
512. Intravenous AAV Gene Therapy Improves Pre- and Post-Mortem Outcomes in a Feline Model of Sandhoff Disease

Anne Maguire[1], Elise Diffie[1], Amanda Gross[1], Hannah Lahey[2], Linh Ta[3], Heather Gray-Edwards[2], Thomas Seyfried[3], Miguel Sena-Esteves[2], Douglas Martin[1]

[1]Scott-Ritchey Research Center, Auburn University, Auburn, AL,[2]Neurology, Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[3]Biology, Boston College, Chestnut Hill, MA

Sandhoff Disease (SD) is a neurodegenerative lysosomal storage disease that results in the death of children before 5 years of age. Because there are no FDA-approved therapies available, current treatment strategies are limited to palliation. SD is a form of GM2 gangliosidosis caused by the absence of ß-hexosaminidase (Hex) and subsequent accumulation of GM2 ganglioside in neuronal lysosomes. In previous studies, intracranial administration of adeno-associated viral (AAV) vector in feline models quadrupled lifespan and increased quality of life. This success, in part, led to AAV treatment of 2 children with GM2 gangliosidosis in an expanded access clinical trial, as well as recent FDA approval of a Phase I/II clinical trial. Our current study investigated the possibility of using a bicistronic AAV vector, reducing the risk of intracranial surgery, and achieving greater systemic vector distribution through the intravenous (IV) treatment of SD cats at one month of age. These cats were divided into four groups: low dose (5e13/kg) short-term (n=4), low dose long-term (n=4), high dose (2e14/kg) short-term (n=3) and high dose long-term (n=4). Animals in the short-term group were euthanized 16 weeks post-treatment, while cats in the long-term group were followed to humane endpoint (inability to stand). While untreated SD cats live to 4.3±0.2 months, cats treated with the low and high doses lived to 8.3±1.2 and 12.4±2.7 months, respectively (Fig 1A). In-life assessments revealed clear clinical benefit of AAV treatment, with the most dramatic improvement seen in the reduction of tremors, the most debilitating feature of feline SD. Treated animals experienced markedly improved quality of life, as indicated by slower weight loss and neurological decline compared to their untreated counterparts. They usually reached the humane endpoint through severe limb paresis due to spinal cord compression by soft tissue within the vertebral column. Cerebrospinal fluid levels of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were decreased, indicating a reduction of cell damage within the central nervous system. Magnetic Resonance images (MRI) and spectroscopy (MRS) acquired on a 7 Tesla scanner indicated that structural pathology and metabolite levels (such as glycerophosphocholine and phosphocholine, GPC+PCh, a marker of membrane turnover) are partially normalized by AAV treatment. Post-mortem assessments revealed dose-dependent reduction of GM2

ganglioside storage (Fig 1B) and increases in Hex activity (Fig 1C) that were more substantial in the caudal regions of the brain and in the spinal cord. These results support the dose-dependent efficacy of AAV delivered IV for partial restoration of clinical metrics and Hex function in a feline model of SD.



513. Drug-Mediated Expansion of Transplanted Hepatocytes In Vivo

Anne Vonada, Amita Tiyaboonchai, Markus Grompe
Oregon Health and Science University, Portland, OR

Hepatocyte transplantation is a promising approach for treating genetic liver disorders. However, it is limited by low rates of engraftment that are below the therapeutic threshold for many diseases. Its successes thus far have relied on creation of a transient selective advantage for transplanted cells though methods such as irradiation of native liver prior to transplantation. However, existing methods are limited in their selection potential. An ideal protocol for expansion of transplanted hepatocytes would allow repeated cycles of selection until a therapeutic threshold of donor cells was achieved. We have previously described a method to selectively expand hepatocytes in vivo by targeting cytochrome P450 reductase (*Cypor*) using gene therapy. Cypor is an obligate cofactor for the metabolism of the drug acetaminophen (APAP) to its hepatotoxic byproduct, NAPQI. Therefore, Cypor deficient-hepatocytes are protected from APAP-induced hepatotoxicity and can be selectively expanded by treatment with high doses of APAP. This method can be used to repopulate up to 40% of the mouse liver from starting rates of less than 1%, and subsequently maintain stable repopulation in the absence of further selective pressure. Utilizing lentiviral vectors expressing a therapeutic transgene and a selectable shRNA or gRNA targeting *Cypor*, transgene expression can be increased by over 100-fold and can correct a mouse model of the metabolic disease phenylketonuria. Here, we report that, in addition to selection of hepatocytes targeted in vivo, this method can also be applied to achieve positive selection of transplanted hepatocytes. Mouse hepatocytes were transfected ex vivo with spCas9-containing ribonucleoprotein complexes targeting *Cypor* and transplanted into congenic wild type mice. Mice were then treated with biweekly APAP injections for 10 weeks. Examination of liver sections after partial hepatectomy revealed clonal expansion of Cypor-deficient hepatocytes characteristic of APAP selection, indicating successful in vivo expansion of transplanted hepatocytes. Clonal expansion was not seen in control animals that were transplanted but not treated with acetaminophen. In addition to selection of transplanted mouse hepatocytes, we are also developing protocols to allow in vivo selection of transplanted Cypor-deficient human hepatocytes using the FRGN mouse model. This mouse can be repopulated with human hepatocytes. Use of this model confirmed that human hepatocytes have a greatly increased resistance to APAP-induced hepatotoxicity as compared to mouse hepatocytes. As an alternative to the prohibitively high dose of APAP that is required to induce hepatotoxicity in human hepatocytes, the use of a glutathione synthesis inhibitor, buthionine sulfoximine (BSO), in conjunction APAP treatment was tested. The combination of BSO and APAP treatment resulted in hepatotoxicity of human hepatocytes. We are applying this method to allow selective expansion of Cypor-deficient transplanted human hepatocytes in vivo. If successful, this method could represent a strategy for allowing therapeutic use of hepatocyte transplantation for a variety of metabolic disorders.

514. Addition of Histone Deacetylase Inhibitor in Human Cell Cultures Generated by Gene Therapy and Synthetic Biology Approaches Inhibits the Secretion of Lysosomal Enzyme

Lílian L. S. Figueiredo[1], Cláudia E. V. Wiezel[1], Daniela P. C. Tirapelli[2], Vania D'Almeida[3], Velia Siciliano[4], Ron Weiss[5], Kuruvilla J. Abraham[6], Stanton L. Gerson[7], Aparecida M. Fontes[1]

[1]Genetics, University of São Paulo, Ribeirão Preto School of Medicine, Ribeirão Preto, Brazil,[2]Surgery and Anatomy, University of São Paulo, Ribeirão Preto School of Medicine, Ribeirão Preto, Brazil,[3]School of Medicine, Federal University of São Paulo, SP, Brazil, São Paulo, Brazil,[4]Center for Advanced Biomaterials for Healthcare, CRIB, Istituto Italiano di Tecnologia, Napoli, Italy,[5]Biological Engineering, Massachusetts Institute of Technology, Synthetic Biology Center, Boston, MA,[6]Economics, University of São Paulo, School of Economics, Business Administration and Accounting at Ribeirão Preto, Ribeirão Preto, Brazil,[7]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH

Enzyme replacement therapy is considered standards of care for patients with type 1 Gaucher disease (GD) and has served as model for the treatment of other lysosomal storage diseases. Here, we investigate whether gene therapy can be combined with synthetic biology and epigenetic approaches for the production and secretion of recombinant enzyme. Previously, synthetic biology approaches and gateway cloning technology were used to generate a lentiviral vector encoding the cDNA with optimized codons of human enzyme β-glucosylceramidase (GCase). This lentiviral vector was used to generate a human transgenic cell line with stable production of GCase. After characterization of individual clones we obtained a cell clone L17_293FT_C15 with highest production of GCase. To investigate an additional strategy for modulating final enzyme production in human cell line systems the L17_293FT_C15 cells were treated with inhibitor of histone deacetylases (HDAC) to block the activity of HDAC enzymes. The inhibtor used was suberanilohydroxamic acid (SAHA) and this drug was tested using a concentration of 2 μM at three different times (8, 16 and 48 h). First, the effect of SAHA on L17_293FT_C15 cell cultures was screened by assessing the average doubling time in untreated cultures and treated by SAHA during 48h. The L17_293FT_C15 transgenic cells grew with similar doubling time of 19.67 + 1.78 h and 19.18 + 1.25 h before or after SAHA treatment ($p = 0.4$). For 293FT virgin cells these value were 20.01 + 1.35 h and 23.78 + 2.32 h and statistically significantly ($p = 0.0089$). To evaluate the amount of lysosomal GCase secreted by these cell populations we measured the amount of fluorescent substrate that is hydrolyzed/h/mL. We observed that L17_293FT_C15 transgenic cells secrete 200.47 (+ 26.13) and 131.53 (+ 14.78) nmol substrate hydrolyzed/ mL/ h after 48 h, without or with SAHA treatment, respectively ($p = 0.0005$). For 293FT virgin cells these values were 4.18 + 0.89 and 1.86 + 0.3 nmol/ mL/h, respectively ($p = 0.0008$). We also screened the cell lysates for GCase specific activity. We observed that L17_293FT_C15 cells produced similar amounts of 601.81 (+ 54.52) and 632.20 (+ 92.41) nmol substrate hydrolyzed/ mg protein/ h after 48 h, in untreated and treated respectively. For 293FT virgin cells these value were 81.57 + 0.89 and 95.37 + 0.3 nmol/mg/h, respectively ($p = 0.014$). These effects were not observed with shorter SAHA treatment times. In conclusion, SAHA toxic effects are not observed in the proliferative potential of transgenic cell lines, but are present in 293FT virgin cells. Regarding the GCase specific activity, SAHA induces the intracellular enzyme production in 293FT virgin cells but is not modulated in transgenic cell line. For secreted enzyme we observed that SAHA inhibits the secretion for both virgin and transgenic cell lines.

515. Development of an AAV-GBA Gene Therapy for the Treatment of Type 1 Gaucher Disease

Ting-Wen Cheng[1], David Souza[1], Ricardos Tabet[1], Katherine Wright[2], Joanne Brodfuehrer[2], Lisa Keyes[3], Sheila Rao-Dayton[3], Patricia Lewis[3], Annette Sievers[2], Douglas M. McCarty[3], Suryanarayan Somanathan[1], Clark Pan[1], Robert D. Bell[1]

[1]Rare Disease Research Unit, Pfizer Inc, Cambridge, MA,[2]BioMedicine Design, Pfizer Inc, Cambridge, MA,[3]Rare Disease Research Unit, Pfizer Inc, Morrisville, NC

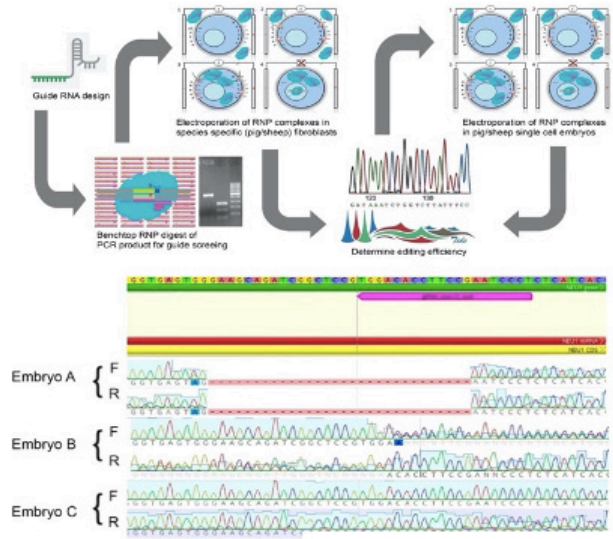
Gaucher Disease (GD) is an autosomal recessive disorder caused by mutations in GBA resulting in the deficiency of glucocerebrosidase (GCase). Loss of GCase results in lipid-filled macrophages that accumulate in bone marrow, spleen, liver and other visceral organs. GD is classified into a non-neuropathic form (Type 1) which represents ~90% of GD patients and neuropathic forms (Type 2 and 3). While life-long and bi-weekly intravenous enzyme replacement therapy is the current standard of care for Type 1 GD emerging gene therapy strategies have the potential to provide a one-time treatment option and address the unmet medical needs in the neuropathic forms of the disease. Here we describe the development of an optimized liver directed AAV gene therapy candidate for the treatment of Type 1 GD. First, we screened multiple transgene cassette designs containing various regulatory elements and GCase variants in vitro and in vivo to identify an optimal construct for persistent expression, secretion into circulation and cross-correction uptake of the GCase enzyme in relevant target cells and organs. In addition to measuring GCase enzyme activity using the standard artificial substrate 4-MUG assay, we have developed a novel immuno-affinity LC-MS/MS method to enable the specific measurement of human GCase protein levels in rodents and non-human primates. Next, we confirm AAV-GBA treatment in the D409V mouse model of GD results in the lowering of serum, liver, and spleen sphingolipids to levels comparable to wild-type healthy animals. Finally, we demonstrate the successful scale-up, in-depth molecular characterization and testing of AAV vectors in translational non-human primate studies. In summary, the data reported here confirms liver-directed AAV-GBA gene therapy, cross-correction of GCase activity, and therapeutic lowering of tissue sphingolipid levels has the potential to be developed as a treatment option for Type 1 GD.

516. Sialidosis: From Gene Editing to Gene Therapy

Jillian Gallagher[1], Judith Gallant[2], Ping Xu[2], Rachael Gately[3], Stephanie Bertrand[4], Kevin Luk[5], Stacy Maitland[5], Camilo Toro[6], Alessandra d'Azzo[7], Cynthia Tifft[6], Scot Wolfe[5], Jaime Rivera[2], Miguel Sena-Esteves[8], Heather Gray-Edwards[9]

[1]Horae Gene Therapy Center & Graduate School of Biomedical Sciences, Neuroscience Program, University of Massachusetts Medical School, Worcester, MA,[2]Pediatrics, University of Massachusetts Medical School, Worcester, MA,[3]Cummings School of Veterinary Medicine at Tufts University, North Grafton, MA,[4]Cummings School of Veterinary Medicine at Tufts University, University of Massachusetts Medical School, North Grafton, MA,[5]Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA,[6]Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD,[7]Department of Genetics, St. Jude Children's Research Hospital, Memphis, TN,[8]Neurology & Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[9]Horae Gene Therapy Center & Radiology, University of Massachusetts Medical School, Worcester, MA

Sialidosis is a rare lysosomal storage disease caused by mutations in the *NEU1* gene that encodes the lysosomal sialidase neuraminidase 1 (NEU1), essential for the catabolism of sialylated glycoproteins in lysosomes. Deficiency in NEU1 causes accumulation of sialylated glycopeptides and oligosaccharides in tissues throughout the body that results in loss of cell and tissue homeostasis and cell death. Type I sialidosis, is the less severe form of the disease with onset in the first or second decade of life with symptoms such as, myoclonus, seizures, muscle twitches and cognitive dysfunctions. Ultimately, type I sialidosis patients become unable to regulate normal bodily functions. Type II is the most severe form and is categorized by congenital or infantile onset that includes facial and bone deformities, hepatosplenomegaly, gingival hyperplasia, hearing loss and severe developmental delay. Those with the congenital form are stillborn, or die within a few years of life, whereas infantile onset patients can live through early childhood or adolescence. There is no effective treatment for sialidosis. We have taken a two-step approach to develop gene therapy for sialidosis. We have developed AAV vectors encoding NEU1, either native or with modifications to enhance secretion. We plan to treat sialidosis mice and WT controls using intravenous or CSF delivery to determine the most efficacious vector design, route and dose. Next, we will test the optimized vectors in a large animal model of sialidosis to further refine dose and evaluate safety and efficacy. As no large animal model of sialidosis currently exists we plan to generate a genetically engineered sheep using CRISPR-Cas9 tehnology. We have optimized our gene editing strategy through electroporation of one cell sheep embryos with Cas9 and RNA guides from exon 2 and exon 5. PCR, Sanger sequencing, and TIDE analysis were used to determine editing rates of electroporated one cell embryos. We have demonstrated 80% editing efficiency in sheep embryos targeting exon 5 of *NEU1*. These edited embryos have been implanted in donor recipient ewes that will be due to give birth in February 2021. We plan to characterize these sheep and select desirable mutations and/or a phenotypes to establish a breeding colony. Efficacy studies in sialidosis sheep in parallel with natural history studies in sialidosis patients will inform future clinical trials in partnership with St. Jude Children's Research Hospital and the National Institutes of Health.



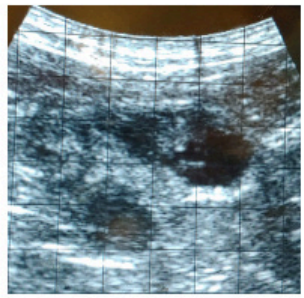


Figure 1. *(Top)* Pipeline for guide RNA selection, validation and editing of embryos. *(Middle)* Sanger sequencing showing CRISPR editing of the *NEU1* gene. Embryo A has a deletion whereas embryo B has a frameshift mutation, Embryo C is a control. *(Bottom)* ultrasound of edited sheep embryos at 4 weeks post implantation.

517. Neonatal AAV Gene Therapy in a Viable Mouse Model of Combined Methylmalonic Acidemia and Homocystinuria, *cblC* Type, Is Comparable to Chronic Hydroxocobalamin Administration

Stefanos A. Koutsoukos[1], Jennifer L. Sloan[1], Kelsey Murphy[1], Madeline Arnold[1], Nathan Achilly[1], Gene Elliot[1], Patricia Zerfas[2], Victoria Hoffmann[2], Charles P. Venditti[1]

[1]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD,[2]Office of the Director, National Institutes of Health, Bethesda, MD

Combined methylmalonic acidemia and homocystinuria, *cblC* type (*cblC*) is the most common inborn error of intracellular cobalamin (vitamin B_{12}) metabolism caused by pathogenic variants in *MMACHC*. The protein encoded by *MMACHC* is responsible for the transport and processing of vitamin B_{12}. Early onset disease can be severe with symptoms including growth failure, anemia, heart defects, neurocognitive impairment, and progressive vision loss. In order to further characterize manifestations of the disease and investigate therapeutic modalities, we generated *Mmachc* mutant alleles [c.163_164delAC p.Pro56Cysfs*4 (del2) and c.162_164delCAC

p.Ser54_Thr55delinsArg (del3)] and studied the resulting homozygous mutants. We observed significant neonatal lethality with decreased number of homozygous mutant pups at birth and median survival of five days (*Mmachc*$^{del2/del2}$ N = 14; P < 0.0001, *Mmachc*$^{del3/del3}$ N = 66; P < 0.0001). Embryos were present in Mendelian ratios at E18.5, but were small compared to littermates and pathology in *Mmachc*$^{del2/del2}$ mutants revealed decreased brown fat, decreased liver glycogen stores, a thin skin with less prominent surface keratin, and decreased bone mineralization. At two weeks, surviving *Mmachc*$^{del3/del3}$ mutant mice weighed 36% less than littermate controls (N = 12; P < 0.0001) and displayed biochemical features characteristic of *cblC* including significantly elevated plasma methylmalonic acid (MMA) and homocysteine (HCYS) and decreased methionine (MET). Pathological examination at one month revealed hydrocephalusand hypoplasia of the corpus callosum in *Mmachc*$^{del3/del3}$ mutants. To better inform relevant tissues to target with AAV gene therapy, we performed RNAScope analysis in wildtype mice which revealed expression of *Mmachc* in several pathologically relevant tissues. To target multiple tissues, we designed two AAV gene therapy vectors with constitutive promoters rAAVrh10-CBA-*Mmachc* (rAAVrh10) and rAAV9-CBA-co*MMACHC* (rAAV9). The AAVs (dose = 1 x 10^{11} GC/pup) were delivered by a single intrahepatic injection on the first or second day of life and compared to weekly hydroxocobalamin (OHCbl) administration. Survival to one year was observed in *Mmachc*$^{del3/del3}$ mutant mice treated with rAAVrh10 (N = 9; P < 0.0001), rAAV9 (N = 11; P < 0.0044), prenatal and chronic OHCbl (N = 16; P < 0.0001), and prenatal OHCbl and rAAV9 (N = 11; P < 0.0001). Long term weights between the different treatment groups were comparable and MMA was greatly reduced six to thirteen months post treatment with prenatal OHCbl and rAAVrh10 (*Mmachc*$^{del3/del3}$ 159.7 ±7.478 μM; N = 3) and prenatal OHCbl and rAAV9 treatments (*Mmachc*$^{del3/del3}$ 136.9 ± 47.52 μM; N = 3), but not with prenatal and chronic OHCbl (*Mmachc*$^{del3/del3}$ 1517 ± 515.1 μM; N = 4). Pathological examination of *Mmachc*$^{del3/del3}$ mutant mice treated with rAAV9, one year after vector administration, established a mitigation of neuropathology. These *Mmachc* mouse models recapitulate key phenotypic and metabolic disease manifestations common among patients living with *cblC*. A single intrahepatic injection of AAV gene therapy, expressing either the mouse *Mmachc* or human *MMACHC* gene, provides a superior clinical and biochemical response as compared to chronic OHCbl administration. Our results demonstrate that AAV gene therapy could be a promising therapeutic modality for individuals living with *cblC*, the most common disorder of intracellular vitamin B_{12} metabolism.

518. Post-Symptomatic Reversal of Muscle Pathology in a Model of Pompe Disease Using Gene Therapy

Ali Ramezani[1], Juliette Hordeaux[1], Steven Tuske[2], Chunjuan Song[1], Nickita Mehta[2], Patrick Tsai[2], Jill Weimer[2], Meardey So[1], Peter Bell[1], Hung Do[2], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]Amicus Therapeutics, Inc. Gene Therapy Center of Excellence, Philadelphia, PA

Background: Pompe disease is a lysosomal disease where mutations in the acid α-glucosidase (GAA) gene cause GAA enzyme deficiency and the accumulation of glycogen in multiple tissues, leading to a neuromuscular disorder characterized by severe weakness and respiratory failure. Enzyme replacement therapy (ERT) is currently the only approved treatment for patients with Pompe disease. Although ERT significantly improves survival in patients with classic infantile Pompe disease, this treatment cannot fully reverse the skeletal muscle pathology, in part due to autophagic buildup, which inhibits the enzyme from reaching the lysosome. We believe that adeno-associated viral (AAV) vector-mediated gene therapy and cross correction through GAA engineering may improve treatment outcomes and reverse the muscle pathology in older patients that are in the advanced stages of the disease. **Objective:** We have previously reported the therapeutic effect of an engineered human acid α-glucosidase gene therapy AAV vector using different routes of administration among aged mice that are in the advanced stages of Pompe disease. Here we aim to further characterize the efficacy of this approach to reverse several features of muscle pathology. **Method:** Seven-month-old Pompe mice (GAA6neo) with advanced disease were administered an optimized gene therapy vector using a pan-tropic AAV capsid carrying a human GAA transgene engineered to improve both secretion and uptake of the enzyme. We tested two dose levels [low dose (LD) or high dose (HD)] of the vector using either intravenous (IV), intracerebroventricular (ICV), or dual routes of administration. Phosphate-buffered saline (PBS)-treated Pompe and wild type mice served as controls. Seven months after administering the injections, we analyzed skeletal muscles for the presence of structural abnormalities such as fiber atrophy, anisocytosis, autophagic buildup, and central nucleation. **Results:** Compared to the Pompe PBS cohort, there were significant increases in the percentage of large muscle fibers in IV HD and ICV+IV HD treated groups, and a significant decrease in the percentage of small muscle fibers in ICV+IV HD treated group. Another structural abnormality in Pompe disease is autophagic build-up in type II fibers. Our findings demonstrate that autophagosome accumulation was completely resolved in aged Pompe mice with pre-existing pathology at treatment, with a significant decrease in the percentages of LC3b+ vacuoles quantified in the quadriceps muscle fibers in IV HD and ICV+IV HD treated groups. **Conclusion:** Our optimized gene therapy candidate reversed pre-existing muscle fiber pathology, including findings that are typically treatment-resistant such as the muscle fiber size and autophagic build-up. The results support the pursuit of AAV gene therapy as a promising clinical approach for treating patients with Pompe disease.

519. Efficacy of Adeno-Associated Viruses Expressing an Engineered Alpha-Galactosidase A Transgene in a Mouse Model of Fabry Disease

Chunjuan Song[1], Juliette Hordeaux[1], Tobias Willer[2], Pai-Chi Tsai[2], Daniel Ellsworth[2], Jean-Pierre Louboutin[1], Peter Bell[1], Hongwei Yu[1], Su Xu[2], Helen Eisenach[2], Jinsong Shen[2], Yanchun Li[1], Ali Ramezani[1], Ting Yu[1], Quyen Q. Hoang[3], Russell Gotschall[2], Jill Weimer[2], Hung Do[2], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]Amicus Therapeutics Inc., Philadelphia, PA,[3]3Department of Biochemistry and Molecular Biology; The Stark Neurosciences Institute, Department of Neurology, Indiana University School of Medicine, Indianapolis, IN

Fabry disease is a rare, X-linked lysosomal storage disorder caused by mutations in the *GLA* gene, which encodes a lysosomal hydrolase: α-galactosidase A (α-Gal A). Deficiency of α-Gal A results in the progressive lysosomal accumulation of globotriaosylceramide (Gb3) or related glycosphingolipids in a variety of tissues. The primary treatment options for patients with Fabry disease consist of 1) regular infusions of recombinant human α-Gal A, termed enzyme replacement therapy (ERT); and 2) the oral pharmacologic chaperone, migalastat. However, migalastat is only used in patients with certain amenable mutations and ERT requires bi-weekly injections and has limited tissue penetration and poor biodistribution. As an alternative, gene therapy has the potential to achieve higher and steadier levels of enzymes in disease-relevant tissues and blood by using a pan-tropic adeno-associated virus (AAV) capsid and a ubiquitous promoter with high activity in key target organs. Human α-Gal A has low physical stability and a short circulating half-life at neutral pH in the blood, which may limit the bystander effect that is achievable with secretion-uptake of enzymes. Cross-correction is desirable in some target organs that are less transduced by AAV, such as kidney or blood vessel walls. We hypothesized that a stabilized human α-Gal A produced *in vivo* through gene therapy would provide a larger window of time for the enzyme to be taken up into the target tissues. By stabilizing the homodimer, several engineered human GLAs (hGLAs) showed improved enzyme stability and half-life under plasma-like neutral pH conditions as well as in the lysosome acidic environment after cellular uptake *in vitro*. Top engineered hGLA candidates, as well as the wild type, were administered to *GLA* knockout (*GLA KO*) mice, a model of Fabry disease, via AAV-mediated gene delivery. We demonstrated that AAV constructs expressing wild-type hGLA or stabilized versions of *hGLA* increased expression and activity of human α-Gal A in the plasma and a variety of tissues in *GLA KO* mice and decreased levels of Gb3 storage after intravenous administration. Importantly, an engineered candidate demonstrated superior clearance of storage in the kidney, heart, and dorsal root ganglia sensory neurons at low doses that were comparatively suboptimal to the wild type version. Collectively, our data show that AAV-mediated systemic gene transfer of stabilized hGLA is more efficient at low doses to correct the kidney, an organ that is traditionally resistant to AAV transduction. We provide the first of its kind proof-of-concept for an enhanced AAV-based gene therapy at low doses of vector, suggesting a safe and translational approach for Fabry disease.

520. rAAV-Mediated Gene Therapy in Combination with Short-Term Nitrogen-Scavenger Treatment Corrects Biochemical and Behavioral Abnormalities and Increases Lifespan in Infant Citrullinemia Type 1 (CTLN-1) Mice

Andrea Bazo[1], Aquilino Lantero[2], Itsaso Mauleón[1], Leire Neri[2], Johannes Häberle[3], Bernard Bénichou[4], Jean-Philippe Combal[4], Gloria Gonzalez-Aseguinolaza[2], Rafael Aldabe[1]

[1]Gene Therapy and Regulation of Gene Expression, CIMA Universidad de Navarra, Pamplona, Spain,[2]Vivet Therapeutics, Vivet Therapeutics SL, Pamplona, Spain,[3]Division of Metabolism and Children's Research Centre (CRC), University Children's Hospital Zurich, Zurich, Switzerland,[4]Vivet Therapeutics, Vivet Therapeutics SAS, Paris, France

Citrullinemia type I (CTLN-1) is a rare autosomal recessive genetic disorder caused by mutations in the *ASS1* gene, which encodes Argininosuccinate Synthase 1 (ASS1) that catalyzes the synthesis of argininosuccinate in the third reaction of the urea cycle. CTLN-1 patients suffer from poor processing and elimination of nitrogen excess in the liver, leading to a toxic accumulation of circulating ammonia and urea cycle byproducts that causes metabolic encephalopathy and death at a very young age. Standard of care (SOC) management of CTLN-1 consists of daily nitrogen-scavenger administration to reduce plasma ammonia concentration and a very restrictive lifelong low-protein, high-calorie diet. Liver transplantation is currently the only curative treatment for CTLN-1; therefore, early and permanent restoration of hepatic ASS1 expression based on recombinant adeno-associated virus (rAAV) gene therapy represents an attractive prospect for alternative treatment. Vivet Therapeutics is developing VTX-804, a rAAV vector expressing the human ASS1 enzyme under the control of a liver-specific promoter. The long-term therapeutic efficacy of VTX-804 (6 months post-vector administration) was evaluated in 3-week-old CTLN-1 mice ($Ass1^{fold/fold}$) treated or untreated from birth to weaning with nitrogen-scavenger agents as per SOC in patients. Untreated CTLN-1 mice had a low survival rate with only 20 % of the animals surviving to 7 months. In contrast, survival was 100 % in CTLN-1 mice who received SOC and VTX-804, and 90 % in mice receiving VTX-804 alone. Accordingly, combination of SOC and VTX-804 normalized body weight gain whereas VTX-804 alone improved but did not normalize body weight gain. In addition, the ammonia levels in mice treated with VTX-804 in combination with SOC showed normal values up to 6 months after treatment, while in mice receiving VTX-804 alone, ammonia levels normalized only transiently, with a re-increase at 6 months post-treatment. Similar to CTLN-1 patients; CTLN-1 mice showed several behavioral abnormalities such as anxiety (open field test) and reduced welfare and innate behaviors (marble burying and nest building tests). All these behavioral alterations were notably improved when CTLN-1 mice were treated with VTX-804, and even more with the SOC in combination. SOC pre-treatment improved rAAV-transduction and *hASS1* transgene expression at 3 and 6 months post-VTX-804 injection. Altogether, our data show that a single dose of VTX-804 administered intravenously to infantile CTLN-1 mice in combination with SOC normalized lifespan and corrected biochemical and behavioral abnormalities up to 6 months

after vector administration (while this was only partially achieved with VTX-804 alone). Therefore, combination of the SOC prior to gene therapy administration represents a very promising therapeutic strategy for very young CTLN-1 patients.

521. A Non-Invasive Bedside Breath Test (BreathID®) for Real Time Monitoring of Liver-Targeted Gene Therapy in Methylmalonic and Propionic Acidemias

Samantha McCoy, Alexandra Pass, Oleg Shchelochkov, Jennifer Sloan, Susan Ferry, Carol Van Ryzin, Irini Manoli, Charles Venditti

National Human Genome Research Institute, NIH, Bethesda, MD

Methylmalonic acidemia and propionic acidemia (MMA and PA) are related inborn errors of metabolism caused by pathogenic variants in genes vital to propionyl-CoA metabolism. Both disorders have high rates of mortality in infancy and childhood, and patients face frequent episodes of metabolic ketoacidosis and hyperammonemia, growth failure, chronic renal disease, intellectual impairment, cardiac manifestations, and other long-term complications. Liver transplantation, the only existing treatment to improve metabolic stability in metabolically fragile patients, is offered at increasingly younger ages to decrease morbidity and mortality and improve quality of life outcomes. However, transplantation is neither curative nor harmless: it is limited by organ availability and poses risks of surgical complications, graft rejection, and lifelong immunosuppression. There are preclinical data indicating that liver-directed genomic therapies in MMA and PA mouse models are highly effective and result in improved survival, weight gain, and reduced circulating disease biomarkers. Additionally, in MMA mouse models, 1-^{13}C-propionate oxidation measured by Isotope Ratio Mass Spectrometry (IRMS) showed robust increases following liver-targeted gene therapy. We translated these studies to humans in MMA and PA patient cohorts under NIH natural history protocols (Clinical Trials IDs: NCT00078078 and NCT02890342) to determine if this could be used as a biomarker of hepatic 1-^{13}C-propionate oxidation. To do so, we used the EasySampler™ Breath Test Kit by Quintron, which requires serial breath sample collection in vacutainers which are shipped to a reference laboratory to measure enrichment through IRMS. Mildly affected participants had comparable levels of 1-^{13}C-propionate oxidation to healthy controls, while severely affected individuals had markedly decreased levels of oxidation. These levels were restored to control range in participants who had undergone liver transplantation (LT). Next, we used IRMS as the standard for comparison to a new breath test method, the BreathID® Exalenz device, which is already cleared by FDA for the diagnosis of H. pylori infection when used with ^{13}C-urea. The machine collects breath by a nasal cannula every four minutes and uses Molecular Correlation Spectrometry (MCS) to calculate Delta Over Baseline ($^{13}CO_2/^{12}CO_2$) in real time. The portability of BreathID® allows it to easily integrate into most clinical settings, and the method used is less labor-intensive than that of IRMS. Nine MMA patients (5 male, 4 female; 2 post-LT; 3.5-18 years) and 13 PA patients (6 male, 7 female; 2 post-LT; 6-36 years) underwent both breath tests simultaneously. The two methods correlated significantly for 1-^{13}C-propionate oxidation at 120 minutes in both the MMA (r=0.996, P<0.0001) and PA (r=0.991, P<0.0001) cohorts. Moreover, the BreathID® method allowed measurement of additional patients who were unable to cooperate with the EasySampler™ method, either due to age or intellectual impairment. In aggregate, our results suggest BreathID® i) will be a convenient tool to identify severely affected and younger patients who will benefit most from early therapeutic intervention, and ii) could, in the setting of an mRNA or gene therapy trial, be used to document improvement in enzyme activity, to monitor kinetics during dose escalations and possible losses during immunosurveillance, and to evaluate long-term durability.

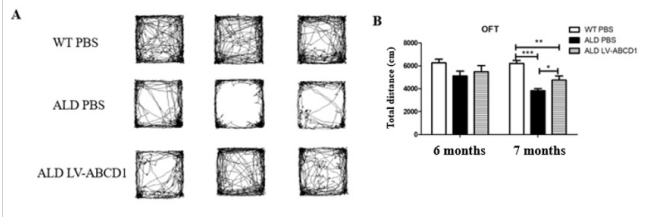
522. Intracerebral Lentiviral Gene Therapy to Improve Neurological Phenotype in a New Mouse Model of X-Linked Adrenoleukodystrophy (ALD)

Jie Gong[1], Liu Xu[1], Yunyun Liu[1], Tsai-Hua Chung[1,2], Lung-Ji Chang[1,2]

[1]Medicine, UESTC, Chengdu, China,[2]Shenzhen Geno-Immune Medical Institute, Shenzhen, China

Introduction: X-linked adrenoleukodystrophy (ALD) is an X chromosome-linked metabolic disease associated with deficiency in the ABCD1 gene. Due to metabolic disturbance of very long chain fatty acids (VLCFA) that accumulates in blood, brain white matter and adrenal cortex and other organs and tissues, the excess of VLCFA results in demyelination of central nervous system (CNS) and adrenal cortical atrophy or dysplasia. Gene therapy is a promising treatment for ALD. Due to the main lesion located in the CNS, direct intracerebral injection of lentiviral (LV) *ABCD1* gene could potentially repair the deficiency. **Methods and Results:** We have previously demonstrated the feasibility and safety of multiple intracerebral LV-*ABCD1* injections in wild type (WT) mice. To investigate the potential for treating the disease, we established a new X-ALD knockout (KO) mouse model and evaluated phenotype correction after intracerebral LV injections. Previous ALD KO mouse models deleted either *ABCD1* exon 1 or 2, which do not show any deficit in motor activities until 20 months of age, quite a deviation from the human diease. In the new model, we deleted the entire exon 3 to 9 of *ABCD1* gene by CRISPR/Cas9 gene editing in the C57BL/6N mouse strain (dl3/9*ABCD1* KO). We injected 4-month-old dl3/9*ABCD1* KO mice in four cerebral sites with LV-*ABCD1* at 1ul of 1.1x10^9 TU/ml per site (treated group, N=5), and included saline injection controls of WT and dl3/9*ABCD1* KO mice. The *in vivo*expression of *ABCD1* was quantitatively determined by direct immunohistochemistry staining after 15 days. Confocal microscopy images illustrated *ABCD1* expression surrounding the injected sites, which was not detected in the control mice. The dl3/9*ABCD1*KO mice showed behavioral deficit in the Open-Field Test (OFT) at six months of age; they spent more time at the border of the field avoiding the central zone of the arena, indicating the existence of anxiety-like behavior (Figure A). Subsequently at seven months, symptoms of reduced locomotor activity appeared in the dl3/9*ABCD1* KO mice, showing significant total distance reduction compared with WT mice (*P < 0.05; **P < 0.01; ***P< 0.001, Figure B). The impaired motor ability of the dl3/9*ABCD1* KO mice was alleviated in the treated group three months after LV-*ABCD1* injection (4,752±741cm), displaying more activities in OFT compared with untreated group (3,821±410cm, P<0.05), whereas

not as fully active as WT mice (6,217±577cm, P<0.01). The direct behavioral tests supported the successful establishment of an early ALD mouse model and demonstrated neurological improvement after LV-*ABCD1* injections. **Conclusion:** We have established a new X-ALD mouse model with early onset of disease phenotype. The intracerebral injection of LV-*ABCD1* demonstrated expression of *ABCD1* with partially corrected behavioral phenotype in the dl3/9*ABCD1* KO mice.



Cardiovascular and Pulmonary Diseases

523. Reversal of Cardiac Hypertrophy with an Optimized MYBPC3 Gene Therapy

Laura Lombardi, Amara Greer-Short, Elena C. Leon, Tawny Neal Qureshi, Anna Greenwood, Christopher A. Reid, Jin Yang, Nathaniel Hunsdorfer, Samir Elmojahid, Mohammad Mandegar, Jaclyn Ho, Stephanie Steltzer, Marie Cho, Chris Alleyne-Levy, Jun Liu, Samantha Jones, Terry Vargas, Charles Feathers, Tae Won Chung, Neshel Getuiza, Frank Jing, Bill Prince, JianMin Lin, Kathryn N. Ivey, Timothy Hoey, Whittemore G. Tingley

Tenaya Therapeutics, South San Francisco, CA

Cardiomyopathy is the number-one cause of sudden cardiac arrest in children under 18. Hypertrophic cardiomyopathy (HCM) affects 0.5 million Americans, potentially resulting in heart failure or sudden death. Loss-of-function mutations in *Myosin Binding Protein C3*, *MYBPC3*, are the most common genetic cause of HCM. The majority of *MYBPC3* mutations causative for HCM result in truncations, via nonsense, frameshift or splice-site mutations. The sarcomeric pathophysiology of the majority of HCM patients with *MYBPC3* mutations appears to be due to haploinsufficiency, as the total amount of MYBPC3 protein incorporated into sarcomeres falls significantly below normal. Decreased sarcomeric levels of MYBPC3 result in decreased myosin inhibition with more myosin heads engaged on the actin filament, resulting in hypercontractility. The clearest path to the treatment of haploinsufficiency is the restoration of the insufficient gene product; in this case wild-type MYBPC3. Thus, we have successfully engineered an AAV vector (TN-201) with superior properties for selective restoration of MYBPC3 to cardiomyocytes upon systemic delivery. Critically, we have demonstrated for the first time with AAV the ability of both a mouse surrogate and TN-201, which encodes the human gene, to reverse cardiac dysfunction and hypertrophy in a symptomatic murine model of disease. Dose-ranging efficacy studies exhibited restoration of wild-type MYBPC3 protein levels and saturation of cardiac improvement at the clinically relevant dose of 3E13 vg/kg. Further, pilot safety studies in adult and infant mice injected with >10X an efficacious dose exhibited no clinical observations, no alterations in cardiac function, and no histopathological findings. Importantly, we have determined that TN-201 produced utilizing the highly scalable Sf9 platform results in similarly potent efficacy as HEK293-produced material in a *Mybpc3*$^{-/-}$ model of disease. Finally, we have established that our observed efficacy is sufficiently meaningful for stable benefit up to one year post-injection, as well as reversal of cardiac dysfunction even in late-stage homozygote disease.

524. Gene Therapy for Autoimmune Pulmonary Alveolar Proteinosis

Claudia I. Juarez-Molina †[1], Helena Lund-Palau†[1], Cuixiang Meng[1], Deborah Gill[2], Stephen Hyde[2], Eric Alton*[1], Uta Griesenbach*[1]

[1]NHLI, Imperial College London, London, United Kingdom,[2]Radcliffe Department of Medicine, Oxford University, Oxford, United Kingdom

Autoimmune pulmonary alveolar proteinosis (aPAP), is a rare lung disease, characterised by the accumulation of surfactant in the alveoli due to anti-granulocyte macrophage colony-stimulating factor (GM-CSF) auto-antibodies. The standard of care is whole lung lavage (WLL), an invasive procedure that only treats symptoms. Recombinant GM-CSF protein has been shown to outcompete the auto-GM-CSF antibodies and stimulates clearance of pulmonary surfactant by alveolar macrophages in some patients, but this treatment is expensive not widely available and a recent Phase 3 trial showed only modest benefit reached. We are, therefore, currently assessing whether gene therapy may be able to overcome some of the limitations of the current therapies. The UK Respiratory Gene Therapy Consortium has developed a lentiviral vector (rSIV.F/HN) designed to transduce a range of lung epithelial cells Lungs of PAP mice were transduced with rSIV/F/HN expressing GM-CSF (1e7-9.2e8 transduction units (TU)/mouse, n=4-13/group) by nasal sniffing leading to dose-related and persistent (11 months) expression of GM-CSF. In addition, we observed a dose-related reduction of disease biomarkers with doses as low as 1e6 TU/animal, which reduced SP-D levels (treated: 114.9, control 502.8 ng/mg, p<0.0001) and surfactant deposition in the lung (treated/control: PAS positive alveoli (%) 0.46/1.5 , p<0.05) (Figure 1 A-C). Biomarkers remained unchanged in mice treated with 1e5 TU, which is consistent with the lack of detectable GM-CSF expression at this dose. In mice treated with 1e7 TU/mouse all biomarkers were significantly reduced s (treated/control: BALF turbidity: 0.48/1.1 OD, p<0.001; SP-D: 20/502 ng/mg, p<0.0001; surfactant deposition: 0.1/1.5 %, p<0.05) (Figure 1 A-C). However, mice treated with doses over 1e6 TU/animal also developed dose-related side-effects over time, due to infiltration and accumulation of inflammatory cells. Our results demonstrate that a single dose of a lentivirus GM-CSF gene therapy persistently ameliorates markers of aPAP disease. The next step will be to repeat these studies in a model that expresses anti-GM-CSF autoantibodies to more closely mimic human disease.

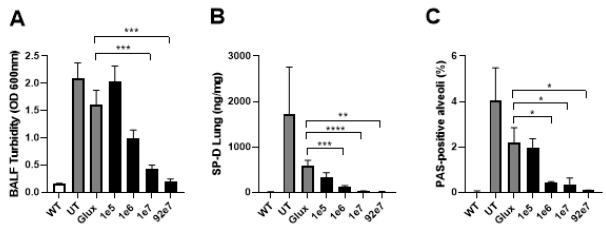


Figure 1: Lentiviral gene transfer of GM-CSF leads to dose-dependent reduction of PAP disease biomarkers. GM-CSF knockout mice were treated with lentivirus expressing GM-CSF at increasing doses (1e5-92e7 TU/mice). Reduced BALF turbidity (A), SP-D levels (B), and surfactant deposition in the lung (C).(i) WT mice untreated (WT), (i) GM-CSF KO mice untreated (UT), GM-CSF KO mice treated with 24e7 TU/mouse of the rSIV.F/HN-Glux control lentivirus (Glux). Data presented as mean ± SEM (n=2-13 per group). Mann-Whitney test. , *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

525. Computational Tools for Airway-Image and Multidimensional-Data Analysis

Anthony Sinadinos, Kyriel Pineault, Aarash Saleh, Uta Griesenbach, Eric W. F. W. Alton

National Heart and Lung Institute, Imperial College London, London, United Kingdom

Introduction: Biological data are increasingly large and multidimensional in scope. The need for fast and accurate imaging and data-analysis workflows in the life sciences is being met by constant improvements in computing power and the development of automatic and semi-automatic processing tools based on novel algorithms. To quantify lentiviral vector transduction efficacy, we have developed medium-to-high-throughput user-friendly pulmonary cell and tissue segmentation and analysis tools for the open-source, Java-based, image-analysis platform, ImageJ. **Results:** Two ImageJ macros were first developed to quantify single-cell fluorescence reporter protein and RNA(scope) signal-strength and distribution across epithelia-derived cytospun cells and lung-tissue sections. Using a custom nuclei-stain facilitated distance-map and Voronoi cell-segmentation approach, a highly accurate, fast, and high-cell-density resilient segmentation algorithm was created. With even modest PC hardware (i7-3330 with 8GB of 1597MHz RAM), a fully accurate quantification of stained molecules (RNA and/or protein) across 20,924 cells could be achieved in 77 seconds. Applied to immunofluorescence-stained lung tissue sections, another ImageJ macro and plugin was developed to semi-automatically define and segment airway epithelia. CPU-multithreading resulted in pronounced algorithm speed-up that is expected to scale well with available system threads, such that a server or virtualisation deployment has great additional potential (currently untested). Using similar underlying methods, a skeletal muscle cross-section plugin was developed to enable fast segmentation and analysis. Additionally, central nucleation percentage can be automatically determined for thousands of muscle fibres simultaneously. Finally, three open-source and publicly licenced dimensionality reduction (DR) Java libraries for PCA, t-SNE, and UMAP were integrated into a single ImageJ plugin. A separate data-clustering library was added and designed to be interoperable with DR. We show how these tools can be applied to both high-dimensional image and non-image data. **Conclusions:** We have designed several image and data-analysis tools for ImageJ to accelerate our pulmonary gene therapy research. These tools may be additionally used in many other cell and tissue contexts. A separate muscle cross-sectional tissue morphometry and central nucleation measurement tool is also showcased, indicating that many of the underlying imaging and processing principals may be further redeployed in even disparate and non-trivial, but still gene and cell therapy related, workflows. Finally, we have leveraged third-party software (Java-)library integration to add cutting-edge dimensionality-reduction (PCA, t-SNE, UMAP) and data-clustering (inc. k-means, (H)DBSCAN, and more) algorithms natively to ImageJ. This allows bioinformatic and mathematically contemporary methods to be seamlessly available to ImageJ users, to be easily applied to imaging and non-imaging problems for a variety of scientific projects.

526. Evaluation of Human Cardiomyocytes for Use in a Potency Assay for XC001, an Adenoviral Gene Therapy Vector for Treatment of Refractory Angina

Alexander Gaidamaka[1], Barb Thorne[2], Francesco Talotta[3], Albert Gianchetti[1], Remi Gloeckler[4], Marco Soriani[3]

[1]XyloCor Therapeutics, Malvern, PA,[2]Independent Consultant, Anacortes, WA,[3]ReiThera s.r.l., Rome, Italy,[4]Independent Consultant, Strasbourg, France

Introduction: Coronary artery disease (CAD), characterized by blood flowlimiting atherosclerotic lesions in the epicardial coronary vasculature, causes inadequate cardiac blood flow (myocardial ischemia) and consequent symptoms of chest pain (angina). XC001 is an adenoviral gene therapy vector being developed to treat refractory angina and potentially other related CAD and is designed to stimulate the formation of new coronary blood vessels by locally expressing multiple natural isoforms of human vascular endothelial cell growth factor (VEGF) in the myocardium. Efficacy and safety of XC001 has been demonstrated in pre-clinical models, and the investigational product is currently in a phase 1/2 clinical trial. A critical aspect of supplying product for future pivotal trials is to use an activity-based potency assay for product lot release and stability testing. Here we describe the first steps in phase 3 potency assay development for XC001, which is the evaluation of biologically relevant cell lines for the assay. **Objectives:** 1) Evaluate multiple candidate human cardiomyocyte cell lines for ability to be transduced by XC001 and express VEGF at levels sufficiently above any endogenous background; 2) Demonstrate dose-dependent expression of VEGF protein; 3) Demonstrate that human cardiomyocyte cell lines express and secrete the expected pattern of VEGF isoforms; 4) Demonstrate that the VEGF expressed in the cardiomyocytes transduced with XC001 is biologically active. **Results:** Three candidate human cardiomyocyte cell lines were evaluated, and all showed significant levels of vector-derived VEGF secretion over background after transduction with XC001, using an ELISA for VEGF protein quantitation. A clear dose response was demonstrated across a three-log range of multiplicities of infection (MOI) for the two most promising cell lines. Using a western blot, both cell lines showed the expected pattern of VEGF isoforms. Finally, vector-derived VEGF secreted by the cardiomyocytes was capable of stimulating a VEGF-responsive cell line similarly to control recombinant VEGF protein. These results establish a firm foundation for the next step

of developing a quantitative relative potency assay for XC001 using human cardiomyocytes, which is a critical aspect of preparing for pivotal clinical trials.

527. Single-Cell Sequencing Analyses of Airway Basal Cell Differentiation Following Lentiviral Transduction

Ashley L. Cooney[1], Andrew L. Thurman[2], Paul B. McCray[1], Alejandro A. Pezzulo[2], Patrick L. Sinn[1]

[1]Pediatrics, University of Iowa, Iowa City, IA,[2]Internal Medicine, University of Iowa, Iowa City, IA

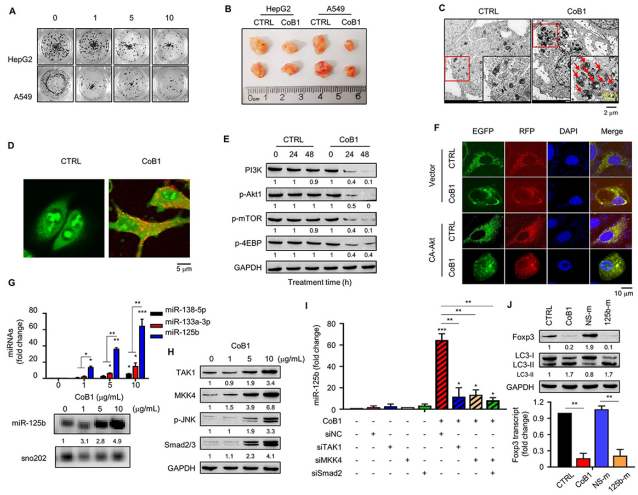
Gene therapy has the potential to be a life-long curativestrategy for all CFTR mutations. To achieve life-long expression of a corrected *CFTR*gene, genetic modification of progenitor cells in the lung islikely required. Monitoring CFTR persistence and stem cell function followingtargeted lentiviral transduction of an airway progenitor cell remainsunexplored. Either vector transduction or ectopic *CFTR* expression in basal cells may have a negative impact ontheir plasticity. Here we use single-cell sequencing to evaluate the geneexpression profile of well-differentiated airway epithelial daughter cellsderived from lentiviral transduced basal cell progenitors. We isolated primarybasal cells from human donor lungs and used single-cell sequencing toinvestigate the ramifications of viral vector transduction and CFTR expressionon cellular gene expression. CF and non-CF basal cells were either transducedor left untransduced by a lentiviral vector carrying CFTR or GFP and werepolarized into well-differentiated airway epithelia. Airway epithelial cultureswere library prepped for single-cell sequencing analysis. In parallel, cultureswere also assayed for anion channel activity by Ussing chamber analysis and theratio of cell types were confirmed by quantitative Real-Time PCR andimmunostaining. Ussing chamber measurements confirmed chloride channelcorrection in CF donor cells. Supraphysiologic ion transport was not observedin non-CF cells transduced with lenti-CFTR. Single-cell sequencing confirmed these observations andquantified the formation of multiple cell types, including ciliated cells,secretory cells, basal cells, ionocytes, and PNECs. Transducing a progenitorcell population with a lentivirus that constitutively expresses a reporter geneor CFTR does not perturb differentiation or cell type distribution. Here, weperformed a thorough analysis at the single cell level to understand theeffects of a gene therapy treatment to airway progenitor cells. These dataallow us to probe other important questions. For example, does thetranscriptome of CF cells that express CFTR more closely resemble CF or non-CFcells?

528. Cochlioquinone Derivative CoB1 Induces Cytostatic Autophagy of Lung Cancer through miRNA-125b and Foxp3

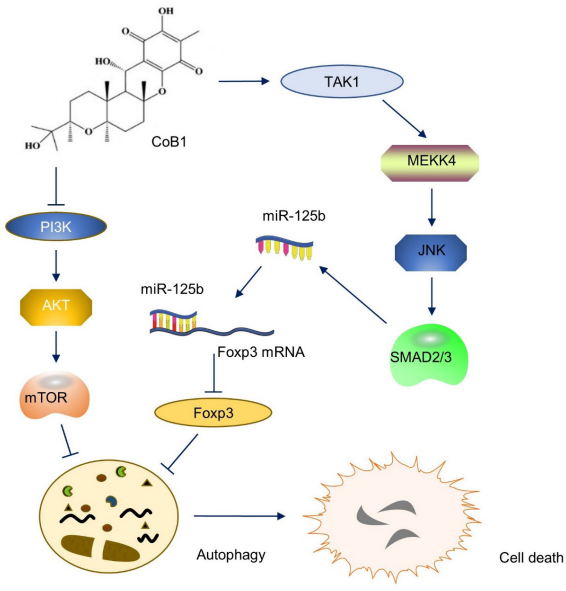
Nana Xu[1,2], Guokai Dong[1,2], Rongpeng Li[1]

[1]School of Life Science, Jiangsu Normal University, Xuzhou, China,[2]Xuhou Medical University, Xuzhou, China

Introduction: Lung cancer is the leading cause of cancer-related deaths worldwide, yet no effective medication of this disease was available. In this study, we purified a Cochlioquinone B derivative (CoB1) from Salvia miltiorrhiza endophytic Bipolaris sorokiniana. We determined that the CoB1 could inhibit the proliferation of lung cancer cells by inducing cytostatic macroautophagy both *in vitro* and *in vivo*. **Methods and Results:** 1. **CoB1 significantly inhibits the growth of lung cancer cells.** In vitro experiments, CoB1 significantly reduced clonogenic survival of A549 cells (Graphic 1A). We then investigated the anticancer effect of CoB1 on lung cancer in vivo. We observed that A549 xenografts in CoB1-treated mice grew at a slower rate than those threated with placebo (Graphic 1B). 2. **CoB1 induces autophagy through PI3K/ Akt/mTOR pathway.** Transmission electronic microscopy observation showed that numerous autophagic vacuoles containing lamellar structures or residual digested material and empty vacuoles were in the CoB1-treated A549 xenograft tumors (Graphic 1C). Acriridine orange staining shown that abundant cytoplasmic AVO formation was readily observed in CoB1-treated cells (Graphic 1D). 3. **CoB1 induces autophagy through PI3K/Akt/mTOR pathway.** PI3K, Akt and mTOR are critical interconnected junctions with the autophagy regulation pathway. As shown in Graphic 1E, CoB1 treatment resulted in inhibition of the Akt/mTOR pathway, as evidenced by decreased phosphorylation levels of Akt, mTOR, and 4EBP1. Akt overexpression reduced the autopahgosome and autolysosome formation (Graphic 1F). 4. **CoB1 induces miR-125b expression by regulating the TAK1/ MKK4/ JNK/ Smad axis.** Three miRNAs were activated by CoB1 in A549 cells, especially for miR-125b, and northern blotting data validated this finding (Graphic 1G). In addition, TAK1/ Smad axis, the signaling pathway that activates miR-125b transcription under the regulation of COB1, was significantly activated in CoB1-treated A549 cells (Graphic 1H). The expression of miR-125b were significantly reduced by blocking this signaling axis (Graphic 1I). 5. **miR-125b targets Foxp3 to induce autophagy.** Bioinformatics analysis predicted Foxp3 possessed a conserved miR-125b seed sequence in its 3'UTR with a high mirSVR score. As shown in Graphic 1J, Foxp3 mRNA and protein levels in A549 cells transfected with 125b-m was lower than that in nonspecific control mimic (NS-m) group. **Conclusion:** CoB1 inhibits lung cancer growth both in vitro and in vivo. As showed in Graphic 2, CoB1, on the one hand, inhibited PI3K/Akt/mTOR axis, on the other hand, induced the expression of miR-125b by activating TAK1/ MKK4/ JNK/ Smad axis, thereby reducing Foxp3 expression. Through the combined effect of these two aspects, CoB1 induced cell autophagy and promoted A549 lung cancer cells death.



Graphic 1. CoB1 induces cytostatic autophagy of lung cancer through miRNA-125b and Foxp3
A, HepG2 and A549 cells were cultured in of CoB1 for 14 days. **B,** HepG2 and A549 xenografts in CoB1-treated or placebo. **C,** Autophagy measured by transmission electron microscope in xenografts. Arrows, autophagosomes. **D,** Autophagy were measured by acridine orange staining, detected by CLSM. **E,** Expression of PI3K/Akt/mTOR/4EBP1 were measured by immunoblotting. **F,** LC3 puncta were detected by CLSM. **G,** Transcripts of miRNA were quantified by qRT-PCR. & Northern blots of miR-125b in cells as previously treated. **H,** Expression of TAK1/MKK4/Smad/JNK were measured by immunoblotting. **I,** Transcripts of miR-125b in A549 cells that treated with siRNA were quantified by qRT-PCR. **J,** Expression of Foxp3 and LC3 were measured by immunoblotting. & Transcripts of Foxp3 in cells as previously treated were quantified by qRT-PCR. Data are mean ± SD from three independent experiments. One-way ANOVA (Tukey's post hoc); *p < 0.05, **p < 0.01, ***p < 0.001.



Graphic 2. Proposed model for the role of CoB1 in activating autophagy to inhibit lung cancer growth.

529. Lentiviral Airway Gene Therapy Correction of CFTR Function in Knockout Cystic Fibrosis Rats

Nikki Reyne[1,2,3], Patricia Cmielewski[1,2,3], Alexandra McCarron[1,2,3], David Parsons[1,2,3], Martin Donnelley[1,2,3]

[1]Adelaide Medical School, The University of Adelaide, South Australia, Australia,[2]Robinson Research Institute, The University of Adelaide, South Australia, Australia,[3]Department of Respiratory and Sleep Medicine, Women's and Children's Hospital, South Australia, Australia

Introduction: Cystic fibrosis (CF) is a recessive genetic disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, resulting in defective ion transport in the airways. The most rational method for preventing or treating airway disease for all CF patients regardless of mutation, is gene addition therapy to add a properly functioning copy of the CFTR gene. We recently generated a CFTR knockout (KO) rat model and preliminary data demonstrated altered ion channel function in the nasal epithelium that was consistent with a CF bioelectric defect. The overall aim of this project was to assess the therapeutic benefit of lentiviral (LV)-CFTR gene vector delivery to the nasal epithelium of KO CF rats, as nasal airways are a widely used surrogate to represent CFTR function in lower airways. Methods: Nasal potential difference (PD) assessments were first optimised in wildtype (WT) and CF rats. The right nostril of adult KO CF rats (n=18) were conditioned with a 5 µl dose of 0.3% LPC, followed one hour later by 20 µl of LV-CFTR vector with a V5 epitope tag. Prior to, and one week following LV delivery, CFTR function was assessed on the same nostril using established nasal PD measurements using a standardised series of KRB solutions in the presence of amiloride. **Results:** Seven days after delivery of LV-CFTR to KO CF rats, amiloride produced a significantly smaller depolarisation compared to pre-treatment, and was similar to a WT animal response. Treated KO CF rats showed a significantly more negative low chloride response compared to the pre-treatment response ($p \leq 0.01$, paired t-test). Functional recovery of CFTR, as shown by a hyperpolarisation in response to low chloride, indicated up to 45% improvement towards the WT level. **Conclusion:** These findings confirm that delivery of a LV vector carrying the CFTR gene can partially correct CFTR function in KO CF rats. Future studies will focus on long-term CFTR correction to further validate the capabilities of our LV vector for translation to the clinic, advancing the potential of this approach as an effective treatment for CF lung disease.

530. Biosafety Risk Assessment: Biodistribution and Environmental Shedding of Topically Administered Lentiviral Vector to the Murine Airway

Kyriel M. Pineault[1,2], Cuixang Meng[1,2], Eric W. F. W. Alton[1,2], Uta Griesenbach[1,2]

[1]National Heart and Lung Institute, Imperial College London, London, United Kingdom,[2]UK CF Gene Therapy Consortium, London, United Kingdom

Background: We have developed a lentiviral vector pseudotyped with the F and HN proteins from Sendai virus (rSIV.F/HN) for cystic fibrosis gene therapy and are now progressing towards a first-in-man clinical trial. Here, we assessed biodistribution in non-target organs

and virus shedding to the environment after topical administration of the vector to the murine lung. **Methods:** Mice were treated with rSIV.F/HN-EGFPLUX (2.2e8 TU/mouse, n=8-16/group) by nasal sniffing. Luciferase expression and vector-specific RNA were quantified 2 weeks and 5 months post-transduction in all major organs. Shedding of infective viral particles in nasal (NALF) and bronchoalveolar lavage fluid (BALF) was measured using an *in vitro* infectivity assay. Key shedding experiments were repeated using clinical vector (rSIV.F/HN-CFTR). **Results:** As expected, transduction efficiency was high in lung (Lux: 213,134 ± 35,810 RLU/mg protein, RNA: 301,608 ± 100,672 copies/ng RNA) at 2 weeks. There was no significant expression above untransduced levels in any other organ, including the gonads. Occasional values above the highest untransduced value were seen in the spleen (untransduced: 77.1 RLU ± 2.8 RLU/mg protein, transduced 132.0 ± 21.4 RLU/mg protein, $p<0.05$) and brain (untransduced: 353.3 RLU ± 59.1 RLU/mg protein, transduced 517.9 RLU ± 83.8 RLU/mg protein) at 2 weeks post transduction but not at 5 months following gene transfer. Low levels (~ 1 TU/µl fluid) of infective vector particles were detectable in NALF and BALF 1 day post administration. Levels close to the limit of assay sensitivity were still detectable in NALF (<0.01 TU/µl fluid) and BALF (<0.1 TU/µl fluid) for at least 3 weeks. The same shedding pattern was demonstrated following administration of the clinical viral vector. Despite the presence of infective particles in secreted fluids, there was no evidence for transmission of the vector between treated and un-treated co-housed animals. Finally, vector viability on plastic was vector concentration dependent (1e9 TU/ml: 100% viability after 4 hr, 1e6 TU/ml: <1% viability after 4 hr). **Conclusion:** These preliminary studies suggest a low risk of both extrapulmonary distribution of the virus and external shedding following pulmonary delivery. Importantly no evidence for gonadal distribution was seen indicating low risk for germline transmission.

531. Assessment of the Air-Liquid Interface Culture Model as a Tool to Validate Efficacy of a rSIV.F/HN-CFTR Vector

Ana Sergijenko[1], Alena Moiseenko[2], Kyriel Pineault[1], Neda A. M. Nafchi[1], Mario Chan[1], Toby Gamlen[3], Deborah R. Gill[3], Stephen C. Hyde[3], Sebastian Kreuz[2], Uta Griesenbach[1], Eric W. F. W. Alton[1]

[1]National Heart and Lung Institute, Imperial College London, London, United Kingdom,[2]Boehringer Ingelheim, Ingelheim am Rhein, Germany,[3]University of Oxford, Oxford, United Kingdom

Introduction: We have developed a lentiviral vector pseudotyped with the F and HN proteins from Sendai virus (rSIV.F/HN) for cystic fibrosis (CF) gene therapy and are now progressing towards a first-in-man clinical trial. Here we assessed the transduction efficiency of rSIV.F/HN expressing EGFP in human bronchial epithelial cells from healthy control (HC) and CF donors grown in air-liquid interface culture (ALI). We also assessed the degree of correction of ion transport by rSIV.F/HN-CFTR in this model. **Methods:** Fully differentiated ALIs (MucilAir, Epithelix) were transduced with rSIV.F/HN-EGFP or Sendai virus (SeV)-GFP and GFP expression was quantified at multiple time points using fluorescence microscopy or flow cytometry. The ion transport in HC, CF, and CF ALIs transduced with rSIV.F/HN-CFTR was measured at 7 days in Ussing chambers (stepwise protocol: chloride buffer as baseline, 100 µM amiloride, 100 µM DIDS, low chloride, 10 µM forskolin/100 µM IBMX, 30 µM ΔCFTR inhibitor-172). **Results:** Transduction efficiency was generally <1% of cells (%GFP area: 0.46±0.05%, Flow cytometry: 0.60±0.14%, n=9/group). There was no difference in transduction efficiency between HC and CF ALIs. Sendai virus, which transduces lung epithelium with high efficiency *in vivo* also only produced low level transduction in these cultures (HC: 2.2±1.1%, CF: 0.72±0.13%, n=6/group).In Ussing chambers, there was no difference in baseline short circuit current between HC and CF ALIs, while forskolin/IBMX-mediated chloride secretion was significantly higher in HC samples compared to CF (HC: 8.9±1.4 µA/cm^2, CF: 1.16±0.33 µA/cm^2, n=14-17/group).We then assessed whether transduction of CF ALIs with rSIV.F/HN-CFTR was able to correct the chloride transport defect. Chloride transport increased significantly ($p<0.05$) in transduced CF ALIs compared to CF controls (CF-CFTR: 3.8±0.7 µA/cm^2, CF: 1.16±0.3 µA/cm^2, n=14-19/group). Corrected CF ALI cultures achieved ~40% of the HC response (CF-CFTR: 3.8±0.73 µA/cm^2, HC: 8.9±1.4 µA/cm^2). **Conclusion:** These data suggest that *ex vivo* transduction efficiency of differentiated human ALIs is low and may not reflect the *in vivo* performance of gene transfer agents. However, even at this low transduction efficiency, functional correction of ~40% of chloride transport was achieved in CF patient-derived ALI cultures following transduction with rSIV.F/HN-CFTR.

532. Evaluating the Use of Fibrinogen Based Scaffolds to Transplant Airway Basal Stem Cells for the Treatment of Cystic Fibrosis

Sriram Vaidyanathan, Dawn T. Bravo, Jayakar V. Nayak, Matthew H. Porteus

Stanford University, Stanford, CA

Cystic fibrosis (CF) affects about 30,000 Americans and 75,000 people globally. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a chloride channel cause CF. CF results in chronic lung infections that cause lung failure and can only be treated by lung transplantation. Although CFTR modulators have benefited many patients, there is a wide variability in patient responses and some patients still cannot be treated using these drugs. A gene therapy that corrects CF causing mutations in airway stem cells and generates a durable layer of corrected airway cells is ideal for CF. *In vivo* viral and non-viral gene therapy strategies have been attempted but success has been limited due to the thick mucus, immune response to viral vectors, the inflamed nature of the epithelial surface and rarity of basal stem cells. Recognizing these challenges, we chose to develop an ex-vivo gene corrected autologous airway stem cell therapy for CF. In addition, we identified the upper airways (nose and sinus) as a readily accessible source of upper airway basal stem cells (UABCs). CF sinusitis is an unmet medical need. This approach enables us to treat CF sinusitis while optimizing transplantation in the sinus while minimizing safety concerns to patients. We recently reported the use of this approach to correct the F508del mutation that affects >70% of CF patients and previously presented our work on inserting the CFTR cDNA in the endogenous locus to restore CFTR function in vitro. Here, we present our efforts to transplant UABCS into the upper airway epithelia starting with the identification of biocompatible cell delivery vehicles. In preliminary in vivo experiments, we discovered that the delivery of cells in saline into the nasal cavity of mice resulted in their expulsion. Thus,

there is a need to deliver the cells using a biomaterial scaffold that will adhere to the tissue and facilitate the engraftment of the gene corrected airway stem cells. Therefore, we evaluated the ability of a biomaterials scaffolds to support the survival and proliferation of airway basal stem cells in vitro. The materials tested include type I collagen (PureCol™), laminin foam-gel (Biosilk™), fibrinogen, alginate, hyaluronan (Hystem C™) and Dextran (Truegel™) and mixtures of these materials. We used basement membrane extract from mouse tumors (Matrigel™) as controls. Among the materials tested, UABCs seeded in Biosilk and Fibrinogen showed 5-10 fold expansion (Figure 1A). In addition, >90% of the UABCs cultured in Biosilk and Fibrinogen maintained the expression of cytokeratin 5 after 4 days in culture (Figure 1B). Subsequent studies will investigate transplantation of gene corrected UABCs seeded in scaffolds into immunocompromised mouse models.

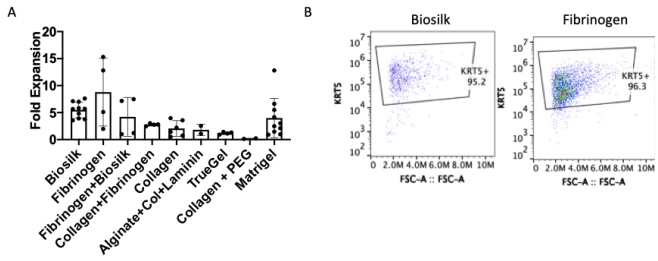


Figure 1: A. UABCs seeded in Biosilk and Fibrinogen gels expanded at levels comparable to UABCs seeded in Matrigel controls. B. UABCs seeded in Biosilk and Fibrinogen maintain the expression of cytokeratin 5 (KRT5), a marker of airway basal stem cells.

Neurologic Diseases

533. A Novel GUCY2D(R838S) Knock-In Mouse Model of Autosomal Dominant Cone Rod Dystrophy (CORD6) Displays Progressive Photoreceptor Degeneration/ Dysfunction

Russell W. Mellen[1], Sanford L. Boye[2], Sean M. Crosson[1], Kaitlyn R. Calabro[1], Diego S. Fajardo[1], K. Tyler McCullough[3], Shannon E. Boye[1]

[1]Department of Pediatrics, University of Florida, Gainesville, FL,[2]Powell Gene Therapy Center, University of Florida, Gainesville, FL,[3]Department of Biomedical Engineering, Duke University, Durham, NC

Purpose: Cone-rod dystrophy 6 (CORD6) is a debilitating, early-onset disorder characterized by progressive loss of cone photoreceptors, profound loss of visual acuity, and photophobia. CORD6 is caused by autosomal dominant mutations in *GUCY2D*, the gene encoding retinal guanylate cyclase-1 (retGC1). The most common gain of function mutations observed in CORD6 patients involve point mutations at residue R838. Currently, there are no murine models that accurately reflect both the genotype and phenotype of CORD6. For this reason, we created the R838S-knock in (R838S-KI) mouse via targeted replacement of endogenous *Gucy2e* exon 13 sequence with exon 13 from human *GUCY2D*. R838S-KI mice have one allele harboring human wild-type (WT) exon 13 *GUCY2D* sequence, while the other allele harbors exon 13 of *GUCY2D* containing the CORD6-causing R838S mutation. The purpose of this study was to characterize this novel mouse model and determine 1) whether it exhibited retinal degeneration/dysfunction that could be used as outcome measures for future therapies and 2) to what degree the phenotype mimics that of CORD6 patients. **Methods:** The R838S-KI mouse was created by injecting a dual-guide RNA (gRNA) CRISPR/Cas9 system and homologous template into mouse zygotes. The two gRNAs targeted (1) the beginning of exon 13 and (2) the beginning of exon 14, respectively. Homologous template encoded the relevant human *GUCY2D* sequence. Zygotes were injected into pseudopregnant female mice, and pups were screened after birth. This process was used to create two distinct mouse lines: one homozygous for the mutant *GUCY2D* exon 13 sequence (contains R838S mutation), and the other homozygous for the WT *GUCY2D* exon 13 sequence. These mice were bred to yield the heterozygous R838S-KI mouse. Electroretinogram (ERG) and optical coherence tomography (OCT) were performed to evaluate retinal function and structure, respectively in both R838S-KI and WT mice at 4, 8, 10, and 12 weeks post-injection (p.i). Cone survivability will also be assessed via whole retina IHC analysis using a cone-specific marker in young (8 weeks) and old (48 weeks old) mice. **Results:** Results collected through week 12 reveal that R838S-KI mice exhibit progressive degeneration of photoreceptors beginning as early as 4 weeks of age. and have reduced rod function by 8 weeks, relative to WT mice. No loss in cone cell function has been observed, however cone-mediated ERG waveforms of R838S KI mice exhibit delayed implicit times relative to WT mice at all ages tested so far. ERG and OCT data collection is on-going. Cone photoreceptor quantification in R838S KI and WT controls is underway. This immunohistochemical method is more sensitive than OCT and will better inform whether differences in cone photoreceptor densities and/or structure exist between these two lines. **Conclusions:** We have generated a novel R838S-KI mouse model with a genomic profile that mirrors human CORD6. Preliminary results reveal that R838S KI mice exhibit loss of rod structure and function, and aberrant cone function. This differs somewhat from disease progression in CORD6 patients. However, the loss of rod structure/function and aberrant cone function in this model still provide useful outcome measures for evaluating therapies. In conclusion, the genetic similarity between these mice and CORD6 patients, and their early onset retinal degeneration/dysfunction make the R838S KI mouse a useful model for pre-clinical testing of AAV-CRISPR/Cas9 therapies for the treatment of CORD6.

534. Towards a Gene Editing Approach for Mitochondrial NeuroGastroIntestinal Encephalomyopathy (MNGIE)

Marta Parés[1], Cristina Fornaguera[2], Ferran Vila[3], Sejin Oh[2], Steven H. Y. Fan[4], Ying K. Tam[4], Natàlia Comes[5], Francisco Vidal[5], Salvador Borrós[2], Jordi Barquinero[1]

[1]Gene and Cell Therapy, VHIR, Barcelona, Spain,[2]Grup d'Enginyeria de Materials (GEMAT), IQS, Universitat Ramon Llull, Barcelona, Spain,[3]Mitochondrial Pathology, VHIR, Barcelona, Spain,[4]Acuitas Therapeutics, Vancouver, BC, Canada,[5]Transfusion Medicine, Blood and Tissue Bank (BST), Barcelona, Spain

Mitochondrial NeuroGastroIntestinal Encephalomyopathy (MNGIE) is a rare metabolic disease caused by pathogenic variants of the TYMP gene, which encode the cytoplasmic protein thymidine

phosphorylase (TP). This enzyme catalyses the phosphorolysis of the pyrimidine nucleosides thymidine (Thd) and deoxyuridine (dUrd) into their respective bases, thymine and uracil. The absence of TP activity causes a systemic accumulation of these two nucleosides and an imbalance in the dNTP pools, which affects mitochondrial DNA synthesis in various tissues. Clinically, the disease is characterized by external ophthalmoplegia, peripheral neuropathy, diffuse leukoencephalopathy on brain magnetic resonance imaging, and gastrointestinal dysmotility that leads to progressive cachexia. To date, both allogeneic hematopoietic stem cell transplantation and liver transplantation have demonstrated long-term clinical efficacy, but high morbidity and mortality associated with these procedures demand the search for safer alternatives. Different gene therapy approaches using lentiviral and liver-targeted adeno-associated (AAV) vectors have provided proof of principle in preclinical studies. However, since these approaches have raised concerns about their potential oncogenicity and expression decline in the long term, safer and more durable strategies need to be developed.This work was aimed at achieving an efficient integration of a TYMP transgene into the introns of the Tymp and Alb loci of hepatocytes by the coordinated action of CRISPR/Cas9 molecules and human TYMP cDNA templates in a murine model of the disease (double KO Tymp-/- Upp1-/-). These templates were inserted downstream the two genomic promoters that drove transgene expression. In the Alb locus the transgene produced a hybrid Alb-hTP protein that was secreted. The CRISPR/Cas9 molecules were delivered as messenger RNA either by lipid or polymeric nanoparticles (LNP or PNP, respectively) or a AAV2/8 viral vector, whereas the DNA templates were packaged in AAV2/8 vectors. The best in vivo results were obtained by using LNP carrying the CRISPR/Cas9 RNAs. These mice showed a consistent and stable biochemical correction that correlated with the presence of TYMP mRNA and functional enzyme in liver cells. Mice edited at the Alb locus displayed high levels of TP activity in plasma, but the overall level of biochemical correction observed was similar in both target loci. Moreover, human TYMP mRNA was detected in mice receiving only AAV vectors containing templates targeting the Alb locus, in the absence on CRISPR/Cas9 nuclease, indicating target gene insertion with AAV alone, albeit with a lesser efficiency than that observed with the addition of nuclease delivered by LNP. Overall, these results demonstrate that liver-directed gene editing is feasible and has potential advantages for long-term correction of this disease.

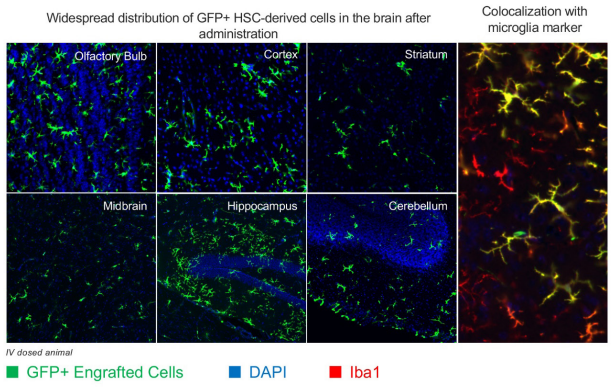
535. A Comprehensive Characterization of Biodistribution and Single-Cell States of HSC-Derived Microglia upon Ex-Vivo Lentiviral Gene Therapy in Murine Models of Neurological Disease

Robert N. Plasschaert[1], Mark P. DeAndrade[1], Mariana Loperfido[1], Cristina Baricordi[1], Luigi Barbarossa[1], John Yoon[1], Fritz Hull[1], Quoc Nguyen[1], Tara Peterson[1], Niek Van Til[2], Oliver Cooper[1], Luca Biasco[3], Chris Mason[4]

[1]AVROBIO, Cambridge, MA,[2]Child Neurology, Emma Children's Hospital, Vrije Universiteit, Amsterdam, Netherlands,[3]Advanced Center for Biochemical Engineering, University College London, London, United Kingdom,[4]Great Ormond Street Institute of Child Health, University College London, London, United Kingdom

Introduction: The genetic modification and administration of hematopoietic stem cells (HSCs) is an established cell and gene therapy platform for the treatment of monogenic disorders. Clinical trials using ex-vivo genetic modification and infusion of autologous HSCs have successfully addressed diseases with hematopoietic, peripheral, and central nervous system (CNS) involvement. We then tested the ability of our platform to induce expression of therapeutically relevant levels of transgene in two mouse models of neurodegeneration. **Methods:** Here, we generated a high throughput biodistribution study of genetically engineered cells upon two routes of administration (intravenous [IV]; and intracerebroventricular [ICV]) in mouse models of HSC gene therapy (HSC-GT) after busulfan conditioning. We studied 6 cohorts of mice across 3 experimental conditions showing that IV-administered HSCs engraft widely in all murine tissues including the CNS, while ICV-administration results in chimerism limited only to the CNS. **Results:** Surprisingly and in contrast with previous reports, IV-administration showed increased total CNS engraftment over ICV administration, a difference confirmed across multiple related endpoints. Using immunohistochemistry, we show that the vast majority of engrafted cells in the brain express canonical markers of microglia and have highly ramified morphology, consistent with these cells carrying out the surveilling role of endogenous microglia. For both routes of administration, we FACS-sorted and analyzed separately endogenous and HSC-derived microglia from the brain via single cell RNA-Seq in 2 male and 2 female mice at 13 months post-dose. By profiling 29,085 single cell transcriptomes, we establish that the CNS-engrafted HSC-derived microglia express bona fide microglial markers and exhibit a transcriptional profile more similar to endogenous microglia than to invading peripheral macrophages. Strikingly, we identify, for the first time at high resolution, a set of distinct transcriptional states and key markers (e.g. *Apoe* and *Mrc1*) that allow clear discrimination of engrafted microglia from endogenous microglia. We then treated Gba^{D409V} Parkinson's disease and GRN^{R493X} FTD mouse models with cells expressing human GBA and GRN respectively. Upon a comprehensive follow up, we observed stable supraphysiological expression of transgene in the periphery and the CNS in both systems at multiple doses in large animal cohorts. In homozygous Grn^{R493X} animals treated with GRN-expressing cells, we observed increased activity levels of GBA an observation consistent with the potential rescue of lysosomal dysfunction associated with GRN-FTD. **Conclusion:** Our findings shed new light into on the

characteristics of HSC-derived microglia and support the application of ex-vivo lentiviral HSC gene therapy as a platform by which a single systemic administration of genetic payloads may address both systemic and neurological pathobiology.



536. Mechanisms of Promoter-Driven AAV Toxicity in the Ear

Gabriela Pregernig[1], Kathy So[1], Monika Kowalczyk[1], Xichun Zhang[1], Arun Senapati[1], Bifeng Pan[1], Ning Pan[1], Lars Becker[1], Leah Sabin[2], Meghan Drummond[2], Jonathon Whitton[1], Adam Palermo[1]

[1]Decibel Therapeutics, Boston, MA,[2]Regeneron Pharmaceuticals, Tarrytown, NY

Background

Adeno-associated virus (AAV) is a promising approach to the treatment of a variety of inner ear disorders. These gene delivery vectors, with which there is deep clinical experience in other tissues, are being developed both for the correction of inner ear genetic disease and for regenerative therapy of common conditions where hair cells are lost. Several products are on their way to the clinic across these areas. An AAV gene therapeutic usually consists of a capsid, a coding sequence, and one or more regulatory elements that may impact when and where the transgene is expressed. We have previously shown that the choice of synthetic promoter can have a substantial impact on the therapeutic efficacy or durability of a gene therapy for genetic disease. First, in the context of Tmc1-mutant mice treated with AAV vectors expressing functional Tmc1, we demonstrated an improvement in ABR threshold recovery when a non-cell-specific CMV promoter was replaced with a hair-cell specific promoter derived from the Myo15 gene. Second, in the context of Otof-mutant mice, we showed that threshold recovery was not durable when a functional copy of Otof was delivered driven by a non-cell-specific smCBA promoter, but was durable when driven by the Myo15 promoter. In both of these contexts, loss of inner hair cells was observed with ubiquitous promoters that was not observed with the Myo15 promoter.

Methods

AAV-Myo15-hOTOF and AAV-smCBA-hOTOF were administered to the inner ear of mice and gene expression profiling of the cochlea was performed one month later.

Results

Consistent with previous results, animals treated with the smCBA-containing vector had impaired long-term hearing relative to those treated with the Myo15-containing vector. Several hypotheses may explain the apparent benefit of using the Myo15 promoter here. First, Myo15 may express at a lower level than the ubiquitous promoters even in the target hair cells, thus reducing the risk of overexpression-driven toxicity. Second, the hair cell-specificity of the promoter may be important through some mechanism. We performed RNA sequencing on cochleae from animals in this study in order to disentangle these two potential mechanisms of toxicity and loss of durability. High quality expression data was obtained, with marked differences between the two treatment groups.

Conclusions

We report the results of these analyses here, including examination of cell pathways and immune system involvement that may drive them.

537. Safety of scAAV9 Gene Therapy for Progranulin Deficiency

Irvin Garza, Sydni Holmes, Janine Prange-Kiel, Joachim Herz, Rachel Bailey

UT Southwestern Medical Center, Dallas, TX

Safety of scAAV9 Gene therapy for Progranulin Deficiency Garza I[1], Holmes S[1], Prange-Kiel J[1], Herz J[1], Bailey RM[1]. [1]UT Southwestern Medical Center Frontotemporal Dementia with Progranulin deficiency is caused by an autosomal dominant loss of function mutations in the *GRN* gene, while complete loss of function causes Neuronal Ceroid Lipofuscinosis type 11 (CLN11). Progranulin has multiple functions in the CNS including regulation of neuroinflammation, lysosomal homeostasis, and cell survival. Several recent publications support the potential benefit of progranulin gene replacement therapy using adeno-associated viral (AAV) vectors in *Grn* heterozygous and knock-out (KO) mice; however, high progranulin levels were also shown to result in T-cell cytotoxicity and hippocampal loss following AAV9 delivery to the brain ventricles of mice. Together, this suggests that while progranulin gene replacement therapy may be beneficial to patients, the route of delivery and expression levels must be carefully considered to avoid potential toxicity. To maximize cell transduction and optimize progranulin expression per cell we developed a novel progranulin gene replacement vector. We designed a codon-optimized human progranulin coding sequence under the control of a weak ubiquitous promoter and packaged it in a self-complementary AAV9 vector (scAAV9/hPGRN). To test for toxicity, WT C57BL6/J mice received an intra-cisterna magna (ICM) injection of vehicle or a high, mid or low dose of scAAV9/hPGRN vector. All vector doses were well tolerated and there were no significant changes in weight or gross behavior up to 6 months post injection. We then injected 6-month-old *Grn* heterozygous and KO littermates with the high dose via the ICM route. *Grn* KO mice recapitulate important features of the human disease, including elevated lipofuscinosis and neuroinflammation throughout the brain and lysosomal pathology. Both KO and heterozygous injected animals showed no signs of toxicity or decreased survival. Weights in all groups remained stable and showed no differences among genotypes. At 11 months of age, brains were collected and histologically assessed for neurotoxicity. In treated KO or heterozygous *Grn* mice, no toxicity was evident in H&E-stained sections and scAAV9/hPGRN KO mice had reduced lipofuscin as compared to vehicle treated KO mice in select brain regions. Assessments are ongoing for neuroinflammation, progranulin expression and lysosome pathology. To test the impact of the delivery-route on toxicity, we injected 9-month-old *Grn* KO and

WT mice with a high and low dose via the intra-cerebroventricular (ICV) or ICM route. Treatment by both routes was well-tolerated as assessed by general activity and survival, however, ICM-treated KO mice lost on average 20% of their body weight. Interestingly, this group of mice had an average starting weight of ~50 grams, so this loss in body weight was well-tolerated and mice remained healthy up to 3 months post-injection. Histological analyses for brain pathology are ongoing. Together, results from this study suggest that scAAV9/hPGRN treatment is safe when delivered via an ICM route. Work is ongoing and results on the potential benefits of this treatment and whether age at treatment or route of delivery affects safety or benefit will be presented.

538. AAV-Mediated Gene Overexpression to Protect Hearing from Noise Exposure and Aging

Wan Du, Zheng-Yi Chen

Massachusetts Eye and Ear/Harvard Medical School, Boston, MA

Permanent damage to and the loss of inner ear hair cells are the major factors underlying noise-induced hearing loss (NIHL) and age-related hearing loss(ARHL). We have previously shown that hair cell overexpression of Islet1, a LIM-homeodomain transcription factor, protects ISL1 transgenic mice from NIHL and ARHL. In this study, we used AAV-mediated local delivery of ISL1 gene to assess if the gene therapy approach could be effective in protection from NIHL and ARHL in wild type adult and aged mice. CBA/CaJ mice were injected with AAV/Anc80-ISL1-GFP at six-week-old age using canalostomy and injected mice were then exposed to noise at 1-20kHz for two hours at 110dB SPL that caused permanent threshold shifts (PTS). One month after injection, mean threshold shifts of the injected ears were significantly lower compared with the uninjected contralateral control ears at low to middle frequencies, which correlated with the transduction pattern in hair cells in middle and apical turns by AAV/Anc80. Subsequently, injected mice were subject to multiple noise exposures at 12 months and 15months of age, respectively. Hearing test after each exposure showed sustained protection against NIHL. Analysis of the injected inner ears detected improved hair cell survival and significant preservation of synapses as the result of ISL1 overexpression. To study AAV-ISL1 in hearing protection in ARHL, C57BL/6J mice were injected with AAV/Anc80-ISL1 at six months of age. Half year after injection, mean threshold shifts of the injected ears were significantly lower compared with the uninjected control ears. We found that p-S6, a mTORC1 signaling effector, was greatly reduced in the outer hair cells of injected inner ear, indicating that ISL1 expression suppresses mTOR activity that leads to attenuation of ARHL. We have thus developed a gene therapy approach by which one-time local delivery of ISL1 in adult mice protects from multiple noise exposures in long term. Our future work aims to reproduce the findings in a large animal model to lay the foundation for the development into clinic in certain patient populations.

539. Alpha-Synuclein Repression Using Zinc Finger Protein Transcription Factors - A Novel Therapeutic Approach for the Treatment of Parkinson's Disease

Asa Hatami[1], Hoang-Oanh Nguyen[1], Theresa Roth[1], Qi Yu[1], Kimberly Marlen[1], David Paschon[1], Elizabeth Mutter-Rottmayer[2], Ken Van[2], Annmarie Ledeboer[2], Angelica Phillips[2], Yanmei Lu[2], Emily Tait[3], Stephen Lam[3], Sarah Hinkley[3], Alicia Goodwin[4], Richard Surosky[4], Edward J. Rebar[5], Kathleen Meyer[2], Bryan Zeitler[1], Amy M. Pooler[1]

[1]Discovery and Translational Research, Sangamo Therapeutics, Richmond, CA,[2]Non-clinical Development, Sangamo Therapeutics, Richmond, CA,[3]Production, Sangamo Therapeutics, Richmond, CA,[4]Vector Core, Sangamo Therapeutics, Richmond, CA,[5]Design and Technology, Sangamo Therapeutics, Richmond, CA

Parkinson's disease (PD) is a progressive neurodegenerative disorder that leads to a wide range of motor and non-motor deficits, as well as dementia in ~50% of cases. Neuropathologically, PD is characterized by the loss of dopaminergic neurons in the substantia nigra and the appearance of intraneuronal inclusions called Lewy bodies and Lewy neurites, which contain alpha-synuclein (aSyn) aggregates. There is a large body of molecular and genetic evidence implicating aSyn as a key mediator of PD pathogenesis, and Lewy pathology is thought to propagate in the brain in a stereotyped prion-like manner as the disease progresses. Repressing the expression of aSyn in the brain thus has the potential to halt or slow PD progression. Zinc finger proteins (ZFPs) are naturally occurring human DNA-binding proteins and may be engineered to specifically bind any genomic sequence. When fused to transcriptional regulatory domains to form ZFP transcription factors (ZFP-TFs), they can be used to modulate the expression level of the targeted gene. We produced ZFP-TFs comprising 1) a ZFP DNA-binding domain targeting the human *SNCA* sequence, and 2) the KRAB domain of the human Kox1 protein. These constructs were screened in human neuroepithelial cells transiently transfected with mRNA coding for each of 384 ZFP-TFs. The screen identified multiple ZFP-TFs with human *SNCA* repression activity ranging from ~40% to >99%. A subset of these constructs was further evaluated in human iPSC-derived and mouse primary neurons transduced with adeno-associated virus (AAV) coding for each of the ZFP-TFs (AAV-ZFP-TFs). Experiments in human iPSC-derived neurons confirmed the on-target activity of the constructs and the durability of the response for 30 days. Transcriptome-wide specificity analyses using the Affymetrix microarray platform were performed in both human iPSC-derived neurons and mouse primary neurons transduced with selected AAV-ZFP-TFs. Highly specific ZFP-TFs were selected for an *in vivo* proof-of-concept study in the PAC synuclein mouse model, which expresses the full human *SNCA* sequence along with its upstream regulatory sequence on a mouse aSyn-null background. AAV vectors expressing each ZFP-TF were bilaterally administered to the striatum at two sites. Three weeks following AAV-ZFP-TF administration, the mice were euthanized and their brains were collected for molecular analyses. AAV-ZFP-TF administration was well-tolerated and led to statistically significant downregulation of aSyn mRNA expression in the brain. Further

optimization of the specificity and on-target activity profiles of the ZFP-TFs is ongoing. Our findings support continued development of ZFP-TFs as a potential aSyn-targeted therapeutic approach for the treatment of PD.

540. Generation of a Lentiviral Based Gene Therapy Vector for the Treatment of Neovascular Age-Related Macular Degeneration (nAMD)

Sharifah Iqball, Efrain Guzman, Michelle Kelleher, Daniel Beck, Gayathri Devarajan, Cheen Khoo, Deirdre O'Connor, Richard Harrop, Kyriacos A. Mitrophanous, Yatish Lad

Oxford Biomedica Uk Ltd, Oxford, United Kingdom

Neovascular age-related macular degeneration (nAMD) is a leading cause of blindness in the aging population, characterised by the abnormal growth of blood vessels in the macula, an area of the retina responsible for central vision. Leakage of blood and fluids from these vessels leads to the progressive degeneration of the macula and ultimately to the deterioration of the central visual field. Vascular endothelial growth factor (VEGF) is a pro-angiogenic factor that is known to have a key role in the development of nAMD and anti-VEGF treatments such as Aflibercept, Ranibizumab and Bevacizumab are the current standard of care for this disease. However, development of new therapies are necessary as current anti-VEGF treatments are only effective for 1-2 months and as such require frequent injections for an extended period of time, causing a significant burden to patients and are associated with side effects such as inflammation, haemorrhage and retinal detachment. An anti-VEGF gene therapy could pave the way for stable, long-term expression of the therapeutic anti-VEGF with a single dose, thereby reducing the treatment burden and potentially improving the clinical outcome. As such, we have developed an EIAV (OXB-203E) and HIV (OXB-203H) lentiviral vector encoding Aflibercept for the treatment of nAMD. Clinical data from RetinoStat®/OXB-201, a predecessor EIAV (equine infectious anaemia virus) gene therapy vector for the treatment of nAMD, expressing endostatin and angiostatin, demonstrated that following a single subretinal injection of this vector in patients, there was stable, long term expression of the transgenes in the aqueous humour out to 6 years, providing proof-of-principle for this type of single-injection gene therapy approach (NCT01301443 (Phase I GEM study) and NCT01678872 (follow-up study)). OXB-203 is the successor to our nAMD programme. Aflibercept derived from the OXB-203 lentiviral vector demonstrated *in vitro* binding characteristics to VEGF and inhibited VEGF induced HUVEC cell proliferation comparable to recombinant Aflibercept. In a preclinical study using a rat choroidal neovascularization (CNV) model for nAMD, a single subretinal administration of OXB-203E demonstrated expression of Aflibercept in the vitreous humour and reduction of laser-induced CNV lesion areas comparable to a bolus of recombinant Aflibercept delivered intravitreally. An *in vivo* head to head comparative study of OXB-203 and an AAV8-Aflibercept vector showed that Aflibercept derived from OXB-203 lentiviral vectors was expressed at significantly higher levels than from the AAV8-Aflibercept in the rat vitreous humour, retina and choroid (with retinal pigment epithelium (RPE)), four weeks following a single subretinal delivery of the viral vectors. These studies demonstrate the comparable efficacy of the OXB-203 lentiviral vector to that of recombinant Aflibercept and the superiority of OXB-203 in terms of the level of Aflibercept expression over an AAV8-based vector, thereby supporting the therapeutic potential of OXB-203 for the management of nAMD.

541. Downregulation of *SNCA* Expression as a Therapoetic Approach for Parkinson's Disease and Other Synucleinopathies: New Target Validation for Next-Generation Drug Discovery

Boris Kantor[1], Wendy Dong[1], Joseph Rittiner[1], Jordan Poe[1], Ornit Chiba-Falek[2]

[1]Neurobiology, Duke University, Durham, NC,[2]Neurology, Duke University, Durham, NC

Elevated levels of *SNCA* are causative in the pathogenesis of Parkinson's Disease (PD) and other synucleinopathies, while, normal physiological levels of *SNCA* are crucial to maintain neuronal function. A so-far unmet need is the development of new therapeutic strategies targeting the regulatory mechanisms of *SNCA* expression to fine-tune *SNCA* levels, versus previous approaches that targeted directly the mRNA or the protein product resulting in robust reduction of *SNCA* levels associated with neurotoxicity. We previously developed a novel strategy targeting the transcription regulation of *SNCA*, based on targeted epigenome editing via lentivirus- mediated delivery of CRISPR/dCas9 fused with the catalytic domain of DNA-methyltransferase3A (DNMT3A). Using human induced pluripotent stem cells (hiPSC)-derived 'aged' dopaminergic neurons from a PD-patient with the *SNCA* triplication as the experimental model, we demonstrated that fine-tuned downregulation of *SNCA*-mRNA and protein levels mediated by targeted DNA-methylation at intron 1 enabled the partial rescue of disease-related cellular-phenotypes characteristics of the *SNCA*-triplication/hiPSC-derived dopaminergic neurons, *e.g.* mitochondrial ROS-production and cellular viability, and nuclear aging signatures. Here, we further optimized, and refined the system to achieve more substantial, robust and long-term editing within the *SNCA* Intron 1 regulatory region. We designed and screened multiple effectors, including heterochromatin proteins 1A and B, methyl-binding proteins MBD1, MBD2, and MeCP2, and Krüppel associated box (KRAB) and nuclear inhibitor of PP1 (NIPP1), paired with both, lentiviral and adeno-associated vector (AAV) delivery platforms. The effectors were paired with dSaCas9 and dCjCas9 to create an all-in-one system compatible with AAV packaging ability. We assessed the repressive capability of those vectors using a dual-reporter cell line harboring the host- chromosome integrated Promoter-Intron1-*SNCA*-luciferase reporter. We report here, that both dSaCas9 and dCjCas9 paired with gRNA/KRAB-MeCP2 shown significantly higher levels of *SNCA*- downregulation compared to that of DNMT3A counterpart. Interestingly, gRNA that targeted a *CpG* island (*CGi*) of the Intron 1 (Kantor et al, 2018 Mol Ther), was found to be ineffective when the DNMT3A effector was replaced with the KRAB-MeCP2, suggesting a different mechanism of the repression. Along the same lines, gRNA/KRAB-MeCP2 systems targeted *SNCA*-Intron 1 at the transcription start site (TSS) shown high-level of *SNCA*- downregulation; albeit were

ineffective when paired with the DNMT3A counterpart. The KRAB-MeCP2 system will be further validated on *SNCA*-triplication/hiPSC-derived dopaminergic neurons as described above. The developed system supplies a strong foundation for advancing the novel epigenetic editing-based system towards PD therapeutic strategy for application in a clinical setting.

542. Time-Dependent Changes in ON Bipolar Cell Transcriptomes before and after Genetic Rescue from Rod Degeneration

Miranda L. Scalabrino[1], Mishek Thapa[1], Emily Davis[1], A.P. Sampath[2], Jeannie Chen[3], Greg D. Field[1]

[1]Neurobiology, Duke University, Durham, NC,[2]Ophthalmology, University of California Los Angeles, Los Angeles, CA,[3]Physiology and Neuroscience, University of Southern California, Los Angeles, CA

Inherited retinal disorders, such as Retinitis Pigmentosa, often lead to blindness due to the death of photoreceptors. This photoreceptor death induces remodeling of bipolar cells (BCs), their synaptic partners. The remodeling is mediated by changes in gene expression that allow the cells to change, but the gene networks that are involved in this BC rewiring are poorly understood. This is a significant hurdle to developing effective retinal gene therapies: visual information can only be transmitted if retinal synapses remain intact. Thus, vision restoration is dependent not only on halting photoreceptor death, but also on preventing or reversing BC remodeling. The purpose of this study was to examine how progressive photoreceptor degeneration impacts the transcriptomes of BCs and how these transcriptomes are altered by genetic rescue in a mouse model of Retinitis Pigmentosa. Mice with a floxed neomycin cassette inserted into the *Cngb1* locus (*Cngb1*$^{neo/neo}$) were crossed with *UBC-cre* and *Grm6-GFP* mice to create a triple transgenic line. These mice express green fluorescent protein (GFP) in BCs and exhibit a slow form of rod degeneration mimicking Retinitis Pigmentosa 45 (all rods are lost ~6 months). Also, this line allows for genetic rescue of rods by tamoxifen-induced cre-recombination that triggers the expression of Cngb1 from the endogenous locus. Littermates heterozygous for the neomycin cassette were used as controls. At designated ages, mice were sacrificed, retinas dissociated using papain, and FACS sorted into GFP+ and GFP- cell populations, which yielded relatively pure populations of sorted BCs for RNA extraction and transcriptomic analysis. We found significant BC gene expression differences between groups that depended on the amount of rod degeneration and the time-point of rod rescue: WT vs degenerating (P30, P90, P210), untreated vs treated, and early treatment (Tx at P30) vs late treatment (Tx at P90). There were fewer differentially expressed genes between late-stage degenerating bipolar cells (P90 untreated) and late treated bipolar cells (Tx at P90, sac at P150), indicating that late therapy is not fully reversing pathology. These results identify key gene networks in postsynaptic BCs that respond to rod degeneration and death. The results also point toward the development of novel therapies to ameliorate blinding conditions and increase the effectiveness of vision restoration, whether through gene therapy or cell replacement.

543. Construction of Vasoactive Intestinal Peptide Encoding Lentiviral Gene Therapy Vector for Retinal Dystrophies

Bahar Akkaya[1], Elif Ozgecan Sahin[1], Serdar Ceylaner[2], Ahmet Burak Bilgin[3], Salih Sanlioglu[1]

[1]The Department of Gene and Cell Therapy, Akdeniz University, Antalya, Turkey,[2]Intergen Center for Genetic Diseases, Ankara, Turkey,[3]The Department of Ophthalmology, Akdeniz University, Antalya, Turkey

Retinal dystrophies are a set of disorders characterized by progressive degeneration of photoreceptors which eventually causes blindness. In the retina, inflammatory signals released from dying cells trigger microglial activation which is one of the known critical mediators of retinal degeneration. Immunohistochemistry of rat retinal sections dissected from a chemical agent induced ($CoCl_2$) animal model of retinal degeneration suggested that microglial cells altered their location in the retinal tissue upon activation and upregulated the expression of surface markers CD11b and CD68. Since the correction of the genetic defect by gene replacement therapy does not necessarily cure or prevent retinal degeneration, inhibition of microglial activation might be necessary to halt the disease progression. In this context, we aimed to develop a lentiviral vector that can provide safe and long term gene expression of anti-inflammatory Vasoactive Intestinal Peptide (LentiVIP). To accomplish this, VIP expressing HIV based transfer plasmid was constructed using Gateway technology, and its protein expression was confirmed by immunocytological staining after transfection into 293T cells. Also, beta- Galactosidase and RFP expressions were analyzed by LacZ staining and fluorescein microscopy, respectively. Then, transfer vector (pLentiVIP) and lentiviral packaging plasmids were co-transfected into 293T cells via $CaPO_4$ transfection to produce lentiviral vectors in roller bottles. The evaluation of the therapeutic efficacy of VIP encoding lentiviral vectors is underway as the next step in our project. TUBITAK-218S543

544. Transplantation of CRISPR/Cas9 Corrected Hematopoietic Stem and Progenitor Cells into a Mouse Model of Friedreich's Ataxia

Joseph N. Rainaldi[1], Celine J. Rocca[2], Manoela A. Abreia[1,3], Stephanie Cherqui[1]

[1]Pediatrics, UCSD, San Diego, CA,[2]Genethon, Évry, France,[3]SDSU, San Diego, CA

Friedreich's ataxia (FRDA) is an autosomal recessive disorder that is predominantly caused by a GAA repeat expansion mutation within the first intron of the frataxin gene (*FXN*). This mutation leads to a reduction of frataxin, a mitochondrial protein involved in iron metabolism. FRDA is characterized by neurodegeneration leading to ataxia, areflexia, sensory loss, muscle weakness, and cardiomyopathy. Currently, there is no treatment for FRDA. We showed that transplantation of wild-type hematopoietic stem and progenitor cells (HSPCs) in the YG8R mouse model of FRDA prevents the development of locomotor deficits, neuronal degeneration in the dorsal root ganglia, and oxidative damage in brain and muscle. We then developed a strategy for autologous transplantation using gene corrected HSPCs ($CD34^+$) relevant for future clinical application. We utilized a CRISPR/Cas9-mediated gene-editing protocol to excise the intronic repeat, leading to an average of 30% gene editing efficiency in conjunction with a therapeutically

relevant increase in frataxin expression, restoration of mitochondrial activity, and no off-target indel events at computationally predicted sites. Now, this study investigates the *in vivo* therapeutic capacity of our clinical product by evaluating YG8s$(GAA)_{>800}$ model mice transplanted with CRISPR/Cas9 gene edited YG8s$(GAA)_{>800}$ murine HSPCs ($Sca1^+$). $Sca1^+$ cells isolated from FRDA model mice exhibit an average of 25% gene editing efficiency in conjunction with a 40% increase in transcriptional frataxin expression 7 days after editing. Edited cells are able to reconstitute transplanted host mice and gene editing can be detected in peripheral blood. Deficits in weight gain and grip strength, both associated with the FRDA phenotype in this model, are monitored weekly in a preliminary cohort of FRDA mice receiving edited cells, with additional locomotor analysis to be done at 3- and 6-months post-transplant. Tissue explant, at 6 months post-transplant, will include histological, biochemical, and genetic investigation to evaluate the impact of gene-edited cells on the FRDA phenotype. With this study, we are laying the foundation for future clinical application of autologous HSPC transplantation for FRDA.

545. Adeno-Associated Virus Capsid-Promoter Interactions in the Brain Translate from Rat to the Nonhuman Primate Using rAAV2-Retro

Martin Bohlen[1], Thomas J. McCown[2], Sara K. Powell[2], Hala G. El-Nahal[1], Tierney Daw[1], Michele A. Basso[3], Marc A. Sommer[1], R. Jude Samulski[2]

[1]Biomedical Engineering and Neurobiology, Duke University, Durham, NC,[2]Psychiatry - Gene Therapy Center, UNC School of Medicine, Chapel Hill, NC,[3]Institute for Neuroscience and Human Behavior, UCLA, Los Angeles, CA

We compared patterns of transduction following rAAV2-Retro infusion into the frontal eye field or superior colliculus in rhesus macaques. In all cases, local gene expression was prominent using the hybrid chicken beta actin (CAG) or human synapsin (hSyn) promoter. However, in frontal eye field cases, only the CAG promoter, not the hSyn promoter, led to gene expression in the ipsilateral claustrum and contralateral FEF. Conversely, infusion of rAAV2-retro-hSyn vectors, but not rAAV2-retro-CAG, into the macaque superior colliculus led to differential and selective retrograde gene expression in cerebellotectal afferent cells. After further analysis, we surmised that the AAV capsids was able to influence transgene expression independent of promoter selection. We also determined that minor changes to the amino acid location for the start of Vp2 was a hot spot for influencing cell type specific expression. Evidence for this capsid/promoter expression influence was observed in rodent with different promoters and extended to primates ruling out species specific transduction results. Our studies strongly suggest that this differential promoter/capsid expression profile is not a byproduct of promoter inactivation from retrograde transport of the rAAV2-Retro vector. In summary, we document the potential for AAV capsid/promoter interactions to impact cell-specific gene expression across species, experimental manipulations, and engineered capsids, independent of capsid permissivity.

546. Optimized Culture Conditions to Generate Fully-Formed 3D Human Retinal Organoids to Model Inherited Blinding Diseases

Patrizia Tornabene, Miriam Centrulo, Elena Marrocco, Elena Polishuchuk, Rossella De Cegli, Sandro Banfi, Alberto Auricchio

Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy

Inherited retinal diseases (IRD) are a major cause of blindness worldwide for which no therapies are currently available. Small and large animal models are available for IRD. Although these models have proven critical to understand the molecular bases of the corresponding IRD and to test potential therapies, they still pose important challenges due to species-specific differences with humans. Human three-dimensional (3D) organ and tissue cultures may provide unique, patient-specific models to study both disease mechanisms and the efficacy of novel therapies.We implemented our 3D retinal organoids differentiation protocol by culturing them in a bioreactor which improves aeration and nutrient distribution resulting in high photoreceptors yields with increased rhodopsin localization and outer segments formation with developing membranous discs. In addition, our Quant-seq analysis revealed that human retinal organoids closely mimic human fetal retina.These findings support retinal organoids as models to phenocopy human retinal diseases and as a reliable and renewable source of transplantable cells for personalized therapies.

547. Targeting Gys1 with SaCas9 Decreases Pathogenic Polyglucosan Bodies and Neuroinflammation in Adult Polyglucosan Body and Lafora Disease Mouse Models

Emrah Gumusgoz, Dikran Richard Guisso, Sahba Kasiri, Jun Wu, Brandy Verhalen, Silvia Nitschke, Sharmistha Mitra, Matthew Dear, Felix Nitschke, Berge Minassian

Pediatrics, UT Southwestern Medical Center, Dallas, TX

Many adult and most childhood neurological diseases have a genetic basis. CRISPR/Cas9 biotechnology holds great promise in neurological therapy, pending the clearance of major delivery and efficiency hurdles. We apply CRISPR/Cas9 genome editing in its simplest modality, namely inducing gene sequence disruption, to one adult and one pediatric disease. Adult polyglucosan body disease is a neurodegenerative disease resembling amyotrophic lateral sclerosis. Lafora disease is a late childhood onset fatal progressive myoclonus epilepsy. The pathogenic insult in both is formation in the brain of glycogen with overlong branches, which precipitates and accumulates into polyglucosan bodies that drive neuroinflammation and neurodegeneration. We packaged *Staphylococcus aureus* Cas9 and a guide RNA targeting the glycogen synthase gene *Gys1* responsible for brain glycogen branch elongation in AAV9 virus, which we delivered by neonatal intracerebroventricular injection to one mouse model of adult polyglucosan body disease and two mouse models of Lafora disease. This resulted, in all three models, in editing of approximately 17% of *Gys1* alleles and a similar extent of reduction of *Gys1* mRNA across the brain. The latter led to approximately 50% reductions of GYS1 protein, of abnormal glycogen and of polyglucosan bodies, as

well as corrections of neuroinflammatory markers in all three models. Our work represents proof of principle for virally-delivered CRISPR/Cas9 neurotherapeutics in an adult-onset (adult polyglucosan body) and a childhood-onset (Lafora) neurological diseases.

548. Developing an AAV Delivered Gene Therapy for Chronic Pain through Inhibition of NaV1.7 Expression

Matt R. Kelley, Wen Jin, Cansu Colpan, Daniella Lu, Ruohong Zhou, Timothy Fiore, Jeyashree Natasan, Li Liu, Ruo Wang, Swati Kumar, Jeffrey M. Brown, Kyle Grant, Jeffrey Thompson, Giridhar Murlidharan, Pengcheng Zhou, Kelly Bales, Todd Carter, Hatim Zariwala

Voyager Therapeutics, Cambridge, MA

Therapeutic innovation is a needed for patients suffering from chronic pain conditions. The neuron localized voltage-gated sodium channel type IX α subunit (NaV1.7) is encoded by the *SCN9A* gene. Mutations within *SCN9A* are associated with both insensitivity as well as hypersensitivity to otherwise painful stimuli. Despite strong genetic evidence for the critical role NaV1.7 plays in gating pain sensation within the peripheral nervous system, the development of selective and efficacious NaV1.7 inhibitors have failed to demonstrate clinical benefit in multiple trials. We hypothesized that suppression of NaV1.7 expression via a selective gene silencing approach would reduce the relative levels of NaV1.7 expression and thereby reduce pain hypersensitivity. To test our hypothesis, we identified a lead siRNA that selectively reduced NaV1.7 expression and optimized an artificial miRNA cassette (miSCN9A). Adeno-associated virus (AAV) mediated delivery of miSCN9A to rat dorsal root ganglion (DRG) neurons *in vitro* resulted in a significant decrease of 85.4% in NaV1.7 mRNA levels with no reduction in the level of the of highly homologous and related voltage-gated sodium channel NaV1.8 (SCN10A) or overt neurotoxicity. To test the potency of miSCN9A *in vivo*, we delivered miSCN9A and measured an 73.6% reduction in NaV1.7 mRNA levels in the rat DRG. Our preliminary results suggest that transduction of miSCN9A *in vivo* results in a significant and selective decrease in NaV1.7 mRNA, supporting this gene based therapeutic approach as a potential strategy to treat chronic pain conditions.

549. Optimal Capsid Selection for Hippocampal Neuron Transduction for Focal Epilepsy

Albena Kantardzhieva, Edward Yeh, Claire Discenza, Jacob Staudhammer, Kathy Anh Lam, Annahita Keravala

CODA Biotherapeutics, South San Francisco, CA

CODA Biotherapeutics' chemogenetic platform aims to control the aberrant activity of neurons to treat neurological disease. Target neuronal populations are modified with AAV-mediated gene transfer to express a tunable ligand-gated ion channel. The ligand-gated ion channel is engineered to be highly responsive to a specific proprietary small molecule but is otherwise inactive. The interaction of the small molecule with the engineered receptor would allow for exquisite, dose-dependent control of the neurons to generate therapeutic effect. CODA's engineered receptors can modulate activity in a variety of neuronal sub-types, resulting in flexibility to treat many neurological disorders with varying underlying pathophysiology. One of CODA's clinical applications is focal epilepsy, a chronic and debilitating neurological disorder that affects over 2 million people in the U.S. and is characterized by unpredictable seizures initiated from a specific location in the brain. Recurrent seizures result in cognitive and emotional deficits, with current interventions offering limited efficacy and multiple side effects. With the aim of expressing our engineered inhibitory receptor in the excitatory cells of the hippocampus, we have tested several AAV capsids- naturally-occurring as well as engineered for their ability to specifically transduce and express in the neurons of our choice. Assessment for tropism and expression strength was done *in vitro* and then *in vivo*. Primary embryonic rat hippocampal cultures were transduced with AAV2, AAV2.5-TV, AAV2.5-2YF, AAV2.5-TV2YF, AAV5, AAV6, AAV9, AAV9-TV, AAV9-2YF, AAV9-TV2YF, and AAV9-PHP.S encoding GFP under a CMV promoter. The GFP expression was measured at RNA (ddPCR) and protein levels (ELISA). Following *in vitro* studies, AAV2.5-TV, AAV2.5-TV2YF, AAV5, AAV6, AAV9, AAV9-TV, and AAV9-PHP.S capsids-carrying GFP were directly dosed, bilaterally into hippocampi of 5-6-week-old male Sprague-Dawley rats. After 4 weeks, these animals were sacrificed, their right and left brain hemispheres excised and further dissected for tissue assessment by ddPCR, ELISA, and immunofluorescence to measure levels of GFP mRNA, protein, and cellular tropism, respectively. Our *in vitro* results demonstrated varying levels of GFP expression depending upon the AAV serotype, with AAV6 mediating the highest level of expression. Results from mRNA, protein analyses, and immunofluorescence of rat hippocampal tissue indicated that AAV6 mediated the strongest expression, closely followed by AAV9 and AAV9-TV. Immunofluorescence analysis of GFP expression along with various cell markers indicated that all serotypes primarily transduced neurons, while AAV5, AAV6 and AAV9-PHP.S also transduced some astrocytes. Based on these studies, a sub-set of AAV capsids were administered directly in hippocampi of African green monkeys in order to make a final selection of the optimal capsid for neuronal transduction in hippocampus for CODA's focal epilepsy program.

550. Restoration of Cholesterol Metabolism as a Therapeutic Approach in ALS

Wurtz Guillaume, Emilie Audouard, Beatrix Meha, Nathalie Cartier, Françoise Piguet

Neurogencell, INSERM U1127 - ICM, Paris, France

Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron disease and is characterized by the progressive loss of upper and lower motor neurons, leading to paralysis and death. Accumulation of cholesterol in the central nervous system (CNS) has been reported to actively contribute to the disease progression in Alzheimer's disease, Huntington's disease, Spinocerebellar ataxia and more recently ALS. Cholesterol is essential for myelin compartment, but also for its functional and structural role in plasmatic membrane. However, in the CNS, cholesterol is synthetized in situ and is not able to freely cross the blood brain barrier (BBB). Cholesterol-24-hydroxylase (CYP46A1) allows the conversion of cholesterol to 24-hydroxycholesterol, able to

cross the BBB, thus regulating cholesterol homeostasis. Furthermore, this enzyme is a key neuronal stress response such as oxidative stress or protein aggregation. Therefore, we hypothesized that CYP46A1 could be relevant for a therapy in ALS to target both familial and sporadic forms of ALS independently from their genetic origin. In the severe SOD1^{G93A} model, we overexpressed CYP46A1 using a new AAV serotype (AAVi) able to cross the BBB after intravenous injection. As a first step, we confirmed that the AAVi viral vector has a specific tropism for the CNS and especially motoneurons. Secondary, we demonstrated a significant and prolonged motor rescue of animals treated pre or post-symptomatically, but also a preventive effect on myelin loss, compared to untreated animals. Evaluation of this therapeutic strategy is ongoing in another model of ALS.

551. Abstract Withdrawn

552. Rod-Derived Cone Viability Factor Provides Trophic Support for Cone Photoreceptors in a Pig Model of Retinitis Pigmentosa

Jennifer M. Noel[1], Archana Jalligampala[1], Myriam Marussig[2], Pierre-Axel Vinot[2], Melanie Marie[2], Melanie Butler[2], Florence Lorget[2], Stéphane Boissel[2], Thierry D. Leveillard[3], Jose A. Sahel[4], Maureen A. McCall[1]

[1]Ophthalmology & Visual Sciences, University of Louisville, Louisville, KY,[2]Sparing Vision, Paris, France,[3]Insitut de la Vision, Paris, France,[4]Ophthalmology, University of Pittsburgh, Pittsburgh, PA

Retinitis pigmentosa (RP) is an example of a rod-cone dystrophy (RCD) as well as an inherited neurodegenerative disease. Over 1.5 million people worldwide are affected by RCD with ~65 genes identified. In RP rod photoreceptors (rods) die and this is followed by cone photoreceptor (cone) degeneration, eventually causing blindness. In photoreceptors, the NXNL1 gene encodes two proteins: rods express rod-derived cone viability factor (RdCVF), whereas rods and cones express a full-length isoform, thioredoxin RdCVFL. Both proteins are hypothesized to support cone survival after rod degeneration by promoting glycolysis and preventing oxidative damage, respectively. In previously published data, a mouse model of RCD, retinal delivery of RdCVF/L via adeno associated viral (AAV) vectors promoted cone survival. We hypothesized that a similar approach could support cones in a large animal model of autosomal dominant RP, the Tg P23H human rhodopsin pig (Tg P23H hRho). We delivered a novel mutation-independent AAV-based drug candidate, SPVN06, encoding both human RdCVF and RdCVFL sequences, using a single AAV vector to the subretinal space of TgP23H hRho piglets at about postnatal day 6. We evaluated cone survival up to 6 months post injection. Neonatal TgP23H hRho pigs received a subretinal injection of SPVN06 and Tg controls receive either vehicle, AAV-GFP, or no injection. We conducted regular ophthalmic exams and fundus imaging to assess tolerability and retinal structure. Euthanasia occurs between 3 or 6 months post injection when retinas are collected and processed for immunohistochemistry and gene expression. We compared SPVN06-treated Tg and control retinas from four transgenic pigs euthanized 3 months post injection. In all treated retinas, SPVN06 is well tolerated. In three out four treated retinas cone inner/outer segment morphology is better preserved compared to control. For example, there is a higher percentage of cones that express medium wavelength opsin and the inner/outer segment lengths of all cones are longer. In the TgP23H hRho pig model of human adRP, SPVN06 subretinal administration preserves cone morphology and m-opsin expression. We expect that SPVN06 will also protect against cone degeneration in RCD patients, independent of the causative mutation.

Neurologic Diseases

553. Cas13d for Ube3a-ATS Targeting in Angelman Syndrome

Ulrika Beitnere[1], Miranda S. Palomares[1], Henriette O'Geen[1], Peter B. Deng[2], David L. Cameron[3], Anna Adhikari[4], Timothy A. Fenton[4], Nycole A. Copping[4], Jill L. Silverman[4], Kyle D. Fink[2], David J. Segal[1]

[1]Genome Center, UC Davis, Davis, CA,[2]Neurology Department, Stem Cell Program, and MIND Institute, UC Davis, Davis, CA,[3]Department of Neurology, UC Davis, Davis, CA,[4]Department of Psychiatry and Behavioral Sciences, UC Davis, Davis, CA

A new class of CRISPR family nucleases, Cas13, was recently characterized that can target RNA directly (Cox et al, 2017, Konermann et al, 2018), suggesting its potential for the development of RNA targeting therapeutics. Further, it was shown to be effective in targeted gene knockdown in the liver in vivo (He at al. 2020). A neurogenetic disorder that could benefit from a therapeutic aimed at the RNA level is Angelman Syndrome (AS). It arises from the genetic loss of the maternal *UBE3A* gene in brain neurons, causing severe communication and motor deficits, seizures, cognitive and physical impairments, and disrupted sleep. Due to a brain-specific long non-coding RNA transcript, the *UBE3A* antisense (*UBE3A-ATS*), paternal *UBE3A* remains silenced. Inactivation of the *UBE3A-ATS* RNA by Cas13 could therefore restore UBE3A expression in the brain. We delivered Cas13d to the brain using AAV-PHP.eB injected either intravenously or intracerebroventricularly. Data will be presented on the safety and distribution of the Cas13d nuclease *in vivo* in the brain of both a paternal *Ube3a* reporter mouse and AS mouse model. The results will be compared alongside delivery of an antisense oligonucleotide (ASO) that targets the same *Ube3a-ATS* RNA. If successful, this RNA directed therapy could be the first Cas13d-mediated therapy for a pediatric neurogenetic disorder.

554. Microglia Behavioral Reprogramming Is Sufficient to Rescue Dopaminergic Neurons from α-Synuclein Accumulation-Mediated Cell Loss

Simone Bido[1], Diana Gambarè[1], Melania Nannoni[1], Sharon Muggeo[1], Serena Giannelli[1], Elena Melacini[1], Luca Massimino[1], Matteo Marzi[2], Francesco Nicassio[2], Vania Broccoli[1,3]

[1]San Raffaele Hospital, Milano, Italy,[2]Center for Genomic Science of IIT, Milano, Italy,[3]National Research Council, Institute of Neuroscience (CNR), Milano, Italy

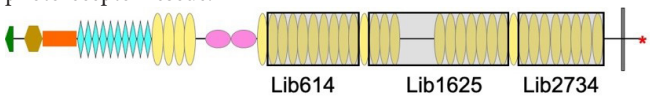
Parkinson's disease (PD) is a complex neuropathology caused by loss of midbrain dopaminergic neurons (DANs). A general assumption is that DAN demise is mainly occurring through cell-autonomous mechanisms while the exact involvement of the glia cell dysfunctions in neurodegeneration has remained largely elusive Evidence supporting neuroinflammation in PD includes post-mortem studies of brain, analyses of pro-inflammatory cytokines in serum and CSF, and epidemiological studies of nonsteroidal anti- inflammatory use. Whether neuroinflammation is the cause of PD pathogenesis or a secondary stress response remain not fully understood. In this work we adopted a cre/lox strategy to achieve the expression of the human mutated a-Syn gene (SCNA*A53T) specifically in the microglia through the nigral lentiviral gene delivery. Our data show a strong activation of microglia in the substantia nigra (SN) accompanied by a massive increase of pro-inflammatory molecules which predicts the loss of the dopaminergic neurons. Single cell RNA sequencing analysis, corroborated by in vitro assays, indicated a detrimental role of microglia in the neurodegenerative process through a mechanism that involved of toxic cues. Therefore, we evaluated the possibility to interfere with the microglia activation by promoting the expression of anti-inflammatory genes by the activated microglia, in the attempt to break down the inflammatory vicious circle. Given the importance of the glia in the brain surveillance, the unspecific and uncontrolled modification of microglia behavior can produce threatening side effect. To this end, a system able to drive the transgene expression only in the inflamed microglia is of clinical relevance. With this in mind, we generated a microRNA regulated lentiviral vector driving gene expression exclusively in activated microglia while silencing its expression in all the other brain cell types. This microRNA de-targeting strategy resulted successful in significantly attenuating the neuroinflammation caused by α-synuclein accumulation after nigral pre-formed fibrils (PFFs) delivery. The reduced neuroinflammation promoted a significant and long-lasting neuroprotection, therefore making this approach promising for its future employment in a clinical setting.

555. Miniaturization of Usher Syndrome Type 2A ene for AAV Mediated Gene Therapy

Weiwei Wang, Michael Florea, Luk H. Vandenberghe

Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Usher Syndrome (USH), an inherited disorder, is the leading cause of deaf-blindness. As a clinically and genetically heterogenous disorder, USH can be categorized into three types based on the severity and progression of vision and hearing loss. In this study, we focus on USH2A, mutations of which cause type2A USH. USH2A encodes protein Usherin (600kDa) with an open reading frame of 15.6kb. Our goal is to engineer minigenes with reduced size and yet sufficient function, to fit within the AAV packaging constraints (<~5kb) for gene augmentation to rescue vision. Usherin localizes to homologous membrane microdomains-- the stereocilia of hair cells in the ear and the periciliary region of photoreceptor connecting cilia in the retina. Full length Usherin consists of many motif repeats: 34 Fibronectin type III motifs (FN3), 10 Laminin EGF motifs (EGF_Lam), and 2 Laminin G domains. We hypothesize that some of the repeats are redundant and aim to miniaturize USH2A through rational design of several clonal minigenes to guide the design of combinatorial libraries with variant motif repeats deletions. The libraires will be subjected to high-throughput screening for candidates with superior readouts. We chose Oc-k1 cell line to be the in vitro model as it displays the characteristic periciliary localization of Usherin and is originated from mouse organ of Corti, an organ that's also affected by USH2A mutation. Our immunocytochemistry data showed that upon transfection, full length human USH2A expressed and localized at the periciliary region of Oc-k1 cell line (USH2A -/-). We also observed that wild type Oc-k1 cells proliferate significantly faster than USH2A -/- cells, and full length USH2A can rescue cell proliferation. This yet to be explained feature is used as our in vitro screening method, and the candidates will be further validated through USH2A mouse model for their function in disease phenotypes rescuing. We designed 7 minigenes to interrogate the motif repeats and screened them by proliferation rate. Three minigenes achieved >60% rescue effect of the full USH2A, two displayed >30% increase and two had no effect compared to control group (vehicle vector). Through comparing the sequences of these minigenes, we discovered that deletion among the FN3 repeats affects cell proliferation less than the other motifs. Thus, we chose the fibronectin region #5~34 as our region of interest to design the libraries shown in the figure below. The acronym describes the range of the fibronectin repeats in each library: Lib614 targets fibronectin repeats #6~14, Lib1625 for #16~25, Lib2734 for #27~34. Each variant contains a unique barcode for batched functionality screening through NGS. We have completed the assembly of Lib614 and Lib2734, with Lib1625 currently under design. These libraries will be subject to cell proliferation assay and NGS for functionality screening. Due to the large ORF size (15.6kb) of USH2A, it's likely that our future candidates exceed the 4.5kb limit for AAV packaging. However, we recognize that some of our minigenes are less than 9kb and thus are suitable for intein-mediated trans-splicing for dual AAV delivery. One of the minigenes is currently under study for this application. Our goal is to deliver this minigene-intein through dual AAV to USH2A mouse model, in order to test its ability for phenotype rescue and to examine the possible correlation between in vitro proliferation and in vivo photoreceptor rescue.



556. Early Delivery of ZF6 Rhodopsin Repressor Unalters Retinal Developmental Transitions and Positively Correlates with Therapeutic Benefit in the Treatment of Rhodopsin-Mediated Autosomal Dominant Retinitis Pigmentosa

Enrico Maria Surace, Rosa Maritato

Dipartimento di Scienze Mediche Traslazionali, University of Naples "Federico II", Napoli, Italy

ZF6 is a synthetic transcriptional factor (TF) engineered to bind a short regulatory sequence of the Rhodopsin (RHO) promoter. ZF6 enables biallelic RHO specific transcriptional silencing upon subretinal injection of adeno-associated viral (AAV: AAV-ZF6) vector. Furthermore, when ZF6 is coupled to Rho gene substitution (*silencing and replacement approach*), supports *mutation independent* therapeutic efficacy in the treatment of *Rhodopsin-Mediated Autosomal Dominant Retinitis Pigmentosa* (RHO-adRP). In this study, we aim at establishing whether ZF6 impacts retinal developmental transitions as a surrogate parameter of safety and to determine the effect of ZF6 treatment at the beginning of RHO-adRP disease presentation. We designed two strategies to express ZF6 to the developing retina, AAV-mediated somatic retinal gene transfer (at post-natal day 4 (P4) and transgenic expression (germline-transmission) by the generation of a transgenic mouse carrying ZF6 (ZF6-TR). The P347S mouse model of RHO-adRP directly injected with AAV-ZF6 or crossed with ZF6-TR shows morphological and functional recovery of retinal activity which outperformed that demonstrated by the injection of AAV-ZF6 in adult mice. We conclude that ZF6 operates orthogonally in photoreceptors by repressing specifically RHO expression without interfering with retinal development and that early expression of ZF6 enhances the therapeutic benefit compared to later stages of delivery.

557. Development of an AAV-Based, RNA-Targeting Gene Therapy for the Treatment of Huntington's Disease

Claire E. Geddes, Daniela Roth, Angeline Ta, Alistair Wilson, Haydee Gutierrez, Nandini Narayan, Dimitrios Angelis, Lydia Fang, Aaron Berlin, Greg Nachtrab, Dan Gibbs, Dimitrios Zisoulis, Ranjan Batra

Locanabio, Inc., San Diego, CA

Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disorder characterized by progressive motor, cognitive, and psychiatric impairments. The genetic cause of HD is a trinucleotide CAG repeat expansion within exon 1 of the huntingtin gene (HTT), which gives rise to a toxic, mutant huntingtin protein. To date, there is no effective treatment for HD and HTT mRNA-targeting strategies currently being evaluated in the clinic (antisense oligonucleotides [ASOs] or miRNAs) require recurring dosing (ASOs), penetrate the subcortical structures poorly (ASOs), do not directly target the CAG repeats, or require and thereby deplete endogenous cellular machinery to function. Here, we describe the development of an AAV-based, mRNA-targeting gene therapy that uses the PUF RNA-binding protein, derived from the naturally-occurring human PUM1 protein, coupled with an endonuclease to directly target and cleave CAG repeats in an allele-preferential manner. *In vitro*, CAG-targeting PUF proteins eliminated CAG-80 repeat mRNA expression in a dose-dependent manner while sparing endogenous HTT mRNA displaying allele preference. We next sought to package our CAG-targeting PUF protein system in an AAV vector for *in vivo* delivery. We evaluated the distribution of five different AAV serotypes (AAV1, AAV5, AAV8, AAV9, and AAVrh10) expressing GFP following intrastriatal delivery in wild-type (WT) mice. AAVrh10 was identified as the preferred serotype based on robust transduction of striatal neurons, transgene expression along the entire striatal anterior-posterior axis, and minimal inflammation that was primarily confined to the injection track. Finally, we demonstrate that our CAG-targeting PUF protein system, when packaged in an AAV vector and delivered to the striatum of WT mice, is strongly and stably expressed at 12 weeks post-injection, with no overt behavioral changes or neuronal loss (NeuN) observed. Lastly, we observe a decrease in mutant human HTT mRNA levels in a mouse model of HD after 4 and 6 weeks of treatment. In sum, we describe a potential therapeutic candidate for the treatment of HD that is meant for one-time delivery, is enzymatically self-contained, and preferentially destroys the mutant mRNA by directly targeting CAG repeats.

558. Subconjunctival AAV8-optHLA-G1&5 for Treatment of Ocular Graft vs Host Disease

Brian Gilger[1,2], Jacob Nilles[1], Darby Roberts[1], Matthew Hirsch[2]

[1]Clinical Sciences, North Carolina State University, Raleigh, NC,[2]Ophthalmology, University of North Carolina, Chapel Hill, NC

Ocular graft versus host disease (OGvHD) is a common complication of allogeneic hematopoietic stem cell transplantation (HSCT) and frequently manifests as keratoconjunctivitis sicca (KCS). AAV-HLA-G1&5 has demonstrated efficacy for reduction of corneal neovascularization, scarring, and uveitis in several animal models; therefore it was hypothesized, based on the direct and indirect immunomodulatory function of HLA-G isoforms, that AAV-HLA-G1&5 may serve as an effective treatment for OGvHD. The aim of this study was to evaluate the efficacy of a single subconjunctival (SC) dose of adeno-associated virus (AAV) gene therapy encoding codon optimized human leukocyte antigen G isoforms (HLA-G1&5) to induce ocular immune tolerance and inhibit clinical signs of OGvHD. To create the murine OGvHD model, bone marrow (BM) and splenocytes were collected from male C57BL/6 (n=4) mice 6-8 weeks of age. Female BALB/c mice (n=12), aged 6-8 weeks, were administered total body X-ray irradiation and mice were injected into a lateral tail vein with 1e7 BM cells and 1e6 splenocytes. Following injection, body weights and tear production (phenol red test) were recorded, and scores (0-4) (observer blinded to treatment groups) of the eyelid margins, corneal opacity, and corneal fluorescein were collected through day 44 after HSCT. At 7 days following HSCT, mice received in both eyes either a single SC injection of AAV8-optHLA-G1&5 (1e9 vg/eye), no treatment (n=4), or 2uL of CsA 1% solution twice daily (n=4). Eyelid scores of eyes dosed with AAV8-optHLA-G1&5 were significantly lower than untreated or CsA treated eyes from 24-44 days after HSCT ($p<0.03$, Wilcoxon). Fluorescein scores of eyes dosed with AAVHLA-G and CsA were significantly lower than untreated eyes from 30-44 days after HSCT ($p<0.02$, Wilcoxon). No statistically significant difference in weight or tear production was observed between any of the three

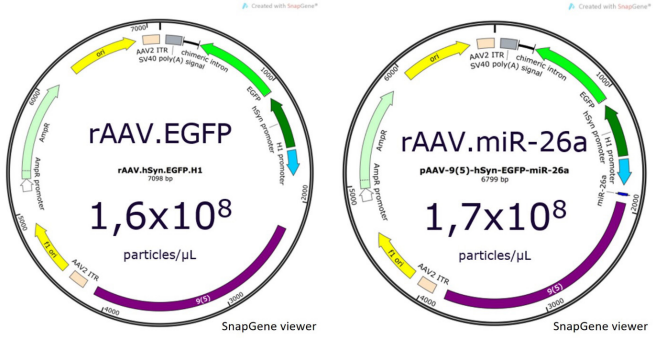
treatment groups. These results further validate the function of HLA-G in the murine model, indicate that the SC therapeutic route targets relevant ocular tissues, and provides strong support that HLA-G-based gene therapy will be an effective single vector treatment for OGvHD. Further evaluation of AAV-optHLA-G based therapeutics are warranted for ocular surface and intraocular immune-mediated diseases.

559. Overexpression of miR-26a Promotes Neurite Regeneration in the Central Nervous System *In Vitro*

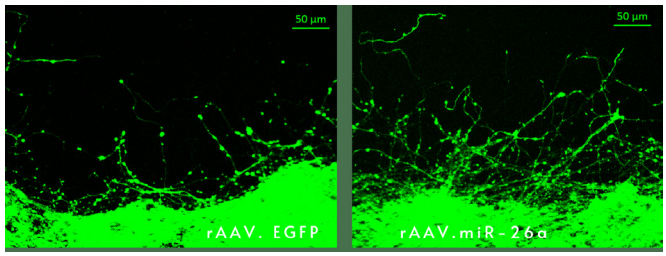
Clarissa Ribeiro de Oliveira Cyrino[1], Carolina Gomes Ferreira[1], Raquel Alves de Almeida Costa[1], Victor Ulysses de Souza Matos[1], Lucas Caldi-Gomes[2], Uwe Michel[2], Vinicius de Toledo Ribas[1]

[1]Morphology, UFMG, Belo Horizonte, Brazil,[2]University of Göttingen, Göttingen, Germany

The central nervous system (CNS) has an intrinsic low capacity for functional axon regeneration due to an imbalance in gene expression. CNS damage, as occurs in neurodegenerative diseases or traumatic lesions, leads to permanent functional impairment. Thus, investigate the role of molecules that control the expression of genes involved in axon regeneration may lead to novel treatments for neurodegenerative disorders. Studies have been conducted to identify the intrinsic mechanisms responsible for controlling axonal regeneration in the CNS and found that microRNAs (miR), non-coding RNAs, that act as post-transcriptional regulators of gene expression, such as miR-26a, are associated with this control. The miR-26a promotes neurite outgrowth and axonal regeneration in the peripheral nervous system (PNS), by targeting mRNAs that encode proteins directly related to regeneration pathways, such as PTEN, GSK3beta. However, the role of miR-26a in axon regeneration in the CNS is still unknown. We aim to test if overexpression of miR-26a promotes axonal regeneration of CNS neurons, using *in vitro* and *in vivo* models. All the animal experiments were approved by the ethics committee for animal experiments of Federal University of Minas Gerais, UFMG (4/2017 and 237/2018). We produced recombinant adeno-associated viral (rAAV) vectors expressing miR-26a (rAAV.miR-26a) or EGFP (rAAV.EGFP), as control. Both vectors express EGFP fluorescent protein for identification of transduced neurons.



Transduction efficiency was performed *in vitro* through primary cortical neuron culture of Wistar rats in embryonic age of 18 days. We also tested retinal ganglion cells (RGC) transduction *in vivo* by intravitreal injections into adult Wistar rats and analyzed neurite regeneration after scratch lesion of primary cortical neurons culture. Finally, we conducted the bioinformatics evaluation of the predicted and validated mRNA targets of the miR-26a. Viral transduction efficiency evaluation showed that rAAV.miR-26a and rAAV.EGFP are working properly and efficiently, since both primary cortical neurons and RGCs showed positive expression of EGFP. In the neurite regeneration analysis (n=3), we found an increase of 49,9% ± 8.3 ($P<0.05$) in neurite length in the group transduced with rAAV.miR-26a compared to the control rAAV.EGFP. We also found that rAAV.miR-26a increases in 81,9% ± 2.9 ($P<0.01$) the number of neurites crossing the distance of 200µm from the scratch border.



For the bioinformatics evaluation we found 33 predicted and 30 already validated mRNA targets of miRNA-26a. These results indicated that miR-26a increases neurite regeneration *in vitro*, however, further actions are required to confirm this pro-regenerative effect in the optic nerve *in vivo*. This study could identify novel therapeutic targets to promote axonal regeneration in the CNS.

560. Intravitreal Immunomodulatory AAV Gene Therapy for Treatment of Autoimmune Uveitis

Brian Gilger[1,2], Elizabeth Crabtree[1], Jacklyn Salmon[1], Liujiang Song[2], Jacquelyn Bower[2], Matthew L. Hirsch[2]

[1]Clinical Sciences, North Carolina State University, Raleigh, NC,[2]Ophthalmology, University of North Carolina, Chapel Hill, NC

Non-infectious uveitis (NIU) is an intractable and painful disease that is a significant cause of vision loss. Current treatments of NIU are non-specific and have serious side effects which limits them to short-term use. A single dose treatment that mediates long-term immunosuppression without side effects is desired. The purpose of this work was to evaluate immunomodulatory gene therapy as an effective long-term treatment for NIU. We investigated the use of intravitreal (IVT) adeno-associated virus (AAV) gene therapy to deliver immunomodulatory transgenes, human leukocyte antigen G (HLA-G) or equine interleukin 10 (eqIL-10), for treatment of a model of experimental autoimmune uveitis (EAU). Naïve Lewis rats received a single IVT injection of saline (3uL) or AAV particles harboring codon-optimized cDNAs encoding HLA-G1&5 or eqIL-10 (1e9 to 1e10vg) seven days prior to EAU induction by subcutaneous injection of inter photoreceptor binding protein. Daily slit lamp examination (EAU inflammatory scores) and optical coherence tomography (OCT) to measure aqueous humor (AH) cell counts were performed with the observer blinded to the treatment groups for 14 days. Genome biodistribution and transduction efficiency were characterized using Q-PCR for ocular tissues. Histology scores of cellular infiltrate and structural damage were recorded. Finally, AAV capsid serum neutralizing antibody assays were performed. Following

IVT AAV-HLA-G or eqIL-10, a significant reduction of EAU scores and AH cells compared to control eyes were observed on days 10-14 post EAU induction ($p<0.05$, Wilcoxon). Furthermore, histologic inflammatory scores were also significantly reduced in AAV-HLA-G or eqIL-10 dosed eyes compared to control ($p<0.05$, Wilcoxon). Vector genome biodistribution studies did not find AAV vector genomes in non-ocular tissues, however, a very low neutralizing antibody response was elicited against the AAV capsid. These studies establish that a single IVT injection of an AAV encoding an immunosuppressive transgene, whether it be HLA-G or eqIL-10, significantly reduces clinical and histologic inflammation in a well-established model of autoimmune uveitis. Results of these studies warrant further study of the IVT route of delivery of AAV gene therapy for a single dose treatment of NIU.

561. Partial Reprogramming Leads to Robust Regeneration of Cochlear Hair Cells in Adult Mice

Yizhou Quan, Zheng-Yi Chen
Mass Eye&Ear Infirmary/Harvard Medical School, Boston, MA

Hair cell loss is the major cause of permanent hearing loss in humans. Spontaneous hair cell regeneration does not occur in the mature mammalian inner ear. Strategies to overcome irreversible cochlear hair cell damage and loss in mammals are of vital importance to hearing recovery. We demonstrated that the combination of *Myc*/*Notch1* activation is sufficient to reprogram adult cochlea and regenerate hair cell-like cells in response to transcription factor *Atoh1* in adult transgenic mice. To move our work towards clinical application it is necessary to reprogram adult inner ear for hair cell regeneration in wildtype adult mammalian inner ear with a clinically relevant approach. We performed screening of small molecule chemical compounds and siRNAs using cultured adult mouse cochlea for reprogramming. We identified a combination that can effectively replace *Myc* and *Notch1* transgenes and sufficiently reprogram adult cochlea shown by the activation/upregulation of Myc, Notch and early inner ear developmental genes. Reprogrammed adult cochlear cell types efficiently transdifferentiated into hair cells in response to the signal by *Atoh1* overexpression. By lineage tracing, regenerated hair cells were found to be derived from both SOX2-positive supporting cells and SOX2-negative cells in the limbus region. Regenerated hair cells were labeled with multiple mature hair cell markers including MYO7A, PVALB, and ESPN. Further, regenerated hair cells were labeled with an outer hair cell maker SLC26A5 (prestin) and an inner hair cell marker SLC17A8 (VGLUT3), respectively, suggesting specification of auditory hair cells. Finally, the new hair cell were found to form connections with adult spiral ganglion neurons and were able to take up FM1-43, an indication of the presence of the transduction complex. Our study identified a combinatory approach by small molecules and siRNAs to regenerate hair cells in wildtype mature mammalian inner ear, laying the foundation to apply the approach for in vivo hair cell regeneration and restoration of hearing.

562. Oxidative Stress Induced Primary Monkey RPE Cells as Model to Evaluate Antioxidative Nanomedicine for Age-Related Macular Degeneration

Kai Wang[1], Martin Bohlen[2], Min Zheng[1], Marc Sommer[2], Zongchao Han[1]
[1]Ophthalmology, The University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Department of Biomedical Engineering, Duke University, Durham, NC

Although the detailed pathology of age-related macular degeneration (AMD) remains elusive, researchers found that several risk factors are associated with the elevated oxidative stress. Retinal pigment epithelium (RPE) is where macular degeneration begins. We previously studied a cellular model using primary mouse RPE cells induced by blue lights to evaluate antioxidative treatment. In this study, we used primary monkey RPE cells as our cellular model to study the antioxidative nanomedicine. We utilized blue light (470 nm wavelength), hydrogen peroxide, and hypoxia incubator chamber (0.5% oxygen containing) to induce oxidative stress. The tight junction of monkey RPE cells was compromised with oxidative stress, and the significantly enlarged nuclei were observed. The level of reactive oxygen species (ROS) was analyzed by MitoSox Red and showed an increased level of ROS-oxidized products. Compared with normal controls, the viability of RPE cells was significantly reduced. When treated with our previously studied glycol chitosan-coated nanoceria (GCCNP) in these oxidative stress-induced monkey primary RPE, our results showed that GCCNP significantly reduced oxidative stress and cell damage as revealed by immunohistochemistry, Mitosox, and live/dead assays. Here we report a relatively easy and fast approach to obtain oxidative damage phenotypes/patterns, which is similar to that of an AMD patient for studying and evaluating AMD treatment using antioxidative nanomedicine.

563. A Chemical Agent Induced Retinal Degenerative Disease Animal Model for Gene Therapy

Elif Ozgecan Sahin[1], Bahar Akkaya[1], Ahmet Burak Bilgin[2], Serdar Ceylaner[3], Salih Sanlioglu[1]
[1]The Department of Gene and Cell Therapy, Akdeniz University, Antalya, Turkey,[2]The Department of Ophthalmology, Akdeniz University, Antalya, Turkey,[3]Intergen Center for Genetic Diseases, Ankara, Turkey

Retinal degenerative diseases, either attained or inherited, are a major cause of visual impairment and blindness in humans. Most retinal degeneration induced by genetic mutations affects the retinal pigment epithelium and retina. A progressive loss of photoreceptors caused by lack of oxygen (hypoxia) will predictably induce metabolic changes in the damaged retina. Activated microglial cells by dead photoreceptors can alter their location in the retinal tissue and further intensify photoreceptor degeneration by virtue of releasing proinflammatory cytokines. As photoreceptor degeneration mechanism and process are mainly governed by the type of mutation, the pathologies observed in hereditary retinal dystrophies are similar. Thus, we aimed to develop a new experimental animal model of retinal degeneration manifesting a disease pathology that is common to all hereditary retinal dystrophies. An *in vitro* retinal degeneration model was established by treating

ARPE-19 cells with a hypoxia mimicking agent, cobalt chloride ($CoCl_2$). Hypoxia was confirmed via western blotting using antibodies against hypoxia-inducible factor-1alpha (HIF-1α). To generate *in vivo* animal model of retinal degeneration, $CoCl_2$ was injected intravitreally into Wistar rats. Hematoxylin&eosin staining and TUNEL assay results indicated that progressive retinal degeneration initiated in the outer segment of the photoreceptors and then further extended into the other retinal layers in time. Moreover, activated microglia cells revealed by CD68 immune staining were staggered throughout the retinal layers. All these results indicate that this chemical agent-induced animal model can be used for gene therapy studies targeting retinal degeneration. TUBITAK-218S543

564. Therapeutic Efficacy of an Investigational AAV Gene Therapy in Alleviating Choroidal Vascularization in Rat Laser-Induced CNV Model

Ting He[1,2], Xiaoyao Hao[3], Yue Gao[3], Pengcheng Zhou[1,2], Jason Zhang[1,2]

[1]GeneLeap Biotechnology LLC, Woburn, MA,[2]Luye Boston Research & Development LLC, Woburn, MA,[3]Nanjing GeneLeap Biotechnology Co., Ltd., Nanjing, China

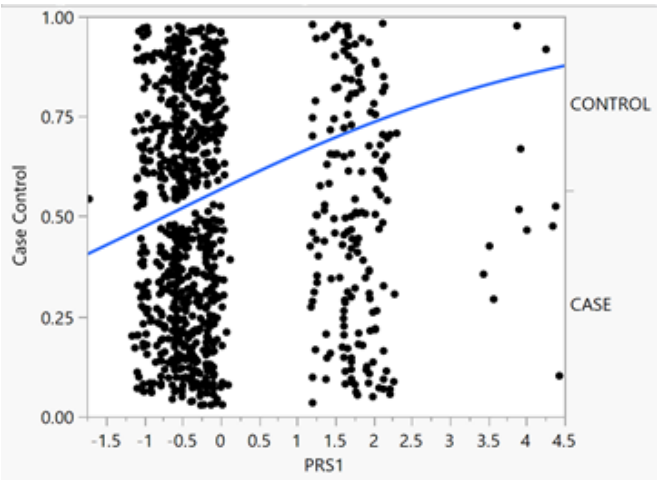
Wet age-related macular degeneration (wet AMD) is a progressive and chronic disease of the retina characterized by the presence of choroidal neovascularization (CNV), which results in leakage of fluid surrounding the retinal pigment epithelium (RPE), and eventually leads to irreversible degeneration of the central retina and blindness in aging population. Vascular endothelia growth factor (VEGF) is the most important angiogenic factor and plays a leading role in CNV pathology. Intravitreal administration of recombinant anti-VEGF Protein is the current standard-of-care of wet AMD patients in the clinic; however, this treatment requires repeated dosing every 4-8 weeks, resulting in increased surgery-associated complications that undermine its therapeutic efficacy. AAV gene therapy has been demonstrated to allow sustainable transgene expression through a single administration in post-miotic tissues and thus holds great potential for long-lasting therapeutic efficacy in treating a variety of human diseases including wet AMD. In this study, a set of AAV constructs and vectors expressing a codon-optimized aflibercept were generated with varied combinations of regulatory elements of molecular structure. The aflibercept expression by different AAV constructs and vectors was evaluated in cultured mammalian cells and then in mouse retina via subretinal injection of AAV vectors. An investigational AAV gene therapy lead expressing aflibercept was successfully selected based on superior expression and its efficacy was further tested in a laser-induced CNV rat model. Our data demonstrated that the lead AAV.pTHVE002 significantly reduced laser-induced choroidal vascularization at all doses tested, as manifested by reduction of vascular leakage and lesion volume assessed by optical coherence tomography (OCT) and fluorescein angiography (FA). The alleviation of choroidal vascularization by AAV-pTHVE002 is comparable to that by intravitreal injection of EYLEA®, which is a standard-of-care in treating wet AMD patients. In line with improved efficacy, dose-dependent VEGF trap protein expression was observed across all retinal layers as detected by immunohistochemistry (IHC) staining and further mRNA expression confirmed in whole eye tissues by RT-qPCR. Vector genomes were quantitatively detected in the whole eye tissue by ddPCR and visualized to distribute primarily in the inner nuclear layer (INL) and the outer nuclear layer (ONL) of retina by in situ hybridization (ISH). Histology analysis by H&E and IHC staining of specific inflammation markers shows good tolerability following subretinal injection of AAV-pTHVE002. Together, these studies support a feasible path towards clinical development of AAV. pTHVE002 gene therapy for treating wet AMD.

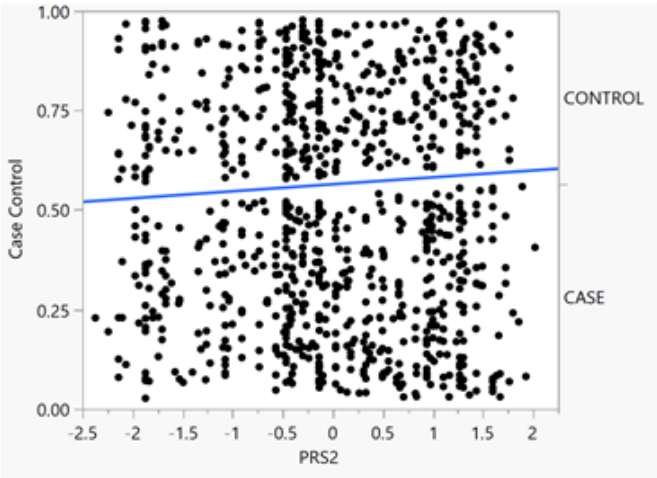
565. Using Functional SNPs to Calculate a Polygenic Risk Score for LOAD

Suraj Upadhya, Michael Lutz, Ornit Chiba-Falek

Division of Translational Brain Sciences, Duke University, Durham, NC

Introduction: Due to the underlying genetic basis for the development of Alzheimer's disease (AD), genome wide association studies (GWAS) have been done to uncover associated single nucleotide polymorphisms (SNPs). Creating a polygenic risk score (PRS) by using only functional, or gene expression altering, SNPs has the potential to predict cases on an individual basis and become a biomarker for clinical studies while exploring potential targets for gene therapies. **Methods:** Two PRS were calculated and evaluated using the ROSMAP dataset (n=1728), where PRS1 included nine functional SNPS and PRS2 excluded two chromosome 19 SNPs due to potential linkage disequilibrium with *APOE*. Normal-logistic curves and receiver operating characteristics (ROC) curves were created to determine efficacy in distinguishing late-onset Alzheimer's Disease (LOAD) cases and controls. Linear regression models were used to evaluate phenotypic associations. **Results:** PRS1 effectively discriminated between cases and controls demonstrated by the case-control plot ($p<0.001$) and an AUC of 0.617 using PRS1, *APOE*, and sex as factors and AUC of 0.580 using PRS1 alone. In addition, PRS1 was strongly associated with amyloid plaques, neurofibrillary tangles, and age at diagnosis (p=2e-13, p=4e-09, and p=1e-05). PRS2 was unable to effectively differentiate between cases and controls with an AUC of 0.519 using PRS2 alone and no significant relationship in the case-control plot. Furthermore, PRS2 did not show any associations with phenotypes. **Conclusions:** Although PRS1 demonstrated predictive power, the *APOE* locus carried much of the weight, as exhibited by the lack of predictive power of PRS2, thus supporting *APOE's* potential as a target for gene therapies. However, among non-*APOEε4* carriers, the method of utilizing functional SNP PRS to predict disease onset remains a viable path in precision medicine, as more functional SNPs can be included to increase the information the PRS possesses.





AUC using different factors	
Factor(s)	AUC
PRS1	0.580
PRS2	0.519
APOE	0.598
PRS1+APOE+Sex	0.617
PRS2+APOE+Sex	0.611

566. Intravenous Gene Therapy Apporach for Metachromatic Leukodystrophy

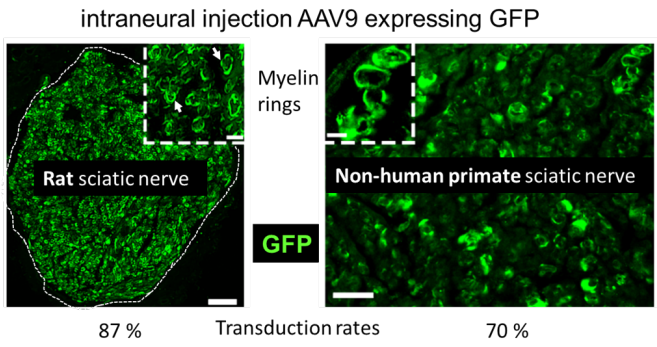
Emilie Audouard, Valentin Oger, Beatrix Meha, Nathalie Cartier, Caroline Sevin, Françoise Piguet
Neurogencell, INSERM U1127 - ICM, Paris, France

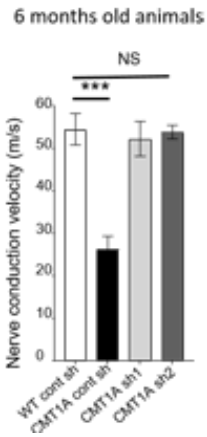
Metachromatic leukodystrophy (MLD) is a rare, autosomal recessive disease caused by deficient activity of the lysosomal enzyme arylsulfatase A (ARSA), resulting in sulfatide accumulation and subsequent demyelination and neuronal loss within the central peripheral nervous systems (CNS, PNS). Three clinical forms of MLD have been described, based on the age of symptom onset. Among them, early-onset forms comprise a continuum between late-infantile MLD (LI-MLD, most frequent accounting for 50-60% of MLD patients) and early juvenile MLD (EJ-MLD). In these early-onset forms, first symptoms typically develop between 1 and 4 years of age and progress rapidly towards severe motor and cognitive regression and premature death. There are currently no approved therapies for early onset MLD once patients are symptomatic. Hematopoietic stem cell transplantation (HSCT) gene therapy with lentiviral vector aiming at overexpressing the ARSA enzyme in bone marrow-derived microglia was shown to be safe and most treated patients exhibit preservation of motor and cognitive functions, associated with protection from CNS demyelination and PNS involvement. Unfortunately, no significant effect was observed once symptoms are already present. Here we proposed a gene therapy approach based on intravenous delivery of an AAVPHP.eB encoding the human ARSA gene with an HA tag. We demonstrated a broad transduction of brain and spinal cord leading to a significant correction of sulfatide storage both in brain and spinal cord in symptomatic animals as well as a significant improvement of neuroinflammation in mouse model of the disease. Ongoing experiments on large animals will also be presented.

567. Proof of Concept for a Local AAV-Based Gene Therapy to Treat CMT1A Disease

Nicolas Tricaud[1,2]
[1]NERVOSAVETX, Montpellier, France,[2]Irmb, INSERM, Montpellier, France

Charcot-Marie-Tooth disease 1A is the main of hereditary peripheral neuropathies. This disease results from the duplication of *PMP22* gene that induces the demyelination of Schwann cells in nerves. This leads to sensory loss and muscular atrophy in limb extremities as main symptoms. We designed a gene therapy based on the use of AAV9 locally injected in nerves whose degeneration lead to the disease symptoms. This vector express a small hairpin RNA targeting PMP22 mRNA in order to decrease the overexpression of the protein and prevent demyelination. Transduction of myelinating Schwann cells by AAV9 after intraneural injection was shown to be highly efficient and specific in sciatic nerves of young and adult mice and rats and in adult macaque. We used CMT1A rats overexpressing mouse PMP22 and mimicking the human disease to perform a proof of concept of this therapy. Two AAV9 expressing different shRNAs targeting mouse/rat PMP22 together with GFP were injected bilaterally in sciatic nerve of young rats and animals were followed for up to 12 months. Either of these treatments were shown to sufficient to reduce PMP22 expression, increase myelination and prevent myelin defects in the injected nerves. Moreover, nerve conduction velocity was maintained in treated CMT1A rats and these animals did not develop the motor and sensory symptoms of the disease on short and long terms. Biodistribution was mostly limited to injected nerves and a limited immune response was observed. These data provide a strong ground for a local gene therapy of peripheral nerves based on AAV9 vectors in the case of demyelinating CMT diseases.





568. Comprehensive Evaluation of an AAV Library in the Mice and Nonhuman Primate Ocular Tissues Following Intravitreal Delivery

Wei-Hua Lee, Devin S. McDougald, April R. Giles, Elad Firnberg, Jenny M. Egley, Samantha A. Yost, Karolina J. Janczura, Justin Glenn, William M. Henry, Kirk Elliott, Randolph Qian, Chunping Qiao, Jared Smith, Ye Liu, Joseph Bruder, Olivier Danos, Subha Karumuthil-Melethil, Andrew Mercer, Xu Wang

REGENXBIO Inc, Rockville, MD

Purpose: Gene therapy is an attractive modality for inherited and acquired vision loss. Adeno-associated virus (AAV) vectors have emerged as a preferred platform to effectively and stably express therapeutic payloads within ocular tissues. The efficiency of gene delivery by intravitreal (IVT) administration has been shown to be limited by immunological and anatomical barriers that reduce transduction of outer retinal cell types with standard AAV serotypes. Advancements in vector discovery and capsid engineering provide new opportunities to identify novel AAV variants with improved ocular tropism. This study aimed to identify AAV variants with improved ocular tropism through IVT administration in mice and nonhuman primates (NHP). **Methods:** An AAV library containing 118 naturally isolated and engineered variants was constructed. AAVs with unique DNA barcodes corresponding to specific capsids were individually packaged, quantified, and pooled as a single vector preparation. C57BL/6 mice received bilateral IVT injections at dose of $1.4x10^{10}$ vg/eye (N = 5) or $1.5x10^{10}$ vg/eye (N = 5). Cynomolgus macaques were prescreened for Nab to AAV2, AAV8, and AAV9 before receiving bilateral IVT injections of the same vector preparations at $3x10^{10}$ vg/eye (N=2) or $4x10^{11}$ vg/eye (N=2). Mouse and NHP ocular samples were harvested three weeks post-injection. DNA and RNA were isolated from these tissues and subjected to next-generation sequencing (NGS) analysis. Vector transduction efficiency was measured as the relative abundance of each barcoded vector adjusted for the corresponding vector input. **Results:** NGS analysis of the retina and retinal pigment epithelium (RPE)-choroid tissue revealed enrichment of previously characterized capsids including AAV2 and an AAV2 variant, in both mouse and NHP tissues. This screen also identified capsids with novel tropisms in mouse retina and RPE-choroid and various NHP ocular tissues, including the retina, RPE, iris, ciliary body, Schlemm's canal, and trabecular meshwork. In the NHP cornea, lens, and optic nerve, only DNA barcodes of capsids were identified, while the transgene mRNA of these capsids was undetectable. **Conclusions:** Collectively, these results provide a comprehensive, high throughput analysis and map of AAV tropism in the eye of mice and NHPs through intravitreal administration. This study suggests that these AAV vectors may enhance gene transfer to selected ocular target tissues and may expand the therapeutic potential of AAV for gene therapy in the eye.

569. Durable Recovery of Auditory Function Following Intracochlear Delivery of AK-OTOF (AAVAnc80-hOTOF Vector) in a Translationally Relevant Mouse Model of Otoferlin Gene (*OTOF*)-Mediated Hearing Loss

Ann E. Hickox[1], Yuan Gao[1], Fan-fan Yu[2], Yuanzhao Darcy[1], Kathleen Reape[3], Shimon P. Francis[1], Ye-Hyun Kim[1], Samantha Davis[1], Pascal Schamber[1], Junaid Syed[1], Katherine D. Gribble[1], Enping Qu[1], Richard M. Churchill Jr.[1], Eva Andres-Mateos[1], Jennifer A. Wellman[1], Michelle D. Valero[1]

[1]Akouos, Inc., Boston, MA,[2]Statistics Collaborative, Inc., Washington, DC,[3]Ark Medical Consulting, LLC, Newtown Square, PA

Mutations in the otoferlin gene (*OTOF*) cause an autosomal recessive form of sensorineural hearing loss, characterized by Severe to Profound sensorineural hearing loss from birth. The lack of normal otoferlin protein in the cochlea impairs synaptic signaling between the cells that sense sound energy (inner hair cells; IHCs) and the cochlear nerve fibers that transmit sound information to the brain. As a consequence, the highly synchronized neuronal responses required for hearing are diminished in individuals with mutations in *OTOF*, often characterized by an absent or abnormal auditory brainstem response (ABR). The concurrent presence of otoacoustic emissions (OAEs), which reflects functioning cochlear outer hair cells (OHCs), indicates that *OTOF*-mediated hearing loss is not a consequence of global cochlear dysfunction but rather is specific to impaired neurotransmission. AK-OTOF, a product candidate in preclinical development, is a dual AAVAnc80 vector encoding the nearly 6 kB cDNA human otoferlin; it is intended to treat individuals with *OTOF*-mediated hearing loss by gene transfer and durable

expression of a normal, functional otoferlin protein to affected cells of the cochlea (IHCs) following intracochlear administration. Gene therapy for *OTOF*-mediated hearing loss is expected to confer the greatest benefit when global cochlear function is normal, i.e., synaptic signaling between the IHCs and cochlear nerve is the primary deficit. Several reports indicate that OAE responses diminish or disappear within the first decade of life in individuals with *OTOF*-mediated hearing loss, suggesting a potential decline in cochlear function. We performed a meta-analysis of 89 peer-reviewed articles, reporting audiologic data in over 300 individuals with biallelic mutations in *OTOF* and covering a broad spectrum of *OTOF* mutations, to describe the natural history of *OTOF*-mediated hearing loss, including a systematic characterization of change in OAE responses as a function of age. Additionally, we demonstrate the translational relevance of an *Otof* knock-out (*Otof* $^{-/-}$) mouse model through characterization of OAE responses up to 10 months of age compared to wild-type conspecifics. Finally, we demonstrate a robust and durable recovery of auditory function in *Otof* $^{-/-}$ mice following administration of AK-OTOF encoding human otoferlin at an age when IHC innervation is mature but prior to the decline of OAEs. Taken together, these data provide support for future clinical development of AK-OTOF for the treatment of *OTOF*-mediated hearing loss.

570. Modeling Friedreich's Ataxia with iPSC-Derived Brain Cells and Characterization of Neuronal Defects Due to FXN Deficiency

Priyanka Mishra[1], Joseph N Rainaldi[1], Emily A Hansen[2], Jacqueline Nguyen[1], Avalon Johnson[2], Nicole Coufal[2], Stephanie Cherqui[1]

[1]Pediatrics, Division of Genetics, University of California San Diego, San Diego, CA,[2]Pediatrics, University of California San Diego, San Diego, CA

Friedreich ataxia (FA) is an autosomal recessive neurodegenerative disease. Most patients carry homozygous GAA expansions in the first intron of the frataxin gene causing reduction in frataxin (*FXN*) expression. FXN is a mitochondrial protein and its deficiency leads to mitochondrial iron overload, defective energy supply and generation of reactive oxygen species.In this study, our aim is to establish a new human model system to advance the understanding of FRDA pathogenesis. We differentiated FA patient-derived induced pluripotent stem cell (iPSC) lines to neurons, microglia cells and cortical organoids and also differentiated isogenic control lines. All iPSC lines have been characterized using well-established immunostaining assays and were found to be karyotypically normal. Interestingly, when we differentiated iPSCs into neurons, we observed a striking phenotype in FXN-deficient neurons. We identified no homogenous microtubule staining along the neurites or in neuronal cell bodies of FA neurons, and instead noted periodic breaks of neuronal immunostaining called blebbing while only few blebbing could be observed in the isogenically corrected cell line. Similar results were obtained in the FA cortical organoids as compared to the isogenically corrected organoids. Dendritic blebbing is a feature of apoptotic neurons. We checked the cell death maker Cleaved-caspase-3 in these cells, and we found that cell death was also higher in FXN-deficient neurons as compared to isogenic control neurons. Overall, this study provides a platform to establish to advance the pathogenesis of FA.

571. ICV Delivery of AAV9-NGLY1 Gene Replacement Therapy Improves Phenotypic and Biomarker Endpoints in Ngly1 Deficient Rats

Lei Zhu, Brandon Tan, Brett Crawford, Selina Dwight, Brendan Beahm, Matt Wilsey, Thomas Wechsler, William Mueller

Grace Science, LLC, Menlo Park, CA

Grace Science has developed GS-100, an AAV9 gene therapy that delivers a functional copy of the full-length human NGLY1 gene for the treatment of NGLY1 deficiency. NGLY1 deficiency is a devastating, ultra-rare, autosomal recessive disease caused by loss of function mutations in NGLY1. Patients suffer from global developmental delay, cognitive impairment, (hypo)alacrima, movement disorders, and other neurological symptoms. No approved therapy for NGLY1 deficiency exists; medications and surgical procedures are often required to manage symptoms. The *NGLY1* gene encodes N-glycanase, a conserved enzyme that cleaves N-glycans from misfolded glycoproteins destined for proteasomal degradation as part of the endoplasmic reticulum-associated degradation (ERAD) pathway. Loss of NGLY1 activity in cells leads to impaired proteotoxic stress response and defects in energy metabolism. Engineered loss of NGLY1 in rats results in decreased survival early in life, severe neurodegenerative phenotypes, and pathological abnormalities in the peripheral and central nervous systems, similar to what is observed in patients. GlcNAc-Asn (aspartylglucosamine, or GNA) is a biomarker of the disease directly related to the activity of NGLY1 and was found to be elevated in both patient and Ngly1 deficient rat samples. *Ngly1*-/- rats were administered GS-100 via intracerebroventricular (ICV), intravenous (IV), or the dual-route of ICV + IV. In the case of ICV and ICV + IV, widespread biodistribution of AAV9 encoding human NGLY1 DNA and corresponding mRNA and protein expression levels were observed in the spinal cord, dorsal root ganglia, hippocampus, cortex, thalamus, brainstem, striatum, and other CNS tissues. ICV + IV administration did not increase NGLY1 transduction or expression levels in the CNS greater than that of ICV administration alone. GS-100 treatment delivered by ICV only or ICV + IV significantly reduced levels of the biomarker GNA in CSF ($P < 0.05$) and brain tissue compared to untreated *Ngly1*-/- rats ($P < 0.05$). Importantly, ICV and ICV + IV GS-100 treated *Ngly1*-/- rats displayed improvement in functional behavioral testing, including significant increases in latency to fall in rotarod testing ($P < 0.001$) and significant improvement in the frequency of rearing in locomotor activity testing ($P < 0.01$). The IV only administration did not provide substantial delivery to CNS tissues, did not consistently reduce GNA accumulation in the CNS tissues, and showed no functional improvement compared to other routes of administration. These data provide evidence that ICV delivery is the preferred route of administration for GS-100 as a therapy for NGLY1 deficiency. Additional non-clinical studies are in progress to identify a safe and efficacious dose for a first-in-human clinical trial.

Neurologic Diseases

572. Policies and Pitfalls: What the Alzheimer's Community Can Learn from the Deployment of Disease-Modifying Therapies for Multiple Sclerosis Patients

Ishika Gupta[1], Boris Kantor[2], Ornit Chiba-Falek[3], Misha Angrist[4]

[1]Duke University, Durham, NC,[2]Department of Neurobiology, Duke University, Durham, NC,[3]Department of Neurology, Duke University, Durham, NC,[4]Initiative for Science and Society and Social Science Research Institute, Duke University, Durham, NC

Alzheimer's disease (AD) is a major public health crisis, affecting more than forty-four million people worldwide. By 2050, that toll is expected to reach 152 million. At present, we have no disease-modifying therapies (DMTs) to address AD. That said, dozens of potential DMTs are in clinical trials and the hope is that one or more will be able to mitigate the enormous burden of AD on patients, families and caregivers. But with effective DMTs comes a slew of concerns. The prices are likely to be exorbitant; even existing generic (and largely ineffective) drugs used for symptomatic AD treatment are expensive. And then there are the questions of how to introduce DMTs; physician and patient acceptance of them; third-party reimbursement and out-of-pocket costs; and surveillance/management of side effects. One approach to anticipating and negotiating these problems is to look to chronic neurological conditions for which there are already multiple DMTs on the market. At present, there are more than 20 FDA-approved drugs designed to safely and effectively treat relapsing forms of multiple sclerosis (MS), none of which are curative and all of which are expensive. We consider historical and current challenges in the development, deployment, and distribution of DMTs for MS and what they might teach the AD community.

573. A Human IPSC-Based Model of Globoid Cell Leukodystrophy Uncovers Early Neurodevelopmental Defects

Elisabeth Mangiameli[1], Anna Cecchele[1], Francesco Morena[2], Francesca Sanvito[3], Vittoria Matafora[4], Angela Cattaneo[4], Lucrezia Della Volpe[1], Daniela Gnani[1], Marianna Paulis[5,6], Lucia Susani[5,6], Sabata Martino[2], Raffaella Di Micco[1], Angela Bachi[4], Angela Gritti[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Chemistry, Biology and Biotechnology, University of Perugia, Perugia, Italy,[3]Pathology Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy,[4]IFOM-FIRC Institute of Molecular Oncology, Milan, Italy,[5]Humanitas Clinical and Research Center-IRCCS, Rozzano, Milan, Italy,[6]National Research Council (CNR)-IRGB/UOS of Milan, Milan, Italy

<Globoid Cell Leukodystrophy (GLD) is a lysosomal storage disorder caused by mutations in the b-galactocerebrosidase (GALC) gene. GALC deficiency causes the accumulation of toxic galactosphingolipids (e.g. psychosine), which promote a rapid and severe CNS and PNS demyelination/neurodegeneration. The mechanisms of disease onset/progression and disease correction upon treatments have been investigated in murine models but are still poorly elucidated in human neural cells. In this work, we studied the impact of GALC deficiency and lentiviral (LV) vector-mediated GALC rescue/overexpression in a panel of GLD hiPSC-derived neural stem/progenitor cells (hiPSC-NPCs) and their neuronal/glial progeny. GLD iPSCs showed undetectable GALC activity. Psychosine accumulation increased during NPCs differentiation and normalized upon lentiviral vector (LV)-mediated delivery of a functional hGALC. Still, LV-mediated rescue of GALC activity only partially rescued the defective oligodendroglial and neuronal cell commitment/differentiation of GLD NPCs, whose severity was cell type- and patient-dependent. These results suggested that multiple mechanisms besides psychosine storage are involved in GLD pathogenesis. Indeed, global lipidomic profiling showed a profound and peculiar unbalance in the relative levels of structural and bioactive lipids in GLD cells. The altered lipidome was associated with a senescence phenotype displayed by GLD neurons/glial cells and possibly contributed to the impaired neural differentiation program. Our data also revealed that GALC overexpression negatively impacts the neuronal/glial differentiation of healthy donor hiPSC-NPCs, suggesting that regulated GALC expression is crucial for proper human neural cell commitment and maturation. This work uncovers unforeseen mutation- and cell-type-specific early pathogenic events that may contribute to GLD neuropathology, paving the way to tailored gene/cell therapy strategies to enhance disease correction in GLD CNS cells.>

574. Dose Response of Dual Route *scAAV9-HexM* Gene Transfer in a Mouse Model of Sandhoff Disease

Brianna M. Quinville[1], Alex E. Ryckman[1], Natalie M. Deschenes[1], Melissa Mitchell[2], Zhilin Chen[2], John G. Keimel[3], William F. Kaemmerer[3], Jagdeep S. Walia[4]

[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada,[2]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada,[3]New Hope Research Foundation, North Oaks, MN,[4]Medical Genetics/ Department of Pediatrics, Queen's University, Kingston, ON, Canada

Sandhoff disease (SD) is caused by the excessive accumulation of GM2 gangliosides in the lysosomes of neuronal cells. Typically, these lipids are hydrolyzed by an enzyme, β-hexosaminidase A (Hex-A), a heterodimer comprised of an α- and a β-subunit. Mutations in the gene encoding either subunit can lead to improper functioning of the enzyme. SD is caused by a mutation in the *HEXB* gene resulting in a deficient or absent β-subunit and subsequent accumulation of GM2 gangliosides. This causes widespread cell death, and consequently progressive symptoms and rapid neurological decline culminating in death by age 5 in the most prevalent, infantile form of the disease. A homodimer formed by a novel hybrid μ-subunit called HexM, an isoenzyme of human Hex-A, has been recently developed and shown to hydrolyze GM2 gangliosides *in vivo*. Previous studies have determined the effectiveness of gene transfer with the gene, *HEXM,* packaged in a self-complementary adeno-associated viral vector, serotype 9 (scAAV9), through increased life span in a SD mouse model ($Hexb^{(-/-)}$). This study aims to determine the dose response of the *scAAV9-HEXM* treatment in the SD mouse model through dual delivery of treatment

via intra-cisterna magna (ICM) and intravenous (IV) routes, along with the ancillary administration of immunosuppressant drugs, rapamycin and prednisone. Treatment for 10 long-term cohorts of 10 mice involved concurrent infusions through both ICM and IV routes. There were three possible infusates for the ICM route (vehicle, low or high vector dose) and six possible infusates (vehicle, 5 different vector doses) for the IV route. The study design allows an efficacy comparison of the total dose when administered either through one route of delivery or split between the two. Mice have been randomly assigned to each cohort. The researcher conducting the procedure and subsequent testing is blinded to which dosage each animal received. Bimonthly behavioural testing and blood collections at specific time points are being done until mice reach their humane endpoint as determined by specific UACC criteria. An additional 4 short-term cohorts of 4 mice were terminated at 16 weeks of age. At termination, blood, gross organs, brain, and spinal cord are collected for analysis of GM2 ganglioside accumulation, vector copy number, enzyme activity, cellular and humoral immune response, rapamycin level and histology. The long-term study is still on-going. However, the survival data from the various cohorts obtained so far allow the following conclusions made by an unblinded data monitor. First, every treatment group has already demonstrated longer survival than the vehicle-only control group ($p < 0.001$ in all cases). Among cohorts receiving the *scAAV9-HEXM* vector by ICM infusion only, there is a clear dose-response relationship between the amount of vector delivered and survival. The higher ICM (2.5e11 vector genomes [vg]) dose group's survival is significantly longer than that of the lower ICM (1e11 vg) dose group ($p < 0.003$). Second, the data so far suggest ($p = 0.082$) that a dual route of delivery (ICM and IV) provides an added survival benefit over the same total dose (5e11 vg) delivered by the IV route alone. More data from this on-going study will determine whether an added survival benefit of the dual route of delivery remains with a higher total dose. Survival data for the long-term cohorts will be presented along with the short-term cohort data.

575. Adeno-Associated Vector Discovery Platform for Inner Ear Disorders

Arnaud PJ Giese, Patrice Vidal, Montserrat Bosch Grau, Audrey Broussy, Selma Dadak, Mickael Deage, Vincent Descossy, Mathieu Petremann, Christophe Tran Van Ba, Julie Duron Dos Reis, Pauline Liaudet, Laurent Désiré

Preclinical, Sensorion SA, Montpellier, France

Around 466 million people worldwide have hearing loss. It is estimated that over 900 million people will have disabling hearing loss by 2050. More than half of the congenital non-syndromic deafness cases have a genetic cause, and 80% are inherited in an autosomal recessive fashion. There are no approved curative therapies for genetic hearing loss and cochlear implantation is the only option proposed to young patients. Even though this solution improves the quality of life and language acquisition, hearing recovery is limited and thus more targeted treatments are overall unmet medical needs. Adeno-associated virus (AAV) is a vector of choice for *in vivo* gene therapy. Many serotypes, either natural or synthetic, allow using the vector for several indications. As the number of clinical trials and successes is increasing, the field is reaching bottlenecks including issues with immunogenicity, toxicity, vector quantity and expression specificity. The inner ear is a closed system which contains a small volume. The organ contains 3 major types of highly specialized functional cells: sensory cells (about 3,500 inner hair cells and 12,000 outer hair cells) but also supporting cells, and spiral ganglion neurons, all of which play an important role in the process of hearing and therefore could be selectively targeted by gene therapy. Therefore, the production of a gene therapy product, especially for the inner ear, requires the development of novel tissue-targeted capsids and cell-specific expression cassettes aiming to reach a therapeutic effect using a minimal dose. Here we describe the newly developed platform at Sensorion, allowing fast screening of AAV candidates for inner ear indications. First, we established an *in vitro* high throughput selection process allowing to sort the best capsids and regulatory element among tens of candidates in few weeks. Using an *ex vivo* approach, the platform allows to culture rodent cochlear and vestibular explants, and either infect them with AAVs or electroporate them with plasmids of interest. The imaging acquisition has been fully automatized for efficient data analysis and quantification, (3D and live imaging). Selected candidates are further validated by *in vivo* injection into the inner ear using a canalostomy or round window injection approach. Afterward, inner ear functions are assessed via Sensorion's audiology platform which includes auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) recordings. Models for testing include genetic models, noise induced hearing loss, synaptopathic and vestibular disorders. The relevant tests are assessed in large groups of animals and are supported by morphological and immunohistochemical examination. The gene therapy platform allows Sensorion to identify and validate novel AAV variants and cell-type specific expression cassettes of interest for new proprietary therapeutic products to treat inner ear disorders.

576. Advances towards a Dual AAV CRISPR-Cas9-Based 'Knockout and Replace' Strategy to Treat Rhodopsin-Associated Autosomal Dominant Retinitis Pigmentosa

Deepak Reyon

Editas Medicine, Cambridge, MA

Mutations in the rhodopsin (*RHO*) gene are the most common cause of autosomal dominant Retinitis Pigmentosa (adRP), a retinal disease that results in blindness due to photoreceptor degeneration. Over 150 autosomal dominant mutations have been identified in the *RHO* gene. Therefore, we are developing a CRISPR-Cas9-based mutation-independent therapeutic strategy that simultaneously 'knocks out' endogenous rhodopsin, and 'replaces' it with exogenous functional rhodopsin using a dual AAV system. Potent, highly specific guide RNAs and an optimized *RHO* expression vector have been identified and used in studies to address the following questions: 1. Could the novel alleles generated as a consequence of on-target editing be dominant negative? 2. What is the optimal ratio of the two AAV vectors? An *in vitro* overexpression system was developed to quantify the effect of novel, editing-induced, alleles on cell viability. Three novel alleles were tested, one in each open reading frame as an indicator of potential dominant negative alleles generated by on-target editing. The system was benchmarked using *RHO*-P23H as the dominant negative allele and WT *RHO* as the normal allele. To confirm *in vitro* results, and to identify the optimal ratio of the two AAVs, the

dual AAV system was tested at multiple ratios in humanized *RHO* mice. The optimal ratio of vectors and percentage of potential novel deleterious alleles was determined by quantifying on-target editing by NGS at 6 and 13-weeks post subretinal injection. Examination of the on-target editing site in humanized mice by NGS revealed low percentage of potential deleterious alleles and a consistent INDEL profile at 6 and 13-weeks post injection, demonstrating that the risk of generating novel dominant negative alleles is minimal. Different ratios of dual AAV vectors showed corresponding change in the level of *RHO* KO and replacement. The configuration that provides the highest level of *RHO* replacement and sufficient on-target editing has been identified as the 'optimal ratio' for future studies. In conclusion, we have demonstrated minimal risk of generating novel dominant negative alleles, and the feasibility of achieving therapeutically relevant levels of editing and replacement using an optimized ratio of the two AAV vectors. We are moving forward to preclinical studies for further characterization of the dual AAV system *in vivo*.

577. Selective Repression of C9ORF72 Repeat Expansion Containing Sense and Antisense Transcripts for the Treatment of ALS

Mohammad Samie[1], Amrutha Pattamatta[2], Dianna Baldwin[1], Sarah Hinkley[1], Emily Tait[1], Nicholas Scarlott[1], David Shivak[1], Lei Zhang[1], Ricardos Tabet[2], Anagha Sawant[2], Robert Bell[2], Robert Moccia[2], John Murphy[2], Christine Bulawa[2], Bryan Zeitler[1], Amy Pooler[1]

[1]Sangamo Therapeutics, Richmond, CA,[2]Pfizer, Cambridge, MA

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease, characterized by the loss of motor neurons in the CNS, leading to paralysis and early death. The most frequent genetic cause of ALS is the expansion of hexanucleotide GGGGCC (G_4C_2•G_2C_4) repeats in the first intron of *C9ORF72* gene. Analysis of patient autopsy tissue shows that G_4C_2•G_2C_4 repeats undergo bidirectional transcription generating sense and antisense expansion containing RNA and RNA foci as well as repeat-derived dipeptide translation products, suggesting a pathological, gain-of-function mechanism. To decrease the levels of expansion-containing transcripts in cells, while maintaining expression of healthy C9ORF72 mRNA levels, we designed a library of engineered transcription factors comprised of a zinc finger protein (ZFP) specifically targeting the G4C2 repeat region fused to a DNA-binding repressor protein (KRAB). Using patient-derived fibroblasts, we were able to identify ZFP-TFs that selectively repress >90% of both sense and antisense G_4C_2 containing transcripts over a wide dose range while preserving the expression of >50% of C9ORF72 mRNA levels. Expression of other G_4C_2-containing genes was minimally affected, and we identified several ZFP-TFs with minimal to no off-target activity. To confirm the effect of these ZFP-TFs in a disease-relevant cell model, we generated motor neurons from patient-derived iPSCs carrying ~1200 G_4C_2 repeats. Similar to the effect observed in fibroblasts, the selected ZFP-TFs, packaged in AAV also displayed >90% reduction of both sense and antisense G_4C_2 containing transcripts over a wide dose range while preserving the expression of >50% of C9ORF72 mRNA levels. Minimal to no modulation of other genes was observed by transcriptomics analysis. These findings illustrate the potential use of ZFP-TFs for the treatment of familial ALS.

578. PS Gene Editing with an Engineered HEXO Construct to Treat Both Tay-Sachs and Sandhoff Diseases

Li Ou, Michael Przybilla, Sarah Kim, Chester Whitley

Pediatrics, University of Minnesota, Minneapolis, MN

GM2-gangliosidoses result from the deficiency of a lysosomal enzyme β-hexosaminidase (Hex) and accumulation of GM2 gangliosides. Genetic deficiency of HEXA, encoding the Hex α subunit, or HEXB, encoding the Hex β subunit, causes Tay-Sachs disease and Sandhoff disease, respectively. We designed a novel HEXX sequence that can also form a homodimer. To improve the efficiency of entering the brain, we performed 3D structural analysis of fusion proteins of HEXX and potential carriers (e.g., ApoE, ApoB, RTB, IGF-2). A HEXO construct composed of HEXX and ApoE was selected for *in vivo* experiments. Then, the *PS Gene Editing* system with HEXO as the cDNA was injected into neonatal Sandhoff mice at three different doses (n=8-14 each group). HEXO achieved 4.0- and 2.1-fold higher plasma enzyme activities than HEXM and HEXX, respectively. Open field test, pole test, mesh test, and rotarod test showed more significant improvements in motor function, coordination, grip strength, and learning ability in mice treated with HEXO than HEXX and HEXM ($p<0.05$). Ganglioside levels will be quantified by HPLC-MS, and histology will be performed to assess correction of pathological cellular vacuolation. As to safety, plasma albumin and creatinine levels of treated mice were not significantly different from controls. No humoral immune responses against Cas9 proteins were detected through ELISA. However, there were significant antibodies against AAV8 capsids in mice treated as adults. Plasma AST and ALT levels, as well as mRNA expression of cytokine (CD4, CD8, TNF-alpha, IFN-gamma) were also not increased, indicating a low risk of cellular immune response. In addition, PacBio Long Read Sequencing identified various on-target gene editing events, including homology directed repair, indels, AAV vector integration, and large deletions. In summary, a novel HEXO transgene, designed for enhanced blood-brain barrier transit, was found to (1) achieve higher *in vivo* levels of catalytic enzyme activity and (2) more significant improvements in neurobehaviors than HEXX and HEXM in Sandhoff mice. Ongoing studies will further assess the biologic response of HEXO expression by the PS Gene Editing System and its potential as a superior approach to treating Tay-Sachs and Sandhoff diseases.

579. Development of an AAV-Based Gene Therapy for Children with Congenital Hearing Loss Due to Otoferlin Deficiency (DB-OTO)

Adam Palermo[1], Xichun Zhang[1], Arun Senapati[1], Joseph Goodliffe[1], Bifeng Pan[1], Tera Quigley[1], Lillian Smith[1], Peter Weber[1], Sarah Cancelarich[2], Danielle Velez[2], Tyler Gibson[1], Lars Becker[1], Ning Pan[1], Leah Sabin[2], Meghan Drummond[2], Jonathon Whitton[1]

[1]Decibel Therapeutics, Boston, MA,[2]Regeneron Pharmaceuticals, Tarrytown, NY

Background

Otoferlin deficiency secondary to biallelic mutations of the OTOF gene causes permanent congenital severe-to-profound deafness. Otoferlin is expressed in the primary sensory receptors of the ear (the inner hair cells) and enables synaptic transmission between the inner hair cells and the dendrites of the auditory nerve by functioning as a calcium sensor for exocytosis during synaptic vesicle release, vesicle trafficking, and replenishment. Of the 1 in 500 neonates who are born with hearing loss annually in the US, 50 to 200 are caused by Otoferlin deficiency. There are no approved therapies for Otoferlin deficiency; infants with biallelic OTOF mutations are currently managed with assistive devices.

Methods

We have developed an Adeno-Associated Virus (AAV)-based gene transfer therapy for rescue of hearing in an Otoferlin deficiency model that mimics a common human OTOF mutation ($OTOF^{Q828X/Q828X}$). Because human OTOF cDNA exceeds the packaging capacity of a standard AAV, we used a dual AAV system to locally deliver Otoferlin to the inner ears of $OTOF^{Q828X/Q828X}$ mice. Key considerations for vector development included the human OTOF isoform that should be expressed, the promoter sequence that determines in which cells the human OTOF will and will not be expressed, the recombinogenic region that enables the dual AAV system, and the capsid in which these elements are packaged for efficient delivery into the target cells.

Results

Using a local dual AAV delivery of hOTOF, pharmacological modulation of Otoferlin expression and durable functional rescue as measured by the Auditory Brainstem Response were demonstrated in the $OTOF^{Q828X/Q828X}$ mice with dosing in mature ears. Safety of efficacious dosing with dual AAV delivery of hOTOF under the control of a hair cell-specific promoter was demonstrated in $OTOF^{Q828X/Q828X}$, $OTOF^{Q828X/wt}$, and $OTOF^{wt/wt}$ mice. Comparison vectors that did not include an engineered promoter to restrict transgene expression to target cells resulted in dose-limiting toxicity, a finding consistent with recent observations for retinal gene therapies and a critical consideration for translation to the clinic. Thus, we selected a synthetic cell-specific promoter to bring forward for therapeutic development. In order to advance DB-OTO (dual AAV Delivery of human OTOF with a cell-specific promoter) to the clinic for children with severe-profound SNHL due to biallelic OTOF mutations, we developed a surgical delivery approach that could be translated into routine otolaryngological practice. This approach involves slow AAV infusion through the RW membrane alongside vestibular fenestration for egress of displaced perilymph by the investigational product. Exploratory studies in non-human primate and human temporal bones suggest the feasibility of this approach for clinical translation.

Conclusions

DB-OTO is a promising emerging therapeutic for genetic hearing loss, and and has the potential to provide the first clinical proof-of-concept for gene therapy in the inner ear.

580. CRISPR-Cas13-Mediated Knockdown of Ataxin-2 in a Stress Granule Cell Model and a TDP-43 Rodent Model

Maria Alejandra Zeballos C., Hayden Moore, Jackson E. Powell, Thomas Gaj

Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL

Amyotrophic lateral sclerosis (ALS) is an incurable and fatal neurodegenerative disorder characterized by the progressive loss of motor neurons in the brain and spinal cord. A major pathological hallmark of the disease, present in more than 90% of ALS cases, including familial and sporadic forms of the disorder, is the cytoplasmic mislocalization and aggregation of TAR DNA-binding protein 43 (TDP-43). TDP-43 toxic deposition can be induced by the formation of highly dynamic cellular structures, termed stress granules (SGs), that assemble transiently in response to cellular stress. Ataxin-2 (ATXN2) is an RNA-binding protein that has been identified as a crucial SG component and a contributor to neurodegeneration and has been found to abnormally localize with cytoplasmic accumulations in motor neurons of ALS patients and associate with TDP-43. Accordingly, antisense oligonucleotide (ASO)-mediated reduction of ATXN2 has been demonstrated to decrease stress-induced TDP-43 pathology in human cells and in a TDP-43 mouse model of ALS, indicating its potential as a therapeutic target. However, the application of ASOs for clinical care has several limitations, including their transient effect and the resulting need to repeatedly administer an ASO over the lifetime of a patient. RNA-targeting CRISPR-Cas13 systems are an alternative technology for gene silencing that can enable long-term knockdown within cells in the nervous system when delivered via a viral vector, such as an adeno-associated virus (AAV). Thus, we have hypothesized that Cas13-mediated ATXN2 mRNA knockdown can be used to reduce the cytoplasmic mislocalization and accumulation of ATXN2 and, consequently, diminish the formation of TDP-43 toxic inclusions in an ALS-like background. To test this hypothesis, we developed a reporter system that links mouse ATXN2 cDNA expression to EGFP fluorescence. Using this reporter, we identified a panel of CRISPR-Cas13 effectors that can knock-down mouse ATXN2 with efficiencies exceeding 90%, which we confirmed by western blot. Owing to homology between the mouse and human ATXN2 cDNAs, we harnessed one of these systems to modulate the dynamics of stress granule formation in human cells, which we tested by first transfecting cells with an ATXN2-targeting CRISPR-Cas13 system and then inducing stress in these cells using an SG-provoking agent. Using immunofluorescence, we found that cells expressing the ATXN2-targeting guide RNA and Cas13 exhibited an ~40% reduction in $ATXN2^{+}$ inclusion formation and an ~30% reduction in the average size of $ATXN2^{+}$ inclusions. Additionally, compared to control cells, we found that CRISPR-Cas13 decreased the average size of $G3BPI^{+}$ SGs by ~43%, indicating that ATXN2 knockdown by CRISPR-Cas13 can inhibit stress-induced assembly of SG cores. We are now evaluating this strategy in a TDP-43 mouse model to further characterize its effect on ALS-like pathology. Thus far our results demonstrate a trend of improved motor function and hindlimb strength, reduced gait impairment and kyphosis, and prolonged survival in mice injected with the ATXN2-targeting platform. Our results therefore indicate that CRISPR-Cas13 technology can be used to modulate the dynamics of ALS-linked SGs and to modify its pathology *in vivo*. In the future, our technology may help to prevent the aggregation of SG-associated RNA binding proteins that contribute to neurodegeneration.

581. Progress toward Complete Treatment of ATP7A-Related Copper Transport Disorders

Andrew T. DiStasio, Stephen G. Kaler
Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH

ATP7A-related copper transport disorders span a broad clinical spectrum, from often fatal infantile-onset illness (Menkes disease), to less severe, childhood-onset phenotypes (mild Menkes disease and Occipital horn syndrome) that primarily involve connective tissue disturbances and dysautonomia due to copper-enzyme deficiencies, to adult-onset isolated distal motor neuropathies that have no overt connection to copper metabolism. These X-linked recessive disorders are caused by mutations in *ATP7A*, an essential copper transporter and have an estimated combined prevalence of 1 in 35,000 live male births. While patients with Menkes disease treated with subcutaneous Copper Histidinate (CuHis) injections often show improved survival and neurodevelopmental outcomes, combining CuHis with adeno-associated virus (AAV)-mediated gene therapy to provide some working copies of the *ATP7A* gene represents an even more promising approach. We are utilizing several different mouse models to evaluate the safety and efficacy of AAV-mediated experimental therapeutics for clinical application, while concurrently developing approaches for population-based newborn screening for ATP7A-related illnesses. The connective tissue aspects of these disorders require correction of lysyl oxidase deficiency, a copper-dependent enzyme resistant to copper treatment alone. The *mottled-blotchy* (*mo-blo*) mouse is a model of Occipital horn syndrome caused by a leaky splice junction mutation (IVS11, DS,+3, A>C) in the mouse homolog, *Atp7a*. Affected *mo-blo* mice manifest premature death due to abnormal aortic morphology (aneurysms in >90% by 6 months of age) and pulmonary emphysema. Efforts to rescue this phenotype using systemic AAV delivery are in progress. This approach carries the potential to improve the connective tissue defects associated with ATP7A dysfunction in human subjects and provides a unique opportunity to glean mechanistic information about lysyl oxidase (LOX) genes. Similarly, characterization and treatment of a mouse model of ATP7A-related distal motor neuropathy will help further illuminate the role of this copper transporter in motor neuron biology. Taken together, potential advances in newborn screening for ATP7A-related disorders by DNA sequencing and progress in small molecule (CuHis) and AAV gene therapies augur well for improved life expectancy and outcomes for affected individuals and their families.

582. Determining the Role of CTCF Loops in Neuron Specific Paternal Imprinting of *UBE3A*

Orangel Gutierrez Fugon[1], Dag Yasui[2], Janine LaSalle[2], David J. Segal[3]
[1]Integrative Genetics and Genomics, UC Davis, Davis, CA,[2]Medical Microbiology and Immunology, UC Davis, Davis, CA,[3]Biochemistry and Molecular Medicine, UC Davis, Davis, CA

Angelman syndrome (AS) is a rare but severe neurogenetic disorder characterized by seizures, ataxia, and a happy demeanor. AS is caused by a deletion of the maternal *UBE3A* allele. *UBE3A* encodes a ubiquitin ligase E3A that regulates synaptic development. It is biallelically expressed in non-neuronal cells but undergoes paternal imprinting in neurons. Previous studies have shown that an antisense transcript is responsible for this silencing, but it is currently unknown how this neuron-specific transcription is regulated. The transcript originates at an upstream *SNRPN* gene, extends past a noncoding gene *PWAR1,* and continues to transcribe antisense to *UBE3A* (*UBE3A-ATS*), however in non-neurons it terminates at *PWAR1*. The presence of binding sites for the loop forming protein CTCF within this region suggest that it may play a pivotal role in performing a boundary function. CTCF has been shown to regulate expression of other parentally imprinted genes. The design of an improved strategy for *UBE3A* unsilencing to treat AS is partly limited by the lack of understanding of how tissue-specific imprinting is controlled at this locus. To address this gap in knowledge we have developed an innovative system to study this critical boundary region using epigenetic editing tools and a human neuronal precursor model. The objective of this research is to characterize the tissue-specific differences in methylation and chromatin structure at *PWAR1* that determine how *UBE3A* imprinting is controlled by CTCF loops. Prior studies showed that CTCF binding at *PWAR1* is reduced upon neuronal differentiation and that methylation inhibits CTCF's binding sites and subsequent looping activity. Therefore, I hypothesize that CTCF creates a chromatin loop in non-neurons that prevents the extension of *UBE3A-ATS* past *PWAR1*. Furthermore, CTCF is evicted by DNA hypermethylation in neurons, allowing extension of the antisense transcript past *PWAR1*. Determining the role of chromatin loops in tissue-specific imprinting utilizing this proposed human cell culture model may hasten the translation of epigenetic unsilencing strategies for novel AS therapies. In order to differentiate transcripts from the maternal allele from transcripts on the paternal in LUHMES cells, allele-specific SNPs were identified using 10X Genomics linked-read sequencing. Parentage was determined using a methylation sensitive restriction enzyme on a differentially methylated region at the *SNRPN* promoter. To assess the differentially methylated state at *PWAR1*, pyrosequencing was performed and showed that methylation was approximately 60% in brain tissue while in HEK293T cells it was about 6%. To determine if CTCF is frequently bound at *PWAR1* in non-neurons, ChIP-PCR was carried out in HEK293T cells demonstrating that DNA from *PWAR1* bound to CTCF is 10 times more enriched in comparison to an IgG control ($2^{-\Delta\Delta Ct}$ = 238 vs 21). Publicly available ENCODE CTCF ChIP-seq data for LUHMES was used to determine possible loop configurations for CTCF bound at the critical boundary region. qRT-PCR was performed to demonstrate that *UBE3A-ATS* is only expressed in differentiated LUHMES neurons and brain tissue but not in undifferentiated LUHMES and HEK293T cells. A timeline of LUHMES cell's *UBE3A-ATS* expression throughout eight days in differentiation media was completed, showing a 10-fold increase in expression of *UBE3A-ATS* after 4 days ($2^{-\Delta\Delta Ct}$ Day 1=5, Day 4=511) of differentiation continuing to increase until harvesting at day 8 (Day 8=11,781). Taken together these preliminary findings support the hypothesis that CTCF binding at *PWAR1* is decreased in neuronal cells in association with increased methylation at its binding site and that LUHMES cells serve as an appropriate model for studying *UBE3A-ATS*.

583. AAV8-*PEX1* Gene Delivery Improves Retinal Functions and Peroxisome Metabolites in a Mouse Model for Zellweger Spectrum Disorder

Catherine Argyriou[1], Ji Yun Song[2], Anna Polosa[3], Devin McDougald[2], Bradford Steele[4], Erminia Di Pietro[1], Frederic Fournelle[5], Pierre Chaurand[5], Joseph Hacia[4], Pierre Lachapelle[3], Jean Bennett[2], Nancy Braverman[1]

[1]Human Genetics, McGill University, Montreal, QC, Canada,[2]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Ophtalmology & Visual Sciences, McGill University, Montreal, QC, Canada,[4]Keck School of Medicine, University of Southern California, Los Angeles, CA,[5]Chemistry, Universite de Montreal, Montreal, QC, Canada

Zellweger spectrum disorders (ZSDs) are a group of autosomal recessive disorders caused by mutations in any one of 13 *PEX* genes whose protein products are required for peroxisome assembly and function. Nearly all affected individuals develop a potentially blinding retinopathy. To test whether we could improve vision in these patients, we performed a proof-of-concept trial for *PEX1* retinal gene therapy using our mouse model homozygous for the murine equivalent of the common human PEX1-G843D mutation (G844D). This model exhibits a gradual decline in scotopic full field flash electroretinogram (ffERG) response, a residual photopic ffERG response, diminished visual acuity (optomotor reflex, OMR), and photoreceptor cell anomalies. AAV8.CMV.HsPEX1.HA vector was administered by subretinal injection to 2 mouse cohorts of 5 or 9 weeks of age, representing earlier and later disease stages; AAV8.CMV.GFP vector was used as a control in the contralateral eye. Successful expression of HsPEX1.HA protein in the photoreceptor layer with no gross histologic side effect was observed by immunohistochemistry. *In vitro* studies using *PEX1*-deficient mouse and human cell lines confirmed vector expression and successful recovery of peroxisome import after *HsPEX1* delivery. Preliminary ffERG analyses on a subset of animals at 8 weeks post injection showed two-fold increase in photopic ffERG in the *PEX1*-injected eyes. ffERGs were improved by 1.6 to 2.5-fold at 25-weeks of age in both cohorts (16 or 20 weeks post gene delivery). At 32 weeks of age (23 or 27 weeks post gene delivery), the average ffERG response in the *PEX1*-injected eyes was double that of GFP-injected eyes or non-injected eyes in both cohorts. LC-MS/MS was used to measure peroxisomal metabolites in whole retinas, revealing that in the younger cohort *PEX1* gene augmentation reduced the average C26:0 lysophosphatidylcholine (lyso-PC) in PEX1-G844D mice, which is elevated due to peroxisome dysfunction. Finally, matrix-assisted laser desorption/ionization (MALDI)-imaging MS was used to resolve the spatial distribution of retinal lipids along retinal sections, and we explore the use of this technique for correlating regions of rAAV delivery with biochemical recovery. This could provide a power new tool for demonstrating metabolic recovery at distinct tissue regions following rAAV gene delivery, without contamination by non- transduced regions. Overall, our results support the potential of retinal gene augmentation to improve vision for ZSD.

584. *In Vivo* Evaluation of Novel Synthetic Promoters for CNS Gene Therapy

Riccardo Privolizzi[1], Maha Tijani[1,2], Enzo Giardina[1], Sinclair Cooper[2], Tony Oosterveen[2], Juan Manuel Iglesias[2], Simon N Waddington[1,3], Michael L Roberts[2], Joanne Ng[1]

[1]Gene Transfer Technology Group, EGA Institute for Women's Health, University College London, London, United Kingdom,[2]AskBio (Europe), Roslin Innovation Centre, Edinburgh, United Kingdom,[3]Wits/SAMRC Antiviral Gene Therapy Research Unit, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Gene therapy for previously untreatable neurological diseases is now a clinical reality. Neurological diseases are diverse with different brain regions and cell types affected and the development of customised design vectors is required before gene therapy can address the diversity of neurological disorders. Using AskBio's Synpromics bioinformatics-based promoter design platform, novel synthetic central nervous system (CNS) promoter candidates were generated and evaluated *in vivo*. GFP reporter gene expression under the transcriptional control of 13 novel promoters was evaluated in rAAV9 vectors. Neonatal CD1 mice received titre matched vector by intracerebroventricular or intravenous delivery and were euthanised at 5 weeks to assess GFP expression. CNS distribution of GFP expression was characterised in detail by free floating whole brain immunohistochemistry and immunofluorescence, and systemic organ biodistribution was evaluated by qPCR. Human Synapsin 1 promoter was used as a control. All novel constructs were active in the CNS with rostrocaudal gradient. Within these novel promoters, we identified a candidate with selective activity in midbrain dopaminergic neurons. We generated a gene therapy construct to validate this novel promoter in the dopamine transporter knockout mouse as a model of dopamine transporter deficiency syndrome: infantile parkinsonism dystonia. Heterozygous mice were time-mated to generate knockout pups that received intravenous gene therapy at P1. Untreated littermates were used as controls (n=10 per group). Here, we present biodistribution of 13 novel CNS promoters, and therapeutic validation data of a novel promoter active in midbrain dopaminergic neurons.

585. In Vivo Genome Editing Using Novel AAV-PHP Variants Rescues Motor Function Deficits and Extends Survival in a SOD1-ALS Mouse Model

Shih-Ching (Joyce) Lo

Neurology, Biogen, Cambridge, MA

CRISPR-based gene editing technology represents a promising approach to deliver therapies for inherited disorders, including the neurodegenerative disease amyotrophic lateral sclerosis (ALS). Toxic gain-of-function superoxide dismutase 1 (SOD1) mutations are responsible for ~20% of familial ALS (fALS) cases. Thus, current clinical strategies for the treatment of SOD1-ALS are designed to lower SOD1 mRNA or protein levels. Here, we utilized AAV-PHP.B variants to deliver CRISPR-Cas9 guide RNAs designed to disrupt the human SOD1 transgene in $SOD1^{G93A}$ mice. A one-time intracerebroventricular injection of AAV.PHP.B-hSOD1-sgRNA into neonatal $H11^{Cas9}$

$SOD1^{G93A}$ mice led to robust and sustained reduction of mutant SOD1 protein in the cortex and spinal cord, and restored motor function. Neonatal treatment also reduced spinal motor neuron loss, NMJ denervation and muscle atrophy, diminished axonal damage and preserved compound muscle action potential throughout the lifespan of the treated mice. Treated mice achieved significant disease-free survival in the $SOD1^{G93A}$ model, extending lifespan by more than 110 days. Importantly, a one-time intrathecal or intravenous injection of AAV.PHP.eB-hSOD1-sgRNA in young adult $H11^{Cas9}$ $SOD1^{G93A}$ mice also extended lifespan by at least 170 days. As we observed substantial protection against disease progression, our results highlight the value of combining robust AAV serotypes with CRISPR-based gene editing as potential therapeutics.

586. Optimal Capsid Selection for Dorsal Root Ganglion Transduction for Neuropathic Pain

Edward Yeh, Claire Discenza, Albena Kantardzhieva, Jacob Staudhammer, Kathy A. Lam, Annahita Keravala
Gene Therapy, CODA Biotherapeutics, South San Francisco, CA

CODA Biotherapeutics, using its chemogenetic platform, seeks to treat people with intractable neurological diseases by controlling the aberrant activity of neurons. Target neurons are modified with optimized adeno-associated virus (AAV) vectors that encode engineered ligand-gated ion channels (chemogenetic receptors) that are highly responsive to specific proprietary small molecules but are otherwise inactive, resulting in tunable efficacy. CODA seeks to treat Neuropathic pain, in particular Peripheral Neuropathy and Trigeminal Neuralgia. The former is a common neurological disorder resulting from the damage to, or dysfunction of, the peripheral nervous system. It is characterized by numbness, tingling and pain, often starting in the hands and feet but can also affect other areas of your body. Trigeminal Neuralgia also known as Suicide Disease, caused by damage to the myelin sheath protecting the trigeminal cranial nerve, brings about extreme, and sporadic shock-like facial pain that can last from a few seconds to a couple minutes. Current pharmacological and surgical approaches provide little relief and a potential for addiction while having significant side effects. Because the dorsal root ganglion (DRG) and trigeminal ganglion (TG) are active structures in the process of pain transmission, we aim to express our engineered chemogenetic receptor in the neurons of the DRG and TG by AAV-mediated gene transfer. For that purpose, we tested several AAV capsids, either naturally occurring or engineered for their ability to specifically transduce neurons of our choice. SK-N-AS cells, a neuroblastoma cell line and human iPSC-derived sensory neuron cultures were transduced with AAV2, AAV2.5, AAV2.5-TV, AAV2.5-2YF, AAV2.5-TV2YF, AAV5, AAV6, AAV9, AAV9-TV, AAV9-2YF, AAV9-TV2YF, and AAV9-PHP.S encoding GFP driven by a CMV promoter. GFP expression was assessed by ddPCR for mRNA and ELISA for protein. Following *in vitro* studies, a subset of AAV serotypes carrying the GFP transgene as a marker were unilaterally injected into L3, L4, and L5 DRGs of 5-6-week-old male Sprague-Dawley rats. After 4 weeks, these animals were sacrificed and ipsilateral as well as uninjected contralateral L3, L4, and L5 DRGs were excised. The tissues were assessed by ddPCR and ELISA, to measure levels of GFP mRNA transcripts and GFP protein, along with immunofluorescence to determine cellular tropism. *In vitro* results demonstrated varying levels of GFP expression mediated by each capsid serotype. Of the 12 serotypes, AAV2.5-TV, AAV2.5-TV2YF, AAV5, AAV6, AAV9, AAV9-TV, and AAV9-PHP.S showed the greatest potential to move forward with *in vivo*. The *in vivo* capsid selection rat study demonstrated that the AAV5 and AAV6 serotypes mediated the highest level of transduction in DRG neurons as assessed by ddPCR for mRNA and ELISA for protein. Immunofluorescence analysis confirmed these two serotypes were most efficient at transducing neurons (cells co-stained for the neuronal marker NeuN). Additional cell marker stains revealed further cellular tropism among the serotypes tested. AAV9 and AAV9-TV transduced the highest percent of NF200 (heavy neurofilament) +ve neurons, a sub-population indicative of larger, myelinated a-fiber neurons. AAV6 transduced the highest percentage of isolectin B4 (IB4) positive neurons, mainly small-diameter c-fibers. Based on these studies, a sub-set of AAV capsids carrying CMV-GFP were unilaterally administered directly into DRGs of African green monkeys to make a final selection of the optimal capsid for transduction in DRGs as well TG for CODA's neuropathic pain program.

587. Creation and Characterization of a Double Knockout Mouse Model for AB-Variant GM2 Gangliosidoses

Natalie M. Deschenes[1], Alex Ryckman[1], Brianna Quinville[1], Melissa Mitchell[2], Jagdeep S. Walia[1,2,3]
[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada,[2]Medical Genetics/Department of Pediatrics, Queen's University, Kingston, ON, Canada,[3]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada

GM2 Gangliosidoses is a group of autosomal recessive neurodegenerative diseases resulting from increased lysosomal storage of GM2 gangliosides. The accumulation results in rapid neurological decline and death by age 4, in its human infantile form; currently, there is no cure. In a properly functioning cell, the metabolism of GM2 ganglioside to GM3 ganglioside is mediated by the β-Hexosaminidase A (HexA) enzyme, and its essential cofactor, the GM2 activator (GM2A) protein. GM2 Gangliosidoses manifests as three forms: Tay-Sachs Disease (TSD), Sandhoff Disease (SD) and AB-Variant (ABGM2). ABGM2, the rarest of the three forms, is characterized by a mutation in the *GM2A* gene, resulting in a deficiency of GM2A protein. Currently, the only commercially available animal model for ABGM2 is the Gm2a-/- mouse model, which lives a normal life span and corresponds to the adult-onset form of the human disease. We postulated that this can be explained by the presence of an alternate sialidase pathway for GM2 breakdown in mice which is absent and/or insignificant in humans. We hypothesized that by knocking out the most likely responsible sialidase gene-Neu3 in Gm2a-/- mice will create a superior ABGM2 animal model. We generated a Gm2a-/-Neu3-/- mouse model by crossing Gm2a-/- and Neu3-/- mouse models. In the Gm2a-/-Neu3-/- mouse model, we demonstrate an increase in GM2 ganglioside accumulation, a decrease in overall behavioural activity and weight, and a significantly compromised life span. Using this new double KO mouse model, we plan to study our previously seen therapeutic effect of scAAV9-GM2A to generate robust survival benefit pre-clinical data as a step towards a clinical trial application.

588. A Mutation-Independent Gene Therapy Strategy Induces Rod Function in a Transgenic Swine Model of Autosomal Dominant Retinitis Pigmentosa

Archana Jalligampala[1], Jennifer Noel[1], Gobinda Pangeni[1], William Hauswirth[2], Micheal Massengill[3], Alfred Lewin[3], Maureen McCall[1]

[1]Ophthalomology and Visual Sciences, University of Louisville, School of Medicine, Louisville, KY,[2]Ophthalomology Research, University of Florida, College of Medicine, Gainesville, FL,[3]Molecular Genetics and Microbiology, University of Florida, College of Medicine, Gainesville, FL

Retinitis pigmentosa (RP) is an inherited neurodegenerative disease, characterized by an initial loss of rod photoreceptors followed by a subsequent loss of cones, leading to irreversible vision loss. 30% of RP cases are autosomal dominant (adRP) and mutations in the rhodopsin (RHO) gene account for about 25% of these. Because there is significant allelic heterogeneity, an efficient therapeutic approach would be allele-independent and could be broadly applied across all rhodopsin mutations. In this study, we applied a gene therapy approach using an AAV2/5 viral vector in a transgenic P23H human RHO (TgP23H) pig model of adRP. This approach has already been successful in a spontaneous rhodopsin mutation in the dog and an engineered mouse RP model. We studied the efficacy and safety of this dual rAAV vector that combines a short hairpin RNA (shRNA), to eliminate expression of both mutant human and wild type pig rhodopsin protein, with an shRNA resistant human RHO cDNA to augment rhodopsin (AAV2/5 shRNA820-hRHO820) . Neonatal Tg and wild-type piglets (P3-7, n=20, 12 respectively) received a unilateral subretinal injection of the dual vector (50µl total volume) at several titers (6.5×10^{11} to 1×10^{13} viral genomes(vg)/ml). The structural and functional integrity of treated and naive retinas were assessed non-invasively at regular time intervals, through ~20 weeks post-injection (wpi), using fundus imaging, optical coherence tomography (OCT), and full-field electroretinograms (ffERG). After the terminal assessment, the animals were euthanized, and immunohistochemistry/confocal was used to assess the retinal morphology. From birth, naïve TgP23H hRHO pigs have no rod isolated ffERG response (Fernandez de Castro et al., IOVS, 2014). In contrast, AAV2/5 shRNA820-hRHO820 injected eyes had a rod isolated ffERG response across all the viral titers, that extended through 20wpi. Treatment with 6.5×10^{11} vg/ml produced a significant rod isolated ffERG b-wave response in comparison to naïve TgP23H hRHO control eyes (p=0.005). We found a reverse correlation of efficacy with viral titer (6.5×10^{11}vg/ml -20-38 uV; $1x10^{12}$vg/ml- 12-15 uV; $1x10^{13}$vg/ml-10-12uV). The treated retinas retain rod photoreceptors that express normally localized rhodopsin and the number of rod photoreceptors correlates well with the scotopic b-wave amplitude. Our results show that AAV2/5 shRNA820-hRHO820 decreases rod degeneration and induces rod function in the TgP23H hRHO swine model of human adRP. Combined with previously published results in dog and mouse models of adRP, our data show that this allele independent approach has the potential to effectively treat rod degeneration in adRP patients.

589. Gene Transfer of *SCN1A* Using a High Capacity Adenoviral Vector Improves the Phenotype of a Dravet Syndrome Mouse Model

Ana Lourdes Ricobaraza

Gene Therapy Program, CIMA University of Navarra, Pamplona, Spain

Heterozygous mutations in the *SCN1A* gene, which encodes the alpha subunit of the Nav1.1 voltage-gated sodium channel, cause more than 80% of Dravet syndrome cases. This disease is characterized by severe epilepsy combined with motor, cognitive and behavioral abnormalities. SCN1A haploinsufficiency is the most accepted physiopathological mechanism, with special impairment in the function of inhibitory interneurons. Current antiepileptic drugs achieve only partial control of seizures and provide little benefit on the patient's neurological development. Novel therapies aim to restore *SCN1A* expression in order to address all disease manifestations. We provide evidence that a High-Capacity Adenoviral Vector harboring the 6 Kb *SCN1A* cDNA is feasible and able to express functional Nav1.1 in neurons. *In vivo*, the best biodistribution was observed after intracerebral injection in basal ganglia, cerebellum and prefrontal cortex. *SCN1A* A1783V knock-in mice received the vector at 5 weeks of age, when most neurological alterations were present. Animals were protected from sudden death, and the epileptic phenotype was attenuated. Improvement of motor performance and interaction with the environment were observed. In contrast, hyperactivity persisted, and the impact on cognitive tests was variable (success in novel object recognition and failure in Morris water maze tests). These results provide proof of concept for gene supplementation in Dravet syndrome and indicate new directions for improvement.

590. Modeling of SCN2A Related Disorders *In Vitro* Allows Exploration of Disease Mechanism and Identification of Potential Therapies for This Neurological Disease

Yacidzohara Rodriguez[1], Cassandra Dennys[1], Annalisa Hartlaub[1], Tracy Bedrosian[2,3], Summer Fair[4], Jennifer Kearney[5], Mark Hester[4], Kathrin Meyer[1,3], Nicolas Wein[1,3]

[1]Gene Therapy, Nationwide Childrens Hospital, Columbus, OH,[2]Institute for Genomic Medicine, Nationwide Children's Hospital, Columbus, OH,[3]Pediatric, The Ohio State University, Columbus, OH,[4]Institute for Genomic Medicine, Nationwide Childrens Hospital, Columbus, OH,[5]Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL

Mutations in the *SCN2A* gene cause severe neurological dysfunction with an estimated incidence of approximately 1:10,000. The *SCN2A* gene encodes the alpha subunit of the voltage-gated sodium channel (Nav1.2), expressed primarily in the brain. This channel belongs to one of the major neuronal sodium channel families that play an essential role in a cell's ability to generate and transmit electrical signals. Loss of function (LOF) mutations are usually associated with autism (ASD) and intellectual disability (ID) while gain of function (GOF) mutations are associated with epilepsy. To date, the mechanisms for the phenotypic heterogeneity, ranging from benign to very severe clinical presentations, are poorly understood. In addition, the genetic analysis does not always

correlate with the disease symptoms which suggests that the effect of the primary mutation is being modified by additional genes, further complicating our understanding of the disease mechanism. Moreover, the broad clinical phenotype of patients makes it difficult to develop therapeutics. Current treatment options are very limited for SCN2A disorders leaving a high unmet need. Here we developed *in vitro* models using neurons and astrocytes, as well as 3D brain organoids to study cell-type specific disease mechanisms. We used different established reprogramming methods (direct conversion and iPSC technology) to evaluate SCN2A mediated neuronal and astrocyte defects in 2D, 3D and co-culture experiments. *SCN2A* expression levels in induced neurons (iNs) and astrocytes (iAs) were compared using immunofluorescence (IF), quantitative real-time PCR and RNA-seq analysis. The non-cell autonomous contribution of iAs to neuron toxicity was assessed using co-culture experiments. Our preliminary results indicate that both neurons and astrocytes from SCN2A patients are defective. Moreover, induced astrocytes demonstrate varying degrees of toxicity towards neurons. Using RNA-seq we identified pathways previously linked to autism and seizure disorders to be altered in multiple cell types and identified potential novel therapeutic targets including mitochondrial activity regulation. Moreover, we found a surprising upregulation of Nav1.2 expression in a patient harboring a supposed loss of function mutation. We identified the small molecule Copper ATSM (CuATSM) that is currently in phase III clinical trials for Amyotrophic Lateral Sclerosis, as a potential treatment option for SCN2A related disorders. CuATSM demonstrated to modify the disease phenotype in 2D and 3D *in vitro* models as well as a mouse model of the disease.

591. AAV1.NT-3 Gene Therapy Improves Motor Function in Accelerated Aging Model SOD1KO Mice

Burcak Ozes, Morgan Myers, Kyle Moss, Alicia Ridgley, Lei Chen, Darren Murrey, Zarife Sahenk

Center for Gene Therapy, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH

Frailty accompanied with sarcopenia cause age-related muscle loss and constitutes a critical health problem mainly affecting older adults. Superoxide Dismutase 1 knock-out (SOD1KO) mice, lacking the antioxidant enzyme, Cu/Zn superoxide dismutase, represent an excellent model of frailty and muscle mass loss. We previously showed that NT-3 increases muscle fiber diameter by directly activating the mTOR pathway, therefore has potential for attenuating age-related changes, muscle wasting, and frailty. For proof of concept, we assessed the therapeutic efficacy of AAV1.NT-3 in the neuromuscular function of this accelerated aging model. Twelve SOD1KO mice of both genders received a $1x10^{11}$ vg dose of AAV1.tMCK.NT-3 at six months of age, via intramuscular injection into the gastrocnemius muscle. Thirteen age- and sex-matched SOD1KO mice were injected with Ringer's lactate as controls. The mice were sacrificed six months post-injection. Animals underwent treadmill and rotarod testing at baseline, three months post-injection, and endpoint. Gait analysis was performed at the endpoint. Quantitative histology included analysis of muscle fiber size and metabolic fiber type composition in the gastrocnemius, tibialis anterior, and triceps muscles using succinic dehydrogenase stain and assessment of peripheral nerve changes. Treadmill outcomes indicate an improvement in the NT-3-treated SOD1KO mice, corresponding to 36%, and 76% longer running distances compared to the untreated cohort at three, and six months, respectively ($p< 0.0001$ for both time points). The rotarod test also showed improved performance in the AAV1.NT-3 injected SOD1KO mice at three, and six months post-injection, 30% ($p= 0.012$) and 54% ($p= 0.0002$) respectively. Additionally, the gait abnormalities observed in the untreated SOD1KO mice were ameliorated in the treated mice at six months post-injection, showing an 11% decrease in the stride width ($p=0.0014$). No significant sex difference was observed in these functional performance tests. Histological analyses of muscle indicated a significant increase in mean muscle fiber diameter in the gastrocnemius (37.2±1.0 μm, n=11 vs 32.3±1.2 μm, n=7, $p=0.01$) and tibialis anterior muscles (37.8±0.8 μm vs 33.7±1.2, n=6 mice for both cohort, $p=0.02$) from the treated cohort compared to untreated SOD1KO mice. Fiber size increase was most prominent in the fast-twitch glycolytic fibers in both muscles (gastrocnemius: NT-3 vs UT, $p=0.004$; tibialis anterior: NT-3 vs UT, $p=0.01$, respectively). No treatment effect was observed in the fiber size of the triceps muscle. Histological examination of peripheral nerves revealed an attenuation of age-related myelin changes and increased myelin thickness in the treated cohort. This study shows that AAV1.NT-3 gene therapy improved neuromuscular function in the accelerated aging model SOD1KO mice. These results are consistent with our previous findings on the naturally aged wild type C57BL/6 mice (two years old) indicating the therapeutic potential of NT-3 for age-related sarcopenia and muscle wasting conditions.

Musculo-skeletal Diseases

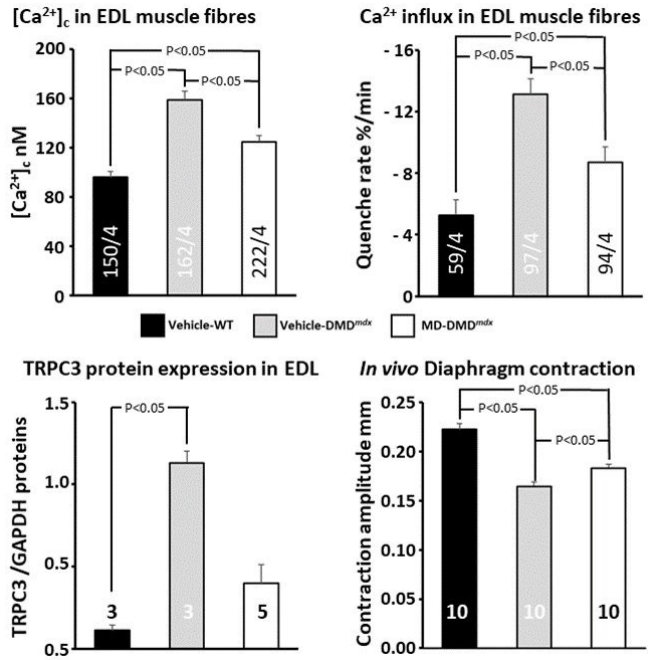
592. Assessment of TRPC1 and TRPC3 as Potential Therapeutic Targets for DMD Treatment in Complement to rAAV-microDystrophin Gene Therapy

Anna Creisméas[1], Claire Gazaille[1], Audrey Bourdon[1], Aude Lafoux[2], Marc-Antoine Lallemand[1], Marine Allais[1], Virginie Le Razavet[1], Mireille Ledevin[3], Thibaut Larcher[3], Gilles Toumanianz[4], Ignacio Anegon[5], Oumeya Adjali[1], Corinne Huchet[1], Caroline Le Guiner[1], Bodvaël Fraysse[1]

[1]Translational Gene Therapy Laboratory UMR1089 INSERM, University of Nantes, Nantes, France,[2]Therassay platform, Capacités, University of Nantes, Nantes, France,[3]INRAE UMR 703, PanTher, APEX, ONIRIS, Nantes, France,[4]INSERM, UMR 1087/CNRS 6291 Institut du Thorax, University of Nantes, Nantes, France,[5]INSERM, UMR 1064-CRTI, University of Nantes, Nantes, France

Duchenne muscular dystrophy (DMD) is an X-linked inherited disease affecting ≈1:5,000 male births that leads to lack of dystrophin expression and muscle degeneration. Patients with DMD become wheelchair-bound in late adolescence and rarely survive beyond the age of 40 due to cardiorespiratory complications. Clinical trials are underway using rAAV-microdystrophin-based gene therapy (rAAV-MD). The goal is to move from DMD to a milder dystrophinopathy, Becker muscular dystrophy (BMD). However, even if this rAAV-MD

gene therapy is currently one of the most promising therapeutic approach for DMD, BMD patients still exhibit muscle decline and they often die before the age of 60. In DMD, muscle cell necrosis is triggered by an increased influx of calcium, which is generally assumed to be largely related to the Transient Potential Receptor (TRP) Ca^{2+} channel amily. However, the specific identity of the TRP channels involved is still unclear. In our study, we evaluated the involvement of TRPC1 and TRPC3 channels in the pathogenesis of DMD in skeletal muscle and assessed their potential as therapeutic targets for complementary or alternative treatments of DMD to rAAV-MD one. The experiments were conducted during the post-natal development of the DMD^{mdx} rat, an animal model that faithfully mimics human DMD disease with, in particular, progressive and severe skeletal muscle necrosis and fibrosis, with significant reduction in muscle strength and decrease in spontaneous motor activity. We demonstrated early increases in $[Ca^{2+}]_c$ and sarcolemmal permeability to Ca^{2+} (SPCa) in fast twitch EDL muscle fibers of DMD^{mdx} rats. This was accompanied by an increase in the expression of the TRPC3 protein, but not mRNA, which instead decreased. TRPC1 mRNA and protein levels increased significantly only 7 months after birth, at an age when the disease was already well established. In addition, the subcellular localization of TRPC1 and TRPC3 was similar at each age between DMD^{mdx} and WT rats. When focusing attention on TRPC3, we found that the apparent molecular weight of TRPC3 channels was slightly but surely higher in DMD^{mdx} rats. This was probably related to post-translational changes since no apparent difference in mRNA isoforms were observed. Most of the increase in SPCa in DMD^{mdx} rats were related to a Pyr10-sensitive calcium influx, supporting the involvement of TRPC3 channels. Finally, we showed that treatment with rAAV-MD induced a high level of MD expression with significant but only partial prevention of alterations in calcium homeostasis, skeletal muscle strength and TRPC3 overexpression (Figure). Altogether, these data suggest that TRPC3 may represent a good therapeutic target for the development of a treatment for DMD, as a complement or alternative to rAAV-MD gene therapy.



593. Improved Transduction of DMD Dog with rAAV-Microdystrophin via Multipotent Mesenchymal Cell Pretreatment

Hiromi Hayashita-Kinoh[1], Yuko Nitahara-Kasahara[2], Mutsuki Kuraoka[3], Shin'ichi Takeda[3], Takashi Okada[1]

[1]Division of Molecular and Medical Genetics, Center for Gene and Cell Therapy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan,[2]Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan,[3]Department of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan

Background: AAV vectors are highly safe and allow long-term expression of therapeutic genes. Gene therapy using rAAV has been attempted for systemic diseases. It is used to treat genetic diseases such as SMA, XLMTM, and DMD. Although adverse events were reported with high-dose systemic administration. When performing intravenous administration of rAAV, a dose of $3x10^{13}$ to $1x10^{14}$v.g./kg BW is common. Systemic administration of $1x10^{14}$ v.g./kg BW and above amount produces direct liver injury, the immune response to viral vectors, and the risk of germline transmission, thus requiring an attempt to reduce the dosage. Duchenne muscular dystrophy (DMD) is a congenital disease causing progressive deterioration of skeletal and cardiac muscles because of mutations in the dystrophin gene. Supplementation of dystrophin using rAAV-microdystrophin is sufficient to reduce the pathogenesis of animal models of DMD. Here we investigated strategies using somatic stem cells in combination with rAAV to induce immune tolerance to the rAAV9 vector and to achieve adequate transgene expression at lower doses. MSCs have been employed in various inflammatory diseases including graft-versus-host disease (GvHD) by their immunosuppressive effects. Furthermore, immune-modulating effects of MSCs on rAAV transduction were examined. **Methods:** Bone marrow-derived MSCs and rAAV9-luciferase or rAAV9-microdystrophin (2×10^{12} v.g./kg BW) were intravenously injected into normal or $CXMD_J$ (DMD) dogs at eight weeks old. Seven days after injection, MSCs were systemically injected again. At eight days after the first injection, rAAV9-luciferase or rAAV9-microdystrophin were intravenously injected into the same dog. To examine the immune response against rAAV9, IFN-γ expression in the purified canine peripheral leukocytes was analyzed using qRT-PCR. Expressions of the transgene in the skeletal muscles of the rAAV9-luciferase or rAAV9-microdystrophin transduced animals were confirmed by immunohistochemistry. Besides, MSCs-treated $CXMD_J$ with rAAV9-microdystrophin transduction and non-treated $CXMD_J$ were compared to assess gait function and lameness of the limb. **Results:** Administration of rAAV9 following MSCs treatment resulted in higher expression of the transgene (luciferase or microdystrophin) at the skeletal and cardiac muscle, compared to the rAAV transduction alone. Expression of IFN-γ in the purified peripheral blood leukocytes after the rAAV9 exposures were not enhanced in the rAAV9 with MSCs, suggesting the immune suppressive effects of the MSCs. The $CXMD_J$ treated with MSCs and rAAV9-microdystrophin showed a better functional improvement than other DMD dogs of the same age. **Conclusion:** Our results demonstrate that rAAV injection with MSCs pre-treatment improved expression of the rAAV-derived transgene in dogs. This strategy would be a practical approach to

analyze the expression and function of the transgene in vivo. These findings also support the future feasibilities of rAAV-mediated protein supplementation strategies to treat DMD and various genetic diseases.

594. Interim Data from the First-in-Human Phase 1 Trial of FX201, an Intra-Articular, Helper-Dependent Adenoviral Gene Therapy for Osteoarthritis - Safety, Tolerability, Biodistribution, and Preliminary Evaluation of Clinical Activity in 5 Patients

Scott Kelley[1], Alan Kivitz[2], Becca Senter[1], David Golod[1], Amy Cinar[1], Emily Walsh Martin[1], Won Hong[1]

[1]Flexion Therapeutics, Inc, Burlington, MA,[2]Altoona Clinical Research, Duncansville, PA

FX201 (humantakinogene hadenovec) is a helper-dependent adenovirus serotype 5 expressing Interleukin-1 receptor antagonist (IL-1Ra) in clinical development as an intra-articular (IA) gene therapy for osteoarthritis (OA). FX201 is designed to produce IL-1Ra in the presence of inflammation via a nuclear factor-kappa B-responsive promoter. Previous work demonstrated safety and efficacy of FX201 in preclinical models of OA. Following IND-enabling toxicology studies and clinical-scale cGMP production, we initiated a Phase 1 open-label, single ascending dose trial (NCT04119687) to assess the safety, tolerability, biodistribution, and preliminary clinical activity of FX201 in patients with knee OA. Here we report on the low-dose cohort treated with FX201 through 24 weeks following administration. Five patients (30-80 y) with moderate-to-severe knee OA (Western Ontario and McMaster Universities Osteoarthritis Index [WOMAC] pain score ≥ 4.0 and ≤ 9.0 [0-10 numeric rating scale], Kellgren-Lawrence Grade 2-3 radiographic severity), and prior failure of ≥2 other OA treatments were enrolled in the low-dose cohort. FX201 (dose of 1.4E10 genome copies) was administered to the index knee via ultrasound-guided IA injection. Safety and tolerability were assessed by adverse events (AEs), physical examinations, index knee assessments, vital signs, ECGs, and clinical lab evaluations. Systemic biodistribution and shedding were assessed via quantification of vector copies in plasma, urine, and injection site swabs. Clinical activity of FX201 was assessed by measuring change in pain (WOMAC-A) and function (Knee Injury and Osteoarthritis Outcome Score [KOOS] Function in daily living). Clinical importance of pain relief after FX201 treatment was assessed using the Initiative on Methods, Measurement, and Pain Assessment in Clinical Trials (IMMPACT) criteria to characterize substantial improvement. FX201 was generally well tolerated in the 5 patients treated. Two patients had self-limited Gr 2 index-knee AEs (pain, swelling, effusion) related/possibly related to treatment managed conservatively. No evidence of systemic biodistribution in plasma or shedding in urine or skin samples was observed in any patient. Clinical activity with reduction in pain and improvement in function was variable (Fig. 1). Substantial improvement in WOMAC-A pain intensity was observed in 3 patients (60%) at Week 8 and 2 patients (40%) at Weeks 12 and 24 (Table 1). Intra-articular FX201 in patients with knee OA was generally well tolerated in this initial cohort. Substantial pain relief persisting to 24 weeks following low-dose FX201 is encouraging and supports evaluating tolerability and responses with higher doses of FX201. Dosing of a mid-dose cohort is complete and follow-up is ongoing. Expansion cohorts are initiating. An update will be presented on data from patients in the study.

Table 1. WOMAC-A Pain Measures and IMMPACT Substantial Responder Status

	WOMAC-A (% Change from Baseline)				Substantial Responder		
Patient	Baseline	Week 8	Week 12	Week 24	Week 8	Week 12	Week 24
1	5.0	5.0 (0%)	4.6 (-8%)	4.2 (-16%)	NO	NO	NO
2	4.0	6.6 (65%)	7.6 (90%)	7.0 (75%)	NO	NO	NO
3	7.6	3.0 (-61%)	2.4 (-68%)	3.6 (-53%)	YES	YES	YES
4	7.0	0.0 (-100%)	0.0 (-100%)	0.0 (-100%)	YES	YES	YES
5	5.2	2.0 (-62%)	3.0 (-42%)	4.0 (-23%)	YES	NO	NO

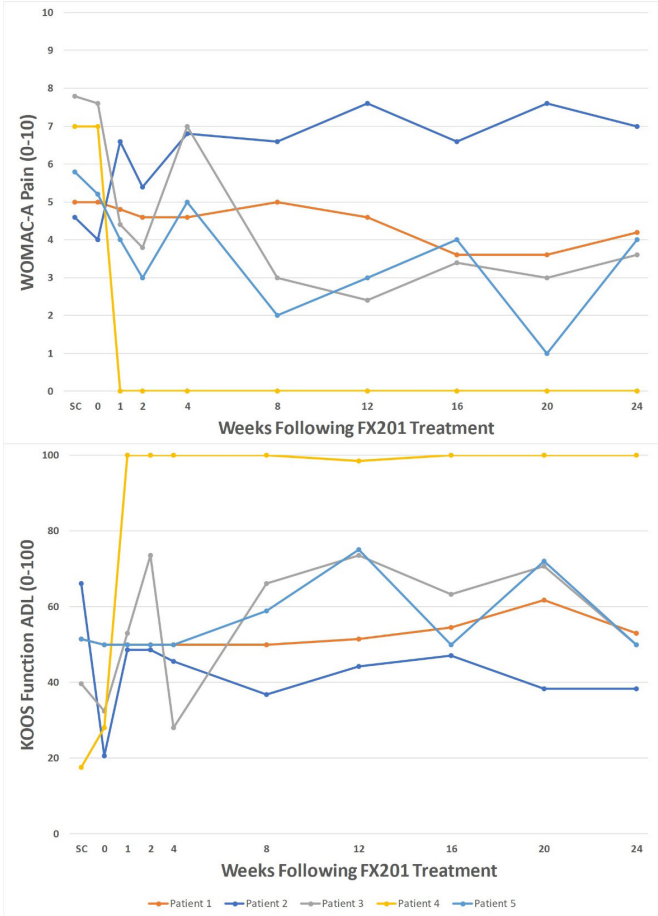


Fig. 1. Clinical measures of pain (WOMAC-A) and function (KOOS Function) prior to (SC- Screening; Baseline - 0) and through 24 weeks following FX201 treatment.

595. Osteoarthritis Gene Therapy: Interim Report from a Phase I Clinical Trial (NCT02790723)

Christopher Evans
Mayo Clinic, Rochester, MN

Introduction: Local gene delivery to joints is a promising approach to treating arthritis and other arthropathies. Not only does this strategy overcome the pharmacokinetic barriers to intra-articular therapy but, by targeting individual joints, it also lowers the amount of vector administered thereby increasing safety and reducing manufacturing cost and complexity. Osteoarthritis (OA) of the knee has few effective therapies, is associated with high morbidity and, as the leading cause of disability, represents a major burden on public health. Interleukin 1 (IL-1) has been implicated in a number of biochemical, genetic, and animal model studies as a major contributor to OA. Here we provide a progress report on a Phase I clinical trial in mild-to-moderate OA using an intra-articular AAV vector encoding the human interleukin-1 receptor antagonist (IL-1Ra) (NCT02790723). **Study Design:** Following screening and appropriate informed consent, subjects with mid-stage OA of the knee (Kellgren-Lawrence score 2 or 3) are treated with a single dose of 10^{11}vg, 10^{12}vg, or 10^{13}vg AAV. IL-1Ra (n=9 total subjects; 3 per cohort). Individuals in each cohort receive an ultrasound-guided, intra-articular injection of the vector and are monitored for 1 year. The primary outcome measure is safety, defined as an absence of drug-related serious adverse events. Additional measures include levels of circulating viral genomes, immune response to the vector, blood and urine analysis and physical examination. Where possible, synovial fluid is withdrawn from knee joints under ultrasound and IL-1Ra concentrations are measured by validated ELISA. Although this study is not powered for efficacy and has no control group, patients report pain via VAS (0-10) and pain and function via WOMAC. Knee joints are imaged by X-ray and MRI upon study entry and after 1 year. **Results:** To date, intra-articular AAV.IL-1Ra has been safe, with no product related adverse events. Blood chemistry and hematology markers have remained normal, with no evidence of the neutropenia that can accompany systemic delivery of IL-1 inhibitors. Immunology and biodistribution studies have been consistent with pre-clinical observations and as expected for local administration of AAV therapy. **Conclusion:** The promising results from this phase 1 trial suggest that further study of AAV.IL-1Ra for the local gene therapy of OA is warranted. **Acknowledgements:** Funded by a Transform the Practice grant from the Mayo Clinic and grant number W81XWH-16-1-0540 from the US Department of Defense. The Clinical Trials Research Unit of Mayo Clinic is thanked for patient-related services.

596. Gene Therapy for Duchenne Muscular Dystrophy Due to Duplications of Exon 2 Using the scAAV9.U7-ACCA Vector: 6-12 Months Clinical Follow Up

Megan Ann Waldrop[1,2], Michael Lawlor[3], Tatyana Vetter[1], Emma Frair[1], Margaret J. Beatka[3], Hui Meng[3], Megan Iammarino[1], Brenna Powers[1], Johan Harris[1], Maryann Kaler[1], Tabatha Simmons[1], Kim McBride[4,5], Nicolas Wein[1], Kevin Flanigan[1,2]
[1]Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Pediatrics and Neurology, Ohio State University, Columbus, OH,[3]Pathology and Laboratory Medicine, Medical College of Wisconsin, Milwaukee, WI,[4]Center for Cardiovascular Research and Division of Genetic and Genomic Medicine, Nationwide Children's Hospital, Columbus, OH,[5]Pediatrics, Ohio State University, Columbus, OH

Exon duplications that cause Duchenne muscular dystrophy (DMD) are promising candidates for exon skipping therapies because skipping a single exon copy should result in wild-type transcript and full-length dystrophin expression. We developed a therapeutic exon skipping viral vector (scAAV9.U7-ACCA) comprising four copies of a modified U7snRNA containing antisense sequences targeting the splice donor (2 copies) and splice acceptor (2 copies) of the *DMD* exon 2, which is the most commonly duplicated exon. Following dose finding studies in Dup2 mice and after demonstrating lack of toxicity in non-human primates, a first-in-human clinical trial (NCT04240314) was initiated. The 2 enrolled subjects were treated with 3.0x10^13 vg/kg infused over 90 minutes and tolerated the treatment well, with only transient nausea and vomiting. No SAEs have been experienced to date and no biochemical measures of hepatic or other toxicity were seen. Serum CK levels decreased from 13,495 U/L to 4,730 U/L at 12 months in subject 1 and from 5,103 U/L to 1,200 U/L at 6 months in subject 2. Muscle biopsy at 3 months shows expression of apparently full-length protein, quantified by Western blot at levels of >6% in the younger (9.0 yrs) subject and 1-2% in the older (13.7 yrs) subject, possibly reflecting differences in myofiber transduction due to differing degrees of dystrophic skeletal muscle changes; 6 month expression analysis is underway. There was no clinically meaningful change from baseline seen in the NSAA, 100 meter timed test or 4 stair climb for either subject at 6 months, although subtle improvements were seen in the 100 meter timed test for subject 1 (54.3% predicted to 58.8% predicted) and the 4 stair climb for subject 2 (4.1 sec to 3.6 sec). Additionally, at 12 months for subject 1 improvements in the 100 meter timed test (61.4% predicted), NSAA (27), and 4 stair climb (2.39 sec) were seen. These findings represent the first therapeutic expression of apparent full-length dystrophin in a human gene therapy trial, continued safety at up to 12 months post gene transfer, and support continued clinical investigation.

597. RGX-202, an AAV8 Vector Encoding an Extended Microdystrophin Efficiently Restores Dystrophin Associated Protein Complex and Corrects Satellite Cells in a Dystrophic Mouse Model

Chunping Qiao, Kirk Elliott, Randolph Qian, SunJung Kim, Nicholas Buss, Ye Liu, Olivier Danos

Regenxbio, Inc, Rockville, MD

To achieve efficient dystrophin replacement gene therapy for Duchenne Muscular Dystrophy (DMD), the large dystrophin protein needs to be truncated to microdystrophin due to the limited packaging capacity of adeno-associated virus (AAV). Several versions of microdystrophin have been shown to correct dystrophic animal models and are currently being tested in the clinic. RGX-202 features a novel design of microdystrophin driven by a muscle specific promoter (spc5-12) and packaged into an AAV8 capsid. The microdystrophin includes 4 rods and 3 hinges and 146 amino acids of the carboxyl terminal (CT) domain. The protein coding sequence has been codon optimized and depleted of CpG dinucleotides. Here, we present data supporting a potential benefit of the added CT domain on microdystrophin expression levels and Dystrophin Associated Protein Complex (DAPC) recruitment at the sarcolemma, and a novel finding of satellite cell correction in the *mdx* mouse model. The CT domain included in RGX-202 contains both α-syntrophin and α-dystrobrevin binding sites. This design was compared to shorter versions with the α-syntrophin binding site only, or without CT domain. All constructs were packaged into an AAV8 capsid and used to transduce differentiated mouse C2C12 myotubes. Expected size microdystrophins were observed on western blot and quantitation showed RGX-202 produced the strongest protein expression. These microdystrophin vectors were further evaluated in *mdx* mice via intravenous administration. Western blot showed that RGX-202 again produced the highest microdystrophin amount in skeletal muscle. Immuno-histochemistry and muscle membrane protein western blot showed that while all constructs can recruit α-dystrobrevin, β-dystroglycan and α-syntrophin to the sarcolemma, RGX-202 was more efficient at recruiting α-syntrophin. We studied the effects of RGX-202 gene therapy on *mdx* skeletal muscle satellite cells using *in-situ* hybridization (RNAScope®). By counting the co-localization of transgene and Pax7 RNA, we estimated the transduction rate of Pax7+ satellite cells to be over 20%. This indicated a significant tropism of the AAV8 capsid and a measurable activity of the spc5-12 promoter in these cells. We also noted that normalized Pax7+ satellite cell counts per image area were reproducibly higher (2.5-fold) in untreated vs treated *mdx*. Consistently, Pax7 mRNA levels measured by digital RT-PCR were also higher (2-fold) in untreated *mdx*. These data indicated an increase of Pax7+ satellite cell count and Pax7 mRNA expression in *mdx* muscle, consistent with active cycles of muscle degeneration and regeneration. Upon RGX-202 treatment, this satellite cell hyperplasia was partially to completely corrected. In conclusion, increased microdystrophin protein level both *in vitro* and *in vivo* were observed with the addition of the CT domain in RGX-202. In *mdx* mouse, treatment with RGX-202 was shown to restore the DAPC at the sarcolemma and prevent the pathological cycle of degeneration / regeneration that ultimately results in muscle wasting. This initial characterization provides a rationale for the further development of RGX-202 as a microdystrophin gene therapy treatment for DMD.

598. A Novel Humanized Knock-In Mouse Modeling a Deep Intronic Mutation in Collagen VI-Related Dystrophy: Characterizing Preclinical Outcome Measures

Fady Guirguis[1], Véronique Bolduc[2], Jun Cheng[3], Lisa Garrett[3], Carsten Bönnemann[2]

[1]NINDS, Neuromuscular and Neurogenetic Disorders of Childhood Section, NIH MRSP, National Institutes of Health, Bethesda, MD,[2]NINDS, Neuromuscular and Neurogenetic Disorders of Childhood Section, National Institutes of Health, Bethesda, MD,[3]NHGRI, Embryonic Stem Cell and Transgenic Mouse Core, National Institutes of Health, Bethesda, MD

Collagen VI is an extracellular matrix protein. A common recurrent dominant-negative deep intronic C>T mutation in the *COL6A1* gene inserts a 72-nucleotide-long pseudoexon between exons 11 and 12 in 50% of the mutant allele transcripts. Patients carrying this mutation manifest early-onset muscle weakness, joint contractures, and respiratory insufficiency. As mouse and human intronic sequences are nonhomologous, using CRISPR/Cas9 technology we have generated a humanized knock-in mouse model carrying either the wild type (HumC) or mutant (HumT) alleles in order to investigate this variant's pathophysiology and to test splice-correction therapies *in vivo*. Here, we comprehensively characterized the phenotype of this new model and identified outcome measures that will enable assessing the efficacy of therapeutic interventions in rescuing phenotype. Grip strengths of male (M) and female (F) $Col6a1^{+/+}$ (n= 8M, 5F), $Col6a1^{+/HumC}$ (n= 7M, 7F), $Col6a1^{HumC/HumC}$ (n= 7M, 7F), $Col6a1^{+/HumT}$ (n= 9M, 8F), $Col6a1^{HumT/HumT}$ (n= 4M, 4F) mice were measured monthly from postnatal day (PND) 28 to PND 140. The mice were weighed every 2 days from PND 14 to PND 42, then weekly to PND 140. Muscles from 11-month-old $Col6a1^{+/+}$, $Col6a1^{+/HumC}$, and $Col6a1^{+/HumT}$ mice were stained with hematoxylin and eosin, and immunohistochemically stained for collagen VI and laminin. For 1 and 2-month-old males and 2, 3, 4, and 5-month-old females, the average grip strengths of $Col6a1^{+/HumT}$ and $Col6a1^{HumT/HumT}$ mice were significantly weaker than those of all other genotypes ($p<0.05$). However, there was no statistically significant difference in weight or percent weight change. The preliminary histological data showed that $Col6a1^{+/HumT}$ quadriceps had more central nuclei than the other genotypes' quadriceps. Additionally, among the $Col6a1^{+/HumT}$ muscles, the tibialis anterior and quadriceps muscles had more central nuclei than the extensor digitorum longus, gastrocnemius, soleus, and triceps muscles. Our findings suggest that central nuclei quantification and grip strength measurement could be appropriate outcome measures to assess the efficacy of human-ready antisense drugs in this humanized knock-in *Col6a1* mouse model.

599. Increasing Allele Selectivity of Small Interfering RNAs to Target a Dominant-Negative Glycine Substitution Causing Collagen VI-Related Dystrophy

Astrid Brull, Apurva Sarathy, Véronique Bolduc, Riley McCarty, Carsten G. Bönnemann
NINDS, NIH, Bethesda, MD

Collagen VI-related dystrophies (COL6-RDs) are a group of frequently severe, congenital-onset muscular dystrophies for which there is no effective treatment. Dominant-negative mutations, in particular glycine substitutions and in-frame exon skips, are common in *COL6A1*, *COL6A2* and *COL6A*3 genes, and they are capable of incorporating into the hierarchical assembly of the encoded collagen α1, α2 and α3 (VI) chains and as a consequence, produce a dysfunctional collagen VI extracellular matrix. RNA directed therapeutics offer great opportunities to silence dominant-negative mutations if designed to selectively target the mutant allele, as haploinsufficiency for the *COL6* genes is not associated with clinical disease. To achieve the potential of RNA directed therapeutics for dominant conditions, the design of highly active, yet allele-specific, antisense oligonucleotides or siRNAs is required. Targeting single missense substitutions proves to be challenging, as the mutant target differs from the normal copy from only one nucleotide. To increase selectivity of siRNA silencing towards a common glycine mutation (G293R) in *COL6A1*, we deliberately introduced a second mismatch into the siRNA design, to destabilize hybridization to the normal allele while maintaining activity on the mutant transcript. A series of siRNAs in which the location of the second mismatch was located at various positions along the siRNA sequence were co-transfected in HEK293 cells with fluorescence reporters for both the normal and the mutant allele. We found at least two siRNA oligonucleotides that retain high silencing activity towards the mutant allele as evidenced by fluorescence and immunoblotting quantification, while preserving the expression of the normal allele. Treatment of patient-derived fibroblasts with these siRNAs effectively rescued COL6 matrix deposition. The design strategy that we tested here increases the targeting potential of specific mutations that were previously difficult to discriminate. This work was supported by CureCMD, the Fundación Noelia, and by NINDS funding.

600. Single Vector AAV Approach to Genome Editing in Pompe Disease

Benjamin Arnson[1], Jason Wang[2], David Courtney[3], Sang-oh Han[1], Songtao Li[1], Bryan R. Cullen[3], Nenad Bursac[2], Dwight Koeberl[1]
[1]Pediatrics Medical Genetics, Duke University, Durham, NC,[2]Biomedical Engineering, Duke University, Durham, NC,[3]Molecular Genetics & Microbiology, Duke University, Durham, NC

Pompe disease is a severe metabolic disorder caused by deficiency of the lysosomal enzyme acid α-glucosidase (GAA) resulting in glycogen accumulation, cardiomyopathy, progressive muscle weakness, and respiratory failure. The current treatment is enzyme replacement therapy which requires frequent injections. New gene therapies based on adeno-associated virus (AAV) vectors are being explored but also lose efficacy over time. Genome editing could permanently correct mutations in *GAA* resulting in stable GAA expression and clearance of glycogen. We have developed CRISPR/Cas9 based editing systems to precisely cleave *GAA* near pathogenic variants and provided a repair template to promote homology directed repair (HDR). This system will be delivered using a single AAV vector and upon successful genome editing, will result in stable, permanent GAA expression. We have developed systems to treat two pathogenic variants in *GAA* that affect the majority of Pompe patients (Fig. 1A): 1) the c.-32-13T->G (IVS1) variant is present in 90% of late-onset Pompe patients and 2) the c.525deltT (ΔT525) variant is associated with the most severe form of infantile-onset Pompe disease. Correction of these variants will benefit the majority of Pompe patients and serve as a model for genome editing in other genetic diseases. We have generated AAV vectors to edit each *GAA* variant and will increase efficiency by delivering all editing components in a single AAV. For the IVS1 variant, we identified three protospacer adjacent motif (PAM) sequences surrounding the variant. The single guide RNA (sgRNA) with the highest rate of cleavage was incorporated into a vector with *Staphylococcus aureus* Cas9 (SaCas9) and the repair template containing the EF1α promoter (Fig. 1A). When the vector was transfected into Pompe patient fibroblasts, we observed 52% of alleles were edited and contained the EF1α promoter (Fig. 1B). For the ΔT525 variant, we tested eight different sgRNAs targeting PAMs near the locus and cloned the most efficient sgRNA into a vector with SaCas9 and a repair template containing the wild type *GAA* sequence (Fig. 1A). Fibroblasts transfected with this construct showed 35% of alleles were edited via HDR and contained the corrected *GAA* sequence (Fig. 1C). Subsequently the IVS1 transgene was packaged into a single AAV9 vector and used to transduce human cardiomyocytes, where up to 7% of GAA alleles were edited via HDR (Fig. 1D). To investigate the physiological effects of genome editing, we will use a novel three-dimensional skeletal muscle model derived from patient muscle biopsies to generate engineered tissues termed myobundles. Pompe myobundles contain elevated glycogen levels as well as reduced GAA and contractile force under metabolic stress compared with healthy controls. We are currently evaluating the single AAV9 vector constructs in this human skeletal muscle model of Pompe disease to achieve gene variant correction through HDR-mediated genome editing. In conclusion, we have shown that we can achieve efficient genome editing of two pathogenic variants in Pompe disease fibroblasts and normal cardiomyocytes. This strategy may restore GAA expression and serve as a model for genome editing in Pompe disease with implications for other genetic diseases.

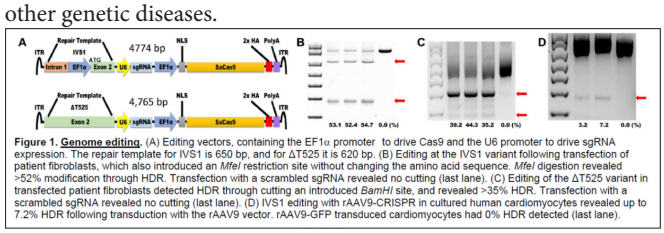


Figure 1. Genome editing. (A) Editing vectors, containing the EF1α promoter to drive Cas9 and the U6 promoter to drive sgRNA expression. The repair template for IVS1 is 650 bp, and for ΔT525 it is 620 bp. (B) Editing at the IVS1 variant following transfection of patient fibroblasts, which also introduced an *MfeI* restriction site without changing the amino acid sequence. *MfeI* digestion revealed >52% modification through HDR. Transfection with a scrambled sgRNA revealed no cutting (last lane). (C) Editing of the ΔT525 variant in transfected patient fibroblasts detected HDR through cutting an introduced *BamHI* site, and revealed >35% HDR. Transfection with a scrambled sgRNA revealed no cutting (last lane). (D) IVS1 editing with rAAV9-CRISPR in cultured human cardiomyocytes revealed up to 7.2% HDR following transduction with the rAAV9 vector. rAAV9-GFP transduced cardiomyocytes had 0% HDR detected (last lane).

601. AAV.U7-snRNA Targeting DUX4 Prevents Muscle Damage in a Facioscapulohumeral Muscular Dystrophy Mouse Model

Afrooz Rashnonejad, Gholamhossein Amini Chermahini, Noah M Taylor, Nicolas Wein, Scott Q Harper

Center for Gene Therapy, The Abigale Wexner Research institute at Nationwide Children's Hospital, Columbus, OH

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant or digenic disorder and among the most common muscular dystrophies, affecting up to 870,000 people worldwide. Classical descriptions of FSHD include weakness in muscles of the face, shoulder girdle, and upper arms, but presentation is non-uniform within the FSHD population. For example, roughly half of individuals have lower limb weakness, and although some people may maintain lifelong ambulation, others become severely debilitated and wheelchair dependent. There are currently no treatments that alter the course of FSHD, and therapy development is an unmet need in the field. FSHD arises from epigenetic changes that de-repress the *DUX4* gene in muscle. DUX4 protein causes cell death and muscle toxicity, and therefore we hypothesize that FSHD therapies should center on inhibiting *DUX4* expression. In this study, we developed a strategy to accomplish *DUX4* inhibition using U7-small nuclear RNA (snRNA) antisense expression cassettes (called U7-asDUX4). These non-coding RNAs were designed to inhibit production or maturation of the full-length *DUX4* pre-mRNA by masking the *DUX4* start codon, splice sites, or polyadenylation signal. In so doing, U7-asDUX4 snRNAs operate similarly to antisense oligonucleotides. However, in contrast to oligonucleotides, which are limited by poor uptake in muscle and a requirement for lifelong repeated dosing, U7-asDUX4 snRNAs can be packaged within myotropic gene therapy vectors and may require only a single administration when delivered to post-mitotic cells in vivo. One construct targeting the DUX4 polyA signal significantly silenced DUX4 *in vitro* in human FSHD myotubes and in muscles of TIC-DUX4 FSHD mice. For the latter, we packaged four copies of our lead U7-asDUX4 expression cassette into AAV6 particles and performed dose-escalation experiments for efficacy and toxicology outcomes. Muscles treated with AAV6-U7-asDUX4 were significantly protected from DUX4-associated deficits while untreated mice were not. DUX4-activated biomarker expression levels were decreased in treated muscles. These results support translation of a new DUX4-targeting gene therapy for FSHD that could be used alone or in combination with other strategies, like RNAi therapy, to maximize DUX4 silencing in individuals with FSHD.**Keywords:** Facioscapulohumeral Muscular Dystrophy, Double Homeobox 4, DUX4, U7-snRNAs, PolyA signal, adeno-associated virus vectors, muscle.This work was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS) Center of Research Translation in Muscular Dystrophy Therapeutic Development grant 1P50AR070604.

602. Safety of Delivery of rAAVrh74.MCK.GALGT2 by Isolated Limb Infusion in Two Boys as a Surrogate Gene Therapy for Duchene Muscular Dystrophy

Kevin M. Flanigan, Tatyana Vetter, Emma Frair, Adrienne Bradley, Johan Harris, Tabatha Simmons, Davin Packer, Megan Iammarino, Deborah Zygmunt, Paul T. Martin

Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH

The severe and progressive X-linked Duchenne muscular dystrophy (DMD) is caused by mutations in the gene encoding the dystrophin protein, absence of which results in instability of the sarcolemmal membrane, myofiber degeneration, and chronic and progressive muscle damage leading to loss of muscle function. Here we present the results of a first-in-human trial of AAV delivery of the *GALGT2* gene, which encodes cytotoxic T cell (CT) βGalNAc transferase, an enzyme that produces the CT glycan on specific glycoproteins and glycolipids. *GALGT2* overexpression protects against muscle loss during eccentric contraction-induced injury in both healthy and dystrophic muscles, as demonstrated in prior *mdx* mouse studies in which it inhibits the development of dystrophic muscle pathology associated with muscular dystrophy. In a phase 1/2, open-label dose escalation trial, we delivered human *GALGT2* encapsidated in AAVrh74, and under the control of the MCK promoter (rAAVrh74.MCK.*GALGT2*) via an intravascular limb infusion approach in which the *GALGT2* gene is delivered bilaterally to the legs via the femoral artery. Two subjects were enrolled: one (age 8.9 yr at dosing) received the minimal efficacious dose (as titered from a supercoiled standard) predicted from preclinical studies (2.5×10^{13} vg/kg per leg, or 5×10^{13} vg/kg total), and the second (age 6.9 yr) a higher dose (5×10^{13} vg/kg per leg, or 1×10^{14} vg/kg total). Each were treated with oral prednisone from day -1 and through the weeks following infusion. No serious adverse events were observed; serum transaminase elevations generally did not exceed baseline values. EliSpot analysis showed no significant positive responses, with only a minimally positive T-cell responses to two of three anti-AAVrh74 peptide pools, and one of two anti-GALGT2 peptide pools in Subject GAL-DMD-04 at their last visit at month 24. Muscle biopsy evaluated at baseline and at month 4 (subject 1; delayed due to family issue) or month 3 (subject 2) revealed increases in GALGT2 positive fibers, in subject 1 from 1.6% to 11.7% (a 7.35-fold increase), and in subject 2 from 0.5% to 5.8% (11.7-fold). Functionally, subject 1 showed a decline in 6 minute walk (6MW) distance; an increase in time to rise and in time to run 100 meters; and a decline in NSAA score until ambulation was lost at the 24 month timepoint. In contrast, subject 2, treated at a younger age and at a higher dose, demonstrated an improvement over 24 months in the NSAA score (from 20 to 23 points), an increase in the 6MW distance (from 405 to 478 m), and only a minimal increase in 100 meter time (45.6 to 48.4 sec), suggesting stability or improvement. These limited data suggest the preliminary safety of delivery of a systemic dose of 1×10^{14} vg/kg, and suggest efficacy at the higher dose in younger patients.

603. Adenine Base Editing to Modulate mRNA Splicing as a Therapeutic Strategy for Duchenne Muscular Dystrophy

Veronica Gough, Claire Engstrom, Joel Bohning, Charles A. Gersbach

Biomedical Engineering, Duke University, Durham, NC

The removal of introns and inclusion of selected exons during mRNA splicing is critical to normal gene function and is often misregulated in genetic disorders. Technologies that modulate mRNA processing and exon selection, such as exon skipping approaches, may be used to study and treat these diseases. Therapeutic exon skipping aims to restore the correct reading frame or induce alternative splicing by blocking the recognition of splicing sequences by the spliceosome, leading to removal of specific exons along with the adjacent introns. For example, Duchenne muscular dystrophy (DMD) is typically caused by deletions of one or more exons from the dystrophin gene, leading to disruption of the reading frame. Expression of dystrophin protein can be restored by correcting the reading frame by inducing the exclusion of one or more additional exons. Studies have shown that by targeting Cas9 to the splice acceptor of exons, the indels produced during DNA repair can disrupt the splice site and induce exclusion of the exon. In contrast to the semi-random indels generated by the conventional CRISPR-Cas9 system, base editing technologies have been developed for the precise modification of a single base pair without inducing double-stranded DNA breaks. Cytosine base editors can change a C directly to a T, while adenine base editors achieve an A to G edit, and they have been targeted to both "GT" splice donors and "AG" acceptors of a variety of exons to modulate mRNA splicing. We first designed gRNAs to target the base editor systems with both *S. pyogenes* and *S. aureus* Cas9 proteins to human dystrophin exons within the hotspot for inherited deletions in the *DMD* gene between exons 45 and 55. We assayed splice acceptor editing at four dystrophin exons by plasmid transfection of human HEK293T cells followed by deep sequencing of the target sites. While some exons and editors showed poor editing efficiency, we found that several recently published adenine base editors, ABE8e and ABE8.17m, resulted in 52.4% and 51.1% edited alleles, respectively, at exon 45. Removal of this exon from the dystrophin transcript could treat the second largest group of DMD patients (~8%) among all single exon skipping approaches. In order to test the effect of splice site disruption on exon skipping, we generated a human iPSC line harboring a deletion of dystrophin exon 44. This pluripotent cell line models an inherited DMD mutation with a disrupted reading frame of the *DMD* gene that is correctable by removal of exon 45. Myogenic differentiation of this Δ44 iPSC line by lentiviral transduction of MyoD cDNA confirms that the mutation ablates dystrophin protein expression, which can then be restored by base editing of the splice acceptor. We also developed a mouse model by excising exon 44 from the human dystrophin gene in the hDMD/mdx mouse. To facilitate in vivo editing, we evaluated both ABE8e and ABE8.17m in AAV-compatible split intein designs. Future studies will evaluate the functional impact this single base pair change can make on the DMD dystrophic phenotype.

604. Allele-Specific CRISPR/Cas9-Guide RNAs Inactivate a Dominant-Negative, Disease-Causing, Single Nucleotide Variant in *COL6A1* through Non-Homologous End Joining

Véronique Bolduc[1], Katherine Sizov[1], Prech Uapinyoying[1,2], Eric Esposito[1], Astrid Brull[1], Grace S. Chen[1], Apurva Sarathy[1], Kory Johnson[1], Carsten G. Bönnemann[1]

[1]NINDS/NIH, Bethesda, MD,[2]Center for Genetic Medicine Research, Children's Research Institute, Children's National Health System, Washington, DC

Targeting dominant-negative pathogenic variants with gene editing tools, with the goal of allele-selective inactivation, is a rational approach to dominant genetic conditions in which haploinsuffiency is tolerated. Here, we explored this simple and straightforward approach of introducing random frameshifting edits via non-homologous end joining, to selectively silence a dominant allele. We focused on a common, single-nucleotide dominant-negative glycine substitution in the *COL6A1* gene (c.868G>A; G290R), that causes dysfunction of collagen VI in the extracellular matrix and is associated with a severe form of muscular dystrophy. We nucleofected primary fibroblasts derived from four patients and one control subject with a spCas9-GFP-expressing plasmid that contained one allele-specific guide RNA (gRNA), without providing a repair template. By expanding clonal populations following CRISPR editing, we found that a collagen VI matrix was re-established when a frameshifting edit was stably incorporated on the pathogenic allele, providing proof-of-principle for this strategy. Using a deep-sequencing method, we examined the indel profile generated by the gRNAs and found that a single frameshifting cytosine deletion accounted for the majority of edits introduced at either allele, across the four patient lines. While the gRNA activity was 2-fold greater at the variant allele, complete allele selectivity was not attained in this experimental framework. Nevertheless, collagen VI matrix deposition was increased in a dish where cells were kept as a mixed population after gene editing, suggesting that the subset of cells that were effectively corrected by this gene editing approach was sufficient to improve the cellular phenotype. To increase allele selectivity, we are currently testing a series of gRNAs that contain a second mismatch. This study strengthens the potential of gene editing to treat dominant disorders, but also underscores the challenge in achieving allele selectivity with gRNAs.

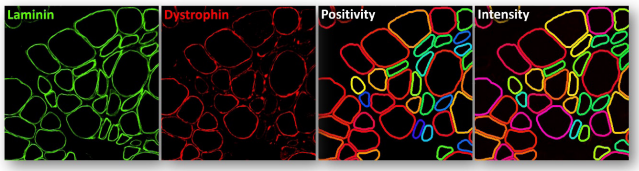
605. Precise and Unbiased Immunofluorescence Quantification Method for Automated Analysis of Muscle Dystrophin Expression

Tatyana A. Vetter[1], Adrienne J. Bradley[1], Emma C. Frair[1], Stefan Nicolau[1], Kevin M. Flanigan[1,2]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Departments of Pediatrics and Neurology, Ohio State University, Columbus, OH

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disorder characterized by muscle degeneration that eventually leads to loss of ambulation and cardiorespiratory failure. DMD is caused by a large variety of mutations in the *DMD* gene that ablate

dystrophin expression at the sarcolemma, where it typically serves to stabilize the membrane during contraction. In recent years, many potential new gene therapies have been developed with the goal of restoring dystrophin expression in patients with a wide range of *DMD* mutations. However, sensitive and reproducible quantification of changes in dystrophin expression continues to be a challenge in assessing efficacy in preclinical and clinical gene therapy studies. We recently developed an automated and unbiased method for precise quantification of dystrophin immunofluorescence in muscle tissue. Images of whole tissue sections co-stained for dystrophin and a sarcolemmal marker (spectrin or laminin) undergo a series of processing, segmentation, and measurement steps that utilize objectively derived thresholds to identify individual muscle fibers and dystrophin-positive sarcolemmal segments belonging to each fiber. Quantification results include precise measurements of dystrophin positivity as a proportion of each muscle fiber's perimeter, dystrophin intensity around each fiber, and fiber morphometric characteristics. Furthermore, the methodology is flexible enough to incorporate additional measurements of sarcoplasmic or nuclear markers. In the present study, we demonstrate the sensitivity and precision of this quantification approach in the context of both preclinical and clinical applications. We present evidence that this analysis method offers the capability to quantify dystrophin expression in terms of both positivity and intensity in a variety of dystrophinopathy biopsy samples, as well as in a DMD mouse model after AAV-mediated dystrophin restoration. As efforts to reestablish dystrophin expression in dystrophic muscle deliver more potential therapies and new clinical trials, this methodology represents a valuable tool for detailed and efficient analysis of muscle markers to reflect the efficacy of these treatments and allow them to be compared across studies.



606. Comprehensive Evaluation of AAV8 and AAV9 Myofiber-Type Preference in Normal and Muscular Dystrophy Dogs Following Systemic Delivery

Matthew J. Burke[1], Dennis O. Pérez-López[1], Chady H. Hakim[1,2], Dongsheng Duan[1,3,4,5]

[1]Molecular Microbiology and Immunology, University of Missouri, Columbia, MO,[2]National Center for Advancing Translational Sciences, Bethesda, MD,[3]Biomedical Sciences, University of Missouri, Columbia, MO,[4]Bioengineering, University of Missouri, Columbia, MO,[5]Neurology, University of Missouri, Columbia, MO

Systemic adeno-associated virus (AAV) mediated gene therapy holds great promise to treat neuromuscular diseases such as Duchenne muscular dystrophy (DMD). Several AAV serotypes have been explored in DMD animal models and clinical trials. Among these, AAV8 and AAV9 are the most commonly utilized serotypes. Muscle consists of myofibers with distinctive molecular and physiological properties. An important question in AAV-mediated muscle gene therapy is to understand myofiber type-specific transduction properties of AAV vectors. We hypothesize that AAV8 and AAV9 have different myofiber type tropism. To test this hypothesis, we evaluated transduction efficiency of AAV8 and AAV9 following systemic gene transfer in normal dogs and in the canine DMD model. A total of 20 systemically injected and 24 un-injected dogs were studied. Half of AAV injected dogs received AAV8 and the other half received AAV9. Half of AAV injected dogs received a therapeutic micro-dystrophin vector and the other half received an alkaline phosphatase (AP) reporter vector. Three muscles were evaluated in each dog. Myofibers were identified by laminin immunostaining. Micro-dystrophin and AP expression were evaluated by immunofluorescence staining and chromogenic immunohistochemical staining, respectively. Myofiber types were determined by immunofluorescence staining using a cocktail of antibodies that recognize different isoforms of myosin heavy chain for type I, type IIa, and type IIb myofibers. Staining protocols were optimized to effectively detect type I, type II, and hybrid myofiber types in AAV-transduced and non-transduced muscle cells. Preliminary data revealed unique transduction profiles of AAV8 and AAV9 in normal and affected dogs. Importantly, we found that micro-dystrophin gene therapy improved myofiber type distribution in affected dogs. This is the first study to comprehensively study myofiber type preference of AAV vectors in a large animal model. Our findings provide important information to better understand systemic AAV gene therapy in large mammals (Supported by NIH, Edgewise Therapeutics, and Jackson Freel DMD Research Fund).

607. Overexpression of JAG1 a Disease Modifier for Duchenne Muscular Dystrophy Therapy

Yuanfan Tracy Zhang, Matthias R. Lambert, Jeffrey J. Widrick, James R. Conner, Janelle M. Spinazzola, Louis M. Kunkel

Genetics and Genomics, Boston Children's Hospital, Boston, MA

The field of gene therapy for Duchenne muscular dystrophy (DMD) has been very active in the past few years with multiple clinical trials going on. The large size of the disease causal gene *DMD* presents a challenge for AAV therapy as the cDNA of dystrophin is 14 kb, well over the carrying capacity of AAV. Thus, different AAV-microdystrophins (truncated forms of dystrophin) has been the main form of gene therapy for DMD in clinical development. While the trials are showing promising results, the limited transgene size and threat of anti-dystrophin immune response indicate the necessity to explore other strategies. Genetic modifiers can be complementary to current therapies and work for a larger patient population. Previous work from our lab has shown that Jagged1, a Notch ligand, as a genetic modifier of DMD pathology in a Golden Retriever Muscular Dystrophy (GRMD) dog colony in which two exceptional "escaper" dogs exhibited a drastically milder phenotype than typical GRMD dogs despite being dystrophin-deficient. The amelioration of dystrophic phenotype was shown in zebrafish models and patient muscle cells. Our present study further assess and validate the therapeutic potential of the genetic modifier Jagged1 to ameliorate DMD pathology using transgenic mouse models as well as AAV. We created a muscle specific overexpression of Jagged1 in a dystrophic mouse model(mdx5cv).

While using AAV, we were able to deliver Jagged1 at different time points in the dystrphic disease progression. Using behavioral, physiological, histological, and molecular assays, we have evaluated the potential of Jagged1 therapy for DMD and related cardiomyopathies.

608. Extracellular Vesicles for Monitoring Micro-Dystrophin Gene Therapy

Lauren M. Sullivan, Ningyan Hu, J. Alex Sizemore, Kevin Liu, Bakhos Tannous, Thurman M. Wheeler
Massachusetts General Hospital, Boston, MA

Objective: To test whether extracellular vesicles (EVs) can be used for monitoring the durability of micro-dystrophin gene therapy in DMD. **Introduction**: Duchenne muscular dystrophy (DMD) is caused by sequence variants in the dystrophin (DMD) gene that lead to absence of dystrophin protein. In the mdx mouse model of DMD, micro-dystrophin gene transfer therapy ameliorates muscle dysfunction and degeneration by increasing sarcolemmal localization and total dystrophin protein in muscle tissue. In ongoing AAV-micro-dystrophin gene therapy clinical trials, measurement of micro-dystrophin expression currently requires that participants undergo serial muscle tissue biopsies, an invasive procedure that requires general anesthesia in pediatric patients. Although the long-term durability of micro-dystrophin in human muscle tissue is unknown, it is doubtful that repeated muscle biopsies is a viable approach to monitor micro-dystrophin expression throughout a patient's lifetime. EVs are membrane-bound particles that include exosomes, microvesicles, and other membrane-encased particles released from and taken up by cells as a form of extracellular communication. EVs in serum and urine contain mRNA and non-coding RNAs, including microRNA (miRNA), termed exRNAs, released from different tissues and can serve as genetic biomarkers of cancers and other disease states. Previous work has identified endogenous DMD mRNA splice products in EVs. **Methods**: We generated DMD myoblasts by co-treatment of primary DMD fibroblasts (GM05017; Coriell) with lentiviral vectors expressing a tamoxifen-inducible MyoD together with a micro-dystrophin/GFP fusion gene (Kimura, 2008). Control cells were treated with the MyoD vector alone or no vector (Lv 0). We confirmed conversion to muscle cells by immunolabeling with antibodies targeting MyoD, myosin heavy chain (MHC), and GFP. We measured EV size in tissue culture medium of differentiated muscle cells using NanoSight particle tracking analysis, isolated exRNA from EVs using ultracentrifugation, and quantified expression of micro-dystrophin and human skeletal actin by droplet digital PCR (ddPCR) and gene-specific primers. For in vivo studies, we treated mdx mice (N = 6) with AAV-CK8-micro-dystrophin for five weeks, quantified the percentage of dystrophin-positive muscle fibers by immunolabeling, and measured micro-dystrophin expression in serum by ddPCR. **Results**: MyoD transfection efficiency was 88 - 98% in growth medium. After one month in differentiation medium, 57 - 94% of treated cells expressed myosin heavy chain. MyoD and MHC expression were absent in Lv 0 treated cells. EV mean and mode size was similar in all three groups (approximately 120 - 150 nm) from one week through one month in differentiation medium. Micro-dystrophin expression relative to reference gene general transcription factor 2B (GTF2B) tended to be higher in EVs (1.8 - 2.7) than in cells (0.76 - 1.3). In mdx mice, micro-dystrophin expression relative to Gtf2b correlated with dystrophin-positive fibers in paraspinal and quadriceps muscle (Pearson r = 0.89; $P < 0.0001$). **Conclusions**: Micro-dystrophin transcripts are measurable in EVs of cultured human myotubes, and in serum EVs of mdx mice correlate with dystrophin-positive fibers. Our results support further investigation of EVs for long-term monitoring of micro-dystrophin in DMD gene therapy patients. **Grant support**: United States Department of Defense.

609. Dissecting Bone Remodelling Mechanisms and Hematopoietic Stem Cell Gene Therapy Impact in Mucopolysaccharidosis Type I Hurler Bone Defects

Ludovica Santi[1], Sara Penna[1], Stefania Crippa[1], Valentina Capo[1], Giada De Ponti[1], Margherita Berti[1], Marilena Mancino[1], Francesca Tucci[2], Mara Riminucci[3], Alessandro Corsi[3], Marta Serafini[4], Thibaut Klein[5], Ivan Martin[5], Bernhard Gentner[1], Alessandro Aiuti[1], Anna Villa[1], Maria Ester Bernardo[1]
[1]San Raffaele-Telethon Institute for Gene Therapy (SR-Tiget), Milano, Italy,[2]Pediatric Immunohematology and Bone Marrow Transplantation Unit, San Raffaele Scientific Institute, Milano, Italy,[3]Department of Molecular Medicine Sapienza University, Roma, Italy,[4]DIMET, University of Milano-Bicocca, School of Medicine and Surgery, Monza, Italy,[5]Tissue Engineering Department of Biomedicine, University Hospital Basel, Basel, Italy

Mucopolysaccharidosis type I Hurler (MPSIH) is a lysosomal storage disorder caused by deficiency of alpha-L-iduronidase (IDUA) enzyme, involved in the catabolism of glycosaminoglycans (GAGs). Progressive accumulation of GAGs in multiple tissues leads to multi-organ dysfunction and a wide range of skeletal abnormalities, known as *dysostosis multiplex*. Although allogeneic hematopoietic stem cell transplantation is considered the current standard of care, skeletal manifestations remain an unmet clinical need, probably due to the insufficient delivery of donor enzyme to bones and/or the irreversibility of the defect at the time of intervention. Preliminary results of the phase I/II clinical trial of autologous hematopoietic stem cell gene therapy (GT) conducted at SR-Tiget show supraphysiologic IDUA levels in peripheral blood, normalization of urinary GAG excretion, maintenance of motor skills, reduced joint stiffness, and growth tracking in-line with baseline percentile. However, as the pathogenesis of bone defects is still not fully understood, we investigated the role and the functional properties of bone cellular components: mesenchymal stromal cells (MSCs), MSC-derived osteoblasts (OBs) and osteoclasts (OCs) isolated from healthy donors and MPSIH patients before and after GT. OC differentiation and bone resorption activity were not impaired by IDUA deficiency, as well as isolation and characterization of patient-derived MSCs before GT were not altered. Indeed, pre-GT osteoblast (OB) and adipocyte differentiation were not affected, although MPSIH-MSCs displayed no enzyme activity. In the GT context, we hypothesized that OCs, deriving from genetically corrected hematopoietic precursors, could deliver the missing enzyme in the bone microenvironment where MSCs and their progeny, including OBs, reside, being the mediators of cross-correction. Notably,

supraphysiologic enzyme activity was found in differentiated OCs after GT and in their culture media. MPSIH-MSCs, normally expressing the mannose-6-phosphate receptor, were able to efficiently uptake the IDUA enzyme when exposed to the supernatant of OCs generated from MPSIH patients after GT. Nevertheless, although MPSIH-MSCs did not display GAG accumulation, if MPSIH-MSCs undergo OB differentiation, at the end of the differentiation, they showed an increase in intracellular GAGs. Importantly, our preliliminary data showed that GAGs engulfment could be rescued by exposure of MPSIH-OBs to the supernatant of post-GT OCs, demonstrating the possibility to cross-correct bone cells of non-hematopoietic origin. We will further investigate the implications and the impact of MPSIH-OB GAG accumulation in terms of functionality, for instance in supporting osteoclastogenesis. In addition, we are investigating chondrocyte differentiation of MPSIH-MSCs to deeply characterize the endochondral ossification process. To this end we are exploiting a 3D model of hypertrophic cartilage from patient-derived MSCs in order to mimic the growth plate. Moreover, we will analyse bone biopsies obtained from MPSIH patients before and after GT by immunohistochemistry and transmission electron microscopy.

610. Abstract Withdrawn

611. AAV Platform Vector Gene Therapy (PAVE-GT) for Congenital Myasthenic Syndomes

Janelle Geist Hauserman[1], Eric Esposito[1], Richa Lomash[2], Julien Oury[3], Sally Spendiff[4], Elizabeth Ottinger[2], PJ Brooks[2], Hanns Lochmüller[5], Steven Burden[3], Donald Lo[2], Anne Pariser[2], Carsten G. Bönnemann[1]

[1]NINDS, National Institutes of Health, Bethesda, MD,[2]NCATS, National Institutes of Health, Bethesda, MD,[3]New York University, New York, NY,[4]University of Ottawa, Ottawa, ON, Canada,[5]University of Ottawa, Bethesda, ON, Canada

Congenital myasthenic syndromes (CMS) are a class of rare genetic diseases that affect approximately 1:100,000 children. They are categorized by early onset diminished motor function due to deficiencies or dysfunction of neuromuscular junction (NMJ) proteins. Adeno-associated virus (AAV)-based gene therapy in principle offers an attractive method for the treatment of CMS, in particular for loss of function situations that would benefit from gene replacement approaches. AAV based therapies for various neuromuscular disorders are in clinical trials with one (for SMA) having reached FDA approval. While promising, the development of each such therapy requires a considerable and complex commitment that will be difficult to realize for ultrarare disorders. CMS as a group are rare but even more so when individual genetic entities are considered, the development of individual gene specific therapies would be very challenging. However, all relevant gene products are localized in the same well-defined structure while their dysfunction results in related clinical manifestations of impaired neuromuscular transmission. Thus, when considering a gene replacement approach using AAV as a tool, various CMS could be grouped and be addressed with the same AAV serotype, expression/promoter construct, outcome measures and trial design. Conceivably, such grouping could extend to production, toxicology and regulatory discussions, making the development of gene therapy for such ultrarare genetic disorders more feasible. This idea is the basis for the Platform Vector Gene Therapy (PaVe-GT) project initiated by the National Center for Advancing Translational Science (NCATS) to explore the feasibility of such grouped platform approaches as public facing and transparent imitative and for which the CMS will serve as one model system (the other being organic acidemias). Two of the most common recessive forms of CMS will be addressed initially and in parallel: deficiencies of Dok7, a regulator of acetylcholine receptor assembly that is necessary for postsynaptic NMJ formation, and of Collagen Q, the collagen tail subunit of acetylcholinesterase, anchored in the synaptic cleft. We have begun to study the use of self-complementary AAV and various vector/expression constructs to target the NMJ's subsynaptic nuclei that will then be used to treat established animal models in order to determine if the gene therapy is successful at restoring NMJ structure and muscle function. We thus plan to demonstrate this platform-based gene therapy approach from conception, preclinical optimization, production, toxicology studies, regulatory filings and interactions, all the way to a clinical trial and make all learnings from this fully available.

612. Novel and Highly Potent Selective Synthetic Promoters for Muscle-Directed Gene Therapy Applications

J. Omar Yanez-Cuna, Ileana Guerrini, Rachael Di Santo, Nicolle Kippen, Flavia Scialpi, Polyxeni Katsoupi, Rinku Rajan, Juan Manuel Iglesias-Gonzalez, Michael Leslie Roberts

AskBio, Edinburgh, United Kingdom

Efficient, durable and targeted expression of therapeutic genes in select tissues is an important pre-requisite for the development of safe and effective gene therapies. The human genome comprises a broad array of regulatory elements that orchestrate the development of multiple cell lineages and tissues. We have found that certain regulatory elements can function in isolation to drive cell-selective transcription and can be incorporated into expression cassettes to drive expression of heterologous genes. We have developed a systematic approach to facilitate the meta-analysis of diverse functional genomics datasets with a view to identify and isolate the important functional regulatory sequences involved in controlling cell-, or tissue-selective gene expression. We have been able to incorporate these identified sequences in the design of novel synthetic promoters that show high levels of durable expression and selectivity. Here we describe the design and construction of a panel of muscle-selective promoters, capable of driving expression of reporter genes in vivo to levels that are several-fold higher compared to other constitutive or muscle promoters that are widely used by the gene therapy community. We have also been able to create selective promoters that target expression either to skeletal muscle groups alone, or to cardiac tissue alone, or to both skeletal and cardiac tissue. Expression from these novel promoters can be further increased by incorporating UTR/intron constructs into the design of the expression cassette. In summary, we have demonstrated that systematic engineering of regulatory elements from the human

genome enables the construction of novel muscle-selective expression cassettes that show improved expression characteristics for different target muscle groups.

613. Application of a Single Vector CRISPRa Therapy for Treatment of Congenital Muscular Dystrophy 1A

Yuanbo Qin, Talha Akbulut, Rajakumar Mandraju, Keith Connolly, Payal Pranami, Claudia Carbone, Mansi Thakkar, Nicole Pandell, Vandhana Chezhiyan, Tetsuya Yamagata

Modalis Therapeutics, Cambridge, MA

Background: MDC1A is a severe muscle disease involving muscle weakness, loss of ambulation, and respiratory failure. It is caused by mutations in both alleles of the LAMA2 gene leading to loss of laminin-α2 protein function. Expression of the Lama1 gene, which encodes a structurally similar protein laminin-α1, ameliorates muscle phenotypes in mouse models of MDC1A, demonstrating a compensatory role for Lama1. Due to the large size of Lama1 and Lama2 genes (cDNA > 9kb) delivery is precluded by the AAV size limit (~4.7 kb). An approach to overcoming this limitation is use of the CRISPR activation (CRISPRa) system, which enables activation of genes from their endogenous loci, to induce the expression of disease compensatory genes that are too large to fit into AAV. Publications that report the use of the CRISPRa system employ bipartite delivery methods, using two-AAV, which impose significant challenges to efficient clinical development. Here we introduce the proprietary CRISPR-GNDM® technology where all necessary components for CRISPRa are packed into a single AAV vector for therapeutic use. **Method:** We screened ~50 gRNAs to identify gRNAs that efficiently up-regulate the expression of mouse Lama1 gene from its endogenous wild-type locus. We packed into a single AAV genome the gRNA expression cassette and the GNDM molecule encoding a potent transactivator moiety driven by the muscle specific CK8 promoter. A single rAAV9 carrying the CRISPR-GNDM® cassette was injected into adult WT mice at 1.5 x 10^{14} vg/kg to evaluate the technical feasibility and potency of our method. We also evaluated effects on muscle function and pathology in disease models for MDC1A, dy2j and dyW mice, at up to 9 weeks post-injection. To identify potential therapeutics we also screened > 400 gRNAs that target human and/or cynomolgus monkey LAMA1 genes. Our proprietary PAM-flex dCas9 platform enabled identification of gRNAs compatible with both human and NHP LAMA1 genes. A gRNA was packaged into the CRISPR-GNDM® cassette and a single rAAVrh74 vector encoding the CRISPR-GNDM® was infused into NHP at 1 x 10^{14} vg/kg to evaluate the primary pharmacology, pathology and immunogenicity of the CRISPR-GNDM® system at 4, 8, 14 and 20 weeks post-injection. Off-target gene expression was also analyzed in muscle tissues in mouse and NHP. **Results:** In WT mice at 12 weeks post-injection, Lama1 mRNA expression was induced up to > 4,000 fold in skeletal muscle tissues and >1,000 in cardiomyocytes over the baseline levels. The Lama1 gene expression relative to the endogenous Lama2 gene expression was induced by > 55% in cardiomyocytes and up to 25% in skeletal muscles. Robust Lama1 protein expression was observed in the basement membrane of the muscle fiber by immunofluorescent staining. The Lama1 expression was only observed in Cas9 expressing cells, confirming the CRISPR-GNDM® mediated modulation. No obvious lymphocyte infiltration or muscle fiber destruction was observed in the histological analysis. In the NHP study, Cas9 and LAMA1 expression was confirmed in the muscle tissues. Isolated PBMCs showed mild *IFNγ* production upon Cas9 peptide stimulation, however, no significant lymphocyte infiltration was observed in muscles, and the Cas9 expression persisted for up to weeks, indicating that no detrimental immune reaction against Cas9 protein was elicited. Whole genome RNAseq analyses demonstrated minimal off-target effects of our gRNAs. **Conclusion:** Our proprietary CRISPR-GNDM® technology offers a single vector treatment modality, with robust potency and high selectivity that is well-tolerated in mice and NHP. The CRISPR-GNDM® technology has broad applicability to a variety of inherited and acquired disorders where the culprit gene is too large to be delivered via AAV-mediated gene therapy.

614. A Novel AAV8 Vector for Microdystrophin Gene Therapy of Duchenne Muscular Dystrophy: Preclinical Studies in the Mdx Mouse

SunJung Kim, Nicholas Buss, Hiren Patel, Lin Yang, Kirk Elliott, Chunping Qiao, Ye Liu, Michele Fiscella, Olivier Danos

REGENXBIO Inc, Rockville, MD

Duchenne muscular dystrophy (DMD) is an x-linked genetic disorder caused by mutations in the dystrophin gene. A deficiency of dystrophin protein results in progressive muscle weakness, degeneration, and waste, eventually leading to premature death due to respiratory and/or cardiac failure. We have developed RGX-202, a recombinant adeno-associated virus of serotype 8 (AAV8) containing an optimized human micro-dystrophin transgene under the control of a muscle specific promoter. The microdystrophin includes an extended coding region of the carboxyl terminal (CT) domain, known to bind α-syntrophin and α-dystrobrevin. The protein coding sequence has been codon optimized and depleted of CpG dinucleotides. To evaluate the therapeutic efficacy of RGX-202, preclinical studies were performed in the *mdx* mouse model of DMD (C57BL/10ScSn-Dmdmdx/J). In a proof-of-concept study, RGX-202 was administered intravenously to male *mdx* mice (5 weeks old, n=10), and muscle functions (grip strength and *in-vitro* force) were measured after 6 weeks. RGX-202-treated *mdx* mice exhibited significant increases in muscle strength compared to the vehicle-treated *mdx* mice. In addition, RGX-202 attenuated the key symptoms of muscular dystrophy in *mdx* mice (i.e., inflammation, degeneration, and regeneration) analyzed in the tibialis anterior (TA) and diaphragm. Biodistribution data and Western blot analysis demonstrated that the microdystrophin transgene was widely expressed in the skeletal and cardiac muscles. To further evaluate the pharmacology of RGX-202, groups of male *mdx* mice (5-6 weeks old, n=10) received a single intravenous injection of RGX-202 at 5 escalating doses. In vivo muscle evaluations by fine motor kinematic gait analysis and T2-magnetic resonance imaging (MRI) mapping were performed at 6 and 12 weeks after RGX-202 administration. RGX-202-treated *mdx* mice showed significant motor function improvement and reduced muscle damage in a dose-dependent manner. Creatine kinase levels as a marker of muscle damage were also dose-dependently decreased

at weeks 7 and 12. Taken together, these results suggest the potential of RGX-202 as a promising candidate for clinical development of microdystrophin gene therapy in DMD patients.

615. Generation and Characterization of GAA Knock Out HepG2 Cell Line

Bikash Shakya, Jie Vanderley, Lester Suarez, Richard J. Samulski

Research and Development, Asklepios BioPharmaceutical Inc, Research Triangle Park, NC

Pompe disease is a life-threatening neuromuscular disease caused by mutations in the gene encoding the lysosomal enzyme alpha-glucosidase (GAA) that converts lysosomal glycogen to glucose. The GAA deficiency leads to the accumulation of the glycogen in lysosomes, particularly in the skeletal muscle, liver, and heart. The inadequate in vitro research models that recapitulate the disease in human tissues is one of the key bottlenecks to study Pompe disease and investigate its treatments. Because of the ability of hepatic cells to efficiently secrete proteins in the blood circulation, the gene therapy using liver-directed AAV vectors is a potential strategy to treat Pompe disease in the entire body and this strategy has been successfully demonstrated in murine models. However, the animal models fail to fully simulate the human condition. For example, the post-translational modifications, such as glycosylation of the transgene-expressed GAA protein vary remarkably between human and mouse cells. The currently available human kidney-derived $GAA^{-/-}$ HEK293 cell does not fully simulate Pompe disease in humans, and thus, a more organ-specific in vitro human liver cell model for Pompe disease was sought. By using CRISPR-Cas9, we generated a $GAA^{-/-}$ human HepG2 cell line, which could be used as an in vitro model to study Pompe disease. A single guide RNA targeting exon 2 of the GAA gene was delivered in complex with SpCas9 into the HepG2 cells, resulting a very high gene editing efficiency of 96%. The pool of 96% GAA knocked out cells was subjected to limiting dilution cloning and a single cell clone with a single nucleotide insertion (Thiamine) at the SpCas9 cut site was obtained, which was confirmed by Sanger sequencing and Next generation sequencing of amplicons from genomic DNA. The $GAA^{-/-}$ cell line was validated by confirming the absence of GAA protein expression, GAA enzymatic activity, and the lysosomal accumulation of glycogen in the cells. We further characterized the cell line by checking the mRNA expression level for the mutated GAA gene, which was found to be >10 fold lower than that for the wild type GAA gene. Transcriptome profiling of the $GAA^{-/-}$ cell line showing the altered pathways resulting from the absence of GAA expression will also be presented. The $GAA^{-/-}$ cell line and the information on the differential gene expression should serve as important resources for elucidating the underlying disease mechanism and screening the therapeutics for Pompe disease.

Cancer - Immunotherapy, Cancer Vaccines

616. Oral Administration of *Bifidobacterium longum* Displaying WT1 Protein Augments Anti-Cancer Activity of Anti-PD-1 Antibody in Mice Renal Cell Carcinoma Model

Hazuki Doi, Mako Kato, Koichi Kitagawa, Shoko Tominaga, Shihoko Yanase, Toshiro Shirakawa

Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Japan

Introduction: Cancer immunotherapy using immune-checkpoint inhibitors such as anti-programmed death-1 (PD-1) has been approved for advanced renal cell carcinoma. Although a dramatic therapeutic effect of PD-1/PD-L1 inhibitor was achieved in a subset of patients, the objective response rate with anti-PD-1 antibody alone was only 25%. Therefore, combining immune-checkpoint inhibitors and cancer vaccines, which can forcibly induce tumor-specific cytotoxic T lymphocytes (CTLs), could be a promising modality to increase response rate in kidney cancer patients. Previously, we developed an oral cancer vaccine using recombinant *Bifidobacterium longum* displaying Wilms' tumor 1 (WT1) protein (*B. longum* 420) and showed anti-tumor effect in mouse prostate cancerin combination with anti-PD-1 antibody. The WT1 gene is the causative gene of renal tumor in children, is highly expressed in various solid tumors including renal cell carcinoma, and is one of the best tumor-associated antigens to induce antigen-specific CTLs. In this study, we investigated the mechanism of the anti-tumor effect of *B. longum* 420 to enhance the therapeutic efficacy of anti-PD-1 antibody in mice renal cancer model. **Methods:** One million cells of Renca, a WT1 and PD-L1-postive murine renal carcinoma cell line, were subcutaneously inoculated into Balb/c mice. One week after tumor inoculation, 1×10^9 of *B. longum* 420, *B. longum* 2012 (negative control), or PBS were orally administered 5 times a week for following weeks. Intraperitoneal injections of anti-PD-1 antibody were combined with *B. longum* 420 administration twice a week. After the treatment, tumors and spleens were collected to analyze the immune responses and tumor infiltrating lymphocytes. **Results:** As the results, the combination of *B. longum* 420 and anti-PD-1 antibody significantly inhibited tumor growth compared to the other treatment groups of anti-PD-1 antibody alone and *B. longum* 420 alone ($p<0.05$). The combination therapy also significantly prolonged the overall survival compared to the other groups after tumor inoculation ($p<0.05$). Intracellular cytokine staining showed that *B. longum* 420 significantly increased multiple WT1-epitope-specific CD4T cells and tumor necrosis factor-α (TNF-α)-producing CD8T cells in Balb/c mice ($p<0.05$, respectively). The number of tumor-infiltrating CD107a-positive CD8T cells were remarkably increased in the combination with *B. longum* 420 and anti-PD-1 antibody compared to the treatment of anti-PD-1 antibody alone. **Conclusions:** This study demonstrated that oral administration of *B. longum* 420 could induce the WT1-specific tumor immune responses and augmented the therapeutic effect of anti-PD-1 antibody. Our findings suggest that *B. longum* 420 break the immune tolerance in the immune checkpoint inhibitors by the forcible induction of tumor-specific activated CD8T cells in tumor

microenvironments. These results indicate that *B. longum* 420 can be a novel therapeutic candidate for kidney cancer in combination with immune checkpoint inhibitors.

617. cytoTILs™ Demonstrate Enhanced In Vivo Persistence in the Absence of IL-2, Paving the Way for Durable Efficacy and Improved Safety in Patients with Solid Tumor Malignancies

Mithun Khattar, Rachel Burga, Alex Storer, Colleen Foley, Scott Lajoie, Dan Thornton, Stanley Tam, Gwen Wilmes, Gabriel Helmlinger, Jeremy Tchaicha, Dhruv Sethi, Michelle Ols, Gary Vanasse, Jan ter Meulen, Shyam Subramanian

Cell Therapy, Obsidian Therapeutics, Cambridge, MA

Tumor-infiltrating lymphocytes (TILs) have generated promising data in clinical trials as therapy for heavily pretreated patients with solid tumor malignancies, such as metastatic melanoma. The IL-2 regimen required for *in vivo* maintenance of TILs poses significant limitations on application of the therapy because roughly one third of patients have co-morbidities or suboptimal Eastern Cooperative Oncology Group (ECOG) performance status contraindicating its use. Furthermore, a significant number of patients who receive IL-2 cannot tolerate the fully planned IL-2 regimen. Moreover, IL-2 increases immunosuppressive regulatory T cells and drives proliferation and exhaustion of terminally differentiated effector T-lymphocytes, which may reduce the long-term persistence and potent anti-tumor cytotoxicity of TILs. Our cytoTIL™ product is comprised of TILs engineered with membrane bound IL-15 (mbIL15) that is regulatable using a drug responsive domain (DRD) designed via our cytoDRiVE™ platform. We have developed a proprietary process for high efficiency transduction and expansion of cytoTILs. Controllable expression of IL-15 results in IL-2-independent expansion *in vitro* and *in vivo* in the presence of orally administered acetazolamide, an FDA-approved small molecule that is the ligand for our DRD. cytoTILs have an immunophenotypic profile that is distinct from conventional IL2-treated unengineered TILs (TIL), and maintain a diverse TCR repertoire, tumor reactivity, interferon gamma production, and cytotoxicity *in vitro* in the absence of exogenous IL-2. We have also demonstrated enhanced persistence of cytoTILs in NSG mice compared to conventional TILs treated with IL-2 dosing regimens analogous to those used in the clinic (Figure 1). In conclusion, cytoTILs pave the way for clinical development of a more potent and persistent TIL product that will not require infusion of IL-2, thereby enhancing the safety and promoting the durable efficacy of TIL therapy for the treatment of patients with metastatic melanoma and other solid tumor malignancies.

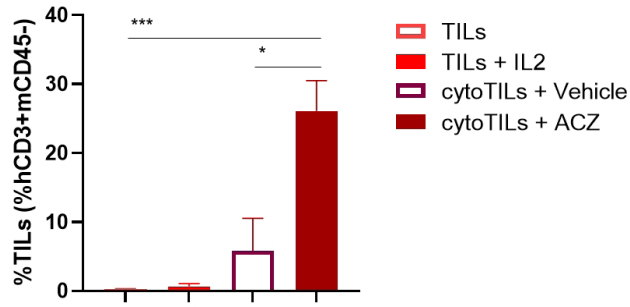


Figure 1: **cytoTILs demonstrate higher expansion and persistence in vivo compared to conventional IL-2 treated unengineered TILs.** Female NSG mice were injected with 1×10^6 TILs in four arms as described (n=5 mice/arm). TILs were enumerated in the peripheral blood of mice using flow cytometry at different time points and data is shown for day 39. The bar graph shows the mean frequency of human $CD3^+$ TILs in mouse blood after lysis of red blood cells. (* P <0.05, *** P <0.0005)

618. Modeling CAR-T Cell Activity against Solid Tumors *In Vitro:* An Assessment of 2D vs 3D Culture

Mary Kathryn McKenna[1], Alexander Englisch[1,2], Mae Louise Woods[1], Malcolm K. Brenner[1]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Department of Pediatric Hematology and Oncology, University Children's Hospital Muenster, Muenster, Germany

Chimeric Antigen Receptor (CAR) T cells can target and kill solid tumor cells *in vitro*, but lack of complete tumor clearance *in vivo* supports the importance of the tumor microenvironment (TME) in protecting tumor cells and allowing tumor progression. To improve the effectiveness of CAR-T cell treatments in solid tumors it is critical to model the behavior and interactions of each component of the TME alone and in combination. For practical reasons, this task is best initially accomplished *in vitro* but standard assays utilizing conventional 2D monolayer culture poorly mimic the cellular interactions with the extracellular matrix, morphology, and growth necessary to recapitulate an *in vivo* system. To address these limitations, we established a scaffold-free 3D *in vitro* culture system with multicellular spheroids to test CAR-T cell cytotoxicity. 3D tumor spheroids are aggregates of cells that can be grown in suspension on minimally adherent surfaces and exhibit a 3D architecture representative of cell-to-cell interactions, cell-produced ECM, proliferative exterior cells, necrotic cores (large spheroids >1mm diameter), as well as an enrichment for cancer stem cells that more closely mimics a solid tumor. Here, we prepare a bed of agarose to create an ultra-low attachment culture dish to create tumor spheroids. Indeed, we are able to observe CAR-T cell destruction of tumor spheroid structures and cell death indicated by Annexin V staining as shown in Figure 1. Additionally, we have incorporated other TME components including M2 macrophages and MDSCs that aggregate to form 3D spheroids with tumor. Incucyte Live Cell Imaging software allows us to visualize specific CAR-T cell interactions with the TME through alterations in 3D structure and quantify tumor cell death. We observe a decrease in Annexin V+ staining of tumor spheroids in the presence of MDSCs and M2 Macrophages suggesting these cells impair CAR-T cell anti-tumor activity. To model the complex TME, we are currently incorporating stromal fibroblasts, endothelial cells and immunosuppressive cellular components to determine their influence on CAR-T cell function as shown in Figure 2. To compare between these TME models, we are applying a Bayesian approach for joint segmentation and tracking to quantify the difference in velocity of T cell infiltration and the largest cross-sectional area of the spheroid. We

hypothesize these 3D models will be invaluable in assessing therapeutic T cell infiltration and overall activity to predict their efficacy in a solid tumor setting.

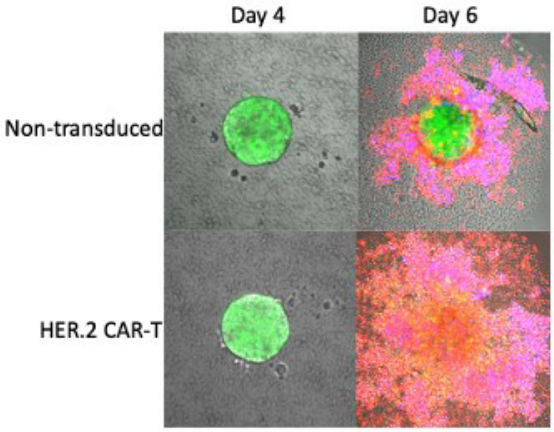


Figure 1: 2x10^3 GFP+ H1650 tumor cells were seeded on an agarose coated tissue culture plate. Four days later, 1x10^3 HER.2 specific CAR-T cells or non-transduced T cells labeled with efluor 670 were added to the culture. Annexin V AF594 was added to the culture with T cells to indicate cell death. Confocal images were acquired on indicated days (10X).

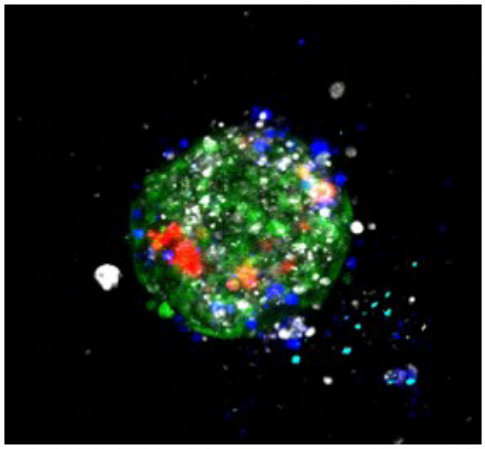


Figure 2: Multicellular 3D tumor spheroid: 2x10^3 GFP+ H1650 tumor cells were cultured with efluor 670 labeled Tig-320 lung fibroblasts (blue), efluor 450 labeled HUVEC cells (white) and RFP labeled mesenchymal stromal cells at a 4:2:2:1 ratio. Confocal image was acquired 96hr post spheroid generation. Maximal projection z-stack shown (20X).

619. Preclinical GMP Development and Testing of High Affinity Antiviral gp350-CAR-T Cells against EBV Malignancies

Renata Stripecke

Regenerative Immune Therapies Applied, Hannover Medical School, Hannover, Germany

Objectives: Epstein Barr virus (EBV) is a highly prevalent herpesvirus worldwide. EBV infection is associated with malignancies such as post-transplant proliferative disease (PTLD), lymphomas, nasopharyngeal carcinomas (NPC) and gastric carcinomas. Therapies against EBV-associated malignancies are still limited by side effects, mechanisms of viral evasion and immunosuppressive effects of EBV lytic reactivations. We previously showed *in vitro* and *in vivo* proof-of-concept of T cells engineered with chimeric antigen receptors (CARs) with high affinity against the EBV glycoprotein 350 (gp350). gp350-CAR-T cells containing the CD28.zeta domain recognized and killed EBV-infected cells *in vitro* (ASGCT-2017 poster presentation) and reduced in fully humanized mouse models EBV lytic infections, EBV$^+$ PTLD and lymphoma development prophylactically and therapeutically (ASGCT-2019 and ASGCT-2020 oral presentations). Here, we show the preclinical GMP development and potency testing of gp350CAR-T cells generated after transduction of T cells with a clinical lentiviral vector (LV-ZT002). Further, we expand our preclinical animal studies validation towards immunotherapy of EBV$^+$ NPC, a malignancy with high incidence in Southern China. **Methods:** HEK 293T/17 suspension cells were adapted to grow in serum free media and a cell bank was developed and characterized. The GMP-compatible processes for production of LV-ZT002 expressing gp350-CAR by transient transfection and purification by chromatography (AKTA) were developed and optimized. For the 10-day cell manufacturing, T cells were activated with Dynabeads CD3/CD28, transduced with LV-ZT002 and expanded with IL-2. LDH-based *in vitro* cytotoxicity assays were performed with the target cell line PCI-1/gp350. *In vivo* studies were performed in B-NDG mice implanted with 5x10^6 C666-1/gp350 NPC tumor cells s.c. and 5 days later treated i.v. with Mock T cells or gp350CAR-T cells. **Results:** LV-ZT002 production batches showed titers averaging 1x10^8 TU/ ml (determined in transduced Jurkat cells). Filling was performed as >5x10^7 TU/ ml and 0.5 ml per vial. Above 80% gp350CAR$^+$ T cells were obtained at LV multiplicity of infection (MOI) of 1 (CAR detectable after staining with immunoconjugated gp350 protein and FACS analyses). A RT-qPCR method was established and 1-4 VCN per cell were detectable depending on different donors. Specific *in vitro* cytotoxicity activity of gp350-CAR-T cells against the cell line PCI-1/gp350 at E:T ratio of 5:1 was in the range of 30%. gp350-CAR-T released IFN-gamma (average 9.0 ng/ml) upon target recognition. Mice pre-implanted with C666-1/gp350 cells (average tumor volume 60 mm^3 at day 5) and infused with 5x10^6 Mock T cells showed tumor growth (263 mm^3 at day 20), which was substantially delayed upon administration of 5x10^6 gp350CAR-T cells (80 mm^3 at day 20). **Conclusions and Outlook:** gp350-CAR-T cells were successfully produced under GMP-compliant conditions and demonstrated *in vitro* and *in vivo* potency. Therefore, we are pursuing further regulatory requirements for development of gp350-CAR-T cells as an Investigational New Drug. A Phase I clinical trial is planned in China to evaluate the safety and tolerability of gp350-CAR-T cells in subjects with relapsed or refractory EBV$^+$ associated malignancies. Conflict of Interest Statement: R.S. is a shareholder and scientific advisor receiving honoraria of Biosyngen/Zelltechs Pte.Ltd.

620. LAG-3, Tim-3 or 2B4 Disruption Differentially Regulate the Anti-Tumor Response of TCR Gene-Edited Human Memory Stem T Cells

Beatrice C. Cianciotti[1], Zulma I. Magnani[1], Barbara Camisa[1], Mattia Di Bono[1], Alessia Potenza[1], Valentina Vavassori[2], Luigi Naldini[3], Pietro Genovese[2], Eliana Ruggiero[1], Chiara Bonini[1]

[1]Experimental Hematology Unit, Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy,[2]Gene Transfer Technology and New Gene Therapy Strategies Unit, SR-TIGET, San Raffaele Scientific Institute, Milan, Italy,[3]SR-TIGET, San Raffaele Scientific Institute, Milan, Italy

Introduction: Adoptive T cell therapy with T lymphocytes genetically engineered to overexpress a tumor-specific T cell receptor represents an effective therapeutic option for cancer patients. However, the lack of long-term persistence of infused tumor-reactive T cells and the immunosuppressive tumor microenvironment represent major drawbacks of ACT. Specifically, chronically stimulated tumor-specific lymphocytes become exhausted, a dysfunctional status characterized by loss of effector functions. The Hallmark of exhaustion is the expression of several inhibitory receptors (IRs), such as PD-1, CTLA-4, Lag-3, Tim-3 and 2B4. Cancer cells often up-regulate the ligands of these inhibitory molecules, thus fostering T cell exhaustion to evade immune attack. The use of monoclonal antibodies targeting IRs, (e.g. anti-CTLA-4 and anti-PD-1), can revert T cell exhaustion but proved effective only in a fraction of patients. Also, autoimmune-related adverse events often occur after immune checkpoint blockade, mainly due to the unleashing of autoreactive clones. **Aim:** Here, we aim to generate innovative cell therapy products, endowed with long-term persisting capacity and resistance to inhibitory signals. **Methods:** Activated primary T cells were electroporated with CRISPR/Cas9 targeting TIM-3, LAG3 or 2B4 and the TCR alpha chain constant region (trac) genes. The frequency of NHEJ was assessed with ddPCR. T cells were activated and kept in culture with a protocol to expand early differentiated stem cell memory (T_{SCM}) and central memory (T_{CM}) cells (Cieri et al., Blood 2013). **Results**: We exploited the multiplexing approach of CRISPR/Cas9 system to simultaneously disrupt the endogenous TCR and one selected IR (Tim-3, LAG-3 or 2B4). We efficiently inactivated the endogenous TCR (>98% of NHEJ). Simultaneously, more than 80% of gene disruption was obtained at LAG-3, Tim-3 or 2B4 locus. TRAC-KO-IR-KO cells were efficiently transduced with the HLA-A2-restricted-NY-ESO1$_{157\text{-}165}$-specific TCR, thus obtaining TCR-edited-IR-KO T cells or TCR-edited-IR$_{COMP}$ T cells. Importantly, TCR-edited-IR-KO T cells retain their expansion capacity and an early differentiated T_{SCM}/T_{CM} phenotype. Upon chronic stimulation with HLA-A2^{pos}-NY-ESO1^{pos} multiple myeloma cells, TCR-edited-Tim-3-KO and TCR-edited-2B4-KO T cells retained a higher degranulation capacity compared to TCR-edited-IR$_{COMP}$ T cells, while TCR-edited-LAG-3-KO T cells showed a limited upregulation of additional inhibitory receptors. In a stress test multiple myeloma model, TCR-edited-Tim3-KO and TCR-edited-2B4-KO T cells did not result in improved anti-tumor activities, but they were enriched in a higher proportion of highly activated T cells compared to TCR-edited-IR$_{COMP}$-treated mice. In the same model, TCR-edited-LAG-3-KO, but not TCR-edited-IR$_{COMP}$, T cells showed a reduced expression of additional IRs and prevented the engraftment of a second infusion of tumor cells, thus indicating an enhanced T cell fitness and resistance to exhaustion of TCR-edited-LAG-3-KO T cells. Overall, our results show that by combining the versatility of multiplex gene editing by CRISPR/Cas9 with culture conditions designed to engineer T_{SCM} cells, we can generate innovative tumor-specific cellular products redirected against tumor antigens and resistant to different inhibitory signals.

621. Targeting Macrophages with CAR-T Cells Delays Progression of a Mouse Model of NSCLC

Alfonso R. Sánchez-Paulete, Jaime Mateus-Tique, Miriam Merad, Brian D. Brown

Icahn School of Medicine at Mount Sinai, New York, NY

Tumor-associated macrophages (TAMs) are one of the most prevalent immune cell types in the microenvironment of solid tumors (TME), and typically exert protumor effects, among which is suppression of antitumor T-cell responses. Although preclinical studies have shown promising results, macrophage-targeted therapies have had limited success in clinical trials. In this work, we have developed a strategy for macrophage depletion using second-generation chimeric antigen receptor (CAR) T cells targeted against the F4/80 antigen. F4/80 CAR-T cells were biologically active and eliminated F4/80+ cells in vitro and in vivo. Administration of CAR-T cells delayed tumor growth and extended survival in the HKP1 orthotopic model of NSCLC. CAR-T cells were observed to infiltrate tumor lesions and reduce the density of tumor-associated macrophages. This was associated with an increase in expression of MHCI/II molecules in tumor cells which was dependent on endogenous T/B cells, potentially suggesting a treatment-mediated sensitization of tumor cells to T-cell killing. This work provides a proof-of-concept cancer therapeutic strategy for targeting of TAMs using CAR-T cells.

622. Engineered Antibody Cytokine Chimera Synergizes with DNA-Launched Nanoparticle Vaccines to Potentiate Suppression of Melanoma Proliferation *In Vivo*

Nicholas J. Tursi[1,2], Ziyang Xu[1,3], Susanne Walker[1], Kevin Liaw[1], Neethu Chokkalingam[1], Michaela Helble[1], Yuanhan Wu[1], April Obeirne[1], Edgar Tello-Ruiz[1], Daniel H. Park[1], Xizhou Zhu[1], Makan Khoshnejad[1], Megan C. Wise[1], Paul D. Fisher[4], Katherine Schultheis[4], Trevor R. F. Smith[4], Kate E. Broderick[4], Laurent Humeau[4], Sonali Majumdar[1], Andrew Kossenkov[1], David B. Weiner[1], Daniel W. Kulp[1]

[1]The Wistar Institute, Philadelphia, PA,[2]Department of Biology, University of Pennsylvania, Philadelphia, PA,[3]Department of Pharmacology, University of Pennsylvania, Philadelphia, PA,[4]Inovio Pharmaceuticals, Plymouth Meeting, PA

Cancer immunotherapy has demonstrated great promise with the checkpoint inhibitors being approved as the first-line therapy for some types of cancer, and engineered cytokines like Neo2/15 have been evaluated in many studies. Here, we designed Antibody Cytokine

Chimera (ACC) scaffolding cytokine mimetics as a full-length tumor-specific antibody to both bind tumor and activate adaptive immunity. We characterized the pharmacokinetic (PK) and pharmacodynamic (PD) properties of first-generation ACC TA99-Neo2/15, which demonstrated synergy with DNA-launched nanoparticle vaccines (DLnano-vaccines) in suppressing *in vivo* melanoma proliferation but caused significant systemic cytokine activation. A novel second-generation ACC TA99-HL2-KOA1, engineered to retain IL-2Rβ/γ binding and possess attenuated but preserved IL-2Rα binding, caused significantly lower systemic cytokine activation and non-inferior protection in murine tumor studies. Additionally, a treatment regimen including TA99-HL2-KOA1 conferred similar protection in a murine melanoma tumor challenge when compared to a regimen utilizing wild-type TA99 and recombinant IL-2. Transcriptomic analyses demonstrated upregulation of Type I interferon responsive genes, particularly ISG15, in dendritic cells, macrophages and monocytes following TA99-HL2-KOA1 treatment. Characterization of additional ACCs in combination with cancer vaccines will likely be an important area of research for therapies for melanoma and other types of cancer.

623. Immunomodulatory Roles of T-Cell Immunoglobulin- and Mucin Domain-Containing (TIM)-3 on Natural Killer Cells in Glioblastoma

Tram Dao

Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN

Natural killer (NK) cells have emerged as a viable alternative to T cells in adoptive cell transfer for cancer treatment. Adoptive NK cell therapy holds potential as an off-the-shelf product, by exploiting NK's ability to engage the target without prior sensitization, and with a limited risk of graft-versus-host disease. However, NK cell activity is driven by inhibitory and activating receptors, many of which remain elusive. One such receptor requiring further elucidation is TIM-3, which has been characterized as both inhibitory and activating, depending on the disease setting. Previously, we discovered that TIM-3 downregulation was associated with decreased cytokine production and a lower cytotoxicity, and that maintenance of expression above a certain threshold was needed for NK cell function. We thus hypothesized that TIM-3 participates in regulation of *ex vivo*-activated NK cell metabolism, which in turn affects the production of the cytokine IFN-γ to sensitize cancer targets to NK cell-mediated lysis. Using TIM-3 *knock-out* human NK cells, we have investigated the role of TIM-3 on NK metabolism and function against glioblastoma targets, quantified in terms of cytotoxicity, degranulation capacity, cytokine production and glycolytic fueling. We also studied the correlation between TIM-3 expression on NK cells isolated from glioblastoma patient tumors and whole blood samples. This information is shedding further light on the immunomodulatory roles of TIM-3, and is aiding in leveraging this receptor usage in future NK cell-based immunotherapies.

624. Unedited CD7 CAR T-Cells Overcome Fratricide and Produce Superior Anti-Tumor Activity in a Preclinical Model of T-Cell Leukemia

Norihiro Watanabe, Feiyan Mo, Royce Ma, Madhuwanti Srinivasan, Mary K. McKenna, Lauren D. Scherer, Maksim Mamonkin

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Development of CAR T-cells for T-lineage malignancies requires solving the problem of self-targeting (fratricide) which often impairs *ex vivo* expansion of CAR-T cells and reduces their therapeutic potency. CD7 is an attractive target highly expressed in T-cell malignancies as well as in a majority of normal T- and NK-cells. We have previously demonstrated the feasibility of generating fratricide-resistant CD7 CAR T-cells in which the CD7 gene expression was disrupted using CRISPR/Cas9 to avoid self-elimination of CAR-transduced T-cells *ex vivo*. While this method yielded highly functional CD7-edited CD7 CAR T-cells, the additional gene editing step increased complexity of GMP manufacturing of CAR T-cell products and produced additional cellular toxicity. Here, we describe an alternative method of overcoming *ex vivo* fratricide of CD7 CAR T-cells by using temporary pharmacologic inhibition of CAR signaling. Unedited CD7 CAR T-cells were expanded in the presence of dasatinib and ibrutinib, potent inhibitors of key signaling tyrosine kinases Lck/Fyn and Itk, respectively. Addition of these pharmacologic inhibitors completely prevented deleterious CAR signaling and minimized fratricide and terminal differentiation of unedited CD7 CAR T-cells while preserving their proliferation and expansion. Inhibition of CAR signaling was fully reversible as CD7 CAR T-cells regained potent cytotoxicity against malignant T-cell lines upon removal of dasatinib and ibrutinib. Expectedly, restoring the CD7 CAR function in unedited T-cells also increased their fratricide and limited the expansion upon coculture with tumor cells. We hypothesized the fratricide of unedited CD7 CAR T-cells was enhanced due to a forced proximity of T-cells *in vitro* and would be attenuated *in vivo* where T-cells are more sparsely distributed and surrounded by tumor cells. We tested this hypothesis using an established *in vivo* model of human T-cell malignancies where NSG mice were systemically engrafted with luciferase-labeled human T-ALL followed by an intravenous injection of CAR T-cells. In this model, mice receiving control unmodified T-cells developed systemic leukemia requiring euthanasia of all animals by day 50. Injection of CD7-edited (CD7KO) CD7 CAR T-cells produced short-term tumor control in most animals and permanently suppressed leukemia in 3/8 mice (38%). Notably, unedited CD7 CAR T-cells produced robust anti-leukemic activity and completely eliminated tumor signal in 7/8 animals (88%) resulting in their long-term survival for >100 days highlighting high anti-tumor potency of these CAR T-cells. To determine the expansion and persistence of unedited CD7 CAR T-cells *in vivo*, we administered CD7 CAR T-cells co-expressing firefly luciferase to mice engrafted with unlabeled T-ALL blasts. Enhanced anti-tumor activity of unedited CD7 CAR T-cells correlated with their increased expansion and longer persistence in mice compared to CD7KO CAR T-cells indicating acquired resistance to CD7-directed fratricide. Mechanistically, the expanded unedited CD7 CAR T-cells retained high levels of CD7 CAR but had no detectable CD7 protein or mRNA expression. These data suggest the long-term anti-tumor

activity of unedited CD7 CAR T-cells was elicited by an outgrowing population of naturally CD7-negative CAR T-cells, which comprised only a small fraction (<3%) of the infused CD7 CAR-T cell product. These results support the feasibility of generating highly functional, non-gene-edited CD7 CAR T-cells that produce complete and durable anti-tumor responses *in vivo* by self-selecting for a fratricide-resistant CD7-negative T-cell subset.

625. Virus-Free Biomanufacturing of CAR T Cell Products Using CRISPR-Cas9

Katherine Mueller[1], Nicole Piscopo[1], Matthew Forsberg[2], Louise Saraspe[3], Amritava Das[1], Brittany Russell[3], Adeela Ali[2], Cicera Lazzarotto[4], Shengdar Tsai[4], Christian Capitini[2], Krishanu Saha[1]

[1]Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI,[2]Department of Pediatrics, University of Wisconsin-Madison, Madison, WI,[3]University of Wisconsin-Madison, Madison, WI,[4]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN

Critical problems in CAR T cell manufacturing with viral vectors arise from imprecise genomic integration, use of animal components, and supply chain challenges. Here, nonviral CRISPR CAR T cells were manufactured within nine days using SpyCas9 protein and nucleic acids, without viral vectors or animal components. We achieve over 25% CAR knockin efficiency, and over 95% concurrent knockout of the target locus (Figure 1). In comparison to retroviral CAR T cells, nonviral CRISPR CAR T cells exhibit TRAC-targeted genomic integration of the CAR transgene, thereby enabling precise control over both copy number and genomic regulatory elements driving expression of the CAR. In some donors, nonviral CAR T cells demonstrated less exhaustion and terminal differentiation at both protein and mRNA levels. Notably, nonviral CAR T cells significantly upregulated CD62L, which is a marker of stem cell and central memory phenotypes that are considered clinically advantageous. These cells also exhibited decreased cytokine production prior to antigen exposure and a higher dynamic range of activation, potentially indicative of a decrease in TCR-mediated tonic signaling, which is believed to drive exhaustion in CAR T cell products. Finally, nonviral CAR T cell products demonstrated potent in vitro and in vivo cytotoxicity against GD2+ neuroblastoma at levels comparable to the clinical standard product, even when a lower proportion of total cells within each dose were CAR positive. These advantageous genomic, transcriptional and phenotypic characteristics, combined with the elimination of viral vectors and animal components, have high biomedical relevance for the flexible and scalable manufacturing of high-quality CAR T cells to treat cancers, including solid tumors.

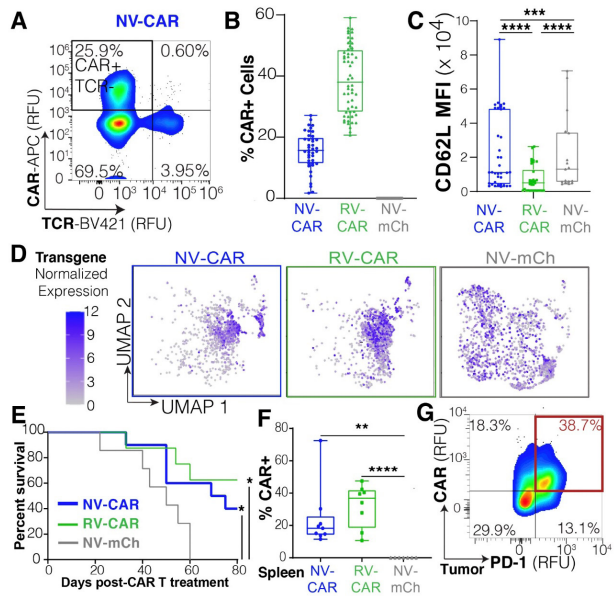


Fig. 1. Production, characterization and potency of non-viral *TRAC*-CART cells. (A) Representative flow cytometry plot for a T cell population after knock-in of a 3rd-gen anti-GD2-CD28-OX40-CAR into *TRAC* Exon 1. 26% of cells had their endogenous T cell receptor replaced with the CAR after genome editing. (**B**) Percent of CAR positive cells in each sample. Non-viral NV-CART (blue) N=31; RV-CAR, retroviral 3rd-gen anti-GD2-CD28-OX40-CAR (green) N=39; NV-mCh, mCherry control knock-in at *TRAC* (gray) N=27. **(C)** Mean fluorescence intensity (MFI) of T_{cm} marker, CD62L. **(D)** scRNA-seq of CART cells after co-culture with GD2+ cells. UMAP projection divided by shows only cells for which transgene-positive cells were detected. Transgene-positive cells cluster similarly for both NV and RV-CART cells, but not NV-mCh T cells. (**E**) Kaplan-Meyer survival curve for NSG mice with CHLA20 tumors that were treated with either 10 million NV-CAR, RV-CAR, or NV-mCh T cells. NV-CAR (blue) N=10; RV-CAR (green) N=8; NV-mCh (gray) N=7. (**F**) Box plots show presence of CAR+CD45+ human T cells in mouse spleens, as measured by flow cytometry. (**G**) Flow cytometry plots show PD-1 on human lymphocytes (CD45+) cells collected from tumors.

626. Combination of Silencing CD38 and Tyrosine Kinase Inhibitor Treatment Can Improve the Quality of CD38-JAK-STAT CAR-T Cells

Sachiko Okamoto[1], Izumi Maki[1], Maiko Sugizaki[1], Yasunori Amaishi[1], Keichiro Mihara[2], Junichi MIneno[1]

[1]Takara Bio Inc., Kusatsu, Shiga, Japan,[2]Dept. International Center for Cell and Gene Therapy, Fujita Health University, Toyoake, Aichi, Japan

Despite recent great successes in chimeric antigen receptor (CAR)-T cancer immunotherapy, there are still considerable challenges to be addressed. Excessive T cell activation leads exhaustion and depletion of naïve/memory subsets important for durable clinical responses. Thus, the CAR construct needs to be optimized so that transduced T cells persist and induce potent antigen-specific response with minimal non-specific activation, which results in maximal efficacy and minimal

toxicity. Recently, a new generation JAK-STAT CAR composed of a truncated cytoplasmic domain of the IL-2 receptor β chain and STAT3/5 binding motifs have been developed, and the JAK-STAT CAR-T cells showed activation of the JAK-STAT signaling pathway, enhanced proliferation, and limited terminal differentiation compared to second generation CAR-T cells, which has the potential to demonstrate improved clinical efficacy. In regard to the target antigen, as there are few targetable tumor specific antigens, the tumor associated antigens which are also expressed weakly in normal tissues are used for the major targets of CAR-T cells, and on-target, off-tumor toxicity may arise the safety concerns. Particularly when targeting antigens expressed on the T cells, CAR expressed on T cells react with the cognate antigen on the CAR-T cells, result in fratricide, limited expansion, cell differentiation, and exhaustion of CAR-T cells during cell manufacturing. To overcome these challenges, various approaches have been attempted, including blocking the target antigen using antibodies and knockout of the target in T cells using genome editing technology. In this study, we have explored the optimal method to produce CAR-T cells targeting shared antigen between T cells and tumor cells. As CD38 is highly expressed in several lymphocyte malignancies including B-cell non-Hodgkin lymphoma and multiple myeloma, CD38 is an attractive target for CAR-T therapy. However, intermediate expression of CD38 in normal cells such as activated T cells, monocyte, and NK cells, may cause fratricide of CAR-T cells, and on-target, off-tumor toxicity. To eliminate CD38 expression on the CAR-T cells, we have developed the vector co-expressing anti-CD38-CAR and siRNAs to knockdown CD38, and also compared the signaling domain of CAR construct. CAR-T cells expressing siRNAs showed the reduction of CD38 RNA expression and exhaustion marker expression, and CD38-JAK-STAT CAR-T cells showed less exhaustion marker expression compared to 2nd generation CD38-28z CAR-T cells. However, CD38-CAR-T cells have still showed higher exhausted and differentiated cell populations compared to control CAR-T cells targeting the antigen not expressed on T-cells. To inhibit the non-favorable CAR-T cell stimulation by CD38 expressed on transduced or non-transduced T cells during manufacture process, we have produced the CD38-CAR-T cells in the presence of the tyrosine kinase inhibitor, dasatinib. The dasatinib treatment could retain the higher proportion of less differentiated T cells and prevent T cell exhaustion. Furthermore, the CD38-JAK-STAT CAR-T cells with CD38 siRNA expression showed the less exhausted state and showed higher cytotoxic activity after repetitive tumor cell challenges compared to CAR-T cells without CD38 siRNA expression. These results suggest that our novel CD38 siRNA co-expressing CD38-JAK-STAT CAR-T cells manufactured with dasatinib treatment may persist longer and induce potent antigen-specific response with minimal excessive unfavorable activation, which results in maximal efficacy. This approach can be applied to other CAR-T cells targeting the shared antigen expressed on T cells.

627. Preclinical Evaluation of B7-H3-Targeted CAR-T Cells against Triple Negative Breast Cancer

Yeongha Jeon[1], Anna Ju[1], Narim Lee[1], Sangeun Lee[1], Song-Jae Lee[1], Sung-Won Min[2], Hye In Park[2], Gong Deuk Bae[2], Ji Chul Lee[2], Seong-Won Song[1]

[1]R&D Center, CellabMED, Seoul, Korea, Republic of,[2]Life Science Laboratory, SG Medical, Seoul, Korea, Republic of

Background: Triple negative breast cancer (TNBC) is known to a highly aggressive breast cancer with a poor prognosis. Targeted and efficient treatment options for TNBC patients are very limited, since currently most breast cancer therapies are based on classical endocrine and Her2-targeted treatments. In addition, due to heterogenic properties with a high mutational burden and metastatic potential of TNBC, its treatment remains challenging and needs a new novel molecular target. B7-H3 (also known as CD276) is one of the immune checkpoint proteins of B7 family and overexpressed on the surface of various tumor cells, which is associated with poor prognosis of cancers including TNBC. Here, we developed a chimeric antigen receptor T (CAR-T) cell targeting B7-H3 and investigated its possibility as a new therapeutic option for TNBC patients. ***Results:*** We generated novel B7-H3 specific monoclonal antibodies and identified that these antibodies bound only to human recombinant B7-H3 proteins (2Ig and 4Ig forms) not to other B7 families including B7-DC (PD-L2), B7-1 (CD80), B7-2 (CD86), B7-H1 (PD-L1), B7-H2 (B7RP-1) and B7-H4. We then developed B7-H3-targeted CAR-T derived from scFvs of these mAbs and confirmed anti-tumor activity through *in vitro* and *in vivo* experiments. We found differently expression of B7-H3 in the seven of eight TNBC cell lines and demonstrated that B7-H3-targeted CAR-T cell efficacy is largely dependent on high surface target antigen density, using MDA-MB-231 and BT-20 cell lines of low and high B7-H3 intensity, respectively. In addition, B7-H3-targeted CAR-T cells showed significant *in vivo* anti-tumor activity in orthotopic xenograft model using BT-20. ***Conclusion:*** B7-H3 is overexpressed in several TNBC cell lines and B7-H3-targeted CAR-T cells preferentially attack tumor cells with high B7-H3 expression. This B7-H3-targeted CAR-T therapy may provide an alternative approach for TNBC patients who have few therapeutic options.

628. CD133 CAR Targeted Natural Killer Cell Therapy for Aggressive Variant Prostate Cancer

Minjing Wang[1,2,3], Emily J. Pomorey[1,2,3], Walker S. Lahr[1,2,3], Hallie M. Hintz[4], Mitchell G. Kluesner[1,2,3], Beau R. Webber[1,2,3], Aaron M. LeBeau[4], Branden S. Moriarity[1,2,3]

[1]Department of Pediatrics, University of Minnesota, Minneapolis, MN,[2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN,[3]Center for Genome Engineering, University of Minnesota, Minneapolis, MN,[4]Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN

Prostate cancer (PCa) is the second-leading cause of cancer deaths for men in the US. Approximately 1 in 9 men will be diagnosed with PCa in their lifetime and a subset will develop aggressive variant prostate cancer (AVPC). There is no treatment option for AVPC and effective therapies

are urgently needed. CD133 is a marker to identify PCa initiating cells and evidence has shown it is overexpressed in AVPC patients, making it a high priority candidate for targeted therapy of AVPC. Natural killer (NK) cell possess a number of unique features that make them a highly promising tool for cancer immunotherapy, including: 1) antibody dependent cell-mediated cytotoxicity (ADCC) via CD16a receptor, 2) induce secondary adaptive immune responses through cytokine and chemokine production, 3) can be used in an allogeneic setting without inducing graft verse host disease, and 4) easy to isolate from peripheral blood (PB) and can be activated and expanded to clinically relevant numbers *in vitro*. Here, we developed a chimeric antigen receptor (CAR) with a novel CD133 scFv bearing a glycosylation-independent epitope, as the highly dynamic glycosylation of the extracellular domains of CD133 could potentially interfere with antibody binding. Our goal is to develop an effective treatment for AVPC using CD133 CAR NK cells in combination with other genetic edits to further improve the effectiveness of CAR NK therapy against AVPC. Using CRISPR/Cas9 and rAAV6 as a DNA donor molecule, we site-specifically delivered the CD133 CAR into human NK cells at the AAVS1 safe harbor locus, as previously described (PMID: 31704085). We achieved a consistent and functional integration rate of CD133 CAR (35-45%, Figure 1) and the CAR-expressing NK cells were enriched to >90% by flow sorting. Activated/expanded CD133 CAR-NK cells were co-cultured with PCa target cells with and without CD133 expression, followed by killing and intracellular cytokine staining (ICS) assays to determine the effectiveness and specificity of the CAR-NK cells against CD133 expressing targets. Our ICS assay demonstrated that the percentage of cell activation is significantly higher among CD133 CAR NK cells upon co-culturing with CD133+ targets compared to that with CD133- targets (Figure 2). The co-culture indicates an effective and specific activation of CD133 CAR NK cells against CD133+ target cells. To further enhance the therapeutic outcome and avoid antigen escape, we developed CAR constructs for two additional PCa targets, namely rearranged during transfection (RET) kinase receptor and fibroblast activation protein alpha (FAP). Constructs are in development for co-delivery of two or more CARs to NK cells using the aforementioned CRISPR/Cas9 based engineering. Moreover, we are working on combining base editing technology to knockout inhibitory genes and create non-cleavable CD16a in NK cells in combination with our CAR constructs. Ultimately, our goal is to go beyond simple CD133 CAR and develop a more effective targeted therapy for AVPC.

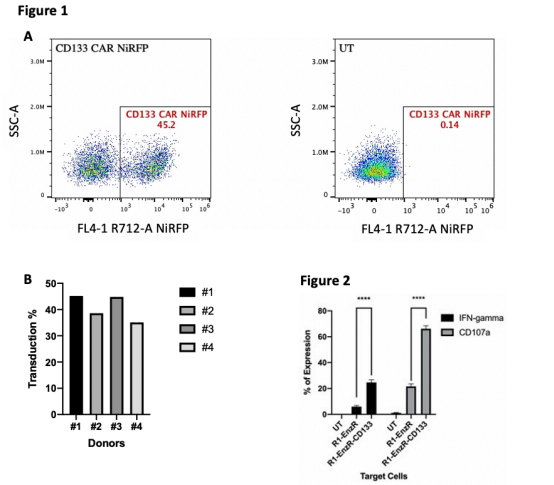


Figure 1. A. Transduction efficiency of rAAV CD133 CAR into primary human NK cells of donor #1. **B.** Summary of transduction efficiencies of rAAV6 CD133 CAR into primary human NK cells of all four donors.
Figure 2. ICS results of CD133 CAR NK and CD133+/- target cells co-culture in two donors.

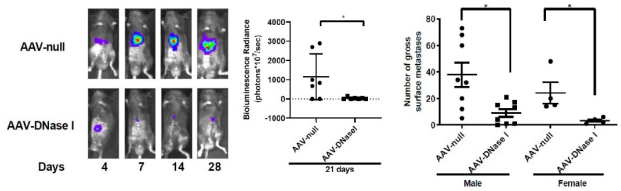
629. First-in-Class Anticancer AAV-Based Gene Therapy Based on the Destruction of NETs with Transgene Expression of DNase I in Liver

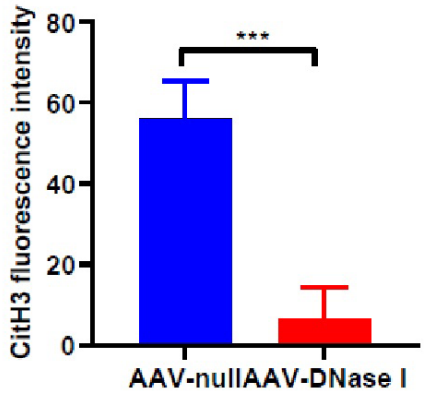
George Tetz[1], Yujia Xia[2], Hongji Zhang[2], Yu Wang[2], Amblessed Onuma[2], Casey Maguire[3], Valeria Ukrainskaya[4], Alexey Stepanov[4], Dmitry Genkin[1], Victor Tetz[5], Hai Huang[2], Allan Tsung[6]

[1]CLS Therapeutics, New York, NY,[2]Division of Surgical Oncology, Department of Surgery, The Ohio State University Wexner Medical Center, Columbus, OH,[3]Molecular Neurogenetics Unit, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA,[4]M.M. Shemyakin and Yu.A. Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, Russian Federation,[5]Human Microbiology Institute, New York, NY,[6]The Ohio State University Wexner Medical Center, Columbus, OH

Background: Patients with cancer are characterized by elevated levels of cell-free DNA (cfDNA) in the bloodstream and is also present extracellularly in primary tumors and metastasis. The origins of cfDNA include release from tumor cells and neutrophils when they form neutrophil extracellular traps (NETs). NETs are increasingly recognized as a critical component of the tumor microenvironment and playing a primary role in tumor progression and metastasis. The destruction of NETs-cfDNA with DNase I enzyme is known to significantly increase the efficacy of the first-line therapy in animal models of different cancers. However, recombinant DNase I protein has a relatively short half-life of 3-4 h with serum concentrations. Strategies that allow for longer term expression of DNase I should allow for a more robust anti-cancer therapy. The purpose of this study was to evaluate the efficacy of AAV-mediated DNase I liver gene transfer in a mouse model of CRC liver metastasis. **Methods:** MC-38 colorectal cancer model was used in 6-w-old C57BL/6 mice. CLS-014 (AAV-DNase I) is an AAV-based vector encoding human DNase I that transduces hepatocytes and triggers them to produce and secrete DNase I. CLS-

014 at 1.05×10e12 GC/mouse was injected through the tail vein on day 4 after MC38 injection. Tumors in the liver were assessed on day 28. **Results:** AAV-DNase I treatment increased serum DNase I concentration ($P < 0.0001$), inhibited the progression of liver metastasis in male and female mice compared to the AAV-null-treated mice ($P < 0.05$), and also significantly decreased the number of metastatic nodules ($P < 0.05$) "$$_graphic_". Flow cytometry of tumors revealed that neutrophil infiltration ($CD11b^+/Ly6G^+$ neutrophils) was significantly reduced in the AAV-DNase I-treated group ($P < 0.05$). The level of CitH3, a marker of NET formation, was markedly lower in the tumors of AAV-DNase I-treated mice compared to AAV-null-treated mice ($P < 0.01$) "$$_graphic_". Similarly, levels of NETs in the circulation as measured by MPO-DNA complexes were significantly reduced in the AAV-DNase I-treated mice. Furthermore, $CD8^+$ T cells in the tumors was greatly increased in the AAV-DNase I-treated group (9.5% CD8+) vs AAV-null (4.75% CD8+) ($P < 0.01$). **Conclusion:** These results suggest AAV-mediated DNase I liver gene transfer induced antitumor immune responses through modulation of both innate and adaptive immune mechanisms. AAV-mediated DNase I liver gene transfer is a safe and effective modality to inhibit metastasis and represents a novel therapeutic strategy for CRC.





630. Establishment of an Innovative Immunopeptidomic Based Pipeline for the Generation of Oncolytic Cancer Vaccines

Sara Feola[1], Beatriz Martins[1], Jacopo Chiaro[1], Salvatore Russo[1], Manlio Fusciello[1], Erkko Ylösmäki[1], Gabriella Antignani[1], Firas Hamdan[1], Michaela Feodoroff[2], Mikaela Gronholm[1], Rui M M Branca[3], Janne Lehtiö[3], Vincenzo Cerullo[1]

[1]University of Helsinki, Helsinki, Finland,[2]University of Helsinki and Institute for Molecular Medicine Finland, FIMM, Helsinki, Finland,[3]Karolinska Institutet, Solna, Sweden

Beside the isolation of peptides MHC-I restricted from the surface of cancer cells, one of the challenges in the generation of anti-tumor CD8+ T cells as part of cancer therapeutic vaccines design is the establishment of a solid pipeline for the downstream selection of clinically relevant peptides followed by the creation of cancer therapeutic vaccines. Indeed, the use of peptides for eliciting specific anti-tumor adaptive immunity is hindered by two main limitations: the selection of the candidate peptides and the use of an immunogenic platform to combine with the peptides in order to induce anti-tumor specific adaptive immune response. Here, we describe for the first time a streamlined pipeline moving from the isolation and selection of peptides candidates for cancer immunotherapeutic approaches to the generation of therapeutic oncolytic vaccines. To this end, we have carefully validated our pipeline in the murine colon tumor model CT26. Specifically, we used state-of-the-art immunoprecipitation and mass spectrometer methodologies to isolate >8000 peptide vaccine targets in the CT26. The prioritization of the target candidates was then based on two separate approaches: RNAseq analysis and HEX software. This latter is a tool previously developed in our lab, able to identify tumor antigens similar to pathogen antigens, in order to exploit molecular mimicry and cross-reactive T-cells in cancer vaccine development. The generated list of candidates (twenty-six in total) was further tested in a functional characterization by interferon-γ ELISPOT (Enzyme-Linked Immunospot), reducing the number of candidates to six. The remanent peptides were then exploited in our technology PeptiCRAd, a vaccine platform that combines an immunogenic oncolytic adenovirus with vaccine peptides. In our work, PeptiCRAd was successfully used for the treatment of mice bearing CT26, showing control over the primary tumor and most importantly on a secondary cancer lesion. These results ruled out the feasibility of applying the described pipeline for the selection of peptides candidates and generation of cancer therapeutic oncolytic vaccine, filling a gap in the field of cancer vaccine and paying the way to translate our pipeline in human therapeutic approach.

631. CRISPR-Mediated Genome Editing to Redirect T Cells against Non-Small Cell Lung Cancer

Daniela Benati[1], Valentina Masciale[2], Giulia Grisendi[2], Matteo Marchionni[1], Tommaso Ferrari[1], Beatrice Aramini[2], Massimo Dominici[2], Alessandra Recchia[1]

[1]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy,[2]Department of Medical and Surgical Sciences for Children & Adults, University-Hospital of Modena and Reggio Emilia, Modena, Italy

Adoptive T cell therapy with T lymphocytes engineered to express tumor-specific T cell receptors (TCR) to redirect T cell antigen specificity and induce antitumor immunity, represents one the most promising strategy of precise cancer therapy for solid tumors. Clinical trials are addressing the therapeutic potential of TCR-engineered T cells to treat lung cancers, the leading cause of cancer-related death worldwide. As an alternative to standard protocols based on the viral transduction of T cells to induce the expression of tumor-specific TCRs, CRISPR-mediated non-viral TCR editing has been recently proposed to replace endogenous TCRs with tumor-specific TCRs (Roth et al. 2018). In this study, we applied the CRISPR-mediated non-viral TCR editing to engineer T cells derived from patients affected by non-small-cell lung cancers (NSCLC), to redirect their specificity toward cancer antigens and induce killing of NSCLC cells. A cohort of NSCLC patients was firstly HLA typed and analyzed by quantitative RT-PCR for the expression of tumor-associated antigens frequently expressed in NSCLC. Guide RNAs targeting alpha or beta chains, in complex to a high-fidelity variant of *Streptococcus pyogenes* Cas9 (Alt-R) were delivered as ribonucleoproteins (RNPs) into primary T cells, to knock out endogenous TCR chains and simultaneously trigger the knock-in of a NY-ESO-1-specific TCR (1G4) in the *TRAC* locus. To this purpose, T cells from healthy donors (HD) and NSCLC patients were co-electroporated with RNPs and HDR donor template carrying a promoterless full-length sequence of 1G4 beta chain, downstream a self-cleaving peptide to ensure the expression under the regulatory elements of *TRA* locus, and a second self-cleaving peptide separating TCR beta chain to the VJ regions and *TRAC* exon 1 of 1G4 alpha chain. The precise integration of the donor cassette in *TRAC* exon 1 allows the in-frame introduction of the alpha chain and expression of tumor-specific TCR. Genomic and cytofluorimetric analyses showed knock-out of endogenous TCR in more than 90% of treated T cells, and precise HDR events leading to TCR replacement. Edited T cells were sorted, expanded and analyzed for their ability to kill tumor cells expressing NY-ESO-1. Co-culture experiments of TCR-redirected T cells from HD with U266 and A375 cell lines, both expressing NY-ESO-1 and HLA-A*0201, demonstrated that TCR engineered T cells were able to recognize and kill cells expressing NY-ESO-1 peptide in complex to HLA-A*0201. Flow cytometric analyses aimed at measuring cell viability confirmed that a higher percentage of U266 and A375 cells were killed by edited T cells, compared to negative controls. TCR-engineered T cells derived from HLA-A*0201-typed NSCLC patients co-cultured with patient-derived cancer cells expressing NY-ESO-1 and analysed by flow cytometry for their killing activity, demonstrated that TCR engineering enabled T cells to recognize and kill NSCLC cells. These data strongly encourage the application of CRISPR-mediated non-viral TCR editing to generate tumor-specific T cells able to kill NSCLC cells, contributing to the preclinical validation of adoptive T cell therapy with CRISPR-mediated engineered T cells for the treatment of lung cancer.

632. Novel coupledCAR™ Technology for Treating Colorectal Cancer

Song Li[1], Chengfei Pu[2], Zhiyuan Cao[2], Xinyi Yang[3], Ning Li[3], Youli Luo[4], Haiyan Zhao[5], Hang Yang[2], Xi Huang[2], Xiaogang Shen[2], Xiuwen Wang[1], Yongping Song[3], Junjie Mao[4], Pengfei Pang[4], Qun Hu[5], Zhao Wu[2], Victor Lu[2], Lei Xiao[2]

[1]Shandong University, Jinan, China,[2]Innovative Cellular Therapeutics, Shanghai, China,[3]Zhengzhou University, Zhengzhou, China,[4]Sun Yat-Sen University, Guangzhou, China,[5]Inner Mongolia Medical University, Neimenggu, China

Chimeric antigen receptor (CAR) T cell therapy has made significant progress in the treatment of blood cancers such as leukemia, lymphoma, and myeloma. However, the therapy faces many challenges in treating solid tumors. These challenges include physical barriers, tumor microenvironment immunosuppression, tumor heterogeneity, target specificity, and limited reactive cell expansion *in vivo*.Conventional CAR T cell therapy has thus far shown weak cell expansion in solid tumor patients and achieved little or no therapeutic responses. Here, we developed CAR T cells based on a novel CoupledCAR® technology to treat solid tumors. In contrast to conventional CAR T cells, CoupledCAR T cells significantly improved the expansion of the CAR T cells *in vivo* and enhanced the CAR T cells' migration ability and resistance to immunosuppression by the tumor microenvironment. The enhanced migration ability and resistance allow the CAR T cells to infiltrate to tumor tissue sites and increase anti-tumor activities. Specifically, we engineered CoupledCAR T cells with lentiviral vectors encoding an anti-GCC (guanylate cyclase 2C) CAR molecule. Furthermore, anti-GCC CAR T cells showed anti-tumor activities *in vitro* and *in vivo* experiments.To verify the safety and efficacy of CoupledCAR T cells for treating solid tumors, we conducted several clinical trials for different solid tumors, including seven patients with colorectal cancer. These seven patients failed multiple rounds of chemotherapy and radiotherapy. In the clinical trial, the patients were infused with autologous anti-GCC CoupledCAR T cells range from 4.9×10^5/kg to 2.9×10^6/kg. All patients using anti-GCC CoupledCAR T cells showed rapid expansion of CoupledCAR T cells and killing of tumor cells. Specifically, we observed that CoupledCAR T cells expanded significantly in the patients and infiltrated tumor tissue sites, demonstrating enhanced anti-tumor activities. PET/CT showed significant tumor shrinkage and SUV max declined, and the ongoing responses were monitored. Patient 3 achieved complete response and the best overall response rate (ORR, include complete remission, complete metabolic response, partial response, and partial metabolic response.) was 57.1% (4/7), complete remission (CR) rate was 14.3% (1/7).The clinical data demonstrated that CoupledCAR T cells effectively expanded, infiltrated tumor tissue sites, and kill tumor cells in patients with colorectal cancer. We used immunotherapy to achieve complete remission in patients with advanced colorectal cancer for the first time. We are recruiting more colorectal cancer patients to further test the safety and efficacy of anti-GCC CoupledCAR T cells. Since our CoupledCAR® technology is a platform technology, we are expanding it to treat other solid tumors using different target tumor markers .

633. GM-CSF Disruption in CART Cells Ameliorates CART Cell Activation and Reduces Activation-Induced Cell Death

Michelle J. Cox[1,2,3], Claudia Manriquez Roman[1,2,4,5], Reona Sakemura[1,2], Erin Tapper[1,2], Elizabeth Siegler[1,2], Sutapa Sinha[2], Dale Chappell[6], Omar Ahmed[6], Cameron Durrant[6], Mehrdad Hefazi[1,2], Kendall Schick[1,2,5,7], Paulina Horvei[1,8], Michael Ruff[1,9], Ismail Can[1,2,5,10], Mohamad Adada[1,2], Evandro Bezerra[1,2], Lionel Kankeu Fonkoua[1,2], Sameer A. Parikh[2], Neil E. Kay[2], Saad S. Kenderian[1,2,4,5,11]

[1]T Cell Engineering, Mayo Clinic, Rochester, MN,[2]Division of Hematology, Mayo Clinic, Rochester, MN,[3]Bioinformatics and Computational Biology, University of Minnesota Graduate School, Minneapolis, MN,[4]Department of Molecular Medicine, Mayo Clinic, Rochester, MN,[5]Mayo Clinic Graduate School of Biomedical Sciences, Rochester, MN,[6]Humanigen, Burlingame, CA,[7]Department of Molecular Pharmacology & Experimental Therapeutics, Mayo Clinic, Rochester, MN,[8]Department of Pediatric Hematology/Oncology, Mayo Clinic, Rochester, MN,[9]Department of Neurology, Mayo Clinic, Rochester, MN,[10]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN,[11]Department of Immunology, Mayo Clinic, Rochester, MN

CD19-directed chimeric antigen receptor T (CART19) cell therapy is limited by toxicities and low overall durable responses. We have previously demonstrated that GM-CSF neutralization prevents CART-associated toxicities through inhibition of myeloid cell activation and their cytokines. We then used CRISPR/Cas9 to generate GM-CSF^{KO} CART19 cells, which exhibited superior antitumor activity in our preclinical model absent of myeloid cells. This suggested that GM-CSF directly affects CART cell functions, independent of its effect on myeloid cells. Given that GM-CSF-producing T cells (Th^{GM}) are known to be sensitive to activation-induced cell death (AICD), we hypothesized that GM-CSF disruption in CART cells refines CART cell activation and prevents apoptosis. First, we generated GM-CSF^{KO} CART cells using CRISPR/Cas9 and confirmed they do not produce GM-CSF (Fig 1A). We then compared CART cell apoptosis by annexin staining on antigen-activated GM-CSF^{KO} to GM-CSF^{WT} CART19, which consistently exhibited lower early apoptosis (Fig 1B). This was confirmed using the BrdU assay (Fig 1C). We then determined the impact of reduced CART cell apoptosis on effector functions. GM-CSF^{KO} CART19 cells exhibited enhanced delayed antigen-specific proliferation (Fig 1D). Additionally, antigen-specific activation of GM-CSF^{KO} CART19 led to lower expression of HLA-DR, CD45, and Trail receptor, indicating reduced early activation (Fig 1E). To validate our findings, we used an *in vivo* xenograft model for relapsed B cell malignancies. Here, NSG mice were engrafted with JeKo-1 and randomized to receive control T cells, GM-CSF^{KO} or GM-CSF^{WT} CART19. GM-CSF^{KO} CART19 led to early amelioration of CART cell activation, enhanced delayed proliferation, and improved antitumor activity (Fig 1F-H). Finally, to determine the mechanisms of reduced AICD following GM-CSF disruption, we ruled out off-target effect for CSF2 disruption. Sequencing of GM-CSF^{KO} CART cells revealed that gRNA precisely targeted the intended CSF2 gene (not shown). We then interrogated the intrinsic and extrinsic regulators of apoptosis. There was no change in GM-CSF^{KO} CART cell apoptosis following blockade of extrinsic death pathways (Fig 1I); however, there was a consistent reduction in Bid following GM-CSF disruption (Fig 1J). In conclusion, we demonstrate that CRISPR/Cas9 GM-CSF knockout in CART cells directly ameliorates CART cell early activation, reduces AICD, and results in enhanced antitumor activity in preclinical models.

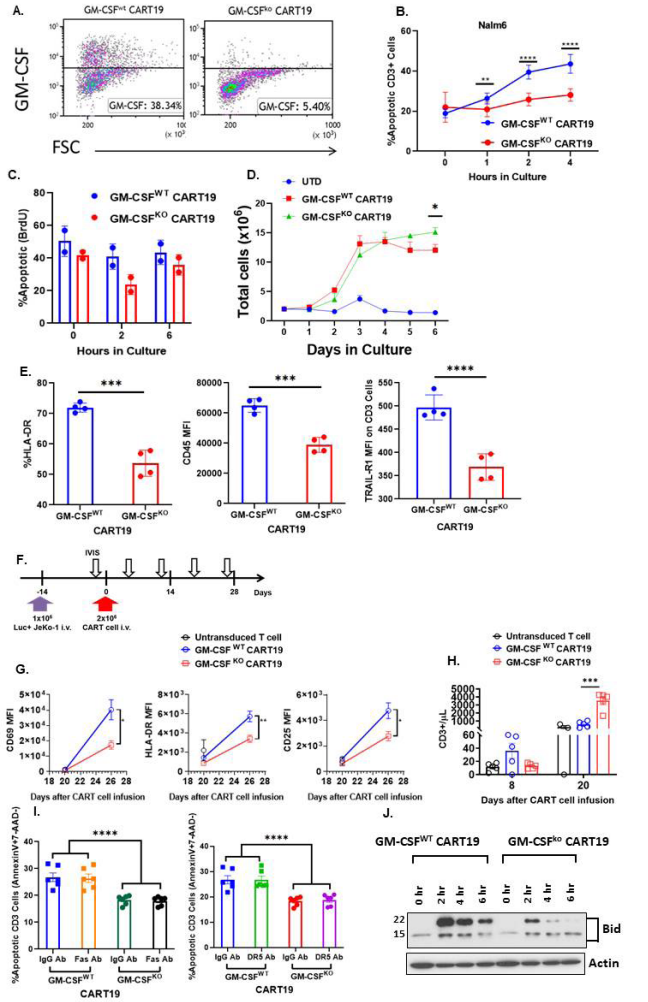


Fig 1. A) GM-CSF^{KO} CART19 cells produce less GM-CSF. Representative figure showing the levels of GM-CSF detected on PMA/ionomycin-stimulated live CD3 cells as measured by intracellular flow cytometric staining. **B) GM-CSF^{KO} CART19 cells undergo significantly less early apoptosis when activated.** GM-CSF^{KO} or GM-CSF^{WT} CART19 cells are co-cultured with CD19$^+$ cell line Nalm6. Flow cytometric staining for Annexin V and 7-AAD is performed at 0, 1, 2, and 4 hours (** p < 0.01, **** p < 0.0001; two-way ANOVA). **C) Trends in apoptosis by BrDU is consistent with Annexin V.** GM-CSF^{KO} or GM-CSF^{WT} CART19 cells are co-cultured with the irradiated CD19$^+$ cell line Nalm6. TUNEL (BrdU assay) is performed at 0, 2, and 6 hours. **D) GM-CSF^{KO} CART19 show enhanced delayed proliferation.** GM-CSF^{KO} and GM-CSF^{WT} CART19 are co-cultured with irradiated Nalm6 and cell counts are obtained daily for 6 days (* p < 0.05; two-way ANOVA. **E) GM-CSF^{KO} CART19 show altered expression of activation markers.** GM-CSF^{KO} or GM-CSF^{WT} CART19 cells are co-cultured with irradiated (HLA-DR and CD45) and intact Nalm6 (TRAIL-R1). Flow cytometric staining is performed at 24 hours (** p < 0.01, *** p < 0.001, **** p < 0.0001; one-way ANOVA. **F-H) GM-CSF^{KO} CART19 shows improved activity *in vivo*.** NSG mice are engrafted with JeKo-1 and then randomized to receive untransduced T cells and either GM-CSF^{KO} or GM-CSF^{WT} CART19 (* p < 0.05, ** p < 0.01 (G), t-test; *** p < 0.001 (H), one-way ANOVA). **I) Blockade of death pathways did not affect apoptosis.** GM-CSF^{KO} or GM0CSF^{WT} CART19 are stimulated with Nalm6 and antibodies to Fas, DR5, or IgG control. Annexin V and 7-AAD are measured at 2 hours (**** p < 0.0001; one-way ANOVA). **J) GM-CSF^{KO} CART19 express lower levels of Bid protein when activated.** CARTs are activated with irradiated Nalm6, and western blot is performed at 0, 2, 4, and 6 hours.

Cancer - Immunotherapy, Cancer Vaccines

634. Bi-Specific Logic Gated CAR T Cells Eliminating CD19+ Tumors While Preventing Neurotoxicity via Pericyte-Induced Inhibition

Darel Martinez Bedoya[1,2,3], Eliana Marinari[1,2,3], Francesca Corlazzoli[1,2,3], Shahan Momjian[4], Valérie Dutoit[1,2,3], Denis Migliorini[1,2,3,5]

[1]Brain Tumor and Immune Cell Engineering Laboratory, University of Geneva, Geneva, Switzerland,[2]Center for Translational Research in Onco-Haematology, University of Geneva, Geneva, Switzerland,[3]Swiss Cancer Center Léman, Lausanne, Switzerland,[4]Department of Neurosurgery, Geneva University Hospital, Geneva, Switzerland,[5]Department of Oncology, Geneva University Hospital, Geneva, Switzerland

Chimeric antigen receptor (CAR) T cell therapy has revolutionized the management of selected hematological malignances. High-rate complete responses were observed with CD19-targeting CAR T cells in patients with refractory B cell malignancies, allowing two anti-CD19 CAR T cell products to become the first FDA-approved genetically engineered immune cell therapeutics. However, these therapies come with significant toxicities, the main ones being cytokine-release syndrome and neurotoxicity. We recently reported a potential mechanism of CD19 CAR T cell-related neurotoxicity, identifying "off-tumor" expression of CD19 in brain pericytes using single-cell transcriptomics, as well as *in vitro* and *in vivo* mouse modeling. Pericytes are a key cell population responsible for the maintenance of blood-brain-barrier (BBB) integrity and their attack triggers BBB disruption and neurotoxicity. Our findings opened the opportunity to engineer a new generation of CAR T cells based on a "logical" CAR design that can only attack CD19+ malignant B cells and spare healthy brain pericytes. To achieve this, we generated different candidates of bi-specific anti-CD19 activatory anti-pericyte marker inhibitory CAR constructs using four different inhibitory intracellular domains derived from T or NK cells. These inhibitory CAR T cells are specific for the canonical pericyte marker CSPG4 and were tested *in vitro* for inhibition of cytotoxicity, proliferation and proinflammatory cytokine production upon co-culture with the CD19+ human B-ALL Raji cell line engineered to express CSPG4 as a proof of concept. In parallel, in an attempt to identify reliable pericyte cell surface markers, we are analyzing human healthy and meningioma brain samples collected in our center after pericytes and endothelial cell sorting, using single-cell transcriptomics. The identified markers will be used to generate novel inhibitory CAR T cell products that will be tested for neurotoxicity prevention.

635. Immunotherapeutic Targeting of TIGIT-CD155 Promotes Both Inhibitory and Activating Interactions with NK Cells in GBM

Kyle B. Lupo, Sandro Matosevic

Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN

Natural killer (NK) cells have emerged as promising effectors to target GBM and other solid tumors, yet remain susceptible to immunosuppressive receptors and interactions within the tumor microenvironment (TME). In particular, the T cell immunoreceptor with Ig and ITIM domains (TIGIT) is expressed on NK cells and interacts with CD155 to induce immunosuppression of NK cell cytolytic functions[1,2]. Although CD155 also binds with activating receptors DNAM-1 and CD96 on NK cells, spurring NK cell activity, TIGIT has predominantly been reported as having an inhibitory effect on NK cells[3-5]. However, data from our and other labs suggest that TIGIT may be involved in more than just NK cell inhibition, and may be linked with a more mature/cytolytic subset of NK cells[6]. We have investigated the TIGIT/CD155 axis to shed light on its functional role in NK cell immunobiology and the potential for targeting these receptors in the context of NK cell-based immunotherapy. Using CD155 knockdown patient-derived GBM cells (GBM43), we studied the role of CD155 in NK cell function both in vitro and in vivo in immunocompetent and immunodeficient mice. In vitro, NK cells exhibited reduced cytolytic function against GBM43-CD155 KD cells when compared with GBM43 WT cells (Figure 1), indicating that, while inhibiting interaction with immunosuppressive TIGIT, targeting CD155 might also impair interactions of NK cells with immunostimulatory ligands CD96 and DNAM-1, thus limiting NK cell effector functions. We next investigated if targeting of TIGIT can be an effective strategy in enhancing NK cell cytolytic function. We found, in agreement with prior data, that antibody blockade of TIGIT alone is insufficient in enhancing NK cell cytolytic functions in vitro[7]. However, our data also indicated that blockade of TIGIT reduced expression of CD16 and increased the expression of 41BB on NK cells (Figure 2). Further, we discovered that TIGIT^{high} NK cells express higher levels of CD16 than either TIGIT^{low} or TIGIT^{-} NK cells. After sorting NK cells based on expression of TIGIT (high, low, negative) we observed that TIGIT^{high} NK cells were able to lyse GBM43 cells, degranulate, and secrete IFNy at higher levels than either TIGIT^{low} or TIGIT^{-} NK cells. Altogether, this suggests that there may be a relationship between TIGIT, CD16, and 41BB, and that TIGIT, while inhibitory, may play a role in NK cell maturation and development of cytolytic functions. To test this, we evaluated the cytotoxicity of NK cells against GBM43 WT tumor cells in combination with dual blockade of TIGIT and 41BB and found that such treatment could enhance NK cell lysis of tumor cells. Overall, we have shown that targeting CD155, while mitigating TIGIT induced immunosuppression, may also inhibit CD96 and DNAM-1 induced NK activation. Further, we have established a relationship between TIGIT, CD16, and 41BB, within the context of GBM, suggesting that TIGIT may be involved in more than just NK cell inhibition. We also show that 41BB and TIGIT dual-blockade is an effective strategy in enhancing NK cell functional activity against GBM tumors cells.

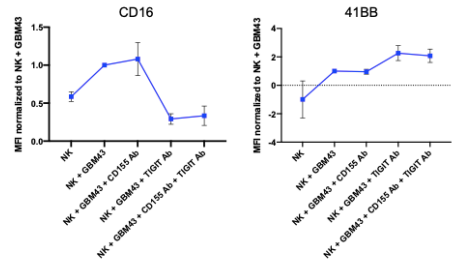


Figure 2: TIGIT blockade induces downregulation of CD16 and upregulation of 41BB in NK cells cocultured with GBM43 cells

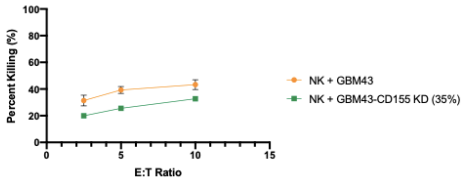


Figure 1: CD155 knockdown inhibits NK cell killing of patient-derived GBM43 glioblastoma cells

636. Combined p19Arf and IFNβ Gene Therapy Induces Superexpression of PD-L1 in Murine Melanoma Models

Ana Carolina M. Domingues, Bryan E. Strauss

Viral Vector Laboratory, Cancer Institute of Sao Paulo, University of Sao Paulo School of Medicine, Sao Paulo, Brazil

Immune checkpoint inhibitors have revolutionized cancer treatment, especially for high-mutational burden tumors such as melanoma, which are frequently associated with higher immune cell infiltration, hence create a good opportunity for the use of immunomodulatory agents. Our group has previously shown that a gene therapy approach using two adenoviral vectors containing p19Arf and IFNβ cDNAs promoted tumor regression in melanoma models, inducing immunogenic cell death in vitro and in vivo. To understand if this combined gene therapy would impact PD-L1 surface expression on tumor cells, two mouse cell lines containing wild-type p53, B16F10 (B16) and TC-1, were treated in vitro with the viruses and, 72 hours later, analyzed for the expression of PD-L1 by flow cytometry. The combined therapy induced an 85% increase in the expression of PD-L1 on B16 cells and 24% on TC-1 cells when compared to control cells transduced with GFP-containing vectors. To understand whether this modulation was mediated by the genes transferred, the B16 cells were transduced in vitro and incubated for either 40 or 72 hours and analyzed for PD-L1 expression by flow cytometry. The results showed that PD-L1 expression is directly proportional to gene expression. When co-cultured with splenocytes from naïve mice, the treated-B16 cells showed higher PD-L1 expression, which was also observed on the immune cells. Nevertheless, we performed in situ gene therapy of subcutaneous B16 tumors in C57bl/6 mice with either the combination or GFP vectors and analyzed PD-L1 expression by flow cytometry and immunohistochemistry. Consistent with the results obtained in vitro, the animals who received the combined treatment had an upregulation of PD-L1 surface expression when compared to the control groups. Together, these results indicate that the p19Arf-IFNβ gene therapy induces a substantial upregulation of PD-L1 expression on murine melanoma cells while promotes immunogenic cell death, thus providing a rational choice for combining our therapeutic strategy with immune checkpoint inhibitors for the treatment of melanoma.

637. UB-VV100, a Novel Platform for In Vivo Engineering of Therapeutic Anti-CD19 CAR T Cells, Shows Effective T Cell Transduction, B Cell Depletion, and Tumor Control in a Humanized Murine Model

Kathryn R. Michels, Alessandra M. Sullivan, Christopher J. Nicolai, Susana Hernandez Lopez, Laurie Beitz, Joshua Whalen, Alyssa Sheih, Blythe Irwin, Anai Perez, Anna Ting, Seungjin Shin, Mark D. Pankau, Shon Green, Rich Getto, Jacob Garcia, Byoung Ryu, Ryan Crisman, Andrew M. Scharenberg

Umoja Biopharma, Seattle, WA

The emergence of chimeric antigen receptor (CAR) T cells as a therapeutic modality to treat refractory B-cell malignancies has inspired a wave of investment in cell-based immunotherapies. However, the field remains hindered by technical, logistical, consistency, cost, and efficacy challenges associated with autologous and allogeneic manufacturing. To overcome these challenges, we developed an *in vivo* CAR engineering platform, VivoVec, which uses a unique vector particle envelope that facilitates effective transduction of T cells *in vivo*. VivoVec particles are pseudotyped with the Cocal glycoprotein, which has been shown to resist complement-dependent inactivation in human serum. In addition, VivoVec particles express a membrane-anchored anti-CD3 scFv that facilitates T cell binding, activation, and transduction. The payload includes a 2nd generation CAR containing 4-1BB and CD3ζ signaling domains that is co-expressed with a novel rapamycin-activated cytokine receptor (RACR) system designed to provide selective cytokine signaling to transduced T cells. Here we show proof-of-concept data for this technology platform utilizing VivoVec particles to deliver an anti-CD19 CAR along with the RACR system as a treatment for B-cell malignancies (termed UB-VV100). We show that UB-VV100 particles induce T cell activation and transduction *in vitro* when applied to unstimulated human PBMCs. The transduced CAR T cells selectively enrich and expand in response to rapamycin, and selectively kill and release cytokines when co-cultured with CD19+ Raji cells. Importantly, *in vivo* administration of UB-VV100 virus particles into CD34-humanized NSG mice resulted in CAR T cell generation, durable B cell depletion, and resistance to B-cell tumor challenge in a dose-dependent manner. These preliminary data support the potential of this platform as a new approach to therapeutic immune cell engineering with the potential to increase the safety and availability of CAR T technology in both hematologic and solid tumors without the need for cell manufacturing or lymphodepletion.

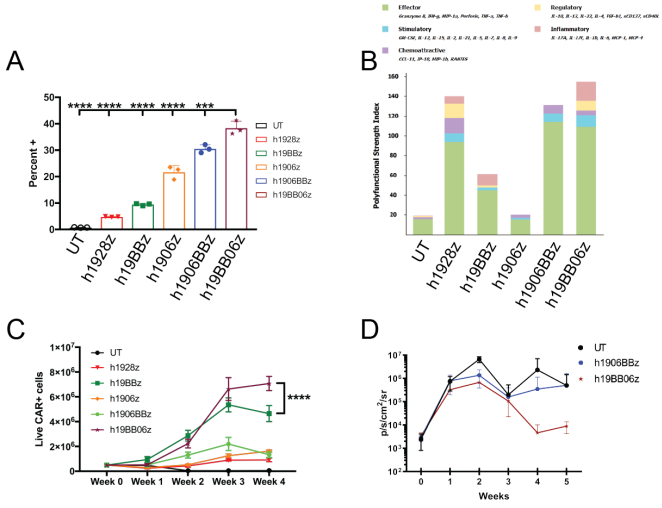
638. Abstract Withdrawn

639. Costimulation with Mutated CD28 and 41BB Enhances Human CAR T Cell Function

Justin C. Boucher[1], Emiliano Roselli[1], Estelle Cervantes[2], Kayla Reid[1], Kristen Spitler[1], Marco L. Davila[1]

[1]Clinical Science, Moffitt Cancer Center, Tampa, FL,[2]Morasani College of Medicine, University of South Florida, Tampa, FL

Co-stimulatory signals regulate the expansion, persistence, and function of chimeric antigen receptor T cells. Most studies have focused on the co-stimulatory domains of CD28 or 4-1BB. CARs containing 4-1BB enhance proliferation and favor longer-term CAR T cell persistence in patients. Our lab has shown the enhanced function of 4-1BB CAR T cells is driven by NF-κB, TRAF1, and TRAF3. Recently we found that mutations in the CD28 co-stimulatory domain of CARs can enhance their function. These insights led us to hypothesize that by combining our mutant CD28 (mut06) with 4-1BB, we could build a CAR that has the most beneficial aspects of both. To test our hypothesis we designed 2 CARs incorporating both 4-1BB and mut06 co-stimulatory domains (h1906BBz and h19BB06z). When we examined the CAR memory phenotypes, we found that h19BB06z and h1906BBz had an increased percentage of naïve and CM T cells compared to other CARs (Figure 1A). Several recent reports have demonstrated CAR T cell polyfunctionality is a substantial predictor of *in vivo* ability. We found that CAR T cells secreting 2 or more cytokines, polyfunctionality, was highest in h1906BBz and h19BB06z. The polyfunctional strength index (PSI) is the percentage of polyfunctional cells multiplied by the intensity of the secreted cytokines. Using this measure h1906BBz and h19BB06z had the highest PSI (Figure 1B). Antigen dependent proliferation of CAR T cells is a vital determinant of *in vivo* expansion and persistence. To examine this, we stimulated CAR T cells with target cells at a 1:10 E:T ratio. Restimulated of CAR T cells with target cells occurred every 7 days for a total of 4 weeks. We observed a significantly greater count of live h19BB06z CAR T cells compared to h1928z and h1906BBz after 4 weeks (Figure 1C). The restimulated h19BB06z cells also had a significant enhancement in cytotoxicity and IL2 secretion. *In vivo,* NSG mouse models show that only when 4-1BB is proximal and mut06 is distal from the cell membrane is tumor-killing enhanced supporting our *in vitro* data showing that the positioning of the 2 co-stimulatory domains was critical to the enhanced functions of the CAR (Figure 1D). These results demonstrate that 4-1BB co-stimulation, in addition to mut06, can enhance CAR T cell function. By incorporating LCK signaling with 4-1BB costimulation we developed a CAR that had a significantly better memory phenotype and polyfunctionality. This resulted in increased persistence and *in vivo* tumor-killing. We are currently incorporating this work into clinical studies to show efficacy in patients.



640. IRP2 Defines the Setpoint of Lymphocyte Proliferation by Controlling Activation Induced Expression of CD71

Jasmin Grählert[1,2], Ilija Brizić[3,4], Greta Giordano Attianese[5], Elisabetta Cribioli[5], Benedikt Meyer[6], Glenn R. Bantug[1], Bojana Müller[1], Maria L. Balmer[1], Robert Ivanek[7,8], Janet Chou[9,10], Lea Hiršl[3,4], Božo Šušak[3,4], Anne-Valérie Burgener[1], Suzanne Welten[11], Nitish Mittal[12], Vanda Juranić Lisnić[3,4], Mihaela Zavolan[12], Veronika Sexl[13], Annette Oxenius[11], Martin Stern[14], Raif Geha[9,10], George Coukos[5], Melita Irving[5], Stipan Jonjić[3,4], Christoph Hess[1,2,15]

[1]Immunobiology Laboratory, Department of Biomedicine, University Hospital of Basel, Basel, Switzerland,[2]Hornet Therapeutics Ltd, London, United Kingdom,[3]Department of Histology and Embryology, University of Rijeka, Rijeka, Croatia,[4]Center for Proteomics, University of Rijeka, Rijeka, Croatia,[5]Department of Oncology, Ludwig Institute for Cancer Research Lausanne, Lausanne, Switzerland,[6]Immunodeficiency Laboratory, Department of Biomedicine, University Hospital of Basel, Basel, Switzerland,[7]Bioinformatics Facility, Department of Biomedicine, University Hospital of Basel, Basel, Switzerland,[8]Swiss Institute of Bioinformatics, Basel, Switzerland,[9]Division of Immunology, Boston Children's Hospital, Boston, MA,[10]Department of Pediatrics, Harvard Medical School, Boston, MA,[11]Institute of Microbiology, ETH Zürich, Zürich, Switzerland,[12]Biozentrum, University of Basel, Basel, Switzerland,[13]Department for Biomedical Sciences, University of Veterinary Medicine Vienna, Vienna, Austria,[14]Roche Glycart AG, Schlieren, Switzerland,[15]Cambridge Institute of Therapeutic Immunology & Infectious Disease, Department of Medicine, University of Cambridge, Cambridge, United Kingdom

Chimeric antigen-receptor (CAR) T cell therapy has been successfully used to treat hematologic malignancies. However, this cell-based therapy has its limitations since not all patients respond and treatment of solid cancers remains inefficient. Whether metabolic engineering of CAR T cells can enhance CAR T cell functionality remains poorly studied. We propose that *pseudo iron deficiency,* a state we discovered in cytokine-enhanced (CE) human NK cells, could be used as a co-engineering strategy to improve CAR T cell proliferation. NK cells are explored as adoptive cell therapeutics and application of CE NK cells has been shown to have better clinical efficacy than their naïve counterparts. We here show that CE NK cells are characterized by a proliferation advantage as a result of a pseudo iron deficient state. In detail, it is well established that iron-depleted cells increase abundance of iron regulatory proteins 1 and 2 (IRP1/2) to stabilize CD71 mRNA, thus enhancing CD71 cell surface expression to augment iron uptake. Upon reinstating iron sufficiency, IRP1/2 are degraded and, along with this, CD71 expression is reduced. However, we identified high levels of IRP1/2 despite low CD71 transcript and protein abundance in quiescent CE NK cells. IRP1/2 are thus expressed as they usually are in iron-depleted cells, yet CD71 remains low - hence the term pseudo iron deficiency. We further demonstrated that expression of CD71 is indeed a prerequisite for NK cell proliferation and that exclusively IRP2 promotes its expression. This newly recognized cellular state enables CE NK cells to more efficiently translate CD71 mRNA upon activation, thus enhancing

CD71-dependent iron uptake, a prerequisite for enhanced proliferation. Importantly, pseudo iron deficiency is a 'transferrable skill', boosting CD71 expression and linked proliferation when genetically enforced in primary T cells and CAR T cells. A particular strength of this finding lies in the fact that IRP2 impacts cellular function only when CD71 mRNA is being transcribed - i.e. in an activation-gated manner. This is in contrast to many ongoing efforts aiming to metabolically enhance cells for adoptive therapy, where enforced changes in cellular metabolism operate out of sync with context-specific cellular needs.

641. *In Vitro* Selection and Engineering of a Human Leukocyte Antigen-Independent T-Cell Receptor Recognizing Human Mesothelin

Martyn J. Hiscox, Alexandra Wasmuth, Chris L. Williams, Jaelle N. Foot, Guy E. Wiedermann, Valeria Fadda, Ian Birch-Machin, Nicholas J. Pumphrey
Adaptimmune, Oxford, United Kingdom

Background: α/β T-cell receptors (TCRs) traditionally bind to human leukocyte antigen (HLA) molecules displaying antigenic peptides to elicit T-cell-mediated cytotoxicity. TCR engineered T-cell immunotherapies targeting cancer-specific peptide-HLA complexes (pHLA) are generating exciting clinical responses, but due to HLA restriction, they are limited to targeting a subset of antigen-positive patients. More recently, evidence that α/β TCRs can target cell surface proteins other than pHLAs has been published, which would negate the disadvantages of HLA restriction. **Methods:** Using phage display, we isolated an HLA-independent TCR (HiT), which directly binds the tumor-associated antigen mesothelin. The binding kinetics of the HiT to mesothelin were analyzed by surface plasmon resonance. T-cells transduced with this HiT were characterized by flow cytometry, ELISpot, supernatant ELISAs, and Incucyte killing assays. **Results:** The mesothelin HiT showed an affinity for recombinant mesothelin in the natural range for pHLA-TCR interactions and demonstrated activation of T-cells in an antigen-dependent manner. Furthermore, when the HiT was transduced into primary T-cells, cytotoxic activity was detected. Mutations of this HiT produced a panel of receptors with different binding kinetics and cytotoxic activities. **Conclusions:** This study demonstrated that a human TCR can target tumor-associated antigens without the need for HLA recognition, and in the context of autologous cell therapy, should allow a wider patient population to be treated. The use of the natural TCR affinity and T-cell signaling mechanisms may be advantageous when compared to chimeric antigen receptors. Additionally, our study showed T-cell activation and tumor cell killing can be modulated by optimizing the HiT's affinity, a key step in developing a safe immunotherapy product.

642. Development of Anti-CD19 Chimeric Antigen Receptor T-Cells with Immune Checkpoint Silencing

Thomas Matthes[1], Audrey Roussel-Gervais[2], Patrick Salmon[3], Sten Ilmjärv[2,4], Antonija Šakić[2], Karl-Heinz Krause[4,5], Marco Alessandrini[2,4]
[1]Translational Research Centre in Oncohaematology, University of Geneva, Geneva, Switzerland,[2]Antion Biosciences SA, Geneva, Switzerland,[3]University of Geneva, Geneva, Switzerland,[4]Pathology and Immunology, University of Geneva, Geneva, Switzerland,[5]University Hospitals of Geneva, Geneva, Switzerland

Anti-CD19 chimeric antigen receptor (CAR) T-cell therapy constitutes an established treatment option for advanced and aggressive B-cell malignancies. However, over 50% of patients with relapsed/refractory diffuse B-cell lymphoma (R/R DLBCL) progress or relapse despite anti-CD19 CAR T-cell treatment. The reasons for this are manifold, with engagement of inhibitory checkpoint receptors expressed on T-cells being a key mechanism exploited by tumor cells. In this situation, the T-cells adopt an exhausted/anergic phenotype due to binding of their inhibitory receptors to cognate ligands over-expressed on tumor cells. We sought to develop anti-CD19 CAR T cells, which would be more resistant to developing an exhausted phenotype. By silencing of relevant inhibitory receptors, we suggest that this would allow CAR T-cells to persist longer in the tumor microenvironment and continue to exhibit an effector phenotype. We designed anti-CD19 CAR T-cells with simultaneous gene silencing of PD1 and TIM3, two inhibitory receptors known to be relevant in the context of R/R DLBCL. We have previously developed microRNA gene constructs to selectively downregulate the expression of one or more target genes with high efficiency. When co-expressed with CAR molecules, these bimodal constructs (miCAR) allow to achieve simultaneous CAR expression and silencing of relevant target genes in the same cell. All constructs were packaged into lentiviral vectors and delivered to primary T-cells to assess CAR expression, gene silencing (PD1 and TIM3 individually, and in combination), and functionality of the miCAR19 T-cells. We first established gene silencing of PD1 and TIM3 in primary T-cells by screening a range of microRNAs directed against each target gene, and selecting those which performed optimally. We then cloned the optimized microRNAs into a miCAR gene construct expressing a second generation anti-CD19 CAR and RQR8 reporter protein. After transduction of primary T-cells, we are able to show CAR expression in over 50% of transduced cells as well as simultaneous silencing of both PD1 and TIM3. Notably, our miCAR19 T-cells uniformly expressed both the CAR and microRNA cassette. We could also demonstrate robust and efficient specific cytotoxicity of a CD19 expressing B-cell cell line (JeKo-1), compared to CAR19 T-cells without immune checkpoint silencing. Our data demonstrates efficient creation of next generation anti-CD19 CAR T-cells via novel miCAR gene constructs to achieve simultaneous CAR expression and gene silencing of relevant inhibitory receptors.

643. Advanced 38 h Automated Manufacturing of Lentivirus-Induced Dendritic Cells for Immunotherapy against WT1$^+$ Cancer

Renata Stripecke

Regenerative Immune Therapies Applied, Hannover Medical School, Hannover, Germany

Objectives: We generated a tricistronic integrase-defective lentiviral vector (IDLV) co-expressing GM-CSF, IFN-α and truncated WT1 tumor-associated antigen. Research Grade (RG) or GMP-Like (GL) IDLV showed similar efficacies inducing self-differentiation of CD14$^+$ cells into highly viable dendritic cells (DCs) in the research laboratory. Here, an automated gene-delivery process was established to enable highly standardized production of iDCtWT1. **Methods:** We performed two GMP-compatible engineering runs, including an adaptation of the T cell transduction (TCT) process, established for the production of CAR-T cells using the CliniMACS Prodigy® system. After collection of the leukapheresis material, the 38 h automated process started with immune magnetic selection of CD14$^+$ cells, followed by media exchange, cytokine stimulation, and transduction with either RG- or GL-IDLV. After 3 washes to remove excess vector, the cell product was formulated in the resuspension buffer, aliquoted, and cryopreserved. Quality control analyses of identity (cell count, viability, cell composition) were carried out in process and on the final product. Characterizations of iDCtWT1 products were performed after thawing (day 0) and at day 7 of culture. *In vivo* testing was performed in humanized NRG mice. **Results:** The frequency of CD14$^+$ cells in the leukapheresis product was approximately 15%. The CD45$^+$CD14$^+$ purity and viability were >90% and > 95%, respectively. After the CD14$^+$ cell selection, a media exchange for culture set-up was performed and stimulation with GM-CSF and IL-4 was carried on for 6 h. For RG-IDLV: 1.5 x 10^8 cells were transduced with 75 x 10^3 ng p24 equivalents of crude vector. For GL-IDLV: 2.0 x 10^8 cells were transduced with 4 x10^3 ng p24 equivalents of purified vector. The cells were transduced overnight for 16 h, washed and harvested. The cell composition of the final product showed >88% CD14$^+$ purity. Viability of the final product was 32.5% for RG-IDLV and 88.9% for GL-IDLV transduction. Correspondingly, 3.0 x 10^7 and 1.49 x 10^8 total viable CD14$^+$ cells were recovered after RG-IDLV and GL-IDLV transductions, with 1.4 and 0.4 copies per genome, respectively. Recovery of viable cells after thawing was >75% (Trypan blue staining). After 7 days in culture, viability was 67% (7-AAD staining and FACS analyses). The iDCs showed high expression of CD11c, HLA-DR and CD86. Expression of WT1 mRNA was within the ranges detectable in the positive control K562-A2 cell line. Analyses of cell supernatants collected on day 7 of culture demonstrated accumulation of transgenic and endogenous cytokines. Fully humanized NRG mice were injected s.c. with 1 x 10^6 thawed iDCtWT1 (GL-IDLV) or with PBS. Compared with controls, immunized mice showed increased frequencies of activated CD8$^+$ TE cells in spleen (average 2.0-fold increase), but no GvHD or other adverse effects. **Conclusions and Outlook:** Automated manufacture of iDCtWT1 was successful. The viability and identity of the thawed products met the expected characteristics and demonstrated *in vivo* potency. iDCtWT1 produced under by this automated method will be further developed for immunotherapy of patients with WT1$^+$ malignancies. Conflict Statement: R.S. is an inventor in a patent describing the iDC technology

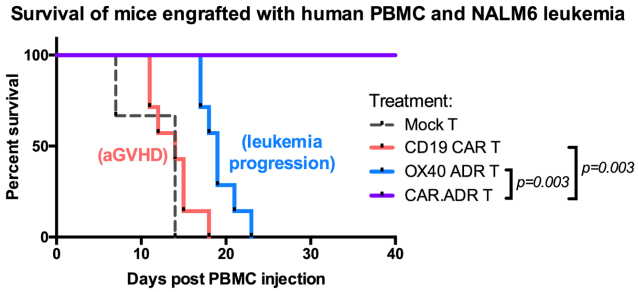
644. OX40-Specific Alloimmune Defense Receptor (ADR) T Cells for the Therapy of Acute Graft-versus-Host Disease

Feiyan Mo, Norihiro Watanabe, Mary K. McKenna, Helen E. Heslop, Malcolm K. Brenner, Maksim Mamonkin

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Adoptive CAR T cell therapies produce remarkable clinical benefit in patients with certain tumors, yet their application to non-malignant diseases is less explored. Acute graft-versus-host disease (aGvHD) is a devastating complication of allogeneic hematopoietic stem cell transplantation (alloHSCT) requiring intense immunosuppressive prophylaxis. It is mediated by alloreactive donor T cells which are stimulated by host alloantigens and can produce widespread tissue damage. We hypothesized targeted elimination of activated pathogenic donor T cells would prevent or reverse aGvHD after alloHSCT. To that end, we utilized alloimmune defense receptors (ADRs) that, when expressed on primary T cells, enable them to recognize and eliminate pathogenic T and NK cells expressing specific activation markers, such as 41BB [PMID: 32661440]. To minimize the damage to donor T cell subsets mediating protective graft-versus-tumor effect (such as CD8$^+$ αβT cells and γδT cells) we chose to target OX40, which is minimally expressed by those T cell subsets. OX40 is primarily upregulated on activated CD4$^+$ T cells which play a central role in initiation and propagation of aGvHD. Indeed, T-cells expressing an OX40-specific ADR preferentially eliminated activated CD4$^+$ T cells while sparing most CD8$^+$ T cells and γδT cells as well as resting lymphocytes. Expression of OX40 ADR protected T cells from alloimmune rejection in a mixed lymphocyte reaction model *in vitro* indicating effective depletion of alloreactive T cells. To evaluate the protective activity of OX40 ADR T cells *in vivo*, we adopted an established model of acute xenogeneic GvHD using NSG mice in which human PBMC produce fatal tissue damage driven by xenoreactive human T cells. This model recapitulates many aspects of aGvHD, enabling evaluation of new therapeutic approaches. Four days after intravenous PBMC administration, mice received a single dose of OX40 ADR T cells. Compared to non-treated control group, which had a median survival of 14 days (N=14), 11 out of 15 mice receiving OX40 ADR T cells survived >40 days. These mice had improved clinical GvHD scores and significantly lower numbers of circulating CD4$^+$ T cells, indicating OX40 ADR T cells effectively protected against xenogeneic aGvHD by targeting xenoreactive T cells. Because both aGvHD and tumor relapse remain the major causes of morbidity and mortality in patients after alloHSCT, we engineered a T cell product that combined both anti-GvHD and anti-leukemic activities by co-expressing OX40 ADR with CD19 CAR. To evaluate its therapeutic efficacy, we developed an animal model of residual leukemia after alloHSCT by co-injecting NSG mice with human PBMC and NALM6 B-ALL cells (Fig 1). Treatment with donor-derived therapeutic T cells expressing either CAR or ADR alone failed to protect mice from fatal aGvHD or tumor relapse, respectively, and all animals required euthanasia by day 25. By contrast, upon treatment with T cells co-expressing CAR and ADR, no mice had detectable tumor signal and maintained body weight for >40 days, suggesting they were well-protected from both leukemia relapse and aGvHD. These results support the feasibility of using donor-derived T cells expressing

OX40 ADR, alone or in combination with a CAR, for the prevention and therapy of aGvHD and tumor relapse. Clinical application of ADR-armed CAR T cells following alloHSCT may reduce morbidity and mortality and improve outcomes.



645. An IL-15 Superagonist Delivered via Replicating Retrovirus Improves Survival and Lymphocyte Infiltration in a Poorly Immunogenic Mouse Model of Glioblastoma

Alexander F. Haddad, Jordan Spatz, Megan L. Montoya, Sara A. Collins, Sabraj Gill, Pavlina Chuntova, Elaina Wang, Jacob S. Young, Noriyuki Kasahara, Manish K. Aghi

Department of Neurological Surgery, University of California, San Francisco, San Francisco, CA

Introduction: Glioblastoma (GBM) leads to severe systemic and local immunosuppression, contributing to the failure of systemically administered single-agent immunotherapies. In addition, systemic immunotherapies can be limited by systemic toxicities and the blood-brain barrier. IL-15 is a promising immunocytokine with the ability to stimulate T cell activation and proliferation. In this study, we evaluate the treatment efficacy of RLI (an IL-15 superagonist) delivered to tumor cells using a tumor-selective retroviral replicating vector (RRV) in the poorly immunogenic murine SB28 model of GBM. **Methods:** First, the ability of RRV-RLI to replicate and spread in cultured SB28 cells was confirmed by intracellular staining and analysis by flow cytometry. Production and function of RLI expressed from RRV-transduced SB28 cells was confirmed by ELISA and T cell proliferation in culture, respectively. Next, tumor growth inhibition *in vivo* was assessed after stereotactic intratumoral delivery of RRV-RLI to luciferase-expressing SB28 orthotopic intracerebral tumors established in syngeneic C57BL/6 mice. Changes in the tumor immune microenvironment were evaluated by flow cytometry. **Results:** RRV-RLI replicates and spreads effectively in cultured SB28 cells. Transduced SB28 cells express high levels of RLI (73.3 ng/mL), which sustained T cell growth in culture. Treatment with RRV-RLI by stereotactic injection into intracerebral murine SB28 tumors significantly reduced tumor growth on bioluminescence imaging, and increased median survival relative to control mice (19 vs. 55 days, p=0.0016), leading to long-term survival in a subset of treated mice. RRV-RLI treated tumors showed significantly increased CD4 and CD8 T cell infiltration, without changes in immunosuppressive cell populations, including myeloid-derived suppressor cells or T regulatory cells. **Conclusions:** Local RLI immunotherapy delivered with an RRV results in anti-tumor changes to the tumor immune microenvironment associated with a significant survival benefit in a poorly immunogenic model of GBM. This localized tumor-specific immunomodulatory gene therapy has the potential to safely reverse the T-cell depleted immunophenotype of GBM.

646. Assessing Biodistribution and Kinetics of Expression of AAV-Luciferase in Multiple Cancer Cell Line Models

Dawn Fellner, Michele Stone, Jiewu Liu, Brian Furmanski

Kriya Therapeutics, Redwood City, CA

In 2020, it was estimated that 1,806,590 new cases of cancer would be diagnosed in the United States and approximately 606,520 people would succumb to their disease. Approximately 39.5% of men and women will be diagnosed with cancer at some point during their lifetimes. For more than a century, scientists and physicians have been interested in using viruses to treat cancer. Oncolytic viruses have long been viewed as tools for directly killing cancer cells, through cell lyses as the primary therapeutic mechanism action. However, a growing body of research suggests that some oncolytic viruses may work in part by triggering an immune response in the body against the cancer. Alternative viral vectors such as adeno-associated viruses (AAVs) can deliver genes expressing proteins to support robust immune responses against tumors, leading to immune memory that can address metastatic disease. When selecting an appropriate viral vector to express an immune potentiating protein, it is necessary to consider the efficiency, durability, and biodistribution of protein expression, as well as the balance of the immune response triggered against the virus versus the response against the cancer antigen itself. AAV-based gene therapies have distinct advantages over other viral vector-based gene therapies (e.g., herpes simplex viruses, adenoviruses and lentiviruses) particularly in the context of cancer therapy, due to their improved safety profile and reduced potential for integration, pathogenicity, and immunogenicity. As compared to other delivery methods, AAVs also have flexibility to carry a wide range of payloads to drive production of varying therapeutic transgene products. In addition, AAV-based gene therapy can drive durable transgene expression, thus enabling the treatment and surveillance of surface and visceral tumor lesions. AAV vectors have been shown to transduce multiple primary human cancer cell lines; however, few reports have included evaluation in murine syngeneic cancer cell lines. To evaluate the potential of AAV for expressing therapeutic modalities in cancer, we examined the transduction of an AAV2-luciferase vector in five different syngeneic mouse cancer models (MC38, B16F10-p436, CloudmanS91, CT26-p928, and A20-p165). The aim of the study was to identify tumor types that were efficiently transduced by an AAV vector. Transduction efficiency and transgene expression levels and kinetics were evaluated both in vitro and in vivo. Transduction was evaluated over 21 days to determine kinetics after intratumoral injection. Luciferase expression was evaluated on multiple days per week for three weeks via whole body bioluminescence imaging. To assess biodistribution or escape of the AAV from the injection site, liver and spleen were taken for evaluation by immunohistochemistry and PCR. Optimal ratios supporting maximal tumor transduction and transgene expression while limiting systemic exposure were assessed. Correlations between in vitro and in vivo transduction were also evaluated. Results from these experiments will be shared in this presentation.

647. Efficacy of B7H3-Targeted CAR T cell Therapy in a Novel Orthotopic Model of Osteosarcoma

Lindsay Jones Talbot[1], Ashley Chabot[1], Amy Funk[2], Heather Tillman[1], Phuong Nguyen[3], Jessica Wagner[3], Andrew Davidoff[1], Stephen Gottschalk[3], Chris DeRenzo[3]

[1]Surgery, St. Jude Children's Research Hospital, Memphis, TN,[2]Animal Resource Center, St. Jude Children's Research Hospital, Memphis, TN,[3]Bone Marrow Transplant and Cell Therapy, St. Jude Children's Research Hospital, Memphis, TN

Background: Novel therapies for metastatic pediatric osteosarcoma (OS) are urgently needed. Chimeric antigen receptor (CAR) T cells offer promise to improve outcomes in OS. However, early phase clinical studies have shown limited efficacy despite potent preclinical effector function, highlighting the need to enhance preclinical models. B7-H3 has emerged as a promising immunotherapeutic target for OS, and we are actively exploring B7-H3-CAR T cell therapies. The goal of this project was to develop an orthotopic implantation technique for high-fidelity metastatic OS modeling and evaluate anti-OS activity of B7-H3-CAR T cells. **Methods/Results:** LM7 OS cells expressing GFP and firefly luciferase were suspended in highly-viscous rat-tail collagen I to create manipulable implants. Eight-week NSG mice underwent orthotopic implantation of LM7 implants by sharp tibial osteotomy. Tumor growth was followed via bioluminescent imaging, and hindlimb amputation was performed at defined functional endpoints. All mice survived osteotomy and implantation; no wound complications, osteomyelitis, or other perioperative complications were observed. Orthotopic tumor engraftment occurred in 22/25 mice (88%). B7-H3.CD28.ζ CAR and nonfunctional control T cells were generated by lentiviral transduction and injected by tail vein 7 weeks post tumor implantation. B7-H3-CAR T cell treatment groups included: control ($3x10^6$), low ($3x10^5$), intermediate ($1x10^6$), intermediate-high ($3x10^6$) and high ($1x10^7$) cells/mouse groups. B7-H3-CAR T cells exhibited dose-dependent antitumor activity, with all control and low dose mice requiring primary amputation and exhibiting progressive systemic disease. Intermediate dose mice exhibited a mixed response, with 3/5 requiring primary tumor amputation and 4/5 succumbing to systemic disease. In the intermediate-high dose group, no mice required amputation, and 2 succumbed to systemic disease. In the high dose group, no mice required amputation, and none developed systemic disease. Pathologic analysis of euthanized mice demonstrated a pulmonary metastatic rate of 58% by H&E and 100% by flow cytometry, and an extrapulmonary metastatic rate of 100% by both H&E and flow cytometry. **Conclusions:** Our data demonstrate the utility of this model for evaluating CAR T cell activity and demonstrate that B7-H3-CAR T cells can control local OS and prevent metastatic disease *in vivo*. The model demonstrates i) robust metastatic activity, ii) accomodates use of modified cells and cell lines, and iii) avoids intramedullary injection, thereby achieving high-fidelity recapitulation of the metastatic process.

648. Bypassing Targeted Immunotherapy Induced Myelosuppression via Adenine Base Editing in HSPCs

Florence Borot[1], Gregory Newby[2,3,4], Sajeev Kohli[2,3,4], Abdullah M. Ali[1,5], David R. Liu[2,3,4], Siddhartha Mukherjee[1,5]

[1]Irving Cancer Research Center, Columbia University Irving Medical Center, Columbia University, New York, NY,[2]Merkin Institute of Transformative Technologies in Healthcare, Broad Institute of Harvard and MIT, Cambridge, MA,[3]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA,[4]Howard Hughes Medical Institute, Harvard University, Cambridge, MA,[5]Myelodysplastic Syndromes Center, Columbia University Irving Medical Center, Columbia University, New York, NY

The translation of antigen directed targeted immunotherapies, including antibody or chimeric antigen receptor T cell (CAR-T) therapy, for acute myeloid leukemia (AML) to clinical use remains a major challenge mainly due to the lack of a unique antigen that is expressed on AML cell but absent on normal cells. Antigens including CD123, CD33, and CLL-1 remains high-priority targets but in pre-clinical and clinical studies, targeting these antigens has been associated with severe myelosuppression (loss of platelets, neutrophils and anemia) with severe life-threatening consequences. We reasoned that transplanting AML patients with targeting resistance donor hematopoietic stem cells (trHSC), one can create an AML-specific antigen, thus enabling maximal immunotherapy by preventing the on-target killing of normal cells. A trHSC can be created either by eliminating the antigen or selectively modifying the epitope. Previously, our group and others published a proof-of-concept study and demonstrated that trHSC can be created by eliminating CD33 protein expression by introducing CRISPR/Cas9 mediated indels via double strand break repair mechanism. $CD33^+$-AML bearing mice transplanted with CD33 trHSPCs and treated with anti-CD33 targeted immunotherapies displayed full remission while trHSPCs repopulated a complete and functional hematopoietic system. While we did not observe any appreciable differences in engraftment, differentiation, and function of trHSC, and we noted low to off target with our approach, those preclinical study observations remain to be validated in the clinic. Moreover, a double-strand break generated by SpCas9 nuclease has raised concerns due to the possibility of inducing a p53 DNA damage response, chromosomal translocations, or large deletions. We reasoned that eliminating only a portion of CD33, specifically a region that is naturally absent in a subset of population, may address the above concerns. We propose to avoid the potential genotoxicity associated with double-strand breaks using a new adenosine base editor (ABE) as a safer approach, exploiting a natural SNP (rs12459419) leading to the skipping of CD33 exon2 which includes the epitopes recognized by several antibody-drug conjugates and CAR-T tested in the clinic. We devised a strategy to achieve high editing efficiency of the CD33 exon2 splicing acceptor site, leading to the generation of $CD33^{\Delta 2}$ HSPCs which express a CD33 lacking exon2. We show that HSPCs $CD33^{\Delta 2}$ are resistant to CD33-targeted immunotherapy cytotoxicity *in vitro* and that the expression of $CD33^{\Delta 2}$ in HSPCs doesn't impair their ability to engraft and to repopulate full hematopoietic system overtime *in vivo*. This demonstration paves the way for safer AML therapies that avoid debilitating immunosuppressive side effects.

649. CD8+ T Cell-Mediated Tumor Rejection by an Adeno-Associated Virus-Like Particle (AAVLP) Vaccine

Lasse Neukirch[1], Patrick Schmidt[1], Inka Zörnig[2], Dirk Jäger[1], Silke Uhrig-Schmidt[1]

[1]Applied Tumor Immunity, German Cancer Research Center (DKFZ), Heidelberg, Germany,[2]Medical Oncology, University Hospital Heidelberg, Heidelberg, Germany

The utilization of adeno-associated viruses (AAVs) as vaccines has primarily been focused on the induction of antibody responses. Displaying antigens derived from HER2 or HPV on the AAV capsid surface lead to strong humoral immune responses. However, the potential of AAVs as T cell inducing vaccines has been poorly investigated. Model antigens have been inserted into the capsid of AAVs to analyze T cell responses, but these studies rather focused on preventing vector immunity for an improved tolerance of gene therapies. By using this as a starting point, we set out to test AAVs as T cell inducing vaccines with the prospect of targeting cancer neoantigens. The viral structure allows the insertion of peptides at certain sites of the AAV capsid sequence without disrupting particle assembly. Such particles, displaying 60 copies of the inserted peptide, are directed to antigen-presenting cells (APCs), followed by efficient ingestion and presentation on MHC molecules. In first experiments, the general properties of the vaccination strategy were estimated by displaying the ovalbumin-derived model antigen SIINFEKL on the surface of adeno-associated virus-like particles (AAVLPs). Upon injection of the AAVLP-SIINFEKL vaccine, mice developed strong CD8+ T cell responses against the displayed antigen. Highest immune responses were achieved by subcutaneous hock injection of AAVLPs, adjuvanted by Montanide ISA 51 VG. SIINFEKL-specific T cell responses peaked around three weeks after vaccination, whereas a memory subset remained present for long term. The anti-tumor efficacy of the vaccine was shown by injecting SIINFEKL-expressing B16F10 melanoma cells subcutaneously into mice, in which the tumor was completely rejected after vaccination. Interestingly, the induction of CD8+ T cell responses and the tumor protection depended on the presence of CD4+ T cells. Accordingly, T helper epitopes were identified in the AAVLP capsid sequence. In addition to the initial tests, a set of murine neoantigens, derived from B16F10 cells, were displayed on AAVLPs. In direct comparison to a peptide vaccine, which did not have an effect on tumor growth, the AAVLP vaccine had a significant impact on the tumor growth rate. In conclusion, AAVLPs show promising effects as T cell vaccines. The vaccination strategy can be used to induce cytotoxic T cell responses in general and anti-tumor effects in particular. AAV capsid-specific helper epitopes is one inherent advantage, since the AAVLP vaccine delivers immune stimulation within the viral particle itself. Thus, antigen-displaying AAVLPs could be an alternative to current gold standards in the field of neoantigen vaccines and have a prospect for future clinical applications.

650. Abstract Withdrawn

651. Humoral Immunity in CD47 Checkpoint Blockade for Efficacious Macrophage Cell Therapies against Solid Tumors

Jason Christopher Andrechak[1], Lawrence J. Dooling[2], Brandon H. Hayes[1], Dennis E. Discher[1]

[1]Bioengineering, University of Pennsylvania, Philadelphia, PA,[2]Chemical & Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA

The macrophage checkpoint CD47-SIRPα has been targeted in over a dozen early phase clinical cancer trials, but efficacy is clear in only one trial to date with a fraction of lymphoma patients responding when anti-CD47 is infused only in combination with tumor-opsonizing rituximab (anti-CD20). CD47 is a ubiquitously expressed marker of 'self' that can potently inhibit phagocytic clearance of cells by engagement of SIRPα expressed on macrophages, among other cell types. Efficacy against solid tumors remains unclear, even in immunocompetent mouse models, with safety also a concern given CD47's ubiquitous expression and a large antigen sink, such as on red blood cells. To better understand the requirements for efficacy against a standard poorly immunogenic solid tumor, we completely ablate CD47 with CRISPR-Cas9 (CD47 KO) in the B16F10 melanoma cell line, and we combine with tumor-opsonizing antibodies. Consistent with monotherapy against CD47 being ineffective, mice with established CD47 KO B16 tumors succumb at control rates, whereas systemic treatment with a monoclonal antibody against surface-expressed melanosomal Tyrp1 cures completely a fraction of mice. When challenging cured mice again with CD47 KO cells, palpable tumors fail to grow completely. Sera analysis shows evolution and long-term persistence of high levels of IgG and pro-phagocytic IgG subclasses that bind specifically to B16F10 surface antigens, indicating epitope spreading beyond the therapeutically targeted antigen. The pro-phagocytic activity of sera IgG is confirmed both in vitro and in vivo, suggesting a phagocytic feedback mechanism. Cured mice also resist B16F10 tumors that have both CD47 and Tyrp1 ablated, indicating broadened recognition of cancer epitopes. To test that macrophages are the key effector cells, we harvest fresh whole marrow from donor mice, block SIRPα on the macrophage side, which also mitigates off-target phagocytosis, and pre-load marrow Fc receptors with tumor-opsonizing anti-Tyrp1. These cells are then re-infused back into tumor-bearing mice within hours of harvest. The approach prolongs survival and enables durable cures in wild-type CD47 B16F10 tumors. We conclude from these data that complete disruption of CD47 is important for efficacy, as even low amounts of CD47 can inhibit phagocytosis. Secondly, humoral immunity can also be generated and can durably protect from tumor engraftment and growth, provided B cells are not also ablated.

652. Engineered Type 1 Regulatory T Cells Have a Cytotoxic Profile and Kill Pediatric Acute Myeloid Leukemia Cells

Ece Canan Sayitoglu[1], Molly Uyeda[1], Jeffrey M. Liu[1], Brandon Cieniewicz[1], Pauline Chen[1], Norman Lacayo[2], Alma-Martina Cepika[1], Maria Grazia Roncarolo[1,2,3]

[1]Institute of Stem Cell Biology and Regenerative Medicine, Department of Pediatrics, Stanford University School of Medicine, Palo Alto, CA,[2]Division of Hematology, Oncology, Stem Cell Transplantation and Regenerative Medicine, Pediatrics, Stanford University School of Medicine, Palo Alto, CA,[3]Center for Definitive and Curative Medicine, Stanford University, Palo Alto, CA

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative approach used for treating many leukemia patients, including chemotherapy-refractory pediatric acute myeloid leukemia (pAML). While replacing the patient's bone marrow with stem cells from a healthy donor, allo-HSCT can cause a life-threatening graft-vs-host disease (GvHD). Type 1 regulatory T cells (Tr1) can dampen the GvHD while killing myeloid blasts, enhancing the graft-vs-leukemia (GvL) response. However, Tr1 cell isolation and *in vitro* expansion capacity is limiting. To study the mechanisms behind this process, we generated a cell therapy product by engineering $CD4^+$ human T cells to over-express *IL10*, $CD4^{IL\text{-}10}$, obtaining the phenotypic and functional properties of Tr1. We performed RNA sequencing on $CD4^{IL\text{-}10}$ and control $CD4^{GFP}$ cells to investigate transcriptional differences between the two cell types. We showed that overexpression of *IL10* drastically upregulated cytotoxicity-related genes in $CD4^{IL\text{-}10}$ without changing the typical Tr1-like cytokine profile of these cells with high IL-10, IFN-γ and low IL-4. As previously reported, high expression of granzyme B was observed in $CD4^{IL\text{-}10}$ compared to control. In addition, four novel molecules, CD244, KLRD1, KLRC1, and FASLG contributing to $CD4^{IL\text{-}10}$ cytotoxicity were identified. We also investigated the killing capacity of $CD4^{IL\text{-}10}$ against primary pAML for the first time. Indeed, pAML could be targeted and killed by $CD4^{IL\text{-}10}$ but with varying sensitivity. RNA-seq analysis showed that resistant pAML have distinct transcriptional profiles and express a surface protein, CD200, which can bind to CD200 receptor (CD200R1) expressed on $CD4^{IL\text{-}10}$ surface. Since CD200R1 triggering is known to induce an inhibitory signal, we investigated whether $CD200^+$ malignant myeloid cells inhibit the function of $CD200R1^+CD4^{IL\text{-}10}$. Importantly, CD200 overexpression in $CD4^{IL\text{-}10}$-killing sensitive AML cells rendered them more resistant to LV-10 cells. In conclusion, $CD4^{IL\text{-}10}$ have a cytotoxic profile and can kill the majority of pAML blasts. Thus, $CD4^{IL\text{-}10}$ is a novel cell therapy which could control GvHD and support GvL in pAML patients transplanted with allo-HSCT.

653. Precise Tumor Targeting with NOT Logic-Gated Chimeric Antigen Receptor Gene Circuits

Nicholas W. Frankel[1], Seunghee Lee[2], Derrick Lee[1], Marcus Gainer[1], Brian S. Garrison[1], Travis Wood[1], Wesley Gorman[1], Russell Gordley[1], Marcela Guzman Ayala[1], Tim Lu[1], Gary Lee[1], Wilson Wong[2]

[1]Senti Biosciences, South San Francisco, CA,[2]Boston University, Boston, MA

Background: The engineering of chimeric antigen receptors (CARs) into T cells or NK cells can redirect them to kill cancer cells that express a specific surface antigen. This technology has led to breakthrough therapies for cancer, but the lack of uniquely cancer-specific antigens has the risk of life-threatening on-target, off-tumor toxicity. CAR-engineered immune cells could be used to treat a broader spectrum of cancers if recognition of a "safety antigen" on healthy cells could selectively block killing. Here, we engineered T and NK cells with a synthetic NOT logic-gated gene circuit: an activating CAR (aCAR) drives killing of targets presenting an activating antigen, while an inhibitory CAR (iCAR) suppresses cytotoxicity against targets expressing a safety antigen (SA). **Methods**: iCARs consisted of an SA-binding domain, hinge and transmembrane domains, and a functional intracellular domain (ICD) derived from the cytoplasmic tails of inhibitory co-receptors containing immunoreceptor tyrosine-based inhibitory motifs. We first constructed a panel of iCARs with different ICDs and performed initial screens for expression and function in T cells. We then developed protocols for transducing aCAR/iCAR pairs in NK cells. Finally, we validated the performance of top aCAR/iCAR combinations in NK cells by measuring cytotoxicity and cytokine release in response to tumor cells that expressed activating antigen +/- SA. **Results**: NOT-gated NK cells with an iCAR inhibiting a second generation aCAR with a CD28 costimulatory domain significantly reduced both cytotoxicity (32%, $p<0.05$) and secretion of TNFa, IFNg, and Granzyme B (50%, 54%, and 18%, respectively; $p<0.05$ for all differences) in response to SA+ targets, while aCAR-only NK cells exhibited no such significant differences. The NOT logic-gated gene circuit did not interfere with aCAR performance since, in the absence of SA on target cells, NOT-gated CAR NK cells showed no reduction in killing performance or cytokine release compared to "conventional" aCAR-only NK cells. When SA+ and SA- target cells were mixed, NOT-gated CAR-NK cells preferentially killed SA- target cells over SA+ target cells, while aCAR-only NK cells killed both subpopulations equally. These data show that this NOT gate gene circuit enables safer killing of SA- target cells, discriminating them from SA+ target cells on a cell-by-cell basis. **Conclusion**: We have developed a best-in-class NOT gate gene circuit technology and demonstrated it in therapeutic CAR-T and CAR-NK cells. NK cells can be used in an allogeneic fashion and can reduce cost and increase patient access relative to autologous products. Therapeutic cells with these NOT-gated CAR gene circuits may improve safety for currently validated cancer antigens, and also enable new therapies for previously unaddressed indications.

Cancer - Oncolytic Viruses

654. Blockade of Type I Interferon Pathway Enables Seneca Valley Virus (SVV-001) Replication and Cell Killing, Broadening SVV-001's Cancer Therapy Indications

Paul L. Hallenbeck[1], Sunil Chada[1], Vincent R. Racaniello[2], Amy B. Rosenfeld[2]

[1]Seneca Therapeutics, Inc., Blue Bell, PA,[2]Columbia University, New York, NY

The picornavirus Seneca Valley Virus (SVV-001) is a single stranded (+) RNA virus being investigated for use as an oncolytic viral therapy. In contrast to many picornaviruses, SVV-001 is not a human virus and its reproduction does not lead to human disease. The cell surface protein TEM 8 (Tumor Endothelial Marker 8, also known as ANTXR1 - anthrax receptor type 1) functions as a receptor for SVV-001. This highly conserved single-pass transmembrane glycoprotein is found on the surface of a number of specialized cells in solid tumors including cancer cells, cancer stem cells, cancer associated fibroblasts and angiogenic endothelial cells. The extracellular domain of TEM 8 interacts with the collagen extracellular matrix, while its intracellular domain interacts with the actin cytoskeleton. These interactions are not only necessary for cell morphology and cell adhesion but also cell migration, modulation of cell chemotaxis, and ECM transition required for metastasis. TEM 8 promotes angiogenesis during tumor disease development and is not required for normal physiology. It is either absent or present at very low levels on the surface of normal cells. Patients with tumors that have enhanced TEM 8 surface presence have worse prognoses and are less likely to respond to intervention. It has therefore been suggested that the presence of TEM 8 within a tumor may be a negative prognostic factor in numerous sold cancers including lung, breast (especially TNBC), colorectal, gastric cancers and sarcomas. Cells that lack TEM 8 on their surface are refractory to SVV-001 infection, suggesting that the receptor confers a high degree of tumor killing specificity with no killing of normal cells. Previously published studies with SVV-001 identified that both TEM 8 surface presence and a low or a non-functional Type I interferon (IFN) response was necessary for efficient SVV-001 replication. Some cancers such as small cell lung cancer are known be TEM 8 positive; yet, have appear to have low expression levels of Type I IFN pathway genes or have defects in the Type I IFN pathway and therefore are susceptible to SVV oncolytic therapy. To further confirm that SVV-001 was sensitive to Type I IFN we pretreated TEM 8 positive permissive cancer cell lines with IFN-α or IFN-β prior to virus infection. A significant reduction in SVV-001 replication was observed. Thus, given the exquisite sensitivity imparted by TEM 8 expression we hypothesized that SVV-001 might replicate safely in any solid cancer expressing the TEM 8 receptor provided that a method could be found to safely and transiently reduce the level of type I IFN in the tumor microenvironment. We evaluated the treatment of TEM 8 positive cancer cell lines with SVV-001 plus small molecule inhibitors of the Type I IFN signaling pathway, including those involved in JAK/STAT and mTOR signaling, and histone deaceylation. Dampening of multiple targets involved in regulation of the type I IFN response enhanced SVV-001 infection and resulted in a significant increase in cell killing. Further studies in vitro and in vivo are analyzing the safety and effectiveness of type I IFN inhibitors as an adjunct therapy for SVV-001. The combination of SVV-001 and Type I IFN inhibitors consequently broadens the applicability of SVV-001 to target the majority of solid cancers.

655. Investigation of Immunological Activity of Interferon alpha Expressing Oncolytic Adenovirus in Hamster Pancreatic Cancer Models

Kazuho Inoko[1], Margarita Romanenko[1], Sacha Robert[1], Shuhei Shinoda[1], Kari Jacobsen[1], Christopher J. LaRocca[1,2], Takuro Noguchi[3], Masato Yamamoto[1,2,4], Julia Davydova[1,2,4]

[1]Department of Surgery, University of Minnesota, Minneapolis, MN,[2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN,[3]Department of Medical Oncology, Hokkaido University, Sapporo, Japan,[4]Institute of Molecular Virology, University of Minnesota, Minneapolis, MN

Despite breakthroughs in cancer immunotherapies, pancreatic ductal adenocarcinoma (PDAC) continues to have a dismal prognosis due to an immunosuppressive tumor microenvironment. Our strategy focuses on the use of oncolytic adenoviruses (OAds) as cancer therapeutics. OAds are designed to selectively replicate and spread within the tumor, resulting in immunogenic cell death, which release damage-associated molecular patterns and evoke strong antitumor immune responses. OAds are also capable of highly efficient delivery of immune-stimulating genes to cancer cells, thereby achieving high intratumoral concentration of the gene products. In the present study, we employed a replication competent OAd expressing syngeneic IFN-α (IFN-OAd), which is one of the most promising cytokines for combination strategies in cancer immunotherapy, and evaluated the therapeutic activity of IFN-OAd in preclinical models of PDAC. When tested *in vitro*, IFN-OAd showed significant cytotoxicity against PDAC cells when compared to its luciferase counterpart and non-replicative IFN-α vectors. The level of IFN-α produced by infected cancer cells increased in a time dependent manner, indicating efficient gene production which depends upon viral replication. *In vivo* experiments using a Syrian hamster syngeneic model demonstrated significant tumor growth inhibition in the IFN-OAd treated group. Following intratumoral delivery, IFN-OAd also achieved significantly high local concentrations of IFN-α, while leaving the serum concentration within the normal rage. Our current goals include investigation of immunological activity induced by IFN-OAd in an immunocompetent, syngeneic hamster PDAC models. The Syrian hamster models are essential for analyzing OAds behavior because, unlike mice, they are permissive to OAds replication. However, there still remains a lack of available tools for analyses of hamster immune responses, resulting in limited characterization of the immune microenvironment. As of now, we are working on establishing and optimizing new procedures and tools to analyze the immune cell subsets and immune-related genes in hamster models. This work will lead to important knowledge which has the potential to contribute to the future use of adenovirus-based oncolytic viroimmunotherapy.

656. NIS-Expressing Oncolytic Adenovirus for Non-Invasive Imaging of Breast Cancer

Sacha Robert, Kari Jacobsen, Margarita Romanenko, Kazuho Inoko, Julia Davydova

Department of Surgery, University of Minnesota, Minneapolis, MN

Breast cancer is the most frequent malignancy affecting women worldwide. Advanced stage associated with metastasis and the heterogeneity of the disease make it challenging to treat and monitor. The sodium/iodide symporter (NIS) expression is a valuable tool used for effective radioiodine treatment and reporter gene imaging of cancers. Encoding the NIS by adenovirus is a promising strategy to facilitate non-invasive imaging and radiotherapy of breast cancer and associated metastasis. However, insufficient levels of NIS expression in tumor cells have limited its clinical translation. To optimize adenovirus-based radiotherapy and imaging, we investigated the effect of Adenovirus Death Protein (ADP) deletion on NIS expression. ADP promotes the release of progeny virus by accelerating the lysis and death of the host cell. Since NIS is a transmembrane protein, ADP deletion could be beneficial for its expression by preserving the membrane. We cloned two sets of oncolytic NIS-expressing adenoviruses that differed only in the presence or absence of ADP. The NIS gene was inserted into the E3 region to allow continual gene expression as the virus replicates. Other modifications included a chimeric Ad5/3 fiber to improve infectivity and the Cox2 promoter to drive tumor-selective replication. Our first finding was that the Cox2 promoter control vectors successfully demonstrated oncolytic effect in all breast cancer cell lines tested. We found that while the presence of ADP expression has more cytotoxic activity on the control lung epithelial adenocarcinoma cell line A549 than their counterpart adenoviruses non-expressing ADP, several breast cancer cell lines from different molecular classification (MCF-7, luminal A, AU565, HER2 positive, MDA-MB-231, triple negative B, MDA-MB-468, triple negative A) are found to be resistant to ADP. However, while ADP deletion had limited impact on the spread and replication of NIS expressing vectors in breast cancer cells, it was found to significantly improve NIS expression in those cells as revealed by immunofluorescence analysis. Our study, in accordance with previous findings in pancreatic cancer, demonstrate that contrary to common thinking, improving oncolysis may hinder the therapeutic effect of oncolytic viruses designed to express NIS. Further investigations will be conducted to comprehend by which mechanism ADP expression could affect NIS expression in adenovirus-infected cancer cells, and if ADP deletion could be as valuable *in vivo* or *ex vivo* for NIS expression and iodine intake and radiotherapy. Another perspective for us is to better control the specificity of replication of our NIS-expressing adenovirus to breast cancer cells. Mucin 1 (MUC1) was found to be aberrantly overexpressed in over 90% of breast tumors and is associated with poor prognosis. Therefore, in the future, we aim to assess the feasibility and specificity of MUC1 promoter in cancer cells by comparing the virus activity between our breast cancer and non-tumorigenic MCF-10A and MCF-12A breast cell lines.

657. CORE1: Phase 2, Single Arm Study of CG0070 Combined with Pembrolizumab in Patients with Non Muscle Invasive Bladder Cancer (NMIBC) Unresponsive to Bacillus Calmette-Guerin (BCG)

Gary D. Steinberg[1], Roger Li[2], Donald Lamm[3], Edward Uchio[4], Vignesh Packiam[5], Ashish Kamat[6], John McAdory[7], Paola Grandi[7], Jee-Hyun Kim[7], James Burke[7]

[1]Urology, NYU Langone Health, New York, NY,[2]Urology, Moffitt Cancer Center, Tampa, FL,[3]BCG Oncology, Phoenix, AZ,[4]UCI, Irvine, CA,[5]Urology, UIHC, Iowa City, IA,[6]MD Anderson Cancer Center, Houston, TX,[7]CG Oncology, Irvine, CA

Introduction: CG0070, an oncolytic vaccine available as an intravesical (IVE) therapy, is a serotype 5 adenovirus engineered to express GM-CSF and replicate in tumor cells with RB gene mutations or pathway defects which result in increased free levels of the transcription factor E2F. The CG0070 mechanism of action includes direct cell lysis in conjunction with immune mediated cell death which is enhanced in the presence of GM-CSF. In an initial phase 1 study as well as a subsequent open label phase 2 study, an overall CR rate of ~45% and a CR at 12 months (m) of 29% have been reported in patients with high risk NMIBC previously treated with BCG. Intravenous pembrolizumab, a PD-1 targeted checkpoint inhibitor, was FDA approved for the treatment of BCG unresponsive NMIBC (CIS with or without papillary tumors) with a complete RR of 41% and a 12 m CR rate of ~20%. This phase 2 study will assess the potential synergy of the two agents in the treatment of BCG-unresponsive NMIBC. **Methods:** 35 patients with BCG-unresponsive CIS with or without concurrent Ta or T1 disease will be treated with IVE CG0070 at a dose of $1x10^{12}$ vp in combination with pembrolizumab at a dose of 400 mg IV q6 weeks. CG0070 will be administered as follows: induction weekly x 6 followed by weekly x 3 maintenance instillations at months 3, 6, 9, 12, and 18. Patients with persistent CIS or HG Ta at 3 m may receive re-induction with weekly x 6 CG0070. Concomitant pembrolizumab will be administered up to 24 m at the above cited dose. Assessment of response will include q 3 m cystoscopy with biopsy of areas suspicious for disease, urine cytology, CTU/MRU, and mandatory bladder mapping biopsies at 12 m. Recurrence of HG disease will be enumerated as disease recurrence. **Results:** The primary endpoint of the study is CR at 12 m. Secondary endpoints will include CR at any time, progression free survival, duration of response, cystectomy free survival and the safety of the combination. Correlate assessments will include changes in the tumor immune microenvironment, systemic immune induction reflected in the peripheral blood and urine, as well as viral replication and transgene expression. Baseline expression of PD-L1, coxsackie adenovirus receptor, E2F transcription factor as well as anti- adenovirus antibody titer will be correlated with tumor response.**Conclusion:** The study is currently enrolling. An update on trial enrollment and preliminary data will be provided at the time of presentation.

658. Investigation of Human Adenoviral Replication Efficacies in Murine Cell Lines for the Generation of an Immunocompetent Mouse Model

Chikako Yanagiba, Mizuho Sato Dahlman, Hideki Yoshida, Kari Jacobsen, Masato Yamamoto
University of Minnesota-Twin Cities, Minneapolis, MN

In current years, oncolytic adenoviruses have shown promising advances in the hope of an effective cancer treatment, by its abilities to induce an oncolytic effect in cancerous cells and stimulate both innate and adaptative immune anti-tumor responses. One of the major limitations involved in testing oncolytic adenoviruses is the lack of a in vivo model. In this study, we focus on identifying murine cells capable of human adenovirus (HAd) replication, and to determine the reason(s) of restricted HAd replication. Several different murine cell lines were tested in vitro assays to determine the binding, killing and replicating efficacies, along with comparison to a cancerous human cell line (A549) and a Syrian Hamster cell line (HP-1) for positive controls. To begin, two different HAds were used to determine higher efficiencies for cellular attachment: Ad5 WT, which that binds to its natural receptor, coxsackievirus-adenovirus receptor, and Ad5 RGD, which binds to the arginyl-glycl-aspartic motif. The murine cells used included: HEPA1.6 (hepatoma), CMT64.6 (lung carcinoma), KPC (PDAC), PAN02 (PDAC), and NMuMG (non-transformed mammary gland cells). Binding assays were performed to test the binding efficacies of both viruses, and also to decide on favorable HAd for the following assays. Cells were infected with 100vp/cell of virus, then incubated at 4C for 2 hours. The cells were lysed and extracted for qPCR analysis with E4 region primers. As a general trend, Ad5 WT had a higher binding efficacy compared to Ad5 RGD. HEPA1.6 had the highest binding efficiency, while CMT64.6 and NMuMG had moderate efficacies, and KPC had low binding efficacy. Thus, Ad5 WT was chosen for further investigation. Crystal Violet Assays were performed to observe the cytolytic effect of the virus in increasing time points (Day 3, 7, 10 and 14). In parallel at the same time points, Replication Assays were used to quantify viral progeny in increasing viral titers (from 0.1vp/cell to 100vp/cell). The supernatant was collected and extracted, and the product was analyzed with qPCR by Ad E4 region primers. Surprisingly, the results for both assays did not correlate with the binding assay. Killing and replication efficacies were the highest in NMuMG, and results showed higher replication rates than in HP-1. We concluded that NMuMG has the highest capabilities of human adenovirus replication compared to other murine cell lines. The next step is for the transformation of NMuMG into a cancerous cell line to induce growth of tumors in immunocompetent mice. Once tumor growth has been observed, the mice will be injected with HAd-WT. Additionally, we will investigate using murine miRNAs in NMuMG to possibly identify gene(s) that may be responsible for decreasing viral replication efficiency.

659. A Phase 3, Single-Arm Study of CG0070 in Subjects with Non-Muscle Invasive Bladder Cancer (NMIBC) Unresponsive to Bacillus Calmette-Guerin (BCG)

Edward Uchio[1], Donald Lamm[2], Paul D. Anderson[3], Ben Tran[4], Ashish Kamat[5], John McAdory[6], Melody Keel[6], Paola Grandi[6], James Burke[6]
[1]UCI, Irvine, CA,[2]BCG Oncology, Phoenix, AZ,[3]Royal Melbourne Hospital, Melbourne, Australia,[4]Peter MacCallum Cancer Centre, Melbourne, Australia,[5]University of Texas MD Anderson Cancer Center, Houston, TX,[6]CG Oncology, Irvine, CA

Introduction: CG0070 is a serotype 5 adenovirus engineered to express GM-CSF and replicate in cells with mutated or deficient RB, with response rates (RR) of approximately 45% observed in patients with recurrent NMIBC after BCG (J Urol. 2012;188:2391-7; Urol Oncol. 2018;36(10):440-7). This single arm phase 3 study was launched to confirm the clinical activity of CG0070 in patients with BCG Unresponsive NMIBC. **Methods:** 110 patients with BCG-unresponsive CIS with or without concurrent Ta or T1 disease will be treated with intravesical (IVe) CG0070 at a dose of 1x1012 vp. CG0070 will be administered as follows: induction weekly x 6 followed by weekly x 3 maintenance instillations at months 3, 6, 9, 12, and 18. Patients with persistent CIS or HG Ta at 3 months (m) may receive re-induction with weekly x 6 CG0070. Assessment of response will include q 3 m cystoscopy with biopsy of areas suspicious for disease, urine cytology, CTU/MRU, and mandatory bladder mapping at 12 m. Detection of high grade disease within the bladder will be enumerated as recurrence or non-response. **Results:** The primary endpoint of the study is CR at any time on study as assessed by biopsy (directed to cystoscopic abnormalities and mandatory mapping at 12 m), urine cytology, and radiography, as above. Secondary endpoints include CR at 12 m, duration of response, progression free survival, cystectomy free survival and safety. Correlative assessments include changes in the tumor immune microenvironment, systemic immune induction as reflected in the peripheral blood and urine, as well as viral replication and transgene expression. Baseline expression of coxsackie adenovirus receptor, E2F transcription factor as well as anti-adenovirus antibody titer will be correlated with tumor response. **Conclusion:** Study enrollment globally is ongoing.

660. Defining the Role of Polycytidine (polyC) Tract Length in Mengovirus Oncolytic Activity

Velia Penza, Autumn J. Schulze, Stephen J. Russell
Molecular Medicine, Mayo Clinic, Rochester, MN

The use of miRNA targeting to restrict replication of Mengovirus, a picornavirus of the Cardiovirus genus, successfully eliminated cardiac and neuronal toxicities in a multiple myeloma mouse model and significantly improved survival, but was not curative. Further attenuation of Mengovirus virulence can be achieved by truncation or deletion of a 55-nucleotides long polycytidine (polyC) tract present in the 5'-noncoding region, a known pathogenicity determinant of the virus. miRNA-targeted (NC) Mengovirus is an ideal platform to explore the impact of polyC tract length on the oncolytic and immunogenic potential of Mengovirus while maintaining the safety

profile. With the goal of improving therapeutic efficacy, we investigated the mechanism behind polyC attenuation in Mengovirus and the effect of virulence and oncolytic potency. Mengovirus variants with polyC tract lengths of C44UC10 (Mwt), C13UC10 (MC24), or no polyC (MC0) show equally robust in vitro oncolytic activity against a panel of human and mouse tumor cell lines. However, the length of the polyC tract significantly impacts Mengovirus replication kinetics in immune cells such as lymphocytes, macrophages, and dendritic cells. This cell type-specific difference in replication also holds true for miRNA-targeted Mengovirus polyC variants. The viral attenuation induced by polyC truncation alters the pro-inflammatory cytokines IFNβ, TNFα and IL-1β release profile in macrophages. However, variations in type I IFN activation does not completely explain the difference in replication kinetics caused by polyC tract truncations, as all polyC variants demonstrate similar susceptibility to IFN response modulation. Similarly, a deleted or truncated polyC tract did not attenuate the oncolytic activity of Mengovirus in IFN responsive nor IFN-non-responsive tumor cell lines in vitro. The presence of a shorter polyC tract in the 5'NCR does not hamper Mengovirus IRES-dependent translation, but rather reduces the synthesis of new negative and positive sense viral genomes during Mengovirus replication in macrophages. These preliminary data suggest that the polyC tract evolved as a specific mechanism to overcome a replication block present in cells of the hematopoietic cell lineage and grant a successful infection in the target tissues. Unlocking the molecular mechanism behind polyC tract-dependent attenuation of Mengovirus in immune cells will be pivotal in exploring new avenues for augmenting its safety and therapeutic efficacy as an oncolytic agent, particularly with regards to immunotherapy combinations.

661. Gut Microbiome Modulates Ad5D24-Mediated Immunogenic Cell Death in a Melanoma Mouse Model

Lorella Tripodi[1], Eleonora Leggiero[2], Valeria D'Argenio[3], Maria Vitale[4], Laura Gentile[4], Giulia Scalia[2], Vincenzo Cerullo[5], Lucio Pastore[4]

[1]European School of Molecular Medicine (SEMM) - University of Milan, CEINGE Biotecnologie Avanzate, Naples, Italy,[2]CEINGE Biotecnologie Avanzate, Naples, Italy,[3]Dipartimento di Promozione delle Scienze Umane e della Qualità della Vita, Università Telematica Sa, CEINGE Biotecnologie Avanzate, Naples, Italy,[4]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federi, CEINGE Biotecnologie Avanzate, Naples, Italy,[5]Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federi, Laboratory of Immunovirotherapy, Drug Research Program, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland

In the last decade, immunotherapy has delivered impressive results in clinical settings, particularly with antibodies that block immune inhibitory pathways, specifically CTLA-4 and the axis between programmed cell death protein 1 (PD-1) and its ligand 1 (PD-L1). However, variable immune responses between individuals are not well understood and the efficacy has not been consistent probably because of additional environmental and genetic factors influencing the outcome. Recent studies have shown that gut microbiome can affect immunotherapy outcome. We therefore hypothesized that modulation of the microbiome could also affect oncolytic virus efficacy in active immunotherapy. We then decided to investigate whether the effect of oncolytic vaccines on tumor regression could be affected by manipulation of the microbial community of gut microbiome. At this aim, we pre-treated a group of C57BL/6J mice with an oral administration of vancomycin. Subsequently, we generated a subcutaneous tumor with syngeneic B16.OVA melanoma cells to these mice and to untreated animals and administered oncolytic adenovirus Ad5D24 to both groups. We observed that the antitumoral effect of Ad5D24 was extremely reduced in mice pretreated with antibiotic with a faster tumor progression compared to the control group. In order to confirm that the effect was gut microbiome-dependent, we cohoused mice pretreated with vancomycin and a control group and then treated them with Ad5D24. We observed that cohousing with untreated animals reduced tumor growth in mice pretreated with vancomycin. In order to further investigate the link between gut microbial content perturbed by antibiotic and systemic immune responses to oncolytic viruses, we analyzed the phenotype of antigen-specific T-cell , and the IFN-gamma secreted by activation of CD4 Th1 and CD8 cytotoxic T-lymphocyte (CTL) effector T-cells in spleen, lymph nodes and tumors in the mice treated.

662. Type II-Specific Internal Ribosome Entry Site Substitution Controls Toxicity of Oncolytic Mengovirus

Yakin Jaleta, Yogesh R. Suryawanshi, Caitessa Venables, Velia Penza, Justin W. Maroun, Rebecca A. Nace, Stephen J. Russell, Autumn Joy Schulze

Mayo Clinic, Rochester, MN

MC_{24}NC, a microRNA-detargeting Mengovirus with atruncated poly(C) tract is a potent and promising oncolytic virus. However, thepotential for escape mutant selection resulting in reversion to a pathogenicvirus may limit its consideration for clinical translation, particularly inimmunocompromised patients. Manipulation of the internal ribosome entry site(IRES), a *cis*-acting genetic element involved in picornavirustranslation and replication, has been used to improve the safety of anoncolytic poliovirus. In this study, we evaluated the ability of IRES elementsfrom human rhinovirus type 2 (HRV2) and foot-and-mouth disease virus (FMDV) toreduce Mengovirus neuro- and cardiotoxicity whilst maintaining oncolyticactivity. MC_{24}FMDV and MC_{24}HRV2 are recombinant strainsof Mengovirus in which the poly(C) tract has been truncated and the IRES regionhas been replaced with the IRES of FMDV or HRV2, respectively. Both virusesexhibited reduced replication capacity in the neuroblastoma cell line SK-N-MC. However,replacement of the Mengovirus IRES, a type II IRES, with that of HRV2, a type IIRES, also significantly diminished virus replication in cell lines used forpropagation and abrogated oncolytic activity *in vivo*. Cell-type specificattenuation in viral growth kinetics, translational capacity, and cytotoxicitywere also observed *in vitro* for MC_{24}FMDV (type II IRES).However, *in vivo* studies demonstrated that FMDV IRES exchangesignificantly reduced viral toxicity while maintaining oncolytic efficacyagainst a murine multiple myeloma (MPC-11) model. Substitution of the entire 5'and/or 3' untranslated regions with that of either HRV2 or FMDV did notalleviate the attenuation, but instead resulted in further inhibition. Studiesaimed at elucidating the mechanism(s) associated with FMDV

IRES-mediatedcell-type specific attenuation including the evaluation of Gemin5, a knowninhibitor of FMDV IRES-mediated translation, are currently underway. Thesestudies demonstrate the potential for IRES-mediated regulation of viral tropismas an ideal mechanism for improving the safety of oncolytic Mengovirus and seta precedent for its continued development for clinical translation againstcertain cancer types.

663. Optimization of Newcastle Disease Virus Production in the Avian EB66 Cell Line

Brenna A. Y. Stevens[1], Lisa A. Santry[1], Jacob E. G. Yates[1], Enzo M. Barachuy[1], Pierre P. Major[2], Byram W. Bridle[1], Sarah K. Wootton[1]

[1]University of Guelph, Guelph, ON, Canada,[2]Juravinski Cancer Centre, Hamilton, ON, Canada

Introduction: Newcastle disease virus (NDV), an avian orthoavulavirus, has demonstrated oncolytic activity against various mammalian cancers, and is a promising viral vaccine vector. Like other avian viruses, NDV is typically produced in embryonated eggs. Egg-based culture is a slow labour-intensive process that produces variable yields, relies on availability of pathogen-free eggs, and is difficult to scale up. Compared to eggs, suspension cell culture provides a flexible production timeline, more reliable yields, and enables large-scale industrial production using high-capacity bioreactors. The EB66® Good Manufacturing Practice-compliant cell line derived from duck embryonic stem cells grows rapidly in suspension and has been used to produce influenza and Modified Vaccinia Virus Ankara-based vaccines. In this study, we evaluated multiple parameters to optimize NDV production in EB66 cultures . The following variables were tested: NDV pathotype, multiplicity of infection (MOI), harvest time, and growth conditions. **Methods:** The lentogenic LaSota strain and its mesogenic derivative, both expressing green-fluorescent protein, were used to infect EB66 cells at various MOIs . Samples of infected culture supernatant were taken at various hours post infection (hpi) for 96 hpi and titered by Median Tissue Culture Infectious Dose ($TCID_{50}$) and converted to plaque forming units (PFU) to establish baseline viral production yields. NDV strains were passaged in EB66 cells eight (mesogenic) or eleven (lentogenic) times and new viral production curves were established with the same MOIs. To increase titers, production of LaSota was evaluated in flasks of varying shapes (T-flask vs Erlenmeyer), infection media (CDM4A vs Dulbecco's Modified Eagle Medium (DMEM)), and calcium chloride concentrations (1, 0.3, or 0 mM). **Results:** For lentogenic NDV, the optimal MOI and harvest time were 10^{-3} and 60 hpi, resulting in the production of 0.14 PFU/cell with titers of 7.3e5 PFU/mL. After eleven passages, the optimal harvest time decreased to 36 hpi, producing 0.23 PFU/cell with titers of 1e6 PFU/mL. For mesogenic NDV, the optimal MOI and harvest time were 10^{-2} and 72 hpi with titers of 6.7e6 PFU/mL. After eight passages, both the optimal MOI and harvest time changed to 10^{-3} and 60 hpi with titers of 2.5e7 PFU/mL. Varying infection media and increasing calcium concentration under optimal infection conditions had a negative impact on NDV production, which dropped below 0.03 PFU/cell. Under optimal conditions, T-flasks produced 0.45 PFU/cell whereas Erlenmeyer flasks produced 0.3 PFU/cell. **Discussion:** In our hands, egg-based NDV culture typically produces ~1.38e7 PFU/mL and ~2.72e7 PFU/mL of allantoic fluid for lentogenic and mesogenic NDV, respectively. Passaging lentogenic NDV in EB66 cells increased production by 1.6-fold, however titers achieved were lower than eggs. Despite the expected lower titers, suspension culture is highly scalable unlike eggs. Passaging mesogenic NDV increased production by 3.7-fold, and titers achieved were similar to egg-based culture. Egg-based NDV production can take up to two weeks, whereas EB66-based culture can be infected and harvested in three days. Additionally, purification of EB66-made NDV is much easier as EB66 media is serum-free, whereas egg-made NDV contains highly immunogenic ovalbumin. EB66-based NDV production offers a valuable alternative to traditional egg-based culture. **Future directions**: In vitro killing assays will be performed with EB66-derived NDVs to ensure they retain oncolytic potential. Comparisons of egg- and EB66-derived NDVs in murine cancer models would ensure that in vivo safety and oncolytic profiles are maintained. These experiments are ongoing.

664. Synergestic Tumor-Killing Effect by Cross-Hybrid IgGA Fc

Firas Hamdan, Erkko Ylösmäki, Jacopo Chiaro, Yvonne Giannoula, Maeve Long, Manlio Fusciello, Sara Feola, Beatriz Martins, Michaela Feodoroff, Gabriella Antignani, Otto Kari, Moon Hee Lee, Petrus Järvinen, Harry Nisen, Anna Kreutzman, Jeanette Leusen, Satu Mustjoki, Thomas McWilliams, Mikaela Grönholm, Vincenzo Cerullo

University of Helsinki, Helsinki, Finland

Despite the success of immune checkpoint inhibitors in the clinic, only a fraction of patients benefit from these therapies. A theoretical strategy to increase efficacy would be to enhance such antibodies with Fc-mediated effector mechanisms. We designed a cross-hybrid Fc-fusion peptide against PD-L1 able to elicit effector mechanisms of an IgG1 but also IgA consequently activating PMNs, a population neglected by IgG1, in order to combine multiple effector mechanisms. Moreover, to prevent toxicity, these Fc-fusion peptides were cloned in oncolytic adenoviruses whose replication is restricted to the tumor. These oncolytic adenoviruses were able to secrete the cross-hybrid Fc-fusion peptides able to bind to PD-L1 and activate multiple immune components enhancing tumor cytotoxicity, compared to FDA-approved immune checkpoint inhibitors, in various cancer cell lines and renal cell carcinoma patient derived organoids. In conclusion, these cross-hybrid Fc-fusion peptides demonstrate that activating multiple immune effector populations increases tumor cytotoxicity potentially leading to improved clinical outcomes.

665. Efficacy of Systemic Administration of Mesothelin-Targeted Oncolytic Adenovirus In Pancreatic Cancer

Mizuho Sato-Dahlman, Praveensingh Hajeri, Kari Jacobsen, Chikako Yanagiba, Masato Yamamoto

Surgery, University of Minnesota, Minneapolis, MN

Pancreatic cancer is an aggressive malignant disease. Despite extensive efforts, systemic therapies have provided only limited efficacy for patients with this disease. Oncolytic Adenovirus (OAd) is a promising

therapeutics, and it is also known for its efficient in vivo gene delivery. However, when adenovirus vectors are injected intravenously into mice, most of the virus goes to the liver and can lead to liver toxicity at high dosage. One of the reason for liver tropism is that hepatocytes express high levels of the primary adenovirus receptor, and non-parenchymal liver cells, such as Kupffer cell and epithelial cell, also capture the viral particle. As a consequence of large sequestration of adenovirus by liver, the tumor transduction rate is low and the in vivo efficacy is limited. Therefore, the improvement of cancer selective transduction and vector distribution to avoid liver sequestration would overcome the obstacles for systemic delivery required for efficient systemic treatment of spread and/or metastatic lesions of pancreatic cancer with OAd. To improve the tumor transduction, we have generated the pancreatic cancer-targeted OAd by high-throughput screening of Ad-fiber library. The pancreatic cancer-targeted OAd binds to mesothelin (MSLN) protein, which is overexpressed on the surface of pancreatic cancer. MSLN-targeted OAd showed selective and powerful anti-tumor effect against MSLN-positive xenograft tumor model in both intratumoral (i.t.) and intravenous (i.v.) injection. Importantly, when we assessed viral distribution after i.v. injection, the liver sequestration of MSLN-targeted OAd was lower than untargeted OAd (Ad5-WT) at 48 hrs after injection. By day 7, the viral copy number of MSLN-targeted OAd in the tumor was significantly higher than Ad5-WT virus. These results suggest that systemic injection of the tumor targeted-OAd showed significantly lower liver sequestration and better tumor accumulation. Next, antitumor effect of MSLN-targeted OAd was assessed in patient-derived xenograft (PDX) model. After i.v. injection, only the MSLN-targeted OAd showed significant antitumor effect compared to the untreated group. Finally, we tested the effect of preexisting immunity to MSLN-OAd in human serum because neutralizing antibodies (nAbs) are one potential *obstacle* in oncolytic virotherapy. In a neutralization assay, the MSLN-targeted OAd was more resistant to human serum, compared to fiber-unmodified OAd (Ad5-WT). These data suggest that OAd with a targeting motif in the AB-loop allow a degree of escape from pre-existing Ab and has a greater capability to facilitate systemic therapy. Our results indicated that tumor targeted-OAd can embody efficient systemic treatment for pancreatic cancer which are mostly found with spread or metastatic lesions.

666. Development and Industrialization of a Cost-Effective Reproducible Oncolytic Virus Production Process Using the iCELLis® Bioreactor System

Toby Price[1], Tim Kellogg[2], Odette Becheau[3], Nathan Hazi[3]

[1]Replimune, Abington, United Kingdom,[2]Replimune, Framingham, MA,[3]Pall Biotech, Westborough, MA

Oncolytic immunotherapies are an emerging class of cancer treatments using modified viruses to selectively replicate in and kill tumors. These powerful therapies are designed to fight cancer in two ways. Firstly, to treat the patient's cancer and secondly to prevent future relapse by generating an immune response. While the clinical studies demonstrate promising results, manufacturing modified viruses in classical adherent formats faces many challenges including a lack of scalability, the need for a large number of manual aseptic handling steps, and open operations which will increase the risk of contamination and batches being lost. The iCELLis bioreactor is a proven technology which overcomes these concerns, enabling fast-track development and industrialization of adherent-based processes. Furthermore, the iCELLis bioreactor drastically reduces the manufacturing footprint while maintaining a closed, controlled, and highly integrated environment. Here, we first demonstrate that the bench-scale iCELLis Nano bioreactor was successfully used to develop a robust and reproducible process to produce engineered herpes simplex virus (HSV) using VERO cells. The development efforts were concentrated on reducing the cell seeding density down to 2,500 cells/cm^2, which would allow for a very manageable seed-train, on limiting the use of serum-containing media improving product safety, and improving the recovery of virus from the bioreactor by the addition of a final salt wash. The data presented also demonstrate that this process was easily and rapidly scaled-up to the iCELLis 500+ bioreactor, producing commercially viable yields of HSV to be used in late clinical studies. This success story is the result of a long-term partnership and is still an ongoing journey to further improve process safety, manufacturability, cost reduction, and product yields.

Cancer - Targeted Gene and Cell Therapy

667. End to End Discovery to Support the Development of Safe and Efficacious Engineered Cell Therapies

Katherine Vousden[1], Sanne Holt[2], Sophie Vermond[2], Monique Hazenoot[2], Sarah Ives[3], Sawsan Youssef[3], Jake Glanville[4]

[1]Charles River, Saffron Walden, United Kingdom,[2]Charles River, Leiden, Netherlands,[3]Distributed Bio, a Charles River company, South San Francisco, CA,[4]Centivax, South San Francisco, CA

The global market for cell and gene therapies, including T-cell/ Natural Killer cell chimeric antigen receptors (CAR-T/ NK), is continuing to grow rapidly - with many clinical success stories for B-cell malignancies in particular. However, the path to clinical success has not been smooth - with early reports of organ damage and even deaths following CAR-T therapy highlighting that there are significant risks involved in their development, and a clear requirement to understand and mitigate for the underlying mechanisms that result in unwanted toxicities. Off-target binding and tonic signalling are indicated in many pre-clinical and clinical cell therapy failures, and it is becoming increasingly apparent that CAR design - particularly the selection of the tumor targeting moiety with exquisite specificity, desirable biophysical attributes and appropriate affinity - is key to developing a successful therapy. These scFv and VHH phage-display libraries (SuperHuman 2.0 and Tungsten) have been designed to be biophysically stable and non-immunogenic. Their size and clonal diversity, coupled with the use of streamlined workflows, enable the rapid identification of large panels of VH CDR3 sequence-unique binders which can then be assessed for CAR suitability by exploiting the integrated approach. Panels of CAR constructs, covering the diversity of sequences and

affinities related to the scFv/ VHH domains isolated, can be triaged through Charles River efficacy and safety work-streams to identify the best therapeutic candidates. For example, co-culture experiments with target-expressing carcinoma cell lines and non-target expressing cell lines can be utilized to investigate and compare activity, specificity and potency of the various CAR constructs. Ultimately, combining antibody discovery with Charles River downstream characterization enables identification of the best lead candidate - balancing efficacy and safety of your CAR-T therapy - for optimal therapeutic outcome.

668. A Recombinant Adenovirus Vector Containing the *synNotch Receptor* Gene Inhibits Tumor Growth in a Mouse Xenograft Model of Triple-Negative Breast Cancer

Shoko Tominaga[1], Naoto Kunimura[1], Koichi Kitagawa[1], Hazuki Doi[1], Ruhan A[1], Masato Fujisawa[2], Toshiro Shirakawa[1]

[1]Graduate School of Science, Technology and Innovation, Kobe University, Kobe, Japan,[2]Kobe University, Kobe, Japan

Introduction: Triple-negative breast cancer (TNBC) is one of the most difficult molecular subtypes of breast cancer to treat. We developed a recombinant replication-deficient adenoviral vector (Ad-CD44-N-HIF3α4) containing a gene encoding a synthetic Notch (synNotch) receptor composed of the extracellular domain of CD44 (CD44-ECD) and the hypoxia-inducible factor (HIF)-3α4 connected by the Notch core regulatory region. CD44 is a cell surface adhesion receptor and a known cancer stem cell marker in breast cancer and other malignancies, and HIF-3α4 is a dominant-negative regulator of HIF-1α. The CD44-ECD in the synNotch receptor acts as a CD44 decoy receptor, and after a ligand binds to the CD44-ECD, HIF-3α4 is released, resulting in the inhibition of both CD44 and HIF-1α signaling pathway in cancer cells. **Methods:** In this study, we employed a human TNBC cell line, MDA-MB-231. Gene transduction with recombinant adenoviral vector containing CD44-N-HIF3α4 (Ad-CD44-N-HIF3α4) and the gene expressions were determined. MDA-MB-231 cells were cultured under conditions of hypoxia (≤1% O_2) with hyaluronic acid (HA). Ad-CD44-N-HIF3α4 and Ad-LacZ were infected into cells at 40 multiplicities of infection (MOIs), respectively. *In vivo* study, MDA-MB-231 cells were subcutaneously inoculated into mice. Intratumoral injections were performed with 1×10^9 PFU of adenoviral vectors Ad-CD44-N-HIF-3α4, Ad-LacZ, and PBS control. **Results:** The expressions of the *HIF-3α4* gene and surface expression of CD44 were significantly increased in MDA-MB-231 cells by infection with Ad-CD44-N-HIF3α4. In addition, the relative gene expressions of *survivin* and *CCL2*, downstream genes of CD44, in the cells infected with Ad-CD44-N-HIF3α4 were significantly lower than in cells infected with Ad-LacZ and control cells under culture conditions of hypoxia with HA. The relative gene expression of *vascular endothelial growth factor* (*VEGF*), a hypoxia target gene, was significantly decreased in the cells infected with Ad-CD44-N-HIF3α4 than that in control cells under culture conditions of hypoxia with HA. Ad-CD44-N-HIF3α4 significantly suppressed tumor growth compared to Ad-LacZ or PBS control in mice. The expression of CD44 was remarkably increased in mice tumors with the treatment of Ad-CD44-N-HIF3α4. **Conclusion:** Ad-CD44-N-HIF3α4 inhibited the both signaling pathways of CD44 and HIF-1α in MDA-MB-231 cells under conditions of hypoxia with HA *in vitro*, and significantly suppressed tumor growth *in vivo*. These findings indicated that Ad-CD44-N-HIF3α4 has a high clinical applicability for invasive types of TNBC.

669. Immunomodulatory Effect of Peptide LL 37 in Breast Cancer

Victor M. Arenas Luna

Molecular Biology Laboratory, Universidad Panamericana School of Medicine, Mexico, Mexico

Breast cancer is one of the most frequent malignancies in women around the world. Breast cancer has been classified into different subtypes, each one defined by the expression of hormonal and growth receptors. In this way, each subtype has unique clinical and histological characteristics. The process of tumor formation and progression is influenced by dynamic interactions of cancer cells and the tumor microenvironment. One of the cellular types present in the tumor microenvironment interacting with cancer cells is mesenchymal stem cells (MSCs). Recently, it has been observed, MSCs could play an important role in tumor development and progression, together with other cells by secretion of multiple soluble factors like cytokines, growth factors and small molecules like peptides. One of these secreted elements in the tumor microenvironment is the peptide LL-37 considered as a host defense peptide with immunomodulatory properties. However, in cancer exerts important functions in chemotaxis, angiogenesis and tumor growth. Curiously in breast cancer, LL-37 is upregulated in high-grade tumors, but their cellular origin inside tumo r and the effect that LL-37 could have on MSC during tumorigenesis it remains to be known. Therefore, studying the LL-37 role in tumorigenesis could help to understand better, the aggressive behavior of some breast cancer types. In this project, we evaluated the expression of LL-37 level on human bone marrow MSCs and their interaction with several cell lines from breast cancer (MCF7, BT-474 and MDA-MB-231) by qRT-PCR, immunofluorescence and western blot. In addition, we determined the effect that LL-37 has on gene expression related to immunosuppressive properties and differentiation potential on MSCs. We observed that conditioned medium from MSCs induce the gene-LL37 overexpression on MCF7 and MDA-MB-231 but not on BT474. However, we did not detect LL-37 peptide at protein level. However, LL-37 induces overexpression of inflammatory molecules as TGF-β and Il-4 on MSCs. In conclusion, LL-37 could induce MSCs immunomodulatory properties and an increase of their differentiation potential that could be determinat for the tumor aggressivity.

670. The HER2-ATAK™Myeloid Cells as a Therapeutic Modality for Solid Tumors

Yuxiao Wang[1], Chetan Rane[1], Patrick Tavares[1], Kyong-Rim Kieffer-Kwon[1], Zheng Lung Ling[2], Namita Bisaria[1], Tyler Nicholson[1], Nicholas King[3], Neha Diwanji[1], Josephine D'Alessandro[1], Kathryn Austgen[1], Siddartha Mukherjee[4], Thomas Prod'homme[1], Daniel R. Getts[1]

[1]Myeloid Therapeutics, Cambridge, MA,[2]School of Medical Sciences Bosch Institute, University of Sydney, Sydney, Australia,[3]School of Medical Sciences Bosch Institute, University of Sydney, Sydney, Australia,[4]Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY

In solid tumors, the tumor microenvironment (TME) remains a formidable barrier to anti-cancer therapies. Myeloid-derived-tumor-associated macrophages (TAMs) are generally the most populous non-tumor cell within the TME. Myeloid cells found within TMEs represent a spectrum of variably polarized phenotypes existing within the M1/M2 paradigm, with M1 showing anti-tumoral activities, while M2 differentiation supports tumor growth. To shift the dynamics of myeloid cell activity in tumors we developed the **A**ctivate, **T**arget, **A**ttack & **K**ill (ATAK™) myeloid cell platform. We proposed that myeloid cells could be engineered to recognize and phagocytose tumor cells, as well as orchestrate a broad anti-tumor immune response. To address this potential, we engineered Human epidermal growth factor receptor 2 (HER2)-ATAK myeloid cells. HER2 is dysregulated in a wide range of solid tumors and is a well-recognized mediator of the cancerogenic process, making it an attractive therapeutic target. Trastuzumab and other HER2-inhibitors have entered into clinical practice for the treatment of breast cancer and, more recently, have been approved to treat HER2-positive metastatic gastric cancers. The HER2 ATAK construct consists in a single-chain variable fragment (scFv) domain derived from an established anti-HER2-monoclonal antibody, associated to a CD8 hinge domain followed by a combination of intracellular signaling domains involved in innate responses, such as Fc gamma receptor and PI3K. Transfection of the mRNA coding for the ATAK construct is associated with high expression in myeloid cells. Following receptor engagement, ATAK-myeloid cells not only show robust phagocytosis, but also increased expression of antigen presentation molecules, co-stimulatory molecules and proinflammatory chemokines and cytokines. In xenograft models of mesothelioma (MSTO) and ovarian cancer (SKOV3), treatment with human-monocyte-derived HER2 ATAK cells was associated with tumor penetration, differentiation into effector cells and the prevention of tumor growth. Together, these data highlight the potential of HER2 ATAK myeloid cells for the treatment of HER2-positive tumors.

671. Modeling Liver Cancer in Humanized Mice

Bin Li, Amita Tiyaboonchai, Tomonori Matsumoto, Markus Grompe

Oregon Stem Cell Center, Oregon Health and Science University, Portland, OR

Hepatocellular carcinoma (HCC) is increasing in frequency in the human population and treatment options remain limited. The molecular architecture of rodent HCC is distinct from human liver cancer and hence better models of the human disease are needed. Patient derived xenografts (PDX) of HCC are difficult to establish. We therefore aimed to transform primary human hepatocytes into transplantable HCC. Healthy (n=7 patients) and cirrhotic (n= 4 patients) liver parenchymal cells (hepatocytes or cirrhosis derived epithelial cells) were transduced with lentiviral vectors encoding the oncogenes cMYC, HRAS and SV40LT-antigen in 2D culture. Morphology and growth curve analysis demonstrated that these parenchymal cells (5/7 normal hepatocytes, 4/4 cirrhotic liver cells) transformed into proliferating cancer-like epithelial cells in vitro. After being expanded for at least 2 passages, the cancer-like cells were transplanted into immunodeficient *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$*NOD* (FRGN) mice to investigate their in vivo properties. As early as one month after transplantation, human donor derived HCC like tumors were found in the host mice by histology. Transcriptome assay by RNAseq and protein assay by immunohistochemistry were performed to characterize the properties of the HCC like tumors. Together, our results indicate that a robust human HCC model can be established by genetically engineering of primary human hepatocytes and transplantation into immune deficient mice.

672. Development of In Vivo Anti-CD19 CAR T-Cell Therapy Based on Chemically Encapsulated Lentiviral Vectors

Frederic Mourlane, Pauline Tesson, Charles Duchene, Maxime Girardon, Marion Lhuaire, Laurence Leclere, Eva Maunichy, Rachel Pacherie, Ozgul Tezgel, Renaud Vaillant, Cecile Bauche

Ixaka, Villejuif, France

With Yescarta, Tecartus and Kymriah marketed therapies for B cell hematological malignancies and more than 300 ongoing clinical trials, genetic modification of T cells with chimeric antigen receptors (CARs) recognizing surface antigens on tumor cells has emerged as a revolutionary therapeutic strategy in immuno-oncology. Despite impressive clinical benefits, the complex logistics required to manufacture *ex vivo* CAR-T cells from individual patients and the associated costs represent major hurdles to the widespread use of these therapies. Ixaka is a preclinical-stage biotechnology company developing *in vivo* CARs based on lentiviral vectors (LV) encoding anti-CD19 CARs encapsulated in oligopeptide-modified poly(beta-amino ester)s (OM-PBAEs) biodegradable polymers. Here we report on a microfluidics-based method that consistently manufactures LV/OM-PBAE nanoparticles able to reprogram peripheral blood mononuclear cells to express a functional CAR directed against the CD19 antigen with a cytotoxic activity. The biodistribution of formulated LV/OM-PBAE nanoparticles encoding Green Fluorescent Protein or Luciferase has been investigated in immunocompetent mice after intravenous administrations or infusions and we could show a different distribution profile compared to pseudotyped LVs, with a pronounced tropism for blood cells. Multiple intravenous administration of these nanoparticles in mice was safe and well tolerated as no obvious sign of distress, body weight loss, change in blood cell count or cytokine levels were reported. Preliminary efficacy data in immunocompetent and humanized mice suggest that *in vivo* reprogramming of blood immune cells with a CD19 CAR can be achieved and it triggers the selective destruction of CD19-postive B cells and lymphoma cells. Based on these promising

properties, OM-PBAE encapsulated LVs have been selected for further preclinical and clinical development of a universal *in vivo* CAR T-cell therapy.

673. Harnessing Myeloid Cells for Peripheral T-Cell Lymphoma Immunotherapy: The CD5 ATAK™ Myeloid Cell Platform

Kyong-Rim Kieffer-Kwon[1], Yuxiao Wang[1], Chetan Rane[1], Xing Du[2], Patrick Tavares[1], Neha Diwanji[1], Namita Bisaria[1], Tyler Nicholson[1], Josephine D'Alessandro[1], Kathryn Austgen[1], Michele Gerber[1], Ron Vale[3], Siddhartha Mukherjee[2], Thomas Prod'homme[1], Daniel R. Getts[1]

[1]Myeloid Therapeutics, Cambridge, MA,[2]Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY,[3]Howard Hughes Medical Institute, Janelia Research Campus, Ashburn, VA

Refractory T-cell lymphoma (TCL) is a lethal disease with few curative treatment options. While engineered T cell therapy has revolutionized other hematological cancer treatments, this has not been realized in the treatment of TCL due to the shared expression of tumor targets with engineered T cells resulting in fratricide or poor product durability. CD5 is a T cell lineage marker commonly expressed on T-cell malignancies, including T-ALL and T cell lymphoma. Given its relatively restricted expression to T cells, it is an attractive target for immunotherapy in TCL. Myeloid cells do not express T-cell tumor antigens and readily accumulate in tumors, in some cases contributing up to 50% of the tumor mass. Furthermore, myeloid cells are potent immune cells capable of orchestrating a broad immune response through phagocytosis, antigen presentation, cytokine production and many other functions. To harness and channel these attributes toward cancer, the **A**ctivate, **T**arget, **A**ttack & **K**ill (ATAK™) platform has been developed to engineer myeloid cells. MT-101 are the first ATAK myeloid cells and consist in an anti-CD5 chimeric antigen receptor (CAR) construct incorporated into autologous myeloid cells for the treatment of CD5-expressing TCL. The ATAK construct contains an anti-CD5 single-chain variable fragment (scFv) domain derived from an established CD5-monoclonal antibody that has been used in previous clinical trials without irreversible off-target effects. The scFv is associated to a CD8 hinge domain followed by a combination of intracellular signaling domains involved in innate responses, such as Fcγ and PI3K. Transfection of the RNA coding for the chimeric ATAK construct is associated with high expression in both the myelomonocytic cell line THP-1, and primary monocytes. The expression of the construct can be detected for up to 9 days at the surface of THP-1 ATAK cells. Antigen-specific stimulation of the CAR leads to Akt phosphorylation. Following receptor engagement, MT-101 cells can robustly phagocytose and produce proinflammatory chemokines and cytokines. Conversely, MT-101 cells did not deplete autologous healthy T cells in a co-culture assay. The phagocytic activity and cytokine secretion are persistent even in immune suppressive M2 polarizing condition. The increased expression of co-stimulatory molecules (CD86) and MHC II over time suggests the ability of CD5-ATAK myeloid cells to present tumor antigens to T cells. Importantly, MT-101 cells demonstrate significant activity in subcutaneous and systemic xenograft mouse models of T cell malignancies. Anti-tumoral response is sustained, with MT-101 myeloid cells being detected for several weeks after treatment. Together these data highlight the potential of MT-101 engineered monocytes for the treatment of TCL.

674. Immune Competent Pediatric Sarcoma Models for the Preclinical Evaluation of B7-H3-CAR T-Cell Therapy

Kenneth J. Caldwell[1], Phuong Nguyen[1], Alexandra Beckett[1], Dalia Haydar[1], Zhongzhen Yi[1], Giedre Krenciute[1], Jason Yustein[2], Stephen Gottschalk[1], Christopher DeRenzo[1]

[1]Bone Marrow Transplant and Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN,[2]Baylor College of Medicine, Houston, TX

Background: Chimeric antigen receptor (CAR) T cells offer promise to improve outcomes for pediatric patients with recurrent/refractory sarcoma, a group of 'poor-prognosis' cancers. However, early phase clinical studies have shown limited activity despite potent antitumor activity in preclinical xenograft models, highlighting the need to develop better preclinical models. B7-H3 has emerged as a promising immunotherapeutic for pediatric solid tumors including sarcoma, and we and other investigators are actively exploring B7-H3-CAR T cell therapies. The goal of this project was to adapt immunocompetent sarcoma models for the realistic pre-clinical evaluation of B7-H3-CAR T cells. **Methods/Results:** We took advantage of sarcoma (rhabdomyosarcoma: KMR19, KMR72; osteosarcoma: F420, F331, F664, Myc9, Myc29) cell lines that were derived from genetically engineered mouse models with relevant human oncogenic mutations including $p53^{+/-}$, Myc^{T58A}, or $KRas^{G12D}$. All seven cell lines expressed B7-H3 as judged by flow cytometric analysis. We generated murine B7-H3-CAR T cells expressing a 2nd generation B7-H3-CD28.ζ CAR by retroviral transduction. B7-H3-CAR T cells recognized all B7-H3-positive sarcoma cell lines as judged by IFNγ production in contrast to B7-H3-negative target cells. B7-H3-CAR T cells also killed B7-H3-posistive sarcoma cell lines. In contrast, CAR T cells recognizing an irrelevant antigen (SP6) did not recognize or kill B7-H3-posistive target cells confirming specificity. Having established that B7-H3-CAR T cells recognize and kill our murine sarcoma cell lines, we selected three cell lines (KMR19, Myc29, F420) for further evaluation based on their *in vivo* growth characteristics. Optimal lymphodepletion was achieved at a dose of 200mg/kg cyclophosphamide without apparent toxicity. In a pilot *in vivo* experiment in the F420 model we could demonstrate that B7-H3-CAR T cells, derived from GFP-ffluc transgenic mice, post lymphodepletion i) expanded *in vivo*, ii) homed to tumor sites, and iii) had significant, albeit transient antitumor activity. Additional *in vivo* studies including analysis of tumor infiltrating immune cells are currently in progress. **Conclusion**: We adapted immune competent rhabdomyosarcoma and osteosarcoma models to evaluate the effector function of B7-H3-CAR T cells. These models should enhance our knowledge of the interplay between sarcoma biology, the host immune system, and CAR T cells, enabling us not only to model but also to improve current CAR T-cell therapy approaches for pediatric sarcoma.

675. Ribozyme-Mediated RNA Replacement with Post-Transcriptional Regulation for Hepatocellular Carcinoma

Seung Ryul Han[1], Chang Ho Lee[2], Ji Hyun Kim[1], Eun Yi Cho[1], Mi Ha Ju[3], Jin Sook Jeong[3], Seong-Wook Lee[1,2]

[1]Rznomics Inc., Seongnam, Korea, Republic of,[2]Integrated Life Science, Dankook University, Yongin, Korea, Republic of,[3]Dong-A University College of Medicine, Busan, Korea, Republic of

Hepatocellular carcinoma (HCC) has high fatality rate and limited therapeutic options. In this study, we propose a new anti-HCC strategy with high cancer-selectivity and efficient anticancer efficacy, based on post-transcriptionally regulated cancer-specific RNA replacement. Adenovirus-mediated *Tetrahymena* group I-based *trans*-splicing ribozymes were developed, specifically inducing suicide gene activity through HCC-specific replacement of telomerase reverse transcriptase (TERT) RNA. To confer potent anti-HCC effects and minimize hepatotoxicity, we constructed post-transcriptionally enhanced ribozyme expression cassettes harnessed with splicing donor and acceptor site and woodchuck hepatitis virus post-transcriptional regulatory element under the control of microRNA-122a, the expression of which is down-regulated in most HCC. Adenovirus encoding the post-transcriptionally enhanced ribozyme improved both *trans*-splicing reaction and inhibition of hTERT RNA level, efficiently and selectively regressing hTERT-positive liver cancers. Adenovirus encoding the microRNA-regulated ribozyme induced selective liver cancer cytotoxicity, the efficiency of which depended on ribozyme expression level relative to the microRNA level. Systemic inoculation of adenovirus encoding the post-transcriptionally enhanced and microRNA-regulated ribozyme caused efficient anti-HCC effects at a single dose of low titers and least hepatotoxicity in intrahepatic xenografted murine model of multifocal HCC. Minimal liver toxicity, tissue distribution and clearance pattern of the recombinant adenovirus were observed in normal animals administered with the virus either systemically or through the hepatic artery. Post-transcriptionally enhanced and microRNA-regulated RNA replacement approach mediated by a cancer-specific ribozyme provides a clinically relevant, safe, and efficient strategy for HCC treatment.

676. Intravenous Cytotoxic Human Induced Neural Stem Cells for the Treatment of Primary Lung and Breast Tumors

Alison Mercer-Smith[1], Wulin Jiang[1], Juli Bago[2], Alain Valdivia[1], Alex Woodell[1], Stephanie Montgomery[3], Kevin Sheets[1], Carey Anders[4], Shawn Hingtgen[1]

[1]Pharmaceutical Sciences, University of North Carolina -- Chapel Hill, Chapel Hill, NC,[2]Hemato-Oncology, University of Ostrava, Ostrava, Czech Republic,[3]Pathology and Laboratory Medicine, University of North Carolina -- Chapel Hill, Chapel Hill, NC,[4]Medicine, Duke University, Durham, NC

Introduction: Human fibroblasts when transdifferentiated into human induced neural stem cells (hiNSCs) exhibit innate tumor tropism. The efficacy of hiNSCs as personalized anti-cancer drug delivery vehicles has been demonstrated in preclinical studies of intracranial tumors, but the potential to treat extracranial, primary tumors remains relatively unexplored. Herein, we investigate the tumor-homing potential, therapeutic efficacy, and safety and toxicity of intravenously infused hiNSCs against two of the most lethal primary tumor types, triple negative breast cancer (TNBC) and non-small cell lung cancer (NSCLC). **Methods:** To study the migration of hiNSCs *in vitro*, we performed time-lapse motion analysis on hiNSCs co-cultured with TNBC and NSCLC. We tested migration *in vivo* by establishing orthotopic human TNBC or NSCLC tumors and intravenously infusing hiNSCs. Post-mortem tissue analysis was used to determine the location and persistence of hiNSCs. The therapeutic potential of these engineered cells was assessed by creating a line of hiNSCs that secreted the cytotoxic protein TRAIL and co-culturing these hiNSC-TRAIL cells with NSCLC and TNBC. Therapeutic efficacy was tested *in vivo* by intravenously infusing hiNSC-TRAIL following establishment of orthotopic TNBC or NSCLC tumors. The safety and toxicity of hiNSCs with and without TRAIL was investigated by following hematology, blood chemistry, and histology for 28 days after mice had received a maximum tolerated dose of either hiNSC or hiNSC-TRAIL. **Results/Conclusion:** Time-lapse motion analysis showed hiNSCs migrated directionally to both human NSCLC and human TNBC in co-culture assays. Kinetic tracking with post-mortem tissue analysis confirmed that intravenously infused hiNSCs co-localized with established orthotopic NSCLC or TNBC tumors within 3 days and persisted through 14 days. Real-time imaging showed hiNSCs secreting the cytotoxic agent TRAIL induced rapid killing of NSCLC and TNBC cells in co-cultures in time- and dose-dependent manners. Using orthotopic tumor models, intravenous hiNSC-TRAIL therapy reduced human NSCLC and TNBC tumor volumes 70% and 80%, respectively. For mice with NSCLC tumors, we observed a modest trend toward improvement in overall median survival (39 days with 95% CI 31 - 46 days for mice treated with hiNSC-TRAIL; 31.5 days with 95% CI 25 - 58 days for control mice). For mice with TNBC tumors, 60% of control mice and 100% of mice treated with hiNSC-TRAIL were still alive at the end of the study.Neither hiNSCs nor hiNSC-TRAIL cells induced significant changes in multiple safety parameters through 28 days post-infusion. These results provide the first evidence that intravenous hiNSC therapy safely and effectively seeks out and kills peripheral tumors, suggesting a potential new strategy for treating aggressive peripheral cancers through personalized cell therapy.

677. Accurate and Sensitive Gene Editing Quantification Using Single-Cell DNA Sequencing

Saurabh Gulati, Shu Wang, Daniel Mendoza, Jacqueline Marin, Kate Thompson, Khushali Patel, Benjamin Schroeder, Manimozhi Manivannan, Kelly Kaihara

Mission Bio, South San Francisco, CA

Cell and gene therapies aim to treat and prevent diseases, including cancer and inherited diseases, by altering the treatment landscape of intractable genetic disorders. For both ex-vivo and in-vivo genome editing approaches, the biggest challenges are editing efficiency and precision. Successful clinical implementation of genome editing products requires precise assessment and reliable quality control at single-cell level. High-throughput single-cell DNA targeted sequencing on the Tapestri Platform enables comprehensive profiling of gene perturbation in thousands of cells, meanwhile provides several advantages over other

currently used techniques which require clonal outgrowth followed by qPCR to assess DNA integrations by transduction, transfection or genome editing nuclease mediated site-specific modification. Multiple targeted sequencing panels were designed to amplify on-target and putative off-target genome editing sites, and to enable detection of translocation in targeted genes. These panels were synthesized and processed through Tapestri single cell DNA platform. Single-cell sequencing was performed on 2-5 replicates for each tested sample. After pre-processing the reads, cells were called using performing amplicons. We developed a workflow for variant calling which works robustly with the various possible outcomes of a gene perturbation. Our workflow is able to identify variants from single nucleotide polymorphisms (SNPs) to large insertions, deletions and also chromosomal translocation events. Custom modules were used to genotype genomic aberrations. Using this workflow we are able to identify and quantify the effects of gene editing experiments. In transduction efficiency study using single-cell DNA targeted sequencing assay, serial dilution experiments showcased the ability to detect and quantify transduced vs. non-transduced cells. The assay demonstrated good reproducibility, low false positive rate (< 0.03%), and dilution series thereafter showed excellent linearity and precision among replicates between the expected and observed transduction percentages. In nuclease mediated site-specific editing study we were able to quantify the percentage of cells which were successfully edited, the zygosity of the edit and also the nature of edit (insertion v/s deletion). Additionally we also quantified the percentage of off-target edits as well as the correlations between on-target edits and off-target edits. Furthermore we classified chromosomal translocation events that occurred due to targeted gene editing. Without the need for any lengthy clonal outgrowth cell culture, single-cell sequencing technology offers an unprecedented level of quantification of DNA integrations in significantly less time compared to bulk approaches and does so at single cell resolution.

678. FDA Authorized Treatment Protocol Using DeltaRex-G with Trastuzumab and Letrozole as First Line Therapy for Early Stage Triple Receptor Positive Breast Carcinoma

Sant P. Chawla[1], Don A. Brigham[1], Frederick L. Hall[2], Erlinda Maria Gordon[1]

[1]Oncology, Cancer Center of Southern California, Santa Monica, CA,[2]Oncology, Delta Next-Gene, LLC, Santa Monica, CA

Background and Rationale: DeltaRex-G is a tumor-targeted, amphotropic, MLV-based retrovector that (a) displays a *Signature (SIG)*-binding peptide on its surface for targeting the tumor microenvironment, and (b) encodes a CCNG1 inhibitor gene for eradicating cancer cells including microscopic disease. When injected intravenously, the DeltaRex-G nanoparticles seek out and accumulate in cancerous lesions where *Signature (SIG)* proteins are abnormally found, in the vicinity of cancer cells, hence augmenting effective drug concentration. DeltaRex-G is currently available via an FDA approved Expanded Access Program for Stage 4 cancers (IND# 19130). In a Phase 1/2 study using DeltaRex-G for chemotherapy-resistant Stage 4 breast cancer, DeltaRex-G induced 17.6% response rate by PET/ Choi criteria, 76% tumor control rate and 83% one-year survival rate, with minimal, if any, systemic toxicity, and with 2 patients still alive 10 years after DeltaRex-G treatment initiation. These data suggest that (1) DeltaRex-G is uniquely safe and exhibits antitumor activity, (2) PET/ Choi are more sensitive indicators of early tumor response to DeltaRex-G and should be used to evaluate efficacy, (3) DeltaRex-G induced >10- year survival in 2 patients with pure bone metastases, (4) DeltaRex-G may prove to be a biochemical and/or antigen modulator when combined with other cancer therapy/immunotherapy, and (5) DeltaRex-G is a viable alternative treatment for patients who choose not to receive standard toxic chemotherapy that may predispose them to secondary malignancies, debilitating peripheral neuropathy and other untoward side effects that impair quality of life while undergoing treatment for breast cancer. Guiding hypothesis: DeltaRex-G will eradicate microscopic disease and prevent recurrence in patients with early stage invasive breast carcinoma. **Objectives:** To evaluate the efficacy and safety of DeltaRex-G in preventing recurrence in early stage triple receptor positive invasive breast carcinoma **Patient and Methods:** A written informed consent will be obtained prior to treatment. Clinical status, hematologic and organ function, EKG, ECHO, will be assessed at baseline and as needed through the course of treatment. **Route of Administration:** The patient will receive DeltaRex-G intravenously (1.2-3-6 x 10e11 cfu/dose) three times a week in combination with trastuzumab i.v. weekly and letrozole po daily. **Safety analysis:** All serious DeltaRex-G therapy related or unexpected adverse events will be reported to the FDA and the IRB within 7 days of incident. All other adverse events will be reported to the FDA and IRB in annual report format and in the final study report. All Grade III or IV toxicities will be reported in the clinical study summary report. In the event of death, an autopsy report will be submitted if an examination was conducted. **Efficacy analysis:** The only treatment outcome parameter is duration of disease-free survival. Molecular residual disease (MRD) using Signatera ctDNA and tumor markers will be obtained at regular intervals for detection of early recurrence and correlation with overt recurrence/metastasis. **Results and Conclusion**: A patient with ER+ PR+ and HER2 amplified Stage 1b invasive breast carcinoma, has received 12 dose infusions of DeltaRex-G, 4 doses of trastuzumab and daily doses of letrozole with no treatment related adverse reactions and no evidence of recurrence since diagnosis. A clinical progress update will be presented. A Phase 2/3 clinical trial is planned to evaluate if DeltaRex-G is equally effective (not-inferior-to) than standard chemotherapy/radiation therapy as first-line therapy for early-stage hormone receptor positive and HER2/ neu amplified breast cancer.

679. CoupledCAR® Technology Strengthens Adoptive T Cell Therapy by Promoting Rapid Expansion

Zhiyuan Cao, Chengfei Pu, Xianyang Jiang, Xiaogang Shen, Ruihong Zhu, Yuzhe Peng, Xi Huang, Zhao Wu, Victor Lu, Lei Xiao

Innovative Cellular Therapeutics, Shanghai, China

CAR T therapy has achieved remarkable results in the treatment of hematological tumors such as leukemia, lymphoma, and multiple myeloma. However, there remains challenges in treating solid tumors. These challenges include physical barriers, tumor microenvironment

immunosuppression, tumor heterogeneity and target specificity. Especially, due to tumor microenvironmental barriers, CAR T cells are not effectively exposed to tumor antigens and cannot activate co-stimulation signals on CAR molecules, thus conventional CAR T cell therapy has thus far shown weak cell expansion in solid tumor patients, achieved little or no therapeutic responses. Here, we developed CAR T cells based on a novel CoupledCAR® technology to overcome the lack of persistence of solid tumor CAR T cells *in vivo*. We used prostatic acid phosphatase (PAP) as an exemplary CAR target for prostate cancer and demonstrated that our CoupledCAR® significantly enhanced the expansion of PAP CAR T cells *in vitro* and *in vivo*. Further, we observed that this expansion showed more memory-like phenotypes, and caused little exhaustion of PAP CAR T cells. Also, we find coupled solid tumor CAR T cells have stronger tumor killing ability. We demonstrated this simple expansion to enable the persistence of solid tumor CAR T cells and can be further applied to other kinds of T cell therapy like TCR T and TILs. We developed a novel platform technology (CoupledCAR®) that allows solid tumor CAR T cells to rapidly expand. This initial CAR T cell expansion enabled enhanced trafficking and infiltration of the tumor tissue whereby further cell expansion occurred and thereby achieved tumor clearance. We have carried clinical trials and obtained early promising clinical data. We will further verify the safety and efficacy of this technology in the treatment of different kinds of solid tumors in the clinic research.

680. CoupledCAR™ Technology for Treating Thyroid Cancer

Xingchen Liu[1], Keshu Zhou[1], Yu Liu[2], Yong Huang[2], Chengfei Pu[3], Zhiyuan Cao[3], Ruihong Zhu[3], Haiyang Tang[3], Zhipeng Huang[3], Hang Yang[3], Xi Huang[3], Yongping Song[1], Renbin Liu[2], Zhao Wu[3], Victor Lu[3], Lei Xiao[3]

[1]Zhengzhou University, Zhengzhou, China,[2]Sun Yat-Sen University, Guangzhou, China,[3]Innovative Cellular Therapeutics, Shanghai, China

Chimeric antigen receptor modified T cells (CAR T) have demonstrated remarkable clinical efficacy in the treatment of B cell malignancies and multiple myeloma. Significant challenges restrict their application across solid tumors due to multiple obstacles, including the lack of robust *in vivo* CAR-T cell expansion and persistence, the immunosuppressive tumor microenvironment.To address these difficulties, we generated CAR T cells using a novel CoupledCAR® technology. Specifically, we engineered CoupledCAR T cells with lentiviral vectors encoding an anti-thyroid stimulating hormone receptor (TSHR) CAR molecule. Immunohistochemistry (IHC) results showed that TSHR was highly expressed in thyroid cancer cells making it an ideal tumor-specific target antigen. *In vitro* co-culture experiments showed that TSHR CAR T cells specifically recognized and subsequently killed TSHR-positive tumor cells. Animal model experiments showed that TSHR CAR T cells inhibited the proliferation of TSHR-positive tumor cells. To evaluate the clinical safety and efficacy of anti-TSHR CoupledCAR T cells on refractory or relapsed thyroid cancer, we treated refractory/relapsed post-thyroidectomy thyroid cancer patients according to an IRB approved protocol. We treated two patients using anti-TSHR CoupledCAR T cells and observed the rapid expansion of CAR T cells and enhanced the killing of tumor cells. One patient's best response was complete remission, and the other was near complete remission.Patient 1 Male, 64Y, Papillary Thyroid Carcinoma. In May 2017, Thyroid cancer was diagnosed, bilateral total thyroidectomy, and right cervical lymph node functional dissection were performed in Jun 2018, followed by iodine 131 isotope therapy. In December 2018, bilateral multiple cervical lymph nodes were enlarged, especially on the right side. In February 2019, right neck lymphadenectomy was performed.Patient 2 Female, 60Y, Thyroid Carcinoma. In Aug 2013, a "double lobectomy of the thyroid gland" was performed. From Oct 2013 to Jan 2014, she received iodine 131 isotope therapy. In Sep 2014, she was diagnosed with iodine - resistant thyroid cancer. From Sep to Jan 2016, 5 cycles of chemotherapy were performed. In Jun 2016, she enrolled in the Anlotinib experimental group. In Mar 2019, multiple metastases in both lungs and multiple enlarged lymph nodes in the mediastinum were observed.Patient 1: One month after infusion (M1), the patient was evaluated as PR. Three months after infusion (M3), the patient was evaluated as CR, and the patient's CR lasted from M3 to M12 after infused anti-TSHR CoupledCAR T cells , and we are still following up.Patient 2: M1, the patient was evaluated as PR (Partial Response): the tumor volume in the right lower lobe of the lung was reduced by approximately 67.51% (decreased from 65*55mm to 42*39mm). Three months after infusion (M3), compared with that before, the tumor volume was reduced by approximately 73.54% and SUV max value decreased from 14.9 to 2.8, therefore, the patient was evaluated as nCR (near complete remission).We show that TSHR is a good target for treating thyroid cancer, and our anti-TSHR CoupledCAR T cells are safe and effective for treating thyroid cancer. Recruitment is ongoing to evaluate the safety and efficacy of our CoupledCAR T cells. Further, since our CoupledCAR® technology is a platform technology, we are developing it to treat other solid tumors using different target tumor markers.

681. A TCR-Like CAR Recruiting Endogenous CD3 Proves Efficacious Tumor Antigen Recognition via Retroviral, IVT-RNA or CRISPR/Cas9 Expression Systems in Human T-Cells and Perceives Costimulatory Signals in Cis

Ralf-Holger Voss[1], Matthias Birtel[2,3], Matthias Theobald[4,5], Petra Oehm[2], Ugur Sahin[1,2,3]

[1]Research Center for Immunotherapy (FZI), University Medical Center (UMC), Mainz, Germany,[2]BioNTech AG, Mainz, Germany,[3]TRON-Translational Oncology gGmbH, Mainz, Germany,[4]Dpt. Hematology/Oncology & Pneumology, University Medical Center (UMC), Mainz, Germany,[5]University Cancer Center (UCT), Mainz, Germany

The concept of chimeric antigen receptor (CAR) design in T-cell based immunotherapy achieved fundamental successes the last decade in the treatment of hematological neoplasia. 1st Generation CARs comprise a polypeptide chain carrying the VH and VL domains of a tumor-reactive antibody and CD3ζ in a single chain format and of note, are C-terminally extended by domains of costimulatory receptors in 2nd Gen. CARs. This one-dimensional arrangement homodimerizes and accomplishes signal 1 and signal 2 of the T-cell signaling machinery towards tumors generally lacking costimulatory ligands. However, scientific literature claimed the importance of all CD3 subunits

for efficient and comprehensive T-cell activation. Thus, we suggested a novel CAR that brings about association with all endogenous CD3 components: A common feature of our TCR-like CARs was the dual fusion of antibody V-domains to the constant TCR Cα/Cβ domains. The latter binds to CD3ζ2, CD3δε (binds to Cα) and CD3γε (Cβ) for proximal T cell signal initiation. The tandem order of identical V-domains in our lead candidate (ie VH-VH-HuCα + VL-VL-HuCβ) provided both, sufficient avidity (i.e. bivalency of antigen recognition) and interchain affinity, which is key to create a stably expressed CAR/CD3-complex. As model tumor antigen, we used the tight-junction protein Claudin 6 (CLDN6). These by either retroviral transduction or IVT-RNA electroporation reprogrammed CAR T-cells revealed exceptional features in proliferation (CFSE), cytokine secretion (IFNγ ELISA), and cytotoxicity (Luciferase-, spheroid-, impedance-based assays) against antigen-titrated immunostimulatory DCs. However, proliferation against CLDN6+ tumor cell lines (t.c.l.s) was less marked. A recently published approach relies on a CAR vaccination strategy that supplies costimulation in trans by introducing tumor antigen RNA-formulated liposomes into DCs located in secondary lymphoid organs. Complementary to such an approach, costimulation in cis can support T cells infiltrating the immunosuppressive tumor microenvironment (TME). However, direct fusion of signaling domains of CD28 or 4-1BB to TCR-like CAR C-domains was prohibitive presumably due to sterical hindrance with CD3. Then, we tested a chimeric costimulatory adaptor (CCA), which incorporates CD28 and 41BB into e.g. the cytosolic sequences of CD3ζ. Along with the TCR-like CAR, it may be co-recruited to the aggregate immunological synapse (IS) upon antigen recognition. Alternatively, we tested chimeric costimulatory receptors (CCR) which in addition to CCAs carry a target cell-specific scFv to facilitate recruitment into IS. We observed better function and viability of these CAR T-cells costimulated in cis. Since Cα/Cβ composite CARs have to compete with endogenous TCRs for the limiting CD3 complex, T-cells were genomically edited by CRISPR/Cas9 utilizing IVT-RNA of Cas9 and synthetic gRNAs targeting the Hu TRAC locus. CARs were then introduced by either IVT-RNA or CRISPR/Cas9-driven homology directed repair (HDR) by coelectroporating a CAR encoding DNA template. This intervention further improved expression and reactivity against CLDN6+ t.c.l.s. In summary, we suggest TCR-like CAR T-cells which may inhere antigen sensitivity of a high-affinity TCR due to physiological T-cell signaling and which may resist activation induced cell-death for better long term persistence in vivo by coexpressing chimeric costimulatory molecules.

682. PeptiCHIP: A Novel Microfluidic-Based Chip Platform for Tumour Antigen Landscape Identification

Sara Feola[1], Markus Haapala[1], Karita Peltonen[1], Cristian Capasso[1], Beatriz Martins[1], Gabriella Antignani[1], Antonio Federico[2], Vilja Pietiäinen[3], Jacopo Chiaro[1], Michaela Feodoroff[3], Antti Ranniko[4], Manlio Fusciello[1], Satu Koskela[5], Jukka Partanen[5], Firas Hamdan[1], Sari Tähkä[1], Erkko Ylösmäki[1], Dario Greco[2], Mikaela Grönholm[1], Tuija Kekarainen[6], Masoumeh Eshaghi[6], Olga L. Gurvich[6], Seppo Ylä-Herttuala[7], Rui M M Branca[8], Janne Lehtiö[8], Tiina Sikanen[1], Vincenzo Cerullo[1]

[1]University of Helsinki, Helsinki, Finland,[2]University of Tampere, Tampere, Finland,[3]University of Helsinki and Institute for Molecular Medicine Finland, FIMM, Helsinki, Finland,[4]Department of Urology and Research Program in Systems Oncology, Helsinki, Finland,[5]Research & Development Finnish Red Cross Blood Service, Helsinki, Finland,[6]Kuopio Center for Gene and Cell Therapy, Kuopio, Finland,[7]A.I. Virtanen Institute, University of Eastern Finland, Kuopio, Finland,[8]Karolinska Institutet, Solna, Sweden

Identification of HLA class I ligands from the tumour surface (ligandome or immunopeptidome) is essential for designing T-cell mediated cancer therapeutic approaches. However, the sensitivity of the process for isolating MHC-I restricted tumor-specific peptides has been the major limiting factor for reliable tumor antigen characterization, making clear the need for technical improvement.Here, we describe our work from the fabrication and development of a novel microfluidic-based chip (PeptiCHIP) and to its use to identify and characterize tumor-speficic ligands on clinically relevant human organoids. Specifically, we assessed the potential of immobilizing a pan-HLA antibody on solid surfaces via well-characterized streptavidin-biotin chemistry, overcoming the limitations of the cross-linking chemistry used to prepare the affinity matrix with the desired antibodies in the immunopeptidomics workflow. Furthermore, to address the restrictions related to the handling and the limited availability of tumour samples, we further developed the concept towards the implementation of a microfluidic through-flow system.Thus, the biotinylated pan-HLA antibody was immobilized on streptavidin-functionalized surfaces, and immune-affinity purification (IP) was carried out on customized microfluidic pillar arrays made of thiol-ene polymer. Compared to the standard methods reported in the field, our methodology drastically reduces the handling, the amount of antibody and the time required for peptide isolation.In this work, we carefully examined the specificity and robustness of our customized technology for immunopeptidomics workflows. We tested this novel platform by immunopurifying HLA-I complexes from as few as 10^6 cells both in a widely studied B-cell line and in patients-derived *ex vivo* cell cultures. After the final elution in mild acid, HLA-I-presented peptides were identified by tandem mass spectrometry and further investigated by *in vitro* methods. These results highlight the potential to exploit microfluidics-based strategies in immunopeptidomics platforms and in personalized immunopeptidome analysis from cells isolated from individual tumour biopsies to design tailored cancer therapeutic vaccines.

683. Tumor Macrophage-Mediated Cytokine Gene Delivery Improves Trafficking and Anti-Tumor Activity of CAR T Cells against Glioblastoma

Federico Rossari[1,2], Melania Cusimano[1], Filippo Birocchi[1], Anna Ranghetti[1], Giorgio Orofino[2], Lucia Sergi Sergi[1], Monica Soldi[1], Stefano Colombo[1], Barbara Costa[3], Peter Angel[3], Nadia Coltella[1], Luigi Naldini[1,2]

[1]Targeted Cancer Gene Therapy, San Raffaele-Telethon Institute for Gene Therapy (SR-Tiget), Milano, Italy,[2]Vita-Salute San Raffaele University, Milano, Italy,[3]German Cancer Research Center (DKFZ), Heidelberg, Germany

Introduction

Despite the identification of promising target antigens, the biological and physical features of the tumor microenvironment (TME) have substantially limited both trafficking and killing capability of chimeric antigen receptor (CAR) T cells for the treatment of solid tumors. The reprogramming of the immunosuppressive TME to enable both endogenous and adoptive immune cells with effective anti-tumor activity is therefore a shared goal in the field. In this regard, we previously exploited a cell and gene therapy approach to deliver interferon (IFN)-α in the TME of preclinical tumor models through TIE2-expressing tumor-associated macrophages (TEM). TEM-dependent IFN-α delivery exerts direct and immune-mediated anti-tumor effects, including increased recruitment and reduced exhaustion of T cells. Since both TEM-based gene therapy and CAR T cells are currently undergoing clinical trials for glioblastoma multiforme (GBM), we reasoned that a combination approach could enhance CAR T-cell specific trafficking and unleash their anti-tumor properties even in the non-permissive GBM TME.

Methods

We modified a new murine GBM cell line named mGB2, closely recapitulating the molecular and morphological features of the human disease, to express the truncated murine CD19 for proof-of-principle studies. C57BL/6 mice were transplanted with lentiviral vector (LV)-transduced hematopoietic stem cells (*Tie2-Ifna* or control). After hematopoietic reconstitution, mGB2 cells were orthotopically injected into the striatum of mice. A LV-based protocol was optimized to efficiently generate syngeneic anti-CD19 CAR T cells. Both IFN-α and control mice received either CAR or untransduced T cells intravenously. Tumor growth was monitored by magnetic resonance imaging, and immunophenotypic analysis was performed on peripheral blood and tumor infiltrate at different timepoints. We then screened a panel of putative human/mouse cross reactive GBM target antigens and identified disialoganglioside GD2 to be expressed by mGB2 cells. A GD2-redirected murine CAR was generated and delivered to syngeneic T cells for *in vivo* evaluation as above.

Results

Combination therapy-treated mice showed longer persistence and enhanced activation of circulating anti-CD19 CAR T cells, mirrored by an increased intratumor localization and decreased expression of exhaustion markers. These changes accounted for reduced GBM growth and prolonged survival over single-treatment and control groups. Importantly, a fraction of mice eradicated the tumor and resisted rechallenge with the parental CD19-negative GBM cell line, suggesting induction of protective immunity against tumor-associated antigens other than CD19. CAR T cells were even detectable 226 days after adoptive transfer in both peripheral blood and spleen of one long-term surviving mouse treated with IFN-α gene therapy. Similar inhibition of tumor growth was observed in mice treated with the combination of GD2-redirected CAR T cells and IFN-α gene therapy over single-treatment and control ones.

Conclusions

TEM-mediated IFN-α gene therapy reprograms the TME to overcome some key limitations of systemically administered CAR T cells against GBM. If safely and effectively translated into the clinic, this approach may rescue CAR T-cell therapies that have conferred poor clinical benefits, so far, and limit antigen escape given the pleiotropic function of local IFN-α.

684. BDP1 Variants Associate with Clinical Outcomes of Neuroblastoma Patients

Lan Sun[1,2], Xiaoqing Li[2], Lingli Tu[1], Andres Stucky[1], Jiang F Zhong[1]

[1]Division of Periodontology, Diagnostic Sciences;, University of Southern California, Los angeles, CA,[2]Department of Oncology, the People's Hospital of Bishan District, Chongqing, China

Neuroblastoma (NB) is the most common tumor in children. The gene BDP1 (B Double Prime 1) may play a role in NB. We analyzed BDP1 mutations in a NB population with 121 patients from the Cancer Genome Atlas database by RNA sequencing, and identified two variants of BDP1 associated with NB, located at chr5:71511131 and chr5:71510884. The prevalence of BDP1 variants, I1264M and V1347M, were 52.9% (64/121) and 45.5% (55/121), respectively. Kaplan-Meier estimates showed a significant difference between subgroups with or without BDP1 variants ($p<0.05$). Multivariate analysis further revealed BDP1 variants were independent prognostic variables in NB ($p<0.05$). Therefore BDP1 variants may associated with significantly improved clinical outcomes in NB.

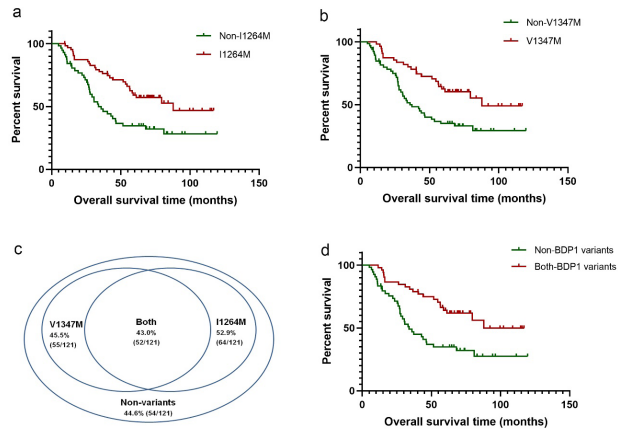


Table 2 Multivariate Cox hazard analysis of risk factors for prognosis.

Characteristics	HR	95% CI	*P* value
Ploidy			0.013
Hyperdiploid	0.526	0.317-0.872	
Diploid	1.0 (Ref)		
MYCN status			0.963
amplified	0.986	0.531-1.829	
non-amplified	1.0 (Ref)		
Age at diagnosis			0.041
≥18 months	20.692	1.13-380.07	
< 18 months	1.0 (Ref)		
Stage			0.019
Not 4	0.148	0.03-0.729	
4	1.0 (Ref)		
COG Risk			0.219
High risk	6.824	0.319-145.76	
Not high risk	1.0 (Ref)		
BDP1			0.035
I1264M	0.580	0.349-0.964	
Non-I1264M	1.0 (Ref)		
BDP1			0.007
V1347M	0.488	0.289-0.826	
Non-V1347M	1.0 (Ref)		

Abbreviations: HR, Hazard Ratio; CI, confidence interval

685. Developing the Quantitative Cell-Based Bioassays for the Discovery and Development of T Cell-Based Therapies

Kara Machleidt[1], Jamison Grailer[2], Julia Gilden[2], Michael Slater[2], Pete Stecha[2], Jim Hartnett[2], Dan Lazar[2], Frank Fan[2], Mei Cong[2], Zhijie Jey Cheng[2]

[1]Promega Corporation, Madison, WI,[2]R&D, Promega Corporation, Madison, WI

T cell-based therapies represent a new paradigm for cancer treatment. There are two main approaches for T cell-based therapies which include molecular T cell redirection by CD3 bispecific molecules such as bispecific T-cell engagers (BiTE) and cellular T cell redirection by genetic modification of T cells with chimeric antigen receptors (CAR) or transgenic T cell receptors (TCR). Molecular redirected T Cell therapy can be induced by CD3 bispecific molecules. Historically, potency determination for CD3 bispecific molecules rely on primary CD8 T cells and measure T cell activation or cytotoxicity through redirected T cell cytotoxicity (RTCC) or cytokine release. However, these methods suffer from lengthy and complex protocols and high assay variability due to the use of primary cells. To overcome those issues, here we report the development of bioluminescent RTCC assay and cytokine immunoassay that can quantitatively measure the potency for CD3 bispecific molecules. In RTCC assay, Thaw-and-Use cytotoxic T cells and target cells stably expressing HaloTag-HiBiT are incubated with a bispecific molecule, resulting in lysis of the target cells and release of HiBiT proteins, which then bind to LgBiT in the detection reagent and form functional Nano-luciferase to generate luminescence. The bispecific antibody-induced cytokine production e.g IL-2 and IFN-γ from the cytotoxic T cells can also be quantitatively measured in the new homogenous NanoBiT Immunoassays. Both HiBiT RTCC assay and LUNIT cytokine immunoassay are homogenous, highly sensitive, and have robust assay windows. For cellular T cell therapy, CAR-T has demonstrated promising results in treating leukemia, while the development of TCR-engineered T cells which can recognize intracellular tumor antigens, is still in very early development. To facilitate the screening and characterization of new transgenic TCRs, we developed three TCRαβ-null reporter T cell lines, CD4+, CD8+ or CD4+/CD8+ double positive. A TCRαβ-null reporter T cell line was first developed by knocking out the endogenous TCR α and β chains in a CD4+ reporter T cell line using CRISPR/Cas9 and the successful knockout is confirmed by phenotypic assays and TCR v chain locus sequencing. A CD4+/CD8+ double positive TCRαβ-null reporter T cell line was generated by exogenous engineering of CD8, and the third CD8+ TCRαβ-null reporter T cell line was generated next by knocking out the endogenous CD4 and exogenous engineering of CD8. We demonstrated that that re-introduction of a peptide-specific TCR α and β chains into TCRαβ-KO reporter T cell lines results in TCR activation and luciferase reporter expression induced by the peptide presented by MHC molecules. The select expression of CD4 or CD8 variants in the TCRαβ-null reporter T cell line could enable the development of transgenic TCRs for both MHCI- and MHCII-restricted tumor antigen targets. Together, these bioluminescent assays represent a new set of tools for the discovery and development of T cell-based immunotherapies.

686. Antibody Displaying Extracellular Vesicles (Fc-EVs) for Delivery of Tumor Targeted Therapeutics

Oscar P.b. Wiklander[1], Rim Jawad[1], Doste R. Mamand[1], Heena Sharma[2], Xiuming Liang[1], Dhanu Gupta[1], Jeremy Bost[1], Antje M. Zickler[1], Wenyi Zheng[1], Joel Z. Nordin[1], André Görgens[1], Samir EL Andaloussi[1]

[1]Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden,[2]Evox Therapeutics Limited, Oxford, United Kingdom

Extracellular vesicles (EVs) can be engineered to display various targeting and therapeutic moieties. Their ability to act as a natural vector to shuttle cargo over biological barriers offers a unique platform for the development of a new class of therapeutics. Here, we engineered EVs to express an Fc-binding moiety (Fc-EVs), so that antibodies (Ab) can be displayed on the surface of the vesicles. We have evaluated the capacity of these Fc-EVs to bind Ab by immuno-electron microscopy, cellular uptake of labelled antibodies/EVs and flow cytometry analysis. As a proof of concept, these Fc-EV can be utilized for decorating EVs with cancer targeting antibodies. Conventional cancer therapies are mainly limited by 1) systemic side effects derived from off target toxicity on healthy tissue and 2) development of resistance. One type of antibody therapy that has been revolutionary in oncology is the use of check-point inhibitors. Immunotherapy (including checkpoint inhibitors) do however still have a sub-optimal response rate (<50%). Here, the aim is to combine EV's ability as a drug vehicle and their bioengineering potential by simultaneously delivering 2 different classes of drug at the same time: checkpoint inhibitors such as anti-PDL1 and conventional chemotherapeutic drugs such as doxorubicin. Displaying PDL1-Ab on Fc-EVs, loaded with cancer therapeutic drugs, should lead to an improved antitumoral effect of the drug and an increased release of neoantigens to stimulate the immune response and act synergistically with the immunotherapy. Our results show that Fc-EVs are taken up to a significantly higher degree by B16F10 (malignant melanoma) cells when PDL1-Ab is displayed on the surface of these EVs. Furthermore, the tumor accumulation of Fc-EVs increased more

than five times in B16F10-tumor bearing mice systemically injected with Fc-EVs, when the Fc-EVs were guided by PDL1-Ab. In addition, B16F10-tumor bearing mice, systemically injected with Fc-EVs loaded with doxorubicin (dox), displayed a significantly decreased tumor progression and increased survival. Following 4 cycles of treatment, the tumor volume of mice treated with dox-Fc-EV:PDL1-Ab was less than 1/3 of the tumor volume of mock treated mice. All ten melanoma mice were still alive at the endpoint (day 20) when treated with dox-Fc-EV:PDL1-Ab, which was the only treatment group with 100% survival. Mock treated mice had 40% survival at the endpoint and mice treated with Fc-EVs:PDL1-Ab without dox had a 60% survival. Overall, the Fc-EV platform offers the prospective of combining antibody and EV technology, with potential applications as a cancer therapy, as well as a plethora of other indications in need of targeted delivery of therapeutics.

687. Neoantigen-Cytokine-Chemokine Multifunctional Natural Killer Cell Engager for the Immunotherapy of Solid Tumors

Xue Yao[1], Sandro Matosevic[1,2]

[1]Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN,[2]Center for Cancer Research, West Lafayette, IN

The effectiveness of natural killer cell-based immunotherapy against solid tumors such as glioblastoma is limited by the lack of specific antigens and the immunosuppressive tumor microenvironment. To improve the clinical efficacy of NK cell therapy, we are developing a new generation of multi-specific killer engagers, which consists of a neoantigen-targeting moiety, together with cytokine and chemokine-producing domains. Neoantigens are new antigens formed specifically in tumor cells due to genome mutations, making them highly specific tools to target tumor cells. Our engager has been designed to target WT-1, a highly specific marker overexpressed in GBM and many other solid tumors. Incorporation of the cytokine IL-15 supports the maturation, persistence, and expansion in vivo, and favors the proliferation and survival of NK cells in the tumor microenvironment, thereby leading to their enhanced clinical efficacy. Additionally, our data indicated that the chemokine CXCL10 plays an important role in the infiltration of NK cells into GBM, however, GBM tumors produce low levels of this chemokine. Incorporation of a CXCL10-producing function into our engager further supports such NK cell trafficking by leading to increased levels of CXCL10 locally in the tumor microenvironment. In summary, we have generated a novel multifunctional NK cell engager, combining neoantigen-cytokine-chemokine elements fused to an activating domain-specific to NK cells, and have investigated its ability to support and enhance NK cell-mediated cytotoxicity against solid tumors in vitro and in vivo. We hypothesize that taking advantage of our multi-functional engager, NK cells will exhibit superior ex vivo expansion, infiltration, and antitumor activity in the treatment of GBM and other solid tumors.

Hematologic and Immunologic Diseases

688. Single-Cell Inference of CRISPR-Cas9-Mediated Antiviral Gene Editing in HIV-Infected Microglia

Mohamed S. Bouzidi[1,2], Zain Y. Dossani[1,2], Joe Hiatt[2,3], Hannah S. Sperber[1,4], Li Du[1,2], Kyle A. Raymond[1], Alexander Marson[2,3], Satish K. Pillai[1,2]

[1]Vitalant Research Institute, San Francisco, CA,[2]University of California, San Francisco, San Francisco, CA,[3]Gladstone Institutes, San Francisco, CA,[4]Freie Universität, Berlin, Germany

HIV gene therapy efforts have mainly focused on the CD4+ T cell compartment. However, myeloid cells harbor HIV provirus during antiretroviral therapy as well, contributing to chronic inflammation and HIV-associated neurocognitive disorder. Moreover, little is known about the actual potency of HIV gene therapy approaches, as existing data describing efficacy have been limited to gel visualization of excision products and DNA sequencing of bulk cell populations. In this study, we investigated the capacity of multiplexed CRISPR-Cas9 ribonucleoprotein complexes (Cas9-RNPs) to 1) excise HIV provirus, and 2) knockout key host dependency factors in microglia (brain-derived myeloid cells) to render them refractory to HIV infection. We evaluated the potency of these antiviral approaches using two methods: population sequencing, and single-cell DNA sequencing enabling precise measurements of allele targeting and genome editing at multiple loci within individual cells. Cas9-RNPs were produced *in vitro* by incubating purified *Streptococcus pyogenes* Cas9 protein and individual guide RNAs (gRNAs) at 37°C for 15 minutes. gRNAs targeted the HIV LTR promoter and the *CCR5* and *CXCR4* host genes encoding viral entry coreceptors. Cas9-RNPs were delivered via nucleofection into HC69.5 cells, a microglial cell line infected with a GFP reporter HIV construct. Flow cytometry was used to measure HIV LTR activity (as indicated by GFP expression), and genomic DNA was extracted, amplified, Sanger sequenced, and analyzed using Tracking of Indels by Decomposition (TIDE). Edited cells were additionally processed through the Mission Bio Tapestri platform to generate targeted single-cell DNA sequencing libraries, characterizing the full-length HIV genome and 50 HIV host dependency genes. Libraries were sequenced on an Illumina MiSeq instrument. Single-cell data were processed using an in-house bioinformatic pipeline developed in Python and CRISPResso2 software. The percentage of CRISPR-induced indels observed in population sequencing data in the *CCR5*, *CXCR4* and HIV LTR loci were 11.3%, 48.9%, and 74.8%, respectively, with an 89% decrease in the frequency of GFP-expressing cells reflecting high-efficiency knockout of HIV provirus. In single-cell data, the percentage of indels in *CCR5*, *CXCR4* and HIV LTR loci were 12.9%, 57.3%, and 74.6%, respectively. Co-editing of *CCR5* and *CXCR4* genes occurred in 8.5% of cells. Bi-allelic editing of the *CXCR4* locus occurred in 26% of cells, while bi-allelic editing of *CCR5* was only observed in 1% of cells (Figure 1).

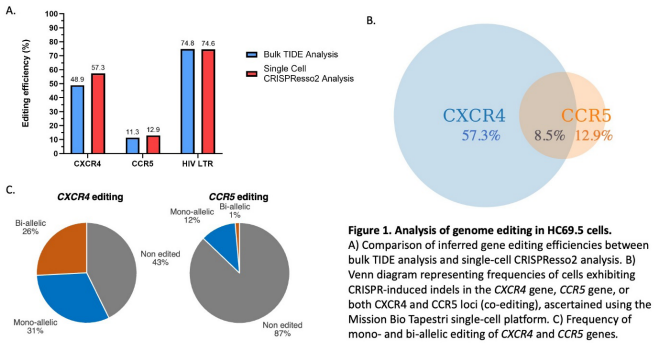


Figure 1. Analysis of genome editing in HC69.5 cells. A) Comparison of inferred gene editing efficiencies between bulk TIDE analysis and single-cell CRISPResso2 analysis. B) Venn diagram representing frequencies of cells exhibiting CRISPR-induced indels in the *CXCR4* gene, *CCR5* gene, or both CXCR4 and CCR5 loci (co-editing), ascertained using the Mission Bio Tapestri single-cell platform. C) Frequency of mono- and bi-allelic editing of *CXCR4* and *CCR5* genes.

Highly efficient, multiplexed antiviral gene editing in myeloid cells can be achieved using the Cas9-RNP system. Our results demonstrate that single-cell sequencing is critical to evaluate the efficacy and therapeutic potential of gene therapy-based HIV cure strategies. Editing efficiencies may be underestimated by population sequencing. Our data also demonstrate that the ratio of mono-allelic to bi-allelic editing varies considerably between targeted host dependency genes. This is of key importance, as knockout of both alleles will likely be needed to fully protect target cells from HIV infection.

689. Engineering of hFIX-Secreting Plasma Cells in Conjunction to a Transgenic BCR as a Novel Therapy for Protein Deficiencies

Tal Akriv, Alessio D. Nahmad, Daniel Nataf, Iris Dotan, Adi Barzel

Dept. of Biochemistry and Molecular Biology, Tel Aviv University, Tel Aviv, Israel

The ability of plasma cells to produce and secrete large amounts of protein has recently placed them as candidates for novel therapies for congenital protein deficiencies. In such disorders, a vital protein is either missing or defective and therefore requires complementation from an exogenous source. The current standard-of-care for several protein deficiencies is a life-long enzyme replacement therapy (ERT), in which the missing protein is intravenously infused to the patients chronically and at a high cost. Plasma cells engineered to express the missing protein can therefore serve as long term 'protein factories', posing a cost-effective alternative to existing ERT. This idea has recently been tested using CRISPR/cas9-engineered plasma cells expressing human factor IX (hFIX) from a 'safe-harbor' locus. We hypothesize that if B cells were alternatively engineered to express the missing protein in conjunction to a transgenic B-Cell Receptor (BCR), secretion could be boosted as needed, by immunizing with the respective antigen. Furthermore, targeting the transgenic cassette to the IgH locus downstream of the variable part will allow replacement of the endogenous BCR with the transgenic one (Fig. 1a). This design will enable better definition and control over the BCR specificity of the engineered plasma cells, preventing secretion of antibodies with polyclonal undesired specificities, as a side-effect of the therapeutic protein secretion. As a proof of concept, we used CRISPR/Cas9 and recombinant adeno-associated viral vectors to target the integration of hFIX and a single-chain version of the 3BNC117 HIV-broadly neutralizing antibody into the IgH locus of murine B-cells (Fig. 1a). 3BNC117 serves as a model antibody and can be replaced by any antibody against a foreign antigen that can be safely delivered to patients. The cassette is expressed under a modified IgH promoter preceded by a poly-adenylation site, for termination of upstream endogenous transcripts. In lipopolysaccharide-activated primary murine B-cells, we achieved membranal expression of 3BNC117 as a BCR in 12% of the cells. Cells were then propagated on feeder cells expressing CD40L and BAFF, with or without sorting for 3BNC117-expression, to induce plasmablast differentiation. After 10 days in culture, the cells were mostly CD19+IgG1+CD38+, while 20% of the sorted cells expressed 3BNC117, compared to less than 2% of the unsorted ones. Following enrichment for engineered cells, hFIX and 3BNC117 were detectable in culture media starting at day 7 of the culture, and increased up to 1,200ng/ml and 300ng/ml for 3BNC117 and hFIX, respectively (Fig. 1b,c). These results show for the first time that upon plasmablast differentiation, engineered B-cells could co-express and secrete therapeutically relevant proteins alongside an antibody with a defined specificity. In future adoptive transfer studies, we aim to demonstrate secretion of hFIX upon immunization with the cognate antigen for 3BNC117, to create novel, better-defined and inducible plasma cell products for protein deficiencies.

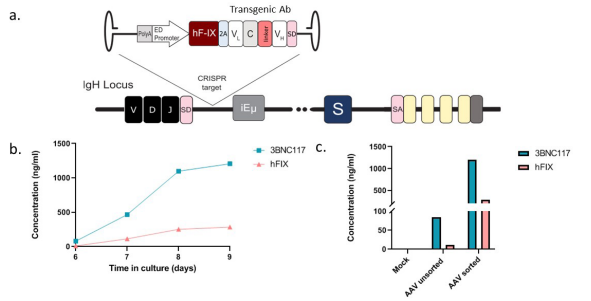


Fig. 1. Engineering of hFIX-secreting plasma cells in conjunction to a transgenic BCR. (a) The targeting scheme of an rAAV encoding hFIX and a transgenic antibody into the IgH locus of B-cells. (b) hFIX and 3BNC117 levels in the media of sorted, engineered cells as a factor of time in culture. (c) hFIX and 3BNC117 levels in culture media of murine B-cells at day 9, with or without enrichment for engineered cells.

690. Dissecting the Non-Cell-Autonomous Effects of Oncogene Activation on Hematopoiesis

Cristina Colleoni, Daniela Cesana, Pierangela Gallina, Eugenio Montini

San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), Milan, Italy

Hematopoietic stem and progenitor cell (HSPC) gene therapy (GT) applications utilize vectors able to insert transgenes into the cells' genome, providing therapeutic benefit upon autologous transplantation. However, the semi-random integration of genetic material may induce oncogenesis, as shown in GT preclinical models and patients. Also, we have previously highlighted that immune-deficient (NSG) mice transplanted with oncogene (BRAFV600E) expressing human HSPCs succumbed to bone marrow failure, consequent to the onset of an aggressive histiocytosis, which occurred even when small numbers of HSPCs were transplanted. Histiocytes expressed classic markers of oncogene-induced senescence (OIS), such as growth arrest and enhanced secretion of pro-inflammatory cytokines, a feature known as senescence-associated secretory phenotype (SASP). SASP factors transmitted senescence to bystander cells and generated an inflammatory microenvironment ultimately causing bone marrow failure. These data indicate that, although OIS dampens the risk of oncogenesis, persisting senescent macrophages are highly detrimental to the

hematopoietic system. Therefore, to uncover the frequency of this phenomenon in GT treatments and to what extent senescent cells persist to immunological clearance, it is fundamental to characterize the interaction between senescent cells and the immune system. Thus, we generated two mouse models of oncogene-induced senescence: immune-competent and immune-deficient. Firstly, HSPCs from CD45.1 wild type (WT) mice, transduced with a lentiviral vector (LV) expressing both human BRAFV600E and GFP, were transplanted into either WT or NSG mice. Peripheral blood (PB) was analyzed by FACS at different time points to determine: engraftment of donor cells (CD45.1), transduction levels (GFP), and lineage composition (CD11b, CD19, CD3). WT mice transplanted with BRAFV600E-expressing HSPCs displayed myeloid skewing, whereas BRAFV600E-expressing lymphoid cells were impaired. A similar counter-selection was observed in NSG mice, yet only after 24 weeks, while bystander B cells slightly expanded when compared to controls. Still, the lethal phenotype observed in the humanized model was not recapitulated, possibly due to defective activity of human BRAF in murine cells. Given these results, we transplanted NSG mice with mouse HSPCs transduced with an LV expressing a constitutionally active form of mouse Braf (lacking the regulatory N-terminal domain), previously identified in insertional mutagenesis studies (mt-Braf). Hematopoietic reconstitution was analyzed by FACS on PB, spleen, and bone marrow harvested at different time points. Control mice were transplanted with HSPCs expressing mouse WT-Braf. At 24 days after transplant, mice from the mt-Braf group showed an expansion of vector-marked B cells, yet without the major impairment on hematopoiesis observed earlier in the humanized model. Of note, at 24 days, mt-Braf expressing B cells showed selective advantage when compared to mWT-Braf expressing B cells. Overall, the transplantation of mouse HSPCs expressing human BRAFV600E is detrimental to lymphoid cells, both in WT and NSG recipients. However, the delayed impairment observed in NSG mice, compared to WT, suggests that the immunological condition of recipient mice has an impact on the tolerance toward oncogene expressing cells. Furthermore, differences between mouse and human biology must be considered, as we showed that mt-Braf expression in mouse HSPCs results in an expansion of oncogene-expressing B cells, as opposed to the humanized model, in which lymphoid cells were impaired. In conclusion, these studies might help to uncover novel effects of insertional mutagenesis beyond oncogenesis and ultimately support the development of improved GT strategies.

691. Preclinical Gene Therapy in Hematopoietic Stem and Progenitor Cells from *RPS19*-Deficient Diamond-Blackfan Anemia Patients

Yari Giménez[1,2,3], Rebeca Sanchez[1,2,3], Christiane Zorbas[4], Manuel Palacios[1,2,3], Laura Ugalde[1,2,3], Omaira Alberquilla[1,2,3], Eva Galvez[2,5], Marion Strullu[6], Jose C. Segovia[1,2,3], Albert Catalá[7], Paula Río[1,2,3], Julián Sevilla[5], Cristina Beléndez[8], Denis Lafontaine[9], Thierry Leblanc[6], Juan Bueren[1,2,3], Susana Navarro[1,2,3]

[1]Hematopoietic Innovative Therapies Division, Centro de investigaciones Energéticas, Medioambientales y Tecnológicas; Centro de Investigación Biomédica en Red de Enfermedades Raras; IIS-FJD, Madrid, Spain,[2]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[3]Instituto de Investigaciones Sanitarias Fundación Jiménez Díaz (IIS-FJD, Madrid, Spain,[4]3RNA Molecular Biology, ULB-Cancer Research Center (U-CRC), Université Libre de Bruxelles, Bruxeles, Belgium,[5]Hospital Infantil Universitario Niño Jesús, Madrid, Spain,[6]Hôpital Robert-Debre, Paris, France,[7]Hospital Sant Joan de Déu, Barcelona, Spain,[8]Hospital Gregorio Marañon, Madrid, Spain,[9]ULB-Cancer Research Center (U-CRC), Université Libre de Bruxelles, Madrid, Spain

Diamond-Blackfan Anemia (DBA) is an inherited bone marrow failure (IBMF) syndrome mainly characterized by red cell aplasia, congenital abnormalities and increased risk of cancer. The incidence of DBA is 5 to 10 per million life births. So far, mutations in 20 DBA genes have been identified, although mutations in *RPS19* are found in about 25% of DBA patients. Hematopoietic stem cell transplantation (HSCT) currently constitutes the unique definitive curative treatment of DBA. Nevertheless, limitations of this therapy, together with the efficacy and safety currently observed in different hematopoietic gene therapy (GT) trials suggest that GT may constitute a novel and efficient therapeutic approach for *RPS19*-deficient DBA patients. Based on our experience in another IBMF syndrome, such as Fanconi anemia (FA), we first evaluated whether the collection of HSC from DBA patients could represent a limitation in the development of a GT trial in this disease. Significantly, numbers of BM $CD34^+$ cells were comparable to HD and significantly higher than numbers observed in FA patients. Concomitantly, although slightly reduced numbers of colony forming cells were observed in DBA patients compared to HDs, these values were also significantly higher compared to FA. Moreover, in contrast to FA, a substantial NSG mice repopulation potential was observed in uncorrected DBA CD34+ cells. With the purpose of correcting the phenotype of RPS19-deficient $CD34^+$ cells, two therapeutic lentiviral vectors (LV) were generated. In these vectors a codon-optimized version of *RPS19* was driven by the *PGK* promoter already used in the FA gene therapy trial, or by the *EF1α(s)* promoter, previously used in experimental DBA studies. Either of these two LVs restored the expression of *RPS19* and also corrected defects in the ribosomal biogenesis of *RPS19*-interferred K562 cells. In subsequent experiments BM $CD34^+$ cells from *RPS19*-deficient patients were transduced with the therapeutic LVs, and then cultured in semisolid medium. The transduction with either therapeutic vector increased the number of hematopoietic colonies, more significantly in the case of the erythroid colonies as compared to samples transduced with a EGFP-LV. Additionally, the therapeutic LVs reverted the red blood cell differentiation defect characteristic of DBA $CD34^+$ cells, and facilitated

the engraftment of NSG mice with corrected cells, showing a safe and polyclonal integration profile. In conclusion, these preclinical studies support that the lentiviral-mediated gene therapy of RPS19-deficient CD34$^+$ cells should constitute an efficient and safe approach for the treatment of the hematopoietic signs characteristic of DBA patients.

692. Improving the Efficacy of Liver Directed Lentiviral Gene Therapy for Hemophilia

Cesare Canepari[1,2], Michela Milani[1], Chiara Simoni[1], Mauro Biffi[1], Fabio Russo[1], Tiziana Plati[1], Markus Grompe[3], Luigi Naldini[1,2], Alessio Cantore[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]Vita Salute San Raffaele University, Milan, Italy,[3]Oregon Health and Science University, Portland, OR

Lentiviral vectors (LV) are attractive vehicles for liver-directed gene therapy, by virtue of their ability to stably integrate into the target cell chromatin, being maintained as cells divide. Over the past years, we developed LV that achieve stable expression of coagulation factor IX (FIX) or coagulation factor VIII (FVIII) in the liver after intravenous (i.v.) administration to animal models of hemophilia and non-human primates (NHP). However, LV-mediated transfer of FVIII transgenes remains more challenging due to the large transgene size and low infectivity of LV-FVIII. Because the low-density lipoprotein receptor (LDL-R), is used as the entry port by LV pseudotyped with vesicular stomatitis virus protein G (VSV-G), we aimed to increase LDL-R expression on hepatocytes in order to improve the efficiency of transduction by VSV-G pseudotyped LV. Since fasting is known to have an impact on LDL metabolism, we first evaluated if it was also relevant on LV-mediated liver gene transfer. We showed a more than 2-fold increase in transgene output when mice were fasted for 24 hours prior to LV administration, compared to mice treated with the same LV dose but fed *ad libitum*. This difference in transgene amounts was maintained overtime, until the end of experiment (3 months post LV). This result was obtained with both FIX and FVIII transgenes and also confirmed in mice treated with phagocytosis-shielded LV (carrying high surface content of the phagocytosis inhibitor CD47). In order to increase the amount of genetically modified hepatocytes *a posteriori* and potentially improve our control on the quantity of transgene product, we set out to endow transduced hepatocytes with a selective advantage over untransduced ones. We exploited a strategy based on acetaminophen (APAP) metabolism previously shown to achieve this outcome. We generated "selectable" LV carrying, next to the FIX or FVIII transgene, a short hairpin RNA (shRNA) against cytochrome P450 reductase (Cypor), responsible for the generation of a toxic metabolite, upon APAP metabolism. We treated adult wild-type mice with selectable LV expressing FIX or newborn hemophilia A mice (FVIII deficient) with selectable LV expressing FVIII, modified as above. LV-transduced Cypor downregulating hepatocytes became resistant to APAP-induced liver toxicity, being unable to convert the drug in its toxic intermediate. We observed 5-fold increase in both FIX and FVIII blood concentration, following 30-35 APAP doses, compared to the those of mice left unselected. At the end of experiment, LV DNA was higher in the hepatocytes of selected compared to unselected mice, reflecting the higher transgene amounts. Overall, improving the efficiency of hepatocytes transduction by LV and selectively expanding genetically corrected cells may allow reducing LV doses to achieve therapeutic efficacy in hemophilia thus easing feasibility and lowering possible dose-dependent toxicities. In addition, these strategies may allow expanding application of liver-directed LV gene therapy to additional cell-autonomous genetic diseases of liver metabolism.

693. Intravenous Infusion of Human Placental Cells Secreting mco-ET3 to Juvenile Sheep Results in Elevation of Plasma FVIII Levels without Induction of an Antibody Response

Brady Trevisan[1], Martin Rodriguez[1], Sunil George[1], Jordan Shields[2], Shannon Lankford[1], Rebecca Combs[3], Michael Gautreaux[4], John Owen[3], Anthony Atala[1], Christopher B. Doering[2], H. Trent Spencer[2], Christopher D. Porada[1], M Graca Almeida-Porada[1]

[1]Fetal Research and Therapy Program, Wake Forest Institute for Regenerative Medicine, Winston Salem, NC,[2]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta and Dpt. of Pediatrics, Emory University, Atlanta, GA,[3]Special Hematology Laboratory, Wake Forest School of Medicine, Winston Salem, NC,[4]HLA/Immunogenetics and Immunodiagnostics Laboratories, Wake Forest School of Medicine, Winston Salem, NC

Currently treatments for hemophilia A include native and recombinant FVIII proteins, engineered clotting factors, and a broad array of new products including antibodies and gene therapy. Mesenchymal stromal cells possess several fairly unique properties that, when combined, make them ideally suited for cellular-based therapies and as vehicles for gene delivery for a wide range of diseases. Since all normal juvenile sheep (n=3) that received weekly intravenous (IV) infusions of human or an expression/secretion-optimized bioengineered (mcoET3) FVIII protein for 5 weeks (20 IU/kg) developed anti-FVIII specific IgG (titers of 1:20 - 1:245) by week 3 of infusion, we hypothesized that the inherent immunomodulatory properties of human mesenchymal placental cells (PLC) could be used to enable the delivery of FVIII without eliciting antibody formation. To answer this question, PLC were transduced with a lentiviral vector (0.5 vector copies/diploid genome equivalent) to produce high levels (4.9-6IU/10^6 cells/24h) of mcoET3 (PLC-mco). Animals then received weekly IV infusions of PLC-mco for 3 weeks (n=3) or a single intraperitoneal (IP) injection of 10^7 PLC-mco/kg (n=3). These cell doses were calculated to mirror the amount of FVIII protein we had infused in our initial studies and provide ~20-60 IU/kg mcoET3 each 24h. Both IV and IP delivery were evaluated to determine whether the route of administration would affect the efficacy and/or safety of this product. These animals were evaluated for % increase in plasma FVIII levels above day 0 by aPTT, and for the development of an immune response, using lymphocytes and plasma that were collected before the first infusion at week 0 (W0), and prior to each subsequent infusion at weeks 1 through 15 (W1-W15). No significant differences were seen between the IP and IV groups with respect to increase in plasma FVIII levels above day 0, with animals in these two groups exhibiting increases of 31% and 34% by W15, respectively. To evaluate humoral immunity, ELISA analysis was performed on serum collected during the course of this study and demonstrated that all sheep in both the IV and IP groups were devoid of mcoET3-specific IgM antibodies at all time points. Similarly, no mcoET3-specific IgG antibodies were ever detected in sheep infused IV with PLC-mco. In contrast, IP delivery of PLC-mco resulted in the generation of a specific IgG response by 2

weeks after administration in 66% of the animals. Nevertheless, none of the animals receiving PLC-mco via either the IP or IV route developed anti-human HLA antibodies against the PLC. Taken together, our results show that human PLC can be transduced to produce high levels of FVIII protein, and that these cells can then be delivered to juvenile animals to yield an increase in plasma FVIII levels. Our findings also support our hypothesis that IV administration of a fVIII transgene via PLC enables avoidance of an immune response to a FVIII protein that is immunogenic to sheep when delivered as an IV bolus.

694. Non-Viral Gene Delivery of Human FVIII to Hemophilia A Mice and Non-Human Primates

Matthew G. Stanton, Deb Klatte, Matt Chiocco, Luke Hamm, Elizabeth Nelson, Greg Feinstein, Nolan Gallagher, Jie Su, Andrew Milstead, Di Bush, Russell Monds, Nicholas Parsonnet, Ashley Penvose, Sarah LaGoy, Erik Hansen, Jonathan Kittel, Anastasia Lymar, Kati Vu, Michelle Rodriguez Joyce, Karl Malakian, Jeff Moffitt

Generation Bio, Cambridge, MA

AAV gene therapy has proven to be an effective modality for the treatment of certain rare, monogenic diseases. Despite these successes, there remain significant limitations to AAV, many of which are attributable to the viral capsid. These include restriction of transgene size to ~4.7kb, development of anti-capsid neutralizing antibodies which limits dosing to a single administration, emerging safety concerns at higher doses, and costly, scale-limited manufacturing that restricts extending gene therapy beyond rare disease. Non-viral gene therapy has been recognized for decades as an attractive solution to address these limitations. However, successful demonstration of preclinical proof of concept (POC) in animal models of disease has remained elusive, largely due to off-target biodistribution to immune cells which results in stimulation of DNA mediated innate immune responses that impact tolerability and limited intracellular trafficking of the DNA payload from the endosome to the hepatocyte nucleus. We have developed a non-viral gene therapy platform that overcomes these challenges to enable a durable, re-dosable, and scalable approach. The system is comprised of an engineered, double-stranded, linear, covalently closed-ended DNA (ceDNA) that can accommodate a transgene expression cassette of at least 12 kb and a cell targeted lipid nanoparticle (ctLNP) that provides for selective hepatocyte delivery and repeat administration. Studies in immunocompetent mice demonstrated that systemic administration of a single dose of ctLNP formulated ceDNA expressing either firefly luciferase or FIX resulted in ceDNA delivery to hepatocytes and durable transgene expression. Administration of a second dose, further increased expression. Hemophilia A is a rare X-linked hereditary bleeding disorder characterized by impaired blood coagulation due to a deficiency in the production or function of coagulation Factor VIII. It affects approximately 16,000 individuals in the United States and 320,000 individuals worldwide. We aim to develop a re-dosable Factor VIII gene therapy that enables all Hemophilia A patients to reach and maintain therapeutic levels of Factor VIII expression. Towards this end, we designed and screened approximately 300 constructs to optimize regulatory and Factor VIII sequences for secretion of functional human Factor VIII. Initial screening was performed *via* transfection into HepG2 cells and administration to mice by hydrodynamic delivery. Selected ceDNA were scaled, encapsulated into ctLNP and systemically administered to Factor VIII null mice. Dose-responsive plasma human Factor VIII levels and activity were observed after a single systemic dose of ctLNP formulated ceDNA. Species translation of human Factor VIII expression was demonstrated after systemic delivery of weight-adjusted doses of ctLNP formulated Factor VIII ceDNA to wild-type mice and non-human primates. In these studies, ctLNP formulated ceDNA was well tolerated at doses up to 2mg/kg, the highest dose tested. These data, coupled with a scalable manufacturing process and the demonstrated ability to re-administer to immunocompetent animals to boost expression establishes preclinical POC and a path toward clinical development of the first non-viral gene therapy for hemophilia A.

695. Novel Vector Systems towards a Cure for HIV/AIDS

Elena Herrera Carrillo, Zaria Andrade dos Ramos, Minghui Fan, Merve Koroglu, Ben Berkhout

Laboratory of Experimental Virology, Department of Medical Microbiology, Amsterdam UMC, Academic Medical Center, University of Amsterdam, Amsterdam, Netherlands

Novel vector systems towards a cure for HIV/AIDS Despite the potency of current antivirals drugs to treat HIV infection, the patient is never cured. Inspired by the cure of the Berlin patient, different gene therapy approaches were proposed as cure treatment. A sterilizing cure can in principle be realized via a CRISPR-Cas attack on the integrated DNA provirus in the HIV reservoir. Our laboratory demonstrated potent and durable HIV inhibition in a combinatorial attack with two guide RNAs (gRNAs), but significant vector production and delivery issues hinder a swift translation to animal and clinical studies. For instance, the relatively large size of CRISPR-spCas9 transgene cassettes impedes their implementation in gene therapy applications with vectors that have a limited packaging capacity, including the lentiviral vector (LV) that we prefer. As such, there is a serious need for more simple/smaller CRISPR-Cas vector designs. We propose to minimize the lentiviral size by: A) incorporating of a smaller Cas9 nuclease from Staphylococcus aureus (saCas9) or Campylobacter jejuni (cjCas9). The protein size of SaCas9 (1,053 amino acids) is smaller than that of *Streptococcus pyogenes Cas9 (*spCas9) with 1,386 amino acids, while cjCas9 is one of the smallest Cas9 orthologues (984 amino acids). B) adaptation of the gRNA and Cas9-encoding mRNA cassettes. The former adaptation is based on our recent finding that the frequently used H1 Pol-III promoter exhibits both Pol-III and Pol-II promoter activity, which allows one to design a combined expression cassette for both the gRNA (Pol-III activity) and the Cas9 mRNA (Pol-II activity). These combined changes should significantly reduce the vector size and consequently increase the production and transduction efficiency (vector titer). We therefore compared different CRISPR-Cas systems for their efficiency in terms of vector titer and antiviral activity. Virus inhibition was tested in HIV replication studies which allowed us to test for prolonged virus inhibition and possible viral escape and ideally for permanent and complete virus inactivation (CURE) in the infected cells. The viral gRNA-target sequences were determined in escape viruses to document the mechanism of escape and the targets

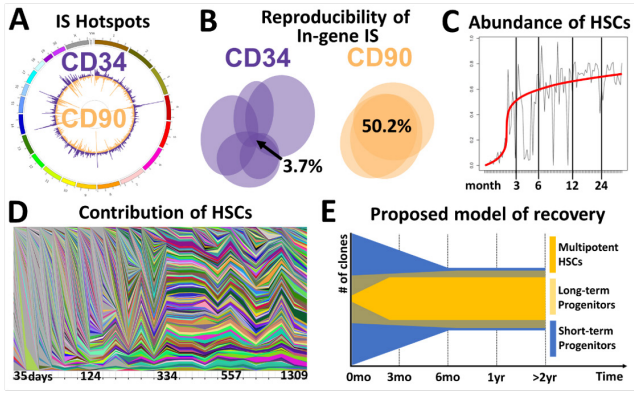
in the proviruses were analyzed to describe the means of provirus inactivation in case a CURE was achieved. We demonstrate that the LV transduction efficiency can be improved by reduction of the LV vector size by incorporating smaller cas9 nucleases, but also by using the dual-polymerase activity of the H1 Pol III promoter. We used the H1 promoter for the simultaneous expression of the small gRNA and the Cas9 protein, and both components were well expressed. This also reduces the complexity of the transgene cassette, which benefits the utilization of viral vectors such as LVs. We report superior antiviral activity of saCas9 (compared to cjCas9) as it can achieve complete HIV inactivation (sterilizing CURE) in cell culture with only a single gRNA. DNA cleavage by the saCas9 and cjCas9 endonucleases and subsequent DNA repair creates an indel mutational profile that is similar to that of spCas9. The general mechanistic features of catalysis by Cas9 orthologs are comparable, but the enzyme kinetics are different. It has been reported that saCas9 is a multiple-turnover enzyme[1] - in contrast to spCas9 which cleaves a stoichiometric amount of DNA - which might explain the increased antiviral activity. This study thus provides new insights and considerations for the future design of CRISPR/Cas9-based applications. 1. Yourik et al. RNA. 2019;25(1):35-44.

696. High Density Clonal Tracking Demonstrates Highly Reproducible Contribution of Multipotent HSCs as Early as 21 Days Post-Transplant in a Nonhuman Primate Model of Gene Therapy

Stefan Radtke[1], Dnyanada Pande[1], Mark Enstrom[1], Margaret Cui[1], Jennifer E. Adair[1,2], Hans-Peter Kiem[1,2]

[1]Fred Hutchinson Cancer Research Center, Seattle, WA,[2]University of Washington School of Medicine, Seattle, WA

Introduction: Retroviral integration site (RIS) analysis is used in hematopoietic stem cell (HSC) gene therapy trials to monitor the clonality/safety profile of engrafted cells. Clonal tracking in patients is further used to study the contribution of multipotent HSCs during reconstitution. However, scarce material remains a bottleneck impacting data quality and complicating the interpretation of datasets. Furthermore, low capturing sensitivity (Adair 2020) and high error rates of RIS require significant data exclusion and sophisticated statistical tests to ensure data reliability. In 2016, Biasco *et al.* reported that increased sampling and purification of peripheral blood (PB) subsets (i.e. high density sampling), from non-myeloablative conditioned gene therapy patients improves the sensitivity of RIS. Results from this study suggest a bi-phasic pattern of reconstitution initially dominated by short-term progenitors and contribution of multilineage HSCs starting ~6-months post-transplant.
Methods: Here, we hypothesized that high density sampling in combination with purification of genetically modified cells could further increase sensitivity, avoid data manipulation, and lead to higher reproducibility of IS patterns across recipients. Three myeloablated nonhuman primates were transplanted with purified HSC-enriched $CD34^+CD90^+$ cells (CD90 animals) after lentiviral gene-modification (Radtke 2017). For 4+ years, fluorophore+ PB white blood cells and gene-modified lineages were purified and analyzed by RIS. Longitudinal RIS data from CD90 animals were compared to 5 historic animals receiving gene-modified bulk $CD34^+$ cells (CD34 animals) where low density sampling was applied.
Results: 46,480 and 155,824 IS were recorded for CD34 and CD90 animals, respectively, with no evidence of differences in IS hotspots (**A**). High density sampling and purification demonstrated a highly reproducible landscape of in-gene IS among the CD90 animals (50.2% identity) as compared to only 3.7% overlap among the CD34 animals (**B**). Furthermore, this approach permitted fewer PCR cycles, resulting in fewer sequence errors and less error correction, and enabled inclusion of low-abundant clones. As proposed by the bi-phasic model, the majority of clones detected in CD90 animals disappeared within 3 months of transplant (**C**). Enhanced sensitivity revealed very clearly and highly reproducibly that HSC-derived clones demonstrating multilineage engraftment were detected as early as 21 days post-transplant. More than 700 HSC clones gave rise to over 50% of all mature gene-marked blood cells as early as 2 to 3-months post-transplant (**D**).
Conclusion: Here, we show that high density sampling of purified gene-modified cells can significantly enhance the quality and reproducibility of clonal tracking data with RIS. Most importantly, our approach revealed the very early and robust contribution of multipotent HSCs suggesting a significantly earlier bi-phasic switch in hematopoietic reconstitution after myeloablation (**E**). These findings should have important implications for the design of ex vivo and in vivo HSC gene therapy and genome editing approaches.



697. Gene Therapy for Fanconi Anemia [Group A]: Preliminary Results of Ongoing RP-L102 Clinical Trials

Agnieszka Czechowicz[1,2,3], Julián Sevilla[4], Claire Booth[5], Rajni Agarwal[1,2,3], Josune Zubicaray[4], Paula Río[6,7,8], Susana Navarro[6,7,8], Phil J. Ancliff[5], Brian C. Beard[9], Kenneth M. Law[9], Grace Choi[9], Miriam Zeini[9], Camilla Duran-Persson[5], Eileen Nicoletti[9], Gayatri R. Rao[9], John E. Wagner[10], Jonathan Schwartz[9], Juan A. Bueren[6,7,8], Maria Grazia Roncarolo[1,2,3]

[1]Center for Definitive and Curative Medicine, Stanford University, Stanford, CA,[2]Div. of Pediatric Hematology/Oncology/Stem Cell Transplantation and Regenerative Medicine, Stanford University School of Medicine, Stanford, CA,[3]Stanford Children's Hospital, Palo Alto, CA,[4]Hospital Infantil Universitario Niño Jesús, Madrid, Spain,[5]UCL Great Ormond Street Institute of Child Health, London, United Kingdom,[6]Instituto de Innovación Biomédica, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain,[7]Centro de Investigación Biomédica en Red de Enfermedades Raras, Madrid, Spain,[8]Unidad Mixta de Terapias Avanzadas, Instituto de Investigación Sanitaria Fundación Jiménez Díaz, Madrid, Spain,[9]Rocket Pharmaceuticals, Inc., Cranbury, NJ,[10]Blood and Marrow Transplant Program, Dept. of Pediatrics, University of Minnesota, Minneapolis, MN

Background: Fanconi anemia (FA) is a genetic disorder characterized by defective deoxyribonucleic acid (DNA) repair, progressive bone marrow failure (BMF), and a predisposition to hematologic malignancies and solid tumors. 80% of FA patients experience BMF within the first decade of life. Allogeneic hematopoietic stem cell transplant (alloHSCT) is a potentially curative treatment for BMF; however, its efficacy is limited by availability of suitable human leukocyte antigen (HLA)-matched sibling donors and transplant-related toxicities. *Ex vivo* lentiviral mediated gene therapy of autologous FA-A CD34+ enriched hematopoietic stem and progenitor cells (HSPCs) has been demonstrated to confer a survival advantage to gene-modified HSPCs in preclinical studies and in the FANCOLEN-I clinical trial. We report updated results from ongoing studies of RP-L102 using "Process B" manufacturing optimizations. **Methods:** Subjects with a confirmed *FANCA* gene mutation ≥1 year, with no HLA-matched sibling donor and at least 30 CD34+ cells/μL in bone marrow (BM) were eligible. Peripheral blood (PB) mononuclear cells were collected via leukapheresis on 2 consecutive days after mobilization with granulocyte-colony stimulating factor (G-CSF) and plerixafor. CD34+ HSPCs were enriched, transduced with a lentiviral vector and infused fresh without antecedent conditioning. Patients are being followed for 3 years post-infusion for safety assessments and to ascertain evidence of efficacy (increasing PB and BM vector copy number (VCN) and mitomycin-C [MMC] resistance in BM colony forming units [CFUs]), along with stabilization/correction of cytopenias. **Results:** As of January 2021, 9 subjects (age 2 to 6 years) have received RP-L102 infusion. Preliminary evidence of engraftment has been identified in at least 5 of 7 subjects with ≥2 months of follow-up as indicated by PB VCN. 2 of 3 subjects with follow up of ≥12 months have shown increasing PB VCN with BM CFU MMC resistance. 1 subject whose course was complicated by influenza B infection 9 months post infusion had progressive BMF requiring alloHSCT. Only 1 subject had a serious Grade 2 transient RP-L102 infusion-related reaction. Updated safety and efficacy data for subjects with ≥12 months of follow up will be presented; drug product (DP) information will be available for all subjects. **Conclusions:** DP has been successfully manufactured for N=9 subjects to meet the required specifications. The safety profile of RP-L102 is excellent. Preliminary evidence of engraftment has been confirmed in 5 subjects as demonstrated by PB VCN; 12+ months of follow-up may be required to observe the proliferative advantage of transduced HSPCs in the absence of conditioning.

698. A Systematic Review and Meta-Analysis on Gene Therapy for the Treatment of Monogenic Disorders

Francesca Tucci[1,2], Stefania Galimberti[3], Luigi Naldini[2,4], Maria Grazia Valsecchi[3], Alessandro Aiuti[1,2,4]

[1]Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[3]Bicocca Bioinformatics Biostatistics and Bioimaging B4 Center, School of Medicine and Surgery, University of Milano - Bicocca, Milan, Italy,[4]Vita Salute San Raffaele University, Milan, Italy

Introduction: Ex-vivo gene therapy (GT) with hematopoietic stem and progenitor cells (HSPC) is a promising treatment for monogenic diseases, but lack of centralized databases is hampering an overall outcomes assessment. The aim of this work is to describe the reported outcomes of HSPC-GT in terms of survival, genotoxicity and engraftment of gene corrected HSPCs across vector platforms and diseases.**Methods:** Searches were conducted in PubMed, Embase, Clinicaltrials.gov and Cochrane Central Register of Controlled Trials from inception to October 2020. Reviews, letters, conference abstracts and communications were also considered. We included HSPC-GT trials in patients affected by monogenic diseases reporting target outcomes. Genotoxicity was intended as the first occurred haematological malignancy related to GT. Engraftment was considered successful when molecular tests reported the presence of gene corrected hematopoietic cells for >1 year post-GT. Studies on cancer or gene editing were excluded. Studies meeting inclusion-exclusion criteria were independently assessed by 2 authors for methodological quality. Random-effects models were used to quantify between-studies heterogeneity and pooled estimates.**Results:** A total of 55 trials for 14 diseases met inclusion criteria and 406 patients treated with HSPC-GT from 1995 to 2020 were analysed. Patients with primary immunodeficiencies (55.2%), metabolic diseases (17%), haemoglobinopathies (24.4%) and bone marrow failures (3.4%) were treated with gammaretroviral vector (yRV) (29.1%), self-inactivating yRV (2.2%) or lentiviral vectors (LV) (68.7%). Twenty-one deaths occurred, but none in the first 100 days. The pooled overall incidence rate of death was 0.9 per 100 person-years of observation (PYO) (95%CI=0.37-2.17), while 5 years survival obtained from the available individual data (n=260) was 91.1% (95%CI=86.8-95.6%). No differences in survival among vector platforms (p=0.2652) and disease subgroups (i.e. PID, metabolic, haemoglobinopathies, Fanconi anemia) (p=0.7264) were observed. There were 21 genotoxic events out of 1,504.02 PYO, with a median time to onset of 2.8 years (min-max=0.7-14.8). Events were limited to yRV trials (0.99 events per 100 PYO, 95%CI=0.18-5.43) and primary immunodeficiencies, with

different incidence among diseases. No genotoxic events were observed with LV (730.7 PYO). Pooled rate of engraftment for gene corrected cells (all hematopoietic subpopulations) was 86.1% (95%CI=66.9-95.0%) for yRV and 99.0% (95%CI=95.1-99.8%) for LV HSPC-GT. **Conclusions:** A variety of conditions can now be treated with HSPC-GT with a positive risk-benefit profile. Results from this meta-analysis summarizing two decades of studies on HSPC-GT in over 400 patients shows stable reconstitution of haematopoiesis with gene-corrected cells in most recipients and superior engraftment and safer genotoxic profile in patients receiving LV-transduced HSPC.

699. Preclinical Safety and Efficacy Validation of $CD4^{LVFOXP3}$ Cells as an Innovative Cell-Based Gene Therapy Approach for IPEX Syndrome

Yohei Sato[1], Abinaya Nathan[2], John Fraser Wright[1,2], Keri Marie Tate[3], Prachi Wani[3], Fariba Fazeli[3], Azadeh Timnak[3], Neehar Bhatia[3], Rajni Agarwal-Hashmi[1,2], Alice Bertaina[1,2], Maria-Grazia Roncarolo[1,2,4], Rosa Bacchetta[1,2]

[1]Pediatrics, Stanford University, Stanford, CA,[2]Center for Definitive Curative Medicine (CDCM), Stanford University, Stanford, CA,[3]Laboratory for Cell and Gene Medicine (LCGM), Stanford University, Stanford, CA,[4]Stanford Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA

FOXP3 is an essential transcription factor for regulatory T cell (Treg) function, and a key regulator of immune tolerance. Genetic or acquired defects in Treg play a key role in many immune mediated diseases including Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) syndrome. Treg have also been explored in the clinic, to prevent Graft-versus-host disease (GvHD) or controlling autoimmunity. We have currently optimized a protocol for the generation of Treg-like cells from CD4+ T cells of healthy donors or IPEX patients, by lentiviral transfer of *FOXP3*($CD4^{LVFOXP3}$cells) which acquire stable and functional regulatory properties. To facilitate pre-clinical safety and efficacy studies, we have developed a humanized-mouse disease model. In this model, the FOXP3 gene is knocked out using CRISPR/Cas9 in human HSPCs, then transplanted into immunodeficient mice. The use of multiple sgRNAs targeting the FOXP3 locus significantly improved targeting rates (73.5%+- 2.8%, Mean+- SEM, n=6) when compared to a single sgRNA (31.1%+- 3.1%, Mean+- SEM, n=7). The immune-deficient mice transplanted with FOXP3 knock-out HSPCs developed lymphoproliferation 10-12 weeks after transplant. This FOXP3 KO humanized mouse model is used to assess the efficacy and safety of $CD4^{LVFOXP3}$cells . We have also tested the stability of $CD4^{LVFOXP3}$cells in the presence of the mTOR inhibitor rapamycin, which is a widely used treatment for patients with IPEX syndrome. $CD4^{LVFOXP3}$cells show similar stability to that of expanded Tregs *in vitro* in the presence of rapamycin. Furthermore, the $CD4^{LVFOXP3}$cells generated in GMP grade conditions are functionally stable after cryopreservation and showed significant improvement in the survival and phenotype of xeno-GvHD mice in a dose-dependent manner. These data complete the IND-enabling studies supporting the clinical use of $CD4^{LVFOXP3}$in a Phase 1 trial to treat patients with IPEX Syndrome and future potential clinical use in other more common immune-mediated diseases caused by insufficient or dysfunctional $FOXP3^{+}$Tregs.

700. Preliminary Results from the Phase 1/2 GuardOne Trial: A First-in-Human Study of *Ex Vivo* Lentiviral Gene Therapy (AVR-RD-02) in People with Gaucher Disease Type 1

L. K. Jacobsen[1], M. N. Trame[1], A. Golipour[1], R. Pfeifer[1], A. Khan[2], C. Mason[1,3]

[1]AVROBIO, Inc, Cambridge, MA,[2]University of Calgary Cumming School of Medicine, Calgary, AB, Canada,[3]Advanced Centre for Biochemical Engineering, University College London, London, United Kingdom

Gaucher disease (GD) is a rare, autosomal recessive lysosomal disorder caused by mutations in the *GBA* gene that encodes glucocerebrosidase enzyme (GCase). This results in the accumulation of toxic substrate in reticular endothelial cells (including macrophages and microglia) leading to chronic inflammation, multiorgan pathology, clinical morbidity and early mortality. Current treatments (enzyme replacement therapy [ERT] and substrate reduction therapy [SRT]) require lifelong use and do not prevent disease progression or all symptoms, especially those relating to bone and the central nervous system (CNS). Furthermore, SRT is not suitable for all patients, and ERT is not a realistic option in many parts of the world. More innovative treatments are therefore required. AVR-RD-02 is an investigational *ex vivo* lentiviral (LV) gene therapy for GD that aims to offer sustained 24/7 production of functional GCase resulting in long-term clinical benefits after a one-time intravenous (IV) infusion. AVR-RD-02 comprises mobilized autologous CD34+ cells transduced *ex vivo* with a third generation LV vector containing an RNA transcript which, after reverse transcription, results in codon-optimized cDNA encoding functional human GCase. Patients receive a single round of personalized busulfan conditioning prior to AVR-RD-02 infusion in order to optimize genetically modified cell engraftment in the bone marrow and brain, while minimizing potential side effects. After AVR-RD-02 infusion, the genetically modified CD34+ cells asymmetrically divide, differentiate and are distributed to sites throughout the body displacing the faulty macrophages and microglia, thus potentially enabling benefits in both body and brain. The ongoing, open-label phase 1/2 GuardOne study is designed to evaluate the safety and efficacy of a single IV infusion of AVR-RD-02 in 8 to 16 adults aged 18-45 years with GD type 1. All patients will be treatment-naïve or will not have received ERT or SRT within the 12 months prior to screening. Key outcome measures include AVR-RD-02 safety and tolerability, engraftment of genetically modified CD34+ cells in the bone marrow, and changes in signals of clinical efficacy, including spleen and liver volume, lyso-Gb1 and chitotriosidase levels, hemoglobin concentration and platelet count. Patients will initially be followed for 52 weeks, followed by an additional 14 years. GuardOne is currently active in Canada and Australia, with additional sites planned for the USA, Israel and the EU in 2021. Despite being off ERT, three-month data from Patient 1 suggest that AVR-RD-02 produces greater reductions in lyso-Gb1 and chitotriosidase levels relative to the patient's ERT baseline, and maintains platelet counts and hemoglobin levels within the normal range. No unexpected trends or safety concerns were identified. Six-month data for Patient 1 will be reported in this presentation.

701. Gene Editing for Congenital Dyserythropoietic Anemia Type II Model in Human Hematopoietic Stem and Progenitor Cells

Mercedes Dessy-Rodriguez[1,2], Sara Fañanas-Baquero[1,2], Veronica Venturi[3], Salvador Payan[4], Cristian Tornador[5], Gonzalo Hernandez[6], Mayka Sánchez Fernández[5,6], Juan A. Bueren[1,2], José Carlos Segovia[1,2], Oscar Quintana-Bustamante[1,2]

[1]Biomedical Innovation Unit, CIEMAT-CIBERER, Madrid, Spain,[2]Unidad Mixta de Terapias Avanzadas, IIS-FJD, Madrid, Spain,[3]Universitat Internacional de Catalunya, Madrid, Spain,[4]Hospital Universitario Virgen del Rocío, Sevilla, Spain,[5]Bloodgenetics, Barcelona, Spain,[6]Universitat Internacional de Catalunya, Barcelona, Spain

Congenital dyserythropoietic anemia type II (CDAII) is a rare inherited disorder that affects red blood cell development. CDAII patients suffer from anemia of variable degrees. CDAII is often accompanied by jaundice and splenomegaly. Bone marrow of CDAII patients is hypercellular, with distinct erythroid hyperplasia and presence of binucleated cells. CDAII is caused by mutations in the *SEC23B* gene. SEC23B is part of coat protein complex II (COPII), which is involved in protein processing and Golgi-reticulum trafficking. Current treatments involve blood transfusions, iron chelation therapy and removal of the spleen and gallbladder. Periodic blood transfusions can worsen the problem of iron overload that must be treated to avoid fatal clinical complications. Until now, the only described definitive therapy for CDAII is allogeneic bone marrow transplantation, which implies additional side effects. Consequently, new advanced therapies for CDAII are urgently required.Lack of a CDAII disease model hampers the development of new therapeutic approaches, since *Sec23b* deficient mice have no anemia phenotype and die shortly after birth with severe pancreatic abnormalities. To circumvent this limitation, we have developed an efficient CRISPR/Cas9 system to model CDAII in human hematopoietic cells. Different single guide RNAs (sgRNA) targeting the start of the coding sequence of human *SEC23B* gene were designed and tested in K562 cell line and in healthy human hematopoietic stem and progenitors (hCB-CD34$^+$) *in vitro* and *in vivo* in NBSGW mice. K562 cells were nucleofected with different sgRNAs independently or in combination. Most indels produced at *SEC23B* gene with the combination of sgRNAs were frameshift mutations. Several oligoclonal cultures were established to study gene editing outcome in depth. SEC23B expression and cell growth were diminished and morphological abnormalities, such as the presence of multinucleated cells, were observed in the majority of oligoclones, mainly in those that were gene edited with the sgRNA combination. Genetic analyses of the oligoclones generated by the combination of sgRNAs showed a low variability of indels, although most of them were able to alter *SEC23B* open reading frame.When CB-CD34$^+$ cells were nucleofected with the most efficient sgRNA or with the combination of sgRNAs, up to 80% knock-out efficiency and close to a 90% reduction of SEC23B protein were obtained. Interestingly, when gene edited hematopoietic progenitors were differentiated *in vitro* to erythroid cells, the percentage of frameshift indels decreased while frequency of wild type alleles increased over time, suggesting a proliferative disadvantage of knock-out cells. A high presence of bi/multinucleated cells and reduction in enucleated cells could be identified in the gene edited cells with the sgRNA combination. Similarly, the *in vivo* erythroid differentiation of these gene edited progenitors in NBSGW mice showed an impairment of terminal human erythroid differentiation with an increment in the percentage of erythroid bi/multinucleated cells without altering other hematopoietic lineages.In summary, CRISPR/Cas9 system has been used to model CDAII in a human cell line and in human hematopoietic progenitors through the knock-out of *SEC23B* gene. Our CDAII model may well be an effective tool for the development of new curative strategies in this disease.

Hematologic and Immunologic Diseases

702. Development of a Novel BTK Lentiviral Vector for Gene Therapy of X Linked Agammaglobulinemia

Noam C. Diamant, Liat Shachnai-Pinkas, Liron Elkouby, Amit Klainberger, Noam Baumatz

Noga Therapeutics, Ness Tziona, Israel

X-linked agammaglobulinemia (XLA) is a rare X linked genetic disorder resulting from mutations in the Bruton's tyrosine kinase (BTK) gene. These mutations lead to the failure of afflicted individuals to generate mature B cells as well as other immunological dysfunctions mainly in NK and myeloid cells. Current therapy consists of immunoglobulin replacement and targeted antimicrobial agents. This therapy is insufficient, as treated XLA patients continue to suffer from low quality of life and recurrent complications. To overcome this insufficiency, Lentiviral (LV)-based gene therapy has previously been tested in various XLA mouse models. Interestingly, in terms of expression cassette design, most efforts focused on the inclusion of generic elements to increase BTK expression with less regard to expression specificity. We hypothesized that by using the human BTK endogenous promoter, we would be able to maintain the tight physiological regulation of BTK gene expression while producing therapeutic Btk protein levels in the desired target cell populations. To study the endogenous BTK promoter in depth, we dissected the proximal and distal regions into several fragments and tested for expression levels and specificity in target (HSCs, myeloid and B cells) and non-target cells (T cells). Next, we designed a BTK lentiviral vector with a codon optimized BTK transgene under the control of the endogenous BTK promoter (hpBTK_BTKco) and tested its function in vitro and in vivo. Transduction of hpBTK_BTKco into lin$^-$ cells derived from the XLA mouse model (Xid) , resulted in high transduction rates (up to 90%) and substantial restoration of *in-vitro* B cell maturation. Engrafting lethally irradiated Xid mice with hpBTK_BTKco transduced Xid lin$^-$ cells led to specific BTK expression in myeloid and B cells with virtually no expression in T cells. Furthermore, engrafted mice successfully restored B cell differentiation and function. Our data demonstrates that the hpBTK_BTKco expression cassette is a good candidate for development of a clinically safe and efficient BTK lentiviral vector for XLA gene therapy.

703. Hematopoietic Stem Cell Collection for the Gene Therapy of Fanconi Anemia Patients

Susana Navarro[1,2,3], Julián Sevilla[2,4], Paula Río[1,2,3], Rebeca Sanchez[1,2,3], Josune Zubicaray[2,4], Eva Galvez[2,4], Eva Merino[2,4], Elena Sebastian[2,4], Carmen Azqueta[5], Jose A. Casado[1,2,3], Jose C. Segovia[1,2,3], Omaira Alberquilla[1,2,3], Massimo Bogliolo[2,6,7], Francisco J. Román-Rodriguez[1,2,3], Yari Giménez[1,2,3], Lise Larcher[8], Rocio Salgado[3], Roser M. Pujol[2,9,10], Raquel Haldun[11], Ana Castillo[12], Jean Soulier[8], Sergi Querol[5], Jesus Fernandez[5], Jonathan Shcwartz[13], Nagore Garcia de Andoin[14], Ricardo Lopez[15], Albert Catala[16], Jordi Surralles[2,9,10], Cristina Diaz de Heredia[17], Juan A. Bueren[1,2,3]

[1]Hematopoietic Innovative Therapies Division, Centro de investigaciones Energéticas, Medioambientales y Tecnológicas, Madrid, Spain,[2]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[3]Instituto de Investigaciones Sanitarias Fundación Jiménez Díaz (IIS-FJD), Madrid, Spain,[4]Hospital Infantil Universitario Niño Jesús, Madrid, Spain,[5]Banc de Sang i Teixits de Catalunya, Barcelona, Spain,[6]IIB-Sant Pau, Hospital Sant Pau, Barcelona, Spain,[7]Universitat Autònoma de Barcelona, Barcelona, Spain,[8]Université de Paris, Institut de Recherche Saint-Louis, Paris, France,[9]Servicio de Genética e Institut de Reserca, IIB-Sant Pau, Hospital Sant Pau, Barcelona, Spain,[10]Departmento de Genética y Microbiología, Universitat Autònoma de Barcelona, Barcelona, Spain,[11]Servicio de Oncología y Hematología Pediátrica, Vall d'Hebron Institut de Recerca. Hospital Universitari Vall d'Hebron, Barcelona, Spain,[12]Análisis Clínicos Hospital Infantil Universitario Niño Jesús, Madrid, Spain,[13]Rocket Pharmaceuticals Inc., New York, NY,[14]Hospital Universitario de Donostia, San Sebastian, Spain,[15]Hospital de Cruces, Bilbao, Madrid, Spain,[16]Department of Hematology/Oncology, Hospital Sant Joan de Déu, Barcelona, Spain,[17]Servicio de Oncología y Hematología Pediátrica, Vall d'Hebron Institut de Recerca, Barcelona, Spain

The collection of clinically relevant numbers of hematopoietic stem and progenitor cells (HSPCs) represents a primary limitation for the development of efficient gene therapy in patients with Fanconi Anemia (FA). In the FANCOSTEM clinical trial we have investigated the safety and efficacy associated with the collection of filgrastim and plerixafor mobilized HSPCs in FA patients. A total of 11 patients were recruited in this study. The mobilization regimen consisted of filgrastim (12 µg/Kg/12 hours) for a maximum of 8 days and plerixafor (240 µg/kg body weight/day; from the fourth day of G-CSF administration). HSPCs were collected by apheresis when at least 5 $CD34^+$ cells/µl in PB were determined after each mobilization treatment. Two to three apheresis procedures were conducted in these patients. No procedure-related serious adverse events were observed. Nine of the eleven patients mobilized beyond the threshold level of 5 $CD34^+$ cells /µL peripheral blood (PB) required to initiate apheresis. Significantly, the eldest two patients, age 15 and 16 years, were the only ones that did not mobilize sufficient numbers of $CD34^+$ cells. In responding patients, a median value of 21.8 $CD34^+$ cells/µL PB was reached at the peak of mobilization, representing a 19.4-fold increase over basal $CD34^+$ cell PB levels. A median value of 4.3 million $CD34^+$ cells/kg was collected in 2-3 apheresis procedures, which decreased to 1.1 million $CD34^+$ cells/kg after immunoselection. In all instances, collected $CD34^+$ cell numbers fulfilled the criteria of the FANCOLEN-I gene therapy trial, and were frequently sufficient to facilitate the progressive engraftment of corrected HSPCs in non-conditioned patients (Rio et al 2019). Interestingly, mobilization of $CD34^+$ cells correlated with patient age and with several parameters determined in PB and BM at time of screening, enabling prospective identification of patients most likely to undergo successful mobilization and subsequent gene therapy.

704. Continuous Delivery of IL-10 Using the Shielded Living Therapeutics™ Platform Leads to Modulation of Immune Cell Function and Prevents Liver Damage in Preclinical Model of Immune-Mediated Hepatitis

Sofia Brites Boss, Tiffany Vo, Jie Li, Janet Huang, Lauren Jansen, David Peritt, Hozefa Bandukwala

Sigilon Therapeutics, Inc., Cambridge, MA

Autoimmune hepatitis (AIH) affects an estimated 70,000 individuals in the U.S. every year. The pathology of this disease results from a breakdown in immune tolerance leading to production of pro-inflammatory cytokines by autoreactive T-cells and subsequent hepatocyte destruction. While AIH patients benefit from immunosuppressive agents such as prednisolone and azathioprine, the chronic nature of this disease requires life-long systemic administration often resulting in serious adverse effects and associated morbidities. Immunomodulatory cytokines, such as IL-10, are well established preclinically as a way to treat autoimmune diseases including AIH. However, while these cytokines have high potency, they have an extremely short half-life, making it challenging to treat with the protein therapeutic. Thus, new approaches are essential for this treatment modality to be beneficial to patients. The use of implanted allogeneic cells, gene modified to express the cytokine transgene, has several potential safety advantages over other therapeutic modes. However, one of the major challenges of such therapies is protection from the host's immune system. While many biomaterials (e.g., hydrogels) can provide a physical barrier to allogeneic cells, they themselves can elicit an immune response which results in build-up of pericapsular fibrotic overgrowth (PFO). PFO effectively renders the physical shield impenetrable to nutrients leading to cell death and inadequate therapy duration. Sigilon utilized a library of proprietary small molecules that avoid PFO when conjugated to alginate biomaterials (Bochenek Nat Biomed Eng 2018) to create a modular, cell-based platform with potential for utilization across a range of chronic diseases. The platform consists of genetically modified allogeneic human cells engineered to produce the therapeutic protein of interest, encapsulated in a two-compartment sphere which supports the function of cells (inner compartment) and shields the cells from the host's immune system and PFO (outer layer) (Barney ASGCT 2020). We hypothesized that the sustained low-dose treatment with immunomodulatory cytokines delivered using this modular platform could restore immune homeostasis providing a functional cure for this disease. First, we engineered allogeneic cells to produce active IL-10 *in vitro*. Second, the cells were efficiently encapsulated in the two-compartment spheres and shown to produce functional IL-10. Next, the spheres were administered intraperitoneally in mice, and we observed sustained delivery of IL-10 which resulted in *in vivo* differentiation of peritoneal macrophages towards the immunomodulatory M2 phenotype marked by increased expression of CD206. Furthermore, we

demonstrated that synthetic steroids can be co-encapsulated with IL-10 producing cells, enabling localized combination therapy approach. Finally, sustained IL-10 production alone or in combination with a synthetic steroid resulted in protection of mice from acute liver injury induced by Concanavalin A. Collectively our data demonstrate that localized and sustained intraperitoneal administration of IL-10 with or without corticosteroids can provide a new, potentially safer, and more effective modality for the treatment of inflammatory liver diseases.

705. Adeno-Associated Virus Expressed Erythroferrone Neutralizing Antibody as a Novel Gene-Therapy Approach for Thalassemia

Reema Jasuja[1], Rajani Shelke[1], Sabra D. Al-Harthy[2], Vicente Pagan[1], Anagha Sawant[1], Dinesh Hirenullar-Shanthappa[2], John E. Murphy[1], Debra D. Pittman[1], Suryanarayan Somanathan[1]

[1]Rare Disease Research Unit, Pfizer Inc., Cambridge, MA,[2]Global Science Technology- Comparative Medicine, Pfizer Inc., Cambridge, MA

β-Thalassemia is an inherited blood disorder caused by a decreased synthesis of β-globin chain of hemoglobin. Mutations or deletions in the HBB gene cause hemolytic anemia with reduced mature red cells, ineffective erythropoiesis and excessive tissue iron accumulation. Iron overload in tissues is associated with multiple endocrine complications, including low bone mass/osteoporosis, and lethal cardio-hepatic consequences. Until recently, the therapeutic options for β-thalassemia were limited to blood transfusions and iron chelation. Progress in understanding the underlying pathophysiology of thalassemia has resulted in identification of Erythroferrone (ERFE) as an erythroid regulator of hepcidin synthesis and iron homeostasis. ERFE is produced in erythroblasts in response to an increased erythropoietic drive, downregulates hepcidin expression and thereby promotes intestinal iron absorption/mobilization. The levels of ERFE are inappropriately high under conditions of ineffective erythropoiesis, in inherited anemias such as β-thalassemia and congenital dyserythropoietic anemias. Regulation of ERFE is an attractive approach to treat hemoglobinopathies, such as β-thalassemia. We have previously shown that inhibition of ERFE by a neutralizing monoclonal antibody (mAb) increases hepcidin expression, reduces iron burden in serum and tissues, and improves hematopoiesis in a β-thalassemia mouse model (Arezes et al, Blood, 2020). Adeno associated virus (AAV) mediated delivery of genes encoding an anti-ERFE mAb has the potential to ameliorate complications of thalassemia as a one-time treatment. To test this hypothesis in a murine model, an AAV8.ERFE-mAb vector was designed to express anti-ERFE mAb 127 from a ubiquitous (CAG) promoter. mAb-127 binds the N-terminal region of human ERFE, neutralizes ERFE activity in vitro and cross reacts with mouse and cynomolgus monkey ERFE. Four-week old male and female thalassemia (th3+) mice were dosed intravenously at 1E10 vg/mouse or saline (n=5-7/group). Longitudinal bone mineral density (BMD) and bone mineral content (BMC) were evaluated every 3 weeks following AAV8.aErfe-mAb 127 administration. Th3+ mice demonstrated significant deficit in BMC and BMD compared to wild-type (WT) littermate controls. Treatment with AAV8.aErfe-mAb 127 resulted in increased BMC and BMD after 6 weeks compared to saline control dosed th3+ mice. In addition, a reduction in reticulocytes and splenomegaly, a measure of ineffective erythropoiesis, was also evident in AAV8.ERFE-mAb dosed th3+ mice (ANOVA $p<0.005$). An increase in hemoglobin and reduction in anemia ($p=0.068$) was also observed in AAV8.ERFE-mAb dosed th3+ mice. Our data support ERFE inhibition by AAV expressed neutralizing antibody as a novel means to improve hematopoiesis and reduce the consequences of iron overload on bone mineral density and content.

706. *In Vivo* HSC Gene Therapy with Base Editors Allows for Efficient Reactivation of Fetal Gamma-Globin in Beta-YAC Mice

Chang Li, Aphrodite Georgakopoulou, Sucheol Gil, Andre Lieber

Division of Medical Genetics, University of Washington, Seattle, WA

Base editors can install precise nucleotide mutations at targeted genomic loci and present the advantage of avoiding double-stranded DNA breaks. Here, we aimed to target critical motifs regulating γ-globin reactivation with base editors delivered via HDAd5/35++ vectors. Through optimized design, we successfully rescued a panel of cytidine and adenine base editors (CBE and ABE) targeting the *BCL11A* enhancer or recreating naturally occurring Hereditary Persistence of Fetal Hemoglobin (HPFH) mutations in the *HBG1/2* promoter. In HUDEP-2 cells, all five tested vectors efficiently installed target base conversion and led to γ-globin reactivation. We observed significant γ-globin protein production (~23% over β-globin) by using an ABE vector HDAd-ABE-sgHBG#2 specific to the -113A to G HPFH mutation in *HBG1/2* promoter. This vector was therefore chosen for downstream *in vivo* hematopoietic progenitor/stem cell (HSPC) transduction studies in mice that carry 248kb of the human β-globin locus (β-YAC mice) and thus accurately reflect globin switching. An EF1α-mgmt^{P140K} expression cassette flanked by *frt* and transposon sites was included in the vector for allowing *in vivo* selection of transduced cells. After *in vivo* HSPC transduction with HDAd-ABE-HBG#2 + HDAd-SB and low doses of chemoselection, an average of over 40% HbF-positive cells in peripheral red blood cells was measured. This corresponded to ~21% γ-globin production over human β-globin. The -113 A to G conversion in total bone marrow cells was on average 20%. Compared to untransduced mice, no alterations in hematological parameters, erythropoiesis and bone marrow cellular composition were observed after treatment, demonstrating a good safety profile of our approach. No detectable editing was found at top-scored potential off-target genomic sites. Bone marrow lineage negative cells, isolated from primary mice at week 16 after transduction, were capable of reconstituting secondary transplanted mice with stable HbF expression. Importantly, the advantage of base editing over CRISPR/Cas9 was reflected by the markedly lower rates of intergenic 4.9kb deletion and no detectable toxicity in human CD34$^+$ stem cells. Ongoing efforts aim to further improve the editing efficacy by using latest versions of base editor variants, such as ABE8e. Our observations demonstrate that base editors delivered by HDAd5/35++ vectors represent a promising strategy for precise *in vivo* genome engineering for the treatment of β-hemoglobinopathies.

707. A Platform for CAR-TREG Generation for Autoimmune Diseases

Matteo Doglio[1], Matteo Bonfanti-Lombardi[1], Zulma Magnani[1], Barbara Camisa[1], Mattia Di Bono[1], Monica Casucci[2], Angelo Andrea Maria Manfredi[3], Chiara Bonini[1]

[1]Experimental Hematology, San Raffaele Scientific Institute, Milan, Italy,[2]Innovative Immunotherapies, San Raffaele Scientific Institute, Milan, Italy,[3]Autoimmunity and Vascular Inflammation, San Raffaele Scientific Institute, Milan, Italy

Introduction: Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by an abnormal inflammatory response against nuclear antigens due to a loss of the immune tolerance with consequent damage to healthy organs. Auto-reactive B cells with autoantibody production and immune complex formation have a fundamental role in the pathogenesis of the disease. Regulatory T cells (Tregs) are a subset of T lymphocytes endowed with immune suppressive capabilities. Tregs control the activation of the immune system, preventing unwanted inflammatory reactions and preserving the immune tolerance against self-antigens. An impaired Treg functionality is involved in SLE, where they do not manage to control the inflammatory response. Adoptive transfer of polyclonal Tregs has been employed to restore the immune tolerance in different autoimmune diseases, achieving unsatisfactory results due to a low frequency of antigen specific suppressive cells. Chimeric Antigen Receptors (CARs) are chimeric molecules capable of redirecting T cell specificity against target antigens, greatly enhancing T cell activity. CAR-Tregs proved effective in controlling the inflammation in pre-clinical mouse models of autoimmune diseases different from SLE. **Aim of the project:** The aim of the project is to employ the CAR technology to redirect and enhance Treg activity in order to exploit their immune suppressive capabilities to restore immune tolerance in Systemic Lupus Erythematous.**Methods:** We sorted CD4+CD25+ cells from peripheral blood of Healthy Donors using magnetic separation beads. Next, we expanded Tregs using anti-CD3/CD28 magnetic beads in the presence of IL-2 and rapamycin. Considering the role of B cells, we transduced CAR-Tregs with a Lentiviral Vector encoding for a second generation anti-CD19 CAR, constituted by the intracellular portion of both CD28 and CD3-zeta to provide a more physiological stimulation. GFP was employed as transduction marker. Starting from day +14 since the initial stimulation, we performed functional assays. Immune and suppression assays were evaluated with flow cytometry. **Results:** We obtained a cell product highly enriched in Tregs and with a transduction efficiency of 50%. CAR-Tregs retained their immune suppressive capabilities and performed equal to untransduced Tregs in suppressing the proliferation of autologous lymphocytes upon polyclonal stimulation. Noticeably, CAR-Tregs, but not untransduced Tregs, suppressed autologous B cell proliferation, demonstrating an antigen-specific suppression capacity. When co-cultured with CD19+ tumor cells, CAR-Tregs did not display killing capacities, but rather an impressive proliferation activity. More strikingly, anti-CD19 CAR-Tregs suppressed conventional anti-CD19 CAR-T cell killing when co-cultured together in the presence of a CD19+ tumor cell line. Finally, we tested the immune suppressive capacities of anti-CD19 CAR-Tregs in a humanized mouse model of lupus. Engineered cells delayed the occurrence of leukopenia compare to untransduced regulatory lymphocytes.**Conclusions:** In conclusion, we efficiently generated anti-CD19 CAR-Tregs and proved their efficacy both in vitro and in an in vivo humanized mouse model of lupus.

708. Exploring the Use of a DSG2-Targeting Adenoviral Junction Opener-Doxorubicin Conjugate for Hematopoietic Stem Cell Depletion

Jiho Kim[1], Darrick Carter[2], Andre Lieber[1]

[1]Division of Medical Genetics, University of Washington, Seattle, WA,[2]PAI Life Sciences, Seattle, WA

HSC transplantations are widely used in oncology and gene therapy. The current methods for myelo-conditioning/ablation in patients that will receive an HSC transplant involve total body irradiation or high-dose chemotherapy (e.g. busulfan). These regimens "to make space" in the bone marrow for transplanted HSCs can cause morbidity and mortality in recipients, because they indiscriminately target all dividing cells in the bone marrow and body. To address this, we identified desmoglein-2, a desmosomal cell junction protein, to be expressed on hematopoietic stem cells (HSC), indicating potential for DSG2 to be a target for HSC-depleting agents. A recombinant adenovirus-type 3 protein, Junction Opener (JO), has been developed to target DSG2 with nanomolar affinity and could act to target DSG2-expressing HSC specifically. We have also synthesized a conjugate of JO to liposomal doxorubicin (JOC-Doxil) and its use in specifically targeting HSCs in the bone marrow could be warranted and further explored. Preliminary experiments of JOC-Doxil in DSG2-transgenic mice suggest a cytotoxic impact on certain HSCs (LSK cells) in the bone marrow, and JOC-Doxil administration in *M. fascicularis* indicated a close colocalization of DSG2-expressing HSC and JO in the bone marrow, indicating JOC-Doxil's specific localization to its targets. An HSC-specific cytotoxic agent such as JOC-Doxil could alleviate many of the toxic side effects caused by broad lymphotoxins or radiation used in hematopoietic cell depletion and represents a promising development in the field of hematopoietic stem cell transplantation.

709. Treatment of Canine Hemophilia A via Intraosseous Delivery of a Platelet-Specific Factor VIII-Lentiviral Vector

Cameron William Rementer[1], Chong Li[1], Timothy C. Nichols[2], Xiaohe Cai[1], Julia Joo[1], Xuefeng Wang[1], Elizabeth P. Merricks[2], Lauren E. Wimsey[2], Hans D. Ochs[1], David J. Rawlings[1], Carol H. Miao[1]

[1]Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]University of North Carolina, Chapel Hill, NC

Hemophilia A (HemA) is a genetic disease resulting from a factor VIII (FVIII) deficiency. Traditional protein infusion to treat HemA is costly, inconvenient, and requires repeated dosing. We demonstrated previously that intraosseous (IO) gene therapy via delivery of lentiviral vectors (LVs) into bone marrow targeting FVIII expression in platelets successfully treated HemA mice, including mice that had developed FVIII inhibitors. To facilitate the translation of this novel strategy to clinical application, we investigated the treatment of HemA dogs using IO gene therapy. The VSVG-pseudotyped G-cF8-LV incorporating a

platelet-specific promoter Gp1bα and canine FVIII gene was injected into the tibia or iliac bones of 4 HemA dogs. Prior to injection, the dogs were treated with cFVIII protein to prevent excessive bleeding and an immune modulation regimen of dexamethasone and prednisone to minimize the immune response. The injection volume for each dog was 4.5 ml at a rate of 0.2 mL/min. Following the procedure, blood samples were taken over time in order to help determine the safety and efficacy of this approach. All dogs recovered well from the procedure and had blood chemistry values within normal ranges. Gene expression of cFVIII was examined in platelets and plasma isolated from LV-treated dogs by ELISA and aPTT assays. Canine FVIII can be detected in platelets with the highest expression around 40-50 days post-procedure and expression persisted for the experimental duration in all treated dogs. No FVIII expression was detected in the dog plasma. Genomic DNA was isolated from peripheral mononuclear blood cells (PBMCs) and used to measure the copy number of the transgene, which was found to be 12.40±6.41 copies/1000 cells. The correction of HemA phenotype was evaluated by whole blood clotting time (WBCT) and thromboelastography (TEG) testing. WBCT was shortened in multiple time points shortly after IO gene therapy, indicating improved hemostasis. Similarly, all four parameters (R time, K time, α angle and MA) examined by TEG indicated partial correction of blood clotting. Furthermore, the IO gene therapy was well tolerated and did not produce any toxicity in treated dogs as evaluated by CBC and blood chemistry analysis. Encouragingly, the first 3 dogs treated experienced fewer bleeding events per year after gene therapy treatment compared with the baseline prior treatment and this effect is being evaluated in the most recently treated dog. We have established an IO-LV gene therapy protocol to treat HemA dogs successfully with persistent effects of treatment over 2+ years. Our study demonstrated a potential strategy for safe and effective application of gene therapy in vivo for treating HemA patients.

710. Use of Retrotransposable mRNA Elements for Stable Non-Viral Gene Delivery

Namita Bisaria, Ece Bapum, Tyler Nicholson, Marie Didiot, Inna Shcherbakova, Yuxiao Wang, Daniel Getts
Myeloid, Cambridge, MA

Efficient delivery of transgenes is to leukocytes is a key step in the successful engineering of these cells for therapeutic applications. Existing technologies for stable gene integration, including viral and CRISPR based approaches can be expensive, inefficient and are not applicable to all cell types due to their immunogenic effects of viral proteins and DNA templates. An ideal tool would use mRNA for stable transgene integration—making use of the safety profile, broad cell-type applicability, and ease of manufacturing of mRNA compared to lentiviruses and CRISPR drug products. Here we present progress in using LINE-1 retrotransposon mRNA to deliver large (>4 kb) gene cargos. We show that expression of optimized LINE-1 retrotransposon mRNAs lead to reverse-transcription of gene cargos placed in the 3' UTR of the mRNA and can be used to delivery chimeric antigen receptors to T cells and monocytes. Importantly, gene integration is stable, with receptors expressed from these transgenes showing functional activity. For instance the expression of CAR in T cell lines imparted tumor cell specific activity. Similarly, retrotransposon delivery of a CAR designed to induce phagocytosis to monocytes, imparted tumor cell recognition and engulfment abilities. Taken together, we show that retrotransposons represent a novel gene delivery tool that can be used to engineer leukocytes. The versatility and cost of this approach have the potential to revolutionize gene and cell therapy and support the continued development of this unique approach.

711. Abstract Withdrawn

712. First-in-Human Gene Therapy Study of BAY 2599023 in Severe Hemophilia A: Long-Term Safety and FVIII Activity Results

Steven W. Pipe[1], Charles Hay[2], John P. Sheehan[3], Toshko Lissitchkov[4], Elke Detering[5], Francesca Ferrante[6]
[1]University of Michigan, Ann Arbor, MI,[2]Manchester University, Manchester, United Kingdom,[3]University of Wisconsin, Madison, WI,[4]National Specialized Hospital for Active Treatment of Haematologic Diseases, Sofia, Bulgaria,[5]Bayer, Berlin, Germany,[6]Bayer, Basel, Switzerland

Introduction: Gene therapy for hemophilia A has the potential to reduce the treatment burden for patients and care providers. It may eliminate the need for factor VIII (FVIII) prophylaxis by facilitating the long-term expression of endogenous FVIII at levels sufficient to normalize coagulation. BAY 2599023 is an adeno-associated virus (AAV) vector with capsid serotype hu37 (AAVhu37), and a genome that directs expression of B-domain-deleted human FVIII under the control of a liver-specific promoter/enhancer combination optimized for liver transgenic expression, used for the first time in humans. Preclinical studies have demonstrated the ability to achieve FVIII expression at therapeutic levels with a good safety profile. This analysis reports the safety and FVIII activity achieved to date with BAY 2599023 for the first six patients enrolled sequentially in three cohorts of the first-in-human study. **Methods:** This phase 1/2, open-label, dosefinding study (NCT03588299) included male patients with severe hemophilia A who each received a single intravenous infusion of AAVhu37. Patients were ≥18 years with >150 exposure days to FVIII products, no history of FVIII inhibitors, and no detectable immunity to the AAVhu37 capsid. Primary endpoints were adverse events (AEs), serious AEs (SAEs) and AEs/SAEs of special interest (S/AESIs). Secondary endpoint was FVIII activity over time. Informed patient consent and ethics committee approval were obtained. **Results:** Three cohorts of two patients each (N = 6) were enrolled sequentially. At the cutoff date (11 January 2021), in Cohort 1 (0.5×10^{13} GC/kg), a follow up of 23 months of safety observation was reported with no SAEs, study-drug-related AEs or S/AESIs. After 12 months from treatment, one patient dropped out for personal reasons. Two additional patients each were sequentially enrolled into the second (1.0×10^{13} GC/kg) and third (2.0×10^{13} GC/kg) dose cohorts, with completion of 17 and 13 months of observation, respectively. In Cohort 2, one AESI, mild elevation in alanine aminotransferase (ALT), was recorded in patient 3. There were no associated clinical symptoms, or loss of FVIII activity levels. A short course of corticosteroid treatment resulted in a rapid return of ALT to the normal range. In Cohort 3, both patients had mild or moderate increases in transaminases without associated symptoms or loss of FVIII expression with ongoing corticosteroid treatment. No SAEs have been reported to date in any cohort. All patients with evaluable FVIII activity data ranging from 9-23 months have shown

durable and sustained FVIII levels over time. No spontaneous bleeds, nor any other bleeds requiring treatment have occurred after achieving FVIII levels >11%. **Conclusion:** In this first-in-human study, six patients were treated with escalating doses of BAY 2599023. Mild to moderate elevations of ALT, managed with corticosteroids, were the only observed AEs related to BAY 2599023. Sustained expression of endogenous FVIII was observed in all patients with indications of hemostatic efficacy.

713. Lentiviral Vector Gene Therapy in *Rag1* Hypomorphic Mice: Treating Immunodeficiency in the Context of Immune Dysregulation

Valentina Capo[1,2], Maria Carmina Castiello[1,2], Elena Draghici[1], Sara Penna[1,3], Martina Di Verniere[1,4], Elena Fontana[2,5], Lucia Sereni[1], Paolo Uva[6], Luigi D. Notarangelo[7], Karin Pike-Overzet[8], Frank J. T. Staal[8], Anna Villa[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Milan Unit, CNR-IRGB, Milan, Italy,[3]Dimet, University of Milano-Bicocca, Monza, Italy,[4]Vita-Salute San Raffaele University, Milan, Italy,[5]Humanitas Clinical and Research Center - IRCCS, Rozzano, Italy,[6]IRCCS Istituto G. Gaslini, Genoa, Italy,[7]Laboratory of Clinical Immunology and Microbiology, NIAID, NIH, Bethesda, MD,[8]Leiden University Medical Center, Leiden, Netherlands

Introduction. Severe combined immunodeficiency (SCID) is a life-threatening rare genetic disease and recombinase activating genes (RAG) 1 and 2 defects are the most frequent. The current treatment is the hematopoietic stem cell transplant, although donor availability is still limited. In the last decade, lentiviral vector (LV) gene therapy (GT) has been proposed as an alternative, effective treatment in *Rag* knock-out models and successful preclinical development for RAG1 was recently published. However, RAG mutations often allow residual activity and patients present with a broad spectrum of clinical manifestations, ranging from Omenn Syndrome to combined immunodeficiency associated with granulomas and/or autoimmunity (CID-G/AI). The GT in these patients poses additional risks, such as worsening of autoimmune manifestations or inflammatory complications. To provide insight on the efficacy of GT in an immune dysregulated context, we applied LV GT to 2 different *Rag1* hypomorphic murine models. *Methods*. We exploited *Rag1F971L* and *Rag1R972Q* murine models that carry mutations described in patients with CID-G/AI. Lin- cells from mutant mice were transduced with a LV encoding *RAG1* under the control of *MND* promoter. GT cells were transplanted into irradiated *Rag1*-mutant recipients. *Results*. Starting 6 weeks after transplant, we observed an increase of white blood cell (WBC) counts in GT mice, compared to mice transplanted with untransduced cells (BMT UT mice). However, WBC remained lower than mice transplanted with WT Lin- cells (BMT WT mice). A similar trend was obtained for B cells and T cells, while myeloid cells markedly decrease, confirming immune subset redistribution. Immune cell functionality was tested by T-dependent antigen challenge. In Rag1F971 GT mice, analysis of TNP-KLH-specific IgM and IgG serum levels showed an amelioration of antibody response. In Rag1R972Q GT mice we detected improvement in TNP-KLH specific IgG response. At sacrifice, mean VCN was 2.4 in the bone marrow (BM), 3.1 in the spleen and 2.1 in the thymus. We observed the appearance of immature and mature recirculating B cells in BM, although at lower counts than BMT WT mice. In thymus we observed the overcome of double negative block and a modest improvement in the medulla/cortex ratio. In the periphery, despite reduced cellularity, splenocytes showed an amelioration of B, T cell proportion and a parallel decrease of myeloid and NK cells. We assayed the autoantibody production in GT mice and analysis is ongoing. *Conclusions*. GT allows the amelioration on lymphopenia in *Rag1* hypomorphic mice models, although absolute counts remain lower than WT-transplanted mice. Autoantibodies production is not exacerbated by irradiation and GT, but deep histological analysis for lymphocyte infiltration remains to be done, especially after viral infection. *Acknowledgements.* This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No. 755170 (RECOMB).

714. NHF Builds a Community-Driven National Research Blueprint for Inherited Bleeding Disorders

Leonard Valentino[1,2], Michelle L. Witkop[1], Maria E. Santaella[1], Donna M. DiMichele[3], Michael Recht[4,5]

[1]National Hemophilia Foundation, New York, NY,[2]Rush University, Chicago, IL,[3]Weill Cornell Medical College, New York, NY,[4]American Thrombosis and Hemostasis Network, Rochester, NY,[5]The Hemophilia Center at Oregon Health & Science University, Portland, OR

Background: The inherited bleeding disorders (IBD) community has witnessed significant advances in recent years, yet important gaps persist, particularly for those with rare disorders and underserved populations. A new initiative shaped by the voices of the patient community is underway to design and implement a national research roadmap to accelerate progress through coordinated collaboration. Objectives: To design and implement a national research blueprint that embraces patient-centric principles to address the priorities of the IBD community. Methods: Virtual community listening sessions were conducted with groups representing adults with IBDs, caregivers, patient organizations, chapter and member organization directors, healthcare providers (HCPs), and industry. These discussions were supplemented by surveys to patients and caregivers as well as the HCP community. Additional insights will be gathered through NHF's community powered registry, Community Voices in Research. The emerging themes will inform action by a newly established Steering Committee and expert working groups, which will convene for a State of the Science (SOS) Research Summit in September 2021. Results: In total, 78 individuals participated in 9 listening sessions: 31% with an IBD, 5% caregivers, 51% (HCPs) and 35% chapter leaders. Additionally, two sessions were conducted with 33 health professionals from a variety of disciplines, and two sessions were hosted with 16 members of industry representing seven global organizations. A survey directed to community members was developed by chapter and member organization executive directors. This was distributed to their members as well as the multidisciplinary HCPs who staff the treatment centers. This survey offered a wider community the opportunity to rank and comment on the priorities identified through the listening sessions. Key themes have emerged to be addressed by multidisciplinary working

groups, (Table 1) including use of new technologies for therapeutic advancements, such as application of precision medicine, genetic testing and point of care diagnostics, as well as continued pursuit of novel therapies for underserved disease segments. In addition, community input raised the need for improvements in comprehensive care, such as mental health, diagnosis, and supportive care; rare IBDs and health disparities; and infrastructure, resource planning and workforce development. During the planned September 2021 State of the Science Summit these themes will be consolidated into actionable, prioritized focus areas that can guide IBD community research. Conclusions: Key themes will continue to be ranked and prioritized throughout the spring and summer by the working groups as they seek to move research gaps and priorities into researchable questions. Actively soliciting the community's views is central in our process to advance research in IBDs. This blueprint will help to guide community research that could fundamentally redefine the experience of living with these disorders. Input and participation from the scientific research community will be key to ensure the success of this process.

Research priorities	
Working Group Scope	**Focus Areas**
1. Research Priorities for Hemophilia A & B	• Across the spectrum of disease Understudied areas including women
2. Research Priorities for von Willebrand Disease, Platelet Dysfunction and other mucocutaneous inherited bleeding disorders	• Across genders and phenotypes Diagnostics and therapeutic
3. Research Priorities for Ultra-Rare Bleeding Disorders	• Across genders and phenotypes Diagnostics and Therapeutics
4, Research Priorities for health of women & girls and those with the potential for menstruation	• Von Willebrand Disease • Platelet Disorders Female biology and phenotype
5. Facilitating Priority Research in the Bleeding Disorders Community	• Infrastructure • Resource Procurement/ Development Workforce Development
6. Diversity, Equity & Inclusion Health Services Research & Implementation Science	• SOC Implementation • DE&I health services research • Telehealth and delivery network development Communications

715. Anti-c-kit CAR-T Cells Afford Effective Eradication of Human AML and Normal Hematopoietic Cells in a Preclinical Model of Safer Non-Genotoxic Stem Cell Transplant Conditioning

Nina Timberlake

Poseida Therapeutics, San Diego, CA

Each year in the U.S. over 8,000 patients with hematological disorders are treated with myeloablative conditioning therapy prior to reconstitution by transplanted hematopoietic stem cells (HSCs). These conditioning regimens typically consist of high doses of genotoxic radiation or busulfan that can lead to life-threatening post-transplant complications. This has prompted the investigation of more targeted and less hazardous approaches to specifically deplete endogenous hematopoietic cells in the bone marrow that thereby provides a vacant stem cell niche for engraftment of donor HSCs. Antibodies broadly directed against HSC antigens, such as c-kit (CD117) and CD45, have been considered for transplant conditioning. This approach, however, is limited by the slow rate of antibody clearance which delays time to transplant, and wide biodistribution which limits efficacy and increases the possibility of toxicity towards non-HSC antigen-bearing cells. Alternatively, short-lived, chimeric antigen receptor (CAR)-T cells with bone marrow-homing capability may provide more effective, selective, and safer depletion of resident HSCs. We have constructed various PB vectors encoding a CAR targeting human c-kit, which is highly expressed on HSCs, as well as myeloid malignancies such as acute myeloid leukemia (AML). In addition to the CAR, the transposon encodes a selection gene for generation of entirely pure CAR+ product, and an inducible and fast-acting safety switch to enable rapid clearance of the reactive CAR-T cell product prior to donor HSC transplant. Initial pilot experiments in humanized (hu)NSG mice, demonstrated that CAR-T cells expressing our lead anti-c-kit binder (CAR 1), become activated and rapidly proliferate in response to target antigen, depleting up to 92% of human CD34+ stem and progenitor cells in the bone marrow within 48 hours. To identify a CAR-T product with further enhanced and consistent stem cell killing capacity, we developed a luciferase-expressing Nomo-1 human xenograft model of AML for rapid screening of our CAR-T cells. Nomo-1 cells endogenously express c-kit and readily engraft and form disseminated disease in NSG mice that is highly localized to the marrow compartment. Using this model and bioluminescence imaging, we screened 6 additional CAR-T product candidates, expressing different c-kit binders. We identified an improved anti-c-kit CAR-T product candidate (CAR 2) that significantly prolongs survival and exhibits enhanced target cell killing. Leukemic cell killing was estimated to be >99% (versus 93% killing with CAR 1), and exceeded the killing achieved following a single dose of 30 mg/kg busulfan. To compare the activity of CAR 1 and CAR 2 in a transplant model we are currently performing a full allogeneic transplant study in huNSG mice involving CAR-T cell-mediated depletion of engrafted human stem cells, safety switch activation to clear the CAR-T cells, followed by engraftment of second donor CD34+ cells. We anticipate our results will provide additional strong evidence for use of stem cell-directed CAR-T cells as an alternative and less toxic conditioning regimen to facilitate engraftment of allogeneic and gene-corrected autologous HSCs and to significantly broaden the number of patients eligible for transplant.

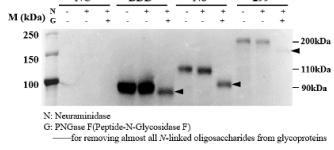
716. Lentiviral Gene Therapy of Hemophilia A - Functional Improvement Based on FVIII B Domain Glycosylation Modification

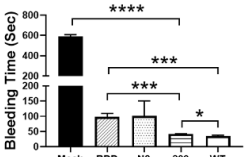
Jie Gong[1], Tsai-Hua Chung[1,2], Lung-Ji Chang[1,2]

[1]Medicine, UESTC, Chengdu, China,[2]Shenzhen Geno-Immune Medical Institute, Shenzhen, China

Background: Hemophilia A (HA) is a coagulation disorder caused by X-linked genetic deficiency of the factor VIII (F8) gene. Although protein replacement therapy (PRT) is the current treatment of HA, it has several limitations including short half-life, high cost

and requirement for life-long treatment. Gene therapy of HA is an attractive solution. **Methods:** We developed an advanced lentiviral vector (LV) system encoding modified F8 transgenes for functional improvement. A codon optimized, B-domain deleted F8 (F8BDD) gene was synthesized with glycosylation modifications. The F8 processing and coagulation functions were investigated *in vitro* and in F8-knockout (KO) mice. **Results:** In an endothelial cell (EC) system, we detected large amount of F8-derived proteins in the supernatants of the F8BDD-transduced ECs but mainly were processed proteins with low coagulation activities. To investigate F8 processing and function, we designed two novel F8 genes with modified glycosylation sites in the B domain, one with an insertion of 8 specific N-cleavage sites (N8) and the other restored all of the N-cleavage sites (299) in the B domain. Western blot analysis detected secretion of the full-length protein from F8BDD-299 (~200 kDa) but not F8BDD or F8BDD-N8 (~90-110 kDa) transduced ECs. Further analysis with neuraminidase and glycosidase inhibitors confirmed the post-translational modification of the two modified F8BDD constructs (left Figure, arrowheads showing band shift after inhibition). We next performed functional assays based on activated partial thromboplastin time (aPTT) test and two-stage chromogenic assay of coagulation process of F8 activity (FVIII:C). The results demonstrated shorter coagulation time and higher coagulation activity per IU/ml (100~800 folds) from F8BDD-N8 and F8BDD-299 ($P<0.001$) as compared with F8BDD. Interestingly, the F8BDD-299 showed improved functional secretion of full-length protein but at reduced levels. To evaluate the therapeutic effect, F8 KO mice were treated with hematopoietic stem cells (HSCs) transduced with the three F8 LVs. The FVIII:C of the 299 gene therapy recipients increased over time from 5% to 10%, and remained stable in plasma, with activities slightly higher than the BDD and N8 recipients. Blood bleeding time from tail clipping test did not differ significantly between BDD and N8 recipients ($P = 0.16$), but 299 recipients bled for a much shorter time, close to WT mice (right Figure). Quantitative analysis of vector DNA in the LV-F8 HSC-transplanted KO mice detected stable LV integration in the liver, spleen and kidney, but not in the heart, lung and testis. There were ~2% transduced cells in the blood after 8 weeks in the 299 recipients, but fewer were detected in the BDD or N8 recipients. Flow cytometry analyses of the liver and spleen showed F8 expression in megakaryocytes and endothelial cells but not macrophages or lymphocytes. Furthermore, anti-F8 antibody inhibitor formation was not detected in the 299 recipients but was markedly increased in the BDD and N8 recipients 60 days after transplantation. Thus, the *in vivo* studies illustrated that the bleeding phenotype was corrected after F8BDD-N8 and -299 modified HSC transplantation, and the 299 gene transfer was associated with reduced immunogenicity. **Conclusion:** The modified F8BDD-299 LV supported synthesis of low immunogenic and increased functional F8 proteins and illustrated increased LV packaging efficiency, which could translate into an improved gene therapy treatment for HA.





*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001

717. Postnatal Challenge with mcoET3 Cell/Gene Product in a Large Animal Model of Prenatal Therapy

Martin Rodriguez[1], Brady Trevisan[1], Sunil George[1], Ritu M. Ramamurthy[1], Jordan Shields[2], Rebecca Combs[3], Jorge Figueroa[4], Michael Gautreaux[5], John Owen[3], Anthony Atala[1], Christopher B. Doering[2], H Trent Spencer[2], Christopher D. Porada[1], M Graca Almeida-Porada[1]

[1]Fetal Research and Therapy Program, Wake Forest Institute for Regenerative Medicine, Winston Salem, NC,[2]Dpt. of Pediatrics, Emory University, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, GA,[3]Special Hematology Laboratory, Wake Forest School of Medicine, Winston Salem, NC,[4]Center for Research in Obstetrics and Gynecology, Wake Forest School of Medicine, Winston Salem, NC,[5]HLA/Immunogenetics and Immunodiagnostics Laboratories, Wake Forest School of Medicine, Winston Salem, NC

We have previously reported that prenatal therapy (PNT) of sheep fetuses (n=14) with human placental cells transduced to express mcoET3, a bioengineered fVIII transgene (PLC-mcoET3), at a dose of 10^7-10^8 cells/kg, increased plasma fVIII activity levels by 57±7.7%, 42±15.6%, and 35±17.2% at 1, 2, and 3 years post-PNT, respectively. We also showed that, throughout the duration of the study, PNT recipients remained tolerant to the therapeutic product as demonstrated by the absence of mcoET3-specific IgM and IgG antibodies, using an ET3-specific ELISA, and mcoET3-reactive T lymphocytes, by ELISpot. To investigate the development of PLC-specific immune responses, a Luminex HLA assay using a panel of human-reactive antigens and one way-MLR were performed. No human HLA-reactive antibodies or T lymphocytes were found. At 6-18 months of age, PNT animals received either weekly i.v. infusions of PLC-mcoET3 (cells producing 20 IU/kg/24h) for 3 weeks (n=3), or weekly i.v. infusions of purified ET3 protein (20IU/kg) for 5 weeks (n=3). These animals were then evaluated for the development of an immune response as described above, using lymphocytes and plasma that were collected before the first infusion at week 0 (W0), and prior to each subsequent infusion at weeks 1 through 10 (W1-W10). All animals that received postnatal weekly infusions of PLC-mcoET3 maintained elevated fVIII activity up to W10, with a 45±25% mean increase above baseline. No mcoET3-specific IgM or PLC-specific antibodies were detected in any of the animals boosted with PLC-mcoET3, and 1 out of 3 animals never developed mcoET3-specific IgG at any time point after boosting. The other 2 animals developed only transient, low titer (1:20) mcoET3-specific IgG antibodies at week 3 which disappeared by week 7. In only one animal, this low titer antibody was weakly inhibitory against hFVIII (1BU). By contrast, in the group of animals that received not the cell based-product, but purified ET3 protein, all recipients developed a robust mcoET3-specific IgG response that appeared at week 3 of infusion at titers of 1:70, 1:245 (anti-mcoET3: 21BU and anti-hFVIII: 20BU), and 1:857 (anti-mcoET3: BU=3 and anti-hFVIII: BU=36), respectively. To define the immune signaling pathways responsible for the apparent immune tolerance to PLCmcoET3 as a result of PNT, a gene expression assay of 165 multiplexed mRNA targets was carried out using mRNA from peripheral blood mononuclear cells at W0, W1, and W5. Although gene expression analysis is still ongoing for animals

infused with ET3 protein, animals boosted with PLC-mcoET3 showed upregulation of genes involved in immune tolerance (FoxP3, CD25, and BTLA) and anti-inflammatory Th2 responses (ICOS, PTGDR2, and NFATC1) starting at W1. In conclusion, postnatal challenge with the same cell/gene product as that used for PNT suggests that immune tolerance to the transgene product (mcoET3) and to the cells (human PLC) is induced by PNT and persists postnatally.Supported by NHLBI R01HL135853 and U01 HL148681

Immunological Aspects of Gene Therapy and Vaccines

718. Gene Therapy for IL1-Mediated Systemic Autoinflammatory Diseases

Mariasilvia Colantuoni[1,2], Raisa Jofra Hernandez[1], Emanuela Pettinato[1], Matteo Zoccolillo[1,3], Luca Basso-Ricci[1], Serena Scala[1], Lucia Sergi Sergi[1], Anna Kajaste-Rudnitski[1,2], Luigi Naldini[1,2], Alessandro Aiuti[1,2,4], Alessandra Mortellaro[1]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]Department of Medicine and Surgery, Tor Vergata University, Rome, Italy,[4]Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy

The central subgroup of systemic autoinflammatory diseases (sAIDs) comprises rare periodic fever syndromes characterized by uncontrolled production of the proinflammatory cytokine interleukin1 (IL1). Patients suffering from these conditions experience severe and recurrent inflammation ranging from fevers to gradual hearing loss, blindness, and organ failure due to amyloid accumulation. IL1 blockade with anakinra, the recombinant form of the IL1 receptor antagonist (IL1RA), is one of the treatments of choice for these patients. However, anakinra has a short half-life, poor tissue distribution, is a life-long treatment, and some patients respond poorly. Here, we propose a gene therapy approach based on autologous hematopoietic stem cells transduced with a lentiviral vector expressing IL1RA. We transduced mouse hemaetopoietic stem-progenitor cells (HSPCs) with a purified human IL1RA encoding lentiviral vector (LV) and a GFP LV as a control vector. We evaluated the transduction efficiency, clonogenic potential, and growth rate of HSPCs transduced with the IL1RA LV compared with the control GFPLV. IL1RA production was measured by ELISA. We generated bone marrow (BM) chimeras by transplanting C57BL6 Ly5.1 lineage negative cells transduced with IL1RA LV or GFP LV in congenic C57BL6 Ly5.2 mice. Engraftment level and immune cell reconstitution were assessed by flow cytometry, transduction efficiency in the peripheral blood by Droplet Digital PCR, and IL1RA production in the plasma by ELISA. LV mediated IL1RA gene transfer in mouse HSPCs did not alter progenitor cells' clonogenic capacity as IL1RA transduced HSPCs proliferated and differentiated similarly to GFP transduced HSPCs in vitro. There was a direct correlation between vectordose and IL1RA levels in HPSC derived supernatants. In IL1RA chimeras, HSPC transduction with IL1RALV led to robust and sustained plasma IL1RA levels. IL1RA expression at a supraphysiological level did not alter the engraftment and the clonogenic capability of HSPCs in vivo. Complete immune reconstitution of lineage-committed cells was achieved in mice transplanted with IL1RA-transduced HSPCs. LV derived IL1RA production efficiently suppressed neutrophilic infiltration in a mouse model of IL1 mediated peritonitis. The IL1RA LV transduction of BM derived mouse dendritic cells and human monocyte cell line THP1 reduced the expression of inflammatory cytokines, such as IL8 and CCL2, compared to untransduced cells. In conclusion, we successfully generated an LV delivering steady IL1RA release by mouse HSPCs in vitro and in vivo. Longterm immunological reconstitution of IL1RA expressing HSPCs was efficiently achieved in vivo and resulted in decreased IL1 mediated inflammation in a mouse model of acute peritonitis. On going studies investigate whether IL-1RA LV HSPC gene therapy transduction suppresses the exaggerated IL1 activity in cells from patients and a mouse model recapitulating sAIDs phenotype.

719. Development of an Effective Anti-Fentanyl Vaccine Based on the Highly Immunogenic Adenovirus Capsid Proteins

Bishnu P. De[1], Matthew C. Hartmann[1], Vikrum Kooner[1], Dolan Sondhi[1], Tristan H. Lambert[2], Ronald G. Crystal[1], Stephen M. Kaminsky[1]

[1]Genetic Medicine, Weill Cornell Medical College, New York, NY,[2]Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Fentanyl, a highly addictive synthetic opioid analgesic, is 50 to 100 times more potent than morphine. In addition to the high costs to society of fentanyl addiction, fentanyl is often added to heroin and cocaine, resulting in accidental death due to respiratory depression. Fentanyl is commonly associated with opioid use, overdoses and death, with >36,000 deaths in the US in 2019 and illegal use of fentanyl is continuing to proliferate with seizures of illegal fentanyl increasing 7-fold from 2012-2014. We have developed a vaccine to stop the reward of fentanyl for users and protect from inadvertent death by overdose. The vaccine is based on the concept that an evoked antibody will bind fentanyl, blocking fentanyl from reaching its cognate receptors in the CNS. The vaccine platform leverages the potent immunogens of the adenovirus capsid, to which fentanyl-like molecules are covalently conjugated. Even though fentanyl itself is not immunogenic, in the context of covalent binding to the adenovirus capsid proteins, we hypothesize that the immune system will respond with anti-fentanyl antibodies sufficient to prevent fentanyl from reaching the brain, thus preventing addictive reward and inadvertent fentanyl overdose in addicts. To assess this hypothesis, Ad5βgal, an $E1^{-}E3^{-}$ serotype 5 adenovirus coding for β-galactosidase was denatured, and the capsid proteins covalently linked to carfentanil, a fentanyl analog. The carfentanil-Ad5 was combined with the Aduplex adjuvant to create dAd-CF, the anti-fentanyl vaccine. Administration of dAd-CF to n=9 mice at 4 -12 μg/dose generated anti-fentanyl antibody titers to greater than 10^5 over 4 to 8 wk. In competition studies, the dAd-CF evoked antibody had equivalent specificity to both fentanyl and carfentanil. Compared to nonvaccinated controls, dAd-CF vaccinated mice had a 64 ± 30% reduction of fentanyl reaching the CNS ($p< 0.02$) and, in parallel, a 403 ± 92% increase of fentanyl sequestered in blood ($p<0.001$). With the knowledge that fentanyl reduces perception of pain, dAd-CF

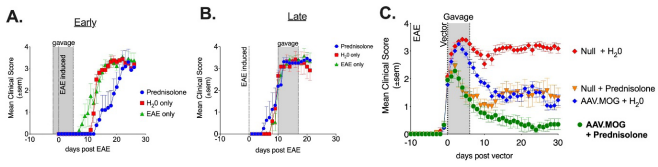
vaccinated mice challenged on a hotplate had a significant decrease in perception of pain compared to controls ($p<0.05$). In summary, based on the high immunogenicity of the $E1^{-}E3^{-}$ adenovirus gene transfer vector capsid, we have developed an anti-fentanyl vaccine that should be useful in preventing addiction reward and fentanyl overdose in addicts.

720. Reversal of Autoimmune Disease with AAV Gene Immunotherapy Is Uninhibited by Prednisolone Immune Suppression

Addelynn S. Sagadevan[1,2], Geoffrey D. Keeler[1], Isabelle Cote[1], Cristina D. Gaddie[1], Madisyn T. Maine[1], Michaela Rechdan[1], Daniel Min[1], Brad E. Hoffman[1,3]

[1]Pediatrics, University of Florida, Gainesville, FL,[2]Genetics and Genomics, University of Florida Genetics Institute, Gainesville, FL,[3]Neuroscience, University of Florida, Gainesville, FL

We previously developed a liver directed AAV gene immunotherapy vector expressing a specific neuro-protein(s) that can selectively induce antigen specific regulatory T cells (Tregs) that not only prevents disease, but can also inhibit/reverse preexisting disease in a murine model of Multiple Sclerosis (MS). Unfortunately, in clinical trials liver directed AAV gene therapies have also been shown to trigger dose dependent immune toxicities against the capsid that can limit the duration of transgene expression in hepatocytes. Corticosteroids such as prednisolone (PSL), have been broadly used across trials to modulate such capsid reactivity. It has also recently shown that the timing of immune suppression can impact the cellular immune response against transduced hepatocytes. The goal of our AAV immunotherapy is to restore immune tolerance so that long-term continuous immunosuppression therapy is unnecessary in MS patients. To achieve sustained transgene expression, it is vital to ensure that PSL's anti-inflammatory properties that mitigate capsid response does not adversely affect the ability of our gene-immunotherapy to induce antigen-specific Tregs and re-establish and maintain tolerance. Using C57BL/6 mice, the effects of early treatment and late treatment with oral gavage of PSL was evaluated in MOG_{35-55} induced EAE. For early treatment, mice received daily gavage of PSL, water only, or nothing (EAE only) beginning 2 days before disease induction for a total of 7 days (Fig A). There was a ~5-day delay in disease escalation in PSL treated mice compared to EAE only control mice. Whereas, when PSL treatment was delayed until 5 days after initial symptoms when mice had well established disease, no difference was seen in mean clinical score of disability (Fig. B). Together these results indicate that short term PSL treatment given very early or very late may delay disease onset more than affect severity.



Next, we evaluated the combined effects of AAV.MOG gene-immunotherapy and PSL in mice on day 2 after severe EAE disease began. Here, as oral PSL treatment began mice also received a single dose of AAV.MOG vector (Fig C). As in previously report data, AAV.MOG alone was able to significantly inhibit and reduce disease compared to control animals. Treatment with an AAV.NULL vector and PSL resulted in slowing the severity of disease until PSL treatment was terminated. In what appears to be a synergistic fashion, AAV.MOG in combination with oral PSL not only slowed the disease progression and severity, but it also maintained significant reversal of disease long after withdrawal of PSL (Fig. C green dots). In conclusion, this series of experiments were designed only to evaluate if the immunosuppressive properties of prednisolone (PSL) would have a negative or adverse effect on our Treg inducing AAV-based immunotherapy. Ongoing studies will evaluate PSL's protective properties against capsid reactivity. Overwhelmingly, the data presented here clearly demonstrates that the anti-inflammatory properties of PSL has no apparent inhibit or negative effect on the function and ability of our AAV.MOG vector to induce antigen-specific Treg tolerance and reverse severe MS like disease, and may in fact be synergistic in disease suppression.

721. EXOVACC™: A Novel Exosome-Based Vaccine Platform That Induces Robust, Tunable Cellular and Humoral Immune Responses in Animal Models

Timothy Soos, Nikki Ross, Christine McCoy, Olga Burenkova, Wei Zhang, Madeleine Youniss, Ke Xu, Kevin Dooley, Russell McConnell, Kyriakos Economides, James Thornton, Jonathan Finn, Sriram Sathyanarayanan

Immunology, Codiak BioSciences, Cambridge, MA

Background: exoVACC™ is a novel exosome-based vaccine platform that is designed to selectively deliver exogenous antigens, adjuvants and immunomodulators to antigen presenting cells to induce robust cellular and humoral immune responses. Exosomes are natural extracellular delivery vesicles but are inherently non-immunogenic. We investigated different antigen-adjuvant designs to better understand the properties that influence exoVACC immunogenicity. Using exosome scaffold proteins PTGFRN and BASP1, we selectively expressed the model antigen OVA either on the exosome surface, by fusion with PTGFRN, or in the lumen, by fusion with BASP1. We co-loaded different adjuvants and assessed both humoral and cellular immune responses in mice immunized through different routes of administration. **Results:** Immunization with OVA expressed on the exosome surface induced anti-OVA IgG antibody responses as early as 14 days after a single immunization which increased following bi-weekly boost immunizations. Antibody responses were induced without an adjuvant and at higher levels than in mice immunized with traditional soluble OVA and adjuvants. Notably, antibody responses were not enhanced when alum, CpG or both alum and CpG adjuvants were loaded onto exosomes. In contrast, mice immunized with exosomes expressing OVA in the lumen had very poor antibody responses even after several boost immunizations. Anti-OVA IgG antibody levels were significantly lower than in mice immunized with the same amount of soluble OVA. However, loading an adjuvant onto exosomes expressing luminal OVA induced antibody responses comparable to mice immunized with exosomes expressing surface OVA. Similarly, immunization of exosomes expressing luminal OVA without adjuvant also failed to induce

antigen-specific T cell responses even after multiple administrations and via multiple routes of administration (RoA). Loading an adjuvant induced robust T effector memory (T_{EM}) and tissue resident memory T cell (T_{RM}) responses after a single immunization via multiple RoA. Notably, the induction of antigen-specific T_{RM} was markedly superior compared to immunization with soluble OVA and adjuvant. Exosomes expressing OVA on the surface also induced robust T cell responses. OVA-specific lung T_{RM} and T_{EM} were induced via IN immunization of STING adjuvanted exosomes but also through a "prime-pull" regimen where mice were first immunized SC with adjuvanted exosomes followed by an unadjuvanted IN boost. In contrast mice immunized with soluble OVA and STING adjuvant using the prime-push regimen did not elicit robust T cell responses. Finally, we assessed the efficacy of exosomes expressing OVA in the lumen and loaded with a STING agonist adjuvant in the E.G7-OVA tumor model. A single prophylactic immunization administered either subcutaneously (SC) or intranasally (IN) significantly reduced tumor growth rate and improved survival compared to mice immunized with soluble OVA and polyI:C. 33% of mice immunized SC with the OVA exosomes had complete responses. **Conclusions:** exoVACC is a versatile vaccine platform that enables antigen-specific immune responses to be modulated through antigen orientation and adjuvant loading. exoVACC induced superior systemic and tissue resident immune responses via multiple routes of administration compared to conventional vaccine formulations in different animal models.

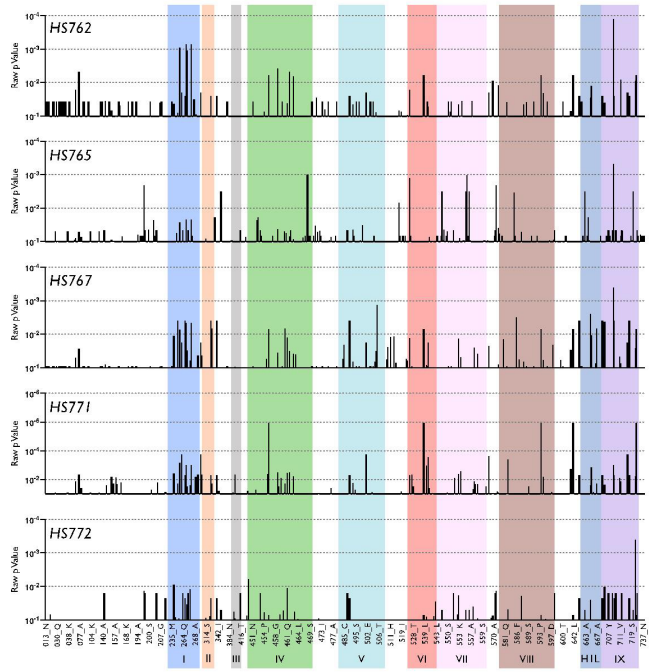
722. High Throughput Phenotype-to-Phylogeny Mapping Identifies AAV Capsid Residues Involved in Neutralization by Human Sera

Pauline F. Schmit, Eric Zinn, Cheikh Diop, Allegra Fieldsend, Ru Xiao, Luk H. Vandenberghe

Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Neutralizing antibodies pose a serious barrier to AAV gene therapy, but human polyclonal responses to natural infection are poorly understood. Reports that analyze different capsids in several non-standardized assays provide a wide range of neutralizing antibody seroprevalence in human populations. These quality of these responses however remain poorly understood, as are the primary antigenic epitopes on the capsid that drive a polyclonal response. Here, we have developed a multiplexed neutralizing antibody assay that uses barcoded AAV libraries and next generation sequencing to rapidly screen a large number of potential capsids. Libraries of putative common ancestors of circulating AAV serotypes (AncAAVs) were used to model functional intermediates between natural AAV capsids used in gene therapy and encompass a diversity of capsid sequences that could be assessed for neutralization by monoclonal antibodies or whole human serum. The multiplexed assay was able to replicate findings from clonal AAVs for both relative capsid neutralization and absolute IC_{50} values. The defined diversity of the libraries allowed us to compute correlation between individual residues and IC_{50} values using a pipeline named BANAnA (Barcoded AAV Neutralizing Antibody Analysis), revealing capsid amino acids specifically associated with differential neutralization. For the monoclonal antibodies ADK8 and ADK8/9, highly correlating, significant residues overlapped with mapped antibody footprints. For human serum, this methodology implicated a diverse range of residues influencing neutralization at different positions on the capsid surface, in particular within variable regions I and IX. This work has a wide range of applications from epitope mapping studies and rational design of the capsid, to AAV epidemiology.

Figure 1: Residues Correlated with Differential Serum Neutralization within AncAAV SuperLibrary. Raw p-values indicating correlation significance for each capsid alignment residue in structural context of variable regions for 5 unique human serum samples. Note that universally conserved amino acids do not appear in alignment, so x-axis is not to scale with primary VP structure.



723. Identification of Hematopoietic Stem Cell-Specific Cyclosporine H Interactors for Improved Gene Therapy Protocols

Giulia Unali[1,2], Ilaria Castiglioni[1], Gornati Davide[3], Vittoria Matafora[4], Monah Abou Alezz[1], Ivan Merelli[1], Marilena De Matteo[3], Romano Di Fabio[3], Angela Bachi[4], Anna Kajaste-Rudnitski[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), Milan, Italy,[2]Vita-Salute San Raffaele University, Milan, Italy,[3]Promidis S.r.l.,Torre S. Michele I, San Raffaele Hospital Science Park, Milan, Italy,[4]FIRC Institute of Molecular Oncology (IFOM), Milan, Italy

We have previously shown that the non-immunosuppressive Cyclosporine H (CsH) potently enhances VSV-G lentiviral vector (LV) based gene transfer and editing in human hematopoietic stem and progenitor cells (HSPC) by counteracting the restriction factor interferon-induced transmembrane protein 3 (IFITM3). CsH overcomes IFITM3 antiviral activity by causing its degradation in the lysosomes. However, the precise molecular mechanism remains unclear and whether CsH could provide additional beneficial effects on LV

transduction uncoupled from IFITM3 is unknown. Interestingly, while IFITM3 is efficiently counteracted by CsH in the pro-monocytic THP1 cell line, it remains insensitive to CsH in the myeloid leukemia K562 cell line indicating that CsH likely requires cell-type specific co-factors for IFITM3 degradation. We sought here to identify these co-factors through comparative CsH interactome and whole cell proteomic studies. In order to functionalize CsH for pull-down assays, a terminal biotin moiety was added to the compound. The best performing biotin-linker moiety was first selected using Cyclosporine A, a stereoisomer of CsH that also counteracts IFITM3 antiviral activity, as bait taking advantage of its well-characterized interaction with Cyclophilin A (CypA). Briefly, a panel of CsA derivatives bearing different spacers linking the olefin moiety present in the CsA side-chain with the COOH group of the terminal biotin molecule were synthesised and purified. These novel modified molecules were tested in terms of their ability to improve LV transduction and to degrade IFITM3 in the CsH sensitive THP1 and HSPC. The best CsA-derivative (dCsA) was then used to set up streptavidin-based pull-down assays. We successfully identified a condition in which the functionalized dCsA immunoprecipitated CypA, as detected by Western blot. Presence of other CsA interactors was verified by Silver staining. Based on these results, the same modifications were made on CsH. The dCsH variant was confirmed to increase transduction in HSPC to comparable levels as the unmodified compound. The CsA and CsH derivatives were used as baits in THP1 and K562 cells followed by comparative Mass-Spec analysis of the immuno-precipitated products. Functional enrichment analysis showed that the THP1-specific interactors common for dCsA and dCsH are involved in lipids and phospholipids binding, lipid metabolism, regulation of endocytosis and vesicles transport and innate immunity. Interestingly, analysis of the impact of CsH on the global proteome of primary human HSC revealed alterations in related pathways. Out of 3136 proteins identified, CsH significantly downregulated 100 and upregulated 140 proteins (T-test FDR<0.05). Pathway enrichment analysis revealed that proteins upregulated by CsH are mainly implicated in metabolic processes such as Kreb-cycle and fatty acid oxidation as well as vesicle mediated transport, whereas downregulated pathways included lipid metabolism and inositol phosphatases activity. Importantly, some of these pathways are also involved in the regulation of IFITM3 antiviral activity. Altogether, these results will help us identify the molecular mechanism behind CsH-mediated enhancement of HSPC gene engineering and contribute to our understanding of the antiviral mechanisms hampering efficient gene therapy in HSPC for the development of improved gene therapies and antiviral strategies.

724. Immunogenicity of Two AAV-Based COVID-19 Vaccine Candidates in Mouse Models of Aging and Obesity

Dawid Maciorowski, Nerea Zabaleta, Urja Bhatt, Cheikh Diop, Wenlong Dai, Reynette Estelien, Dan Li, Allison Cucalon, Abigail Sheridan, Ruchi Chauhan, Luk H. Vandenberghe

Department of Ophthalmology, Harvard Medical School, Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Immunization efforts of the SARS-CoV-2 pandemic will largely depend on the efficacy of vaccines, as well as their logistical actualization. We have developed two adeno-associated virus (AAV)-based vaccine candidates expressing different SARS-CoV-2 Spike antigens (AAVCOVID vaccines). The candidates, called AC1 and AC3, express a full-length stabilized Spike and a secreted subunit 1 (S1) of Spike, respectively. These vaccines demonstrated potent immunogenicity in mouse and nonhuman primates following a single injection. This approach is novel in many aspects since AAVs are poorly characterized for their use as vaccines and, the serotype rh32.33 of the AAVCOVID vaccine, to which no relevant pre-existing immunity exists in humans, has not been tested in human trials. Vaccine efficacy is known to be impaired in obese and aged populations, which are two of the most vulnerable groups to serious COVID-19 complications. In the context of the current pandemic and a novel vaccine platform, it is critical to understand whether we observe these same efficacy limitations for these populations. To model these conditions, 18-week and 2-year-old mice of both genders were immunized with AAVCOVID. We found that the immunogenicity of AAVCOVID is influenced by age, potent antibody responses were measured in young mice while lower titers and seroconversion percentage were detected in 2-year-old mice. In addition, we found that AC1 showed improved immunogenicity to that observed for AC3 in aged mice. To model obesity, a diet-induced C57BL/6 obesity (DIO) mouse model was used to study vaccine efficacy in inducing SARS-CoV-2 Spike-specific antibodies in overweight animals. 12-week-old C57BL/6 and C57BL/6 DIO (n=10) mice were vaccinated with 10^{10} and 10^{11} gc of our vaccine candidates. Interestingly, we found that overall obesity was not a major factor in reducing AAVCOVID immunogenicity. However, AC3 at low dose induced significantly lower binding antibody responses in obese mice compared to lean controls and failed to elicit neutralizing antibodies. This data suggests that AAVCOVID should be further evaluated in the clinical setting, especially in obese and aged populations; those that are most at risk during this pandemic.

725. Immune Suppression with Rapamycin May Cause Prolonged IgM Formation against AAV Vector

Kentaro Yamada[1], David M. Markusic[1], John S. S. Butterfield[1,2], Tom Stephen[3], Hong Wang[3], Xinyu Huang[3], Pengcheng Zhou[3,4], Jason Zhang[3,4], Roland W. Herzog[1]

[1]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN,[2]College of Medicine, University of Florida, Gainesville, FL,[3]Luye Boston R&D, Woburn, MA,[4]GeneLeap Bio., Woburn, MA

Background:

Adeno-associated virus (AAV) vector-mediated gene transfer to the liver has drawn attention to its recent success in clinical trials for various genetic diseases including hemophilia. However, there are still limitations that need to be overcome toward delivering safe, durable and reliable therapies to patients. One of the major limitations is immune responses, including formation of neutralizing antibodies (NAb) against AAV vectors. Immune suppressive drugs such as rapamycin and cyclosporine has long been utilized to mitigate immune responses

in transplantation. Rapamycin incorporated into nanoparticles has recently been shown to blunt NAb formation to AAV vectors.

Results:

We sought to identify alternative nanoparticle formulations for co-delivery of lower doses of rapamycin that could effectively prevent NAb formation against AAV in liver gene transfer. We tested three nanoparticle-encapsulated rapamycin formulations (NP1-Rapa, NP2-Rapa, and NP3-Rapa) along with free rapamycin as control to prevent anti-AAV antibody formation and allow for successful AAV vector re-administration. An AAV8 vector expressing chicken ovalbumin (ova) was intravenously injected to 8-weeks old male BL/6J mice (n=6 per group) at the vector dose of $1x10^{11}$ vg per mouse on Day 0 with or without the three different NPs formulated with 50 or 200 ug of rapamycin. Three weeks later (Day 21), mice were injected with an AAV8 vector expressing human coagulation factor IX (hFIX) with or without rapamycin loaded NPs to test for vector re-administration. 50-67 % of mice in all high-dose NP-Rapa or free rapamycin groups showed almost complete suppression of anti-AAV8 IgG2c, the IgG class formed against AAV capsid in BL6 mice, on Day 20 (i.e. the day before the re-administration). Despite this, hFIX expression levels from the second vector (AAV8-hFIX) of NP2-Rapa or NP3-Rapa treated mice were less than 10% compared to those (~46,680 ng/mL) of the mice in the control group, which received only the second vector (measurements were performed on Day 34 of the experiment). These results indicate that the re-administration of AAV8 vector was not successful. Similarly, expression of hFIX was nearly undetectable in the high-dose NP1-Rapa or free rapamycin groups. To explore a possible cause for this unsuccessful re-administration despite suppression of anti-AAV8 IgG2c, we hypothesized that other immunoglobulin subclasses may have formed against AAV8, including IgG1, IgG2b, IgG3, and IgM, and contributed to the inability to re-administer AAV vector. While the titers of IgG1, IgG2b and IgG3 were equivalent or lower in NP-Rapa or free rapamycin treated mice compared to the control group, the IgM titers in the NP-Rapa or free rapamycin groups were found to be significantly higher, despite the fact that IgM titers typically wane 14-21 days after initial antigen exposure. These results suggest that rapamycin, either in a free or nano-particle encapsulated forms, may suppress IgG2c formation but cause prolonged neutralizing IgM formation. Therefore, rapamycin prevented immunoglobulin class switch in B cells rather than completely suppressing B cell responses. Nonetheless, we were able to achieve effective re-administration by repeat administration of rapamycin in free form or formulated in nanoparticles. Nanoparticle formulation reduced IgM formation as compared to free rapamycin, indicating that nanoparticles can help reduce the duration of rapamycin-based immune suppression regimens. We are now further investigating the mechanism by which rapamycin causes the increase in IgM formation.

726. AAV Intramuscular Gene Transfer in the Non-Human Primate Model Leads to an In Situ Immune Transcriptomic Signature

Malo Journou, Mickaël Guilbaud, Marie Devaux, Nicolas Jaulin, Jean-Baptiste Dupont, Virginie Pichard, Oumeya Adjali

University of Nantes, Nantes, France

Recombinant adeno-associated virus-derived vectors (rAAV) provide a clinically relevant platform for efficient and sustained gene therapy. However, studies in large animal models have shown that rAAV-mediated gene transfer can result in a potential immune-induced loss of the transgene expression, in particular following intramuscular (IM) vector delivery. Following IM delivery of a rAAV expressing an immunogenic transgene in the nonhuman primate model, we report here that transgene expression loss can be only transitory. Despite the detection of humoral and cellular immune responses against the transgene product and the presence of CD8 T cell infiltrates in the injected muscle, we were able to recover gene expression after an initial transient loss. Functional viral genomes (vg) were detected at the site of rAAV injection until at least five years following gene transfer. Interestingly, the transcriptomic analysis of muscular cell infiltrates at the site of rAAV administration reveals a particular profile that is different from the one observed for post-injection peripheral mononuclear cells. Our results show that rAAV-mediated gene transfer can lead to an immunomodulatory in situ immune signature and that conventional monitoring of peripheral immune responses using IFNg secretion marker does not predict the fate of gene transfer.

727. AAV3B Seroprevalence Study in Human and Non Human Primates

Carmen Unzu, Amany Awad, Hongbin Xu, Florie Borel

Apic Bio, Cambridge, MA

Alpha-1 antitrypsin (AAT) deficiency is an autosomal codominant disease affecting an estimated 100,000 individuals in the United States alone. It is the most common genetic cause of emphysema, which results from low levels of active AAT. To address AAT deficiency, we have adopted a *Silence & Replace* strategy using a dual-function AAV3B vector (THRIVE™), that simultaneously silences endogenous AAT using an artificial miRNA and expresses an active AAT protein as replacement. AAV3B is a primate specific Adeno-associated virus (AAV) serotype with narrow tropism for the liver, which makes it a top candidate for AAT gene therapy. Unfortunately, one of the main limitations of AAV gene therapy is pre-existing immunity against a specific capsid, which makes patients ineligible to the treatment. To confirm that Alpha-1 patients would benefit from the dual-function AAV3B therapy, we run a seroprevalence study to identify neutralizing antibody titers in more than 100 samples from healthy donors of representative American populations (caucasian, african-american and hispanic) and Alpha-1 patients. In addition, we studied the seroprevalence of AAV3B serotype in non-human primates of different origins to inform preclinical studies. Our results showed an AAV3B seroprevalence below 20% in the human population. In addition, we were able to identify a high non-human primate inter-population variability, which is a determinant factor in animal selection for preclinical studies. In conclusion, our seroprevalence results showed low immunogenicity of the AAV3B capsid compared to other clinically relevant liver serotypes, which expands enrollment opportunity in AAV3B-mediated gene therapy clinical trials.

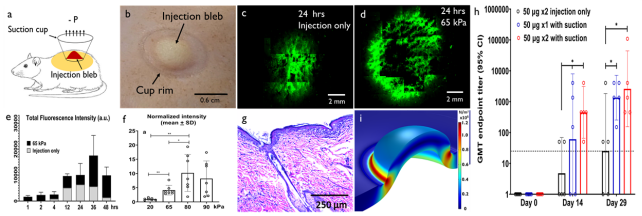
728. Abstract Withdrawn

729. A Novel, Suction-Based Transdermal DNA Vaccine Delivery Platform Induces Immunogenicity to SARS-CoV-2 in Rats

Emran O. Lallow[1], Nandita C. Jhumur[1], Ijaz Ahmed[2], Sagar B. Kudchodkar[3], Christine C. Roberts[3], Moonsup Jeong[3], Juliet Melnik[4], Sarah Park[1], Jerry W. Shan[1], Jeffrey D. Zahn[2], David I. Shreiber[2], Jonathan Singer[1], Young Park[3], Joel N. Maslow[3], Hao Lin[1]

[1]Mechanical and Aerospace Engineering, Rutgers, The State University of New Jersey, USA, Piscataway, NJ,[2]Biomedical Engineering, Rutgers, The State University of New Jersey, USA, Piscataway, NJ,[3]GeneOne Life Science, Inc., Seoul, Korea, Republic of,[4]Graduate School of Biomedical Sciences, Rutgers, The State University of New Jersey, USA, Piscataway, NJ

A critical barrier in the development of nucleic acid vaccines is the need for an effective means to deliver the mRNA or DNA vaccines to the cells. For mRNA this has required encapsulation into lipid nanoparticles to allow for target cell transfection. Methods of delivery of DNA to target cells in vivo include electroporation (EP), Gene Gun, or pressurized delivery of microdroplets, with EP being the most common technique used in recent years. EP devices generate strong electric fields of several hundreds of volts per centimeter to transiently permeabilize the cellular membrane, that can cause strong muscle contractions, pain, and tissue damage. In order to overcome the limitations of existing DNA delivery devices, as well as to address both cost and manufacturing scalability, we developed an alternative and effective device for transdermal cellular delivery. DNA plasmid delivery is accomplished through application of negative pressure of several tens of kilopascals to the skin of vaccine delivery administered ID by Mantoux injection (Fig. 1a, b). This simple method efficiently resulted in cellular uptake evidenced by GFP expression in which we compared top-view fluorescence (Fig. 1c, d). Expression was observed as early as 1 hour (Fig. 1e) whereas injection on or injection followed by EP demonstrated expression only after significant time delay (Fig. 1f). Moreover, in contrast to other methods of DNA vaccine delivery such as EP that cause significant tissue damage, histology revealed no damage to tissue structures in the epidermis and upper dermal layers caused by our novel device (Fig. 1g). Importantly, use of the device to deliver a candidate SARS-CoV-2 vaccine induced strong immune responses (Fig. 1h) characterized by rapid seroconversion. Of note, single vaccination appeared to be as effective as a two-vaccination regimen suggesting that this method of DNA plasmid delivery may provide clear benefit as part of an epidemic outbreak response. A simulation with COMSOL Multiphysics produces a stress pattern (Fig. 1i), in corroboration with the observed expression pattern and suggests novel means of endocytosis of DNA uptake into cells. graphic_{Fig. 1. a. Schematic. b. Rat-skin post suction treatment. c. and d. Top view GFP expression 24 hours on control (injection of the same plasmid only) and suction-treated skin, respectively. e. Time evolution of total fluorescence (n=3). f. Fold fluorescence enhancement as a function of pressure (n=7). g. Skin H&E staining indicates no damage post suction treatment. h. Humoral response to a SARS-CoV-2 vaccine candidate as quantified by the geometric mean titer (GMT). x1: treatment only on day 0; x2: dosage repeat on day 14. i. von Mises stress distribution from a COMSOL simulation.}$$.



730. Larkin: FDA Emergency Use Authorization of DeltaRex G for Severe COVID-19

Ellie Soheili[1], George Michel[1], Don A. Brigham[2], Erlinda Gordon[2]

[1]Internal Medicine, Larkin Community Hospital, South Miami, FL,[2]Aveni Foundation, Santa Monica, CA

Background and Rationale: Coronavirus Disease 2019 (COVID-19) causes life threatening complications such as ARDS. These complications are caused by an exaggerated immune reaction to the infection. Histopathologic examination of lung biopsies from patients with COVID-19/ARDS and lung cancer shows striking similarities including presence of abnormal Signature (*SIG*) proteins in both the damaged lung environment (DLE) of COVID-19 and the tumor microenvironment (TME) of lung cancer, and the presence of rapidly dividing cells (i.e., immune cells in DLE and cancer cells in TME). Based on these similarities, DeltaRex-G could work as a treatment for severe COVID-19/ARDS. DeltaRex-G is a disease-targeted replication-incompetent, amphotropic MLV-based retrovector that displays a *SIG*-binding peptide on its surface for targeting areas of pathology and encodes a cytocidal dominant negative human cyclin G1 construct for killing rapidly dividing cells. Injected intravenously, DeltaRex-G nanoparticles seek out and accumulate in the DLE or TME where abnormal *SIG* proteins are exposed, in the vicinity of activated cells. Guiding hypothesis: DeltaRex-G would then enter the cells via their native viral receptors, integrate into the genome of rapidly dividing T cells and activated macrophages, and kill them by blocking the G1 phase of the cell division cycle, hence, reducing cytokine release and the severity of ARDS. DeltaRex-G is currently available via an FDA approved Expanded Access Program for Stage 4 cancers (IND# 19130). **Objectives:** To evaluate the efficacy and safety of DeltaRex-G in improving survival and hastening recovery from severe COVID-19 when standard therapy has failed. **Patient and Methods:** A written informed consent will be obtained prior to treatment. Clinical status, hematologic and organ function, chest Xray/CT, acute phase reactants and IL-6 will be assessed at baseline and as needed through the course of treatment. **Route of Administration:** The patient will receive DeltaRex-G intravenously at a dose of 1.2 x 10e11 colony forming units (cfu) daily for 7 days. The dose of DeltaRex-G was calculated based on FDA approved Calculus of Parity: # circulating lymphocytes x MOI of 100. **Efficacy analysis:** Treatment outcome parameters based on NCT04292899 Odds of Ratio for Improvement on a 7-point Ordinal Scale on Day 14. The odds ratio represents the odds of improvement in the ordinal scale. The ordinal scale is an assessment of the clinical status at a given day. Each day, the worst score from the previous day will be recorded. The scale is as

follows: 1. Death; 2. Hospitalized, on invasive mechanical ventilation or Extracorporeal Membrane Oxygenation (ECMO); 3. Hospitalized, on non-invasive ventilation or high flow oxygen devices; 4. Hospitalized, requiring low flow supplemental oxygen; 5. Hospitalized, not requiring supplemental oxygen - requiring ongoing medical care (coronavirus related or otherwise); 6. Hospitalized, not requiring supplemental oxygen - no longer requiring ongoing medical care (other than per protocol DeltaRex-G administration; 7. Not hospitalized. **Safety analysis:** Treatment-emergent adverse events from first dose date up to 10 days plus 30 days will be reported. Further, vector-related adverse events including detection of RCR in PBLs, vector neutralizing antibodies, and vector integration in non-target organs at 3, 6, 12 months will be reported. If negative, yearly test samples will be archived for 15 years. All Grade III or IV toxicities will be reported in the clinical study summary report. In the event of death, an autopsy report will be submitted if an examination was conducted. **Results**: will be presented.

731. Establishment of Serum Cytokine Profiles in Normal and Duchenne Muscular Dystrophy Dogs for Gene Therapy Studies

Dennis Ossie Lopez[1], Matthew Burke[1], James Teixera[1], Chady Hakim[1,2], Dongsheng Duan[1,3,4]

[1]Molecular Microbiology and Immunology, University of Missouri-Columbia, Columbia, MO,[2]National Center for Advancing Translational Sciences, NIH, Bethesda, MD,[3]Department of Neurology, University of Missouri, Columbia, MO,[4]Department of Biomedical Sciences, University of Missouri, Columbia, MO

Duchenne muscular dystrophy (DMD) is the most common lethal muscle disease caused by dystrophin gene mutation. It affects 1 boy per 5,000 male birth worldwide. Adeno-associated virus (AAV)-mediated gene therapy is one of the most promising approach to treat DMD. A problem using AAV-mediated gene therapy is the immune response against the viral vector and the therapeutic protein. Different strategies have been developed to study the AAV immune response. Among these is the quantification of serum cytokines as it provides information on bodywide immune responses. To set up a baseline for understanding cytokine changes following AAV gene therapy, we compared the serum cytokine profiles between normal and DMD canines. The Milliplex® MAP canine cytokine magnetic bead panel is based on the Luminex® xMAP® technology. It provides a simultaneous quantification of 13 different cytokines (GM-CSF, IFN-γ, IL-2, IL-6, IL-7, IL-8, IL-15, IP-10, KC-Like, IL-10, IL-18, MCP-1, TNF-α) from a single sample. Serum samples from dogs of 0-3, 4-6, 7-9, 10-12, 13-24, and 25-36 months of ages were evaluated. Specifically, IL-8, MCP1 and KC like were significantly increased in young but not old DMD dogs. Interestingly, some cytokines showed no significant differences between normal and DMD dogs. The data presented here have set a foundation to study cytokine changes following AAV gene therapy in canine models in the future (Supported by NIH, Jackson Freel DMD Research Fund, University of Missouri Life Science Fellowship, and University of Missouri Molecular Biology T32 training grant).

732. Differential Activation of Innate Immune Response Following Intrastromal Administration of Adeno-Associated Viral Vectors at Variable Doses in Human Corneas

Zhenwei Song, Liujiang Song, Chengwen Li

Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Gene delivery approaches using Adeno-associated virus (AAV) are currently the leading method of human gene therapy and have demonstrated success in clinical trials for diverse diseases including ocular diseases. However, both the innate and adaptive immune responses against AAV remain the major challenges that limits its utility and efficacy. While dose-dependent adaptive immune responses have been reported in numerous preclinical and clinical studies; activation of innate immune response right after or at a later time point following AAV administration has also been well elaborated in our previous study; yet no study exists regarding the AAV doses-related effects on the innate immune response in human organs. Herein, this study explores the role of the initial administrated doses on the activation, level and duration of innate immune response following intrastromal injection of single stranded AAV vectors in human cornea context. Following the injection, the human corneal discs were cultured *ex vivo* for 8 days, and several vital metrics were then quantitatively evaluated including: 1) the viral genome and transgene expression levels and kinetics; 2) the activation of innate immune responses and duration; and, 3) the dose effects on the transgene expression and the innate immune responses. As was foreseeable, the transgene expression (luciferase activity) from both low (1e8 vg per 4 mm corneal disc) and high (1e9 vg per disc) AAV doses peaked around day 3 in a dose-dependent manner. However, while the expression in the low dose group sustained in a moderate level until the experimental endpoint, the expression from the high dose fluctuated over time. Notably, the hypothesis of higher dose resulted in linear increase of transgene expression was refuted as results revealed that compared to the high dose group, the low dose resulted in significant higher transgene expression when normalized to the viral genome numbers (RLU/vg). Following transduction, the innate immune response determined by IFNβ mRNA transcript was initially observed in the next day post injection (the earliest testing time point) in all corneas. Although tremendous individual difference existed, which resulted in up to hundredfold difference in innate immune response among different donors, the activation and duration of the innate immune response appeared to be in a dose-dependent manner. The high dose trended to result in higher innate immune response in general and the IFNβ levels fluctuated over time which may find relevance to the real-time changes in transgene expression and viral genomes in the tissues. Interestingly, in low dose group, 9 out of 12 corneas, which showed relatively stable levels of transgene expression and the viral genomes, the IFNβ levels decreased over time, which indicates that the innate immune response induced by the low dose of AAV was not negatively affecting the transgene expression. Collectively, the data derived from these studies contributes to the understanding of the influences of AAV doses on the onset, intensity and duration of innate immune responses along with the transgene expression abundance, and may reveal potential strategies to circumvent the innate immune response in future clinical trials.

733. Prophylactic Prednisolone and Rapamycin Improve AAVrh10-hFVIII Gene Therapy in Cynomolgus Macaques by Reducing Plasma Cells

Barbara Anne Sullivan[1], Jessica Chichester[2], Alex Kistner[1], Lili Wang[2], Roberto Calcedo[2], Jenny A. Greig[2], Sunjay Sethi[3], Brian E. McIntosh[4], Kathleen McKeever[1], Sam Wadsworth[1], James M. Wilson[2], Emil Kakkis[1]

[1]Ultragenyx Pharmaceutical Inc., Novato, CA,[2]Gene Therapy Program, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Charles River Reno, Reno, NV,[4]Covance Madison Inc., Madison, WI

Adeno-associated viral (AAV) gene therapy (GT) is a promising and established approach to treat rare genetic disease. Prophylactic immunosuppression (IS) may lead to improved clinical outcomes by reducing innate and adaptive immune responses. Multiple combination prophylactic IS regimens were tested in two cynomolgus macaque studies using an AAVrh10 GT vector expressing human FVIII (hFVIII). In study 1, we compared no IS with prednisolone alone, rapamycin alone, rapamycin and cyclosporin, or cyclosporin and azathioprine. IS was administered 14 days before, and for 85 days following, GT administration, and was tapered 25% by week on days 85-113. Subsequently, study 2 compared no IS with prednisolone alone or in combination with rapamycin or methotrexate using a double-taper design. Full strength IS regimens were administered 14 days before, and for 28 days following, GT administration. Prednisolone was tapered 25% by week from days 29 - 57 following GT; rapamycin and methotrexate were tapered 25% by week from days 85-113 following GT. Both studies used a single dose of AAV vector at 1e13 GC/kg in male cynomolgus macaques (n=3 or 4 per group). Animals were pre-screened and those with AAVrh10 neutralizing antibody titers 1:5 or lower were selected for study inclusion. Transgene protein (hFVIII), anti-hFVIII antibodies, anti-AAVrh10 capsid neutralizing antibodies, anti-AAVrh10 capsid IgM and IgG, total IgM and IgG, Type 1 Interferon (IFN) gene signature and plasma cell (PC) gene signature were assessed in plasma, serum or whole blood samples. In study 1, the prednisolone group demonstrated higher hFVIII expression than the no-IS control group; however, anti-hFVIII antibodies developed in 2/3 animals. In study 2, the prednisolone/rapamycin group demonstrated an increase in hFVIII expression compared with the no-IS group and the prednisolone-only group. A significant reduction in the PC gene signature was observed in the prednisolone/rapamycin group (compared to pre-IS dose levels and to the other groups) during the rapamycin dosing window. One animal (of 4) in the prednisolone/rapamycin group had sustained hFVIII and no detectable anti-hFVIII antibodies through end of study (day 217). The combination of prednisolone/rapamycin led to the absence of anti-capsid IgG in 3/4 animals during the rapamycin treatment window as compared with 1/8 in Groups 1 & 2 (no-IS and prednisolone alone). Two animals in the prednisolone/rapamycin group did not develop anti-capsid IgG after IS withdrawal. One animal in the prednisolone/rapamycin group developed high anti-AAV rh10 NAbs and IgG following GT administration. There were no changes in total IgM or IgG in the prednisolone/rapamycin group. An increase in the Type 1 IFN gene signature was observed in all study groups 24 hours following GT dosing, with no apparent impact of IS. In summary, the combination of prednisolone and rapamycin improved hFVIII expression and reduced anti-capsid neutralizing antibodies. The reduction in neutralizing antibody levels, anti-capsid IgG development, and PC gene signature suggest that rapamycin's tolerogenic effects may include an impact on B cell development and PC differentiation through direct or indirect mechanisms that can alter the response to AAV gene therapy.

734. Similar DTX301 (scAAV8OTC) Gene Therapy Outcomes in Female and Male Cynomolgus Macaques

Lili Wang[1], Claude C. Warzecha[2], Alex Kistner[3], Jessica Chichester[2], Peter Bell[2], Zhenning He[2], Sunjay Sethi[4], Kathleen McKeever[3], Emil Kakkis[3], James Wilson[2], Sam Wadsworth[3], Barbara A. Sullivan[3]

[1]Gene Therapy Program, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA,[3]Ultragenyx Pharmaceutical Inc., Novato, CA,[4]Charles River Reno, Reno, NV

Ornithine transcarbamylase (OTC) deficiency is a rare X-linked genetic urea cycle disorder. Patients with OTC experience episodes of acute hyperammonemia that can result in progressive neurocognitive damage, and, in some cases, death. Current treatment options are limited to protein-restricted diet, ammonia scavengers and liver transplantation. DTX301 is a promising gene therapy for OTC deficiency. The impact of sex and prophylactic immunosuppression (IS) on OTC gene therapy outcomes is not well known. A single 1e13 GC/kg dose of DTX301 was assessed in adult, sexually-mature female and male cynomolgus macaques, with and without prophylactic IS. Animals (N=3/group) were pre-screened and those with neutralizing antibody titers less than 1:5 were selected for study inclusion. Prednisolone IS (1mg/kg/day) was administered 4 days before, and for 28 days following, DTX301 administration, and was tapered 25% by week from days 28 - 56. At regular intervals through the study, blood samples were assessed for chemistry, hematology, vector persistence, anti-capsid neutralizing antibodies and IFNγ ELISPOT. In-life liver biopsies were collected 28 and 84 days following DTX301 administration and terminal liver samples were assessed on day 140. Liver samples were analyzed for Vector Genomes (VG) by quantitative PCR (qPCR), human codon optimized OTC transgene (hOTCco) mRNA by RT-qPCR, vector DNA/RNA by In Situ Hybridization (ISH), OTC expression by immunohistochemical (IHC) staining, and histopathology evaluation. DTX301 with and without IS was well tolerated. Half of the animals had a >2-fold elevation in ALT levels at variable time points from study day 7 to 140, with no clear group association, that resolved without intervention. There were no other remarkable findings in the chemistry and hematology results. Most vector DNA in blood was cleared by day 7, but the residual vector DNA persisted in blood and slowly decreased towards the end of the study. All study animals developed sustained, high titer anti-capsid antibodies. There was no significant differences between groups, at all three time points, for liver VG or hOTCco mRNA. On day 28, the mean VG levels and hOTCco mRNA levels in male and female groups with IS were higher than those without IS. Mean VG levels and hOTCco mRNA levels decreased over time in all animals, with

similar mean levels of VG and hOTCco mRNA on days 84 and 140 in all four groups. These data are consistent with ISH and IHC results. Most animals showed positive ELISPOT responses to a capsid peptide pool (AAV8-C), with weak, transient responses in PBMCs on day 70, and stronger positive response in liver lymphocytes on day 140. Few/weak ELISPOT responses were observed in the liver to the transgene peptide pools and none were observed in PBMCs. In summary, there were no apparent differences between female and males cynomolgous macaques in immunologic parameters or in DTX301 effectivity.

735. AAV Capsid Proteins Fused with SARS-CoV-2 RBD or RBM: Expression in *E. coli*, *In-Vitro* Assembly, and Characterization

Dinh To Le[1], Olaf Behrsing[2], Claire Rothschild[1], Marco T. Radukic[1], Katja M. Arndt[2], Kristian M. Müller[1]

[1]Cellular and Molecular Biotechnology, Bielefeld University, Bielefeld, Germany,[2]Molecular Biotechnology, Institute for Biochemistry and Biology, University of Potsdam, Potsdam, Germany

In the current COVID-19 pandemic, the SARS-CoV-2 virus has infected over a hundred million people and contributed to millions of deaths. The SARS-CoV-2 spike protein, in particular its receptor-binding domain (RBD) containing the receptor-binding motif (RBM), mediates virus binding to the cellular receptor angiotensin-converting enzyme 2 (ACE2) and subsequent viral internalization. Therefore, RBD and RBM are lead candidates for SARS-CoV-2 subunit vaccine development. Virus-like particles (VLPs) have been widely used for vaccine development due to their ability to induce a strong immune response and adeno-associated virus-like particles (AAV VLPs) produced in mammalian cells have been tested as a scaffold for presenting antigens. However, mammalian cell culture is expensive and particles may contain cellular components. Recently, we reported that AAV2 VP3 capsid proteins produced in *E. coli* can be assembled to AAV2 VLPs *in vitro*. In the current work, we explore the potential of *in-vitro* AAV2 VLPs for coronavirus vaccine development. SARS-CoV-2 RBD and RBM were incorporated into the 587-loop of AAV2 VP3 proteins and these fusion proteins were expressed in *E. coli* using T7 promoter (pET) plasmids. The proteins formed inclusion bodies in *E. coli* and were successfully purified under denaturing conditions of 8 M urea. Refolding and concomitant *in vitro* assembly of VP3_RBD VLPs or VP3_RBM VLPs was attained by serially dialyzing initial denatured proteins against different buffers containing 4, 2, 1, 0.5, and finally 0 M urea. After dialysis, atomic force microscopy (AFM) of AAV2 VP3_RBD and VP3_RBM samples identified particles with the expected size. This hints that the incorporation of RBD or RBM into the 587-loop is compatible with *in vitro* AAV2 VLP capsid assembly, though irregular aggregates and smaller structures, potentially single and intermediate assemblies, were also recorded. In ELISAs, an RBD conformation-specific antibody readily recognized coated protein samples indicating at least partial correct folding of VP3_RBD. Next, mice were immunized and boosted with VP3_RBM and VP3_RBD VLPs mixed with adjuvants, and serum was collected at various timepoints. The immune response was examined by ELISA with coated antigens (VP3_RBD and VP3_RBM VLPs) as well as GFP_RBD produced in 293-F cells as a control for SARS-CoV-2 specificity. Immunization of mice with the motif VP3_RBM VLPs resulted in a high level of the desired RBD-specific antibodies and a low level of VP3 VLP-directed antibodies. Unexpectedly, the domain VP3_RBD VLPs induced a low level of RBD-specific antibodies, but an intermediate level of VP3 VLP antibodies. Taken together our data suggest that *in vitro* AAV VLPs bear potential as vaccine scaffolds and that AAV VLP production using *E. coli* lends itself as a versatile strategy for vaccines and drug delivery.

736. Presence of Pre-Existing Anti-Adeno-Associated Virus (AAV) Serotype 5 Neutralizing Antibodies (NABs) in Serum of Huntington Disease (HD) Patients Was Not Associated with Detectable Anti-AAV5 Nabs in Cerebrospinal Fluid (CSF)

Anna Majowicz[1], Floris van Waes[1], Astrid Valles[1], Sander van Deventer[1], Mette Gilling[2], Anka Ehrhardt[3], Pavlina Konstantinova[1], Valerie Sier-Ferreira[1]

[1]uniQure N.V, Amsterdam, Netherlands,[2]Enroll-HD Platform Team, European Huntington's Disease Network (EHDN), University Hospital of Ulm, Ulm, Germany,[3]CHDI Foundation, Princeton, NJ

Background AAV-based therapies are under investigation in early clinical trials for several neurodegenerative diseases. We have previously reported that serum anti-AAV5 NABs titers up to 340 in humans and as high as 1030 in primates did not interfere with the therapeutic efficacy of intravenously administered AAV5 vector. However, it remains unclear whether naturally acquired pre-existing systemic immunity to AAV would impact the therapeutic efficacy of AAV vectors delivery to the Central Nervous System (CNS). This knowledge is of importance for the interpretation of AAV-based gene therapies for Huntington Disease (HD), since the blood-brain barrier in HD patients might be compromised. To address such concern the prevalence of NABs against AAV serotype 5 was correlated between matched serum and CSF samples obtained from a same individual, either HD patients at different stages of the disease or healthy controls participating in HDClarity. **Methods** Serum and plasma samples from HD patients and healthy donors were analyzed for the presence of anti-AAV5 NABs with the use of luciferase-based assay. The sensitivity (lowest sample dilution) of the assay was set at 50. Consequently, CSF samples were obtained from all HD patients and healthy donors that tested positive for serum/plasma anti-AAV5 NABs and 3 that tested negative (controls) to analyze for presence of CSF anti-AAV5 NABs. **Results** Overall, serum anti-AAV5 NABs were not associated with detectable NABs in CSF; remarkably, this was true even in the presence of the highest serum titers (5364 in HD patient, 5129 in healthy donor). In HD patients, 37 (21.5%) of 172 serum samples had detectable anti-AAV5 NABs titers; none (0%) of the 37 HD patients had detectable anti-AAV5 NABs titers in CSF above threshold. Similarly, in healthy controls, 12 (18.2%) of 66 serum samples had a detectable anti-AAV5 NABs titer; none (0%) of those 12 donors had detectable anti-AAV5 NABs titers in CSF. **Conclusion** Pre-existing serum anti-AAV5 antibodies have not impacted clinical efficacy of intravenous AAV5 therapies. The current analysis of HDClarity samples suggest patients with detectable serum NABs do not have CSF NABs. Therefore, we conclude that there is minimal risk for reduced therapeutic efficacy of intrathecal or intraparenchymal administration of therapeutic AAV5 vectors by pre-existing serum or CSF AAV5 NABs in HD patients.

737. Bispecific T-Cell Engager Adoptively Transduced T Cells Is a Multivalent Targeting Strategy Intensively and Specifically Responding to Gliomas

Yibo Yin[1,2], Zev A. Binder[2], Nannan Li[1], Radhika Thokala[2,3,4,5], Li Hu[1], MacLean P. Nasrallah[3], Logan Zhang[2], Jesse L. Rodriguez[2,3,4,5], Jiasi Vicky Zhang[2], Hongsheng Liang[1], Yu Long[1], Devneet K. Kainth[2], Leila Haddad[2], Jiping Qi[6], Xiangtong Zhang[1], Avery D. Posey Jr.[4,5], Zhiguo Lin[1], Donald M. O'Rourke[2]

[1]Department of Neurosurgery, First Affiliated Hospital of Harbin Medical University, Harbin, China,[2]Department of Neurosurgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[4]Center for Cellular Immunotherapies, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[5]Parker Institute for Cancer Immunotherapy, Philadelphia, PA,[6]Department of Pathology, First Affiliated Hospital of Harbin Medical University, Harbin, China

Background Bispecific T-cell engagers (BiTEs) are bi-specific antibodies that bind T cells to tumor cells expressing target antigen. Previous studies have demonstrated BiTEs can be consistently secreted by T cells to recruit naïve T cells against antigen positive tumor cells. We hypothesized that BiTE secreting T cells could be a promising T cell therapy with distinct properties in mono- or multi- valent strategies against solid tumors. **Methods** Expression of EGFR, EGFRvIII, and IL13Rα2 was detected in freshly resected glioma tissues and glioma stem cell lines. A conformation-specific EGFR targeting antibody (806), able to bind tumor related EGFR conformation and EGFRvIII, was adopted to generate 806BiTE. Hu08, an IL13Rα2-targeting antibody tested in a CAR construct in our previous work, was adopted to generate Hu08BiTE. 806CAR T cells and Hu08CAR T cells were included to compare with 806BiTE T cells and Hu08BiTE T cells (Figure 1, upper) by CD69, indicating T cell activation, cytokine secretion (IFNγ, IL2 and TNFα), and bioluminescent based cytotoxicity assays when co-cultured with wild type or target overexpressed glioma stem cell line (5077), U87 glioma cell line, and normal astrocytes. Bivalent targeting T cells (806CAR-Hu08CAR T cells, 806BiTE-Hu08CAR T cells, and 806BiTE-Hu08BiTE T cells; Figure 1, lower) were generated and tested in the previously mentioned co-culture experiments, along with the D270 implanted glioma mouse model. **Results and Conclusion** Simultaneous targeting of EGFR, EGFRvIII, and IL13Rα2 expands coverage of glioma tumor populations, given their heterogenous nature. We successfully generated 806BiTE T cells and Hu08BiTE T cells targeting EGFR, EGFRvIII, and IL13Rα2 positive tumors with superior responding activity than corresponding CAR T cells. BiTE T cells were shown to be more sensitive in responding to low target expressing glioma cells than CAR T cells or BiTE recruited naïve T cells. The responses of BiTE T cells and BiTEs were also shown to be more restricted to the binding property of target binding scFv than CAR T cells, which decreased off target toxicity. Superior responding activity was also demonstrated in BiTE secreting bivalent targeting T cells compared with bivalent targeting CAR T cells, which controlled tumor growth more quickly in a glioma mouse model. In summary, BiTE integrated mono- or multi- valent targeting T cells intensively respond to tumor cells *in vitro* and *in vivo* with superb sensitivity and specificity, demonstrated a promising strategy in tumor therapy.

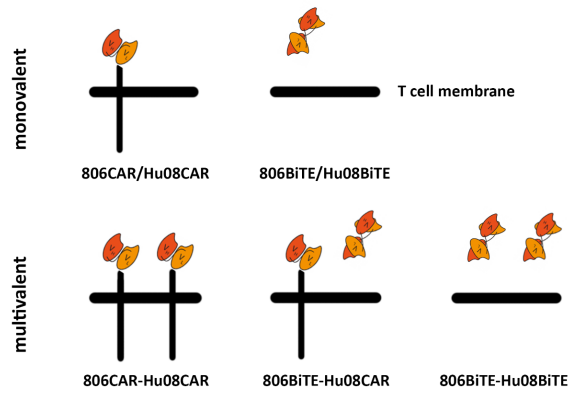


Figure 1 Schematic diagram of tested mono- and multi- valent targeting T cells

738. LV.InsB9-23-Based Therapy to Arrest T1D and Suppress Its Recurrency Post Allo-Islets Transplant

Fabio Russo[1], Antonio Citro[2], Francesca Sanvito[3], Paolo Monti[2], Silvia Gregori[1], Maria Grazia Roncarolo[4], Andrea Annoni[1]

[1]SR-Tiget, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Diabetes Research Institute (DRI), IRCCS San Raffaele Scientific Institute, Milan, Italy,[3]Pathology Unit, Department of Oncology, IRCCS San Raffaele Scientific Institute, Milan, Italy,[4]Department of Pediatrics, Stanford School of Medicine, Stanford, CA

The induction of antigen (Ag)-specific tolerance represents a therapeutic option for autoimmune diseases. Autoimmune Type 1 Diabetes (T1D) is caused by T cell mediated elimination of pancreatic islets' beta cells. Specific haplotypes and inadequate immune regulation predispose to the disease. A successful strategy to cure diabetic patients and to prevent the disease in at risk subjects still represents an unmet need. Insulin is the primary beta cell-related Ag targeted by T cells. Spreading of the response to a wide family of beta cell Ags occurs in humans and Non-Obese Diabetic mice (NOD), which spontaneously develop the disease resembling human T1D. We demonstrated that a single systemic administration of lentiviral vector (LV) enabling expression of insulinB9-23 (LV.InsB) in hepatocytes, hampers immune-mediated beta cell destruction in pre-diabetic NOD mice, by generating InsB9-23-specific Foxp3+T regulatory cells (Tregs). The combination of LV.InsB with a suboptimal dose of non-mitogenic anti-CD3 mAb (combined therapy, 1X5μg-CT5) reverts diabetes at the onset and suppress recurrence of autoimmunity after islets transplantation in ~50% of NOD mice. Based on these results we investigated how CT could be optimized to achieve abrogation of recurrence of autoimmunity post islets transplantation. Therefore, allo-islets were transplanted after optimized CT tolerogenic conditioning (1X25μg-CT25). Diabetic NOD mice conditioned with CT25, when glycemia was <500mg/dL, remained normoglycemic for 100 days after allo-islets transplantation independently from the graft survival, showing significantly reduced infiltration of endogenous islets. Accordingly, cured mice showed T cell unresponsiveness to InsB9-23

stimulation and increased Tregs in islets infiltration and pancreatic LN. Further investigation to understand the mechanism of Ag-specific immune regulation driven by CT25 revealed that both Tregs and PDL1 co-stimulation pathway cooperate and are necessary to control autoimmunity, while transplanted islets play a crucial role, although transient, in attracting diabetogenic cells to an alternative site, thus alleviating the immune pressure to endogenous pancreatic islets. Therefore, InsB9-23 gene transfer to hepatocytes may represent the base of an Ag-specific therapy for T1D. It can be adapted and potentiated, in combination with minimal doses of anti-CD3, to be applied at different stages of the disease to arrest its progression preserving endogenous beta cells mass and normalizing glucose homeostasis.

739. AAV-Mediated Delivery of Soluble Blockers of SARS-CoV-2 Entry into Host Cells

Ivana Trapani[1,2], Mariangela Lupo[1], Edoardo Nusco[1], Arjun Padmanabhan[1], Alberto Auricchio[1,3]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy,[2]Medical Genetics, Department of Translational medicine, Federico II University, Naples, Italy,[3]Medical Genetics, Department of Advanced Biomedicine, Federico II University, Naples, Italy

The outbreak of COVID-19 pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is demanding unprecedented worldwide efforts to prevent further spread of the virus. A key event for SARS-CoV-2 entry into the host cells is the interaction of its spike glycoprotein with the angiotensin-converting enzyme 2 (ACE-2) cell surface receptor. Therefore, therapeutic options against COVID-19 which interfere with this step, including convalescent plasma, monoclonal antibodies and a variety of vaccines based on introduction of the spike protein into the body, are being actively developed and tested. However, each of these approaches has its own limitations, including manufacturing, long-term immunity, vaccine (or "antibody-dependent") enhancement of the disease, need for the host to respond immunologically. As an alternative to these approaches, we have planned to explore gene transfer to non-hematopoietic tissues (i.e. liver and muscle) of soluble blockers of SARS-CoV-2 entry. Specifically, we developed adeno-associated viral (AAV) vectors encoding either: i. antibodies described to neutralize (nAbs) SARS-CoV-2 to induce host passive immunization; or ii. a soluble form of ACE2 (sACE2) which binds spike and blocks its interaction with ACE2 receptors on the host cells. Upon transfection of these AAV constructs in HEK293 cells, we have found efficient production and secretion in the media of both nAbs and sACE2. Additionally, either intramuscular injection of AAV1-CMV vectors or intravenous injection of AAV8-TBG vectors (that target liver) in 3-4 weeks old C57BL/6 mice result in high levels of both nAbs and sACE2 in the sera of mice up to 6 weeks post-injection, the last time point of the observation. Further investigation of long-term expression and neutralization activity on SARS-CoV-2 entry is ongoing.

740. Single-Stranded rAAV Vectors Are Unable to Trigger Intracellular RNA Sensors That Mediate Innate Immunity

Jennifer G. Chen*[1], Manish Muhuri*[1,2,3], Wei Zhan[1,2,3], Yukiko Maeda[1,3,4], Phillip Tai[a1,2,3], Guangping Gao[a1,2,5]

[1]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, Worcester, MA,[3]VIDE Program, University of Massachusetts Medical School, Worcester, MA,[4]Department of Medicine, University of Massachusetts Medical School, Worcester, MA,[5]Li Weibo Institute for Rare Diseases Research, University of Massachusetts Medical School, Worcester, MA

Recombinant adeno-associated viruses (rAAVs) have emerged as powerful tools for *in vivo* gene therapy. Despite the recent clinical successes of rAAV-based therapeutics, host immune responses mounted against the vector and transgene product can effectively limit the therapeutic potential and safety outcomes of these therapies. Therefore, investigating and overcoming the immunogenicity of rAAV is critical towards advancing rAAV-based gene therapies, particularly for long-term treatments. There has been substantial research on the adaptive immune response against AAV. However, in light of the adverse events encountered in recent rAAV-based gene therapy clinical trials, the innate immune response also warrants detailed investigation. RNA sensors are one of the many ways rAAV can be sensed by the host to activate the innate immune system. Upon binding to the appropriate viral transcripts, cytosolic RNA sensors retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated protein 5 (MDA-5) interact with mitochondrial antiviral signaling protein (MAVS) and initiate a series of pathways leading to the downstream production of type 1 Interferons (IFNs), hallmarks of innate immune activation inflammation. Previously, it had been shown that the double-stranded RNA (dsRNA) sensor MDA-5 was activated in response to self-complementary AAV (scAAV) administration, stimulated production of IFNβ, and subsequently lead to suppressed transgene expression. In our study, we explored the activation of intracellular RNA sensors by single-stranded AAV (ssAAV) infection. Given the variability of immune response activation on cell type, AAV serotype, and vector dose, the induction of MDA5, RIG-I, and MAVS was examined in cell lines of immune and non-immune lineages. rAAV infection at higher doses stimulated transcript levels of RNA sensor around eight days following infection. However, increased expression of RNA sensors showed no correlation with transgene expression and activation of the inflammatory cytokines, IFNβ, IFNα, Il-1β, Il-6, and TNFα. Since intramuscular (IM) rAAV administration can serve as a promising target tissue for the sustained production of therapeutic proteins, activation of RNA sensors was further investigated in the context of skeletal muscles. IM injections of ssAAV1.A1AT vectors to the tibialis anterior muscle in mice can neither activate RNA sensors significantly nor increase the production of inflammatory cytokines compared to controls. Measurement of serum A1AT levels showed that transgene expression was not affected as well. Taken together, our results demonstrate that ssAAV vectors do not significantly activate innate immunity via RNA sensor induction and do not affect long-term transgene expression. In order to develop effective and safe solutions

to overcome the innate immune response, further investigation is necessary to dissect the differences between single and double-stranded AAV vectors in activating RNA sensors.

741. Dynamics, Features and Cross-Reactivity of IgG Pool Induced after AAV5 Gene Therapy for Hemophilia B

Giorgia Squeri, Johannes P. F. de Laat, Karin Kwikkers, Ricardo Dolmetsch, Melvin Evers, Valerie Ferreira
uniQure N.V, Amsterdam, Netherlands

In the AAV gene therapy scenario, AAV5-based vectors have a privileged immunological profile. Anti-AAV5 antibodies can be found in the healthy population with a relatively low prevalence and their neutralization titer and binding affinity for the antigen have been shown to be on average lower when compared to antibodies/antigen complexes of other AAV serotypes. These features justified the choice of including patients with detectable levels of AAV5-neutralizing antibodies in the uniQure-sponsored HOPE-B phase 3 clinical trial for hemophilia B. 26 weeks data was recently presented and showed that safety and efficacy of the AAV5-hFIX treatment was not impaired by the presence of anti-AAV5 pre-existing antibodies at generally prevalent titers before AAV5-vector administration. Given the capsid similarity between AAV serotypes, we analyzed plasma from 9 hemophilia B patients enrolled in a uniQure-sponsored phase I/II clinical trial for immunological cross-reactivity. Samples from before and 1, 2, 4, 8 and 26 weeks after AAV5-hFIX gene therapy treatment were tested to monitor the magnitude and features of their humoral response. Total IgG and IgG subclasses against AAV5 as well as against four unrelated serotypes (AAV1, 2, 6 and 8) were determined using a Meso Scale Discovery (MSD) assay. An *in vitro* luciferase-based cellular assay was used to measure the capacity of plasma samples of neutralizing transduction with the five different capsids. Before AAV5-hFIX administration, 4 patients tested positive for IgG binding to one or more AAV serotypes, of which 2 patients had IgG against AAV5 (positivity threshold: 400 ECL units, tested by MSD at 1:50 dilution). The other 5 patients tested negative for IgG specific for all tested AAV serotypes. Four weeks after AAV5-hFIX administration, all patients developed high levels of AAV5 binding (ECL range 6079-200081, tested by MSD at 1:500 dilution) and neutralizing (IC50 range 5426-204800) IgG, and remained positive until week 26. The ability of the AAV5 gene therapy-induced antibodies to cross-reactively bind and neutralize between AAV serotypes could be related to the pre-immunization profile of the subjects before AAV5-hFIX treatment. In details: cross-reactivity between AAV serotypes was observed in the 4 patients where detectable levels of antibodies binding one or more AAV serotype were present before treatment. Four weeks after gene therapy, these cross-reactive antibodies were able to efficiently neutralize (IC50 > 8000) all tested serotypes. On the contrary, the other 5 subjects with no detectable pre-existing antibodies developed high levels of IgG specifically binding and neutralizing AAV5 and no or low amounts of antibodies against AAV1, 2, 6 and 8. Analysis of the IgG subclasses showed different levels of expression and cross-reactivity depending on the IgG subtype analyzed. At both pre and post AAV5-hFIX administration and for all AAV serotypes, IgG1 was the most represented and cross-reactive subclass. Interestingly, IgG3 subclass was detected only after AAV5-hFIX administration and showed low cross-reactivity with a preferential affinity for AAV5. Further studies on post gene therapy samples and deeper epitope mapping analysis are envisaged to define the nature of the cross-reactivity between AAV5 and other serotypes and thus confirm the peculiar immunological profile of AAV serotype 5.

Immunological Aspects of Gene Therapy and Vaccines

742. Lentiviral-Mediated Gene Therapy for the Treatment of Adenosine Deaminase 2 Deficiency

Matteo Zoccolillo[1,2], Immacolata Brigida[1], Raisa Jofra Hernandez[1], Federica Barzaghi[3], Serena Scala[1], Luca Basso-Ricci[1], Mariasilvia Colantuoni[1,4], Lucia Sergi Sergi[1], Francesca Conti[5], Simone Cesaro[6], Francesca Schena[7], Marco Gattorno[7,8], Luigi Naldini[1,4], Maria Pia Cicalese[3], Alessandro Aiuti[1,3,4], Alessandra Mortellaro[1]
[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Medicine and Surgery, Tor Vergata University, Rome, Italy,[3]Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy,[4]Vita-Salute San Raffaele University, Milan, Italy,[5]Azienda Ospedaliero-Universitaria S. Orsola-Malpighi, Bologna, Italy,[6]Pediatric Hematology-Oncology, Ospedale della Donna e del Bambino, Verona, Italy,[7]UOSD Centro Malattie Autoinfiammatorie ed Immunodeficienze, IRCCS Istituto G. Gaslini, Genova, Italy,[8]UOC Clinica Pediatrica e Reumatologica, IRCCS Istituto G. Gaslini, Genova, Italy

Background. The deficiency of adenosine deaminase 2 (DADA2) is a rare inherited disorder due to autosomal recessive mutations in the ADA2 gene. Clinical manifestations include vasculitis, early-onset ischemic strokes, hematologic abnormalities (pure red aplasia), and immunodeficiency (neutropenia and decreased mature B cells). ADA2 protein is an adenosine deaminase mainly secreted by macrophages. Anti-TNF therapy controls strokes and inflammatory episodes, while allogeneic hematopoietic stem progenitor cell (HSPC) transplantation is the treatment of choice for patients with hematologic manifestations. However, the morbidity and mortality associated with this procedure make this treatment only recommended for the most severe patients, and compatible donors are not always available. Hence lentiviral vector (LV) mediated HSPCs gene therapy based on transplantation of patients' corrected HSPCs may represent a valid therapeutic option for treating DADA2 patients who are not eligible for allogeneic transplantation.
Methods. We generated a third-generation lentiviral vector (LV) encoding human ADA2 under a ubiquitous promoter. The ADA2-LV was used to transduce mouse and human HSPCs at different vector concentrations. Transduction efficiency, clonogenic potential, and growth rate of HSPCs transduced with the IL-1RA-LV and control GFP-LV were assessed in vitro. The ability of ADA2-LV transduced HSCPs to engraft in vivo was evaluated in humanized NSGW41 and C57BL/6 mice. Transduction efficiency and ADA2 reconstitution were assessed in BM $CD34^+$ HSPCs isolated from two adult patients. We also estimated the correction of ADA2-deficient macrophage immune defects in patients' macrophages and an ADA2-deficient myelomonocytic cell line.

Results. Transduction efficiency evaluated on individual vector-positive granulocyte-macrophage colony-forming units and erythroid burst-forming units (BFU-E) was ≥75% in all conditions. The median vector copy number measured in CFU-GM and BFU-E colonies ranged 1.82-2.69 and 1.58-3.7, respectively, at all vector concentrations. ADA-LV transduction led to the supra-normal increase of ADA2 expression and enzymatic activity in healthy donors' and patients' of HSPCs. Although patients' BM contained fewer amounts of various HSPC subsets, ADA2-LV transduction did not alter their viability, clonogenic potential, or differentiation capacity. ADA2 expressing human and mouse HSPCs engrafted and normally differentiated in mice. ADA2 restoration in patients' macrophages and ADA2-deficient myelomonocytic cell line led to a complete correction of the exaggerated cytokine response. **Conclusion.** Our data support LV-mediated gene transfer in HSPCs as a new advanced therapy for patients with ADA2 deficiency and warrant further studies in support of its clinical development.

743. Depletion of Pre-Existing Anti-AAV Neutralizing Antibodies by a Combination Immunosuppressive Treatment with Bortezomib and CD20 mAb Allows Successful Vector Re-Administration in Mice

Su Jin Choi, John Yi, Thomas F. Tedder, Dwight D. Koeberl, Priya S. Kishnani, Baodong Sun
Duke University, Durham, NC

Background: Recombinant adeno-associated virus (AAV) is a preferred vector for gene therapy to treat genetic disorders in both preclinical studies and clinical trials. However, preclinical studies indicate that even low titers of pre-existing anti-AAV capsid neutralizing antibodies (NAb) can block viral vector transduction and prevent efficacy. Consequently up to 40% of patients who have pre-existing NAb are excluded from clinical trials. We previously reported that a 8-week immunosuppression (IS) treatment using bortezomib and a mouse-specific CD20 mAb (MB20-11) along with AAV injection effectively depleted blood and spleen $CD19^{+}B220^{+}$ B cells and prevented the formation of anti-AAV NAb, allowing re-administration of an AAV vector of the same serotype in a mouse model of Pompe disease (GAA-KO mice). This study examines the ability of this combination IS treatment to deplete pre-existing NAb and to enable re-administration of an AAV8 vector in GAA-KO mice. **Methods:** Three-month-old GAA-KO mice were pre-immunized by intravenous injection of an AAV8 vector (AAV8-CB-hGAA, $5x10^{12}$ vg/kg) that ubiquitously expresses human acid α-glucosidase. The AAV-treated mice were divided into two groups after 24 weeks: **Group 1** received IS treatment (intraperitoneal administration of MB20-11 monthly and intravenous injection of Bortezomib twice a week) for 8, 12, 16, or 20 weeks, to define an optimal IS treatment duration; **Group 2** did not receive IS treatment. An additional group of treatment-naïve mice was used as positive controls for AAV re-dosing (**Group 3**). After the IS treatment period, half the mice from each treatment group were euthanized for analyzing plasma anti-AAV8 NAb titers. The remaining mice in each group were injected with a second AAV8 vector that contained a liver-specific promoter (LSP) driving human secreted alkaline phosphatase (hSEAP) expression (AAV8-LSP-hSEAP, $5x10^{13}$ vg/kg). Following AAV8-LSP-hSEAP injections, plasma activities of secreted hSEAP were monitored for up to 6 weeks. **Results:** Plasma hSEAP activity was undetectable in **Group 1** mice receiving IS for 8-weeks, but increased moderately in mice receiving IS for 12 or 20 weeks. Mice in **Group 1** that received the 16-week IS treatment demonstrated the highest hSEAP activity, equivalent to naïve mice that received only AAV8-LSP-hSEAP in **Group 3**. This suggests that IS for 16 weeks was an optimal treatment duration that enabled successful re-administration of the AAV8 vector. By contrast, hSEAP activity was not detected in **Group 2** mice that received no IS. As expected, anti-AAV8 NAb titers were low in **Group 1** mice (16 weeks IS; titers 1:15±16), high in **Group 2** mice (no IS; titers 1:248±244), and below the limit of detection in **Group 3** mice (naïve; titers 1:5). **Summary:** These data indicate that a combination treatment of bortezomib with CD20 mAb will effectively deplete anti-AAV8 NAb. Choosing the optimal duration of the immunosuppression is critical to the success of anti-AAV NAb depletion to enable AAV-mediated gene therapy in patients with pre-existing anti-AAV NAb and the efficacious readministation of AAV vectors.

744. Effect of Peptide-Based Inhibition of DNase II on AAV Transduction and TLR9 Signaling

Audry Fernandez, Amaury Pupo, Lester Suarez, Richard J. Samulski
Asklepios BioPharmaceutical, Research Triangle Park, NC

Enhancing adeno-associated virus (AAV) transduction efficiency and impairing AAV recognition by the immune system are two major challenges for achieving successful gene therapy treatments in the patients. During AAV transduction, the viral particles are internalized by receptor-mediated endocytosis, followed by endosomal trafficking and subsequently escape from late endosomes or lysosomes to reach the nucleus where the viral genome is delivered. In this work we evaluated the effect of DNase II inhibition on AAV transduction. GM16095 human cells were transduced *in vitro* with AAV8 in the presence or absence of inhibitory peptides targeting DNase II and luciferase transgene expression was detected. One of two anti-DNase II peptides tested increased AAV8 transduction by 3-fold at the maximum concentration used. The higher effect was observed when the cells were pre-incubated with the peptides 1 hour before adding AAV8. Additionally, it has been shown in the literature that DNA digestion by DNase II is required for TLR9-mediated recognition of CpG ODN and bacterial DNA. Therefore, we studied the modulation of TLR9 signaling with DNase II inhibitory peptide *in vitro* using the HEK-Blue hTLR9 reporter cells. Although a deeper characterization is needed, our results suggest that peptide-mediated blocking of DNase II activity could be a strategy worth exploring for potentiating the efficacy of AAV-mediated gene delivery.

745. Higher Seroprevalence of Anti-AAVNAb among Racial Minorities in the United States

Arpana Khatri
RDRU, Pfizer Inc, Cambridge, MA

Pre-existing immunity against AAV is a major challenge for AAV directed gene therapy, resulting in exclusion of patients from different

clinical trials. Even low level NAb can completely block vector transduction in both animal models and in the clinical. Appropriate assessment of anti-AAV immunity is necessary to conduct better clinical trials and AAV based product development. Addressing this need, we undertook an effort to characterize NAb in a panel of AAV vectors. A cell-based assay was used to assess prevalence of different AAV capsids (AAV1, 3B, 5 and SPARK100) among 100 healthy donors in the United States. The samples included even numbers of Caucasians, African Americans and Hispanics (~33%) and the final data set was normalized based on the US demographics to arrive at an estimated seroprevalence in the US population. Seroprevalence against different capsids varied and ranged between 25-40% and was consistent with previously published reports. Moreover, there was considerable cross-reactivity, although capsids belonged to diverse clades. There was no gender difference and seroprevalence was similar among male and female donors. Interestingly, NAb incidence was highest among Blacks and Hispanics that approached close to 80% for some capsids. Seroprevalence was also higher in subjects aged (> 30 and below 60 years). An extended study was then undertaken with a second larger cohort of donors that further confirmed our initial findings of increased seroprevalence among minorities. While the reasons for this increase are unclear we discuss the impact of geographical and socio-economic conditions that may impact the increased NAb incidence in these subjects. Finally, our data demonstrates a previously underappreciated link between higher AAV seroprevalence among ethnic minorities in a population and underscores the need for NAb testing in the target patient population while selecting capsids with lower seroprevalence for drug development.

746. Anti-CAR Antibody Detected after Infusion of Autologous CD4-MBL-CAR/CXCR5 CAR T Cells in SIV-Infected Rhesus Macaques

Brianna C. Davey, Emily K. Cartwright, Mary S. Pampusch, Pamela J. Skinner

Department of Veterinary and Biomedical Sciences, University of Minnesota, Falcon Heights, MN

Human immunodeficiency virus (HIV) continues to be a global problem, despite substantial research over the past four decades. Previous treatments have been unsuccessful in eliminating HIV infection and there is still the need to find a cure. We are investigating an HIV-specific CAR T cell immunotherapy that targets concentrated viral replication in lymphoid B cell follicles for the sustained remission of HIV. We are using CD4-MBL-CAR/CXCR5 CAR T cells for this approach. Preliminary work in a simian immunodeficiency virus (SIV)-infected animal model of HIV has shown that CD4-MBL-CAR/CXCR5 T cells accumulate in B cell follicles and are associated with decreased viral loads. However, these engineered T cells do not persist long term in vivo. We hypothesized that persistence could be inhibited by an antibody mediated clearance of the CAR T cells. To test this hypothesis, we investigated the frequency of anti-CAR antibodies in the serum of CAR/CXCR5 treated rhesus macaques. Serum from treated and control animals was incubated with CAR transduced PBMCs and analyzed by flow cytometry. We found an IgG antibody response to the CD4-MBL-CAR post-infusion in 8 out of 9 treated animals. When we removed the MBL motif from the CAR construct (CD4noMBL CAR T cell), we were still able to detect the antibodies, suggesting antibodies are directed at the CD4 motif of the CAR. Preliminary data suggests these antibodies are functional and can activate antibody dependent cell-mediated cytotoxicity (ADCC) via NK cells. Importantly, though we were able to detect these functional antibodies, we find no correlation between the amount of antibody detected and the persistence of CAR T cells. However, an antibody response against the CAR construct makes a second infusion improbable due to antibody-mediated clearance and potential unfavorable reactions in vivo. These findings support the need for more research regarding immune responses to CAR T cell therapy before they can be considered as a functional cure for HIV.

747. AAV9 Capsid-Anti-AAV9 Antibody Immune Complexes Promote Complement Activation and Cytokine Release In Vitro

Sophie Song, Guanrong Huang, Meghan Soustek-Kramer, Lauren D. Wood, Qian Chen

Solid Biosciences Inc., Cambridge, MA

Several high dose systemic gene therapy clinical trials have observed heightened innate immune response manifested by complement activation, decreased platelets and red blood cell counts, and acute kidney injury. These acute immune toxicities have not been detected in preclinical animal studies so mechanistic understanding of the innate immune response to gene therapy in humans is limited. We investigated innate immune activation including complement activation stimulated by rAAV vectors using several in vitro systems. We showed that neither empty rAAV9 capsids or transgene-containing rAAV9 capsids activated complement in human serum or whole blood that lacked anti-AAV9 antibodies. However, in AAV9 seropositive serum and whole blood, and following the addition of purified anti-AAV9 IgG3 to AAV9 seronegative serum, complement activation was observed. Detection of increased levels of complement activation split products C3a & sC5b-9 occurred in a dose-dependent manner for both anti-AAV9 antibody levels and AAV9 capsid levels. Furthermore, a transgene-containing rAAV9 capsid induced robust type-I IFN production only in AAV9 seropositive human whole blood suggesting an immune complex mediated mechanism. AAV9 did not stimulate NF-κB activation, type-I IFN or proinflammatory cytokine production in TLR/IFN/NF-κB reporter cells, human PBMCs and pDCs suggesting that whole blood provides a more sensitive in vitro system to evaluate innate immune activation by AAV gene therapy. These results indicate that the AAV9 capsid/anti-AAV9 antibody immune complexes promote classical complement activation as well as type-I IFN production. Further studies are being carried out to evaluate other AAV serotypes and to determine if TLR9 signaling, FcR signaling or complement system are involved in the induction of type-I IFN and proinflammatory cytokine production in AAV9 seropositive whole blood.

748. AAV6-Mediated hACE2 Expression as a Versatile Model for SARS-CoV-2 Infection and synDNA Vaccine Efficacy

Ebony N. Gary[1], Bryce Warner[2], Elizabeth Parzych[1], Bryan Griffin[2], Xizhou Zhu[1], NIkesh Tailor[2], Mable Chan[2], Robert Vandramelli[2], Nicholas J. Tursi[1], Mansi Purwar[1], Emma Reuschel[1], Kevin Liaw[1], Ali Ali[1], Sarah K. Wootton[3], Ami Patel[1], Stephanie Ramos[4], Trevor Smith[4], David B. Weiner[1], Darwyn Kobasa[2]

[1]Vaccine and Immunotherapy Center, The Wistar Institute, Philadelphia, PA,[2]Public Health Agency of Canada, Winnipeg, MB, Canada,[3]Ontario Veterinary College, University of Guelph, Guelph, ON, Canada,[4]Inovio Pharmaceuticals, San Diego, CA

Severe acute respiratory syndrome coronavirus 2 (**SARS-CoV-2**) emerged in the human population in late 2019 and is the causative agent of coronavirus disease of 2019 (**COVID-19**). To date, more than 17 million people have been infected with SARS-CoV-2 and COVID-19 has caused over 500,000 deaths worldwide. We developed a DNA vaccine encoding the SARS-CoV-2 spike glycoprotein (INO-4800) which has since entered clinical trials (NCT04336410, NCT04447781). As wild-type mice do not express angiotensin-converting enzyme 2 (**ACE2**) which serves as the receptor for the SARS-CoV-2 spike glycoprotein, easily accessible mouse models of SARS-CoV2 infection are limited. Here, we characterized immune responses to a similar DNA vaccine antigen encoding full-length SARS-CoV-2 spike glycoprotein (**pS**), and employed a mouse model based on the transduction of the respiratory tract of wild type BALB/c mice with an adeno-associated virus 6 vector carrying the gene for human ACE-2 (**hAAV6-ACE2**) to evaluate vaccine efficacy *in vivo*. Immunization with pS induced robust spike-specific T cell responses as measured by IFNy ELISpot and intracellular cytokine staining. pS immunization resulted in the rapid development of robust anti-spike IgG titers in the serum of immunized mice. IgG2a was the predominant isotype induced by pS immunization and resulted in an increased IgG2a:IgG1 ratio. pS-immunization elicited antibody responses that persisted more than 100 days post-final immunization. Serum from pS-immunized mice neutralized SARS-CoV-2 spike-pseudotyped viruses *in vitro*. pS immunized mice transiently expressing ACE2 had decreased infectious SARS-CoV-2 virus titers and reduced viral RNA in their lungs as compared to unimmunized after challenge with SARS-CoV-2. Finally, using antibody depletion confirmed that CD8+ effector cells play an important role in protection from challenge in this model. These data demonstrate that spike-encoding DNA vaccines are immunogenic and protective *in vivo*, support the continued translation of these constructs to the clinic, and demonstrate that the hAAV6-ACE2 mouse transduction model represents an easily accessible, versatile small-animal model for wild-type SARS-CoV-2 infection which can be used to evaluate anti-SARS-CoV-2 vaccine efficacy.

749. Pharmacokinetic Analysis of Plasma fVIII Exposure Identifies an Immunogenicity Threshold in the Context of Liver-Directed AAV-FVIII Gene Therapy

Taran Lundgren, Trent Spencer, Christopher Doering

Pediatrics, Emory University, Atlanta, GA

Preclinical studies of liver-directed, fVIII-encoding adeno-associated viral (AAV) vector gene therapy products have demonstrated a high incidence of anti-factor VIII (fVIII) neutralizing antibody (inhibitor) development. This is unsurprising, as fVIII is immunogenic in approximately 30% of severe hemophilia A patients when delivered intravenously as protein replacement therapy. However, the parameters governing the preclinical fVIII immune response following AAV delivery remain unclear. All AAV-FVIII vectors in late-stage clinical testing are bioengineered to contain codon-optimized, B-domain-deleted, human fVIII transgenes expressed from synthetic liver-directed promoters. To date, no inhibitors have been observed in clinical trials, but enrollment has been limited to subjects who have been extensively treated with fVIII replacement products and have no history of an inhibitor response. Therefore, the immunogenicity risk in the previously untreated hemophilia A population remains unknown. The goal of this study was to investigate the vector-specific parameters governing the immune response to liver-directed AAV-FVIII gene therapy. To achieve this, an extensive AAV-FVIII dose response study was performed using an exon 16 disrupted hemophilia A mouse model. AAV2/8 vectors incorporating combinations of two liver-specific promoters of different strengths (E06.TTR > HCB) with two engineered fVIII transgenes of different protein secretion efficiencies (ET3 > HSQ) were produced, yielding four vectors with a range of potencies (E06.TTR-ET3 > HCB-ET3 > E06.TTR-HSQ > HCB-HSQ). The dose range contained six doses, starting at 6.00x10^13 vector genomes (vg)/kg and decreasing at 3-fold intervals to 2.46x10^11 vg/kg (N=4 mice per vector/dose group). Injections were performed according to a pre-randomized, rolling enrollment study design, and plasma was collected at 20 time points through week 40. Each sample was analyzed by chromogenic assay for fVIII activity, by anti-fVIII IgG ELISA when fVIII activity decreased, and Bethesda (inhibitor) assay in the event of a quantifiable IgG titer. FVIII activity vs. time data from 67 AAV-FVIII treated mice revealed a direct correlation between the vector potency and the initial peak and rate of increase in plasma fVIII activity. A clear threshold in the fVIII activity levels was observed between mice that developed an inhibitor response and mice that did not. A single-compartment, first-order elimination pharmacokinetic model assuming a constant fVIII infusion rate demonstrated that an approximately 1 IU/day increase in the rate of plasma fVIII exposure (k_o) with a total exposure (AUC) of at least 50 IU in the first 10 days indicates a threshold for an inhibitor response [Ranges: Day 5 k_o = 3.5 - 5 IU/day (AUC = 8.7 - 12.7 IU), Day 10 k_o = 8.5 - 14 IU/day (AUC = 33.6 - 67.9 IU)]. Subsequently, mice that did not develop inhibitors were challenged with 5 weekly injections of 1ug purified ET3 protein to test whether further exposure would induce an inhibitor response or if immune tolerance had been established. Those mice that had higher steady-state fVIII activity levels demonstrated a trend toward immune tolerance, while those with lower fVIII levels trended toward inhibitor formation. These data suggest that there may be an ideal rate and level of fVIII exposure just below the proposed immunogenicity

threshold that is most effective at generating immune tolerance to fVIII in the previously untreated patient population. Studies in non-human primates seem to follow this fVIII exposure kinetics principle, with perhaps an even lower immunogenicity threshold than that observed in the hemophilia A mice. Indeed, it will be of interest to determine if this pharmacokinetic model applies generally to gene therapy approaches for hemophilia A and other monogenic disorders.

750. Co-Administration of Plasmid-Encoded IL-12 Enhances Humoral and Cellular Responses to SARS-CoV-2 synDNA Antigens and Promotes *In Vivo* Protection

Nicholas J. Tursi[1,2], Ebony N. Gary[1], Bryce Warner[3], Bryan Griffin[3], Ali R. Ali[1], Stephanie Ramos[4], Trevor R. F. Smith[4], Kate Broderick[4], Sarah K. Wootton[5], Darwyn Kobasa[3], David B. Weiner[1]

[1]The Wistar Institute, Philadelphia, PA,[2]Department of Biology, University of Pennsylvania, Philadelphia, PA,[3]Public Health Agency of Canada, Winnipeg, MB, Canada,[4]Inovio Pharmaceuticals, Plymouth Meeting, PA,[5]Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

SARS-CoV-2, the causative agent of coronavirus disease 2019 (**COVID-19**) has caused a global pandemic resulting in over 100 million cases and over 2 million deaths worldwide. Recently, several vaccine candidates have been successfully deployed into the clinic. However, continued mutations in the receptor binding domain (**RBD**) of the spike glycoprotein have been reported. These mutations may increase transmissibility and attenuate vaccine-mediated protection, thus second-generation vaccines are necessary. Here, we investigate the effect of co-immunization with plasmid-encoded IL-12 (**pIL-12**), which has been successfully used in the clinic, on the magnitude and durability of synDNA antigens expressing the spike glycoprotein (**pS**) of SARS-CoV-2. Co-immunization with pIL-12 significantly enhanced humoral responses against RBD including binding and neutralizing antibodies. We detected RBD-specific IgG in the serum of immunized animals for greater than 100 days post-immunization. In addition, co-administration of pIL-12 significantly enhanced cellular responses to the spike protein as measured by interferon gamma (IFNγ) ELISpot and intracellular cytokine staining (**ICS**) for CD4 and CD8 T cells. Importantly, a single co-immunization with pIL-12 promoted complete protection from SARS-CoV-2 infection in a novel AAV6 transduction challenge model. These initial findings support that pIL-12 co-administration can have dose-sparing effects on a SARS-CoV-2 DNA vaccine regimen supporting the continued evaluation of this regimen in the context of next-generation SARS-CoV-2 vaccines.

751. Adenosine Deaminase as a Molecular Adjuvant to Enhance SARS-CoV-2 synDNA Vaccine Responses in Young and Aged Mouse Models of Immunization and Challenge

Ebony N. Gary[1], Nicholas J. Tursi[1], Bryce Warner[2], Jennifer Connors[3], Shiyu Zhang[4], Gabriela Canziani[4], Irwin Chaiken[4], Sarah K. Wootton[5], Michele Kutzler[3], David B. Weiner[1], Darwyn Kobasa[2], Elias Haddad[3]

[1]Vaccine and Immunotherapy Center, The Wistar Institute, Philadelphia, PA,[2]Public Health Agency of Canada, Winnipeg, MB, Canada,[3]Microbiology, Drexel University College of Medicine, Philadelphia, PA,[4]Cellular and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA,[5]Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

SARS-CoV-2 is responsible for a global pandemic claiming over 2 million lives and infecting over 100 million people. Elderly patients display increased COVID-19 morbidity and mortality. Vaccine candidates have been studied and deployed in the clinic. However, second generation vaccines which are single-dose and promote long-lived immunity in young and aged populations are necessary. Germinal center follicular helper T cells (T_{FH}) promote affinity maturation of B cell receptors and memory B cell differentiation and are defined by expression of adenosine deaminase-1 (ADA-1). We investigated the adjuvant properties of plasmid-encoded adenosine deaminase (pADA) in the context of a SARS-CoV-2 spike synDNA antigens in young and aged mice. pADA co-immunized animals had increased frequencies of lymph node GC T_{FH}. A single co-immunization with pADA elicited RBD-specific antibodies equal to those observed with 2 doses of pS alone. pADA co-immunized mice sustained increased spike-specific IgG and neutralizing antibodies for >100 days following a single immunization. When immunized with pS alone, aged mice had decreased spike-binding IgG and neutralization titers. However, young and aged animals co-immunized with pADA had similar humoral responses. pADA co-immunization also rescued age-associated decreases in spike-specific IFNy secretion in the spleens and lungs of co-immunized aged mice as measured by ELISpot. Finally, co-immunization with pADA significantly impacted viral load in a novel model of SARS-CoV-2 infection after a single immunization. These data suggest that pADA enhances antigen-specific cellular and humoral immunity in young and aged mice and supports further study of this molecule as an immunoadjuvant for vaccines, specifically those targeting elderly populations.

752. Toward Persistent Control of HIV Infection by Expressing a Highly Potent HIV Decoy Receptor after In Vivo HSC Transduction

Chang Li, Hongjie Wang, Sucheol Gil, Andre Lieber

Division of Medical Genetics, University of Washington, Seattle, WA

Despite the success achieved by antiretroviral HIV-1 therapies, long-term and repeated drug administration is associated with toxicity, virus evasion, and high cost. We aim to develop a gene therapy approach for persistent control against HIV-1 infection by delivering the transgene expressing a decoy receptor (eCD4-Ig) to hematopoietic stem cells (HSCs) by in vivo transduction. In this approach, HSCs are mobilized

from the bone marrow into the peripheral blood stream and transduced with intravenously injected virus vectors. We use an integrating, helper-dependent adenovirus (HDAd5/35++) vector system that targets human CD46, a receptor that is abundantly expressed on primitive HSCs. Transgene integration is achieved by a hyperactive Sleeping Beauty transposase (SB100x) and transgene marking in peripheral blood cells can be increased by *in vivo* selection. The efficacy and safety of our in vivo HSC transduction/selection strategy has been previously demonstrated for the treatment of β-hemoglobinopathies, hemophilia A, and cancer in murine disease models. In non-human primates, we have showed safety and over 90% transgene expression in peripheral blood cells using this strategy. eCD4-Ig, containing a CCR5 mimic (PMID: Farzan) has the potential of broad neutralization activity against HIV-1, HIV-2 and SIV isolates. We have designed and produced two versions of HDAd5/35++ vectors expressing rhesus eCD4-Ig (rh-eCD4-Ig; for the purpose of test in rhesus macaques), driven by a constitutive and highly active EF1α promoter and an immunoglobulin gene promoter (KIE-Ig) specific for B-cells, respectively. In vitro assay demonstrated that both designs led to successful expression of rh-eCD4-Ig, with EF1α promoter showing over 100-fold higher transgene expression in HEK293 cells. The KIE-Ig promoter exhibited higher activity in a B-cell line (BJAB), consistent with its lineage specificity. Neutralization assay showed that the produced rh-eCD4-Ig effectively inhibited HIV-1 and SIV infection. We have performed in vivo HSC transduction in CD46 transgenic mice with the produced vectors. The animals are currently under in vivo selection for eCD4-IG expressing HSPGs. We are going to study rh-eCD4-Ig plasma levels and potential side effects in these animals. In vivo HSC transduction of a rhesus macaque is scheduled on this March 1st. We will report preliminary data from this animal. Additionally, we are exploring the therapeutic potential of our approach by targeting long-lived plasma cells (LLPCs). Using an GFP reporter, we have observed that in vivo transduction in mice with HDAd5/35++ vectors led to 7% transgene expression in total bone marrow LLPCs. Since no selection is needed, this application could generate high level eCD4-Ig production shortly after vector injection.

753. CRISPR-Cas9 Mediated Insertion of HIV LTR within BACH2 Promotes Expansion of T Regulatory-Like Cells

Sam Scharffenberger[1], Michelle Christian[1], Michael Dapp[2], Hank Jones[1], Chaozhong Song[2], Kristen Miller[2], Lisa Frenkel[3], Ann Duerr[4], Anthony Krumm[2], James Mullins[2], David Rawlings[1]

[1]Center for Immunity & Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]Microbiology, University of Washington, Seattle, WA,[3]Seattle Children's Research Institute, Seattle, WA,[4]Fred Hutchison Cancer Research Institute, Seattle, WA

A barrier to HIV cure is the limited understanding of the mechanisms that allow HIV to persist despite Antiretroviral Therapy (ART). It has been shown that HIV provirus integration sites (IS) within the transcription factor BACH2 are common, persist for years in cells from patients on ART, and almost all integration sites mapped to date have been located upstream of the start codon in the same transcriptional orientation as the gene. Our study utilized two distinct experimental approaches: 1) longitudinal analysis of samples from HIV infected individuals and 2) gene editing using CRISPR-Cas9 in primary human T cells - to better understand how one of the most commonly identified HIV integration events leads to CD4 T cell expansion and persistence. First, we performed an extensive, longitudinal RNA analysis in a large cohort of HIV infected individuals that had initiated suppressive ART early in infection. Indeed, we detected low levels (~ 1 copy per million CD4) of hybrid HIV LTR-*BACH2* transcripts in 41% of HIV infected individuals. Of note, the frequency is likely even higher than reported here, because only integrations in the sense orientation and within a limited portion of the gene (75%) would have been detected. Thus, *BACH2* is likely a near ubiquitous target for HIV integration early in infection, and with continued detection of hybrid transcripts out to 3-4 years, this implies that T cells with this IS proliferate *in vivo* and likely play an important role in HIV persistence. Next, we used homology directed repair (HDR) based- gene editing using CRISPR-Cas9 in primary human CD4 T cells to directly model these events and the effects of HIV integration within *BACH2*. Integration of the HIV LTR and major splice donor (MSD) increased levels of BACH2 RNA and protein and promoted selective outgrowth of activated T regulatory (T_{reg})-like cells. In contrast, introduction of the HIV-LTR alone, or an HIV-LTR-MSD construct into another IS hotspot *STAT5B,* had no functional impact. Thus, HIV LTR-driven *BACH2* expression modulates T cell programming and leads to cellular outgrowth; findings that support a direct role for IS-dependent HIV persistence. Our findings provide the first demonstration that targeting of the HIV LTR to a defined genomic location can modulate the biological behavior of primary human T cells. This primary T cell HDR knock-in system more closely recapitulates the endogenous transcriptional control at key HIV IS compared to previous over-expression studies, and implicates two key retroviral elements, the HIV LTR and MSD as sufficient to manipulate cellular function and phenotype through insertional mutagenesis. Taken together, these novel clinical and gene-editing findings provide a major advance to explain the persistence and orientation bias of HIV proviruses at the *BACH2* locus, and furthermore propound a provocative model in which a byproduct of certain HIV infection events may transform cells to adopt a more T-reg, immunosuppressive phenotype thereby exacerbating HIV disease progression.

754. Treatment Using SOLVx-COVID-19 in Preclinical Animal Model with a Broad-Surveillance Pooled-Peptide Therapeutic Strategy against Multiple Viral Proteins of SARS-COV2

John A Catanazaro, Anton Yuryev, Md Shamsuddin Sultan Khan

R&D, Neo7Logix LLC, Dallas, TX

This article describes the development of a pooled-peptide therapeutic, known as SOLVx-COVID-19 for the treatment of SARS COV2. The therapeutic was developed by profiling global human leukocyte antigen (HLA) susceptible populations and multiple viral protein targets to identify virus-specific short peptide sequences that initiate defense and eradication of the virus. In preclinical studies, transgenic BALB/c mice infected with a mutant human strain of SARS-COV2

virus were treated and neutralized with SOLVx-COVID-19. This pooled-peptide therapeutic design eradicated 99.8% of the viral load with no adverse effects. This proof of concept also revealed remarkable immunomodulatory stimulation of T and B cells in mice. SOLVx-COVID-19 induced no Antibody-Dependent Enhancement (ADE), a complication associated with antibody vaccine designs and the disease. The SOLVx-COVID-19 design induced only CD8+ / CD4+ T-Cell responses with concomitant reduction in IL-6, IL-10 and TNF-alpha response (commonly elevated in cytokine storm) without autoantibodies generated by B-cell responses and a significant favorable increase in Interferon gamma response. SOLVx-COVID-19 also considerably reduced the local inflammation normally associated with a cytokine storm.

755. Immunogencity and Informed Consent in Pediatric Gene Therapy

Lesha D. Shah

Psychiatry, Icahn School of Medicine at Mount Sinai, NY, NY

INTRODUCTION: Gene therapy trials utilize adeno-associated viral vector with related clinical sequelae due to issues of immunogenicity via innate and adaptive responses as well as longer-term and broader consequences. While researchers examine these immunological processes and responses across organ systems on a background of transgene mutations, other areas of study include immune-monitoring both pre- and post-treatment via assays of antibodies, identification of neutralizing factors, and methodology of cellular immune response monitoring. These complexities of immunogenicity due to adeno-associated virus (AAV) directly impact individual trial eligibility, dosing considerations during participation, and general exclusion from future access to gene therapy.METHODS: The direct impact of AAV-mediated immunogenicity on eligibility, enrollment and participation in pediatric gene therapy clinical trials is examined. *Eligibility:* Because antibodies to any component of a gene therapy may pose potential risk to patient safety and limit therapeutic potential, patients with pre-existing AAV antibodies are excluded from GT trials. Screening assays measure product-directed immune responses often with variable cutoffs for inclusion. The presence of AAV antibodies may exclude patients from current eligibility and also future eligibility for other trials or for access to future approved GT resulting in patients and families making significant efforts to isolate and modify daily activities in an effort of mitigate risk for exposure. *Enrollment:* Upon enrollment, patient and families are informed of the risks and benefits related to viral vector administration including notable risk of enhanced immune response with repeat administration of GT which generally precludes repeat administration. AAV-mediated GT limits re-dosing due to immunological consequence, impacting dose selection and individual subjects' prospect of benefit. As early-phase studies aim for potentially therapeutic with innovative dose escalation approaches, phase of trial may be crucial to patient/families' consideration of enrollment, and thereby integral to informed consent. *Participation:* Innate and adaptive immune responses directed against components of GT products may impact product safety and efficacy, and development of neutralizing and non-neutralizing immune responses that are directed against the product are monitored. Additionally, patients who have received AAV therapies may shed virus following administration. RESULTS: AAV-mediated GT's challenge transitional frameworks of consent for human subjects' research. Elements of informed consent include: consent being voluntary, disclosure of benefits to the prospective subject or others (specifically prospect of direct benefit for children), reasonable foreseeable risks or discomforts, appropriate alternative procedures or course of treatment, and, and expected duration of participation with ability to withdraw. In Pediatric GT trials, however, guardians generally consent often without pediatric assent for young children. Immunogenicity in GT results in concern regarding therapeutic dosage and prospect of direct benefit and toxicity related to dose and immune suppression. Moreover, patients receiving AAV-mediated GT are generally excluded from other clinical trial (GT and non-GT), and may be ineligible for receiving future approved GT. Additionally, consent (or participation) is life-long without the actual ability to withdraw from the therapeutic intervention following viral vector introduction. CONCLUSION: AAV antibodies directly impact current and future eligibility for GT trials as well as eligibility for alternative trial participation and for future approved gene therapies, and informed consent requires patients and families to understand these implications.

756. An Anti-CD32B/CD79B Bispecific DART® Molecule Modulates B Cells, Inhibits Antibody Responses to rAAV9 Vector, and Improves GAA Vector Survival and Transgene Efficiency in a Mouse Model of Pompe Disease

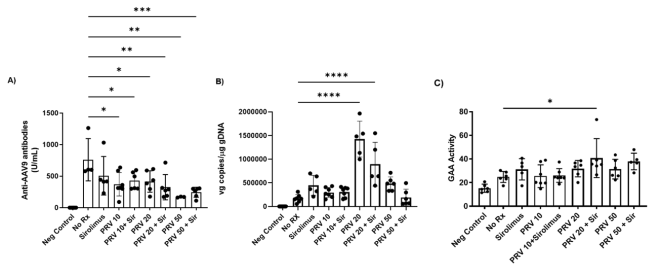
Paul J. Dunford[1], Barbara A. Perez[2], Francisco Leon[1], Manuela Corti[2], Barry J. Byrne[2]

[1]Provention Bio, Red Bank, NJ,[2]University of Florida, Gainesville, FL

Introduction: A critical challenge for the success of gene therapy, affecting both safety and efficacy is the host immune responses to both the vector capsid and transgene product. Immune mediated toxicities and clearance of vector have been attributed to both humoral and adaptive immune responses. B cells are a key contributor to the humoral response, for example, rapidly responding to AAV capsid antigens with anti- AAV antibody production and initiating adaptive immune responses via presentation of antigen. Inhibition of B cell mediated responses is therefore a potential therapeutic immunomodulatory modality to be used during gene therapy. PRV-3279 is a bi-specific antibody-based bispecific DART (dual-affinity retargeting) molecule that specifically targets B Cells by co-engaging the inhibitory Fc gamma receptor IIb (FcyRIIb or CD32B) and the B Cell receptor subunit CD79B, resulting in rapid inhibition of B cell responses without depletion. In Phase 1 studies, PRV-3279 has been shown to be safe and effective in reducing B cell responses to a viral antigen challenge. Here, the efficacy of a PRV-3279 murine surrogate in modulating immune responses and improving outcomes to a Pompe disease AAV9 vector/transgene is evaluated. **Method:** PRV-3279 bi-specific surrogate molecule, recognizing human CD32B and murine CD79B was administered daily by IP injections at 10, 20 or 50 mg/kg, starting 3 days prior to injection of the Pompe disease rAAV9-DES-GAA vector/transgene construct in to huCD32B transgenic mice. Sirolimus, a T cell suppressing agent, was also administered to some groups to evaluate additional benefit, and was dosed daily by oral gavage at 4 mg/kg starting 3 days prior to AAV injection. After administration of the AAV vector/transgene, samples were obtained from recipients at various timepoints to assess anti-AAV9 IgG levels, circulating viral

vector levels, and the efficiency of gene transfection (GAA activity) in tissue. **Results:** As seen in Figure 1, administration of PRV-3279 surrogate was able to improve multiple aspects of gene therapy-related efficiency and immunogenicity. A dose-dependent and significant reduction in immunogenicity, as detected by anti-AAV9 IgG antibodies, was observed at Day 7, with no additional benefit of sirolimus observed (Figure 1A). As a result of diminished anti-AAV9 responses, the viral vector load was also increased by PRV-3279 surrogate on Day 14, most notably and significantly at the 20 mg/kg dose level, with no additional benefit of sirolimus observed (Figure 1B). Finally, improved transfection efficiency of the GAA gene was also noted at Day 21, with a significant increase in GAA gene product activity observed in skeletal muscle with a dose of PRV-3279 at 20 mg/kg in conjunction with sirolimus (Figure 1C)**. Conclusion:** Taken together, these results suggest that PRV-3279 treatment, via modulation of antigen-specific B cell responses, can reduce the deleterious antibody response to AAV-transgene vectors, increasing their survival with consequent improvement in transgene delivery to target tissues. Importantly, inhibition of antibody mediated immunity should also ameliorate downstream immune-mediated toxicities. Combination with sirolimus may improve T cell dependent outcomes. Clinical assessment of PRV-3279 as an immunomodulatory adjunct to gene therapy is warranted.

Figure 1.



HuCD32B mice (n = 6-7/group) were administered PRV-3279 Surrogate (PRV) at 10, 20 or 50 (mg/kg) +/- sirolimus (Sir) daily 3 day prior to AAV9-DES-GAA vector/transgene administration and daily thereafter.
A: Effect of drug treatment on Anti-AAV9 IgG antibodies at Day 7.
B: Effect of drug treatment on viral vector copy in blood at Day 14.
C: Effect of drug treatment on transgene activity in skeletal muscle at Day 21.
Treatment groups were compared with those of the no treatment (No Rx) group using one-way ANOVA with Dunnett's multiple comparison test: * = P < 0.05, ** = P < 0.01, *** = P < 0.001, **** = P < 0.0001

757. Characterization of Anti-AAV Neutralizing and Binding Antibodies

Cara West[1], Joel Federspiel[2], Arpana Khatri[1], Kara Rogers[3], Sandra Casinghino[3], Laurence Whiteley[2], John E. Murphy[1], Suryanarayan Somanathan[1]

[1]RDRU, Pfizer, Cambridge, MA,[2]DSRD, Pfizer, Andover, MA,[3]DSRD, Pfizer, Groton, CT

Gene replacement therapy using recombinant Adeno-Associated Viral (AAV) vectors has been approved to treat dyslipidemia, neuromuscular disease and retinal blindness. While it is relatively safe, humoral and adaptive immune responses to the viral capsid proteins as well as the encoded transgene have been described in some clinical trials. Preexisting anti-AAV neutralizing antibodies (NAb) developed from natural infections are able to block vector transduction, and thus are a major hurdle to AAV administration. Patients with preexisting NAb titers are often excluded from clinical trials. Additionally, some antibodies are capable of binding to the AAV capsid, but do not neutralize vector transduction. The downstream effects of these binding antibodies (BAb) are not well characterized in AAV transduction. In these studies, we first sought to identify serum from healthy human donors that contain NAbs or BAbs. Preliminary studies demonstrated that donors could be stratified into those who had either NAb, BAb or both. Next, we sought to characterize these anti-AAV antibodies *in vitro.* We noted that incubating viral capsids with an excess of non-neutralizing BAbs blocked antibody-mediated neutralization. Our data demonstrate anti-AAV BAb and NAb antibodies may recognize capsid epitopes that are close in three-dimensional space. Mass spectrophotometry was used to examine factors that bound vector from different donor serum. Our studies identify proteins with high capsid affinity to AAV vectors that were common among different donors. We discuss the effect of differential binding of serum factors to capsids and the effect on vector transduction.

758. Micro-Dystrophin Gene Therapy Delivery and Therapeutic Plasma Exchange in Non-Human Primates

Ellyn L. Peterson[1], Rachael A. Potter[1], Danielle Griffin[1], Sarah Lewis[1], Eric Pozsgai[1], Aaron Meadows[2], Louise R. Rodino-Klapac[1]

[1]Sarepta Therapeutics, Inc., Cambridge, MA,[2]Wexner Medical Center, The Ohio State University, Columbus, OH

Background Adeno-associated virus (AAV)-mediated gene transfer therapy is being extensively studied as a treatment for Duchenne muscular dystrophy (DMD) and other monogenic diseases. As gene therapies rapidly advance through clinical development, it is essential to optimize their safety and efficacy, as well as determine the best dosing strategies for individuals with preexisting antibodies against the vectors used for systemic delivery. **Objective** This non-human primate (NHP) study consisted of 2 parts. Part 1 investigated the safety and efficacy of various immunosuppressive strategies for gene transfer therapy. Part 2 analyzed the safety and efficacy of Therapeutic Plasma Exchange (TPE) as a pre-treatment for individuals with preexisting immunity. **Methods** In Part 1, we intravenously (IV) dosed 5 cohorts (n=3 each) of treatment-naïve NHPs at 2x1014 vg/kg titer (supercoiled qPCR titer method) with rAAVrh74.MHCK7.micro-dystrophin (SRP-9001). We trialed different immunosuppressive regimens in each cohort pre- and post-vector administration. Cohort 1 (control cohort) received no immunosuppressive treatment. Cohorts 2-4 received prednisone at different timepoints (cohort 2: 1 day pre, 30 days post; cohort 3: 1 day pre, 60 days post; cohort 4: 14 days pre, 60 days post). Cohort 5 received rituximab, sirolimus and prednisone (14 days pre, 60 days post). We analyzed transduction and micro-dystrophin expression in muscle pre- and post-vector administration. To evaluate safety and immune response, we monitored serum chemistries and immunology throughout the study. In Part 2, we treated 7 NHPs who were dosed in Part 1 and were positive for AAVrh74 antibodies with TPE prior to a second SRP-9001 dose. These NHPs received prednisone 1 day pre- and 30 days post-vector administration. As with Part 1, we assessed safety and efficacy. **Results** After 2-3 consecutive rounds of TPE, the NHPs had reduced levels of circulating antibodies against AAVrh74 and were successfully re-dosed. The procedure was well tolerated, with no abnormal clinical or immunological observations. Additionally, NHPs that underwent TPE showed robust levels of transduction and expression in skeletal muscle, heart, and diaphragm. **Conclusion** These

findings establish TPE as a safe and effective strategy for AAVrh74 vector delivery in NHPs. The results may inform human studies of individuals with preexisting immunity.

759. PITPMN3 Is a Potential Gene Therapy Target to Treat Rheumatoid Arthritis in Fibroblast-Like Synoviocytes by Inhibiting CCL18 Associated Effect, Which Might Activate Toll-Like Receptor Pathway

Yuebin Tan[1], YingQian Mo[2]

[1]The Department of Biochemistry and Molecular Biology, Georgetown University, Washington, DC,[2]Adeno-Associated Virus Biology Section, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD

Background: Rheumatoid Arthritis (RA) is an immunological disorder that damage joints (and bones in worse cases) with chronic inflammation. CC chemokine ligand 18 (CCL18) is a secretion chemokine that is mainly produced by macrophages. Our prior study suggested that elevated CCL18 (500 ng/mL) in synovial fluid associated with RA pathological development. It has been reported that Toll-like Receptors (TLR) pathway has been activated significantly in RA patients' synoviocytes, resulting synoviocytes proliferation and migration. Both CCL18 and TLR pathway have been identified roles in RA development. However, biological connection of CCL18 with TLRs in RA is stilled unknown. **Objective:** This study focused on the potential role and connection of CCL18 and Toll-like receptors pathway in RA synoviocytes and identified a potential drug target. **Methods:** Primary RA-FLS were obtained from Parker-Pearson needle biopsy of synovial tissues from 5 cases of RA patients. Primary normal HFLS cells were purchased from Cell-Applications Inc (USA) and cultured with recombinant CCL18 (500ng/mL) or vehicle. Total RNA was extracted and isolated from sampling groups (RA-FLS, HFLS, and HFLS+CCL18). RNA-Seq were performed using Illumina SBS technology. DESeq2 was implemented to assess differentiation expression genes (DEGs). Gene-Set-Enrichment-Analysis (GSEA) were conducted to identify the pathways activities and gene ontological regulation. **Results and Discussion:** compared with HFLS, RA-FLS has significant activation of TLR pathway via Kyoto Encyclopedia of Genes and Genomes (KEGG) GSEA (Figure 1A). Upset plot indicates intersection gens in Chemokine signaling pathway (CCL18 related), RA and TLR signaling pathway has shown upregulated in 3 folds (Figure 1B). Similarly, TLR pathway was activated significantly in HFLS+CCL18 compared with HFLS. Marker genes (such as TLR4 and NFKB1) were upregulated significantly in HFLS+CCL18 (Figure 2A). Gene ontological analysis indicated multiple cellular biological processes were activated (such as cell-substrate adherents' junction and focal adhesion) which matched the activated TLR pathway biological regulation and RA molecular symptom (e.g., FLS migration) (Figure 2B). Similar TLR gene profiles in RA-FLS and HFLS+CCL18 indicates CCL18 might play positive role in RA pathological development via TLR pathway regulation. PITPMN3 (as one of the major receptors of CCL18) was upregulated in HFLS+CCL18 (Figure 2C), it could be a potential gene therapy target for RA treatment. Downregulated expression of PITPMN3 in RA-FLS by designed gene therapy compound (e.g., RNAi and CRISPR) might show potential therapeutic efficiency with further studies. **Conclusion:** Toll-like receptor pathway potentially activated by CCL18 in RA-FLS. PITPMN3 could be potential drug target by gene therapy design to treat RA.

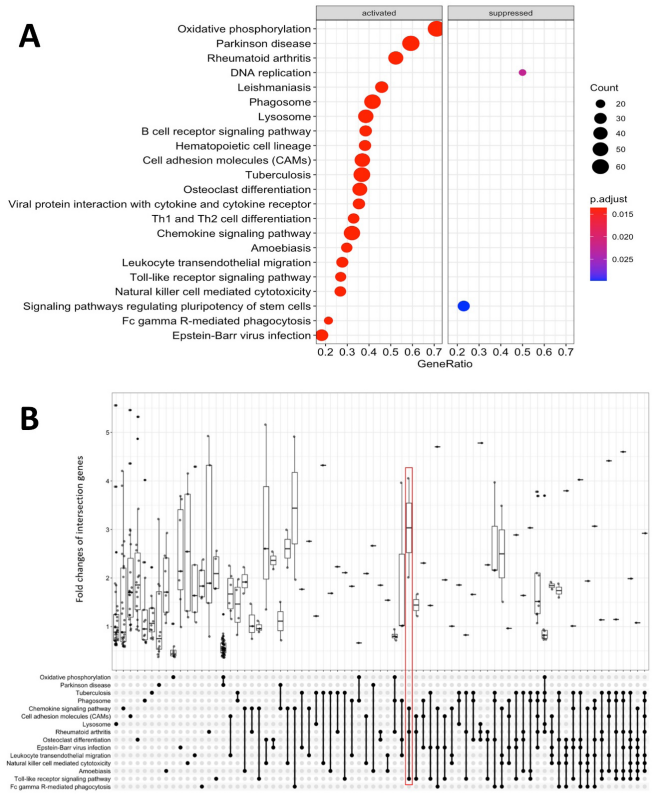


Figure 1: TLR pathway is activated in Gene-Set-Enrichment-Analysis (KEGG) of RNA-Seq in RA-FLS and HFLS. (A) Dot plot shows the top 20 KEGG pathway with significant enrichment scores (*p*.adjust <0.05). (B) Upset plot depicts the fold changes of unique and shared transcripts in each pathway from KEGG-GSEA (red box highlighted intersection fold changes of genes among Chemokine signaling pathway, Rheumatoid Arthritis and TLR signaling pathway).

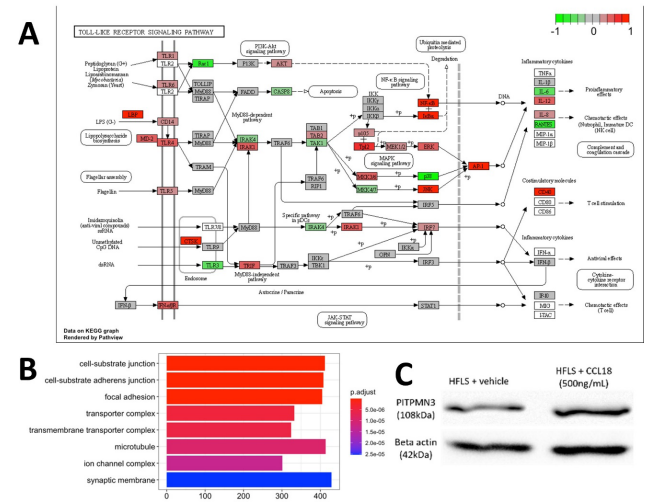


Figure 2: TLR pathway is activated in HFLS+CCL18 compared with HFLS. (A) TLR signaling pathway (KEGG) genes regulation in HFLS+CCL18. (B) Top upregulated terms in cellular components by gene ontology annotation in HFLS+CCL18 (x-axis: gene set size). (C) PITPMN3 is overexpressed in HFLS+CCL18.

760. Treatment Using SOLVx-ZIKA in a Preclinical Animal Model with a Broad-Surveillance Pooled-Peptide Therapeutic Strategy against Multiple Viral Proteins of Zika Virus

John A Catanazaro, Anton Yuryev, Md Shamsuddin Sultan Khan

R&D, Neo7Logix USA LLC, Dallas, TX

There is no effective Zika treatment despite substantial efforts after the 2015-16 outbreak. This article describes the development of a pooled-peptide therapeutic, known as SOLVx-ZIKA for the treatment of ZIKA virus. The therapeutic was developed by profiling global human leukocyte antigen (HLA) susceptible populations and multiple viral protein targets to identify virus-specific short peptide sequences that initiate defense and eradication of the virus. In preclinical studies, transgenic BALB/c mice infected with ZIKA virus were treated and neutralized with SOLVx-ZIKA. This pooled-peptide therapeutic design eradicated 93% ($P<0.01$) of the viral load with no adverse effects. This proof of concept also revealed remarkable immunomodulatory stimulation of T and B cells in mice. SOLVx-ZIKA induced no Antibody-Dependent Enhancement (ADE), a complication associated with antibody vaccine designs and the disease. The SOLVx-ZIKA design induced only CD8+ / CD4+ T-Cell responses with concomitant increase of IgM and IgG total antibody response with reduction in IL-6, IL-10 and TNF-alpha response (commonly elevated in cytokine storm) without autoantibodies generated by B-cell responses and a significant favorable increase in Interferon gamma response. It was observed that the % of B cells were reduced when compared to un-infected mouse, 21.32 ± 0.94 and infected mouse with 8.86 ± 0.32 % cells. However, the analysis showed that there is a distinct increase in % T cells and the data shows that there has been an increase in NK cell population as well in mice treated with SOLVx-ZIKA independently. Therapeutic vaccine showed eradication of virus from the brain which is potential to treat the spectrum of congenital diseases including microcephaly in newborn and Guillain-Barré syndrome (GBS) in adults. Overall, the results suggest that the SOLVx-ZIKA has reduced the virus infectivity and showed better activation of T-effector cells.

761. ImmTOR Nanoparticles Enhance the Level and Durability of AAV Transgene Expression after Initial Dosing and Mitigate the Formation of Neutralizing Antibodies in Nonhuman Primates

Takashi K. Kishimoto, Sheldon Leung, Stephanie Elkins, Teresa Capela, Gina Rizzo, Petr Ilyinskii

Selecta Biosciences, Watertown, MA

Achieving durable therapeutic activity is a major challenge for systemic AAV gene therapies. Transgene expression can be adversely affected by immune responses to the capsid or transgene product. Moreover, transgene expression may wane over time due to dilution of the nonreplicating AAV vector during cell turnover in response to injury or inflammation of the target organ or cell proliferation in a growing pediatric patient. The loss of transgene expression is exacerbated by the inability to re-treat patients due to the formation of neutralizing anti-AAV antibodies. We have previously demonstrated that tolerogenic ImmTOR nanoparticles encapsulating rapamycin selectively mitigate anti-AAV T and B cell responses and enable vector redosing in mice and in a small nonhuman primate (NHP) study (Meliani et al., Nature Commun, 2018). Here we extend those findings in a larger NHP study and evaluate the effects of ImmTOR on the level and durability of transgene expression after a single dose of AAV vector. Five cohorts of NHP (n=3 per cohort) each received 2e12 vector genomes (vg)/kilogram (kg) of AAV8 expressing human secreted embryonic alkaline phosphatase (SEAP) either alone or in combination with ImmTOR. Cohort 1 received a single dose of AAV vector alone. Cohort 2 was treated the AAV vector in combination with a single dose of ImmTOR, administered as sequential IV infusions. Cohort 3 was the same as Cohort 2, except the two components were admixed together prior to infusion. Cohort 4 received a single dose of AAV vector on day 0 and three monthly doses of ImmTOR on days 0, 28, and 56. Cohort 5 was the same as Cohort 4 with the addition of a low dose (0.2e12 vg/kg) of vector administered on days 28 and 56. Expression of the SEAP transgene product increased over time and peaked at day 28 in Cohort 1 animals receiving vector alone. All of the ImmTOR treated groups showed higher levels of transgene expression compared to Cohort 1, with an average of 60% higher levels of SEAP expression at day 28. Strikingly, SEAP expression declined precipitously after day 28 in Cohort 1, indicating an anti-SEAP immune response. In contrast, all of the cohorts treated with ImmTOR showed stable SEAP expression through day 84, with the exception of Cohort 5 which showed increasing expression of transgene from day 28 to day 84, attributable to the additional low doses of AAV vector administered on days 28 and 56. As expected, all three Cohort 1 animals treated with the AAV vector alone developed high levels of anti-AAV IgG antibodies with neutralizing antibody (NAb) titers greater than 1:3400 at day 84. Cohorts 2 and 3 showed suppression of anti-AAV IgG antibodies through day 56, although some animals exhibited a late antibody response at day 84. In contrast, Cohorts 4 and 5 animals treated with three monthly doses of ImmTOR showed suppression of anti-AAV IgG antibodies through day 84. Five of 6 animals in Cohorts 4 and 5 showed NAb titers <1:5 and the sixth animal showed a low titer of 1:8. Taken together, these results indicate that ImmTOR may enhance the level and durability of transgene expression after initial treatment of AAV vector, while inhibiting the formation of neutralizing antibodies that would enable re-administration of AAV therapy if needed.

762. Phenotype and Function of SIV-Specific CAR T Cells with CD28 and 4-1BB Intracellular Signaling Domains

Emily K. Cartwright[1], Mary S. Pampusch[1], Edward A. Berger[2], Aaron Rendhal[1], Pamela J. Skinner[1]

[1]Veterinary and Biomedical Sciences, University of Minnesota, Minneapolis, MN,[2]NIH-NIAID, Bethesda, MD

Despite a robust antiviral CD8 T cell response to human immunodeficiency virus (HIV) infection, most individuals are unable to control HIV viral load without antiretroviral therapy (ART). Chimeric antigen receptor (CAR) T cell therapy is under intensive investigation to achieve durable remission of chronic HIV infection. Ligand independent tonic signaling can lead to dysfunctional CAR T cells and costimulatory domain selection can impact tonic signaling.

Characteristics of tonic signaling include increased expression of CAR, decreased expansion and increased apoptosis in vitro, increased effector memory population, as well as increased expression of co-inhibitor expression. We used flow cytometry to examine the phenotype and function of rhesus macaque PBMCs transduced with a bi-specific SIV-CAR encoding either CD28 or 4-1BB costimulatory domains. We find evidence of tonic signaling in 4-1BB CAR T cells measured by increased expression of CAR and CD95. Though we find evidence of tonic signaling in 4-1BB CAR T cells, we did not find decreased expansion or increased apoptosis compared to CD28 CAR T cells, suggesting the tonic signaling does not have a negative impact on CAR T cells in this system. Consistent with previous findings, we find that in vitro cytokine production is greater in CD28-CAR T cells but both CAR T cell populations are capable of suppressing SIV in vitro. Taken together, these data suggest both CD28- and 4-1BB CAR cells are valid for suppression of SIV and provide rationale for pursuing studies comparing CD28- and 4-1BB CAR T cells for in vivo efficacy

763. Systemic Anti-Transgene T-Cell Immune Response Induced by Subretinal AAV Gene Transfer Can Be Inhibited by a Simultaneous Co-Injection of Peptides from the Transgene Product in Pathophysiological Conditions

Chauveau A. Gaelle[1,2], Julie Vendomèle[1], Deniz Dalkara[2], Anne Galy[1], Sylvain Fisson[1,2]

[1]Inserm UMR_S951, Université Evry, Université Paris Saclay, GENETHON, Evry, France,[2]Inserm UMR_S968, Institut de la Vision, Paris, France

Subretinal injection of adeno-associated virus (AAV) gene therapy vectors can successfully treat several inherited retinal diseases. However, some patients display inflammatory events. The eye is known as an immune-privileged site but anti-capsid and anti-transgene immune responses have been reported in some patients, possibly contributing to the loss of transduced cells. We previously reported in wild type conditions that a subretinal injection of AAV8 triggers a systemic anti-transgene T-cell response in a dose-dependent manner, and that peptides from the transgene product introduced into the subretinal space can provide a systemic antigen-specific immunosuppression referred to as subretinalassociated immune inhibition (SRAII). Here, we wanted to explore this SRAII inducer efficacy when introduced simultaneously with the AAV in a pathophysiological condition. A single subretinal injection of AAV8 with peptides from the transgene product was performed into rd10 female mice displaying an inherited retinal degeneration. The transgene cassette encoding GFP and HY male antigen, containing MHC class I- and MHC class II-restricted T cell epitopes (UTY and DBY peptides immunodominant in H-2b background), was packaged into AAV8 under the ubiquitous PGK promoter and injected subretinally with or without HY peptides at day 0 in female rd10 mice. ELISpot and multiplex assays were done 3 to 14 days post injection for systemic anti-transgene specific primary Tcell response evaluation, or at day 21 (following a subcutaneous challenge at day 14 with HY peptides adjuvanted in CFA) for memory T-cell response analysis. We found that: (i) subretinal injection of 2.10e9 or 5.10e10 vg of AAV8-PGK-GFP-HY triggered a dose-dependent systemic primary and memory anti-transgene Th1/Tc1 responses, (ii) the simultaneous co-injection of AAV8 and of HY peptides inhibited both CD8+ and CD4+ T-cell specific primary and memory responses against HY even at high dose (5.10e10 vg) of AAV. SRAII phenomenon seems to be a powerful systemic immunosuppressive mechanism specific to a transgene expressed in an eye. Since we have confirmed these results in the rd10 pathophysiological context, co-injection of the transgene product and the therapeutic vector may be considered as a new immunomodulatory strategy to control inflammatory reactions in the context of ocular gene therapy.

764. Cleavage of IgG Antibodies with the Bacterial Protease IdeS in Mice and Non Human Primates: A Potential Strategy to Circumvent Pre-Existing Immunity to AAV Vectors

Jennifer Sullivan[1], Jasmine Bloom[1], Bindu Nambiar[1], Alexandra Talotta-Guignard[2], Celine Gommet[3], Elodie Bouchoux[3], Fabienne Gallen[4], Christophe Boixel[4], Christian Mueller[1], Catherine O'Riordan[1]

[1]Sanofi, Framingham, MA,[2]Sanofi, Montpellier, France,[3]Sanofi, Vitry-Sur-Seine, France,[4]Sanofi, Chilly-Mazarin, France

Neutralizing antibodies to adeno-associated viral (AAV) vectors are highly prevalent in the human population and represent a major challenge to successful gene therapy in the clinic. Specifically, neutralizing antibodies can negatively affect the efficiency of AAV transduction in vivo, reducing the therapeutic efficacy of a gene therapy vector and preventing vector re-administration if warranted. Notably, patients with pre-existing neutralizing antibodies can be excluded from participation in clinical trials and availing of a gene therapy product once marketed. We are exploring strategies to enable successful gene transfer in the presence of an immune response to AAV. One such strategy is the use of IdeS, a protease that specifically degrades circulating IgG, currently approved for use in kidney transplantation in the clinic. Treatment with IdeS results in a transient reduction in serum IgG providing a window of time to administer a gene therapy vector. We evaluated the efficacy of IdeS to enable successful gene transfer in non-human primates with pre-existing neutralizing antibodies to AAV-SNY001, an AAV capsid that is tropic for the liver. NHPs seropositive for the AAV-SNY001 capsid received a dose of IdeS (0.5 mg/kg) two days prior to an intravenous administration of an AAV-SNY001-sFLT02 vector (5E12 vg/kg). Systemic delivery of an AAV-SNY001 vector results in liver transduction and secretion of the sFLT02 transgene into the serum. Neutralizing antibody titers to the AAV-SNY001 capsid and sFLT02 expression were measured in the serum of treated NHPs, prior to and after AAV-SNY001 vector administration for a period of three-months. IdeS treatment of NHPs seropositive for the AAV-SNY001 capsid resulted in undetectable levels of AAV-SNY001 neutralizing antibodies within 48 hours of treatment, providing a window of time for the successful administration of the AAV-SNY001-sFLT02 vector. Importantly, NAb titers to the AAV-SNY001 capsid were restored five days post AAV-SNY001 vector administration, demonstrating that the antibody suppression afforded by IdeS was transient. The levels of sFLT02 in the IdeS treated seropositive NHPs was similar to that measured in the seronegative control NHPs and was significantly higher than seropositive NHPs that did not receive IdeS treatment. sFLT02 expression in all groups

persisted over the course of the three month study. These data suggest that administration of IdeS could provide a safe and effective strategy for addressing the challenge of pre-existing neutralizing antibodies to AAV in the clinic.

Cell Therapies

765. Enhanced Differentiation Efficiency and Contractility of Human iPSC-Derived Cardiomyocyte in Xeno-Free Condition

Hokyung Oh, Yong Guk Kim, Dabin Seong, Soyeong Kang, Hyunju Kim, Joon Ho Eom, Ki Dae Park, Misun Park

Advanced Bioconverence Product Research Division, National Institute of Food and Drug Safety Evaluation, Cheongju-si, Korea, Republic of

Background: Cardiovascular disease is the most common cause of death globally. Drug treatment and surgical intervention could not address the root problems of impaired cardiac contractile function due to major loss of cardiomyocytes after ischemia. Biological intervention using human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) has emerged as a promising therapeutic approach for the treatment of heart disease. Although hiPSC-CM shows robust potential for remuscularization of injured heart, animal-derived xenogenic materials such as FBS, porcine trypsin, Matrigel in differentiation process hampered to reach the clinical reality. To overcome this concern, we established xeno-free differentiation methods for hiPSC-CM having enhanced functional myocyte contractility, and performed characteristic analysis for assessment of quality control. **Methods:** To establish xeno-free differentiation method for hiPSC-CM, all process for hiPSC expansion and further differentiation into cardiac-lineage cells was performed on feeder-free, vitronectin-coated plate in optimized xeno-free media instead of Matrigel and FBS contained media. We compared the characteristics of hiPSC cultured under xeno-free condition (XF-hiPSC) with non-xeno-free condition (non-XF-hiPSC). We examined microscopic cell morphology of hiPSCs, cell survival & proliferation ratio, undifferentiated state by alkaline phosphatase (AP) staining, and detected the expression of pluripotent markers by immunostaining and qPCR. To evaluate functional contractility of hiPSC-CM, we measured electrophysiological properties using multi-electrode array (MEA assay). **Results:** We successfully expanded hiPSCs and differentiated into cardiomyocytes in xeno-free condition. From characteristic analysis of hiPSCs, we detected high expression of AP and stem cell markers (NANOG, OCT-4, and SOX2) in both XF-hiPSC and non-XF-hiPSC. However, we found that the colony growth rate, cell viability, and total cell number of hiPSC were significantly higher for XF-hiPSC compared to non-XF-hiPSC at day 5 after seeding. According to our xeno-free differentiation, XF-hiPSC-CM cultured on vitronectin showed more vigorous beating activity at 2 wk after differentiation compared to non-XF-hiPSC-CM cultured on Matrigel or Geltrex, suggesting the importance of scaffold-materials. The electrophysiological properties such as BPM, field potential amplitude (FPA), and FPDcF which can be assessed by MEA assay was significantly increased at 4 wk compared to 2 wk after differentiation in both XF- and non-XF-hiPSC-CM. Interestingly, the mean FPA was higher in XF-hiPSC-CM (1.04 mV) than non-XF-hiPSC-CM (0.4 mV) at 2 wk after differentiation, implying early onset of differentiation process into XF-hiPSC-CM with enhanced efficiency. The expression of cTnT, a unique cardiomyocyte marker, was also detected in XF-hiPSC-CM by flow cytometric analysis. **Conclusion:** In this study, we established xeno-free differentiation method to enhance differentiation efficiency and contractility of hiPSC-CM. We also demonstrated that functional XF-hiPSC-CM can be generated in shorter period by using our XF-differentiation method compared with non-XF-differentiation condition. These results could be contributed to develop a new therapeutic source of cardiomyocyte and provide consideration points for quality control of hiPSC-CM based cell therapy product.

766. Anti-HER2 CAR Monocytes Demonstrate Targeted Anti-Tumor Activity and Enable a Single Day Cell Manufacturing Process

Linara Gabitova, Daniel Blumenthal, Andrew Best, Brett Menchel, Rashid Gabbasov, Stefano Peirini, Kayleigh Ross, Yumi Ohtani, Sascha Abramson, Thomas Condamine, Michael Klichinsky

Carisma Therapeutics, Philadelphia, PA

Engineered cell therapies have demonstrated significant clinical activity against hematologic malignancies, but solid tumors remain an intractable challenge. We have previously developed a human CAR macrophage (CAR-M) platform for adoptive cell therapy and demonstrated potent anti-tumor activity in pre-clinical solid tumor models[1]. CAR-M overcome critical solid tumor challenges such as tumor infiltration, immunosuppression within the tumor microenvironment, lymphocyte exclusion, and target antigen heterogeneity. Currently, CAR-M are generated *ex-vivo* by differentiating peripheral blood monocytes into macrophages prior to genetic manipulation - a 1 week process. In order to streamline manufacturing and reduce the cost of goods associated with cell manufacturing, we evaluated the use of engineered CD14+ monocytes, the precursors to macrophages, as a platform for adoptive cell therapy. Using the chimeric adenoviral vector Ad5f35, we engineered human monocytes to express a CAR targeted against HER2. Both CAR expression and cell viability exceeded 90%, and cells efficiently differentiated into CAR-expressing macrophages within 3-5 days of treatment with various maturation factors and cytokines. Anti-HER2 CAR-monocytes showed production of pro-inflammatory cytokines in response to antigen, phagocytosed HER2-overexpressing target cells, and eradicated HER2-overexpressing tumor cells in a time and dose-dependent manner. CAR monocytes demonstrated anti-tumor activity in both the monocyte and macrophage state. The adenoviral based gene modification method led to upregulation of several pro-inflammatory markers on CAR-monocytes and resulted in pre-conditioning of these cells towards an M1 macrophage phenotype, even in the absence of exogenous M1 polarization factors. This M1 phenotype was maintained upon exposure to immunosuppressive environments *in-vitro*. CAR-monocytes carried normally expressed myeloid chemokine receptors and responded to a panel of chemotactic factors. Notably, CAR-M originating from CAR-monocytes demonstrated potent effector function, regardless of

exposure to GM-CSF, M-CSF, or immunosuppressive factors. Finally, a large scale, closed-system, same-day manufacturing process for primary human CAR monocytes was established. By reducing the manufacturing time to 12 hours, the CAR monocyte process represents a significant translational advance in the myeloid cell therapy field.
[1]Klichinsky M, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. Nature Biotechnology. March 2020.

767. Forced HLA-F Expression Represses Nature Killer Cell Induced Cytotoxicity

Kai Wang, Xiuling Chi, Alfonso Farruggio, Ruby Y. Chen-Tsai

Applied StemCell, Inc., Milpitas, CA

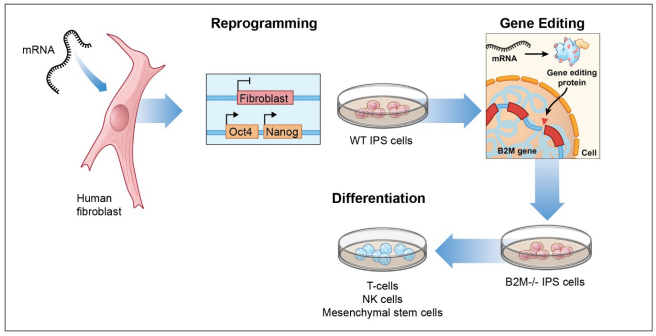
Repressing/inhibiting the host innate immunity (e.g. nature killer cell induced cytotoxicity) is part of the key strategy of building "universal cell" therapy. Forced expression of HLA-G or E in donor cells represses the host innate immunity activity in vitro and in vivo, and is used in the current "universal cell" strategy. Similar with HLA-G and E, HLA-F is in the category of nonclassical antigens (HLA-Ib). However, its function is still ambiguous. The objective of the present research is to explore HLA-F function in repressing innate immunity. We over-expressed HLA-F and HLA-F fusion with B2M (HLA-FB), as well as HLA-G as a positive control in HEK293 cells line using our H11-locus-specific TARGATT system and evaluated their immune-repression activity using nature killing cell (NK) cytotoxicity assay. We found that, similar to HLA-G, both HLA-F and HLA-FB can repress NK cytotoxicity activity in HEK293 cells. Further to verify HLA-F function in other cell lines, we expressed HLA-FB in human iPSC line (our proprietary TARGATT iPSC line) and confirmed similar immunorepression activity in these stem cells using NK cytotoxicity assay. In conclusion, this is the first research showing HLA-F`s immunorepression function similar to other HLA-Ib members, HLA-G or E. Further investigation on its mechanism and potential synergistic effect with other HLA-Ib members are in progress. This can enrich our tool box of manipulating host innate immunity and building more efficient "universal cells" based cell therapy.

768. High Efficiency Generation of Biallelic Gene Knockout iPSC Lines Using mRNA Gene Editing

Mitchell Ryan Kopacz[1], Jasmine K. Harris[1], Franklin Kostas[1], James Pan[2], Christopher B. Rohde[1], Matthew Angel[1]

[1]Factor Bioscience Inc., Cambridge, MA,[2]Exacis Biotherapeutics Inc., Cambridge, MA

Autologous engineered cell therapies such as autologous chimeric antigen receptor T-cell (CAR-T) therapies have revolutionized the treatment of hematologic cancers, however they are limited by manufacturing time and variability, the requirement for lymphodepletion, and side effects related to cytokine release. Allogeneic cell therapies derived from gene-edited induced pluripotent stem cells (iPSCs) are being developed to address the challenges associated with autologous engineered cell therapies. These "off-the-shelf" cell therapies contain specific edits designed to reduce immune rejection and to confer enhanced therapeutic properties and greater safety. However, efficient, footprint-free, biallelic targeting of defined loci in iPSCs remains technically challenging with current gene-editing approaches. We demonstrate efficient targeting of defined loci in iPSCs using novel messenger RNA (mRNA)-encoded gene-editing endonucleases comprising DNA-binding domains containing novel linker regions. We targeted exon 3 of beta-2 microglobulin (B2M), a key component of MHC class I molecules, and confirmed targeted editing in 10/12 lines, with 6/12 lines containing a desired biallelic deletion. Gene knockout in iPSCs was confirmed via RT-PCR and immunofluorescence in the context of B2M upregulation following exposure to interferon-γ. We show differentiation of $B2M^{-/-}$ iPSCs into $CD34^{+}$ hematopoietic progenitor cells using both 2D and 3D directed-differentiation protocols. This mRNA gene editing platform could serve an important tool for the development of minimally-immunogenic cell lines for future allogeneic cell therapies.



769. Advantages of the Cell Squeeze® Technology for Multiplex Gene Editing

Emily McNally, Jonathan Gilbert, Marija Tadin-Strapps, Armon Sharei, Jacquelyn Hanson

SQZ Biotechnologies, Watertown, MA

Effective gene engineering in sensitive cell types such as primary immune cells is often hampered by toxicity, cargo limitations, and scalability challenges of current delivery approaches. Microfluidic deformation using the Cell Squeeze® technology, effectively delivers a variety of cargo to cells while resulting in less cell toxicity and off-target effects than seen by other methods such as electroporation. These consequences of other editing methods are a challenge in the context of multiplexed editing, which has become of increased interest for cell therapies. This study investigated the advantages of using the Cell Squeeze® technology for editing of multiple targets and highlights the unique benefits that this technology offers for genome engineering in cell-based therapy development. We assessed the use of the Cell Squeeze® technology to deliver pre-complexed CRISPR/Cas9 RNPs specific to multiple loci in primary human T cells. Gene editing efficiency and downstream cell functionality were assessed. Editing performance of CRISPR/Cas9 RNPs delivered using the Cell Squeeze® technology was similar to electroporation across multiple loci. When comparing functional effects of the Cell Squeeze® technology to optimized electroporation, we found significant differences in transcriptional changes between the two treatments. Cells treated with electroporation corresponded with increased cytokine production, potentially resulting in premature T cell exhaustion. However, the Cell Squeeze® technology did not disrupt transcriptional profiles

or cytokine production and squeezed T cells readily expanded post treatment with robustness similar to untreated cells immediately after delivery. Transcriptional changes induced by electroporation contributed to poorer cell recovery post-delivery, characterized by a deficiency in expansion which slowed the expansion of the culture at 1 week. This work highlights the advantages of using the Cell Squeeze® technology for cell engineering. We demonstrate a clear distinction in cell performance when the Cell Squeeze® technology is compared to electroporation which results in large gene expression disturbances and functional deficiencies. The Cell Squeeze® technology is well tolerated to achieve multiple, distinct edits. These results lead us to conclude the advantages of our technology over other engineering methods in applications for therapeutic and clinical cell therapy that could potentially include a reduction in off-target expression and more viable and functional cells.

770. Up to 151.10^6 Cumulated Fold Expansion of Encapsulated hiPS Cells in Bioreactor over 28 Days, and Comparison with 2D Culture and Standard Spheroid Culture

Philippe Cohen[1], Elisa Luquet[1], Justine Pletenka[1], Elise Warter[1], Lucie Remichius[1], Eddy Quelennec[1], Andrea Leonard[1], Camille Fialin[1], Fabien Moncaubeig[1], Nathalie Lefort[2], Kevin Alessandri[1], Maxime Feyeux[1]

[1]TreeFrog Therapeutics, Pessac, France,[2]Institut Imagine, Paris, France

Stirred tank bioreactors constitute an obvious path to scale up the manufacturing of stem cell-based therapies to treat millions of patients with millions to billions of cells. Nevertheless, the mechanical agitation which is necessary to constantly homogenize the media has negative impacts on cell viability and integrity. Here we propose to encapsulate human pluripotent stem cells (iPSCs) in core-shell alginate capsules to protect them from impeller-induced mechanical damages in bioreactors. Pluripotent stem cell encapsulation is performed at high-throughput - 1,000 capsules per second - using a proprietary microfluidic device designed to meet industrial requirements (GMP-ready, automated, closed and single-use system). Once encapsulated, cells grow in a protected microenvironment without direct contact with bioreactors mechanical stressors. Oppositely to bead encapsulation, liquid core capsules provide stem cells with space to grow. Each micro-compartment allows for the self-organization of a biomimetic epiblast-like 3D stem cell colony. Following cell amplification inside the capsule, cells can easily be harvested by dissolving the hydrogel shell. Here we demonstrate:

#1 A very robust weekly amplification factor: over 100x / 7 days

#2 Similar performance in static or stirred cultures

#3 Straightforward scale-up from 3mL static culture to 1L bioreactor (10L pending)

#4 Proven maintenance of stemness: OCT4/NANOG+/+ superior to 92% (over 4 iPS cell lines)

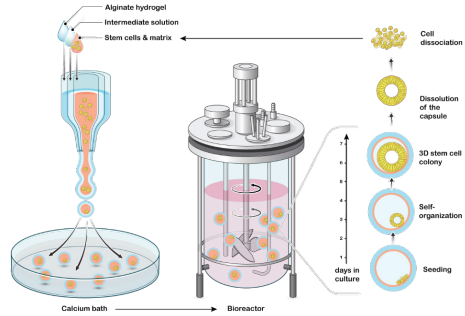
#5 Extremely low cell mortality during encapsulation and bioreactor culture: less than 2%

#6 Serial encapsulation passaging: 4 encapsulations in a row, over 28 days of dynamic suspension culture *in capsulo*

- 151 million cumulated amplification factor over 28 days

- OCT4/NANOG+/+ : over 99 % at day 28

#7 Differentiation inside the capsules enables an integrated process from stem cell amplification to cell therapy product. In summary, the scale-independence of the cell micro-environment enables rapid development of large-scale cultures of stem cell or differentiated cells. Beyond scalability and improved "time to clinic", we anticipate that cultivating stem cells in a biomimetic stress-free 3D micro-environment will significantly improve cellular quality.



771. A Novel Booster Molecule Improves the Manufacture of TCR Gene-Edited Allogeneic CAR-T Therapies

Devon Shedlock, Maximilian Richter, Stacey Cranert, Min Tong, Yening Tan, Rebecca Codde, Leslie Weiss, Julia Coronella, Eric Ostertag

R&D, Poseida Therapeutics, San Diego, CA

Genetic disruption of the T cell receptor (TCR; >99% of cells) during manufacture of allogeneic (Allo) chimeric antigen receptor (CAR)-T therapies is required to prevent graft-versus-host disease in non-HLA-matched patients but can nevertheless severely affect overall CAR-T activation, expansion, quality and/or yield. The TCR is commonly used as a key receptor for T cell stimulation in most autologous CAR-T manufacturing strategies whereby activation via binding by anti-CD3 agonist monoclonal antibodies (mAbs), oftentimes in conjunction with CD28 ligation, results in stimulatory signals crucial for the generation of multiple cellular responses, including proliferation and differentiation. However, in Allo strategies, knockout of any single component of the TCR (e.g., CD3 or either TCR chain) causes loss of the entire TCR complex from the surface of the engineered T cell, thereby eliminating or significantly reducing its responsiveness to anti-CD3 mAbs during manufacture.We designed a novel chimeric stimulatory receptor (CSR), or "booster molecule", to deliver critical primary stimulation to TCR-negative Allo CAR-T cells during manufacture to help rescue and/or improve their growth, phenotype and function. Several T cell surface proteins with commercially available agonist mAbs were identified and then engineered to also express the intracellular CD3 zeta chain. In this way, when expressed in TCR-negative cells, incubation with the cognate agonist mAb during manufacture may result in binding and activation of both the cell surface fusion protein and the CD3 zeta chain, which could ultimately act as a surrogate for optimal TCR stimulation. We assessed both stable and transient expression of candidate boosters during Allo CAR-T cell manufacture; use of the nonviral piggyBac® (PB) DNA Modification System enabled integration and stable

expression of candidate CSR molecules, while transient expression was achieved by co-delivery of mRNA encoding candidate CSRs. Lastly, since boosters could also possibly function as "natural-ligand" CARs capable of killing receptor-positive cells, we performed mutagenesis studies to identify CSR mutants that were incapable of mediating target cell killing but retained receptivity to agonist activator mAbs.A novel booster was developed that resulted in enhanced expansion and yield, allowing for the production of more than 35 billion functional Allo CAR-positive cells from a single manufacturing run (e.g., hundreds of doses at a standard dose effective in autologous CAR-T clinical trials). CAR-T cells maintained equivalent functional activity as non-edited CAR-T cells and exhibited an exceptionally high level of proliferative capacity and percentage of desirable stem cell memory (TSCM). Furthermore, mutagenesis studies revealed a single point mutation within the receptor binding domain of a CSR that completely abrogated killing of receptor-positive cells while fully retaining receptivity to the cognate agonist activator mAb. This mutant version is not only safer, as it cannot mediate killing, but also eliminates the possibility of prolonged auto/trans-stimulation via binding of natural ligands on activated T cells during manufacture. Importantly, a novel booster enabled manufacture and development of several off-the-shelf Allo CAR-T product candidates, including P-BCMA-ALLO1, which are engineered using PB in combination with the high-fidelity Cas-CLOVER™ Site-Specific Gene Editing System. These products have demonstrated superior potency in NSG xenograft tumor models and are rapidly advancing towards the clinic.

772. Robotic Automation of T Cell Generation for the Treatment of Acute Myeloid Leukemia (AML)

Sherket Peterson[1], Anastasiya Smith[2], John D'Aigle[1], Tara Shahim[2], Derian Salas[1], Thorsten Demberg[2], Aditya Tandon[1], Sam Chang[2], Alex Riley[1], Jeannette Crisostomo[2], Jaime Avalos[1], Tsvetelina Hoang[2], Jose-Manuel Collados[1], Juan Vera[2]

[1]Division of Healthcare, Consumer Segments & Service Robotics, ABB Inc, Houston, TX,[2]R&D, Marker Therapeutics, Houston, TX

Two of the most critical challenges in cell therapy, after achieving clinical efficacy, are scalability and reproducibility. Multi-tumor-associated antigen (mTAA)-specific T cell therapy developed by Baylor College of Medicine has been administered to over 150 patients with blood-derived malignances and solid tumors and has shown clinical efficacy without toxicity. In AML or myelodysplastic syndromes (MDS), 11 of 17 enrollees in the adjuvant group never relapsed [median leukemia-free survival (LFS) not reached at the median follow-up of 1.9 years)]. 11/15 patients remained alive [estimated 2-year overall survival (OS) of 77%], which compares favorably with HSCT outcomes for risk-matched patients [median LFS of 9-15 months and 2-year OS of 42%]. In the active disease setting 8 enrollees were treated. 1 patient developed a complete response (CR) that was durable for 13 months and 1 had a partial remission (PR) with reduction in blasts from 50% to 15%. Additionally, 1 patient had reduction in blasts from 70% to 45%. Due to these encouraging clinical data, Marker Therapeutics licensed this technology to commercialize this therapy. To streamline and automate the manufacturing process for commercialization, Marker Therapeutics and ABB healthcare robotics have established a unique collaboration aimed at developing the first GMP robotic assistant that can be placed in a standard biosafety cabinet to collaborate with a human operator during mTAA-specific T cell manufacturing. We have simplified the manufacturing process by decreasing the number of interventions from ~3000 to ~13 and decreasing the culture time from 38 to 16 days. These changes resulted in improved T cell phenotype and function - a 12-fold increase in the proportion of naïve/stem cells, a 13-fold decrease in the percentage of terminally-differentiated effector cells and broader antigen specificity. The improved process provided the basis for process automation. To assess if a robotic prototype could aid in manufacturing, ABB has adapted a single-arm YuMi robotic arm, which has a small footprint, 7-axis dexterity, high speed, accuracy and exchangeable grippers to interact with a serological pipette. The robot was more precise and accurate than human operators in pipetting 25 mL aliquots of liquid to achieve a target volume of 250 mL. It was also more consistent in the number of cycles performed over a 1-hour test period, while the human operator's performance deteriorated over time. The combination of significant process simplification with robotic automation should address some of the challenges associated with cell therapy and make this therapy readily available to a larger patient population.

773. Base Editors as a Singular Platform for Polyfunctional Multiplex Engineering of Immune Cells for Cancer Immunotherapy

Walker Lahr, Mitchell Kluesner, Cara-Lin Lonetree, Nicholas Slipek, Branden Moriarity*, Beau Webber*

Department of Pediatrics, University of Minnesota, Minneapolis, MN

The efficacy of adoptive cell therapy remains limited against solid and epithelial cancers. As the circuitry of immune function becomes more clear, increasingly complex engineering is being pursued to improve outcomes. We previously implemented base editors for multiplex knockout (KO) in T cells, demonstrating improved efficiency and reduced genotoxicity over Cas9 nuclease[1,2]. However, introduction of tumor-specific receptors relies on randomly integrating vectors that pose safety risks and hinder function. Using an orthogonal system where SpCas9-BE4 mediates KO and Cas12a stimulates knockin (KI) via homology directed repair (HDR), we achieved one-step KI+KO in T cells with high efficiency (>50% KI, >90% KO). To implement base editors as an all-in-one tool for multiplex KO+KI, we tested the ability of SpCas9-BE4 and ABE8e nickase activity to stimulate HDR. Although both editors activated HDR (BE4=11.4%, ABE8e=25.3%), efficiencies were significantly lower than that of spCas9 nickase (74.2%). By incorporating a novel gRNA design strategy and a modified AAV template, we increased the efficiency of ABE8e-stimulated HDR by >2-fold, achieving KI rates >60% in human T cells. By employing gRNAs targeted to disrupt splice sites we believe that we will be able to combine these high rates of ABE8e mediated KI with efficient multiplex gene KO[2]. These findings establish a platform that expands the utility and safety of base editing platforms, while providing a simplified platform for complex cellular manufacturing.

1. Webber, Lonetree, Kluesner, et al. (2019). Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. Nat. Commun. 10, 5222 (PMID: 31745080)

2. Kluesner, Lahr, Lonetree, et al. (2020). CRISPR-Cas9 cytidine and adenosine base editing of splice-sites mediates highly-efficient disruption of proteins in primary cells. bioRxiv.

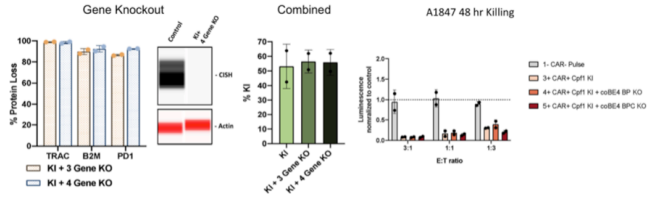


Figure 1. Orthogonal (Cas12a) HDR combined with BE4 knockout efficiently mediates gene KI and KO. Cas12a CAR KI combined with BE4 KO leads to efficient killing at multiple effector to target ratios.

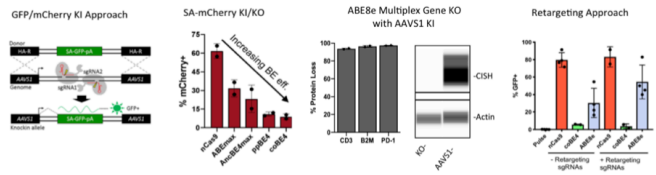


Figure 2. Base editor mediates knockin of SA-GFP and SA-mCherry. Multiplex ABE8e engineering is an efficient method of targeted gene KO. Combining ABE8e mediated nicking with gRNAs that retarget base edited DNA increases KI efficiency.

774. A Robust and Semi-Automated HIPSC Gene Editing Workflow

Claire Richards[1], Philip D. Manos[2], Ian Taylor[1]
[1]Solentim Ltd., Wimborne, United Kingdom,[2]EverCell Bio, Natick, MA

Human induced pluripotent stem cells (hiPSCs) have the potential to address a broad range of research and clinical applications, including disease models, cell reprogramming and therapeutics. However, the inability to robustly manipulate hiPSCs as single cells has, until recently, been a significant biological and technical hurdle. We have recently shown the robustness and significant advantage of using the VIPS™ system in combination with MatriClone™ matrix (in solution) to achieve clonal outgrowth with 30-40% efficiency compared to under 10% achieved with limited dilution (LD). This significantly decreases total workflow time and cost by reducing the need for large quantities of plates or high reagent consumption. Critically, in contrast to typical LD techniques, the VIPS system ensures clonality during the droplet formation at the time of seeding and then once more after the addition of media, providing a "double-lock" of assurance and establishing new standards in efficient single cell cloning. This study translates the improvements that the VIPS and MatriClone bring to a hiPSC gene-editing workflow. Using CRISPR-Cas9 via RNP delivery we aimed to disrupt the *EMX1* gene locus in an hiPSC line. Following nucleofection, cells were single cell seeded via VIPS or limiting dilution (LD) and grown out in media containing MatriClone. Plates underwent daily whole-well imaging on the VIPS system to confirm clonality and track outgrowth of the colonies. As previously described, the VIPS demonstrated a 3-4 fold improvement in numbers of colonies successfully derived from single cells per plate when compared to the LD control. From the VIPS-seeded plates, 100 clones were selected and genotyped to confirm indel formation in the *EMX1* locus. Three clones containing a confirmed indel were then expanded and underwent characterisation. Selected clones exhibited pluripotency marker expression of Oct4 and Tra-1-60 via ICC/IF and normal karyotype, validation that pluripotency and genomic integrity is maintained. Here we have demonstrated successful gene editing with VIPS using *EMX1* as proof of concept. Importantly, these data show the advantages of using VIPS with MatriClone over traditional LD methods. By increasing efficiency, we estimate a 50% reduction in typical gene-editing workflows, providing a platform for improving low-efficiency editing projects. Moreover, the VIPS system provides quality data assurance and reporting that allows for real time assessment and regulatory compliance.

775. NK-Xpander: Generation of a Feeder-Free NK Cell Expansion Medium for Cell Therapy

Erica Heipertz, Andrew Hungler, Elise Gill, Mohan C. Vemuri, Navjot Kaur
Cell Biology, Thermo Fisher Scientific, Frederick, MD

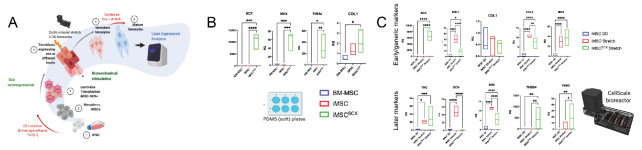
Natural killer (NK) cells are gaining importance as powerful effector cells for adoptive immunotherapy of cancers. They have the potential to be safer, less expensive, and more effective than current engineered T-cell therapies. As part of the innate immune response, NK cells respond to anything they perceive as "non-self", including malignant cells. Unlike T-cells, NK cells are able to provide an anticancer response in an antigen independent manner, allowing NK cells to be a potential "off the shelf" allogeneic therapeutic product. Genetically modified feeder cells are typically used to successfully expand NK cells. However, despite being lethally irradiated before use, the feeder cells do pose safety concerns. Generating clinically relevant numbers of NK cells, without the use of feeder cells, continues to be a major challenge. We have developed a "feeder-free" NK cell culture medium that expands functional primary human NK cells to clinically relevant levels in two to three weeks. NK cell expansion does vary depending on the donor, however qualified donors have shown up to 1000-2000 fold expansion within 2 weeks. The expanded NK cells are functional and able to kill K562 cancer cells within 2 hours of co-incubation. NK-Xpander directly increases the ability to generate large numbers of functionally viable NK cells needed for translational researchers to run phase I and II clinical trials; ultimately improving the quality of life of cancer patients.

776. Directing Stepwise iPSC MSC Differentiation into iTenocytes Using Combined Transcription Factor Overexpression and Cyclic Loading in 2D Bioreactor

Angela Papalamprou, Victoria Yu, Tina Stefanovic, Angel Chen, Khosrowdad Salehi, Juliane Glaeser, Dmitriy Sheyn
Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA

Tendon and ligament injuries are the main reasons for all musculoskeletal consultations worldwide, associated with high morbidity, prolonged disabilities, while secondary ruptures often ensue. The mechanical/structural properties of repaired tissue are permanently altered and fail to reach those before injury. Development of a cell therapy, which could be applied to an injured tendon, could dramatically alter patient

outcomes. Bone marrow mesenchymal stromal cells (BM-MSCs) have been the main source for tendon repair strategies due to their abundance, multipotency and regenerative potential *in vivo*. However, their major disadvantages are their limited self-renewal capacity, their heterogeneity and potential for ectopic bone formation, which limits their clinical application. Additionally, their tenogenic potential might be restricted. Induced pluripotent stem cells (iPSCs) have been successfully differentiated to MSCs (iMSCs) by our group and others. The main advantage to the use of iMSCs is that they potentially represent an unlimited source for tenocytes. However, without additional modification, similarly to BM-MSCs, naïve iMSCs have limited tenogenic potential. Development of tenocytes progresses through at least two stages: first, tenocyte progenitors (tenoblasts) express scleraxis (Scx). Second, tenocyte maturation results in tissue formation. However, overexpression of Scx in BM-MSCs was not found sufficient for tenogenesis. Mechanical cues are known to affect the embryonic tendon development, differentiation of stem/progenitor cells and healing. Uniaxial cyclic stretching has been used to model these cues and drive maturation of the tenocyte phenotype. Tenocytes can actively sense external loading. Tenoblasts/tenocytes are able to retain their phenotype when they are stretched but were shown to dedifferentiate when cultured statically. Therefore, combinatory mechanical and biological stimulation may be essential for the differentiation of stem/progenitor cells and the maturation of tenoblasts. We hypothesized that iMSCs can be efficiently differentiated to tenocytes by stable overexpression of Scx and biomechanical stimulation *in vitro* (Fig. 1A). First, we generated 2nd generation lentiviral vectors expressing Scx tagged with GFP. BM-MSCs and iMSCs were cultured *in vitro* using plates coated with polydimethylsiloxane of varying rigidity. Following 14 days, BM-MSCs cultured on 32kPa stiffness resulted in upregulation of Scx and Mkx, while iMSCs upregulated Scx, Mkx, Bgn and Dcn (Fig. 1B). Cyclic stretching of BM-MSCs, BM-MSC^{SCX+}, iMSCs and iMSC^{SCX+} for 3 days resulted in upregulation of the early mechanoresponsive tendon transcription factors Egr-1 and Scx, suggesting that the cells responded to the loading stimulus acutely. However, later markers of tendon differentiation (Tnc, Bgn, and Dcn) were detected in all cell types only after 7 days of cyclic stretching. Interestingly, Tnmd and Thbs4 upregulation were only observed in the iMSC^{SCX+} group (Fig. 1C). Thus, combined stable overexpression of Scx and mechanical stimulation were able to induce tenogenic marker expression in iMSCs but not in BM-MSCs. Taken together, our data suggest that iMSCs might be more developmentally plastic compared to BM-MSCs, which would make those cells a better candidate for tendon cell therapy applications.



777. Use of a Predictive Yield Calculator to Promote Raw Material Standardization and Donor Stewardship for Allogeneic Cell Therapies

Jennifer L. Chain[1], Jessica Raley[2], Rogelio Zamilpa[2], Jeanie Goree[1], Blaire Rus[1], DeAnn Cross[3], Tuan Le[4], Charles Mooney[1]

[1]BioDevelopment, Oklahoma Blood Institute, Oklahoma City, OK,[2]GenCure, BioBridge Global, San Antonio, TX,[3]Apheresis Collections, Oklahoma Blood Institute, Oklahoma City, OK,[4]Medical, Oklahoma Blood Institute, Oklahoma City, OK

Indications for cell therapies are expanding and so is the demand for human-derived starting material. A high-quality donor retention program focused on maintaining biologically ideal donors is key to standardizing and sustaining the apheresis-derived raw material needed to manufacture allogeneic cell therapies. Blood centers have sophisticated donor recruitment and loyalty programs and are leading an effort to standardize apheresis raw material to provide consistent product characteristics and promote donor stewardship. Using a highly refined and predictive yield calculator, a retrospective analysis was performed on 25 apheresis mononuclear cell (MNC) collections and 71 peripheral blood stem cell collections at two blood centers to predict the number of MNCs, CD3+ T cells, and CD34+ stem cells collected. The calculator factors in starting white blood cell count, total blood volume processed, and the starting MNC%, CD3% or CD34%. Strong and significant correlations were found between the number of cells collected and predicted by the calculator at 50% collection efficiency. For MNCs, the Pearson correlation coefficient (r) is 0.910 (P<0.0001), for CD3+ T cells, r is 0.902 (P<0.0001), and for CD34+ stem cells, r is 0.916 (P<0.0001). These data indicate that this refined predictive yield calculator is a useful tool to help achieve a target cell yield during allogeneic apheresis collections. Since each donor has a different total blood volume, hematocrit, pre-collection WBC count, and responds differently to medications to mobilize progenitor cells, performing collections using total blood volumes processed guarantees variability in every apheresis product. Additionally, maximizing the number of blood volumes processed when collecting apheresis products puts unnecessary strain on volunteer donors. Donors are exposed to more citrate as collection times increase, which can cause cramping, fatigue, nausea, and low blood pressure. Although rare, severe hypotension and cardiac arrythmias have been reported due to apheresis-related citrate toxicity. If 20 liters of blood were processed for all 71 stem cell collections analyzed, the predicted CD34 yields would have ranged from 24 million to 2.9 billion CD34+ cells. For the 25 MNC collections analyzed, CD3+ T cells would have ranged between 3 and 18 billion if 20 liters of blood were processed for each donor. These extended collections expose every donor to unnecessary risks and create non-standardized final products, which is not ideal for use in validated manufacturing processes. To meet the CD3+ targets for each of the analyzed MNC collections, only an average of 10 liters of blood (5.3 blood volumes) was processed from each donor. Therefore, to process the minimum blood volume possible to reach the targeted cell yield, we propose the use of a predictive yield calculator to help collect a pre-determined number of MNCs, CD3+ or

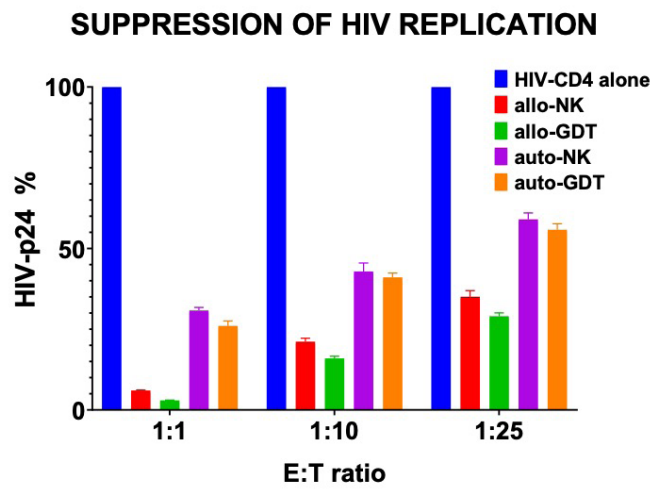
CD34+ cells. Minimizing the time of apheresis collections will promote donor safety and increase the likelihood of biologically ideal donors returning for future donations.

778. Immunotherapeutic Potential of Inhibitory KIR/HLA Mismatched Allogeneic NK and gamma-delta T Cells as an HIV Cure Strategy

Serhat Gumrukcu[1,2], David W. Hardy[3], Mark Dybul[4], Tung X. Nguyen[1,2], Gregory T. Howell[1], Phillip Musikanth[1]

[1]Seraph Research Institute, Los Angeles, CA,[2]Enochian Biosciences, Los Angeles, CA,[3]The Johns Hopkins University School of Medicine, Baltimore, MD,[4]Georgetown University, Georgetown, DC

Background: Certain current HIV cure strategies are based on targeting latent reservoirs activating viral replication and simultaneously neutralizing the virus with immunotherapeutic approaches. These strategies focus on cellular antiviral responses and face challenges including limited antigen presentation, insufficient cytotoxic T cell responses, and immune exhaustion or tolerance. Both natural killer (NK) and gammadelta (γδ)T cells are innate immune cells known to be important in HIV responses. Because antigen recognition is achieved outside of MHC restriction in these cell subsets, we studied inhibitory KIR/HLA mismatched allogeneic NK and γδT cells as a potential adoptive cell therapy for HIV cure. **Methods:** PBMCs were isolated from two ART-controlled persons with HIV (PWH) and two seronegative KIR/HLA mismatched individuals. γδT cells were expanded using IL-2 and zoledronic acid, and NK cells were isolated via immunomagnetic separation. In vitro killing assays were run with 1:1, 1:10, 1:25, 1:50 effector/target cell ratios in co-cultures with allogeneic or autologous NK or γδT cells and CD4+ T cells from PWH superinfected with HIV JRCSF strain *in vitro*. Same experiments were done with latent CD4+ T cells that were unstimulated or treated with latency reversal agent vorinostat (VOR). To study the killing of latent cells, latent target cells were treated with VOR after killing experiments. HIV p24 antigen was measured in cell cultures via ELISA and in cells with intracellular p24 staining by FACS. **Results:** Allogeneic NK and γδT cells inhibited HIV replication in superinfected CD4+ cells substantially at 1:1 (94% and 97%) and 1:10 ratio (79% and 84%) compared to autologous cells (69% and 74% at 1:1, 57% and 59% at 1:10) (Fig 1). Similar results were obtained with VOR-treated latent target cells. Unstimulated latent cells co-cultured with either allogeneic cell at 1:1 ratio demonstrated no p24 production after VOR treatment, potentially suggesting the mismatched cells' ability to recognize latently infected cells. **Conclusion:** Utilization of allogeneic NK and γδT cells could be an important strategy to target HIV latently-infected cells as a potential pathway to curing HIV. An IRB-approved, exploratory pilot clinical study testing this concept is ongoing.



779. Multi-Engineered, iPSC-Derived NK Cells for the Treatment of B-Cell Malignancies

Mark A. Wallet, Barry A. Morse, Chiamin Bullaughey, Michael Naso, Buddha Gurung, Shelby Keating, Liam R. Campion, Mark Mendonca, Luis Ghira Borges

Research, Century Therapeutics, Philadelphia, PA

Induced-pluripotent stem cells (iPSCs) offer a unique opportunity to create novel cell therapies for cancer with advantages over conventional cell therapies that are derived from leukapheresis products. These advantages include: 1) ability to perform multiple genetic editing steps at the renewable iPSC stage, 2) ability to select and fully characterize single cell clones for genetic integrity and functionality before selecting final product candidate, and 3) ability to generate a clonal master cell bank that is used to produce highly uniform drug products at low cost. Here we describe a highly-engineered iPSC derived natural killer cell (iNK) with six precise genetic modifications intended for the treatment of cancer. These modifications include the use of stealth edits to reduce alloreactivity, a safety switch for product elimination if necessary, the expression of a homeostatic cytokine for improved functionality and persistence, and a chimeric antigen receptor (CAR) targeting CD19 to mediate tumor cell engagement and killing. For resistance to CD8 T cell-mediated allogenic immune rejection, the beta-2-microbulin (β2M) gene is disrupted with simultaneous insertion of a transgene encoding the HLA-E protein with tethered β2M and a peptide. HLA-E was introduced to prevent NK cell cytotoxicity against the engineered cells, which lack HLA-I. For resistance to CD4 T cell-mediated allogenic immune rejection, the class II major histocompatibility complex transactivator (CIITA) gene is disrupted with simultaneous insertion of a transgene encoding both the extra-cellular and transmembrane domains of EGFR, and the NK cell growth factor IL-15. EGFR provides an elimination tag that can be engaged by clinically approved anti-EGFR antibodies, such as Cetuximab. Finally, a CAR targeting the CD19 antigen is encoded by a transgene delivered into a safe harbor locus. From the engineered, clonal iPSC bank, iNK cells are differentiated using a 30-day long feeder cell-free process. These iNK cells resemble peripheral blood derived $CD3^{-}$ $CD56^{+}$ NK cell lymphocytes based on cell surface phenotype. They eliminate HLA-I negative tumor cells comparable to peripheral blood NK cells and

when exposed to CD19+ target cells, CAR-iNK cells, they demonstrate potent antigen specific killing that is comparable to conventional CART cells. The multi-engineered iNK cell platform provides a new paradigm for cancer cell therapies through an off-the-shelf product that can be delivered in repeated doses with reduced risk of allo-rejection.

780. Directly Link T Cell Phenotype and Function to Genotype with the Opto Cell Therapy Development 1.0 Workflow

James Lovgren
Berkeley Lights, Emeryville, CA

Background: The key challenges to developing T cell-based therapies center on the fact that T cell-mediated tumor death relies on complicated cell-cell interactions and several complex mechanisms. These therapies have also been associated with significant side effects related to cytokine release syndrome (CRS) and neurotoxicity, placing importance on understanding T cell anti-tumor functions like cytokine release and killing kinetics. Ideally, T cell therapies would be tailored to mediate the rapid destruction of multiple tumor cells while reducing these side effects. **Methods:** The Berkeley Lights Opto™ Cell Therapy Development Workflow is a collection of software capabilities, reagents, and protocols that allow scientists to selectively measure cytokine secretion, visualize killing behavior, and sequence TCRs from individual cells in parallel. Here, we demonstrate its use for CAR-T cell phenotypic and functional screening as well as the discovery of TCRs associated with specific T cell behaviors. **Results:** The cumulative percentage of pens with tumor cell caspase-3 activity increased over time in pens loaded with CD19+ tumors, peaking at 50% tumor cell death after 16 hours of incubation. This is in contrast to only 10% of pens displaying tumor cell death in control pens loaded with CD19- tumor cells; control pens also exhibited slower killing kinetics. The single-cell resolution of the OptoSelect™ microfluidic chip enabled us to analyze each significant T cell-tumor cell interaction. We were able to directly compare differences in killing kinetics of individual T cells and link this tumor killing behavior to IFNγ secretion. We identified fast-killing and slow-killing CAR-T cells in a single-day experiment, which could then be exported for genomic analysis. We highlight an example where TCR alpha and beta sequences are recovered from single T cells after export. **Conclusion:** The Opto™ Cell Therapy Development Workflow on Berkeley Lights systems enables researchers to correlate cytokine secretion to target cell killing behavior in CAR-mediated antigen recognition, discriminate CAR-T cell subsets based on kinetics of target cell killing, and link cytokine secretion and target cell killing behavior to TCR sequence in TCR-mediated antigen recognition.

781. Production of Recombinant Protein after Transplantation of Ex Vivo Transduced Macrophages

Nora K. Clarke, Anthony Sinadinos, Cuixang Meng, Eric W. F. W. Alton, Uta Griesenbach
National Heart and Lung Institute, Imperial College London, London, United Kingdom

The UK Respiratory Gene Therapy Consortium has developed a novel vector for *in vivo* gene therapy delivery to the lung; a lentivirus virus pseudotyped with Sendai virus envelope proteins (F and HN). Given the large surface area of the lung and the extracellular barriers that exist, there would be obvious cost and efficiency benefits to undertaking transduction *in vitro*. We are, therefore, in parallel assessing an *ex vivo* allogeneic cell therapy approach for secreted protein delivery. Recently a mouse study by Arumugam et al. 2019 demonstrated that following lung delivery, macrophages derived from peripheral mononuclear cells mimic the long-term stability of alveolar macrophages. We aim to develop a system where macrophages function as transgenic protein factories that secrete therapeutic protein within the lung. To determine transduction efficiency, we first transduced murine bone marrow-derived macrophages (BMDMs) with an F/HN lentiviral vector carrying a green fluorescent protein (EGFP) reporter gene. We assessed multiplicity of infections (MOIs) ranging from 1-100 and demonstrated dose-related expression that peaked at an MOI of 50 with an average of 3.6±1.4% of cells being GFP positive (n=6). Next BMDMs were transduced with a F/HN lentiviral vector carrying a secreted Gaussia luciferase reporter gene at MOI 50 and transplanted at a dose of $2x10^6$ or $6.3x10^6$ cells to the mouse lung via oropharyngeal aspiration (n=4/group). Mice were culled 1 week after transplantation and dose related Gaussia luciferase was detectable in BALF and lung homogenate harvested 7 days post-delivery. (BALF: 9,100 [min/max: 6,717/14,816] RLU and 26,313 [min/max: 22,787/34,955] (p=0.01) RLU at low and high dose respectively, Lung: 10,257 [min/max: 6,403/22,699] RLU/mg protein and 28,377 [min/max: 21,862/45,093] (p=0.01) RLU/mg protein). We next repeated the study and showed that expression levels were similar 1 and 2 weeks after treatment and that transplanted Ly5.1 positive BMDMs were still detectable in BALF 2 weeks post-transplantation. We will next assess the efficacy of BMDMs expressing GM-CSF in a mouse model of acquired pulmonary alveolar proteinosis. In preparation for these studies we delivered BMDMs which were transduced with a lentiviral vector expressing GM-CSF (MOI 50) to the lungs of healthy mice ($2x10^6$ BMDMs/mouse), and quantified GM-CSF expression 1-week post transplantation. GM-CSF was detected in the BALF of mice that received transduced macrophages (n=4) (11.42 [min/max: 5.6/22.2] pg/ml) but was undetectable in mice receiving untransduced cells (n=3). We are currently assessing whether transplantation of BMDMs expressing GM-CSF can ameliorate PAP biomarkers in a mouse model. In summary, we have shown, for the first time, that F/HN lentiviral vectors can transduce BMDMs and following transplantation lead to expression of a therapeutically relevant protein in the murine lung.

782. Development of a Feeder-Cell-Free and Serum-Free Culture Method for Primary Natural Killer Cell Expansion

Christopher Johnson[1], Eric Frary[1], Caroline Nazaire[1], Jamie Van Etten[2], Bora Faulkner[2], Sookyong Joo[3], Brian Astry[3], David Hermanson[2], Kevin Flynn[2], Nithya Jesuraj[1], Joseph Lomakin[1]
[1]Bio-Techne, Woburn, MA,[2]Bio-Techne, Minneapolis, MN,[3]Bio Techne, Minneapolis, MN

Introduction: Generation of clinical scale doses of natural killer (NK) cells is an important challenge for Chimeric Antigen Receptor (CAR)-NK cell therapies. Current manufacturing methods to expand NK cells use irradiated feeder cells and human or bovine serum. However, these methods are restrictive due to high costs, scale-up challenges, licensing restrictions, and clinical translation challenges. Feeder-free workflows offer defined reagents to reduce variability and improve consistency for clinical scale manufacturing. In this study we investigated a method to expand NK cells in a feeder-free and serum-free system that enables cell expansion for cell therapy manufacturing. **Methods**: An NK cell culture expansion method was developed with dissolvable microspheres conjugated to CD2 and NKp46 antibodies part of the Cloudz™ Human NK Cell Expansion Kit. Expansion in serum-free media was compared to a serum-containing culture method. For the serum-containing culture, human peripheral blood mononuclear cells (PBMCs) were seeded at a density of 4,800 NK/cm^2 in T25 flasks. Serum-containing cultures were maintained in SCGM media, 10% FBS, 1% penicillin/streptomycin, for 10-days with cytokines: 27 ng/mL IL-2, and 10 ng/mL each of IL-12, IL-18, IL-21. The cytokines and media were refreshed every 3 days. We previously determined that seeding density is an important parameter for NK culture. For serum-free culture, PBMCs were seeded at a range of densities from 25,000 - 400,000NK/cm^2 in 24-well plates and cultured for 14 days in Excellerate™ NK media with the same cytokines. On the final day, PBMCs were analyzed using a flow cytometer for CD3$^-$/CD56$^+$ NK cells. **Results and Discussion**: Preliminary experiments showed that NK cells expanded from PBMCs required a higher seeding density when expanded in serum-free media compared to expansion in serum-containing media. Therefore, the seeding density was increased and assayed from 25,000-400,000 NK cells/cm^2. The culture time was increased from 10 to 14 days to accommodate the slower growth rate in serum-free cultures. Since donor variability is an important factor in NK expansion, the results were repeated in 3 separate donors (Figure 1). The results suggest that, after 14 days in culture, NK purity is maximized at a seeding density of 100,000 NK/cm^2. However, NK cell expansion decreases as the initial seeding density is increased. Together, these data indicate that the total number of expanded NK cells does not increase proportionally as the seeding density is increased. In our newly evaluated protocols, 100,000 NK/cm^2 seeding density offered the highest combination of NK expansion and purity in serum-free conditions. The results from the serum-containing Cloudz NK Cell expansion protocol resulted in 79±14% purity and 239±110-fold expansion (15 donors, 322 samples). Growth in the serum-free ExCellerate™ media expansion resulted in 80±20% NK cell purity and 94±36-fold expansion (7 donors, 30 samples). These results provide an effective serum-free and feeder-cell free cell expansion method as a step toward a GMP-compatible clinical workflow for NK and CAR-NK cell therapy.

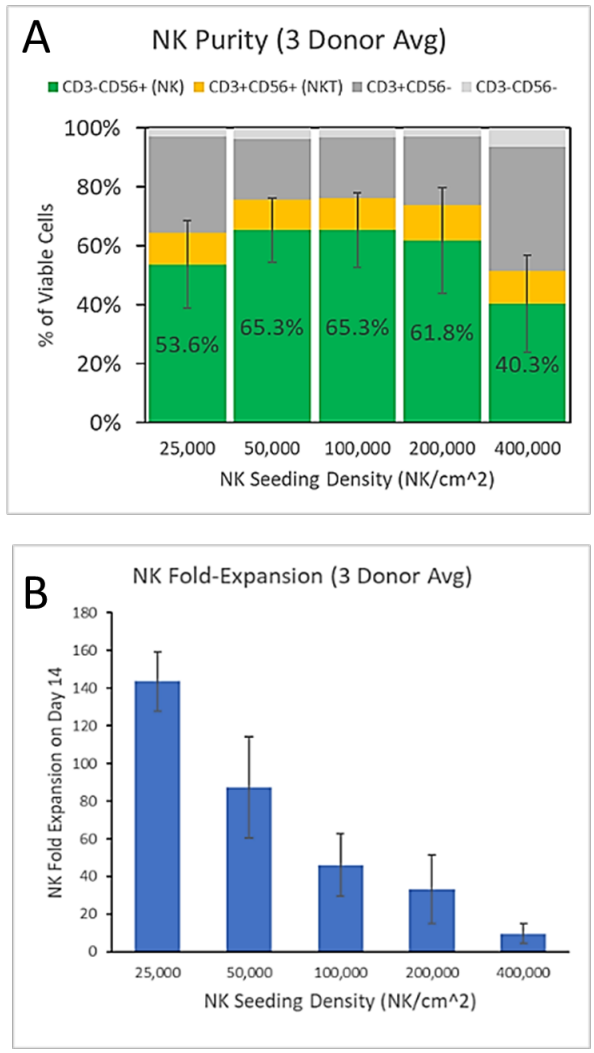


***Figure 1**: The effect of seeding density on (A) the distribution of viable cells and (B) the fold-change in viable NK cells after 14-days of culture in serum-free Excellerate™ NK media. Results show the mean±standard deviaiton from 3 independent donors.*

Cell Therapies

783. Characteristic Analysis of Neural Stem Cell Directly Reprogrammed from Human Fibroblast

Soyeong Kang, Nari Seo, Ji Won Park, Hokyung Oh, Hyunju Kim, Ki Dae Park, Joon Ho Eom, Misun Park
NIFDS, Cheongju-si, Korea, Republic of

Background: Neurodegenerative diseases are caused by a loss of neurons within the central or pheripheral nervous system. Because of the regenerative potential of neural stem cell(NSC), cell-ased therapy using NSC is an emerging therapeutic approch targeting neurodegenerative diseases. NSC can be obtained from embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC). However, ethical issues and potential risk of tumorigenicit still remain as major concerns before clinical reality. To overcome these issues, we directly reprogrammed human fibroblasts into neural stem cells and performed characteristic analysisfor assessment of quality controls.Methods: To generate human induced neural stem cells (iNSC), human fibroblasts were directly reprogrammed by introducing pluripotent factors (Oct4, Sox2, Klf4, c-Myc) with Sendai virus using optimized media and growth factors at each step. After obtaining of stabilized iNSC colonies, Sendai virus inactivation was performed by further incubation at 39 celsius degree in CO2 incubator for 15 days. To identify the properies of iNSCs, we assessed microscopi cll morphology, doubling time, Short Tandem Repeat(STR) profiling. The expression of iNSC-specific markers was also detected by immunostainig and qPCR. To examine the differentiation ability of iNSC into neuronal-lineage cells, we further measured neuron-specific eletrophysiological activity of differentiated cells using patch clamp assay.Results: We successfully generated iNSC from human fibroblasts by direct reprogramming. The iNSC showed traditional round-shaped pluripotent stem cell morphology after 14 days culture with reprograming protocol. In doubling time analysis, fibroblast and iNSC has different doubling time. The STR profiling results showed same genetic identity between fiboblast (starting cell) and iNSC, suggesting genetic stability after direct reprogramming. Furthermore, we detected the expression of NSC-specific markers (Nestin, SOX1, SOX2, PAX6, N-cadherin) in iNSC by immunostaining and qPCR. However, iNC did not express CoL1A1, a fibroblast marker, and NANOG, one of the pluripotent marker. To examine differentiation abililty of iNSC, we differentiated iNSC into neuronal-lineage cells b adding supplments such as BDNF, GDNF and compound E. The cell morphology was changed from day 3, and the growth of dendrites was observed from day 7. After 20 days of differentiation, the expression of neuron-specific markers(Microtubule-associated protein 2, Tuulin beta-3 chain) was detected by immunostaining. We also confirmed the neuron-specific electrophysiological activit of iNSC-derived neuronal lineage cells by patch clamp assay.Conclusion: In this study, we demonstrated that iNSC was successfully generated from human fibroblasts by direct reprogramming. The results from the characteristic analysis of iNSC and differentiated neuronal-lineage cells infer a potential usage of direct-reprogrammed iNSC for treatment of neurodegenerative disease. We also suggest that this characteristic analysis can be applied to quality control assays for human iNSC-based cell therapy products.

784. Temporal Gene Regulation of T Cell Enhancers by Locus Targeted Engineering Enables Cytokine Autonomy and Augments Anti-Tumor Efficacy of iPSC Derived Off-the-Shelf CAR-T Therapy

Yijia Pan, Mochtar Pribadi, Matthew Denholtz, Bjoern Gaertner, Bi-Huei Yang, Gloria Hsia, Hui-Yi Chu, Emily Carron, May Sumi, Eigen Peralta, Janel Huffman, Joyee Yao, Dave Robbins, Emily Driver, Chia-Wei Chang, Cokey Nguyen, Tom T. Lee, Bahram Valamehr
Fate Therapeutics, San Diego, CA

Adoptive cell therapy has shown great promise in treating various cancers. However, challenges with the current autologous and allogeneic therapies remain, including product consistency, cost, complexity of manufacture, and inherent genetic engineering variability seen with patient- and donor- derived immune cells. Induced pluripotent stem cells (iPSCs) are a unique renewable starting material for the manufacture of homogenous off-the-shelf immune cells. We recently developed FT819, a first-of-kind off-the-shelf chimeric antigen receptor (CAR)-T cell therapy derived from a renewable master iPSC line engineered to uniformly express a novel CD19 1XX-CAR driven by the endogenous (TCR) promoter at the T-cell receptor α constant (TRAC) locus. Such features provide access to off-shelf availability, antigen specificity and improve safety by knocking out TCR to eliminate the possibility of graft versus host disease. To further enhance the effectiveness of iPSC-derived CAR-T cells (CAR-iTs), especially in solid tumor settings, we sought to develop a novel off-the-shelf CAR-T cell product engineered to express T cell enhancers (TCEs, e.g. a cytokine receptor signaling complex) under the endogenous promoter at a desired T cell locus (TCL), where the gene expression is tightly regulated during the course of T cell differentiation and subsequent activity. This engineering strategy would enable the CAR-iTs to precisely express TCEs in a temporally regulated manner, therefore boosting the potency of CAR-iTs without inducing undesired outcomes such as early-stage exhaustion or checkpoint inhibition. To identify the ideal TCLs, we examined gene expression profile by RNA-seq throughout the lifecycle of CAR-iTs. We identified a list of candidate loci where gene expression is not upregulated until the late stage of differentiation, including TRAC, CD25 and CD38. We then designed strategies to insert TCEs into candidate TCLs by CRISPR engineering without disrupting the endogenous gene expression. As a proof of concept, we tested IL-15 receptor fusion protein (IL15RF) as the TCE at a selected TCL and demonstrated improved CAR-iT activity. Utilizing our single cell derived iPSC engineering platform, a clonal iPSC established to express a CAR19 1XX-CAR at the TRAC locus was then engineered by CRISPR/Cpf1 mediated knock-in of IL-15RF into the selected TCLs. Successfully engineered iPSC clones were validated for specific targeted integration of IL15RF without random donor template integration, and the transgene copy number was confirmed by droplet digital PCR as well as genome stability by karyotype analysis. Next the function of these engineered CAR-iTs was examined during in vitro differentiation. We demonstrated that IL15RF can be tightly regulated by the endogenous promoter of TCLs in a stage specific manner, while the endogenous gene expression remains mostly unaffected. Furthermore, CAR-iTs with these engineered modalities

exhibited improved expansion during differentiation. Functional studies including antigen specific cytokine release assays, cytotoxic T lymphocytes assays, and serial restimulation assays demonstrated enhanced efficacy. Our preliminary data validate the potential of engineering multiple modalities at desired loci of clonal iPSCs to enable the creation of functionally enhanced CAR-T cells. Ongoing work is focused on extending these studies into disease specific models, as well as further investigating other TCE and TCL candidates for the development of next-generation iPSC derived CAR-T therapy with better efficacy and safety profiles.

785. Rationally Designing Multi-Domain Proteins in High-Throughput for Cell Therapies, without a Protein Structure

Martin Jacko, Chen Yu, Aaron Goebel, Brian Buttrick, Yana Gofman, Kenny Workman, Paul Duyker, Winode Handagama, Diego Vargas, Colin Farlow, Justin Farlow

Serotiny, San Francisco, CA

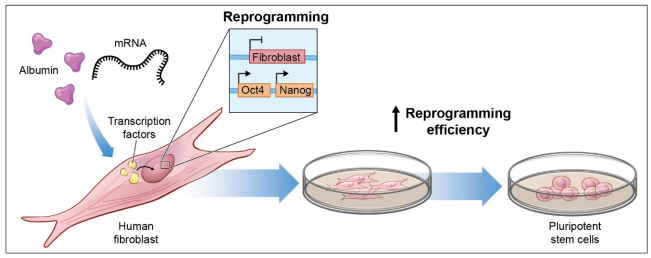
Rationally designing transmembrane signaling proteins is a process that requires overcoming constrains different to those related to enzyme or antibody protein engineering. In Cell Therapies, the primary clinical impact of the protein payloads is to improve the functional capabilities of the modified cell. Importantly, these new cell functions are not solely defined by protein-intrinsic properties like binding coefficients or catalytic efficiencies of the payload protein. We have used our high-throughput system to design, construct and characterize libraries with over 100,000 different Chimeric Antigen Receptors (CARs) for their effect on cellular function. We have identified arrangements and identities of novel domains in the CAR scaffold, other than the binding region, that effect T cell function. We have used machine learning to integrate empirical data from prior designs in order to build subsequent generations of CARs with novel regulatory, costimulation and activation domains. We are currently utilizing this domain-driven protein design pipeline to address current issues in CAR T cell therapies, aiming to reduce tonic signaling, limit exhaustion, increase persistence, and expand the available IP landscape. With our high-throughput data we aim to show generalized effects of different protein architectures on T cell function.

786. Lipid-Stripped Albumin Enables High-Efficiency mRNA Reprogramming of Adult Human Fibroblasts

Jasmine K. Harris[1], Mitchell Kopacz[1], Franklin Kostas[1], Mackenzie Parmenter[2], Christopher B. Rohde[1], Matthew Angel[1]

[1]Factor Bioscience Inc., Cambridge, MA,[2]Novellus, Inc., Cambridge, MA

Induced pluripotent stem cells (iPSCs) have emerged as an exciting platform for developing personalized cell therapies. However, clinical applications are limited by the low efficiency of viral and episomal reprogramming methods and safety concerns related to vector integration and genomic scarring. Using messenger RNA (mRNA) to reprogram cells avoids these issues, however previously reported mRNA-based reprogramming methods required lengthy transfection schedules and suffered from the need to use proprietary media formulations. Here we report a seven-day, high-efficiency, immunosuppressant-free mRNA reprogramming protocol for dedifferentiating adult human dermal fibroblasts into iPSCs. To achieve this, we designed a chemically defined, animal component-free and xeno-free reprogramming medium that contains specially treated albumin, which we show enhances transfection efficiency and supports efficient reprogramming. We measured reprogramming efficiency by counting the number of SSEA4 positive colonies generated from a starting culture of $1x10^3$ adult human dermal fibroblasts. Using this approach, we measured a 6% reprogramming efficiency, which is a 60-fold improvement to the <0.1% efficiency typically obtained with viral and episomal reprogramming methods. We analyzed iPSCs for expression of the pluripotency markers OCT4 (POU5F1), SSEA4, and NANOG, as well as by differentiating the cells in vitro into mesenchymal stem cells, hematopoietic stem cells, osteoblasts, adipocytes, chondrocytes, T cells, natural killer (NK) cells, cardiomyocytes, and neurons. We also explored the role of other media components, including TGF-β, in mRNA reprogramming and evaluated protein expression, immunogenicity, and reprogramming efficiency of various novel mRNA chemistries and molecular designs.



787. Urine-Derived Stem Cells for Treatment of Acute Kidney Injury in Mouse and Human Organoid Models

Julie Bejoy[1], Richard C. Welch[1], Felisha M. Williams[1], Matthew H. Wilson[1,2,3], Neal Paragas[4,5], Lauren E. Woodard[1,2,6]

[1]1Department of Medicine, Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, TN,[2]Department of Veterans Affairs, Nashville, TN,[3]Department of Pharmacology, Vanderbilt University, Nashville, TN,[4]Department of Radiology, University of Washington, Seattle, WA,[5]InVivo Analytics, Inc., New York, NY,[6]Department of Biomedical Engineering, Vanderbilt University, Nashville, TN

Urine-derived stem cells (USCs) are adult human stem cells that propagate in tissue culture in media containing growth factors on gelatin-coated plates. We have isolated, expanded, transfected, and tracked these cells. Because the cells are renal in origin, we hypothesized that they may be able to treat acute kidney injury. FACS characterization confirmed the characteristic marker panel (CD44, CD73, CD90, & CD146 + / CD31, CD34, & CD45 -). When luciferase-modified USCs were injected into healthy kidneys, they migrated to the surgical wound scar. Luciferase-modified USCs also migrated to the surgical wound scar if a sham operation was performed with an IP injection of cells, suggesting that the cells are able to home to sites of injury. The InVivoPLOT plug-in for the IVIS enabling quantitative tomographic optical live animal imaging allowed us to quantify the luciferase signal

within the individual organs of the live mice. Luciferase-modified USCs were injected into the peritoneum of NSG mice on Day 3 after acute kidney injury was induced by ischemia-reperfusion. When one kidney was injured and the other kidney was healthy, the luciferase-modified USCs did not home to the injured kidney. However, when the healthy kidney was removed to enhance the injury, the luciferase-USCs homed to the injured kidney. This signal appeared within two hours following IP injection, suggesting that homing of the USCs to sites of injury occurs rapidly. Histologic studies found USCs in the tubules and glomeruli of mice subjected to rhabdomyolysis-induced acute kidney injury that were sacrificed the day of injection. To evaluate the therapeutic effect of USCs on injured human kidney tissue *in vitro*, we cultured kidney organoids from human induced pluripotent stem cells. The derived organoids expressed the kidney markers ECAD (distal tubule), GATA3 (collecting duct), LTL (proximal tubule) and NEPHRIN (glomeruli). We co-cultured labeled USCs with healthy kidney organoids and found efficient incorporation within two days of the culture. We established a nephrotoxic injury model by adding Cisplatin (5 μm) at day 21 of kidney organoid culture. The organoids treated with 5 x 10^4 USCs at day 22 for 48 hours showed significantly reduced expression of kidney injury molecule-1 (KIM-1) compared to the organoids without USCs, suggesting a positive therapeutic impact of USCs. In conclusion, USCs are an easily isolated, clinically relevant cell type that can be genome engineered and migrate from their injection site to the injury. Incorporation of USCs into injured kidney organoids reduced the tubular injury within the organoids. In future studies, we plan to compare markers of acute kidney injury in mice with and without USC treatment.

788. Pooled Single Cell Knockin Screens Identify Synthetic Cellular States for Improved Cellular Therapies

Theodore Roth

Microbiology and Immunology, UCSF, San Francisco, CA

Genetically-engineered immune cell therapies have proven effective to treat some types of cancer, but most tumors still cannot be cured. Forward genetics with CRISPR loss-of-function screens in T cells have identified some mutations to enhance cellular therapies, however these methods do not yet take full advantage of the power of gain-of-function knock-in targeting to engineer cell functions. We developed a robust new platform to assess the functional effects of pools of knock-in constructs in parallel. We used high-efficiency nonviral gene editing to introduce large panels of candidate therapeutic knockin constructs into a defined genome position in human T cells allowing us to compete the cells against each other and test which constructs enhance T cell function. Pooled knock-ins combined with single-cell sequencing also revealed high-dimensional cellular phenotypes induced by each construct ex vivo and in an in vivo tumor microenvironment. Finally, large scale pooled knockin screens identified synthetic cellular states induced by specific therapeutic knockin constructs that correlated with improved anti-tumor functionality and reduced exhaustion phenotypes. Overall, these studies demonstrate the power of pooled knockin technology to discover and functionally characterize complex synthetic gene programs that can be written into targeted genome sites to generate more effective cellular therapies.

789. P-BCMA-ALLO1 — A Fully Allogeneic Stem Cell Memory T Cell (T_{SCM}) CAR-T Therapy Targeting BCMA for the Treatment of Multiple Myeloma Shows Potent Anti-Tumor Activity

Maximilian Richter, Stacey Cranert, Deepak Patil, Yan Zhang, Yening Tan, Min Tong, Christine Domingo, Leslie Weiss, Karl Marquez, Jessica Sparks, Elvira Argus, Christopher Martin, Eric Ostertag, Sumiti Jain, Julia Coronella, Devon Shedlock

Immuno-Oncology, Poseida Therapeutics, San Diego, CA

Emerging data from the clinic highlight a great potential of autologous CAR-T cell therapies in the treatment of refractory/relapsed multiple myeloma (MM). Despite their promise, autologous CAR-T cell therapies face many manufacturing challenges including consistency, product variability, toxicity and cost. However, the production of allogeneic off-the-shelf CAR-T cell therapies from healthy donor T cells can address these concerns.Using Poseida's proprietary non-viral piggyBac® (PB) DNA Delivery System, in combination with the high-fidelity Cas-CLOVER™ (CC) Site-Specific Gene Editing System, we have engineered a fully allogeneic P-BCMA-ALLO1 product candidate for MM. P-BCMA-ALLO1 is generated by editing healthy donor T cells using CC to eliminate surface expression of both TCR and MHC class I, with little to no off-target activity for optimal safety. Moreover, CC efficiently edits resting T cells that have not yet undergone activation, a critical element of our process that uniquely yields a T_{SCM}-enriched allogeneic CAR-T product candidate. Tscm have been correlated with best responses and superior safety in the clinical trial of our P-BCMA-101 autologous product candidate. Inclusion of a "booster molecule" in our allogeneic manufacturing process further improves the expansion of gene-edited cells and enables the production of hundreds of patient doses from a single manufacturing run, thereby reducing the manufacturing cost per dose into the same range as that of a monoclonal antibody. In addition to the CAR, the PB system enables the delivery of a safety switch, which can be used to rapidly deplete some or all of the administered cells in case of an adverse event, as well as a selectable marker allowing the generation of a final cell product that is >95% CAR-positive. In addition to selective CAR+ enrichment during expansion, our final product also undergoes depletion of residual CD3+/TCR+ cells in a robust clinical scale manufacturing process, resulting in a safe T_{SCM} enriched allogeneic BCMA CAR-T product for MM.Preclinical in vitro evaluation of P-BCMA-ALLO1 showed effective target-dependent killing, cytokine secretion as well as high proliferative capacity in serial restimulation assays, as we expect from Poseida's unique high percentage T_{SCM} product candidates. Importantly, the gene-edited allogeneic product exhibited similar or superior performance compared to an autologous product, both in vitro and in multiple in vivo immunodeficient mouse (NSG) models. In an aggressive systemic MM.1S model, P-BCMA-ALLO1 from multiple donors induced long-term tumor control after a single dose administration. Notably, anti-tumor efficacy and in vivo CAR-T expansion observed for P-BCMA-ALLO1 was comparable to that of non-gene edited healthy donor CAR-T cell controls.The P-BCMA-ALLO1 product is generated using multiple proprietary components of Poseida's technology that aim to confer efficacy and persistence,

safety and cost effective and consistent manufacturing of our product candidate in the clinic. P-BCMA-ALLO1 could potentially make a significant impact on the treatment of MM.

790. Microfluidic Facilitated CRISPR-Cas9 RNP Transfection

Ailin M. Goff, Ian Sicher, Jocelyn Loo, Tiffany Dunn, Nicole Clary, Benjamin Chang, Alla Zamarayeva, Miguel Calero-Garcia

Research and Development, CellFE, Alameda, CA

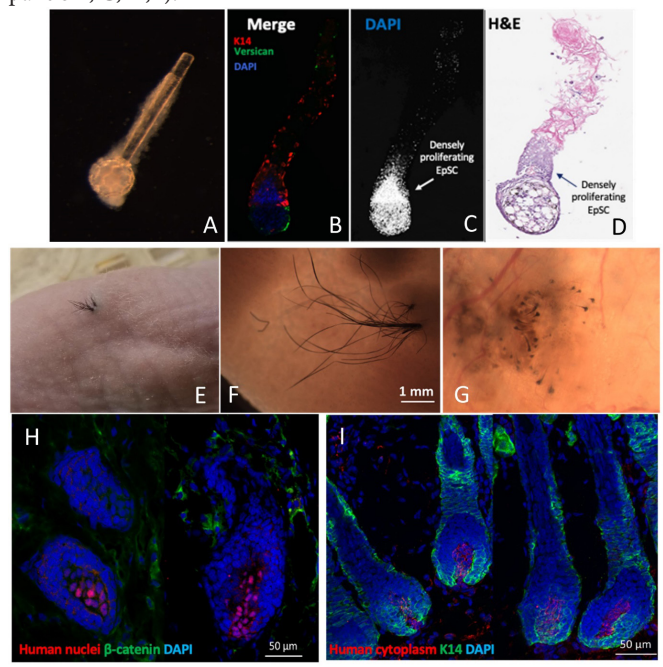
The gene editing of T cells has given rise to new gene therapies such as Chimeric Antigen Receptor (CAR) T and T Cell Receptor (TCR) T-cell therapies. These innovative therapies offer hope for patients with otherwise untreatable cancers and can be engineered with gene editing, using CRISPR-Cas9 ribonucleoprotein (RNP) complexes. We have developed a microfluidic transfection device which allows volume exchange for convective transfer (VECT). The device uses non-viral means to efficiently deliver the CRISPR-Cas9 payload, leading to high levels of TCR knock-out (60-80% TCR knockout; n=3), while retaining cell functionality. In the present work, we have demonstrated that the device has a large operational range and is able to successfully transfect cells with varying cell density (1×10^6 - 8×10^6 cells/mL) and RNP concentrations (6 - 30µg/mL). Additionally, the co-transfection of multiple guide RNAs induced a double knock-out of the TCR and CD5 genes (~30% co-transfection). These results demonstrate a proof of concept that the microfluidic device can be utilized for the development of gene editing-based T-cell immunotherapies.

791. Derivation of Folliculogenic Organoids from Human iPSC

Antonella Pinto[1], Yulia Shamis[1], Lisa McDonnell[1], Alison Chambon[1], Michael Jung[1], Joan Valls Cuevas[1], Richard Chaffoo[2], Meghan Samberg[1], Cenk Sumen[1], Alexey Terskikh[1,3]

[1]Stemson Therapeutics, San Diego, CA,[2]Plastic Surgery and Cosmetic Dermatology, La Jolla Skin, La Jolla, CA,[3]Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA

Background & Aim Induced pluripotent stem cell (iPSC) research has enabled a new class of clinical cell therapies to address unmet medical needs. Although hair loss is a benign disorder, even the most negligible amount of hair loss can be devastating to a patient's self-esteem and quality of life. For the hundreds of millions of people globally who have hair loss because of androgenic alopecia, medication, scarring, or autoimmune conditions, there exist very few options for hair restoration. The aim of this research is to provide a solution to hair loss for those patients that neither respond to medications nor are good candidates for hair transplantation due to extensive hair loss. We are developing a regenerative medicine approach to hair restoration based on patient-derived induced pluripotent stem cells (iPSC) transplanted in a biodegradable scaffold.
Methods, Results iPSC differentiated into dermal papilla cells (iPSC-DPs) and epithelial stem cells (iPSC-EpSCs) were combined and cultured in 3D to generate hair follicle organoids. Organoids were cultured both *in vitro* and transplanted *in vivo* in mice and monitored for folliculogenesis. For ease of transplantation and directed cell growth, the organoids were loaded into degradable scaffolds and transplanted into the backs of mice. To additionally study the individual contribution of each iPSC-derived cell type on folliculogenesis, iPSC-DPs were combined with mouse E18.5 keratinocytes (mKCs) and iPSC-EpSCs were combined with mouse E18.5 dermal cells (mDCs). As scaffold controls, the cell combinations were injected into the dermis of mice via "patch assay." Cultured and transplanted organoids were harvested at various timepoints and analyzed via immunohistochemistry. *In vitro*, the 3D co-culture of iPSC-DPs and iPSC-EpSCs resulted in spontaneous formation of organoids. iPSC-DP and iPSC-EpSC aggregated within the scaffolds after 24 hours (image panel A). Over time, the cells self-organized, migrated, and proliferated to form a hair follicle-like structures, with specific arrangements of dermal and epithelial cells that expressed human hair follicle biomarkers as analyzed by flow cytometry, RNA sequencing, and immunocytochemistry (image panels B-D). *In vivo*, transplantation of organoids resulted in de novo hair follicle generation with multiple hair shafts, as observed after 40 and 90 days (image panels E-G). Analysis of the hair follicles documented expected stereotypic positions occupied by human iPSC-DPs and iPSC-EpSCs within newly generated hair follicles (image panels B, C, H, I).



Conclusion We have demonstrated de novo generation of hair follicles by co-culturing iPSC differentiated cells within a biodegradable scaffold. This technology will eventually provide an unlimited source of patient-specific hair follicles to address a range of hair loss disorders.

792. Evaluation of Competing Methodologies for In Vivo Tracking of Human Regulatory T Cells Intended for Adoptive Cell Therapy in Transplantation

Jacinta Jacob[1], Suchita Nadkarni[2], Alessia Volpe[3], Qi Peng[3], Sim Tung[1], Rosalind Hannen[2], Cristiano Scotta[1], Robert I. Lechler[1], Federica Marelli-Berg[4], Lesley Smyth[5], Giovanna Lombardi[1], Gilbert O. Fruhwirth[3]

[1]School of Immunology and Microbial Science, King's College London, London, United Kingdom,[2]Centre for Cell Biology & Cutaneous Research, Queen Mary University of London, London, United Kingdom,[3]Comprehensive Cancer Centre, King's College London, London, United Kingdom,[4]William Harvey Research Institute, Queen Mary University of London, London, United Kingdom,[5]School of Health, Sport and Bioscience, University of East London, London, United Kingdom

Background: Regulatory T cells (Tregs) are emerging as a new cell-based therapy in solid organ transplantation. Adoptive transfer of Tregs was shown preclinically to protect from graft rejection, and the safety of Treg therapy has been demonstrated in clinical trials. Despite these successes, the *in vivo* distribution and persistence of adoptively transferred Tregs remained elusive which hampers clinical translation. Here, we explored options for *in vivo* long-term tracking of adoptively transferred human polyclonal Tregs. **Methods:** Human Tregs enriched via a GMP-compatible protocol were either directly labelled using ^{89}Zr-oxine or lentivirally transduced with the reporter human sodium iodide symporter (NIS) fused to a fluorescent protein (FP) for preclinical evaluation. Tregs were characterized *in vitro* for phenotype, survival/expansion, suppressive capacity/activatability, and NIS radiotracer uptake if NIS-transduced. BALB/c Rag2$^{-/-}$ γc$^{-/-}$ mice were transplanted with HLA-A2^{+} human skin and six weeks later traceable Tregs were i/v administered. Transplants were monitored by SPECT/CT up to 40 days for Treg recruitment/presence. *Ex vivo* tissue histology was employed to support *in vivo* data. **Results:** Genetic modification of human Tregs with the NIS-GFP reporter successfully conferred radiotracer uptake while it did not alter Treg phenotypes and function. This was in contrast to direct Treg radiolabelling with ^{89}Zr-oxine. ^{89}Zr-Tregs radiolabelled at cellular radioactivity amounts sufficient for the required experimental periods of up to six weeks, did not survive neither *in vitro* nor *in vivo* in the long term. NIS-GFP-expressing Tregs were traced *in vivo* for up to 40 days in human skin grafts. Applied to the human skin transplant model setting, we found early trafficking of Tregs to skin grafts to be affected by innate immune cells. **Conclusion:** Our findings suggest utility of the reporter gene-afforded cell labelling strategy for long-term *in vivo* Treg tracking in Treg therapy development, while the required high radiolabelling amounts necessary for long-term tracking were incompatible with Treg function. Notably, the NIS approach is exploiting a host reporter gene and, in the absence of the GFP portion, compatible with future clinical translation for the purpose of Treg therapy monitoring.

793. Development and Application of a Pluripotent Stem Cell Platform to Generate Precision-Engineered Single Cell-Derived Renewable Clonal Master Cell Lines for Therapeutic Use

Hui-Yi Chu, Mochtar Pribadi, Shohreh Sikaroodi, Lauren Fong, Greg Bonello, Yi-Shin Lai, Janel Huffman, Chelsea Ruller, Steven Castro, Karma Farhat, Megan Robinson, Ellen Liu, Ramzey Abujarour, Tom T. Lee, Bahram Valamehr

Fate Therapeutics, Inc, San Diego, CA

Induced pluripotent stem cells (iPSCs) are an attractive source of cellular material for various therapeutic applications due to their unique capabilities of unlimited self-renewal and differentiation capacity to various cell types. To further leverage the unique attributes of iPSCs for therapeutic product development, we established a cellular platform that is amenable to multiplexed and complex engineering applications. To this end, we describe an iPSC platform that involves precision-engineering, single-cell derivation, and clonal line screening, leading to the generation of expanded clonal iPSC banks with multifaceted engineered modalities, lack of off-target effects, and maintenance of genomic stability. Through a series of examples, we show that our cellular platform is extremely versatile and efficient for supporting multi-nuclease, multi-locus, and multi-step engineering. **T**argeting the AAVS1 safe harbor locus using a zinc-finger nuclease, we genetically edited iPSCs with a multi-gene cassette containing inducible caspase9 and GFP transgenes. All selected GFP-positive single cell-derived clones showed targeted editing and the ability to undergo robust chemical induced cell death (100%, 20 of 20 clones). We next utilized another nuclease, TALEN, to insert two separate transgene expression cassettes into a second safe harbor locus and again achieved efficient engineering and stable transgene expression (~89% transgene+) following enrichment. **W**ith the advancement of CRISPR technologies, we tested the efficiency and applicability of multiple Cas nucleases with different DNA recognition and cleavage mechanisms. Using Cas9 and a donor template, we engineered T cell-derived iPSCs to contain biallelic integration of a chimeric antigen receptor into the T-cell receptor α constant locus, for development of allogenic T cell products. The knock-in frequency was efficient enough (6.5%) to conduct single-cell selection to derive homogenously engineered clones, but not efficient enough to support population level engineering to derive a homogenous product, a common challenge with population engineering platforms. Moreover, since targeting multiple loci is a necessary step to support sophisticated engineering, we performed targeted integration of two separate gene expression cassettes into two different loci. With this design and in a single round of engineering, multiple endogenous genes can be disrupted, the expression of transgenes can be controlled separately, and previously established master cell lines can be further engineered leading to simplified manufacturing process of complex engineered products. To this end, starting from a clonal iPSC line with transgene expression cassette integrated at the CD38 locus, we successfully performed a second round of engineering for an additional transgene integration mediated by lentivector transduction or Cpf1/donor template targeting at a different locus. Importantly,

pluripotency and genomic stability were maintained after subcloning, freeze/thaw cycle and prolonged culturing, validating that the feasibility of sequential engineering with our platform. Furthermore, we continued to expand our toolset to include a novel nuclease, MAD7. Tested in three different loci, MAD7-mediated engineering allowed us to obtain clones with specific transgene integrations. In summary, the results demonstrate that our cellular platform supports a wide range of engineering needs and allows for generation of precision-engineered, single cell-derived iPSC clonal master cell banks for the mass manufacture of off-the-shelf cellular therapeutics.

794. Double Suicide Gene Strategy for the Safety of Cell Therapies

Lauren Smith[1], Corey Falcon[1], Isaac Abu Zaanona[2], Mustafa AL-Obaidi[2], Kentaro Minagawa[2], Katelyn Purvis[2], Ravi Bhatia[2], Frederick Goldman[1], Antonio Di Stasi[2]

[1]Pediatrics, University of Alabama at Birmingham, Birmingham, AL,[2]Medicine, Hematology-Oncology, University of Alabama at Birmingham, Birmingham, AL

The strategy to control the expansion of infused gene-modified cells should permit flexibility; the cells could be either downregulated to manage moderate toxicities or eliminated in case of severe toxicities, such as organ damage or insertional mutagenesis. Activation of the inducible Caspase 9 (diC9) is a feasible and effective strategy. However, it is necessary to refine the approach to reliably eliminate the infused cells as required. The issues include (1) optimization of the strategy to address the inability of the diC9 system to induce apoptosis of 10-20% modified cells; and (2) the potential for inter-individual variations in responsiveness and acquisition of resistance in initially responsive cells. Inhibiting the effects on the X-linked inhibitor of apoptosis (XIAP) using the XIAP inhibitor Embelin or mutating the binding region on the diC9 molecule did not result in the complete elimination of gene-modified cells. Therefore, we sought to combine the diC9 activated by a homodimerizer with a diC8 (Caspase 8 without prodomain) triggered by a heterodimerizer, with activation of the mitochondrial apoptotic pathway. We found (i) leakiness of each dimerizer in activating the respective drug-binding domain and (ii) incomplete elimination of Jurkat gene-modified cells expressing both constructs with regrowth, albeit with reduced mean fluorescence intensity of the selection marker. Interestingly, the killing was superior when the two transgenes were introduced by co-transduction (95% cell elimination), and the heterodimerization of diC8 was superior to homodimerization. Next, we combined the diC9 with the RQR8 compact suicide gene incorporating a CD20 mimotope and the QBEND10 selection marker. The goal was to achieve complete elimination using the anti-CD20 monoclonal antibody Rituxan, resulting in CDC/ADCC, a non-overlapping killing mechanism. Rituxan alone granted 91±8% efficient elimination of gene-modified Jurkat cells in vitro, which increased to complete elimination (99.6±0.3%) when combined with the diC9 (N:3). We are currently investigating this strategy in Wharton jelly derived mesenchymal stromal cells (MSC), MSC differentiated progenies, and primary T-cells coexpressing a chimeric artificial receptor targeting CD19. To avoid off-target effects, achieve complete cell elimination by gene therapy means while counteracting some mechanisms of resistance, we coexpressed an inducible Caspase with a doxy-inducible shRNA targeting an anti-apoptotic molecule. A non-targeting shRNA was used as a control. We performed co-transduction of a retroviral vector encoding the diC8 or diC9 and a lentiviral tet-on shRNA vector. Cells were enriched for transgene expression by flow cytometry and puromycin selection. After a dose-response assessment, we chose 3 ug/mL as the doxycycline concentration for our experiments. We assessed apoptosis on day 3 after the addition of doxy and overnight after the dimerizer in the appropriate condition. The killing of control cells was less than 10%. We found a statistically significant difference (P=0.004) in cell elimination when activating the diC8 alone (75±4%) or together with a doxy-inducible shRNA targeting BCL-2 (95±1.9%). We also found improvement when activating the diC9 together with a tet-on shRNA targeting BCL-XL (95±4 vs. 82±2%) (N: 3). Our future goal is to validate this strategy in primary T-cells for GVHD or MSC for regenerative medicine. In conclusion, diC9 and RQR8 co-activation resulted in regulatable up to complete elimination of gene-modified cells. We are further optimizing our model with an inducible Caspase and a tet-on shRNA, which is also valuable to interrogate apoptotic pathways.

795. Understanding the Effects of IL-2, IL-7, and IL-15 Supplementation on the Growth and Signaling of CD4 Naïve T Cells

William J. Kelly, Zuyi Huang, Canaan Copolla, Brooks Hopkins

CBE, Villanova, Schwenksville, PA

While CAR-T therapy is a growing and promising area of cancer research, it is limited by high cost and the difficulty of consistently culturing T-cells to therapeutically relevant concentrations *ex-vivo*. Cytokines IL-2, IL-7 and IL-15 have been found to stimulate the growth of T cells, however, the optimized combination of these three cytokines for T cell proliferation is unknown. In this study, we designed an integrated experimental and modeling approach to optimize cytokine supplementation for rapid expansion in clinical applications. We assessed the growth data for statistical improvements over no cytokine supplementation, and used a systems biology approach to identify genes with the highest magnitude of expression change from control at several time points. Further, we developed a predictive mathematical model to project the growth rate for various cytokine combinations, and investigate genes and reactions regulated by cytokines in naïve $CD4^+$ T cells. The highest growth rates were observed where either IL-2 or IL-7 was at the highest concentration tested (15 ng/ml for IL-2 and 80 ng/ml for IL-7) while the other was at the lowest (1 ng/ml for IL-2 and 6 ng/ml for IL-7), or where both IL-2 and IL-7 concentrations are moderate. This suggests a synergistic interaction of IL-2 and IL-7 with regards to promoting optimal proliferation and survival of the naïve $CD4^+$ T cells. Transcriptomic data analysis identified the genes and transcriptional regulators up/down-regulated by each of the cytokines IL-2, IL-7, and IL-15. It was found that the genes with persistent expressing changes were associated with major pathways involved in cell growth and proliferation. In addition to influencing T cell metabolism, the three cytokines were found to regulate specific genes involved in TCR, JAK/STAT, MAPK, AKT and PI3K-AKT signaling. The developed Fuzzy model that can predict the growth rate of CD4+ naïve T cells for various combinations of cytokines, along with identified optimal cytokine

cocktails and important genes found in transcriptomic data, can pave the way for optimizing CD4 naïve T cells by regulating cytokines in the clinical setting.

796. Development of a Novel Rat Bioreactor to Facilitate Manufacture of Human Hepatocyte Cell Therapies for the Treatment of Patients with Severe Liver Diseases

Raymond D. Hickey, Elizabeth M. Wilson, Rafal P. Witek, Tin Mao, Kristen Darrell, Michael Wong, Glen Mikesell, Gabriel Peixoto, Alan Mendoza, Prativa Sharma, Craig Wise, Peter Lee, Tiffany Tse, Douglas Matje, Charity Juang, Janice Kim, Carol Alvarez, Gaetano Faleo, Jason Hulse, Abeba Demelash, Chy-Anh Tran, Kenneth Dorko, Glenn F. Pierce, Fei Yi, Stanley J. Hollenbach, Michael C. Holmes

Ambys Medicines, South San Francisco, CA

Introduction: Orthotopic liver transplantation remains the only curative treatment for liver disease. Hepatocyte transplantation is a potential alternative therapy for acute and chronic liver diseases. The safety and short-term efficacy of hepatocyte transplantation have been demonstrated in the clinic, but this approach has been hindered by the poor availability of high-quality livers and low yield of well-characterized, functional hepatocytes. Given no methods exist to expand transplantable hepatocytes ex vivo, we hypothesized that generation of an immune-deficient rat model of hereditary tyrosinemia type 1 ($Fah^{-/-}$, $Rag1^{-/-}$, $Il2rg^{-/-}$ [FRG] rats) could be used for the engraftment and large-scale expansion of functional primary human hepatocytes. These expanded hepatocytes could be purified, cryopreserved, and extensively characterized prior to infusion to provide on-demand therapy for patients with severe liver diseases. **Results:** FRG rats were maintained on the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3 cyclohexanedione (NTBC) that prevents accumulation of toxic metabolites during tyrosine catabolism in FAH-deficient animals. FRG rats exhibited normal fecundity and remained healthy while on NTBC. We next characterized the phenotype of FRG rats off NTBC. As expected, animals exhibited hypertyrosinemia (>1000μM) and accumulated succinylacetone (>0.1μM) in the blood. FRG rats off NTBC displayed a failure-to-thrive phenotype resulting in liver and kidney damage, and ultimately death. Transplantation of wild-type FAH+ rat hepatocytes, combined with NTBC cycling to provide selective pressure for FAH+ cells to expand, rescued the lethal phenotype and normalized tyrosine and succinylacetone levels. FAH+ rat hepatocytes were shown to repopulate FRG rat livers (>95%) by immunohistochemistry. Next, we undertook a series of experiments to optimize engraftment and expansion of primary human hepatocytes in FRG rats. Immunodeficiency of FRG rats, needed for xenograft maintenance, was confirmed by flow cytometry which demonstrated severely reduced T, B, and Natural Killer cells in peripheral blood. Repopulation of FRG rat livers was achieved with multiple human hepatocyte donors based on human albumin ELISA (>5mg/ml) and FAH immunohistochemistry (>50%). Purified expanded human hepatocytes exhibited typical hepatocyte transcription profiles based on multiple analyses, including scRNA-Seq. Expanded cells retained their synthetic (e.g. albumin expression), metabolic (e.g. cytochrome P450 activity), and detoxification (e.g. ammonia clearance) functions. Finally, FRG rat-derived human cells demonstrated robust engraftment and expansion in a mouse model of hereditary tyrosinemia. Transplanted hepatocytes normalized tyrosine and succinylacetone levels, preventing onset of liver failure, and demonstrating therapeutic efficacy of bioreactor-expanded human cells. **Conclusion:** Ongoing experiments are being conducted to optimize the procedures for the engraftment and expansion of human hepatocytes to support maximal repopulation of FRG rat livers with the ultimate goal of generating large numbers of high-quality primary human hepatocytes for studies in patients with severe liver diseases. We expect development of the FRG rat bioreactor will enable clinical testing of hepatocyte transplantation as a potential alternative to liver transplantation.

797. Development of an Optimized Lentiviral Transduction Medium and Process to Manufacture Genetically Modified MSC Working Cell Banks

Therese Willstaedt, Amy Walde, James Brennan, Jon Rowley, Katrina Adlerz

RoosterBio, Frederick, MD

Background & Aim Mesenchymal stem/stromal cells (MSCs) are widely used in clinical development and have an excellent safety profile demonstrated in over 1,000 human trials. Their innate therapeutically beneficial functions include being angiogenic, mitogenic, immunomodulatory, anti-microbial, and having the ability to home to injury sites, make them useful for many indications. The safety profile of hMSCs also makes them ideal candidates for cell-based gene therapies and there is growing interest in cell therapy using genetically modified hMSCs. hMSCs may be genetically engineered to improve their inherent therapeutic properties or to secrete specific proteins for new applications. A critical challenge, however, is efficiently modifying the cells needed for therapy within the context of scalable cGMP manufacturing, especially given the historically low transduction efficiency of many primary cells. Here we describe the development of a manufacturing process using a novel medium optimized for maximum lentiviral transduction to generate expandable working cell bank vials of genetically modified hMSCs. **Methods** Various lentiviral promoters, MOI, and transduction processes were investigated for the efficient transduction of human umbilical cord MSCs (hUC-MSCs) and human bone marrow MSCs (hBM-MSCs). Briefly, cells were isolated from fresh bone marrow or cord tissue, expanded in a xeno-free expansion medium (RoosterNourish-XF, RoosterBio) and cryopreserved to create Master Cell Banks (MCB). Cryopreserved MCBs were thawed and seeded at 3,000 - 5,000 cells/cm^2. After proliferating for 2 days, hMSCs were transduced with lentiviral vectors expressing Green Fluorescent Protein (GFP) or ZsGreen at MOIs ranging from 2 to 50. 24 hours post-transduction, viral supernatants were aspirated and replaced with RoosterNourish-XF. Transduction efficiencies were assessed by fluorescent microscopy and quantified by flow cytometry. Working Cell Banks (WCBs) of transduced cells were created and further expanded to a final product PDL to monitor protein expression and to characterize MSC Critical Quality Attributes (CQAs) including phenotypic markers (CD90 and

CD166), multilineage differentiation, and cytokine secretion (HGF, IL-8, TIMP-1, TIMP-2 and VEGF). **Results & Conclusions** Two hUC-MSC lots and two hBM-MSC lots were efficiently transduced with lentiviral vectors expressing a fluorescent reporter gene using a novel genetic engineering medium. The use of this medium resulted in a 2-5 fold increase in transduction efficiencies above growth medium alone. The technique of Spinoculation in which the virus and cells are centrifuged together did not markedly increase transduction efficiencies. Significant differences were observed in the transduction efficiency of lentivirus from different vendors ranging from 20% to 90%. Although the use of higher MOIs resulted in increased efficiency, MOI as high as 50 showed decreased cell health in some cases. Overall, the use of our novel medium resulted in WCBs with over 90% transduction efficiency. This medium optimizes a critical unit operation of gene modification and provides a useful tool for the generation and expansion of engineered hMSCs for use in clinical therapies.

798. Strategies to Align Evidence Generation for Cell Therapies

Rebecca Ray, Beena Bhuiyan Khan

Duke-Margolis Center for Health Policy, Washington, DC

Introduction

Cell therapies are poised to disrupt the treatment paradigm for genetic disorders, cancers, and other chronic conditions. These therapies may provide durable responses, or even cures. While cell therapies hold the promise of improved health and reduced downstream health care costs, the long-term safety, effectiveness, and durability of treatment effect is often unknown at the time of market approval. These research questions are addressed through post market evidence development that is driven by stakeholders such as regulators, providers, and payers. Regulators, such as the US Food and Drug Administration (FDA) will require post market surveillance to monitor for long term safety signals. Providers will drive evidence development through specialty registries to asses long term effectiveness and subpopulation effects. Payers, on the other hand, impact evidence generation through data collection requirements to inform cost effectiveness assessments, quality indicators, and site distinctions. Efforts to generate evidence for novel cell therapies across stakeholders are not aligned which can result in duplicative efforts and increased administrative burden which can hamper evidence development. Given the importance of establishing the value of new and potentially cost cell therapies, along with the proliferation of data collection approaches, there are opportunities to align evidence generation strategies across multiple stakeholders. The Duke-Margolis Center for Health Policy convened experts across sectors of the cell therapy space to formulate a coordinated policy approach towards establishing an interoperable and efficient, post-market evidence infrastructure that addresses multiple stakeholders' evidentiary needs. Stakeholders included regulators, payers, manufacturers, providers, patient advocates, and registry leaders. Through a series of convenings participants engaged in moderated panels, presentations, and open discussions to align evidence generation strategies to support three areas: long term data collection on safety and effectiveness, care coordination, and sustainable financing. **Long Term Data Safety and Effectiveness** A coordinated approach to develop evidence through post market registries can ensure long term data collection on the safety and effectiveness of cell therapies. **Supporting Care Coordination** Engaging the clinical community by providing incentives to collect and report data as well as utilize modernized data systems will be key in enabling continuous improvement in patient management.**Sustainable Financing** Value based payment modes are sustainable financing models that encourage evidence generation as well as align stakeholder priorities.

799. Subconjunctival Administration of Low Dose Murine Allogeneic Mesenchymal Stromal Cells Promotes Corneal Allograft Survival in Mice

Thomas Ritter[1], Kevin Lynch[1], Nick Murphy[1], Xizhe Chen[1], Aoife Canning[1], Ellen Donohoe[1], Paul Lohan[1], Georgina Shaw[1], Gerry Fahy[2], Aideen Ryan[3], Oliver Treacy[3]

[1]Medicine, National University of Ireland Galway, Galway, Ireland,[2]Ophthalmology, Galway University Hospital, National University of Ireland Galway, Galway, Ireland,[3]Pharmacology and Therapeutics, National University of Ireland Galway, Galway, Ireland

Background: Systemic administration of mesenchymal stromal cells (MSCs) has been efficacious in many inflammatory disease settings, however, little data are available on the potential immunomodulatory effects following local MSC administration in the context of corneal transplantation. The purpose of this study was to assess the potential of subconjunctival injection of MSCs to promote corneal allograft survival. **Methods:** MSCs were isolated from female C57BL/6 (H-2^k) or Balb/c (H-2^d) mice and extensively characterised. An allogeneic mouse corneal transplant model was used with Balb/c mice as recipients of C57BL/6 grafts. A dose finding study starting with $5x10^5$ MSCs injected subconjunctivally at day -7 was tested first followed by a more clinically translatable low-dose single or dual injection strategy on day -1 and day +1 before/after transplantation. Graft transparency served as the primary indicator of rejection while neovascularisation was also recorded. Lymphocytes (from draining lymph nodes) and splenocytes were isolated from treatment groups on day 2 post-transplantation and characterised by flow cytometry and qRT-PCR. **Results:** Both high- and low-dose injection of allogeneic MSCs on day -7 led to 100% graft survival over the observation period. Moreover, low-dose dual subconjunctival injection of $5x10^4$ allogeneic MSCs on day -1 or day +1 led to 100% allograft survival in transplant recipients (n=7). We also demonstrate that single administration of allogeneic MSCs on either day -1 or day +1 promotes rejection-free graft survival in 100% (n=8) and 86% (n=7) of transplanted mice, respectively. Early time-point *ex vivo* analysis suggests modulation of innate immune responses towards anti-inflammatory, pro-repair responses by local MSC administration. **Conclusion:** This work demonstrates that low-dose subconjunctival injection of allogeneic MSCs successfully promotes corneal allograft survival and may contribute to refining future MSC immunotherapies for prevention of corneal allograft rejection.

800. Stem Cell Derived-Islets Edited with Gene Deletions and Bearing an Immunomodulatory Transgene Have Reduced Immunogenicity

Mike Conway[1], Fanny Sierra[1], Sara Tezza[1], Niloofar Rasouli[1], Felicia Pagliuca[2], George Harb[1]

[1]Stem Cell Research, Vertex, Cambridge, MA,[2]T1D Disease Strategy Team, Vertex, Cambridge, MA

Widespread therapeutic application of allogeneic cell therapies requires a strategy to prevent immune rejection that is both safe and does not impair cell function. Current approaches include either immunoisolation (encapsulation) or immunosuppression. A third option is to develop an immune evasive cell line that is hypoimmunogenic and, therefore, can be used to derive cell therapy products suitable for allogeneic transplantation. To achieve this goal, we have edited our human pluripotent stem cell (hPSC) line to disrupt a set of genes to reduce host immune system interactions. This edited line showed reduced T-cell activation, and when successfully differentiated into stem cell derived-islet (SC-islet) cells, maintained this reduced T-cell activation phenotype. We further ectopically expressed an immunomodulatory transgene to mitigate NK cell mediated cytotoxicity. Coupling transgene expression with gene deletions reduced NK cell activation while maintaining reduced T-cell activation. Transgene expression did not impair SC-islet differentiation nor negatively impact normal cell function. Together, these data illustrate how genetic edits designed to reduce immunogenicity do not impair normal SC-islet differentiation or function and can endow SC-islets with a less immunogenic phenotype.

Vector Product Engineering, Development or Manufacturing

801. Development of a Scalable AAV Purification Process and an SPR Titer Assay

Asa Hagner-McWhirter, Mats Lundgren

Cell and Gene Therapy, Cytiva, Björkgatan, Uppsala, Sweden

To meet the needs for sufficient high-quality adeno-associated virus (AAV) for gene therapy, purification processes must be robust, scalable, and cost-efficient. A successful process reduces empty capsids and removes impurities efficiently while achieving high overall yields. In this study, AAV2 and AAV5 serotypes were produced by triple-plasmid transfection of HEK 293T cells in suspension. Each purification step — harvest, clarification, concentration and buffer exchange, affinity capture, and polishing — was evaluated and optimized. At the time of harvest cells were lysed using detergent, and DNA was fragmented by DNA nuclease. A final low-pH treatment induced a precipitate that was removed by sedimentation prior to clarification by normal flow filtration. The concentration and buffer exchange step was performed using ultrafiltration/diafiltration. Comparing 300 MWCO and 100 MWCO filters, the 300 MWCO filters gave similar high yields but better impurity reduction. The retentate was applied to Capto AVB resin for affinity chromatography; the virus was eluted with high recovery and excellent impurity reduction. To reduce empty capsids, different ion exchange and multimodal resins were evaluated in a polishing step. Viral genome titer was determined by qPCR, and viral capsid titer was assessed by ELISA. The results of both were used to determine the % full capsids. An SPR assay for viral capsids was developed. This Biacore assay gave comparable results to the ELISA assay, with improved reproducibility and ease of use. A cell-based transduction assay was used to analyze the functional virus titer. Multiplex fluorescence SDS-PAGE and Western blot were used to simultaneously analyze HCP and virus content of fractions from the different chromatography steps. In conclusion, a scalable and efficient AAV purification process was developed using filtration, chromatography, and new analytics.

802. Development of an Assay for Detection of Replication-Competent Adeno-Associated Virus

Samuel Getchell[1], Katherine Marotte[1], Heather Beyer[1], Marian McKee[2]

[1]Viral Safety Testing Services, Eurofins Lancaster Laboratories, Lancaster, PA,[2]Eurofins Lancaster Laboratories, Lancaster, PA

Adeno-associated virus (AAV) vectors are used in gene therapy applications in order to transfer therapeutic genes into patients. AAV contains many properties that make it an ideal gene delivery system. It is replication defective by nature and requires the presence of a helper virus, such as adenovirus or HSV, in order to replicate. Although replication competent AAV (rcAAV) particles are not typically harmful to patients, there are risks. rcAAV can increase cellular immune responses, increase the likelihood of vector mobilization, and cause significant changes to the biology of the vector. Therefore, rcAAV in a vector product is highly undesirable. Eurofins BioPharma Product Testing has developed an assay to detect rcAAV in AAV vector preparations. First, a wild type AAV positive control was created which contains *rep* and *cap*, the genes that are required for a vector to be replication-competent. Using this control, a few key parameters were evaluated during assay development, included determining a feasible assay design, establishing an ideal MOI for the helper virus, evaluating the sensitivity of the assay with and without the presence of recombinant AAV matrix, and assessing targets for the qPCR endpoint. In this talk, we will elaborate and show data from each of these key parameters from the assay development process.

803. Transient Transfection-Based Lentivirus (LV) Production Process Scaled to 25 L in Single-Use Bioreactors

Zahra Sheikholeslami[1], Rajan Ramanuj[2], Philip Beaudette[1], Azher Razvi[2]

[1]Bridge, CCRM, Toronto, ON, Canada,[2]Bridge, Cytiva, Toronto, ON, Canada

Promising results in gene therapy clinical studies are driving demand for late-stage clinical and commercial manufacturing capabilities. Efficient large-scale lentivirus (LV) production is needed to translate LV-based therapeutic strategies to the clinic, where several factors can impact process performance and product quality. The aim of this work was to develop a scalable, cost-effective transient-transfection system with HEK293 cells grown in serum-free suspension culture. First, we developed a scalable approach for preparing the transfection

complex. Then, we performed small-scale experiments (125 mL shake flasks) to study factors that impact large-scale preparation of the transfection mix, including incubation time, agitation rate, and volume. Next, we transferred this process to 3 L bench-scale glass bioreactors and achieved comparable infectious titer to the shake flask cultures. Finally, we scaled the process to Cytiva's Xcellerex XDR-50 platform of stirred-tank bioreactors, completing two 25 L runs. In the first run, we scaled the process from 3 L bench-scale glass bioreactor to the single-use XDR-50 bioreactor. In the second run, we performed the entire process in the XDR-50 bioreactor. Infectious titers were comparable in both runs. In summary, our production process of LV by transient transfection of suspension HEK293 cells is scalable to 25 L with a viral titer yield between 7×10^7 and 1×10^8 TU/mL.

804. Wildtype AAV2 Rep Protein Produces Higher Titer AAVHSC Vectors with Improved Packaging Profiles Compared to Clade F Associated Chimeric Rep

Laura van Lieshout

Homology Medicines Inc, Bedford, MA

A chimeric adeno-associated virus (AAV) Rep gene associated with AAV9 RepCap packaging plasmids has been observed in Genbank sequences in addition to plasmids from vector cores and DNA vendors. Compared to wild type AAV2 Rep, this chimeric Rep contains 14 nucleotide mutations resulting in seven silent mutations and four amino acid changes within the 3' end of the open reading frame. The chimeric Rep functions to produce AAV vectors of various serotypes, including AAVHSCs, Clade F vectors originally isolated from human hematopoietic stem cells. When compared, these of the wild type (WT) AAV2 Rep sequence results in higher vector yields and improved vector packaging profiles. Vector production utilizing WT AAV2 Rep resulted in up to an 80% increase in vector genomes and up to a 40% increase in full capsids generated in crude lysate when compared to vector production using the chimeric Rep. A substantial increase in DNA containing vectors by analytical ultracentrifugation was observed as well as a reduction in partial genomes packaged. In addition, vector produced with WT AAV2 Rep resulted in an increase of vector infectivity and up to a 50% reduction in packaged host cell DNA compared to production with the chimeric Rep. Furthermore, vector quality attributes including aggregation, purity, residual host cell protein and residual packaged plasmids remained in process range. Similar trends were observed for both adherent and suspension HEK293 production platforms ranging across scales up to 500L and using multiple gene of interest constructs.

805. High Efficiency Purification of Multiple AAV Serotypes Using AAVX Column Chromatography

Michael Florea[1,2,3], Fotini Nicolau[3], Simon Pacouret[3], Julio Sanmiguel[3], Eva Andres-Mateos[4], Carmen Unzu[3], Amy Wagers[5,6,7], Luk H. Vandenberghe[3]

[1]Harvard Stem Cell Institute, Cambridge, MA,[2]Ph.D. Program in Biological and Biomedical Sciences, Division of Medical Sciences, Harvard University, Boston, MA,[3]Grousbeck Gene Therapy Center, Mass Eye and Ear, Harvard Medical School & The Broad Institute of Harvard and MIT, Boston, MA,[4]Grousbeck Gene Therapy Center, Mass Eye and Ear, Harvard Medical School & The Broad Institute of Harvard and MIT, Cambridge, MA,[5]Department of Stem Cell and Regenerative Biology, Harvard University, Harvard Stem Cell Institute, Cambridge, MA,[6]Paul F. Glenn Center for the Biology of Aging, Harvard Medical School, Cambridge, MA,[7]Section on Islet Cell and Regenerative Biology, Joslin Diabetes Center, Boston, MA

Recombinant adeno-associated viruses (AAVs) are widely used as gene therapy vectors in research and medicine. Increasingly more applications require manufacturing of high doses of AAV, which has led to the need to develop simpler, cheaper and more scalable production methods than the currently used ultracentrifugation-based methods. One such method is high-performance liquid chromatography, which uses AAV-binding columns to purify virus directly from cell lysate. This has been shown to be scalable, efficient and produce pure virus, but has been limited by the small number of serotypes that can be efficiently purified without extensive serotype-specific optimization. Here we characterize the recently developed POROS CaptureSelect AAVX resin for purification of multiple AAV serotypes. We find that at room temperature, AAVX efficiently binds several AAV serotypes, including the commonly used AAV2, AAV8, AAV9, PHP.B and Anc80, and that chromatographic purification with AAVX resin can be used to efficiently purify AAV preparations at all scales tested. We also find that AAVX resin can be reused multiple times without loss of purification efficiency or contamination from previous purifications, and that the purity and in vivo gene transfer efficacy of the resulting vectors are comparable to iodixanol ultracentrifugation purified vectors. This establishes chromatographic purification of AAV using POROS AAVX resin as a versatile and efficient method for purification of a range of AAV serotypes.

806. Subretinal Rd9 Mouse Study to Compare RPGR Expression Pre- and Post-Manufacturing Process Improvements

Judith A. Newmark[1], Adrian M. Timmers[1], John O'Grady[2], David R. Knop[2], Mark S. Shearman[1]

[1]Pre-Clinical R&D, AGTC, Cambridge, MA,[2]Process Development, AGTC, Alachua, FL

Purpose: AGTC's proprietary HAVE (herpes-assisted vector expansion) platform has been used to manufacture AAV vector for multiple clinical trials. The benefits of HAVE compared to other AAV production platforms include scalability, yield, efficiency and cost. Recently, several process improvements have been introduced to further maximize those benefits. The most significant change involves substitution of an anion exchange column for the original cation exchange column. The anion

exchange column allows significant improvement in the full/empty capsid ratio. Other process improvements include optimization of the clarification and affinity steps. The objective of this study was to compare transgene expression of rAAV-RPGR vectors produced by both the original (Process 1.0) and improved (Process 3.0) platforms, following subretinal injection in Rd9 mice, to determine if the final vector product maintains a similar expression profile. **Methods:** rAAV-RPGR vector was manufactured by HAVE Process 1.0 and two batches of rAAV-RPGR were manufactured by HAVE Process 3.0. Vectors were dosed by bilateral subretinal injection into Rd9 mutant mice lacking RPGR (n=10/group, 1 µL of $1x10^{12}$ vg/mL per eye). Mice were sacrificed at 8 weeks post-dose. One eye from each animal was collected, fixed, processed for H&E and immunohistochemistry of RPGR expression, and scored by a board-certified veterinary pathologist. The other eye was collected, frozen, and processed for capillary western blotting of RPGR expression. **Results:** Process changes from 1.0 to 3.0 resulted in an improved full/empty capsid ratio from 30% to greater than 80%, reduced process residuals (Host cell DNA and Protein), and an improved ratio of infectious particles (< 4.0 total particles/infectious particles). Clinical observations and body weights measured during the study remained normal. H&E staining showed minimal findings related to the injection procedure. Immunohistochemistry staining of RPGR expression was scored by the pathologist as equivalent in retinal area (most showed > 50% photoreceptor positivity) and intensity of staining (2.2-2.8 on a 5-point scale) among the groups. RPGR expression by western blotting (normalized to GAPDH) was measured as similar among the groups. **Conclusions:** HAVE process changes resulted in improvements to the full/empty capsid ratio, a reduction of process residuals, and an improved ratio of infectious particles. The resulting rAAV-RPGR vectors produced similar levels of RPGR expression in the Rd9 mouse model, verifying that the process changes did not change the resulting expression from the vectors. These findings support that our novel, more efficient production method yields similar, high quality gene therapy product for use in future clinical trials.

807. Demonstration of Transient Transfection Scalability for a Suspension AAV9 Viral Vector Production Process up to 500 Liter Manufacturing Scale

Anne MacIntyre[1], Tim Erlandson[1], Benedict Kang[1], Nathan Hazi[1], Todd Sanderson[1], Benjamin I. Ingersoll[2], Michael McLaughlin[2], Phillip B. Maples[2]

[1]Pall Corporation, Westborough, MA,[2]Abeona Therapeutics, Cleveland, OH

Viral vector-based gene therapies are bringing life changing treatments to many patients and families. One of these treatments is a novel hSGSH gene replacement therapy for the treatment of Sanfilippo syndrome type A (MPS IIIA). This gene replacement therapy is based on rAAV9 (adeno-associated virus serotype 9) viral capsid for delivery of a functional hSGSH gene. A scalable upstream production process is needed to provide the adequate manufacturing capacity to enable this vector to be a viable treatment for patients and families suffering from this devastating disease. We evaluated the Allegro™ STR bioreactor family for its performance as a scalable vector production platform. This design offers scalable performance from 50 L to 2000 L working volume. Here we evaluated the scalability of a PEI mediated transfection process between the 50 L and 500 L scale for production of this AAV9 vector. Performance of these bioreactors were evaluated based on cell growth, metabolic profile, and vector production as determined by qPCR. The results demonstrate scalable vector production process between the 50 L and 500 L scale while controlling key process parameters.

808. Engineering Exosomes with Altered Cellular Tropism for Neuronal Cell Targeting

Wei Zhang, Palak Shah, Jing Zhou, Ke Xu, Jonathan Finn

Codiak BioSciences, Cambridge, MA

Exosomes are a type of extracellular vesicle that can transfer complex molecules between neighboring and distant cell types, enabling cell to cell communication. Translational research efforts have been focusing on harnessing this communication mechanism to deliver exogenous payloads to treat a variety of diseases.Codiak's unique engEx™ platform utilizes proprietary scaffold proteins to generate engineered exosomes functionalized with a variety of structurally and biologically diverse proteins. The goal of the work presented here is to engineer exosomes to direct tropism to neuronal cells for the treatment of neurological disorders.We designed exosomes that contain ligands that have previously been demonstrated to target neuronal cells, transferrin (to target various cells, including Schwann cells), and a peptide from the rabies virus glycoprotein (RVG) (for neuron targeting), both of which were fused to PTGFRN for display on the exosome surface. The engineered exosomes contained high levels of the specific ligand. For example, engineered exosomes displaying transferrin have more than 2000 copies per particle.In both primary mouse Schwann cells and human Schwann cells, exosomes engineered to display Transferrin(exoTF) targeting the transferrin receptor resulted in preferential uptake when compared with un-targeted exosomes. ExoTF also demonstrated increased uptake in both mouse and human neuroblastoma cell lines. Exosomes engineered to express RVG (exoRVG) demonstrated preferential uptake in a mouse neuroblastoma cell line.Our current results demonstrate the potential of our engEx™ platform to modulate exosome tropism by generating novel exosome therapeutic candidates with high density ligand display and target various cell types of interest in the nervous system.

809. Expanding the Utility of the ExpiSf™ Protein Expression System into AAV Production for Gene Therapy: A Scalable Chemically-Defined Sf9/Baculovirus System for Adeno-Associated Virus Production

Kenneth Thompson[1], Maya Yovcheva[1], Natalie Lindo[1], Bria Flowers[1], Katy Irvin[1], Mark Bundy[1], John Li[1], Natasha Lucki[2], Jonathan Zmuda[1]

[1]Cell Biology, Thermo Fisher Scientific, Frederick, MD,[2]Cell Biology, Thermo Fisher Scientific, Carlsbad, CA

The ExpiSf™ Protein Expression System is the first chemically-defined insect system for high-yield production of recombinant proteins, virus-like particles (VLPs) and membrane proteins. This system is suspension-based, readily scalable, and uses animal-origin free (AOF)

reagents and chemically defined medium, which allows for consistent cell growth of ExpiSf9 cells with high lot-to-lot consistency. The system also includes a chemically-defined expression booster in the form of an enhancer, and an improved transfection reagent, Expifectamine Sf™, which has enabled the baculovirus production protocol to be adapted to a suspension format. In this format, high baculovirus titers are achieved without time-consuming amplification steps enabling faster time to protein. Recently, insect expression has been employed by researchers for the cost-effective production of adeno-associated virus (AAV) for gene therapy applications. Here, we demonstrate the optimization of the ExpiSf system to produce high titer, high quality AAV. AAV production runs were performed using a dual infection approach with two baculoviruses; one containing the Rep and Cap genes, and the other containing green fluorescent protein (GFP) flanked by the AAV inverted terminal repeat sequences. Expression of a panel of AAV serotypes in the ExpiSf system achieved AAV titers of ~1-2x10^{11} vg/mL, and comparable titers were observed in shake flasks and bioreactors. AAV particles produced at all scales were infectious, as HT1080 cells inoculated with AAV crude lysates could be visualized expressing GFP. Using our POROS™ GoPure™ AAVX affinity resin we observed up to 89% recovery of pure AAV6 post-purification and total yields of 1-2x10^{14} AAV6 vector genome containing particles per liter of culture. Transmission electron microscopy and anion exchange chromatography showed that the AAV6 preparation contained ~50-70% full particles, with expected levels of infectivity in the final preparation. Data on additional analytical methods for quantification and characterization of AAV particles produced in the ExpiSf system, consisting of analytical ultracentrifugation (AUC) for Empty:Full ratios and mass spectrometry for capsid sequence verification and VP1/VP2/VP3 protein ratios will be reported. Taken together, we demonstrate the versatility of the ExpiSf™ system beyond protein expression, highlighting the use of this platform for the scalable production of recombinant AAV.

810. Scalable, High-Titer Production of Adeno Associated Virus (AAV) in the Gibco™ AAV-MAX Helper-Free AAV Production System

Chao Yan Liu[1], Julia Braun[2], Yanfei Zou[2], Mark Bundy[1], Katlyn Irvin[1], Kenneth Thompson[1], Kyle Williston[1], Collin Rasmussen[1], Namritha Ravinder[2], Jonathan Zmuda[1]

[1]Thermo Fisher, Frederick, MD,[2]Thermo Fisher, Carlsbad, CA

Adeno Associated Virus (AAV) has emerged as the leading platform for gene therapy to treat human disease with two AAV-based therapeutics gaining regulatory approval in Europe or the United States. As gene therapy progresses past use for rare diseases, one critical constraint for the use of AAV is the large number of viral particles required to be delivered to the patient. Until recently, AAV production has taken place primarily in adherent cultures using 293T cells in the presence of fetal bovine serum (FBS); however, such adherent systems suffer from a number of significant drawbacks including difficulty in scaling up, the presence of the SV40 large T antigen in the producer cell line as well as cost, consistency and regulatory considerations stemming from the use of animal sera. To address these shortcomings, we have developed the Gibco™ AAV-MAX Helper-Free AAV Production System, a chemically defined, suspension based AAV production system that allows for scalable, high titer production of AAV viral vectors in a non-293T cell lineage. The AAV-MAX system comprises all of the components required for scalable AAV production in mammalian cells including: (1) a clonally-documented, high-titer 293F-derived producer cell line, (2) a chemically defined growth and expression medium, (3) a production enhancer, (4) a cationic lipid-based transfection reagent and booster, (5) a protein-free complexation buffer, and (6) a Polysorbate 20-based lysis buffer. The AAV-MAX system delivers AAV titers at ≥5x10^{10} viral genomes per mL (vg/mL) across multiple AAV serotypes with excellent scalability and reproducibility across multi-well plates, shake flasks and stirred tank bioreactors in a triple-transfection setting. The AAV-MAX system uses streamlined protocols and is based on high-density (3x10^{6} cells/mL) and high-efficiency transfection that requires 50% less plasmid DNA than alternative reagents on a per cell basis, a critical benefit that can reduce overall production costs. To further streamline the AAV-MAX workflow, downstream purification of high titer AAV is attained using Thermo Scientific™ POROS™ CaptureSelect™ AAVX, AAV8 and AAV9 Affinity Resins enabling singlestep purification with high purity and yield at higher flow rates and improved throughput. Host cell DNA is detected via the Applied Biosystems™ resDNASEQ™ Quantitative HEK293 DNA Kit allowing for sensitive and specific quantitation of HEK293 cell DNA. Here, we provide data on the production of AAV in the AAV-MAX system from milliliter scale in multi-well plates and shake flasks to multi-liter scale in stirred tank bioreactors along with downstream purification strategies and fine characterization of the resultant AAV particles. In summary, the Gibco™ AAV-MAX Helper-Free AAV Production System allows for scalable, high-titer AAV production in a streamlined protocol that maximizes viral titers while reducing time and cost of production.

811. Economic Analysis and Cost Optimization of Adenovirus Purification Processes

Karina Kawka[1], Ian Gough[1], Nicholas Graham[2], Yuki Abe[2], Maria F. C. Medina[3], Brian Lichty[3], Prashant Mhaskar[1], Raja Ghosh[1], David R. Latulippe[1]

[1]Chemical Engineering, McMaster University, Hamilton, ON, Canada,[2]Biopharm Services Ltd., Chesham, United Kingdom,[3]Robert E. Fitzhenry Vector Laboratory, McMaster Immunology Research Centre, McMaster University, Hamilton, ON, Canada

Downstream purification of gene therapy viral vectors necessitates a complex series of unit operations. To meet regulatory requirements, purification processes must remove product- and process-related impurities, including host-cell DNA (HCD), from the viral vector product. The U.S. Food and Drug Administration (FDA) requires gene therapy products to contain no more than 10 ng HCD/dose and no DNA fragments greater than 200 base-pairs in length. Presently in viral vector manufacturing processes, HCD is typically separated via a combination of enzymatic DNA digestion and chromatography. Purification-related challenges often cause significant product losses - as high as 80% - by the end of the manufacturing process, which indicates the need for more efficient and economic processes. To address the strict HCD regulatory requirement and product loss challenge, we developed an efficient adenovirus purification process via an integrated, multi-unit operation optimization approach.[a] This study investigated the HCD and vector concentration response to the

order of unit operations, HCD digestion enzyme, and digestion and chromatography parameters. By optimizing and accounting for the interactions between unit operations, the process was scaled-up by a factor of 10 while recovering 73% of vectors and reducing the HCD/dose by a factor of 80. This study is presently focused on assessing the manufacturing and economic feasibility of the adenovirus purification process. To perform this analysis, the upstream and downstream adenovirus manufacturing process was modeled in BioSolve Process (Biopharm Services) and the scale-up was simulated from tens to thousands of litres. The model incorporates data collected during the process development and data reported in the literature to generate the capital, material, consumable and labour costs associated with each unit operation. Significantly, the work presented here quantifies the impact of DNA removal to meet FDA standards on the overall production cost. The effect of process parameters including the source of the nuclease (i.e. Benzonase and Denarase), DNA digestion time and enzyme concentration are being evaluated to determine their contribution to the cost of goods under the different scenarios. This study shows that implementing such economic analysis as early as possible in vector process development will ultimately benefit patients that rely on safe and affordable biotherapeutics. [a]Reference: Kawka, K., et al. "Integrated development of enzymatic DNA digestion and membrane chromatography processes for the purification of therapeutic adenoviruses." Separation and Purification Technology 254 (2021): 117503.

812. Novel Inducible Packaging Cells for the Production of Lentiviral Vectors Using a Combination of the Cumate and Coumermycin Gene-Switches

Sophie Broussau, Mélanie Leclerc, Claire Guilbault, Viktoria Lytvyn, Mélanie Simoneau, Nathalie Coulombe, Rénald Gilbert

Production Plateforms & Analytics, National Research Council Canada, Montreal, QC, Canada

Lentiviral vectors (LVs) are powerful tool for cell therapy because they efficiently integrate their genome into the chromosomes of the targeted cells and therefore can stably modify them. LV are commonly produced by transient transfection of plasmids using HEK293 cells, an efficient but cumbersome process, particularly if large volumes of vectors are needed. To facilitate LV production, we have generated packaging cells that contain all the components necessary for the assembly of LV. Because some of these components, such as the envelope glycoprotein of vesicular stomatitis virus (VSVG) and the protease, are cytotoxic, their transcription needs to be tightly controlled. To construct our packing cells, we first generated a cell line (293SF-CymR/λR-GyrB), derived from HEK293 cells previously adapted to serum-free suspension culture, that expresses the repressor (CymR) and the transactivator (λR-GyrB) of the cumate and coumermycin gene-switches respectively. Transcription of CymR is under the control of the constitutive CMV promoter, whereas λR-GyrB is regulated by CMVCuO promoter. In the absence of cumate, CymR binds to the CuO operator of CMVCuO and prevents transcription. Addition of cumate releases CymR from CuO allowing transcription of λR-GyrB. In the presence of coumermycin, λR-GyrB dimerizes and transactivates the coumermycin promoter. The packaging cells were generated by transfecting 293SF-CymR/λR-GyrB simultaneously with four plasmids encoding respectively the resistance for hygromycin, Gag/Pol (under the control of the strong CAG constitutive promoter), Rev and VSVG (both regulated by the coumermycin promoter). Hygromycin resistant clones were generated by limiting dilution into nanowells followed by isolation using a robotic cell picker (CellCelector™). LV Production was tested by transient transfection with a plasmid encoding a LV producing GFP (LV-CMV-GFP) and induction with cumate and coumermycin. The titer of the best packaging clones varied between 2 to 5 x 10^7 transduction units (TU)/ml from the culture medium. Producer clones were generated by transfecting one of our best packaging cell line with a plasmid encoding LV-CMV-GFP and a second plasmid for the neomycin resistance. Producer clones were isolated as described above and tested for the production of LV-CMV-GFP after induction with cumate and coumermycin. The best titers ranged between 4 to 8 x 10^7 TU/ml from the culture medium. For some clones, LV production was maintained to that level after being subcultured for up to 10 weeks in the absence of selective agent. In conclusion, the combination of the cumate and coumermycin gene switches provides transcriptional regulatory control that is sufficiently tight to generate packaging cells for LV. The fact that our packaging cells are stable and adapted to serum-free suspension culture should facilitate the scale-up of LV for clinical application.

813. XLenti Stable Cell Lines for Lentivirus Manufacturing: New Development, Characterisation, and Future Goals

Corinne Branciaroli

Biomanufacturing - Viral Cell Line Development, Oxford Genetics Ltd, Oxford, United Kingdom

Lentiviral vectors (LV) present huge potential for cell and gene therapy. After the first LV-engineered CAR-T cell therapies gained approval by the FDA in 2017 (Yescarta and Kymriah), more LV-based therapies are being investigated and more than 600 trials are active in the US. The demand for more efficient LV manufacturing continues to increase, but the technology still presents challenges associated with process robustness and scalability, as well as high cost-of-goods. To overcome these manufacturing obstacles, OXGENE has developed suspension HEK293-based packaging cell lines, stably engineered with Lentivirus packaging components. Building on our existing XLenti pre-packaging cell line which expresses VSV-G and GagPol under the control of a proprietary Tet inducible system, OXGENE developed XLenti packaging cell lines with addition of the Rev gene. The newly developed cell lines show a 3-fold titre improvement compared with the pre-packaging cell line, consistently achieving the titre of ~3E7 TU/mL. The cell lines are characterised for transgene copy number and verified by Targeted Locus Amplification (TLA) analysis. Cell line stability of growth and vector production was monitored for over 20 passages (>50 generations) and further upstream process development is ongoing. In parallel, next-generation strategies for LV packaging and producer cell lines are being explored aiming toward high throughput cell line development with shorter turnaround time and further enhanced vector production capabilities.

814. Self-Attenuating Adenovirus TESSA Enables Production and Propagation of AAV for High Manufacturing Yield without Contamination

Leonard W. Seymour[1], Weiheng Su[1], Maria I. Patricio[2], David W. Brighty[2], Ryan Cawood[2]

[1]University of Oxford, Oxford, United Kingdom,[2]OXGENE Ltd, Oxford, United Kingdom

Adeno-associated virus (AAV) is the vector of choice for many types of gene therapy, particularly for diseases where prolonged transgene expression is required in post-mitotic tissues. Despite showing impressive clinical activities, however, scalability, yield and quality of AAV produced remain significant issues. Here we describe an AAV manufacturing strategy based on a self-silencing 'helper' adenovirus known as TESSA (Tetracycline-Enabled Self-Silencing Adenovirus). Inserting a tetracycline repressor (TetR) binding site into the major late promoter (MLP) and encoding the TetR under transcriptional control of the MLP allowed normal adenovirus replication in the presence of doxycycline but only genome amplification and early gene expression (including the essential 'helper' functions) in its absence. Using this negative feedback system, we demonstrate efficient delivery of adenoviral helper functions, AAV rep and cap genes, and the AAV transfer vector to yield over 1000-fold increase in production of infectious AAV2 compared to standard helper-free plasmid systems. TESSA vectors (which are attenuated by both E1 deletion and MLP inactivation in the absence of doxycycline) were found at typically only 2-15 particles per ml of total cell lysate, with no evidence for wild type virus. This system also allows propagation of existing AAV without transfection, a process that depletes reverse packaging and improves AAV batch quality. We propose this as a high-yielding, contaminant-free system suitable for scalable AAV manufacture.

815. Improving Downstream Purification of Plasmid DNA for Gene Therapy Applications

Alejandro Becerra[1], John Li[1], Matthias Knoedler[2], Johannes F. Buyel[2]

[1]Thermo Fisher Scientific, Bedford, MA,[2]Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen, Germany

The field of gene therapy has made significant advancements in the last decade and the pipeline of therapies in clinical trials is rapidly growing year over year. Delivery of the therapeutic gene is most often accomplished through viral vectors such as Adeno-associated Virus (AAV) or Lentivirus (LV). Transient transfection of host cells with plasmid DNA (pDNA) encoding the required genes is most commonly used today in the production of viral vectors. Recently, mRNA has also emerged as an efficient therapeutic tool and pDNA vaccines continue being developed. Plasmid DNA plays a critical role in the mRNA workflow as a template in an in vitro transcription reaction. The pDNA required in all these applications needs to be of high purity which is accomplished through a downstream process typically consisting of anion exchange (AEX) and hydrophobic interaction chromatography (HIC) steps. Nevertheless, low binding capacity, RNA removal and separation of pDNA isoforms remain as challenges. In this work a 5.3 kb plasmid was used as a model to optimize the purity and recovery of both chromatography steps using POROS™ AEX and HIC resins. A Design of Experiments (DoE) approach using MiniColumns was used to determine the optimal conditions on each unit operation. Additional studies with 1 mL columns showed a high dynamic binding capacity (>5 g/L) for some of these chromatography media. The performance of the optimized conditions was confirmed using a representative manufacturing scale-down model. These results demonstrate the potential of these resins to address the existing pDNA purification challenges as well as facilitate the development and implementation of a scalable and flexible process.

816. How to Mitigate Contamination Risk for Viral Vector Large Scale Production

Keen Chung

Pall Biotech, Westborough, MA

Adeno-associated virus (AAV) vectors are potent gene therapy vectors, used to deliver therapeutic transgenes to target tissues. Gene therapy clinical trials often require high titer vector preparations to adequately deliver the therapeutic transgene, in great excess of research-level production utilized in many laboratories. To bring the virus into the pre-clinical and clinical phases, Pall Biotech simplified, optimized and scaled-up the current upstream and downstream process of viral vector production to industrial scales using the fully-closed, single-use Xpansion® multiplate seed train bioreactor and the production packed-bed iCELLis® 500 single-use bioreactor. In these processes, it is important to ensure that steps are built into the process to ensure adequate control of adventitious agents. Cell culture media filtration is one important technique that can be used for this purpose. Pegasus™ Prime virus removal filters can be used to mitigate the risk of viral contamination of cell culture media. They show robust performance, are easy to implement and use, making them an ideal technology to minimize the risk of upstream and downstream viral contamination. High-flux viral filtration allows for rapid, scalable processing from laboratory scale to commercial manufacturing. In this webinar, we will address how to mitigate the risk of contamination by adventitious agents to ensure viral vector safety used in biotherapeutics.

817. Novel Next-Generation Sequencing Methodologies to Analyze Viral-Vector Integrity and Purity for Gene Therapy

Alexei Saveliev, Yu Qiu, Andrea O'Hara, Ritu Mihani, Elizabeth Louie, Lindsay Kendall, Haythem Latif, Ginger Zhou

GENEWIZ, A Brooks Life Sciences Company, South Plainfield, NJ

Recombinant adeno-associated viruses (rAAV) have become the vector of choice for virus-mediated gene therapy due to their non-replicating nature, high-titers, low immunogenicity, and low genotoxicity. Extensive quality control (QC) throughout the entire manufacturing process is essential. A robust QC process expedites the safe and effective commercialization of the final product. Compared to existing QC methods, next-generation sequencing (NGS) offers an effective high-throughput approach for monitoring rAAV quality, from initial construct assembly to analysis of the final encapsulated product. Both, Illumina® short-read and PacBio® long-read sequencing technologies

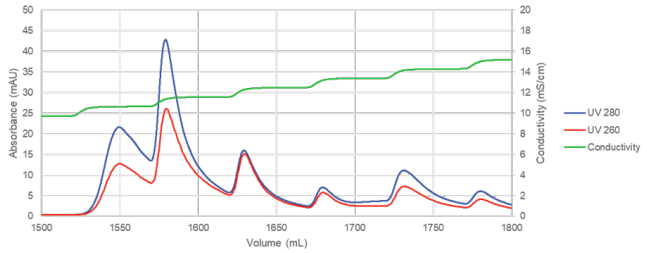
offer distinct advantages including sequencing of the entire rAVV genome, with the inverted terminal repeat (ITR) regions, detection of potential mutations, truncations and, contaminants. Workflows for both platforms require conversion of the single-stranded genome to double-stranded DNA prior to library preparation; however, robust protocols for this step are lacking. Additionally, an efficient bioinformatics pipeline is needed to convert the massive amounts of NGS data to interpretable results. Here, we describe our novel, end-to-end workflows, across multiple NGS platforms, that alleviate current constraints for high-throughput rAAV sequencing and thereby enhance the overall QC process.

818. Empty and Full Separation of Adeno-Associated Virus Vectors by Anion Exchange Membrane Chromatography

Aydin Kavara, Adam Hejmowski, Kurt Boenning, Julio Huato, Mark Schofield

R&D Bioprocessing, Pall Corporation, Westborough, MA

The approval of adeno-associated virus (AAV) gene therapies has led to increased research into AAV production and the burgeoning of promising clinical trials. However, significant challenges persist in AAV purification as AAV harvests typically contain a majority population of empty capsids that generate an immune response without delivering the therapeutic payload. In addition, subtle differences between therapies restrict platformability. Here we assess the performance of anion exchange (AEX) membrane chromatography as the polishing stage of an AAV platform process following affinity purification. Utilizing a novel 1 mS/cm step gradient approach, we are able to demonstrate separation of empty and full AAV capsids of serotypes 5, 8, and 9 with the Mustang® Q XT membrane. This process maximizes the high flow rate benefits of membrane chromatography relative to traditional column chromatography, while providing improved separation. Distinct populations in the UV 260/280 chromatogram, analytical trends with PCR and ELISA tests, and capsid standards prepared by ultracentrifugation reaffirm separation. This technique is scalable between the 0.86 mL Mustang Q XT Acrodisc® unit and 5 mL Mustang Q XT capsule, and effectively clears residual host cell protein and DNA contaminants.



819. The Corning® Ascent™ Fixed Bed Reactor (FBR) System: Designed for High Yield, Scalable Viral Vector Production for Gene Therapies

Zara Melkoumian, Vasiliy Goral, Yue Zhou, Jennifer Weber, Ann Ferrie, Yulong Hong, Lori Romeo, Kathleen Krebs, Joel Schultes, Vinalia Tjong, Abhijit Rao, Yujian Sun, Stephen Caracci, Daniel Manning, Todd Upton

Corning Research and Development Corporation, Corning, NY

Gene therapies hold great promise for one-time treatments that may alleviate or cure certain genetic conditions. Recombinant adeno-associated viral (AAV) and lentiviral vectors have emerged as leading gene delivery methods for *in vivo* and *ex vivo* gene therapies, respectively. With more than 500 gene therapy clinical trials underway, there is an urgent industry need for scalable, cost-efficient manufacturing of viral vectors. To address this need, Corning developed the Ascent FBR System, which is specifically designed for viral vector manufacturing. Key features of the Ascent FBR System include specially treated and packed polymer mesh that enables uniform low-shear fluid flow through the bioreactor bed, uniform high-density HEK293(T) cell growth, >90% transfection efficiency, and 3-fold higher rAAV2-GFP vector yield/m^2 compared to other fixed bed reactors. The ability to harvest viable cells from the Ascent FBR System enables its use in seed train, results in a significant reduction in the viral vector harvest volume that needs to be processed through downstream purification, and provides the flexibility of using chemical (detergent) or mechanical (freeze/thaw, microfluidization) cell lysis methods for release of cell-associated viral vectors. Collectively, these benefits will enable significant reduction in viral vector manufacturing cost. The Ascent FBR System is designed to provide a broad range of cell growth surface areas to meet various viral vector yield needs: small size (1-5m^2), medium size (20-100m^2) and large size (200-500m^2). The Ascent FBR System consumable set is equipped with disposable sensors for key process parameters (pH, dissolved oxygen, temperature), gamma irradiated and ready-to-use. We are confident that the Ascent FBR System will enable scalable manufacturing of high-density adherent cells for multiple applications, such as viral vectors, oncolytic viruses, stem cells, extracellular vesicles, and cell-based meat.

Vector Product Engineering, Development or Manufacturing

820. A Novel Fixed Bed Reactor That Enables High AAV Vector Yield, Cell Harvest and Flexibility for Downstream Processing

Jie Wang[1], Kirsten L. Cooper[1], Lori E. Romeo[2], Ann M. Ferrie[2], Zara K. Melkoumian[2], Christopher Jaquin[3], Yang Su[3], Kelley McCabe[3]

[1]Corning Research and Development Corporation, Bedford, MA,[2]Corning Research and Development Corporation, Corning, NY,[3]Microfluidics International Corporation, Westwood, MA

Recombinant adeno-associated viral (AAV) vector has emerged as a leading gene delivery vector for *in vivo* gene therapy due to its robust, long-term *in vivo* transgene expression and low toxicity. Currently, the adherent HEK293 cells in combination with transient transfection methods using calcium phosphate or polyethyleneimine (PEI) reagents is considered the industry gold standard expression system for clinical AVV vector manufacturing. Adherent platforms represent a less risky path to clinical trials due to a precedent of FDA/EMA approved gene therapies manufactured using adherent cells. In addition, adherent platforms provide higher specific productivity (VG/cell) and enable easier process manipulations such as media change and perfusion. However, the major challenge of current adherent platforms is the lack of scalability for clinical and commercial manufacturing. To address the industry challenge with large scale (>10^{18} vector genomes) viral vector manufacturing while leveraging high specific productivity of adherent cells, Corning has developed the Ascent™ FBR System, a novel fixed bed reactor specifically designed for viral vector production. The key features of the Ascent FBR System include uniform fluid flow across the fixed bed, enabling uniform cell attachment and growth, high transfection efficiency, and high viral vector yield (2x10^{10} bulk rAAV2-GFP VG/cm2). The ability to harvest viable cells from the Ascent FBR System makes it suitable for seed train and cell therapy applications. In this study, we demonstrate compatibility of the Ascent FBR System with both chemical and mechanical AAV vector harvest methods. More than 90% of transfected cells were harvested from Ascent FBR System with >90% cell viability. Three different cell lysis methods were compared: freeze/thaw, Microfluidizer™ homogenization and detergent. Microfluidizer processor, a scalable mechanical cell lysis technology was used to efficiently release cell-associated AAV vector from up to 50x10^{6} cells/ml without the need for regulatory unfriendly detergent or costly DNase. Viral vector yield was comparable between Microfluidizer process and non-scalable freeze/thaw method. In summary, Corning's novel Ascent FBR System enables high AAV vector yield, cell harvest and flexibility of using different scalable cell lysis options. In combination with Microfluidizer technology, the Ascent FBR System provides scalable, detergent-free process for AAV vector manufacturing.

821. Development of a 96-Well Plate-Based High-Throughput System for rAAV Manufacturing Platform Optimisations and Candidate Selection

Bettina Prieler, Britta Voland, Martina Ohme, Rupert Derler, Sandra Göbert, Florian Sonntag, Markus Hörer, Andreas Schulze

Freeline, Stevenage, United Kingdom

We have developed an iCELLis®-based recombinant adeno-associated virus (rAAV) manufacturing platform that provides very high cell-specific productivity, overall yields, vector quality and potency. To overcome the serum dependence of this adherent system, we developed a fully single-use suspension cell-based manufacturing platform free of animal-derived components based on the HEK293 cell family. This process is capable of producing AAVS3 of comparable quality and potency as our iCELLis-based benchmark manufacturing system. We demonstrated scalability from shake flask to bioreactor formats resulting in good productivity and quality attributes across different scales. Further optimization of our suspension cell-based manufacturing platform, as well as candidate selection for new programs in our gene therapy pipeline, requires a fast, reliable, and robust system for direct comparison. Here we present the current development status of a 96-well plate-based high-throughput system using our proprietary HEK293 suspension cell line and manufacturing process. We will present data outlining parameter adaptation and optimization of the HEK293 suspension cells to the 96-well format with the goal of obtaining comparable/improved growth kinetics and viabilities in comparison to larger scale formats. Furthermore, we will show implementation and optimization of rAAV production using a transient transfection with our proprietary two-plasmid system, resulting in reproducible, high transfection efficiencies and robust particle production. The established 96-well high-throughput suspension platform facilitates robust comparisons and candidate screening enabling the further expansion of our state-of-the-art gene therapy pipeline and continuous improvement of the platform manufacturing process.

822. Engineered Herpes Simplex Virus Type 1 (HSV-1)-Based Vectors as a Platform for Localized Delivery of Therapeutic Antibodies in the Treatment of Skin Disorders

John C. Freedman, Peipei Zhang, Branimir Popovic, Alexandra Collin de l'Hortet, Pooja Agarwal, Trevor Parry, Suma Krishnan

Krystal Biotech, Inc., Pittsburgh, PA

HSV-1 has emerged as a promising gene therapy vector for the treatment of disorders of the skin and other organ systems. Recently, Krystal Biotech, Inc. (Krystal) has leveraged its HSV-1-based STAR-D (Skin-Targeted Delivery) platform to deliver therapeutic transgenes to patients with debilitating diseases, including vectors beremagene geperpavec ("B-VEC") - encoding human *COL7A1* for the treatment of dystrophic epidermolysis bullosa (ClinicalTrials.gov Identifier: NCT04491604), and KB105 - encoding human *TGM1* for the treatment of autosomal recessive congenital ichthyosis

(Clinicaltrials.gov Identifier: NCT04047732). Krystal is continually evaluating opportunities to expand beyond rare, autosomal recessive skin diseases using its STAR-D platform and has embarked upon a discovery program to assess the potential for our vectors to deliver and locally express therapeutic single chain antibodies (sc-Abs). Localized expression of therapeutic antibodies offers several potential advantages over systemic antibody exposure including improved tissue penetration and decreased systemic side effects. To this end, we engineered two vectors - KB501 (expressing a TNF-α targeting antibody) and KB502 (expressing an IL-4Rα targeting antibody)-designed to neutralize the indicated effector and downregulate immune pathways involved in the development and/or maintenance of focal skin lesions. Molecular efficacy and disease correction of these recombinant vectors was then demonstrated *in vitro* (in 2D cell culture) and/or *in vivo* (in two well-established models of atopic dermatitis (AD)). *In vitro,* KB501 was shown to express its transgene in a dose-dependent manner in immortalized human keratinocytes and fibroblasts. Cell culture supernatants harvested from these KB501-infected keratinocytes were found to inhibit the detection of recombinant human TNF-α in an ELISA, demonstrating proper binding of the antibody to the targeted epitope. Anti-TNF-α sc-Ab purified from transfected cell supernatants was demonstrated to bind TNF-α with picomolar affinity (~400 pM), comparable to clinically approved anti-TNF-α monoclonal Abs. Next, the *in vivo* efficacy of KB501 and KB502 was assessed in two murine models of AD, one highlighting molecular efficacy (in the case of KB501), and the other highlighting phenotypic correction (in the case of KB502). To determine efficacy on a molecular scale, an oxazolone-induced murine model of TNF-α induction was utilized, whereby intradermal administration of KB501 led to a decrease in TNF-α transcript and protein levels at the site of injection, as well as a decrease in expression of IL-10, a downstream marker of TNF-α signaling, as compared to vehicle-treated animals. To evaluate the ability of a vector-encoded antibody to reduce an AD-like phenotype on a macro-scale, KB502 was applied topically in an MC903-induced murine model of AD. In this system, topical administration of KB502 drastically and quantitatively reduced MC903-induced ear skin thickening. Taken together, these data demonstrate the potential of STAR-D as a platform for inducing disease correction following the localized administration of vectors expressing therapeutic antibodies. At this time, Krystal has expanded its STAR-D-based antibody portfolio to at least 12 unique products, including vectors expressing anti-CD20, anti-IL-17, and anti-CCR4 antibodies. The studies described herein warrant the further investigation of STAR-D as a platform for localized delivery of therapeutic antibodies, including for the treatment of diseases of skin and other organ systems amenable to immunomodulatory therapeutics.

823. Empty Full AAV Capsid Comparability Study by AUC and TEM Using AAV Reference Standard Materials

Jeffrey Hung
Vigene Biosciences, Rockville, MD

The empty full capsid ratio is of great importance for both safety and potency reasons for AAV based gene therapies. Currently there are a plethora of assays for the empty: full capsid assays. But the correlation between the results by different methods are not well established. Here we report for the first time the empty full AAV capsid comparability study by Analytical Ultracentrifugation (AUC) and Transmission Electron Microscopy (TEM) using AAV reference standard Materials. The AAVs are the AAV reference standard materials from Vigene Biosciences. The empty: full ratio data detected by the AUC and TEM methodologies are remarkably consistent for AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9 standard reference materials. In addition, we discovered that AUC can potentially have more resolution to distinguish partially vs. fully packaged capsids. Additional data regarding other interesting findings and assay results will be presented.

824. Addressing HEK293 Cell Lineage Diversity through Basal Media Optimization

Gino Stolfa, Sarya Mansour, Jennifer Schieber Schieber, Amanda Zunic, Matthew T. Smonskey
Thermo Fisher Scientific, Grand Island, NY

Background: Adeno-associated virus (AAV) has become an attractive vector for gene therapy; however, generally low titer yield limits its viability as a therapeutic. Various methods have been employed to achieve increased titer, including cell engineering, expression platform optimization, transfection optimization, and media development. One of the preferred methods to produce recombinant AAV (rAAV) is helper-free triple transfection using HEK293 cells. In this method AAV is produced by transfection of three plasmids: a transfer construct encoding the therapeutic gene, a RepCap construct encoding the replication and capsid genes, and a helper construct encoding adenovirus genes into HEK293. We aimed to develop media that addresses the lower titer of the HEK293 system, but the high diversity of HEK293 cells and processes used for gene therapy would make it difficult to develop one medium that suites all uses. Therefore, we developed a panel of media to support AAV production by helper-free triple transfection using HEK293 cells with increased viral titers agnostic of client manufacturing processes or cell lineage. **Methods:** In order to develop a panel that captures the diversity of HEK293, a total of fifty-eight internal media were assessed by principal component analysis. Seventeen diverse media were selected for screening in three HEK293 suspension cell lines. The media were screened for promoting growth, transfection efficiency, and improved physical titer (vg/mL) producing GFP-encoding AAV2 in PEI-based helper-free triple transfections at the shake flask scale. **Results:** Of the seventeen media initially screened we selected five media that promoted growth and high transfection across three cell lines. Three of the five panel media resulted between 2- and 10-fold increases over the industry standard Freestyle F17 in two out of three cell lines. In the third cell line these three media were similar to F17. **Conclusions:** The media panel we developed attempted to address HEK293 diversity with five diverse formulations that contain a wide range of components at various concentration. While the media result in comparable growth among three different HEK293 suspension cell lines, the titer increases for each panel medium compared to F17 are largely cell line dependent indicating that these media address the diversity HEK293 cells to some degree.

825. LVV Production At-Scale: From Cell Factory™ Systems to iCELLis® 500 Bioreactor

Francesca Bellintani, Margherita Neri, Luca Crippa, Samuele Corbetta, Federico Lorenzetti, Manuela Cota, Martina Brunati, Luca Allievi, Marco Rausa, Giuliana Vallanti

AGC Biologics, Milan, Italy

Evolving from the well-established GMP 48L Nunc™ Cell Factory™ (CF) process, AGC Biologics has developed a robust, scalable, high quality and quantity process for the industrial-scale production of lentiviral (LV) vectors for use in gene-modified cell therapy or *in vivo* gene therapy. This platform includes vector production in the iCELLis® bioreactor, downstream purification and concentration by chromatography and tangential flow filtration (TFF) steps and sterile filtration and filling in different formats. Upstream process was developed leveraging the scale down iCELLis® Nano system where key process parameters were screened in order to increase productivity, producing a bulk vector with consistently low contaminant profile. Data obtained on full-scale iCELLis® 500 system confirmed full scalability and equivalent performance of the process.

	Infectious viral titer (TU/mL)	Physical viral titer (ng p24/mL)	HCPs (ng/mL)	DNA (µg/mL)	
Bulk vector iCELLis® Nano	Average ± St. dev.	1.7E+07 ± 0.7E+07	330 ± 84	1073 ± 380	Negative
Bulk vector iCELLis® 500	Average ± St. dev.	2.5E+07 ± 0.8E+07	428 ± 221	1173 ±734	Negative

The downstream process was designed to remove main process related contaminants (Host Cell Proteins, Host Cell DNA, plasmid DNA and BSA), thus maintaining vector infectivity. The process consists of an anion exchange chromatography step that captures and concentrates the vector, enabling 80% recovery while removing HCPs, DNA and BSA. Eluted vector is then concentrated and diafiltered with TFF using hollow fibers. With this step, the vector is further concentrated with 80% recovery, and additional contaminant removal is achieved. The vector is then 0.2µ sterile filtrated and filled in vials. Total process recovery is approximately 30%.

	Infectious viral titer (TU/mL)	Physical viral titer (ng p24/mL)	HCPs (ng/mL)	DNA (µg/mL)	
Purified vector iCELLis® Nano	Average ± St. dev	3.3E+08 ± 1.4E+08	4820 ± 1274	negative	1.7 ± 0.8
Purified vector iCELLis® 500	Average ± St. dev	4.9E+08 ± 1.7E+08	9951 ± 3238	negative	1.5 ± 0.8

Final purified vector data shows a similar impurity profile as the established 48L CF's process. The system has already been successfully implemented for the manufacture for different vectors with different Gene of Interest (GoI). With the ability to produce a volume of 4x without a loss in quality, the iCELLis® 500 platform offers a scalable and cost-effective solution with regards to the number of patients treated versus the cost of production and quality control. Moreover, the possibility to leverage the analytical platform already in use for the GMP 48L CF's process accelerates transition from development to GMP clinical and commercial production with reduced comparability exercises.

826. Intensified Production of Vaccinia-Based Oncolytics in the High Density Cell Respirator (HDCR) Bioreactor Improves Vaccine Logistics and Economics

Colin A. Cook[1], Seonah Kang[1], Jianming Lu[1], Yu-Chong Tai[2], Saswati Chatterjee[1], Yuman Fong[1]

[1]Surgery, City of Hope, Duarte, CA,[2]Medical Engineering, Caltech, Pasadena, CA

Intensification of cell-based production processes is key to improving oncolytic vaccine logistics and economics by saving on GMP space, time, labor, and feedstock. Many promising vectors, including those based on vaccinia virus (e.g. CF33, JX-594/Pexa-Vec) are still produced using flask-based culture due to the cost, effort, and uncertainty involved in adapting to stirred-tank or perfusion processes. Based on prior success culturing adherent cells (e.g. HEK293, A549, CV-1) to high densities ($10^{7\text{-}8}$ cells/mL) using the scalable high-density cell respirator (HDCR) bioreactor, we hypothesized that the platform could support orders-of-magnitude-intensified production of replication competent viruses. Here we report on CF33 virus production as a proof-of-concept for oncolytic virotherapy. Bioreactor cartridges were produced based on a 4-stack of 600 cm^2 HDCR membranes, featuring a proprietary gas perfusable and permeable microarchitecture that optimally oxygenates cells even at high densities. Media perfusion was feedback-controlled based on glucose measurements in the waste stream. A549 cells were seeded into the bioreactor on microcarriers, expanded 10-fold to mid-10^7 cells/mL densities, infected with CF33 virus (MOI of 0.1), and harvested 48 hours post-infection. CF33 virus was gradient purified and titered for functional virus by plaque forming assay. Multiple production runs using different strains of CF33 virus validated the reproducibility of the process, as summarized in Table 1. Importantly, cell specific titers (PFU/cell) remained comparable to conventional flask-based production, leading to significant intensification due to the higher cell densities supported in the HDCR bioreactor. Volumetric productivity is on the order of 100× that of cell factories. The efficient usage of media due to the gas-media decoupled operation of the HDCR bioreactor enables gradient-purified virus costs of around $\$500/10^{10}$ PFU. This is significantly below the $\$10{,}000/10^{10}$ PFU charged by CMOs and supports our mission of democratizing access to life saving medicines. The straightforward adaptation of CF33 virus production from a flask-based to a high yield, intensified process highlights the logistical and economical advantage of the HDCR platform for oncolytics.

Table 1: Production metrics for CF33 virus produced in HDCR bioreactor.				
Virus Strain	**Crude Yield (PFU/Run)**	**Specific Yield (PFU/Cell)**	**Media Usage (Liters)**	**Cost Per 10^{10} Purified PFU**
CF33-hNIS-αPDL1	6E10	28	10.5	402
CF33-mCD19t	2.6E11	130	10.9	275
CF33-tk	2.8E10	18	10.6	540

827. Comparative Analysis of Endotoxin Removal Protocols for AAV Purification

Samantha Desmarais, Misha Chittoda, Swetha K. Iyer, Junghae Suh, Davide Gianni

Gene Therapy Accelerator Unit, Biogen, Cambridge, MA

Endotoxin, otherwise known as lipopolysaccharide (LPS), is the major component of the outer membrane of Gram-negative bacteria. Endotoxin can be released by bacteria and co-purify with AAV therapeutics. *In vivo*, endotoxin can cause sepsis that leads to excessive inflammation, leading to multiple organ failure and potentially death. As such, endotoxin must be monitored as an adventitious agent that can adversely affect the safety and efficacy of AAV drug products. Preclinical research animal model endotoxin limits based on the threshold of 5 EU/kg for human doses are calculated to be approximately 1.5 EU/mL. We have investigated three endotoxin removal strategies to clear AAV vectors of endotoxin for potential use in mouse and primate studies. AAV viral preps with titers of 1 e13 GC/mL and 6 e12 GC/mL were spiked up to 980 EU/mL with LPS. A commercial endotoxin removal kit was compared to published endotoxin removal protocols based on detergents Triton X-100 and Triton X-114. The commercial product reduced endotoxin content by 30-fold but sacrificed 30 % of the titer. Triton X-100 reduced endotoxin by 3,500-fold but sacrificed 60% of the titer. Triton X-114 was the most efficient at removing LPS, up to 60,000-fold for final levels less than 0.100 EU/mL, while maintaining the highest titer recovery of 75%. Endotoxin removal using Triton X-114 is recommended for quick processing of LPS-contaminated AAV therapeutics.

828. Development and Optimization of Transient Transfection Process for Adeno-Associated Viral Vector Production

Le You

Novartis, San Diego, CA

Transient transfection is presently the primary approach widely used for recombinant adeno-associated virus (rAAV) production. In this study, we have explored the critical process parameters for rAAV production. We have evaluated multiple polyethylenimine (PEI)-based transfection reagents and identified PEIpro® as the lead candidate. We have also compared various cell culture media formulations and observed high compatibility of PEIpro® with different media types. Key transfection parameters, including transfection cell densities, plasmid DNA amounts, transfection complex volume, transfection/ harvest timings as well as bioreactor settings were also screened through DoE studies to optimize the manufacturing process. The optimized production process shows high robustness and improved batch-to-batch consistency and reproducibility.

829. Stealthed, Retargeted HIV-1 Vectors Incorporating Darpin-Displaying Canine Distemper Virus Envelope Glycoproteins without Cytoplasmic Tail Truncations

Miguel Á. Muñoz-Alía, Stephen J. Russell

Molecular Medicine, Mayo Clinic, Rochester, MN

Receptor-targeted lentivirus vectors (LV) are emerging as a key technology for selective gene delivery to key target cell populations both during *ex vivo* and *in vivo* transduction protocols. Currently, the best developed approach is to pseudotype the LV with paramyxovirus Fusion (F) and Hemagglutinin (H) glycoproteins whose natural tropisms are ablated and whose attachment specificity is redirected via cell specific polypeptide binding domains (eg. scFv) displayed on the C-terminus of the H protein. LV pseudotyped with engineered measles F and H glycoproteins can be efficiently retargeted but pre-existing anti-measles antibodies render them ineffective for *in vivo* gene delivery. An addittional limitation of current pseudotying strategies with receptor-targeted *paramyxovirus* glycoproteins is the requirement for extensive engineering of their cytoplasmic tail regions to allow for incorporation into lentiviral particles. Here we show that fusion-competent, full-length canine distemper virus (CDV) glycoproteins (ie without engineered cytoplasmic tails) can be efficiently pseudotyped onto LVs. Cytoplasmic-tail truncations of the CDV envelope glycoproteins did not improve efficiency of pseudotyping, while swapping the cytoplasmic tail of CDV-H with the heterologous tail from the measles virus H protein completely abolished it. To generate an EpCAM-targeted lentiviral vector, we engineered CDV glycoproteins to ablate their tropisms for cognate receptors, canine SLAMF1 and human/canine nectin4, and fused a designed ankyring repeat proteins (DARPin) specific for EpCAM to the C-terminus of the CDV-H protein. The receptor-targeted CDV-LVs showed improved EpCAM-specific transduction efficiency when compared with their MeV-based counterparts. Our current effort focus on the generalizability to other receptors of choice. Lentiviral vectors pseudotyped with full-length retargeted CDV glycoproteins might provide a valuable addition to the family of targeted LVs for gene therapy purposes.

830. Comparative Analysis of Anion Exchange Chromatography for the Enrichment of Adeno-Associated Virus Serotype 9 Full Particles Using a Conductivity Step Gradient

A. Hejmowski[1], J. Huato[1], A. Kavara[1], M. Schofield[1], Victoria Bensimon Maharaj[2], Brittny Schnur[2], Lindsey O'Heron[2], Allison S. DeGroat[2], Phillip B. Maples[2]

[1]Pall Biotech, Westborough, MA,[2]Abeona Therapeutics, Cleveland, OH

Adeno-Associated Viruses (AAVs) are a well-established vector for gene therapy applications due to their ease of use and low pathogenicity. However, their production yields empty particles, that lack the genetic material required for the therapy, at ratios up

to 100:1 empty to full particles. As a result, the enrichment of full particles is a necessary step in downstream processing of these vectors. In this collaboration study, we tested various anion exchange chromatography media to enrich for full AAV9 particles. By using a recently developed step conductivity gradient elution method, we were able to explore the separation potential of resin, membrane, and monolith media formats for our rAAV9 construct. Distinct populations of E/F particles were identifiable while measuring A260/A280 ratios during elution steps; these eluates were further quantitated using QPCR and ELISA allowing us to characterize each format's recovery and enrichment potential. While we found all formats performed similarly, faster flow rates using membranes and monoliths are a noteworthy advantage. Lastly, we were able to reduce the number of elution steps as an optimization strategy for large scale and manufacturing processing. Our results showcased the effectiveness of the step gradient as an alternative to linear gradient elution methods and opportunities for the method in downstream process development.

831. Container Closure Systems for Viral Vector: From Research to IND

Claudia Lee, Sam Molina, Matthew Gehrmann, Olga Laskina

West Pharmaceutical Services, Inc., Exton, PA

In January 2020, FDA issued the final version of the guidance document on Chemistry, Manufacturing, and Control Information for Human Gene Therapy Investigational New Drug Applications (INDs) and requested the evaluation of container closure system compatibility with gene therapy drug substances. Seeking to understand the development necessary from research to clinic in terms of the container closure system (CCS), we first studied the particulate level of three common container closure systems: 1) parenteral grade stoppers paired with glass; 2) parenteral grade stoppers paired with Crystal Zenith (CZ) vials and 3) polypropylene screw-cap vials typically used in preclinical studies. Selected vials were filled in an ISO 5 clean room to mimic a cGMP compliant drug fill and finish process. The vials and their content were examined using USP <788> and <790>. While particles found in water from all three systems were under the USP limits, the polypropylene vials were found to have a higher particulate level. We also examined the potency of adeno-associated viral vectors before and after storage at -80°C in the three types of vials. By flow cytometry, the mean fluorescence intensity of HEK293 cells transduced using the AAV2-EGFP was found to be higher in the CZ vial systems. Furthermore, we examined the gas permeability of the glass and the CZ systems. Both systems were filled with nitrogen. Over two months, the amount of atmospheric oxygen ingress in the headspace of the vials was measured by frequency modulation spectroscopy. Glass and CZ systems at -80°C were found to be comparable. Lastly, we examined the impact of shipping over dry ice on pH stability. Polymers are known to be gas permeable, and the pH fluctuation was found to extend beyond the duration of shipping. However, the use of mylar bags as secondary packaging material was sufficient to address the concern of pH fluctuation due to CO_2 ingress from dry ice sublimation. In summary, for gene therapy using viral vectors, we recommend shifting to a parenteral grade CZ containment system and defining shipping practices according to the acceptable pH range prior to IND filing to address the FDA CCS request and to minimize product risk during clinical development.

832. Development of a Scalable Adeno Associated Virus Production Process by Transient Transfection in Suspension Cells

Ann-Christin Magnusson, Eva Blanck, Josefin Thelander, Albin Larsson, Sebastian Persson, Viktor Björklund, Elin Monie, Daphne Areskoug, Mats Lundgren

Cytiva, Uppsala, Sweden

Viral vectors have become increasingly used as means of gene transfer for specific tissue or cell type modifications. Several viruses have been investigated for their use in cell and gene therapy with Adeno Associated Virus (AAV) as the main vector for gene therapies. In this study, an efficient and scalable cell culture process for AAV production was developed by evaluation and optimization of each process step. In this study, HEK 293T cells were successfully adapted to suspension culture in a serum-free medium without any animal derived components. Triple plasmid transfection using polyethylenimine (PEI) was optimized for different parameters and conditions using a Design of Experiments (DoE) strategy in shake flasks. The optimal concentrations and ratio of plasmids, PEI, temperature, transfection volume and incubation times were evaluated for transfection efficiency and virus productivity. The conditions were further developed for production in single-use bioreactor systems. A qPCR assay was used for viral genome titer. For total virus capsid titer, a commercial ELISA was used for quantification. In parallel a Biacore-based method was developed for total virus capsid titer quantification. The percentage of full capsids was calculated as the ratio between viral genomes and viral capsids. A transduction assay based on flow cytometry was used to evaluate the infectious virus titer. An optimized transfection protocol in shake flasks was generated based on the DoE studies for AAV2, with titers above 10^{14} viral capsids/L. The protocol has also been evaluated with other serotypes, such as AAV5, AAV8 and AAV9 and was confirmed to generate similar productivities. A stirred tank single-use bioreactor process was designed and optimized based on the shake flask results. Furthermore, a second process was developed in WAVE bioreactors and gave similar productivities. Thus, scalable, robust and reproducible production of AAV was developed from small scale shake flask production up to 20L in single-use bioreactor systems.

833. Developing a Suspension Transfection Platform to Produce Adeno-Associated Viruses

Kory M. Blocker, Jason L. Rush, Boris N. Petkov, Simin F. Zaidi, Julie C. Johnston, James M. Wilson

University of Pennsylvania, Philadelphia, PA

Through the delivery of recombinant adeno-associated virus (rAAV) vectors, gene therapy has the potential to treat and/or cure many genetic disorders. Currently, most rAAV vector production processes employ triple transfection in an adherent production vessel, which has limited scalability that results in a low batch yield. Improving process

yield is critical for enabling the treatment of large disease indications and lowering manufacturing costs. One way to increase rAAV vector production is to employ suspension cell culture, which would allow for a larger process scale. In this work, we developed a suspension transfection process through media selection, optimizing transfection and process parameters, and demonstrating process scalability. Media selection allowed vector generation to transition into a completely chemically defined, animal-derived component-free system that alleviated potential regulatory concerns. We then took a Quality-by-Design (QbD) approach to explore the transfection and process parameters utilizing multi-factor Design of Experiments (DoEs). The DoE model established in the Ambr250 accurately predicted upstream productivity in the scaled up 2 L and 50 L bioreactor systems. With a clinically relevant product, this process has demonstrated the potential to yield up to 8e13 GC/L. By increasing single-batch productivity by up to 10-fold from a comparable adherent process, this process significantly decreases the timeline for gene therapy treatments.

834. Strategies in AAV Upstream Process and Downstream Purification for High Yield and High Separation of AAV Empty and Full Capsids

Heather Mallory, Xiaojun Liu, Jun Jiang, Jing Zhu, Jason Zhang

Gene Therapy, Luye R&D, Woburn, MA

One of the most common AAV process platforms uses adherent HEK cells with triple plasmid transfection and subsequent downstream purification. Despite early success in the feasibility stage, it remains a great challenge to achieve the desired specific productivity (AAV per cell) and a good separation of full AAV capsid from other impurities. Typical production yield is limited between 10^{12}-10^{14} vg per L with typical final recovery of downstream process between 15%-40%. Considering a single patient dose can be as high as $1x10^{15}$ vg AAV product for some gene therapies, 30-40L of AAV harvest material at current process capacity is required to meet the single dose demand. Therefore, an AAV production process with high productivity and high purity is critical to meet the AAV material demand, supporting both clinical and commercial efforts. To optimize AAV process steps, we conducted a series of experiments in both the AAV upstream and downstream process. A 1L batch scale with adherent HEK293 cell culture was used. Key parameters which impact the process performance were identified and further explored for significant process improvement. Specifically, optimization of post transfection media replacement time was performed, comparing 5hrs vs 24hrs. A cell lysis step was performed to recover virus bound inside cells. Results for upstream optimization show >2.5x higher production with 24-hr post transfection media change compared with 5hr post transfection media change. The lysis step reclaimed >70% of the total harvest viral genomes (vg) and the total yield for the most recent 6000 cm^2 batch, transfected at 3 x 10^5 cells/cm^2 harvested at 72 hrs (media + lysate) was 2.3 x 10^{14} vg (specific productivity ~1.3 x 10^5 vg per cell). For the downstream process, AEX steps targeted high load challenge and separation of empty vs full capsid by separation of empty capsid by elimination in the flowthrough fraction to prevent binding of this impurity to the column. High load challenge resulted in high step recovery of 73% vg and an excellent separation of AAV full capsid purity with greater than 95% purity in the column eluate. In addition, it was demonstrated that by adjusting pH + conductivity of the AEX step load, AAV empty capsid can be excluded from binding to the column, resulting in better separation of empty and full capsid and increasing column capacity. By excluding empty capsid from binding to the column, full capsid purity is improved. Empty capsid can be targeted for the flowthrough fraction at the AEX polishing purification step and can be precisely controlled at either the flowthrough step, wash step or elution fractions. This modification builds in process flexibility based on various final needs. These successes in process optimization were evaluated and confirmed via characterization of the AAV products at individual experimental steps with HPLC, ddPCR, ELISA and electron microscopy measurements. Together, these new strategies in the AAV upstream and downstream process offer great feasibility for a novel AAV production process yielding high vg recovery and excellent separation of AAV empty and full capsid. Extra development and scale-up efforts will be performed to fully build up AAV production capacity supporting our clinical development to treat Hemophilia B.

835. Ultrafiltration Behavior of Adeno-Associated Viral Vectors (AAVs) in Gene Therapy: Process Considerations for High Concentration UF/DF

Abhiram Arunkumar[1], Jacob Cardinal[2], Nripen Singh[1], Danielle Ladwig[1]

[1]Voyager Therapeutics, Cambridge, MA,[2]Biogen, Cambridge, MA

Ultrafiltration and Diafiltration (UF/DF) is the final step in the purification of biotherapeutics to concentrate the vector and place it in the final formulation buffer. While UF/DF has been extensively studied for highly concentrated monoclonal antibody solutions, little published quantitative data exist for viral vectors like AAV, where there is a need for higher dose concentrations to meet the requirements for new indications being evaluated by AAV gene therapy. This work examines the bottlenecks and processing challenges to reach concentrations exceeding 1×10^{14} viral genome copies (vg)/mL. In particular, a systematic analysis on the impact of process parameters and their ability to help achieving these concentrations is examined. The manufacturing implications of this choice of process parameters for the 200L, 500L and 2000L scales are discussed. Finally, this talk quantitatively explores the importance of the final plug flow recovery flush that is implemented to recover the product and the implications of identifying the proper configuration for the same.

836. A Scalable and Robust Platform Technology for AAV Purification

Michael Dzuricky, Kelli Luginbuhl, Jennifer Haley, James Jones, Melissa Callander

Isolere Bio, Durham, NC

Biomanufacturing of engineered viruses is a challenging and rate-limiting process which impacts the cost, safety, and efficacy of gene therapies. Traditional ultrafiltration and chromatography methods are currently employed for purification but they are inefficient, costly, and impractical when applied to gene therapy viral vectors. Isolere

Bio, Inc. has developed ViraTag™, a novel non-chromatographic purification process capable of affinity-capture of adeno-associated viruses (AAV) and their subsequent separation from contaminants via liquid-liquid phase transition. AAV capsids can be sequestered and concentrated into ViraTag™ droplets away from contaminants and then subsequently released from the droplets in a user-triggered manner for collection of pure product. The entire process can be performed with a standard tangential-flow filtration (TFF) setup using hollow fibers with 0.2 micron pores and a three-step process: concentrate, wash, elute. ViraTag™ is remarkably robust, capable of >95% capture efficiency across a wide range of serotypes, a 3-log range in titer, and with diverse starting medium which contains high levels of detergent lysis components. During the initial continuous fed-batch capture and concentration step, losses are <2% by qPCR and total capsid ELISA, while transmission of contaminants remains high. During washes, the losses are <1%. Highly predictable removal of contaminants enables simple process design a priori, achieving product specifications by simply adjusting the number of wash diavolumes. With ViraTag™, 2E14 purified vg per liter of AAV9 starting material is routinely achieved at a productivity of ~3.5E15 vg/m2/hr. Our process yields highly pure AAV whose in vitro infectivity is comparable to AAV purified by affinity chromatography. As we continue to optimize, validate, and scale the technology, we expect ViraTag™ to offer a true "plug and play" approach to viral vector processing, addressing the current bottleneck that is slowing development and commercialization of these important therapeutics. Furthermore, the technology is a platform, with other applications underway to deliver much needed solutions for other viral vectors, nucleic acids, and protein therapeutics.

837. Ethnographic Social World and Actor-Network Analysis of the Gene Therapy Commercialization Landscape

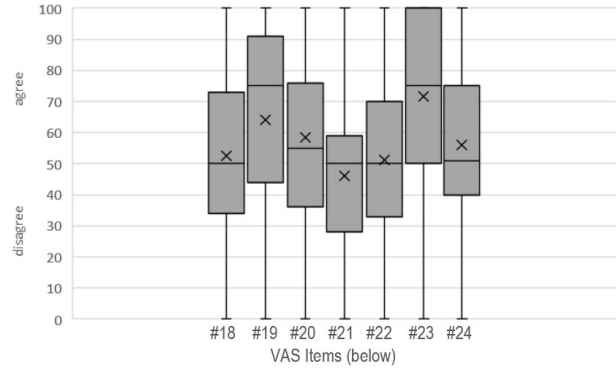
Angela Nicole Johnson

Regulatory Affairs, Northeastern University, Boston, MA

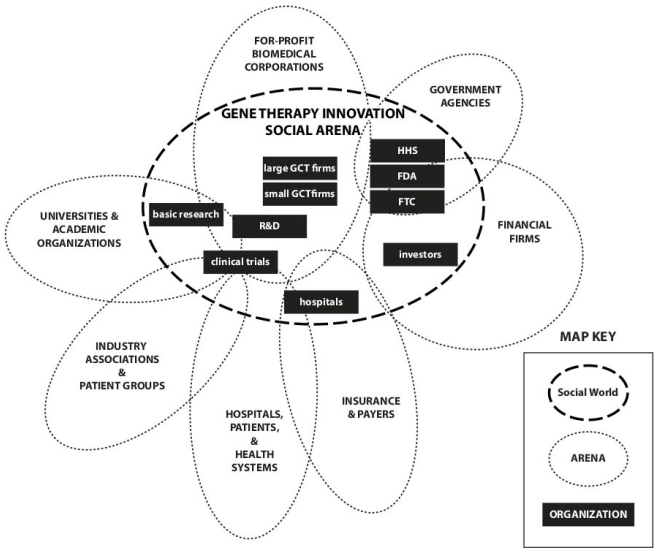
Background: Technological issues, dynamic global policy landscape, and a complex supply chain pose challenges to regulation and commercialization of gene therapies. As a field, systematic data on how organizations involved in gene therapy development collaborate are necessary to inform industry strategy, pedagogy, and policy-making. **Methods**: A total of 83 survey responses were collected across 7 countries, with balanced respondents from biopharmaceutical industry, private/governmental institutions, and health care organizations. Of these, 15 volunteers were selected (5 from each group) for further unstructured ethnographic interviews. Data on perceptions of intraorganizational communication in gene therapy were analyzed qualitatively and qualitatively (NVivo, QSR), and social world, actor-network maps were generated. **Results**: Results of visual analog scale (VAS) survey items were analyzed (Fig. 1; Table 1), showing that the majority of respondents outlines clear gaps in communication of governmental policy on gene therapy to industry leaders involved in its development and only 51% saw the United States as equal or more favorable for the development of gene therapies compared to other global regions. Scores showed that participants on average strongly agreed with the sentiments that small firms face more difficulties than larger firms due to regulatory and policy hurdles (#19) and that government policy is critical in helping biomedical innovations reach doctors and patients (#23). A series of visual social world, actor-network analysis maps were constructed based on the most commonly noted problematic social interfaces between organizations involved in gene therapy development (Fig. 2), of potential utility in mapping policy as well as training and pedagogical approaches to novel biopharmaceutical development. **Conclusions**: This research provides insights into interfaces between organizations engaged in commercialization of novel gene therapies, highlighting discrepancies based on organization size, region, and professional competencies. It also provides systematic data to support future policy, education, and industry initiatives aimed at facilitating commercialization in this dynamic field.

Table 1. Mean, SD, and Median for Survey Gene Therapy Policy Preferences on Visual Analog Scales

Item#	Mean	SD	Median
#18	52.51	25.80	50
#19	64.07	31.37	75
#20	58.35	27.80	55
#21	46.10	25.85	50
#22	51.16	26.53	50
#23	71.53	27.42	75
#24	55.93	26.32	51



18. The government does not do enough to help biomedical start-ups and product developers
19. The regulatory process for new products is harder for small firms than large pharmaceutical companies
20. Policy changes that affect my work are communicated within my organization so that those are affected understand the change(s)
21. Communication about policy changes between government/policymakers and investors/investment firms is clear and effective
22. Communication about policy changes between biomedical business leaders and investors/investment firms is clear and effective
23. The governmental biomedical policy is critical in helping innovative products reach everyday doctor and patients
24. Biomedical policy in my region is more friendly to new product development than in other countries and global regions



838. Efficient Lentiviral Vector Production in a Chemically Defined, Blood-Free and Serum-Free Medium, Scalable to the iCELLis® Technology

Sofia A. Pezoa[1], Randall Alfano[1], Atherly Pennybaker[1], Nathan Hazi[2], Odette Becheau[2]

[1]Invitria, Aurora, CO,[2]Pall Biotech, Westborough, MA

Retroviral vectors are a promising candidate for the treatment of rare, monogenic diseases. Lentivirus — a type of retrovirus based on HIV — is currently being clinically evaluated in stage 3 trials for the treatment of rare blood disorders in addition to the genetic modification of human T cells in oncology applications. While the efficacy looks promising in the clinic, numerous questions surrounding the feasibility of large-scale manufacturing of lentivirus remain. Traditionally, production of these retroviral vectors has been performed using adherent platforms that rely on the use of fetal bovine serum for the adherence and growth of HEK cells used to produce lentivirus vectors. At scale, fetal bovine serum presents numerous problems including but not limited to lot-to-lot variation, constraints on the global supply chain, and increasing cost due to global demand. To overcome these limitations, we have developed OptiPEAK• HEK serum-free, chemically defined cell culture medium that is free from any blood-derived proteins and supports adherent HEK cells in 2D and 3D formats. With OptiPEAK HEK cell culture medium, we are able to achieve equivalent growth kinetics and viral titer compared to medium supplemented with serum. We also demonstrate that OptiPEAK HEK medium can be readily scaled up to an iCELLis Nano bioreactor, achieving high viral titers without the addition of serum. The presented data here demonstrate that high titer, retroviral vectors can be manufactured without the constrictions brought on by the inclusion of serum in cell culture medium. •OptiPEAK is a trademark of InVitria

Vector Product Engineering, Development or Manufacturing

839. Development of a HEK293-Based Fully Stable, Helper Virus-Free ELEVECTA Production System for rAAV

Ben Hudjetz, Sabine Schmidt-Hertel, Tamara Grabeck, Nikola Strempel, Nicole Faust, Silke Wissing

CEVEC Pharmaceuticals GmbH, Köln, Germany

We and our colleagues have recently developed a stable helper-virus free rAAV production platform named ELEVECTA®. This AAV production platform consists of mammalian suspension cells which have stably integrated all components necessary to produce AAV. Initially, this platform was based on immortalized human amniocytes (CAP cells). However, the application of this unique stable helper virus-free AAV production method is not restricted to CAP cells but is also be applicable to other mammalian cell lines. In order to expand our portfolio, HEK293 cells were chosen for the generation of a second ELELVECTA AAV production platform. In the gene therapy field, HEK293 cells represent a widely used system for the production of rAAV-based viral vectors by transient transfection and the growing number of HEK293-derived products in the gene therapy market underlines the trust that is placed in this cell substrate. Adherent HEK293 cells were adapted to serum-free suspension growth in animal component-free and chemically defined culture medium. Subsequent single cell cloning of these newly generated suspension 293 cells resulted in a clonal cell line, which grows in single cell suspension mode to high density. Benchmarking experiments against competitor 293 cell lines revealed favorable rAAV production characteristics of this cell line in a fully transient production setting. For the generation of the suspension HEK293 ELEVECTA cell line, cells were modified to express AAV Rep as well as adenoviral helper functions under control of a Tet-inducible promotor system. Subsequently, single cell clones were generated, which displayed high titer rAAV production upon activation of the inducible promotor system and transient transfection of packaging as well as transgene elements. In a final step, a proof of concept 293 producer cell line was generated by genetically integrating capsid and transgene elements into the cellular genome.

840. Regulations and Science: Common Sense Approaches to Accelerating Patient Access to Novel Gene and Cell Therapies

Tanya M. Scharton-Kersten, Tirzah Cherian Pathicheril

Kersten Compliance Services, LLC, Dobbs Ferry, NY

New products plus predicate data equals faster product development for cell and gene therapies. Informatics related to Clinical Trial and Marketing Applications is an emerging, often overlooked tool in product development that can be used by all sponsors - academic, industry, non-profit to accelerate gene and cell therapy therapeutic interventions. In 2019 FDA anticipated that by 2020 it would be receiving more than 200 INDs per year and by 2025 would be approving 10 to 20 cell and gene therapy products a year based on an assessment of the current pipeline and the clinical success rates of these products

(1) . The use of converging technologies for different gene and cell therapy indications is an unprecedented opportunity for leveraging predicate data for clinical trial initiation and product licensure. Robust compilation of evidence-based data for safety, efficacy and quality facilitates patient access to therapeutic interventions. Sponsors that are not aware of predicate CMC, clinical and non-clinical data are at risk of performing unnecessary development activities, delay in timelines for concept to IND/CTA to BLA/MAA submission, inefficient use of sponsor and health authority resources and, most importantly, delay of patient access to new medicines. Example questions that can be readily addressed for most programs, by any SME in an organization include: How many trials were conducted to license product X, what country was the phase 1 trial conducted in, what cell line was the product manufactured in, what is the dosage form, what is the final container, what is the storage condition for drug product, how long can it be stored, what changes were made to the product during development, which changes required additional non-clinical or clinical studies? Strategies for compiling what is known and not known in the public domain for gene and cell therapies are available and accessible but may be underutilized due to lack of awareness of the databases and regulatory repositories available on the internet. Both the US and European regulatory agencies (FDA and EMA) provide public information on most aspects of the approval process for Gene Therapy, Cell Therapy and Vaccines in their public assessment reports (EPAR in Europe and SBAR in the US). Relevant details on the Phase 1, 2 and 3 clinical studies including the number of subjects and endpoints, a description of the manufacturing process, and the animal studies (safety, biodistribution and efficacy) are listed for each product with redaction of proprietary details. Issues encountered during product development, risks highlighted during the marketing application review, and commitments between the manufacturer-sponsor and the health authority are highlighted. Transcripts of advisory committee meetings where product risks are discussed with experts, the sponsor-manufacturer, and the health authority are provided in the US and provide rationales for reviewer concerns and thought processes during product application review. These health authority repositories complement the international clinical trial databases, company investor reports for public traded companies, grant agency repositories, and the growing number of scientific literature databases available through the internet. Example resources: FDA: https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products Europe: https://www.ema.europa.eu/en/medicines/field_ema_web_categories%25253Aname_field/Human/ema_group_types/ema_medicine_en (1) Statement from FDA Commissioner Scott Gottlieb, M.D. and Peter Marks, M.D., Ph.D., Director of the Center for Biologics Evaluation and Research on new policies to advance development of safe and effective cell and gene therapies (January 2019).

841. Transgene Protein Engineering for Increased Gene Therapy Efficiency

Benjamin J. Doranz, Kyle Doolan, Ross Chambers, Riley Payne, Trevor Barnes, Joseph Rucker
Integral Molecular, Philadelphia, PA

Transgene engineering has the potential to significantly advance the field of gene therapy by optimizing target expression, trafficking, functionality, stability, immunogenicity, and transgene size. Any one of these enhancements can boost efficacy and delivery efficiency by several fold, and combinations of these independent elements have the potential for logarithm increases. Transgenes are often multipass transmembrane proteins or other difficult proteins whose structural complexities make them challenging to engineer, such as ion channels, transporters, GPCRs, viral proteins, and enzymes. Here we present four different platforms that we have developed and used for engineering such proteins. These approaches use both proprietary and widely available engineering and screening approaches, including Shotgun Mutagenesis, phage display, Comprehensive Activity Mutagenesis, and FACS. Combined with proprietary algorithms and machine learning, thousands to billions of variants can be screened and selected for desired properties. We will discuss select case studies that have resulted in proteins with 10 to 1,000 fold improvements of desirable properties. We anticipate that these engineering strategies will significantly improve the success of gene therapy.

842. Meeting Market Needs with Scalable AAV Manufacturing

Giuliana Vallanti, Margherita Neri, Francesca Bellintani, Sylvia Ungari, Emanuele Simonetti, Veronica Meraviglia
AGC Biologics, Milan, Italy

Adeno Associated Virus (AAV) vectors are used in gene therapy for both *in-vivo* and *ex-vivo* applications. Because the application of AAV products is so diverse across indications and virus volumes needed, there is a market need for a platform offering that allows for manufacture at different scales. The availability of an efficient and scalable AAV vector production process is often a critical manufacturing challenge, for both the clinical and commercial phases. To address this, AGC Biologics is developing a platform for various scales (50L-500L) of AAV production with different serotypes (AAV-6; AAV-8; AAV-2; AAV-9), based on transient transfection in HEK293 and HEK293T cells. This will be achieved on an adherent cell line using Pall iCELLis® fixed-bed disposable bioreactors. The upstream process will be optimized by evaluating the producer cell line and identifying the best transfection setting, harvest and lysis strategy and timing for each step. The downstream process will consist of a capture step with affinity chromatography, followed by concentration and formulation steps based on tangential flow filtration (TFF). When needed, a polishing step will be added to increase the full to empty particle ratio, in accordance with the serotypes features. AGC Biologics will evaluate and present process development data, including potency and vector purity at each production step for the different serotypes to ensure quality standards. This ready-to-use platform will be extremely flexible, both in terms of AAV serotypes and Gene of Interest (GOI) and will be available for clients at scales ranging from 50L-500L.

843. Development and Scale Up of a Suspension Cell-Based AAV Manufacturing Process

Ahmed Youssef, Christina Weiss, Sarah Hanselka, Leena Heel, Jared Babic, Katja Kondratenko, Johanna Weizenegger, Regina Staffler, Melanie Langhauser, Karl Heller, Marcin Jankiewicz

Freeline, Stevenage, United Kingdom

We currently have two adeno-associated virus (AAV) gene therapy products in clinical trials, and two programs for which clinical candidates have been identified. For commercial-scale manufacturing of AAV gene therapies, we have developed an iCELLis-based manufacturing platform. Whilst this platform offers the advantages of adherent cell culture with high vector quality and potency, it requires the addition of serum to the culture medium. We have therefore started to develop a suspension cell manufacturing platform completely free of animal-derived components (ADC). Our major development objectives are to increase cell-specific yields and to reduce the payload of undesired DNA packaging in addition to maintaining vector quality and potency. We developed a fully single-use (SU) serum-free suspension USP platform based on the HEK293 cell family, capable of producing AAV of comparable quality and potency to the currently established adherent platform technology. This SU platform showed linear scalability up to 200L SU bioreactors, with comparable metabolic, cell growth, titer, impurity, and potency profiles. The advantages of this platform would include a reduction in cost of goods (COGs) and the elimination of ADC and related process variability, as well as a reduced risk of supply shortages. Secondly, suspension process implementation would decrease process complexity and increase flexibility for scaling up the process. Here we present the current status of the development of this platform.

844. Development of a Transfection-Based, Scalable, Serum-Free, Single-Use Suspension Platform for Manufacturing Lentiviral Vector

Pratima Cherukuri, Sara Bennett, Conrad Triebold, Saikat Chakraborty, William Vincent

Genezen Laboratories, Indianapolis, IN

Lentiviral vectors (LVV) are commonly used as an efficient and safe tool to deliver a transgene into dividing and nondividing cells enabling long-term gene expression. Production methods must be capable of making large quantities of high-quality functional LVV in an efficient and cost-effective way. The global supply of cGMP LVV is constrained due to the limited scalability of adherent HEK293T production systems. The industry is adapting lentiviral vector production to suspension-based cultures to meet large-scale clinical-grade vector production needs. Suspension cultures can be volumetrically increased to meet Phase-III and commercial needs from a single batch of production. In order to support LVV production in our upcoming GMP facility, Genezen has developed a HEK293T-based suspension cell line (GL293Ts) that is clonally selected to grow at high densities (1.5e7cells/mL) in suspension and serum-free conditions. Using transient transfection, GL293Ts produces titers above 1E7 transforming units per ml of culture and these titers have been observed in multiple suspension culture formats suggesting a robust cell line and transient transfection procedure. We have developed and optimized a process that is closed and compatible with cGMP production. Additional development of our closed downstream purification process and analytical platform to support our suspension platform will present our clients with a uniquely efficient solution for their LVV cGMP production and testing needs. To summarize, our GL293Ts cell line enables high-titer lentiviral vector production in adherence and serum-free conditions resulting in a highly efficient LVV manufacturing process.

845. Increasing Baculovirus/Sf9 Platform Productivity by Developing a Fed-Batch Production Process

Krishanu Mathur, Zeynep Guillemin, Charles Ly, Peter Slade

Voyager Therapeutics, Cambridge, MA

Accelerated development of novel therapeutic targets combined with demand for therapies that require high-dose systemic administration necessitates corresponding manufacturing processes that are robust and can provide a sufficient supply of gene therapy vectors with desirable quality attributes. One approach to meet increased rAAV demand is by increasing the productivity of current manufacturing platforms. The Baculovirus/Sf9 production platform has many advantages over mammalian systems for rAAV production, and yet it continues to be used in batch mode with little intensification since its introduction over 20 years ago. Cell culture medium is an important raw material that can significantly impact cell culture productivity. However, most commercially available media solutions cannot meet nutrient demands of high-density, Sf9 cell cultures, thereby limiting volumetric productivities. This poster highlights Voyager Therapeutics' efforts to develop a customized basal media formulation and feed concentrate to promote high-density Sf9 cell growth and increased AAV productivity. Voyager has identified specific nutrients which have a notable effect on culture performance. The Voyager cell culture group has also accelerated media development by using high-throughput multivariate study designs and optimization-focused statistical models. The new fed-batch strategy intensified Sf9 cell growth, baculovirus replication, and rAAV production. Work is continuing to further improve the process and to better characterize the effects of an intensified process on AAV product quality.

846. Ixaka Versatile Platform for the Bioproduction and Characterization of Lentiviral Vectors: From R&D Quality up to Pilot-Grade Batches

Thibaut Dempton, Estelle Zhang, Yvana Kouadio, Sarra Ezzine, Noemie Charron, Aurelie Perier, Carla Da Cunha, Sarah Duquesne, Amandine Chapot, Renaud Vaillant, Cecile Bauche

Ixaka, Villejuif, France

Recent market approval of CAR-T cell therapies and the continuously growing number of gene-based *ex vivo*and *in vivo* therapies evaluated in clinical trials has created a huge constraint on the supply of high quality grade lentiviral vectors (LV). This results in higher manufacturing

costs and longer timelines that are not sustainable for early-stage development programs.Ixaka has built up a versatile manufacturing platform for the manufacturing of high-quality grade LV material designed to cover customers' needs from R&D *in vitro* and *in vivo* evaluation. Our 350 m2 facility based in Paris employs 10 USP and DSP experts and offers manufacturing capacities in mammalian cell suspensions from 150mL to 3L of bulk produced with synthetic medium (no bovine serum albumin needed). The facility complies with the European regulations on the handling and use of genetically modified organisms. After two purification and concentration steps, the vector batches are sterile-filtered and stored at -80°C in adapted vials. Here we report improvements on process optimization and in process control on our routine process that allows the bioproduction of 60 mL drug product within 5 days with increasing physical titers and process handling. We succeed transferring the cell culture steps from Shake Flask to Single use, fully automated Bioreactors, enabling us to increase our production capacity from 3L to 6L of Bulk, and to implement in line parameter monitoring and process regulation. We developed a new intensified cell culture process further optimized with the use of small scale deep well and Design of Experiment enabling us to increase our physical viral titer 2-3 fold from 5.10^9 part.mL^{-1} to 2.10^{10}part.mL^{-1} (measured by RTqPCR). Adapted quality controls have been developed, analytically validated and implemented to ensure the characterization of the products. These controls are used to define identity, potency (p24, qPCR…), purity (HCP, residual DNA…), safety (standard sterility tests, endotoxin, mycoplasma…), and stability (pH, osmolality, aggregates…) of the products. In addition, we have implemented biophysical methods compatible with in-process and in line control (Biacore, Videodrop, Metabolites monitoring, turbidity, pressure, etc.) that allow a tight management of operations and bioproduction costs. With the implementation of new in process controls and in line parameter monitoring we will be able to increase process automation (Fed batch implementation, CPP monitoring), reliability and batch to batch comparability. With the input of an internal Quality Assurance service, we are able to constantly improve our bioproduction process, enable a process control strategy driven by Quality by Design approach and enhance product safety, quality and efficacy. Gearing up for clinical GMP manufacturing. Ixaka is thus able to provide an integrated solution to support biotech companies and academic laboratories throughout the development of their disruptive gene therapy programs.

847. Expression of AAV Rep78 and Rep52 from Distal Baculovirus loci for Production of Gene Therapy Vectors: Proof-of-Concept and Initial Optimization

Christopher C. Nguyen, Zeynep M. Guillemin, Amanda Ibe-Enwo, Jeffrey M. Slack, Peter G. Slade

TechOps, Voyager Therapeutics, Inc, Lexington, MA

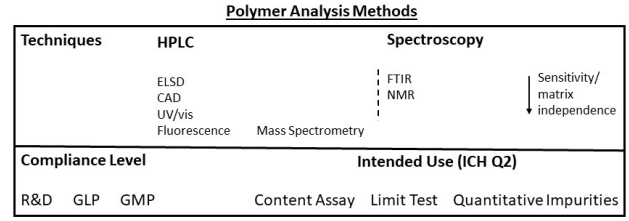
Recombinant adeno-associated virus (rAAV) vectors are a promising mode of therapeutic DNA delivery with desirable characteristics for patient safety. However, large-scale manufacturing of rAAV remains challenging due to the complexity of these vectors relative to other biologic therapies. Sf9/baculovirus-based production of rAAV requires expression of AAV replicase proteins Rep78 and Rep52 for replication and packaging of transgene DNA into transduction-competent rAAV particles. Conventional Rep expression strategies involve the use of a devoted baculovirus vector, dual head-to-tail expression cassettes, or bicistronic Rep78/52 designs. Voyager explored an alternative Rep expression strategy involving the distal placement of two cassettes in the same baculovirus vector - one cassette at a first locus expressing Rep78 and the other cassette at a second locus expressing Rep52. We successfully generated transduction-competent rAAV using this baculovirus vector. Voyager also explored upstream modifications which can moderate the suppression of Rep78 levels to enhance rAAV yield, potency, and percent-full capsid amounts. These alternative designs provide opportunities for transcriptional and translational control of Rep78 and Rep52, while also placing these similar sequences at distal loci to reduce the rate of recombination.

848. Analysis of Polymeric Excipients and Process Residuals in AAV Gene Vector Products

Andrew Hanneman, Marc Plante, Matthew Costa, Pranitha Naiki, Jennifer Ricardo, Joseph Pearce

Biologics Testing Solutions, Charles River Laboratories, Shrewsbury, MA

Various polymeric excipients and process residuals in Adeno Associated Virus (AAV) drug products, potentially present at very low-levels, require advanced analytical test methods to confidently establish their presence and content in AAV in-process samples and final drug product. HPLC methods utilizing detection by UV/Vis, fluorescence, CAD, and ELSD are employed. Various spectroscopies are also presented, providing data required to meet Method Validation parameters supporting Content and Impurities assays. Requirements for the analytical methods applied to polymeric analytes used in AAV production, many lacking UV-chromophores, are discussed in the context of adaptability to variable matrices, and achieving robustness where establishing low limits of quantification (LOQs) is a principal challenge. Among the methods presented include HPLC approaches to measure residual polyethyleneimines (PEI), both linear and branched, antifoaming agents including simethicone, Antifoam 204 and polypropylene glycol (PPG), as well as Triton-X and pluronics which are used as AAV excipients. Specific challenges discussed are selection of the appropriate HPLC stationary phase, detection modes, and sample treatments needed for various analytes in different matrices, including options for on-line sample cleanup. The approaches developed and applied are placed within the context of low AAV sample availability and the need for method optimization and pre-validation steps prior to Method Validation in a GMP environment based on the Intended Use of each method according to the ICH Q2(R1) guidance criteria.



849. Purification of Novel Synthetic AAV Vectors and Library Preparations by Capture Affinity Chromatography

Simon Pacouret, Ken Y. Chan, Qingxia Zheng, Pam Brauer, Albert T. Chen, Helen Huang, Thomas Beddow, Benjamin E. Deverman

Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA

Adeno-associated virus (AAV) vectors are moving to the market as therapeutics for intractable genetic diseases, as illustrated by the recent approvals of Zolgensma and Luxturna. In the years to come, high-throughput screening of AAV capsid libraries in relevant models will yield novel synthetic gene therapy vectors with improved safety and efficacy profiles. AAV capsid libraries are routinely produced in adherent HEK293 cells and purified by cesium chloride or iodixanol gradient ultracentrifugation. This purification approach is agnostic to structural variant and serotype, but is not scalable, and hence is impractical in the context of AAV library screening in large animal models. In this work, we investigate an alternative purification pipeline for novel synthetic serotypes and AAV libraries, based on depth filtration and POROS AAVX or AAV9 capture affinity chromatography. First, we demonstrate that this approach is suitable for the purification of various synthetic variants engineered via peptide display. Second, we compare this method side-by-side with iodixanol gradient ultracentrifugation, for the purification of a novel synthetic, AAV variant capable of widespread central nervous system transduction. Following titration by a ddPCR assay, calibrated using the Reference Standard Material serotype 8 (RSM8), we show that both preparations have comparable purity, thermostability and in vivo transduction profiles. Last, we use this pipeline to purify an AAV library of 40k variants and show by NGS that the AAV capsid variant distribution in the purified product is comparable to that of (1) the clarified lysate and (2) the same library purified by iodixanol gradient ultracentrifugation. The scalability and limits of this alternative purification pipeline will be discussed.

850. Impacts of Storage Conditions on rAAV Vector Stability: Addressing Challenges towards Clinical Practice

Tierra Bobo[1], Preston Samowitz[1], Michael Robinson[2], Cesar Pelaez[1], Xinghua Zeng[3], Haiyan Fu[1]

[1]Gene Therapy Center, Department of Pediatrics, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Gene Therapy Center, Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC,[3]Vector Core, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC

As rAAV gene therapy products advance toward routine treatment of diseases in humans, new issues are emerging as potential critical challenges to clinical practice. This study addresses concerns over whether rAAV vectors are stable after long-term storage under different temperature conditions. An AAV vector product was divided into 200µl aliquots in airtight tubes, and 10 aliquots each were stored at 4°C, -80°C, and room temperature (RT). The vector product was titered by qPCR, assayed by transmission electron microscopy (TEM), and tested *in vivo* for biopotency in C57BL/6 mice, prior to and at 3, 6, 12 and 18 months storage. At each time point, mice were treated with an IV injection of 1e13vg/kg rAAV vector (n=3-6/group). At 1wk pi, liver tissue samples were assayed by qPCR for vector genome (vg) copy numbers. Data at different storage times were compared to values from samples prior to the storage. Interestingly, the results showed no significant changes in liver vg levels in mice treated with the vector stored at 4°C through the entire 18m study duration. In mice receiving the vector stored at -80°C, the liver vg copy numbers were reduced approximately 2-fold at each time point compared with the pre-frozen sample, but with no significant differences during 3m - 18m storage time. In contrast, there was an approximately 10-fold decrease in liver vg copies in mice treated with vector stored at RT for just 3m. Notably, the results showed no significant differences in the vector titer or morphology over time during this study. These data demonstrate the optimal temperature for rAAV vector storage is 4°C, at which rAAV vectors remain potent for at least 18m. While rAAV vectors appear to be stable at -80°C, a single freeze-thaw cycle reduces the biopotency by 2-fold. This study offers important information in facilitating rAAV vector storage and may ease the burden in transportation and storage of clinical vectors for end users.

851. Next-Generation Transfection Reagent for Large Large Scale AAV Manufacturing

Mathieu Porte

Polyplus-Transfection, Illkirch-Graffenstaden, France

The number of ATMP therapeutic-based medicines for inherited genetic disorders is in constant growth, with a global 32% increase in new clinical trials in the last 4 years. ATMPs have demonstrated their success with already more than ten approved for commercialization. The success of AAV as the most promising viral vector for gene therapy is due to low immunogenicity, broad tropism and non-integrating properties. One major challenge for translation of promising research to clinical development is the manufacture of sufficient quantities of AAV. Transient transfection of suspension cells is the most commonly used production platform, as it offers significant flexibility for cell and gene therapy development. However, this method shows some limitations in large scale bioreactors: inadequate transfection protocol, reduced transfection efficiency and lower productivity. To address this concern, we present data on the novel transfection reagent showing: *i)* increased AAV titers, *ii)* improved transfection protocol for large scale bioreactors and *iii)* reproducibility of viral titers at different production scale. The aforementioned optimized parameters make this novel transfection reagent ideal for cell and gene therapy developers by combining the flexibility of transient transfection with scalability and speed to market.

852. Improving AAV Productivity by Transient Transfection by Implementing Small Molecule Medium Additives

Jenny Shupe

Biogen, Cambridge, MA

Recombinant adeno-associated viral vectors (rAAV) are the vehicle of choice for therapeutic gene delivery in the gene therapy field. The transient transfection method of production remains one of the most attractive processes for the manufacture of AAV due to its fast-

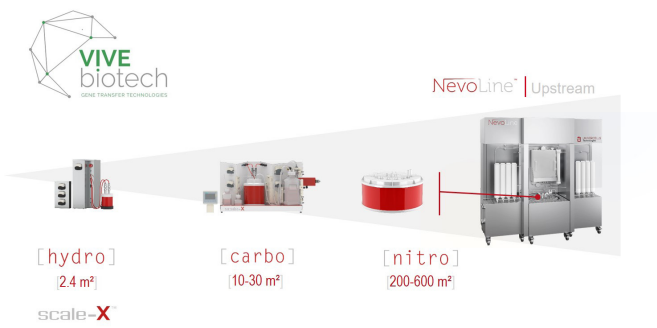
turnaround and versatility towards the production of a wide-range of rAAV constructs. To meet the increasing drug substance demand, further improvement on productivity has been one of the primary focuses of process development. Multiple small molecule medium additives were screened and several compounds demonstrated significant improvement on productivity. The favorable effects of these medium additives have been confirmed in the lab scale as well as manufacturing scale bioreactors. The fundamental mechanisms of the leading compounds were also studied.

853. Lentiviral Vector Manufacturing: Success in Scaling up to Various Batch Sizes Using Univercells Technologies' Production System

Filipe Cristóvão[1], Juan Carlos Muñoz[1], María José Dávila[1], Alex Chatel[2], Gonzalo Mesado[1]

[1]USP Development, VIVEbiotech, San Sebastian, Spain,[2]Univercells Technologies, Nivelles, Belgium

VIVEbiotech is a GMP CDMO specialized in lentiviral vector (LVV) manufacturing meeting both EMA´s and FDA´s standards. This abstract herein is specifically based on the LVV production by using adherent HEK293T cells growing in fixed bed bioreactors for clients in EU, US, Asia and Australia, some of them already demanding large virus quantities. For being able to meet this exponentially growing market demand, VIVEbiotech has started to scale-up its production process using Univercells Technologies' production systems which presents great advantages such as the availability of a wide range of scales from 2.4 sqm to 600 sqm cell growth surface. This allows easily adapting the process to the different phases a LVV may require. In this work the results for 2.4 sqm to 30 sqm scales are shown, in which very good results considering both product quantity and/or quality can be observed. A LVV codifying for green florescent protein (GFP) was produced, and quantities were determined by measuring p24 protein concentration by ELISA, and by quantifying transfection units (TU) by flow cytometry. Additionally, the performance (viral particles/TU) of each batch was analyzed (viral particle = p24 x 1,25x10E07). The results show that the levels of p24, TU and performance for the 2.4 sqm, 10 sqm and 30 sqm scales are highly comparable demonstrating therefore a robust scalability of the process. These results are very relevant for VIVEbiotech´s coming scale up using the biggest version of reactor available in the market, the scale-X nitro integrated in the NevoLine Upstream platform. VIVEbiotech has been the first company using Univercells Technologies´ reactors for LVV GMP manufacturing which is dedicatedly working in the scale-up of its process up to commercial scales.



VIVEbiotech's USP manufacturing platform

854. Scale up of a Lentiviral Production Process from the iCELLis® Nano Bioreactor to the iCELLis 500 + Bioreactor

Isabelle Pelletier[1], Volga Pasupuleti[1], Pragati Agnihotri[1], Young Do[1], Ziv Sandalon[1], Kaitlynn Bayne[2], Odette Becheau[2], Nathan Hazi[2]

[1]Advanced BioScience Laboratories, Rockville, MD,[2]Pall Biotech, Westborough, MA

Lentivirus is a type of retrovirus that has a unique ability to infect non-dividing cells, giving it the potential to be used in a wide range of applications. Traditional adherent methods of growing cells and producing lentivirus are cumbersome at the large scales required to obtain enough doses for patients. In addition, traditional flatware methods often have open handling steps that increase the likelihood of contaminations. The iCELLis bioreactor technology addresses the need for a scalable adherent system that has process controls and is closed, reducing the risk contamination risk from open handling. Further, by keeping factors such as linear speed, perfusion rates, fixed bed height, and fixed bed compaction constant, processes can be scaled from the bench-scale iCELLis Nano bioreactor to the large-scale iCELLis 500 + bioreactor. The data presented here shows that a process producing lentivirus was effectively scaled from a 0.53 m^2 iCELLis Nano bioreactor to the 66 m^2 iCELLis 500 + bioreactor. Utilizing perfusion, the iCELLis Nano achieved a titer of $1.01x10^8$ copies/cm^2 and the iCELLis 500 + achieved a titer of $3.47x10^8$ copies/cm^2, showing that an efficient large-scale process can be adapted quickly using the iCELLis fixed bed bioreactor technology.

855. AAV Xpress ELISAs - Finish It Faster

Hueseyin Besir, Dana Holzinger, Iris Queitsch, Katharina Hammer, Caroline Odenwald, Katja Betts

PROGEN Biotechnik GmbH, Heidelberg, Germany

A growing number of academic and industrial labs are using AAV vectors for the development of gene therapies. This leads to an increase in the demand for effective and reliable analytical AAV tools for R&D and manufacturing. To enable safe and effective AAV gene therapies, a dependable and reproducible quantification of accurate rAAV titers is needed to ensure secure and reliable gene transfer. PROGEN's AAV ELISAs are widely used for the quantification of intact AAV capsids, thus to determine the total capsid titer including full & empty capsids.

In combination with additional quantification methods, characterizing different AAV titers (e.g. genomic titer), PROGEN´s AAV ELISAs are a robust and reliable tool for the comprehensive characterization of AAV preparations. In order to reduce the assay time from 4-5 hours (standard AAV ELISA) to less than 2 hours, we have adjusted the kit components and the protocols for the ELISAs for AAV2, AAV8 and AAV9. The corresponding adjustments resulted in the shortening of the incubation steps from 60 to 20 minutes each, thus leading to a reduction of the assay time of more than 50%. This was achieved by adapting reagent concentrations without changing the composition of the kit's core characteristics, allowing a seamless and risk-free transition of users from the standard AAV ELISAs to the Xpress version. Here, we demonstrate that these Xpress ELISAs show no significant differences in accuracy and matrix effects, reproducibility and recovery as the corresponding standard titration ELISA kits but save a significant amount of time for the users. With these faster AAV ELISAs, users can rely on the well-established quality of PROGEN's reagents for accurate titer determination of AAV samples.

856. Characterizing the Impact of Shear on SF9 Cells Used in the Baculovirus Expression System for Recombinant AAV Gene Therapy Vector Production

Harvir Grewal, Christian Gagnon, Shamik Sharma, Peter Slade

Cell Culture Development, Voyager Therapeutics, Lexington, MA

The Sf9 insect cell-baculovirus expression system is a highly productive platform for the manufacture of recombinant adeno-associated viruses (rAAV) for gene therapy. Productivities can be further increased by increasing manufacturing cell densities. To meet the increased oxygen demand of a high cell density process and to ensure consistent high productivity as the process is scaled up, the impact of shear on Sf9 host cells needs to be considered. The negative impact of shear stress on Sf9 cultures can be observed in growth and productivity differences between low shear shake flask cultivation and higher shear sparged stirred-tank bioreactor cultivation. Two main sources of shear in suspension culture are mechanical shear and bubble shear in bioreactors. To understand and mitigate the negative impact of shear, a well characterized small scale bioreactor model was developed. Initially, theoretical models for estimating mechanical shear stress were created and experimentally verified to identify the mechanical shear limit for Sf9 cells. Following this, the impact of mechanical and bubble shear was investigated by separately varying agitator configuration as well as bubble size. Results showed that bubble shear had a greater effect on cell growth and productivity compared to mechanical shear. Further studies were completed to provide improved concentrations of shear protectant for the desired agitation and sparger configuration, so that oxygen demand of higher density cultures could be met without incurring the harmful effects of bubble shear. Study results were then incorporated into a final scale down model that mimicked cell growth observed in a low shear environment while offering the benefits of operating in a stirred tank configuration that can be more readily scaled up.

857. Application of Aber's FUTURA⬥ Biomass Probes to Inform Transfection and Cell Lysis in iCELLis® Bioreactor-Based AAV Manufacturing

Randall Alfano[1], Sofia A. Pezoa[1], Atherly Pennybaker[1], Nathan Hazi[2], Odette Becheau[2]

[1]Invitria, Aurora, CO,[2]Pall Biotech, Westborough, MA

Utilization of classical adherent formats in large scale viral vector manufacturing can have significant setbacks due to the lack of scalability of production vessels typically used at small scale. The iCELLis fixed bed bioreactor has emerged as an enabling technology to efficiently scale adherent-based processes in a controlled and highly integrated environment. This technology has been developed for the clinical manufacture of lentiviral and adeno-associated vectors with commercially viable yields. Aber's FUTURA Biomass probes, which induce polarization of cells and measures the resulting capacitance of the medium in pF/cm, can be employed with the iCELLis bioreactor to provide direct online information on cell biomass during a viral vector production run. Routine utilization of these probes can provide invaluable online information regarding cell growth and health, control of feed/perfusion rate, and the identification of optimal time for transfection or harvest. Here, we utilized Aber's FUTURA Biomass probes in iCELLis bioreactor runs to produce an AAV-2 GFP vector in OptiPEAK⬥ HEK293t chemically defined media, using different feeding strategies and harvest protocols of the bioreactor. Data obtained from the biomass probes are correlated with overall functional vector titer to identify ideal capacitance trends that are predictive of bioreactor productivity. Taken together, these data suggest the Aber's FUTURA Biomass probes can be used to identify ideal capacitance ranges to commence major unit operations of the manufacturing process to maximize vector yields. ⬥FUTURA is a trademark of Aber Instruments Ltd and OptiPEAK is a trademark of InVitria

Cell Therapy Product Engineering, Development or Manufacturing

858. Rigorous Assessment of Off-Target Editing by CRISPR/Cas9 in VOR33, an Engineered Hematopoietic Stem Cell Transplant for the Treatment of Acute Myeloid Leukemia

Dane Hazelbaker, Meltem Isik, Azita Ghodssi, Matthew Ung, Amanda Halfond, Shu Wang, Kit Cummins, Gabriella Angelini, Gabriela Zarraga-Granados, Julia Etchin, Brent Morse, Sadik Kassim, John Lydeard, Gary Ge, Elizabeth Paik, Tirtha Chakraborty

Vor Biopharma, Cambridge, MA

Introduction: VOR33 is an engineered allogeneic hematopoietic stem cell transplant for treatment of AML in which the CD33 surface antigen is removed by CRISPR/Cas9 gene editing. This removal

enables post-engraftment immunotherapeutic targeting of leukemic cells that display CD33 while sparing the *CD33* gene-edited graft (Borot et al. 2019; Humbert et al. 2019; Kim et al. 2018). To ensure safety of gene-edited CD34+ hematopoietic stem and progenitor cells (HSPCs), off-target editing must be minimized. However, established paradigms for off-target analyses of gene-edited *ex vivo* therapies are not well defined. Here, we used an assay ensemble to enable rigorous assessment of unintended and off-target events by CRISPR/Cas9. **Methods:** Assays of unintended and off-target events (>2300 genomic sites) were performed, including assessment of on-target structural variants (SV) such as large (>50 bp) insertions and deletions (indels), and inversions. To identify on-target SVs that may potentially impact post-engraftment safety and efficacy of VOR33, long-range PCR of a 10-kb region of CD33 was performed, followed by long-read sequencing. For *in silico* prediction, sites containing a 5'-NRG-3' PAM and (i) up to 5 mismatches with no indels, or (ii) up to 3 mismatches and a one-base insertion, or (iii) up to 3 mismatches and a one-base deletion were predicted via a pipeline based on Cas-OFFinder (Bae et al. 2014). For unbiased query of off-target sites, GUIDE-seq was performed on 4 VOR33 batches manufactured at research-scale, followed by hybrid capture-based NGS of 7 batches manufactured at clinical-scale. To evaluate the genomic stability of VOR33, surveillance of gross chromosomal abnormalities by G-banded karyotyping was performed. **Results:** Analysis of on-target SVs by long-read sequencing revealed total SV frequencies of 7-9% across 3 VOR33 research-scale batches. Large deletions accounted for the majority of events, while inversions and large insertions were detected at very low frequencies, similar to those reported for CRISPR/Cas9 (Kosicki et al. 2018). Fine mapping of the SVs suggests they have no perceivable impact on the safety or efficacy of VOR33, as the primary mechanism of action by CD33 disruption is preserved. Through *in silico* prediction and GUIDE-seq on 4 research-scale batches, a total of 29 sites were identified, with 10 showing high homology to the on-target site (≤5 mismatch/gaps). The remaining 19 sites had moderate/poor homology (≥7 mismatch/gaps). In 7 VOR33 batches manufactured at clinical-scale, indel frequencies were assessed by NGS at 2369 *in silico* and GUIDE-seq identified sites (reads ≥500). Across batches, no significant and reproducible off-target sites were observed.Importantly, our findings show the VOR33 manufacturing process is robust, with no discernable differences in off-target frequencies or patterns in batches generated using multiple guide RNAs and manufacturing scales, or cell delivery methods. Lastly, karyotyping revealed no detectable abnormalities across multiple batches, indicating VOR33 displays preserved genomic stability. **Conclusion:** Through karyotyping, long-read on-target sequencing, and quantification of indel frequencies, we achieved an expansive appraisal of off-target events across multiple VOR33 products. This assessment of off-target editing by CRISPR/Cas9 establishes a rigorous and clinically applicable framework to evaluate off-target events in CD34+ HSPC-based cell therapies.

859. Abstract Withdrawn

860. *Analyzing the Effectiveness of CD8-Receptor Targeted Transduction for CAR T Cell Therapy Using Single Cell Transcriptomics*

Filippos T. Charitidis[1], Elham Adabi[1], Frederic B. Thalheimer[1], Csaba Miskey[2], Colin Clarke[3], Christian J. Buchholz[1]

[1]Molecular Biotechnology and Gene Therapy, Paul Ehrlich Institut, Langen, Germany,[2]Medical Biotechnology, Paul Ehrlich Institut, Langen, Germany,[3]Systems Biology Research Group, National Institute for Bioprocessing Research and Training, Dublin, Ireland

The development of chimeric antigen receptor (CAR) T cell therapies has led to improved outcomes for a number of indications, particularly B cell malignancies. However, the complicated and multifactorial CAR T cell manufacturing process involving gene delivery often by lentiviral vectors (LVs) can potentially affect clinical efficacy and emerge issues ranging from compromised product quality to post-transfusion side effects. In this study (funded by H2020 Agreement No. 813453 - STACCATO), we investigated transcriptomic changes associated with the manufacture of CD19-targeted CAR T cell products via single cell RNA sequencing (scRNA-seq). We performed targeted scRNA-seq using the BD Rhapsody system to enrich for a panel of genes involved in immune response. In addition, we designed primers for the woodchuck hepatitis virus post-transcriptional regulatory element (*WPRE*) residing at the 3'-end of the CAR transgene mRNA to permit the identification of individual vector-exposed cells. For this experiment we incubated human PBMCs with either non-cell-specific VSVG pseudotyped LV or with a previously described CD8-targeted LV (CD8-LV) for 6 days. Untransduced PBMCs were used as negative control. Following single cell isolation, library preparation and Illumina sequencing, the raw reads were preprocessed, demultiplexed and mapped using the BD Rhapsody bioinformatics pipeline to produce a cell-gene UMI (unique molecular index) count matrix. Low quality cells were removed and the final cell-gene count matrix comprised of 400 genes across 21,344 cells. The portion of transduced CAR T cells identified by the expression of *WPRE* in scRNA-seq was found to be in agreement with flow cytometry analysis. Furthermore, the CD8-LV was proved to have excellent selectivity for CD8+ T cells (confirmed via the expression of *CD8A* mRNA) demonstrating the utility of scRNA-seq for evaluating the vector specificity. Next, we conducted a global analysis of the gene expression data using uniform manifold approximation and projection (UMAP) method in the Seurat R package. The 2D UMAP visualization revealed the presence of multiple cell clusters, as expected from such a complex cell therapy product. B cells characterized by *CD20* gene expression were observed only in the untransduced sample, while the CAR T cells in the transduced samples had completely eradicated any remaining B cells. Differential gene expression analysis revealed significant differences in expression profiles (WRS test, log2(FC) +/- 0.25; adjusted p-value < 0.05) between cell populations that were either transduced with VSVG-LV or CD8-LV (CD8+CAR+) either non-transduced cells (CD8+CAR-) or untransduced negative control. Genes associated with proliferation, activation, cell exhaustion, regulatory genes of TCR signaling and inhibitors of apoptosis were found to be increased in expression in transduced CD8+CAR+ cells, while genes associated with negative regulation of T cell activation and proliferation, along with IFN-induced and pro-apoptotic genes, were found to be upregulated in non-transduced CD8+CAR- cells,

suggesting gene profile alterations and the activation of intrinsic mechanisms that possibly influence the transduction efficiency of LVs. The findings of this study demonstrate the potential of scRNA-seq to enable comprehensive characterization of CAR T cells, identification of genes and key pathways for product development as well as confirming the specificity of a receptor-targeted LV.

861. Monitoring the Variations of Critical Components in T-Cell and HEK293 Media

Ji Young L. Anderson, Kerin E. Gregory, Glenn A. Harris

Life Sciences, 908 Devices Inc., Boston, MA

Cell culture groups manage hundreds of bioreactors and cell media formulations throughout a project. Many samples and vessel volume restrictions slow down the ability to get timely and useful process analytics. Here, we show how a new integrated at-line cell media analyzer (the REBEL) assists with cell media analysis for cell therapy and viral vector production. The analysis is label-free, supporting high-throughput media analysis while only requiring approximately 10 microliters of sample. Each run simultaneously assays over 30 standard cell media nutrients and metabolites like amino acids, biogenic amines, and vitamins. With the impressive growth of cell and gene therapy research, particularly T-cell-based immunotherapies, many organizations are investigating how to boost T-cells' cultivation and expansion. Classically, T-cells have been cultured in cell media like RPMI with serum. For clinical applications, there is a desire to use cell media that allow for higher expansion rates, have lot-to-lot consistency, and exclude animal-derived components. We've observed that various off-the-shelf T-cell media vary considerably in their nutrient profiles compared to the standard RPMI+10% serum formulation. Our analysis found that the traditional RPMI+10% serum and a serum-free chemically-defined base media had the lowest levels of the essential amino acids of the five media evaluated. Additionally, we identified that the conventional RPMI media with serum had anywhere between 2 to 3-times more arginine than all the chemically-defined media types tested in this study. This is exceptionally relevant for T-cells since various research teams have identified the importance of arginine for growth and efficacy. We extended this analysis to HEK293 media used for viral vector production. With a long history of stable growth and easy transfection, HEK293 cells have been a workhorse cell line in cell biology and bioprocessing labs. Therefore, there has been a desire to improve both process productivities and consistencies of HEK293 cells grown in serum-supplemented media. When evaluated, there was a high variation in the fresh medias' nutrient composition for four vendors' serum-free media used for HEK293 growth. For instance, the dipeptide Ala-Gln was only detected in one media, whereas hydroxyproline was only detected in another media. Asparagine was 25x higher in one vendor's media compared to the next highest composition. This media also had the highest concentration of roughly two-thirds of all the detected components in the study. In contrast, another vendor's media had the lowest concentration of over half of the components. In its entirety, researchers can utilize this type of quick media screen to assess cell media options before investing in a particular platform for their cell therapy and viral vector processes.

862. The Manufacturing Process for Lisocabtagene Maraleucel (liso-cel) Enables Robust Clearance of Non-T Cell Impurities across B-Cell Malignancies

Jeffrey J. Teoh, Wissam Charab, Kurt Van Gunst, Sara Cooper, Calvin Chan, John Wesner, Ryan P. Larson

Bristol Myers Squibb, Seattle, WA

Introduction: Liso-cel is an investigational, CD19-directed, defined composition, 4-1BB CAR T cell product administered at equal target doses of $CD8^+$ and $CD4^+$ CAR^+ T cells for the treatment of multiple B cell malignancies (Abramson et al. *Lancet*. 2019). Variance in patient age, prior therapies, and baseline hematologic parameters leads to heterogeneous cellular composition of patient starting material, and the manufacturing of individual patient lots may introduce the potential risk of non-T cell product-related impurities in the CAR T cell product. Here, we describe strategies employed in the liso-cel manufacturing process to generate a highly pure T cell product across diverse patient populations and disease indications. **Methods:** $CD8^+$ and $CD4^+$ CAR^+ T cells were isolated from leukapheresis material using immunomagnetic selection, and each T cell lineage was separately activated, transduced, expanded, formulated, and cryopreserved as independent $CD8^+$ and $CD4^+$ CAR^+ T cell components. Cellular phenotypes and compositions of the leukapheresis, postselection T cell material, and final CAR^+ T cell product components were assessed using flow cytometry. All cell populations are reported as frequencies of viable $CD45^+$ leukocytes. Cell phenotypes are shown for lots manufactured in support of NCT02631044 and NCT03484702 (NHL) and NCT03331198 (CLL) clinical studies, with characterization data for paired leukapheresis, postselection T cells, and CAR^+ T cell product (N = 486 lots). **Results:** Selecting for $CD8^+$ and $CD4^+$ T cells early in the liso-cel manufacturing process reduced the variability of $CD3^+$ T cell frequencies, relative to leukapheresis, before activation and transduction. The median $CD3^+$ T cell frequency of postselection T cell material was 98.3% of viable leukocytes for NHL (minimum, 62.3%) and 99.2% for CLL/SLL (minimum, 81.0%). The frequency of $CD19^+$ B cells was significantly reduced through the selection process in both NHL and CLL, with median values below the limit of quantitation (LOQ, 0.2%). Non-T cell populations present in postselection T cell material that exhibited <99% $CD3^+$ T cell purity were predominantly $CD3^-CD56^+$ natural killer (NK) cells and monocytes. $CD3^+$ T cell composition was increased with ≥99% $CD3^+$ T cell purity of viable leukocytes exhibited in the final drug product for 426 of 430 NHL lots (minimum, 94.2%) and 56 of 56 CLL/SLL lots (minimum, 99.2%). Cell characterization strategies were used to confirm removal of non-T cell cellular impurities in the CAR T cell product, including B cells, monocytes, NK cells, invariant NK T cells, hematopoietic progenitor cells, granulocytes, and dendritic cells. In the 4 NHL cases where $CD3^+$ T cell purity was <99% in the CAR T cell product, non-T cell populations were confirmed to be NK cells (maximum frequency, 3.8%). All other non-T cell drug product-related impurities were removed to levels below LOQ. **Conclusions:** Despite substantial heterogeneity of leukapheresis material among patients and across B-cell malignancies, the liso-cel manufacturing process demonstrates a consistently purified $CD3^+$ T cell drug product. The purification of T cells from leukapheresis material substantially reduces the amount of non-T cells that advance through the manufacturing

process. Activation and transduction steps were optimized for specific transduction of T cells, which further reduces the potential of transducing unintended cell types. Analytical methods for screening non-T cell impurities have consistently confirmed process clearance of non-T cells below LOQ in the CAR T cell product. Together, these data demonstrate the robustness of the liso-cel manufacturing process.

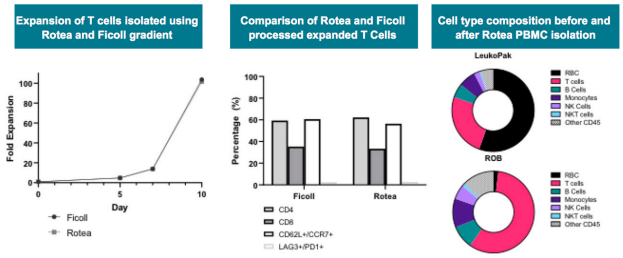
863. Building New Cell Therapy Workflow Solutions by Leveraging the Flexibility of Rotea's Closed Automated Cell Processing System

Carl Dargitz, Steffany Dunn, Sarah Daoudi, Xavier de Mollerat du Jeu, Namritha Ravinder

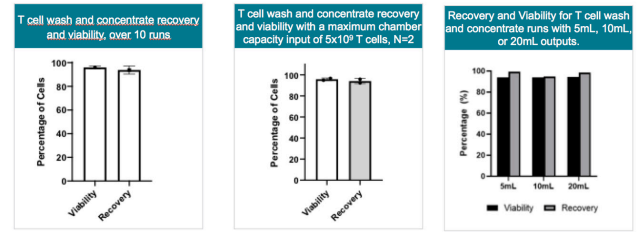
Cell Biology, Cell & Gene Therapy, Thermo Fisher Scientific, Carlsbad, CA

The Rotea Counterflow Centrifugation System has the ability to revolutionize cell therapy workflows by providing automated, closed system solutions. PBMC isolation and T Cell wash and concentrate are the core capabilities for Rotea. These features make it suitable for cell therapy workflows during multiple steps in the process including initial PBMC isolation from leukopak as previously mentioned, buffer exchange in preparation for electroporation, and media or other buffer exchange or concentration throughout the process. Beyond these processes the flexibility of the system allows for a myriad of additional applications with user programmable protocols and adjustable workflow set-ups. Recently, we have shown Rotea's ability to cryopreserve T cells by concentrating and exchanging growth media with cryopreservation media without significant loss of cells post-processing and post-freeze-thaw, providing utility in yet another step within traditional cell therapy workflows. Additionally, we have explored NK cell wash and concentrate while maintaining greater than 80% recovery, high viability, and maintenance of phenotype. We have also experimented with alternative methods for PBMC isolations using Rotea as well alternative starting material for initial cell isolations . As these new data and show, the versatility of Rotea will continue to be a vital tool in the cell therapy developer and producer's tool belt.

PBMC Isolation Characterization & Ficoll Comparison



T Cell Wash and Concentrate and Input and Output Specifications



864. Analytical Development Considerations for the Shielded Living Therapeutics™ Platform: A Novel Encapsulated Cell-Based, Non-Viral Platform for Delivery of Therapeutic Proteins

Zoe Yin, Martha Rook

Sigilon Therapeutics, Inc., Cambridge, MA

Allogeneic cell-based therapy has the potential for application across a wide range of diseases. One of the major challenges of this approach is cell-cell interaction and resulting immune rejection by the host. While many biomaterials (e.g., hydrogels) can provide a physical barrier, they themselves can elicit an immune response resulting in build-up of pericapsular fibrotic overgrowth (PFO), which severely limits the longevity of such therapy. Sigilon utilized a library of proprietary small molecules that avoid PFO when conjugated to alginate biomaterials (Bochenek Nat Biomed Eng 2018) to create a modular, cell-based platform with potential for utilization across a range of chronic diseases, including rare blood disorders, lysosomal storage disorders and type 1 diabetes. The platform consists of genetically modified allogeneic human cells engineered to produce the therapeutic protein of interest, encapsulated in a two-compartment sphere which supports the function of cells (inner compartment) and shields the cells from the host's immune system and PFO (outer layer) (Barney ASGCT 2020). The sphere simultaneously facilitates oxygen and nutrient influx and therapeutic protein efflux. Appropriate analytical development strategy and techniques are essential throughout the development phases of cell-based therapeutic products. As novel and cutting-edge technology emerges, bringing great potential to advance the care of patients with chronic diseases, analytical strategy and techniques must also evolve accordingly to support evaluation and development of product safety, effectiveness, critical quality attributes, specifications, and shelf life. In this presentation, two cases will be exemplified to demonstrate opportunities and considerations in analytical development to support this innovative platform. The first case presents method development to assess critical mechanical properties, such as initial fracture and elasticity of the spheres, and to design appropriate *in vitro* conditions to correlate with *in vivo* performance. The second case illustrates method development strategy to explore design space of the Afibromer™ biopolymer (small molecule chemically linked to modified alginate resulting in minimization of PFO) leading to optimized *in vivo* performance. Critical quality attributes such as modification level and viscosity need to be understood and require appropriate analytical

methods to analyze. More details will be discussed in the presentation to demonstrate the criticality of appropriate analytical strategy and methods in the product development process.

865. A Scaled and Semi-Automated Cell Encapsulation Process for a Shielded Cell-Based Platform for Chronic Diseases

Lauren Barney[1], Jacob Schladenhauffen[1], Lauren Jansen[1], Mark Lalli[1], Michael Beauregard[1,2], Devyn Smith[1], Martha Rook[1], Susan Drapeau[1]

[1]Sigilon Therapeutics, Inc., Cambridge, MA,[2]Currently at Rice University, Houston, TX

Cell-based therapy is an attractive option for the treatment of various chronic diseases due to the ability to modify the cell genome to express therapeutic molecules. With allogeneic cell therapy, the main challenge is to avoid the attack by the recipient's immune system. We have reported previously (Barney ASGCT 2020) on a modular, innovative platform to address this challenge through encapsulation of allogeneic cells within a 1.5 mm, two-compartment alginate sphere which is a physical barrier that also contains a proprietary small molecule to evade the host's immune system. The platform consists of 1) genetically modified allogeneic cells that produce human therapeutic protein(s), 2) an inner compartment optimized for therapeutic protein production, and 3) an outer layer optimized to evade the host's immune system and minimize pericapsular fibrotic overgrowth (PFO) on the surface of the sphere. The most advanced product candidate, SIG-001, contains genetically modified allogeneic cells that produce human factor VIII (hFVIII) for the treatment of hemophilia A (HA). SIG-001 produces active hFVIII in a dose-dependent manner at steady state, corrects the bleeding phenotype in HA mice and demonstrates viability of encapsulated cells *in vivo* through at least 6 months (Carmona ASH 2019). SIG-001 was granted orphan designation by the FDA and the EMA and is currently in an active clinical trial (Shapiro ASH 2020, NCT04541628). To supply the product for this first-in-human clinical trial, we developed a semi-automated, scaled encapsulation process to streamline the manufacturing. Cell encapsulation processes often require significant manual adjustments to produce a consistent product. The process used to demonstrate preclinical proof-of-concept for Sigilon spheres employs a syringe pump which extrudes alginate through a needle into a barium bath for crosslinking (Bochenek Nat Biomed Eng 2018). A high voltage is applied to the needle tip to accelerate the rate of droplet formation, and the voltage, alginate flow rate, and needle gauge are selected and manually adjusted as needed to control the resulting sphere size. Here, we report an improved encapsulation process that has been scaled and semi-automated to produce sufficient supply of drug product for a clinical trial. First, we built a system to control the droplet rate via a feedback control loop. The system is comprised of an optical sensor capable of measuring the sphere production rate in real time, coupled with a control loop to modulate the applied voltage across the needle to the crosslinking bath. Removing the manual control of the droplet formation rate allowed the system to produce consistent spheres at rates several-fold higher than possible with the manual system. The increased sphere formation rate achievable in this system allowed an order of magnitude increase in the volume of spheres that can be produced while maintaining a short residence time in the crosslinking bath, without impact to sphere strength, viability, or potency. The semi-automated manufacturing method described above is being used for the ongoing first-in-human clinical trial of SIG-001 in hemophilia A. There is continuing work to further scale and automate the manufacturing process for future manufacturing for the hemophilia A program, and for our pipeline programs in other rare blood disorders, lysosomal storage disorders, type 1 diabetes, and other serious chronic illnesses.

866. Screening Cloudz Are a Useful Tool in Determining Optimal Ligands for T Cell Expansion and Manufacturing

Caroline Nazaire, Ryan Spooner, Eric Frary, Christopher Johnson, Nithya Jesuraj, Joseph Lomakin

Bio-Techne, Woburn, MA

Introduction: Reproducible T cell expansion is a critical step in development and commercialization of immunotherapies. While there are products for T cell activation and expansion on the market, there are few tools to optimize the phenotype of expanded cells for basic research and control in clinical applications. Cloudz™ products successfully expand human T cell, T regulatory, or Natural Killer cells in culture. Traditionally, Cloudz are directly conjugated (DC) with ligands. Conjugating Cloudz with DC can be a multiple-day process, limiting a user's ability to screen many ligands. Screening Cloudz allow for simplified addition of biotinylated ligands to Cloudz for faster ligand screening. Here, we demonstrate the utility of Cloudz as user-friendly screening platform by rapidly prototyping Cloudz functionalized with various configurations of CD3 and CD28 antibodies. The Screening Cloudz were evaluated in a model $CD3^+$ cell culture and demonstrated ability to tune T cell CD25/CD69 co-expression, CD3+ expansion, and final ratio of $CD8^+/CD4^+$ cells across a clinically relevant range. **Methods:** Single-ligand Screening Cloudz and DC Cloudz were functionalized with CD3 or CD28 (Clone 1 or Clone 2) antibodies and combined in two ratios (Ratio 1 and Ratio 2). Double-ligand Cloudz were produced by simultaneous functionalization with CD3 and CD28, using DC. The produced Cloudz and Cloudz blends were evaluated in $CD3^+$ cell culture. Cloudz were seeded together with $5x10^5$ $CD3^+$ selected T cells and cultured for 9 days in 24-well plates and T25 flasks. GMP Cloudz CD3/CD28 and Nanomatrix based commercial product (Nanomatrix Supplier) were used to stimulate cultures as control conditions. The cells were cultured with RPMI media, 10% FBS, 5% PenStrep and recombinant human (rh) IL-7 (10 ng/mL) and rh IL-15 (5 ng/mL). Alternatively, cells were cultured in serum-free ExCellerate™ Human T cell Expansion Media. Flow cytometry was used to assess cells for viability, T cell activation, and phenotype following expansion on days 0, 2 and 9 of culture. **Results:** All Cloudz combinations resulted in increased CD25/CD69 co-expression after 2 days (Figure 1A) and 20-80 fold expansion after 9 days in culture (not shown). Of the Single Ligand Screening Cloudz, Ratio 1 of the Clone 2 condition showed the highest CD25/CD69 co-expression at 69%±5%. Of the Single Ligand DC Cloudz, Ratio 1 of the Clone 1 and Ratio 1 of the Clone 2 condition performed similarly, at 66%±4% and 66%±2% co-expression, respectively. Of the Double Ligand DC Cloudz, Ratio 1 of the Clone 1, and Ratio 1 of the Clone 2 condition again performed best at 68%±4% and 69%±1% co-expression, respectively. Similar trends were seen between

conjugation methods. Additionally, a range of CD8/CD4 ratios were achieved on day 9 with the GMP Cloudz CD3/CD28 delivering the most CD8 rich phenotype of 55%±4% $CD8^+$ cells/43%±4% $CD4^+$ cells, and Ratio 1 of the Clone 2 condition enabling the most CD4 rich phenotype of 20%±1% $CD8^+$ cells/78%±1% $CD4^+$ cells (Figure 1B). **Conclusions:** These results demonstrate the utility of Screening Cloudz as a rapid method for identifying effective ligand combinations to achieve a 20 - 80 fold expansion of T cells with control over expanded cell CD8/CD4 ratios. The rapid prototyping capability of screening cloudz is useful for identifying ligands and combinations to improve T cell culture workflows for research and clinical applications.

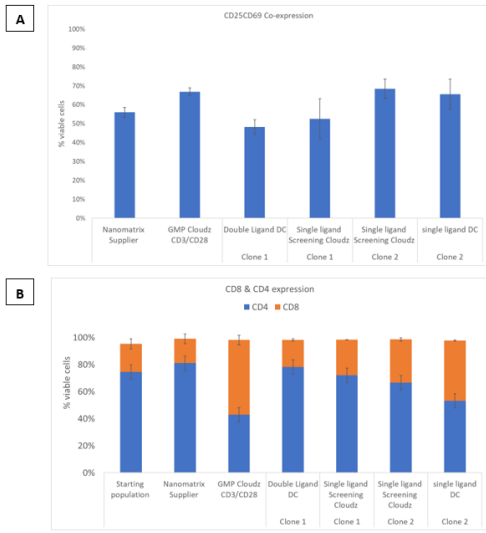


Figure 1 (A) Day 2 Co-expression of CD25 and CD69 in Cloudz activated cultures. $CD3^+$ cells were activated and expanded with Controls, Screening Cloudz, DC Cloudz in single and double ligand formats, with Ratio 2. **(B) CD8/CD4 ratio on day 9 of culture.** Cloudz activated cultures showed a wide range of CD8/CD4 ratios on day 9. Results represent the mean ± standard deviation.

867. Microfluidic-Enabled Delivery of mRNA into PBMCs for Multiplex Transfection of Naive T Cell, Natural Killer Cell, and Other Lymphocytes

Jocelyn Loo, Benjamin Chang, Tiffany Dunn, Ockchul Kim, Sewoon Han, Todd Sulchek, Alla Zamarayeva, Miguel Calero-Garcia

CellFE, Alameda, CA

Chimeric antigen receptor (CAR) therapy is an innovative adoptive immunotherapy where cell types of interest are equipped with synthetic receptors to better recognise, fight, and eliminate malignancies. $CD4^+$ T, $CD8^+$ T, and Natural killer (NK) cells are promising cell carriers in the CAR therapy space. At present, only CAR T-cell therapies have achieved FDA approval after displaying great success in eliminating B-cell malignancies. CAR NK cells, under evaluation in clinical trials, can be used in an allogeneic fashion to produce cost-efficient "off-the-shelf" products. Moreover, NK cells equipped with CARs are able to target malignancies in both a CAR-dependent and a CAR-independent manner. Literature implies a potential synergistic effect when combining CAR NK and CAR T therapy to eliminate high tumour burden. In gene therapy, mRNA transfection is being developed as a modality for cancer immunotherapies, contrasting transgene integration through viral vectors. Gene addition with viral vectors carries risk of insertional mutagenesis and potential downstream safety issues caused by long-term persistence of engineered CAR lymphocytes. Transient mRNA delivery removes these risks. Presently, the gold standard technique for mRNA transfection into lymphocytes is electroporation, reported to cause irreversible damage compromising cell viability. Mechanoporation proposes a new platform with the potential to overcome the boundaries of current transfection techniques. We have developed an updated version of a microfluidic device capable of volume exchange for convective transfect (VECT) in cells, resulting in cell transfection with payloads of interest. In this project, we set ourselves to test if the device was capable of efficiently transfecting a heterogeneous mix of lymphocytes currently researched in CAR therapy. We tested different cell starting materials, concluding that peripheral blood mononuclear cells (PBMCs) were the most cost-efficient option for obtaining a heterogeneous mix of lymphocytes. Our results show that the new device is able to process PBMCs, resulting in efficient multiplex mRNA transfection of naive $CD4^+$ T, naive $CD8^+$ T, and NK cell compartments while maintaining high cell viability and recovery. We aim to translate our multiplex transfection process into CAR mRNA for the rapid manufacturing of a mixed anti-CD19 CAR lymphocyte product.

868. Enabling the Advancement of Non-Viral CRISPR-Based Cell Therapy with a New Large-Scale Electroporation Platform

Nektaria Andronikou[1], Way Xuang Lee[2], Steven Yeo[2], Yanfei Zou[1], Monique LaCourse[1], Michael Neal[1], Xavier de Mollerat du Jeu[1], Namritha Ravinder[1]

[1]Thermo Fisher Scientific, Carlsbad, CA,[2]Thermo Fisher Scientific, Singapore, Singapore

Crispr-Cas9 based gene modification is gaining traction in the field of cell-based immunotherapy as a safe non-viral alternative to lentiviral vectors which are currently utilized to manufacture the approved FDA Car-T therapies. However, the performance benefit and specificity of the Cas9 protein can only be realized with a delivery platform that can efficiently and safely deliver the ribonucleoprotein complex (RNP) to the target cells. In addition, with the speed at which *ex vivo* modified cell therapies are moving into process development and manufacturing phases, it is imperative to not only achieve performance targets but also be able to quickly integrate into regulated and controlled environments. A newly developed large-scale electroporation platform has shown efficient gene editing in human primary t-cells. Targeting multiple genes, to show the flexibility of the platform, knock-out of the target loci was achieved reproducibly and in a scalable manner from small scale to large scale. In addition, newly developed knock-in enhancers, are able to achieve knock-in efficiency with a single electroporation delivering the Cas9 RNP and donor DNA all at once. Data will be presented to show the integration efficiency of various size donor DNA constructs at multiple loci with primary t-cells that have been isolated from fresh leukapheresis, activated with CD3/CD28 Dynabeads™, cultured with OpTmizer™ media and electroporated 3-days post activation. Data will also be presented showing the downstream process optimizations that were performed to increase total edited cell counts such as post-electroporation cell seeding density, culture format and feed schedule.

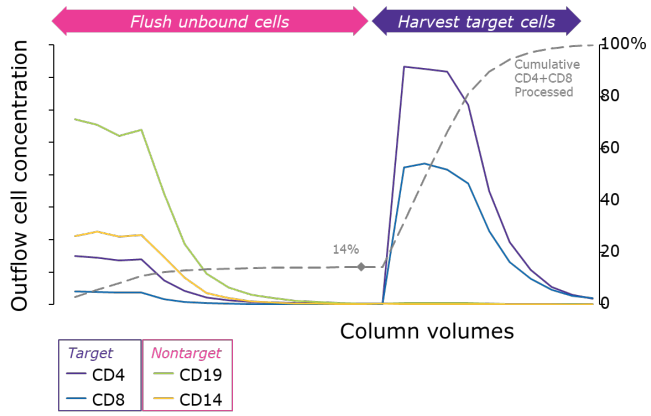
The promising results of gene delivery and cell engineering in primary T-cells are helping to advance new platforms and cell therapies forward with unprecedented speed. The technological advancement of CRISPR-Cas9, in combination with electroporation is able to provide researchers and drug developers the ability to quickly and seamlessly scale their therapeutic platforms from early research and development to clinical manufacturing. Speed, efficiency and proper quality control are critical to ensure that more patients are able to benefit from the promising cell and gene therapies being developed.

869. Acoustic Affinity Cell Selection: A Non-Paramagnetic Scalable Technology for T Cell Selection from Unprocessed Apheresis Products

Changjie Zhang, Jack Saloio, Kimberly Stewart, Theodore Schultz, Tyler Campbell, Kimberly Wojeck, John Cushman, Krishna Kumar, Rui Tostoes, Bart Lipkens

Acoustic R&D, MilliporeSigma, Wilbraham, MA

Acoustic Cell Processing is a unique acousto-fluidics platform technology for minimal manipulation of cells using ultrasonic waves. The platform has broad applications in the field of cell and gene therapy, e.g., cell concentration and washing, acoustic affinity cell selection and label-free cell selection. The acoustic radiation force exerted by the ultrasonic field on the suspended cells in combination with fluid drag forces and gravitational forces is used to manipulate the cells and perform a certain cell processing unit operation, e.g., separate, concentrate, wash or select. The technology is single-use, continuous, and can be scaled up, down or out. It therefore allows for a flexible and modular approach that can be customized to process a desired cell count, cell culture volume or cell concentration within a given required process time. Utilizing its proprietary multi-dimensional standing wave platform, MilliporeSigma has been developing the Acoustic Affinity Cell Selection (AACS) system for closed and automated Cell and Gene Therapy manufacturing, e.g., CAR-T immunocellular therapies. The AACS technology is an acoustic affinity cell selection method using acoustic (non-paramagnetic) affinity beads for positive or negative cell selection. A multi-dimensional acoustic standing wave is then used to separate the affinity bead-cell complexes from the unbound cells, thereby completing the process of a negative or positive cell selection. In this work the AACS system has been used to capture CD4+ and CD8+ cells from unprocessed apheresis products. The AACS column and acoustic section allow for a continuous flow of the initial cell population (pre-labeled with biotinylated antibodies) while acoustically retaining the acoustic affinity particles in the column. The affinity particles are functionalized with an avidin-capture biomolecule and thus capture the target cells that are kept inside the column, while the non-target cells are washed out of the column. The figure below illustrates the typical chromatogram obtained during an AACS run, where non-target cells are washed out and target cells are retained and later recovered with a higher than 70% yield and at least 95% purity.



870. Automated Clinical-Scale Non-Viral Transfection of T Cells in a Functionally Closed System

Lisa O'Flynn, Fernando Oliveira, Siobhan Leonard, Emily McFadden, Alan Pearson

Avectas, Maynooth, Ireland

The next-generation cell therapy products now in development are aimed at improving response rates in hematological cancers as well as tackling solid tumors and progressing towards allogeneic treatment options. Many of these products will require complex engineering that will not be addressable with viral vectors alone. Therefore, non-viral delivery platforms that are flexible and can integrate into manufacturing processes are required. We have previously reported the development of a novel SOLUPORE technology for non-viral engineering of immune cells and described the SOLUPORE Research Tool which is an open, laboratory instrument-grade device for discovery and feasibility studies. The technology uses reversible permeabilization to achieve rapid intracellular delivery of cargos by precisely controlling the contact of a permeabilizing delivery solution containing the cargo with the target cells. The process causes minimum cell perturbation and maintains high cell viability and functionality. We have now further developed the technology as an automated, cGMP compliant system using a closed, sterile, single use assembly. Here we demonstrate successful non-viral transfection of primary human T cells using this SOLUPORE SUS (Single Use System). The SOLUPORE SUS, as shown in **Figure 1**, is a single use system comprised of a Multi-Use Controller (MUC), a sterile Single Use Assembly (SUA) and a Delivery Solution (SOLUPORE DS). The MUC is a non-product contacting production controller used to control all aspects of the processes in the SUA using customized software which guides user operation. Target cells are transferred to and from the system via sterile, weldable bags. The process which addresses 10^7 - 10^8 cells per transfection is simple and rapid, completed in less than 8 min. SOLUPORE SUS has been designed to integrate into autologous and allogeneic manufacturing workflows. To evaluate the system, human T cells in culture medium were transferred from sterile bags into the SUA and transfected with GFP mRNA. Cells were then removed and placed in culture. The viability of cells at 24 h post-transfection is consistently >90% and proliferation of T cells post-transfection is similar to that of untreated cells over a 7 day proliferation time course. Importantly, we have demonstrated

equivalent transfection efficiency across the major T cell subtypes within the population. In summary, having previously reported that the SOLUPORE technology causes minimum perturbation to T cells, we demonstrate here that this critical attribute of the SOLUPORE technology is replicated in the SOLUPORE SUS. This system will enable *ex vivo* transfection of cells for the manufacture of cell therapy products in an automated, cGMP compliant system using a closed, sterile, single use assembly.

Figure 1: SOLUPORE Single Use System for non-viral ex vivo cell engineering. The system comprises a delivery solution, a sterile, disposable single use assembly and a multi-use controller.

871. CRISPR/Cas9 Inducible Systems for Neurodegenerative Disease

Natalie Asmus, Boris Kantor
Neurobiology, Duke University, Durham, NC

CRISPR/Cas9 has, in recent years, taken the scientific community by storm as a novel, targeted, mechanism by which to edit the genome. Employing a variety of approximately 20-nucleotide single guide RNAs (sgRNAs) designed to introduce site-specific double-stranded breaks (DSBs) in the target sequence of DNA following Cas9 nuclease activity, DSBs are then repaired by the host cells using either non-homologous end joining (NHEJ) or homology directed repair (HDR). Depending on which method is used, CRISPR/Cas9 editing can be employed to generate functional knockouts of a targeted gene in order to study its function, or knock in a donor DNA sequence at a specific locus in the genome in order to repair a mutated, or otherwise dysfunctional gene. However, the role of removing or replacing entire genes is less widely applicable in the context of several prominent neurodegenerative diseases, such as late-onset Alzheimer's disease (LOAD), for which GWAS associated genes that confer risk of developing the disease, such as APOE, also have important physiological functions. Here, the goal is not to get rid of these genes entirely, but instead to downregulate their pathologic effects and repress expression in a highly specific and controlled manner. To this effect, our lab has primarily focused on the use of catalytically deactivated Cas9 (dCas9) tethered to a diverse range of epigenetic effectors and transcriptional repressors, such as DNMT3A and KRAB-MeCP2, as a means to control gene expression. With this strategy, we have produced preliminary results which suggest effective downregulation of APOE4 expression in HEPG2 cells transduced with lentivirus, as measured by Western Blot and RT-qPCR analysis when compared to controls. Still, challenges with this methodology remain. In comparison to sequence editing, epigenetic approaches that utilize Cas9 are often much less precise, and the delivery of CRISPR/Cas9 systems through viral vectors carries with it the inherent risk of off-target effects - especially when the system is constituently active. Therefore, the development and use of inducible systems combined with CRISPR/Cas9 technology presents an effective way to enhance the precision and specificity of genome editing, allowing for high temporal control of Cas9 mediated gene modification and fine tuning expression. This can be done using one of two general mechanisms: either by regulating the expression, stability and nuclease activity of Cas9, or by modulating the availability and conformation of the sgRNA. Inducible systems can also be divided into two categories, depending on whether chemical control occurs at the level of transcription or protein expression. A literature review was conducted in order to compare existing tamoxifen and tetracycline systems on the basis of their ability to minimize background editing and modify gene expression, as well as the extent to which each allowed for temporal and spatial control. From this analysis, it is clear that the tradeoff between specificity and efficiency will require further investigation and development. Nevertheless, by combining elements from both tamoxifen and tetracycline systems into a hybrid model it may be possible to generate an optimized system that does not compromise on either specificity or efficiency.

872. Electroporation of a Novel Non-Integrative DNA Nanovector for Efficient, Semi-Automated, GMP Manufacturing of CAR T Cell Therapies

Matthias Bozza
Postdoctoral Researcher, DNA Vector Laboratory, Heidelberg, Germany

Recombinant T cell expression of Chimeric Antigen Receptors (CARs) has shown extraordinary efficacy in numerous clinical trials as an adoptive cell therapy to treat hematological malignancies. Still, CAR T therapy faces significant challenges, ranging from long lead times and expensive manufacturing to complicated vector-engineering. Also CAR T cell production routinely employs random integration of viruses or transposons, which carries an inherent risk of genotoxicity and costly, long-term patient follow-up. CAR engineering by transient mRNA transfection could be safer but more cost-prohibitive, requiring several doses per patient. DNA is a promising alternative but can cause sensitive T cells to lose functional capacity or induce apoptosis. Here we describe Nano-S/MARt, a novel DNA Vector platform for stable CAR expression with minimal disruption of T cell activity. This antibiotic-free, nanovector technology uses scaffold/matrix attachment regions (S/MARs) for DNA vector maintenance and replication and transfects primary human T Cells efficiently and without toxicity. When combined with GMP-compliant MaxCyte Flow Electroporation®and CliniMACS Prodigy ™ automated cell processing, Nano-S/MARt enabled the production of recombinant T cells with stable CAR expression and enhanced anti-tumor activity in only five days. The result was a shortened manufacturing protocol, producing safer cell therapeutics for up to thousands of patients from a single batch.

873. Gammaretroviral Vector CD19 CAR-T Manufactured in the Cocoon® Platform: An Automated and Closed Workflow

Kelly A. Purpura[1], Rivka Gal Malka[2], Elad Jacoby[3], Abraham Avigdor[4], Anastasia Rodin[2], Hadar Brayer[5], James Morrissey-Scoot[1], Karen Fargo[1], Michele Vicentini-Hogan[1], Nicholas Ostrout[6], Matthew Hewitt[6], Eytan Abraham[6], Nuala Trainor*[1], Michal Besser*[2,5,7]

[1]Octane Biotech - A Lonza Company, Kingston, ON, Canada,[2]ABC GMP Facility, Sheba Medical Center, Ramat Gan, Israel,[3]Division of Pediatric Hematology, Oncology and BMT, The Edmond and Lily Safra Children's Hospital, Sheba Medical Center, Ramat Gan, Israel,[4]Division of Hematology, Sheba Medical Center, Ramat Gan, Israel,[5]Ella Lemelbaum Institute, Sheba Medical Center, Ramat Gan, Israel,[6]Lonza Walkersville Inc., Walkersville, MD,[7]Department of Clinical Microbiology and Immunology, University of Tel Aviv, Tel Aviv, Israel

Chimeric antigen receptor T cell (CAR-T) therapies have gained increasing attention due to the novel personalized medicine approach and robust therapeutic efficiency in hematological malignancies. However, clinical utilization of these cell therapies is limited due to complex workflows using open, manual, labour intensive cell culture systems with high manufacturing costs and variability. Enabling widespread cell therapy will require employing closed, automated solutions to produce cost effective, high quality therapies. One solution is the Cocoon® Platform which automates multiple cell therapy manufacturing process steps within a closed system coupling aseptic in-process sampling capabilities (i.e. cell counts & identity) with a fully customizable, single-use cassette. This study focused on translating an open, manual, clinical CD19 CAR-T process to the Cocoon. Activities were performed in a collaborative fashion between the Cocoon R&D, Process Development, and Medical Center teams. All process development and translation activities utilized healthy donor material. Once process translation was complete and tech transferred, hospital staff completed manufacturing processes using healthy donor cells and patient (clinical) material as part of a clinical product bridging study. In brief, after processing fresh leukopaks, $4x10^8$ viable PBMCs were seeded into the Cocoon cassettes and activated with soluble OKT3. Following activation, approximately $6x10^7$ PBMCs were transduced with a retroviral vector to insert a CD19 CAR. Cells were expanded in IL-2 and human serum supplemented media, using an automated feeding strategy throughout the 10 day process. In-process sampling was performed to assess cell yield, viability, transduction efficiency, cell purity/identity, and sterility. Potency was assessed via IFNγ secretion and final product was formulated at $1x10^6$ CD19 CAR-T per kg in infusion media. Healthy donor cells used during process development manufacturing runs yielded an average of $1.06x10^9 \pm 0.25x10^9$ cells (n=4) on Day 10 with 69±9% (n=3) transduction efficacy, 98±1% CD3+ purity (n=4), and 96±3% viability (n=4). Clinical product bridging studies using patient material split between the Cocoon and manual process yielded an average of $1.71x10^8 \pm 0.77x10^8$ CAR-T cells and $5.07x10^8 \pm 3.05x10^8$ with a transduction efficacy of 45±25% and 63±16%, and viability of 90±7% and 82±16% respectively (n=6). In summary, a clinical CD19 CAR-T process was successfully translated into the Cocoon. Once translation activities were complete, the Cocoon process was successfully transferred to the Medical Center for on-site manufacturing at the clinical center. Results from the clinical product bridging study demonstrated the Cocoon produced a comparable final product, as defined by the critical quality attributes required for final product release, to the original open, manual process.

874. Designing the Lisocabtagene Maraleucel (liso-cel) Manufacturing Process for Consistency across Large B-Cell Lymphoma (LBCL), Mantle Cell Lymphoma (MCL), and Chronic Lymphocytic Leukemia (CLL) Indications

Lauren F. Brown, Jeffrey Teoh, Rachel Yost, Amanda Pace, Erica Brust, John Wesner, Brian Christin, James Powell, Kathleen Mehigan, Roland Ashton, Paul Guptill, Ryan P. Larson, Mary Mallaney, Christopher G. Ramsborg

Bristol Myers Squibb, Seattle, WA

Introduction: We describe how the liso-cel manufacturing process was designed to generate a highly pure T-cell product across disease indications and heterogeneous patient populations. **Methods:** For the liso-cel manufacturing process, $CD3^+$ T cells were purified through immunomagnetic selection for $CD8^+$ and $CD4^+$ T cells from leukapheresis starting material, followed by independent activation, transduction, expansion, formulation, and cryopreservation of $CD8^+$ and $CD4^+$ CAR^+ T cell drug product components. These components were cryopreserved separately at high cell concentrations and low-formulated drug product volumes in cryopreservation vials. Phenotypic and functional analyses of liso-cel and process intermediates were performed using flow cytometry and cell-based assays. Cell composition frequencies are reported as frequency of viable leukocytes. Liso-cel was manufactured in support of the TRANSCEND NHL 001 (NCT02631044) and TRANSCEND CLL 004 (NCT03331198) clinical studies. **Results:** Risk-based assessments and process development studies performed at the manufacturing scale were used to identify unit operation adjustments that improve product consistency without compromising quality or function of CAR T cell product across malignant B-cell indications. T-cell purification from leukapheresis reduced between-lot variability despite the heterogeneous composition of starting material across LBCL, MCL, and CLL indications (eg, circulating B-cell tumor burden, CD8:CD4 ratio, T-cell frequency, absolute lymphocyte count) (Teoh et al, *Mol Ther*. 2020;28:31-32). The variance of $CD3^+$ T-cell frequencies was significantly reduced from leukapheresis (LBCL, MCL = 79.2%; CLL = 94.2%) to post-selection T-cell materials (LBCL, MCL = 37.7%; CLL = 18.9%). Selection unit operations for $CD8^+$ and $CD4^+$ T cells significantly reduced variance in process intermediates relative to starting leukapheresis material and controlled the number of T cells forward processed to the activation unit operation. Independent manufacture of $CD8^+$ and $CD4^+$ CAR^+ T cell product components enabled optimization of all unit operations for each T-cell lineage and enabled administration of equal target doses of viable $CD8^+$ and $CD4^+$ CAR^+ T cells, maintaining a defined ratio of infused cell types. Modifications to activation and transduction

protocols, as well as media and culture conditions, supported optimal growth and product quality for the $CD8^+$ and $CD4^+$ T-cell components. Furthermore, the final drug product formulation and container closure configuration supported a stable, highly viable cell concentration (maximum, 66.9×10^6 viable cells/mL; n = 235) and enabled low-dose volumes (minimum, 0.8 mL; n = 235). Liso-cel drug product components have been thawed up to 13 months after cryopreservation with no loss of viability ($P > 0.05$) or functionality ($P > 0.05$); both drug product components maintained cellular homogeneity upon thaw, ensuring that defined doses are withdrawn and delivered to patients. Together, these manufacturing process optimizations resulted in robust production of CAR T cell products between patients and across B-cell malignancies, with high manufacturing success rates. **Conclusion:** Despite the diverse starting material across heterogeneous patient populations and different disease indications, the liso-cel manufacturing process has been developed to deliver a consistent product containing highly pure T cells, with long-term storage stability of liso-cel product in a cryopreservation vial and the ability to deliver a defined dose with low volume and high cell density.

Pharmacology/Toxicology Studies or Assay Development

875. Sonication Linker Mediated-PCR (SLiM-PCR), an Efficient Method for Quantitative Retrieval of Vector Integration Sites

Fabrizio Benedicenti[1], Andrea Calabrìa[1], Daniela Cesana[1], Alessandra Albertini[1], Erika Tenderini[1], Giulio Spinozzi[1], Victor Neduva[2], Ashkenaz Richard[2], Martijn H. Brugman[2], David J. Dow[2], Chiara F. Magnani[3], Samantha Scaramuzza[1], Maximilian Witzel[4], Christoph Klein[4], Valérie Zimmermann[5], Marie Pouzolles[5], Naomi Taylor[5], Andrea Biondi[3], Steven J. Howe[2], Giuliana Ferrari[1], Luigi Naldini[1], Alessandro Aiuti[1], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy,[2]GlaxoSmithKline, Stevenage, United Kingdom,[3]Tettamanti Research Center, Monza, Italy,[4]Dr. von Hauner Children's Hospital, LMU, Munich, Germany,[5]Institut de Genetique Moleculaire de Montpellier, CNRS, Montpellier, France

Sonication Linker mediated- PCR (SLiM-PCR) is a method for quantitative Integration Site (IS) retrieval where the genomic DNA is fragmented by sonication, ligated to a linker cassette, and amplified by PCR with primers complementary to vector and linker cassette sequences. Each vector IS can be quantified by counting the number of different fragments containing the same vector/genome junction. We validated this method on samples composed by DNA extracted from two purified cell clones carrying one and six lentiviral vector (LV) integrations in known genomic positions and DNA extracted from a polyclonal LV-marked cell population, mixed at different ratios. The abundance estimates of the IS of the 2 purified clones in the different dilutions closely correlated with theoretical estimations (±5%). When compared to other established techniques, SLiM-PCR was ~10-fold more efficient in IS retrieval and showed a high correlation between the number of retrieved and expected IS (R2≃0.9). SLIM-PCR was successfully applied on different vector platforms including gamma-retroviruses (γRV), LV, Sleeping Beauty Transposon and adeno-associated viruses (AAV). We investigated if SLiM-PCR would allow the detection and quantification of premalignant or malignant clonal expansions triggered by insertional mutagenesis. For this purpose, we analyzed peripheral blood mononuclear cells collected at different time points from two patients enrolled in a γRV-based clinical trial for Wiskott-Aldrich syndrome, who developed T-acute lymphocytic leukemia because of insertional mutagenesis. From these samples we retrieved >36,000 IS, and, in agreement with previous reports, the leukemic clone in a patient was marked by two insertions targeting LMO2 and TAL1, while in the other patient, the leukemic clone was marked by nine integrations targeting LMO2, TAL1, C11orf74, TMEM217, UBB, ST8SIA6, CPSF6, CD46 and RIN3. In both patients the clonal abundance of γRV-IS specific for pre- and leukemic clones was <2% before leukemia onset and reached 85% and 75% at the time of leukemia diagnosis, in line with the estimates made using multiple state-of-the-art methods for quantitative or semiquantitative IS retrieval. SLiM-PCR was highly efficient also in retrieving AAV IS from lymphoid tissues collected from Zap70-/- immune-deficient mice treated by intra-thymic AAV injection. Overall, we retrieved >1,300 AAV IS, many of which clustered within the TCR loci, suggesting that RAG mediated recombination of the T-cell receptor genes may enhance the insertion of AAV genomes. SLiM-PCR allowed to study the response to tumor overtime of CD19 CAR T-cells engineered by the sleeping beauty transposon in a clinical trial for B cell acute lymphoblastic leukemia. Overall, we retrieved >200,000 IS from in vitro and in vivo samples and found that the progeny of infused CAR T cells was able to engraft, expand, and persist over time. At later time points, the clonal repertoire was markedly reduced, in agreement with the contraction phase after tumor clearance. In summary, our results show that SLiM-PCR is a powerful method for IS retrieval and accurate clonal abundance estimation and that can be applied to a broad spectrum of gene therapy platforms.

876. Development of PCR Methods to Assess Vector Biodistribution and mRNA Production in Muscle Tissue Dosed with AAV1-Rat-Glucokinase

Jiewu Liu, Michele Stone

Kriya Therapeutics, Redwood City, CA

Glucokinase is a hexokinase isoenzyme that plays a critical role in glucose metabolism. It acts as a glucose sensor by catalyzing the phosphorylation of glucose to glucose-6-phosphate and has been considered as an outstanding drug target for diabetic treatment. AAV-mediated co-delivery of glucokinase and insulin into skeletal muscle is a potential novel therapeutic option for the treatment of type I diabetes. Here we report the development and qualification of a Quantitative real-time PCR assay (qPCR) and a relative quantitative Reverse Transcription-qPCR (RT-qPCR) assay that have been used in support of AAV based therapeutic biodistribution and vector-derived transgene expression analyses. In the case of local administration to skeletal muscle, sampling that is proximal to the injection site

can cause variations in the analytical results. Traditionally, multiple portions of target tissue are processed for DNAs extraction to mitigate potential sampling bias. RNAs used for transgene expression analysis are usually extracted separately from different tissue portions due to the intrinsic differences between DNAs and RNAs. This strategy is not only time consuming and labor intensive, but also vulnerable to variations between analytical runs. In the case of small animals such as mice, small tissue size adds a further challenge. In this case, mouse skeletal muscle tissues were processed as a whole piece whenever possible. The procedure of DNA/RNA extraction was optimized based on the protocols of the DNeasy Blood and Tissue kit and RNeasy Mini kit to simultaneously extract high quality DNAs and RNAs from the same tissue lysates. For distribution analysis to assess the glucokinase transgene DNA copy numbers, 3 sets of primer/probe were designed, synthesized, and tested in the assay. The primer/probe sets were subjected to BLAST against the genomic DNA and RNA databases of mice for specificity check. One set of primer/probe showed superiority to the others regarding PCR efficiency and specificity and was consequently selected for the assay. Annealing temperatures were tested with the final optimized temperature determined based on PCR efficiency and specificity. In order to circumvent the significant PCR inhibition introduced by the matrix gDNA, 4 different qPCR master mixes were evaluated and compared. The one that demonstrated high tolerance to the matrix gDNA was selected. The qPCR method was subjected to assay qualification encompassing accuracy, precision, sensitivity, linearity, specificity, and stability assessments. The assay qualification results demonstrated good accuracy with %RE < 25, and precision with %CV <25 for both intra-assay and inter-assay variability. The assay is sensitive enough to measure < 50 copies of target gene per 1ug mouse skeletal genomic DNA, which meets the recommended FDA guidance. The same set of primer/probe was further used to develop a 2-plex RT-qPCR assay for the assessment of glucokinase mRNA expression. The 2-plex assay demonstrated high compatibility with either Hprt or β-actin as the normalization gene. The annealing temperature was optimized to accommodate the dual PCR reactions to ensure efficient amplification and specificity of both the glucokinase and normalization gene (β-actin) were achieved. The 2-plex RT-qPCR assay was then subjected to assessment of relative sensitivity, precision, linearity, specificity, and matrix effect. The assay showed good precision with %CV < 25 for both intra- and inter-assay runs. No matrix effect was observed using RNAs extracted from skeletal muscle tissues of three naïve individual animals. The successfully qualified 2-plex RT-qPCR and qPCR assays demonstrate the ability to precisely correlate the AAV DNA copy number and transgene mRNA expression based on therapeutic efficacy observed in the animals from a single tissue.

877. A Case Study on Viral Clearance for a Downstream AAV Process Using a Model Virus Panel and Non-Infectious MVM Surrogate

Shaojie Weng
REGENXBIO Inc., Rockville, MD

During the past 5 years, significant advancements in the realm of AAV purification have been achieved; however, one elusive aspect of process development is viral clearance in AAV purification. Viral clearance validation is a key regulatory requirement governing all recombinant biopharmaceuticals, however, due to the nature of AAV being a derivative of the family parvoviridae, the industry will need to depend more on chromatographic modes of separation rather than nanofiltration. This study shows viral spiking studies within an AAV8 downstream process. The two-step process utilized POROS™ CaptureSelect™ AAVX affinity resin and CIMmultus™ QA monolith. A wide range of viruses with different sizes, molecular makeups, and physiochemical properties were selected as spiking agents. In addition, a non-infectious MVM surrogate which could be used as an economical and rapid way to predict MVM clearance was also tested. The results demonstrated effective clearance at center point and worst case conditions and comparability between MVM and the non-infectious surrogate.

878. Defining a Reliable Quantification Assay Strategy for Adeno-Associated Virus (AAV)-Based Gene Therapies

Felicia Thoennissen, Marina Magerl, Andreas Schulze, Allison Dane, Markus Hörer, Sonya Schermann, Renée Kober
Freeline, Stevenage, United Kingdom

AAAV-based vectors are among the most promising forms of gene therapy to treat genetic diseases. We have developed investigational gene therapies based on a proprietary, rationally designed, liver-directed AAV capsid (AAVS3), which shows significantly higher transduction efficiency in human liver cells compared with natural AAV serotypes used in many other gene therapy programs. Here, we explored the utility of various quantitative assays with the goal of identifying an assay allowing us to quantify, for a given AAV batch, the number of transgene-containing recombinant vector genomes. The ideal assay would detect all vector genomes contributing to overall vector potency, allow for comparison of vector integrity during process development and serve as a validated assay for release of clinical drug product. First, we compared vector genomes based on results from quantitative polymerase chain reaction (qPCR) and droplet digital PCR (ddPCR) assays using two differently sized transgenes (Transgene 1 [TG1]: 2.7 kb; Transgene 2 [TG2]: 4.7 kb) and multiple primer positions. Using qPCR, we observed trends toward higher titers in the middle of vector genome cassettes for both TG1 and TG2. With ddPCR some differences in titer across the vector genome cassette were observed but to a lower extent than with qPCR. We investigated whether different efficiencies in qPCR obtained for plasmid standard and samples might account for some of the position effects observed by analyzing cycle threshold (Ct) values. We found very little difference in Ct values on the packaged vector genome cassettes across the different primer positions tested. However, for the double-stranded plasmid standard also containing the vector genome cassette, decreased Ct values toward the 3´and 5´region of the transgene and increased Ct values near the ITRs were observed, explaining the strong position effects observed with qPCR compared to ddPCR, which does not require a plasmid standard for quantification. We then compared AAV vector genome titers using qPCR vs. ddPCR of the same vector carrying a 4.7 kb transgene produced by two different manufacturing platforms and found that vector integrity profiles were strongly dependent on the manufacturing platform, resulting in different vector genome titres with titre values obtained

further dependent on the primer position chosen. Since the titer value also determines the vector dose applied to *in vitro* potency testing, primer position must be chosen carefully to allow an accurate comparison of quality attributes obtained by different manufacturing processes. Finally, we conducted a 2-dimensional ddPCR and found it useful for assessing and comparing vector integrity of AAV samples. The classical qPCR is a robust and accepted method as an AAV gene therapy release assay, however ddPCR is now widely implemented. Based on this work, ddPCR, while more costly, is the preferred method for quantification of transgene for future development programs due to fewer position effects and independence from the need for a plasmid standard and associated issues of amplification efficiency. We suggest a primer positioning study and usage of complementary methods to characterize each product to allow accurate comparisons of different batches. This study also highlights the importance of using the same manufacturing platform and dosing assay throughout the development of AAV-based gene therapies to get a consistent understanding of the potency of viral genomes in *in vitro* and *in vivo* disease models, and for dosing of patients in clinical trials.

879. Method Validation for a One-Step Duplex RT-qPCR Bioanalysis Assay

Kristin N. Bell

Northern Biomolecular Services, Kalamazoo, MI

The emergent field of cell and gene therapeutics requires the need for cost-effective and efficient bioanalytical testing. Investigational new drug applications require data not only for the biodistribution of viral and cellular test articles, but also the accompaniment of correlating gene expression data to demonstrate transcriptional functionality within the test subject after administration. For analysis of the vector-derived mRNA by RT-qPCR, traditional practice includes establishment of a plasmid DNA standard curve for quantitation of target mRNA copy numbers and monitoring reference gene transcripts for the overall RNA integrity. Under these conditions, total RNA is first converted into cDNA, which is subsequently analyzed for both target and reference transcripts in separate qPCR assays. This approach potentially ensures a time-consuming and costly process when considering hundreds, or even thousands, of samples to be analyzed. Further, the use of a DNA standard for quantification of mRNA is more relative than using an RNA standard which undergoes the same process of reverse transcription prior to amplification. To alleviate some of these burdens of mRNA bioanalysis, a one-step RTqPCR duplex assay had been developed and validated. Using a serially diluted RNA standard containing 100ng of liver RNA matrix, five accuracy and precision runs were completed. Amplification of both the target and reference genes were included in each assay run. The completed validation met the inter- and intra-assay acceptance criteria for target gene amplification (Qty %CV ≤ 25% and Qty %RE within ± 25% for copies > 100; Qty %CV ≤ 45% and Qty %RE within ± 45% for copies ≤ 100) and reference gene amplification (Ct %CV ≤ 2.0%). Matrix effect and freeze-thaw stability testing during validation ensured that quantitation of the target mRNA copy numbers would not be affected by total RNA purified from various sample types or hindered by multiple freeze-thaw cycles. Additionally, for each run, the reference gene showed similar Ct values regardless of the amount of target gene included. Finally, master mix prepared without reverse transcriptase, used to quantitate potential vector DNA contamination within RNA samples, was compared to master mix containing the enzyme. Results indicated a mean Qty %RE difference of less than 30%. These data demonstrate an efficient, accurate, and reproducible duplex one-step RT-qPCR assay for gene expression bioanalysis.

880. Development of a Functional Reporter Assay to Identify and Assess Off-Target Risk for CAR T Cell Therapies against a Panel of >6,200 Human Proteins

Jim Freeth[1], Jo Soden[1], Yen Ho[2], Gabriela Hernandez-Hoyos[2], Kyle Kolaja[2]

[1]Retrogenix Limited, Chinley, High Peak, United Kingdom,[2]Bristol Myers Squibb, Seattle, WA

Target-related safety risks for CAR T cell therapies occur either through unanticipated on-target/off-tumour binding, or via lack of specificity of the CAR targeting element which results in off-target activation. Specificity to the primary target can be assessed by screening for binding against thousands of human proteins that are individually over-expressed on cell microarrays. As well as exposing potential non-specificity, the cell microarray platform also reports the identities of any off-targets allowing for focused follow up studies to assess the implications of any observed binding. *Aim:* Here we describe the development of a functional assay to identify and/or validate CAR-related off-target interactions using cell microarray technology. *Methods:* A reporter cell line, engineered with a CAR that targets a specific haematological cancer antigen ("CAR-reporter cells"), was used throughout the study. Interaction of the CAR-reporter cells with cells that express the known target, activates a reporter gene, resulting in expression of a red fluorescent protein. Investigation of cell densities, cell incubation periods, buffer conditions, cell fixation and washing steps were all required to optimise the screening of CAR- reporter cells using the cell microarray platform. *Results:* Cell microarray expression of the known primary target antigen (in HEK293 cells), induced reporter gene expression in the CAR-reporter cells, providing a clear functional read-out for this CAR-target interaction. This was observed in parallel to the binding-based signal generated using previously established protocols. *Impact:* Cell microarray screening is widely used to demonstrate CAR T specificity, generating key data for IND submissions and providing additional confidence in target safety. Where off-targets are uncovered, this latest development demonstrates a novel approach for functional validation of such off-targets. Furthermore, this functional read-out could be applied across the full panel of over 6,200 expressed human proteins, identifying only those off-targets that lead to a CAR T cell activation. These strategies could enhance the value of cell microarray technology in the overall assessment of target safety prior to clinical studies.

881. Biodistribution and Antigen Expression Kinetics of AAVCOVID Vaccine in Mice

Julio Sanmiguel, Nerea Zabaleta, Wenlong Dai, Urja Bhatt, Reynette Estelien, Maya Kim, Cheikh Diop, Dan Li, Luk H. Vandenberghe

Grousbeck Gene Therapy Center, Schepens Eye Research Institute, Mass Eye and Ear, Boston, MA

AAVCOVID is an experimental AAV-based preventative vaccine for COVID-19. Characterization of AAVCOVID candidates in mouse and non-human primates indicate a highly robust immunogenicity from a single dose. Moreover, AAVCOVID was shown to be stable and functional after storage at room-temperature for at least one month. These attributes have the potential to address some of the primary logistical barriers to effective global COVID-19 vaccine campaigns. Given the novelty of the AAV platform for preventative immunization, and to elucidate the basis for the potency of this vaccine candidate, we characterized the biodistribution and expression kinetics following intramuscular administration of a single low dose of AAVCOVID in C57BL/6 mice. Vector persistence at the injection site, systemic expression levels, and correlates of the kinetics of the immune response were analyzed over a 4-month period for two AAVCOVID candidates at 10^{11} vector copies per animal: AC1, an SV40 promoter-driven full-length pre-fusion stabilized codon-optimized SARS-CoV-2 spike gene and AC3, a CMV-driven S1 subunit of the same gene. Detectable vector genomes were found at the injection site on day 7 for both groups. Systemic biodistribution was minimal with levels 100-fold or more reduced compared to the injected muscle. Measurements following day 7, at day 60 and 120 illustrate a decline of vector genomes over time. AC1-injected males lost 10 times more vector than females by day 28, while AC3-injected males lost only 3 times more than females in the injected muscle. By days 60 and 120, AC1 and AC3 levels were reduced by over 20-fold from the 7 day timepoint. The gene expression data offered starker differences between AC1 and AC3 vector performance. Remarkably, AC1 is expressed significantly less than AC3, likely due to the use of different promoters, however, was shown to be more immunogenic in mice and non-human primates. These differences were also observed *in vitro* were AC3 outperformed AC1 by about 500-fold. Systemic expression levels were detectable for AC3 at early timepoints but diminished over time and minimal for either candidate in comparison with the early expression at the injection site. Binding IgG antibodies against codon-optimized spike transgene appeared faster with AC3 vectors than with AC1 vectors concordant with the gene expression kinetics. However, humoral responses to AC1 exceeded those of AC3 at the one month timepoint, indicating the qualitative superiority of the AC1 antigen. In summary, these data indicate that AAVCOVID candidates AC1 and AC3 demonstrate a focal biodistribution to the site of injection with limited biodistribution. Over a course of 2 to 4 months, vector genomes diminish dramatically in all tissues. AC3 provides more robust expression compared to AC1 which is minimally detectable at the intramuscular site of injection, and concomitant with the declining vector genomes, reduces over time in a substantial manner. At these doses for AC1 and AC3, these biodistribution and vector genome persistence profiles provide further evidence on the safety of AAVCOVID and AAVrh32.33 as a preventative vaccine.

882. Unlocking the Potential of Viral Vectors through with Functional Titer Assays

Leyla Diaz, Nikolay Korokhov

BioReliance, MilliporeSigma, Rockville, MD

Ensuring the quality of virus vectors used in gene therapy is achieved through a multi-tiered approach that examines several factors to establish manufacturing consistency and product safety. The steady increase in the use of virus vectors to produce ground-breaking gene-based therapies has intensified the need for novel approaches to virus testing that improve upon well-established techniques and streamline testing. Understanding the functional characteristics of gene therapy vectors is crucial in establishing their potential as an effective gene delivery system. In particular, consistent delivery of the appropriate amount of virus vector to the correct tissue is critical in designing accurate dosing strategies to ensure efficacy and safety. Based on the unique nature of virus vectors used for gene-based therapies, careful design and often customization of test methods is necessary. In this presentation, we discuss two virus-based gene delivery platforms - adeno-associated virus (AAV) and Lentivirus (LV) - focusing on the development, validation, and implementation of titration methods used to measure functional titer. We will review factors to consider for method selection and discuss strategies to accelerate method development and validation.

883. Failing Fast: A Pharmacokinetic Model for Predicting Plasma Levels of Therapeutic Proteins via AAV Gene Therapy

Alexandra M. Burr, Patrick Erickson, Kariman Shama, Biju Parekkadan

Biomedical Engineering, Rutgers University, Piscataway, NJ

As gene therapy modalities expand to include tissue-specific promoters, immune-evading mechanisms, targeted delivery, and potent transgene expression for complex diseases, the needs to accurately and precisely dose patients also rise. AAV gene therapy dosing has been largely empirical relative to small molecule or recombinant protein drugs, but translational understanding of how expression relates to the viral dose continues to improve. Hemophilia is the most prevalent example of AAV gene therapies in development for the purpose of a secreted factor. While dosing continues to be a challenge immunologically, significant clinical improvement such as reduced bleed incidents can be seen without serum levels reaching normal physiologic range. With future potential to target diseases with more narrow efficacy windows or even those that are more complex and dynamic in nature such as endocrine and metabolic applications, dosing need to shift from empirical to predictive. It would be economically beneficial to predict the failure to achieve therapeutic levels of a protein *in vivo* when at the *in vitro* stage. Our predictive model provides a transferrable platform for researchers to test their probability of success with a few basic experiments before the need to perform timely and costly disease model studies. Utilizing a model secreted protein, *gaussia luciferase*, we are using experimental data along with computational modeling to predict the stable plasma levels of a protein based on viral dose in mice. The goal of this model is to create a system which can be adapted for therapeutic proteins or other animal models using fundamental experiments such as *in vitro* transductions and existing *in vivo* data. Generally, the

compartment model is viewed as having a dose input (VG/kg) and a concentration output (pg/mL). We have exploited the necessary pharmacokinetic parameters when delivered with AAV to characterize the system as a function of other inputs. By using a specific protein and AAV serotype, we have characterized how viral dose, protein half-life, or route of administration affects the plasma concentration kinetic profile. Additionally, we utilized existing literature data to show how including these parameters increases the correlation of fit between model and literature data. Data will be shown to demonstrate how the model was developed and how experimental data was used throughout the process to test failure and success. This model platform would allow researchers to perform a few basic experiments to derive the experimental parameters necessary to predict the feasibility of reaching therapeutic protein levels based on viral dose. Having an adaptable system such as this creates a transferrable resource that would save time and money by predicting failure to achieve therapeutic levels of expression at a very early stage.

884. Expression Assay Development for rAAV Vector Encoding Retinitis Pigmentosa GTPase Regulator (RPGRco)

Geeta Iyer[1], Ben Avery[2], Anthony Monroe[2], Judith A. Newmark[3], Mark S. Shearman[3], Adrian M. Timmers[3]

[1]Pre-Clinical R&D, AGTC, Alachua, FL,[2]Bioagilytix, Durham, NC,[3]Pre-Clinical R&D, AGTC, Cambridge, MA

Purpose: Potency assays for clinical gene therapy products are multifaceted, requiring continuous efforts throughout preclinical and clinical phases. Gene therapy potency measurements face many unique challenges including transduction, transcription, translation, protein modification, cellular localization, and ultimately, protein function. A potency test, along with several other tests, assesses product conformance: release testing, stability programs and comparability studies when manufacturing changes are made. Potency tests are critical to measure rAAV product attributes such as quality, identity, purity, strength, and stability. Here, we report the establishment of a sensitive and specific *in vitro* cell-based assay to measure hRPGRco (codon optimized human retinitis pigmentosa GTPase regulator) mRNA expression from our ophthalmic gene therapy vector, rAAV2tYF-GRK1-RPGRco, using multiplexed qPCR. **Method:** Human epithelial cells (HEK293T) seeded in a 96-well plate at 20K cells per well were co-transduced with rAAV-RPGRco at multiplicities of infection across 5 orders of magnitude and human adenovirus 5 (Ad5) helper virus. Cells were incubated for 48 hours with a media supplementation after 24 hours. Next, the cells were lysed and total RNA was purified using a silica-based membrane spin column extraction method. Total RNA was measured via UV-Vis and normalized to 100 ng/μL. Multiplexed gene-specific RT-qPCR (quantitative reverse transcription PCR) was performed on 200 ng of total RNA. Primers designed for 5' sequences in the vector were used to discriminate between hRPGRco mRNA and ssAAV vector DNA to confer assay specificity. Finally, deltaCt data analysis provided hRPGRco gene expression relative to GAPDH housekeeping gene levels. **Results:** Measurement of hRPGRco mRNA expression after transduction with our AAV-RPGR vector was achieved in three phases: cell transduction, cell harvesting/RNA isolation, and detection via gene-specific RT-qPCR. We successfully optimized this gene expression assay and qualified the assay for use as a functional potency assay. Qualification evaluated the assay's sensitivity, specificity, accuracy, precision, RT-qPCR linearity, and total assay linearity. **Conclusions:** We have described design and development of an *in vitro* cell-based assay to measure hRPGRco expression from our ophthalmic vector using multiplex qPCR. This robust assay performed well and was determined to be highly reproducible, sensitive, and specific. The assay has been qualified for use as an expression assay according to the ICH guidelines and will be applied to support clinical phase product release for our XLRP clinical Phase 2/3 studies.

885. Abstract Withdrawn

886. Advancements in Adeno-Associated Virus (AAV) Analytical Toolset for Improving Process and Product Quality

Anthony Hassall

OXGENE, Oxford, United Kingdom

There is increasing need for accurate and robust analytical methods to validate the quantity and quality of the recombinant AAV (rAAV) vectors used in gene therapies. Here we report an automated method for AAV qPCR titration and Nanopore sequencing based approaches to quantify virus and identify DNA contaminants. One of the standard analytical methods for AAV is to assay the viral genome titre by qPCR. The process requires repetitive and accurate liquid handling, and there is a need for higher throughput (e.g. 384-well format) formats to improve cost-effectiveness and reduce inter-plate variability. We configured a Hamilton STAR line liquid handling platform to perform qPCR plate setup in 96- and 384-well format. The automated process significantly reduced labour cost and operator variability, improving efficiency, accuracy and precision of the qPCR-based rAAV viral genome titre assay. Additionally, we developed the capability to identify the process-related DNA impurities in rAAV materials by utilising the MinION sequencing platform.; This tool enabled us to determine relative sequence abundance, identify host cell DNA contaminants and assess AAV sequence fidelity in a quick and cost-effective way. Sequencing was not only useful as a product quality test, but also presented the opportunity to optimise conditions to reduce DNA impurities in AAV preparations. We also took advantage of transmission electron microscopy (TEM) imaging to directly assess the critical product quality attribute of empty and full particle distribution. Along with stability and aggregation profile data, this information was critical for optimisation of downstream rAAV processing, improving final product quality.

887. Utilizing a QF-PCR Platform to Detect Replication Competent Lentivirus and Product-Related Impurities in Gene and Cell Therapy Products

Sung Ryeol Park, Meghan E. Dolan, Lyndsey M. Kelly, Douglas B. Brown, Michael J. Hantman, Altaf S. Kazi

Charles River Laboratories, PA Biologics, Malvern, PA

Lentiviral vectors have become increasingly promising tools for gene modification of mammalian cells in biomedical research and gene and cell therapy, due to their capability to stably express genetic elements

of interest in target cells and to transduce vector sequences into both dividing and non-dividing host cells with high efficiency. Additionally, Vesicular Stomatitis Virus G-protein (VSV-G)-pseudotyped lentivirus vectors broaden the target cell ranges. However, despite their utility and ubiquitous use, one of the major theoretical safety concerns is the formation of replication competent lentivirus (RCL), potentially by homologous or nonhomologous recombination. Although the frequency of potential recombination events leading to RCL is strongly decreased with multiple modifications, the lentiviral vector systems still present a small chance of generating RCL. Incorporation of an envelope gene sequence would be required to generate a replication competent virus and therefore envelope gene, VSV-G is a suitable target for reliable detection of RCL. In addition to characterizing the presence of the RCL sequences in transduced cell products, assessing risk for the presence of product-related variants, such as undesirable carryover of plasmid DNA into the lentiviral preparation, should be determined because these impurities can cause problems with most downstream applications. Purification processes are commonly employed in the production scheme to remove the vector DNAs, however, verification of the quantity of residual plasmid DNA impurities is necessary to meet regulatory guidelines. In this presentation, Charles River Laboratories (CRL) PA Biologics (PAB) evaluates a QF-PCR-based platform as a sensitive and robust method to detect VSV-G DNA sequences for both RCL and product-related impurities in cell and gene therapy products. Our results demonstrate assay sensitivity as the Limit of Detection (LOD) and Limit of Quantitation (LOQ) as well as specificity by various controls per ICH Q2 (R1) guidance. The linearity, accuracy and precision will be also demonstrated

888. Cell Therapy Assessments in the NCG Mouse Model: Study Design and Success Factors

Viktorija Smutova[1], Stephen Festin[2], Camila Para[1], Jenny Rowe[2], Simon Authier[1]

[1]Charles River Laboratories, Laval, QC, Canada,[2]Charles River Laboratories Research Models and Services, Wilmington, MA

Cell therapy requires a combination of safety and efficacy assessments which are often performed in mice as a single species. Regulatory submissions in support of clinical trials require adequate characterization of the test system. Herein, we report historical control data including engraftment/flow cytometry, clinical pathology and pathology using the NCG mouse model for cell therapy investigations. The triple immunodeficient NCG mice lack functional/mature murine T, B, and NK cells, and have reduced macrophage and dendritic cell function. They are on a NOD/Nju background, in which *Prkdc* and *Il2rg* genes were edited by CRISPR/Cas9. The objectives of this study were to optimize the irradiation dose for myeloablation in NCG mice and to qualify the NCG mouse model for toxicity, engraftment/biodistribution and tumorigenicity assessments of cell therapies, using mobilized adult $CD34^+$ human Hematopoietic Stem and Progenitor Cells (hHSPCs) with various conditioning regimens. To assess conditioning regimens, mice were irradiated with 1.4 to 2.0 Gy (X-ray or Cobalt-60) or administered Busulfex® (15 mg/kg for males and 20 mg/kg for females, on Days -2 and -1, intraperitoneally) and received 0.1-1.0 x 10^6 cells/mouse $CD34^+$ hHSPCs or PBS (control) via the tail vein. A positive control group was included with NCG receiving 2 x 10^6 HL-60 cells/mouse after irradiation. Mobilized $hCD34^+$ cells from individual donors were injected into 4-6 week old female or 8-9 week old male and female irradiated NCG mice (group size n=8-20). $hCD34^+$ cells were obtained from different donors (D) each receiving a unique regimen of immunostimulatory agent: donors stimulated with either Mozobil® (MOZ), G-CSF (Neupogen®) or a combination of both. Blood samples were collected to assess engraftment rates by flow cytometry and body weights were recorded periodically up to 20 - 30 weeks post-injection (wpi) of mobilized $hCD34^+$ cells. At time of injection, animal body weights were 16.8 g ± 0.3 (D1), 16.7 g ± 0.5 (D3) and 17.1 g ± 0.3 (D2). Body weights were 25.4 g ± (D1), 24.6 g ± 11.2 (D3) and 25.2 g ± (D2) by 26wpi. Growth curves were comparable between all groups with minimal effects from conditioning. Clinical signs were observed at a low incidence, were generally mild and were expected in a normal mouse population given the experimental procedures. At week 18 post-injection, the percentage of human $CD45^+$ cells of total lymphocytes was 48.4 ± 29.0 (D1), 56.4 ± 11.1 (D2) and 17.9 ± 12.6 (D3) in comparison with 84.5 ± 9.84 in animals injected with cord blood derived $hCD34^+$ cells (standard control). At 26 weeks post-injection the percentage of human $CD34^+$ cells of total lymphocytes was 34.2 ± 24.0 (D1), 58.8 ± 20.1 (D2) and 10.2 ± 8.4 (D3). In the tumor cell line positive control group (i.e. HL-60), mortalities due to tumorigenesis were observed between days 27 and 40 post-dose and affecting 80% of the animals. Based on the data obtained, the NCG mouse model was successfully conditioned and transplanted with $CD34^+$ hHSPC adult mobilized cells. Toxicology endpoints were characterized for safety assessments. The procedure did not significantly affect the animals body weight or survival and engraftment rates, confirming the relevance of this mouse model for cell therapy efficacy and safety studies.

889. Assessment of Inflammatory and Neurodegenerative Biomarkers in NHPs with DRG Toxicity

Ryan M. Reddinger[1], Kwi Hye Kim[1], Lisa G. Lanigan[2], Jillynne Zeller[3], Eric Adams[3], Olivier Danos[1], Nicholas Buss[1], Michele Fiscella[1]

[1]Regenxbio Inc., Rockville, MD,[2]StageBio, Frederick, MD,[3]Northern Biomedical Research, Grand Rapids, MI

Recent studies in non-human primates (NHPs) have shown lesions in the dorsal root ganglia (DRG) including neuronal degeneration and subsequent axonal degeneration following intrathecal administration of AAVs. Currently, these findings have not been associated with any adverse clinical signs in NHPs, and the pathogenesis and relevance to humans are unknown. Aspart of the continued characterization of these observations , we assessed a panel of inflammatory and neurodegenerative biomarkers in samples of cerebrospinal fluid (CSF) and serum from cynomolgus monkeys administered AAV9. Groups of cynomolgus monkeys were administered AAV9 or vehicle control into the cisterna magna (CM) or lumbar (IT-L) with serum and CSF (via an implanted IT-L catheter) collected on Days 4, 15 and 29 followed byeuthanasia. Serum and CSF samples were used for an exploratory assessment of cytokines, chemokines, neuroinflammatory or neurodegenerative biomarkers. Samples were analyzed using the Luminex MILLIPLEX MAP Non-Human Primate Cytokine Magnetic Bead Panels 1 (GCSF, GM-CSF, IFN-γ, IL-1ra, IL-1β, IL-2, IL-4, IL-5,

IL-6, IL-8, IL-10, IL-12/23 (p40), IL-13, IL-15, IL-17, IL-18, MCP-1, MIP-1α, MIP-1β, sCD40L, TGF-α, TNF-α, VEGF) and 2 (sCD137, Eotaxin/CCL11, sFasL, FGF-2, Fractalkine/CX3CL1, Granzyme A, Granzyme B, IL-1α, IL-2, IL-4, IL-6, IL-16, IL-17A, IL-17E/IL-25, IL-21, IL-22, IL-23, IL-28A, IL-31, IL-33, IP-10/CXCL10, MIP-3α/CCL20, Perforin and TNFβ/LTA), Human Neurodegenerative Disease Magnetic Bead Panels 1 (α2 Macroglobulin, Apo Al, Apo CIII, Apo E, Complement C3 and Complement Factor H) and 4 (Amyloid β40, Amyloid β42, GDNF, sRAGE and S100B) and Human Neuroscience Magnetic Bead Panel 1 (GFAP, NSE, α-Synuclein, PARK5 and TGM2). Additionally, samples were analyzed using the Quanterix Simoa Neurology 4-Plex A (GFAP, Neurofilament light, Tau and UCHL1). At the end of the study, AAV9-related adverse findings were seen in the DRG, spinal nerve roots, spinal cord, and sciatic nerve from AAV9-treated animals. Interestingly, in the animals with morphological changes, there were no detectable differences in the analytes evaluated in either the CSF or serum on Days 4, 15 or 29. The data from this study suggest that these morphological effects do not lead to any notable changes in a panel of inflammatory cytokines and chemokines despite increased inflammatory cell infiltrate. Furthermore, the observed axonal degeneration was not associated with any classical biomarkers of neurodegeneration after 4 weeks.

890. Novel AAV Infectivity Assay Mediated by Tetracycline Enabled Self-Silencing Adenovirus (TESSA)

Weiheng Su[1], Maria Patricio[1], Anthony Hassall[1], Claire Greenwood[1], Matt Burridge[1], Simon Pollack[1], David W. Brighty[2], Ryan Cawood[1]

[1]Oxgene, Oxford, United Kingdom,[2]

Infectivity of rAAV vectors is a key attribute impacting theefficacy of AAV-mediated gene therapy, but it has been challenging to determineAAV infectious titres due to their low transduction efficiency *in vitro*.A common solution is the use of replication-competent adenoviruses and specificengineered cell lines (e.g. HeLa RC32) for AAV infectious titre assays. Theseplatforms raise concern around safety and how representative the titres are inthe final target cell types. OXGENE has overcome the above limitations by usinga Tetracycline-Enabled Self-Silencing Adenovirus (TESSA) system encoding bothRep and Cap AAV genes. These TESSA vectors can infect a wide range of celltypes similar to wild type adenovirus, but they only replicate the early-phaseadenoviral genes to help amplify the rAAV vectors for titration withoutproducing any adenovirus in the absence of induction. This system allows titrationof various serotypes of rAAV vectors in a range of cell lines, providing more informativeand representative infectious titre data without contaminating adenovirus. AAV infectious titres are frequently determined via Median TissueCulture Infectious Dose (TCID50) assay, involving the preparation of multiple96-well plate replicates per sample with a number of dilutions of each viralvector. This process is laborious at scale and can be susceptible to humanerrors and operational variability due to the intensive liquid handling. We deployeda Hamilton liquid handling platform to automate the workflow, further increasingefficiency, accuracy and reproducibility of the TESSA assisted process, while reducinglabour cost and operator variability.

891. Quantification of Double-Stranded RNAs Produced by AAV Vectors: A Methodological Reassessment

Chunping Qiao, Samantha Yost, Randolph Qian, Kirk Elliott, Devin McDougald, Justin Glenn, Elad Firnberg, April Giles, Jenny Egley, Andrew Mercer, Nicholas Buss, Joseph Bruder, Olivier Danos, Ye Liu

REGENXBIO, Inc, Rockville, MD

Introduction: Antisense transcription of AAV vector genomes may result in the formation of intracellular double-stranded (ds) RNA species that trigger an innate immune response. Significant amounts of transcripts initiated from the 3'ITR have been measured and strategies to avoid their accumulation have been proposed. We have used digital PCR to quantify the amounts of dsRNA produced by different AAV expression cassette designs following transduction of HEK293T-AAVR cells as well as in the skeletal muscle of mice. **Results:** To differentiate between sense and antisense RNA, we used strand-specific or control primers during the reverse transcription (RT) step. When Superscript IV (Thermo) reverse transcriptase was used, we detected similar cDNA counts in digital PCR reactions regardless of which primers were used in the RT reaction. This indicated that the detected cDNA was primer-independent, possibly due to RNA secondary structures and self-priming or non-specific primer annealing and extension during cDNA synthesis. To avoid non-strand-specific cDNA synthesis, we explored alternative reverse transcription formats. We found that performing RT at high temperature (55 to 60°C) and using Omniscript® reverse transcriptase (Qiagen) can reduce non-specific self-priming and achieve strand-specific cDNA synthesis. With this improved method, we quantified antisense RNAs in HEK293T-AAVR cells transduced by several AAV9 vectors. Sense strand RNA transcripts driven by the strong, ubiquitous CAG promoter (AAV9-CAG-EGFP) were 6000-fold more abundant than antisense strand RNAs. Signals for both sense and antisense strand RNAs were barely above background level in cells transduced with a promoter-less AAV9 vector, indicating that RNA transcripts produced by the AAV ITRs accumulate at a very low level. Sense and anti-sense RNAs were then measured in mouse muscle after intravenous dosing with AAV8 vector containing a muscle-specific promoter. Sense RNA transcript was about 300-fold higher than antisense RNA. **Conclusions**: We have identified a potential pitfall of measuring dsRNA produced by AAV vectors with a traditional RT-PCR method, and proposed an improved method using Omniscript reverse transcriptase and digital PCR. With this method, we observed that sense RNA produced by transgene promoter is about 300- and 6000-fold higher than antisense RNA initiated by 3' ITR *in vivo* and *in vitro*, respectively. This is consistent with a recent report comparing CBA promoter and ITR promoter activity in mouse by Earley et al.

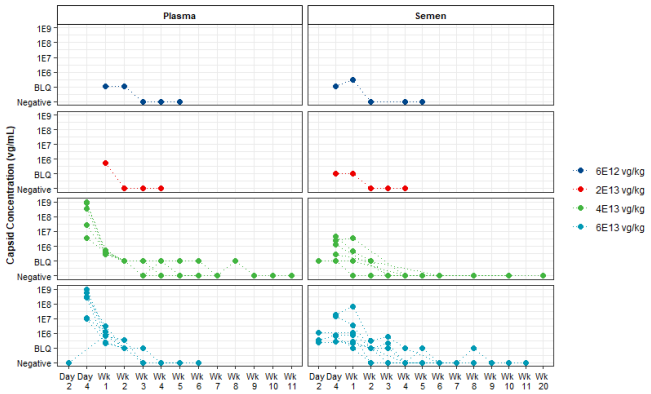
892. Ultra-Sensitive AAV Capsid Detection by Immunocapture-Based Quantitative Polymerase Chain Reaction Following Factor VIII Gene Transfer

Krystal Sandza[1], Annie Clark[2], Elli Koziol[1], Hassib Akeefe[3], Fan Yang[1], Jennifer Holcomb[1], Kathryn Patton[1], Kevin Hammon[1], Nina Mitchell[4], Wing Y. Wong[5], Stephen J. Zoog[1], Ben Kim[4], Joshua Henshaw[1], Christian Vettermann[1]

[1]Translational Sciences, BioMarin Pharmaceuticals Inc., Novato, CA,[2]Clinical Pharmacology, BioMarin Pharmaceuticals Inc., Novato, CA,[3]Research & Development, BioMarin Pharmaceuticals Inc., Novato, CA,[4]Clinical Sciences, BioMarin Pharmaceuticals Inc., Novato, CA,[5]Medical Affairs, BioMarin Pharmaceuticals Inc., Novato, CA

Introduction: Adeno-associated virus (AAV)-based gene therapy (GT) vectors are replication-incompetent and pose minimal risk for horizontal transmission or environmental release. In studies with AAV5-FVIII-SQ (valoctocogene roxaparvovec), an investigational GT for hemophilia A, biodistribution and shedding of vector DNA was characterized in blood, secreta and excreta using conventional qPCR, but it remained unclear how long intact AAV5 vector capsids were present. ***Methods:*** We developed a new method (termed iqPCR) utilizing capsid-directed immunocapture followed by qPCR amplification of encapsidated DNA. Intact AAV5 vector capsids are immunoprecipitated from samples, then treated with Benzonase to digest DNA external to the capsid or within structurally damaged capsids, followed by heat denaturation/elution and qPCR detection of vector DNA. Due to antibody-based capsid enrichment and inclusion of Benzonase treatment, the developed method was highly sensitive and specific for encapsidated, potentially infectious, vector DNA. ***Results:*** The sensitivity of iqPCR was compared to existing AAV5 capsid detection methods, including cell-based transduction assay and capsid immunoassays (ELISA, ECLA, and SMC). The transduction , ELISA, ECLA, and SMC assays had limits of detection (LODs) of 6.40 E+10, 7.01 E+08, 3.42 E+08, 2.50 E+07 capsids/mL in plasma, respectively. The iqPCR method had an LOD of 1.17 E+04 and 2.33 E+04 vg/mL in plasma and semen, respectively, approaching the high sensitivity of qPCR methods used to detect total vector DNA in extracted specimens. Acceptable precision, accuracy, specificity, and selectivity were verified as non-encapsidated vector DNA was detected up to 1.00 E+09 vg/mL. Anti-AAV5 antibody plasma concentrations >141 ng/mL interfered with AAV5 capsid detection, suggesting that iqPCR mainly detects free capsids, not complexed with antibodies. AAV5-FVIII-SQ capsids were observed in plasma and semen but became undetectable in all subjects (n = 15) within nine weeks after vector administration at doses from 6.00 E12 to 6.00 E13 vg/kg (Figure 1). ***Conclusions:*** This iqPCR method is a novel approach to monitor biodistribution and shedding kinetics of intact AAV5-FVIII-SQ vector capsids, instead of vector DNA fragments with conventional PCR method, following GT. Intact vector capsids were found in semen and plasma but rapidly became undetectable, restricting the minimal risk of horizontal transmission to a period after dose administration far shorter than the duration of vector DNA shedding. The iqPCR method provides significant improvements in sensitivity and specificity over alternative capsid detection methods, making it broadly applicable across GT programs and AAV serotypes.

Figure 1. Encapsidated Vector DNA Detected in Plasma and Semen Following AAV5-FVIII-SQ Administration



893. Stereotactic Convection Enhanced Delivery Infusions Directed by Real Time MRI in NHP Models

Erik A. Larson[1], Jerneja Stare[1], Karen Wong[1], Michael V. Accardi[1], Simon Authier[1,2]

[1]Charles River Labs (Laval), Laval, QC, Canada,[2]Faculty of Veterinary Medicine, University of Montreal, Montreal, QC, Canada

Non-human primates (NHP) are important models for cell and gene therapies as it relates to proof-of-concept investigations but also regulatory testing requirements. As recent advances have provided new approaches for the application of targeted therapies, translational considerations need to be addressed in safety and efficacy studies by using similar technologies as will be applied in clinical trials. Accordingly, we have implemented real-time MRI and the Clearpoint-guided drug delivery system to precisely and stereotacticly dose brain regions in NHPs. This technique was used for bilateral conventional enhanced delivery infusion and for targeting of up to six regions per animal surgery. To accurately plan the appropriate trajectories for the desired brain structures, a high resolution T1 MRI scanning was performed to ensure the cannula trajectory was appropriate for the targeted brain regions prior to insertion. A gadolinium-containing solution was then infused to act as a contrasting agent which allowed for real-time MRI confirmation of the dosing site and dose diffusion monitoring. The dosing components that were used are the same as for clinical dose delivery to ensure translational value. Dependent on the targeted brain structure, an infusion rate of 30-300 µL/hour was used. Following infusion, gadolinium was observed to cover most of the targeted brain structure while further post-hoc analysis was used to calculate the estimated coverage of the subcortical structures. During surgery recovery, animals presented generally mild expected procedure-related clinical signs which resolved within four days of the procedure. Since the use of NHP models is particularly important for testing the delivery of cell specific gene therapies, this approach can provide a clinically relevant preclinical methodology for targeted brain CED with high structural accuracy.

894. Development of an Assay to Measure Transduction Efficiency of Adeno-Associated Virus (AAV)-Based Gene Therapies

Anita Heinlein, Alexandra Schoberth, Katja Haslauer, Matthias Lee, Sonya Schermann, Markus Hörer
Freeline, Stevenage, United Kingdom

We have developed several investigational gene therapies based on a proprietary, rationally designed, liver-directed AAV capsid (AAVS3), which shows significantly higher transduction efficiency in human liver cells compared with natural AAV serotypes used in many other gene therapy programs. In the current work, we describe the development of a sensitive and universal transducing titer assay using a commercially available mRNA detection kit. The transducing titer describes the vector titer required to transduce a given number of cells. The PrimeFlow™ RNA Assay (Thermo Fisher™) allows detection of mRNA transcribed in cells at very high sensitivity and has potential for use in monitoring functional AAV vector transduction on the level of transgene transcription. Since Freeline AAV vectors carry both sense and antisense single-stranded (ss) DNA, the probe set used to detect mRNA can cross-react with the antisense strand of the viral input DNA to some degree resulting in a potential overestimation of transducing titers. Therefore, novel strategies are needed to develop PrimeFlow™ RNA-based transducing titer assays for ssAAV vectors. We systematically developed a method to overcome this limitation. We demonstrate that specific probe sets allow us to differentiate between the codon-optimized gene carried by AAV versus its genomic wild-type counterpart. To differentiate between single-stranded input AAV DNA and mRNA transcribed from successfully transduced AAV, we designed two probe sets labelled with two different fluorophores. Using these two probe sets, we could distinguish between cells expressing mRNA and those carrying only AAV DNA but not expressing mRNA. This tool enabled us to determine the optimal time for detection of mRNA expression without interference by input DNA. We obtained dose-response curves across a range of multiplicities of infection using our novel method. In summary, we have developed a robust, sensitive and universally applicable transducing titer assay. Standard *in vitro* potency assays measure either the total amount of protein produced and/or its functional activity upon AAV transduction of cells at a given dose. However, they do not provide information about the number of cells transduced and, therefore, contributing to the overall protein levels/activity. This assay is complementary to traditional *in vitro* potency assays and provides a useful orthogonal characterization tool to improve our understanding of functional AAV transduction.

895. Rare Genomic Integrations of AAV5-hFVIII-SQ Occur without Evidence of Clonal Activation or Gene-Specific Targeting

Lorraine Sullivan[1], Marco Zahn[2], Irene Gil Farina[2], Theresa Kasprzyk[1], Charles A. O'Neill[1], Kevin Eggan[1], Stephen J. Zoog[1], Gabor Veres[1], Manfred Schmidt[2], Christian Vettermann[1]
[1]BioMarin, Inc., San Rafael, CA,[2]GeneWerk GmbH, Heidelberg, Germany

Introduction: Recombinant adeno-associated virus (AAV)-based vectors are considered to be non-integrating vectors, since chromosomal insertion is not an essential part of their regular lifecycle. However, sporadic integrations into the host genome may occur at low levels, and there is a need to better understand the nature of these events as well as their potential impact, in particular for clinical AAV gene therapy candidates. **Methods**: Valoctocogene roxaparvovec (AAV5-hFVIII-SQ) is an investigational gene therapy for treatment of Hemophilia A. To characterize AAV5-hFVIII-SQ vector integrations, we performed target enrichment sequencing (TES) on non-human primate (NHP) liver tissue. Liver samples were collected from 12 male NHPs either 13 or 26 weeks after vector administration, with dose levels ranging from 2 E13 to 6 E13 vg/kg. The liver was selected, since it represents the target organ for AAV5 vectors, and previous nonclinical studies had demonstrated long-term retention of transgene DNA in the liver. **Results**: The vast majority of the AAV5-hFVIII-SQ vector (>99.9%) showed no signs of integration, consistent with a predominantly episomal presence. Rare vector integration events occurred on average in less than 1 in 600 liver cells. This integration frequency is in line with expectations for AAV vectors and several orders of magnitude lower than the annual rate of natural mutations in humans (0.5 mutations per E9 base pairs, or approximately 3 mutations/diploid cell). Across all doses and time points, individual integration sites showed no evidence of clonal expansion or gene-specific targeting, as determined by the frequency distribution of sequencing reads in each NHP subject: most integrations (93-96%) were low in abundance and detected by only 1-2 reads; the few integrations detected by multiple (>2) reads did not exceed an absolute read count of 7 and a relative frequency of 1.6%. These data strongly suggest that individual integration sites were restricted to single cells or small groups of progeny cells. Overall, integration sites were broadly distributed across the whole genome. Less than 10% of all integrations occurred within 100 kb from the transcriptional start site of known human cancer genes and all such integrations showed relative read frequencies <0.6%, indicating absence of clonal enrichment both 13 and 26 weeks after dose administration. In accordance, no pre-neoplastic lesions, tumors or malignancies were noted in this study. Across all NHPs, genomic regions showing recurrent integrations were located near active genes, such as the liver-restricted albumin locus, suggesting that open chromatin is conducive to AAV vector integration. **Conclusions**: While sporadic integrations can occur with AAV5-hFVIII-SQ similar to other gene therapies, the identified integration sites were not associated with preferential targeting of oncogenes or clonal activation of affected liver cells, at all time points examined in this non-clinical study. Furthermore, the observed vector integration frequency remained substantially below the annual rate of natural mutations in humans. Continuous scientific evaluations are required to better characterize integration profiles across AAV gene therapy products, improve our understanding of potential impact, and elucidate product-specific risk factors.

896. Development of a High Content Imaging Assay for CLN5 Protein Expression as a Platform for Quantitative AAV Mediated Production of Lysosomal Proteins *In Vitro*

Radhika Desai, Ruda Cui, Wei Liao
Neurogene Inc, New York, NY

Introduction: Lysosomal storage disorders (LSDs) are a group of inherited monogenetic diseases caused by disrupted protein function within the lysosome, and are prominent targets for gene therapy. As part of gene therapy product development, it is important to characterize protein expression as one of several key critical quality attributes. We have developed a high content imaging platform to assess AAV-mediated protein expression and localization with utility for multiple LSD targets. Here we describe the development of an assay for the CLN5 lysosomal protein using this platform. **Methods:** Several candidate cell lines were screened using an AAV expressing a fluorescent reporter for optimal AAV permissiveness. The glioblastoma cell line U87 MG was selected based on this screen for further development. Cells were co-plated with a serial dilution of NGN-CLN5 with MOIs ranging from 500 to 5E+07 genome copies per cell in a 96-well plate that was incubated for three days. Cells were fixed with paraformaldehyde and permeabilized using a mild detergent to maintain lysosomal and CLN5 protein integrity. CLN5 protein levels were detected using a primary antibody and a fluorescently tagged secondary antibody. A nuclear counterstain was used for normalization. The assay plate was imaged using an automated imaging system and images were processed using analysis software. Assay response was normalized to cell number and plotted as %CLN5 positive cells versus NGN-CLN5 concentration. **Results:** In its GxP application, a relative protein expression assay was developed for evaluating purified NGN-CLN5. A dose response curve of NGN-CLN5 vector was generated and compared to reference material. The assay showed precision with a coefficient of variation of less than 20% and demonstrated linearity over a range of 50-150% relative protein expression. To assess its utility as a stability indicating assay, heat stressed NGN-CLN5 was generated. NGN-CLN5 heated at 55C for 15-60 minutes showed a temperature and time dependent loss in relative protein expression. **Conclusions:** These method development results demonstrate that the assay is suitable for GMP method qualification for lot release and stability testing for early clinical lots of NGN-CLN5. This approach could be extended to other therapeutic targets where reagents for standard approaches like ELISA are not available.

897. Detection and Quantitation of Cytokines Using a Simple, 10-Minute Assay

Thomas P. Quinn, Lily Lee, Mei Fong, Matt Rowe, Baz Smith, Michael Haugwitz, Andrew Farmer
Takara Bio USA, Mountain View, CA

Cytokines are a broad category of proteins that regulate the responses of specific immune cell populations by binding to cell receptors and modulating intracellular signaling pathways. Recently, CAR-modified CD4+ and CD8+ T cells were reported to be equally effective in direct killing of target tumor cells, and the cytotoxic activity of these populations was associated with increased expression of both Th1 and Th2 signature cytokines (1). It has been demonstrated that the differing cytokine profiles of engineered T cells and native T cell receptor-expressing cells reflect varying mechanisms of activation, underscoring the importance of cytokine measurement and related assays as means for characterizing the functional properties of CAR-T cell products. Current methods for measuring cytokines include bead platforms (e.g. Luminex and CBA), solid-phase arrays, and intracellular cytokine staining, but the most ubiquitous means of cytokine analysis is the ELISA. ELISAs enable accurate, precise, and sensitive measurement of specific cytokines in an array of different biological samples, but are both labor intensive and time consuming, taking from 90 minutes to 4 hours to complete. In addition, cytokine ELISAs are formatted in a way that compels researchers to collect and accumulate samples over days or weeks to minimize hands-on time and costs by analyzing several samples in parallel and occupying an entire ELISA plate, rather than analyzing samples as they are obtained. In this work, we present lateral flow-based immunoassays for the analysis of both Th1 (IL-2, IFN-γ, and TNF-α) and Th2 (IL-6, IL-4, IL-10) cytokines that employ an iOS- and Android-compatible smartphone application to deliver accurate, quantitative results in approximately 10 minutes. The simplicity of these assays facilitates quick measurement of cytokine levels to enable real-time monitoring during experiments, and they can also be used to minimize the number of samples subjected to further downstream processing and analysis using more traditional immunoassays. Each two-step assay involves adding a small amount of culture supernatant or diluted serum to a lateral-flow cassette, followed by a brief 10-minute incubation. Imaging and densitometric analysis of resulting assay bands on the cassette are then performed with a smartphone running the intuitive GoStix™ Plus software application, which compares the results to an automatically downloaded, lot-specific standard curve to determine the quantity of analyte present in the sample. The GoStix Plus app provides a unit value (pg/ml) that generally falls within the same range as ELISA-based measurements but is generated in a fraction of the time. Analysis of the performance of the assay using recombinant control proteins yielded R^2 values greater than 0.99 and coefficients of variation less than 15%, indicating comparable precision to ELISA-based approaches. To demonstrate the utility of GoStix Plus assays as compared to ELISAs, we used both methods to analyze T cell culture supernatants at various timepoints following activation with either anti-CD3/CD28 conjugated beads or anti-CD3/CD28 tetramers. Both analysis methods revealed differing cytokine profiles for T cells activated by the respective methods, providing evidence of a relationship between activation strength and the conformation in which primary and secondary signals are provided. In summary, by combining the convenience of lateral-flow technology with smartphone-based image processing, GoStix Plus assays enable accurate and precise cytokine quantitation in approximately 10 minutes. These assays provide researchers with a powerful alternative to existing methods, enabling a reduction in expenses associated with labor and materials while accelerating the pace of immunology research and the development of novel therapies. (1)doi.org/10.1016/j.gpb.2019.03.002

898. Detection of SARS-CoV-2 Using LAMP and CRISPR/Cas13a

Gabriel Lamothe, Jacques Tremblay
Medicine, Laval University, Québec, QC, Canada

Background: The ongoing COVID-19 pandemic has lasted longer and was more devastating for many countries than was initially anticipated. While the development of several vaccines will hopefully control this pathogen, adequate distribution of the vaccines remains a valid concern. Until under-developed countries have sufficient access to the vaccine, diagnostic tests will remain at the forefront of their fight against COVID-19. We have therefore sought to create a simple detection test that does not require specialized equipment. Based on the SHERLOCK test which used Reverse-Transcriptase Recombinase Polymerase Amplification (RT-RPA) to amplify viral sequences, followed by transcription and detection with Cas13a to resolve a signal, we have sought to create a functional test with components that have reliable supply chains. Having had difficulties at the beginning of the pandemic to obtain the materials required for an RPA reaction, we sought adapt the SHERLOCK test to better reflect existing supply chain capacities to bolster future distribution. The result was a test that is simple to use, requires common and inexpensive equipment, and is easy to modify. With this test it will be possible to flag emerging SARS-CoV-2 variants and thus help health officials track dangerous variants such as the London variant (B.1.1.7), the South African Variant (501Y.V2), and the Brazillian variants. **Methods:** This diagnostic test is based on a two-pot amplification and detection system. Extracted viral RNA is first added to a Reverse Transcription Loop-Mediated Isothermal Amplification (RT LAMP) reaction to amplify a section of the N gene. LAMP primers from Zhang et al.'s protocol (Zhang et al. 2020) have been modified to include a T7 promoter in the FIP primer for downstream applications. After the incubation at a constant temperature (65°C) is complete, the amplified sequence is transferred to the second reaction containing a T7 RNA polymerase and an LwaCas13a protein. The RNA produced from the polymerase activates the Cas13a and induces the protein to indiscriminately cleave surrounding RNAs. Among the surrounding RNAs there is RNAse ALERT which contains a FAM fluorochrome on one end and a quencher on the other. The non-specific cleavage of this RNA reporter by the LwaCas13a protein following the latter's specific activation by the N gene induces a fluorescent signal that indicates the presence of the virus in the patient's sample. **Results:** 1. LAMP with the T7 insert in the FIP promoter is possible with a limit of detection of 10 copies/µL (Figure 1A). 2. T7 transcription can be performed on these amplicons to produce RNA bands in the characteristic LAMP pattern. 3. The reaction time for the LAMP reaction is crucial to avoid false positives. 4. There is a limit to the volume of the amplified LAMP reaction that can be added to the T7-Cas13a reaction before the fluorescent signal begins to weaken. 5. A strong binary signal is produced with this test to allow clinicians to verify by visual inspection whether a patient is infected or not (Figure 1B). 6. The detection test can be performed with only two heat blocks, two pipettes, and a UV light making it ideal for areas lacking specialized equipment such as thermocyclers. **Conclusion:** We have developed a detection test that is faster to use than the current RT-qPCR gold standard. It can target RNA or DNA pathogens and is easy to adapt to target emerging viruses and strains. This test does not require specialized equipment making it ideal for low resource settings. Future work will involve targeting the emerging SARS-CoV-2 variants.