

Report from the ASGT Ad Hoc Committee on Retroviral-mediated Gene Transfer to Hematopoietic Stem Cells

Review of murine data on insertional mutagenesis and predisposition to tumorigenesis following gene transfer in HSCs

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- I. Introduction
- II. What is the frequency of tumor formation associated with GT in HSCs?
Reviewers: John Tisdale, Isabelle Riviere and Michel Sadelain
- III. What are the mechanisms of tumor formation associated with GT in HSCs?
 - a. Transgene/promoter combinations implicated in tumor formation and non-malignant proliferative responses
 - b. Transduction with drug resistance genes or HoxB4 have not led to adverse events except for one study on MDR1. Reviewer: Tony Blau
 - c. Review of murine models of insertional mutagenesis leading to hematological malignancies. Reviewer: David Bodine
- IV. Is there a higher risk of developing tumors in SCID mice?
 - a. Studies on γc and jak3 gene transfer in murine hematopoietic chimeras.
Reviewer: Brian Sorrentino
 - b. Review of tumorigenesis in transgenic and naturally occurring immune deficient mice.
Reviewer: Jan Nolta
- V. Recommendations
- VI. Tables 1-7 and references

I. Introduction

This report is an overview of the literature on retroviral-mediated gene transfer in murine hematopoietic chimeras, with emphasis on studies in mouse models of Severe Combined Immune Deficiency (SCID). The report was commissioned by the ASGT in response to two serious Adverse Events in the human X-linked SCID trial, in which two patients developed a T-cell malignancy after receiving gene therapy to treat X-SCID.

This report is focused on particular topics from the vast literature on mouse models for gene therapy, and should not be regarded as a complete database. Its ultimate objective is to address the following three questions:

- What is the frequency of tumor formation associated with gene transfer in HSCs?
- What are the mechanisms of tumor formation associated with gene transfer in HSCs?
- Is there a higher risk of developing tumors in SCID mice?

We know that there is not as yet a complete answer to the first two questions, but we begin to address them based on the available data that we could review in a short time span. Several individuals were co-opted to undertake this analysis and assist David Bodine and Michel Sadelain: Tony Blau, Jan Nolta, Isabelle Riviere, Brian Sorrentino, and John Tisdale. The organization of the review process is shown in Appendix 1. The personal communications in this report are to be considered confidential and for ASGT to use to inform the public about new information relevant to the recent Adverse Events. The work may be described in general terms,

but all studies are on going or in preparation for publication and great pains should be taken to protect the confidentiality of the work.

II. What is the frequency of tumor formation associated with GT in HSCs?

This section is a broad survey of the frequency of tumors reported in hematopoietic chimeras engrafted with retrovirally transduced bone marrow cells.

Search process

Two literature searches were performed and merged:

- In PubMed, Embase (European database), Biosis (Reports and Meetings Subset), CancerLit Database Search (no date restrictions):
Hematopoietic stem cells and Gene/ Genes/ Genetic* and Vector*
And Retrovirus/ Retroviral/ Retroviridae and Mice/ Mouse
- In PubMed:
Mouse and gene transfer/gene therapy and bone marrow, and fetal liver/stem cell/ hematopoietic chimera, and retrovirus/retroviral vector/lentiviral vector, and tumor/leukemia/lymphoproliferative disorder.

Summary of findings

Compilation of citations after excluding repetitions: 785 original articles

After excluding studies in NOD/SCID xenochimeras, studies on retroviral-mediated gene transfer of oncogenes, and short-term studies under 50 days: 233 original articles.

Thirty four of these papers are reviewed separately below (24 papers on drug resistance and HoxB4 gene transfer in III.b; 10 papers on γ_c and jak3 gene transfer in IV.a). Of the remaining 199 papers, 78 papers have been reviewed (see Tables 1-3).

| | Mice: | Studies: |
|-----------------------------|-------|----------|
| Bone marrow chimeras: | 2620 | 78 |
| Primary recipients >3 mo: | 1658 | 70 |
| Primary recipients >6 mo: | 460 | 24 |
| Secondary recipients >3 mo: | 423 | 9 |
| Secondary recipients >6 mo: | 138 | 3 |

The average duration of follow-up is 24 weeks.

About 25% of the hematopoietic chimeras are followed for 6 months or more.

There are essentially no reports past 14 months.

About 35% of the studies include secondary transplants.

Integration site analysis was employed in only one manuscript to directly address the issue of insertional mutagenesis (Li, Science, 2002).

Adverse events

Seven studies with adverse events were identified (see IIIb for MDR1 studies):

- muIL3, MLV LTR: no tumors; fatal non-neoplastic myeloproliferative disorder
- muTPO, MLV LTR: leukemia in 2 out of 32 mice declared 3-4 months post-transplant
- mu flt3/flk2 ligand, MSCV LTR: B cell or myeloid tumors in all long-term chimeras (n=7) and in 20 out of 24 secondary recipients; all primary recipients (n=18) are healthy at 5 months
- huΔLNGFR, MLV LTR: Li et al. (2002) described a mouse with a myeloid leukemia associated with the integration of a provirus in the Evi-1 locus. The BXH-2 mouse strain has a high frequency of myeloid leukemias and Evi-1 is the most common site of proviral insertion in BXH-2 leukemias (Mucenski et al., 1988). These data indicate that at least in this form of murine leukemia, recombinant and replication competent provirus insertions can cause leukemia by the same process.
- muCD40L: 12 of 19 CD40L^{-/-} mice (X-linked hyperIgM syndrome) developed T lymphoproliferative disorders 7-9 months after transplantation; hematology normal at 5 months; no tumor in 7 vector treated control mice. Tumors were mostly lymphoblastic lymphomas of thymic origin.
- muΔ4 Notch ligand
- muIL2: lymphocytic infiltration causing hepatomegaly was noted 12 weeks after BM transplantation and not further characterized (Khur, Exp Hem, 2000).

Conclusions

- Together with the 24 studies listed in IIIb and 10 studies listed in IIIc, we have surveyed 78 + 24 + 10 = 112 studies, about half of the 233 deemed to be relevant.
- The combined number of mice is 4172, including 1038 followed for at least 6 months.
- Adverse events including tumors or abnormal hematopoietic proliferation are reported in 7 studies; regrettably, most studies do not include specific studies or statements on the health status of long-term chimeras
- There is only one report of tumorigenesis linked to insertional mutagenesis (Li, Science, 2002); the 2 cases of leukemia noted in 32 mice transduced with muTPO have not been explored (Villevall, Blood, 1997).
- The leukemia found by Li et al. was uncovered in 6 of 10 secondary transplant recipients engrafted with pooled marrow from several donors; the unique integration site found in all 6 cases establish that the disease is linked with a single integration event. There remains a doubt whereas to a possible role played by DLNGFR itself in this transformation (see Li, Science, 2002; Sadelain and Riviere, Mol Ther, 2002).
- The frequency of transformation can therefore be estimated to be 1/4172. However, this frequency is underestimated because of possible under-reporting and insufficient follow-up. Based on follow-up of at least 6 months, the frequency is 1/1038.

IV. What are the mechanisms of tumor formation associated with GT in HSCs?

a. Transgene/promoter combinations implicated in tumor formation and non-malignant proliferative responses

Tumors have been found with the following transgene/promoter combinations:

- MDR1
- CD40L
- ΔLNGFR
- TPO
- Δ4 Notch ligand
- flt3 ligand

Non-malignant proliferative responses were found with the following transgene/promoter combinations:

- IL3
- IL6
- G-CSF
- IL2

b. Transduction with drug resistance genes or *HoxB4* have not led to adverse events except for one study on *MDR1*.

This section aims to summarize the preclinical studies performed in mice that have investigated gene transfer methods with the intention to select or amplify at the level of the hematopoietic stem cell.

Methods

Articles were identified through searches using the National Library of Medicines PubMed search engine (<http://www.ncbi.nlm.nih.gov/entrez>). Table 4 and Table 5 list the papers that were identified. A total of 28 papers are included in the analysis. The majority of papers involve stem cell selection via transfer of a chemotherapeutic resistance gene (n=24). These are listed in Table 4. The second category of selection uses gene transfer to deliver a pro-proliferative signal. Only four papers were identified that meet the criteria for inclusion. These four papers are listed in Table 5.

Findings

Stem cell selection.

Multiple different strategies have been tested in mouse transplantation experiments with the intention of providing gene-modified stem cells with a selective advantage. These strategies are dependent on the expression of one of the following genes 1) dihydrofolate reductase (DHFR), 2) multi-drug resistance (MDR1), 3) methyl-guanine methyl transferase (MGMT), and 4) cytidine deaminase (CD). There is an additional paper that describes a combined DHFR and aldehyde dehydrogenase (ALDH-1) method (Takebe, Zhao et al. 2001). The identified papers are listed in Table 4 under the appropriate heading.

The use of antifolates dates to 1987 when Williams et al., used Moloney-based oncoretroviral vectors to transfer the DHFR gene to mouse bone marrow followed by transplantation (Williams, Hsieh et al. 1987). Six additional studies are listed in Table 5. As with most studies examining stem cell selection using chemotherapy resistance, studies were designed to demonstrate survival differences between animals transplanted with transduced bone marrow and control animals. This study design resulted in expected animal deaths noted in many of the studies. A key study demonstrated the ability of trimetrexate and the inhibitor NBMPR-P to select at the stem cell level for the expression of a mutated DHFR (Allay, Persons et al. 1998). In all, a total of 268 mice are reported that had been transplanted with DHFR transduced marrow cells and then exposed to selective pressure. Animals were followed for an average of 25.1 weeks (range 8 to 60 weeks). No incidence of malignancy has been reported.

MDR1 has been used extensively in mouse experiments as well as in clinical trials. 11 studies are listed in Table 1. Due to the ability of p-glycoprotein to protect cells from a number of chemotherapeutic agents, there is great variability in the studies. A total of 370 experimental mice have received bone marrow transduced with the selectable MDR1 gene. Animals were followed for an average of 19.1 weeks (range 4 to 54 weeks).

The use of MGMT to confer resistance to nitrosureas is a more recently developed strategy. Six papers are summarized in Table 4. These studies include data on 182 experimental mice with a significant number of deaths, primarily from dose finding experiments with BCNU. Animals were followed for an average of 22.5 weeks (range 17 to 24 weeks). No other unexpected side effects are described in these studies.

Cytidine deaminase (CD) has been studied in cell culture assays but only one paper that involved mouse transplantation could be identified (Eliopoulos, Bovenzi et al. 1998). This study involved 37 mice and followup for 13 months. No adverse events were described.

Stem Cell Amplification

Though a number of strategies for in vivo expansion of hematopoietic stem cells exist, only two methods have been tested in the mouse transplant model. A third method has been recently presented in abstract form (Hara et al., ASH 2002). The four published papers using this approach are listed in Table 5.

Hox B4 is a homeobox protein involved in pattern development as well as hematopoiesis. Three papers describe the results of retroviral mediated transfer of HOXB4 to hematopoietic stem cells followed by transplantation (Sauvageau, Thorsteinsdottir et al. 1995; Thorsteinsdottir, Sauvageau et al. 1999; Klump, Schiedlmeier et al. 2001). These studies demonstrate clear expansion of transduced stem cell activity. 147 mice are included in the experimental arm in these three papers though 142 mice are included in two of the three papers (Sauvageau, Thorsteinsdottir et al. 1995; Thorsteinsdottir, Sauvageau et al. 1999). No adverse events have been reported with follow up as long as 13 months.

Our group has published a single paper using a dimerizable form of mpl to direct in vivo expansion. This study did not demonstrate selection at the stem cell level. Data from 15 mice are presented that were treated with the chemical inducer of dimerization (CID) and followed for up to 6 months. We have a manuscript in preparation that will include follow up to 2 years. The treatment is well tolerated without adverse events. We have transplanted 85 mice that have received as much as 7 weeks of CID without the appearance of leukemia. However, cells that underwent long-term (>6 months) expansion in vitro did develop a leukemia phenotype that was transplantable (data not shown). It is anticipated that constitutive mpl signaling would result in a myeloproliferative disease (Cocault, Bouscary et al. 1996). A significant side effect was reported

in the Mdr studies under unique selection conditions (Bunting, Galipeau et al. 1998; Bunting, Zhou et al. 2000). Mice transplanted with Mdr transduced cells that had been expanded ex vivo before transplantation developed a myeloproliferative syndrome that could progress to acute leukemia (Bunting, Galipeau et al. 1998). The authors pointed out that removal of a cryptic splice site improved expression of the Mdr gene. The myeloproliferative syndrome was observed at a much lower frequency in the absence of prolonged ex vivo expansion (Bunting, Zhou et al. 2000). Another report did not demonstrate the development of malignancy in transplanted mice following ex vivo expansion (Licht, Goldenberg et al. 2000). The report of the myeloproliferative syndrome with MDR1 is the only documented malignancy in published mouse transplant studies employing in vivo selection.

Conclusions

- A wide variety of strategies have been employed in an attempt to endow genetically modified stem cells with a selective advantage. This review has identified 28 papers which present data from 1464 individual mouse transplants. These animals were followed for an average of 23.1 weeks (median time was 21.45 weeks). The design of many of the presented studies involved the use of chemotherapeutic agents at doses for which toxicities were predictable.
- Sorrentino and colleagues described the only unexpected adverse event in two papers. The appearance of a myeloproliferative disorder was associated with MDR1 expression and an increase in cell copy number. The predisposition to development of the myeloproliferative syndrome occurred in the absence of ex vivo expansion but with a longer latency time.

c. Review of murine models of insertional mutagenesis leading to hematological malignancies

The alteration of gene expression in hematopoietic cells caused by the insertion of replication competent oncoretroviruses is a well-known and studied mechanism for leukemogenesis (Lagaespada, 2000). In mouse strains with competent endogenous proviruses, such as AKR and HRS, leukemias develop within 9 months of birth in nearly 100% of the animals (Jackson Laboratory website). Several recombinant inbred lines of mice derived from an initial cross of AKR/J X DBA/2J mice were developed to study the effects of specific genes on leukemia development. These animals are highly susceptible to the development of B and T cell malignancies, and the mapping of proviral insertions within the tumors has identified many genes important in leukemia development (Suzuki et al., 2002, Hansen et al., 2000, Joosten et al., 2002). In one complete study by Suzuki et al, 1336 proviral insertions in 194 distinct B and T cell malignancies, and a similar number of myeloid leukemias were isolated, sequenced and mapped to the draft sequence of the mouse genome. 493 of the insertions mapped to one of 152

loci in 2 or more tumors, while the remaining 843 sequences were present only in one tumor. Several loci were particularly frequent targets. The Sox4 locus was targeted in 55 tumors, the Hhex locus in 23 tumors and 64 loci were targeted in 10 to 20 tumors (Suzuki et al., 2002). In contrast, integration into the Lmo-2 locus was detected in only one tumor (N. Copeland, personal communication), indicating that at least for replication competent mouse oncoretroviruses, the Lmo-2 locus is a less frequent target for provirus insertion. This study and three related studies are summarized in Table 6.

The finding that the IL2RG provirus had integrated near the Lmo-2 locus in both patients who have developed T-cell leukemia (Hacein-Bey et al., 2003) prompted the committee to review the literature concerning the relationship between Lmo-2 and leukemia in the mouse. As noted above, there is only one documented replication competent oncoretrovirus insertion into the Lmo-2 locus among 1336 insertions identified in approximately 300 hematologic tumors (N. Copeland, personal communication). Of note, however, that most of these tumors were either B cell or myeloid malignancies. 72% of transgenic mice that constitutively express Lmo-2 develop a T-cell leukemia between 6 and 18 months of age (Larson et al., 1994). During the latency period it was demonstrated that while the hematology was essentially normal, T-cell proliferation is highly increased in Lmo-2 transgenic mice (Neale et al., 1995, 1997). The latency period indicates that Lmo-2 contributes to leukemia, but requires additional mutations before the development of T-cell leukemia.

Conclusions

- Retroviral insertional mutagenesis is causally linked with hematopoietic oncogenesis in mice.
- The mechanisms of tumor formation are complex. The relationship between insertional mutagenesis and oncogenesis is therefore complex too.

IV. Is there a higher risk of developing tumors in SCID mice?

a. Studies on γc and jak3 gene transfer in murine hematopoietic chimeras.

The most relevant studies in mice have involved oncoretrovirus mediated transfer of the IL2RG gene into autologous hematopoietic cells of the mouse model of X-SCID. There are no fewer than 6 reports in the literature demonstrating that this approach can rescue the immune system in the mutant animals, including studies using the identical virus backbone used in the human trials (Cavazzana-Calvo et al., 2000, Hacein-Bey et al., 2002). To summarize the published literature, approximately 88 XSCID animals have been transplanted with autologous cells transduced with various IL2RG containing oncoretrovirus vectors with no adverse events. The follow up period ranged from 16-32 weeks in these studies, about 10-15% of the normal mouse life span (Aviles Mendoza et al., 2001, Lo et al., 1999, Otsu et al., 2000a,b, 2001, Soudais et al., 2000). These studies are summarized in Table 7. A minority of the mice were followed for 6 months.

The XSCID studies summarized in Table 7 differ significantly from the human trial in several ways. First, the X-SCID mouse mutation is a deletion of the IL2RG gene, while the human mutations are primarily point mutations. Secondly, X-SCID mice are both T and B-cell deficient, while X-SCID patients are T-cell deficient but produce large numbers of non-functional B-cells. Thirdly, most of the transplant experiments in the mouse used full marrow ablation, whereas the human trial infused transduced cells into untreated recipients. Fourth, the frequency of gene transfer into primitive mouse hematopoietic cells is more than 10 fold higher than the rates observed in human trials or large animal models. Finally, the mouse studies were done on young adult animals while the human trial involved neonatal patients. It is clear that any of these factors could be responsible for the differences observed in the detection of adverse events.

Adverse events

Unpublished data provided by Dr. Fabio Candotti of NHGRI has examined an additional 53 animals for 12-30 weeks, including 10 animals in which transduced autologous cells were transplanted into non-myeloablated X-SCID mice. Among these animals 5 were noted to have an enlarged spleen at necropsy, and two of the five animals were sick enough to require euthanasia. These data are being reported to the FDA. Dr. Candotti noted that the finding of an enlarged spleen is relatively common among unmanipulated X-SCID mice (F Candotti, personal communication).

A second series of related studies described the transplantation of autologous JAK3 deficient hematopoietic cells transduced with an oncoretrovirus containing the JAK3 gene into myeloablated JAK3 deficient mice. Mutations in the JAK3 gene produce a Severe Combined Immune Deficiency syndrome similar to that cause by mutations in the IL2RG gene. The expression of the proviral JAK3 fully restored the immune system of the treated animals, and no adverse events were described in the published work (Bunting et al. 1998, 1999, 2000). Ongoing work by Drs. Bunting and Sorrentino, which is being prepared for publication, has discovered several incidents of T-cell leukemia, which have been reported to the FDA. Specifically, when JAK3 deficient cells were transduced with the JAK3 vector and transplanted into JAK3 deficient mice, T-cell leukemia was identified in 13 of 61 transplant recipients (21%). In a second experiment, neonatal liver cells from JAK3 deficient mice were transduced with the JAK3 vector and transplanted into unirradiated newborn JAK3 deficient mice. Two incidents of T-cell

leukemia were identified in 36 transplant recipients (6%) (BP Sorrentino, personal communication).

To test whether the T-cell leukemia was due to the integration of the JAK3 oncoretrovirus vector, a control experiment in which autologous JAK3 deficient hematopoietic cells were cultured under identical conditions but without any oncoretrovirus vector and transplanted into irradiated JAK3 mice. An identical T-cell leukemia was detected in 8 of 29 animals (28%). An investigation of approximately 100 unmanipulated JAK3 deficient adult animals revealed 3 cases of T-cell leukemia (BP Sorrentino, personal communication).

As noted above these data have been reported to the FDA. These data strongly support the hypothesis that the T-cell leukemia is intrinsic to the animal model and while perhaps accelerated by the myeloablation and bone marrow transplantation associated with gene transfer, the leukemia is NOT caused by the integration of the JAK3 vector.

Conclusions

- Tumors were reported following gene transfer of CD40L in CD40L^{-/-} mice (in 12 of 18 mice), with declared disease 7-9 months after engraftment.
- No tumors were reported in 8 studies investigating γ c; however, the longest reported follow-up is only 7 months.
- A high incidence of T cell leukemias is found in long-term JAK3^{-/-} mice, which does not correlate with the retroviral transduction of the JAK3 cDNA.

b. Review of tumorigenesis in transgenic and naturally occurring immune deficient mice.

Reviewer: Jan Nolta

i. Immune deficient mice are more susceptible to the endogenous development of leukemia/lymphoma than are immune competent strains.

Mice with the severe combined immune deficient (SCID) mutation in the DNA-dependent protein kinase (DNA-PK) lack T and B lymphoid cells, but they do have some NK cell function. Abnormal B and T receptor rearrangements were detected by Schuler et al. in spontaneous thymic lymphomas from C.B-17scid mice. SCID mice are known to spontaneously develop thymic lymphoma with low incidence and long latency. It was more recently demonstrated that low-dose irradiation of SCID mice dramatically increases the frequency and decreases the latency of thymic lymphomagenesis. The lymphocyte-specific endonuclease encoded by the recombinase-activating genes (RAG-1 and RAG-2) is required for radiation-induced thymic lymphomagenesis in SCID mice. Williams et al demonstrated, in 2001, that irradiation induces a DNA-PK-independent non-homologous end joining pathway that facilitates V(D)J joining, but also promotes aberrant and often oncogenic misjoining of RAG-1/2-induced breaks in SCID T-cell precursors (Williams *et al.*, 2001).

Gladdy et al further showed, in 2003, that RAG-1 or RAG-2-induced double-strand DNA breaks (DSB) induced complex, clonally heterogeneous and amplified IgH/c-Myc translocations in leukemic pro-B cells from p53/Prkdc-deficient mice (Gladdy *et al.*, 2003). The PRKDC mutation denotes the knockout of the DNA-PKcs, the DNA repair protein kinase that is deficient in SCID mice. The genomic instability conferred by the p53/Prkdc disruption efficiently transformed pro-B cells lacking RAG-1/2-induced DSB. Unexpectedly, RAG-2/p53/Prkdc-deficient mice were also shown to develop leptomeningeal leukemia (Gladdy *et al.*, 2003).

In addition to the defects described above for SCID mice, additional incidences of lymphoma were observed after transfer of the SCID mutation from the C.B-17 congenic strain background onto the diabetes-susceptible nonobese diabetic (NOD) background. An unusually high incidence of spontaneous thymic lymphoma development was observed in the resultant NOD-scid/scid mouse. Thymomagenesis in the NOD-scid/scid mouse was determined by Prochazka et al to be caused by a NOD mouse-unique endogenous ecotropic murine leukemia provirus locus (Emv-30, mapped to proximal region of chromosome 11) not expressed in the parental NOD/Lt thymus (Prochazka *et al.*, 1992). Prochazka et al proposed that the unusual features of T-cell ontogeny characteristic of the NOD inbred strain synergize with the block in thymocyte development in SCID mice, leading to activation of the NOD-unique Emv-30 virus to initiate thymomagenesis. Since the lymphoma cells could not be rejected due to the extreme immune deficiency of the mice, death rapidly ensued once the transformed cells began to expand in each mouse, severely shortening the lifespan.

Unpublished data from the Nolta laboratory (submitted for publication) further demonstrates the high incidence of neoplasia in mice that lack an immune system. A study was undertaken to follow a large breeding colony of beige/nude/XID mice, which lack T, B, and NK cells, and to monitor the colony for the development of leukemia and solid tumors during their 2-year lifespan. The mice are not known to harbor the Emv-30 virus which shortens the lifespan of NOD/SCID mice, as described above. However, spontaneous transformation of cells that cannot be rejected is observed frequently in mice that lack an immune system, and specifically lack NK cells, as described in more detail in the following section. The incidence of development of adverse events, ranging from severe patches of skin discoloration similar to melanoma, to the development of solid tumors or leukemia, in the unmanipulated bnx breeder colony was 268 in

954 mice. The number is higher than for normal mice, probably at least partially due to the fact that rearrangement of immunoglobulin genes cannot rearrange properly in *bnx* mice, due to the deficiency in Bruton's tyrosine kinase. The failure of successful gene rearrangement usually leads to death of the cells, but may also lead to leukemogenesis in some cases. Also, the animals have no innate NK cell function to eliminate leukemic cells as they arise. The incidence of adverse events in transduced human stem cell – injected mice of the same strain (117/412) was not significantly higher than in the unmanipulated breeding colony (268/954).

The MMR gene, in addition to the SCID mutation described above, is essential for DNA mismatch repair and has been found to cause predisposition to cancer in hereditary nonpolyposis colorectal cancer (HNPCC). Loss of the wild-type allele generates a MMR-deficient cell compartment with a high propensity to oncogenic transformation. Jansen et al demonstrated that the combination of MMR deficiency and exposure to genotoxic agents in mice strongly accelerated lymphomagenesis (Jansen *et al.*, 2000).

Poly(ADP-ribosyl)ation occurs as an immediate cellular response to DNA damage, and is catalyzed by poly(ADP-ribose) polymerase (PARP, PARP-1). PARP-1 knockout mice are generally not prone to the development of tumors, but an enhanced tumor development was observed when Tong et al. introduced the PARP-1 null mutation into severely compromised immune-deficient mice (Tong *et al.*, 2001).

Mice with inactivation of the gene encoding the suppressor of cytokine signaling-1 (SOCS-1) die soon after birth from an IFN-gamma-dependent inflammatory disease. Survival was made possible by also deleting the IFN-gamma gene. SOCS-1(-/-) IFN-gamma(-/-) mice exhibited a functional T cell deficiency and an increased predisposition to the development of T lymphoid leukemia, either spontaneous or radiation-induced (Metcalf *et al.*, 2002).

B cell malignancies arise with increased frequency in aging individuals and in patients with genetic or acquired immunodeficiency or autoimmune diseases. Davidson et al. (Davidson *et al.*, 1998) determined that mutations at the Fas (*lpr*) and FasL (*gld*) loci, which prevent Fas-mediated apoptosis and cause an early onset benign lymphoid hyperplasia and autoimmunity, also predispose mice to malignant lymphomas later in life. They showed that a significant proportion of C3H-*lpr*, C3H-*gld*, and BALB-*gld* mice 6-15 mo old developed B cell malignancies which resembled immunodeficiency-associated B cell lymphomas. By 1 yr of age, approximately 60% of BALB-*gld* and 30% of C3H-*gld* mice had monoclonal B cell populations that grew and metastasized in *scid* recipients but in most cases were rejected by immunocompetent mice (Davidson *et al.*, 1998).

Mice deficient in the expression of retinoid-related orphan receptor gamma (RORgamma) demonstrated that this receptor plays a crucial role in the regulation of thymopoiesis and lymph node organogenesis. Ueda et al demonstrated that over half of the RORgamma-deficient died within the first 4 months as a result of thymic lymphomas (Ueda *et al.*, 2002). The lymphoblastic cells metastasized frequently to spleen and liver. No other tumor types were detected in any of RORgamma-/- mice that died during the course of the experiment, and none of the heterozygous mice developed thymic lymphomas (Ueda *et al.*, 2002). This data indicates that changes in homeostasis in the thymus of mice are associated with a high incidence of T-cell lymphomas. Lymphoma formation was associated with increased cellular proliferation, which may enhance the probability of individual cells to acquire genetic alterations that make them escape negative selection and normal differentiation programs and as a consequence lead to increased susceptibility to the development of T-cell lymphoma.

Collectively, these data show that the incidence of spontaneous leukemia and lymphoma is significantly higher in immune deficient mice than in their normal counterparts. The section

below demonstrates that in addition to the lack of T and B cells, it is the lack of NK cells which is critically important in immune deficiency to allow the development of neoplasia.

ii. The importance of Natural Killer cells in the growth and metastasis of leukemia and lymphoma cells in immune deficient mice.

Haliotis et al compared spontaneous tumor development and primary oncogenesis in a large number of NK4-deficient, homozygous C57Bl/6-bg/bg mice and their normal, heterozygous +/bg littermate controls (Haliotis *et al.*, 1985). In a group of 167 retired breeders followed for spontaneous tumors, the probability of survival for mice eventually dying with a tumor was greater for the NK-competent, +/bg than for the NK-deficient, homozygous C57BL/6-bg/bg mice ($p = 0.0019$). This data demonstrated that NK impairment in beige mutant mice early in life may lead to significantly greater rates of death with spontaneous malignant tumors late in life. The results were among the first to suggest that NK cells play a role in surveillance against spontaneously arising tumors.

The Involvement of NK cells in the control of growth and metastatic spread of tumors was further studied by Gorelik et al. (Gorelik *et al.*, 1982). The authors found that the ability of BALB/c nude and C57BL/6 mice to eliminate tumor cells from the blood stream was severely impaired after a single inoculation of anti-asialo BMI (asGMI) serum, which targets NK cells. Tumor cells surviving in the lungs of BALB/c nude mice pretreated with anti-asGMI serum was 28 times higher than in the control nude mice. In C57BL/6 +/+ mice treated with anti-asGMI and in C57BL/6 beige mice, i.v. inoculation of B16 melanoma cells induced 10 times more metastatic foci in the lungs than in the control mice. However, in nude mice which lack thymic – selected T lymphocytes but have extremely high levels of NK reactivity, metastatic growth was suppressed 7-fold in comparison with intact C57BL/6 +/+ mice. These data demonstrated that NK cells play an important role in resistance to the dissemination of tumor cells, and therefore contribute to the control of metastasis formation in mice.

The calcium-dependent phosphatase calcineurin and its downstream transcriptional effector nuclear factor of activated T cells (NFAT) are important regulators of inducible gene expression in multiple cell types. In T cells, calcineurin-NFAT signaling represents a critical event for mediating cellular activation and the immune response. Bueno et al deleted the gene encoding the predominant calcineurin isoform expressed in lymphocytes, calcineurin A beta (CnA beta). CnA beta(-/-) mice were viable as adults, but displayed defective T cell development characterized by fewer total CD3 cells and reduced CD4 and CD8 single positive cells (Bueno *et al.*, 2002). Total peripheral T cell numbers were significantly reduced in CnA beta(-/-) mice and were defective in proliferative capacity and in activation. CnA beta(-/-) mice also were permissive to allogeneic tumor-cell transplantation in vivo, unlike wild-type littermates (Bueno *et al.*, 2002).

TNF-related apoptosis-inducing ligand (TRAIL) is important in innate immune surveillance against tumor development. TRAIL gene-targeted mice were shown to be more susceptible to experimental and spontaneous tumor metastasis than their non-targeted littermates (Cretney *et al.*, 2002).

The transporter for antigen presentation (TAP) protein is involved in class I MHC (MHC-I) antigen processing and has been shown to be deficient in some human tumor cells. Antitumor responses by CD8 T cells allow outgrowth of cells with defective processing of tumor Ags. Inoculation of C57BL/6 mice with TAP1-negative cells by Johnsen et al. (Johnsen *et al.*, 1999) produced large and persistent tumors. In contrast, TAP1-positive cells did not generate lasting tumors. Both TAP1-positive and TAP1-negative cells produced tumors in athymic mice,

confirming that TAP-dependent differences in tumorigenicity were due to T cell-dependent immune responses.

Perforin-dependent cytotoxicity is a major effector function of CD8+ MHC class I-restricted T cells and of NK cells. Van den Broek et al. used perforin-deficient C57BL/6 (PKO) mice to study the involvement of perforin and Fas ligand in tumor surveillance in vivo (van den Broek *et al.*, 1996). Tumors were induced in PKO and normal C57BL/6 mice by injection of different syngeneic tumor cell lines or by administration of chemical carcinogens. The tumor cell lines tested were eliminated 10-100-fold better by C57BL/6 mice in an unprimed situation, and after priming the differences were even more pronounced. Perforin-dependent cytotoxicity was shown to be a crucial mechanism of cytotoxic T lymphocyte- and NK-dependent resistance to injected tumor cell lines, and to chemical carcinogenesis in vivo (van den Broek *et al.*, 1996).

Christianson et al backcrossed the severe combined immunodeficiency (scid) mutation onto the C57BL/6J background to examine the role of natural killer (NK) cells in rejection of normal and malignant human lymphohematopoietic cells (Christianson *et al.*, 1996). C57BL/6J-scid/scid mice have very low numbers of T and B cells but higher levels of NK1.1+ cells and myeloid cells than in the C57BL/6J strain (+/+) controls. Injections of an anti-NK1.1 antibody were administered weekly and resulted in eradication of NK cell activity in the C57BL/6J-scid/scid mice, over 8 weeks of treatment. Human CEM-C7 T lymphoblastoid tumor cells were injected into the unmanipulated C57BL/6J-scid/scid mice, and grew slowly. However, the anti-NK1.1 treatment resulted in increased growth of the CEM cells, accompanied by metastasis of human lymphoma cells to multiple organs (Christianson *et al.*, 1996).

The authors further backcrossed the beige (bgJ) mutation onto the C57BL/6-scid/scid strain, to decrease NK cell activity. C57BL/6-scid/scid (bgJ/bgJ) mice showed metastasis of human CEM-C7 cells to the brain and other organs (Christianson *et al.*, 1996). These studies further demonstrate that NK cells, in the absence of an adaptive immune system, function in resistance to growth and metastasis of human lymphoma cells.

iii. Occurrence of Leukemia and Lymphoma in mice transgenic for growth factors that utilize the common γ_c as a part of their receptor: IL-2, IL-7, IL-9, and IL-15.

IL-2: Baldassarre et al. found an increased expression of IL-2 and IL-15 proteins and their receptors in natural killer (NK)-T/NK cell lymphomas starting from 12 months of age in mice transgenic for a truncated high mobility group protein I-C construct (HMGI-C/T) (Baldassarre *et al.*, 2001). Lugasi et al described a spontaneously occurring BALB/c-derived murine T-cell leukemia that constitutively overexpresses the IL-2 receptor and also produces IL-2 (Lugasi *et al.*, 1990). The autocrine loop may have played a role in the initial steps of leukemogenesis. As discussed below, for the IL-7 transgenic mice, early overproliferation in the lymphoid cells that must undergo repair of double strand breaks for maturation can predispose them to aberrant rearrangements with translocation, generating a malignant lymphoma.

IL-7: Fisher et al observed a high incidence of severe lymphoproliferative disease in a newly generated strain of mice carrying the murine IL-7 transgene under the control of the E alpha (MHC class II) promoter (Fisher *et al.*, 1993). Cells from lesions in these mice showed the selective expansion of cells at an early stage of B cell development. Barata et al showed a causative link between IL-7-mediated proliferation and p27kip1 down-regulation to drive cell cycle in malignant T cells (Barata *et al.*, 2001). In the presence of IL-7, T-ALL cells up-regulated bcl-2 expression to escape apoptosis. They also displayed enhanced activation of cyclin-dependent kinase (cdk)4 and cdk2 and hyperphosphorylation of Rb, to drive them

continually through the cell cycle (Barata *et al.*, 2001). Valenzona et al further characterized the B lymphoid tumors that develop in IL-7 transgenic mice (Valenzona *et al.*, 1996). In the marrow of IL-7 transgenic mice, the number and proliferative activity of cells in each of the pro-B and pre-B cell populations were markedly increased, with mature B lymphocytes increased to a lesser extent. These results demonstrate that overexpression of IL-7 causes excessive proliferation of a wide range of precursor B cells in marrow and spleen. Such prolonged stimulation at early stages of B cell development, prone to genetic errors due to Ig gene rearrangement, was proposed to be the factor which predisposes the cells to neoplastic transformation.

IL-9: Approximately 7% of transgenic mice overexpressing the interleukin 9 gene developed thymic lymphomas at the age of 3-9 months (Renauld *et al.*, 1994). The tumor cells were clonal with unique T cell rearrangements and expressed both CD4 and CD8. The incidence of malignancy suggested that there was a requirement for additional transforming events. Indeed, the transgenic animals were highly susceptible to injections of low doses of N-methyl-N-nitrosourea, a chemical carcinogen with a thymic tropism. The authors proposed that dysregulated IL-9 expression could be involved in the development of some T cell malignancies, causing initial hyperproliferation followed by a “second hit” or transforming translocation during TCR gene rearrangement (Renauld *et al.*, 1994).

To support the “second hit” hypothesis, Lange et al (Lange *et al.*, 2003) used IL-9 transgenic mice and corresponding wild-type mice (FVB/N) and transplanted them with bone marrow cells transduced to express NPM-ALK, which is the t(2;5)(p23;q35) translocation product resulting from fusion of the nucleophosmin (NPM) gene to the anaplastic lymphoma kinase (ALK) gene. The NPM-ALK protein is expressed in over 50% of anaplastic large-cell lymphoma (ALCL), which comprises approximately 25% of all non-Hodgkin lymphomas (NHL) in children and young adults, and up to 15% of high-grade NHL in older patients. IL-9 transgenic mice, serving as a control group, received pLXSN (vector only)-infected marrow.

The combined overexpression of NPM-ALK and IL-9 led to the transformation of murine lymphoid cells with accelerated and enhanced development of T-LB in 46% of the mice, which only very rarely occurred in IL-9 transgenic mice only, and in the controls. 33% of the mice developed plasmacytic/plasmoblastic neoplasms, which can share many features with anaplastic/plasmoblastic diffuse large-B-cell lymphoma (Lange *et al.*, 2003). Since IL-9 is known to be expressed at high levels in some cases of human ALCL, its overexpression is thought to drive early lymphoid cells to divide rapidly and this can allow the t(2;5)(p23;q35) translocation to occur in some cases, generating lymphoma.

IL-15: Fehniger et al. discovered that the fatal leukemia that invariably occurs in interleukin 15 transgenic mice follows early expansions in natural killer and memory phenotype CD8⁺ T cells. The target cells that were transformed in the IL-15 transgenic mice had a T-NK phenotype (Fehniger *et al.*, 2001). The transgenics had early overproliferation in natural killer (NK) and CD8⁺ T lymphocytes. Later, these mice invariably developed a fatal lymphocytic leukemia with a T-NK phenotype (Fehniger *et al.*, 2001). These data again provided evidence that leukemia can arise as the result of chronic stimulation of early lymphocytes by a growth and expansion - inducing cytokine.

Summary:

- The studies briefly described in this section demonstrate that the incidence of leukemia and lymphoma are significantly higher in immune deficient mice than in their normal counterparts.

- Factors that predispose cells to chromosomal rearrangements such as defects in DNA repair pathways, or mutagens, have a much higher chance of causing a neoplastic event that cannot be eradicated in immune deficient mice than in normal mice.
- Natural Killer cells are critically important in detecting and eradicating neoplastic cells as they arise, and in preventing their spread throughout the body.
- Finally, driving T or B cells to proliferate more rapidly than normal by overstimulation of growth factor pathways initially causes a lymphoproliferative syndrome that, when coupled with a transforming event, can easily become an aggressive malignant disease in immune deficient mice.

V. Recommendations. This is only a draft

The observations noted above lead to consider the following recommendations.

- Mice investigated in disease models should be extensively followed up; the classic 4 month follow-up is minimally informative with respect to toxicities; authors should strive to follow cohorts of mice for at least one year and this should be highly valued by reviewers and editors; papers should clearly indicate the number of mice and duration of follow-up (these parameters are often difficult to obtain).
- Hematological analyses and necropsies should be systematically performed in long-term hematopoietic chimeras (extent to define-should a standard be recommended).
- A proportion of treated animals should be maintained for at least one year and followed with standard hematological measurements including a CBC and white blood cell differential. It would be reasonable to request that pre-clinical studies used to support proposed gene therapy trials perform a set of standard hematological analyses.
- Integration sites should be monitored, at least in long term chimeras. Now that the mouse genome is publicly available, the determination of insertion location should become part of the experimental plan in selection studies. At the least, DNA should be stored from all animals at sacrifice so that analysis can be performed in the setting of unexplained adverse events.
- The integration sites should be related to target cell type, transduction conditions, clonal expansion, duration of transgene expression, and toxicities.
- Investigators in the field of gene therapy should share and centralize the information with the goal of establishing a large database of integration sites to assess the frequency of retroviral integration sites and their risk.
- Therapeutic genes should be tested in relevant disease models.
 - Preclinical animal studies should appropriately mimic the human trials. The experiments should attempt to use age appropriate animals, lower frequencies of gene transfer, and eventually without myeloablation.
- Novel transgenes should be tested in transgenic mice as well as in hematopoietic chimeras. The effect of increased expression of the gc chain in the development of the T-cell leukemias is not known. More studies are needed to determine the need for tighter regulation of g-c expression (temporal regulation, level).

Table 1.

| Author | Year | Journal | vector | Transgene and control (# mice) | Total # Mice+ | Transcriptional control element | Primary transplant (#mice), time exam. | Secondary transplant (#mice), time exam. | leukemia,lymphoproliferation; other; RCR status |
|-------------|------|--------------------------|--------------------|--|---------------------|------------------------------------|---|---|--|
| Sabatino D, | 2000 | PNAS | Mo-MuLV? | hu γ -globin gene | 10 | ankyrin-1 promoter | (10) 16 wks | N/A | no |
| Sauvageau | 1995 | Genes and Development | MSCV2.1 | HOXB4 (53), Neo (34) | 87 | MSCV LTR(HOXB4), PGK (Neo) | (7) Neo, (7) HOXB4 12-20wks | (17)Neo,(13)HOXB4,12wks (4) Neo,(16) HOXB4,20wks Note: Tertiary transplant (6) Neo, (17)HOXB4, 5wks? | see comment N°1 |
| Sawai | 2001 | Mol Ther | MSCV | P140K MGMTires EGFP | 48 | MSCV LTR | (36) 12 wks (9) 20wks | (3) 14wks | no, RCR- |
| Schiffmann | 1995 | Blood | LN (LG) | hu glucocerebrosidase | 7 | Mo-MuLV LTR | >6-9 months | N/A | see comment N°2 |
| Shah | 2002 | J Immunol | MSCV | TGFbR IIDNires GFP | | MSCV-LTR | | | dramatic expansion of myeloid cells, primarily monocytes/macrophages |
| Shah | 2002 | Cancer Res | MSCV | TGFbR IIDNires GFP(15) GFP (15) | 30 | MSCV-LTR | 3- 4 months | N/A | no |
| Sorrentino | 1995 | Blood | Ha-MuSV Mo-MuLV | HaMSV-hu MDR1(16) MoMuLV-huMDR1 (4) | 20 | HaMSV-LTR MoMuLV-LTR | (16) 8wks, (4) 8 months | | no, RCR- |

| Author | Year | Journal | vector | Transgene and control (# mice) | Total # Mice+ | Transcriptional control element | Primary transplant (#mice), time exam. | Secondary transplant (#mice), time exam. | leukemia,lymphoproliferation; other; RCR status |
|------------------|------|-------------|----------------------------|---|------------------|------------------------------------|---|---|---|
| Spain | 1992 | PNAS | Mo-MuLV? | Neo | 7 | Mo-MuLV-LTR? | (7)12 wks | N/A | no |
| Spencer | 1996 | Blood | Ha-MSV | L22Y-DHFR (16),F31G-(1) MDR (9), ± Neo | 26 | Ha-MSV-LTR ± TK promot-Neo | 26 to 30 wks | N/A | see comment N°3 |
| Tahara-Hanaoka | 2002 | Exp Hematol | pCS-CG ±WPRE | GFP (29) | 29 | CMV promoter | (20) 4-8 months | (9) 12wks | no |
| Takebe | 2001 | Mol Ther | SFG-MPSV | ALDH-IRES-F/S(17) ALDH-IRES-Neo(14) | 31 | MPSV 3'LTR | 50 days | 11days | no |
| Thorsteinsdottir | 1999 | Blood | MSCV2.1 | HOXB4 (25), Neo (6) | 31 | MSCV LTR(HOXB4), PGK (Neo) | (10) 32 to 52 wks | (21) 12 to 15 wks | see comment N°1 |
| Vassilopoulos | 2001 | Blood | hu foamy virus (HFV) | Alkaline phosphatase AP(17) GFP (8) | 25 | MSCV (AP) Mo-MuLV (GFP) | (22) 1-6 mnths | (3) 4-8 wks | no |
| Villeval | 1997 | Blood | MPZen | TPO (62), Neo (>8) | >70 | MPSV-LTR | (40) 3-10 mnths | (30) 10-12 mnths | see comment N°4 |
| Wahlers | 2001 | Gene Ther | pSHX (SFa11GFP) | EGFP (14), d2EGFPm (6) | 20 | MPSV Enh | (8) 13wks (12) 9-11 wks | N/A | no |
| Whartenby | 2002 | Blood | | FasL | | | | | |
| Yan | 1995 | Blood | MSCV 2.2 | MGDF-PGKNeo(117) | >152 | MSCV LTR | (25) 7-16wks | (40) 7-18 wks | see comment N°5 |

| Author | Year | Journal | vector | Transgene and control (# mice) | Total # Mice+ | Transcriptional control element | Primary transplant (#mice), time exam. | Secondary transplant (#mice), time exam. | leukemia,lymphoproliferation; other; RCR status |
|--------|------|-------------|--------|-----------------------------------|---------------------|------------------------------------|---|---|---|
| | | | | PGK-Neo (>35) | | | | | |
| Yan | 1999 | Exp Hematol | MSCV | c-mpl (58), neo (36) | 94 | MSCV LTR | 16 weeks | N/A | see comment N°6 |

Comments:

1. In mice overexpressing HOXB4 (MSCV-HOXB4) after retroviral transduction of BM, Sauvageau et al (Genes Dev, 9: 1753, 1995) specifically mentioned that they have not seen any leukemic transformation in their mice after 1 year observation and despite persistent level of high HOXB4 in at least 53 mice. Thorsteinsdottir et al (Blood, 94:2605, 1999) also report no evidence of leukemia in at least 25 mice despite persistent HOXB4 over-expression for up to 52 weeks.
2. In non ablated mice overexpressing human glucocerebrosidase (Mo-MuLV based LG vector) after retroviral transduction of BM, Schiffman et al (Blood, 86:1218, 1995) report that 5 out of 11 mice show lymphocytic cuffs in lungs from repeated transplantation .
3. In mice overexpressing hu-MDR1 and L22Y-DHFR (Ha-MSV based vector) after retroviral transduction of BM, Spencer et al (Blood, 87:2579, 1996) report that 3 out of 6 MDR⁺ mice and 3 out of 5 L22Y⁺ died from gastrointestinal bleeding and neutropenic sepsis after treatment with TMTX.
4. In mice overexpressing TPO cDNA (MPZen-TPO) after retroviral transduction of BM, Villeval et al. (Blood, 90: 4369, 1997) found hyperplasia of megakaryocytes and granulocytes in the spleen and BM and hypoplasia of erythrocytes in the BM (7-9 weeks posttransplant). From 10 weeks posttransplant and thereafter, WBC, platelets and RBC numbers declined dramatically. The absolute numbers of progenitors were very low in the spleen and BM. Extramedullary hematopoiesis was observed in several organs. The mean survival was 7 months (mice died of pancytopenia). **Notably, two mice (2/32) died between 3 and 4 months transplant with a leukemic transformation. This disorder was transplantable into secondary recipients who developed an attenuated form of the disease similar to the one previously described (Yan et al, Blood 86: 4025, 1995). The blasts could not be identified (B220-, Mac-, CD4-, CD8-, 4A5-, Gr1-, Ter119-)**
5. In mice overexpressing MGDF (MSCV-MGDF-PGK-Neo) after retroviral transduction of BM, Yan et al (Blood, 86:4025, 1995) found that the platelets levels increase 4 to 8 fold above normal baseline levels and remained elevated in these animals, which are alive and well at more than 4 months transplantation. Increased megakaryocyte numbers were detected in BM, spleen, liver and lymph nodes. Prolonged overexpression led to decreased marrow hematopoiesis with a shift to extramedullary hematopoiesis in the spleen and liver. All mice developed myelofibrosis and osteosclerosis. **No significant effect on other hematopoietic lineages was found in these overexpressing MGDF mice.**

6. In mice overexpressing c-mpl (MSCV-c-mpl) after retroviral transduction of BM, Yan et al (Exp Hematol, 27:1409, 1999) 75% of the mice died by week 16 due to decreased numbers of MK-CFC and thrombocytopenia. 25% survivors were anemic and thrombocytopenic.

Table 2.

| Author/Journal/Year | Transgene | Control | # | duration | Reported AEs |
|---------------------------------|----------------------------|------------------------|---------|-----------------|--------------|
| Ohoshi PNAS 92 | G.C. | MFG | 20 | 5mo | No |
| Pawliuk Blood 94 | CD24 Neo | MPSV tk | 25 | 1-4 mo | No |
| Kang J Immunol 94 | Mtv-7 sag | b-actin | 73 | 3 mo | No |
| Riviere PNAS 94 | ADA | Moloney MPSV CMV | 93 | 7mo-1yr | No |
| Lieht Blood 95 | MDR1 | Harvey | 22 | 9 weeks | No |
| Moritz Blood 96 | Neo HADA | Tk PGK | 47 | 1 year | No |
| Maze PNAS 96 | MGMT | PGK | 61 | 4mo | No |
| Raftapoulous Blood 97 | B-globin Neo | Moloney PGK | 33 | 4mo Some 8mo | No |
| Maze J Immunol 97 | MGMT | PGK | 42 | 5mo | No |
| Lange Gene Ther 97 | HyTk | Moloney | 50 | 12mo | No |
| Robbins PNAS 98 | Neo | Moloney | 176 | 2-4mo | No |
| Raftapoulous Ann NY Acad Sci 99 | B-globin Neo | LCR PGK | 33 | 4-9mo | No |
| Okimoto J Virol 99 | Neo B-gal | Moloney SV40 | 5 | .5 mo | No |
| Morel Hum Gen Ther 99 | RevM10 Anti-Pol NGFr | Moloney IRES | 32 | 4mo | No |
| Michaels Human Gen Ther 99 | MDR | PGK | 165 | 9mo | No |
| Lorincz JBC 99 | Glucoronidase GFP | Moloney | No data | 2 mo | No |
| Kume Stem Cells 99 | GFP Neo | CMV Sra CAG | 13 | .5 mo | No |
| Relander Exp Hem 00 | GFP NGFr | Moloney IRES | >52 | 4mo | *1 |
| Ragg Can Res 00 | MGMT EGFP | MSCV IRES | 40 | 4mo | No |
| Patel Blood 00 | HENT2 EGFP DHFR | MSCV IRES | >28 | 3mo | No |
| Onai Blood 00 | SDF-1 GFP | Moloney | No data | No data | No |
| Lung Blood Cells Mol Dis 00 | g-globin Neo | HS40 LTR (Mol) | 4 | 4mo | No |
| Kume Gene Ther 00 | EGFP Neo CD24 | MSCV PGK IRES | 17 | 6mo | No |

| Author/Journal/Year | Transgene | Control | # | duration | Reported AEs |
|------------------------|-------------------------|-----------------------|--------------|---------------------|--------------|
| Kuhr Exp Hem 00 | IL-2 Neo | Moloney Tk | Not given | Not given (?3mo) | *2 |
| Klug Blood 00 | GFP | MFG | 27 | 6mo | No |
| Kalbeer PNAS 00 | EGFP b-globin | MSCV PGK (PPT) | 7 | 5.5-9.5mo | No |
| Jin Nat Gen 00 | F36V-mpl Neo Egfp | MSCV | 11 | 6mo | No |
| Richard Mol Ther 01 | Ferro- chetalase | HIV Ankyrin/SV40 | 14 | 4mo | No |
| Purton J Hemato 01 | GFP | MND | 56 | Up to 12mo | No |
| Pawliuk Science 01 | Delta-b- globin | Lenti LCR | 16 | 3mo | No |
| Moreau-Gaudry Blood 01 | GFP | HIV LCR | 8 | 3mo | No |
| Kobune Can Res 01 | Fapy GFP | MSCV LTR IRES | 20 | 3mo | No |
| Klump Gen Ther 01 | HoxB4 GFP | MSCV LTR IRES | 15 | Up to 12 mo | No |
| Kang Hum Gen Ther 01 | GFP Neo | Moloney | 30 | 6mo | No |
| Puig Gen Ther 01 | GFP | FMEV | 98 96 | 1mo 5mo | No |
| Pawliuk Mol Ther 02 | Endostatin GFP | MSCV IRES | 28 | 4mo | No |
| May Blood 02 | b-globin GFP | HIV LCR CMV | 15 | 10 mo | No |
| Lotti J Virol 02 | GFP NGFr | HIV cPPT WPRES PGK | Not given | 3mo | No |
| Li Leukemia 02 | HCD34 | Moloney | 12 | 7mo | No |
| Li Science 02 | NGFR | Moloney | 75 | 7mo | *3 |
| Leiming Blood 02 | PPCA | Moloney | Not given | 10mo | No |

*1 Deaths due to emboli at high infused cell dose

*2 Intense lymphocytic liver infiltration seen, attributed to IL-2 expression

*3 Leukemia observed in secondary (n=10) and tertiary (n=8) recipients, insertion in Evi-1 gene

Table 3

| First author, Year, Journal | Transgene ± marker Control transgene | Transcriptional control elements | Primary recipients Number, observation period | Secondary recipients Number, observation period | Reoprted adverse event, Comments |
|--------------------------------|---|-------------------------------------|--|--|-------------------------------------|
| Hannenber, 1996 Nature Med | huADA | huPGK | 19 mice, 6 mo | no | no |
| Dick, 1985 Cell | neoR | MoMLV LTR with mu IgH enhancer | 7 mice, 11-17 wks | no | no |
| Ally, 1995 J Immunol | LCMV gp neoR | MSCV LTR | 14 mice, 3-5 mo 06 mice, 3-5 mo | no | no |
| Bernad, 1994 Br J Haematol | neoR | MoMLV LTR | 04 mice, 5 mo | no | no |
| Dolnikov, 2000 J Hematother | LacZ | MoMLV LTR | 02 mice, 4 mo 01 mouse, 6 mo | 01 mouse, 3 mo | no |
| Corey, 1990 Blood | muDHFR | Friend/Mo-MLV LTR | 24 mice, 24 wks | 30 mice, 10 wks | no |
| Einerhand, 1993 Blood | huADA | MoMLV LTR | 07 mice, 6 mo | no | no |

| First author, Year, Journal | Transgene ± marker Control transgene | Transcriptional control elements | Primary recipients Number, observation period | Secondary recipients Number, observation period | Reported adverse event, Comments |
|--------------------------------|---|--|--|--|---|
| Correll, 1992 Blood | huGC | MoMLV LTR | 04 mice, 10 mo | no | no |
| Chang, 1989 Blood | muIL3 | MPZen (?LTR) | 12 mice, 11 wks | 137 mice, 5 mo 48 mice, 6 mo | fatal but non-neoplastic myelo- proliferation; no tumors |
| Apperley, 1991 Blood | huADA hu ADA, neoR | hu PGK hu PGK, TK | 06 mice, 6 mo 04 mice, 5 mo | no | no |
| Hawley, 1996 PNAS USA | muIL11 | MSCV LTR | 20 mice, ? | II: 12 mice, ? III: 12 mice, 5 mo | no reported tumors see comment 1 |
| Dunbar, 1996 PNAS USA | huMDR1, neoR | MoMLV LTR | 64 W/W ^v mice, 4 mo | no | no |
| Imren, 2002 PNAS USA | huβ-globin | β-globin promoter lentiviral vector | 08 Hbbth1/th1 mice, 6mo | no | no |
| Bodine, 1994 Blood | huMDR1 | MoMLV or HaMSV LTR | 127 mice, 4 mo | no | no |

| First author, Year, Journal | Transgene ± marker Control transgene | Transcriptional control elements | Primary recipients Number, observation period | Secondary recipients Number, observation period | Reported adverse event, Comments |
|--------------------------------|---|-------------------------------------|--|--|---|
| Hawley, 1998 Blood | muf1t3/flk2 ligand with huPGKneoR | MSCV LTR | 18 mice, 12 mo | 24 mice, 6 mo | tumors in all long-term primary recipients; see comment 2 |
| Clapp, 1995 Blood | neoR and LacZ | ? MLV LTR | 03 rats, 6-20 mice | no | fetal liver transduction in situ no AE reported |
| 2002 Blood | FANC-A FANC-C | CMV IE E/P lentiviral vector | 06 mice, 8 mo 06 mice, 8 mo | 05 mice, 2 mo 05 mice, 2 mo | no |
| Halene, 1999 Blood | eGFP with SV40neoR | MND LTR (MPSV-based) | 33 mice, 8 mo | 15 mice, 2 wks | 11 from 5 mice after 2-6 mo no reported AE |

Table 4. Stem cell selection

| Study | Target | Transduction | Transplant | # animals (test/control) | Observation time | hematological analysis | adverse events |
|----------------------------------|--------|---|------------------|-------------------------------------|------------------|---|-----------------------------|
| DHFR | | | | | | | |
| (Williams, Hsieh et al. 1987) | bm | Ampho./5fu/Wehi/co-cult./poly. | 1400 cGy | 24/12 | 56 days | CFC/bm cellularity | Expected MTX related deaths |
| (Corey, DeSilva et al. 1990) | bm | Ampho./5fu/Wehi/co-cult./poly. | 1150 cGy | 24/24 2°-30/30 | 60 to 72 days | CFC/Hct | Expected MTX related deaths |
| (Zhao, Li et al. 1994) | | | | | | | |
| (Li, Banerjee et al. 1994) | bm | Ampho./5fu/co-cult. | 900 cGy | 17/15 | 12 weeks | Southern/CFC | MTX associated deaths |
| (Spencer, Sleep et al. 1996) | | eco/5fu/3/6/co-cult | W/W ^v | 23 | 30 weeks | CBC/retic. | TMTX associated deaths |
| (Allay, Persons et al. 1998) | bm | eco/5fu/s/3/6/co-cult | Lethal radiation | 12/15 11/9 19/9 2° – 18/12 | 225 days | CD24 and GFP expression/CBC CFUs 2° transplants | TMTX associated deaths |
| (Havenga, Valerio et al. 1999) | bm | Ampho./3/IL1a/co-cult./poly. <i>Multiple infusions of bm cells</i> | 925 | 120/40 | 6 months | CBC/expression/CFC | Expected deaths from MTX |
| MDR | | | | | | | |
| (Podda, Ward et al. 1992) | bm | Eco/5fu/6/S/ co-cult /poly | 975 cGy | 13 | 14 m | PCR/Flow | None |
| (Sorrentino, Brandt et al. 1992) | bm | eco/5fu/s/3/6/co-cult | W/W ^v | 35/22 | 4 –8 m | Neutrophil/PCR/Southern | None |

| Study | Target | Transduction | Transplant | # animals (test/control) | Observation time | hematological analysis | adverse events |
|---|-----------------|--|------------------------------------|--------------------------------------|-----------------------------------|----------------------------------|--------------------------------------|
| (Hanania and Deisseroth 1994) | bm | Ampho/5fu/3/6/co-cult/poly | 900-1000 cGy | ~26 | 7 weeks, 6 successive transplants | Wbc | Chemotherapy deaths in control group |
| (Hegewisch-Becker, Hanania et al. 1995) | bm | ampho/5fu/3/6/co-cult | | 5 | 4 weeks | Wbc/flow cytometry | none |
| (Sorrentino, McDonagh et al. 1995) | bm | eco/5fu/s/3/6/co-cult./poly. | W/Wv | 13? | 11 weeks | rtPCR | None |
| (Licht, Aksentijevich et al. 1995) | Lin-MHCII-sca1+ | Eco/3/6/S/IL-1 β /co-cult. X 6 - 8 days | 350 cGy/SCID | 30 12 2 $^{\circ}$ transplants | 6 months | PCR/Flow | None |
| (Bunting, Galipeau et al. 1998) | Bm | eco/5fu/s/3/6/co-cult <i>expanded x 12 days</i> | No conditioning Or 1000 rads | 56 | 14 months | Cbc/morphology/flow/ southern | Myeloproliferative disorder/leukemia |
| (Qin, Ward et al. 1999) | bm | 3/6/S x 24hr 3/6/S/supe./poly x24 hrs | 100 cGy | 42 | 160 days | Pcr of cfu | none |
| (Bunting, Zhou et al. 2000) | bm | Eco./S/3/6/co-cult/poly. | 1100 cGy | 43 | 25 weeks | CBC/Southern/side pop. analysis | Myeloproliferative disorder |
| (Licht, Goldenberg et al. 2000) | bm | Ampho or eco/5fu/S/3/6/IL1 β /co-cult/poly Ex vivo selection | 900 cGy | 65/7 | 45 days | Flow expression/CFC | Deaths related to ex vivo selection |
| (Carpinteiro, Peinert et al. 2002) | bm | Ampho./5fu/s/3/6/co-cult. or supe. | 950 cGy | 30/30 | 36 days | CBC/CFU | Deaths from paclitaxel toxicity |
| MGMT | | | | | | | |
| (Allay, | bm | eco/5fu/s/3/6/co-cult | 1050 | 32 | 54 weeks | AGT activity/CFC | Expected deaths |

| Study | Target | Transduction | Transplant | # animals (test/control) | Observation time | hematological analysis | adverse events |
|------------------------------------|--------|------------------------------|------------|--------------------------|------------------|---|---|
| Dumenco et al. 1995) | | | | | | | from BCNU |
| (Allay, Davis et al. 1997) | bm | eco/5fu/s/3/6/co-cult | 1050 | 6/8 | 17 weeks | PCR/CFC/AGT expression | Expected deaths from BCNU |
| (Davis, Reese et al. 1997) | bm | eco/5fu/s/3/6/co-cult/prot. | 1040 | 12/9 | 120 days | PCR/CFC/AGT expression | Expected deaths from BCNU |
| (Chinnasamy, Rafferty et al. 1999) | bm | Ampho/5fu/S/3/6co-cult/poly. | 1520 | 15/15 | 5 weeks | CFU-S/CFC/smear | None |
| (Davis, Koc et al. 2000) | bm | eco/5fu/s/3/6/co-cult/prot. | BG/BCNU | 78/19 | 6 months | CBC/CFC/ AGT expression | Expected deaths from BCNU |
| (Sawai, Zhou et al. 2001) | bm | eco/5fu/s/3/6/co-cult | 1100 cGy | 39/6 | 18 weeks | GFP expression/CBC,ANC, BM cellularity, myeloid progenitors | Deaths from TMZ/BG toxicity at high doses |
| CD | | | | | | | |
| (Eliopoulos, Bovenzi et al. 1998) | bm | eco/5fu/wehi/co-cult | 1050 | 37/6 | 13 months | PCR/hCD activity | none |
| ADH/DHFR | | | | | | | |
| (Takebe, Zhao et al. 2001) | bm | Ampho/5fu/S/3/6/co-cult | 950 | 14/14 2° 6/6 | 50 days | CBC/weight | Expected deaths from chemotherapy |

Table 5. Amplification strategy

| Study | Target | Transduction | Transplant | # animals | Observation time | hematological analysis | adverse events |
|---|--------|-----------------------------|---|----------------------------------|------------------|---------------------------------------|----------------|
| <i>CID</i> | | | | | | | |
| (Jin, Zeng et al. 2000) | bm | eco/5fu/s/3/6/co-cult/poly. | 1050 cGy | 15/4 2° 10 | 6 months | CBC/CFC/Flow cytometry | none |
| HoxB4 | | | | | | | |
| (Sauvageau, Thorsteinsdottir et al. 1995) | bm | Eco/5fu/s/3/6/co-cult/poly. | 950 cGy | 7/7 CRU:33/32 2° CRU:27/13 | 20 weeks | CFC/CFU-S/CRU | none |
| (Antonchuk, Sauvageau et al. 2001) | bm | eco/5fu/s/3/6/co-cult/prot. | 900 cGy or 450 cGy/ W ⁴¹ | 75/16 | 8 months | Lineage analysis by flow/CRU/Southern | none |
| (Klump, Schiedlmeier et al. 2001) | bm | VSV/5fu/s/3/6/poly | 1000 cGy | 5/5 | 52 weeks | cbc | none |

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Table 6

| # tumors | type of tumor | total # insertions | # of insertions common to 2 or more tumors | # of loci with common insertions | Reference |
|----------|--------------------------------------|--------------------|--|----------------------------------|----------------------|
| ~300 | Lymphoid (B- and T-cell) and myeloid | 1336 (1 Lmo-2) | 493 (41%) | 152 (none Lmo-2) | Suzuki et al. 2002 |
| 107 | Lymphoid | 217 | ND | 31 (none Lmo-2) | Hansen et al. 2000 |
| 107 | Lymphoid and myeloid | 126 | ND | 41 (none Lmo-2) | Joosten et al., 2002 |

Table 7

| Vector | Donor | Recipient | conditioning | # animals | Length of follow up | Adverse Events | reference |
|---------|--------------------|---|--------------|-----------|---------------------|----------------|-----------------------------|
| MPSV | g _c -/Y | g _c -/Y | irradiation | 8 | 6-10 weeks | 0 | Lo et al., 1999 |
| none | g _c -/Y | g _c -/Y | irradiation | 4 | 6-10 weeks | 0 | Lo et al., 1999 |
| MFG | g _c -/Y | g _c -/Y | irradiation | 7 | 8-12 weeks* | 0 | Soudais et al., 2000 |
| | | g _c -/Y secondary transplant | irradiation | 8 | 24 weeks | 0 | Soudais et al., 2000 |
| MND | g _c -/Y | g _c -/Y | irradiation | 8 | 24-32 weeks | 0 | Otsu et al., 2000a |
| MND | g _c -/Y | g _c -/Y | irradiation | 8 | 18-22 weeks | 0 | Otsu et al. 2000b |
| MND | g _c -/Y | g _c -/Y | None | 12 | 12 weeks | 0 | Otsu et al., 2000b |
| Various | g _c -/Y | g _c -/Y | irradiation | 33 | 16-28 weeks | 0 | Aviles Mendoza et al., 2001 |

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Appendix 1: Organization of review

Review of gene therapy data in murine models of SCID

Reviewer: Brian Sorrentino

Summary

Study analysis and conclusions

Overall recommendations

Review of gene therapy data in marking studies in murine hematopoietic chimeras, as well as gene therapy data in non-lymphoid murine disease models

Reviewers: Michel Sadelain (w/John Tisdale and Isabelle Riviere)

Objectives:

Indicate database or other data source, key words, years covered

Experimental summary: Transgene, transcriptional control elements

Number of animals, length of observation

Insertion site analysis, adverse events

Study analysis and conclusions

Overall recommendations

Review of tumorigenesis in transgenic and naturally occurring immune deficient mice

Reviewer: Jan Nolte

Summary

Study analysis and conclusions

Overall recommendations

Review of stem cell expansion in murine hematopoietic chimeras

Reviewers: C. Anthony Blau, Robert Richard

Summary

Study analysis and conclusions

Overall recommendations

Review of murine models of insertional mutagenesis leading to hematological malignancies

Reviewer: David Bodine

Summary

Study analysis and conclusions

Overall recommendations

Review of leukemogenesis/lymphomagenesis in transgenic mice and hematopoietic chimeras induced by oncogenes and fusion genes

Reviewer: Pier Paolo Pandolfi

Experimental summary (Excel spread sheet or Table)

Transgene, transcriptional control elements

Number of animals, length of observation, time to overt disease

Study analysis and conclusions

Overall recommendations