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Challenges in Vector and Trial Design Using Retroviral Vectors for Long-Term Gene Correction in Hematopoietic Stem Cell Gene Therapy

Retroviral and Lentiviral Vectors for Long-Term Gene Correction: Clinical Challenges in Vector and Trial Design

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Over the past two decades, incredible progress has been made using gene therapy for inherited severe immunodeficiency disorders, such as X-linked severe combined immunodeficiency disorder (SCID-X1) and adenosine deaminase deficiency–severe combined immunodeficiency disorder (ADA-SCID).[1–3] However, for reasons that remain unclear, gene transfer for SCID-X1 has also been associated with some cases of vector-induced leukemia whereas no cases have been seen in the ADA-SCID trials despite the common use of γ-retroviral vectors. The first case was reported in a French gene transfer trial for SCID-X1.[4] Over the next six years, an additional three cases were reported in that trial and one in a second SCID-X1 trial that enrolled a combined total of 20 subjects.[2] Unfortunately, genotoxicity would not remain confined to SCID-X1. Recent reports of insertional mutagenesis leading to myelodysplastic syndrome in a trial for chronic granulomatous disease and a case of leukemia in a trial for Wiskott-Aldrich syndrome (WAS), both of which used γ-retroviral vectors, underscored that this type of toxicity can also apply to other disease settings.[5–7] In all these cases, insertion of the γ-retroviral vector near known proto-oncogenes led to enhancer-mediated expression of these proto-oncogenes.

To address this toxicity, investigators have developed new vectors that, in experimental models, can achieve long-term gene correction with less risk of insertional mutagenesis. The field is also seeking to develop new animal models and *in vitro* assays that can accurately predict whether these vectors will indeed reduce the risk of insertional mutagenesis.

In light of these innovations and new trials, the National Institutes of Health (NIH) Office of Biotechnology Activities, in partnership with the NIH Recombinant DNA Advisory Committee and the European Network for the Advancement of Clinical Gene Transfer and Therapy (CliniGene; http://www.clinigene.eu) hosted a conference in December 2010 to examine new developments in this field so as to inform the scientific community, regulators, and the public. The focus of the conference was not only on the scientific advances but also on the potential development of

Table 1 Clinical experience with vectors

Speaker	Disorder	Vector type	Conditioning	Result	IO	Other outcomes
Malech	SCID-X1 (infants)	γ-RV	None	Most with normal T-, low-normal B-, and NK-cell reconstitution	5/20 T cell LPD LMO2	17 subjects with good growth and development; 2 deaths[1,21,52]
	SCID-X1 (children)	γ-RV	None	Minimal engraftment gene-marked cells	0/5	Minimal; immune improvement
	SCID-X1 (infants)	SIN γ-RV SIN LV	None	Opening 2011–2012	–	–
	Chronic granulomatous disease	γ-RV	None	Low frequency, transient oxidase (+) neutrophils for a few months	0	Ref. 53
	Chronic granulomatous disease	γ-RV	Busulfan (medium)	>10% oxidase-positive neutrophils	2/2 MDS *Evi1*	Infections cleared; one death[6,8]
Aiuti	ADA-SCID (Milan)	γ-RV	Busulfan (low)	Multilineage marking (0.1–10% myeloid cells) polyclonal T-cell reconstitution, low-normal B- and NK-cell reconstitution; ADA metabolic detoxification	0/17	15 subjects off PEG-ADA, good growth and development; no deaths; 3 delayed neutrophils recovery[3] (A. Aiuti, unpublished data)
	ADA-SCID (London)	γ-RV	Melphalan (low) or busulfan (low)	Multilineage marking (0.1–10% myeloid cells) polyclonal T-cell reconstitution, low-normal B- and NK-cell reconstitution; ADA metabolic detoxification	0/6	4 subjects off PEG-ADA; good growth and development[54]
	ADA-SCID Los Angeles/Bethesda	γ-RV	4 with no conditioning 14, busulfan (low)	Multilineage marking polyclonal T-cell reconstitution, low-normal B- and NK-cell reconstitution; ADA metabolic detoxification	0/18	4 subjects no conditioning: minimal gene marking; 3/6 with busulfan, off PEG-ADA (F. Candotti, unpublished data)
	Wiskott-Aldrich syndrome	LV	Busulfan (medium); fludarabine	Stable gene marking over first 6 months	0/1	Treated recently
Naldini	Metachromatic leukodystrophy	LV	Busulfan (full)	Stable gene marking over first 6 months	0/1	Treated recently
von Kalle	Wiskott-Aldrich syndrome	γ-RV	Busulfan (medium)	Immune reconstitution, platelet recovery	1/10 T cell LPD *LMO2*	Ref. 55
	X-adrenoleukodystrophy	LV	Busulfan (full); cyclophosphamide	Neurological stabilization; polyclonal gene marking	0/2	Ref. 8
Leboulch	β-thalassemia	LV	Busulfan (full)	Transfusion independent; clonal dominance	1/2 *HMG2A*	Ref. 10

ADA-SCID, adenosine deaminase deficiency–severe combined immunodeficiency disorder; IO, insertional oncogenesis, frequency, affected lineage, implicated *trans*-activated cell gene; LPD, lymphoproliferative disorder; MDS, myelodysplastic syndrome; PEG-ADA, pegademase bovine; SCID-XI, X-linked severe combined immunodeficiency disorder.

predictive assays, regulatory oversight challenges, and ethical questions regarding the design of new trials with these vectors. This article reviews the key issues raised during that conference. (The presentations and the webcasts for the conference are available at http://oba.od.nih.gov/rdna/rdna_symposia.html#CONF_003h.)

Clinical experience with retroviral vectors for long-term gene correction and insertion-site analysis

Some of the earliest trials using γ-retroviral vectors for long-term gene correction focused on the severe combined immunodeficiency disorders including ADA-SCID and SCID-X1 and on the neutrophil oxidase–deficiency chronic granulomatous disease (**Table 1**).

Review of these γ-retroviral clinical trials demonstrated that gene transfer has the potential to provide long-term gene correction that is comparable to alternative therapies, such as bone marrow transplant (BMT), and—depending on the disease, the age of the patient at diagnosis, and the availability of alternative therapy—gene transfer may prove equally efficacious with potentially less toxicity. However, the factors leading to development of genotoxicity from integration of the vector in a particular trial or subject are not completely understood.

Early data on trials using lentiviral vectors were also discussed. Lentiviral vectors are being used in trials for leukodystrophies, including adrenoleukodystrophy (ALD), metachromatic leukodystrophy (MLD), and WAS. In MLD, a defect in the enzyme arylsulfatase A leads to impaired development of the myelin sheath that insulates nerve fibers. ALD, an X-linked disorder, is caused by a mutation in the ALD protein resulting in disruption of myelin

and early death due to demyelination. In 2009, in a trial administering autologous CD34+ hematopoietic cells transduced with a lentiviral vector expressing the ALD protein, two subjects achieved polyclonal reconstitution with 9–14% of granulocytes, monocytes, and T and B lymphocytes containing the transferred *ALDP* gene and that, beginning at 14–16 months, progressive demyelination stopped.[8] Luigi Naldini reported the results of the first treated patient in the MLD trial who received autologous CD34+ cells transduced with a self-inactivating (SIN) lentiviral vector containing arylsulfatase A complementary DNA following busulfan preconditioning. At 3- to 6-month follow-ups, there was sustained gene marking, and expression of the arylsulfatase enzyme at levels 5- to 10-fold above normal were detected in the peripheral blood cells. Eugenio Montini, via analysis of unique integration sites in that subject, demonstrated polyclonal hematopoiesis and displayed a genomic integration profile similar to that of ALD patients and in human mouse hematochimeras.[9] Alessandro Aiuti presented the preclinical data and rationale supporting a novel clinical trial for WAS using a lentiviral vector encoding WAS protein under the control of its homologous promoter. A clinical trial using lentiviral vector-transduced autologous CD34+ cells combined with reduced-intensity conditioning has started at his institution, and the first subject was treated in June 2010.

Whereas both of these trials reported polyclonal hematopoiesis, another trial, using a lentiviral vector for β-thalassemia, reported the development of an oligoclonal population of vector-containing cells. An HIV-based SIN vector expressing a functional β-globin gene was introduced into hematopoietic stem cells with the goal of correcting deficiencies in hemoglobin production, the underlying defect in β-thalassemia. The first study subject engrafted with the transduced cells has shown improved hemoglobin production and displayed significant clinical improvement.[10] However, a clone carrying the gene transfer vector has become dominant. The clone overexpresses the gene encoding *HMGA2*, a protein that regulates gene transcription. Analyses reveal that cells bearing this insertion express a truncated form of *HMGA2* messenger RNA (mRNA) lacking 3′ binding sites for a regulatory microRNA (miRNA) that arises as a consequence of aberrant splicing to a cryptic splice acceptor site in the vector insulator sequences. In contrast to findings in the earlier γ-retrovirus vector-based SCID-X1 trials, in which five study subjects developed cases of leukemia associated with vector-mediated activation of LMO2, the subject in the β-thalassemia trial has remained healthy for nearly four years. In addition, there is no evidence of other abnormalities, including karyotype changes, abnormal blood counts, or cytology. Aberrant expression of *HMGA2* has been associated with lipomas and some hematological disorders including malignancies.[11,12] However, integrations similar to the one discussed here have also been detected in the SCID-X1 trials in study subjects who remain healthy.[13] The mechanism(s) that led to partial clonal dominance of this clone are not fully understood. A stochastic event related to low transduction of hematopoietic stem cells could be responsible. Alternatively, the integration affecting *HMGA2* may confer a homeostatic advantage to cells. A recent article demonstrated that *HMGA2* overexpression could result in benign hematopoietic stem and progenitor cell expansion in a murine model.[14]

Beyond enhancer-mediated genotoxicity

Although the clonal population of cells detected in the β-thalassemia trial has remained clinically benign, it is illustrative of how vector insertion can affect adjacent cellular gene expression through

Table 2 Non-enhancer-mediated mechanisms by which integrating elements affect expression of adjacent cellular genes

Integrating element	Gene	Outcome	Mechanism	Ref.
MLV	*c-myb*, Notch-1	Leukemia, lymphoma	Produce truncated mRNA; protein with increased stability, activity	56–58
SB	*Braf*, intron 9	Fibrosarcoma	Chimeric transcript produces kinase-only BRAF acting as dominant oncogene	59
SB	*Egfr* C-terminal exon	Hepatocellular carcinoma	Loss of C-terminal regulatory domain of EGFR, resulting in constitutively active receptor	60
MLV	*Gfi1* 3′ untranslated region	Lymphoma	Loss of binding sites for miRNA that negatively regulates Gfi1, increasing expression level	61; analogous to clinical dominance with lentiviral vector integration into the HMGA2 gene in a β-thalassemia subject[10]
Friend MLV, Friend SFFV	*p53*	Erythroleukemia	Insertional inactivation of disrupted tumor suppressor gene	62
MLV	*NF1*	Myeloid leukemia		63
MLV in p19ARF and p53-deficient mice	Variety of putative tumor suppressors	Lymphoma		64
SB	*Nfi1, PTEN*	Peripheral nerve sheath tumors	Disruption of tumor suppressor gene	D. Largaespada (unpublished data)
SB	*Wac*	Colorectal carcinoma		
MLV	*Ikaros*—intron between exons 2 and 3	Lymphoma/acute lymphoblastic leukemia	Expression of truncated dominant negative isoforms	65, 66

non-enhancer-mediated mechanisms. There are essentially four "alternative" mechanisms other than oncogenic activation by which retroviruses and, more recently, transposons have been shown to induce cancer in animal models: alternative splicing, gene inactivation, truncation of cellular mRNA or protein, and miRNA activation.

Drawing from *in vitro* and *in vivo* preclinical models, numerous examples were presented, in which each of the four alternative mechanisms was responsible for transformation by both replication-competent retroviruses and translocating *Sleeping Beauty* (SB) transposons (**Table 2**). In contrast to murine leukemia viruses (MLVs), which primarily cause hematopoietic lineage cancers, genetically engineered SB transposons have been used to induce tumors in many different cell types. Linda Wolff presented several murine models in which infection with replication-competent MLV causes a variety of hematopoietic cancers, including erythroleukemias, myeloid tumors, and T- and B-cell lymphomas/leukemias, by non-enhancer-mediated mechanisms. David Largaespada, using the SB system, provided evidence that alternative mechanisms of oncogenesis are not limited to hematopoietic tumors.

A common theme gathered from research on retroviruses, transposon systems such as SB, and oncogenesis is that it may take multiple hits to different cellular genes (genetic or epigenetic) to convert a normal cell into a tumor cell. Many mice transgenic for a single proto-oncogene have long latency periods for the development of tumors, and crossing two lines carrying "cooperating" oncogenes (e.g., *c-myc* and *Runx2*) can result in more rapid tumor development.[15] Because of the requirement for multiple events, many models, including those discussed by Wolff and Largaespada, incorporate the use of mice with genetic predisposition to particular cancers (e.g., tumor suppressor gene knockouts or transgenics that express different oncogenes), chemical mutagens, or inflammatory agents (e.g., pristane) to accelerate transformation by the integrating elements (retroviruses/transposons). However, malignancies secondary to retrovirus-induced changes still occur in wild-type animals, albeit with lower incidence and longer latency.

Oncogenesis by retroviral gene therapy vectors may also be influenced by age and genetics. Marc Sitbon reviewed MLV leukemogenesis and pointed out that both age and genetic background influence the efficiency and specificity of disease. In humans, individual genetic makeup may also influence the likelihood of developing a tumor after retroviral gene therapy. MLV infection of young (neonatal) animals leads to much more efficient development of leukemias than infection with the same virus in adults. This could reflect the increased availability of target cells with the potential to become leukemic in young animals or to more robust antiviral immunity in older animals. These findings in mice suggest that human gene transfer by retroviral vectors in neonates or young children may entail a higher oncogenic risk than in older subjects. However, younger age may also underlie the better clinical responses that have been seen for gene transfer to hematopoietic stem cells (HSCs) with integrating vectors, such as the infants with SCID.

Lee Ratner discussed human T-lymphotrophic virus type 1, which has direct transforming activity and causes adult T-cell leukemia in humans,[16] and the long latency between infection and oncogenesis. Interestingly, almost all infections that result in leukemia occur early in life through vertical transmission (predominantly breastfeeding),[17] but the time between infection and development of leukemia is typically several decades, and only about 5% of infected individuals ultimately develop adult T-cell leukemia. By contrast, infection of adults is associated with a neurological disease that occurs with a shorter latency. Of relevance to the field of gene transfer is that even when a retrovirus carries genes with transforming potential, the lag between infection and development of a tumor can be decades, again underscoring the importance of long-term follow-up of subjects in gene correction trials. If activation or inactivation of a single cellular gene does not immediately result in development of oncogenesis, tumorigenicity may take years to develop, leading to a false presumption of safety.

Altogether, these observations of the cooperative effects of multiple integrations into different genes have implications for considering oncogenic risk from integrating vectors in gene transfer trials. On the positive side, a single genetic hit (e.g., insertional activation or mutagenesis of one cellular gene) may not be sufficient to induce a tumor cell by itself. Minimizing the numbers of vector integrants per target cell while achieving sufficient percentages of gene-modified cells should be a goal in clinical applications. On the negative side, it has long been recognized that human cancers are the result of multiple genetic changes; dysregulation of cellular gene expression by vector insertion could be an initiating event, and long-term follow-up of subjects may be required.

Approaches to improving design and safety of gene transfer vectors

Following the reports of severe adverse events directly attributable to gene transfer vectors integration, several approaches have been pursued separately or in combination, as follows: (i) genetic insulator elements (GIEs) to act as both enhancer-blockers and boundary against potential silencing; (ii) inhibition of integration by knocking down in target cells lens epithelium-derived growth factor (LEDGF), the cell partner of HIV integrase, which may also have the potential to allow integration to be redirected toward defined genetic regions; (iii) selection of genetic regions as safe harbors for integration; (iv) alternative integration systems with a more random integration profile in human cells, such as foamy virus vectors or nonviral vectors derived from transposons; and (v) inhibition of unwanted expression of the transgene in nontarget cells.

Odile Cohen-Haguenauer reviewed studies of safety modifications in MLV and lentiviral vectors to determine whether new short synthetic transcriptional insulator modules, identified in collaboration with Nic Mermod (University of Lausanne), could inhibit *cis*-acting transcriptional effects.[18] Further studies of a particular GIE that showed sustained and robust expression were undertaken to establish how the system would perform in primary cells. Of note, in a study with David Klatzmann (Paris, France), a strong CD4-specific promoter was constrained by the insulators in human kidney 293 cells, and expression in Jurkat T-cell lines was observed. Comparative high-throughput integration-site analysis

performed with Christof von Kalle in collaboration with Montini, using an *in vivo* genotoxicity assay on *Cdkn2a*$^{-/-}$ tumor-prone mice,[19] provided evidence that this new GIE system might lead to a lower risk of genotoxicity.[20] Insulated lentiviral vectors expressing the Fanconi anemia A complementary DNA are currently being developed. Additional experiments are exploring the retargeting of insulated vectors to transcriptionally inactive chromatin through use of chimeric integrases.[21]

Frederic Bushman gave an update on data his laboratory has accumulated from studies of integration sites using high-throughput 454 sequencing of sequences flanking HIV-1 integration sites. Integrase determines viral integration–site preference, as demonstrated by studies in which substitution of the MLV integrase into HIV-1 vectors resulted in integration profiles similar to those of MLV. Host cell factors are also involved; in particular, knockdown of a transcriptional co-activator LEDGF that binds to HIV-1 integrase drastically reduces titer, and the remaining integration events are not targeted into transcriptional units.[22] In proof-of-principle experiments (with Zeger Debyser and Rik Gjisbers, Katholieke Universiteit Leuven, Belgium) using LEDGF knockdown cells with a chimeric LEDGF fusion to HP1-β that binds heterochromatic regions, integration was redirected to nontranscribing regions.

Naldini elaborated on studies using zinc-finger nucleases to direct integration into safe genomic sites, which may allow robust transgene expression without disrupting endogenous transcription.[23] Initially, *CCR5* and AAVS loci were selected, as zinc-finger nucleases for these targets have been developed and extensively characterized. Integration of an enhanced green fluorescent protein (EGFP) expression vector was targeted to the *CCR5* locus, and when EGFP-positive cells were then sorted, 90% of cells had a site-specific integration. Upregulation of the *CCR5* transcript was observed when phosphoglycerate kinase or spleen focus-forming virus promoters were used in the *CCR5*-targeted integrating expression cassette, and some flanking genes were also upregulated. By contrast, targeting into the AAVS1 site did not result in deregulation of flanking genes, demonstrating locus- and promoter-dependent effects that do not correlate with the strength of the promoter. Ultimately this site-specific gene modification technology may allow for the correction of a mutation at an endogenous locus.

Other vector systems with different integration profiles may also have less mutagenic properties, and Scott McIvor and Thierry VandenDriessche described advances with the transposon vector SB. This bipartite system consists of a source of transposase (typically a plasmid) and a transposon. Although the integration sites for SB are spread uniformly across the genome with no bias toward transcribed genes, some approaches are also in the pipeline to target integration of transposons. For example, the group of Zoltán Ivics (Max Delbrück Center for Molecular Medicine, Berlin) has used a fusion protein that contains a DNA-binding moiety and the SB transposase.[24] In addition, VandenDriessche presented an overview of recent advances with the SB and piggyBac (PB) transposons systems for gene transfer, including the development of a hyperactive transposase, SB100

Table 3 Bioassays of vector safety

Investigator	Model	Type	End point	Strengths	Weaknesses
Baum	*In vitro* insertional mutagenesis	*In vitro*	Clonal growth/fitness	Rapid, predictive	Myeloid-restricted
Sorrentino	Jurkat/targeted *LMO2* insertion	*In vitro*	LMO2 *trans*-activation	Rapid, inexpensive	Only tests one gene/site
Baum	C57BL6J/BMT	*In vivo*	Tumorigenesis/ leukemogenesis	Multilineage	Long time (>1 year), rare event; expensive (costs can be about 2000 Euros per mouse); large *n* needed
Baum, Grez, von Kalle	C57BL6J/BMT	*In vivo*	Clonal skewing	Multilineage, shorter time	Biological meaning of clonal dominance unclear
Sorrentino	SCID-X1 mouse serial transplant	*In vivo*	Tumorigenesis	Disease-specific	Long time (>1 year); expensive (costs can be about 2000 Euros per mouse); high background of tumors; low sensitivity with + control
Montini	Tumor-prone *Cdkn2a*$^{-/-}$ HSPC transplant	*In vivo*	Acceleration of tumor onset	Multilineage; shorter time; statistically validated risk assessment	Background of oncogenesis may hamper the detection of subtle genotoxicity events.
Montini	*Rag*-/γc- mouse; human CD34^{+}	*In vivo*	Integration-site analysis for identification of common insertion sites and clonal abundance estimation	Human cell-based; reproduces with the vector-integration profile described in clinical trials	No oncogenicity readout
Kiem	Canine; nonhuman primate	*In vivo*	Tumorigenesis, long-term multilineage integration-site analysis	Long-term evaluation, multiple lineages, relevant species, disease models, autologous transplant setting, and intact immune system	Cost; duration

(ref. 25). Integration-site analysis of SB100 integrants confirmed no bias of integration into genes. A side-by-side comparison of the SB system with the PB system in HSCs showed that the SB100 system was slightly more efficient than the PB system. However, the PB system has been reported to carry larger transgenes more efficiently. Several disease models and target organs are being examined, including targeting the liver for hemophilia A and B, and hereditary tyrosinemia and human T cells for Fanconi anemia. Hydrodynamic delivery of a factor IX–encoding transposon and SB100 transposase resulted in prolonged and stable expression from the liver in mice. The SB system can also be used to mediate gene transfer into induced pluripotent stem cells, and the cells retain pluripotency to differentiate into neurons, glia, muscle, and hepatocytes.[26]

Naldini presented a novel approach that relies on incorporating an miRNA targeting sequence into the 3′ untranslated region of lentiviral vectors so that the vector mRNA is degraded if expressed in a cell type that expresses the cognate miRNA. Based on a bicistronic vector system to screen candidates, miRNA-126 was defined as specific for HSC expression in murine and human models. Because overexpression of galactocerebrosidase (GALC) in differentiated cells has been proposed for correction of globoid cell leukodystrophy but is limited by the toxicity of GALC in human HSCs, the target sequence for miRNA-126 was incorporated into a GALC-expressing lentiviral vector. While allowing expression in differentiated progeny, as required, GALC expression was successfully prevented in HSCs. This approach resulted in significant survival in a mouse model.[27]

Is it safer? Defining an optimal preclinical testing strategy

One of the key questions for the field is what preclinical testing needs to be done before bringing a new vector into the clinic, and, more specifically, how one can predict the risk of genotoxicity of a new vector. Several of the available *in vitro* and *in vivo* systems and their relative benefits and limitations were described (**Table 3**).

For example, *in vitro* assays using immortalized C57BL/6J bone marrow cells can be used to determine the incidence of mutants based on the number of cells that need to be exposed before development of a transformant.[28] Evaluation of the fitness of the mutant is an important factor. In general, a weaker enhancer in the vector tends to reduce the fitness of the insertional mutants, perhaps by lowering proto-oncogene upregulation levels. This assay has also been used to test the impact of different insulators on development of a clone, and it revealed that, in comparison to the γ-retroviral integration pattern, the lentiviral integration pattern reduces the risk of insertional transformation. When SIN vectors contain cellular promoters that lack strong enhancer activity, such as the intronless promoter fragment of the human elongation factor 1α gene, both γ-retroviral and lentiviral vectors did not induce transformation in this assay. Of note, in this assay the culture conditions and cell density impact the results, requiring standardized operating procedures to obtain reproducible results.

Numerous models have been developed in which murine or human HSCs are transduced and transplanted into mice, allowing for *in vivo* observations. End points are leukemogenesis in the more severe cases or "clonal skewing," in which integrants near certain subclasses of genes involved in cell growth, proliferation, and survival are relatively amplified, which may lead to clonal expansion even to the point of clonal dominance. Although providing the comfort of having performed an *in vivo* assessment, these assays may be of low sensitivity, given that they test only a small fraction of a patient dose, take a long time to perform, and are costly when performed under good laboratory practice (GLP) conditions.

An unanswered question is whether large-animal models would be better able to predict the risk of genotoxicity. Hans-Peter Kiem presented studies in large animals with follow-up of more than five years—in some cases, more than seven years. A major advantage of large-animal studies is the ability to follow gene marking or correction levels in multiple lineages for a very long time. Analysis reveals that both lentiviral and γ-retroviral vectors show increased frequency of integration near proto-oncogenes and also suggested that foamy virus vector may have a more favorable integration-site profile.[29–31] However, despite this observation with γ-retroviral and lentivirus vectors, there has been long-term polyclonal hematopoiesis in the animal studies using vectors with lower-risk transgenes. The substitution of a growth-promoting gene, however, can lead to cases of leukemia in these models. Large-animal studies allow for the evaluation of engraftment kinetics of gene-modified cells using different conditioning regimens, impact of stem cell dose, and long-term follow-up of expression. In addition, the vector performance can be studied in the presence of an intact immune system in contrast to the immunodeficient mouse models. Disease-specific large-animal models are also available.

von Kalle summarized the advances in methods for vector insertion-site analyses and the associated bioinformatics. The advent of high-throughput sequencing has allowed characterization of the number of γ-retroviral and lentiviral integration events in one afternoon that previously required years of sequencing. The common core technology currently used by many laboratories is 454 pyrosequencing because of the usefulness of its relatively long sequence reading, allowing up to half a million amplicons in two to three days. But improved methods such as a combination of linear amplification-mediated polymerase chain reaction (LAM-PCR) with next-generation sequencing promise a two-log yearly growth or greater of unbiased sequence capability in the immediate future. Those next advances face technical obstacles standing in the way of a truly nonrestricted approach to defining the integration events and their consequences. Many of these issues have been addressed through the development of bioinformatical integration-site sequence analyses performed by QuickMap (http://www.gtsg.org/quickmap.jsp), HISAP (von Kalle laboratory, unpublished data, or SeqMap (http://seqmap.compbio.iupui.edu). These bioinformatical data-analysis pipelines allow the characterization of integration sites, including compression of sequence-read information and quick clustering to reduce redundant reads and cross-sample clone-tracking and "read-count" assessment. Such tools permit simplified and automated tracking of clonal inventories and provide support for trends toward a read-count interpretation. The result is large-scale chromosomal integration site distribution, gene-related integration site distribution, functional integration site analysis, and ingenuity pathway analysis.

One of the important impediments to integration site retrieval is the limitation imposed by the use of restriction enzyme sites to chop DNA into fragments of more than 500 base pairs. As underscored by Bushman, the use of restriction enzymes in older studies led to bias based on the restriction enzyme–site distributions in the genome, and there is currently no method to fully sequence any integration-site population. The limits of current methods were highlighted in an article first published online in November 2008 (ref. 32) and precisely addressed in 2009 by von Kalle's group.[33] It is therefore important to develop a nonrestrictive integration-site approach that combines LAM-PCR methods (nrLAM-PCR)[33] to reach a more comprehensive integration-site analysis. This approach, which is under development, promises to remove DNA-processing biases due to restriction enzymes and reduce the biases to those associated with polymerase errors, as recently described.[34]

Another important issue regarding the use of these insertion site–analysis methods is that they require trained labs and in inexperienced hands may yield discrepant results. Christopher Baum noted the results of an interlaboratory comparison of a protocol in which DNA from a K562 clone that had a number of insertions was sent around, as well as primers for the PCR; surprisingly, all the labs produced different results. Hence, although the assays may be a powerful tool, the experience of the lab may also need to be taken into account. If such assays are to be widely used, the operating procedures will need to be standardized to ensure uniform results. Standardization would apply equally to any animal model that might become widely adopted. Currently, there is no consensus on the optimal model(s) that can definitively answer the question of whether a new vector will be safe for a particular condition. A combination of models may be used to add to the risk assessment, but even when *in vitro* assays are combined with *in vivo* animal models, the results may not be entirely predictive. Moreover, the appropriate end point for an animal model and assay is unclear. If one chooses as the end point a rare event such as the development of leukemia or other tumors, it is likely to take considerable time and resources to reach. Alternatively, one could look at the development of clonal skewing, which will allow for a more rapid readout, but clonal dominance is not always predictive of an adverse clinical outcome. As Janis Abkowitz's review of mathematical models demonstrated, even in the absence of any oncogenic event related to insertional mutagenesis, when transplanting limiting stem cell doses, clonal dominance is a very common outcome.[35]

Although most of the models build on data obtained in clinical trials to try to develop methods for comparing relative risks of insertional mutagenesis, a model may not always be able to recapitulate the event. For example, after the development of an *HMGA2*-insertion-site clone in the β-thalassemia trial, the investigators performed extensive studies in thalassemic mice, including secondary transplants with the GMP vector that was used in the subject at a very similar multiplicity of infection and dose of cells and the identical type of conditioning (adapted to a mouse), and yet there was no skewing in the population of cells as seen in the clinical trial.[36]

Quantitative assay systems will be essential to monitor the outgrowth of premalignant clones and to assess the practical value of preventive actions. Data obtained in murine models of BMT in combination with an *in vitro* immortalization assay suggest that the modification of the vector's *cis*-active elements (most importantly, transcriptional enhancers and splice sites) is even more important to prevent insertional oncogene activation than a modification of the integration pattern of semirandomly integrating vectors.[37] For newer vectors lacking strong enhancers, it remains to be determined which of the currently established semirandomly integrating vectors (lentiviral, γ-retroviral, foamy viral, α-retroviral, transposons) has the safest integration pattern. Furthermore, there is growing evidence for the importance of milieu factors, probably connected to the underlying disease, in the selection of insertional mutants.

The development of rational prevention strategies thus depends on the identification of the specific mutations forming premalignant clones and a better knowledge of the mechanisms underlying their creation, expansion, and homeostatic control.[37] Finally, it was noted that, given the diversity of models and assays being developed to test new vectors, it is critical that investigators using such models to support new clinical trials also have the opportunity to publish these data, both positive and negative, because public distribution of these data will foster more rapid comparison of the preclinical models and development of the field. One potential opportunity for this exchange will be through the National Gene Vector Biorespository (NGVB), which currently has a listing of GLP pharmacology and toxicology results. NGVB leadership is proposing to expand this database to include non-GLP studies that have been submitted as part of an investigational new drug application. A European Commission–funded counterpart currently is under consideration.

Monitoring for clonality: regulatory paradigms

In the United States, the Food and Drug Administration (FDA) has codified recommendations regarding monitoring for insertional mutagenesis into their guidance on long-term follow-up of subjects in gene transfer trials.[38] Peripheral blood mononuclear cells can be used for monitoring in a protocol that used transduced $CD34^+$ cells. It is recommended that vector sequences should be tested at least every 6 months for the first 5 years and then yearly for the next 10 years or until no vector is detected.

When at least 1% of the surrogate cells have detectable vector by PCR or another sensitive method, then the pattern of vector integration should be assessed. The FDA does not prescribe one specific method, only that the method should be shown to be specific, sensitive, and reproducible and be based on data with appropriate positive and negative controls, such as a target cell with known number and sites of integrated vector versus target cells with no vector integrants. LAM and ligation-mediated PCR have typically been used.

If this integration analysis reveals the development of a predominant clone or monoclonality, the investigator is asked to identify the integration site(s) in that clone. A predominant clone is not defined by the FDA, but some investigators use a cutoff of >20% of gene-modified cells being derived from a single clone. Analysis should include determining whether the vector is integrated in the vicinity of a

known proto-oncogene, monitoring for signs of malignancy, and performing additional clonality analysis within three months. The optimal methods for this analysis are not yet prescribed.

European gene transfer trials are subject to individual country regulation and approval required for early phases of clinical trials as well as to regulations of the European Medicines Agency (EMA) when applying for market authorization. For gene transfer, new regulations for advanced-therapy medicinal products, which includes gene and cell therapies, were issued in 2007 (ref. 39). Out of these regulations arose a consolidated regulatory framework that included the establishment of a Committee for Advanced Therapeutics (CAT), a multidisciplinary scientific committee of experts representing all members of the European Union and countries from the European Economic Area and the European Free Trade Association as well as patient and medical associations.[39] The CAT is primarily responsible for evaluation of applications for marketing authorization of advanced-therapy medicinal products for the EMA. Several European regulatory guidance documents address the issues relevant to monitoring for development of insertional mutagenesis and malignancy in gene transfer trials. The risk of integration of the vector and the risk of oncogenicity are used to develop an appropriate monitoring plan. Although close monitoring is expected, current guidelines are not detailed in terms of the type of monitoring and time frames for particular events.[40] A monitoring plan with stopping rules is part of the application to begin the clinical trial and is reviewed on a case-by-case basis. Each application dossier is expected to state the rationale for the proposed monitoring plan based on a risk-centered approach. Dedicated guidelines have been issued that are consistent with this[41,42] because both the EMA-CAT and national-level regulatory bodies consider such an approach paramount when considering both first-in-human administration and later clinical trials.

Putting it all together: design of new clinical trials and ethical and scientific considerations

Perhaps the greatest challenge in any first-in-human trial is dealing with uncertainty. Despite strong preclinical models, uncertainty about toxicity in humans remains. A key ethical question is how one determines the point at which the potential benefit of proceeding into the clinic outweighs the risks of unforeseen toxicities.[43–45] Because many diseases targeted for long-term gene correction are pediatric diseases, an additional consideration is that if the intervention presents greater than minimal risk, which a new gene transfer trial does, current regulatory standards in the United States require that there be the prospect of direct benefit to the subject (45 CFR 46 Subpart D). Finally, in the face of uncertainty about risks of insertional mutagenesis, what is the optimal consent process?

The ethical questions of when to move into the clinic, for which diseases, and in which populations, are often addressed in scientific ways by minimizing uncertainty and risks of harm through preclinical testing and by implementing plans to detect and address rare events, such as insertional mutagenesis. Notably, determining whether and when a study has sufficient value to go forward is both a scientific and an ethical determination.[46] As in science, much in ethics is about anticipating issues likely to be encountered later. Thus, it is important for researchers to begin thinking early about the harm–benefit balance that would be appropriate in order to move forward at different research stages.

When patients are selected as research subjects, their selection should reflect the goals of the research. Because safety is the foremost consideration in first-in-human trials, it may not always be possible to start with the most informative patient group if the research poses undue risks for them. If it becomes necessary to choose between the most informative subjects and the subjects for whom the risks can best be minimized, the latter are often chosen; nonetheless, those two considerations should be balanced, and it is critical to develop a more systematic sense of what goes into that balance.[46]

For pediatric subjects, the calculus is more complex because US regulations require that, even in an initial trial involving pediatric subjects, there must be a prospect of direct benefit. What investigators *hope for* is often different from what they can *meaningfully expect* from the data.[47] This distinction is difficult not only in the consent process but in the harm–benefit calculus itself. Moreover, there may be reasonable disagreements about the meaning of the available data, about what the harm–benefit balance should look like, and about how to value the risks of harm and the chance of benefit under the particular circumstances of a given trial.[48] In the context of phase I trials, the prospect of direct-benefit requirement is particularly problematic because it can result in "benefit creep"; that is, researchers and oversight bodies may perceive themselves as having to invent or inflate a potential direct benefit in order to do important research in children. Although there is some debate about the usefulness of this regulatory requirement,[49] it must be addressed so as to help develop a clearer consensus on what "the prospect of direct benefit" means, especially in early-phase research.

There is irreducible uncertainty in predicting insertional mutagenesis in these studies. The key question is when that uncertainty is small enough that it becomes fair to ask subjects to be involved in the research. Ethical research demands a robust discussion of uncertainty with potential subjects and their families, at the levels of both science and policy.[50,51] Patients with few options may have unrealistic expectations of the potential benefit and perhaps minimize the risks of harms—that is, the therapeutic misconception. It is not problematic when researchers' expectations for what is likely to happen in a study and patient-subjects' hopes for what will happen to them differ as long as the risks are minimized and clear information is shared. The key question, then, is how to share the right amount of information in the right amount of detail to facilitate informed decisions about research participation.

The investigator is the most appropriate person to facilitate the information sharing required in the consent process for a given trial, given his or her in-depth understanding of the trial; moreover, the investigator is ultimately responsible, both ethically and legally, for the adequacy of the consent form and process. However, this does not mean that the investigator should be the only participant in the process. When patients have few therapeutic options for a serious or fatal disease, the patient's desire to enroll in a trial strongly

Table 4 Summary of key observations

- Gene therapy trials in X-linked severe combined immunodeficiency disorder and adenosine deaminase deficiency–severe combined immunodeficiency disorder have demonstrated that long-term gene correction is feasible when using retroviral vectors and in some patients may provide equal clinical benefit with less risk than standard treatments.
- Initial data from other indications demonstrate that integrative vectors, and lentiviral vectors in particular, translate into promising results in other classes of genetic diseases, such as adrenoleukodystrophy, metachromatic leukodystrophy, and Wiskott-Aldrich syndrome.
- Our understanding of the mechanisms by which γ-retroviral vectors cause leukemia has continued to grow and offers new avenues to develop vectors that maintain the efficacy seen in the first-generation retroviral vectors but offer a safer alternative.
- Despite the great strides made in this understanding and in developing animal models and *in vitro* assays to predict the safety of new vectors, there are limits to our ability to accurately predict the risk of genotoxicity with a new vector. This may be a function of the assays or models or may indicate that clinical data will always remain the true test.
- This is not to minimize the importance of continued refinement of preclinical models. Ideally, standard assays or platforms might be developed to use across trials. Although there may be some standard models, disease-specific models may also be helpful and each gene construct may be considered unique, in particular with respect to cryptic splice sites and polyadenylation signals.
- Further ongoing studies address the impact of the target cell biology and disease background on the risk of oncogenic complications induced by integrating gene vectors. At present, it is unknown whether the risks documented in the context of clinical trials targeting primitive hematopoietic cells in diseases with a high degree of "stress" hematopoiesis are predictive for other applications of integrating vectors. In the absence of quantitative data addressing the impact of oncogenic cofactors related to target cell biology and disease-specific milieu conditions, care must be taken in extrapolating results from a given experimental or clinical setting to other conditions.
- A stated goal is to develop validated models or assays that may be able to use biological markers that are predictive of genotoxicity allowing sufficient data to assess the risk but that do not require such expense and long-term follow-up as does the double-transplant mouse model, which requires >1 year of follow-up.
- As these assays and models are developed, it is important to assess whether they can be consistently used; verification across labs will be key in establishing their validity.
- The risk of genotoxicity will need to continue to be acknowledged, discussed with patients, and monitored. Given the latencies between dosing and the cases of leukemia seen in these trials, it may take several years of clinical experience to appreciate whether a vector offers a "safer" alternative.
- In addition, as vectors are redesigned to increase efficacy and address the mechanism of insertional mutagenesis seen to date, it is important to understand and be vigilant for alternative mechanisms that may have already been detected in other studies, where these viruses are used to study oncogenesis. Although it is premature to focus research on developing models to assess the risks of these alternative mechanisms, it does underscore the possibility that the follow-up needed to detect enhancer-mediated insertional mutagenesis may be shorter than what might be needed to detect insertional mutagenesis mediated by another mechanism.

aligns with the investigator's goal of enrollment, and it may be beneficial to separate the recruitment process from the consent process, the latter being where the investigator's input is critical. Waiting periods between recruitment, screening, consent, and dosing may also provide mechanisms to promote careful consideration of the risks of harm and potential benefits of participation. Finally, including other parties who do not have a direct interest in the research—for example, clinicians involved in alternative treatments such as a BMT—and even using consent monitors to help test how effectively information has been conveyed to potential subjects may facilitate an effective informed-consent process.[48]

Because long-term follow-up is critical for gene transfer trials that use integrating vectors for long-term gene correction, the rationale for follow-up, the length of follow-up, and the testing that will be undertaken need to be understood at the outset. Although subjects can always opt out of long-term follow-up, enrolling subjects who are not willing to participate in long-term follow-up at the outset would be a matter for concern. Long-term follow-up is an ethical obligation as well. As a result, it is important to determine how long-term follow-up will be implemented and, in particular, who will undertake long-term follow-up if a commercial or other sponsor is no longer able to support the trial or if an investigator leaves the institution. For NIH-supported research or research carried out at institutions that receive NIH support for recombinant DNA research, the responsibility for long-term follow-up falls on the principal investigator. This does not preclude an institution from placing the responsibility on the department and not just the principal investigator or from devising other arrangements that provide mechanisms to share this responsibility.

There was agreement that the NGVB (previously National Gene Vector Laboratories) plays a critical role in allowing investigators to carry out their responsibilities in long-term follow-up. Without the banking of specimens that the NGVB offers and the LAM-PCR analysis, only very affluent institutions would have the resources to engage in this type of research.

Conclusion

Trials in several blood cell diseases have now demonstrated that long-term gene correction is feasible and in some patients may provide equal clinical benefit with less risk as compared with standard treatments (**Table 4**). With these therapeutic benefits has also come recognition of real clinical risks, such as leukemogenesis. The increasingly sophisticated understanding of the mechanisms by which these vectors caused leukemia provides new avenues to develop vectors that maintain or even increase the efficacy seen in the first-generation retroviral vectors but that potentially offer a safer alternative. These vectors must undergo preclinical testing before moving into the clinic, but despite great strides made in developing animal models and *in vitro* assays to predict the safety of new vectors, there are limits to our ability to accurately predict the risk of genotoxicity with a new vector. Ideally, standard assays or platforms might be developed to use across trials. Verification across multiple labs will be key for establishing validity. It is important to understand and be vigilant for alternative mechanisms that may have already been detected in other studies, in which these viruses are used to study oncogenesis. Further ongoing research should address the impact of the target cell biology and disease background on the risk of oncogenic complications induced by integrating gene vectors. Given the latencies between dosing and the cases of leukemia seen in these trials, it may take several

years of clinical experience to appreciate whether a new vector truly offers a "safer" alternative. Until a greater understanding is in hand, risk will need to be taken into account in determining whether integrating vector-based gene therapy is appropriate for the disease and patient population being targeted.

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