

Chance or necessity? Insertional Mutagenesis in Gene Therapy and Its Consequences

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Recently, unusual forms of leukemias have developed as complications following retroviral transfer of potentially therapeutic genes into hematopoietic cells. A crucial component in the pathogenesis of these complications was the upregulation of a cellular proto-oncogene by random insertion of the retroviral gene transfer vector. These findings have great implications for the genetic manipulation of somatic stem cells in medicine. This review discusses the extent to which the random oncogene activation may have required disease-specific stimuli of the transgene and the hematopoietic milieu to become leukemogenic. Based on these considerations, we propose approaches to risk prediction and prevention.

INTRODUCTION

Selection operates on the products of chance (hasard) ... within a domain of rigorous demands (Jacques Monod, *Le Hasard et la Nécessité*, 1970).

The recent paper by Hacein-Bey-Abina *et al.* represents a turning point in the development of human gene therapy [1]. Serious adverse events (SAEs)¹ were observed in an otherwise successful clinical phase I trial: two similar cases of uncontrolled lymphoproliferation occurred after hematopoietic cells had been engineered with retroviral vectors to express a therapeutic transgene. In clinical terms, the diseases presented as acute lymphoblastic leukemia. Before these complications developed, the patients were cured by the genetic intervention of a life-threatening X-linked severe combined immunodeficiency (SCID-X1) [2]. Preclinical animal models of SCID-X1 gene therapy failed to predict this risk [3–7]. A related side effect was previously reported in just one mouse study, using a different retroviral gene transfer vector that was designed for diagnostic cell marking experiments [8]. In all cases, gene transfer vectors based on simple mouse leukemia viruses

(MLV) were used to transduce repopulating hematopoietic cells *in vitro*, and random insertion of a single vector copy into a cellular chromosome resulted in the activation of a proto-oncogene. With a latency of 3 years in the clinical trial and 1 year in the murine studies, diseases with an unusual phenotype developed, indicating that novel types or combinations of genetic alterations were involved in their pathogenesis.

These data emphasize a profound risk in the genetic manipulation of somatic cells. Elucidating context-dependent factors and specific prevention strategies will be of crucial importance for the future of the field, since chromosomal transgene insertion is a prerequisite to numerous potentially powerful future treatment options. Here, we discuss these SAEs from the perspective of current concepts of human stem cell and tumor development and develop perspectives for future research.

CHANCE? THE FREQUENCY OF INSERTIONAL MUTATIONS

Many potentially genotoxic agents are being successfully used in the treatment of severe diseases, according to a clinical risk–benefit evaluation. Gene transfer vectors

¹ Abbreviations used: SAEs, serious adverse events; SCID-X1, X-linked severe combined immunodeficiency; MLV, mouse leukemia viruses; T-ALL, T-cell acute lymphocytic leukemia; BMT, bone marrow transplantation.

that direct sequence insertion into cellular chromosomes represent a new class of potentially therapeutic mutagens. Insertion of viruses into the cellular genome is a known risk factor for tumor development and as such not unique to retroviruses, which integrate into cellular chromosomes as a default event in their life cycle [9–11]. Accordingly, mutagenesis has always been a major concern in the development of human gene therapy [12]. However, the frequency of severe phenotypic alterations related to the insertion of a replication-defective gene vector appeared to be extremely low based on the experience of animal models and clinical trials [13].

Retroviral insertion has been investigated in greater detail than any other eukaryotic gene insertion process. It occurs with high efficiency but without clear preference for specific target sequences or loci; therefore, each transduced cell is clonally marked by the insertion site. However, open chromatin (euchromatin), which is associated with transcriptionally active regions and therefore dependent on cell cycle and differentiation, is the preferred target of the retrovirus integration machinery [14]. Following mitosis, transcriptional control regions probably are among the first chromatin areas that decondense. This may explain why simple MLV-based vectors, which depend on mitosis for access to chromosomes, appear to have a propensity to insert in the vicinity of cellular promoters [15]. Even gene transfer vectors based on the lentiviruses such as HIV-1, which are capable of active nuclear transport into cells resting in the G1 phase of the cell cycle, prefer active genes for insertion although without obvious preference for promoter regions [14,15]. Interestingly, the likelihood of insertions into active genes seems to be even more pronounced when using adeno-associated viruses as vectors, which represent an interesting alternative to retroviruses for several nonhematopoietic target tissues [16].

Depending on the architecture of the affected gene, activation and silencing may be triggered from several positions upstream or downstream of the promoter, while disruption requires insertion downstream of the promoter. Probably depending on its potency and specific composition, the transgene enhancer may act over large distances (as much as 100 kb). However, target gene deregulation possibly occurs only from a distinct subset of insertion sites which coincide with allele-specific regulatory regions. Promoter replacement or inappropriate RNA processing may also result from random transgene insertion.

Gene disruption due to transgene insertion usually produces a monoallelic, recessive defect that requires an independent mutation in the second allele to become phenotypically relevant. Importantly, there are also cases of dominant oncogene formation resulting from the truncation of coding sequences. Of note, it is as yet unclear to what extent differential insertion patterns of

various types of integrating viruses, as recently described [15,16], increase or decrease the risk of oncogene activation (by upregulation or truncation) or tumor suppressor gene inactivation (by silencing or truncation). For any type of vector, the genotoxic risk increases with both the number of cells manipulated and infused into the recipient and the number of insertion events per cell.

Considering that the patients who developed the SAEs in the SCID-X1 gene therapy trial received about 30 million transduced CD34⁺ cells, the entire trial involving nine successfully treated patients may have generated more than 10⁸ random transgene insertions. Based on a random insertion model and cumulative risk calculation, almost every 10th base pair of the human genome may thus have served as a target of transgene insertion. The risk for introducing a transgene within a problematic distance from any of the potential known human proto-oncogenes (estimated at >200) that may be accessible in the available open chromatin of a single cell (~10⁹ bp) may range between 10⁻³ and 10⁻² [17]. If we assume that a particular allele offers 10 kb of vulnerable sequence within a total potentially accessible (“open”) genome size of 1 million kb, a risky insertion may occur once in about 100,000 insertions.

NECESSITY? THE FATE OF GENE-MODIFIED CLONES

The above calculated risk of oncogene activation (10⁻³ to 10⁻²) is several orders of magnitudes higher than the transformation frequency of ~10⁻⁷ determined in growth-factor-dependent cell lines observed *in vitro* [18]. Several biological filters are expected to reduce the chance of a clinically relevant malignancy following insertional mutagenesis [17]:

- (A) Only a small subset of cultured hematopoietic cells engrafts long term. Engraftment is a consequence of clonal selection and as such directed by micro-environmental as well as cellular factors, which are incompletely understood. Highly sensitive PCR assays have been introduced for monitoring the survival and expansion of gene-modified cells by enumerating and characterizing transgene insertion events [19,20]. Data from recent preclinical models, large animal experiments, and clinical trials have demonstrated progress in increasing the efficiency of retroviral gene transfer into long-lived hematopoietic cells [2,13,21,22], with evidence for polyclonal reconstitution (still less than 100 clones) of hematopoiesis in a primate model [20] and some ongoing clinical trials.
- (B) The oncogene-related signal alterations either may not affect cellular homeostasis or may induce extinction rather than expansion. Single oncogene

activation in otherwise normal cells may lead to short-term expansion of cells followed by cellular senescence and crisis, as shown by retroviral transfer of the AML-ETO translocation product into primary human cells [23]. Other oncogene activations may be compensated for without phenotypic consequences or trigger apoptosis when occurring in a normal or nonpermissive genetic environment, as shown for MYC [24].

- (C) To promote uncontrolled outgrowth of transformed clones, cooperating genetic alterations and environmental stimuli will be required. Clinical and experimental findings of cancer development in humans provide evidence for a multistep process requiring up to six independent and specifically cooperating genetic lesions [10,25]. Induction of leukemia is often based on the cooperation of at least two genetic alterations: one that disturbs differentiation and another that promotes proliferation [26]. In human carcinogenesis, maintenance of telomerase activity is crucial to overcome proliferative senescence of transformed clones. TERT, the reverse transcriptase subunit of telomerase, is the only known enzyme that is capable of elongating telomeres and its expression underlies a tight developmental control. Thus, *TERT* expression is downregulated with age and with cellular differentiation [27,28].
- (D) Further important mechanisms controlling promotion of malignant clones are related to immune surveillance [29] and insufficient nutritional supply [30].

Thus, we need to distinguish between the frequency of insertional hits in (the vicinity of) cellular growth regulatory genes, the frequency of functional gene alterations following insertional mutagenesis, and the frequency of insertional oncogenesis, which is highly context-dependent and would therefore be predicted to be several orders of magnitude lower. To avoid generalization of the oncogenic complications noted so far, and to prevent their repeated occurrence, it will be important to understand what enabled the known cases of insertional leukemogenesis following the use of vectors designed for human gene therapy to overcome the four major barriers described above (engraftment, induction, promotion, and systemic surveillance).

Engraftment

The degree to which engraftment of gene-modified cells was favored may have been unique in the setting of SCID-X1. As calculated by Hacein-Bey-Abina *et al.* [1], each patient of the SCID-X1 trial may have been infused with up to 10 (or more) clones harboring a random transgene insertion in the vicinity of *LMO2*. Proliferating hemato-

poietic cells typically have a profound engraftment deficit. However, this defect seems to be less pronounced in the early stages of postnatal hematopoiesis [31], a status that may still apply to the two affected patients who were both treated within the first trimester after birth. Further, it is possible that the insertional *LMO2* activation occurred in a population of committed T cell precursors whose frequency and engraftment conditions are disease-specific. In SCID-X1, the pool of repopulating cells may be significantly enlarged because common γ_c -chain (γ_c)-deficient precursors accumulate before treatment and their engraftment after γ_c reconstitution may be more efficient than in other conditions, due to the presence of a hypocellular thymus presenting an otherwise intact niche.

The aim of treating high cell numbers in SCID-X1 gene therapy is justified by the observation that the kinetics and quality of immune reconstitution are cell-dose-dependent. However, it may not be a coincidence that the two patients receiving the largest number of infused cells were those who developed the SAEs.

Accordingly, it is possible that the other patients were grafted with limiting numbers of cells carrying an activated *LMO2* allele, such that not every treated individual is at risk for secondary leukemogenesis. These considerations suggest three consequences: (i) Further improving cell engraftment without reducing the risk of the gene transfer procedure cannot be recommended for SCID-X1 gene therapy. (ii) Similar to small-molecule pharmacology, we may be confronted with a limited therapeutic window of cell dosage for gene transfer in this condition. (iii) Mouse models may underestimate the risk of insertional mutagenesis because the cell numbers infused per recipient are much smaller (and the observation periods shorter) than in humans.

Induction

Precisely cooperating genetic lesions are likely to be present in cases of cellular transformation [24]. In gene therapy, the transgene itself (irrespective of its insertion site) represents a potential lesion, because expression from such transgenes is usually not regulated in a physiologic manner using current technology [17]. In fact, nonphysiologic transcriptional control of γ_c currently represents the only default genetic alteration that can be identified in the patients suffering from the SAEs other than the random activation of *LMO2*. Potential side effects of ectopic transgene expression may escape detection in preclinical investigation unless cooperation with insertional mutagenesis occurs.

Interestingly, the few known instances of vector-induced oncogenesis all involved transgenic expression of potentially growth-promoting signaling molecules. In the mouse study, a truncated variant of the p75 neurotrophin receptor served as a cell-surface marker

[8]. However, this artificial mutant has a highly context-dependent growth-stimulatory potential [32]. In the SCID-X1 trial, the therapeutic vector encoded the wild-type version of the γ_c to correct the inborn genetic deficiency of this protein [1]. Members of the interleukin (IL)-2 family of cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) share this signal-competent receptor subunit, which associates with one or two other specific receptor subunits for high-affinity cytokine binding and signal transfer [33]. γ_c -deficient cells fail to respond to survival and proliferation signals mediated by these ILs. Therefore, SCID-X1 patients suffer from a life-threatening failure to produce T, B, and natural killer lymphocytes. Retroviral γ_c restoration corrects the cellular survival deficiency and therefore generates a strong selective advantage, explaining the striking recovery of immune function achieved in most patients of the phase I gene therapy trial [2]. This is not only because γ_c expression restores signaling by IL-2 (and IL-7 in the thymus), but also because a block in development is alleviated, which allows other proliferative signals (such as pre-TCR and Wnt signaling) to proceed [34].

At present, one cannot exclude that transgenic expression of γ_c may disturb cellular homeostasis by mechanisms that could have escaped the sensitivity of the preclinical models [3–7]. In retrospect, subtle abnormalities may be a concern, including the potential for insufficient downregulation of transgenic γ_c following stimulation by ILs [35], interference of altered γ_c expression with IL-receptor cross talk [36], disturbed feedback mechanisms in downstream cascades involving antiapoptotic or proliferation-promoting signals [37], or nonphysiologic expression of soluble γ_c , which is considered a negative regulator of IL signaling [38]. While the findings of Hacein-Bey-Abina *et al.* exclude the presence of a constitutive γ_c signal [1], potential alterations in negative feedback mechanisms have not so far been addressed. However, it should be noted that these potential signal alterations of transgenic γ_c , if occurring, are not alone sufficient to produce obvious phenotypic consequences, such that all SCID-X1 patients successfully engrafted with gene-modified cells have experienced a disease correction by the gene transfer.

How can we explain that the same oncogene (*LMO2*) was found to be activated in independent cell clones of both patients [1]? Such a coincidence typically reflects a stringent selection for a precise cooperating event in oncogenesis, as previously shown in studies on insertional mutagenesis elicited by replication-competent retroviruses in birds and mice [10,11]. The *LMO2* proto-oncogene, encoding a hematopoietic transcription factor, is one of the most frequently involved oncogenes in childhood acute T-ALL (T-cell acute lymphocytic leukemia), activated by chromosomal translo-

cation t(11;14) (p13;q11) or t(7;11) (q35;p13) into T cell receptor genes (*TCRA* being located on 14q11 and *TCRB* on 7q35). In mice, transgenic overexpression of *LMO2* in the T cell lineage results in clonal T cell tumors with a latency of approximately 1 year [39]. In human cells, *LMO2* expression is high in CD34⁺ cells in the bone marrow and moderate in the most immature cells in the human thymus, but very low or absent in later stages of thymic development (Table 1, Fig. 1). This suggests that, similar to mice, *LMO2* downregulation is required for normal T cell development in the human thymus. Of note, T-ALL clones observed in children with *LMO2* translocations often are CD3⁻, indicating that in these cases the transformation occurs before completion of TCR rearrangement and development of mature thymocytes. However, the blasts of the SCID-X1 patients with insertional upregulation of *LMO2* were CD3⁺ with fully rearranged $\alpha\beta$ or $\gamma\delta$ T cell receptors [1]. This suggests a unique mechanism of transformation, pointing to transgene effects or other disease-specific factors (Fig. 1).

In the mouse study demonstrating vector-mediated insertional leukemogenesis, a monocytic leukemia arose following insertional upregulation of *Evi1* [8]. Ectopic activation of this transcription factor is involved in human myelodysplastic syndromes and acute myeloid leukemias and in murine leukemogenesis following infection with replicating MLV. Transgenic mice overexpressing *Evi1* show a predisposition to myeloid leukemias, which typically do not present with a monocytic phenotype [40]. Based on the above frequency calculation, it is likely that this oncogene has been hit in several of the numerous other mouse studies of retroviral gene therapy, obviously without inducing malignancy [13]. As above, the phenotypic peculiarity of leukemia development observed in the

Table 1: Expression levels of *LMO2* and γ_c -related genes in human subpopulations

Gene	CD34 ⁺ bone marrow	CD34 ⁺ CD1a ⁻ thymus	CD34 ⁺ CD1a ⁺ thymus
<i>LMO2</i>	17,000	1,500	<100 to A
γ_c	6,500	25,000	20,000
<i>IL2Rα</i>	A	A	A
<i>IL2Rβ</i>	A	A	A
<i>IL4Rα</i>	A	A	A
<i>IL7Rα</i>	<100 to A	11,000	18,000
<i>IL9Rα</i>	A	A	A
<i>IL15Rα</i>	A	A	A
<i>IL27Rα</i>	ND	A	A

A, absent; ND, not determined. Data are presented as background-subtracted, arbitrary fluorescence units and obtained from Affymetrix microarray studies on FACS-sorted subpopulations. Each population has been repeated at least once. The thymic subpopulations have been analyzed in six replicate experiments.

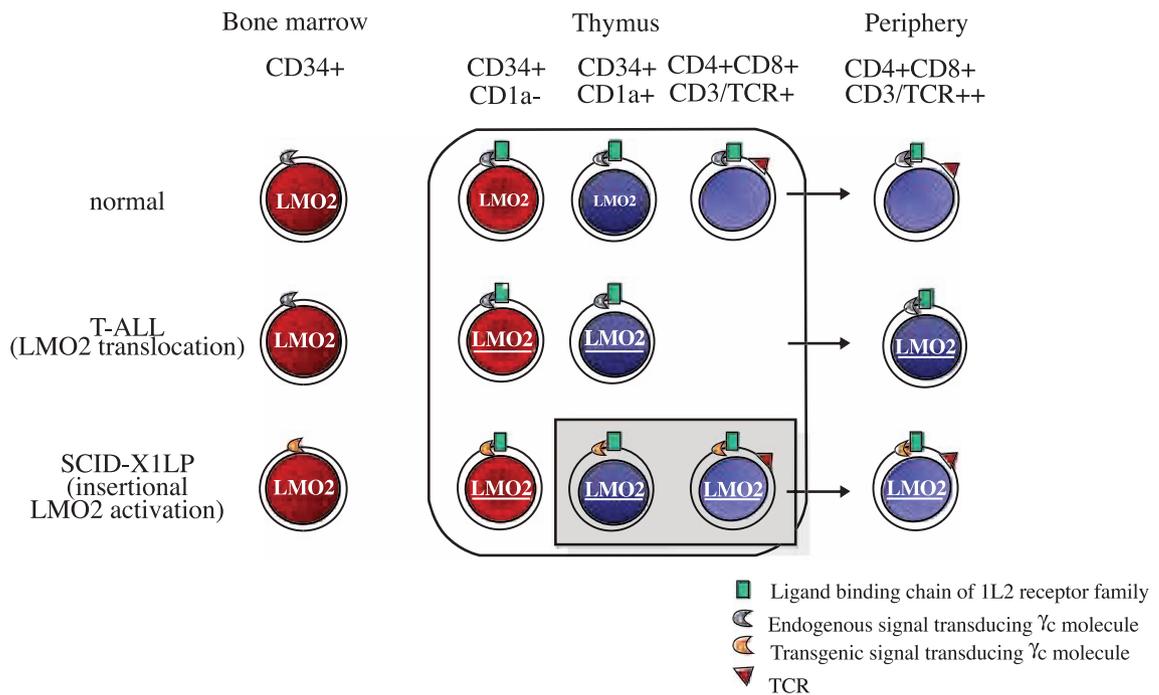


FIG. 1. Specific pathophysiology of a gene therapy side effect. The lymphoproliferations (LP) observed in SCID-X1 patients following retroviral vector-mediated upregulation of *LMO2* show some similarity with T-ALLs due to *LMO2* translocations, but are unusual with respect to the presence of a mature fully rearranged T cell receptor (TCR)/CD3 complex. This may indicate that in SCID-X1 LP a different set of cooperating genetic alterations is involved in the final stages of T cell maturation and expansion (gray box), of which dysregulated transgenic expression of γ_c could be one factor. For details on expression data of *LMO2*, γ_c , and related receptor subunits, see also Table 1. Ectopic *LMO2* expression is underlined.

case associated with vector-dependent *Evi1* activation [8] suggests a context-specific oncogene cascade.

This discussion crystallizes into competing hypotheses (Fig. 2). The first postulates that *LMO2* and *Evi1* represent members of a yet poorly defined class of high-risk proto-oncogenes whose activation (in primitive hematopoietic cells) is sufficient to induce the

dangerous track to transformation. Depending on chance and circumstance (growth rate and environmental mutagens), this model would predict that cooperating lesions will sooner or later accumulate. Alternatively, *LMO2* and *Evi1* could represent oncogenes that cooperate with subtle signal alterations related to the nonphysiologic transgenic expression

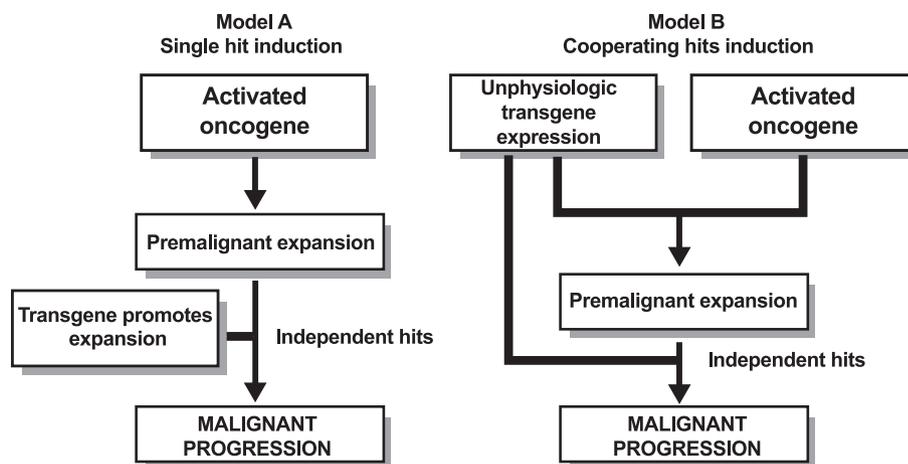


FIG. 2. Two alternative scenarios of oncogenic complications in retroviral gene therapy. In scenario A, *LMO2* and *Evi1* represent high-risk oncogenes whose activation is sufficient to place the cells on a premalignant expansion track. Tumor manifestation will depend on the kinetics of clonal expansion and random replication errors or mutations. The transgene may contribute by promoting expansion. In scenario B, nonphysiologic transgene expression evokes a signal alteration that specifically cooperates with the activated oncogenes in the initiation of disease. Subsequent hits are also required in this model. Importantly, model B suggests that oncogenic complications can be avoided by more physiologic transgene expression and that oncogenic complications will be even more context-dependent than in model A.

of signaling proteins, and without such a cooperation existing from the very beginning, the clone would be much less likely to survive and induce a malignant cascade.

Promotion

As discussed above, it is likely that each of the patients of the SCID-X1 trial potentially received several clones with insertions near *LMO2*. Despite this, most patients to date have not developed SAEs. Therefore, leukemia progression does not seem to be a necessary consequence of engraftment of mutagenized cells. Cytogenetic analyses of leukemic cells from both patients revealed additional chromosomal aberrations that cannot be attributed to the retroviral insertion [1]. Forced clonal expansion may be an independent risk factor promoting genetic instability. In the mouse study [8], this may have been elicited by serial bone marrow transplantation (BMT). In the patients, the combination of BMT, the enormous cell proliferation (1000- to 10,000-fold) undergone by T cell precursors that seed the thymus, and their subsequent antigen-mediated expansion has to be considered, especially in view of the mature T cell phenotype of the malignant clones. To identify the antigens that are potentially involved, it will be interesting to study T cell receptor specificity of the leukemic clones. A potential role for antigens in the promotion of T cell malignancies is supported by several mouse models [41]. This may explain the strong evolutionary pressure for control mechanisms such as activation-induced cell death, and any interference with these pathways is expected to increase the risk for transformation.

It is important to emphasize that clonal expansion per se is not sufficient to produce leukemias or preleukemic phenotypes, as also demonstrated in the context of retrovirus-transduced murine hematopoietic stem cells [42,43].

Target Cell Type

These considerations lead to another important parameter in the risk evaluation of genetic interventions: the developmental stage and age of the target cells. Although the data are limited, it is somewhat striking to note (in retrospect) that the two patients who developed insertion-related leukemias were those who were treated at the earliest time point after birth. Fetal-like target cells may have an increased risk of insertional mutagenesis due to three sets of specific genetic circumstances: the presence of preleukemic cytogenetic alterations, the activation levels of oncogenes that suppress differentiation (such as *LMO2*), and the increased activity of *TERT*. Considering that distinct preleukemic cytogenetic alterations have been detected by PCR in up to 0.1% of fetal hematopoietic cells [44], there remains a remote possibility that insertional activation of *LMO2* coincided with

a preexisting lesion. The considerable discrepancy between the frequency of mutations in fetal cells and the subsequent development of a malignant disease could have been altered by dysregulated expression of *LMO2*. Ontogeny-related differences in *LMO2* transcription (and thus in the susceptibility of this locus to transgene insertion) are speculative. However, there is clear evidence for an increased *TERT* activity in young postnatal hematopoietic cells and even higher expression in earlier fetal stages [45,46]. Acknowledging that hematopoietic and lymphoid cells are exquisitely sensitive to the persistence of *TERT* function to clonally expand [27,47], *TERT* activity may represent a crucial component in the risk classification of stable transgene insertion. Adult mice show an extent of *TERT* activity that may be more similar to human fetal cells than to subsequent steps of human development [48]. Therefore, mouse models already represent a high-risk scenario with respect to *TERT*-dependent escape from senescence [25]. This point may have to be addressed in studies with nonhuman primates.

Systemic Surveillance

There is considerable evidence for immune surveillance of malignant clones. This level of control is likely to be compromised in SCID-X1 patients and in other approaches involving conditioning treatments prior to infusion of gene-modified hematopoietic cells [49]. Another level of systemic control is provided by the tumor microenvironment. Neovascularization and nutritional supply may be growth limiting in later stages of leukemia development [30]. However, it appears unlikely that the SCID-X1 setting is especially permissive in this respect.

THE FUTURE: APPROACHES TO RISK EVALUATION AND PREVENTION

Most of the above hypotheses related to the disease-specific impact of engraftment, induction, promotion, and surveillance (summarized in Table 2) can be addressed in well-designed cell culture experiments and animal models. Systematic research may produce a risk classification and approaches to risk reduction. Potential side effects need to be addressed in the context of the biology of the underlying disease, where possible.

Unfortunately, before the data will have accumulated, a reliable risk assessment of malignant adverse events for patients undergoing insertional gene transfer cannot be offered. In the absence of appropriate alternatives, an individual risk-benefit ratio still needs to be derived, taking into account that malignant disorders are multifactorial and gene therapy involves rather heterogeneous technologies and biological conditions.

Table 2: Risk factors potentially contributing to insertional leukemogenesis in SCID-X1 gene therapy

Level	Potential mechanism	Consequence	Solution
Transgene vector insertion (MLV)	(A) Propensity for open chromatin close to promoters of active genes (B) Strong viral enhancer/promoter with increased chance of random gene activation	(A + B) Increased chance to activate oncogenes in primitive hematopoietic cells	(A) Alternative viral or nonviral delivery system (B) Alternative transcriptional control elements
Transgene (γ_c)	(A) Growth advantage (B) Nonphysiologic expression of signal molecule	(A) Selective expansion of transduced lymphocytes (essential for disease correction) (B) Disturbed T cell homeostasis (may trigger induction and promotion)	(A) Not applicable (B) More physiologic transcriptional control
Target cells	High numbers of progenitors available for gene transfer	Increased risk of random oncogene activations	Reduce target cell number
Young age of patient	(A) Cell cycle restriction of engraftment reduced (B) Increased risk of preexisting mutations (C) Increased TERT activity	(A) Increased engraftment (B) Cooperation of spontaneous and induced mutations (C) Longevity of mutated clones	(A–C) Treatment after first postnatal trimester
Surveillance of malignant clones	Disturbed immunity	Tolerance to transformed clones	Immunotherapy of malignancy using antibodies or allogeneic T cells

None of the individual risk factors would be considered sufficient for leukemia development. Approaches to risk prevention need to be evaluated in appropriate functional assays.

For SCID-X1, only patients with matched related donors available have a high chance of a satisfactory outcome following stem cell therapy without genetic engineering. For the population of patients without matching family donors (which this trial was designed for), treatment alternatives such as matched unrelated or haploidentical donor transplantation involve significant risks of treatment-related morbidity and mortality [50]. As both gene-transfer-related malignant complications have been successfully treated, the SCID-X1 gene therapy trial's current follow-up shows a 2-year survival rate of 100% and a freedom from treatment failure rate of 80%, undoubtedly a favorable outcome [1,2].

Using existing technology, the danger of insertional mutagenesis is predicted to decrease by lowering the numbers of treated and infused cells. As discussed above, this may be of special concern for treating CD34⁺ cells of infants. In the case of SCID-X1, cell dose reduction could be achieved by an adaptation of the transduction protocol, step by step, starting from the lowest number of cells that was successful in the patients observed so far. Incomplete reconstitution of B cell function appears to be the first consequence of lowering the cell dose, but may be acceptable due to the availability of immunoglobulin treatment. Of note, a 10-fold reduction of input cell dose can be compensated for by only three to four self-renewal divisions. Translating better purification procedures for hematopoietic stem cells into clinical use could lead to a 100-fold reduction in the numbers of exposed cells and a similar reduction of risk, if otherwise

short-lived progenitors contribute to the risk of insertional leukemogenesis.

Alternative gene delivery technologies will need to be assessed carefully for potential risks and specificity of insertion site distribution [51,52], type and frequency of postinsertion recombinations [16], toxicity of the enzymes mediating transgene insertion [53], and the number of divisions a corrected (stem) cell needs to undergo to generate mature progeny. There are numerous options to change the vector backbone and use alternative *cis*-acting sequences, including those potentially leading to insulation of the transgene from the neighboring chromatin [17]. Especially for transgenes whose deregulated expression may produce adverse effects, vectors that direct more physiologic expression need to be developed [17]. However, many of these alterations have implications for the production technology of viral vectors, and the hypothesis of superior safety would therefore have to be validated using appropriate experimental systems.

The latency period of a malignant adverse event would be expected to increase (many years) and the frequency to decrease (even to undetectable levels) when the selective advantage of the transgene can be controlled by exogenous signals, when cooperative transgene effects are irrelevant despite ectopic expression, and when the genetic background and environmental conditions do not foster genome damage. Importantly, more mature target cell populations may be less susceptible to insertional oncogenesis, because their proliferative capacity is typically lower, the number of oncogenes potentially

contributing to cellular transformation may be smaller, and some of these loci may be less prone to retroviral insertion due to transcriptional silencing (as indicated for *LMO2* in Table 1). For diseases that remain at increased risk for oncogenic side effects of integrating vector technology, and for which current BMT protocols are still insufficient, a new approach that involves clonal selection of transgenic cells, prescreened for “safe” insertion sites, needs to be developed. Although proof of principle has been provided in a mouse model, this approach is currently beyond available technology for human applications [54].

Given the documented potential and the present results, gene therapy continues to be a reasonable investment. The history of medicine has provided numerous examples in which dose finding and physical modifications have been the keys to converting potential poisons into life-saving agents.

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