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Solving the Problem of γ-Retroviral Vectors Containing Long Terminal Repeats

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Results from a clinical gene transfer trial for the X-linked hematological disorder Wiskott–Aldrich syndrome (WAS), carried out by Klein and colleagues at Hannover Medical School in Germany, were reported in the 11 November 2010 issue of the *New England Journal of Medicine*.[1] The article described the clinical outcome and three-year follow-up of two young boys treated with autologous CD34$^+$ cells transduced with a γ-retroviral vector encoding the WAS protein. Following submyeloablative conditioning, long-term engraftment levels of vector-modified CD34$^+$ cells of 9% and 20% were observed in the bone marrow of the two patients, with similar or higher levels of corrected cells found in multiple peripheral blood hematopoietic lineages. Correction of immune abnormalities as well as resolution of autoimmunity and thrombocytopenia were observed in both patients. Although this was a remarkable success, almost as important was the announcement by the investigators in a press release,[2] but absent from the publication, that one of what is now a total of 10 treated patients has developed an acute T-cell leukemia related to a vector insertion. Of note, in the *New England Journal of Medicine* article, significant clonal imbalances were reported for both patients, with clones observed with increased frequency having insertions in proto-oncogenes such as *LMO2*, *MDS/EVI1*, *PRDM16*, and *CCND2*. This WAS trial thus combines the insertional risk profile reported for the induction of myelodysplastic syndromes (connected with *MDS/EVI1*) and acute lymphoblastic leukemia (*LMO2*, *CCND2*) in previous clinical trials to correct chronic granulomatous disease and X-linked severe combined immunodeficiency (X-SCID).[3,4]

Preliminary data indicate that the development of an acute T-cell leukemia in the WAS trial was associated with an insertional event upstream of *LMO2* and additional chromosomal aberrations (C. Klein, Hannover Medical School, personal communication, 29 November 2010), highly reminiscent of the leukemic adverse events observed in the Paris and London SCID-X1 trials. Again, this highlights the now well-recognized insertional gene activation activity of first-generation γ-retroviral vectors containing transcriptionally active long terminal repeats (LTRs) based on various forms of murine leukemia virus. With this event, there have now been leukemias or pre-leukemias reported in four different trials using LTR-driven γ-retroviral vectors to target CD34$^+$ hematopoietic cells (X-SCID, Paris and London trials; chronic granulomatous disease, Germany; WAS, Germany).[3-6] In response to the new adverse events, principal investigators in Germany have put on hold two phase I clinical trials using LTR-driven γ-retroviral vectors for gene transfer into hematopoietic stem or progenitor cells (HPSCs): the WAS trial and another trial exploring the safety of a novel anti-HIV principle in the context of AIDS-related lymphoma (C. Klein, Hannover Medical School, and B. Fehse, University Medical Center Hamburg-Eppendorf, Hamburg, Germany, personal communication, 29 November 2010).

Still puzzling, and without adequate explanation, is the observation that approximately 20 patients with adenosine deaminase (ADA)-SCID treated with γ-retroviral vector gene transfer in the Milan and London trials have done exceedingly well, with no patient developing leukemia.[7] Although a trend to clonal skewing was observed in many patients, no clonal outgrowth has been observed.[8] Similarly, an ongoing ADA trial in Los Angeles utilizing an MLV-based γ-retroviral vector has reported no adverse events due to vector insertion (D. Kohn, University of California, Los Angeles, personal communication, 24 November 2010). Furthermore, numerous clinical trials targeting mature T cells utilizing LTR-driven γ-retroviral vectors have not yielded evidence for insertional adverse events despite long-term persistence of transduced cells. These data suggest that disease background factors and cell-intrinsic mechanisms may modify the risk of insertional mutagenesis. To complicate matters further, an ongoing trial for adrenoleukodystrophy utilizes a self-inactivating (SIN) lentiviral vector containing the MND viral LTR as an internal promoter. In the two patients reported on last year, no clonal imbalances were observed.[9] Although results from more patients are needed, this suggests that the lentiviral vector system may mitigate the deleterious effects of viral LTR enhancers, perhaps through integration-site preferences.

On the basis of these results, a strong argument can be made that γ-retroviral vectors that retain transcriptionally active LTRs containing a battery of strong enhancers should not be used in future clinical gene transfer trials involving genetic modification of HPSCs unless this intervention offers the only reasonable salvage therapy. An exception may be the open trials for ADA-SCID, but even here, new clinical trials should use a potentially safer vector design. This conclusion seems to be supported by the field, as evidenced by the preferential use of alternative vectors (such as SIN lentiviral or γ-retroviral vectors) and designs in recently opened or planned clinical gene transfer trials (**Table 1**). Specifically, the use of SIN lentiviral and γ-retroviral vectors—and in the future maybe even transposon-based approaches, in which the viral LTR elements have been removed—will

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Table 1 Open or planned clinical gene transfer trials using hematopoietic cells

Disease	Trial location	Source of HSCs	Vector	Gene to be transferred	Promoter	Trial status
ALD	Paris	mPB CD34⁺ cells	SIN lentiviral	ABCD1	MND viral LTR	Open
WAS	Boston, Milan, London	mPB CD34⁺	SIN lentiviral	WAS	Endogenous WAS elements	Open
β-Thalassemia	Paris	Bone marrow and mPB CD34⁺	SIN lentiviral	β-Globin	Globin gene regulatory elements	Open
Metachromatic leukodystrophy	Milan	mPB CD34⁺	SIN lentiviral	Arylsulfatase A	PGK	Open
ADA-SCID	Los Angeles, NIH	Bone marrow CD34⁺	γ-Retroviral	ADA	MND viral LTR	Open
HIV/AIDS	City of Hope	mPB CD34⁺	SIN lentiviral	Anti-HIV	Pol III U6 and Va-1	Open
X-SCID	Memphis	Bone marrow CD34⁺	SIN lentiviral	γ-Chain	Elongation factor1-α	Not yet open
X-SCID	Boston, Los Angeles, London, Paris	Bone marrow CD34⁺	SIN γ-retroviral	γ-Chain	Elongation factor1-α	Not yet open
β-Thalassemia	New York	mPB CD34⁺	SIN lentiviral	β-Globin	Globin gene regulatory elements	Not yet open
β-Thalassemia	Memphis	Bone marrow CD34⁺	SIN lentiviral	γ-Globin	Globin gene regulatory elements	Not yet open
Sickle-cell disease	Cincinnati	Bone marrow CD34⁺	SIN lentiviral	γ-Globin	Globin gene regulatory elements	Not yet open
β-Thalassemia	Milan	N/A	SIN lentiviral	β-Globin	Globin gene regulatory elements	Not yet open

ADA-SCID, adenosine deaminase–severe combined immunodeficiency disease; ALD, adrenoleukodystrophy; HSC, hematopoietic stem cell; LTR, long terminal repeat; mPB, mobilized peripheral blood; N/A, not available; NIH, National Institutes of Health; SIN, self-inactivating; WAS, Wiskott-Aldrich syndrome.

be favored. These vectors utilize internal enhancer–promoter elements to drive transgene expression, and investigators have invested significant work to define internal promoters and RNA processing tools that mediate sufficient levels of gene expression while avoiding long-distance enhancer-mediated interactions with cellular promoters. In extensive nonclinical studies that address the insertional risk profile of such redesigned vectors, consistent data were reported from independent investigators using different experimental platforms that SIN vectors with selected promoters have a significantly reduced potential to transform hematopoietic cells by insertional mutagenesis.[10–14] However, so far it remained unclear which insertion profile is safer for a vector with a low risk of enhancer-mediated crosstalk: the tendency of γ-retroviral vectors to integrate close to transcriptional start sites or the preference of lentiviral vectors to integrate into active transcription units. Another approach to improving safety might include the addition of a suicide gene to therapeutic vectors for use in the setting of evolving monoclonality. In the context of gene-modified T cells, this strategy has shown promise in feasibility and efficacy.[15,16]

Interestingly, one ongoing trial for β-thalassemia, utilizing a lentiviral vector containing a β-globin gene driven by erythroid enhancer elements, has reported clonal dominance within the gene-modified cell population, but no hematopoietic abnormalities have developed thus far.[17] Long-term follow-up and the treatment of additional patients are needed to understand this event. Only through a combination of improved nonclinical assay systems and well-designed human clinical trials will further understanding be gained regarding the relative risk of the new approaches that eliminate inclusion of viral LTRs in the vector design.

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AAV6-Mediated Gene Silencing fALS Short

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The truth is rarely pure and never simple.
—Oscar Wilde

Amyotrophic lateral sclerosis (ALS) is a devastating progressive motoneuron disease that is invariably fatal within 3–5 years after diagnosis.[1] Approximately 2% of ALS cases are due to an autosomal dominant mutation in the gene encoding Cu/Zn superoxide dismutase1 (SOD1). Because SOD1 enzyme activity is not lost in familial ALS (fALS) and transgenic expression of pathogenic G93A SOD1 (muSOD1), in addition to expression of the normal endogenous mouse SOD1, faithfully recapitulates human fALS pathology, muSOD1 expression likely induces pathology via a toxic gain of function. Therefore, muSOD1-related fALS is a potential target for gene therapy aimed at silencing the mutant SOD1 allele in motoneurons. fALS is also attractive for gene therapy from a regulatory perspective, because no therapy is currently available that alters the progression of the disease.

In this issue of *Molecular Therapy,* Towne *et al.* report the findings of a study utilizing recombinant adeno-associated viral vector 6 (rAAV6) to deliver a small hairpin RNA (shRNA) to silence muSOD1 in muscle and motoneurons as a potential therapy in a mouse model of fALS.[2] Even though they report no effect on the primary outcome measure—longer lifespan of the fALS mice—the study represents a significant attempt to translate an allele-specific gene-silencing strategy to treat fALS. The results stand in stark contrast to an earlier study that reported a remarkable extension of life span in fALS mice following delivery of an equine infectious anemia virus (EIAV) lentiviral vector encoding an identical therapeutic gene.[3] Studies that report negative results, such as the one by Towne *et al.*, are rarely published, but this impressive study may nevertheless move the field forward. Because the two studies employed methods that are as similar as could possibly be expected from two different laboratories, exploring the potential reasons for the vast difference in outcome between the two is crucial because fALS is an excellent target for gene therapy.

By any measure, the study by Towne *et al.* is well designed and carefully executed, albeit ostensibly reporting a negative result. These workers made use of rAAV serotype 6 to deliver shRNA to muscle and motoneurons via retrograde transport to silence expression of muSOD1 messenger RNA (mRNA) in the fALS mouse model. Each motoneuron innervates one or more muscle fibers. Intramuscular injection of vector results in transduction of a motoneuron when the virus infects the axon and the virion is transported back to the motoneuron's nucleus. Retrograde delivery of the vector and subsequent motoneuron transduction is the preferred route for several important reasons. First, a direct injection of the vector into the spinal cord is impractical because the numerous injection sites that would be required would greatly increase the probability of morbidity. Second, transduction specificity for motoneurons would be lost; third, there are advantages to silencing muSOD1 in muscle as well, which would not be targeted by this strategy.[2] Finally, as discussed below, systemic vector injections do not lead to sufficient motoneuron transduction for a therapeutic effect.[4] Furthermore, the experiments were extremely well controlled, comprising large numbers of wild-type, vehicle-injected, and control (receiving rAAV6 encoding a control shRNA that contains only two mismatches, which is a strict control for shRNA experiments) mice. Transduction of all targeted muscle groups was shown by green fluorescent protein fluorescence, and the sample sizes of the treatment groups were far greater than those seen in most preclinical studies.

Beneficial effects of knocking down the G93A muSOD1 mRNA were previously established by direct intra–spinal cord injections[5] and via retrograde transport after intramuscular injection of a rabies glycoprotein-pseudotyped lentiviral vector (EIAV).[3] The latter results indicated that widespread muSOD1 knockdown via global muscle transduction using a vector that can be retrogradely transported from muscle to spinal cord motoneurons might be a potent treatment for fALS mice.[4,5] Towne and co-workers had previously performed systemic injections of rAAV6, which had been shown to transduce motoneurons after intramuscular injections in rodents[4,6] and in nonhuman primates.[7] Unfortunately, this strategy affected neither the onset of disease nor survival of fALS mice, apparently because of low transduction

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