Gene Therapy for β-Thalassemia

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Gene transfer for β-thalassemia requires gene transfer into hematopoietic stem cells using integrating vectors that direct regulated expression of β globin at therapeutic levels. Among integrating vectors, oncoretroviral vectors carrying the human β-globin gene and portions of the locus control region (LCR) have suffered from problems of vector instability, low titers and variable expression. In recent studies, human immunodeficiency virus–based lentiviral (LV) vectors were shown to stably transmit the human β-globin gene and a large LCR element, resulting in correction of β-thalassemia intermedia in mice. Several groups have since demonstrated correction of the mouse thalassemia intermedia phenotype, with variable levels of β-globin expression. These levels of expression were insufficient to fully correct the anemia in thalassemia major mouse model. Insertion of a chicken hypersensitive site-4 chicken insulator element (cHS4) in self-inactivating (SIN) LV vectors resulted in higher and less variable expression of human β-globin, similar to the observations with cHS4-containing retroviral vectors carrying the human γ-globin gene. The levels of β-globin expression achieved from insulated SIN-LV vectors were sufficient to phenotypically correct the thalassemia phenotype from 4 patients with human thalassemia major in vitro, and this correction persisted long term for up to 4 months, in xeno-transplanted mice in vivo. In summary, LV vectors have paved the way for clinical gene therapy trials for Cooley's anemia and other β-globin disorders. SIN-LV vectors address several safety concerns of randomly integrating viral vectors by removing viral transcriptional elements and providing lineage-restricted expression. Flanking the proviral cassette with chromatin insulator elements, which additionally have enhancer-blocking properties, may further improve SIN-LV vector safety.

Hemoglobin disorders were among the first diseases for which the gene therapy was envisioned. However, the complexities of the globin gene expression and regulation made this a formidable task. Making gene therapy a therapeutic option in β-thalassemia requires: 1. efficient, safe and high-level gene transfer into target hematopoietic stem cells (HSCs), 2. regulated erythroid lineage-specific expression, and 3. therapeutic levels of β-globin gene expression. A variety of non-viral and viral vector systems have been tried to achieve this goal. While viral components in some vector systems, like the adenovirus and adeno-associated virus, can evoke an immune response to the viral proteins from the host, non-viral methods of gene delivery cause a relatively low immune response and can deliver relatively larger sizes of DNA than can be accommodated by viral vectors. But they are limited by a low rate of gene transfer, a low rate of permanent integration into the host genome and poor long-term expression. In contrast, natural viruses have evolved over millions of years to equip themselves with the ability to enter, transfer and express viral genetic information in the host cells. Retroviruses reverse-transcribe their RNA genome to DNA and integrate permanently into the host genome. Hence, they have been widely exploited as vehicles for gene therapy for diseases requiring life-long correction in the progeny of HSCs.

The secondary murine bone marrow transplant model has been used to assess gene transfer into the HSC and the longevity of gene expression. In this model, murine bone marrow stem/progenitor cells are subjected to gene transfer followed by transplant into lethally irradiated syngeneic/congenic mice. Three to six months later, marrow from these primary recipients is transferred into another set of lethally irradiated mice (secondary transplant). Mouse models of disease are very valuable in this regard, such as the β-thalassemia intermedia mice. Additionally, xenotransplantation of transduced human bone marrow progenitors into highly immune-deficient mice, such as the NOD-SCID and the β2-microglobulin null NOD-SCID mice, have been used to assess the therapeutic efficiency of the transferred gene into the progeny of highly primitive human hematopoietic progenitor cells. Although, the short life-span of these immune deficient mice restricts expression in human HSC progeny for 3-5 months, the information obtained from long-term in vivo analysis of human cells is ultimately used to assess therapeutic efficacy and is the most relevant and critical preclinical requisite to gene transfer into humans.

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Insights into Elements Required for High-Level Regulated Expression of β-Globin

The β-globin gene is tailored for a remarkably high-level, erythroid-specific expression. The work of numerous investigators on globin regulation and globin gene therapy

has provided important insights into the elements essential for expression in globin genes in vectors:

1. High level of globin gene expression requires distal locus control elements: Expression of human β-globin gene driven by an internal β-globin promoter within a retroviral vector is very low.[1] A high-level regulated globin gene expression requires presence of a series of distal control DNAse hypersensitive sites (HS) in the 21 kb of DNA upstream from the β-globin locus, the LCR, that plays a role in maintaining the β-globin gene locus in an open configuration to allow for high level expression of the globin genes.[2-4]

2. The LCR is too large to be incorporated into oncoretroviral vectors; therefore, small LCR fragments, consisting of 200-300 bp fragments from the HS, containing only the core sequences have been used. However, inclusion of the core LCR elements into Moloney leukemia virus (MLV)-based oncoretroviral vectors leads to low levels of expression that is prone to position effects.[5-7] Inclusion of larger LCR elements in transgenic mice results in position-independent, copy number-dependent, high-level transgene expression.[8] However, due to the size constraints of MLV vectors, individual HS site cores or a few combinations of these have been tested in MLV vectors with variable, position-dependent expression.[6,9,10]

3. Oncoretroviral long terminal repeats (LTRs) transcriptionally interfere with LCR elements within MLV vectors, resulting in unstable proviral transmission and/or poor transgene expression.[7,11] Transcriptional interference from the LTR[12] can be overcome by self-inactivating (SIN) vectors. In SIN vectors, the LTR promoter/enhancers are deleted upon integration of the provirus. However, SIN MLV vectors have a loss in titers resulting from the LTR deletion. Replacement of the viral LTR with enhancers from other erythroid genes (the GATA-1,[13] or HS-40,[14,15] the distal control element from the α-globin locus) has met with reasonable success.

4. β-globin is an intron-dependent gene that destabilizes oncoretroviral vectors, if placed in antisense orientation: Retroviral vectors are limited to incorporating cDNA, since during production of the viral RNA genome, vector plasmids containing introns would be spliced. This becomes a serious handicap in expressing intron-dependent genes, such as β-globin. The β-globin cDNA is very poorly expressed, as β-globin introns contain elements that enhance transcription, allow proper 3′ end processing and the nuclear export of transcripts.[16,17] The β-globin gene is, therefore, inserted in reverse orientation relative to the open reading frame of the viral LTR transcript. This results in vector instability, as several splice sites and polyadenylation signals are introduced into the open reading frame of the vector transcript.

5. Measures taken to overcome instability of globin-containing retroviral vectors: 1) Deletion of a 372-bp fragment of the intron-2 of the β-globin gene,[18,19] which contains most of the 'instability elements,' helps stabilize the viral genomic RNA. 2) Leboulch et al have mutated numerous deleterious sequences in an MLV vector containing the core LCR elements HS2, 3 and 4 driving expression of β-globin, showed stable transmission in the majority of mice, but low and variable expression.[19] 3) We have attempted to improve expression from the β-globin cDNA, by including viral elements that would replace the functions of the globin introns and show that the woodchuck hepatitis virus post-transcriptional regulatory elements improves expression by 5- to 10-fold,[20] although levels of β-globin expression were still subtherapeutic. In summary, efforts to impart stability to the β-globin mRNA in MLV vectors by modifying various 'instability elements' and adding RNA export element vastly improved upon previously reported results, but were still suboptimal to be of therapeutic benefit.

Revival in Gene Therapy for Hemoglobinopathies with Lentiviral Vectors

LV vectors, derived from the HIV-1 virus[21] have several favorable features for gene therapy: (1) The LV vectors can enter a fully intact nuclear membrane and integrate into non-dividing HSC chromatin without prestimulation with cytokines. (2) They are able to package full-length, unspliced RNA due to the presence of a strong RNA export element, the rev response element (RRE). In this regard, May et al have shown that β-globin gene cassettes that were unstable in MLV vectors were stably transmitted in LV vectors.[7] (3) Furthermore, LV vectors can hold a much larger cargo (~9-10 kb) than oncoretroviral MLV vectors, allowing for the larger LCR elements to be incorporated into these vectors. LV vectors, therefore, were able to overcome the hurdles encountered with MLV vectors.

Generation of lentiviral vectors

A three-plasmid system was initially used to create the first generation LV vectors.[21] It included a packaging plasmid containing the viral *gag* and *pol* and accessory genes. A second plasmid encoded an alternative envelope protein, the G glycoprotein of vesicular stomatitis virus (VSV-G), to allow for concentration by ultracentrifugation and broaden host cell infectivity. A third plasmid encoding the expression cassette for the transgene contained all the *cis*-acting sequences required for encapsidation, reverse transcription and integration. The transgene also contained the CMV internal promoter, intact 5′ and 3′ LTR, the packaging sequence from the *gag* gene and RRE.

Subsequent generations of LV vectors have attempted to reduce HIV elements and split the packaging gene into different plasmids. Second generation LV vectors improved on biosafety by deleting accessory HIV-1 genes. The third generation vectors deleted HIV regulatory genes, *tat* and *rev*, from the packaging construct and expressed the rev protein from a fourth plasmid. Most importantly, a SIN-design of the vector backbone was achieved by deleting the promoter enhancer in the U3 region of the 3′ LTR without significant loss in titers or infectivity; during reverse

transcription of the viral RNA, this U3 deletion is transferred to the 5′ LTR, resulting in transcriptional inactivation of the LTR in integrated proviruses.[22,23] Another advantage of SIN-LV vectors was that the level of expression from internal promoters was higher and lineage-specificity was enhanced probably because of the lack of interference from the LTR transcriptional sequences.[24]

Erythroid-specific expression from SIN lentiviral vectors

All current clinical gene therapy trials with MLV vectors have utilized the viral promoter and enhancer to drive expression of the therapeutic gene. This results in unregulated ubiquitous expression of the target gene, which is unnatural and can even be dangerous, as was seen in the X-linked severe combined immune deficiency trial. Moreover, β-globin gene expression is restricted in the erythroid progeny of HSC. Therefore, it would be important to design vectors that would express in a regulated manner in the desired erythroid lineage. Several SIN-LV vectors have been tested that contain heterologous enhancer/promoters to drive erythroid-specific expression of cDNAs from GFP and human ferrochelatase (FC) or a hybrid human β/γ-globin gene.[25,26] Vectors driven by the human ankyrin-1 gene promoter and carrying the HS-40 element from the α-globin locus as an enhancer were found to be erythroid-specific. The cDNA was expressed at high levels and transduced cells showed sustained long-term expression.[25,26] The levels of expression of FC from SIN-LV vectors were sufficient to correct the phenotype in a murine model of erythropoietic protoporphyria in secondary mice, suggesting that heterologous promoter enhancers in these vectors are capable of driving expression at clinically relevant levels.[26] Both the GFP-expressing and FC-expressing mice showed an apparent lack of vector silencing. This was unlike the silencing of proviral gene expression, from methylation of the viral LTR, typically observed with MLV vectors. There was no methylation of the human ankyrin-1 promoter in the SIN-LV vectors in secondary mice, suggesting that lentiviral vectors, unlike oncoretroviral vectors, resisted silencing. However, the expression of the vector was highly variable in different mice, or clones derived from cell lines or HSC, since it was dependent on the structure and influences of the surrounding chromatin, where the vectors integrated. This variability in expression of vectors resulting from integration into different chromosomal sites is termed position effects. Therefore, the SIN-LV vectors resisted gene silencing that is typically seen in oncoretroviral vectors, but suffered from chromatin position effects.[27]

Genetic correction of β-thalassemia intermedia phenotype with lentiviral vectors

The mouse β-globin cluster has two adult β-globin genes, β^{minor} and β^{major} globin. The first animal model of β-thalassemia was found in a DBA/2J mouse due to spontaneous deletion in the β-major gene. The resultant mice had a phenotype resembled human thalassemia intermedia. Subsequently, models were generated with deletion of both the β^{major} and β^{minor} on one allele, $Hb\beta^{th3/+}$ mice. These heterozygous mice also have a thalassemia intermedia phenotype. The embryonic to adult switch in mice occurs at day 14-15 of gestation, making deletion of both the β^{minor} and β^{major} globins on both alleles (equivalent to human β^0 thalassemia major) embryonic lethal, because unlike humans, the switch to adult globin production occurs during embryonic life in mice.

Sadelain and colleagues[28] were the first to show stable transmission and high-level β-globin expression using a first generation LV vectors backbone containing a large human LCR configuration in a mouse model of β-thalassemia intermedia. They tested two LV vectors termed RNS1 (carrying minimal core LCR elements) and TNS9 (with large LCR fragments encompassing HS2, HS3 and HS4; approximately 3.2 kb in size). Cells transduced with the larger TNS9 vector maintained higher human β-globin transcript levels; the same vector achieved marked improvement in the hematocrits, RBC count, reticulocyte count and hemoglobin levels in β-thalassemia mice ($Hb\beta^{th3/+}$ mice). This correction was sustained in secondary mice.

Imren et al[29] used a similar lentiviral vector, carrying a modified anti-sickling β-globin (mutated at β^{T87Q} to prevent sickle polymer formation) to produce long-term correction in a murine β-thalassemia intermedia model.[30] Notably, they observed no correction if there were single proviral integrants, which was attributed to chromatin position effects. The vector used by this group carried a 2.7 kb LCR, which may have contributed to the lower β-globin expression per vector copy, as compared to the results published by May et al,[28] although simultaneous comparisons of these vectors have not been done.

In a different approach, Persons and colleagues[31] used a human γ-globin containing SIN-lentiviral vector under the control of the β-globin promoter and a smaller LCR fragment (1.7 kb in length) in $Hb\beta^{th3/+}$ mice and showed an increase of hemoglobin by 2.5 g/dL, and normalization of RBC morphology with approximately 2 copies of the provirus per cell. One important advantage of using the γ-globin gene, normally expressed only during fetal life, is that high level γ-globin expression would be therapeutic not only for β-thalassemia, but also sickle cell anemia. While this was the highest level of γ-globin expression in adult (postnatal) RBCs, the vectors produced much less hemoglobin/vector copy than reported by others,[28,29] likely due to the smaller LCR elements or difficulties inherent in achieving high level expression of the γ-globin gene in adults. Like other groups, their vectors also showed significant position effects. They subsequently used a vector with a larger LCR and showed much higher expression of γ-globin in thalassemic mouse, that was less prone to position effects. **Table 1** compares the different LV vectors carrying the human β- or γ-globin expression and LCR fragments.

Gene Therapy for Thalassemia Major

Rivella et al[32] developed an elegant mouse model of β-thalassemia major by transplanting fetal liver cells from thalassemia major fetuses, prior to embryonic fatality, into lethally irradiated normal adult mice. They showed that fully engrafted mice die of severe anemia (hemoglobin levels 2-3 g/dL) within 6-8 weeks following the transplantation, while genetic correction of the β-thalassemia major fetal liver cells with the same vector, previously shown to correct thalassemia intermedia mice,[28] rescued the lethality. In this model, the TNS9 vector increased the hemoglobin up to 6.5 ± 2.9 g/dL in 6 long-term chimeras with an average copy number of 1.6 ± 0.6. One chimera achieved a hemoglobin level of 12 g/dL with a vector copy number of 2.2, but most other mice were converted to a severe thalassemia intermedia phenotype.

Of note, gene therapy in thalassemia mice[29,31,32] with β- or γ-globin-based LV vectors suggests that vectors with higher and more predictable expression may be necessary for correction of human thalassemia major. Another notable feature in murine studies is the highly variable erythroid-specific expression amongst different mice,[27,29,31,32] despite the presence of β- or α-globin regulatory elements, which are known to confer position independent expression. Hanawa et al addressed the position effects by using a vector carrying a 3.2 kb LCR and were able to show reduction in position effects that were attributed to modifications in the cryptic splice sites.[33]

Our laboratory used a different approach to address chromatin position effects, increasing β-globin expression while at the same time trying to improve vector safety. A SIN-lentiviral vector termed BGI, carrying the human β-globin gene, a 3.2 kb LCR and the chicken hypersensitive site-4 (cHS4) insulator in the SIN-LTR, was used to transduce $CD34^+$ cells from bone marrow of 4 patients with thalassemia major.[34] The chromatin insulator element was inserted to reduce chromatin position effects and reduce the probability of the vector LTR activating a surrounding cellular gene or vice-versa. There was high-level transduction of bone marrow $CD34^+$ progenitor cells (86 ± 5%) from 4 patients with transfusion-dependent thalassemia major with a complete phenotypic and functional correction of human thalassemia erythropoiesis (**Figure 1A**; see Color Figures, page 547), and levels of β-globin expression were similar to those derived from normal bone marrow progenitors (**Figure 1B**; see Color Figures, page 547). These results were confirmed in vivo 10-16 weeks after transplantation of $\beta 2m^{null}$ NOD-SCID mice with transduced thalassemia bone marrow. Transplanted mice achieved multilineage human cell engraftment and HbA production, similar to mice transplanted with only normal bone marrow. Apoptotic cells were only found in the marrow of mice with untransduced thalassemia major xenografts, showing ineffective erythropoiesis from untransduced thalassemia major bone marrow progenitors. Effective human erythropoiesis was achieved with circulating β-globin-producing human erythroid cells in the genetically corrected xenografts, at levels comparable to normal bone marrow xenograft controls (**Figure 1C**; see Color Figures, page 547). This was the first report of complete correction of human thalassemia major model.

It is possible that an incomplete correction in mice (where human β-globin forms tetramers with mouse α-globin) underestimates the degree of correction that would be achieved with similar vectors in human cells (where natural tetramers of human α and β globins would be formed), and this may partially explain the success of our study.[34] However, it is likely that the increased β-globin expression by the BGI vector was due to incorporation of the insulator element, shown to reduce position effects and thereby improve expression of γ-globin from MLV vectors.[35] Similar results on increased transgene expression have been recently reported in vitro with SIN-lentiviral vectors by Hawley and colleagues.[36] We have subsequently analyzed expression from erythroid-specific SIN-LV vectors with and without cHS4 insulator, carrying either the GFP reporter or the β-globin gene in MEL cells and in mice, and observed reduced clonal variegation and signifi-

Table 1. Comparisons of different lentiviral vectors used for gene therapy of β-thalassemia.

	Transgene	Rise in Hb/ Vector Copy	Summary of Findings
May et al[28]	β-globin	3.8 gm	Correction of anemia in thalassemia intermedia mice
Imren et al[29]	β^{87}-globin	1.5 gm	Correction of anemia in thalassemia intermedia mice
Rivella et al[32]	β-globin	2.3 gm	Rescue of lethality in a thalassemia major mouse model
Persons et al[31]	γ-globin	1.1 gm	Correction of anemia in thalassemia intermedia mice
Hanawa et al[33]	γ-globin	2.0 gm	Correction of anemia in thalassemia intermedia mice Reduced position effects
Imren et al[42]	β^{87}-globin	—	High level expression of an antisickling β^{87} globin in human erythroid cells derived from cord blood progenitors in immune deficient mice Integration of vector near potential oncogenes
Puthenveetil et al[34]	β-globin	—	Correction of Human β-thalassemia major phenotype in vitro and in immune deficient mice Reduced position effects

cantly increased expression from insulated vectors (unpublished results, Malik et al).

Safety of Randomly Integrating Vectors for Gene Therapy of β Thalassemia

The recent development of leukemia in 3 patients successfully treated for X-linked severe combined immune-deficiency (SCID) due to insertional oncogene stimulation with an MLV-based oncoretroviral vector[37] has caused a profound rethinking in the design of safe gene therapy vectors. Subsequent investigations showing preferred intergenic/intragenic integration of MLV and LV vectors[38,39] suggest that the enhancers in the vector expression cassette may have played a significant role in activating cellular genes within/around which the vector integrated. In the SCID study, the MLV vector was driven by the viral LTR enhancer/promoter that imparted constitutive expression to the common γ-chain in stem cells and all hematopoietic progeny.

Unlike MLV, the risk of insertional oncogenesis from LV vectors in humans has not been studied in clinical trails, since these vectors are relatively new. Also, unlike MLV,[40] no insertional oncogenesis has been reported in murine studies with LV vectors, likely due to lack of functional viral promoters and enhancer elements with these vectors. Only one phase I trial using a LV vector has been performed for gene therapy for HIV,[41] and short-term preliminary findings to date show that it has resulted in no adverse events. Imren et al[41] transduced human cord blood $CD34^+$ cells with the LV vectors carrying a modified antisickling β-globin (mutated at β^{T87Q}) and showed high levels of expression in the erythroid progeny with ~2 copies/cell. Genomic sequencing of vector-containing fragments showed that 86% of the proviral inserts had occurred within genes, including several genes implicated in regulation of hematopoieisis and human leukemia, pointing to a need for ensuring that the β-globin expression cassette does not transactivate surrounding cellular genes.

LV vectors carrying the β-globin cassette have several 'built in' safety features: a) regulated, erythroid lineage-specific expression[25,28] restricts their expression to differentiated erythroid cells rather than stem or other hematopoietic cells, b) a self-inactivating (SIN) lentiviral design[25,31,33,42] renders the integrated provirus devoid of viral transcriptional elements,[22,23] and c) insertion of the cHS4 insulator such that it flanks the integrated vector would additionally reduce position effect variegation (PEV) and have enhancer blocking effects in SIN-LV vectors.[34] Our studies with the BGI vector suggest that 'insulated' LV vectors reduce the PEV. Whether they have a reduced propensity to activate surrounding cellular genes needs to be tested. If the cHS4 is found to have enhancer blocking effects in the BGI provirus, it should result in reduced genotoxicity of the vector.

While extensive data have emerged showing the propensity of retroviral vectors to integrate in and around genes, one perplexing feature is integration of the RV provirus in the LMO2 oncogene in all 3 X-SCID patients, while no integrations in this locus occurred in children treated with a similar LTR containing RV vector in ADA-deficient SCID. These data suggest that there may be may be disease- or transgene-specific predilection or presence of 'integration hot spots' into this locus due to varying transcriptionally open domains in progenitors. Whether there are specific loci that are integration hot spots in thalassemia progenitors versus normal bone marrow progenitor cells needs to be determined. While the cHS4 insulator results in more predictable expression due to reduced clonal variegation, whether this element truly has enhancer blocking effect when it flanks an erythroid-specific cassette in a lenti-provirus remains to be determined.

Summary

Lentiviral vectors have resulted in unprecedented levels of β-globin expression and high levels of transduction of human hematopoietic stem cells,[34,42] resulting in correction of mouse and human models of thalassemia intermedia[28,29,31,33] and major.[32,34] With the correction of human thalassemia major cell phenotype, it needs to be determined what is necessary to take gene therapy to the clinic. What amount of corrected thalassemia major stem cells is needed to correct the disease phenotype? What preconditioning regimen will be necessary for adequate engraftment of gene modified cells? Persons et al have shown correction of thalassemia intermedia phenotype with 10%-30% normal cells[43] and improved engraftment of gene-modified donor cells after in vivo drug selection with a vector containing the mutant methyl guanosyl methyl transferase gene.[31] Will an ex vivo expansion or an in vivo selection of genetically corrected HSC be necessary to ensure sufficient HSC are engrafted for therapeutic effects? Lentiviral vectors have already gone into phase I trials as an anti-HIV gene delivery into patients with HIV and have proven safe in the short term so far, in an environment where there would be a high propensity to recombination events and adverse effects. Whether these preliminary results from the HIV trial suggest that these vectors may conceivably be safe to use for gene therapy of hemoglobinopathies remains to be seen.

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