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Gene therapy for hemoglobinopathies: progress and challenges

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Abstract

Hemoglobinopathies are genetic inherited conditions that originate from the lack or malfunction of the hemoglobin (Hb) protein. Sickle cell disease (SCD) and thalassemia are the most common forms of these conditions. The severe anemia combined with complications that arise in the most affected patients raises the necessity for a cure to restore hemoglobin function. The current routine therapies for these conditions, namely transfusion and iron chelation, have significantly improved the quality of life in patients over the years, but still fail to address the underlying cause of the diseases. A curative option, allogeneic bone marrow transplantation is available, but limited by the availability of suitable donors and graft-vs-host disease. Gene therapy offers an alternative approach to cure patients with hemoglobinopathies and aims at the direct recovery of the hemoglobin function via globin gene transfer. In the last 2 decades, gene transfer tools based on lentiviral vector development have been significantly improved and proven curative in several animal models for SCD and thalassemia. As a result, clinical trials are in progress and 1 patient has been successfully treated with this approach. However, there are still frontiers to explore that might improve this approach: the stoichiometry between the transgenic hemoglobin and endogenous hemoglobin with respect to the different globin genetic mutations; donor cell sourcing, such as the use of induced pluripotent stem cells (iPSCs); and the use of safer gene insertion methods to prevent oncogenesis. With this review we will provide insights about (1) the different lentiviral gene therapy approaches in mouse models and human cells; (2) current and planned clinical trials; (3) hurdles to overcome for clinical trials, such as myeloablation toxicity, insertional oncogenesis, and high vector expression; and (4) future perspectives for gene therapy, including safe harbors and iPSCs technology.

Hemoglobinopathies are conditions that result from defects in the genes that control the expression of the hemoglobin protein. Sickle cell disease (SCD) and the thalassemias are the most common hemoglobinopathies worldwide.¹ The extremely high frequency of hemoglobin disorders compared with other monogenic diseases reflects natural selection mediated by the relative resistance of carriers against *Plasmodium falciparum* malaria.² Structural defects in the hemoglobin molecule characterize SCD, whereas diminished production of α or β -globins, the 2 subunits of the hemoglobin molecule, can lead to α - or α -thalassemia, respectively.^{3–5}

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Palliative therapeutic options to correct patients' anemia are red blood cell transfusion and iron chelation. Even in the less severe cases, anemia worsens with aging and can become symptomatic, therefore, blood transfusion becomes mandatory. In general, the level of chronic stable anemia determines the transfusion regimen. In addition to life-threatening anemia, patients may present with intrinsic and treatment-related features that exacerbate the pathology. For patients with SCD, common complications are painful episodes, acute chest syndrome, and stroke; whereas in patients with thalassemia, they are represented by hepatosplenomegaly, recurrent infections, and spontaneous fractures. In both cases, transfusion-associated infections and organ damage are side effects of long-term treatment and unsatisfactory iron chelation.^{3,4} Iron overload is observed also in patients that do not require blood transfusion for survival because of ineffective erythropoiesis. Ineffective erythropoiesis triggers increased iron absorption by reducing the expression of hepcidin, the hormone that controls iron absorption.^{6,7} Although both transfusion and iron chelation treatments have remarkably improved over the years and, thus, improved quality of life, they do not provide a definitive cure, as they lack the ability to address the intrinsic genetic defect. To this end, hematopoietic stem cell (HSC) transplantation is the only presently available cure. Allogeneic bone marrow (BM) transplantation can cure hemoglobinopathies, but only a small proportion of patients have suitable donors. Myeloablative HSC transplantation carries a 5%–10% mortality rate and is poorly tolerated in adults; in addition, graft-vs-host disease may limit the success of the procedure.^{8,9} Given the limitations associated with allogeneic BM transplantation, gene therapy using a patients' own HSCs represents an alternative and potential cure because it aims at the direct recovery of the hemoglobin protein function via globin-gene transfer. The development of gene therapy tools for hemoglobinopathies has been the object of research of the last few decades and has been proved successful in mouse models in many studies. The following sections will provide insights about these studies and how gene therapy has paved its way to become the object of clinical investigations.

WHY GENE THERAPY FOR HEMOGLOBINOPATHIES?

Gene therapy offers a new approach to cure patients with hemoglobinopathies. As reported in the sections that follow, many studies based on animal models have shown that both SCD and β -thalassemia can be reversed using this alternative and novel approach. Although some studies utilized adenoviruses,¹⁰ most studies were focused on the use of onco-¹¹ and lentiviral vectors, whose success have lead to considering their clinical implication in the setting of these diseases.^{12–15}

Research on the field has come a long way in the last decade, moving the gene therapy approach from the bench to the bedside. The fundamental conditions that must be met for a safe and efficacious gene transfer been can be summarized by the following points^{16–28}:

- highly efficient and stable transduction
- effective targeting of HSCs
- controlled transgene expression (erythroid-specific, stage-restricted, elevated, position-independent, and sustained over time)
- absent or low genomic toxicity
- correction of the phenotype in preclinical models and in transgenic mice

Researchers have addressed these requirements and also applied their knowledge to correct abnormal globin gene function in induced pluripotent stem cells (iPSCs) generated from adult cells of β -thalassemia and SCD patients.^{29–31}

Promising progresses of gene therapy studies for hemoglobinopathies have led the medical field to initiate clinical trials (see Clinical Trials section). However, the original studies in animal models of β -thalassemia utilized mice characterized by partial or complete deletion of the mouse β -globin genes. In contrast, β -thalassemia in humans is characterized by nearly 300 mutations, mostly associated with 1 or a limited number of nucleotides. Genetic heterogeneity adds a further level of complexity in the identification of the right conditions for each patient; conditions such as optimization of vector copy number, transgenic-chimerism (the relative ratio between lentiviral-transduced and non-transduced HSCs), and the level of transgenic-hemoglobin necessary to correct each patient's phenotype. Further concerns are the potential genome toxicity associated with the random integration of the current gene transfer vectors and the limited homogeneity of gene transfer in HSCs (further elaboration provided in the following several sections of this article).

PRECLINICAL STUDIES IN ANIMAL MODELS AND HUMAN CELLS

Earlier gene therapy studies utilized recombinant oncoviruses as carriers to introduce a functional copy of the β -globin gene. These vectors have the ability to transfer the β -globin gene into murine HSCs without transferring any viral genes but did not produce high and stable expression of the β -globin gene in mice.^{32–35} The incorporation of the core elements of the hypersensitive sites (HS) 2, 3, and 4 of the human β -globin locus control region (LCR) was key to increased β -globin expression and overcame the inability to reach therapeutic hemoglobin levels. However, it failed to prevent position-effect variegation and achieve therapeutic levels of β -globin expression.^{36,37} Later work focused on blocking the activation of splicing sites within the LCR sequence of the viral RNA, which caused rearrangements. These efforts were effective yet did not satisfy the requirement of maintaining high β -globin gene expression. Additional studies introduced the α -locus HS40 regulatory region in place of the large LCR,^{38,39} alternative erythroid specific promoters, such as ankyrin,^{40,41} and mutant hereditary persistence of fetal hemoglobin (γ -globin) promoters.^{42,43} Results from these studies failed to show their therapeutic relevance but ultimately pioneered the way for successful gene therapy.

In the mid-1990s, the HIV-1 based lentiviral vector became available and offered a real alternative to oncoviral vectors as possible carriers of the globin genes.⁴⁴ As opposed to onco-vectors, lentiviral vectors have the ability to infect quiescent cells⁴⁵ and can carry large transgene cassettes with introns and regulatory LCR elements with none or limited sequence rearrangement.^{46–48} Incorporation of different promoters, enhancers, and other large regulatory elements from the β -globin locus in lentiviral vectors have shown to be particularly suitable to achieve high and specific expression of α - and β -like genes in several mouse models for hemoglobinopathies.

Using a lentiviral vector in which the α -globin gene and promoter were flanked by the HS2, 3, and 4 of the human LCR, Han and colleagues were able to rescue murine fetuses affected by α -thalassemia. Three months after birth, treated mice sustained a 20% α -globin transgenic expression compared with normal mice, although transgene expression started to decline at 7 months.⁴⁹ Several laboratories including ours have shown that lentiviral vectors, carrying the human β or γ -globin gene and its fundamental regulatory elements, were able to cure and rescue animal models for β -thalassemia intermedia and major, respectively.^{25–27,50–52} With a lentiviral vector with enhanced anti-sickling features, Pawliuk and colleagues corrected the phenotype of beta S-Antilles-D Punjab (SAD) and Berkeley (BERK) SCD mouse models.⁵³ The BERK mouse model was also treated in a study in which Pestina and colleagues used a γ -globin based lentiviral vector with a β -globin 3' untranslated region (UTR) and enhancer.⁵⁴ The group of Miccio showed that genetically corrected thalassemic erythroblasts undergo *in vivo* selection in mice, indicating

that the corrected cells have a survival advantage over the thalassemic ones.²² From a clinical point of view, this advantage could imply a lower myeloablative regimen for a patient, who could benefit even when some chimerism persists after BM transplant.

Much progress has been made in the treatment *in vitro* of both human SCD and β -thalassemic cells with lentiviral vectors. Samakoglu and colleagues corrected human sickle cells using a vector that combined a γ -globin gene with a small hairpin RNA targeting the sickle β -globin messenger RNA (mRNA) to, respectively, increase fetal hemoglobin expression and down-regulate production of the sickle β -chains.⁵⁵ Our laboratory and others utilized lentiviruses that incorporate insulators to maximize β -globin expression at the random integration site and protect the host genome from possible genotoxicity. Insulators can in fact shelter the transgenic cassette from the silencing effect of non-permissive chromatin sites and, at the same time, protect the genomic environment from the enhancer effect mediated by active regulatory elements (like the LCR) introduced with the vector. Puthenveetil and colleagues utilized the 1.2 kb chicken β -globin hypersensitive site 4 (cHS4) insulator to rescue the phenotype of thalassemic CD34⁺ BM-derived cells.²⁸ The study by Wilber and others showed that fetal hemoglobin can be synthesized in human CD34⁺-derived cells after treatment with a lentiviral vector encoding the γ -globin gene, either in association with the 400 bp core of the cHS4 insulator or a short hairpin RNA targeting the γ -globin gene repressor protein BCL11A.⁵⁶ We recently showed⁵⁷ that using a 200 bp insulator, derived from the promoter of the ankyrin gene, a significant amelioration of the thalassemic phenotype was achieved in mice and high level of expression was reached in both human thalassemic and SCD cells. The group of Miccio⁵⁸ instead used the HS2 enhancer of the GATA1 gene to achieve high β -globin gene expression in human cells from patients with β -thalassemia. A scheme of the most successful gene therapy tools is provided in Fig 1.

Several studies have focused on nonviral vectors as potentially safer method to genetically modify cells.⁵⁹ Their application is, however, challenged by relatively low gene transfer efficiency and the difficulty of maintaining long-term stable expression.⁶⁰ For the purpose of providing a stable genomic integration mechanism and long-term transgene expression transposons have been developed.^{61,62} The *Sleeping Beauty* transposon system has become increasingly useful in the field of gene therapy over the last decade and the recent development of the SB100X transposase and its utility in modifying primary CD34⁺ cells are evidence of the system's continued improvements and potential clinical significance.^{63,64} In a recent study, Sjeklocha and colleagues used the SB100X transposase system in combination with the IHK- β -globin gene and the cHS4 insulator to produce erythroid specific expression of the β -globin gene integrated into primary CD34⁺ cells, and differentiated into multiple lineages.⁶⁵ The results of this study support the potential use of a SB IHK- β -globin expression system for the gene therapy of sickle cell disease in human clinical trials.

TARGETED REACTIVATION OF FETAL HEMOGLOBIN

The switch from fetal (HbF) to adult hemoglobin (HbA) is critical to the pathogenesis of SCD and the β -thalassemias. Persistence of HbF in adults reduces the severity of both thalassemia and SCD traits. Three major loci (*XmnI-HBG2* single nucleotide polymorphism, *HBS1L-MYB* intergenic region, and *BCL11A* encoding gene) have been identified as major players. Other loci are expected to be involved,^{66,67} but their contribution seems to be only marginal. Given that these loci are associated with putative binding sites for transcription repressors, studies are undergoing to characterize individuals carrying genetic variations associated with increased production of HbF and its correlation to disease severity.^{68,69} Short oligonucleotides have been employed to prevent the binding

of these factors to their target sites or target additional transcriptional factors (TFs). The group of Xu used a double-stranded 22 bp decoy oligonucleotide containing the *Oct-1* consensus sequence to demonstrate its ability to bind *Oct-1* and cause significant increases in the level of the γ -globin mRNA when administered to K562 cells. Recent publications focused on the direct manipulation of γ -globin genes transcription. To this end, both Gräslund⁷⁰ and Wilber⁷¹ engineered artificial TFs to facilitate their interactions with specific DNA sequences and modulate endogenous gene expression within cells. Following this approach, several zinc finger-based transcriptional activators were designed to target the sites proximal to the -117 position of the γ -globin promoter. The results obtained by the 2 groups demonstrate that it is possible to enhance HbF production in primary erythroblast by using a zinc finger transcriptional activator designed to interact with the γ -globin promoter. More recently, targeted reactivation of HbF induced by BCL11A repression was proven successful in correcting the phenotype of transgenic knock out mice, which express exclusively human sickle hemoglobin.⁷² Altogether these studies showed new insights in translating HbF silencing into mechanism-based, improved therapy for the major hemoglobinopathies.

CLINICAL TRIALS

The first successful gene therapy trial for β -thalassemia was reported by Leboulch in 2010.⁷³ Using a β T87Q LentiGlobin vector, Leboulch and colleagues were able to achieve transfusion independence in 1 adult patient with severe β E/ β 0-thalassemia. The patient is a compound heterozygote (β E/ β 0), in which one allele (β 0) is nonfunctioning and the other (β E) is a mutant allele whose mRNA may either be spliced correctly (producing a mutated β -globin) or incorrectly (producing no β -globin).

The β T87Q LentiGlobin vector expressed a mutated β -globin distinguishable from transfused β -globin because of an anti-sickling mutation at the 87th amino acid. This vector also contained 2×250 bp copies of the cHS4 insulator. Analysis of the patient's transduced cells revealed an intact vector, however, with the loss of 1 copy of the cHS4. Of the 24 chromosomal integration sites (IS) found, 1 of the sites, high mobility group AT-hook 2 (*HMGA2*), caused transcriptional activation of *HMGA2* and conferred clonal dominance. Overexpression of *HMGA2* can lead to a clonal growth advantage and is found in a number of benign and malignant tumors.⁷⁴ In the majority of these cases, overexpression of *HMGA2* is associated with mutations affecting the 3'UTR, which contains binding sites for the regulatory micro RNA (miRNA) let-7.⁷⁴ Binding of let-7 miRNAs to the 3'UTR of *HMGA2* negatively regulates *HMGA2* mRNA and protein expression.⁷⁵ Transgenic mice carrying a 3' UTR-truncated *HMGA2* expressed increased levels of *HMGA2* protein in various tissues including hematopoietic cells and exhibited an increased number of peripheral blood cells in all lineages, splenomegaly and erythropoietin-independent erythroid colony formation. Furthermore, BM cells derived from these animals had a growth advantage over wild-type cells. Thus, overexpression of *HMGA2* is associated with clonal expansion at the stem cell and progenitor levels.⁷⁴

Cells from the clinical trial patient with a *HMGA2* IS showed a loss of the 3' UTR of *HMGA2*, preventing the binding of the let-7 miRNAs to complementary sequences. Erythroid cells from this IS exhibited a dominant, myeloid-biased cell clone. The expression was reported to be erythroblast-specific, as *HMGA2* mRNA was undetectable in granulocyte-monocytes. However, the clonal dominance of *HMGA2* is represented in all populations in similar proportions (erythroblasts, granulocyte-monocyte, and LTC-IC cells). Leboulch et al hypothesize that this dominance is due to a transient expression of *HMGA2* in a myeloid-restricted LT-HSC during β -LCR priming, before the β -LCR becomes restricted to the erythroid lineage. At the time of reporting, the patient had been transfusion-

independent for 2 years and showed stable Hb levels from 9–10 g/dL. Therapeutic Hb- β T87Q LentiGlobin, however, only accounted for one-third of the total Hb, with endogenous HbE and HbF making up the rest. Without the additive effect of these endogenous Hbs, this first trial might not have been a success. This suggests that we not only need a predictive *in vitro* model with which to evaluate potential trial patients, but better vectors that can achieve higher therapeutic Hb expression.

Currently, the first US phase I clinical trial has received Food and Drug Administration approval and is enrolling patients. The strategy was briefly described by Sadelain and colleagues in 2010⁷⁶ with the main goals of assessing insertional oncogenesis and replication-competent lentivirus safety, and determining levels of engraftment and vector expression. The study will use CD34⁺ cells mobilized by granulocyte colony-stimulating factor (G-CSF). In 2002 Li et al⁷⁷ studied G-CSF peripheral blood stem cell mobilization in β -thalassemia patients and found up to a 21.5-fold increase in CD34⁺ cells could be collected. Using G-CSF, Sadelain et al have already achieved successful mobilization of CD34⁺ cells in 3 patients with β -thalassemia in amounts sufficient for transduction. The previously described TNS9 vector^{26,50} will be used to induce transgenic expression of β -globin. Small unpublished modifications have been made to this vector to increase titer, but the gene, promoter, enhancers, and LCR remain intact. For myeloablation, a lower intensity-conditioning regimen of busulfan at a total dose of 8 mg/kg will be used.

The success of conditioning regimen intensity depends on a balance between toxicity and the amount of mixed transgenic-chimerism (ie, the amount of BM made up of transplanted cells that carry the vector (Fig 2)). Full myeloablation can, theoretically, result in a complete transgenic-chimerism, where the BM is made entirely of transplanted cells, with a greater amount of therapeutic vector-transduced cells than in a partial myeloablation setting. However, full myeloablation is more toxic and puts the patient at greater risk, especially in the case of graft failure. A reduced-intensity conditioning regimen can be less toxic, however, it can also lead to a lower composition of therapeutic vector-transduced cells because of mixed transgenic-chimerism. When a reduced-intensity conditioning regimen is used, β -globin expression from the vector must be high enough to give transduced cells a survival advantage compared to untransduced cells. In studies done of traditional allogeneic BM transplantation, patients with as low as 20% donor contribution were still able to achieve transfusion independence and normal hemoglobin levels. However, a high initial engraftment (>90% at 60 days post-transplant) is necessary for good chances of stable mixed chimerism.⁷⁸ Lucarelli and colleagues identified 3 Pesaro risk classes for β -thalassemia patients based on previous iron chelation, hepato- and splenomegaly, and liver fibrosis.⁷⁹ Those patients with less iron overload and liver damage fall into class 1 and class 2. Those patients with irregular iron chelation and more liver damage fall into class 3. Full myeloablation for class 1 and class 2 patients involves obliteration of the BM by 14–16 mg/kg busulfan, followed by 20 mg/kg of cyclophosphamid to suppress the immune system. An assessment of 886 patients with β -thalassemia who received transplants from HLA-matched siblings or parents showed a 91% and 84% probability of thalassemia-free survival with this regimen for class 1 and class 2 patients, respectively.⁸⁰

As reviewed by Bertaina et al,⁸¹ there has been a recent trend to lower-intensity, nonmyeloblastic conditioning regimens based on the following data: (1) lower morbidity and mortality is associated with these regimens, (2) patients not eligible for the traditional full myeloblastic regimen have been safely transplanted with these regimens, and (3) mixed chimerism can be sustained and still lead to amelioration of disease in patients with allografts. Multiple groups have had success with reduced-intensity regimens with lower doses of busulfan, or by using alternatives such as thiotepe, treosulfan, fludara-bine, busulfex, or antithymocyte globulin (as reviewed in the references below).^{81,82} These reports, however,

relate to β -thalassemia transplants with HLA-matched donors and not to autologous transplants done with vector-transduced cells. The US trial with vector-transduced cells will use a reduced-intensity regimen based off of data from successful allogeneic transplants⁷⁶ and data from autologous transplants with vector-transduced cells in immunodeficiency disorders.^{83,84}

GENOME TOXICITY

Random integration of transgenes might be associated with genome toxicity and oncogenesis, a phenomenon already observed in several patients with X-linked severe combined immune deficiency.⁸⁵ Hargrove and colleagues investigated gene expression in genomic regions adjacent to the site of integration of lentiviral vectors containing enhancer elements of the β -globin LCR.¹⁸ The analysis investigated gene expression changes following viral transduction in primary clonal murine β -thalassemia erythroid cells, where globin regulatory elements such as the LCR are highly active. The study revealed an overall incidence of perturbed expression in 28% of the transduced clones, with 11% of all genes contained within a 600-kilobase region surrounding the vector-insertion site, demonstrating altered expression. Studies by Arumugam and colleagues utilized both γ -retroviral and lentiviral vectors containing LCR elements and their potential to transform primary hematopoietic cells.⁸⁶ In this study, lentiviral vectors exhibited approximately 200-fold lower transforming potential compared with the conventional γ -retroviral vectors. In an effort to shield cellular chromatin in the vicinity of the sites of chromosomal integration from enhancer effects brought upon by the vector, the cHS4 insulator was included,⁸⁷ showing that a further 2-fold reduction in transforming activity was observed when the LCR elements were flanked by this element. However, the failure to control genomic insertion still remains the main vulnerability of gene therapy.

PHENOTYPIC VARIABILITY AND GENE TRANSFER IN PATIENTS AFFECTED BY HEMOGLOBINOPATHIES

An additional concern associated with the attempt to cure hemoglobinopathies by gene transfer is the pheno-typic variability of patients affected by hemoglobinopathies. The 2 major categories of the inherited hemoglobin disorders are structural hemoglobin variants and the thalassemia syndromes. The structural hemoglobin variants result primarily from amino acid substitutions in α - or β -globin chains.⁸⁸ The most widespread variants are associated with missense mutations, and the main structural changes are classified as HbS, HbC, and HbE.⁸⁸ In contrast, thalassemias are characterized by almost 300 mutations. An updated list of these mutations is accessible at the Globin Gene Server website: <http://globin.cse.psu.edu>. Furthermore, the phenotype of the patients affected by hemoglobinopathies can be profoundly impacted by the presence of modifiers. A major modifier of severity in both β -globin associated hemoglobinopathies is the reduced or increased number of α -globin genes. Additionally, mutations and/or allelic variants associated with increased fetal hemoglobin expression can also dramatically ameliorate these disorders.⁸⁹ In fact, patients with the same set of pathogenic globin mutations can have dramatically variable clinical courses based on modifier conditions.⁹⁰

The vast majority of β -thalassemias are caused by point mutations within the gene or within its immediate flanking sequences and are classified according to the mechanism by which they affect gene regulation: transcription, RNA processing, and mRNA translation.⁸⁸ Some transcription mutations involve either the conserved DNA sequences that form the β -globin promoter ($-80T > A$) or the stretch of 50 nucleotides in the 5' UTR. Generally, they result in a mild to minimal deficit of β -globin output and can be phenotypically *silent* carriers (Fig 3). An additional set of mutations introduces premature termination codons (PTCs)

attributable to frameshift or nonsense mutations, and nearly all terminate within exon 1 or 2. Mutations that result in PTCs are typically associated with minimal steady-state levels of β -mRNA in erythroid cells because of rapid degradation of the mutant mRNA by non-sense mediated mRNA decay (Fig 3).^{91–93} One of the most prevalent mutations in Western Mediterranean populations is the PTC at base 118 (C > T), which historically was named β^0 -39 since it introduces a PTC in codon 39 (Fig 3). In heterozygotes, no β -chain is produced from the mutant allele, but activity from the normal allele is sufficient to result in a typical asymptomatic phenotype.^{89,94,95} Another subset of mutations abolishes or limits the use of the proper splice sites or creates novel aberrant cryptic splice sites. All these mutations lead to unspliced or improperly spliced products with varying severities of phenotype. When the mutations involve the invariant di-nucleotides at the splice junctions (GTat 5' and AG at 3') normal splicing is completely abolished (intervening sequence 1 [IVS1]-5; Fig 3). Other mutations within the splice consensus sequences only reduce the efficiency of normal splicing and produce phenotypes that range from mild to severe (IVS1-110 and IVS2-654; Fig 3). Furthermore, mutations that produce PTCs later in the β -globin sequence are not subjected to non-sense mediated mRNA decay and result in amounts of mutant β -mRNAs comparable to those from the normal allele (β^0 -121; Fig 3).⁹⁶ Heterozygotes for such late termination mutations tend to have a much more severe phenotype. Frequently these aberrant mRNAs are stable, can be identified in patients, and can generate abnormal β -globin proteins leading to dominant forms of β -thalassemia (Fig 3).⁹⁷

As mentioned previously, several preclinical studies showed that it is possible to rescue mice with β -thalassemia by lentiviral-mediated β -globin gene transfer. However, the original studies in animal models of hemoglobinopathies utilized mice characterized by only a handful of mutations^{22,25–27,50–54} (Fig 1), whereas the complexity of the mutations and modifiers within the population of individuals affected by hemoglobinopathies is a source of great phenotypic variability. Therefore, some patients, based on their genetic profile and endogenous hemoglobin production, might be better candidates for lentiviral-based therapies than others. This is especially important in light of the intrinsic ability of the specific vectors used in the different clinical trials to make hemoglobin. In SCD, a further limitation of this curative approach through “gene addition” is due to the fact that the β -globin encoded by the vector must replace the mutant β -globin protein in the adult hemoglobin tetramer without increasing the total amount of β -globin chains (normal + sickle) produced. Increase in the total amount of β -globin chains could potentially lead to a phenotype similar to that observed in α -thalassemia.

Thus, studies on the correlation between types of mutations in the β -globin gene and expression of globin from the nonaffected gene have great potential to increase our understanding of gene expression in general and globin expression in thalassemia specifically. In addition, this knowledge may greatly improve the therapeutic options based on gene transfer, gene replacement, or gene correction. Based on these premises, we developed an in vitro protocol using CD34⁺ cells isolated from peripheral blood mononuclear cells (PBMCs) to predict if a patient has the potential to become transfusion-independent with a given vector. In β -thalassemia, mutant alleles that are associated with no β -globin synthesis are classified as β^0 , whereas those that allow some protein synthesis are designated β^+ . Patients with thalassemia are therefore generally classified as β^0/β^0 , β^0/β^+ , or β^+/β^+ , based on the combination of these 2 alleles.⁹⁸ Using a wide-range of samples from β^0/β^0 , β^0/β^+ , and β^+/β^+ patients, we investigated the correlation between genotype, endogenous levels of hemoglobins, and vector copy number (VCN), with the phenotypic outcome.⁵⁷ We used a novel lentiviral vector, AnkT9W, that carries both the human β -globin gene and the erythroid-specific ankyrin 5' HS barrier insulator.⁹⁹ This vector was able to maintain high, yet stable levels of Hb synthesis in MEL cells and in β -thalassemic (*th3/+*) mice. Compared with β -thalassemic mice transplanted with BM cells transduced with a vector that did not

incorporate the ankyrin element (T9W), *th3/+* animals treated with AnkT9W showed a greater improvement in Hb synthesis and he-matologic parameters.⁵⁷ Using 30 mL of blood, we developed a protocol for evaluating the correlation between the VCN of AnkT9W, the number of β -globin mRNA molecules, and the level of Hb synthesis in PBMC-derived human CD34⁺ and erythroid progenitor cells, following in vitro β -globin gene transfer and erythroid differentiation. Another goal of this study was to identify an in vitro protocol which could predict correction of both the thalassemic and SCD phenotype in a large pool of patient samples. In this regard, AnkT9W increased the synthesis of β -globin protein in most thalassemic and SCD specimens to levels comparable to those observed in carriers and control samples. However, after treatment a subset of thalassemic samples, mostly $\beta^0/0$, exhibited the lowest amount of total HbA (transgenic and endogenous). In contrast, $\beta^0/+$ and $\beta^{+}/+$ benefited from the expression of the endogenous HbA and reached curative levels at lower VCNs. Overall these results indicate 3 major endpoints.

First, $\beta^0/0$ specimens, which exhibited the lowest level of β -globin chain synthesis, required higher amounts of vector to reach therapeutic levels of Hb, compared with specimens from $\beta^0/+$ and $\beta^{+}/+$ patients. For this reason, patients should be carefully investigated to determine if they would be good candidates for a clinical trial based on this approach, especially if the level of gene transfer achieved might not be sufficient.

Second, when adding transgenic β -globin into SCD HSCs, there is a concern that after gene transfer, the total amount of β -chains (sickle + normal) might exceed the amount of α -chains, leading to an α -thalassemia-like phenotype. However, recent data from Khandros and colleagues indicate that erythroid cells are equipped with a series of protein quality control responses that can eliminate, to a certain extent, the excess of α -chains produced in β -thalassemia.¹⁰⁰ In fact, in SCD specimens, the treatment with AnkT9W was able to correct the phenotype by modifying the proportion of sickling vs functional Hb, without changing the overall Hb content, suggesting that a moderate excess of globin chains might be tolerated and eliminated efficiently by the erythroid cells.⁵⁷

Third, considering these results and the additional thought that irrespective to the β -globin mutations, additional mechanisms, potentially associated with genetic modifiers may contribute to the variability observed following gene transfer, analysis of erythroid progenitors transduced with different amounts of lentiviral vectors could be extremely useful for testing the potential of each lentiviral construct prior to myeloablation and transplant.

FUTURE PERSPECTIVES: SAFE HARBORS, HOMOLOGOUS RECOMBINATION, AND GENE TRANSFER IN IPS CELLS

Lentiviral vectors are currently utilized in clinical trials, however, the fact they integrate semirandomly throughout the genome is associated with several drawbacks. The expression of the transgene can be influenced by activating or repressing elements flanking the integration site, leading to expression variegation or silencing.¹⁰¹ Some insertions may disrupt genes or perturb their transcription, altering cycle and survival of the transduced cell, and eventually lead to cancer.^{102–104} Site-specific integration may limit these risks, achieving safe and uniform gene expression. Integrase-defective lentiviral (IDLV) or non-integrating lentiviral vectors that carry a mutant integrase are under development to facilitate such a task.^{105–107} Following reverse transcription, the DNA from these vectors does not integrate into the host genome but forms episomal circles. The approach consists in generating 2 IDLV vectors. One carries the sequences to target a specific genomic region by homologous recombination (HR)¹⁰⁷ and the second IDLV vector expresses zinc finger nucleases, which are target-specific endonucleases and induce site-specific DNA double-strand breaks, facilitating HR. With this approach, in principle a curative sequence could be

utilized to target specific mutant genes to achieve gene correction or for gene insertion into 'safe harbors'.¹⁰⁷ Safe harbors are genomic regions in which a transgene, following integration, exhibits elevated and stable expression in different cell types or tissues, does not show position effect variegation, and does not disrupt coding or regulatory sequences due to the integration. Lombardo and colleagues identified one of such safe harbors in the AAVS1 genomic locus of human cells.¹⁰⁸ The same group also demonstrated that this approach can be utilized to generate genetically modified stem cells and human lymphocytes.^{108,109}

Alternatively, somatic cells reprogrammed to iPSCs might also provide a possible new approach to treat β -thalassemia and SCD.³¹ Currently, robust generation of iPSCs requires the introduction and integration of genes encoding the TFs octamer-binding transcription factor 3/4 (OCT3/4), and sex determining region y-box 2 (SOX2) with either Kruppel-like factor 4 (KLF4) and c-MYC or NANOG and LIN28.^{110–112} Recently, iPSCs with a single viral integration were generated by transduction with a single lentiviral vector (known as STEMCCA) expressing a "stem cell cassette" composed of the 4 TFs (OCT4, KLF4, SOX2, and c-MYC) and a combination of self-splicing peptides and internal ribosome entry site technology.^{113,114} iPSCs generated in this manner display embryonic stem celllike morphology, express embryonic stem cell markers, and exhibit *in vivo* pluripotency, as evidenced by their ability to form all 3 germ layers in teratoma assays and their robust contribution to mouse chimeras.

Chou and colleagues investigated epigenetic and gene expression signatures of multiple cell types such as fibroblasts and blood cells.¹¹⁵ Their analysis suggested that newborn cord blood and adult PBMCs display unique signatures that are closer to iPSCs and human embryonic stem cells than age matched fibroblasts, thus, making PBMCs an easy and attractive cell choice for the generation of iPSCs. They generated iPSCs from unfractionated blood cells using 2 episomal vectors expressing the reprogramming factors and the SV40 Large T antigen. With this approach, they were able to generate between 3 and 12 iPSCs from 2×10^6 PBMCs. Ye and coworkers have shown that iPSCs can be generated from cells derived from skin fibroblasts, amniotic fluid or chorionic villus sampling of patients with β -thalassemia and subsequently be differentiated into hematopoietic cells that synthesized hemoglobin.

To minimize the oncogenic potential attributable to the presence of these TF and random integrations, several precautions can be taken. Lentiviral vectors can be used to generate iPSCs and add therapeutic β -globin at the same time. Once the reprogramming has occurred, the genes encoding for the iPSC TFs can be excised from the cells, minimizing their role in potential future oncogenic events. Alternatively, iPSCs can be generated with episomes,¹¹⁶ nonintegrating viruses, synthetic RNA, or proteins, as reviewed by Yanamaka's group.¹¹⁷ Moreover, iPSCs can be analyzed to identify whether the β -globin gene integration events occurred in 'safe harbors' within the genome.¹¹⁸ Safe harbors can be identified based on insertion site. A safe harbor is typically considered to be more than 50–100 kb away from regions transcribing microRNAs, coding gene regions and ultraconserved regions. Analysis of iPSCs infected with lentiviruses has demonstrated that ~10% of integrations occur in safe harbors. This analysis can include monitoring of the expression of genes in *cis* to the integrations sites, to evaluate whether their regulation has been altered. Clones without insertional mutagenesis can be selected for high levels of transgene expression. However, this approach still relies heavily on cellular manipulation and random integrations. Furthermore, in SCD patients this approach does not eliminate the issue of overproducing β -globin chains (therapeutic + sickle).

In another method, patient iPSCs could be corrected by HR techniques. This strategy has several potential advantages over conventional gene therapy because it avoids the risk of

insertional mutagenesis by therapeutic vectors and maintains β -globin expression by endogenous control elements rather than a constitutive promoter. The Kan and Townes laboratories utilized HR to correct mutations and generate iPSCs from, respectively, a patient with β -thalassemia and sickle cell mice. , Using a humanized SCD mouse model, Jaenisch's laboratory demonstrated that mice can be rescued after transplantation with hematopoietic progenitors obtained *in vitro* from autologous iPSCs. This was achieved after correction of the human sickle hemoglobin allele by gene-specific targeting.²⁹ Further proof of principle that the β -globin gene can be repaired in human iPSCs was provided by 2 additional groups.^{120,121} They achieved correction of the β -globin gene by HR in iPSCs derived from a SCD patient using β -globin zinc finger nucleases engineered to specifically stimulate HR at the β -globin locus.

Therefore, development of iPSCs technology combined with safe harbors or HR might represent a safer approach for the cure of hemoglobinopathies than traditional gene therapy. However, limitations with this approach are associated with the observations that red cells derived-iPSCs have limited ability to express adult Hb or home to the BM. These are important problems that will need to be solved before the strategy can be utilized for therapeutic purposes.

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Abbreviations

BM	bone marrow
Hb	hemoglobin
HR	homologous recombination
HSC	hematopoietic stem cell
iPSC	induced pluripotent stem cell
IVS	intervening sequence
PBMC	peripheral blood mononuclear cell
SCD	sickle cell disease
TF	transcription factor
VCN	vector copy number

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Fig 1.

Lentiviruses (LV) expressing the β or γ -globin gene. A prototypical vector and its main components are represented on the top. Constructs are divided in 3 major categories: β -globin, β -globin anti-sickling, and γ -globin LVs. In each box they are represented in chronological order of publication, starting from the most recent. Names of vectors, first authors, and publication date are indicated. Hypersensitive sites (HS) of the locus control region (LCR) are indicated with the respective sizes. Specifics of each construct are described in the text.

Fig 2.

Schematic of full myeloablation vs reduced-intensity myeloablation. Left panel, BM before myeloablation. Middle panel, BM after myeloablation. Right panel, BM after transplant with transduced cells; numbers indicate vector copy number (VCN). Transduced and not transduced cells are represented by grey and white circles, respectively.

Fig 3.

Structure of the human β -globin gene and of the normal and alternative spliced forms for each mutant allele. Only the coding regions (dark boxes) and the introns (IVS, light boxes and lines) are indicated with their respective length. The size of the coding sequences + introns is equal to 1424 bp. Circles indicate stop codons in the coding sequence. Aberrant mutant forms present premature termination codons (PTCs) due to point or frame-shift mutations. The effect of the mutation on β -globin synthesis is indicated as $\beta 0$ or $\beta +$ (in grey or black, respectively). Aberrant messenger RNA sensitive to NMD.