

Development and recent progresses of gene therapy for β -thalassemia

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Abstract

β -thalassemias are among the most common inherited monogenic disorders worldwide due to mutations in the β -globin gene that reduce or abolish the production of the β -globin chain resulting in transfusion-dependent chronic anemia. Currently, the only curative treatment is allogeneic hematopoietic stem cells (HSCs) transplantation, but this option is limited by the availability of HLA-matched donor. Gene therapy, based on autologous transplantation of genetically corrected HSCs, holds the promise to treat patients lacking a compatible bone marrow donor. Initial attempts of gene transfer have been unsuccessful due to limitations of available vectors to stably transfer a globin gene in HSCs and reach high and regulated expression in the erythroid progeny. With the advent of lentiviral vectors (LVs), based on human immunodeficiency virus, many of the initial limitations have been overcome. Since 2000 when Sadelain and co-workers first demonstrated successful globin gene transfer in murine thalassemia models with improvement of the phenotype using a recombinant β globin/LV, several other groups have developed different vectors encoding either β , γ or mutated globin genes and confirmed these results in both murine models and erythroid progeny derived from patient's HSCs. In light of these encouraging results, research has recently moved into clinical trials that are ongoing or soon to begin. One participant in an ongoing gene transfer trial for β -thalassemia has achieved clinical benefit with elimination of his transfusion requirement. Here, development and recent progress of gene therapy for β -thalassemia is reviewed.

β -thalassemia

β -thalassemias represent the most common monogenic disorders worldwide with 80 million

carriers of the trait and a global birth-rate incidence of 40,000/year, most prevalent in the Mediterranean region, the Middle East, India, and South East Asia. In Italy alone, there are over 5000 patients and about 1.5 million carriers of the trait.^{1,2} These syndromes arise from more than 200 autosomal recessive mutations that affect the human β -globin gene leading to the absence or insufficient production of hemoglobinA (HbA) β -chains. This in turn leads to an imbalance of α - and β -globin chains, resulting in excess of α -chain molecules which precipitate in red blood precursors, leading to impaired erythrocyte maturation, mechanical damage, and ultimately to apoptosis.³

Homozygotes or heterozygotes compound harboring either null or very low expressing alleles present severe anemia that becomes evident a year after birth, and that is lethal early in life if left untreated.

Current therapies

Palliative therapies consist of regular blood transfusions in combination with daily iron chelation life-long. Hydroxyurea treatment for fetal hemoglobin (HbF) induction has also been employed in β thalassemia patients, but only some patients, with thalassemia intermedia showed significant improvement in total hemoglobin levels and for a short time of treatment.⁴

Although an appropriate scheme of transfusion and chelation have improved life expectancy of patients and significantly delayed the onset of iron-related organ failure, the lifelong treatment severely compromises the quality of life of thalassemia patients. Treatment noncompliance is common and many patients suffer from cardiac, hepatic and endocrine complications.⁵ Moreover, higher incidence of new complications, as hepatocarcinoma, has been described probably as a result of improvement in thalassemia outcomes.⁶ The management of the disease and its complications constitutes an enormous financial effort affecting in particular the developing countries;⁷ in fact a proper medical therapy for thalassemia requires expertise for associated complications and the support of an increasing number of new technologies (*e.g.*, cardiac magnetic resonance imaging, etc.).⁸

Considering that the vast majority of patients today lives in non-industrialized countries, where these effective therapies are improving but are still severely curtailed, the problem of relative costs between a lifelong medical therapies *versus* a therapy intended to cure is crucial.

Allogeneic hematopoietic stem cell transplantation

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only possible curative

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treatment for β -thalassemia major. The defective gene is replaced by the normal hematopoietic cells from donors. Since the first report of a successful HSCT from an HLA-identical sibling in a child with homozygous β -thalassemia in 1982⁹ more than 3000 patients worldwide have received allogeneic HSCT as curative treatment for β -thalassemia major. This therapeutic treatment was developed and grew into accepted routine clinical practice primarily thanks to the Pesaro group experience during the 1980s and early 1990s, that developed a prognostic scheme to predict transplant outcome in patients. A remarkable result of 82-93% of disease-free survival is achieved if a matched sibling donor is available, and if the patient belongs to the class 1 and 2 of the Pesaro's classification and is younger than 17 years of age. This favorable result cannot be reproduced in older, more heavily iron-overloaded patients. Transplantations from matched-unrelated donors, haploidentical donors, or in patients with disease in later stage are associated with significantly lower disease-free survival (21-70%) and higher morbidity and mortality (25-30%).^{10,11}

The development of new techniques to improve the management of graft versus-host

disease (GVHD), and to perform HSCT from unrelated donors or cord blood stem cells may expand the pool of potential donors in the near future; currently they remain largely investigational.¹²⁻¹⁴ Because of these significant disadvantages, and shortage of suitable donors, HSCT is limited to less than one third of the patients; for the vast majority of thalassemic subjects lacking an HLA-matched donor, clinical improvement or even a final cure might be achieved by gene therapy approaches.

Gene therapy of β -thalassemia

The goal of globin gene therapy is to restore the capacity of the thalassemic subject's own HSCs to generate red blood cells containing normal hemoglobin level which will reverse the ineffective erythropoiesis and correct the inherited anemia. This therapeutic way consists of a normal globin gene transfer into autologous HSCs. The transfer is virtually applicable to all patients with no risk of GVHD or immunological complications since the graft is autologous. HSCs recovered from bone marrow (BM) or mobilized peripheral blood (PBMCs) are thus the target for *ex-vivo* globin gene transfer, as these cells are the only ones capable to self-renew and generate all hematopoietic lineages including the erythroid one.^{15,16}

The gene therapy treatment plane consists of the follow steps summarized in Figure 1: i) collection of the subject's hematopoietic CD34+ stem and progenitor cells from BM or PBMC; ii) *ex-vivo* transduction with globin gene/recombinant vectors under appropriate culture conditions; iii) reinfusion of the transduced CD34+ cells after a preparative regimen able to promote the engraftment and expansion of the corrected HSCs; and iv) follow up the post-treatment period to monitor the safety of the procedure, the occurrence of engraftment and the expression of the globin transgene. The development of gene transfer methods for the treatment of thalassemia by various types of vectors has been a challenge for more than three decades; to date most efforts have focused on the development of recombinant retroviral vectors as mean of gene delivery into stem cells.^{17,18}

These vectors, based on γ -retroviruses (γ -RV) or lentiviruses (LV), are widely used in the setting of hematopoietic gene therapy because of their ability to efficiently integrate therapeutic genes into the target cell genome, resulting in long term gene transfer even after many rounds of cellular division.¹⁹

Even if β -thalassemias are among the first diseases for which gene therapy was envisioned, the addition of a globin gene, mediated by retroviral vectors, raises major challenges in terms of controlling transgene expression. In fact, to made gene therapy of β -thalassemia effective, safe and realistic as therapeutic

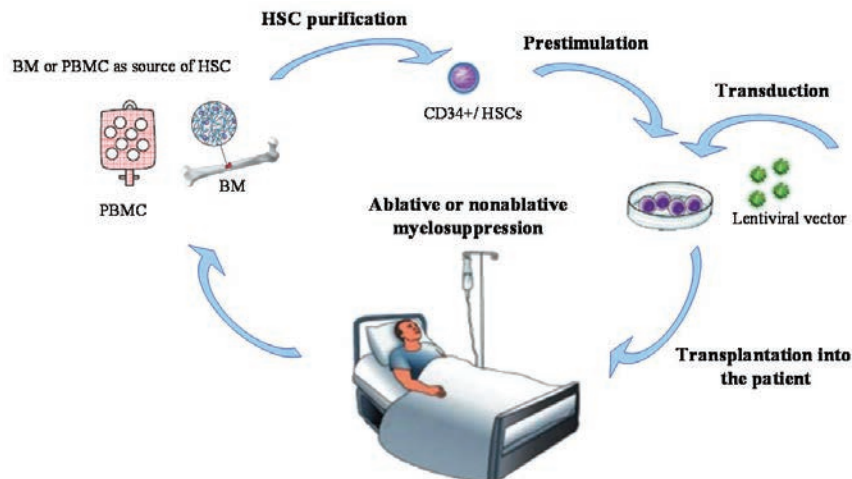


Figure 1. Schematic representation of hemopoietic stem cell (HSC)-based gene therapy for β -thalassemia. HSCs collected from bone marrow (BM) or from peripheral blood mononuclear cells (PBMC) after mobilization, are processed to purify CD34+ hematopoietic stem cells (HSC purification). The cells are cultured in early-acting cytokines (prestimulation) and transduced with a therapeutic globin lentiviral vectors designed to express high levels of normal human β -globin (transduction). The patient is treated with ablative or non-ablative myelosuppression, and then engrafted with the vector-modified cells (transplantation).

approach, some fundamental conditions have to be fulfilled: i) the therapeutic vector should exhibit high titer and ability to efficiently transduce HSCs and the genome of the vector should be stably transmitted; ii) the expression of the transgene must be controlled through the use of regulatory elements in order to be erythroid-specific, stage-restricted, elevated, independent, and sustained over time; iii) the therapy itself must be safe (e.g., absent or low vector genotoxicity).

Development of effective vectors for β -thalassemia gene therapy

γ -retroviral vectors

Early attempts back in the 1980s and 1990s utilized γ -RV vectors derived from murine leukemia virus (MLV) to drive expression of β -globin genes into murine HSCs. The transfer of a complete globin gene into murine stem cells has been accomplished, but the level of expression remained relatively low, reaching 0%–2% of the endogenous RNA level.²⁰⁻²²

In an attempt to increase β -globin expression, Novak *et al.*²³ incorporated the newly identified powerful DNA-enhancer elements from the β -globin locus control region (LCR) into MLV vectors; This genomic element was discovered in transgenic mice experiments by virtue of its functional activity in providing copy number-dependent, integration site-

independent globin gene expression in erythroid cells.²⁴ In its native configuration, the β -globin LCR is too large to be included in retroviral vectors, therefore, the functional hypersensitive sites (HS2, HS3, and HS4) upstream from the β -globin gene cluster has been adapted to minimize the size of vectors by focusing on the core.

Unfortunately, the inclusion of LCR core elements, although significantly increase the expression levels in murine erythroleukemia cells, significantly lowers the titer of the vectors and makes them genetically unstable.^{25,26} The latter, frequently occurs due to activation of cryptic splice sites of the LCR sequence in the RV RNA, and additional attempts were done to eliminate these sites. Leboulch *et al.*, after extensive mutagenesis studies of the transduced β -globin gene, identified a 372 bp intronic segment and multiple polyadenylation and splicing sites, that were responsible for low viral titers and instability of proviral transmission, upon infection.²⁷ Even when small segments of the LCR element were included without these problems, the resulting vectors exhibited variable expression patterns, indicating sensitivity to position effects.²⁸ Incorporation of chromatin insulators improved the expression and stability of RV vectors, but their expression still remained well below therapeutic levels.²⁹

Another major limitation is that the γ RV vectors need to infect cells before or close to their division, because the viral RNA cannot pass through the nuclear membrane. Since the majority of hematopoietic stem cells are in a

quiescent state, it is necessary to stimulate them with cytokines to induce cell division and achieve higher transduction efficiency; on the other hand, the stimulation of quiescent hematopoietic stem cells impairs the long-term repopulating capacity.³⁰

In general, the extensive efforts over nearly two decades for the development of effective β -globin/ γ RV vectors have had limited success,³¹ but eventually paved the way for the success of gene therapy.

Lentiviral vectors

In the mid-1990s, the HIV-1 based LV vector became available and offered a real alternative to γ -RV vectors as possible carriers of globin genes. As opposed to γ -RV, LV vectors have the ability to transduce non-dividing cells and can carry large transgene cassettes (9-10 kb) with introns and regulatory LCR elements with none or limited sequence rearrangement. Therefore, LV vectors are thus likely to be selected as vectors of choice for the stable delivery of regulated transgenes in stem cell-based gene therapy.^{32,33}

Since their introduction, these vectors underwent many improvements, including the reduction of HIV sequences including the viral enhancer; a self-inactivating (SIN) vector design that eliminates expression of the viral sequences by deleting the U3 enhancer region of the viral long-terminal repeat (LTR) was introduced.³⁴ With such a SIN vector design, vector expression can thus be driven exclusively by erythroid-specific enhancers and promoters; in turn, this allows for elevated, tissue restricted globin gene expression and increases safety profile by reducing the potential for vector sequences to activate the expression of cellular genes in most tissues.^{35,36}

A prototypical β globin/LV self-inactivating vector is depicted in Figure 2.

In addition, as documented both in cell lines and in large animal models, HIV-derived vectors possess a safety advantage over those derived from MLV because of their natural propensity to integrate all along transcription units without preference for promoter regions.³⁷⁻³⁹ These differences in integration patterns have now been verified in human clinical trials via large-scale insertion site analyses.⁴⁰⁻⁴³

The use of chromatin insulators to improve the expression and safety of integrating gene transfer vectors

LV vectors, although to a lesser degree compared to the γ -RV vectors, are subject to chromosomal position effects leading to transgene silencing or variegated expression. The reduced versions of the β -globin LCR are apparently insufficient to overcome position-dependent variability of gene expression in erythroblasts.

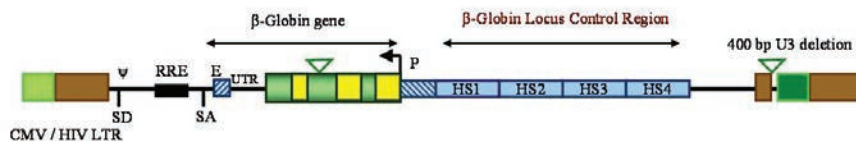


Figure 2. General scheme of a prototypical β -globin lentiviral self-inactivating vector. The vector consists of viral and human β -globin gene sequences. Viral sequences: viral packaging signal (ψ); donor (SD) and acceptor (SA) splicing sites; viral Rev responsive element (RRE). β -globin gene sequences: a combination of 2 to 4 core sequences from DNase hypersensitive (HS) sites (HS1, HS2, HS3, HS4); β -globin gene body including from 200 to 400 bp of promoter sequences (P); a stabilizing 372 bp *RsaI* deletion of IVS2 (*RsaI*); variable length of the 3' untranslated region (UTR); the enhancer region contained in a *PstI* fragment 3' to the β -globin gene (E). Safety modifications include stop codons in the ψ signal and a 400 bp deletion in the U3 region of the 3' long terminal repeat (LTR).

The use of chromatin insulators offers a potential solution for generating vectors more effective and safer; insulator elements can act as barrier elements to dampen position effects and enhancer blockers to prevent nearby genes deregulation. The prototypic chromatin insulator from chicken β -globin gene locus, cHS4, reduces the silencing chromosomal position effects providing a more consistent/uniform expression, and therefore leading to superior genetic correction in both animal models and cell cultures.⁴⁴ Moreover, this element alone or in combination with other elements has been shown to have a lower propensity to perturb gene expression of neighboring genes.⁴⁴ Unfortunately, the caveat is that LV vectors carrying the full length (1.2 bp) cHS4 element dramatically reduces vector titers which is another very important aspect in hematopoietic stem cell gene transfer; smaller versions of this element do not retain the full insulator activity. In the attempt to expand the repertoire of available elements that lead to higher, potentially therapeutic levels of the human globin genes, several groups, including ours, have identified new sequences functioning as chromatin insulators.⁴⁴⁻⁴⁸ Our group focused on an element derived from the sea urchin histone gene locus, termed *sns5* (462 bp long); its inclusion in a γ -RV reporter vector made it even more resistant to silencing position effects than the cHS4 insulator itself in an erythroid cell line model. As the cHS4 element, *sns5* displays enhancer-blocking insulator activity and has the ability to recruits epigenetic markers of active chromatin in erythroid milieu, making it potentially an ideal candidate to improve globin/recombinant vectors efficacy.⁴⁷

Preclinical studies

Correction of β -thalassemia on mouse model

The major breakthrough in the correction of

β -thalassemia came from the group of Sadelain in New York. They reported the first recombinant β -globin/LV vector, termed TNS9, capable to accommodate more complex transcription units without compromising the titer. The TNS9 vector carried a much larger (3.2 kb) LCR than that previously carried by globin/ γ RV vectors; it results from a combination of the most transcriptionally active elements HS2, HS3, and HS4 linked to a β -globin gene plus its 3' enhancer.⁴⁹

The greater functionality of this larger β LCR element combination gave rise to a much higher average level of β -globin expression (4-6 g/dL per vector copy) resulting in amelioration of severe β -thalassemia intermedia and thalassemia major on mouse models.^{49,50}

Since these initial reports, several other groups have confirmed and extended these results in models of thalassemia using LV encoding β - or γ - or mutated globin gene.^{36,51-53}

In addition, Miccio and co-workers showed that genetically corrected thalassemic erythroblasts undergo to *in vivo* selection in mice, indicating that the corrected cells have a survival advantage over the thalassemic ones.⁵⁴ From a clinical point of view, this advantage could imply a lower myeloablative regimen for a patient undergoing to gene therapy, who could benefit even when some chimerism persists after BM transplant with genetic modified cells.

Correction of human thalassemic erythroid cells

In vitro model of human erythropoiesis have been developed for preclinical evaluation of β -globin/LVs to assay for production of HbA after gene delivery into CD34⁺ cells obtained from mobilized peripheral blood of normal adults or steady-state BM from patients with β -thalassemia major. Production of normal amounts of β -globin with correction of ineffective erythropoiesis has been achieved in erythroid progeny of β -thalassemic CD34⁺ cells and

effective human erythropoiesis was documented 3-4 months after transplantation of these gene-corrected primitive cells into immune-deficient mice.⁵⁵ Another study that used CD34+ cells collected from a large cohort of patients provided additional evidence that the β -thalassemia major phenotype may be corrected by gene transfer.⁵⁶

An additional concern associated with the attempt to cure hemoglobinopathies by gene transfer is the number and complexity of the mutations within the thalassemic population that is source of great phenotypic variability: higher levels of globin transgene expression are required to treat β^0 -thalassemia compared with milder form of thalassemia.

Furthermore, in the same set of globin mutations additional mechanisms, potentially associated with genetic modifiers and/or other unknown factors, can determine dramatically variable clinical courses of the disease (Renzo Galanello and Antonio Cao with their valuable work have greatly contributed to knowledge in this field).⁵⁷⁻⁵⁹

This raises concern that some patients, based on their genetic profiles 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 express sufficient amount of the therapeutic protein to cure severe thalassemias at a low vector copy.

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. With this aim, the group of Rivella recently developed an *in vitro* protocol, which could predict the total amount of hemoglobin and the subsequent number of vector copies/cell required to reverse the thalassemic phenotype in a large pool of patient's samples that exhibited phenotypic variability of the disease.⁴⁶

Clinical trials

Altogether, these results from several laboratories demonstrated the efficacy of globin gene transfer in the treatment of animal models and cell cultured of β thalassemia, paving the way to assess globin gene transfer in selected patients who lack an HLA-matched donor. The first clinical trial using a SIN-LV vector to transfer a globin gene into bone marrow cells from patients with β -thalassemia was initiated in 2007 in Paris by the group of P. Lebouch and is still open.^{60,61} The lentiviral vector that was used, termed LentiGlobin, encodes a mutated adult β -gene (βA^{T87Q}) distinguishable from transfused β -globin because of an anti-sickling mutation at the

87th amino acid. The vector was flanked by two copies of the 250 bp core of the cHS4 chromatin insulator. To date two patients have been treated: autologous cells were harvested from bone marrow, and the recipients were conditioned with full myeloablation with busulfan. The treatment was unsuccessful for the first patient, in that, owing to delayed hematological recovery and cytopenia related toxicity, he received non-manipulated back-up cells for rescue and was reconstituted by unmodified thalassemic marrow cells. The second participant, a 18-year old male at the time of treatment, was affected by severe β^E/β^0 -thalassemia and began on regular transfusion therapy at age 3 because of severe and poorly tolerated anemia. The patient received the transduced bone marrow cells in early June of 2007 and became transfusion independent one year after gene transfer. The transduction efficiency of bulk CD34+ cells was approximately 30% and an average of 0.6 vector genomes/ transduced cell were found in his transduced bone marrow CD34+. The contribution of vector-encoded β -globin total Hb was estimated to be 3 g/dL, consistent with preclinical mouse studies. Although this patient stabilized at 9-10 g/dL of Hb, the outcome constitutes only a partial success of the gene therapy. This is due to the fact that the therapeutic Hb- βA^{T87Q} in this patient contributed only one third of the total Hb synthesized, while HbE and HbF accounted equally for the remaining Hb.⁶¹ In addition, up to 50% of the LV-derived Hb chains were produced from a single clonally expanded myeloid progenitor cell. This clonal dominance resulted from an insertional mutagenesis event in which the vector had integrated within the third intron of *HMG2* gene (high mobility group AT-hook 2) and gave rise to aberrant splicing of the third exon of the host gene onto a cryptic splice acceptor site within the core cHS4 insulator elements present in the LV-LTRs.

The aberrant splicing resulted in production of a truncated HMG2 mRNA devoid of let-7 miRNA regulatory target sequences and in a protein product encoded by only the first three exons of the gene at highly elevated levels, with consequent downstream disturbances in gene function. The expression was reported to be erythroblast-specific, as HMG2 mRNA was undetectable in granulocyte-monocyte cells. However, the clonal dominance of *HMG2* gene is represented in all populations in similar proportions (erythroblasts, granulocyte-monocyte and LTC-IC).

The authors have hypothesized that this dominance is due to a transient expression of HMG2 in a myeloid-restricted LT-HSC during β -LCR priming, before the β -LCR becomes restricted to the erythroid lineage.⁶²

It is possible that, in this case, the oligoclonal reconstitution of the bone marrow has contributed to the clonal expansion of one of the few cells containing the vector (only 24

insertion sites have been highlighted). In this case, the frequency of the clone should stabilize or even decrease as a result of the exhaustion of the clone itself. As consequence, also the high-level expression associated to this clone should run out with the consequent loss of effectiveness of the treatment. In contrast, the clone could continue to expand emphasizing the nature of insertional oncogenesis of this event. Only a long-term follow-up of the patient will be able to shed light on these two hypotheses.

This expansion has been stable over several years to the present time, the patient is currently leukemia-free despite the prolonged clonal expansion and continues to produce an additional 2 to 3 g/dL of hemoglobin comprising the vector-encoded β^{T87Q} -globin chain.

Given the special nature of outcomes in this case it is therefore not possible to deduce whether the vector used is capable to eventually ameliorate the condition of patients with β -thalassemia major, who possess little or no β -globin chains. Nevertheless, the gene therapy approach has great potential for success, as evidenced by the fact that this patient no longer needs transfusion therapy, does not manifest any malignant or pre-malignant state and has significantly improved quality of life.

At least two other groups have announced plans to carry out similar trials in the United States (<http://oba.od.nih.gov/oba/rac/>).

The source of HSCs, the conditions of *ex vivo* cell manipulation and transduction are among the parameters that influence the outcome of the treatment. Although steady state bone marrow CD34+ cells were used as the source of HSCs for gene transfer in the first globin gene transfer trial,⁶¹ others group, including our own, are evaluating whether cytokine mobilization of peripheral blood CD34+ cells in patients with β -thalassemia might be safe and effective as a way to collect a greater number of HSCs.

In a pilot trial of CD34+ cells mobilization in adults with β -thalassemia, recently conducted at Memorial Sloan-Kettering Cancer Center (MSKCC) in New York, Sadelain and co-workers have demonstrated the feasibility of collecting a sufficient number of CD34+ cells mobilized with G-CSF in thalassemia major patients and validated an effective procedure for β -globin gene transfer.⁶³ These findings provided the basis for the implementation of the first gene therapy trial in the United States in patients with severe hereditary globin disorders. The trial (registered at ClinicalTrials.gov under NCT01639690) already started at the beginning of 2013 at MSKCC and it is a multicenter phase I/II clinical trial that involves other European centers including our own. The trial is offered to patients with transfusion-dependent β thalassemia who lack a matched, related donor.⁶⁴

Conclusions

The severe β -thalassemias are still invalidating, often lethal disorders, for which a curative therapy is justified. The allogeneic HSCT from HLA-matched sibling results in a high likelihood of long-term disease-free survival mostly in pediatric patients. The remaining patients who lack of HLA-matched donor or those affected by mild or moderate iron overload and liver complications have a higher risk of morbidity or mortality if they pursue allogeneic HSCT. For these patients, the globin gene transfer in autologous stem cells offers the prospect of a cure since the efficient delivery of globin genes using LVs led: to amelioration of β -thalassemia murine models, and to the success of the first clinical trial in France. Nevertheless, more patients and a longer follow-up are necessary in order to establish whether the gene therapy may be safer than conventional allogeneic HSC transplantation. The unique pathology of β -thalassemia makes necessary to pay more attention to assess the optimal level of ablation, the source of HSCs, and cell dose in order to ensure effective engraftment of gene-engineered cells, reducing both the hematopoietic and the non-hematopoietic toxicity.

The risk of insertional oncogenesis in stem cell gene therapy with lentiviral vectors needs to be addressed together with vector configurations for increasing biosafety. Great efforts are being made to make realistic other future perspectives such as safe harbors and homologous recombination. Regardless the need for constantly improving vector design, a lot of attention has been drawn also towards strategies that result in higher numbers of genetically modified HSCs, that will in turn contribute to the HSC pool in the patient.

The latter, together with extensive research towards alternative HSC, such as iPSC cells, will undoubtedly put the bases for more successful clinical trials.

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