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EXPERT OPINION

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Globin gene regulation for treating β -thalassemias: progress, obstacles and future

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Introduction: β -thalassemias result from mutations in the β -globin gene, leading to a reduced or absent production of β -globin chains. The treatment of β -thalassemias has been historically based on blood transfusions and iron chelation therapy. The only curative therapy currently available is allogeneic hematopoietic stem cell transplant (HSCT) from suitable donors. With the limited pool of suitable donors, HSCT remains unavailable for many thalassaemic patients. They may instead benefit from globin gene therapy and other modalities, which exploit recent advances in understanding of globin gene regulation.

Areas covered: The objective of this review is to discuss the relevance of novel treatment modalities based on globin gene regulation, including globin gene transduction therapy, which is currently being studied in clinical trials. We also discuss globin gene editing, microRNA therapy, hypomethylating agents, hydroxyurea and butyrate derivatives.

Expert opinion: In the future and as a result of ongoing clinical trials, gene therapy, using lentiviral-based or other vectors, could be an alternative to allogeneic HSCT in patients with β -thalassemia. Gene editing is also a promising avenue that has not been explored in clinical trials yet.

Keywords: gene therapy, genome editing, globin, hematopoietic stem cells, thalassemia

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1. Introduction

β -thalassemias result from mutations in the β -globin gene resulting in reduced or absent production of β -globin chains. Consequently, an excess of α -globin chain molecules is present and precipitates in erythroid cells resulting in impaired red blood cell maturation and hemolysis [1]. This further leads to a state of chronic anemia with varying phenotypes necessitating different supportive transfusion regimens. β -thalassemias are among the most common monogenic disorders around the globe. Until allogeneic hematopoietic stem cell transplantation (HSCT) came into the picture, the treatment of β -thalassemias had been traditionally focused on supportive measures, such as blood transfusions and fetal hemoglobin (HbF) inducers, and on therapies addressing the resulting complications, such as iron chelation therapy. HSCT is the only currently available procedure capable of definitively curing this disease [2,3]. Nevertheless, the lack of availability of human leukocyte antigen (HLA)-matched donors remains one of the rate-limiting steps to the widespread use of allogeneic HSCT in thalassemia [4]. This strengthened the interest in potential novel therapies, grounded in the exploitation of the molecular understanding of thalassemia, with the intent of curing the disease or alleviating its phenotypic severity.



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Article highlights.

- Globin gene regulation, whether through gene transduction, genome editing, or other pharmacologic approaches, holds a great promise in the treatment of β -thalassemia.
- Requirements for the success of gene therapy in β -thalassemia include the safe and effective harvest and transfer of hematopoietic stem cells and a high level of erythroid-specific, position-independent expression of the β - γ -globin gene with a minimal risk of mutagenesis.
- Compared to γ -retroviral vectors, lentiviral vectors show a lower degree of genotoxicity, are capable of integrating into non-dividing cells, and are currently being tested in β -thalassemia patients.
- While still at the preclinical stages, genome editing techniques, including the use of zinc finger nucleases, TALENs and CRISPR/Cas9, do not require gene transduction and, consequently, alleviate the risk of genotoxicity.
- More intensive systematic research is needed to evaluate the effects of agents that target increased HbF production, including hypomethylating agents (5-azacytidine and decitabine), hydroxyurea and butyrate derivatives, in β -thalassemia patients.

This box summarizes key points contained in the article.

2. Current established therapies in β -thalassemia

2.1 Supportive treatment

The mainstays of current management in β -thalassemias are regular blood transfusions, especially for β -thalassemia major (TM), and iron chelation therapy – both of which have improved morbidity and mortality in β -thalassemia [5]. HbF induction with hydroxyurea has been also used to improve hemoglobin levels in β -thalassemia intermedia (TI) [6]. The cost of the aforementioned lifelong or long-term therapies is added to the cost of the advanced testing techniques, such as MRI for the assessment of liver iron concentration (LIC) and cardiac T2*, currently recommended for follow up of iron overload in patients with β -thalassemia [7,8]. In addition to the financial burden imposed by these new useful modalities, these tools require advanced expertise that might not be readily available in areas of the world where β -thalassemia is prevalent [1,9]. Therefore, the idea of a curative therapy that not only improves the quality of life of patients with β -thalassemia but also achieves better cost-effectiveness has come into light.

2.2 Allogeneic hematopoietic stem cell transplant

In 1982, Thomas *et al.* reported the first successful HLA-matched allogeneic HSCT for a patient homozygous for β -thalassemia in Seattle [10]. Until today and with the progressive accumulation of experience, allogeneic HSCT remains the only therapeutic option proven to be curative for

thalassemias [11,12]. The Pesaro group's efforts in the 1980s and 1990s resulted in the acceptance of allogeneic HSCT as routine clinical practice [11-15]. The source of the hematopoietic stem cells has been traditionally the bone marrow of an HLA-matched sibling. In patients with TM who lack a compatible sibling donor, high-resolution molecular typing of HLA has been used to identify potential alternative unrelated volunteer donors [4,16-19]. Another alternative source of hematopoietic stem cells is cord blood from an HLA-identical sibling [20-24]. Hematopoietic stem cells from umbilical cord blood were first successfully used in 1988 to cure Fanconi anemia [25]. A recent study comparing outcomes in patients with hemoglobinopathies between those who received cord blood stem cell transplant (CBT) versus those who received bone marrow stem cell transplant (BMT) showed similar 6-year disease-free survival (DFS) and 6-year overall survival but lower incidence of acute graft-versus-host disease (GVHD) [24]. In this large retrospective study, Locatelli *et al.* suggested that patients with TM have excellent outcomes with both HLA-matched sibling cord blood transplant and bone marrow transplant [24]. Six-year DFS in TM patients was shown to be 86 and 80% after BMT and CBT, respectively [24].

With the limited pool of suitable donors, HSCT remains unavailable for many thalassaemic patients who still lack a chance of cure. On the other hand, HLA-matched unrelated CBT and HLA-mismatched related donor HSCT remain experimental with lower DFS and OS [26-29]. Consequently, gene therapy may be an alternative curative approach that relies on the genetic modification of autologous hematopoietic stem cells from patients with β -thalassemia [30]. Table 1 summarizes the advantages and disadvantages of different potential therapeutic modalities based on globin regulation.

3. Current understanding of globin gene expression

The β -, ϵ -, γ - and δ -globin genes are all located on chromosome 11 [31,32]. While, in embryonic life, erythropoiesis takes place in the yolk sac and ϵ -globin gene predominates, a switch into the fetal phase mandates a transition of erythropoiesis into the liver with an accompanying shift to γ -globin expression [33]. Ultimately in the post-partum stage, γ -globin chain expression gradually wanes and is superseded by the adult β -globin gene expression [34]. The persistence of the fetal form of globin expression has been reported to appease the symptoms of globin chain defects, whether quantitative or qualitative [35,36]. Based on our knowledge that the persistence of HbF may alleviate the phenotypic severity of concomitantly inherited β -globin mutations, current research has been aiming to further understand this process and develop agents to reactivate the production of HbF as a therapeutic target in hemoglobinopathies [37-40].

During different stages of development, the expression of the globin genes is regulated by transcription factors (TFs),

Table 1. Advantages and disadvantages of novel modalities of globin gene regulation.

Methodology	Advantage	Drawback
Gene transduction with γ -retroviruses	Successful use in immunodeficiencies Long experience	Insertion site mutagenesis Inability to carry large genes Integration in genes rich with promoters Clonal predominance
Gene transduction with lentiviruses	Integration in quiescent cells Ability to carry large transgenes	
Gene transduction with foamy viruses	Nonpathogenicity Ability to carry large transgenes	Failure of integration into quiescent cells
Gene editing	No risk of genotoxicity High precision	Absence of clinical studies in thalassemia
Hydroxyurea	Safe	Heterogeneous study populations Inferior clinical outcomes when compared to sickle cell disease
Butyrate derivatives	Safe	Inferior clinical outcomes in thalassemia when compared to sickle cell disease
Hypomethylating agents miRNA	Safe Safe	Myelotoxicity Absence of clinical studies in thalassemia

such as GATA1, FOG1, BCL11A, KLF1, SOX6 and Ldb1, and the locus control region (LCR) [41-43]. Located *in cis* to the globin genes, The LCR appears to play an important role in switching between HbF and HbA. Deletions in the LCR are associated with the inactivation of the β -globin gene despite the absence of other mutations [37,44-51]. Among the aforementioned TFs, BCL11A and KLF1 seem to be among the smart targets to increase HbF in β -thalassemia [52].

For instance, by binding to the LCR and controlling loop formation, BCL11A induces silencing of γ -globin expression in the adult life in response to high-mobility-group (HMG)-box-containing TF SOX6 and in concert with GATA1 [53,54]. Therefore, downregulation of BCL11A leads to increased HbF expression in adult human erythroid cells [41,55]. This increase in HbF production has been shown to ameliorate the thalassaemic phenotype in clinical studies [8,56].

On the other hand, while *KLF1* null mice suffer from defective hematopoiesis and failure of β -globin gene activation and die during fetal development, reduced synthesis of KLF1 results in increased HbF production and improved survival [57-60].

Finally, Ldb1 protein acts at the β -globin locus via LMO2 and is needed for looping of the β -globin LCR to the active β -globin promoter [61-63]. The tethering of the self-association domain (SA) of Ldb1 to the β -globin promoter via zinc fingers induces LCR-promoter looping [64]. Moreover, SA targeting to a developmentally silenced embryonic globin gene in adult murine erythroblasts triggered its transcriptional reactivation [46]. These findings demonstrate the importance of Ldb1 in the transcription of genes at the β -globin cluster. Figure 1 shows chromosome 11 with the β -, ϵ -, γ - and δ -globin genes and explains the basic steps of physiological globin gene modulation.

4. Current progress in globin gene regulation: gene transduction therapy

Gene therapy relies on the ability of retrovirus vectors to reverse transcribe their RNA into cDNA, which, in turn, is integrated into the genome of a host cell [65]. In hemoglobinopathies, including thalassemia, hematopoietic CD34⁺ stem cells are mobilized from peripheral blood or isolated from the bone marrow of the affected patient, modified *ex vivo*, then returned back to the patient using the principles and techniques of HSCT [66,67]. Figure 2 shows the basic stages of gene transduction therapy. The viral vectors used are modified viral particles in which the gene of interest replaces the genetic elements needed for pathogenicity and replication [65]. Successful gene therapy in β -thalassemia requires not only the safe and effective hematopoietic stem cell harvest and transfer but also a high level of erythroid-specific, position-independent expression of the β -/ γ -globin gene [65]. Successful gene therapy also necessitates the safe gene expression with a minimal or absent increase in risk of mutagenesis [65]. A major asset of gene therapy is its reliance on autologous hematopoietic stem cells, which eliminates the risk of GVHD and other immunological complications associated with allogeneic HSCT [68].

Retroviruses are unique in their capacity to infect a wide range of cells all while retaining a low toxicity margin via permanent integration into the host cell genome [69]. For many years, retroviral vectors have proven efficacy for transduction of genes into mammalian cells in the laboratory as well as the clinical trial setting [70]. Cancer, immunodeficiency and hereditary diseases are the prevailing indications for which retroviral based gene therapies have been investigated [71,72]. Being the first to be studied in clinical trials, retroviral vectors have shown great promises in providing hope for cure from different diseases, including cancers [73,74].

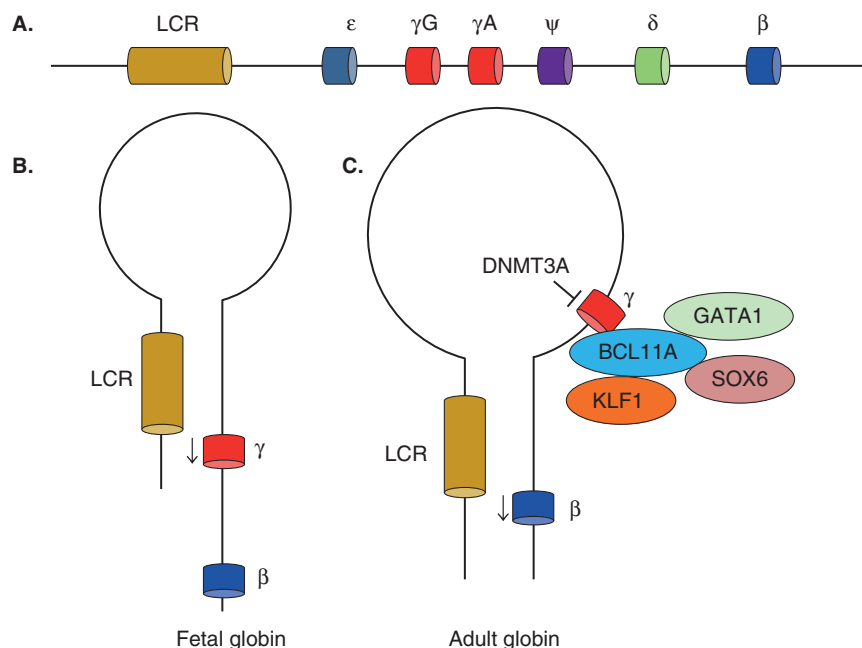


Figure 1. (A) Chromosome 11 and its accompanying β -, ϵ -, γ - and δ -globin genes all in cis with the LCR; (B) *In utero*, erythropoiesis shifts from the yolk sac where ϵ -globin gene expression predominates during embryonic stage to the liver where γ -globin expression predominates during fetal stage; (C) After delivery, γ -globin gene expression normally decreases gradually to be replaced by an increasing expression of β -globin gene. However, under the effect of variable transcription factors (TFs), γ -globin gene expression may be modulated to either increase or decrease influencing the level of HbF presence or absence and ultimately affecting the patient's clinical course. KLF1 activates BCL11A, which, in turn, suppresses the expression of γ -globin gene in collaboration with SOX6 and other TFs.

LCR: Locus control region.

In the realm of the β -hemoglobinopathies, retroviruses held the theoretical potential of modulating the erythroid genetic program via gene transfer in order to boost hemoglobin production and meet oxygen needs. Retroviruses contain a single strand of a positive sense RNA. Retroviruses replicate via reverse transcription using intermediate DNA and dependence on the target host cell machinery for protein translation. The newly formed DNA, the provirus, has the potential to intercalate between the host DNA and is equipped with a set of genes for reverse transcription, polymerization and membrane synthesis. Transmission of retroviruses from humans to humans involves transfer of cells and biological fluids and has been associated with transmission of an HIV and the human T-cell lymphotropic virus [75].

4.1 γ -retroviruses: the rise and the fall

The gammaretroviridae was one of the earliest genera used in genetic therapy, as they were the first to be isolated and cloned. A common feature to γ -retroviruses is the presence of core encapsidation signal, an RNA segment critical for genome assembly and virus packaging [75]. Examples of γ -retroviruses include the murine leukemia virus, the feline leukemia virus, the gibbon ape leukemia virus and the xenotropic murine

leukemia virus-related virus. The role of γ -retroviruses in therapeutic globin gene transfer was first explored in the late 1980s. Dzierzak *et al.* reported the successful transduction of human β -globin chain into irradiated murine hematopoietic stem cells by means of retroviruses [76]. The β -globin expression was observed exclusively in erythrocytes. In the same year, Karlsson *et al.* reproduced similar findings by transfecting the intact genomic human β -globin gene into murine bone marrow cells [77]. Authors demonstrated the expression of β -globin in splenic colony-forming units. They later transplanted the β -globin transfected bone marrow cells into anemic mice and found the expression of β -globin in circulating erythrocytes to be persistent at 3 – 8 weeks after transplantation.

However, the use of γ -retroviruses as vectors for globin gene transduction therapy was not free of complications. Many reports pointed to the risk of insertion site mutagenesis and the inadvertent activation of proto-oncogenes [78,79]. The most notorious experience was in patients with X-linked severe combined immunodeficiency enrolled in gene therapy trials, whereby four patients were reported to have new incidence of leukemia [80]. In a separate trial, patients with X-linked chronic granulomatous disease sustained

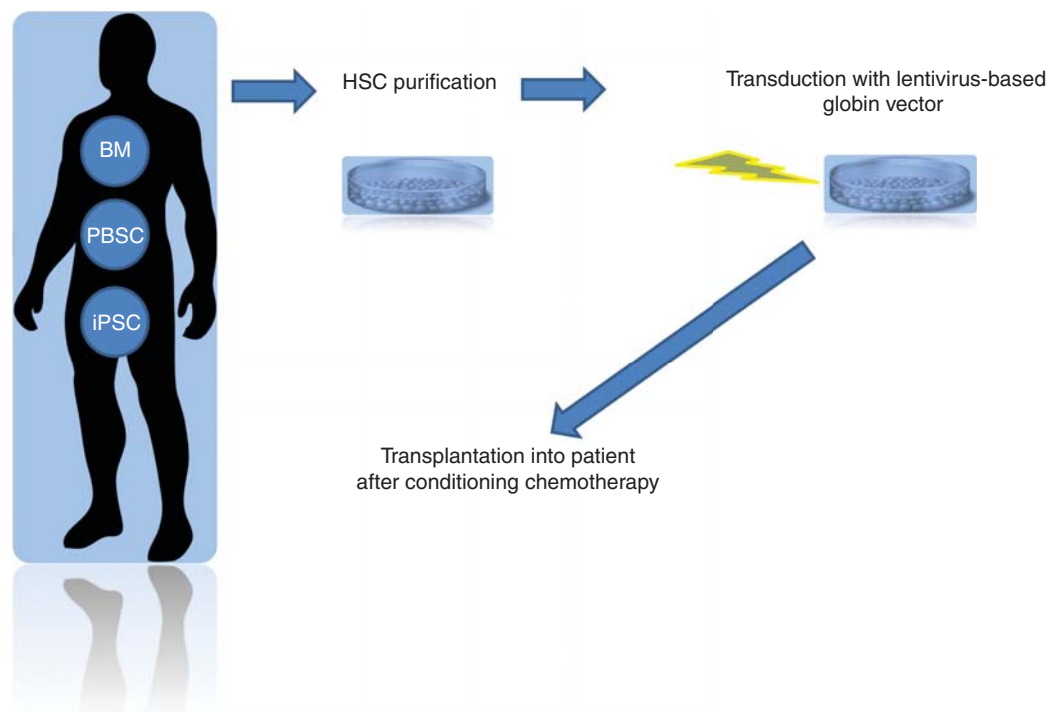


Figure 2. The basic stages of gene therapy. HSC are harvested from the BM or from mobilized peripheral blood (PBSCs). Induced pluripotent stem cells could be potentially used in the future. HSC are subsequently purified then transduced with a lentivirus-based globin vector. After receiving conditioning chemotherapy, the transduced autologous HSC are transplanted into the patient.

BM: Bone marrow; HSC: Hematopoietic stem cells; iPSC: Induced pluripotent stem cell; PBSC: Peripheral blood stem cell.

myelodysplasia after undergoing γ -retrovirus-based genetic transfer [81]. The concern for the oncogenic risk with γ -retroviruses triggered the search for other viral vectors with less ominous side effects in terms of oncogenicity [82]. In addition and specifically for hemoglobinopathies, another limitation of γ -retroviruses is their inability to carry the large load of the globin gene and its regulatory elements needed for therapeutically high levels of globin expression [65]. γ -retroviruses also lack the ability to infect quiescent hematopoietic stem cells [65]. Finally, γ -retroviruses depend on long terminal repeats (LTRs), repetitive sequences of DNA that allow integration of provirus into host DNA by means of LTR-specific integrase [65,83]. The presence of LTRs was indispensable for their successful use in gene therapy but was hypothesized to be implicated in their high oncogenicity [84,85]. Lentiviruses, another genus of retroviruses, seemed to offer solutions to these challenges.

4.2 Lentiviruses: toward safer and more effective gene therapy

In contrast to the γ -retroviral vectors, the lentiviral vectors, based on HIV-1, exhibit a much lower degree of genotoxicity and have the ability to integrate into quiescent, non-dividing cells [65,86]. This observation is accounted for

by a few factors. Lentiviruses preferentially integrate within active genes while γ -retroviruses exhibit a predilection for sites rich with promoters and enhancers [87-89]. The tendency of lentiviral vectors to integrate throughout transcription units without predilection to promoter regions may grant them a safety lead when compared with γ -retroviruses [90-92]. With myriad improvements in lentiviral vectors since their introduction, the self-inactivating (SIN) lentiviral vector currently allows for erythroid-specific and elevated globin gene expression with lower risks of insertional mutagenesis [93-95]. The SIN lentiviral vector design includes a deletion of the U3 enhancer region of the lentiviral 3' LTR. Furthermore, other advantages of lentiviruses include the ability to transduce quiescent cells and the ability to carry large transgenes [96-98].

In 2000, May *et al.* were able to use a lentiviral vector in a murine thalassemia model (Hbb^{th3/+} mice that phenotypically resemble TI in humans), demonstrating improvement in hemoglobin levels and correction of red cell indices [87]. However, it was reported that a clone dominates in a murine TI model into which a human β -globin gene was transferred [87]. Other studies on murine thalassemia models also showed correction of the thalassemia phenotype with the use of a lentivirus but required multiple vector copies owing to variability in

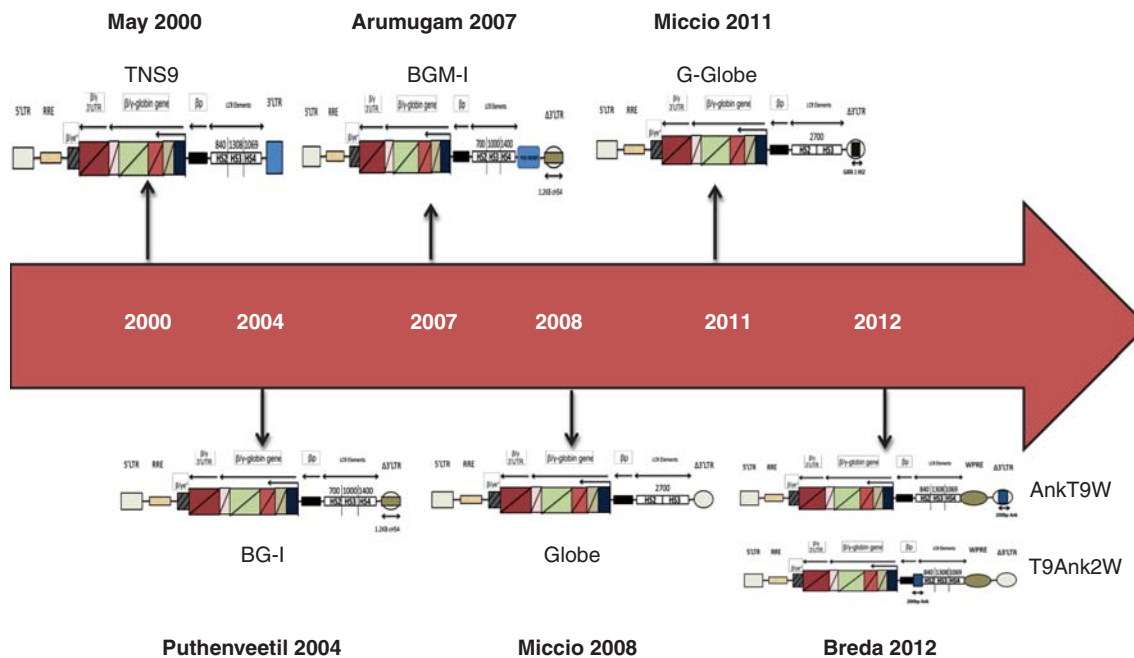


Figure 3. Different Lentivirus vectors expressing β -globin genes presented in chronological order of publication, along with the name of the vector, the first author and the year of publication.

β p: β -globin promoter; Ankyrin: Ankyrin insulator; cHS4: Chicken β -globin hypersensitive site 4 insulator; GATA1 HS2: Globin transcription factor 1 hypersensitive site 2 enhancer; HS: Hypersensitive site; LCR: Locus control region; LTR: Long terminal repeat; PGK MGMT: Murine phosphoglycerate kinase-1 promoter and O6-methylguanine DNA methyltransferase gene; RRE: Rev response element; WPRE: Woodchuck posttranscriptional regulatory element.

the expression of the transferred gene [99,100]. Following these promising results, phenotypic correction of β -TM was shown in an *in vitro* human model and in a xenograft model in mice [101].

Advances at the level of the LCR led to improvement in globin expression through the inclusion of HS1 in addition to extended HS2-4 elements of the LCR [102,103]. Other improvements in lentiviral vectors include the use of chromatin insulators that can protect genes from their chromatin environment [94,104]. Chromatin insulators simultaneously prevent gene silencing caused by heterochromatin and activation of gene promoters by adjacent enhancers [105,106]. While insulated lentiviral vectors improved globin expression, the most widely used Chicken β -globin hypersensitive site 4 (cHS4) insulator was associated with a decrease in lentiviral vector titer [104,107]. It is therefore necessary to overcome the effect of cHS4 on viral titers to benefit from its enhancer-blocking effects and the associated improvement in expression of globin genes [94,104,107,108]. On another hand, Globe, one of the lentiviral vectors that lack HS4 of the LCR and harbor a larger deletion of intron 2 of the β -globin gene, was able to achieve titers high enough to correct thalassemia in a murine model and in hematopoietic stem cells of pediatric patients [109,110]. Other attempts at optimizing the use of lentiviral vectors include the use of rapamycin to facilitate the transduction of the provirus into the host cell, the

supplementation with a chromatin opening element, and the inclusion of an ankyrin insulator [30,111-114]. **Figure 3** shows the various lentivirus vectors and the improvements introduced to the original prototype.

Based on different studies, the efficacy and safety profile of lentiviral globin gene transfer were quite encouraging for the use of lentiviral vectors [87,115]. One of the initial steps in the long march toward institution of human gene therapy was the pioneering trial of lentiviral β -globin gene transfer to three patients with HbE/ β -thalassemia by the group of P. Leboulch, initiated in Paris in 2007 [116,117]. The investigators designed a lentiviral vector equipped with a replicate of cHS4 insulator at the LTR as a safety measure to prevent insertion mutagenesis (β T87Q LentiGlobin vector). While the first patient failed to engraft and required the infusion of backup bone marrow cells, the clinical course of the second patient unveiled interesting results. While achieving the primary clinical goal of transfusion independence, a clonal predominance was noted at 35 months after transplant. This observation was explained by the inadvertent insertion of the provirus next to the HMG AT-hook 2 (HMGA2) locus in both erythrocytes and granulocytes. This clonal predominance peaked at 4 years of treatment then dropped later on until it reached only 6.8% at 7 years of treatment. Another startling aspect of the trial is the major contribution of fetal globin to the total hemoglobin pool and that was almost

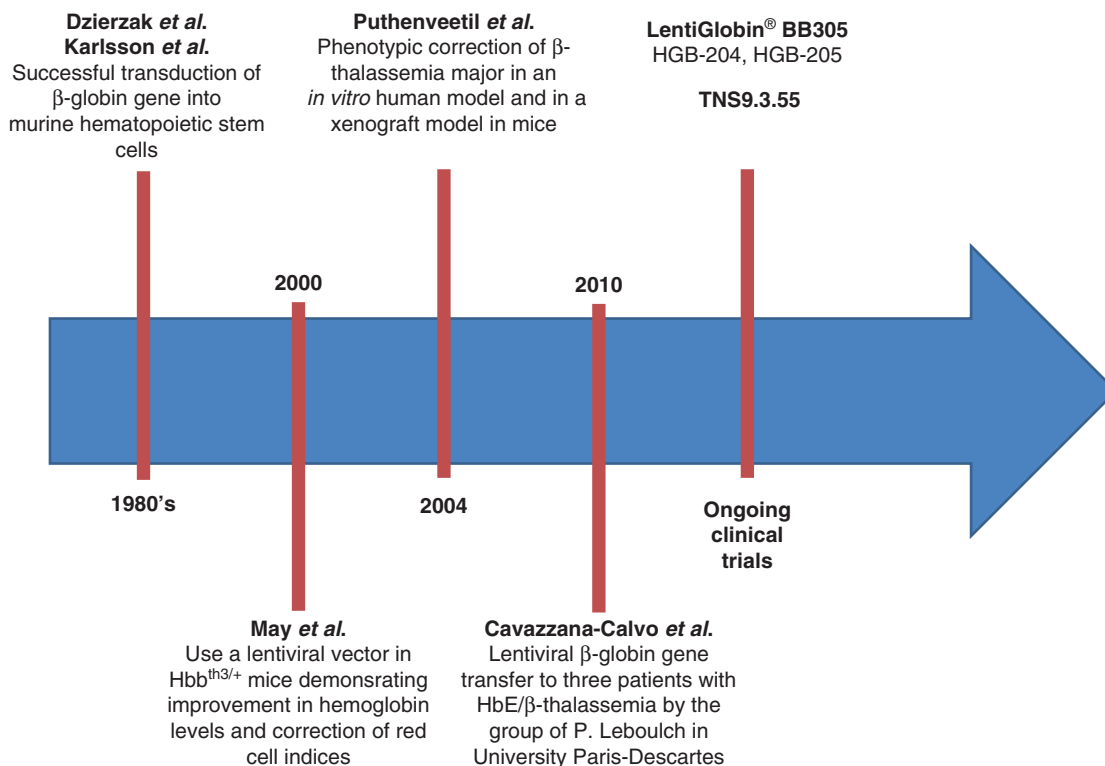


Figure 4. This timeline highlights major advances in the clinical application of gene therapy in β -thalassemia. The years and names mentioned refer to the details of the manuscripts reporting these advances.

double that of the endogenous HbE, thus highlighting the pivotal role of γ -globin induction in recovery of globin production.

Other clinical trials are currently investigating gene therapy in β -thalassemia (NCT00669305, NCT01639690, NCT01745120 and NCT02151526) [118]. One of these clinical trials (NCT01639690) is investigating TNS9.3.55, a lentiviral vector that contains the cHS4 insulator [119]. Trials HGB-204 (NCT01745120) and HGB-205 (NCT02151526) are evaluating LentiGlobin® BB305 after encouraging safety and efficacy data from preclinical studies in murine models [120]. **Figure 4** showcases the major advances in the clinical application of gene therapy in β -thalassemia.

4.3 Foamy virus vectors: promising preclinical results

Vectors based on foamy virus have special advantages when compared to retrovirus-based and lentivirus-based vectors. Foamy virus-based vectors are nonpathogenic, can be used to package a larger transgene, and have a tendency to integrate into nongenic regions [121]. However, like retrovirus-based vectors, they fail to integrate into a quiescent hematopoietic stem cell. Based on experiments on erythroid cell lines, murine thalassemic models and human CD34⁺ cells, Morianos *et al.* suggested that foamy viruses with the α -globin HS40 element can be used as efficient vectors for human β -globin gene expression [122].

5. Novel trends in gene regulation: the role of HbF induction

5.1 Gene editing

Another approach to genetic modulation consists of fine-tuning the DNA without gene transduction, thus mitigating the risk of genotoxicity associated with DNA insertion. Though attractive, this strategy remains in its early investigational phases, and successes have been mainly achieved in the field of immunodeficiencies [123-125].

A notable example is targeting *BCL11A*, a TF that plays a pivotal role in switching from HbF to HbA [52]. By deleting a specific erythroid enhancer, suppression of *BCL11A* might be achieved in erythroid cells and, therefore, might be a potential target for therapeutic genome engineering for hemoglobinopathies [126].

The advent of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) was pivotal in expanding therapeutic options for genome editing. Both ZFNs and TALENs induce breaks in the DNA double strand by activating dimerization of an enzyme FokI, a double-stranded DNA nickase [127-133]. In addition, clustered regularly interspaced short palindromic repeats (CRISPR) linked to Cas9 nuclease are being actively explored as promising modalities for optimization of therapeutic outcomes in genetic editing. CRISPR/Cas9, on the other hand, can leave

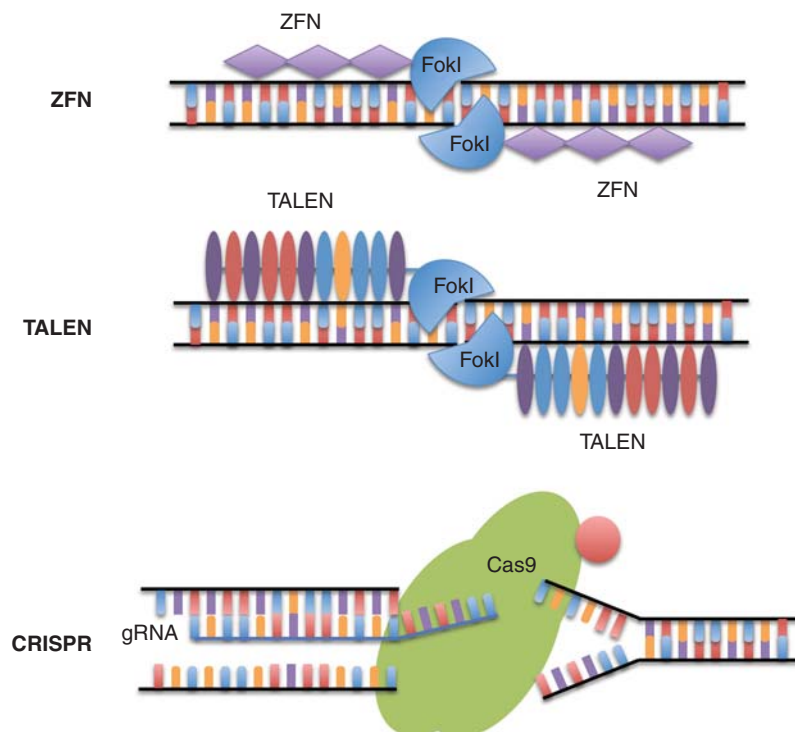


Figure 5. This figure schematically represents ZFN, TALEN and monomeric CRISPR. A dimeric FokI nuclease introduces a nick in the target DNA can introduce a double-strand break (DSB). A monomeric CRISPR, associated with Cas9 nuclease and guided by guide RNA (gRNA), can bind to double-stranded DNA and introduce a DSB. The DSB may then be used to modify the β -globin gene in induced pluripotent stem cells from thalassemia patients.

CRISPR: Clustered regularly interspaced short palindromic repeats; TALEN: Transcription activator-like effector nucleases; ZFN: Zinc finger nucleases.

the double-stranded DNA all without dimerization [134]. Using TALENs to correct β -globin mutations in situ, Ma *et al.* generated two integration-free, gene-corrected induced pluripotent stem cell (iPSC) lines without TALEN-induced off-target mutations [135]. These iPSCs can be eventually induced into erythroblasts that express normal β -globin. While all of these advances and others may hold great promise for the future, there are no clinical studies on genome editing in β -thalassemia yet. Figure 5 schematically represents ZFNs, TALENs and CRISPR/Cas9.

5.2 microRNA

MicroRNA (miRNA) are small RNA strands that silence cDNA sequences. miRNA strands are effectors of gene silencing and are well conserved in different species [136]. For instance, *let-7* miRNA is known to interact with a protein LIN28B that downregulates its activity and consequently suppresses γ -globin gene expression in adult life. Overexpression of LIN28B in human cord blood and adult CD34⁺ cells downregulated the expression of the *let-7* miRNA family and suppressed BCL11A, subsequently resulting in a rise in HbF and hemoglobin [137]. In addition, in hematopoietic stem cells, miR-486-3p, miR-23a/27a, miR-15a and miR-16-1 were reported to modulate the expression of

BCL11A and increase the expression of fetal globin [138-140]. These findings shed the light on the potential of using miRNA for therapeutic purposes in β -thalassemia in the future [141].

5.3 Hypomethylating agents, hydroxyurea and butyrate derivatives

Increased HbF production has been linked to an improved clinical course in sickle cell disease and β -thalassemia [8,56,142]. In the following section, we will discuss hypomethylating agents (5-azacytidine and decitabine), hydroxyurea and butyrate derivatives.

5.3.1 Hypomethylating agents

The mainstay of epigenetic regulation is modifying the reading of genes according to a set of checks and balances. Epigenetic modifiers appear to tag specific genes and, therefore, modulate their expression. One of the earliest described epigenetic modifiers is the DNA methyltransferase (DNMT). Among the novel methylases, DNMT3A transfers methyl groups to cytosine-phosphate-guanine on both strands of unmethylated DNA [143]. In thalassemia, DNA methylation contributes to the silencing of γ -globin expression in the post-partum life. 5-azacytidine, a hypomethylating agent,

antagonizes the enzyme DNMT3A [144,145]. In the 1980s and early 1990s, the therapeutic role of azacytidine in promoting human fetal γ -globin production in β -thalassemia was suggested by reports showing significant improvement in hemoglobin [146-148]. The wide use of 5-azacytidine was limited, however, by safety concerns related to myelotoxicity despite a marked improvement in serum hemoglobin among a few patients with β -thalassemia [149,150]. In thalassemia, the beneficial effect of azacytidine in HbF induction is not attributed to their global hypomethylation but rather to a specific demethylation of the promoter of γ -globin [36].

Decitabine demonstrates superior safety given its selective integration into DNA strands in contrast to azacytidine that integrates into both DNA and RNA strands [151,152]. The subcutaneous form, given twice a week for 12 weeks in five patients with β -TI, was effective in significantly raising the total hemoglobin and the absolute HbF and in improving red blood cell indices [153]. In this sense, a trial of low dose of decitabine in patients with β -thalassemia was successful in inducing a surge in HbF all without causing any cytotoxicity or genotoxicity.

5.3.2 Hydroxyurea

Hydroxycarbamide, an inhibitor of ribonucleotide reductase, is a well-established therapy in the treatment of sickle cell disease [154]. While it increases mRNA expression in β -thalassemia patients, its impact on serum HbF remains to be modest [155]. In fact, hydroxyurea has a pleiotropic effect in sickle cell disease. In addition to its potential HbF-inducing effect, hydroxyurea improves a set of factors including increased red cell flexibility, decreased hemolysis, reduced generation of reactive oxygen species and decreased counts of immature erythrocytes in peripheral blood [156]. A closer look into this effect might explain the differential benefit from hydroxyurea between sickle cell disease and thalassemia. One of the unique aspects regarding the myelotoxicity of hydroxyurea in β -thalassemia patients is the state of intramedullary inflammation promoted by different cytokines mediating interaction among bone marrow stromal cells and red cell precursors [157]. Although hydroxyurea has been studied in thalassemia in multiple clinical trials over the past decades with good hematological responses, these studies were limited by the heterogeneity of study populations in terms of transfusion dependence and the heterogeneity of study endpoints [8].

5.3.3 Butyrate derivatives

Butyrate are short-chain fatty acids that induce the expression of fetal globin [158]. The role of butyrate derivatives was explored in small cohort studies in patients with β -thalassemia. In patients with transfusion-independent β -thalassemia, administration of butyrate resulted in induction of HbF, but there was no impact on the ratio of β -globin to α -globin or on any other parameter related to ineffective erythropoiesis [159,160]. However, arginine butyrate, given intravenously for 10 weeks, failed to produce a hematologic response in

patients with β -thalassemia [161]. In a study on 11 patients by Collins *et al.*, sodium phenylbutyrate was also associated with an improvement in hemoglobin in transfusion-independent β -thalassemia patients [162]. Cappellini *et al.* also suggested that oral isobutyramide therapy can stimulate HbF in a study on 12 patients with TI, but results did not reach significance [163]. In a study on eight patients with transfusion-dependent thalassemia, oral butyrate doubled the percentage of HbF and prolonged transfusion intervals in some patients with transfusion-dependent β -thalassemia [164].

Overall, the inferior efficacy of butyric acid derivatives in thalassemia compared to sickle cell disease is attributed to its differential action on α -globin chain in each disease. In fact, butyric acid upregulates α -globin chain expression and further aggravates the α -/ β -chain imbalance in thalassemia [35]. Sodium-2,2-dimethylbutyrate (HQB-1001) is a butyrate derivative that has been associated with promising outcomes in sickle cell disease. In a small Phase I/II trial conducted in patients with non-transfusion-dependent thalassemia, HQB-1001 was well tolerated and was effective at a dose of 20mg/kg to increase HbF by 6.6% above baseline and total hemoglobin by mean of 11g/dL in 45% of subjects [165]. The optimal dose was administered to 10 patients with β -TI was tolerated for all subjects except one who experience worsening fatigue. HQB-1001 increased HbF by 4.8% and total hemoglobin by 0.47g/dL [166]. In patients with HbE/ β -thalassemia, when given the same dose, HbF was increased by 0.96g/dL and total hemoglobin by 0.93g/dL [167].

6. Conclusion

While the field on globin gene regulation is promising, the majority of its aspects remain in the preclinical or very early clinical stages. Recent advances in viral vector design for gene therapy and the use of iPSCs are among the promising areas to be further exploited. Gene editing as a concept may also hold opportunities for globin gene regulation and curative therapy without adverse effects encountered in gene transduction therapy. Results from ongoing clinical trials in gene therapy will be the first clinical checkpoint that will decide the bifurcations globin gene regulation will take in the field of thalassemia.

7. Expert opinion

The ultimate goal in the field of thalassemic disorders is curing the disease without therapy-related complications. This not only reduces the cost of care for thalassemic patients but also significantly improves their quality of life as they achieve independence from transfusions and iron chelation therapy. In fact, the economic burden of treatment of thalassemia has been a major concern especially in resource-limited settings where thalassemia is most prevalent. All of these factors have driven the efforts for searching a long-term cure for thalassemia. Allogeneic HSCT represents a true opportunity for

thalassemia patients to become transfusion independent; however, it is associated with several complications during and post-transplant and its application depends on finding a matched donor. The complications of the procedure and the scarcity of suitable donors shed the light on gene transduction therapy or genomic editing, with or without HbF-induction therapies, as safer and more effective alternatives.

One of the biggest challenges in the novel therapies exploiting our knowledge of globin gene regulation is the smart and safe design of clinical trials. While gene therapy trials are currently ongoing, close attention should be put into the process of monitoring patients undergoing these therapies based on the lessons learned from preclinical and earlier clinical experiences. Special attention should be given to vector-induced oncogenesis and leukemogenesis. However, these early road-blocks should not hinder the progress of clinical research in the field of gene therapy especially with the crystal-clear clinical benefits in patients with congenital immunodeficiencies who have undergone gene therapy in the previous two decades [168]. The prospects of gene therapy are even brighter when the relatively poorer outcomes from allogeneic HSCT from HLA-mismatched donors are taken into consideration. In trial design, there is a need for reinforcing international consortia for genetic research in thalassemia accruing the highest number of patients and joining local expertise with referral centers in management of thalassemia. With such international structures established, the stakes of gene therapy will be higher and funding agencies and sponsors will be more enthusiastic about supporting genetic research in thalassemia [169]. This will catalyze progress in this area.

Addressing major challenges in gene therapy has been an ongoing mission. The search continues for the ideal vector that can provide therapeutic globin transgene expression levels with minimal or no risk of insertional mutagenesis. Lentivirus-based vectors seem to partially meet this objective; however, it remains crucial to optimize these vectors by using alternative chromatin insulators and understanding the mechanisms for integration site specificity [170]. Another challenge to be faced is the isolation, transduction and long-term repopulation of autologous hematopoietic stem cells in the process of gene therapy. In addition, improvement in the transplant conditions is also needed to allow gene therapy procedures with milder conditioning. Finally, a disease-specific hurdle in β -thalassemia remains the need for strict transcriptional outcomes (stage-specific, erythroid-specific, elevated transcription). Achieving globin expression that is elevated enough is essential to cure thalassemia. This challenge has been addressed by attaining therapeutic levels of hemoglobin synthesis in the progeny of virally transduced hematopoietic stem cells [87,100-102,171-176].

In parallel to the advances in gene transduction, HbF induction has witnessed major strides in its different components. The area of gene editing is gaining an accelerating

momentum, and expertise in gene cleavage, activation and suppression are rapidly growing. Altering the methylation patterns on BCL11 using hypomethylating agents is an interesting therapeutic opportunity. Despite growing concerns of myelotoxicity associated with 5-azacitidine, decitabine represents a feasible alternative with a better safety profile. The role of butyrate derivatives in HbF induction has been under investigation for long years. With variable rates of success, these agents are still in early phases of development for the widespread clinical use. In the realm of miRNA-based therapy, developing means to deliver pre-miRNA or miRNA to erythroid cells is a major rate-limiting step to overcome. In this venture, supporting these potential treatments with hydroxyurea or other HbF inducers may be helpful in achieving more favorable results.

As in other areas of research in hemoglobinopathies, we should be careful when extrapolating clinical data from sickle cell disease to β -thalassemia. There exist outstanding differences in the pathophysiology of the different hemoglobinopathies. Despite the similarities in treatment intent with these diseases, nuances should be kept in mind when outcomes of globin gene therapy or regulation are extrapolated among hemoglobinopathies.

Looking at all the morbidity-related, mortality-related and financial burdens of β -thalassemia, globin gene therapy remains to be one of the most promising strategies to achieve a permanent cure. The first step is proving, through ongoing clinical trials, that gene therapy is an option for thalassemic patients who lack suitable donors for allogeneic HSCT. Nevertheless, farther in the horizon lies the promise of gene therapy and/or gene editing, alone or coupled with old or novel HbF inducers, being a curative option that is as valid as allogeneic HSCT.

Looking at the 'genetic battle' with thalassemia at an earlier stage, screening for thalassemia and genetic counseling remain at the forefront of prevention especially in areas of the world where these approaches may be much more affordable than novel therapies.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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