

Combining gene therapy and fetal hemoglobin induction for treatment of β -thalassemia

Expert Rev. Hematol. 6(3), 255–264 (2013)

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β -thalassemias are caused by nearly 300 mutations of the *β -globin* gene, leading to a low or absent production of adult hemoglobin (HbA). Two major therapeutic approaches have recently been proposed: gene therapy and induction of fetal hemoglobin (HbF) with the objective of achieving clinically relevant levels of Hbs. The objective of this article is to describe the development of therapeutic strategies based on a combination of gene therapy and induction of HbFs. An increase of *β -globin* gene expression in β -thalassemia cells can be achieved by gene therapy, although *de novo* production of clinically relevant levels of adult Hb may be difficult to obtain. On the other hand, an increased production of HbF is beneficial in β -thalassemia. The combination of gene therapy and HbF induction appears to be a pertinent strategy to achieve clinically relevant results.

KEYWORDS: β -thalassemia • erythroid progenitor cells • gene therapy • hemoglobin • high persistency of fetal hemoglobin • sickle cell anemia

β -thalassemias are a group of hereditary hematological diseases caused by mutations of the human *β -globin* gene, leading to a low or absent synthesis of adult β -globin and an excessive production of the α -globin protein in erythroid cells, and are characterized by ineffective erythropoiesis and low or absent production of adult hemoglobin (HbA) [1–8]. Together with sickle cell anemia (SCA), thalassemia syndromes are the most inherited and debilitating disorders in developing countries. Lack of genetic counseling and prenatal diagnosis as well as limited clinical management have also contributed to maintaining the frequency of these genetic diseases at very high levels [7]. Furthermore, due to the insufficient therapeutic options and negative consequences associated with the current treatments, novel therapeutic approaches are urgently needed [7,8].

β -thalassemia is caused by decreased production of β -globin chains and concurrent formation of hemichromes, which interfere with the structure and function of the plasma membrane of erythroid progenitor cells (ErPCs), ultimately causing cell death, ineffective erythropoiesis and severe anemia. The focus of new therapies is to increase the production of β globin or β -like globin chains and simultaneously decrease free

α -globin chain formation. The reduction of the excess of α -globin production *per se* can ameliorate some clinical features of β -thalassemic cells. Decreased α -globin synthesis leads to milder symptoms, exemplified in individuals or transgenic mice with coinherited α - and β -thalassemia [9,10].

SCA is due to a single mutation (A–T) of the *β -globin* gene, resulting in glutamic acid being substituted by valine at position 6. This mutation triggers the sickling of red cells, decreasing their flexibility, which can lead to vaso-occlusive crises and various acute and chronic complications, some of them with potential lethal consequences [11]. In newborns, fetal hemoglobin (HbF) is almost completely substituted by HbA by approximately 6 months postbirth. Hereditary persistence of HbF (HPFH) may alleviate or prevent the severity of the clinical conditions associated with hemoglobinopathies [12]. For this reason, HbF reactivation in adults could be helpful in the treatment of diseases such as β -thalassemia and SCA.

In addition, expression of a transgenic normal *β -globin* gene would probably be beneficial for correcting the unbalanced ratio between α - and β -globin chains in β -thalassemia and reduce the

ability of the red cells to sickle in SCA [13]. This is the rationale for treating these disorders by gene transfer.

Treatment & management of the β -thalassemia patient & objectives of gene therapy

Management of β -thalassemia patients

Thalassemia patients present with several complications, including endocrine abnormalities [14], delayed sexual maturation and impaired fertility [15], growth retardation [16], hypogonadism [17], hypothyroidism [18], impaired carbohydrate metabolism [19] and chronic liver disease [20]. The conventional treatment of patients affected by severe forms of β -thalassemia is based on regular blood transfusions and iron chelation therapy [6]. Accurate and safe transfusion of blood, together with an optimal chelation therapy, is the most important therapeutic option for β -thalassemic patients [21]. In many countries, blood transfusion therapy has considerably increased the lifespan of patients due to: identification of the optimal period to initiate the infusion of blood; careful evaluation of the patients' transfusion requirements; improvement of the procedures to collect and preserve the blood safely; and identification of the optimal hemoglobin (Hb) levels to minimize the side effects associated with the chronic anemia. Despite such progression, transfusion therapy is associated with serious complications and requires iron chelation therapy. The primary goal of chelation therapy is to maintain body iron at safe levels, preventing excessive iron concentrations from damaging organs. However, iron chelation may be associated with undesirable effects and scarce compliance. Moreover, once iron overload accumulation has occurred, its removal is slow and inefficient, because only a small proportion of body iron is available for chelation at any given time [21]. For all of these considerations, the development of new therapies is extremely desirable and may prevent some of the problems associated with intensive blood transfusion and chelation therapy.

Gene transfer techniques for the cure of β -thalassemia have been the object of several reviews [22–28]. Although several adeno-associated viral vectors have been described [29], most of the studies aimed at the translation of basic research to the clinical setting are based on the use of retroviral [30–32] and lentiviral vectors [33–57]. The efforts of the groups working in this field have been dedicated to produce highly efficient and stable transduction [30,34]; effective targeting of the hematopoietic stem cells (HSCs) [24]; controlled transgene expression (erythroid-specific, differentiation and stage restricted, elevated, position independent and sustained over time) [24,31,35]; low or absent genomic toxicity [36,37]; *in vivo* selection of the transduced HSC [38–41,43,45]; correction of the β -thalassemia phenotype in preclinical models, including transgenic mice (see section on 'Targeting TFs that are negative regulators of γ -globin gene transcription') [40,42,44,46–50]; and application of the knowledge on gene therapy for corrections of β -globin gene expression in induced pluripotent stem cells generated from somatic cells of β -thalassemia patients [24].

General considerations on gene therapy of thalassemia

Many patients with thalassemia require lifelong transfusions and iron chelation. Only allogeneic bone marrow transplantation is

potentially curative. However, as in the case of SCA, the availability of this treatment is often subject to finding a donor with an identical HLA, and even then, there are severe risks and complications [58]. In consideration of the above, new approaches aiming at treating patients with severe β -thalassemia are greatly needed. Hemoglobinopathies, therefore, are candidates for genetically based therapies involving autologous HSCs. As discussed in the next section, many studies showed that it is possible to rescue mice with β -thalassemia using lentiviral-mediated β -globin gene transfer. Based on these studies, clinical trials have been proposed or are underway. However, the original studies in animal models utilized mice characterized by partial or complete deletion of the mouse β -globin genes. By contrast, β -thalassemia in humans is characterized by nearly 300 mutations, mostly associated with one or a limited number of nucleotides. This increases the complexity to identify the right conditions for each patient to optimize the vector copy number, the chimerism and the level of transgenic Hb that needs to be produced to achieve a clinically significant benefit from this approach. Furthermore, the present limitations in achieving homogeneous gene transfer in HSCs and the potential genome toxicity associated with the random integration of the current gene transfer vectors limits this approach. Therefore, the use of additional therapeutic tools such as fetal Hb inducers, in concert with gene therapy, may help clinical investigators to achieve full correction of the thallemic phenotype in a large cohort of patients, even if the gene transfer may not be optimal.

Retroviral & lentiviral vectors

The initial pilot gene therapy studies for β -thalassemia utilized recombinant oncoviruses as carriers. Despite their ability to transfer the β -globin gene into murine HSC without transferring any viral gene, oncoviruses did not succeed at producing high and stable expression of the β -globin gene in mice [59–62]. Modifications of the β -globin gene regulatory elements were necessary to overcome the inability to reach therapeutic Hb levels. To this end, the incorporation of the core elements of the hypersensitive sites 2, 3 and 4 of the human β -globin locus control region (LCR) was crucial to increase expression levels but still did not prevent position-effect variegation [63,64]. Subsequent studies focused on preventing rearrangement events that are due to the activation of splicing sites within the LCR sequence of the viral RNA. These attempts, although successful, failed to maintain high β -globin gene expression. Therefore, later studies aimed at introducing alternative regulatory elements, promoters and even mutations that lead to HPFH in patients [65,66]. Results from these studies did not support the use of oncoviruses as an ideal gene therapy tool but they pioneered the way for other more successful gene therapy studies. Owing to all the limitations described so far and to overcome the inability of oncoretroviruses to infect quiescent cells, lentiviral vectors were subsequently utilized. In addition to their ability to transduce nonreplicating cells, lentiviruses can accommodate large genomic elements in their genome with limited or no sequence rearrangement [67]. 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.

Han *et al.* were able to rescue fetuses affected by α -thalassemia, using a lentiviral vector in which the α -globin gene and promoter were flanked by the hypersensitive sites (HS) 2, 3 and 4 of the human LCR [68]. Three months after birth, treated mice exhibited 20% expression of the transgenic α -globin gene compared with normal mice. However, transgene expression started to decline at 7 months. Subsequent studies of the authors' and other groups demonstrated the ability of lentiviral vectors, carrying the human β -globin gene and its fundamental regulatory elements, to cure and rescue animal models for β -thalassemia intermedia and major, respectively [47,48,69]. With a similar lentiviral vector with enhanced antisickling activity, Pawliuk *et al.* corrected the phenotype of SAD and BERK sickle mouse models [67–70]. Miccio *et al.* showed that genetically corrected thalassemic erythroblasts undergo *in vivo* selection in mice, indicating that the corrected cells have a survival advantage over the thalassemic cells [43]. From a clinical point of view, this advantage could imply a lower myeloablative regimen for the patient, who could benefit even when some chimerism persists after bone marrow transplant. Both human sickle and β -thalassemic cells have been successfully treated with lentiviral vectors. Samakoglu *et al.* corrected human sickle cells using a vector that combined a γ -globin gene with a short hairpin RNA (shRNA) to increase HbF expression and downregulate sickling β -globin at the same time [71]. Other groups as well as the authors' focused on the correction of human thalassemia and sickle cells using lentiviruses that incorporate insulators to maximize β -globin expression at random integration site and protect the host genome from possible genotoxicity. Insulators can in fact shelter the transgenic cassette from the silencing effect of nonpermissive chromatin sites and, at the same time, protect the genomic environment from the enhancer effect mediated by active regulatory elements (e.g., the LCR) introduced with the vector. While Puthenveetil *et al.* utilized the cHS4 insulator [49], Miccio *et al.* utilized the HS2 enhancer of the *GATA1* gene to achieve high β -globin gene expression in human thalassemic cells [72]. The authors recently showed that using a 200bp insulator, derived from the promoter of the *Ankyrin* gene, a significant amelioration of the thalassemic phenotype was achieved in mice and a high level of expression was reached in both human thalassemic and sickle cells [73]. A summary of the most impactful murine and human studies using a lentiviral-based gene therapy approach is provided in TABLE 1.

Genomic toxicity

Although conclusive evidence is not available, preliminary data suggest that great caution should be taken in performing lentiviral-based gene therapy. Hargrove *et al.* investigated gene expression in genomic regions adjacent to the site of integration of lentiviral vectors containing enhancer elements of the β -globin LCR [36]. The study focused on 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. They found an overall incidence of

perturbed expression in 28% of the transduced clones, with 11% of all genes contained within a 600-kb region surrounding the vector-insertion site, demonstrating altered expression. This is the first direct evidence that lentiviral vectors can cause insertional dysregulation of cellular genes at a frequent rate. An additional study by Arumugam *et al.* utilized both γ -retroviral and lentiviral vectors containing LCR elements and their potential to transform primary hematopoietic cells [74]. They demonstrated that lentiviral vectors exhibited approximately 200-fold lower transforming potential compared to 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 [75], showing that a further twofold reduction in transforming activity was observed when the LCR elements were flanked by this element.

Clinical trials using gene therapy protocols based on viral vectors

Due to the successful employment of lentiviruses in mouse models of both β -thalassemia and SCA, clinical trials based on gene therapy of β -thalassemia patients have been encouraged and initiated [55,57,76–78]. In 2010, a report documented a successful case of gene therapy treatment of a patient with $\beta\text{E}/\beta\text{O}$ thalassemia. The patient showed Hb levels that no longer required transfusion after a year from the treatment and is still healthy. In this patient, erythropoiesis is supported by equal levels of HbA (produced by the transgenic β -globin), HbF and HbE (both produced by the endogenous genes). This evidence suggests that the gene therapy treatment alone may not have been curative and that the additive effect of HbF activation may have been crucial for the restoration of physiological erythropoiesis in this patient. Unexpectedly, the insertion of the lentiviral vector in the *HMG2* gene has promoted selective growth of a clonal population in the bone marrow of the patient [77]. In a similar fashion, a more severe event that developed into leukemia was observed in the retroviral vector-based gene therapy trial for the X-linked combined immunodeficiency disease [78]. Together, these observations indicate that gene therapy needs to achieve its curative goal by the minimum number of integrations to avoid genome toxicity.

Subsequently, Ronen *et al.* utilized the same lentiviral vector to correct the phenotype of a β -thalassemia murine model [79]. Five mice treated with this vector showed almost normal levels of Hb and normalization of spleen weight. Mapping of integration sites prior to transplantation showed the expected preferred integrations in transcription units. The numbers of gene-corrected long-term repopulating cells deduced from the numbers of unique integrants indicated oligoclonal reconstitution, although clones with integration sites close to cell-cycle-related genes were not enriched during growth. In addition, no integration near *HMG2* was detected. Given the limitations of this model (the lifespan of the animals and the number of mice analyzed), these data indicate that gene correction in thalassemic mice can potentially be achieved without expansion of cells harboring vectors integrated near genes involved in growth control.

Table 1. Summary of murine/human gene therapy studies and fetal hemoglobin reactivation studies .

Classification	Summary of results	Ref.
Murine gene therapy studies	α -thalassemia fetuses are rescued by a lentiviral vector carrying the α -globin gene and promoter flanked by the HS2, 3 and 4 of the human LCR	[68]
	Lentiviral vectors, carrying the human β -globin gene and its fundamental regulatory elements, cure/rescue animal models for β -thalassemia intermedia and major	[47,48,69]
	A lentiviral vector with enhanced antisickling activity corrects the phenotype of SAD and BERK mice	[67]
	Genetically corrected thalassemic erythroblasts undergo <i>in vivo</i> selection, indicating their survival advantage over the thalassemic erythroblasts	[43]
	Compared to the conventional γ -retroviral vectors, lentiviral vectors exhibit approximately 200-fold lower transforming potential, which is further reduced in the presence of the cHS4 insulator	[71,72]
	Anemia in mice transplanted with β -thalassemic cells transduced with a γ -globin/MGMT vector improves after selection with the DNA alkylating agent 1,3-bis-chloroethyl-1-nitrosourea followed by increased expression of the transgenic γ -globin gene	[85]
Human gene therapy studies	A vector that combines the γ -globin gene with a shRNA to increase HbF expression and downregulate sickling β -globin corrects sickle cells	[71]
	The cHS4 insulator and the HS2 enhancer of the <i>GATA1</i> gene improve high β -globin gene expression in thalassemic cells	[49,72]
	A 200-bp ankyrin insulator promotes higher level of β -globin expression in thalassemic and sickle cells	[73] [†]
	A patient with β E/ β 0-thalassemia is cured with a lentiviral-based gene therapy treatment	[77]
HbF inducers/repression of negative TF approach studies	Pomalidomide and lenalidomide are potent inducers of HbF in human CD34 ⁺ progenitor cells from healthy and SCA individuals	[87]
	HU is the most used HbF inducer in both moderate and severe forms of β -thalassemia and 50% of patients present clinical amelioration after treatment	[7,8,89,90]
	A thalassemia major patient carrying a IVS1-6/cd44-C genotype responds to treatment with thalidomide with high increase of HbF production and high levels of total Hb content, supporting the findings of a previous study	[91,92]
	A double-stranded 22-bp decoy oligonucleotide targeting Oct-1 increases the level of the γ -globin mRNA in K562 cells	[102]
	Reduced level of BCL11A in human and mouse ErPCs, triggered via knockdown of KLF1, increases human γ -globin/ β -globin expression ratios	[110]
	A lentiviral construct with a shRNA against BCL11A successfully increases HbF levels in β -thalassemic erythroid cells, preserving erythroid differentiation	[50]
	Zinc finger transcriptional activator designed to interact with the γ -globin promoter enhances HbF production in primary erythroid erythroblasts	[111,112]

[†]This study has produced similar results in murine β -thalassemia.

ErPC: Erythroid progenitor cells; Hb: Hemoglobin; HbF: Fetal hemoglobin; HS: Hypersensitive sites; HU: Hydroxyurea; LCR: Locus control region; MGMT: Methylguanine methyltransferase; SCA: Sickle cell anemia; TF: Transcription factor.

Induction of HbF production

Induction of HbF: *in vitro* studies

Several research articles and reviews have been published on the topic of HbF induction [5,7,8,80–85]. The basic idea is to replace HbA function with HbF (which is highly synthesized in the fetus but typically switched off in adult life).

Several original inducers have been proposed in the past few years, including small molecules, naturally occurring proteins, small DNA fragments and artificial transcription factors (TFs) [8,86]. For instance, Moutouh-de Parseval *et al.* reported a study showing the effects of pomalidomide and lenalidomide

on erythropoiesis and HbF production using human CD34⁺ progenitor cells from healthy and SCA individuals. They found that both compounds regulated Hb transcription, resulting in potent induction of HbF without the cytotoxicity effects usually associated with other HbF inducers [87]. When combined with hydroxyurea, pomalidomide and, to a lesser extent, lenalidomide were found to have synergistic effects on HbF induction. A completely different approach has been recently described by Boosalis *et al.*, which aimed at identifying novel therapeutic candidates for HbF induction [88]. The authors studied 13,000 compounds within a chemical library by molecular modeling,

employing interactive pharmacophores, pseudo-binding site modeling and *in silico* high-throughput screening and were able to identify 23 novel, structurally unrelated compounds. Among these candidates, four were found to be very effective in inducing HbF in ErPCs, and two of them exhibited pharmacokinetic profiles favorable for clinical applications.

Novel inducers of HbF: in vivo studies

While several reports describe *in vitro* experimental systems to test the efficacy of HbF inducers, *in vivo* trials are still scarce. Hydroxyurea is the most used HbF inducer in both moderate and severe forms of β -thalassemia [89,90]. More than 500 patients with β -thalassemia have been treated with hydroxyurea worldwide and approximately 50% of them responded to the treatment, exhibiting a clear amelioration of the clinical parameters [7,8,90]. Other inducers of HbF synthesis, such as butyrates, 5-azacytidine and, more recently, decitabine, have also been employed. Another example of *in vivo* HbF induction was recently described by Masera *et al.*, which reported the remarkable response to thalidomide in a thalassemia major patient carrying a IVS1-6/cd44-C genotype and resistant to conventional therapy [91]. This was a clinically unique case in which blood transfusion was no longer possible, in consideration of the occurrence of severe post-transfusion reactions. This patient showed an outstanding response to thalidomide, by reaching a high increase of HbF production and high levels of total Hb content, supporting the findings of a previous study on the use of thalidomide therapy in a patient with β -thalassemia major [92].

Despite these encouraging outcomes, more HbF-induced agents are required, especially those able to induce HbF without the need of high doses or intravenous infusion.

Novel approaches: induction of expression of γ -globin genes & HbF by gene therapy

Therapeutic interventions on thalassemic mice using retroviral and lentiviral vectors carrying the human γ -globin gene have been reported by several groups [85,93–97]. The work by Zhao *et al.* demonstrated the amelioration of β -thalassemia features in mice through drug selection of HSCs transduced with a lentiviral vector encoding both γ -globin and the methylguanine methyltransferase (MGMT) drug-resistance gene, which confers resistance to DNA alkylating drugs [85]. Mice transplanted with β -thalassemic cells transduced with a γ -globin/MGMT vector improved their anemia after selection with the DNA alkylating agent 1,3-bis-chloroethyl-1-nitrosourea followed by increased expression of the transgenic γ -globin gene.

Targeting TFs that are negative regulators of γ -globin gene transcription

This issue was the subject of a recent review paper by Thein and Menzel, which reported the progress made in understanding the HPFH phenomenon in adults [98]. Three major loci (Xmn1-HBG2 single nucleotide polymorphism, HBS1L-MYB intergenic region on chromosome 6q and BCL11A) contribute

to high HbF production. Other loci are expected to be involved [99,100] but their contribution to the HPFH phenomenon seems to only be marginal. Although these three major loci have been described, a therapeutic approach based on their function has not been developed. Given that these loci are associated with putative binding sites for transcription repressors, studies are underway to characterize individuals carrying genetic variations associated with increased production of HbF and its correlation to disease severity [98,101].

Putative repressors of γ -globin gene transcription described in the literature include Oct-1 [102], MYB [103] and BCL11A [104–107]. Short oligonucleotides have been employed to prevent the binding of these factors to their target sites or target additional TFs. Xu *et al.* used decoy oligonucleotides that target the Oct-1 TF consensus sequence for inducing increased expression of this gene [102]. They 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. Western blot analysis further demonstrated significant increases of HbF in the Oct-1 decoy oligonucleotide-treated cells. Therefore, the disruption of the binding site of the Oct-1 transcriptional factors with the decoy oligonucleotide offers a novel approach for inducing the expression of the γ -globin genes [102,108]. In a recent publication, Sankaran *et al.* showed that persistency of HbF and increased embryonic Hb in newborn with trisomy 13 is caused by elevated expression of two miRNAs, miR-15a and -16-1 [109]. Moreover, they showed that downregulation of the MYB gene, which is the target of miR-15a and -16-1, leads to similar effects, suggesting that this additional pathway can be targeted to increase HbF in patients with hemoglobinopathies. Additionally, it has been observed that reduced levels of BCL11A in human and mouse ErPCs, triggered via knockdown of KLF1, increases human γ -globin/ β -globin expression ratios [110]. This study suggests that KLF1 is a key regulator of globin gene switching, which implicates that its controlled repression may also provide a method to increase HbF in thalassemia and SCA patients.

Based on some of these findings, it has been shown that a lentiviral construct with a shRNA against BCL11A successfully increased HbF levels in β -thalassemic erythroid cells, preserving erythroid differentiation [50]. In addition to studies targeting repressor TFs, other recent publications focused on the direct manipulation of γ -globin genes transcription. To this end, both Gräslund *et al.* [111] and Wilber *et al.* [112] engineered artificial TFs in order 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 these two groups demonstrate that is possible to enhance HbF production in the primary erythroid erythroblast by using a zinc finger transcriptional activator designed to interact with the γ -globin promoter. A summary of the most impactful studies focused on the development of HbF inducers is provided in TABLE 1.

Coexpression of β -like globin genes in β -thalassemia

Recently, new approaches based on the activation of multiple globin genes, have been proposed. In 2011 Zhu *et al.* obtained upregulation of the δ -globin gene to increase HbA2 synthesis, with a series of EKLF–GATA1 fusion constructs made of the transactivation domain of EKLF and the DNA-binding domain of GATA1 [113]. With this strategy, a significant upregulation of δ - and γ -globin RNA transcript and protein expression in CD34⁺ cells were obtained, demonstrating that different globin genes can be upregulated and expressed concurrently.

It is well established that HbF inducers can produce therapeutic levels of Hb in ErPC isolated from β -thalassemia intermedia patients who are able to synthesize some β -globin. For instance, in the study by Musallam *et al.*, 63 thalassemic patients who did not require transfusions showed that the proportion of HbF correlated positively with total Hb levels [114]. In addition, the median HbF levels were significantly lower in patients presenting with the majority of evaluated morbidities than in those without, leading to the conclusion that there is a strong association between HbF levels and morbidity in the subset of untransfused patients with thalassemia intermedia.

Combination of gene therapy for *de novo* HbA production & HbF induction for β -thalassemia

As a complete correction of the α -globin and β -like globin unbalance may not be achieved following gene therapy alone, the authors have recently conducted a study aimed at testing the efficacy of lentiviral-based gene therapy combined with stimulation of HbF induction and the ability of the combined approach to reduce α -globin excess in ErPCs. For this purpose, the authors treated ErPCs with a lentiviral vector carrying the β -globin gene and mithramycin at the same time. All thalassemic specimens were obtained from homozygous β^{039} -thalassemia patients. The major finding of this study is the formal demonstration that concurrent expression of the transgenic β -globin mRNA and induction of the γ -globin genes by gene transfer and HbF induction, respectively, exhibit an additive effect profoundly improving Hb synthesis in β -thalassemic ErPC cells. The increased β - and γ -globin syntheses were associated with a significant reduction or complete depletion of free α -globin chain aggregates. The restoration of a balance between α -globin and β -like globin chains was also associated with clear amelioration of the phenotype of thalassemic cells [115]. Similar findings were observed in experiments performed using ErPCs from two additional patients carrying a $\beta^{039}/IVS110$ genotype [ZUCCATO C ET AL., UNPUBLISHED DATA].

While it is expected that the gene transfer will be done only once due to the ability of the vector to integrate in HSCs, it is expected that administration of the fetal Hb inducer will require a chronic approach. As mentioned above, this combined approach should be envisioned only if the gene transfer leads to insufficient levels of *de novo* Hb to correct the phenotype of the patients. In this case, administration of fetal Hb inducers may be sufficient to eliminate the need for blood transfusion. In addition, if the synthesis of the *de novo* Hb is substantial, the administration of the fetal Hb inducers may require limited

doses to achieve therapeutic levels, reducing toxicity-related complications. Nevertheless, this approach will need to be carefully evaluated in animal models to exclude that combining the two methods may not lead to unexpected side effects.

Expert commentary

The data reported in our previous studies support the idea that combining a gene therapy approach with HbF induction may have a much higher impact in resolving the anemia in thalassemia than the single approaches alone. Many studies also demonstrated the beneficial effect of HbF expression in thalassemia intermedia patients [87–90,116–118]. Based on these observations, we propose and showed that the combination of gene therapy with HbF induction may be especially useful to eliminate the excess of α -globins present in ErPC from β^0 -thalassemia patients in which the transgenic Hb levels were insufficient to correct the α - versus β -like globin unbalance. This appears to be a major therapeutic goal for the treatment of β -thalassemic cells since it is expected to effectively improve their pathophysiological features.

In conclusion, our data suggest that the cotreatment of target ErPCs with a lentiviral vector carrying a therapeutic β -globin gene and the HbF inducer mithramycin leads to increased *de novo* accumulation of HbA and HbF production, ultimately suppressing the excess of free α -globin chains. These results may be relevant for establishing a protocol to maximize the production of clinically therapeutic Hbs in thalassemic erythroid cells.

Five-year view

This article emphasizes the importance of a combined approach for the treatment of β -thalassemia. Our findings, along with those of others, attest that gene transfer of the β - or γ -globin gene as well as induction of HbF synthesis in preclinical studies have largely succeeded and paved the way for clinical trials. We have shown that the combination of these approaches may have an additive effect, overcoming the limitation of the two strategies used individually. In this article, the development of new constructs for the expression of the β -globin gene in association to shRNAs against γ -globin gene repressors holds great therapeutic potential. Alternatively, HbF inducers and repressors of factors that are negative regulators of HbF could be utilized to complement gene transfer when therapeutic Hb levels fail to be achieved. Mithramycin analogues with a lower toxicity feature are currently under testing as potential candidates for the use of these compounds in the long-term treatment for HbF induction.

Acknowledgments

The authors would like to thank the Associazione Veneta per la Lotta Contro la Talassemia, the Talassaemia Modular Stratification System for personalized therapy of β -thalassemia and the THELETHON grant #GG10214.

Financial & competing interests disclosure

S Rivella is a consultant for Novartis, Bayer, Biomarin, Merganser, Imago and Isis Pharmaceuticals, owns equity of Merganser Pharmaceuticals, is a co-inventor for the patents US8058061 B2 C12 20111115 and US7541179

B2 C12N 20090602, and is supported by the NIH grant RO1 HL102449-03. The consulting work and intellectual property of S Rivella did not affect in any way the design, conduct or reporting of the research described in this manuscript. The authors have no other 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 apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Key issues

- β -thalassemias are inherited disorders characterized by chronic insufficiency of the β -globin chain.
- In the most severe cases (thalassemia major), lifelong treatment with blood transfusion and iron chelation is required but lack of a definitive cure leads to the development of complicated comorbidities.
- High persistency of fetal hemoglobin can ameliorate a patient's phenotype leading to an asymptomatic life.
- Gene transfer of the β -globin gene using lentiviruses is a curative approach that has proven successful in preclinical and clinical studies.
- Many studies are elucidating pathways that regulate fetal hemoglobin activation and repression.
- Induction of HbF is an alternative approach to overcome the anemia in hemoglobinopathies.
- The combination of the gene transfer and fetal hemoglobin induction could maximize the therapeutic potential of the two approaches used separately.

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