

# Recent trends in the gene therapy of $\beta$ -thalassemia

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**Abstract:** The  $\beta$ -thalassemias are a group of hereditary hematological diseases caused by over 300 mutations of the adult  $\beta$ -globin gene. Together with sickle cell anemia, thalassemia syndromes are among the most impactful diseases in developing countries, in which the lack of genetic counseling and prenatal diagnosis have contributed to the maintenance of a very high frequency of these genetic diseases in the population. Gene therapy for  $\beta$ -thalassemia has recently seen steadily accelerating progress and has reached a crossroads in its development. Presently, data from past and ongoing clinical trials guide the design of further clinical and preclinical studies based on gene augmentation, while fundamental insights into globin switching and new technology developments have inspired the investigation of novel gene-therapy approaches. Moreover, human erythropoietic stem cells from  $\beta$ -thalassemia patients have been the cellular targets of choice to date whereas future gene-therapy studies might increasingly draw on induced pluripotent stem cells. Herein, we summarize the most significant developments in  $\beta$ -thalassemia gene therapy over the last decade, with a strong emphasis on the most recent findings, for  $\beta$ -thalassemia model systems; for  $\beta$ -,  $\gamma$ -, and anti-sickling  $\beta$ -globin gene addition and combinatorial approaches including the latest results of clinical trials; and for novel approaches, such as transgene-mediated activation of  $\gamma$ -globin and genome editing using designer nucleases.

**Keywords:** Thalassemia, gene therapy, HbF induction, transcription factors, induced pluripotent stem cells, genome editing, TALEN, CRISPR, ZFN

## Introduction

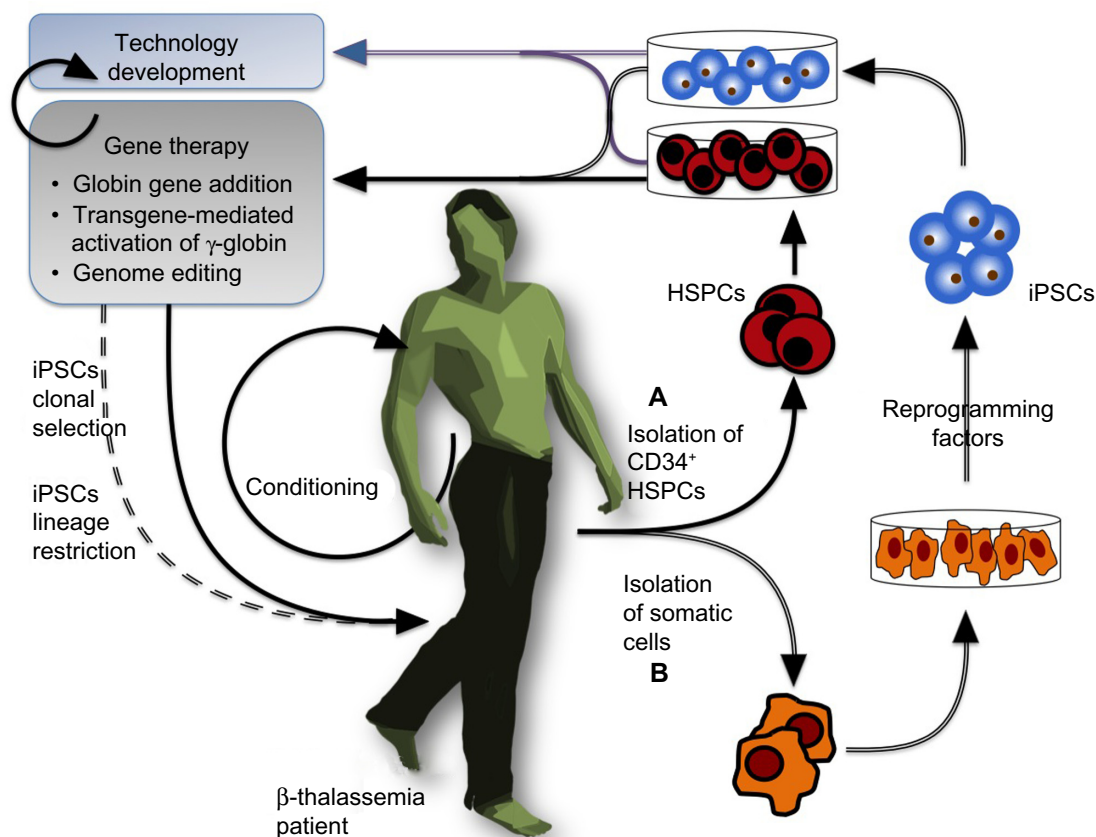
The  $\beta$ -thalassemias are a group of hereditary hematological diseases caused by over 300 mutations of the adult  $\beta$ -globin gene,<sup>1</sup> with excellent reviews providing background information outlining genetics,<sup>2-4</sup> pathophysiology,<sup>5,6</sup> and therapeutics<sup>7</sup> of  $\beta$ -thalassemia that is beyond the scope of this review. In brief,  $\beta$ -thalassemia is brought about by mutations reducing or abrogating  $\beta$ -globin expression, which thus lead to reduced adult hemoglobin ([HbA] an  $\alpha_2\beta_2$  heterotetramer) and excess  $\alpha$ -globin content in erythroid cells, in turn resulting in ineffective erythropoiesis and apoptosis in the erythroid lineage.<sup>3,8,9</sup> Most  $\beta$ -thalassemia patients therefore require lifelong clinical management by blood transfusion and chelation therapy,<sup>10-12</sup> with a few having the option of curative but potentially hazardous allogeneic transplantation of hematopoietic stem and progenitor cells (HSPCs) instead.<sup>13,14</sup> This indicates the need for alternative therapies, and the observation that high levels of the fetal  $\beta$ -globin-like  $\gamma$ -globin chain result in an ameliorated  $\beta$ -thalassemia phenotype<sup>15</sup> has prompted the search for  $\gamma$ -globin-inducing chemical agents.<sup>16-21</sup> Patient response to known  $\gamma$ -globin inducers, however, is varied,<sup>22</sup>

and the search continues for reagents with higher efficiency, consistency, and tolerability in chronic application,<sup>23</sup> if not to cure the disease, then to reduce transfusion requirements and the significant cost of disease management. Of note, hemoglobinopathies, such as the thalassemia syndromes and sickle cell anemia (SCA; caused by the toxic  $\beta$ -globin<sup>E6V</sup> mutation), most severely affect low-income countries, where the lack of prevention programs and an underlying high carrier rate bring about high disease frequencies,<sup>24</sup> although global migration has now turned hemoglobinopathies into a concern for many nonendemic countries as well.<sup>25</sup> Globally,  $\beta$ -thalassemia mutations introducing gene deletions, aberrant splicing, or premature stop codons have the greatest impact in terms of global disease burden and clinical severity.<sup>26,27</sup> Recent progress in the research of disease modifiers,<sup>28</sup> chemical modulation of gene expression,<sup>22,29</sup> and tools and approaches for DNA-based therapies<sup>30,31</sup> have opened new avenues toward novel and more personalized strategies to manage or cure  $\beta$ -thalassemia, as we have reviewed recently.<sup>23,32</sup> Particularly with regard to curative approaches by gene therapy, the field

has come to a crossroads, with the initiation of clinical trials, the possible plateauing off of incremental improvements to gene augmentation therapy, and the increasing preclinical application of novel genome-editing tools. The objective of the present manuscript is to review the most relevant findings published in the period 2005–2014 concerning the preclinical and clinical application of gene therapy for  $\beta$ -thalassemia. To this end, we will describe the pertinent model systems,  $\beta$ -like-globin gene-addition strategies, gene addition in combination with chemical inducers of  $\gamma$ -globin, transgene-mediated activation of endogenous  $\gamma$ -globin, and the emerging use of designer nucleases for  $\beta$ -thalassemia gene therapy. The general flow of gene-therapy-based approaches for  $\beta$ -thalassemia is outlined in Figure 1.

## Experimental model systems

Several experimental systems have been developed to establish the suitability of and provide proof of principle for gene-therapy approaches to  $\beta$ -thalassemia. Erythroid cell lines, such as human and murine erythroleukemia cells, allow cost-effective



**Figure 1** General view of a gene-therapy approach for  $\beta$ -thalassemia.

**Notes:** Adult hematopoietic stem and progenitor cells (HSPCs) or induced pluripotent stem cells (iPSCs) can be the object of gene-therapy approaches. (A) The commonly used CD34<sup>+</sup> HSPCs and subpopulations may be corrected directly by gene therapy. (B) Alternatively, somatic cells can be isolated and reprogrammed to pluripotency, with the resulting iPSCs then being a patient-specific substrate for gene therapy, clonal selection, and lineage-specific differentiation. Excepting circular arrows, solid arrows indicate procedures for HSPCs and hollow arrows those for iPSCs. Circular arrows apply to HSPCs and iPSCs alike. Application of  $\beta$ -thalassemia iPSCs to patients is still pending, as indicated by dashed arrows.

high-throughput assessments in the erythroid lineage,<sup>33–36</sup> and cancer-prone mouse models have been instrumental in gauging the genotoxicity of integration and genome-modification events for vector classes applied to  $\beta$ -thalassemia.<sup>37–39</sup> The most informative functional studies of candidate therapies toward their clinical application, however, instead rely on thalassemic human stem cells for in vitro assessments of authentic human responses and on thalassemic murine models for long-term systemic assessments in vivo.

### **In vitro experimental systems: erythroid precursor cells from $\beta$ -thalassemia patients**

HSPCs are the substrate for clinical gene-therapy application, so that in vitro assessment of HSPC-derived erythroid precursor cells (ErPCs) is highly informative for toxicity and efficacy of any therapeutic intervention (Figure 1A). ErPCs from peripheral blood are widely used, while access to bone marrow and mobilized blood,<sup>40,41</sup> which, incidentally, contain the cells preferentially used in clinical applications,<sup>42,43</sup> is more restricted. Using peripheral-blood-derived ErPCs, it is possible to obtain large cultures of relatively pure and synchronized erythroid cell populations in which compounds can be added at specific stages of maturation. In the procedure developed by Fibach et al,<sup>44,45</sup> the culture is divided into two phases: first, an erythropoietin (EPO)-independent proliferation phase, in which peripheral blood cells are first cultured in the presence of a combination of growth factors, but in the absence of EPO; and, second, a differentiation phase, when the culture, supplemented with EPO, generates orthochromatic normoblasts and enucleated erythrocytes, with cells decreasing in size and accumulating hemoglobin (Hb) and large cellular clusters assuming a reddish color and giving brown-red pellets upon centrifugation.<sup>45,46</sup> This system recapitulates many aspects of in vivo erythropoiesis, including globin RNA metabolism, cell cycle kinetics, expression of cell surface antigens, iron and ferritin metabolism, and recruitment of transcription factors,<sup>45</sup> and allows analysis of Hb content by a variety of techniques, such as alkaline denaturation, benzidine staining, capillary electrophoresis, cation-exchange high-performance liquid chromatography for hemoglobins, and reversed-phase high-performance liquid chromatography for globin chains.<sup>45,46</sup>

### **In vitro experimental systems: human embryonic stem cells and induced pluripotent stem cells**

Human embryonic stem cells (hESCs) have been used extensively to study the early phases of hematopoietic and

erythroid development.<sup>47</sup> In this approach, after 5 to 7 days of in vitro cell culture, a blastocyst is generated, showing a clearly visible and easily accessible inner cell mass, from which pluripotent stem cells can be isolated, giving rise to in vitro hESC lines. From these cell lines, embryoid bodies can be developed and used for further tissue-specific differentiation. hESCs themselves have only a minor role in the preclinical study of therapies for hemoglobinopathies,<sup>48,49</sup> and their clinical application would suffer due to the ethical repugnance of their origin and from the same incompatibilities seen for allogeneic HSPC transplantations. However, the underlying hESC methodology is being reemployed in the culture of induced pluripotent stem cells (iPSCs),<sup>50–53</sup> which closely mimic hESCs and represent a potential cornucopia for cell-based therapies in general. The creation of iPSCs from somatic cells with the use of reprogramming factors (originally Oct3/4, Sox2, c-Myc, and Klf4<sup>50</sup>) represented a paradigm shift in our understanding of developmental biology and in the conception of novel therapeutic approaches, not least because their use avoids the ethical concerns associated with hESCs and creates a patient-specific, histocompatible substrate for cell therapy. Human iPSCs retain embryonic and fetal characteristics of gene expression even upon erythroid differentiation in vitro, so that the hope arose that patient-derived iPSCs for  $\beta$ -thalassemia or SCA might be therapeutic in their own right via the maintenance of high levels of  $\gamma$ -globin expression (Figure 1B).<sup>54,55</sup> However, according to recent in vivo findings after transplantation into immunodeficient mice, in which a gradual switch to the adult  $\beta$ -globin gene was observed,<sup>56,57</sup> this hope appears to be unfounded. Notwithstanding this apparent setback, iPSCs are a promising substrate for gene therapy, as they can be amplified in vitro indefinitely (where they are, alas, still subject to the same mutation rates and potentially undesirable changes as any other cell type) and thus allow the clonal selection of rare events of therapeutic interest. Since its inception, iPSC technology has been used extensively in innovative studies on  $\beta$ -thalassemia and other hemoglobinopathies, as will be detailed for specific gene-addition and genome-editing approaches.

### **In vivo experimental systems: mouse models**

Thalassemic mouse models provide the most economical option for gauging the putative and systemic effects of gene-therapy approaches in thalassemic patients. Of note, the regulation of  $\beta$ -like globin chains in humans comprises a switch in utero from the embryonic ( $\epsilon$ ) to the fetal ( $\gamma$ ) chain, followed by an HbA switch perinatally up to 6 months after birth,<sup>58,59</sup>

which also allows the birth and early postnatal development of homozygous  $\beta^0$  patients without disease management. In contrast, the murine  $\beta$ -globin locus encodes four functional  $\beta$ -like globin genes:  $\beta h1$  and  $\epsilon^\gamma$  (transcribed only during the embryonic phase of development up to E14–E15 of a total gestation period of approximately 21 days), and the  $b1$  ( $\beta^{\text{major}}$ ) and  $b2$  ( $\beta^{\text{minor}}$ ) genes, which are transcriptionally activated in utero around 11 days after conception.<sup>59</sup> Accordingly, mice homozygous for ( $\beta^0$ ) mutations that prevent expression of the adult  $\beta$ -globin genes die perinatally, owing to a complete lack of expression of any Hb.<sup>59</sup> The most widely used, non-humanized adult murine models of  $\beta$ -thalassemia therefore need to retain some  $\beta$ -globin expression and thus show features similar to those observed for  $\beta$ -thalassemia intermedia patients, who carry moderate to mild ( $\beta^+$ ) mutations,<sup>60</sup> although a  $\beta^0$  surgical model of murine  $\beta$ -thalassemia major has also been developed.<sup>60,61</sup>

In order to test the activity of novel mutation-specific approaches in vivo, humanized mouse models needed to be developed,<sup>58</sup> with those combining absence of murine  $\beta$ -like globin genes with the presence of a human  $\beta$ -globin gene cluster and mutated  $\beta$ -globin gene being of the greatest utility. For instance, Vadolas et al<sup>62</sup> reported generation of a humanized mouse model carrying the common  $\beta^+$  IVSI-110 splicing mutation on a bacterial artificial chromosome carrying the human  $\beta$ -globin locus. Comparison of heterozygous  $\beta$ -globin knock-out mice carrying either the IVSI-110 or the normal human  $\beta$ -globin locus showed a 90% decrease in human  $\beta$ -globin chain synthesis in the IVSI-110 mouse model. The model, moreover, accurately recapitulates the splicing defect found in  $\beta$ -thalassemia patients and is thus a suitable platform on which to test approaches for the restoration of normal splicing. Similarly, a humanized mouse model carrying the common G26A (HbE) mutation, frequently co-inherited with  $\beta$ -thalassemia in Southeast Asia, has been developed, which allows in vivo analysis in mouse of therapies for HbE/ $\beta$ -thalassemia.<sup>63</sup> Mouse models (whether of a wild-type or thalassemic background) carrying all or parts of the human  $\beta$ -globin locus have also proven an essential resource for the analysis of globin switching and therapeutic approaches for  $\beta$ -thalassemia.<sup>64–66</sup> Finally, a keen interest in the study of developmental gene regulation,  $\gamma$ -globin induction, and therapies for  $\beta$ -thalassemia major has prompted the development of further humanized transgenic mice as models for  $\beta$ -thalassemia major.<sup>67</sup> These mice carry a mutated human  $\beta$ -globin gene and are born viable due to the prolonged expression of human fetal hemoglobin (HbF), but require chronic transfusion for survival and are not yet widely available in the community.<sup>67–69</sup>

## Globin gene addition

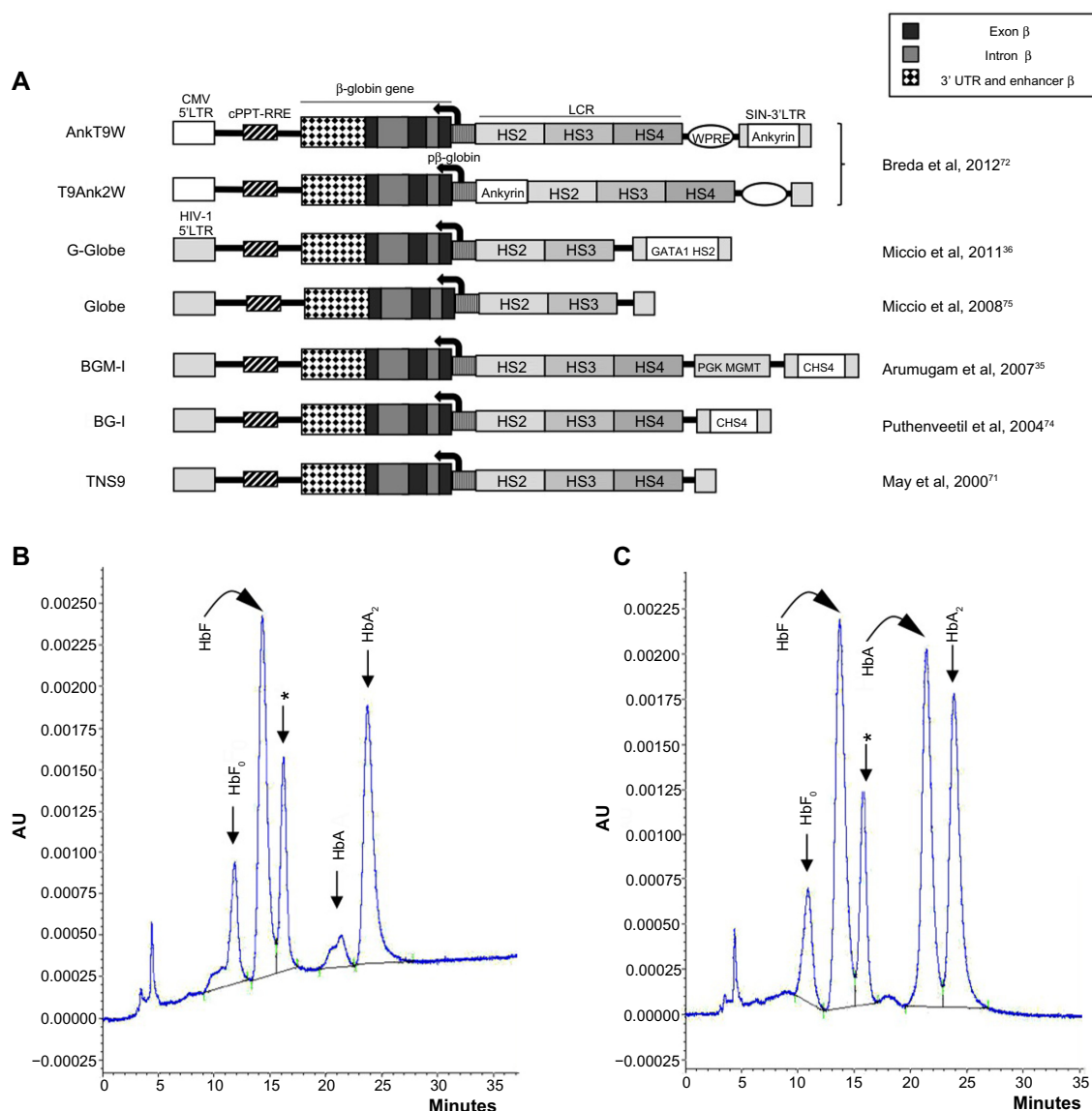
Over the last 2 decades, major efforts have been made to achieve therapeutic levels of exogenous  $\beta$ -like globin chains in  $\beta$ -thalassemia and SCA. These finally came to fruition when a switch from  $\gamma$ -retroviral vectors to lentiviral vectors allowed the efficient transduction of nondividing cells with a sufficiently large expression cassette,<sup>70</sup> encouraging numerous research groups to work toward vectors expressing  $\beta$ -globin, anti-sickling variants of  $\beta$ -globin and  $\gamma$ -globin.

## Lentiviral expression of exogenous $\beta$ -globin

The efforts of the groups working in this field have been dedicated to achieving highly efficient and stable transduction of HSPCs, to optimizing transgene expression (erythroid- and stage-specific, elevated, position-independent, and sustained over time), and to correcting the  $\beta$ -thalassemia phenotype in preclinical models with minimal genotoxicity.<sup>35,36,40,42,71–75</sup> While the field has reached a high level of optimization, incremental improvements to procedures and vectors continue to be made. These include the use of rapamycin to enhance LV transduction<sup>76</sup> and the recent inclusion of chromatin opening elements<sup>77–79</sup> or an ankyrin insulator<sup>72</sup> for improved vector-derived expression, with an ongoing search for and evaluation of alternative insulators<sup>80</sup> to prevent transgene silencing and minimize host gene perturbation while avoiding the reduction of vector titer during production that is associated with the most widely used chicken HS4 insulator.<sup>81</sup> It has also been demonstrated that, in order to avoid insertional mutagenesis, it is possible to select suitable clones with insertions in inert (“safe harbor”) genome sites, in combination with iPSC technology.<sup>82</sup> Several recent reviews on gene therapy of thalassemia and related hemoglobinopathies point out the state of the art with respect to the structure of  $\beta$ -globin-carrying lentiviral vectors,<sup>70,83,84</sup> and Figure 2A depicts a number of therapeutic lentiviral vectors that produce high levels of  $\beta$ -globin in human or murine  $\beta$ -thalassemic erythroid cells.<sup>35,36,40,42,71–75</sup> Figure 2B and C illustrates how one such vector, T9W, generates high-level HbA production in ex vivo culture of HSPC-derived cells isolated from a  $\beta^0$ -thalassemia patient upon differentiation.<sup>46</sup>

## Lentiviral expression of anti-sickling $\beta$ -globin and exogenous $\gamma$ -globin

With a view to applying the same vector for  $\beta$ -thalassemia and SCA,  $\beta$ -globin vectors have also been modified to approach or even exceed the anti-sickling activity shown by  $\gamma$ - and  $\delta$ -globin. Of note are the HPV569 and BB305



**Figure 2** Lentiviral vectors expressing exogenous  $\beta$ -globin.

**Notes:** (A) Structure of the lentiviral vectors proposed for gene therapy of  $\beta$ -thalassemia and carrying a therapeutic human  $\beta$ -globin gene.<sup>35,36,71,72,74,75</sup> Not to scale. (B and C) Representative experiment demonstrating de novo production of HbA in erythroid precursor cells from a  $\beta^0$ -thalassemia patient after gene therapy. Asterisks indicate the peak corresponding to aggregates of free  $\alpha$ -globin. (B) Untreated control cells; (C) cells treated with the T9W vector.<sup>114</sup>

**Abbreviations:** Ankyrin, ankyrin insulator; AU, absorbance units; cHS4, chicken  $\beta$ -globin hypersensitive site 4 insulator; CMV, Cytomegalovirus; GATA1 HS2, globin transcription factor 1 hypersensitive site 2 enhancer; HIV-1, human immunodeficiency virus; HS, hypersensitive site; LCR, locus control region; LTR, long terminal repeat; p $\beta$ -globin,  $\beta$ -globin promoter; PGK MGMT, murine phosphoglycerate kinase-1 promoter and O<sup>6</sup>-methylguanine DNA methyltransferase gene; SIN, self-inactivating; UTR, untranslated region; WPRE, woodchuck posttranscriptional regulatory element; cPPT, central polypurine track; RRE, Rev response element.

LentiGlobin<sup>®</sup> vectors,<sup>85,86</sup> which feature in  $\beta^E/\beta^0$  clinical trials<sup>86–89</sup> (see Clinical trials below) and carry  $\beta$ -globin<sup>T87Q</sup>, and which are expected to provide some anti-sickling activity<sup>90</sup> and thus be suitable for SCA therapy. Particularly important in this context, however, are the anti-sickling  $\beta$ (AS3)  $\beta$ -globin designed by Townes et al<sup>91,92</sup> and pertaining lentiviral vectors.<sup>93</sup> The combination of three amino acid changes (creating the artificial  $\beta$ -globin<sup>G16D, E22A, T87Q</sup> variant) confers anti-sickling activity exceeding that of  $\gamma$ -globin to  $\beta$ (AS3) and therefore renders the mutant

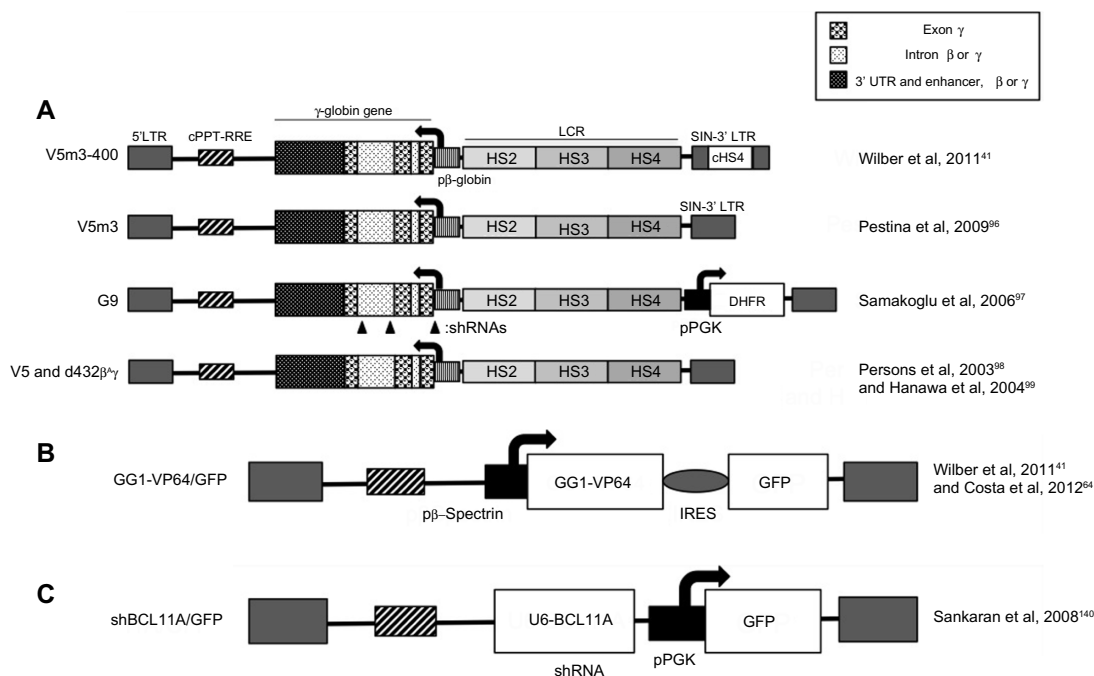
transgene particularly suitable for the therapy of SCA and  $\beta$ -thalassemia/ $\beta$ -globin<sup>E6V</sup> compound heterozygotes. Independently, and primarily with clinical application for SCA in mind, numerous groups have also developed retroviral vectors encoding  $\gamma$ -globin instead of  $\beta$ -globin (Figure 3).<sup>41,94–101</sup> Of note, Wilber et al<sup>41</sup> used lentiviral vectors encoding the human  $\gamma$ -globin gene with or without an insulator, which were tested on erythroid progeny of normal CD34<sup>+</sup> cells and resulted in high levels of HbF production, suggesting that lentiviral-mediated treatments have

the potential to provide therapeutic HbF levels to patients. These findings are corroborated by several independent research groups that work on  $\gamma$ -globin-based lentiviral (and  $\gamma$ -retroviral) therapy of SCA and  $\beta$ -thalassemia.<sup>41,94–101</sup> Figure 3A shows the structure of some of the corresponding vectors.<sup>41,96–99</sup> All vectors intended for gene augmentation described here, be it for the expression of  $\beta$ -globin, anti-sickling  $\beta$ -globin, or  $\gamma$ -globin, have overlapping fields of application. Further preclinical and clinical studies will show which vector may be most suitable for specific disease conditions, with the vector itself as a key factor, but with all components of the treatment protocol, including conditioning, HSPC source and isolation, transduction protocol, and general culture conditions, playing a critical role in the outcome and in the comparison of vector performance.

## Combination therapy of gene addition with HbF inducers

Induction of endogenous HbF is one of the most widely applied therapeutic strategies for  $\beta$ -thalassemia and SCA, as indicated by several recent studies and reviews.<sup>102–110</sup> Lending additional significance to preclinical studies, it has been shown that the level of  $\gamma$ -globin mRNA and in vitro induction of HbF in primary ErPCs isolated from

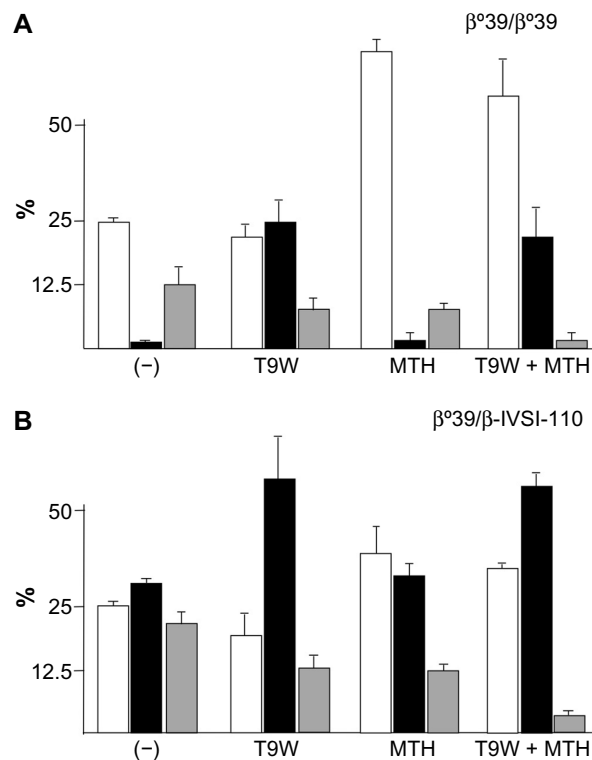
$\beta$ -thalassemia patients is predictive of the hydroxyurea response in vivo.<sup>111,112</sup> While most of the recent studies in the field still focus on low-molecular-weight HbF inducers,<sup>102–110,113</sup> the innovative strategy of combining them with vector-derived  $\beta$ -globin has lately been investigated and reviewed.<sup>30,114</sup> The combined treatment induces an increase of both HbA (by gene addition) and HbF (by chemical HbF induction) with important therapeutic implications, given that  $\beta$ -like globin transfer in some  $\beta$ -thalassemia major ErPCs has been unable to reach physiological levels of Hb in vitro and might thus only lead to partial phenotypic correction in vivo as well. Since increased production of HbF in  $\beta$ -thalassemia is undoubtedly beneficial, the one-off application of gene therapy combined with chronic application of HbF inducers appears to be a pertinent strategy to achieve clinical benefits not achievable with either strategy alone. Representative results for this approach are depicted in Figure 4 on ErPCs from a  $\beta^0$ -thalassemia patient carrying the codon-39 stop codon mutation ( $\beta^039$ ) (Figure 4A) and a  $\beta^039/\beta$ -IVSI-110-thalassemia patient (Figure 4B). The results demonstrate that this combination strategy achieves high levels of functional Hb in  $\beta$ -thalassemic cells and a concomitant sharp decrease of excess  $\alpha$ -globin, with significant scope for further improvements for what is as yet a nascent field of research.



**Figure 3** Lentiviral vectors expressing exogenous  $\gamma$ -globin or inducing endogenous  $\gamma$ -globin.

**Notes:** (A) Structure of the lentiviral vectors proposed for gene therapy of  $\beta$ -thalassemia and carrying a therapeutic human  $\gamma$ -globin gene. (B) Example of a lentiviral vector carrying a  $\gamma$ -globin gene artificial transactivator. This approach has been applied to the control of  $\gamma$ -globin gene expression by Gräslund et al,<sup>123</sup> Wilber et al,<sup>41</sup> and Costa et al.<sup>64</sup> (C) Example of a lentiviral vector carrying an shRNA for BCL11A, driven by the constitutive RNA polymerase III U6 promoter.

**Abbreviations:** cHS4, chicken  $\beta$ -globin hypersensitive site 4 insulator; HS, hypersensitive site; LCR, locus control region; LTR, long terminal repeat; p $\beta$ -globin,  $\beta$ -globin promoter; shRNA, short hairpin RNA; SIN, self-inactivating; cPPT-RRE, viral sequences harboring the central polypurine tract and the rev response element; pPGK, PGK promoter; DHFR, the *DHFR* gene, providing partial resistance to myelosuppression and thus potentially in vivo selection for transduced cells; U6-BCL11A, an shRNA expressed from the U6 promoter and targeting BCL11A mRNA; IRES, internal ribosome entry site; GFP, green fluorescent protein.



**Figure 4** Combination therapy using lentiviral-vector-derived  $\beta$ -globin expression and chemical  $\gamma$ -globin induction.

**Notes:** Combination of gene therapy and HbF induction leads to high production of both HbA and HbF and reduction of excess  $\alpha$ -globin chains, as shown for representative experiments performed with erythroid precursor cells from (A) a  $\beta^0$ -thalassemia patient homozygous for the codon-39 stop codon mutation (genotype  $\beta^0\beta^0/\beta^0\beta^0$ ) and (B) a  $\beta^0\beta^0/\beta\text{-IVSI-110}$ -thalassemia patient. Cells were cultured without treatment (-) and in the presence of T9W, mithramycin (MTH), and T9W with MTH, respectively, as indicated. The % of HbF (white boxes), HbA (black boxes), and  $\alpha$ -globin peak (gray boxes) is shown (Zuccato et al, unpublished data, 2014; and data from Zuccato C, Breda L, Salvatori F, et al. A combined approach for  $\beta$ -thalassemia based on gene therapy-mediated adult hemoglobin (HbA) production and fetal hemoglobin (HbF) induction. *Ann Hematol.* 2012;91:1201–1213.)<sup>14</sup>

## Clinical trials

To date, there are a total of seven patients who have been treated successfully or for whom longer follow-up is pending in three clinical trials for  $\beta$ -thalassemia, all of which have used  $\beta$ -globin-expressing lentiviral vectors. The first successful gene therapy trial for  $\beta$ -thalassemia<sup>85</sup> was reported in the manuscript published by Cavazzana-Calvo et al in 2010<sup>87</sup> and commented on by Kaiser.<sup>115</sup> The pertaining  $\beta$ -globin<sup>T87Q</sup> vector (LentiGlobin<sup>®</sup> HPV569) holds a tandem copy of the 250-bp cHS4 insulator in its 3' long terminal repeat (LTR) as a safety feature and bears a T87Q amino acid, which, besides its conferring anti-sickling activity,<sup>90</sup> makes it distinguishable from transfusion-derived  $\beta$ -globin and thus allows the quantification of vector-derived  $\beta$ -globin during follow-up. Three patients with severe  $\beta^E/\beta^0$ -thalassemia have been treated to date. In the first patient, engraftment of treated bone marrow failed after full myeloablation, requiring reinfusion of backup bone marrow. For the second patient, however, transfusion independence was

achieved at 12 months after treatment and continues to date. At 36-month follow-up, of 24 detectable clones in peripheral blood, one clone with cross-lineage dominance held a proviral integration in the high mobility group AT-hook 2 (*HMGA2*) gene, whose expression is associated with tumor metastasis and proliferation,<sup>116,117</sup> in a position that removed posttranscriptional control elements and thus increased *HMGA2* mRNA stability. This clone, moreover, showed a recombination event that had removed one of the cHS4 copies and possibly exacerbated transcriptional enhancement of *HMGA2* from the proviral  $\beta$ -globin locus control region (LCR), with transcriptional and posttranscriptional effects combined resulting in 10,000-fold *HMGA2* expression. Clonal dominance of this clone (peaking at 22% of nucleated cells after 48 months) dropped to 6.8% 7 years after treatment. Notably, at 36 months, only one-third of the total Hb was vector derived, with endogenous HbE and unexpectedly high HbF constituting the other two-thirds, so that the patient might have failed to become transfusion independent in the absence of endogenous HbE and elevated  $\gamma$ -globin expression and if mild conditioning instead of full myeloablation had been applied. Finally, engraftment with HPV569-treated cells of the third adult patient for this trial was also successful. However, the patient remains transfusion dependent, with a low vector copy number (VCN) in the originally engrafted cell material (VCN 0.3) and a low VCN in nucleated cells (VCN in neutrophils 0.016), and with vector-derived Hb accounting for only approximately 5% of total Hb more than 2 years after engraftment.<sup>88</sup> Engraftment failure for the first patient, a low VCN for the third patient, and oligoclonal reconstitution, vector recombination, and low vector-derived gene expression for the second patient provide important pointers for necessary improvements in future trials and vectors and, moreover, call for ex vivo preclinical assessment in cells from prospective trial participants, as we argue elsewhere.<sup>72</sup>

A second clinical study (HGB-205) and follow-up to the trial described above has been initiated by bluebird bio Inc. in France and utilizes the third-generation lentiviral LentiGlobin<sup>®</sup> BB305 vector.<sup>86</sup> Compared to HPV569, BB305 holds a cytomegalovirus (CMV) promoter instead of the U3 promoter/enhancer in its 5' LTR and no longer bears cHS4 insulator elements in its 3' LTR. Preliminary results obtained for two  $\beta^E/\beta^0$ -thalassemia patients, who had both been transfusion-dependent for most of their lives, were encouraging, with a VCN of 1.5 and 2.1, respectively, in the engrafted material and with a reported transfusion independence at 3.5 and 6.5 months, respectively, after treatment.<sup>88</sup> This success has most recently also prompted the application of BB305 for gene therapy of SCA.<sup>89</sup>

Finally, an independent trial for globin gene transfer in adult patients with  $\beta$ -thalassemia major has been initiated

(NCT01639690) by the Sadelain group and associates<sup>118,119</sup> to study safety and efficacy, representing the first US trial for  $\beta$ -thalassemia. The  $\beta$ -globin vector used for the trial, TNS9.3.55, holds the *chs4* insulator and minor unpublished modifications compared to TNS9.<sup>60,71</sup> A preclinical study testing TNS9.3.55 in patient HSPCs in vitro, by BFU-E assays, and, in vivo, using NOD-scid IL2r<sup>null</sup> mice, indicated high vector-derived expression (73% to 100% of normal hemizygous levels) and long-term repopulation potential (69% retention after 7 months) for vector-positive cells.<sup>42</sup> In the ongoing clinical trial, five patients have been enrolled and three treated to date, using G-CSF-mobilized CD34<sup>+</sup> cells and mild conditioning (8 mg/kg busulfan). Possibly owing to the latter, which reduces the risk for patients but also the level of donor chimerism and thus the overall efficiency of the approach, transfusion independence had not been reached 12 months after treatment in the first three patients, albeit with an ongoing rise of the average VCN in peripheral blood mononuclear cells (from, initially, 1% to 7%–9%) and without the emergence of clonal dominance. As of this writing, treatment of additional patients has been postponed until fuller evaluation of the first three patients can indicate whether dose escalation of the conditioning treatment might be required.

## Transgene-mediated activation of endogenous $\gamma$ -globin genes

Inspired by chemical induction of HbF as a therapeutic approach,<sup>102–109</sup> and enabled by the burgeoning fields of engineered transcription factors and RNA interference,<sup>23,32</sup> a relatively novel approach to the therapy of  $\beta$ -thalassemia is the transgenic activation of  $\gamma$ -globin, either by the overexpression of  $\gamma$ -globin-activating transcription factors or by the stable knockdown of  $\gamma$ -globin repressors.

## Overexpression of $\gamma$ -globin-activating transcription factors

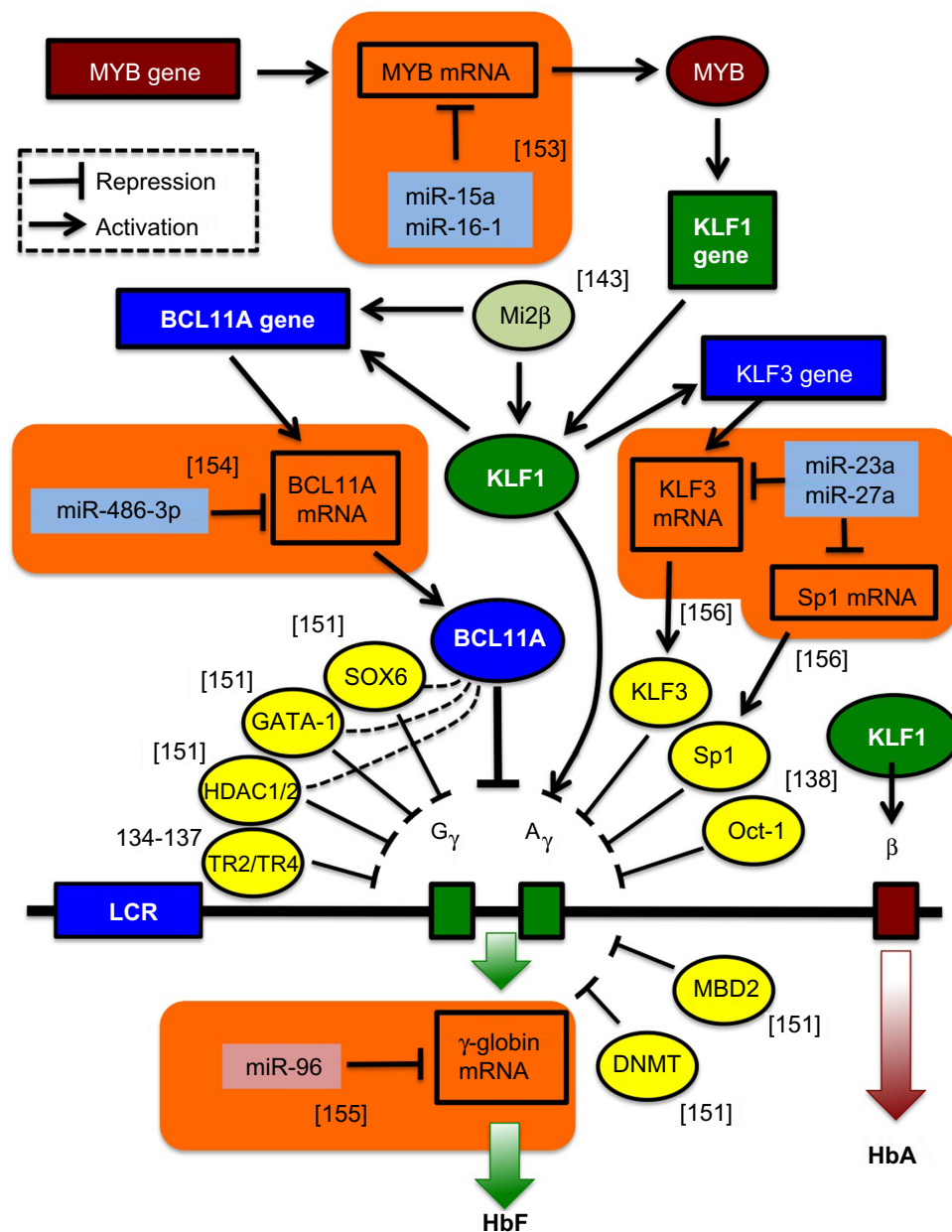
The  $\beta$ -type globin genes are activated through dynamic interactions with a distal upstream enhancer, the LCR. The LCR physically contacts the developmental stage-appropriate globin gene via chromatin looping, a process partially dependent on the protein Ldb1. Deng et al showed that tethering Ldb1 to the murine  $\beta$ -globin promoter with a custom-designed zinc finger protein (ZF-Ldb1) can induce loop formation and  $\beta$ -globin transcription in an erythroid cell line.<sup>120</sup> Further work using a similar approach showed that forced chromatin looping can be exploited to potently reactivate fetal globin gene expression in adult human erythroid

cells.<sup>121</sup> For this work, a fusion protein that brings together a zinc finger protein, which recognizes a specific sequence at the  $\gamma$ -globin promoter, and Ldb1 was created.<sup>120,122</sup> Insertion of a lentiviral vector carrying this fusion protein into adult primary human erythroid cells strongly activated the  $\gamma$ -globin gene, whose transcription accounted for nearly 90% of total  $\beta$ -like globins and led to concomitant reduction of  $\beta$ -globin. This approach would therefore be particularly suitable for the therapy of SCA, by increasing anti-sickling  $\gamma$ -globin, while at the same time reducing  $\beta^S$  expression (see also<sup>97</sup>).

Alternatively, engineered zinc-finger-based transcription factors can be used to reactivate developmentally silenced  $\gamma$ -globin genes in adult cells. Figure 3B shows the structure of a lentiviral vector expressing the artificial zinc finger protein GG1-VP64, which was designed to interact with the –117 region of the A $\gamma$ -globin gene proximal promoter and led to a significant increase in  $\gamma$ -globin gene expression in K562 cells.<sup>123</sup> Moreover, Wilber et al<sup>124</sup> and Costa et al<sup>64</sup> reported increased  $\gamma$ -globin gene expression following transfection with GG1-VP64 constructs, with significantly increased HbF levels in CD34<sup>+</sup> erythroid progenitor cells from normal human donors and  $\beta$ -thalassemia patients. These results provide new insights into the mechanism of  $\gamma$ -globin silencing and may translate into mechanism-based, improved therapies for  $\beta$ -thalassemia and related SCA.

## Transgene-mediated silencing of $\beta$ -thalassemia modifiers

With the advent of the concept of RNA interference, efforts began to utilize short interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) in the therapy of  $\beta$ -thalassemia. Early efforts, mindful of the amelioration of  $\beta$ -thalassemia pathology by a reduction in  $\alpha$ -globin excess, knocked down  $\alpha$ -globin mRNA to achieve a moderate but significant reduction in disease parameters,<sup>34,125–127</sup> an approach superior to the alternative strategy of sequestering excess  $\alpha$ -globin protein by overexpression of its private chaperone, AHSP.<sup>128</sup> Recently, regulation by RNA interference has also become an option for the activation of endogenous  $\gamma$ -globin expression. This was made possible through a growing understanding of the regulation of globin switching, also by regulatory microRNAs (miRNAs), and of transcriptional repressors of  $\gamma$ -globin as therapeutic targets (see Figure 5).<sup>129–144</sup> Among the candidate target genes for knockdown is the zinc finger transcription factor Krüppel-like factor 1 (KLF1, also known as the erythroid Krüppel-like factor, EKLF), which acts as an erythroid-specific master switch of globin gene expression<sup>145</sup> and whose autonomy in directing globin gene expression is



**Figure 5** Novel targets for  $\gamma$ -globin gene transcriptional activation.

**Notes:** Transcription factors directly or indirectly affecting (either positively or negatively)  $\gamma$ -globin gene transcription are listed, together with known regulatory microRNAs. High expression of miR-15a,<sup>153</sup> miR-16-1-1,<sup>153</sup> miR-486-3p,<sup>155</sup> miR-23a,<sup>154</sup> and miR27a<sup>154</sup> might lead to downregulation of repressors of  $\gamma$ -globin genes (MYB, BCL11A, KLF3, Sp1) and increased HbF production.

**Abbreviation:** LCR, locus control region; HbF, fetal hemoglobin; HbA, adult hemoglobin.

underlined by the observation that the mere insertion of a KLF1 binding site into the human  $\delta$ -globin promoter confers developmental inducibility and a reduction of the thalassemia phenotype in mice.<sup>146</sup> Besides KLF1, Oct-1,<sup>138</sup> MYB,<sup>139</sup> and BCL11A<sup>129,133,140</sup> have been identified as repressors of  $\gamma$ -globin gene transcription. For instance, the zinc finger transcription factor BCL11A has recently been shown to function as a repressor of HbF expression, with transgenic deactivation of BCL11A reactivating HbF and correcting a humanized sickle

Hb mouse model<sup>147</sup> and with BCL11A knockdown leading to significant HbF induction in human cells,<sup>129,133,140,144</sup> similar to knockdown of its positive regulator KLF1.<sup>132</sup> Moreover, compound Klf1::Bcl11a mutant mice that carry the human  $\beta$ -globin locus showed further enhanced  $\gamma$ -globin expression compared to single-mutant animals,<sup>148</sup> indicating that a strategy targeting both genes together (without affecting non-erythroid functions of BCL11A) might have additional therapeutic benefits in  $\beta$ -thalassemia. In order to move

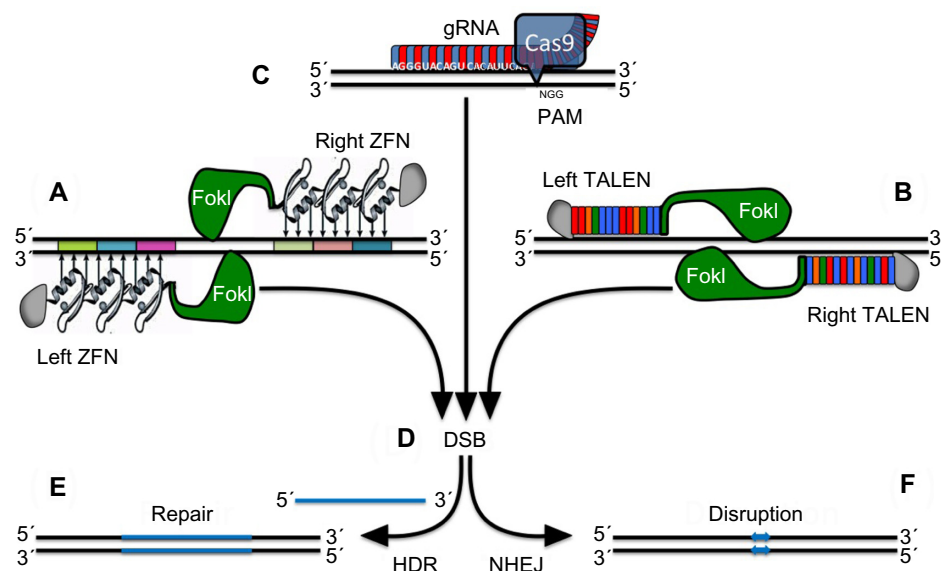
transgene-mediated activation of  $\gamma$ -globin from concept to therapeutic application, shRNA expression from constitutive RNA polymerase III promoters, such as the commonly used U6 promoter (see Figure 3C), needs to be avoided. To this end, Renella et al has surrounded a BCL11A-specific shRNA with the flanking sequences of a naturally occurring miRNA (miR223), allowing its (potentially regulated) expression from RNA polymerase II-driven promoters.<sup>149</sup> Using lentiviral vectors for spleen focus-forming virus (SFFV)-promoter-driven BCL11A shRNAmiR expression in murine erythroleukemia cells, approximately 50% of control embryonic  $\epsilon^{\gamma}$  levels were achieved compared to the equivalent positive U6 shRNA control,<sup>150</sup> so that controlled and stable shRNA-mediated HbF induction has achieved an efficiency of potential clinical relevance. Figure 5, in addition to transcription factors negatively regulating the expression of the  $\gamma$ -globin genes,<sup>151,152</sup> shows examples of miRNAs validated as regulators of  $\gamma$ -globin gene expression,<sup>153–156</sup> either directly<sup>155</sup> or through interactions with relevant target transcription-factor mRNA,<sup>153,154,156</sup> such as miR-15a and miR-16-1 (targeting MYB),<sup>153</sup> miR-23 and miR-27a (targeting KLF3 and Sp1, respectively),<sup>156</sup> and miRNA-486-3p (targeting BCL11A).<sup>154</sup> Lentiviral vectors carrying sequences of these miRNAs are expected to lead to inhibition of  $\gamma$ -globin gene transcription-factor repressors and induction of HbF.<sup>41</sup>

## Genome editing

In contrast to gene-augmentation approaches, the direct DNA-level repair of primary mutations would achieve physiological levels of gene expression for each corrected cell and, in the absence of off-target activity, would altogether avoid the risk of insertional mutagenesis inherent to integrating vectors. Genome-editing approaches, however, still suffer from low efficiencies in HSPCs, which, without selection (eg, of iPSC clones) or enrichment steps, mostly precludes their clinical application for gene therapy. However, Genovese et al<sup>157</sup> have recently achieved high-efficiency targeted DNA replacement in HSPC from controls and patients with X-linked severe combined immunodeficiency, reaching efficiencies of 3%–11% depending on the subpopulation, thus moving homology-directed gene repair of HSPCs into the realm of clinical application.

## Repair of causative mutations

Endogenous genomic loci can be altered efficiently and specifically using engineered zinc finger nucleases (ZFN)<sup>158–163</sup> and transcription activator-like effector nucleases (TALENs), as recently reported by Voit et al for the human globin locus.<sup>164</sup> Moreover, besides ZFN and TALEN, clustered regularly interspaced short palindromic repeats (CRISPR) linked to Cas9 nuclease are now also being investigated for



**Figure 6** Alternative strategies for targeted gene repair.

**Notes:** Schematic representation of (A) ZFNs, (B) TALENs, and (C) a monomeric clustered regularly interspaced short palindromic repeat associated with Cas9 nuclease bound to double-stranded target DNA. (D) The FokI nuclease introduces a nick in the spacer region of the target DNA, necessitating a dimer of two modules to introduce a DSB in the target, while monomeric Cas9 suffices to introduce a DSB preceding its PAM site. Depending on the intended application, the DSB may then be repaired (E) by the template-dependent HDR mechanism (eg, for the repair of a causative mutation) or (F) by error-prone NHEJ (eg, for the disruption of  $\gamma$ -globin repressors or their binding sites). The HDR approach has already been applied by Zou et al<sup>158</sup> and Ma et al<sup>168</sup> to correct the  $\beta$ -globin gene in induced pluripotent stem cells from sickle cell anemia and  $\beta$ -thalassemia patients, respectively.

**Abbreviations:** DSB, double-strand break; gRNA, guide RNA; HDR, homology-directed repair; NHEJ, nonhomologous end joining; PAM, protospacer adjacent motif; TALEN, transcription activator-like effector nuclease; ZFN, zinc finger nuclease.

their utility in modifying  $\beta$ -globin.<sup>165,166</sup> ZFN (Figure 6A), TALEN (Figure 6B), and CRISPR (Figure 6C) comprise a specifically engineered DNA binding domain fused to a nuclease.<sup>31</sup> Binding of a ZFN or TALEN pair at contiguous sequences flanking a target site leads to the dimerization of the FokI (a double-stranded DNA nickase) domain, resulting in a targeted DNA double-strand break, while CRISPR/Cas9 introduces double-strand breaks as a monomer.<sup>31</sup> To increase target-site specificity and thus reduce off-target activity, CRISPR linked to nickases and thus requiring dimerization for genome modification are also being investigated.<sup>167</sup> The resulting double-strand break (Figure 6D) can be repaired by error-prone nonhomologous end joining or by high-fidelity homology-directed repair in the presence of a homologous DNA donor template (Figure 6E and F). In their study, Voit et al engineered a pair of highly active TALENs that induce modification of about 50% of human  $\beta$ -globin alleles near the site of the sickle mutation. These TALENs stimulate targeted integration of therapeutic, full-length  $\beta$ -globin complementary DNA to the endogenous  $\beta$ -globin locus in about 20% of K562 erythroleukemia cells.<sup>164</sup>

Using patient-specific iPSCs, Ma et al<sup>168</sup> have recently applied this technology to  $\beta$ -thalassemia, with Sun and Zhao<sup>169</sup> likewise applying it to SCA patient-specific iPSCs, both groups following the idea that correction of disease-causing mutations offers an ideal therapeutic solution when iPSCs are available. In the  $\beta$ -thalassemia study, Ma et al described a robust process combining efficient generation of integration-free patient-specific  $\beta$ -thalassemia iPSCs and TALEN-based universal correction of *HBB* mutations in situ. Integration-free and gene-corrected iPSC lines from two patients carrying different types of homozygous mutations were generated. These iPSCs are pluripotent, have normal karyotype, and, more importantly, can be induced to differentiate into hematopoietic progenitor cells and then further to erythroblasts expressing normal  $\beta$ -globin. Interestingly, and of importance for any clinical application of genome-editing tools, the correction process did not generate TALEN-induced off-target mutations.<sup>168</sup>

## HbF activation by genome editing

In contrast to SCA, wherein a single mutation is present in all patients,  $\beta$ -thalassemia is caused by a large variety of mutations, each of which would have to be corrected by an individually validated designer nuclease. The alternative and universally applicable approach of using designer nucleases to induce HbF instead is therefore an attractive option. While results for this strategy as a gene-therapy approach have, to our knowledge, not yet been published in peer-reviewed journals, it is already being employed, as patent applications for

corresponding ZFN and TALEN indicate.<sup>170,171</sup> Intriguingly, and depending on the target (such as a  $\gamma$ -globin repressor or its binding site), this approach might use nonhomologous end joining to disrupt the target sequence in HSPCs and thus achieve high levels of efficiency that would allow a direct translation to clinical applications for  $\beta$ -thalassemia.

## Toward personalized therapy of thalassemia

With hundreds of primary mutations, disease modifiers, and polymorphisms linked to hereditary persistence of HbF,<sup>1</sup>  $\beta$ -thalassemia patients can be stratified into clinically distinct subgroups. It is expected, therefore, that the management of  $\beta$ -thalassemia patients will increasingly be customized for stratified classes of  $\beta$ -thalassemia patients, which will also hold for intervention by gene therapy. For instance, with the objective of reaching therapeutic levels of hemoglobins, an optimized gene-therapy protocol might differ between patients with  $\beta^0$  genotypes (without endogenous  $\beta$ -globin expression, such as  $\beta^0\beta^0$  and  $\beta^0$ -IVSI-1 homozygotes or compound heterozygotes) and those with  $\beta^+$  genotypes (with residual  $\beta$ -globin expression, such as  $\beta^+$ -IVSI-110 and  $\beta^+$ -IVSI-6 homozygotes or compound heterozygotes). In this respect, the response of patients with compound heterozygote  $\beta^0/\beta^+$  genotypes to exogenous  $\beta$ -globin expression might need careful study. Moreover, in the case of mutation-specific genome editing, considerations of personalization are inherent in the approach, while, for other approaches, these considerations might be less obvious but similarly critical. For instance, the efficiency of gene therapy based on exogenous  $\gamma$ -globin gene expression or on the activation of endogenous  $\gamma$ -globin by any of the means discussed above may be in doubt in cases where the patients involved are already expressing high endogenous levels of HbF. These considerations also hold for the combination of gene therapy and pharmacological induction of HbF detailed in the section titled "Combination therapy of gene addition with HbF inducers", because the individual genetic composition is an important cause of variations in the response and tolerance to drug treatment, as recently reviewed.<sup>30</sup> Pharmacogenomic-based studies have clearly demonstrated that several genomic variations (not restricted to the human  $\beta$ -globin gene cluster) are significantly associated with differential responses of  $\beta$ -globinopathy patients to treatment with chemical HbF inducers, such as hydroxyurea.<sup>172</sup> This insight renders the use of genomic/transcriptomic analysis to predict the in vivo response and to guide the personalization of any such therapy a logical conclusion.<sup>111,112</sup> With the same rationale, the analysis of patient-specific responses in cell culture before

therapy, and, in particular, before permanent therapeutic intervention, is strongly recommended<sup>83</sup> and will become increasingly common. This trend, combined with the ex vivo approach used for the therapy itself and with an increasing use and creation of patient-specific iPSCs (in particular for gene-correction approaches), is expected to lead to a dramatic increase in biobanking of patient-derived cells, with all the regulatory, management, and ethical issues involved.<sup>173</sup>

## Conclusion

In summary, gene therapy is one of the most promising approaches for the future treatment of  $\beta$ -thalassemia patients and comprises several, at times complementary, strategies. The clinically most advanced approach, that of substituting nonfunctional endogenous  $\beta$ -globin genes with a normal  $\beta$ -globin gene carried by lentiviral vectors, leads to de novo production of HbA. This approach can be enhanced, as in vitro evidence indicates, by additional treatment with inducers of endogenous HbF, which is firmly established as clinically beneficial. In the same vein, numerous gene-therapy approaches also draw on HbF as a positive disease modifier, either by expressing exogenous HbF from a lentiviral vector or by inducing endogenous HbF with a variety of approaches, including the expression of exogenous artificial transcription factors or the disruption of  $\gamma$ -globin repressors or their binding sites. This latter approach has been made possible by an increasingly detailed understanding of globin gene regulation and by the development of rationally designed artificial nucleases for genome editing. Designer nucleases in turn now also allow gene editing of the human globin locus and thus the correction of altered  $\beta$ -globin genes as the most direct gene-therapy approach. As for the cellular targets of gene therapy, human erythropoietic stem cells have been considered in most studies and are still the substrate of choice for clinical applications. However, it can be expected that iPSCs from  $\beta$ -thalassemia patients will play an increasing role in preclinical, and possibly clinical, gene-therapy studies in the future.

As a result of all these developments, and after decades in the making, gene therapy of  $\beta$ -thalassemia has reached a critical phase and is beginning to live up to its long-held promise. At this privileged moment in time, the model systems and protocols are in place to test gene-therapy approaches, and the first clinical trials show therapeutic efficiency and guide our decisions for future developments, such as the choice of conditioning regimen (full or mild), the HSPC source (bone-marrow-derived or mobilized), and the inclusion of insulators for gene augmentation. Ongoing optimization of extant

gene-augmentation tools and combinatorial approaches with chemical reagents are approaching therapeutic efficiency, even for severe forms of the disease. At the same time, fundamental insights into globin switching and new tools for cellular reprogramming, transcriptional regulation, post-transcriptional silencing, and genome editing have opened up as-yet uncharted territory in what has become a fast-moving and highly competitive field of research. While there is no telling which approach will win out for widespread clinical application in the course of time, vigilance, widespread competence in shared methodology, and the availability of diametrically different treatment strategies will provide the pressure and scope for fast improving efficacy and safety, for the good of the field and for the benefit of the patients.

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## Disclosure

The authors report no conflicts of interest in this work.

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