EXPERT OPINION

Recent trends for novel options in experimental biological therapy of β-thalassemia

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Introduction: β-thalassemias are caused by nearly 300 mutations of the β-globin gene, leading to low or absent production of adult hemoglobin. Achievements have been recently obtained on innovative therapeutic strategies for β-thalassemias, based on studies focusing on the transcriptional regulation of the γ-globin genes, epigenetic mechanisms governing erythroid differentiation, gene therapy and genetic correction of the mutations.
Areas covered: The objective of this review is to describe recently published approaches (the review covers the years 2011 – 2014) useful for the development of novel therapeutic strategies for the treatment of β-thalassemia.
Expert opinion: Modification of β-globin gene expression in β-thalassemia cells was achieved by gene therapy (eventually in combination with induction of fetal hemoglobin [HbF]) and correction of the mutated β-globin gene. Based on recent areas of progress in understanding the control of γ-globin gene expression, novel strategies for inducing HbF have been proposed. Furthermore, the identification of microRNAs involved in erythroid differentiation and HbF production opens novel options for developing therapeutic approaches for β-thalassemia and sickle-cell anemia.

Keywords: fetal hemoglobin induction, gene therapy, induced pluripotent cells, microRNA, thalassemia, transcription factors

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1. Introduction

β-thalassemias are relevant hereditary hematological diseases caused by nearly 300 mutations of the β-globin gene [1-3]. In β-thalassemia patients, the mutations of the β-globin gene lead to low or absent production of adult β-globin and excess of α-globin content in erythroid cells, causing ineffective erythropoiesis and low or absent production of adult hemoglobin (HbA) [4,5]. Background information on β-thalassemia is available in excellent reviews outlining the genetics [1,3,6], physiopathology [4,7,8] and therapeutics [9-18] of this disease [2-9]. Together with sickle cell anemia (SCA), thalassemia syndromes are the most important problems in developing countries, in which the lack of genetic counseling and prenatal diagnosis has contributed to the maintenance of a very high frequency of these genetic diseases in the population [19].

Presently, clinical management of β-thalassemia patients includes lifelong blood transfusions associated with chelation therapy to remove the excess transfused iron [13-15] and, in some cases, bone marrow transplantation [16-18]. However, considering limitations and side effects of the currently available therapeutic approaches and management of the thalassemia patients, novel alternative options for therapy are needed [11,12,19]. We have recently reviewed the available literature concerning the development of DNA-based therapeutic strategies for β-thalassemia [19]. The objective of the present review is to comparatively evaluate the most relevant

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Article highlights.

- β-thalassemias are a group of hereditary human diseases caused by nearly 300 mutations of the human β-globin gene, causing low or absent production of adult hemoglobin (HbA).
- Modification of the β-globin gene expression in β-thalassemia cells can be achieved by gene therapy, correction of the mutated β-globin gene, and RNA repair.
- DNA-based targeting of α-globin gene expression has been also demonstrated to reduce the excess of α-globin production by β-thalassemia cells, one of the major causes of the clinical phenotype.
- Therapeutically relevant production of fetal hemoglobin (HbF), beneficial for β-thalassemia, has been achieved with DNA-based approaches.
- HbF can be induced following treatment of the cells with zinc finger artificial promoters.
- HbF can be induced with miRNAs targeting mRNA coding transcriptional repressor of γ-globin genes.
- microRNAs are involved in regulation of γ-globin genes and HbF production and are expected to be novel important targets for molecular interventions.

This box summarizes key points contained in the article.

findings published in the period 2011 – 2014 and outlining key areas of progress in molecule-based (including DNA, RNA and engineered proteins) experimental approaches for β-thalassemia. The different types of molecule-based therapies described are targeted at the thalassemia mutation itself (at the DNA or other levels) and/or at modulating the expression of the α-globin gene, in order to minimize α/β chain imbalance, which damages the erythroid cells [4,7,8]; moreover, the described therapeutic strategies include those aimed at increasing fetal hemoglobin (HbF) production, which can satisfactorily compensate for decreased or absent β-globin production [11,12,20]. In fact, as reviewed by Fibach and Gambari [11], by Bianchi *et al.* [20] and by Testa *et al.* [12], high level of HbF production is beneficial to the β-thalassemia patients and, ultimately, might abrogate the need for transfusions by replacing the lacking biological activity of HbA with HbF, as verified in the case of Hereditary Persistence of HbF and hydroxyurea (HU)-treated patients [11,12,20].

2. Novel therapeutic approaches to repair the defective β-globin genes: nuclease-mediated gene editing by homologous recombination of the human globin locus

Endogenous genomic loci can be altered efficiently and specifically using engineered zinc finger nucleases (ZFNs) [21-23] and Tal-effector nucleases (TALENs) [24,25], as recently reported by Voit *et al.* [26]. ZFNs and TALENs are composed of a specifically engineered DNA-binding domain fused to the FokI endonuclease domain as shown in **Figure 1** [27-33]. Binding of a pair of ZFNs or TALENs to contiguous sites leads to the dimerization of the FokI domain, resulting in a targeted DNA double-strand break. Repair of the break can proceed by mutagenic nonhomologous end joining or by high-fidelity homologous recombination with a homologous DNA donor template.

Compared to ZFNs, TALENs seem to cause lower levels of cytotoxicity [26,34]. In their study, Voit *et al.* engineered a pair of highly active TALENs that induce modification of about 50% of human β-globin alleles near the site of the sickle mutation. These TALENs stimulated targeted integration of therapeutic, full-length β-globin cDNA to the endogenous β-globin locus in about 20% of cells [26].

Ma *et al.* have recently applied this technology to patient-specific induced pluripotent stem cells (β-Thal iPSCs) [35], following the idea that correction of the disease-causing mutations offers an ideal therapeutic solution when iPSCs are available [36,37]. In this study they have described a robust process combining efficient generation of integration-free β-Thal iPSCs from the cells of patients and TALEN-based universal correction of hemoglobin beta chain (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 β-globin. Interestingly, the correction process did not generate TALEN-induced off-targeting mutations [35].

3. Enhancing the potential benefits of gene therapy with HbF inducers

The gene therapy approach for β-thalassemia has been the subject of several studies and reviews [38-46] and its extensive description is not a major aim of the present review. However, it should be mentioned that the efforts of the groups working in this field have been dedicated to the following major issues: i) highly efficient and stable transduction; ii) targeting of the therapeutic vector to hematopoietic stem cells (HSCs); iii) control of transgene expression (erythroid-specific, differentiation and stage-restricted, elevated, position-independent, and sustained over time); iv) low or absent genomic toxicity; v) selection of the transduced HSC *in vivo*; vi) correction of the β-thalassemia phenotype in preclinical models, including transgenic mice; and vii) application of the knowledge on gene therapy for corrections of β-globin gene expression in iPSCs generated from adult cells of β-thalassemia patients [12,22,39-48].

Furthermore, experimental trials based on gene therapy were recently directed also on possible reactivation of the γ-globin genes. This is particularly attractive for SCA in consideration of the anti-sicking activity of γ-globin. Wilber *et al.* forced the production of HbF after gene delivery into CD34 (+) cells obtained from mobilized peripheral blood of normal adults or steady-state bone marrow from patients with

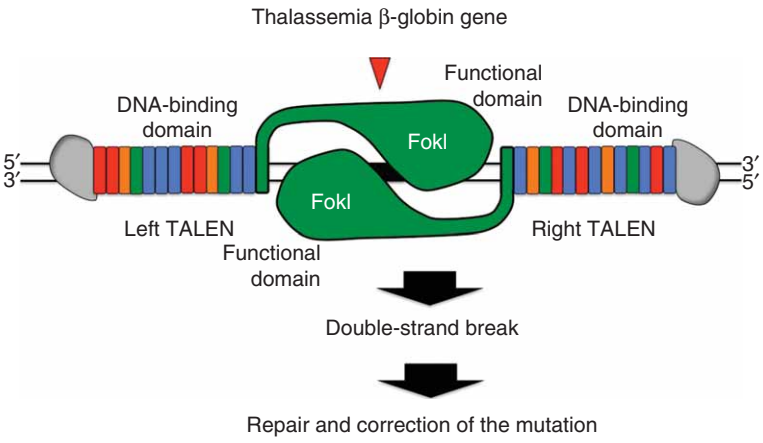

Figure 1. Schematic representation of TALEN bound to double-stranded DNA of the β-globin gene. The mutation to be corrected is shown by the arrowhead. Each TALEN unit is designed to recognize one single nucleotide. After binding of both TALEN modules (left and right modules) to DNA, the FokI endonuclease dimerizes and cleaves DNA at the spacer region, facilitating homologous recombination and gene correction. This approach has been used by Sun *et al*. [25] and by Voit *et al*. [26].

TALEN: Transcription activator-like effector nucleases.

β-thalassemia major [38]. Lentiviral vectors encoding: i) a human γ-globin gene with or without an insulator; ii) a synthetic zinc-finger transcription factor (TF) designed to interact with the γ-globin gene promoters; and iii) a short-hairpin RNA targeting the γ-globin gene repressor, B-cell lymphoma/leukemia 11A (BCL11A) were tested. The obtained results suggest that both lentiviral-mediated γ-globin gene addition and genetic reactivation of endogenous γ-globin genes have potential to provide therapeutic HbF levels to patients with β-globin deficiency [49-53].

Accordingly, combined strategies based on gene therapy for *de novo* β-globin production together with induction of HbF have been undertaken [54] and recently reviewed [55]. As expected, the combined treatment induces both increase of HbA (due to the gene therapy intervention) and HbF (due to the exposure to γ-globin gene inducers). This might be of interest, since, as well established, an increase of β-globin gene expression in β-thalassemia cells can be reproducibly obtained by gene therapy, but several limitations are present in achieving homogeneous gene transfer in HSCs and clinically relevant levels of HbA [56-58]. In fact, the potential genome toxicity associated with the random integration of the current gene transfer vectors suggests the use of low infectious protocols based on the use of therapeutic lentiviral vectors [55-58].

On the other hand, as already mentioned, an increased production of HbF is beneficial in β-thalassemia. Accordingly, the combination of gene therapy and HbF induction appears to be a pertinent strategy to achieve clinically relevant results. Specifically, this strategy allows reaching high levels of functional hemoglobin in β-thalassemic cells, together with a sharp decrease of the excess of α-globin. It should be underlined that even in the absence of a direct correction of the β-globin gene defect or in the lack of induction of HbF, the clinical parameters of β-thalassemia cells can be ameliorated by simply reducing the excess of α-globin production by the cells. Excess α-globin precipitates in erythroid progenitor cells resulting in cell death, ineffective erythropoiesis and severe anemia. Decreased α-globin synthesis leads to milder symptoms, exemplified in several pilot experiments [59-63].

4. Targeting α-globin gene expression

Within this context is the recent work by Voon *et al.*, aimed at the investigation of the feasibility of utilizing short-interfering RNA (siRNA) to mediate reductions in α-globin gene expression [60]. After comparative testing of several siRNA sequences targeting murine α-globin mRNA, one highly effective siRNA sequence (si-α 4) was identified and found to be able to reduce α-globin by ~ 65% at both RNA and protein levels. Electroporation of si-α 4 into murine thalassemic primary erythroid cultures restored α:β-globin ratios to balanced wild-type levels and resulted in detectable phenotypic correction, indicating that siRNA-mediated reduction of α-globin has potential therapeutic applications in the treatment of β-thalassemia, as also pointed out by Xie *et al.* [61].

5. Induction of HbF by synthetic γ-globin zinc finger activators

5.1 The proof of concept: HbF induction with hydroxyurea and other low-molecular-weight compounds

Induction of HbF is one of the most appealing therapeutic strategies for β-thalassemia and SCA, as reviewed in several recent papers [64-72]. Most of the recent findings in this field

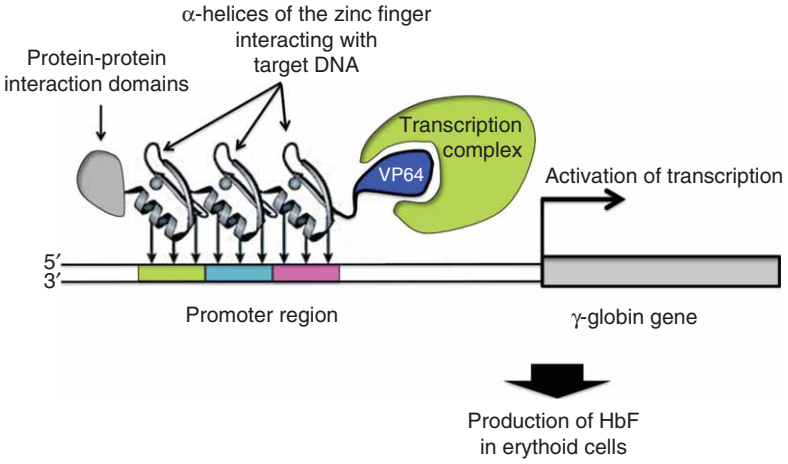

Figure 2. ZF can be engineered to bind desired sequences of DNA and, when fused to transcriptional effector domains, can act as artificial transcription factors that regulate the expression of target genes. Furthermore, additional components can be added to either end of the zinc finger, including protein–protein interaction domains (for recruiting other proteins) and chromatin remodeling proteins, to enable more complex forms of gene regulation. This approach has been applied to the control of γ-globin gene expression by Gräslund *et al*. [85], Wilber *et al*. [38,86] and Costa *et al*. [87].
ZF: Zinc fingers.

of investigation have been focused on small-molecular-weight drugs as HbF inducers [65,66,68,71]. Of great interest is the demonstration that the level of γ-globin mRNA and *in vitro* induction studied on primary erythroid precursors isolated from β-thalassemia patients is predictive of HU response *in vivo* [73,74]. Despite the fact that HU might exert activity also on other factors, such as changes in the RBC cytoskeleton and numbers of WBC and platelets [75-78], the strategies described in this review, aimed at specifically increasing HbF production, might be combined with HU treatment in HU-responsive cells in order to achieve higher therapeutic levels of HbF production.

5.2 HbF induction using artificial promoters

Recent studies have also confirmed selective γ-globin gene activation following approaches targeting the transcription machinery. In this specific field of investigation, the use of synthetic zinc-finger transcriptional activators (ZF-TF) [79-81] or transcription activator-like effectors (TALE-TF) [82-84] is of great interest, since these TFs (either activators or repressors) can be forced to bind the promoter of interest. In fact, both ZF and TALE can be designed to interact with a specific DNA sequence; if the carried TF is a transactivator, this might lead to a strong activation of gene expression [79-84]. Data from studies in cell lines indicated that synthetic activators targeted to the proximal promoter of the A γ-globin gene have successfully induced γ-globin gene expression [38,85-87]. The outline of this very interesting experimental strategy is summarized in **Figure 2** and it is based on bringing a transactivator protein (or domain) at the level of the γ-globin gene promoter in order to induce a specific transcriptional upregulation. Among the very first examples is the work published by Gräslund *et al.*, who reported data of great interest for HbF induction in β-thalassemia. In this study, the artificial ZF gg1-VP64 was designed to interact with the -117 region of the A γ-globin gene proximal promoter, leading to significant increase in γ-globin gene expression in K562 cells [85]. Despite the fact that K562 cell lines express mostly the embryonic hemoglobin Hb Gower 1 ($\zeta_2\varepsilon_2$) and Hb Portland ($\zeta_2\gamma_2$) [11,12], these findings support the concept that HbF should increase following this strategy in other erythroid cell systems. Interestingly, increased γ-globin gene expression was also observed following transfection of the gg1-VP64 construct into immortalized bone marrow cells isolated from human β-globin locus yeast artificial chromosome (β-YAC) transgenic mice [38]. Finally, the proof of concept of a strict relationship between increased γ-globin gene expression and HbF was published by Costa *et al.*, who reported that the gg1-VP64 activator significantly increased HbF levels in CD34+ erythroid progenitor cells from normal human donors and β-thalassemia patients [87].

6. HbF induction by targeting TFs negatively regulating γ-globin gene expression

Several publications that appeared in the last 3 years have confirmed that the γ-globin gene expression is under a strong negative transcriptional control [88-104]. Apart from the theoretical importance, this conclusion indicates the potential therapeutic use of targeting these TFs to treat hemoglobinopathies [90-94]. The ZF TF BCL11A was recently shown to function as a repressor of HbF expression [89,93,94]. When erythroid Kruppel-like factor 1 (KLF1), an adult β-globin gene-specific ZF TF, was knocked down in erythroid progenitor CD34+

cells, γ-globin expression was induced [88,103]. In another set of studies, direct repeat (DR) erythroid definitive factor was shown to be a repressor complex that binds to the DR elements in the ε- and γ-globin gene promoter. Two of the components in this complex are the orphan nuclear receptors TR2 and TR4 [95-97]. Enforced expression of TR2/TR4 increased fetal γ-globin gene expression in adult erythroid cells from β-YAC transgenic mice [97] and also in adult erythroid cells from the humanized SCD mice [98]. These studies clearly demonstrate that manipulation of TFs efficiently reactivates γ-globin gene expression during adult definitive erythropoiesis.

The issue of the relationship between γ-globin gene repressors and levels of HbF in erythroid cells was also the subject of a recent review paper by Thein and Menzel [90], reporting the progress in the understanding of the persistence of HbF in adults. Three major loci (Xmn1-hemoglobin gamma chain (HBG2) single-nucleotide polymorphism, HBS1L-myb proto-oncogene protein (MYB) intergenic region on chromosome 6q, and BCL11A) contribute to high HbF production. As far as the intergenic region HBS1L-MYB it was recently found by Stadhouders *et al.* [105] that several HBS1L-MYB intergenic variants affect regulatory elements that are occupied by key erythroid TFs within this region. These elements interact with MYB, a critical regulator of erythroid development and HbF levels. They found that several HBS1L-MYB intergenic variants reduce TF binding, affecting long-range interactions with MYB and MYB expression levels. These data provide a functional explanation for the genetic association of HBS1L-MYB intergenic polymorphisms with human erythroid traits and HbF levels. In conclusion, according with the review by Thein and Menzel [90], and in agreement with several additional studies, putative repressors of γ-globin gene transcription are Oct-1 [99], MYB [100] and BCL11A [89,93,94,101,102].

In agreement with these conclusions is the study by Borg *et al.*, who performed a pharmacogenomic analysis of the effects of HU on HbF production in a collection of Hellenic β-thalassemia and SCA compound heterozygotes and in a collection of healthy and KLF1-haploinsufficient Maltese adults, to identify genomic signatures that follow high HbF patterns. According with their results, KLF10 emerged as a top candidate for important predictive information, that is, the responsiveness to HbF-inducing treatment, constituting a pharmacogenomic marker to discriminate between response and nonresponse to HU treatment [104].

As a final comment, the list of putative repressors (and co-repressor) of γ-globin gene transcription is expected to increase in the near future, especially when these proteins are studied in the context of chromatin-modifying complexes, including MBD2-NuRD and GATA-1/FOG-1/NuRD complexes, which are known to play a role in γ-globin gene silencing [106]. In a recent study, Amaya *et al.* demonstrated that Mi2β is a critical component of NuRD complexes. They observed that knockdown of Mi2β relieves γ-globin gene silencing in CD34(+) progenitor-derived human primary adult erythroid cells. Furthermore, Mi2β binds directly to and positively regulates both the KLF1 and BCL11A genes, which encode TFs critical for γ-globin gene silencing during β-type globin gene switching. Remarkably, even a partial knockdown of Mi2β is sufficient to significantly induce γ-globin gene expression without disrupting erythroid differentiation of primary human CD34(+) progenitors. These results indicate that Mi2β is a potential target for therapeutic induction of HbF [106].

The research on transcriptional repressors of γ-globin genes is of great interest, since several approaches can lead to pharmacologically mediated inhibition of the expression of γ-globin gene repressors, leading to γ-globin gene activation. Among these strategies, we underline direct targeting of the TFs by aptamers [107] or decoy molecules [99], as well as inhibition of the mRNA coding γ-globin gene repressors with antisense molecules [19,89], peptide nucleic acids [108] and short hairpin RNAs [38].

Furthermore, the transcriptional regulation of γ-globin gene repressors is becoming an hot issue in the recent years, as demonstrated by the very interesting work by Bauer *et al.*, who found by genome-wide association studies that common genetic variation at BCL11A associated with HbF levels lies in noncoding sequences decorated by an erythroid enhancer chromatin signature. Fine-mapping uncovers a motif-disrupting common variant associated with reduced TF binding, modestly diminished BCL11A expression and elevated HbF. The surrounding sequences function *in vivo* as a developmental stage-specific, lineage-restricted enhancer [109].

7. MicroRNAs, erythroid differentiation and expression of globin genes: microRNA therapeutics for β-thalassemia?

7.1 MicroRNAs

MicroRNAs (miRNAs, miRs) are a family of small noncoding RNAs that regulate gene expression by targeting mRNAs in a sequence-specific manner, inducing translational repression or mRNA degradation [110-113] at the level of the RNA-induced silencing complex [113]. This complex is responsible for the gene silencing. miRNAs have been found deeply involved in the control of erythroid differentiation [114-123].

7.2 miRNAs and HbF production

The number of relevant studies on the possible effects of miRNAs on HbF production by erythroid cells is growing. The first very intriguing observation in this field of investigation was reported by Sankaran *et al.*, who observed that, in human trisomy 13, there is delayed switching and persistence of HbF and elevation of embryonic hemoglobin in newborns [124]. In partial trisomy cases, this trait maps to chromosomal band 13q14; by examining the genes in this region, two miRNAs, miR-15a and miR-16-1, appear as top candidates for the elevated HbF levels. Indeed, increased expression of

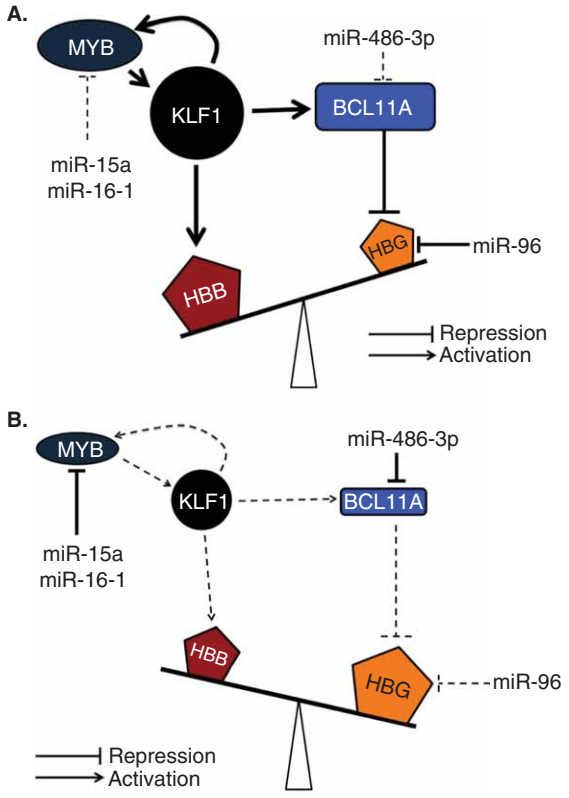

Figure 3. Regulation of γ-globin gene expression by miRNA. Panel A reports a scheme outlining the low expression of miR-15a (mRNA target: MYB), miR-16-1 (mRNA target: MYB) and miR-486-3p (mRNA target: BCL11A) and the high expression of miR-96 (mRNA target: γ-globin) in erythroid cells in which γ-globin gene expression and, consequently HbF (HBG), is downregulated and HbA (HBB) upregulated. In panel B, higher expression of miR-15a, miR-16-1 or miR-486-3p and lower expression of miR-96 might lead to increase of expression of γ-globin gene and, consequently, increase of HbF (HBG). The higher expression of miR-15a, miR-16-1 or miR-486-3p might lead to downregulation of the γ-globin gene repressor network, as reported by Sankaran *et al*. [124], Lulli *et al*. [127] and Ma *et al*. [129].
BCL11A: B-cell lymphoma/leukemia 11A; HBB: Hemoglobin beta chain; HBG: Hemoglobin gamma chain; KLF1, Kruppel-like factor-1; miR: microRNA; MYB: Myb proto-oncogene protein.

these miRNAs in primary human erythroid progenitor cells results in elevated fetal and embryonic hemoglobin gene expression. Moreover, this group showed that MYB mRNA is a direct target of these miRNAs; interestingly, MYB, as already discussed, plays an important role in silencing the fetal and embryonic hemoglobin genes [124].

The list of miRNAs proposed to be involved in downregulation of γ-globin gene repressors and, consequently, in upregulation of γ-globin gene transcription is increased in the last few years (**Figure 3**) [125-130]. For instance, Lulli *et al*. showed that miR-486-3p regulates BCL11A expression by binding to the extra-long isoform of BCL11A 3' untranslated region. Overexpression of miR-486-3p in erythroid cells resulted in reduced BCL11A protein levels, associated to increased expression of γ-globin gene, whereas inhibition of physiological miR-486-3p levels increased BCL11A and, consequently, reduced γ-globin expression [127]. The data obtained indicate that BCL11A, one of the major repressor of γ-globin gene expression, is a molecular target of miR-486-3p; accordingly, pharmacologically mediated upregulation of miR-486-3p might lead to BCL11A downregulation and, consequently, activation of the γ-globin gene expression [127].

If the research in this field of investigation will confirm that miRNAs upregulated in HbF-expressing erythroid cells recognize mRNA-coding TF repressors of γ-globin gene expression, the strategy of design molecules able to mimic activity of those miRNAs for reactivation of the γ-globin genes could be very appealing (as outlined in **Figure 4**). Other miRNAs found upregulated in association with γ-globin gene expression were miR-210 [115,116], miR-26b [130] and miR-451 [118]. On the contrary, an interesting effect on γ-globin gene expression was found for miR-96, which directly binds to γ-globin mRNA, inhibiting γ-globin production; therefore, in this case, molecules able to interfere with miR-96 are expected to play a role in HbF production [126].

In conclusion, the findings that miRNAs are involved in γ-globin anticipate the possibility that their pharmacological alteration might be a key strategy for increasing HbF in erythroid cells.

7.3 miRNAs and α-globin gene expression

Moreover, miRNAs have been found to be involved also in the regulation of α-globin gene. For example, Fu *et al*. found that the erythroid lineage-specific miRNA gene, miR-144, expressed at specific developmental stages during zebrafish embryogenesis, negatively regulates the embryonic α-globin, but not embryonic β-globin gene expression, through physiologically targeting Klfd, an erythroid-specific Krüppel-like TF. Klfd selectively binds to the CACCC boxes in the promoters of both α-globin and miR-144 genes to activate their transcriptions, thus forming a negative feedback circuitry to fine-tune the expression of embryonic α-globin gene. The selective effect of the miR-144-Klfd pathway on globin gene regulation may thereby constitute a novel therapeutic target for improving the clinical outcome of patients with thalassemia [114].

8. Expert opinion

Molecular interventions based on DNA, RNA and engineered proteins appear as the most promising approaches for the development of novel protocols starting from *de novo* induction of HbA and/or HbF to personalized therapy performed on stratified patients [11,12,90]. We have already presented a first review on alternative options for DNA-based therapy of β-thalassemia, underlining that several very interesting and novel approaches are available to the researchers, including modification of β-globin gene expression in β-thalassemia cells,

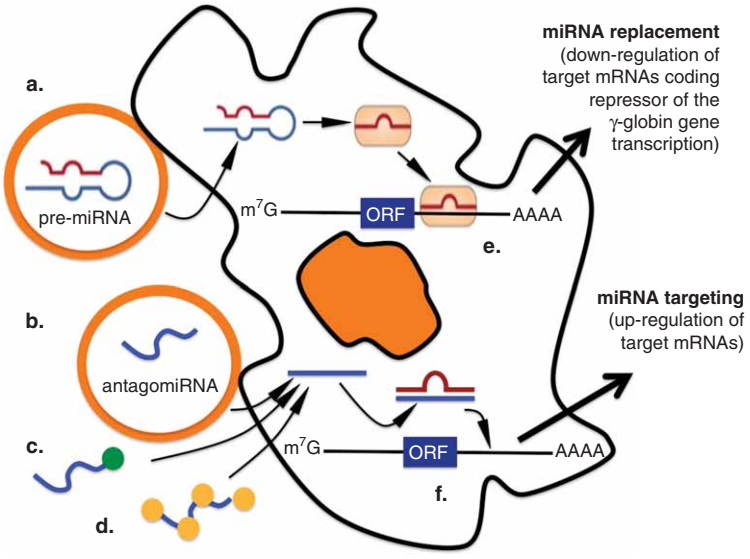

Figure 4. miRNA replacement and miRNA targeting therapy. In miRNA replacement therapy, downregulation of the expression of miRNA target mRNAs is expected. Vice versa, in miRNA targeting, the miRNA targets are expected to be upregulated. In the scheme pre-miRNA (a) or antagomiRNA (b) molecules are delivered to recipient cells using exosome-like or liposomal structures. AntagomiRNAs can be also delivered when functionalized with permeable peptides (c) or when suitably modified within their backbone (d).

miR: microRNA; ORF: Open reading frame.

achieved by gene therapy, correction of the mutated β-globin gene and RNA repair. In addition, reports were reviewed on cellular therapy for β-thalassemia, including reprogramming of somatic cells to generate iPSCs to be genetically corrected [19]. The papers commented in the present review further support new hopes for the future concerning the therapy of β-thalassemia. This pathology is at present treated using blood transfusion regimen and iron-chelation therapy [13,15]. The data here discussed introduce the possibility to design novel therapeutic protocols rendering the β-thalassemia independent from heavy transfusion regimen and chelating therapy.

First of all, recent published papers clearly demonstrate the possibility to predict response to HU of β-thalassemia patients [73,74]. This possibility is of great interest for the patients, as well the clinicians involved in patient management. There is no question on the fact that total (or partial) independence from blood transfusion is the major objective in the field of the management of the β-thalassemia patients, especially in developing countries in which blood is scarcely available and often contaminated. There is a general agreement on the fact that the possibility to stimulate clinically relevant levels of HbF can be useful [8,9,65-72]. Accordingly, HU treatment in HU-responsive cells can be combined with several of the strategies described in this review, in order to achieve higher therapeutic levels of HbF production.

In this respect, while several papers were focused on novel HbF inducers or on clinical trials of already proposed modifiers of γ-globin gene expression [65,70,72], a great interest concerns novel and very exciting studies on dissection of the regulation of γ-globin gene expression in erythroid cells [91,92]. The robust demonstration that γ-globin genes are under a strong negative transcription regulation allows to identify novel targets for HbF-inducting strategies, that is, the transcription repressors BCL11A, MYB and KLF1 (and additional factors belonging to the same family) [94,100-104]. Remarkably, the research is also rapidly moving from γ-globin gene repressors to their upregulators, such as, for example, Mi2β, which was found to directly bind and positively regulate both KLF-1 and BCL11A genes [106]. These last classes of regulatory genes are, of course, promising targets for HbF-inducing therapy.

In this context, we are witnesses of a real burst of information of miRNA-dependent regulation of gene expression leading to miRNA-therapy strategies for most of the human pathologies recognized to be directly or indirectly regulated by miRNA pathways [110-112]. The most recent findings confirm that miRNAs are involved in erythroid differentiation. Of great interest is the finding that several γ-globin gene repressors (for instance, MYB and BCL11A) are targets of different miRNAs, including miR-15a, miR-16-1, miR-486-3p and miR-23a/27a [124,127,129]. Treatment of target erythroid cells with these pre-miRNAs or lentiviral vectors expressing the same miRNAs might lead to downregulation of γ-globin gene repressor with concomitant increase of HbF production. These treatments are expected to be carried out also in combination with HbF inducers in order to achieve the highest effects possible.

In our opinion, this specific field of investigation can be of interest also to research groups studying gene therapy of β-thalassemia finalized to *de novo* β-globin production. In

this case the reach of clinically relevant levels of HbA can be achieved with difficulty, rendering co-treatment with HbF inducers a very appealing approach, as recently discussed [55].

A final comment concerns the development of novel approaches based on the possibility to drive protein functions at the level of specific gene regions. This is achieved using engineered ZF modular DNA-binding domains to bind desired sequences of DNA [79-81,27-29]; alternatively, transcription activator-like effectors (TALEN) can be used to specifically bind to double-stranded DNA [82-85,30,31]. This approach allows the driving of transcription complexes at the level of the γ-globin gene promoter for inducing γ-globin gene transcription (as schematically represented in **Figure 1**) [87], or the driving of nucleases at the level of β-globin gene mutations to be corrected by increasing homologous recombination [26].

This specific field of investigation is expected to provide novel and more efficient strategies to correct the gene defect in erythroid cells, also in the case of iPSCs isolated from β-thalassemia patients.

The issue of management of β-thalassemia patients in developing countries is becoming a key issue in consideration of the very high costs of these patients for the local health system on one hand, and the high costs of the therapeutic protocols (with special consideration of the costs of the chelating drugs). Moreover, β-thalassemia is becoming an important issue also in developed countries, where this disease was not frequent, thanks to genetic counseling and prenatal diagnosis. In these countries, the distribution of carriers and affected people is rapidly increasing in relation to the migration of populations from endemic areas to countries where their prevalence in indigenous populations had been extremely low (USA, Canada, Australia, South America, the United Kingdom, France, Germany, Belgium, the Netherlands and, more recently, Scandinavia). These deep changes have encouraged most of the health systems of these countries in facilitating access to the prevention and treatment services available for these hemoglobin disorders [19].

Declaration of interest

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