Gene Editing-Based Technologies for Beta-hemoglobinopathies Treatment

Ilnaz Rahimmanesh 1,†, Maryam Boshtam 2,†, Shirin Kouhpayeh 3, Hossein Khanahmad 4, Arezou Dabiri 10,*, Ali Zarrabi 9,*, Rajender S. Varma 10,*

Abstract: Beta (β)-thalassemia is a group of human inherited abnormalities caused by various molecular defects, which involves a decrease or cessation in the balanced synthesis of the β-globin chains in hemoglobin structure. Traditional treatment for β-thalassemia major is allogeneic bone marrow transplantation (BMT) from a completely matched donor. The limited number of human leukocyte antigen (HLA)-matched donors, long-term use of immunosuppressive regimen and higher risk of immunological complications have limited the application of this therapeutic approach. Furthermore, despite improvements in transfusion practices and chelation treatment, many lingering challenges have encouraged researchers to develop newer therapeutic strategies such as gene editing. One of the most powerful arms of genetic manipulation is gene editing tools, which have been recently applied to improve β-thalassemia symptoms. Nevertheless, several obstacles, such as off-target effects, protospacer-adjacent motif requirement, efficient gene transfer and expression methods, DNA-damage toxicity, and immunotoxicity issues still need to be addressed in order to improve the safety and efficacy of the gene editing approaches. Hence, additional efforts are needed to address these problems, evaluate the safety of genome editing tools at the clinical level and follow the outcomes of gene editing tools-mediated therapeutic approaches in related patients.

Simple Summary: β-thalassemia syndromes are clinically and genetically heterogeneous blood disorders presented by β-chain deficiency in hemoglobin production. Despite improvements in transfusion practices and chelation treatment, many lingering challenges have encouraged researchers to develop newer therapeutic strategies such as gene editing. One of the most powerful arms of genetic manipulation is gene editing tools, which have been recently applied to improve β-thalassemia symptoms. Nevertheless, several obstacles, such as off-target effects, protospacer-adjacent motif requirement, efficient gene transfer and expression methods, DNA-damage toxicity, and immunotoxicity issues still need to be addressed in order to improve the safety and efficacy of the gene editing approaches. Hence, additional efforts are needed to address these problems, evaluate the safety of genome editing tools at the clinical level and follow the outcomes of gene editing tools-mediated therapeutic approaches in related patients.

Correspondence: l.shariati@amt.mui.ac.ir (L.S.); varma.rajender@epa.gov (R.S.V.)

† These authors contributed equally to this work.

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addition, regulating the transcription factors involved in expression of endogenous γ-globin such as KLF1, silencing of γ-globin inhibitors including BCL11A, SOX6, and LRF/ZBTB7A, and gene repair strategies. In this review article, we present a systematic overview of the appliances of gene editing tools for β-thalassemia treatment and paving the way for patients’ therapy.

**Keywords:** beta-thalassemia; gene therapy; ZFN; TALEN; CRISPR

1. Introduction

β-thalassemia syndromes are clinically and genetically heterogeneous blood disorders presented by β-chain deficiency in hemoglobin production [1]. Phenotypes of β-thalassemia are highly varied, from an asymptomatic disorder to severe anemia, β-thalassemia major or Cooley’s anemia, which causes death before the age of 10 and the only therapeutic approach available is regular transfusion of red blood cells (RBCs) [2,3]. The annual incidence rate of symptomatic β-thalassemia is estimated to be 1 per 100,000 live births in the world. The origins of β-thalassemia were found to be in the Mediterranean, while its major types are mostly seen in the Middle East, Southeast Asia, India, and China. Moreover, increased prevalence of β-thalassemia has been reported in malaria endemic countries. Notably, human migration has contributed to the further spread and establishment of β-thalassemia all around the world [4].

Allogeneic bone marrow transplantation (BMT) from a matched donor is the traditional treatment for β-thalassemia major. However, significant disadvantages of BMT, including the limited HLA-matched donors, the need for a long-term immunosuppressive regimen, the limited application of BMT in young patients, and further immunological side effects, have limited its use [5,6] (Figure 1).

![Figure 1. Current and future therapeutic approaches for β-thalassemia major. Among the various kinds of treatments used for treatment of β-thalassemia patients, nanomedicine and gene therapy are the new emerging ones and have provided new hope in treatment of patients. Their application does not require immunosuppression.](image-url)
The β-thalassemia major management procedure is a combination of lifelong RBC transfusion and chelation therapy to restrict iron deposition, and full observance of this treatment greatly increases the life expectancy of the patients [7]. This therapeutic approach is too expensive in many countries; therefore, novel therapeutics should be introduced in treatment of this disease. The majority of patients with β-thalassemia major die from heart failure due to secondary hemochromatosis following sub-optimal iron chelation, or hepatocarcinoma in older patients [8] (Figure 1).

Other strategies for β-thalassemia major treatment are epigenetic approaches that intervene in the fetal gamma globin reactivation. Increased synthesis of hemoglobin F (HbF) is accompanied by significant decrease in intensity of thalassemia major’s symptoms [9]. There are several chemical compounds inducing gamma globin reactivation including 5-azacytidine as a demethylating agent and derivatives of small-chain fatty acids, such as arginine butyrate, which are more effective when administered with erythropoietin (EPO) [9] (Figure 1). The conventional therapies for β-thalassemia major suffer from a number of adverse impacts, such as neurological complications and dramatically increased levels of platelets, thus encouraging researchers to develop new strategies for β-thalassemia major treatment including nanomedicine and gene editing [10].

Nanomedicine as an appropriate and new treatment with high efficacy for treatment and diagnosis of disease [10–12] can be used in the management of various disorders, particularly blood disease disorders (BDDs) [13]. It switches the conventional drugs/treatments into nano-platforms carrying small therapeutic molecules as newer strategies in disease treatment [14,15]. The application of nanomedicine for curing BDDs was first approved by FDA in 2018. Considering mRNA-based approaches as the first approved techniques for therapy to treat BBDs, and the risk of degradation and cellular uptake of unprotected mRNA, nanomedicine is able to protect and control mRNA function for in vivo application [16]. Direct whole blood transfusion is still the ideal treatment method for the rare BDDs [15], although, short half-life and the possibility of bacterial infection, are referred as the limitations of whole blood storage. Consequently, another possible application of nanomedicines could be synthesis/designing of artificial blood components [15]. For instance, in view of the main role of RBCs as a gas (oxygen and carbon dioxide) transporter, RBC alternatives might be considered as a solution for urgent situations in BDDs and particularly thalassemia treatment. Nanomedicine can imitate the major features of the RBCs by incorporating the functional parts of the molecule into the nanoplatforms [15]. Despite the advantages of nanomedicines, the possible toxicity and complications in size determination, as well as the generalization of the functional modules, also need to be considered [17] (Figure 1).

Gene therapy is an effective one-time treatment method, which does not require immunosuppression and graft versus host disease (GVHD) prophylaxis and can be employed for every patient. Gene therapy is mainly applied for β-thalassemia treatment to attain stable functional globin genes or manipulation of transcription factors regulating gamma chain-expression in the patient’s own hematopoietic stem cells (HSCs) to modify inefficient erythropoiesis and to treat hemolytic anemia [18]. One of the most powerful arms of genetic manipulation is gene editing tools (GETs), which have been recently applied to improve β-thalassemia symptoms. Herein, we deliberate a systematic overview on the applications of GETs for β-thalassemia treatment in recent years.

2. β-Thalassemia: Molecular Basics

β-globin is typically codified by a group of β-like globin genes including ε (HBE), Gγ (HGB2), Aγ (HGB1), δ (HBD), and β (HBB). These genes are located on the chromosome 11(11p 15.15) in order to make various tetramers of Hb such as embryonic Hbs (Hb Gower-1 (ε2ε2), Hb Gower-2 (α2ε2), and Hb Portland (ζ2β2)), fetal Hb (α2γ2), and adult Hbs (HbA, α2β2 and HbA2, α2δ2) [7]. Hemoglobin genes are expressed at distinct growth phases via a hemoglobin switching from embryonic to fetal and finally to adult. Moreover, the globin gene expression including fetal genes relies on vital regulatory regions within the
globin domain, including local promoter sequences and the control region of β-globin locus located at the upstream β-globin containing sites that are hypersensitive to DNase 1. Indeed, some particular erythroid transcription factors including GATA-binding factor 1 (GATA-1) and -2, Kruppel Like Factor 1 (KLF1), Nuclear Factor, Erythroid 2 (NF-E2), Erythroid 2 (NF-E4), Stem Cell Leukemia (SCL), BAF Chromatin Remodeling Complex Subunit BCL11A (BCL11A) and different cofactors such as p300 and Friend of GATA (FOG) bind to the regulatory regions of the globin gene [19]. Furthermore, a number of proteins, including the DRED complex, IKAROS, and GATA-1, have been demonstrated to bind to the -globin promoter region and suppress transcription of fetal genes. BAF Chromatin Remodeling Complex Subunit BCL11A (BCL11A) and SRY (sex determining region Y)-box 6 (SOX6) are also involved in the fetal and embryonic globin genes being silenced. These two proteins are most likely part of a bigger protein complex that also includes the NuRD and GATA-1 corepressor complexes.

There are various molecular mechanisms underlying the β-globin downregulation. Typically, mutations could arise at the early fetal stage, leading to the complete deletion of a globin gene and causing β₀-thalassemia. Based on the level of deletion in the β-chain, various mutations produce β-globin subunits with different reduced expression levels which are classified as β⁺ or β⁺⁺ (“silent”) thalassemia. In fact, the reduced β-globin chain production causes excess unassembled α-globin chains in erythrocyte precursors to aggregate, and further drives the pathophysiology of the disorder [20]. Thus, the severity of the disorder is typically dependent on the balance of α- and β-globin chain production as well as the amount of the free α-chain. Moreover, structurally abnormal β-chain variants following point mutations in the β-globin β-globin gene are extremely unstable and cause a type of β-thalassemia (HbE [β26 Glu→Lys]), known as “thalassemic hemoglobinopathies” [21]. Likewise, some variants of β-globin chain are not capable of generating a stable form of hemoglobin tetramers, resulting in a defect in β-globin function. Notably, these β alleles are rare, with a dominant inheritance pattern leading to severe anemia. In contrast, common types of β-thalassemia are inherited as haploinsufficient Mendelian recessives, where two copies of β-thalassemia alleles are essential for developing the disorder [22].

Most of the β-globin downregulating-related mutations occur in the β-globin locus alleles, which are known as cis-acting regulatory elements, while mutation in trans-acting elements modifies the β-globin gene expression and leads to a phenotype that segregates independent of the β-globin cluster. Nearly 300 alleles of β-thalassemia have been identified to date [23]. Although the majority of the phenotypes in α-thalassemia are related to deleterious mutations in the α-globin gene cluster, the majority of β-thalassemia phenotypes appear by mutations in one or more nucleotides in the β-globin cluster or instant flanks [24]. Figure 2 illustrates the summary of molecular mechanisms underlying β-thalassemia.
Figure 2. Molecular mechanisms underlying β-thalassemia. (a) Role of fetal globin repressors including BCL11A, SOX6 and KLF1 in the expression of γ and β-globin gene: In the fetus, chromatin factor Friend of Prmt1 (FOP) expression is low. Hence, fetal globin repressors including BCL11A, SOX6 and KLF1 did not have any function, and transcription factors such as NF-E4 bind the coding region of the gene and fetal globin (HbF) is synthesized. In adults, expression of FOP is high and fetal globin repressors are activated, bind to the coding site of the gene and β-globin is produced in erythroid progenitors. (b) In β-thalassemia, some mutations cause β-globin gene to downregulate, which are known as cis and trans acting elements, leading to downregulation of β gene expression. Mutations in GATA-1, TFIIH, and KLF1 are known as trans acting regulatory elements, while mutations in alleles of β-globin locus are known as cis acting elements.
3. Gene Editing Tools

Engineered bacterial nucleases and the creation of the programmable nucleases, has made possible editing of genome sequences. These tools include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeat (CRISPR)–Cas-associated nucleases (Figure 3) [25].

![Gene editing tools](image)

**Figure 3.** Gene editing tools. This figure reveals the mechanisms of targeted nucleases. From top to bottom: Meganucleases or homing endonucleases are nuclease enzymes that do not have separate DNA binding and cleavage domains, and recognize a 20–40 bp DNA sequence. Meganucleases may be utilized in all genome types to repair damaged genes in gene therapy by interrupting their DNA substrates as dimers.

3.1. Zinc Finger Nucleases

ZFNs are a group of engineered and chimeric nucleases which were developed by combining a bacterial endonuclease of FokI (a double-stranded DNA nickase,) with DNA-binding zinc finger domains to target and cut a particular 3–4 bp DNA sequence site of genome [26,27]. The ZFNs are used to modify targeted sequence through creation of a double stranded nick on target DNA and induction of an indel mutation for improving the gene function. The ZFNs do not function specifically for the target sequence, and the same genomic sequences off-targets can be affected and modified by the DNA molecule of donor leading to undesired genome modification. However, these systems are capable of elevating the specificity of DNA targeting and attenuating off-target effects using dimerized FokI. [28].
3.2. TALENs

TALENs were initially introduced in 2011 based on the application of transcription activator-like effector (TALE) proteins found in nature (Xanthomonas species). These GEts are artificial proteins including a nonspecific nuclease (FokI) cleavage domain connected to a DNA-recognition TALE region. TALEN activity is associated with two DNA binding sites flanking an undedicated 12–20 bp spacer sequence. Based on spacer length and TALE construction, the DNA cleavage efficiency in TALENs is different.

As with ZFNs, TALENs are chimeric nucleases with a DNA binding and an effector domain. Both the ZFN and TALEN nucleases act as dimers, so a pair of ZFN or TALEN should be developed that is capable of targeting a genomic site [29]. Unlike ZFNs, which are only commercially available, engineered TALENs can be easily synthesized in any molecular biology laboratory; however, cloning of TALEN constructs can be laborious and time-consuming. The main advantages of TALENs over ZFNs are their enhanced specificity and predictability of binding to a particular sequence and cost effectiveness of TALENs. Both ZFNs and TALENs constructions require in vitro verification to demonstrate an adequate level of cleavage efficiency before they can be employed in experiments. Since each gene editing with ZFN or TALEN requires the synthesis of a new engineered nuclease, both techniques are relatively expensive and complicated [30].

3.3. CRISPR

CRISPR is a new GET strategy that was initially introduced with considerable ability for targeted genome engineering [31,32]. CRISPR-associated protein (Cas) is a genetic engineering technique for the correction of genomes of living organisms. This system includes three components, an endonuclease (Cas9), a sequence-specific CRISPR RNA (crRNA), and a trans-activating crRNA (tracrRNA), that links Cas9 with crRNA [33]. The CRISPR-Cas9 system has been simplified by fusion of two RNA sequences into a single 100-nucleotide chimeric RNA called gRNA or sgRNA, which can competently lead Cas9 to recognize and cut foreign DNA strands in a special target site, and a specific array of tandem reiterative elements which is separated with short variable 20 nucleotide sequences. CRISPR technology has advantages over previous systems. The most important advantage is the shorter assembly time and simplicity due to application of artificial sequences which are much easier than protein engineering required in the ZFN and TALEN systems [34]. The major problem concerning CRISPR/Cas9 application is the off-target effects. The specificity of CRISPR/Cas9 system in mammalian cells can be increased with adding two “nickase” CRISPR/Cas9 complexes, which have a specific binding region. Nickase Cas9 is created by mutation in one of the two Cas9 nuclease domains and can cleave only one of the DNA strands. The adjacent DNA site could be targeted by a pair of nCas9s to create a double-strand break (DSB). Both of these nCas9s have been applied to improve the Cas9-based genomic editing specificity [35].

4. HbF or Gamma Globin Induction Using Gene Editing Tools

Based on preclinical studies, HbF re-induction can improve the sickle cell disease (SCD) and β-thalassemia severity. The initial clinical observations have revealed, in the case of natural mutations in the HBB gene cluster or related genes, expression of γ-globin and, subsequently, HbF would modify this deficiency [36].

Moreover, β-thalassemic infants show symptoms after attenuating HbF production. Recently, researchers have verified the effect of high HbF synthesis on the improvement of clinical symptoms of β-thalassemic patients [37–39]. All together, these observations lead to further investigation of HbF inducers with emphasize on the role of naturally higher levels of HbF in β-thalassemia patients [40,41].

The fact is that β-thalassemia and SCD patients suffer from a less severe disorder when HbF is increased; therefore, HbF re-expression could be considered as a beneficial therapeutic approach for these diseases. Some animal and human studies have reported the usage of GETs for HbF elevation [42–44]. Strategies for HbF induction in adult RBCs
deploying GETs can be summarized as inducing natural mutations regarding enhanced HbF level, knocking out repressors of HbF and modifying intermediates of epigenetic to control HbF synthesis [45].

4.1. Targeting the HbF Repressors

Recent advances in our knowledge of β-globin locus regulation at transcriptional level have revealed a strict negative control of γ-globin gene expression, which is mediated by epigenetic modification and different transcription factors [46]. Several experimental and clinical treatments have been used to increase HbF levels by direct targeting of fetal globin repressors including BAF Chromatin Remodeling Complex Subunit BCL11A (BCL11A), SRY-Box Transcription Factor 6 (SOX6), HBS1L-MYB, KLF1 and Zinc Finger and BTB Domain Containing 7A (ZBTB7A). Re-induction of HbF using GETs could possibly delete or reduce the expression of the HbF repressors; however, various types of side effects may occur since transcription factors often have broad spectrum functions to regulate multiple target genes (Figure 4A(1)).

![Figure 4. Molecular mechanism of β-globin repair in beta-hemoglobinopathies patients by genome editing tools.](image)

(A) HbF or gamma globin induction
1) Targeting the HbF repressor
KLF1, Sox6, BCL11A, and ZBTB7A
In erythroid cells: High KLF1, Low KLF1
Erythroid-specific elements

(B) Reproducing HPPF mutation
KLF1
BCL11A
ZBTB7A
NHEJ
HDR/base editing

4.1.1. BCL11A

The genome wide association studies have identified BCL11A as the master repressor of HbF production in both the mouse models and human cells [47]. Erythroid-lineage Bcl11a knockout mouse SCD model showed pancellular HbF induction and phenotypic modi-
fication in the mice with minimal effects on erythropoiesis [48]. Another study revealed that microdeletions in the BCL11A locus of haploinsufficient patients led to considerable neurocognitive phenotypes, in addition to increased HbF level to a therapeutic extent, which modify the pathologic and hematologic deficiencies [49]. In principle, BCL11A knockout through exerting frameshift null alleles in BCL11A coding region could be a feasible treatment approach for these diseases. However, the role of BCL11A in B-cell development and HSC function are identified as the major obstacles, which has limited the application of the BCL11A knockout strategy. As BCL11A plays a critical role in lymphoid and neural development, Bcl11a deletion causes neonatal mortality [50,51]. Moreover, different supportive evidence indicated a lymphopoiesis defect in Bcl11a-knockout HSCs [52]. Based on the findings of a recent study, due to role of BCL11A coding site in the lymphoid lineage synthesis and function of hematopoietic stem cells, its genetic editing could not be considered as an efficient treating approach [53]. Thus, recent strategies have mostly focused on the deletion of BCL11A-binding sequences in the proximity of γ-globin gene or using erythroid-restricted expression of genome-editing components that have been applied for erythroid specific repression of BCL11A [54].

A recent HbF-associated GWAS demonstrated a BCL11A erythroid intronic enhancer comprising three DNase I hypersensitive sites, named +55, +58 and +62 based on the distance from the BCL11A transcription start site in kilobases [54,55]. The deletion of orthologous regions in a mouse erythroid cell line led to the deletion of BCL11A at both the RNA and protein levels, while BCL11A expression was saved in a B-cell line with the same deletion. Deletion in all intronic positions of BCL11A enhancer, but especially in the +58 position demonstrated a significant reduction in BCL11A expression and increase in γ-globin levels in human erythroid cells [54,56]. In a direct comparison between application of ZFNs to disrupt the BCL11A coding region and the erythroid-specific enhancer of BCL11A, it was revealed that the bi-allelic disruption of GATAA motif in BCL11A erythroid enhancer led to increasing levels of fetal globin expression. Simultaneously, targeting the BCL11A enhancer appears to be more tolerated within the erythroid lineage since the residual low levels of BCL11A are insufficient to repress g-globin, while promoting cellular fitness. Therefore, directed deletion of the 12-kb erythroid enhancer of BCL11A could function as an alternate strategy for targeting BCL11A coding sequence [57]. Aiming a 200 bp region within the human erythroid-enhancer of BCL11A (including GATAA motif) by CRISPR/Cas9 showed increased expression of γ-globin in the K562 cell line [58]. The investigational application of CRISPR/Cas9-based gene editing to treat a patient with β-thalassemia and the other patient with SCD has been reported in a clinical trial. In this study, the patient who received the autologous CD34+ cells edited by CRISPR/Cas9 targeting the BCL11A enhancer showed an increased level of HbF that was pancellularly distributed [59]. Recently, during a clinical trial, Franguol et al. administered CTX001 (an autologous CD34+ cells edited by CRISPR/Cas9 targeting the BCL11A enhancer) to a beta-thalassemia patient and a SCD patient and followed them up for 12 months. They succeeded to increase HBF level considerably and continuously in both patients who received CTX001 [60].

Ma et al. examined the effects of plasmid length and structure on electroporation efficiency in HSPC as the primary method used to transfect these cells. As a result, they investigated the use of a minicircle (MC-DNA) vector without a bacterial backbone to supply the CRISPR/SaCas9 tool into HSPCs to reactivate γ-globin expression as a potential therapeutic approach for β-thalassemia patients. They found that the transfection efficiency of CD34+ hematopoietic stem cells depends on plasmid length and linearization. Furthermore, the MC transgene expression without major plasmid sequences was excellent compared to conventional plasmids in vitro and in vivo. In this research, MC DNA was used to deliver the cassette of Staphylococcus aureus Cas9 (SaCas9) into HSPCs, and a single-guide RNA targeting the erythroid enhancer region of BCL11A was chosen. After electroporation with MC-DNA, an apparent efficiency of gene editing and reactivation of γ-globin expression was obtained in unsorted HSPC-derived erythroblasts. The developed
MC-DNA vector offered a potential strategy to deliver SaCas9 cassettes and reactivate γ-globin expression to alleviate β thalassemia syndromes [61].

4.1.2. SOX6

The SOX6 transcription factor plays a critical role in gene switching of β-globin in erythroid cells as well as BCL11A [62]. The first described role for SOX6 in gene regulation of β-globin was documented by the evaluation of the SOX6-knockout mice model. The expression of mice embryonic β-like globins (ε and βh1) was markedly increased at the fetal liver stage of Sox6-knockout mice model, p100H [63]. The silencing effect of BCL11A for the γ-globin gene is suggested to be through long-range cooperation with SOX6. The BCL11A-mediated suppression of γ-globin genes is exerted through both the local interactions with SOX6 chromatin-associated proteins and within the human β-globin gene. Throughout hemoglobin switching, SOX6 interacts with γ-globulin proximal promoters, thus mediates BCL11A recruitment to the γ-genes proximal regions [62]. In adult human erythroid lineage, γ-globin gene expression, which is mediated by stem cell factor, is also related to SOX6 downregulation. Furthermore, CRISPR/Cas9 and ZFN technologies have recently been used to generate a mutation in the binding site of SOX6 for γ-globin gene reactivation. These studies showed that induction of mutation in the binding domain of SOX6 gene gives rise to γ-globin reactivation [9,64]. Altogether, SOX6 appears to function as a potentially promising target for HbF reactivation.

4.1.3. LRF/ZBTB7A

Another transcription factor, LRF/ZBTB7A (Pokemon), has newly been documented as a major repressor of γ-globin gene expression. LRF-knockout mice model exhibits elevated levels of Hbb-bh1 as the embryonic globin while maintaining normal levels of Hbb-γ. This phenomenon is in contrast to the Bcl11a-null mice models, in which both embryonic globins, Hbb-γ and Hbb-bh1, are overexpressed. Zbtb7a-/-mice models are embryonic lethal due to anemia, whereas the conditional knockout of Zbtb7a in adult mice leads to mild macrocytic anemia following inefficient terminal erythropoiesis [48,65]. CRISPR/Cas9-mediated knockout of LRF in an erythroid cell line resulted in significant upregulation of γ-globin gene expression. The LRF/BCL11A double knockout model was indicated to upregulate HbF expression up to of 90%, suggesting that the role of LRF in γ-globin regulation is moderately independent of Bcl11a. Further analysis confirmed a slight delay in erythroid lineage differentiation upon LRF knockdown in primary human CD34 HSPCs differentiated down to the erythroid lineage with a subsequent γ-globin induction [66]. Although the effect of LRF knockdown on γ-globin upregulation is prominent, the LRF role in cell fate decisions in multiple hematopoietic lineages, and specifically for terminal erythropoiesis, can limit the LRF application as a potential therapeutic strategy [67].

4.1.4. KLF1

The KLF1 has long been appreciated, as a main factor in the switching of γ- to β-globin and HbF reinduction processes. Thus, some investigations have targeted KLF1 as a genetic regulator by GETs including CRISPR/Cas9 to reactivate the expression of HbF; KLF1 gene manipulation leads to a profound effect on KLF1 promoter sequence [68]. Our previous study was conducted to study the ability of an engineered CRISPR/Cas9 system to target KLF1 gene to induce KLF1 disruption and eventually stop the γ to β hemoglobin switching process in the K562 cell line.

Our results showed that the γ-globin level was significantly raised in differentiated K562 cells treated with CRISPR/Cas9 [69]. In a parallel investigation, we effectively applied the ZFN technology to the KLF1 gene knockout through targeted genome deletion induction. We envisaged that targeted induced mutations in the KLF1 gene coding region, lead to β-globin chain synthesis reduction and BCL11A gene down regulation, thus removing the inhibitory effect of BCL11A on the expression of the γ-globin gene [64]. Furthermore, in another comparative analysis, KLF1, BCL11A, and HBG1/2 were targeted in a parallel
manner in CD34+HSPCs by CRISPR/Cas9 to induce fetal hemoglobin [70]. The results were compared to assess the impact of each targeted gene on HbF induction and safety measurements by molecular analyses to select the most effective candidate for clinical investigation. The successful downregulation of KLF1 and BCL11A transcripts led to prominent γ-globin mRNA expression, and significant HbF levels, comparable to Hereditary Persistence of Fetal Hemoglobin (HPFH) mutations. Although the elevated level of HbF (up to 25%) after KLF1 gene disruption was associated with no off-targets (verified by GUIDE-seq), the negative effect of KLF1 knockdown was detected in further analysis, which could be a major safety concern in the clinical application of this strategy. When compared to non-edited reference cells, RNA-seq analysis indicated that targeting the KLF1 gene with CRISPR/Cas9 dysregulated many genes with various biological activities [70]. Recent investigations have documented the reduced KLF1 expression, the influence of the profile expression of genes complicated in cancer (FLI-1) and some biological processes, including microcytosis (AQP1) and cell–cell interaction (CD44 and ITGA2B) [71,72].

4.2. Reproducing HPFH Mutations Recapitulates A Mutation Associated with A Benign Genetic Condition

Hereditary persistence of fetal hemoglobin (HPFH) is a benign condition in which some mutations naturally occur in the fetal globin promoter sequence, leading to HbF reactivation in adulthood [73]. These natural mutations consist of small deletions and/or single point mutations in the proximal promoter of fetal γ-globin gene [74].

A recent therapeutic strategy for β-thalassemia is to mimic HPFH mutations to reactivate HbF (Figure 4A(2)). One of the HPFH mutations is British HPFH, in which a point mutation of the −198 T > C in the fetal globin gene promoter has occurred. As HbF expression level elevates up to 20% in these thalassemic patients, the symptoms of β-hemoglobinopathies are considerably improved. In an in vitro and in vivo combinational study by Wienert and his colleagues, CRISPR/Cas9 technology was applied to exert a similar mutation to ameliorate the β-hemoglobinopathies symptoms by increasing HbF level. Subsequent to introduction of the −198 T > C mutation into the fetal globin promoter of the clonal WT HUDEP-2 cells, the mRNA expression percent of γ-globin [γ/γ + β] elevated from ~0.5–1% to 4–6%. Moreover, the number of HbF-immunostained cells improved in the −198 T > C mutated cells. Hence, deployment of CRISPR/Cas9-mediated homology-directed repair system to induce the −198 T > C mutation in clonal cells, leads to the creation of a new binding site for KLF1 as the major erythroid gene activator, which further enhances the HbF level [75].

Another type of HPFH naturally occurs through −175 T > C single point mutation, which leads to significant increase in fetal γ-globin to erythroid cell lines ratio. The elevated fetal globin level in these individuals is related to de novo recruitment of the activator TAL1 to promote chromatin looping of distal enhancers to the modified γ-globin promoter. In this study, the −175 T > C substitution into the Aγ and Gγ-globins genes promoters of murine erythroid and human erythroid K562 cells was induced using TALEN-based homologous recombination strategy. In both the cell lines, TAL1 could bind to the promoters and activate γ-globin in the mutated erythroid cells [76]. Generally, the results ascertained that the percentage of the HbF was significantly increased between 16 and 41% of total hemoglobin. In a recent study on β-thalassemic Egyptians, mutation in the β-globin first intron (IVS-1-110 [G > A]), which is the most prevalent mutation in this country [77], was directly corrected using CRISPR/Cas9-edited non-homologous end joining (NHEJ) technology [78]. The researchers succeeded in knocking out the mutation in peripheral blood CD34+ hematopoietic stem cells of the β-thalassemic patients, and the edited cells were then differentiated to erythroid lineage in culture media in the presence of erythropoietin [78].

One of the other point mutations which is responsible for HbF re-induction and improvement of β-thalassemic patients sign and symptoms, is related to the direct HbF gene repressors, BCL11A and ZBTB7A. In wild-type species, BCL11A as well as ZBTB7A directly join with the γ-globin gene promoter at the positions −115 and −200 bp, respectively, and
repress the fetal globin gene expression. Martyn and his colleagues employed CRISPR/Cas9 technology to exert the homozygous HPFH-associated mutations of −117 G > A, −114 C > A, Δ 13 bp, and −195 C > G in γ-globin gene promoter of erythroid cells where BCL11A and ZBTB7A can bind to the gene, to prevent the repressors binding and elevate γ-globin gene expression; results showed increased γ-globin mRNA and HbF levels in −117 G > A, −114 C > A, Δ 13 bp, and −195 C > G mutated populations [79].

5. Gene Repair Strategies

Gene repair, as a precise approach that potentially corrects the mutations in the native β-globin locus, is a novel non-pharmacological therapeutic method for the β-hemoglobinopathies treatment (Figure 4B). Over 200 various point mutations are known to cause β-thalassemia [80], while in contrast, a single point mutation of Glu > Val in the coding site (position 7) of the β-globin is responsible for SCD. Effective specific modifications exert on endogenous genomic loci by GETs, as recently reported for the human globin locus [81].

The binding of a ZFN or TALEN pairs to contiguous sequences flanking a target region causes dimerization of the FokI domain and ultimately a targeted DNA DSB, whereas CRISPR/Cas9 develops DSBs at a particular sequence [82]. The subsequent DSB is corrected using error-prone NHEJ or high-fidelity homology-directed repair (HDR) in the presence of a homologous DNA donor template [83].

To modify pathogenic mutations, an exogenous donor fragment is applied as a pattern. The major obstacle for developing an ideal HDR-based gene repair procedure is selection of the optimum exogenous template. The eligible donor fragment is transferred to HSPCs via various means including transfection as single-stranded oligodeoxynucleotide (ssODN), transduction using a viral vector-like integrase-defective lentiviruses (IDLVs) or recombinant adeno-associated viral vectors serotype 6 (rAAV6). The rAAV6 has long been considered as the most effective system for HDR-mediated gene repair transferring to HSPCs [84,85]. Despite rAAV6 efficiency in vitro, recent comparative studies revealed HSC engraftment decrease in animal models of immunodeficiency using rAAV6 compared to ssODNs [86,87].

HDR-based gene repair strategy could be efficiently applied in SCD as a monogenic and prevalent model. In the last decade, various strategies including ZFNs, TALENs, and CRISPR/Cas9 have been applied to induce HDR-mediated gene repair in order to correct the pathogenic variant in pluripotent stem cells (iPSCs) [88,89] and SCD patient-derived HSPCs (Figure 4) [90,91]. On the other hand, in vitro studies revealed that the correction efficiency was 7% to 50% (based on various editing technology and/or donor delivery systems), which is appropriate for HbA production up to 50% and improving the SCD phenotype [92]. However, transplantation of edited SCD cells presented a reduced gene repair frequency up to 10% when applied in vivo [93]. The incomplete HDR-mediated gene repair and the minority of HSCs in HSPC population are the main known reasons for insufficiency of HDR gene repair system in vivo. This led to generating an appropriate level of NHEJ-mediated INDELs in the HBB gene, which causes the S-globin gene to inactivate and leads to an appearance of undesired β-thalassemic phenotype [94].

HDR-mediated gene repair approaches have also been utilized to correct some mutations of β-thalassemia. Several studies have been performed on modifying specific point mutations of β-thalassemia in the patient derived iPSCs [95–100]. In a noticeable study, a wild-type HBB gene-complementary DNA is considered as a donor template for targeted integration. This plan is capable of repairing various point mutations involved in β-thalassemia [101]. However, the main limitation of this strategy is deficiency of a sufficient protocol for the production of adequate population enriched for HSCs as described earlier.

Regarding the inefficiency of HDR-based repair system, the non-specific NHEJ strategy was applied for long-term repopulation of HSCs. The imprecise disruption of DNA using NHEJ generated mutations in the non-coding region of the HBB gene, including IVS1-110G > A and IVS2-654C > T. These mutations were associated with the generation of new
binding sites in the \textit{HBB} introns, which led to synthesis of unusual mRNA and formation of immature termination codon [102].

In parallel studies conducted in 2019, TALEN- and CRISPR/Cas9-based editing strategies were applied for disruption of the unusual binding sites created by the mutations of IVS1-110G > A and IVS2-654C > T in patient HSPCs, which led to normal HBB binding and eventually elevated the expression of \textit{HbA} gene [102,103].

Recently, a pair of engineered TALENs were used in order to induce targeted integration of a full-length \(\beta\)-globin complementary DNA subsequent to mutation induction of about 50% of human \(\beta\)-globin alleles near the site of the sickle cell-related mutation (Figure 4) [81].

In line with \(\beta\)-thalassemia studies, Ma et al. combined two methods of TALEN-based repair strategy of \textit{HBB} mutations with effective production of edited patient-specific \(\beta\)-thalassemia iPSCs. Based on pluripotency, normal karyotype, lack of off-targets, and capability of iPSCs to differentiate into hematopoietic progenitor cells and further to erythroblasts with normal \(\beta\)-globin expression, this strategy seems the most ideal approach among GETs for further clinical applications [104].

In research published in 2021, the b039 thalassemia mutation was corrected using CRISPR-Cas9 technology. Consequently, erythroid progenitor cells formed and homozygous b039-thalassemia patients express normal \(b\)-globin genes. In terms of efficiency, a crucial point of this research is that the CRISPR Cas9-corrected cells have a significantly high production of HbA and were associated with a considerable reduction in free \(a\)-globin chains. The protocol could be the starting point for developing an efficient edition of CD34+ cells derived from b039 patients and designing combined therapies using the CRISPR/Cas9 editing of the \(b\)-globin gene [105].

6. Conclusions

Alongside the advances in gene therapy, gene editing tools offer an innovative approach for treating the \(\beta\)-hemoglobinopathies, which has recently emerged in clinical trials. Animal and human studies have presented the efficacy of the gene therapy approaches, currently based on GETs, in particular for \(\beta\)-thalassemia. Recent advances in genome sequencing methods, improvements in gene delivery systems, understanding the molecular mechanism involved in regulation of the \textit{HBB} locus, and the progresses in gene-editing technology made a basis for new achievements in the treatment of hemoglobinopathies concomitant with significant clinical benefit.

Several significant challenges are yet to be effectively addressed for in vivo genome editing via CRISPR-Cas technology to be clinically translatable to different fields. CRISPR-Cas guide RNAs and nuclease must be optimized for potent and straightforward on-target consequences with minimal off-target consequences, and they should be delivered efficiently to specific human cells and have minimal antigenic properties so that they are accepted by human immune systems. Novel CRISPR-Cas enzymes and delivery systems are being developed to overcome these obstacles. To enhance the specificity of CRISPR-Cas9, researchers modified the Cas9 construct, optimized the configuration of sgRNA, and developed a CRISPR-Cas9 double nickase system that presents only single-strand nicks at target zones.

The more efficient approach using transient delivery systems instead of viruses for in vivo applications could reduce safety concerns and off-target effects of sustained expression of CRISPR editing components. Nanoparticles have been designed to deliver CRISPR components such as plasmid DNA, mRNA, and ribonucleoproteins, and polymeric formulations encapsulating chemotherapeutic agents could also be engineered to deliver CRISPR molecular components for combination therapy. Hence, additional efforts are needed to address these problems, evaluate the safety of genome editing tools at the clinical level and follow the outcomes of GETs-mediated therapeutic approaches in related patients.
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Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| BCL11A       | BAF Chromatin Remodeling Complex Subunit BCL11A |
| BDDs         | blood disease disorders |
| BMT          | bone marrow transplantation |
| Cas          | CRISPR-associated protein |
| CRISPR       | clustered regularly interspaced short palindromic repeat |
| crRNA        | sequence-specific CRISPR RNA |
| EPO          | erythropoietin |
| FOG          | Friend of GATA |
| FOP          | Friend of Prmt1 |
| GATA-1       | GATA-binding factor 1 |
| GATA-2       | GATA-binding factor 2 |
| GVHD         | graft versus host disease |
| GETs         | gene editing tools |
| HbF          | hemoglobin F |
| HDR          | homology-directed repair |
| HLA          | human leukocyte antigen |
| HPFH         | hereditary persistence of fetal hemoglobin |
| HSCs         | hematopoietic stem cells |
| IDLVs        | integrase-defective lentiviruses |
| iPSCs        | in pluripotent stem cells |
| KLF1         | Kruppel Like Factor 1 |
| NF-E2        | Nuclear Factor, Erythroid 2 |
| NHEJ         | non-homologous end joining |
| rAAV6        | recombinant adeno-associated viral vectors serotype 6 |
| RBCs         | red blood cells |
| SCD          | sickle cell disease |
| SCL          | Stem Cell Leukemia |
| SOX6         | SRY-Box Transcription Factor 6 |
| ssODN        | single-stranded oligodeoxynucleotide |
| TALENs       | transcription activator-like effector nucleases |
| tracrRNA     | trans-activating crRNA |
| ZBTB7A       | Zinc Finger and BTB Domain Containing 7A |
| ZFNs         | zinc-finger nucleases |

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