

INVITED REVIEW ARTICLE

Genome editing strategies for fetal hemoglobin induction in beta-hemoglobinopathies

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Abstract

Genome editing to correct a defective β -globin gene or induce fetal globin (HbF) for patients with beta-hemoglobinopathies has the potential to be a curative strategy available to all. HbF reactivation has long been an area of intense interest given the HbF inhibition of sickle hemoglobin (HbS) polymerization. Patients with HbS who also have high HbF tend to have less severe or even minimal clinical manifestations. Approaches to genetically engineer high HbF include *de novo* generation of naturally occurring hereditary persistence of fetal hemoglobin (HPFH) mutations, editing of transcriptional HbF repressors or their binding sites and/or regulating epigenetic intermediates controlling HbF expression. Recent preclinical and early clinical trial data show encouraging results; however, long-term follow-up is lacking, and the safety and efficacy concerns of genome editing remain.

Introduction

Beta-globin disorders, including sickle cell disease (SCD) and beta-thalassemia, are the most common monogenic disorders worldwide, with more than 600 000 newborns affected each year (1,2). Supportive care, vaccines and antibiotic treatment in the developed world have improved life expectancy and quality of life for SCD; however, the majority of the disease burden is in the developing world, particularly sub-Saharan Africa and the Middle East, where mortality remains high. Currently, the only curative option is allogeneic hematopoietic stem cell (HSC) transplantation, and it is mostly utilized for patients with severe disease and a matched-sibling donor. The scarce availability of having a human leukocyte antigen (HLA)-matched donor (<15%) limits the wide use of this approach (3,4). Research to expand donor options is underway; however,

allogeneic transplantation carries the risk for graft-versus-host disease (GVHD), graft rejection, and transplant-related morbidity and mortality. Genetic modification of autologous HSCs is, therefore, an appealing option that would be available to all patients and would avoid the morbidity and mortality associated with GVHD. Such systems include gene addition versus gene editing strategies, with the former currently having more available outcome data. Recent advances in viral gene delivery systems have demonstrated the efficient transfer of healthy adult β -globin, γ -globin or modified version of β -globin (β T87Q-globin) with enhanced anti-sickling properties (5,6). Although gene addition approaches are beyond the scope of the review, clinical trials with β T87Q-globin gene addition show encouraging results, demonstrating circumvention of the disease phenotype and transition to a transfusion-independent stage (7,8).

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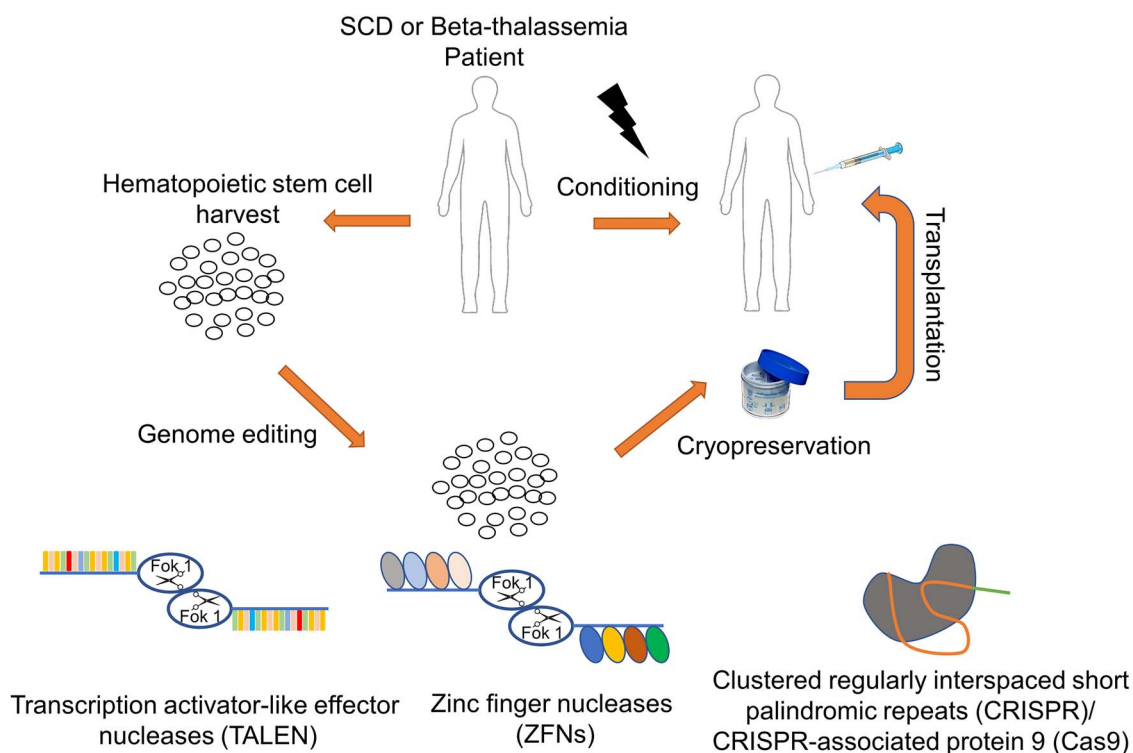


Figure 1. Ex vivo genome editing with TALENs, ZFNs and CRISPR/Cas9 for autologous hematopoietic stem cell transplantation for beta-hemoglobinopathy patients.

The field of genome editing has grown quickly given technological advances, specifically with the introduction of the safer, more efficient and relatively inexpensive clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system. The CRISPR/Cas system is, therefore, attractive for *ex vivo* correction of defective β -globin genes, allowing for autologous HSC correction and transplantation (Fig. 1). However, this process is challenging as homology-directed repair (HDR) is less efficient compared to non-homologous end joining (NHEJ), and therefore, requires more optimization in *ex vivo* culture conditions and animal models before transitioning into clinical trials (9).

Naturally occurring elevated fetal hemoglobin ($\alpha_2\gamma_2$, HbF) termed hereditary persistence of fetal hemoglobin (HPFH) occurs rarely in some individuals. When co-inherited with a hemoglobinopathy (i.e. SCD) the disease phenotype is significantly less severe (10). While beta-hemoglobinopathies have been studied for more than a century, there are only four FDA-approved drugs for the treatment of SCD, three of which were only recently approved between 2017 and 2019. The most utilized therapy, hydroxyurea, induces partial benefit due to HbF induction; however, no drug fully eliminates the complications of the disease, and there is significant patient-dependent variability (11). Genetic strategies aimed at reactivating HbF, recapitulating the phenotype seen in patients with naturally occurring HPFH, offer a potential definitive solution for disease- and treatment-free survival.

Globin switching and the importance of HbF for beta-hemoglobinopathies

HbF is the most abundant hemoglobin produced by the fetus in utero and is gradually replaced by adult globin ($\alpha_2\beta_2$, HbA)

after birth. By approximately 6 months of life, the contribution of HbF is generally less than 1%. The concentration of HbF is not pan-cellular; HbF is not evenly distributed among red blood cells (RBCs); rather it is concentrated in particular cells (termed F-cells). The protective effect of high HbF was first noted in 1945 when a delay in RBC sickling was noted in newborns with SCD compared to their affected mothers (12) and was confirmed when milder clinical outcomes were noted in patients with HPFH (13). High HbF offsets defective β -globin production in hemoglobinopathies and additionally exerts a protective anti-sickling effect in SCD. γ -globin reduces sickling in RBCs of SCD patients by reducing the overall concentration of sickle hemoglobin ($\alpha_2\beta^S_2$, HbS) and by forming either non-polymerizing HbF tetramers ($\alpha_2\gamma_2$) or hybrid tetramers ($\alpha_2\gamma\beta^S$), which enter sickle polymerization with a much lesser extent than HbS (14).

Given the protective effects of high HbF in hemoglobinopathies, research efforts have focused on approaches that induce HbF. Multiple strategies are being explored and include the use of chemical inducers or small molecules, silencing transcriptional γ -globin repressors, *de novo* generation of elevated HbF-related mutations or reactivation γ -globin expression using forced chromatin looping vectors (5). Here we discuss the current status of the research on HbF induction through genome editing applications.

Genome editing for HbF induction

Genome editing relies on the creation of a double-strand break (DSB) in a sequence-specific manner followed by repair by either NHEJ or HDR in the presence of a donor template. NHEJ changes the genomic sequence through small insertions/deletions (INDEL), resulting in interrupted expression of the targeted gene or by changing the sequence motif required for protein binding.

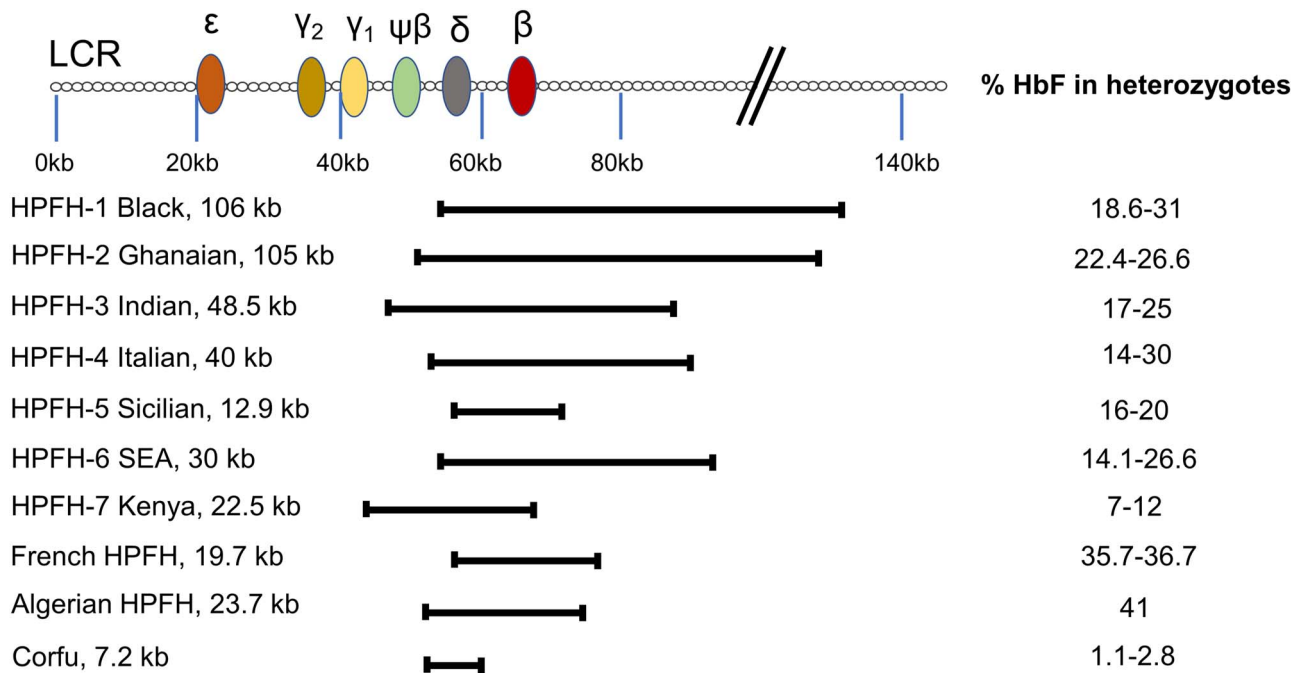


Figure 2. Common hereditary persistence of fetal hemoglobin (HPFH) mutations and relative fetal hemoglobin (HbF) levels in adults. The figure is adapted from (15,70).

Given cell preference for NHEJ over HDR, and that the creation of INDELS is sufficient for the various approaches to induce HbF, derepressing HbF has been a natural first choice for gene editing in hemoglobinopathies. The most commonly used genome editing tools are zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALENs) and the CRISPR/Cas9 system. While all three systems have their own limitations, the biggest advantage of the CRISPR/Cas9 system is its ease of use, low relative cost and general efficiency (11). Approaches to induce HbF in adult RBCs using these tools include creating natural mutations related with high HbF, knocking out HbF repressors and regulating epigenetic intermediates controlling HbF expression.

Generation of HPFH mutations

The human β -globin locus is composed of five globin genes (β -, $A\gamma$ -, $G\gamma$ -, ϵ - and δ -globin) located on a short region of chromosome 11. Naturally occurring HPFH mutations present with either small or large deletions within the human β -globin locus, leading to pancellular expression of elevated HbF levels (~30%) (Fig. 2). Early attempts to mimic the naturally occurring Sicilian HPFH mutation in human CD34+ cells demonstrated significant HbF induction by two mechanisms: generation of the ~13 kb deletion of the β -globin locus and creation of a 13 nt deletion in the γ -globin promoter sequence (15,16). A more detailed investigation deleted a 3.5 kb γ - δ intergenic region containing the putative HbF repressor protein (*BCL11A*) binding site, a 7.2 kb Corfu region and a larger 13.6 kb deletion, starting from the 5' breakpoint of the Corfu deletion and extending further to 3' to include the promoter and first exon of the β -globin gene (17). In patients with SCD, this 13.6 kb deletion would simultaneously upregulate γ -globin synthesis and inactivate the β^S -globin gene. Disruption of just the putative HbF silencer resulted in a mild increase in γ -globin expression, whereas deletion or inversion of

the 13.6 kb region caused a robust reactivation of HbF synthesis in adult erythroblasts. This work confirmed that gene competition between fetal and adult globins may limit the degree of HbF induction and that chromosomal configuration plays a critical role given that the inversion of the 13.6 kb area was as effective as the deletion of the site. Further mechanistic dissection studies revealed that point mutations at -115 (*BCL11A* binding site) and -200 bp (*ZBTB7A*, also known as *LRF*, binding site) upstream of the transcription start site of γ -globin gene enhanced robust HbF induction in the immortalized RBC progenitor cell line, HUDEP-2 (18). As a different strategy, a -115 and -114 bp sequence alteration was created using a base editor (*ha3A-BE3*) to avoid the deleterious effect of DSBs and reported potent HbF induction from 6.8-13.7% to 44.2-58.1% in differentiated edited cells (19).

To test the feasibility of the *de novo* HPFH mutation in progenitor cells, NBSGW mice were transplanted with CD34+ cells with a 13 nt *BCL11A* binding motif deleted (20). There was durable CD34+ engraftment (60%), HbF activation to potentially therapeutic levels (~30%) in RBC progeny, with no detectable genotoxicity reported. In a recent study, disruption of the *LRF* binding site led to γ -globin induction, correction of the sickling phenotype in SCD patient-derived CD34+ cells and persistence of high editing efficiency in NSG mice (21). In a rhesus-macaque model utilizing a 13 nt deletion of rhesus CD34+ cells, engraftment of 18% of edited cells (>1-year follow-up) with 1-5% γ -globin expression *in vivo* was recently shown (22). Stable engraftment of edited cells and detectable levels of HbF in rhesus macaques is encouraging; however, optimization is needed to improve engraftment of edited cells, and higher HbF expression is needed to achieve therapeutically meaningful levels.

Editing of transcriptional repressors

Globin expression is controlled by a developmentally regulated mechanism known as globin switching and includes the interaction of transcriptional factors with the upstream enhancer

cluster and gene promoters (23). In the last decade, significant research has been devoted to elucidating the underlining mechanism for globin switching. Specific globin expression is thought to be the result of chromosomal looping created through complex interactions of transcriptional factors. Genome-wide association studies (GWAS) identified crucial genomic locations regulating globin expression, for example, that *HBS1L-MYB* intergenic region and *BCL11A* are associated with elevated HbF levels (24,25).

In targeting *BCL11A* as an important repressor for HbF expression, various transcription factors including *GATA1*, *SOX6*, *KLF1* and *MYB* have been identified as forming a multiprotein complex interacting with *BCL11A* in the chromosomal reorganization of globin synthesis (26). Among these, the erythroid-specific transcription factor, *KLF1*, is proven to exert a dual effect on HbF expression by both direct involvement in the looping construct (27) and acting as an indirect silencer through regulating *BCL11A* expression (28). Significant HbF activation has been reported in *ex vivo* models (primary human erythroid cells, K562 cells) for these transcription factors either through direct gene editing (29–31) or disruption of their binding sites (18,32–34). *KLF1* and *MYB* have critical roles in erythropoiesis and hematopoiesis, respectively; however, strategies aimed at most of these transcription factors have not been evaluated *in vivo* in mice or large-animal models and do not appear to be as strong as *BCL11A* for the robust HbF induction needed for clinical application (35).

Many studies validate *BCL11A* as a master HbF regulator (28,36,37). Inactivation of *BCL11A* prevented clinical complications in a mouse model of SCD through high-level pancellular expression of HbF (28.3 versus 1.3% of total β -like human globins in adult SCD/*Bcl11a*^{-/-} mice compared with SCD mice) (38). A non-human primate model investigating *BCL11A* knockout using TALEN mRNA reported a much lower level of indel persistence (~0.3%) and negligible γ -globin induction (~0.05%) at 1-year post-transplantation (39). As rare *BCL11A* haploinsufficiency in humans is associated with a mild clinical course due to elevated HbF levels (40,41), optimized and efficient *BCL11A* editing has become an attractive and promising clinical approach. Importantly, however, *BCL11A* expression is essential for HSC function and B-cell development (42–44); therefore, focus is directed toward knocking down erythroid-specific expression. Identification of the erythroid-specific enhancer for *BCL11A* expression, intron-2, as sufficient to specify developmentally restricted, erythroid-specific gene expression made such an approach feasible (45). A follow-up study determined pivotal minimal features of the enhancer by targeting a series of gRNAs. Here, the investigators demonstrated that editing of the enhancer using specific gRNA targets induced substantial HbF expression in RBCs without affecting erythropoiesis and the expression levels of *BCL11A* in other lineages (46). Encouragingly, CD34⁺ cells edited at the *GATA1* binding site of the enhancer by either CRISPR/Cas9 or ZFN systems displayed multilineage engraftment potential ($\leq 95\%$) in the immunocompromised mouse model (47–49). Ribonucleoprotein (RNP) base editing of the *BCL11A* enhancer in human HSCs as an alternative to nuclease editing (and therefore the destructive effects of DSB) was validated using an A3A (N57Q)-BE3 base editor and provided comparable levels of HbF induction compared to standard Cas9 application (50).

To test the feasibility and safety of erythroid-specific enhancer editing, we have conducted a rhesus macaque model verifying the persistence of high-level edited cells (up to 80%) with robust γ -globin induction (up to 29%) (51). Consistent with the clinical trials performing ZFN-mediated *BCL11A* enhancer editing (NCT03653247) (52), and

erythroid-specific knockdown of *BCL11A* expression using a lentiviral vector shmiR (NCT03282656) (53), we have observed a peak in the HbF levels in rhesus macaques at early time points of transplantation followed by a significant drop, possibly due to stress erythropoiesis HbF induction overestimating editing effectiveness at early time points. Another clinical trial evaluating CRISPR/Cas9-based *BCL11A* enhancer editing has released early data (NCT03745287), demonstrating 10.1 g/dL HbF at 9 months post-transplantation for a patient with β -thalassemia and a total hemoglobin level of 11.3 g/dL with 46.6% HbF at 4 months post-transplantation for a patient with SCD (54). Given concerns for the long-term persistence of HbF induction, long-term follow up data from preclinical and clinical trials are essential to determine the feasibility, safety and efficacy of enhancer editing.

Epigenetic factors are another protein family involved in the regulation of globin expression through chromatin structure reorganization, histone modifications and DNA methylation (55). The majority of this approach uses pharmacological inhibitors to induce HbF by targeting epigenetic mediators (i.e. NuRD/HDAC, DNMT, EHMT and KDM1A) rather than creating permanent gene editing. Oral drugs including decitabine as a DNMT1 inhibitor (NCT01375608), INCB059872 as LSD1 inhibitor (NCT03132324), and panobinostat (NCT01245179) and pomalidomide (NCT01522547) as histone deacetylase inhibitors have been tested for SCD patients in clinical trials with some clinical benefit reported (56). However, long-term safety and efficacy of these drugs are needed. Genome editing tools, in fact, have helped elucidate the HbF potential of these various genes. It was shown that CRISPR/Cas9 based knockout of *MBD2* gene of the NuRD complex reactivates HbF in HUDEP-2 cells but not *MBD3* (57). A recent study reported a more detailed investigation of the NuRD family protein members for their HbF activation potential using CRISPR/Cas9 comprehensive mutagenesis in HUDEP-2 cells (58). The results revealed that only 5 members of the complex (*CHD4*, *GATAD2A*, *HDAC2*, *MBD2* and *MTA2*) were required for HbF silencing. The major hurdle in epigenetic editing, however, seems to be cell specificity as various side effects including neutropenia, thrombocytopenia or thrombophilia have been reported (59) in addition to the various contributions of these intermediates in hematopoiesis (60). Though inhibition of LSD1 has been associated with elevated HbF levels in cell culture conditions, erythroid-specific inhibition could be biologically problematic, for instance, in mouse and large animal models where LSD1 is important for erythroid maturation (61,62).

Challenges and Prospects

Given the hurdles in gene correction efficiency, including a preference for NHEJ over HDR, genome editing by knockout appears to be as effective for HbF induction (63) and is a potential clinically applicable treatment modality for beta-hemoglobinopathies. The erythroid-specific *BCL11A* enhancer is currently the most sought after candidate for clinical translation; however, long-term data and continued research may identify new targets or combination approaches with higher HbF induction, safer editing or improved engraftment. Cell culture models with reporter globin genes (64), CRISPR/Cas9-based genome-wide screening protocols (29) or nuclease-dead mutants of Cas9 that either block (CRISPRi) or activate (CRISPRa) gene expression (65) may uncover new, improved or potentially synergistic targets.

The safety and specificity of genome editing approaches are critical parameters that will ultimately determine how and

when these therapeutic tools become widely clinically available. Recently, two studies demonstrated a significant portion of adults with a pre-existing adaptive immune response to Cas9 (66,67). While these reports did not address the effects of these antibodies on the efficiency of CRISPR/Cas9-based treatments and host safety, these concerns should be addressed in large-animal models and clinical trials. Another critical consideration regarding genome editing is off-target activity. The majority of reports demonstrate no off-target effects in their experimental models; however, off-target detection sensitivities and false-negatives of *in vitro* off-target assays remain a significant hurdle that must be improved to avoid unwanted phenotypic outcomes.

For most of the editing applications, electroporation of the RNP complex is preferred due to its superior editing efficiency and faster kinetics. However, electroporation-induced HSC toxicity may limit the application for patients with SCD whose HSC quality and quantity are of concern (68). One potential solution to this problem would be the development of toxicity-reducing electroporation solutions (49). Another definitive solution would be the establishment of targeted *in vivo* delivery of RNPs that does not require stem cell harvest or transplantation. Given *ex vivo* manipulation and culture of HSCs reduces stem cell property, function and engraftment potential, successful *in vivo* genome editing would increase the impact of the application (69). An *in vivo* model may also expand access of these curative strategies to developing countries where resources are less widely available to carry out transplantation approaches.

Recent improvements in genome editing approaches demonstrate HbF induction as a feasible and potentially clinically meaningful treatment modality for beta-hemoglobinopathies. Long-term data showing long-term engraftment with high gene expression is currently lacking and limits the broad application of this therapy. Whether these expensive applications will be successful is yet to be determined, as is the ability to implement such modalities in resource-limited regions such as sub-Saharan Africa where the burden of disease is highest. To be applicable for the largest number of patients, editing systems must be proven safe and effective prior to a transition into a large-scale, cost-effective system that is needed to provide this disease-modifying and potentially curative strategy to patients.

Conflict of Interest Statement. None declared.

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