

## GENOME EDITING IN HEMATOLOGY

Customizing the genome as therapy for the  $\beta$ -hemoglobinopathiesMatthew C. Canver<sup>1</sup> and Stuart H. Orkin<sup>1-4</sup><sup>1</sup>Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA; <sup>2</sup>Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA; <sup>3</sup>Department of Pediatrics, Harvard Medical School and Harvard Stem Cell Institute, Harvard University, Boston, MA; and <sup>4</sup>Howard Hughes Medical Institute, Boston, MA

Despite nearly complete understanding of the genetics of the  $\beta$ -hemoglobinopathies for several decades, definitive treatment options have lagged behind. Recent developments in technologies for facile manipulation of the genome (zinc finger nucleases, transcription activator-like effector nucleases, or clustered regularly interspaced short palindromic

repeats-based nucleases) raise prospects for their clinical application. The use of genome-editing technologies in autologous CD34<sup>+</sup> hematopoietic stem and progenitor cells represents a promising therapeutic avenue for the  $\beta$ -globin disorders. Genetic correction strategies relying on the homology-directed repair pathway may repair genetic defects,

whereas genetic disruption strategies relying on the nonhomologous end joining pathway may induce compensatory fetal hemoglobin expression. Harnessing the power of genome editing may usher in a second-generation form of gene therapy for the  $\beta$ -globin disorders. (*Blood*. 2016; 127(21):2536-2545)

## Introduction

The  $\beta$ -hemoglobinopathies, namely sickle cell disease (SCD) and  $\beta$ -thalassemia, result from genetic mutations in the  $\beta$ -globin gene and are among the most common monogenic diseases in the world.<sup>1</sup> SCD results from a nonsynonymous A to T mutation in codon 6 of the  $\beta$ -globin gene leading to a Glu-Val replacement,<sup>2,3</sup> whereas  $\beta$ -thalassemias are caused by diverse point mutations or deletions.<sup>4-9</sup> Treatment options are largely supportive. Transfusion and iron chelation are mainstays in the thalassemias whereas pain management, hydration, and hydroxyurea are used in SCD.<sup>10-16</sup>

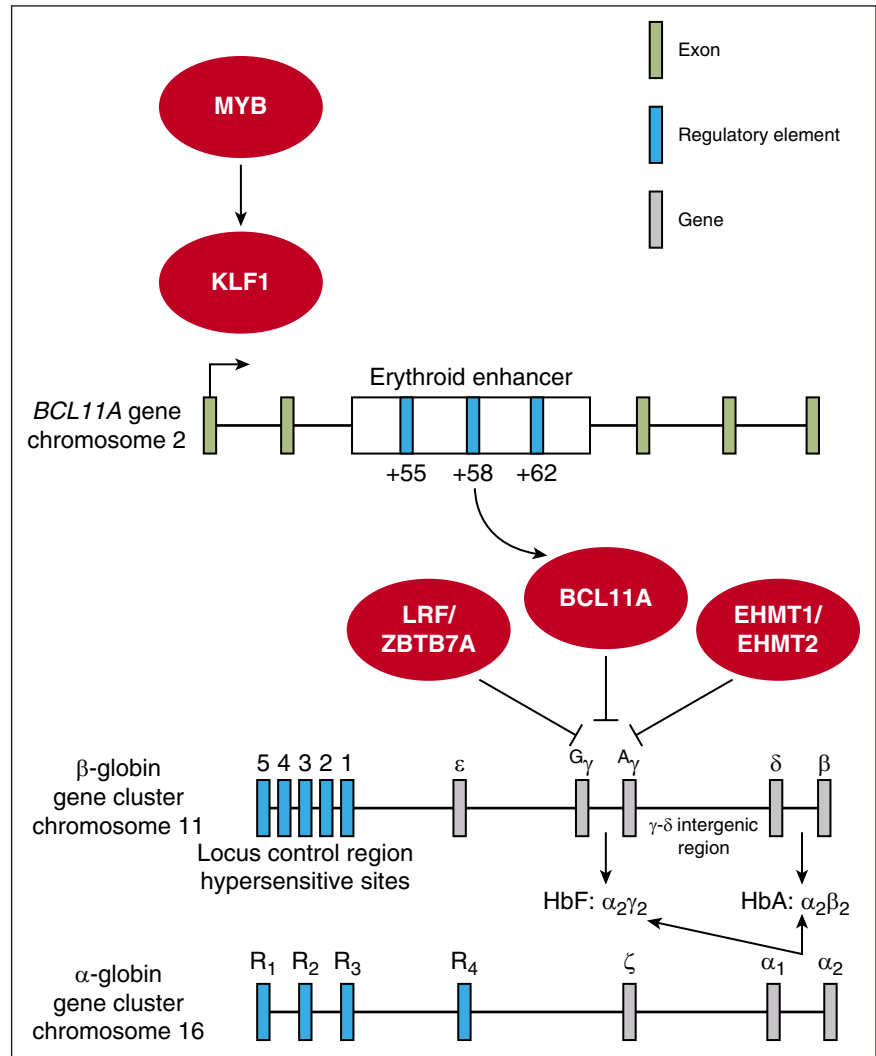
The hemoglobin tetramer is composed of 2  $\alpha$ -like globin chains encoded by any of the 3 genes in the  $\alpha$ -globin cluster on chromosome 16 and 2  $\beta$ -like globin chains encoded from any of the 5 genes in the  $\beta$ -globin locus on chromosome 11. The expression of the 3 genes at the  $\alpha$ -globin locus ( $\zeta$ ,  $\alpha_1$ ,  $\alpha_2$ ) and the 5 genes at the  $\beta$ -globin locus ( $\epsilon$ ,  $\zeta$ ,  $\gamma$ ,  $\delta$ ,  $\beta$ ) are developmentally regulated. It has been appreciated for many years that levels of fetal hemoglobin (HbF;  $\alpha_2\gamma_2$ ), subject to developmental silencing in the months after birth, is a modifier of disease severity in patients with  $\beta$ -globin disorders.<sup>16-23</sup> This protective effect of HbF has motivated the therapeutic strategy to reinduce its expression in adult life. Hydroxyurea, a cytotoxic agent that inhibits ribonucleotide reductase, induces HbF modestly through an unknown mechanism. However, it has dose-limiting myelosuppressive effects and some patients are non-responders to therapy.<sup>10-13</sup> Although bone marrow transplant (BMT) represents the sole established curative option for patients, its use is limited by donor availability and graft-versus-host disease (GVHD). A clinical trial has demonstrated successful gene addition of an antisickling form of  $\beta$ -globin to a transfusion-dependent  $\beta^E\beta^0$  thalassemia patient who gained transfusion independence as a result of gene transfer.<sup>24</sup> Several additional somatic gene therapy trials for  $\beta$ -thalassemias and SCD are ongoing.<sup>25</sup> Despite a deep understanding of molecular defects and gene control mechanisms, treatment options for the majority of patients remain limited.<sup>3</sup>

The emergence of designer nucleases for eukaryotic genome editing has ushered in an era of unprecedented control over the genome. The

development of zinc finger nucleases (ZFNs),<sup>26-35</sup> transcription activator-like (TAL) effector nucleases (TALENs),<sup>36-40</sup> and meganucleases<sup>41-44</sup> established genome editing as a valuable laboratory technique. The emergence of the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) nuclease system,<sup>45-49</sup> which utilizes a single guide RNA (sgRNA) to direct the Cas9 nuclease for site-specific cleavage, has engendered tremendous excitement about potential clinical applications. The breakneck speed at which new variations on the general theme are developed is truly remarkable. Other Cas9-like systems include the CRISPR/Cpf1 nuclease platform,<sup>50</sup> dimeric RNA-guided FokI nucleases,<sup>51,52</sup> and use of Cas9s derived from a variety of prokaryotic species.<sup>53,54</sup> It is unlikely that the discovery of novel CRISPR-based systems and Cas9-like nucleases capable of eukaryotic genome editing will end soon.<sup>55</sup> The relative benefits of the newly developed CRISPR-based systems, ZFNs, and TALENs are still subject to debate. Although CRISPR-based systems are often cited as the most efficient,<sup>56</sup> ZFNs are the only editing technology that has been brought thus far to a clinical trial. The *CCR5* gene has been targeted by ZFNs in autologous CD4<sup>+</sup> T cells from patients with HIV. The gene-modified cells were subsequently reinfused, which led to a decrease in the blood level of HIV in most patients.<sup>57</sup> Notably, this study demonstrated that reinfusion of autologous genome-edited primary human cells could be achieved, well tolerated, and possibly lead to clinical benefit.

Genome-editing-based therapies rely on gene correction or disruption. Double-strand break (DSB) induction by an engineered nuclease is repaired by the endogenous repair pathways of homology-directed repair (HDR) or nonhomologous end joining (NHEJ).<sup>58</sup> Genetic correction strategies exploit the HDR pathway to insert custom sequences into the genome through codelivery of an extrachromosomal repair template in conjunction with an engineered nuclease. The creation of a DSB improves HDR frequency.<sup>59</sup> As such, wild-type (or customized) sequences can be provided as an extrachromosomal donor for repair following site-specific cleavage by the nuclease. In contrast, genetic disruption strategies rely on the NHEJ pathway following nuclease-induced

**Figure 1. Network of potential targets for genome-editing-based therapy of the  $\beta$ -globin disorders.** Therapeutic genome-editing strategies rely on genetic correction through the HDR pathway or genetic disruption through the NHEJ pathway. Genetic correction/repair strategies involve direct modification of the  $\beta$ -globin gene cluster through (1) correction of the sickle mutation in the  $\beta$ -globin gene or (2) insertion of the HPFH-associated single-nucleotide polymorphisms (SNPs) into the  $\gamma$  or  $\delta$  promoters. Genetic disruption strategies involve targeted disruption of (1) *BCL11A* coding sequence, (2) the minimal critical sequences in the +58 DHS within the erythroid-specific *BCL11A* enhancer, (3) the HbF-associated sequences within the  $\gamma$ - $\delta$  intergenic region, or (4) other genes with a known role in  $\gamma$ -globin regulation such as MYB, KLF1, LRF/ZBTB7A, or EHMT1/EHMT2. HbA, adult hemoglobin.



DSB to produce local insertions/deletions (indels).<sup>47,48,58</sup> Introduction of 2 engineered nucleases can result in targeted deletion or inversion, duplication, local indels at nuclease cleavage sites, or translocations/chromosomal rearrangements.<sup>60-73</sup> Here, we review genome-editing approaches for genetic correction and disruption strategies for the  $\beta$ -hemoglobinopathies as summarized in Figure 1 and Table 1.

## General strategies for SCD and $\beta$ -thalassemia therapeutic genome editing

### Correction of underlying genetic defects

The most appealing and theoretically straightforward application of genome editing for monogenic disorders is correction of a mutant DNA sequence and, in that manner, preservation of all intrinsic regulatory mechanisms acting on the gene of interest. Precise gene correction relies on HDR from an extrachromosomal template containing a wild-type gene sequence. Typically, the frequency of HDR is relatively low, and particularly low in CD34<sup>+</sup> hematopoietic stem and progenitor cells (HSPCs) as discussed in the next section. However, gene correction strategies may benefit from mixed chimerism allogeneic transplant studies suggesting that low levels of chimerism can produce clinical benefit.<sup>74</sup> Clinical development of such strategies requires optimizing efficiency and safety

of correcting the sickle mutation, whereas the diverse spectrum of  $\beta$ -thalassemia point mutations and deletions necessitates optimization for each unique genetic target, a significant challenge for clinical translation.

### Gene editing for reactivation of HbF in SCD and $\beta$ -thalassemia

Elevated HbF is beneficial in SCD and  $\beta$ -thalassemia. Targets for manipulation include sequences lying within the  $\beta$ -globin cluster or within the genes encoding transcriptional regulators of globin gene expression (Figure 1; Table 1). Depending on the target, editing would rely on HDR or NHEJ. Suitability of each target relates to the ease with which the desired gene modifications can be generated and the extent to which the modifications reactivate HbF expression. In SCD, the goal is to induce sufficient HbF to prevent sickle hemoglobin (HbS) polymerization. In  $\beta$ -thalassemia, the aim is to replace deficient  $\beta$ -globin and thereby reduce globin chain imbalance.

## Possible targets for editing

### Genetic correction of the SCD and $\beta$ -thalassemia mutations

Classical gene-targeting approaches have been used to repair the SCD mutation in embryonic stem cells,<sup>75</sup> but this approach cannot be applied

**Table 1. Potential targets for therapeutic genome editing for the  $\beta$ -globin disorders**

Target	Repair strategy	Efficiency	Advantages/Disadvantages
Repair of SCD allele	HDR	Low-moderate	Single target allele; inadvertent generation of $\beta$ -thalassemia alleles
Repair of $\beta$ -thalassemia allele	HDR	Low-moderate	Heterogeneous target alleles
Recreation of nondeletional HPFH	HDR	Low-moderate	Inadvertent generation of $\gamma$ -null alleles; identified HPFH patients support mutation tolerance/clinical benefit
Recreation of deletional HPFH	NHEJ	Low-moderate	Insufficient efficiency of targeted deletion; identified HPFH patients support mutation tolerance/clinical benefit
Other targets in $\beta$ -globin cluster	NHEJ	—	Targets unknown
BCL11A	NHEJ	High	HSC/B-cell dysfunction due to BCL11A requirement; haploinsufficient patients have significant HbF induction
BCL11A enhancer	NHEJ	High	Erythroid-specific BCL11A loss; haploinsufficient patients have significant HbF induction
$\alpha$ -Globin	NHEJ	Moderate-high	Balance $\alpha$ - $\beta$ chains; inadvertent generation of $\alpha$ -thalassemia cells
KLF1	NHEJ	High	Broad role in cell proliferation and cellular development
MYB	NHEJ	High	Broad role in cell proliferation and cellular development
LRF/ZBTB7A	NHEJ	High	Broad role in cell proliferation and cellular development
EHMT1/EHMT2	NHEJ	High	Role in hematopoiesis unknown
LIN28B pathway	NHEJ	High	Role in hematopoiesis unknown

to CD34<sup>+</sup> HSPCs due to low efficiency and the necessity to isolate and propagate faithful recombinants. Correction of genetic defects in cultured cells with an engineered nuclease and a donor repair template has been achieved for multiple disorders, including cystic fibrosis,<sup>76-79</sup> Duchenne muscular dystrophy,<sup>80,81</sup> ornithine transcarbamylase deficiency,<sup>82</sup> hereditary tyrosinemia,<sup>83</sup> and other diseases.<sup>71,84-87</sup> Gene correction for SCD and  $\beta$ -thalassemia has also been accomplished in a laboratory setting.<sup>75,88-91</sup> Of note, a recent study reported correction of an SCD allele at nearly 20% gene modification in CD34<sup>+</sup> HSPCs upon delivery of a repair template via integration-deficient lentivirus or by DNA oligonucleotide electroporation in the presence of a  $\beta$ -globin-targeted ZFN. Similar levels of correction were observed in bone marrow cells isolated from SCD patients.<sup>88</sup> Despite successful HDR in bulk cells in vitro, the levels of HDR were 10- to 20-fold reduced in the spleen and bone marrow of transplanted immunodeficient mice, suggesting that HDR within long-term engrafting hematopoietic stem cells (HSCs) was far less efficient than in downstream progenitors. Another study reported HDR rates of 17% to 43% at 2 genomic loci in fetal liver-derived or mobilized peripheral blood-derived cells via electroporation of ZFN messenger RNA (mRNA) in conjunction with an adeno-associated virus (AAV) donor repair template.<sup>92</sup> These rates of HDR were maintained in vivo, suggesting the ability to perform HDR in primitive repopulating cells. Studies using AAV in conjunction with megaTALs,<sup>93</sup> TAL effectors coupled to a sequence-specific homing endonuclease, demonstrated ~14% rates of HDR in CD34<sup>+</sup> HSPCs.<sup>94</sup> Although megaTALs may enhance HDR through generation of 3' DNA overhangs in HSPCs, the rate of HDR in repopulating HSCs has not been examined.

The relative efficiency of HDR vs NHEJ is critical to potential use of gene editing for gene correction. High rates of NHEJ-mediated indel formation are suboptimal for clinical translation of  $\beta$ -globin gene correction as the process creates the possibility of disruption of  $\beta$ -globin production and inadvertent generation of  $\beta$ -thalassemia alleles. Another consideration is that mutagenesis has also been observed in the highly homologous  $\delta$ -globin gene in  $\beta$ -globin gene correction experiments, which may result in deletions and rearrangements affecting  $\beta$ -globin

that may be difficult to detect by standard polymerase chain reaction (PCR)-based genotyping approaches.<sup>88</sup>

It is possible that small molecules that enhance HDR and/or inhibit NHEJ may improve the efficiency of gene correction within CD34<sup>+</sup> HSPCs, so long as they do not impair cell engraftment capability.<sup>95-98</sup> Another possibility is the use of asymmetric donor template DNA to enhance rates of HDR.<sup>99</sup> NHEJ is the dominant pathway in G1, S, and G2 phases of the cell cycle, whereas HDR preferentially occurs during late S-phase and G2 phase when sister chromatid templates become available.<sup>100</sup> Because HSCs, the rare long-term repopulating cells within CD34<sup>+</sup> HSPC preparations, are largely quiescent, HDR is not favored. These observations are supported by the roles of BRCA1, PALB2, and BRCA2 in DSB repair. BRCA1 creates single-strand DNA through end resection and interacts with PALB2 to recruit BRCA2 and RAD51 to mediate HDR at sites of DSB. Identification of the cell cycle's role in suppressing BRCA1 in the G1 phase supports the dominance of NHEJ repair in quiescent cells.<sup>101</sup> However, restoration of the BRCA1-PALB2 interaction during the G1 phase can support HDR. Therefore, it may be possible to enhance HDR in quiescent HSCs through modulation of the BRCA1-PALB2-BRCA2 pathway.<sup>101</sup> Moreover, 1 study demonstrated enhanced rates of HDR in HEK293T and nonhematopoietic primary cells through cell cycle synchronization to achieve nuclease-mediated cleavage during the optimal portions of the cell cycle for HDR.<sup>100</sup> However, triggering proliferation in HSCs tends to impair their ultimate repopulating potential. Whether expansion of HSPC populations with small molecules such as SR1<sup>102,103</sup> or UM171<sup>104</sup> will allow for improved HDR efficiencies with concomitant retention of stem cell activity in vivo is as yet unknown.

#### Modification of the $\beta$ -globin locus to recreate hereditary persistence of fetal hemoglobin

As would be anticipated from the existence of rare hereditary persistence of fetal hemoglobin (HPFH) alleles, genome-wide association studies (GWAS) have linked the  $\beta$ -globin cluster itself to HbF levels.<sup>105-110</sup>

This corroborated previous human genetic studies that identified HPFH patients with elevated HbF levels resulting from large deletions within the  $\beta$ -globin cluster.<sup>111-113</sup> Re-creating the larger deletional HPFH alleles is impractical given their large size.<sup>60</sup> However, opportunities may exist for targeting discrete regions of the  $\beta$ -globin gene cluster by NHEJ. Comparison of large deletions in the cluster that generate either HPFH or  $\delta\beta$ -thalassemia phenotypes has implicated sequences in the  $\gamma$ - $\delta$  intergenic region as harboring silencers of  $\gamma$ -gene expression. Notably, study of 3 families with overlapping deletions in the  $\beta$ -globin cluster identified a 3.5-kb region between the  $\gamma$  and  $\delta$  genes that may be essential for  $\gamma$ -globin repression. Additional indirect support was derived from chromatin immunoprecipitation–PCR experiments that suggest BCL11A binding within this region.<sup>111,114-117</sup> At present, the optimal sequences in the cluster amenable for targeted deletion by editing and NHEJ have not been identified.

Several point mutations or small deletions in the  $\gamma$  or  $\delta$ -globin gene promoters lead to persistence of HbF into adult life. HbF levels in heterozygotes with these nondeletional HPFH mutations may be as high as 30%.<sup>116,118-120</sup> One of the strongest HPFH alleles ( $-175\text{T>C}$  in the  $\gamma$  promoter) was recently created in cultured K562 cells with TALENs. Increased  $\gamma$ -globin production resulted, most likely through de novo generation of a TAL1-binding site that facilitated increased chromatin looping between the  $\gamma$  promoter and the locus control region.<sup>121</sup> An HPFH allele with a small deletion in the  $\gamma$  promoter was re-created in CD34<sup>+</sup> HSPCs with sgRNA and Cas9 expression, presumably due to microdeletion of a repeated sequence.<sup>122</sup> Therapeutic genome editing to generate HPFH mutations is an attractive strategy as the effects of these mutations are known through study of families with these rare beneficial alleles. The approach, however, faces many of the same challenges as precise gene correction, given the apparent dominance of the NHEJ pathway at the expense of HDR efficiency in HSCs.

### BCL11A targeting

**BCL11A gene disruption.** The GWAS-implicated transcription factor BCL11A is a validated repressor of HbF.<sup>105-110,114,123</sup> Erythroid-lineage Bcl11a knockout in a mouse model of SCD led to pancellular HbF induction and phenotypic correction of a mouse model of SCD without perturbing other hematologic parameters.<sup>124</sup> Haploinsufficient patients with microdeletions within the BCL11A locus have significant neurocognitive phenotypes as well as elevated HbF at levels near or above therapeutic thresholds.<sup>125,126</sup> In principle, the genetic knockout of BCL11A by targeting *BCL11A* coding sequence in order to create frameshift null alleles represents a potential therapeutic strategy. Roles of *BCL11A* in nonhematopoietic lineages, including the neural lineage,<sup>127,128</sup> pancreatic progenitors,<sup>129</sup> and the breast epithelium,<sup>130</sup> would not be problematic upon modification of *BCL11A* in autologous CD34<sup>+</sup> HSPCs. However, this strategy is limited by extraerythroid roles of *BCL11A* in the hematopoietic system, including its requirement for B-cell development<sup>127,131-133</sup> and HSC function.<sup>134,135</sup> These roadblocks might be circumvented by use of erythroid-restricted expression of genome-editing components. A variation of this approach involves erythroid-specific, short hairpin RNA–mediated knockdown of *BCL11A* expression, which is under development as a gene-therapy strategy.<sup>136</sup> Delivery of genome-editing tools stably to CD34<sup>+</sup> HSPCs would be inadvisable due to potential insertional mutagenesis<sup>137</sup> as well as elevated risk of off-target mutagenesis over time. Furthermore, the effects of long-term expression of ZFNs, TALENs, or CRISPR/Cas9 on CD34<sup>+</sup> HSPCs are unknown.

**BCL11A gene enhancer.** Recent fine mapping of HbF-associated GWAS variants led to the identification of a developmental stage-specific, erythroid-restricted 12-kb region bearing a characteristic

enhancer chromatin signature. This enhancer region is composed of 3 DNaseI hypersensitive sites (DHS), termed +55, +58, and +62 as their distance in kilobases from the *BCL11A* transcriptional start site. Deletion of the orthologous element in a murine erythroid cell line resulted in a complete loss of BCL11A at both the RNA and protein levels whereas expression was spared in a B-cell line with the same deletion.<sup>67</sup> Subsequent deletion studies demonstrated a similar requirement for this element for *BCL11A* expression in human erythroid cells.<sup>138,139</sup>

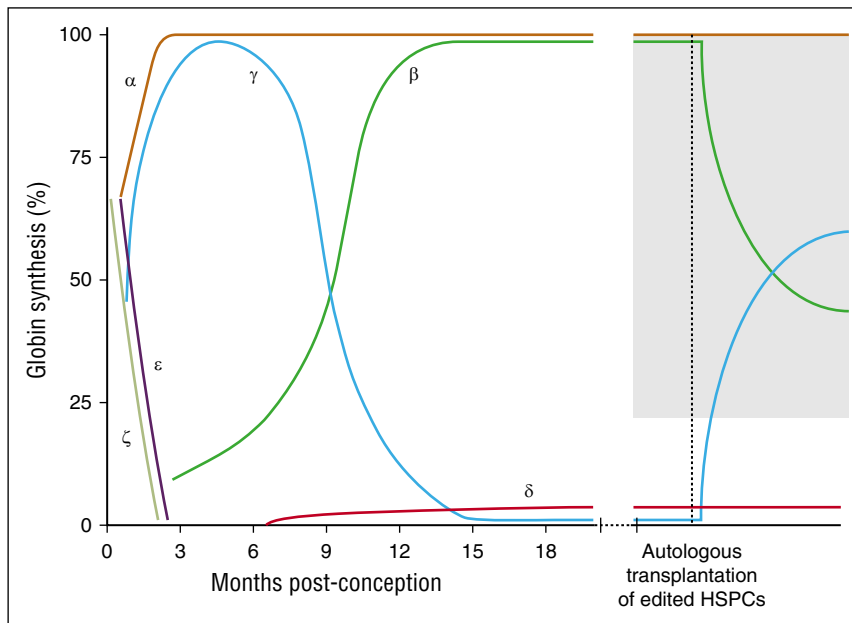
*BCL11A* enhancer targeting has several distinct advantages over coding sequence disruption: (1) GWAS studies have demonstrated that variation in the *BCL11A* enhancer is associated with elevated HbF levels and is both common and well tolerated.<sup>67</sup> (2) Targeted deletion of this element in a human erythroid cell line leads to loss of *BCL11A* expression and subsequent HbF induction nearly comparable to *BCL11A* null clones.<sup>138</sup> (3) Targeted deletion of the murine +62 DHS within the *Bcl11a* erythroid enhancer results in delayed hemoglobin switching sparing expression in the brain and nonerythroid hematopoietic lineages.<sup>138</sup> The +62 DHS knockout mice were viable and born in normal Mendelian ratios as compared with *Bcl11a*<sup>-/-</sup> knockout mice that are perinatal lethal likely due to neural defects.<sup>123,138</sup> These results further highlight the erythroid specificity of this element in vivo.<sup>138</sup> (4) Targeting the *BCL11A* enhancer has been shown to be better tolerated even within the erythroid lineage as compared with targeting the *BCL11A* coding sequence, suggesting a residual low level of BCL11A present after enhancer targeting is insufficient to repress  $\gamma$ -globin, but promotes cellular fitness.<sup>138,139</sup>

Therefore, an alternative approach to targeting *BCL11A* coding sequence might be targeted deletion of the 12-kb *BCL11A* erythroid enhancer.<sup>67</sup> However, although targeted deletions from  $\sim$ 1 kb to 1 Mb have been demonstrated to occur at an appreciable frequency, these are unlikely to occur at a sufficient frequency at clinical scale with current genome-editing technologies due to competing outcomes to deletion when using a dual nuclease strategy including scarring (multifocal indels), inversions, and duplications.<sup>47,48,60,68,140</sup> Furthermore, the heterogeneous population of cells resulting from a dual nuclease strategy would be suboptimal for clinical translation.

Functional footprinting–informed targeting by ZFNs/TALENs within the *BCL11A* enhancer and comprehensive functional mapping of the *BCL11A* enhancer by CRISPR/Cas9-mediated saturating mutagenesis has revealed an “Achilles’ heel” to the *BCL11A* enhancer within the +58 DHS.<sup>138,139</sup> Disruption of this minimal functional sequence at the core of the DHS +58 by CRISPR/Cas9 or ZFNs/TALENs resulted in  $\gamma$ -globin induction comparable to targeting coding sequence in CD34<sup>+</sup> HSPCs subject to erythroid differentiation conditions.<sup>138,139</sup> The core region has been fine-mapped to an  $\sim$ 20-bp region including a GATA1-binding motif which appears to be essential for *BCL11A* expression and subsequent HbF repression.<sup>138,139</sup> As previously discussed, the erythroid specificity of the regulatory element would not require erythroid-specific expression of the genome-editing components, as would be necessary with a *BCL11A* coding sequence targeting approach. Taken together, targeting of the *BCL11A* enhancer at the functional core of +58 DHS in autologous CD34<sup>+</sup> HSPCs followed by BMT represents a promising therapeutic strategy to induce HbF expression in patients with the  $\beta$ -globin disorders (Figure 2).

### LRF/ZBTB7A gene disruption

Another transcription factor LRF/ZBTB7A (also referred to as Pokemon) has more recently been recognized as a major repressor of  $\gamma$ -globin.<sup>141</sup> LRF-knockout mice exhibit elevated levels of the embryonic globin *Hbb- $\beta$ h1* with normal levels of *Hbb- $\gamma$* . This contrasts from *Bcl11a*-null mice that exhibit elevation of both embryonic globins,



**Figure 2. Reversal of hemoglobin switching to induce therapeutic levels of HbF.** Reversal of hemoglobin switching can be accomplished through autologous bone marrow transplantation of genome-edited CD34<sup>+</sup> HSPCs. The gray region indicates the hypothesized levels of HbF required for clinical benefit.

*Hbb-γ > Hbb-βh1*.<sup>123,124</sup> *Zbtb7a*<sup>-/-</sup> mice are embryonic lethal due to anemia, whereas conditional knockout of *Zbtb7a* in adult mice leads to inefficient terminal erythropoiesis resulting in a mild macrocytic anemia.<sup>142</sup> CRISPR/Cas9-mediated knockout of LRF in an erythroid cell line resulted in dramatic upregulation of  $\gamma$ -globin. LRF/BCL11A double knockout in this system was near additive and resulted in HbF of >90%, suggesting LRF's role in  $\gamma$ -globin regulation is partially independent of BCL11A. Subsequent analysis demonstrated a mild delay in erythroid differentiation upon knockdown of LRF in primary human CD34<sup>+</sup> HSPCs differentiated down the erythroid lineage with a corresponding induction of  $\gamma$ -globin.<sup>141</sup> Although the effect of LRF loss on  $\gamma$ -globin is striking, the role of LRF in cell fate decisions in multiple hematopoietic lineages and its requirement for terminal erythropoiesis may limit its therapeutic potential.<sup>143</sup>

### Reducing chain imbalance for $\beta$ -thalassemias

The physiologic hallmark of  $\beta$ -thalassemia is globin chain imbalance, such that deficiency of  $\beta$  chains leads to precipitation of unstable, free  $\alpha$  chains, membrane damage, hemolysis, and ineffective erythropoiesis.<sup>20,144-146</sup>  $\alpha$ -Thalassemia serves as a genetic modifier of  $\beta$ -thalassemia, as chain imbalance is reduced.<sup>146</sup> This is supported by a milder disease course in patients with the co-inheritance of  $\alpha$ -thalassemia and  $\beta$ -thalassemia.<sup>146-148</sup> In principle, therefore,  $\alpha$ -globin genes or their regulatory elements constitute potential targets for genome editing. Targeting an  $\alpha$ -globin gene itself could result in a heterogeneous population of cells including those null for  $\alpha$ -globin which might not support erythropoiesis. This approach could become a viable option if technological advancements allow for the precise control of the number of  $\alpha$ -globin null alleles generated. However, with present editing tools, the inability to control the number of  $\alpha$ -globin null alleles created through targeting  $\alpha$ -globin coding sequences makes targeting known regulatory elements of  $\alpha$ -globin or RNA interference-mediated knockdown of  $\alpha$ -globin more attractive alternatives in this context.

### Other potential therapeutic targets

Transcription factors KLF1 and MYB have previously been considered potential targets for HbF reactivation, but are not attractive due to their

broad roles in cell proliferation and cellular development.<sup>112,113</sup> Other genes such as EHMT1/EHMT2 and the LIN28B pathway have been implicated in the regulation of  $\gamma$ -globin; however, the selectivity of these targets and roles in hematopoiesis need further investigation.<sup>149-151</sup>

## The platform: autologous bone marrow transplantation of genome-edited cells

Significant obstacles to wider use of BMT for cure of patients with  $\beta$ -globin disorders are the availability of compatible donors and risk of GVHD. Donor availability is particularly severe for SCD patients. The most persuasive rationale for therapeutic genome editing of  $\beta$ -globin disorders rests with the use of autologous CD34<sup>+</sup> HSPCs as the cellular target. Through use of the patient's own cells for therapy, donor availability and GVHD are avoided. As with more "conventional" somatic gene therapy with modified viruses, delivery of the requisite editing components to the target cells is the principal hurdle to be overcome in achieving clinical success.<sup>152</sup> Delivery of therapeutic genes to CD34<sup>+</sup> HSPCs has been accomplished with integrating and nonintegrating viral vectors (such as lentiviral, adenovirus, and AAV vectors), as well as physical methods (eg, electroporation).<sup>88,153-155</sup> The optimal method for gene editing is currently unknown but is likely related to the specific technology used. High-efficiency delivery at clinical scale, roughly  $>10^8$  CD34<sup>+</sup> HSPCs cells, presents a practical challenge. However, recent studies have taken promising steps forward with electroporation of mRNA to CD34<sup>+</sup> HSPCs at clinical scale ( $>1 \times 10^8$  cells).<sup>139,156</sup> Robust cellular delivery is required for clinical translation of any envisioned therapeutic genome-editing approaches.

Genome editing is generally more difficult in primary cells as compared with immortal cell lines for reasons that are not entirely well understood, but may reflect inefficient delivery, diminished promoter activity of constructs, interferon responses, exonuclease activity, and host mechanisms of DNA repair.<sup>152</sup> Electroporation of ZFNs, TALENs, and CRISPRs as DNA, RNA, and/or protein is an efficient delivery strategy to CD34<sup>+</sup> HSPCs in a laboratory setting.<sup>88,92,140,152,157</sup> For the CRISPR/Cas9 system, mRNA or ribonucleoprotein electroporation may obviate

toxicity associated with DNA delivery, as well as yield higher rates of editing in cell lines and CD34<sup>+</sup> HSPCs.<sup>100,152</sup> The identification of novel Cas9 proteins isolated from diverse prokaryotes or other Cas9-like nucleases that are smaller than the widely used *Streptococcus pyogenes*-derived Cas9 may facilitate delivery efficiency, particularly for viral vectors.<sup>53</sup> In addition, chemical modification of sgRNAs enhances editing efficiency in primary hematopoietic cells and CD34<sup>+</sup> HSPCs.<sup>152</sup>

BMT poses risks to SCD and  $\beta$ -thalassemia patients beyond GVHD as reviewed in Lucarelli et al.<sup>158</sup> Myeloablative or submyeloablative conditioning will be required to allow for engraftment of edited HSPCs. The inverse relationship between conditioning and engraftment rates suggests that myeloablative regimens maximize the likelihood of clinically beneficial engraftment rates. However, a myeloablative approach has elevated risk of BMT-associated morbidity and mortality.<sup>158</sup> The optimal conditioning regimen for autologous BMT of genome-edited HSPCs requires investigation and may vary from patient to patient depending on the extent of end-organ damage resulting from vaso-occlusive events in SCD and/or iron overload in  $\beta$ -thalassemia patients. Given the risks associated with BMT, clinical guidelines for treatment may be stratified based on clinical disease severity for both SCD and  $\beta$ -thalassemia patients. BMT represents a viable therapeutic avenue in developed countries in spite of the associated risks, albeit the price of allogeneic BMT exceeds \$200 000 (in the United States).<sup>159</sup> The extent of infrastructure and resources required for BMT restricts its wide use in developing countries,<sup>160,161</sup> which have the highest prevalence of  $\beta$ -globin disorders.<sup>162</sup>

## Steps to clinical translation

Although clinical translation of therapeutic genome editing for the  $\beta$ -hemoglobinopathies is appealing, several steps must be taken before the vision can become a reality. (1) Target selection, (2) delivery of editing reagents to HSCs, and (3) empirical testing of off-target potential must all be addressed and optimized. Targets that may be chosen for clinical development are summarized in Figure 1 and Table 1. (1) Strategies that rely on NHEJ are likely to be the first attempted using current technologies due to the dominance of NHEJ in quiescent HSCs and overall high efficiency of NHEJ as compared with HDR. At present, disruption of the core *BCL11A* enhancer sequences within the +58 DHS by NHEJ appears quite favorable in terms of potency of an effect on HbF expression and sparing of consequences for nonerythroid lineages. It may also be possible to combine an NHEJ-based approach with an HDR strategy or delivery of antisickling adult hemoglobin to enhance potential clinical benefit.<sup>163,164</sup> (2) Transient delivery (electroporation or nonintegrating viral vectors) represents a safer alternative to stable integration of genome-editing components due to reduced risk of insertional mutagenesis and risk of off-target cleavage, as well as freedom from the uncertainty of long-term expression of genome-editing tools in CD34<sup>+</sup> HSPCs. Transient delivery also necessitates high levels of on-target editing within a shorter window of time prior to loss of the genome-editing components through cell division. One possibility would be to enrich for edited cells prior to BMT of autologous cells,<sup>60</sup> which could be further enhanced by strategies to expand HSCs ex vivo.<sup>102-104</sup> (3) Off-target cleavages represent a legitimate concern for therapeutic genome editing. Newly developed techniques allow for unbiased genome-wide identification of off-target mutagenesis.<sup>165,166</sup> Various methods have been reported that aim to enhance on-target vs off-target specificity. These include use of Cas9 nickase, truncated guides, dimeric RNA-guided FokI nucleases, and rationally engineered enhanced specificity Cas9.<sup>51,52,68,167-169</sup> In addition, alternative RNA-directed nucleases (Cpf1) or modified Cas9

derivatives with reduced off-target cleavage potential appear to be steps toward “clean” editing reagents. It will be necessary to empirically test the optimized editing reagents for off-target cleavage potential and assess the associated risk of inappropriate DSBs within the genome. As methods to predict and detect off-target cleavages continue to improve, it may become possible to screen autologous genome-edited cells prior to BMT for possible pathogenic off-target mutations. “CD34<sup>+</sup> humanized” mice, NOD-SCID- $\gamma$  mice with bone marrow-engrafted human CD34<sup>+</sup> HSPCs, can be used to evaluate the safety of genome-editing tools as these models can demonstrate multilineage reconstitution, self-renewal, and the ability to monitor leukemogenesis. However, due to the inability to model all human hematopoietic lineages, notably the erythroid lineage, and general limitations of chimera mouse models, humanized mice have limitations in assessing safety of genome-editing treatments in vivo. Furthermore, most off-target cleavages will exhibit a neutral effect on cellular fitness, whereas only rare off-target cleavages will be pathogenic, including induction of myelodysplastic syndrome or leukemic disorders. It will be important to enhance off-target cleavage prediction and detection methods to minimize risk of these rare pathogenic mutations.

One additional challenge for clinical development is harvesting CD34<sup>+</sup> HSPCs for autologous stem cell transplantation from patients with  $\beta$ -globin disorders. Sufficient numbers of CD34<sup>+</sup> HSPCs for BMT can be harvested from 2 sources, peripheral blood or bone marrow. Harvest of CD34<sup>+</sup> HSPCs from peripheral blood is preferred due its minimal invasiveness and higher yield of CD34<sup>+</sup> HSPCs following mobilization by granulocyte colony-stimulating factor (G-CSF).<sup>170</sup> Use of G-CSF as a mobilizing agent is generally well tolerated for healthy adults and cancer patients. However, there are significant risks of G-CSF administration for patients with  $\beta$ -globin disorders. SCD patients have significant risk of vaso-occlusive events, acute chest syndrome, multi-organ system failure, and death,<sup>171</sup> whereas  $\beta$ -thalassemia patients are susceptible to splenic rupture, hyperleukocytosis, and thrombosis.<sup>170</sup> Plerixafor is an alternative mobilizing agent that may provide a safer option to G-CSF.<sup>170,172,173</sup> Although the effect of plerixafor in SCD patients requires investigation, it has been shown to be safe and effective in both splenectomized and nonsplenectomized  $\beta$ -thalassemia patients. In contrast, although G-CSF was well tolerated in nonsplenectomized patients, it resulted in hyperleukocytosis and lower yield of CD34<sup>+</sup> HSPCs as compared with plerixafor in splenectomized  $\beta$ -thalassemia patients.<sup>172</sup> Combination of plerixafor with a reduced dose of G-CSF to avoid adverse effects has been shown to be superior to either agent alone.<sup>170,173</sup> Therefore, plerixafor or combination G-CSF/plerixafor mobilization may provide a safe avenue for peripheral blood CD34<sup>+</sup> HSPC harvests for  $\beta$ -thalassemia patients. Until acceptable protocols for mobilization of CD34<sup>+</sup> HSPCs are established for SCD patients, traditional bone marrow harvesting may be required. It may be advisable as well to test ex vivo editing efficiencies and maintenance of modified cells upon transfer into suitable immunodeficient mice for CD34<sup>+</sup> HSPCs obtained by different methods to ensure optimization for clinical use.

## Conclusions

The technological advances in genome manipulation are breathtaking in terms of the speed with which they have been reported in the past several years. The potential of genome-editing approaches for clinical benefit in the  $\beta$ -globin disorders is immense. Besides the choice of the editing platform and its delivery to repopulating cells within CD34<sup>+</sup> HSPC harvests, a major factor in considering application to these conditions is the target sequences to be modified. If the goal is precise

gene correction, the desired sequence alteration is clear. This strategy relies on HDR, and at the moment must await improved protocols for HDR in bona fide repopulating cells for clinical implementation.

Reactivation of HbF is an attractive approach, as it might be “one size fits all” in principle, suitable for both SCD and the  $\beta$ -thalassemias. The precise levels of pan-cellular HbF necessary for clinical benefit remain elusive, but is hypothesized to be  $\geq 20\%$  for SCD and likely somewhat higher in  $\beta$ -thalassemia (Figure 2). Due to the inability to precisely model HbF control experimentally, it may be difficult to assess the minimal threshold for clinical benefit in a laboratory setting. Furthermore, it is unlikely that HSCs undergoing therapeutic genome editing based on the strategies reviewed here will have a selective advantage in vivo. However, results from mixed chimerism allogeneic transplant demonstrate that low levels of chimerism can produce clinical benefit due to the survival advantage of normal red blood cells.<sup>74</sup>

Given the current state of genome-editing technologies, HbF induction mediated by NHEJ repair may provide a long-sought “silver bullet” for therapy. As such, harnessing the power of genome-editing tools may finally allow for therapeutic exploitation of the deep understanding of the genetics of hemoglobin and lead to a genome-editing-based therapeutic option for the  $\beta$ -hemoglobinopathies in the near future.

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## Authorship

Contribution: M.C.C. reviewed the literature; and M.C.C. and S.H.O. wrote the manuscript.

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