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Emerging Genetic Therapy for Sickle Cell Disease

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Abstract

The genetic basis of sickle cell disease (SCD) was elucidated >60 years ago, yet current therapy does not rely on this knowledge. Recent advances raise prospects for improved, and perhaps curative, treatment. First, transcription factors, BCL11A and LRF/ZBTB7A, that mediate silencing of the β-like fetal (γ-) globin gene after birth have been identified and demonstrated to act at the γ-globin promoters, precisely at recognition sequences disrupted in rare individuals with hereditary persistence of fetal hemoglobin. Second, transformative advances in gene editing and progress in lentiviral gene therapy provide diverse opportunities for genetic strategies to cure SCD. Approaches include hematopoietic gene therapy by globin gene addition, gene editing to correct the SCD mutation, and genetic manipulations to enhance fetal hemoglobin production, a potent modifier of the clinical phenotype. Clinical trials may soon identify efficacious and safe genetic approaches to the ultimate goal of cure for SCD.

INTRODUCTION

Using a custom-made electrophoresis apparatus, nearly 70 years ago, Pauling and colleagues detected a charge difference in the hemoglobin of individuals with sickle cell disease (SCD) and anointed SCD the first "molecular disease" (1). Ingram's (2) discovery eight years later of the β-globin chain position-6 glutamic acid>valine substitution validated Pauling's finding and set the stage for biophysical and structural studies of how a single amino acid change initiates sickling by rendering the hemoglobin tetramer ($\alpha_2\beta^s_2$) prone to forming rod-like polymers that deform and damage red blood cells. Despite knowing the genetic basis of SCD for seven decades, we are humbled by the recognition that no current therapies rely on molecular or genetic understanding. The structures and DNA sequences of the human α- and β-globin gene clusters were determined >35 years ago, yet a positive impact of this science for patients with SCD is unrealized. Recent progress on the molecular genetics of hemoglobin expression and gene therapy offers new hope that patients with SCD, and also those with β-thalassemia, may finally receive benefit.

SICKLE CELL DISEASE: ORIGINS, CLINICAL HETEROGENEITY, AND CURRENT MANAGEMENT

The high prevalence of SCD in endemic regions is attributed to relative protection of SCD carriers from malaria. Classic studies in the 1980s relying on different haplotypes of human β-globin gene clusters defined by restriction fragment length polymorphisms (RFLPs) were consistent with multiple, independent origins of the β^S mutation over the course of human evolution (3). Nonetheless, whole-genome-sequence-based haplotype analysis has recently challenged this conclusion and argues instead for a single origin of SCD 7,300 years ago in a malaria-endemic region of Africa (4). Within ~2,400 years, selection drove the frequency of the β^S allele to ~12% at equilibrium. Although SCD is a simple, Mendelian disorder, the clinical presentation and course of the disease are notoriously unpredictable and variable in severity. Apart from the episodic nature of many consequences of SCD, the major genetic modifiers are the level of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) and reduced α-globin production (α-thalassemia).

The impact of SCD is global and increasing. In the United States, current prevalence estimates for SCD are 70,000–100,000 which is associated with ~$1 billion in health expenditures. In sub-Saharan Africa, ~300,000 children are born with SCD each year and face 75–90% mortality by age five due to infectious disease. In the coming decades, a marked increase in disease prevalence will further strain health systems (5).

Management of patients with SCD is supportive, consisting principally of pain control and penicillin prophylaxis for functional asplenia. For those patients who have a stroke, intractable chronic pain, or repeated splenic sequestration, chronic transfusion is indicated. Standard of care also includes oral administration of hydroxyurea (HU), a chemotherapeutic agent previously given for chronic myeloid leukemia and polycythemia vera. HU was first proposed for use in SCD after experiments in primates showed that its administration led to increased HbF (6). Approval by the US Food and Drug Administration (FDA) in 1998 was based on reduction of painful crises (7). While HU elevates HbF modestly in primates and in many patients with SCD, the mechanism(s) by which it affects disease severity is unclear and most likely is not merely a reflection of HbF level. HU treatment leads to an average increase in red blood cell size, which may reduce sickling due to a decrease in cellular hemoglobin concentration, and alters cell kinetics. HU is beneficial for many patients and is probably underutilized. However, not all patients respond to or can be maintained on HU, and the drug does not correct the underlying hematology of SCD. As such, it is a halfway measure for a disorder that cries out for better management.

Bone marrow transplantation is curative but requires donor hematopoietic stem cells (HSCs) from a histocompatible donor and myeloablative preconditioning of the host. These procedures are associated with graft-versus-host disease and fertility loss, among other generic complications of bone marrow transplantation. In addition, donor availability is limited in populations most affected by SCD due, in part, to the ethnic makeup of existing stem cell banks. Gene therapy offers a means of definitive treatment with the patient's own stem cells and without the risk of graft-versus-host disease.

All clinical features of SCD can be traced to the properties of HbS and its effects on red blood cell lifespan. To reverse the underlying pathophysiology, one must block polymerization or provide nonsickling hemoglobin. Yet, current therapies are aimed largely at reducing the effects of secondary or tertiary clinical consequences, including pain, inflammation, and leukocyte adhesiveness.

HEMOGLOBIN: THE CENTRAL PROBLEM IN SICKLE CELL DISEASE

Efforts to circumvent the inherent property of HbS to polymerize are of two types. One approach seeks to develop small-molecule inhibitors of the β^S polymer formation, a strategy that leverages detailed structural knowledge of the hemoglobin tetramer. Early attempts along these lines were pioneered by Cerami & Manning (8), who discovered that potassium cyanate blocked HbS polymerization. The impracticability of administering these agents to patients prevented further development. Recently, Global Blood, Inc., has revived this approach through screening for chemicals that shift the oxygen-dissociation curve, since only deoxyhemoglobin is susceptible to polymerization in SCD. Clinical trials are under way to determine tolerability and efficacy of a lead compound, GBT440 (9).

The second broad category addresses the central physiology of SCD by altering the hemoglobin composition of the red cell. Several approaches are being explored. In what may now be considered traditional gene therapy, an additional globin gene—either a nonsickling β-chain variant or γ-chain (for HbF)—is expressed by lentiviral transduction of $CD34^+$ donor cells and marrow reconstitution (10). Alternatively, one might repair the β^S mutation through gene editing (11). A different strategy relies on the potent antisickling effects of HbF and seeks to enhance endogenous γ-globin gene expression through manipulation of the normal factors and pathways responsible for HbF silencing in the adult (12).

Here, we focus specifically on genetic approaches aimed at improved treatment for patients with SCD. As the rationale for much current activity in the field centers on enhancement of HbF expression, we first discuss advances in our understanding of how HbF is normally silenced in the transition from fetal to adult life. Transformative developments in genetics, namely genome-wide association studies (GWAS) (13) and gene editing with clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology (14), have driven recent work. Following a discussion of HbF regulation, we review progress in gene therapy and gene editing.

GENETIC CONTROL OF FETAL HEMOGLOBIN

HbF is a tetramer of two α-globin and two γ-globin chains, each the product of duplicated genes within the α- and β-globin gene clusters residing on 16p and 11p, respectively. In the β-globin cluster, the embryonic (ε), fetal (Gγ and Aγ), and adult (β) globin genes are transcribed sequentially during ontogeny under the control of a powerful enhancer, the locus control region (LCR), which must physically loop to each for it to be active. The switch from γ- to β-globin gene expression initiates slowly during the fetal liver stage of development and is complete only

several months into the postnatal period. In 1948, Watson (15) inferred a salutary effect of HbF on the phenotype of SCD from the clinical observation that newborns destined to suffer from the consequences of SCD as older children were unaffected. While HbF normally constitutes ~1% of total hemoglobin in adults, the level varies and is subject to strong genetic control. Such common variation, though, is associated with small changes in level, on the order of a few percent. Greater elevation of HbF, termed hereditary persistence of fetal hemoglobin (HPFH), is less common and, in rare examples, may be 10–30% in heterozygotes. The uncommon varieties of HPFH are due to substantial deletions within the β-globin gene cluster, including the β-globin gene itself, and single base substitutions, or smaller deletions, in either of the duplicated γ-globin gene promoters. These latter rare variants fall into three groups: a cluster of mutations at approximately −200, a single base substitution at −175, and a cluster of mutations at approximately −115 relative to the transcriptional start site (16). Until this year, a molecular explanation for the effects of these mutations was elusive.

REGULATORY FACTORS RESPONSIBLE FOR REPRESSION OF FETAL HEMOGLOBIN

Efforts to determine how the γ- and β-globin genes are selectively regulated date back to comparisons of the DNA sequences of their promoters and surrounding regions obtained >30 years ago. Although GATA1, TAL1/SCL, KLF1, and NF-E2 all constitute erythroid transcription factors (TFs), none exhibits the stage selectivity that would account for HbF silencing in the adult. The application of GWAS to HbF expression constituted a turning point and provided the first genetic evidence for loci controlling HbF (17, 18) (**Figure 1**). An unanticipated finding was association of HbF with the *BCL11A* locus on chromosome 2p. Associations with a broad region around the *c-Myb* locus (called *HBS1L-MYB*) and within the β-globin cluster were also observed. Taken together, these three common associations, which are found throughout all populations worldwide (though at varying frequencies), explain 25–50% of the genetic contribution to HbF level, a remarkably high fraction as compared with GWAS for most other traits or disorders (19, 20). Subsequent efforts to find additional associations by expanding cohort size are being undertaken but as yet have not identified other validated hits.

The *BCL11A* Locus

As a newcomer on the scene, the *BCL11A* locus was attractive as a candidate. *BCL11A*, a locus first discovered as the site of retroviral insertion in experimental murine leukemia (21), was reported as an essential gene for B lymphocyte development in knockout mice (22). No hint of a role in erythroid cells preceded the GWAS association with HbF. Knockdown of BCL11A by short hairpin RNA (shRNA) in cultured primary human $CD34^+$ stem and progenitor cells (HSPCs) undergoing erythroid differentiation reactivated HbF expression without substantial effects on cell growth or differentiation, or major changes in the RNA transcriptome (23). Consistent with a postulated role in HbF silencing, expression of BCL11A mRNA and protein is restricted to adult erythropoiesis and absent from embryonic erythroid cells of mouse and human. At the time the initial functional studies of BCL11A were performed, experience with manipulations that influence HbF level during ex vivo erythroid differentiation of HSPCs was limited. Indeed, we now appreciate that many perturbations retard erythroid differentiation or perturb cell cycle kinetics, leading to changes in HbF. The "forgiving" nature of this cell system may account for the historical lack of success in translating effects of small molecules or drugs that appear to

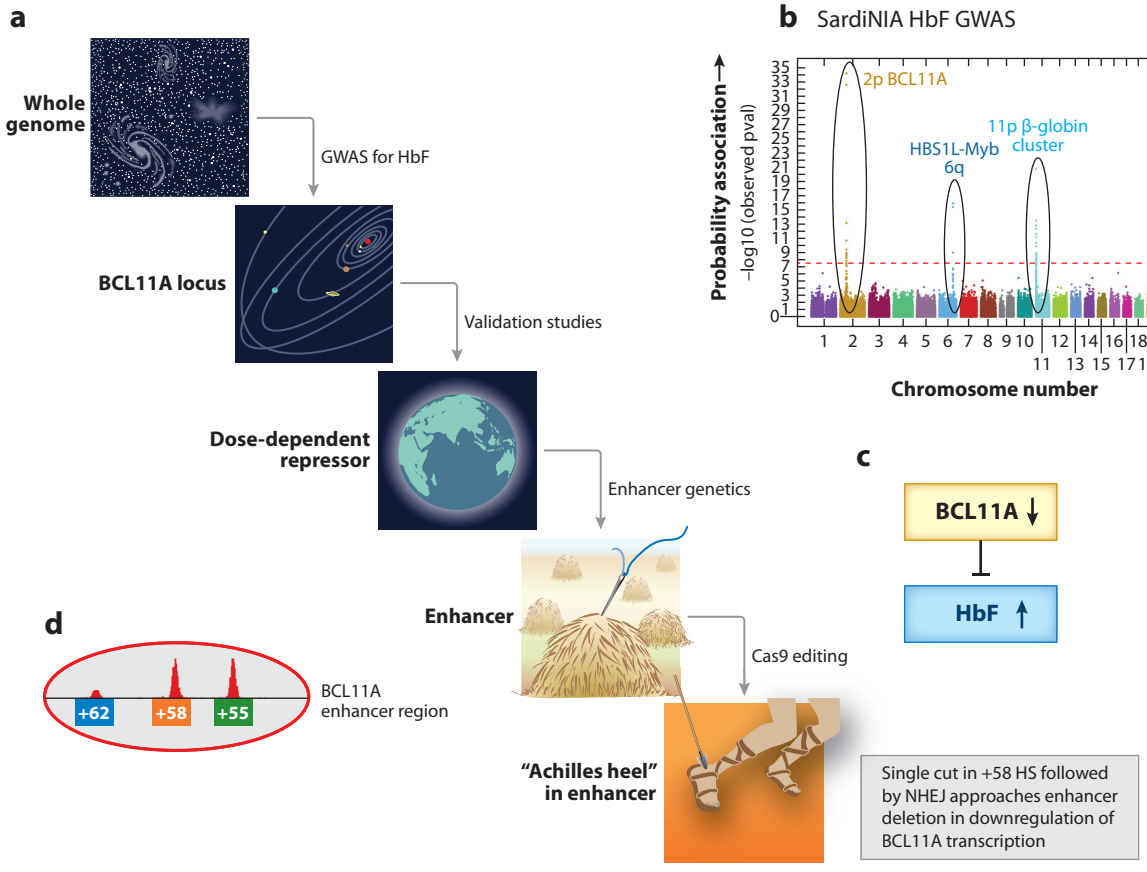

Figure 1

The path from GWAS to the "Achilles heel" in the *BCL11A* enhancer (*a*). GWAS in the Sardinian population revealed association of HbF level with three loci: *BCL11A*, *HBS1L-MYB*, and the β-globin cluster (summarized in the Manhattan plot in *b*) (18). Subsequent studies firmly established that BCL11A levels inversely correlate with HbF levels (*c*). SNPs in GWAS that associate most strongly with HbF level overlap three DNA hypersensitive sites (+62, +58, and +55 kb relative to the TSS) within intron-2 of the *BCL11A* gene (*d*) (25). Dense CRISPR/Cas9 gene editing localized the major activity of the enhancer to a discrete sequence of +58, a region that is favorable for gene editing by NHEJ to enhance HbF in patients (28). Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; GWAS, genome-wide association studies; HbF, fetal hemoglobin; NHEJ, nonhomologous end joining; SNP, single-nucleotide polymorphism; TSS, transcription start site.

reactivate HbF in culture to the in vivo setting or clinical trials. Fortunately, more rigorous tests have established a central role for BCL11A in HbF regulation.

Ablation of BCL11A in the erythroid lineage in engineered SCD mice is sufficient to correct red cell lifespan and hematology through reactivation of HbF, thereby establishing the substantial contribution of this single factor (24). The most highly genetically associated single-nucleotide polymorphisms (SNPs) in GWAS reside in an intronic region of *BCL11A* that is marked by three erythroid-specific DNAse I hypersensitive sites (HSs) (25). A causal SNP reduces allele-specific binding of GATA1 and TAL1/SCL, leading to a ~40% decrease in BCL11A expression and a modest HbF increase. The HSs are contained within a 10-kb segment of the intron that exhibits enhancer-associated histone modifications, erythroid-specific TF binding, and erythroid-specific

enhancer activity in transgenic mice. The enhancer is essential and strictly erythroid-specific in vivo (26). Near-saturating CRISPR/Cas9 mutagenesis of the enhancer in HUDEP-2 cells, an adult-type transformed progenitor cell line (27), revealed a discrete small region within the +58 HS, including a GATA1 binding site that confers the major activity of the entire enhancer (28). As noted below, this "Achilles heel" in the enhancer is a favorable target for clinical gene editing to boost HbF in SCD or β-thalassemia patients. The path from GWAS to identification of an "Achilles heel" is summarized in **Figure 1**.

Rare haploinsufficient individuals with microdeletions or point mutations on 2p encompassing and within the *BCL11A* gene suffer from an autistic-like developmental syndrome, yet are remarkably informative in revealing the in vivo phenotype of 50% expression (29–31). The mean HbF level among haploinsufficient individuals is ~13%, approximating classical HPFH. Overall hematopoiesis, including B lymphocyte maturation, is normal, despite a requirement for BCL11A in both HSCs and B cells (32). Experimental knockdown by shRNA shows that BCL11A is a continuous, quantitative regulator of HbF level (33). As expression of BCL11A is reduced to ~40% of the wild-type level, HbF expression rises to a therapeutic range for SCD.

The LRF/ZBTB7A Protein

The BTB-family protein LRF/ZBTB7A appears similar to BCL11A in potency of HbF repression, as revealed by knockout in HUDEP-2 cells (34). Loss of either BCL11A or LRF in these cells leads to ~45–50% HbF and combined knockout ~90–100%, suggesting that the two factors account for the bulk of HbF silencing. Both factors physically interact with the nucleosome remodeling and deacetylase (NuRD) chromatin remodeling complex, although the mechanisms of interaction differ (34, 35). BCL11A protein has a canonical NuRD-binding motif at its extreme N terminus that interacts with RBBP4, as defined by X-ray crystallography (36). The precise mode of interaction of LRF with NuRD is unknown. Although both repressors associate with NuRD, they are not found within common protein complexes.

The Nucleosome Remodeling and Deacetylase Complex

The multiprotein NuRD complex is unique in possessing both ATP-dependent chromatin remodeling and histone deacetylase (HDAC1/2) activities (37). The complex comprises several subunits, each of which has at least one alternative paralog, e.g., CHD3/4 (Mi2α/β), MBD2/3, RbAp46/48 (RBBP7/4), HDAC1/2, MTA1/2/3, and GATAD2A/B (p66α/β). Ginder and colleagues proposed that physical interaction between p66α and MBD2 recruits CHD4 for globin repression (38, 39). Potential recruitment of NuRD by BCL11A via its N-terminal NuRD-binding motif is consistent with this model (40). Moreover, CRISPR/Cas9 mutation of specific components of NuRD in HUDEP-2 cells leads to derepression of HbF (D.E.B. and S.H.O., unpublished data; 41). Remarkably, only one of the two paralogs for each subunit of NuRD is required for repression, hinting at combinatorial assembly of an active NuRD complex for HbF repression.

A PARSIMONIOUS MODEL FOR FETAL HEMOGLOBIN REPRESSION

Numerous TFs have been indirectly implicated in HbF silencing. Yet, recent findings converge on two principal repressor proteins (BCL11A and LRF) and the NuRD complex. Whether there exist additional repressors that contribute directly, and significantly, to HbF silencing is unknown, but prospects for other major TFs are diminished by recent observations relating BCL11A and LRF to rare HPFH mutants (**Figure 2**).

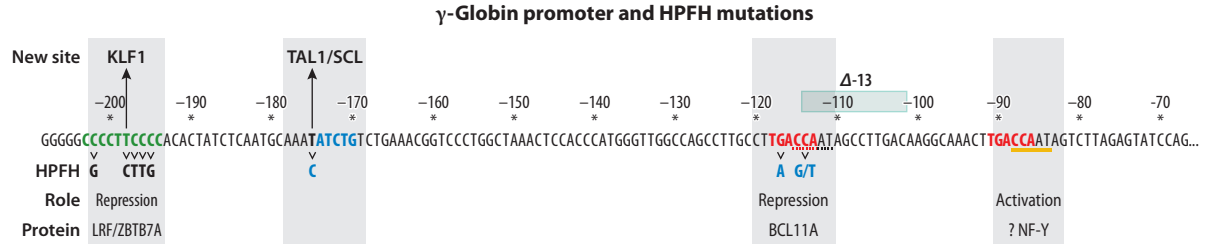

Figure 2

The DNA sequence of the human γ-globin gene (either Gγ or Aγ) promoter from approximately −210 to −67 relative to the transcription start site is shown. Mutations identified in rare individuals with hereditary persistent fetal hemoglobin (HPFH) are shown below the wild-type sequence. Regions bound by LRF/ZBTB7A and BCL11A are boxed, with their recognition motifs shown in green and red, respectively. Blue indicates the recognition motif for TAL1/SCL. The naturally occurring 13-base-pair HPFH deletion (70) is depicted as a box above the wild-type sequence. Two HPFH mutations generate new binding sites for KLF1 and TAL1/SCL (44, 45). The TGACCA motif at −86 to −91 is a potential binding site for BCL11A but is not occupied in vivo in chromatin (40). It may constitute a site at which the ubiquitous transcription factor NF-Y activates γ-globin expression.

Using an innovative candidate approach, Crossley and associates asked if BCL11A and LRF might recognize sequences in the −200 and −115 regions of the γ-globin promoters where clustered single-base HPFH mutants lie (42). They reasoned that independent mutations within a discrete region might disrupt binding of a repressor. In confirmation of this prediction, they observed that nuclear extracts of heterologous cells expressing zinc-finger domains of BCL11A and LRF bound nucleotide sequences derived from the −115 and −200 regions, respectively, and failed to bind HPFH mutant sequences in gel shift assays.

Our studies provide additional insight into the direct action of BCL11A at the γ-globin promoters (40). An unbiased screen identified TGACCA as the DNA recognition sequence of BCL11A. Remarkably, this motif is present in all embryonic and fetal-expressed globin promoters of human and mouse, and it is duplicated in the γ-globin promoters. The −115 HPFH region includes the distal motif of the duplication. The TGACCA motifs in the promoter overlap CCAAT, the recognition sequence for the ubiquitous factor NF-Y. The TGACCA motif within the embryonic/fetal promoters may have escaped prior attention due to overlap with the canonical CCAAT boxes. Application of a new method for high-resolution mapping of protein occupancy in chromatin [CUT&RUN (43)] revealed that in vivo binding of BCL11A occurs principally at the distal motif of the γ-globin promoters, consistent with the absence of known HPFH mutations within the proximal motif. Editing of the distal motif by CRISPR/Cas9 in HUDEP-2 cells prevents BCL11A binding and leads to increased chromatin accessibility of the promoter and high-level HbF expression.

Taken together, these data show that HPFH alleles with substitutions in the −200 and −115 regions of the promoters fail to bind LRF and BCL11A, respectively. Thus, repression of γ-globin expression occurs largely through direct action of these factors at the promoter. This straightforward model contrasts with earlier suggestions that invoked long-distance chromatin interactions within the β-globin locus as most critical for HbF silencing. Crossley and colleagues also reasoned that the single HPFH T>C mutation at −175 of the promoter might create a new site for a positive acting factor, rather than displace a repressor, and have shown that TAL1/SCL binds the variant HPFH sequence, thereby confirming this hypothesis (44). Furthermore, the −198T>C HPFH promoter variant creates a new binding site for the transcriptional activator KLF1 (45). These recent studies represent a turning point in the search for the TFs that mediate the effects of these HPFH mutations.

DEFINITIVE GENE THERAPY APPROACHES

A hemoglobin-centered view of SCD, suggesting β^S globin gene correction, globin gene addition, or induction of HbF as definitive approaches, has inspired diverse genetic therapeutic approaches at various stages of translation to the clinic. These strategies are also applicable to the β-thalassemias, characterized by quantitative β-globin deficiency.

Advances in cell therapy and genetic manipulation have laid a strong foundation for curative therapies. The paradigm is to obtain HSCs, genetically manipulate them ex vivo, and return them to the patient, yielding a lifelong hematopoietic autograft that produces modified red cells.

Acquiring cells for autologous therapy of SCD patients presents special challenges, since conventional granulocyte colony-stimulating factor mobilization of HSPCs is contraindicated due to the risk of precipitating serious vaso-occlusive events (46) and to the need for multiple marrow harvests under anesthesia to obtain enough cells for hematopoietic reconstitution (47). Recent studies indicate that plerixafor, a CXCR4 antagonist, can be safely used to mobilize HSPCs to peripheral blood in SCD patients (48–50a). Hypertransfusion in advance of gene therapy may facilitate collection and enhance engraftment of HSCs in SCD while avoiding risk of vaso-occlusive events (49, 51).

Globin Gene Addition

Transfer of globin genes to HSCs for therapy has been contemplated since the dawn of the molecular biology era. Initial work demonstrated that globin gene regulatory elements could be harnessed to drive high-level erythroid-restricted expression of a globin transgene. Over the past decade, the field of HSC gene therapy has shifted from γ-retroviral to lentiviral vectors. With the current generation of lentiviral vectors, promising clinical results for a variety of genetic disorders have been obtained without evidence of insertional toxicity (52).

Results in preclinical studies nearly 20 years ago demonstrated cure in mouse models of SCD and β-thalassemia (53, 54). After fits and starts (55), an international group of investigators recently reported experience with lentiviral gene therapy in both SCD and β-thalassemia (51, 56). Trials used a vector expressing an antisickling β-globin variant, T87Q, distinguishable from endogenous HbA or HbF by high-performance liquid chromatography. β-Thalassemia patients of three broad genotypes received ex vivo HSC lentiviral gene therapy (56). Of eight patients with the most severe genotype, $\beta^0\beta^0$, six remained transfusion dependent. Two patients who stopped transfusions remained anemic, with hemoglobin levels of 9–10 g/dL. Of nine patients with the milder $\beta^E\beta^0$ genotype who stopped transfusions, two maintained hemoglobin levels below 10 g/dL and one required intermittent transfusion. Of five patients with other β-globin genotypes, four stopped transfusions, though two remained anemic. Beyond β-globin genotype, the major predictor of response was vector copy number. In the reported trial, the ranges of vector copy numbers were 0.3–2.1 copies/genome in the infused cell product and 0.1–4.2 copies/genome in peripheral blood after a year. In summary, results of the trial were encouraging, but variability of response, particularly in patients with more severe genotypes, indicates that therapy is not yet optimal.

The response to lentiviral gene therapy in SCD has been inconsistent (51). A satisfactory outcome in a single patient is reported. However, of seven other patients treated with a similar protocol, none showed sustained production of transgenic hemoglobin. The poor response was attributed to low rates of transduction and low doses of infused cells. Two trials at academic centers are open, although results have not yet been reported (**Table 1**) (12).

A critical determinant of clinical outcome is the degree of gene transfer, which is influenced predominantly by the vector copy number in the infused cell product. Also important are the

Table 1 Gene therapy approaches in clinical development for sickle cell disease

Title	ClinicalTrials.gov identifier	Status[a]	Molecular strategy	Sponsoring institution	Notes
Gene Transfer for Patients With Sickle Cell Disease	NCT02186418	Open, recruiting	Lentiviral globin addition	Cincinnati Children's Hospital Medical Center	
Gene Transfer for Sickle Cell Disease	NCT03282656	Open, recruiting	Lentiviral anti-BCL11A shRNA	Boston Children's Hospital	
Stem Cell Gene Therapy for Sickle Cell Disease	NCT02247843	Open, recruiting	Lentiviral globin addition	University of California at Los Angeles	
A Study Evaluating the Safety and Efficacy of the LentiGlobin BB305 Drug Product in Severe Sickle Cell Disease	NCT02140554	Open, recruiting	Lentiviral globin addition	Bluebird bio	Related open studies in β-thalassemia: NCT03207009, NCT02906202
Gene-Edited Cell Therapy BIVV003 to Treat Sickle Cell Disease		FDA IND accepted	Gene editing *BCL11A* enhancer	Bioverativ/Sangamo	Related open studies in β-thalassemia: NCT03432364
CRISPR/Cas9 Gene Edited Treatment for Sickle Cell Disease and β-Thalassemia		FDA IND planned	Gene editing *BCL11A* enhancer	CRISPR Therapeutics/ Vertex	European Clinical Trial Application submitted for β-thalassemia

[a]Status is based on review of **https://www.clinicaltrials.gov** on May 27, 2018.
Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; FDA, US Food and Drug Administration; IND, investigational new drug.

number of CD34 cells infused and intensity of preparative conditioning. The distribution of transgene in cells, both in the cell product and in the engrafted cells, is an associated variable that is typically not reported but could influence outcome, since widely distributed transgene copies might be more beneficial than concentration of more transgenes in fewer cells. As compared to a prior $\beta^E\beta^0$-thalassemia lentiviral gene therapy subject treated a decade ago who showed oligoclonal hematopoiesis, the recent set of β-thalassemia subjects has exhibited more polyclonal hematopoiesis (56).

These results underscore the need to develop gene transfer protocols that ensure efficient and consistent delivery of the therapeutic globin gene cargo to HSCs. Long-term monitoring of patients to evaluate both safety and efficacy is necessary, as are thorough data collection and transparent data reporting to avoid selective presentation of favorable findings.

Gene Modification Technology

The extraordinary advances in gene editing provide new tools that expand possible therapeutic options. Gene editing is a set of technologies in which programmable DNA-binding factors (endonucleases) are directed to a particular genomic sequence where they make enduring modifications. The nucleases produce targeted double-strand breaks (DSBs) in mammalian genomes

that in turn result in insertions and deletions (indels) by imprecise end joining at the cleavage site [nonhomologous end joining (NHEJ)] and, in the presence of extrachromosomal donor DNA sequences, enable competing templated homologous repair [homology-directed repair (HDR)] (57, 58). Designer zinc-finger nucleases and transcription activator-like effector nucleases (TALENs) employ an arrayed assembly of peptide modules to bind specific trinucleotide and mononucleotide sequences, respectively. In the CRISPR platform, Cas9 (or a variety of related nucleases) is guided to specific genomic regions by RNA (guide RNAs). A dazzling assortment of Cas9-like nucleases with different specificities, targeting ranges, and efficiency is available, based on both naturally occurring and synthetic forms. For example, tethering various DNA- or chromatin-modifying moieties to catalytically inactive or nickase forms of Cas9 may direct biochemical modifications to specific gene loci. Of these strategies, base editing is most exciting, as DNA base changes are produced in the absence of DSBs (59, 60).

Each of the major classes of genome editing outcome—NHEJ, HDR, and HDR-related pathways (microhomology-mediated deletions, typically 5–50 base pairs in length)—could potentially be exploited in therapy of SCD.

Globin Gene Repair

The most straightforward approach would be correction of the SCD mutation (A>T, resulting in Glu6>Val) by HDR. In a homozygous cell, correction of just one of the two alleles would convert a patient's genetic condition from SCD to sickle trait ($\beta^A\beta^S$). A single strategy would apply to all SCD patients; this is not the case with β-thalassemia and many other genetic diseases, where diverse genotypes underlie similar phenotypes. Correction of the β^S mutation has been achieved with various nucleases, including zinc-finger nucleases and Cas9, and DNA donor templates, including single-stranded oligonucleotides and adeno-associated viruses (11, 61, 62). The efficiency of correction was very low in initial efforts. With improvements in gene editing technology and careful attention to experimental conditions, substantial frequencies of gene repair have been reported. Since DSBs precede HDR, imprecise NHEJ alleles represent competing outcomes that reduce overall correction. Thus, gene disruption converts β^S- to β-thalassemic alleles, thereby complicating precise repair. Additionally, HSCs, which are largely quiescent, favor NHEJ at the expense of HDR even more than progenitors do (63, 64). Therefore, gene repair frequencies, as measured in bulk $CD34^+$ HSPCs, overestimate the fraction of corrected alleles in engrafting HSCs. Currently, gene editing in HSCs is best quantified by human-to-mouse xenotransplantation, though the predictive power of this assay for human autotransplant performance is uncertain. Stimulation of the cell cycle or inhibition of NHEJ repair may favor HDR. But the impact of such strategies on long-term repopulating potential and DNA damage accumulation is unknown. Base editing is an attractive strategy as it avoids issues related to DSBs. To date, however, this technology does not allow for transversion mutations, such as T>A, which are required to correct the β^S mutation (59, 60). Another approach for DSB-free mutation correction utilizes peptide nucleic acids (65). In this method, binding of the peptide nucleic acid to target sequences stimulates templated repair from a DNA donor without DSB (66). The modest efficiency of repair by this method presently limits its clinical application.

HbF Induction

A variety of genome editing outcomes could be harnessed to achieve HbF induction (**Table 2**). An attractive feature of HbF induction leverages the reciprocal expression of β- and γ-globin genes within the same globin cluster due to competition for the LCR. Hence, increased HbF benefits

Table 2 Therapeutic genome editing strategies for sickle cell disease

Locus	BCL11A enhancer	HBB cluster	HBG promoter	HBB
Repair mode	NHEJ	NHEJ	MMEJ	HDR
Cell cycle restricted	–	–	+	+
Efficient in HSCs	+	?	?	+/–
Extrachromosomal donor DNA required	–	–	–	+
Cleavages	1	2	1	1
Competing edits	–	Indels, inversion	Deletion, inversion	Indels
Natural variant mimicked	–	Deletional HPFH	Nondeletional HPFH	β^A

Abbreviations: HDR, homology-directed repair; HPFH, hereditary persistence of fetal hemoglobin; HSC, hematopoietic stem cell; MMEJ, microhomology-mediated end joining; NHEJ, nonhomologous end joining.

SCD therapy by two mechanisms: HbF inhibition of HbS polymerization and HbF substitution for HbS.

Paired DSBs can yield large precise deletions by joining of semisynchronously produced DNA ends (67). Several investigators have reported proof-of-principle for genome editing with two cleavages to produce HPFH-associated large deletions or similar rearrangements of the β-globin gene cluster (68, 69). The efficiency of these maneuvers in engrafting HSCs has not been reported. Competing genome editing outcomes, such as multifocal small indels and inversions, accompany these deletions and therefore may limit clinical application (67).

As reviewed above, HPFH is also caused by mutations in the DNA-recognition sequences for BCL11A and LRF in the γ-globin promoters (**Figure 2**) (40, 42). Disruption of these sequences would mimic such rare genetic variation. Weiss and colleagues (70) reported that following Cas9-mediated cleavage within the γ-globin promoters at approximately −115, the most frequent outcome is a 13-base-pair naturally occurring HPFH-associated deletion, itself the product of microhomology-mediated end-joining repair (**Figure 2**). Edits at this target site may occur at either of the duplicated Aγ- and Gγ-globin genes, as well as interstitial deletions and inversions between these. In a given edited cell, many combinations of these edits might ensue. Among genome editing alleles, the 13-base-pair deletion appears to be the most potent HbF inducer, although even shorter indels might disrupt binding of BCL11A and induce HbF (40, 42). The efficiency with which these alleles may be produced in HSCs is uncertain. One of us (D.E.B.) has observed at other loci following genome editing of $CD34^+$ HSPCs that microhomology repair alleles are relatively disfavored in HSCs as compared to progenitor cells.

The most tractable gene editing approach to HbF induction at this time relies on generation of short indels by NHEJ at the "Achilles heel" of the erythroid enhancer of *BCL11A*, which includes a GATA1 binding site (**Figure 1**) (28, 71, 72). Since NHEJ is active at all stages of the cell cycle, including in nondividing cells, this strategy is free of concerns that apply to HDR gene correction. The approach is being advanced for clinical development. Investigational new drug (IND) applications of two biotechnology entities are approved or under submission (see **Table 1**).

Hybrid approaches that combine lentiviral gene therapy and HbF induction are yet other options. Blobel and colleagues have proposed a strategy based on lentiviral expression of a synthetic DNA-binding protein that forces a loop between the γ-globin promoter and the LCR (73). An alternative approach relies on erythroid-restricted expression of shRNA to knock down BCL11A transcripts. Potent HbF induction has been observed in preclinical studies of mice engrafted with transduced SCD patient HSPCs. This novel clinical trial is active and enrolling at Boston Children's Hospital (33).

Looking Ahead

Approaches that successfully employ globin gene addition or correction, or induction of HbF, ensure production of red cells that have a longer half-life than SCD red cells. Therefore, correction of only a subset of HSCs would likely have a major therapeutic benefit, given selective erythrocyte advantage. Indeed, clinical experience in recipients of allogeneic transplant with mixed chimerism suggests that sequelae of SCD are not observed above a threshold of ~20% normal HSCs, an observation consistent with mathematical modeling (74, 75).

Although initial clinical trial subjects are adults and adolescents, it is expected that younger SCD patients might benefit the most, as they have less accumulated, irreversible organ damage. However, the risks of late effects from conditioning for transplant, in addition to any intrinsic risks of gene modification, may also be greatest in such patients. Future advances in autologous therapy could minimize the toxicity of myeloablative preconditioning (76), or perhaps even allow in vivo gene modification.

As gene therapies demonstrate clinical efficacy and advance toward FDA approval, challenges of reimbursement for these resource-intensive procedures must be addressed. Although the economic benefits of curing a serious lifelong disease cannot be underestimated, the cost of expensive one-time therapy poses a challenge to payers. To demonstrate a value proposition, therapy should be curative, consistent, and durable. If so, novel payment structures will be developed, including amortized or subsidized payments or incentivized pay-for-performance mechanisms (77). The greatest challenge ahead is the scaling problem of making genetic therapies available in geographic areas where the global burden of disease is greatest but resources the scarcest (5).

DISCLOSURE STATEMENT

D.E.B. holds issued patents on BCL11A as a target for HbF induction. S.H.O. serves on the Scientific Advisory Board of Syros, Inc.; serves as a compensated consultant for Epizyme, Inc.; and holds issued patents on BCL11A and reactivation of HbF for therapy of hemoglobin disorders.

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