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*Annual Review of Medicine*

# Diverse Approaches to Gene Therapy of Sickle Cell Disease

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Annu. Rev. Med. 2023. 74:473–87

First published as a Review in Advance on September 6, 2022

The *Annual Review of Medicine* is online at med.annualreviews.org

https://doi.org/10.1146/annurev-med-042921-021707

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## Keywords

sickle cell disease, hematopoietic stem cell transplant, gene therapy, gene editing, lentiviral vector, CRISPR

## Abstract

Sickle cell disease (SCD) results from a single base pair change in the sixth codon of the β-globin chain of hemoglobin, which promotes aggregation of deoxyhemoglobin, increasing rigidity of red blood cells and causing vaso-occlusive and hemolytic complications. Allogeneic transplant of hematopoietic stem cells (HSCs) can eliminate SCD manifestations but is limited by absence of well-matched donors and immune complications. Gene therapy with transplantation of autologous HSCs that are gene-modified may provide similar benefits without the immune complications. Much progress has been made, and patients are realizing significant clinical improvements in multiple trials using different approaches with lentiviral vector–mediated gene addition to inhibit hemoglobin aggregation. Gene editing approaches are under development to provide additional therapeutic opportunities. Gene therapy for SCD has advanced from an attractive concept to clinical reality.

# INTRODUCTION

## Genetics, Biochemistry and Clinical Manifestations of Sickle Cell Disease

Sickle cell disease (SCD) is a hemoglobinopathy caused by a single common mutation in the β-globin chain of hemoglobin in all affected persons, leading to an abnormal hemoglobin protein. It is most prevalent in sub-Saharan Africa, India, Saudi Arabia, and Mediterranean countries; it affects 300,000 to 400,000 newborns per year and about 20 million people worldwide (1). While the prevalence in the United States is significantly lower, it is estimated that approximately 100,000 persons have SCD in this country (2). SCD causes life-long complications leading to significant morbidity and a shortened life expectancy.

The classical mutation causing SCD, also known as hemoglobin SS disease, is a homozygous point mutation (A-T) in the sixth codon of the β-globin gene on chromosome 11. This mutation leads to a single amino acid substitution of glutamic acid to valine at position six within the β-globin chain. This single substitution characterizes sickle hemoglobin (HbS) and leads to the many clinical manifestations. Persons with hemoglobin SS disease do not synthesize hemoglobin A (HbA) and instead have >75% HbS. Deoxygenation of HbS leads to polymerization of the hemoglobin, followed by the formation of a gelatinous network of fibrous polymers and the transformation of red blood cells (RBCs) into rigid, sickle-shaped cells. Coinheritance of hemoglobin C, hemoglobin E, or β-thalassemia alleles (including β-thal$^0$ or β-thal$^+$, among others) leads to forms of SCD with varying phenotypes (3, 4).

The sickled RBCs, being less deformable, cause capillary blockage, sustain cell membrane damage, and undergo hemolysis, all of which contribute to the clinical manifestations of SCD. The phenotypic presentations of patients with SCD range significantly in severity, including both acute and chronic complications. Most individuals in developed nations are diagnosed early in infancy via newborn screening. If undiagnosed at birth, SCD may begin to manifest as early as 6 months of age, coinciding with the natural decline of fetal hemoglobin (HbF) synthesis and the onset of production of adult hemoglobin containing β-globin.

Persons with SCD experience life-long intravascular hemolysis and chronic anemia. The most common acute complication is veno-occlusive events (VOEs), which are episodic microvascular occlusions (5). These events may manifest as dactylitis (mainly in infants and toddlers); bone pain and abdominal pain due to microvascular occlusion of mesenteric blood vessels; and infarction of the liver, spleen, or lymph nodes. Other acute complications include acute chest syndrome, stroke, priapism, splenic sequestration, and transient pure RBC aplasia.

Great progress has been made in preventing childhood mortality; now >95% of children in developed countries survive to adulthood (6). With an increasing number of persons with SCD living longer, the chronic sequelae of the disease have become clinically salient. These chronic manifestations of SCD are largely secondary to chronic tissue damage and vasculopathy with secondary end-organ damage. Any organ system can be affected. The chronic complications most commonly noted include effects from silent stroke, pulmonary hypertension, renal and liver dysfunction, avascular necrosis of bones, retinopathy, and functional hyposplenism.

HbS can also be coinherited with α-thalassemia (7). Coinheritance is now well documented, as about one-third and one-half of patients of African and Middle Eastern or Indian descent, respectively, are noted to have α-thalassemia. This coinheritance is most often seen with deletion of one or two of the α-globin genes and leads to an overall decreased concentration of hemoglobin in the RBCs. Because the rate of HbS polymerization is dependent on the concentration of hemoglobin within the erythrocytes, this decrease in hemoglobin level results in a decrease in cellular damage. The phenotype of patients with coinheritance is characterized by higher hemoglobin levels,

lower mean corpuscular volume, less hemolysis, and therefore fewer complications associated with hemolysis.

When HbF levels are higher, such as at birth, patients with SCD have fewer or no clinical manifestations. Most patients do not have complications until after the decline in HbF synthesis that follows birth. It has also been observed that persons with coinciding hereditary persistence of fetal hemoglobin (HPFH) have a milder disease phenotype (8). HbF interferes with polymer formation of the HbS within RBCs (9). The known benefits of increasing HbF in patients with SCD has been the basis for development of various treatment options.

## Standard Care for Sickle Cell Disease

Much of the routine care for patients with SCD involves close follow-up for early indications of known serious complications or active management of acute or chronic complications. Routine screenings include frequent laboratory monitoring for anemia and hemolysis markers, transcranial Doppler ultrasounds for monitoring of intracranial blood vessel flow velocities, urinalysis to evaluate for micro-albuminurea and close monitoring for signs of infection, among others. Although there has been much progress in reducing early childhood mortality in developed countries, this has largely been due to early detection with newborn screening, penicillin prophylaxis, and vaccination against encapsulated microorganisms, which can cause sepsis in SCD patients who develop functional asplenia early in life.

Until recently, hydroxyurea was the only disease-modifying treatment option for patients with SCD (**Table 1**). Hydroxyurea is an oral chemotherapeutic that raises levels of HbF. First tested in SCD in 1984, it has been shown to decrease frequency of pain episodes, acute chest syndrome, transfusions, acute stroke, and hospitalizations (10). However, hydroxyurea does not completely ameliorate symptoms and does not fully prevent progression to the chronic complications of SCD.

Transfusion therapy is a management option with the purpose of decreasing the circulating HbS. There are various indications for either simple or exchange transfusions. Simple transfusions are indicated in patients with acute symptomatic anemia, aplastic crisis, symptomatic acute

**Table 1 Current approved pharmacologic therapies for sickle cell disease**

| Medication (year of FDA approval) | Approved age group | Mechanism of action | Physiologic effects | Clinical effects |
|---|---|---|---|---|
| Hydroxyurea (1998 in adults and 2017 in children) | >9 months | Ribonucleoside diphosphate reductase inhibitor | Increases HbF production<br>Decreases intracellular HbS polymerization | Decreases pain crises<br>Decreases acute chest syndrome episodes<br>Decreases blood transfusions<br>Decreases overall mortality |
| L-Glutamine (2017) | >5 years | Increases NADPH | Increases RBC reducing potential | Decreases pain crises<br>Decreases hospitalizations<br>Decreases acute chest syndrome episodes |
| Crizanlizumab (2019) | >16 years | Monoclonal antibody against P-selectin | Decreases erythrocyte and leukocyte adhesion | Decreases pain crises |
| Voxelotor (2019) | >12 years | Increases Hb affinity for oxygen | Delays production of deoxyhemoglobin<br>Decreases HbS polymerization | Increases baseline Hb > 1 g/dL<br>Decreases hemolysis markers; indirect bilirubin levels and % reticulocyte count |

Abbreviations: FDA, US Food and Drug Administration; Hb, hemoglobin; NADPH, nicotinamide adenine dinucleotide phosphate; RBC, red blood cell.

chest syndrome associated with anemia below baseline, splenic sequestration, and severe anemia, as well as in patients being prepared for anesthesia (11, 12). Exchange transfusions are indicated in patients with acute or impending cerebrovascular accident and symptomatic severe acute chest syndrome. Some patients also require continued, chronic simple or exchange transfusions based on the severity and frequency of SCD complications. Multiple or chronic transfusions can have serious side effects, including alloimmunization and transfusional hemochromatosis, and often require concurrent iron chelation therapy (12).

There had been little progress in the development of disease-modifying therapeutics until recent years (**Table 1**). L-Glutamine, an oral powder, was approved in 2017, almost 20 years after hydroxyurea (13, 14). The exact mechanism of action is unknown; L-glutamine increases nicotinamide adenine dinucleotide synthesis, which should help in preventing oxidative damage to RBCs and subsequently decreasing hemolysis. It has been shown to decrease VOEs and hospitalizations, although nearly three-quarters of patients in the phase III trial were also on hydroxyurea, suggesting that it should be used in combination (13, 14).

Voxelotor, a HbS polymerization inhibitor, was approved in 2019 (15). It is an oral medication, taken daily. Voxelotor binds reversibly to hemoglobin, stabilizing the oxygenated form and therefore preventing HbS polymerization by increasing hemoglobin's affinity to oxygen (16). Voxelotor has demonstrated a dose-dependent increase in hemoglobin affinity and an increase in baseline hemoglobin by at least 1.0 g/dL and a decrease in hemolysis markers. While this may be beneficial in helping patients to discontinue chronic transfusions, it has not been shown to decrease VOE frequency (15).

Crizanlizumab is another recently introduced disease-modifying option for patients with SCD. Crizanlizumab is a humanized, anti-P-selectin monoclonal antibody that is given intravenously. P-selectin is a cell adherence molecule that is expressed on the surface of endothelial cells and platelets when activated and is involved in the complex process that results in VOEs. Crizanlizumab was shown to decrease the annual rate of VOEs, regardless of concomitant hydroxyurea use (17).

All of the disease-modifying agents in **Table 1** have a benefit in the short term and act to prevent acute complications. The agents newer than hydroxyurea have not yet been evaluated longitudinally to evaluate long-term prevention of chronic complications of SCD.

## Allogeneic Hematopoietic Stem Cell Transplant for Sickle Cell Disease

Allogeneic hematopoietic stem cell transplant (HSCT) is currently the only proven curative option for patients with SCD. Highest overall survival (OS) rates are seen when HSCT is performed with a matched sibling donor (MSD); however, <20% of patients have a MSD available (18–20). Along with the highest OS (93–97%), MSD HSCTs also have the highest disease-free survival (82–100%), as well as the lowest rates of graft rejection (8–18%) and graft-versus-host disease (GVHD) (6–35%) (6). The lack of availability of MSDs for most patients with SCD has led to multiple studies evaluating the use of matched unrelated donors (MUD) and haploidentical donors. In general, results have been consistent, with haploidentical HSCT resulting in lower rates of GVHD and higher rates of graft rejection, while MUD HSCTs have demonstrated the reverse: lower rates of rejection and higher rates of GVHD (6). Use of umbilical cord blood as a stem cell source is typically limited by the fixed number of stem cells within a product, which makes them less likely to be utilized in adult patients given that the required stem cell dose is dictated by weight of the recipient. The SCURT trial (NCT00745420), published in 2016, aimed to evaluate unrelated donor sources in children with SCD; the umbilical cord blood arm of the trial was closed early due to high rates of rejection (21).

Overall, age has been shown to have a significant impact in prognosis and outcome following allogeneic HSCT. Increasing age (16 years and older) has also been associated with lower OS and event-free survival; increasing age has been correlated with graft failure and death. The rate of development of GVHD has also been significantly higher in patients over the age of 16 years.

While HSCT is the only curative option, it carries significant potential risks. Given that the phenotypic variation in SCD patients is so broad and that there are not good predictive models of life-long disease severity, evaluation for HSCT eligibility is largely based on current disease severity, and transplant is often reserved for those with severe disease (22). Severe disease is not typically present in pediatric patients, thus leading to frequent transplant in adult patients, who are known to be at higher risk for HSCT complications.

## GENE ADDITION: γ-RETROVIRAL AND LENTIVIRAL VECTORS

Autologous HSCT with gene-modified cells (gene therapy) has become a relevant approach as a curative option for SCD. One strategy involves genetically modifying hematopoietic stem cells (HSCs) with viral vectors to incorporate globin genes including γ-globin, γ/β-globin hybrids, and anti-sickling β-globin (1, 23) (**Figure 1**). Initially, γ-retroviruses were commonly used for clinical gene therapy. Their many advantages included stable and efficient integration; flexibility of target cell types that can be transduced; and the simplicity of their genomes, which facilitated manipulation to produce replication-incompetent vectors and stable packaging cell lines (24–25).

β-Globin γ-retroviral vectors that were developed and studied first in murine HSCs suffered from minimal transgene expression following hematopoietic reconstitution (26). These vectors were further optimized by the inclusion of the β-globin locus control region (LCR), an erythroid super-enhancer containing major regulatory elements that induced high levels of β-globin expression in murine erythroleukemia cells and in murine bone marrow cells in vivo (27). Apart from expression challenges, γ-retroviral vectors have limited capacity to carry the complex β-globin transgene intact; they require ex vivo culture of the target stem cells for several days to induce mitosis, due to the fact that integration can only occur during cell division; and they carry risks for insertional mutagenesis and oncogene activation (28). γ-Retroviral vectors tend to integrate near transcriptional start sites of genes and, due to their strong enhancer elements, they have the potential to alter expression of endogenous genes adjacent to sites of vector integration (29).

As gene therapy progressed, there was a shift in technology from γ-retroviral to lentiviral vectors (LVs). LVs, typically derived from HIV-1, showed greater promise for clinical application due to their ability to deliver larger and more complex DNA cassettes. This ability is crucial for globin vectors to achieve a high level of expression. Lentiviruses can also transduce and integrate into nondividing HSCs, promoting stable transgene expression, and they have a better safety profile than retroviruses due to their preference of integration across gene units and their ability to produce LVs at high titer without strong enhancer elements (30). The integration profile of LVs tends to be across regions of actively expressed genes with increased chromatin accessibility (29).

However, even with a safer integration profile, LVs still raise safety concerns due to the potential for insertional mutagenesis. To combat this, LVs have been modified to be self inactivating: The viral enhancer and promoter sequences have been removed, limiting *cis*-acting effects of the long terminal repeat (LTR) on cellular genes adjacent to vector integration sites (31, 32). A further modification to the 5′ LTR has also been implemented in the vector plasmid, where the U3 region is deleted and replaced with the cytomegalovirus promoter (31). Both of these changes

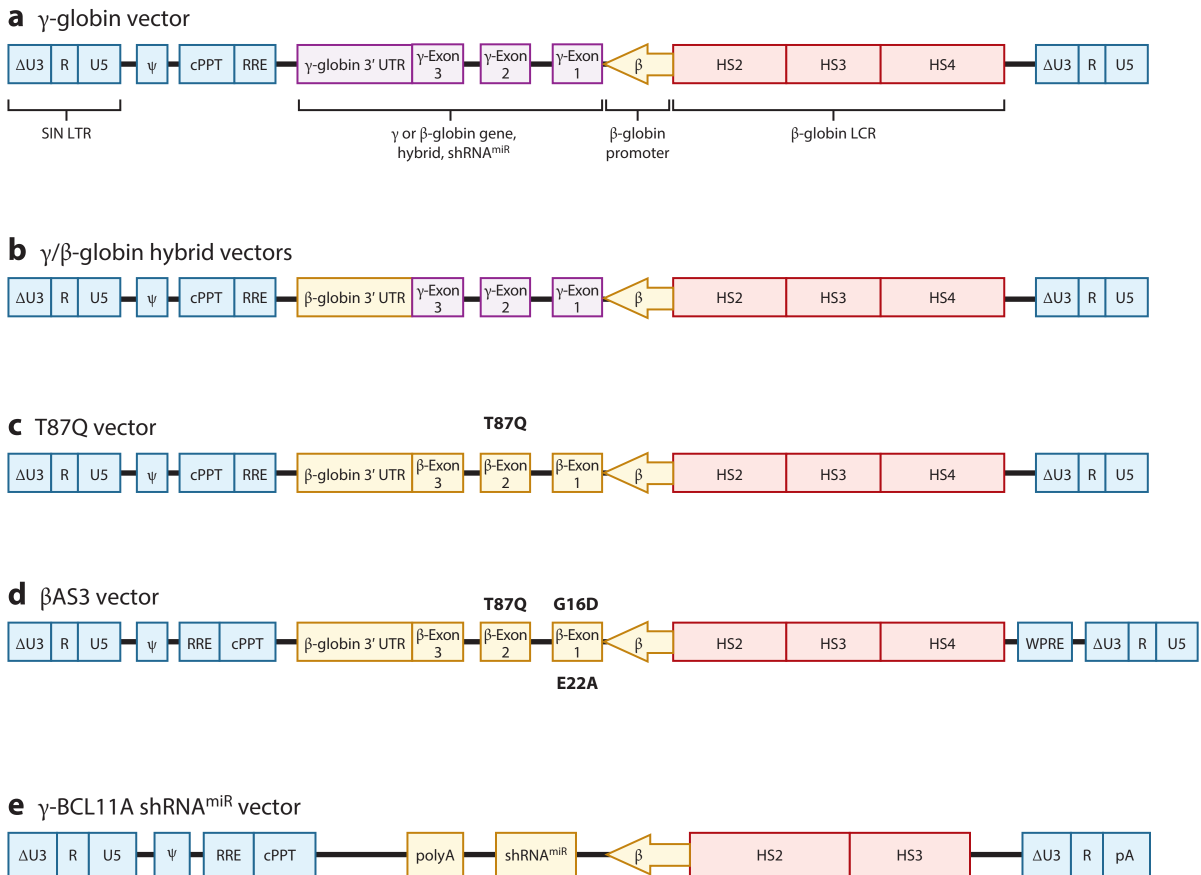


**Figure 1**

Lentiviral vector approaches for gene therapy of sickle cell disease (not drawn to scale). (*a*) γ-Globin lentiviral vector containing γ-globin gene coding and noncoding regions, β-globin promoter, and modified LCR elements HS2, HS3, HS4. (*b*) γ/β-Globin hybrid lentiviral vector containing γ-globin gene coding regions, β-globin 3′ UTR, β-globin promoter, and modified LCR elements HS2, HS3, HS4. (*c*) T87Q lentiviral vector containing a modified β-globin gene with a T87Q amino acid change to promote anti-sickling properties, β-globin promoter, and modified LCR elements HS2, HS3, HS4. (*d*) βAS3 lentiviral vector containing three anti-sickling amino acid changes (G16D, E22A, T87Q) in the β-globin gene, β-globin promoter, and modified LCR elements HS2, HS3, HS4. (*e*) *BCL11A* shRNA$^{miR}$ lentiviral vector containing *BCL11A* shRNA$^{miR}$, synthetic polyA, β-globin promoter, and modified LCR elements HS2 and HS3. All vectors are SIN lentiviral vectors (ΔU3). Definitions: Ψ, packaging signal; cPPT, central polypurine tract; HS, hypersensitive site; HS2, HS3, HS4, DNase hypersensitive sites 2, 3, and 4 from the β-globin LCR; LCR, locus control region; LTR, long terminal repeat; polyA, polyadenylation signal; RRE, rev-response element; shRNA$^{miR}$, microRNA-adapted short hairpin RNA; SIN, self-inactivating; UTR, untranslated region; WPRE, woodchuck hepatitis virus posttranscriptional regulator element.

enhance safety and decrease the chance of recombination to produce replication-competent lentivirus.

Apart from lentiviral safety modifications, β-globin vectors faced challenges due to low expression of the transgene, which limited therapeutic applicability. Recombinant LVs incorporating the key transcriptional regulatory elements of the β-globin LCR [DNase hypersensitive sites (HS): HS2, HS3, HS4] into the β-globin cassette addressed this limitation by allowing for stable and long-term expression in transduced bone marrow of β-thalassemic mice in primary and secondary transplant studies, which was the first time therapeutic levels of β-globin expression were demonstrated (33).

## γ-Globin-like Transgenes

Induction of HbF expression has been a popular strategy to combat SCD. The idea first arose from observations of SCD and β-thalassemia patients who also had HPFH, where RBCs have higher levels of HbF. Individuals with HPFH were shown to have less severe forms of SCD or β-thalassemia in comparison to individuals without HPFH (34). A genome-wide association study evaluated levels of HbF in patients with β-thalassemia. Variants in the *BCL11A* erythroid enhancer region led to HPFH, and the higher HbF expression resulted in less severe forms of β-thalassemia (35). Further studies showed that generating a *BCL11A* knockout in SCD mouse models corrected the pathogenic defects associated with SCD through increased HbF expression (36).

One strategy to elevate HbF expression is to design LVs to deliver the γ-globin gene. Vectors containing the γ-globin gene along with β-globin regulatory elements were shown to induce therapeutic levels of HbF to ameliorate SCD in Berkeley sickle mouse models (BERK) (37). Other modifications to γ-globin vectors involved replacing the γ-globin 3′ untranslated region (UTR) with the β-globin 3′ UTR to enhance γ-globin expression (38). Overall, γ-globin vectors with the β-globin 3′ UTR achieved better correction of SCD in BERK mice through increased mRNA stability, HbF expression, and RBC counts, compared to γ-globin vectors with the γ-globin 3′ UTR (38). **Table 2** summarizes clinical trials evaluating γ-globin vectors.

**$\beta^{A\text{-}T87Q}$-Globin.** Utilizing LVs expressing β-globin in RBCs already producing HbS is another potential approach to ameliorating SCD. One drawback of overexpression of the β-globin gene is the need for very high levels of expression of HbA to prevent HbS polymerization.

Generating a mutated codon 87 ($\beta^{A\text{-}T87Q}$) in β-globin that changes the amino acid from threonine to glutamine promotes anti-sickling activity by disrupting lateral contacts in the sickle hemoglobin fibers. This codon change is derived from the amino acid from γ-globin that is responsible for its ability to inhibit HbS polymerization (39). In vivo and kinetic studies using turbidimetry of RBC lysates showed that BERK mouse bone marrow transduced with $\beta^{A\text{-}T87Q}$-globin lentivirus had delayed HbS polymerization, and also that $\beta^{A\text{-}T87Q}$-globin corrected hematological parameters in mice (40). Currently, clinical trials conducted by Bluebird Bio are utilizing $\beta^{A\text{-}T87Q}$-globin (HBG-206) for patients with severe SCD (**Table 2**). $CD34^+$ cells were transduced with the LentiGlobin BB305 LV and, after autologous transplantation, patients had an average $HbA^{T87Q}$ expression of ∼40% of the total hemoglobin after infusion (median 17.3 months). A single infusion of LentiGlobin BB305-transduced autologous hematopoietic stem and progenitor cells (HSPCs) reduced hemolysis and completely resolved severe veno-occlusive crises and acute chest syndrome in the 25 patients that could be evaluated (41).

**$\beta^{AS3}$-Globin.** Additional anti-sickling modifications were studied and incorporated into LVs. Mutating glutamic acid at amino acid position 22 to alanine further enhances anti-sickling properties by disrupting axial contact in the sickle fiber (42). E22A in combination with T87Q significantly inhibits HbS polymerization. An additional modification (G16D) increases the affinity for the α-globin polypeptide, which gives the anti-sickling β-globin subunit a competitive advantage over the sickle subunits for forming hemoglobin tetramers. An anti-sickling globin containing all three anti-sickling codon modifications ($\beta^{AS3}$-globin) was incorporated into LV (Lenti/$\beta^{AS3}$) (43). This vector corrected hematologic and clinical findings in a mouse model of SCD and was also shown to efficiently transduce SCD patient bone marrow $CD34^+$ cells and induce therapeutic levels of $Hb\beta^{AS3}$-globin to correct RBC physiology (44). Clinical trials are currently utilizing $\beta^{AS3}$ self-inactivating LVs for patients with SCD (**Table 2**).

One major challenge for clinical applications of gene therapy that utilizes β-globin LVs is their low titer due to the large vector proviral length (45). It has been shown that smaller

**Table 2 Ongoing or completed gene therapy trials for sickle cell disease**

| Sponsor | Approach | ClinicalTrials.gov identifier | Status (phase) |
|---|---|---|---|
| ***Lentiviral vectors*** | | | |
| Bluebird Bio | LV encoding the human $\beta^{A\text{-}T87Q}$ globin gene | NCT02151526 | Completed (I) |
| Bluebird Bio | LV encoding the human $\beta^{A\text{-}T87Q}$ globin gene | NCT02140554 | Active/not recruiting (II) |
| Bluebird Bio | LV encoding the human $\beta^{A\text{-}T87Q}$ globin gene | NCT04293185 | Recruiting (III) |
| Aruvant Sciences GmbH | LV to express γ-globin | NCT02186418 | Recruiting (I/II) |
| Donald B. Kohn (UCLA) | LV to express an anti-sickling ($\beta^{AS3}$-globin) gene | NCT02247843 | Recruiting (I/II) |
| David Williams (BCH) | LV containing a short-hairpin RNA targeting *BCL11A* | NCT03282656 | Active/not recruiting (I) |
| Assistance Publique – Hôpitaux de Paris | LV expressing the $\beta^{AS3}$-globin gene | NCT03964792 | Recruiting (I/II) |
| ***Gene editing*** | | | |
| Vertex Pharmaceuticals | Autologous HSPCs modified with CRISPR-Cas9 at the erythroid lineage-specific enhancer of *BCL11A* | NCT03745287 | Active/not recruiting (II/III) |
| Novartis Pharmaceuticals | Two genome-edited HSPC products to reduce the biologic activity of BCL11A and increase HbF | NCT04443907 | Recruiting (I/II) |
| Mark Walters (UCSF) | Autologous HSPCs with sickle allele modified by the CRISPR-Cas9 ribonucleoprotein | NCT04774536 | Not yet recruiting (I/II) |
| Graphite Bio | CRISPR-Cas9 edited and sickle mutation-corrected HSPCs to convert HbS to HbA | NCT04819841 | Recruiting (I/II) |
| Editas Medicine | Autologous CRISPR gene-edited HSPCs | NCT04853576 | Recruiting (I/II) |

Abbreviations: BCH, Boston Children's Hospital; CRISPR-Cas9, clustered regularly interspaced short palindromic repeats–CRISPR-associated nuclease 9; HbA, hemoglobin A; HbF, fetal hemoglobin; HbS, sickle hemoglobin; HSPC, hematopoietic stem and progenitor cell; LV, lentiviral vector; UCLA, University of California Los Angeles; UCSF, University of California San Francisco.

LVs can be produced at 10–100-fold higher titers than β-globin LVs (46). In addition to larger genomes, β-globin LVs tend to contain complex expression cassettes that also have a negative impact on vector titer. Low titers make producing clinical-scale vector preparations under good manufacturing practices more expensive, leading to increased costs per patient dose. β-Globin LVs have also been shown to have reduced efficiencies for transduction of primary human HSCs, compared to simpler LVs. Less efficient transduction can lead to suboptimal gene transfer and transgene expression levels, resulting in suboptimal therapeutic benefit (46). A $\beta^{AS3}$-globin LV (GLOBE-AS3) designed with a short human β-globin promoter and a reduced LCR containing HS2 and HS3 elements enabled a higher titer while driving high transgene expression (47). $\beta^{AS3}$-Globin LVs have been even further optimized to reduce the proviral length (∼4.7 kb) by modifying the LCR size, which produced higher titers and gene transfer to HSPCs, and these smaller $\beta^{AS3}$-globin LVs are able to ameliorate the sickle phenotype in SCD mouse models (45).

## siRNA to BCL11A

Another approach to increasing HbF expression is through knockdown of *BCL11A* through RNA interference. Erythroid lineage–restricted knockdown of *BCL11A* is essential for allowing successful engraftment of HSPCs, while ubiquitous knockdown of *BCL11A* causes toxicity in HPSCs, leading to poor reconstitution after transplantation (48). Vectors containing a *BCL11A* microRNA-adapted short hairpin RNA ($shRNA^{miR}$) (BCH-BB694) expressed under the control of the β-globin promoter and regulatory elements derived from HS2 and HS3 of the LCR ameliorated the sickle phenotype in mice and induced 40% HbF induction in erythroid-differentiated SCD $CD34^{+}$ cells (49). A phase I clinical trial (BCH-BB694) showed a sustained increase of HbF levels, with a median of 30.5% of all hemoglobin levels, in six patients; none of the patients have had a severe veno-occlusive crisis or stroke post transplantation (**Table 2**) (50).

# GENE EDITING

In the past decade, new methods have been developed to perform direct editing in the genome of cells, which are being applied in a multitude of approaches for the treatment of SCD (**Figure 2**). Genetically engineered site-specific nucleases (SSNs) have been developed that can direct editing, ideally to a single base pair in the entire genome. These SSNs include ZFNs (zinc finger nucleases), TALENs (transcription activator-like effector nucleases), and CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats–CRISPR-associated nuclease 9) (51–55). Each of these SSNs can be introduced into target HSPCs by electroporation of expression plasmids, by in vitro transcribed mRNA, or as preformed ribonucleoprotein complexes of recombinant Cas9 protein with the single-guide RNA (sgRNA) that will be present transiently to initiate editing, but the SSNs do not persist in the cells long term.

Upon introduction to the HSPC, these SSNs produce DNA double-stranded breaks (DSBs) at their target site in the genome, which induces cellular DNA repair pathways. One repair pathway that is active throughout the cell cycle is non-homologous end joining, which reanneals the ends of the DNA, but often in an error-prone manner, leading to the insertion or deletion of some bases (indels) at the repair site. The indels may cause disruption of the target gene, disrupting the translational reading frame or adding or deleting some amino acids in the encoded protein when multiples of three bases have been added or deleted.

Another DNA repair pathway that may be induced is homology-directed repair (HDR), which repairs the break gap using a DNA template that is provided by a sister chromatid during cell division or provided as an additional nucleotide sequence reagent to serve as a donor of the intended sequence change. HDR is restricted to the S and G2 phases of the cell cycle, when DNA replication occurs, and thus gene editing using HDR is also restricted to cells that are actively cycling. Inducing a DSB near a target site for editing greatly increases the efficiency of HDR (56).

More recently, base editing has emerged as a highly precise method to modify single base pairs (57). Base editors use a fusion protein between Cas9, to locate the genomic target using a sgRNA, and an enzyme capable of deaminating a nucleotide (cytosine deaminase or adenosine deaminase). The Cas9 is modified to eliminate one of its nuclease domains, so that a single-stranded nick, not a DSB, is made. Instead, the deaminase enzyme is "parked" near the target base to modify it by deamination. During repair of the single-stranded nick made on the opposite strand by the modified Cas9, deaminated cytosine is interpreted as uracil and deaminated adenosine as guanosine. Thus, base editing can produce transversion type base pair changes (C:G → T:A or A:T → G:C). Serial improvements of the architecture of the Cas9-deaminase molecules have led to highly active

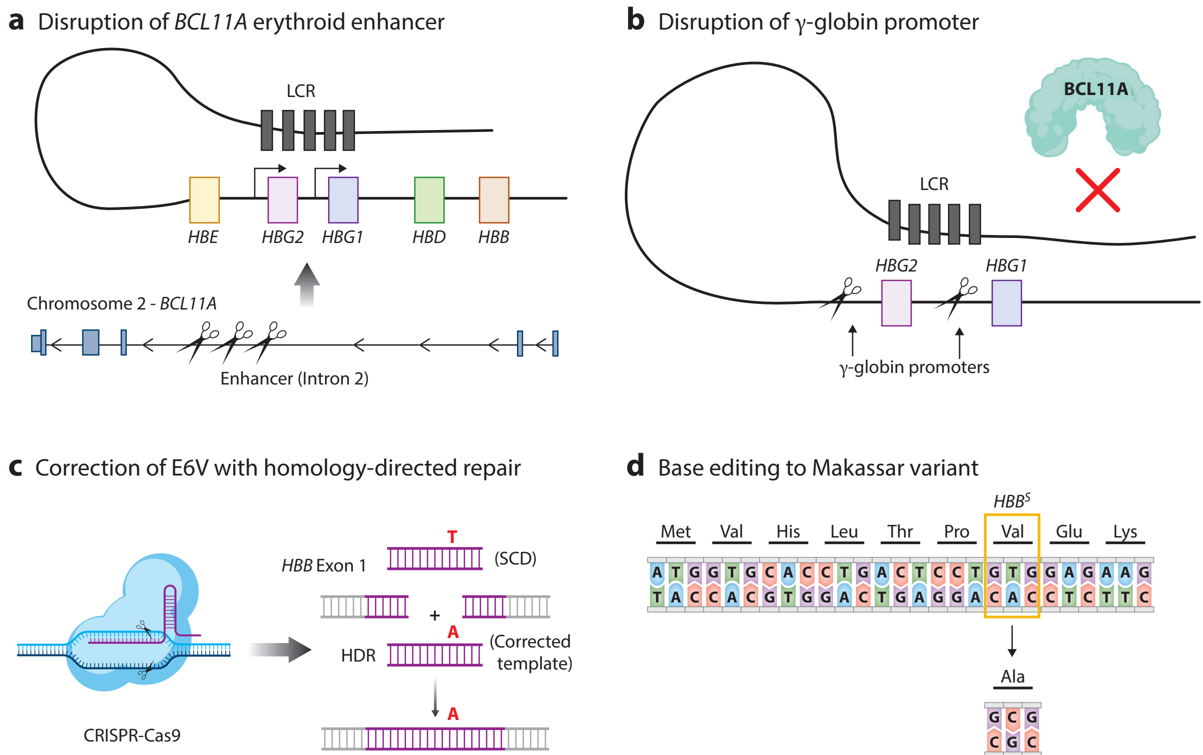


**Figure 2**

Gene editing approaches for gene therapy of sickle cell disease. (*a*) Disruption of *BCL11A* erythroid enhancer. Applying CRISPR-Cas9 to disrupt the erythroid enhancer region in intron 2 of *BCL11A* on chromosome 2 leads to BCL11A downregulation. Without BCL11A repression of γ-globin, the LCR will interact with *HBG2* and *HBG1*, promoting γ-globin expression. (*b*) Disruption of γ-globin promoter. Altering the *BCL11A* binding sequence in the γ-globin promoters of *HBG2* and *HBG1* will inhibit BCL11A binding and γ-globin repression. The LCR will interact with *HBG2* and *HBG1*, promoting γ-globin expression. (*c*) Correction of E6V with homology-directed repair. CRISPR-Cas9 and sgRNA are used to target the point mutation (T), and a corrected template is supplied to incorporate the wild-type nucleotide (A) with homology-directed repair. (*d*) Base editing to Makassar variant. Adenine base editing converts the GTG (Val) to the codon GCG (Ala) to produce a nonpathogenic variant ($HBB^G$). Abbreviations: CRISPR-Cas9, clustered regularly interspaced short palindromic repeats–CRISPR-associated nuclease 9; HDR, homology-directed repair; LCR, locus control region; SCD, sickle cell disease.

enzymes that act largely at the intended target site (58). Further advances in base editing technology are continuing, with prime editing and other variations allowing complex sequence overwrites to be made, and these will likely be applied to gene editing for SCD.

## BCL11A: A Prime Target for Gene Therapy of SCD

A highly studied target for gene editing in SCD is the *BCL11A* gene, encoding a transcriptional factor that serves to repress expression of fetal (γ-)globin, as described above (35, 59). Clinical efforts using ZFN and CRISPR-Cas9 are targeting the erythroid enhancer of *BCL11A* to preserve its expression in nonerythroid blood cell lineages, where it is needed for proper stem cell function and multilineage differentiation (60–62). Using optimized CRISPR editing reagents and HSPC manipulation techniques can lead to highly efficient disruption of the *BCL11A* erythroid enhancer in a large percentage of treated HSPCs that retain engraftment capacity and stem cell function (63).

Results from one clinical trial using CRISPR-mediated *BCL11A* disruption were reported by Frangoul et al. (64) (**Table 2**). Two patients, one with SCD and one with severe β-thalassemia, were transplanted with autologous HSPCs that were edited ex vivo using CRISPR-Cas9 to disrupt the erythroid enhancer of *BCL11A*. Both patients developed high levels of fetal globin in circulating erythrocytes, and the SCD patient did not have further VOEs. Other trials with this approach are expected to open soon. Base editing has also been used to mutagenize the *BCL11A* erythroid enhancer to block its repression of γ-globin (65).

A parallel approach to induce γ-globin expression uses CRISPR-Cas9 to introduce indels in the promoters of the γ-globin genes to disrupt the binding sites for BCL11A protein (66, 67). These deletions mimic a subset of naturally occurring HPFH-associated γ-globin gene promoter deletions. This approach may have the advantage of maintaining all functions of BCL11A protein except its repression of γ-globin.

## Direct Correction of the E6V Amino Acid Substitution in $HBB^S$

Several groups have developed HDR-mediated approaches to correct the canonical E6V glutamic acid–to–valine amino acid change in β-globin that causes SCD (68–71). Using ZFN or CRISPR to introduce a DSB near the sickle-causing mutation and providing a homologous repair template that contains the wild-type base at the mutation site can revert the mutation of $HBB^S$ to $HBB^A$, if the donor is used for HDR. Different methods have been used to provide the homologous donor, such as single-stranded oligodeoxynucleotides, or viral vectors that do not persist, such as adeno-associated virus 6 (AAV6), integrase-defective LVs, adenovirus, and others. Because of the restriction of HDR to the S and G2 phases of the cell cycle, with non-homologous end joining occurring at all times, it is challenging to achieve high levels of HDR-mediated gene correction in long-term primitive HSCs. Clinical trials of direct correction of the SCD-causing mutation are expected to begin in 2022.

## Base Editing to Convert E6V to an Alternative Amino Acid That Does Not Cause Sickling

While current base editing methods are not capable of performing the transition base pair change from T:A to A:T needed to revert the sickle-causing mutation in $HBB^S$, Newby et al. (72) reported using an adenine base editor to convert the GTG sickle codon (encoding valine) to the novel GCG codon, which encodes alanine. Alanine is present in a known nonpathogenic hemoglobin Makassar ($HBB^G$) variant (73). They reported highly efficient base editing in a mouse model of SCD and $CD34^+$ cells from patients with SCD, largely preventing sickling manifestations. A clinical trial is expected to be performed in the near future.

## In Vivo Gene Therapy for Sickle Cell Disease

The ultimate mode for using gene editing of HSCs to treat SCD will be to perform the editing in vivo, rather than through ex vivo HSC isolation with chemotherapy conditioning, as is currently done in all of the approaches discussed above. If it were possible to administer gene editing reagents systemically and achieve efficient editing in HSCs in vivo, the treatment would be much more widely applied and not reliant upon the high-acuity medical setting of the bone marrow transplant unit (74). The dream of a gene therapy that may be distributed world-wide, especially to the majority of patients with SCD in lower-resourced areas, is highly motivating, and a great deal of effort is being brought to bear for developing solutions (75).

## CONCLUSIONS

It is not possible to identify the "best" approach for gene therapy for SCD at this point. LVs are working effectively in patients, with several different anti-sickling genes. There have been no vector-related clinical adverse events; in the two patients who developed myelodysplastic syndrome and acute myeloid leukemia after LV gene therapy, the LV did not appear to be causal. Other approaches have only early clinical data (CRISPR-Cas9) or are just now entering clinical trials (E6V correction, base editing). LVs do have risks of causing insertional genotoxicity, albeit relatively small based on the safety profile in the current population of treated patients. Risks of genotoxicity from various editing approaches are not fully known. Off-target editing may cause genotoxicity, and CRISPR-Cas9 may have risks from translocations or loss of chromosomal arms. The long-term disease-modifying efficacy and safety of high levels of HbF or hemoglobin Makassar will take several decades of observation to assess. Meanwhile, certainly there will continue to be new approaches that may be even better.

## DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

## ACKNOWLEDGMENTS

K.H. is supported by U2CDK129496 KUH-ART: Advanced Research Training in Kidney Disease, Urology and Hematology and S.L.W. is supported by NIH T32 HL086345 Training Grant in Developmental Hematology.

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