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Sickle Cell Disease: From Genetics to Curative Approaches

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Abstract

Sickle cell disease (SCD) is a monogenic blood disease caused by a point mutation in the gene coding for β-globin. The abnormal hemoglobin [sickle hemoglobin (HbS)] polymerizes under low-oxygen conditions and causes red blood cells to sickle. The clinical presentation varies from very severe (with acute pain, chronic pain, and early mortality) to normal (few complications and a normal life span). The variability of SCD might be due (in part) to various genetic modulators. First, we review the main genetic factors, polymorphisms, and modifier genes that influence the expression of globin or otherwise modulate the severity of SCD. Considering SCD as a complex, multifactorial disorder is important for the development of appropriate pharmacological and genetic treatments. Second, we review the

characteristics, advantages, and disadvantages of the latest advances in gene therapy for SCD, from lentiviral-vector-based approaches to gene-editing strategies.

INTRODUCTION

Sickle cell disease (SCD) is a genetic disorder that causes the production of an abnormal hemoglobin (Hb) molecule, sickle hemoglobin (HbS). HbS tends to polymerize, which leads to the deformation (sickling) of red blood cells (RBCs) and thus blood vessel obstruction (55). The SCD mutation is thought to have arisen between 1100 and 200 BC, probably in several places in Africa and Asia (101). Four specific haplotypes have been defined in Africa (the Senegal, Benin, Bantu, and Cameroon haplotypes), and one has been defined in Asia (the Arab-Indian haplotype).

SCD is the world's most prevalent genetic disease (19) and the most common life-threatening genetic disorder in Africa (32). Every year, more than 300,000 children are born with SCD (85). The majority of cases are found in sub-Saharan Africa, the Middle East, and India (117). This distribution is explained partly by the high level of protection against malaria conferred by the sickle cell trait: It is possible to carry a mutated allele without being clinically affected by SCD. The sickle cell gene appeared in areas where malaria was (and still is) widespread, and genetic selection has operated since then (82, 86). Migration might explain the current prevalence of SCD in people of Mediterranean, Middle Eastern, Caucasian, Indian, Hispanic, Native American, and other ancestries (117).

CLINICAL CHARACTERISTICS AND SOCIAL IMPACT OF SICKLE CELL DISEASE

The main manifestations of SCD are anemia (due to the short half-life of sickle RBCs) and particularly painful vaso-occlusive crises (VOCs) that lead to poor blood circulation and poor tissue oxygenation. Some of the signs and symptoms of SCD (such as acute chest pain syndrome, stroke, and priapism) constitute medical emergencies.

The chronic manifestations of SCD are due to the combination of the characteristic hemolytic anemia and functional impairments of the organs affected by the VOCs. For example, functional asplenia caused by splenic infarctions means that patients with SCD are abnormally susceptible to infections by encapsulated germs (*Streptococcus pneumoniae*, *Neisseria meningitidis*, etc.).

The clinical expression of SCD varies radically from one patient to another. Some patients develop very few complications before the age of 60 or 70, while others develop many episodes of acute chest pain syndrome and VOCs very early in life. A third category of patients presents cerebrovascular disease with a high risk of stroke during childhood or adolescence (98).

SCD can lead to stigma, isolation, social exclusion, and discrimination. The disease not only affects the person with SCD but also has repercussions for the family as a whole (47, 107). Furthermore, several studies have shown that SCD is associated with a considerable economic burden and a great impact on public health (42). The mean annual cost of SCD has been estimated at US$10,000 for a child and US$34,000 for an adult (88, 103).

Newborn screening for SCD has been implemented in several countries; the goal is to improve outcomes and reduce mortality by detecting the disease early and allowing rapid treatment (54, 65, 113). The country-scale benefit of newborn screening is likely to be confirmed in coming years, and clinical protocols for the comprehensive care of all affected newborns in the various countries are likely to be shared. In parallel, genetic counseling and prenatal diagnosis for SCD carriers might help to raise awareness of the potential consequences of carrying abnormal Hb traits, assess the risk of recurrence, and promote family testing (59, 93, 102).

SCD: sickle cell disease

Hb: hemoglobin

RBC: red blood cell

VOC: vaso-occlusive crisis

GENETICS OF SICKLE CELL DISEASE

LCR: locus control region

SNP: single-nucleotide polymorphism

The gene clusters encoding the human α-like and β-like globin chains are located on chromosomes 16 and 11, respectively, and are arranged in the order of their expression during development. The α-globin cluster consists of three genes [ζ (*HBZ*), α2 (*HBA1*), and α1 (*HBA2*)], and the β-globin cluster consists of five genes [ε (*HBE1*), Gγ (*HBG2*), Aγ (*HBG1*), δ (*HBD*), and β (*HBB*)]. The main Hb variants in fetuses and adults, respectively, are fetal hemoglobin (HbF, composed of two α-globin chains and two γ-globin chains) and adult hemoglobin (HbA, composed of two α-globin chains and two β-globin chains). Fetal-to-adult Hb switching occurs soon after birth.

Many genetic factors are involved in the regulation of globin gene expression, including promoters and locus control regions (LCRs) containing DNase-hypersensitive sites (HSs). The α-globin LCR contains four enhancers, referred to as multispecies conserved sequences R1–4. The β-globin LCR contains five HSs (HS1–5), which are the regulatory regions involved in the stimulation of β-like globin gene transcription (17). In the β-globin locus, the β-globin LCR loops with the target gene promoters to activate and regulate the expression of the β-like genes (105).

From a historical standpoint (**Figure 1**), the research leading to the discovery of SCD was started by James Herrick in Chicago in 1910, with the identification of sickle-shaped RBCs in a blood smear from a student of Caribbean ancestry (95). Further research (12, 50, 68, 77, 84) led to the discovery that SCD is an autosomal recessive disease caused by a single point mutation in the β-globin gene located at 11p11.5. A mutation in codon 6 (GAG6GTG) results in the replacement of glutamic acid by valine (E6V). The HbS formed by the combination of α-globin with the mutant sickle β-globin (β^S) subunits polymerizes when it is in a deoxygenated state, leading to deformation (sickling) of the RBCs (4).

Several studies have demonstrated that SCD corresponds to a set of inherited blood disorders rather than to a single disease (116). Several different genotypes are associated with SCD because the disease results from the combination of two alleles of the β-globin gene, at least one of which carries the E6V mutation. Depending on the concomitant presence of other common Hb variants, three genotypes can be defined, which differ in their clinical presentation: HbS/HbS (accounting for 70% of cases of SCD), HbS/hemoglobin C (HbC) (25%), and HbS/Hb-thalassemia (5%). Heterozygous HbA/HbS individuals are healthy carriers. The type of β-thalassemia variant has a great impact on the severity of the disease, with severe clinical symptoms in HbS/β^0-thalassemia (i.e., when a β^0-thalassemic mutation results in a lack of β-globin chains) and some variants of HbS/β^+-thalassemia [such as IVS-I-5 (G>C), when β^+-thalassemic mutations allow the synthesis of the β-globin chain] and milder disease for the other types of HbS/β^+-thalassemia.

Many other factors contribute to the pleiotropic clinical manifestations in SCD, including the presence of several polymorphisms (at least five different haplotypes) in the β-globin gene cluster (75). It has long been known that the haplotype is a prognostic factor in SCD. The haplotypes are related to the phenotypic expression and severity of SCD, together with the patient's residual level of HbF. The HbF level is lowest in patients with the Bantu haplotype and then increases in the following order: Cameroon, Benin, Senegal, and Arab-Indian (2, 73, 75). In fact, the HbF level is directly correlated with the ability to inhibit the polymerization of deoxygenated HbS (20, 92, 120). Indeed, the presence of HbF reduces the HbS concentration, and the γ-globin chains, once incorporated into the Hb tetramer, prevent Hb polymerization. As mentioned above, a high concentration of HbF is present in the Senegal and Arab-Indian haplotypes in general and in carriers of the single-nucleotide polymorphism (SNP) C>T mutation [located 158 bp 5′ to the γ-globin *HBG2* gene (rs7482144) and determining the generation of a restriction site for the XmnI enzyme] in particular. Patients carrying this mutation present higher reticulocyte counts and total Hb levels (10, 30).

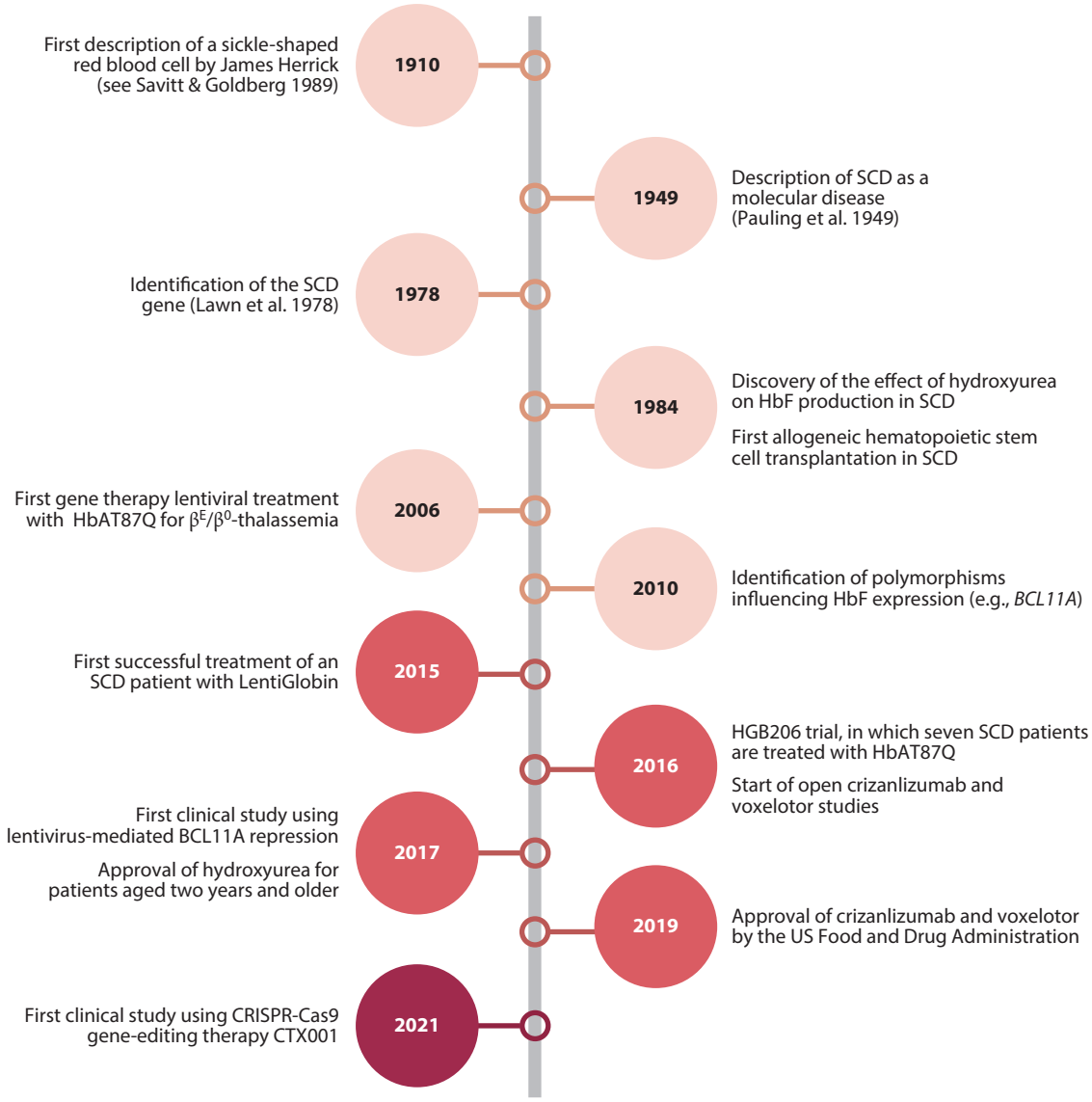


Figure 1

Milestones in research and clinical progress on SCD. Abbreviations: HbF, fetal hemoglobin; SCD, sickle cell disease.

The coinheritance of SCD mutations with α-thalassemia is also associated with favorable SCD clinical outcomes in Africa, including a low intracellular Hb concentration that decreases the likelihood of Hb polymerization, reduces hemolytic anemia, delays the onset of clinical manifestations, and increases survival times (94, 109).

In addition to the β-globin locus, SNPs mapping to other two loci (the *BCL11A* locus and the *HBS1L-MYB* intergenic region) are associated with higher levels of HbF. First, the *BCL11A* gene is located on chromosome 2p16 and encodes a transcription factor that inhibits expression of the γ-globin gene (71). The repression complex formed by the BCL11A protein and various

cofactors binds to several sites in the β-globin locus, including the *HBG* promoters (64, 69). Several polymorphisms mapping to the *BCL11A* gene (rs4671393, rs10189857, and rs11886868) limit its transcription and therefore are associated with higher HbF levels in people with SCD (1, 2, 119). Second, the *HBS1L-MYB* intergenic region is located on chromosome 6q23.3 and contains the *MYB* gene (an oncogene from the myeloblastic transcription factor family). The gene product acts on erythropoiesis by controlling the differentiation/proliferation balance. MYB contributes to HbF silencing. The SNPs rs9399137 and rs6665037 in this region reduce MYB expression and increase γ-globin expression (51).

HPFH: hereditary persistence of fetal hemoglobin

Importantly, coinheritance of the SCD-causing mutation and the genetic variants in the β-globin locus causing hereditary persistence of fetal hemoglobin (HPFH) in adult life ameliorates the clinical status of HbSS individuals. For example, some HPFH mutations map to the *HBG* promoter and either create de novo binding sites for transcriptional activators (TAL1, GATA1, and KLF1) or disrupt binding sites for transcriptional repressors (BCL11A and LRF) (36).

Polymorphisms in genes not involved in erythropoiesis can also influence the clinical presentation of SCD and the response to treatment. These include polymorphisms in the promoter region of the *UGT1A1* gene, encoding uridine diphosphate glucuronosyltransferase (11, 111). Polymorphisms in the *UGT1A1* gene reduce the production of this enzyme and thus increase levels of nonconjugated bilirubin, which are already elevated in people with SCD because of hemolysis; this results in a greater incidence of gallstones and symptomatic biliary disease in people with SCD (even in childhood). Other polymorphisms in genes encoding inflammatory proteins are also associated with a specific clinical presentation in people with SCD. For example, polymorphisms in the gene encoding TGF-β are known to be associated with vascular disease and notably increase the incidence of vascular complications (such as priapism, pulmonary hypertension, and osteonecrosis) in people with SCD (8, 15).

Lastly, polymorphisms in genes involved in oxidative stress [*SOD* (rs4880), *MPO* (rs2333227), *XO* (rs207454), and *NFE2L2* (rs35652124)] can also account for the great interindividual differences in the clinical profile seen in SCD. Oxidative stress is high in SCD and has a major role in the pathophysiology of the disease by promoting RBC damage, inflammation, and endothelial–vascular dysfunction (81). Therefore, polymorphisms in pro- or antioxidant genes can modulate the severity of the disease.

Hence, although SCD is a monogenic disease, its clinical phenotype is influenced by many other genetic factors. Determining the respective contributions of these factors to clinical variability in SCD is essential for gaining a better understanding of the pathophysiology and for developing or optimizing therapeutic strategies.

THERAPEUTIC STRATEGIES IN SICKLE CELL DISEASE OTHER THAN GENE THERAPY

The management of SCD is based on specific background (chronic) treatments and the control of acute complications. The most frequent acute complications are VOCs. In addition to treatment for the VOC trigger (warming, hydration, oxygen therapy, antibiotic therapy, etc.), pain is treated mainly with opioids (e.g., morphine derivatives, in a hospital setting). In cases with severe complications, donor blood transfusion or exchange transfusion may help to reduce the quantity of circulating, HbS-expressing RBCs. The long-term prevention of these events may require the implementation of a transfusion program, together with chelation treatment (to limit posttransfusion hemochromatosis).

Several drugs are used to reduce the frequencies of VOCs. Hydroxyurea was the first drug to be approved on the basis of SCD's pathophysiology (**Figure 1**). It reduces the frequency of

painful crises in most patients and extends their life expectancy. The drug's mechanism of action is based on the reactivation of HbF synthesis and potentially other processes, such as a reduction in excessive adhesion of erythrocytes to the endothelium and the mild leukopenia induced by treatment with hydroxyurea (21, 46).

HLA: human leukocyte antigen

HSPC: hematopoietic stem and progenitor cell

LV: lentiviral vector

In the last few years, a number of new therapeutic targets and treatment options have emerged. Crizanlizumab (a humanized monoclonal antibody against P-selectin) acts by inhibiting the adhesion of both RBCs and leukocytes to endothelial cells (43). The antioxidant amino acid L-glutamine is able to penetrate the RBC membrane (31, 50, 80) and can reduce oxidative stress and decrease the frequency of episodes of SCD-related pain. Moreover, L-glutamine and crizanlizumab can be combined with hydroxyurea. Lastly, the Hb oxygen-affinity modulator voxelotor is able to stabilize oxygenated Hb and thus prevent polymerization (100, 112).

All of these new therapeutics have a palliative role, and the only currently approved curative treatment for SCD is a human leukocyte antigen (HLA)–matched bone marrow transplant. However, the likelihood of HLA identity between siblings is approximately 25%, which limits the application of this treatment (115). Considerable progress has been made in improving the efficacy of HLA-matched, unrelated donor transplants, reducing the risk of graft-versus-host disease and reducing the toxicity of chemotherapy—notably thanks to reduced-intensity conditioning (18, 56).

In the absence of a fully HLA-identical donor, two novel strategies can be envisioned: an HLA haploidentical allograft and gene therapy. In the first case, successful engraftment is still associated with a higher risk of mortality and graft-versus-host disease as a result of the necessarily high-dose myeloablative conditioning regimen (9, 63). Pioneering work in drug-induced immune tolerance via posttransplant administration of high doses of cyclophosphamide has mitigated severe acute and chronic graft-versus-host disease and has reduced (but not eliminated) the incidence of graft rejection. The latter event is more frequent in patients with transfusion-dependent hemoglobinopathies, due to alloimmunization through recurrent transfusions (83). Gene therapy is therefore a promising option for curative treatment without the risk of graft-versus-host disease and graft rejection and is discussed in the following sections.

GENE THERAPY FOR SICKLE CELL DISEASE

Ex vivo gene therapy consists of the collection of autologous hematopoietic stem and progenitor cells (HSPCs) and then in vitro gene correction (**Figure 2**). First, $CD34^+$ HSPCs are mobilized in the blood and collected by apheresis. The mobilization results from stimulation of the interaction between HSPCs and the bone marrow niche; for example, plerixafor (an antagonist of the CXCR4 receptor present on HSPCs) binds to stromal cell–derived factor 1 in the bone marrow and is an effective, safe mobilizing agent in people with SCD (58). After the HSPCs have been collected, they are genetically cryopreserved and tested to evaluate the efficiency of the gene correction and the quality of the drug product (e.g., sterility). The genetically modified cells are then thawed and infused into the patient after myeloablation. The clinical effectiveness of gene therapy is proportional to the number of corrected HSPCs that engraft into the patient.

To date, two gene correction techniques have been used in clinical trials of infused, ex vivo genetically modified HSPCs in the setting of SCD: lentivirus-based vectors and genome-editing approaches.

GENE THERAPY STRATEGIES BASED ON LENTIVIRAL VECTORS

The first gene therapy strategy consists of adding a therapeutic gene to the HSPC genome via a lentiviral vector (LV). Lentivirus is able to reverse transcribe its RNA genome (thus carrying

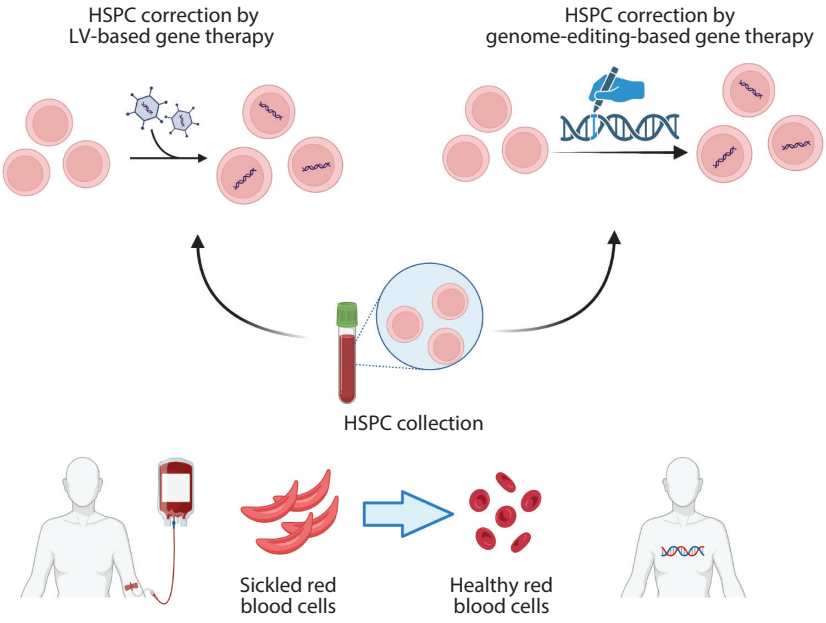


Figure 2

Ex vivo gene therapy approaches to treating SCD using LV-based gene therapy or genome editing. HSPCs are genetically modified to correct the sickling in their red blood cell progeny. Abbreviations: HSPC, hematopoietic stem and progenitor cell; LV, lentiviral vector; SCD, sickle cell disease. Figure adapted from images created with BioRender.com.

the transgene into the DNA) and permanently integrate it into the genome of the target cells (**Figure 3**). This strategy was first tested in 2006, in a phase 1/2 clinical trial in β-thalassemia patients (LG001, NCT01745120) (78). The HPV569 vector contained a transgene encoding the $\beta^{A\text{-}T87Q}$-globin, which has the same amino acid in position 87 as the fetal γ-globin. This residue enables the vector-derived β-globin to be distinguished from the endogenous or transfusion-derived wild-type β-globin. Furthermore, the amino acid in position 87 confers antisickling properties on the therapeutic protein and thus promoted effectiveness in SCD. This vector was further optimized to increase transduction efficiency. The first successful gene therapy procedures using an improved vector (BB305) were reported in 2017 and 2018 for SCD and β-thalassemia, respectively (67, 91, 104). The phase 3 study in SCD is ongoing (HGB-210, NCT04293185, sponsored by bluebird bio); a total of 49 people with SCD have been treated with this investigational treatment (LentiGlobin) in the United States and Europe (**Table 1**).

Similarly, a lentivirus coding for a modified fetal γ-globin is being tested in a phase 1/2 clinical trial in people with SCD. Thanks to a G16D amino acid substitution, the modified fetal γ-globin binds to α-globin with high affinity and can therefore outcompete the mutant sickle β-globin for incorporation into the Hb tetramer. The trial's preliminary results show a reduction in VOCs following treatment (40).

Two other transgene vectors (GLOBE-AS3 and βAS3-FB) encoding the modified AS3 β-globin are now being tested in phase 1/2 clinical trials (NCT02247843 and NCT03964792) (**Table 1**). The β-globin contains three beneficial antisickling amino acids that inhibit the contact required for HbS polymerization (T87Q and E22A) or increase the affinity for α-globin (G16D).

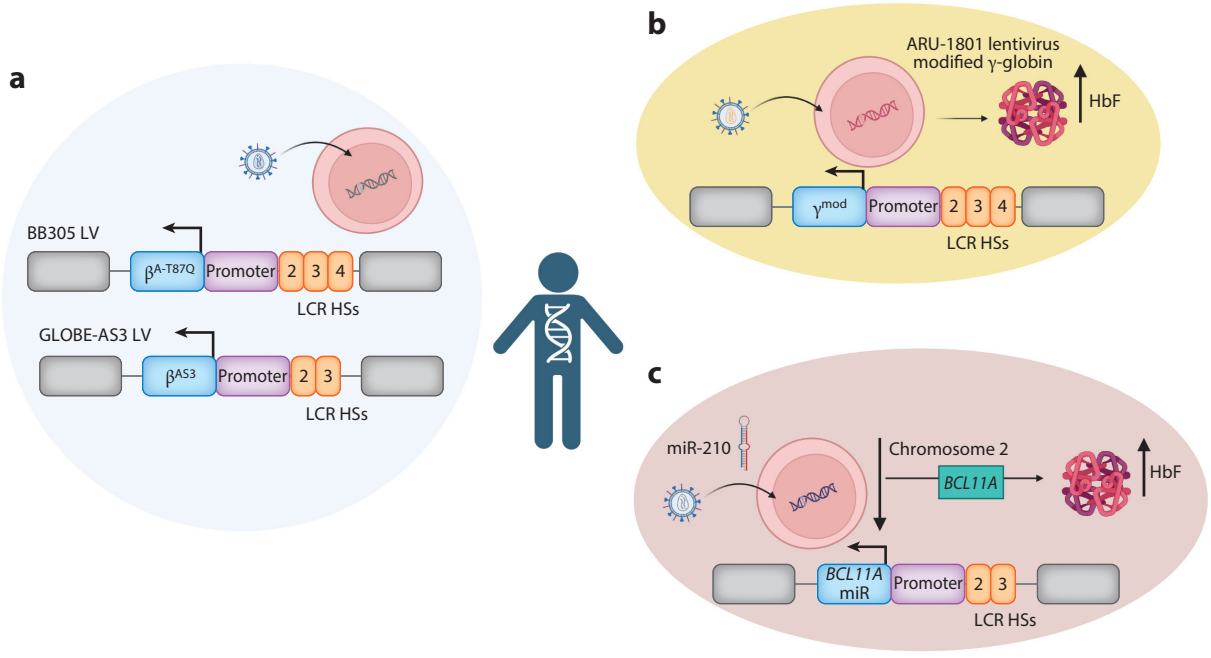


Figure 3

Current strategies for treating SCD with LV-based gene therapy. LV-based strategies aim at expressing (*a*) a β-globin antisickling transgene, (*b*) a modified γ-globin transgene, or (*c*) the endogenous γ-globin genes by downregulating *BCL11A*. Abbreviations: HbF, fetal hemoglobin; HS, DNase-hypersensitive site; LCR, locus control region; LV, lentiviral vector; SCD, sickle cell disease. Figure adapted from images created with BioRender.com.

Lastly, an LV coding for a short hairpin RNA that reduces BCL11A expression has shown its effectiveness in reactivating HbF expression and reducing the signs and symptoms of SCD in six patients (NCT03282656) (33) (**Table 1**).

Despite very encouraging results, gene addition by a viral vector has some limitations. The amount of Hb resulting from a single vector copy is far from that required for a positive clinical outcome (53, 67). If the level of transduction is low (i.e., <1), the clinical response might be satisfying only if the SCD is combined with other mutations, such as α-thalassemia or HbF-related genetic polymorphisms (67). Vector manufacture and conditioning have been optimized to ensure a large supply of modified cells, a high level of transgene expression, and thus more consistent clinical results (53). However, increasing the number of lentiviral integration events might increase the risk of insertional mutagenesis (i.e., preferential integration into genes), the disruption of oncosuppressor genes, and thus clonal proliferation (24). Recently, three SCD patients in the HGB-206 study developed myelodysplastic syndrome or acute myeloid leukemia; however, this was probably linked to the accumulation of mutations in hematopoietic stem cells (HSCs) prior to gene therapy, rather than vector insertion (39, 48, 53). It should be noted that the risk of malignant hematological conditions is 2–11 times higher in people with SCD than in the general population (16, 97). Identifying and characterizing risk factors for the development of malignant hematological conditions is therefore crucial for considering which people with SCD should be included in gene therapy trials.

HSC: hematopoietic stem cell

Table 1 Gene therapy interventional clinical trials for SCD (as of November 2022)

NCT number	Title	Gene therapy strategy	Drug	Study phase	Gene therapy approach
NCT02151526	Study Evaluating the Safety and Efficacy of LentiGlobin BB305 Drug Product in β-Thalassemia Major (Also Referred to as Transfusion-Dependent β-Thalassemia [TDT]) and Sickle Cell Disease	LV-based gene therapy	bb1111 (also known as LentiGlobin BB305)	Phase 1 Phase 2	Autologous $CD34^+$ cell transduced with a β^{T87Q}-globin LV
NCT02140554	A Study Evaluating the Safety and Efficacy of bb1111 in Severe Sickle Cell Disease				
NCT02247843	Stem Cell Gene Therapy for Sickle Cell Disease	LV-based gene therapy	Lenti/βAS3-FB	Phase 1 Phase 2	Peripheral blood $CD34^+$ cells transduced with an AS3-FB vector
NCT02186418	Gene Transfer for Patients with Sickle Cell Disease	LV-based gene therapy	ARU-1801	Phase 1 Phase 2	Ex vivo gene transfer using γ-globin LV ARU-1801 into $CD34^+$ hematopoietic stem cells
NCT03282656	Gene Transfer for Sickle Cell Disease	LV-based gene therapy	BCH-BB694	Phase 1	Single infusion of autologous bone marrow–derived $CD34^+$ HSCs transduced with an LV containing a short hairpin RNA targeting *BCL11A*
NCT03964792	Safety and Efficacy of Gene Therapy of the Sickle Cell Disease by Transplantation of an Autologous $CD34^+$ Enriched Cell Fraction That Contains $CD34^+$ Cells Transduced Ex Vivo with the GLOBE1 Lentiviral Vector Expressing the βAS3 Globin Gene in Patients with Sickle Cell Disease (DREPAGLOBE)	LV-based gene therapy	DREPAGLOBE	Phase 1 Phase 2	Autologous $CD34^+$ HSCs transduced with the GLOBE1 LV, which carries the β-AS3-globin gene
NCT04091737	CSL200 Gene Therapy in Adults with Severe Sickle Cell Disease	LV-based gene therapy	CSL200	Phase 1	Autologous enriched $CD34^+$ cell fraction that contains $CD34^+$ cells transduced with an LV encoding human β-globin G16D and short hairpin RNA734
NCT04293185	A Study Evaluating Gene Therapy with BB305 Lentiviral Vector in Sickle Cell Disease	LV-based gene therapy	bb1111 (also known as LentiGlobin BB305)	Phase 3	HSC transplantation using bb1111
NCT05353647	A Gene Transfer Study Inducing Fetal Hemoglobin in Sickle Cell Disease (GRASP, BMT CTN 2001)	LV-based gene therapy	GRASP	Phase 2	Autologous $CD34^+$ HSCs transduced with an LV containing a short hairpin RNA targeting *BCL11A*
NCT03653247	A Study to Assess the Safety, Tolerability, and Efficacy of BIVV003 for Autologous Hematopoietic Stem Cell Transplantation in Patients with Severe Sickle Cell Disease	Gene editing	BIVV003	Phase 1 Phase 2	NHEJ zinc finger nuclease approach with reexpression of γ-globin by zinc finger nucleases to disrupt an enhancer of *BCL11A*

(*Continued*)

Table 1 (*Continued*)

NCT number	Title	Gene therapy strategy	Drug	Study phase	Gene therapy approach
NCT03745287	A Safety and Efficacy Study Evaluating CTX001 in Subjects with Severe Sickle Cell Disease	Gene editing	CTX001	Phase 2 Phase 3	NHEJ CRISPR-Cas9 nuclease approach with reexpression of γ-globin by CRISPR-Cas9 targeting of the *BCL11A* erythroid-specific enhancer
NCT04443907	Study of Safety and Efficacy of Genome-Edited Hematopoietic Stem and Progenitor Cells in Sickle Cell Disease	Gene editing	OTQ923	Phase 1 Phase 2	NHEJ CRISPR-Cas9 nuclease approach with reexpression of γ-globin by targeting of the *BCL11A* gene
NCT04819841	Gene Correction in Autologous $CD34^+$ Hematopoietic Stem Cells (HbS to HbA) to Treat Severe Sickle Cell Disease (CEDAR)	HDR CRISPR-Cas9 nuclease approach using AAV	GPH101	Phase 1 Phase 2	Correction of a single-nucleotide mutation (A>T) in autologous HSCs by CRISPR and DNA's natural HDR mechanisms delivered via an AAV6 vector
NCT04853576	EDIT-301 for Autologous HSCT in Subjects with Severe Sickle Cell Disease	Gene editing	EDIT-301	Phase 1 Phase 2	NHEJ CRISPR-Cas9 nuclease approach with reexpression of γ-globin by Cas912a ribonucleoprotein targeting of the *HGB1/2* promoters
NCT05456880	BEACON: A Study Evaluating the Safety and Efficacy of BEAM-101 in Patients with Severe Sickle Cell Disease	Gene editing	BEAM-101	Phase 1 Phase 2	Ex vivo base-editing approach with an ABE that incorporates A>G base edits in the *HBG1* and *HBG2* gene promoters to enhance the expression of HbF
NCT04774536	Transplantation of Clustered Regularly Interspaced Short Palindromic Repeats Modified Hematopoietic Progenitor Stem Cells (CRISPR_SCD001) in Patients with Sickle Cell Disease	Gene editing	CRISPR_SCD001	Phase 1 Phase 2	Ex vivo HDR CRISPR-Cas9 nuclease approach using single-stranded oligodeoxynucleotides to correct the SCD mutation

Abbreviations: AAV, adeno-associated virus; ABE, adenine base editor; HbF, fetal hemoglobin; HDR, homology-directed repair; HSC, hematopoietic stem cell; LV, lentiviral vector; NCT, National Clinical Trial; NHEJ, nonhomologous end joining; SCD, sickle cell disease.

DSB: double-strand break

gRNA: guide RNA

NHEJ: nonhomologous end joining

Indel: small insertion or deletion

HDR: homology-directed repair

GENE THERAPY STRATEGIES BASED ON GENOME EDITING

Genome editing consists of the replacement, deletion, or insertion of a genomic DNA sequence. CRISPR-Cas9 is an adaptive defense system used by prokaryotes to cleave nonself nucleic acid sequences. In particular, it has been exploited to develop genome-editing tools capable of generating double-strand breaks (DSBs) at a target locus within the genome. In the CRISPR-Cas9 system, a small RNA molecule [a guide RNA (gRNA)] drives the Cas9 nuclease to a specific region in the genome. Other nucleases (such as zinc finger nucleases) have also been developed and used as genome-editing tools. DSB repair through nonhomologous end joining (NHEJ) results in small insertions or deletions (indels) around the target site and can be exploited to inactivate genes or regulatory sequences. By contrast, the addition of an exogenous DNA template with homology to the target sequence triggers homology-directed repair (HDR) and thus makes it possible to precisely replace one sequence with another.

Genome-editing strategies have been designed to either correct the mutations responsible for β-hemoglobinopathies and restore endogenous *HBB* expression or induce high levels of

endogenous γ-globin expression. While lentiviruses cannot contain the entire *HBB* LCR regulatory sequence (due to packaging limitations), genome-editing-mediated reexpression of the endogenous *HBG*/*HBB* genes occurs in the endogenous globin genes' physiological environment and produces high levels of therapeutic globin expression. Moreover, genome-editing strategies do not require the semirandom insertion of exogenous DNA and therefore avoid the risk of insertional mutagenesis (28).

AAV: adeno-associated virus

In genome-editing approaches, researchers mostly use electroporation to increase the permeability of the cell membrane and allow the Cas9 protein/gRNA to enter HSCs directly. Alternatively, mRNA can be transfected into target cells for production of the editing machinery (89). Virus-based methods [such as adeno-associated virus (AAV) vectors and integrase-deficient LVs] can be used to deliver the donor template for HDR-based modifications. Nonviral delivery using single-stranded oligodeoxynucleotides has also been explored.

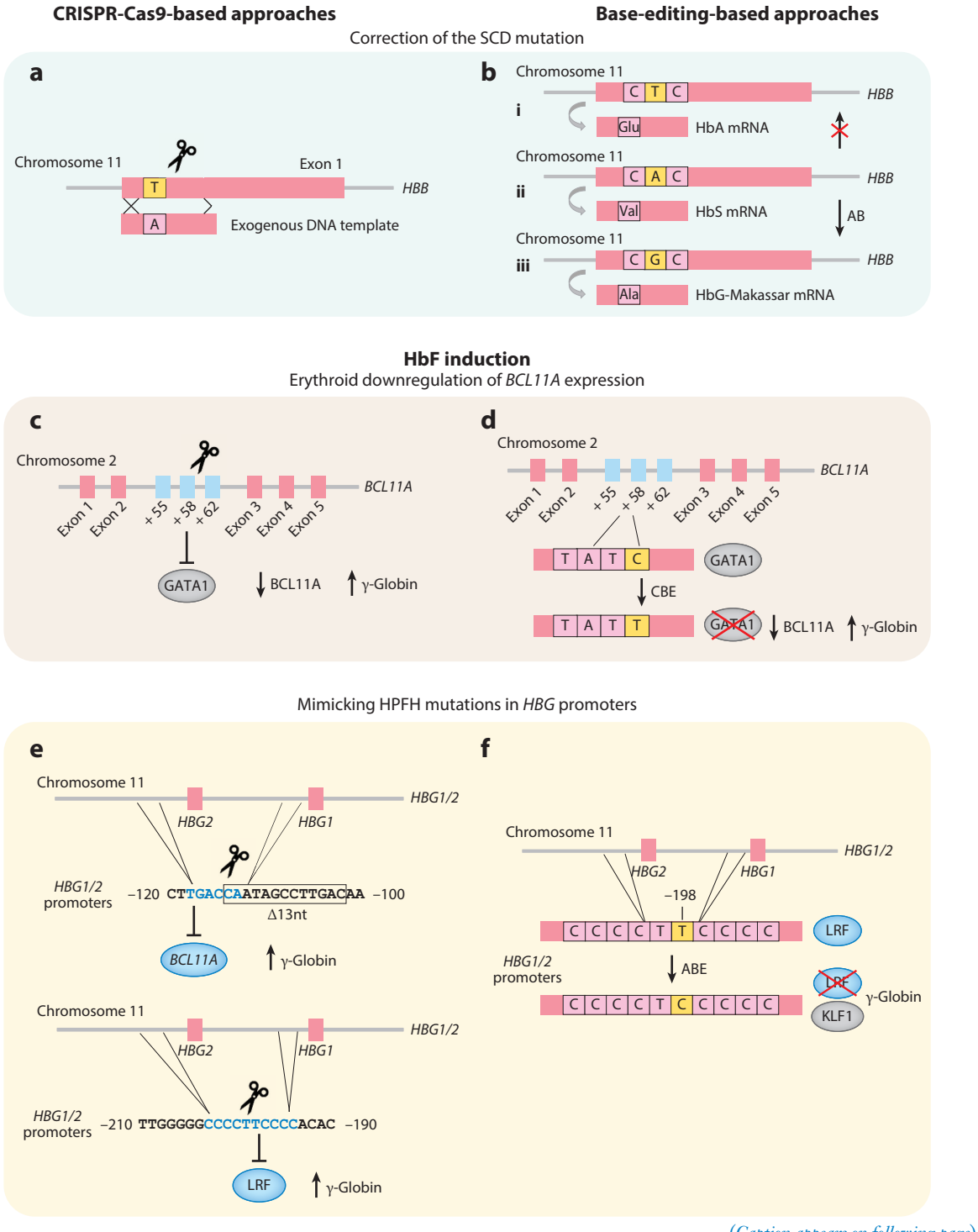
Genome Editing Using Nucleases

Due to its high prevalence and single causal mutation, SCD is a good target for the development of gene correction strategies. Efficient *HBB* gene correction by CRISPR-Cas9 and HDR has been demonstrated (25, 26, 60) (**Figure 4*a***). However, HDR competes with other repair mechanisms, such as NHEJ. Correction of the mutation can therefore be accompanied by a high frequency of alleles harboring indels. The indel-induced frameshift mutations can abolish β-globin expression and thus generate a β-thalassemic phenotype. Furthermore, the HDR pathway is weakly active in quiescent cells, such as long-term repopulating HSCs targeted in gene therapy approaches (60).

In 2021, two phase 1/2 clinical trials of the safety of a nuclease-based editing strategy for correcting the SCD mutation in HSCs were announced. First, a clinical trial conducted by the University of California will include 9 patients (NCT04774536) and will be based on the use of single-stranded oligodeoxynucleotides as DNA donor templates (26, 66). Second, a trial sponsored by the company Graphite Bio will include approximately 15 patients (NCT04819841) and will use the AAV6 vector to deliver the donor template. AAV6 is particularly effective for stimulating HDR to correct mutations in the *HBB* gene, but some studies show that it reduces HSC engraftment (34).

Based on the observation that mutations producing high HbF levels relieve the signs and symptoms of SCD, several research groups have sought to reactivate HbF expression by either decreasing the expression of transcription factors that repress *HBG* genes (e.g., BCL11A) or disrupting the BCL11A and LRF factors' binding sites at *HBG* promoters.

One therapeutic strategy has been designed to downregulate *BCL11A* by targeting its erythroid-specific enhancer. This region is located in the second intron of *BCL11A* (58 kb downstream of the *BCL11A* transcription start site) and contains a binding site for the transcriptional activator GATA1. Cas9 nuclease can be used to disrupt this binding site by introducing indels. This strategy specifically represses BCL11A expression in the erythroid lineage and thus decreases inhibition of *HBG* expression (72, 106) (**Figure 4*c***). A clinical trial of a treatment based on existing zinc finger nucleases was launched by Sangamo Therapeutics in patients with SCD (NCT03653247), with promising results (38). Another trial (based on the use of CRISPR-Cas9 technology to target the *BCL11A* enhancer) was sponsored by Vertex Pharmaceuticals (NCT03745287). The results were encouraging because the first patient with SCD to be treated presented a high level of editing in bone marrow cells and peripheral blood nucleated cells more than one year after treatment, which confirmed the engraftment of gene-edited HSCs (35). Interestingly, the patient showed pancellular expression of HbF after treatment. The results were confirmed in 31 SCD patients and prompted the initiation of a phase 3 clinical trial in a dozen pediatric patients (NCT05329649). Lastly, an observational study of patients from these two studies is set to evaluate efficacy and



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

CRISPR-Cas9 nuclease and base-editing strategies for treating SCD. (*a*) Correction of the SCD mutation by homologous recombination. The Cas9 nuclease, delivered with an exogenous DNA template homologous to the cleavage site, allows the insertion of specific mutations into the targeted sequence. Thus, the mutation responsible for SCD in the β-globin gene can be corrected by HDR with a donor DNA template carrying the wild-type allele. (*b*) Correction of the SCD mutation by an ABE (A>G), resulting in the production of the HbG-Makassar variant. (*i*) The wild-type β-globin gene encodes for a glutamic acid at the sixth position of the protein. (*ii*) The mutated β^S-globin gene encodes for a valine at the sixth position of the protein. (*iii*) The β^G-globin variant gene encodes for an alanine at the sixth position of the protein. (*c*) CRISPR-Cas9 nuclease indel induction at the erythroid-specific enhancer of *BCL11A* to derepress the γ-globin genes. Disruption of the GATA1 binding site by Cas9 nuclease in the *BCL11A* erythroid-specific enhancer decreases its expression and thus induces HbF production. (*d*) Use of a CBE to disrupt the GATA1 binding site in the *BCL11A* erythroid-specific enhancer. This strategy allows HbF reactivation by decreasing the expression of the transcriptional repressor BCL11A. (*e*) CRISPR-Cas9 induction of indels in *HBG1/2* promoters. CRISPR-Cas9 can be used to disrupt HbF repressor binding sites in order to reactivate HbF expression. Here, indels are generated in the −115 region (*top*) or in the −200 region (*bottom*) of HBG promoters, in order to disrupt the BCL11A or LRF repressor binding site, respectively. (*f*) Introduction of HPFH mutations in the promoter of the γ-globin genes. ABEs generate de novo a DNA motif recognized by transcriptional activators (e.g., KLF1) or disrupt binding sites for transcriptional repressors (e.g., LRF) by inducing HPFH mutations in the HGB promoters. Abbreviations: ABE, adenine base editor; CBE, cytidine base editor; HbA, adult hemoglobin; HbF, fetal hemoglobin; HbG, hemoglobin G; HbS, sickle hemoglobin; HPFH, hereditary persistence of fetal hemoglobin; SCD, sickle cell anemia.

toxicity in the 15 years after treatment (NCT04208529). Novartis has also launched a clinical trial using a similar strategy, although no results had been reported at the time of writing (NCT04443907).

Another CRISPR-Cas9 nuclease strategy under investigation is the introduction of indels into *HBG* promoters in order to mimic the effect of known HPFH mutations. Several HPFH mutations found in the −115 and −200 regions of the *HBG* promoters disrupt the binding sites of the potent HbF repressors BCL11A and LRF, respectively (69). Several reports have shown that Cas9 nuclease efficiently induces indels in these regions, reactivates HbF, and corrects the SCD phenotype (106, 118) (**Figure 4*e***).

Limitations of Nuclease-Based Genome-Editing Strategies

Despite the remarkable advances in nuclease-mediated strategies for SCD, several limitations and concerns must be addressed to make genome editing safer.

Off-target effects are still a major concern in therapeutic applications. Although nucleases target specific sequences, they can nevertheless erroneously cleave similar sequences (e.g., those with only one or two mismatches). In fact, gRNA hybridization can tolerate a few mismatches, especially in the 5′ part of the gRNA (37). The resulting unwanted mutations might modify (via local mutagenesis or genomic rearrangements) the expression of nontargeted genes. Off-target DSBs raise some safety concerns; they not only might be deleterious per se if they occur at a sensitive site (e.g., a gene or a regulatory sequence) but also might trigger complex chromosomal rearrangements, such as translocations between on-target and off-target sites (14, 57). Importantly, off-target effects can be limited by using high-fidelity Cas9 nuclease (110).

The on-target genotoxic events are unwanted genomic modifications at the target site. These range from a few base-pair edits to large chromosomal rearrangements. The most frequent unwanted modifications are caused by the involvement of the wrong DNA repair mechanism. In particular, genome-editing strategies designed to correct a gene sequence through HDR can suffer from a low editing rate when the NHEJ pathway is involved. This can even inactivate (and not correct) the target gene. Furthermore, it has been demonstrated that CRISPR-Cas9 can also induce large deletions, chromosomal loss, and chromothripsis (extensive chromosome rearrangements restricted to one or a few chromosomes known to be involved in cancer) (3, 27, 87, 108, 110, 123).

Lastly, CRISPR-Cas9 nuclease treatment can trigger the activation of the p53-mediated DNA damage response pathway (23, 96). In particular, CRISPR-Cas9-induced DSBs can activate p53-dependant cell cycle arrest (34, 49). This arrest affects HSC function and reduces the cells' repopulating potential (76, 96). Thus, genome-edited cell populations might be counterselected due to DNA damage response activation. Furthermore, edited cells presenting a deficient p53 pathway might have a selective advantage (96). Given p53's tumor suppressor function, this advantage would be problematic because it might promote tumorigenesis by edited cells (44, 96).

SSB: single-strand break

CBE: cytidine base editor

ABE: adenine base editor

Genome Editing Using Base Editors

Base editing is a CRISPR-Cas9-based technology that can insert a point mutation without generating a DSB and without the need for an exogenous DNA template (6). A base editor is composed of a deaminase that converts the base and a Cas9 nickase that nicks the targeted strand to generate a single-strand break (SSB). Base editing might be a safer, less complex genome-editing technology compared with the original CRISPR-Cas9 system. Two major classes of base editors have been developed: cytidine base editors (CBEs) for C>T conversions and adenine base editors (ABEs) for A>G conversions. Base editing is a promising therapeutic strategy for genetic diseases caused by point mutations, such as SCD with its GAG to GTG mutation. ABEs can convert AT base pairs to GC, i.e., conversion of the GTG codon (valine) to the GCG codon (alanine). Although this conversion does not restore the original amino acid, alanine is found in the hemoglobin G (HbG)–Makassar nonpathogenic variant of HbA (13, 74, 114) (**Figure 4*b***). In 2021, Newby et al. (79) achieved an 80% mutation correction rate in SCD HSPCs and demonstrated the correction of the SCD phenotype in vitro and in vivo after the autologous transplantation of edited HSCs from a mouse model of SCD.

Base editing has also been explored as a means of reactivating HbF. *BCL11A* expression has been downregulated by using a CBE to convert a C into a T within the GATA1 binding site of the *BCL11A* erythroid-specific enhancer (121) (**Figure 4*d***). This editing strategy was highly efficient in SCD HSPCs, with substantial reactivation of HbF in their erythroid progeny and thus subsequent correction of the SCD phenotype in vitro.

Base editing has been used to introduce HPFH mutations that disrupt the binding sites of the HbF repressors BCL11A and LRF or create de novo binding sites for transcriptional activators such as TAL1, GATA1, and KLF1 (5, 36) (**Figure 4*f***). Some of these strategies are effective in HSPCs and have led to the reexpression of HbF in their erythroid progeny (70). Mayuranathan et al. (70) demonstrated the correction of the SCD phenotype by creating a TAL1 binding site in the *HBG* promoters. Similarly, Antoniou et al. (5) demonstrated efficient generation of a KLF1 binding site in the *HBG* promoters and correction of the SCD phenotype. Lastly, a recent publication highlighted the ability of simultaneous T>C base editing in positions −123/−124 of the *HBG* promoters to increase HbF expression by recruiting the KLF1 transcription activator (89). A clinical trial of base-editing HbF reactivation in SCD patients has been announced by Beam Therapeutics (BEAM-101, NCT05456880).

Limitations of Base-Editing Strategies

Even though base editing has advantages over nuclease-based approaches, the former is not devoid of unwanted off-target and on-target events.

Base editors can induce deamination at off-target DNA or RNA sites. Off-target editing is either gRNA dependent (as observed with the CRISPR-Cas9 nuclease system) or gRNA independent. gRNA-dependent off-target effects are caused by Cas9's recognition of sequences that are similar to the on-target locus; to limit these edits, high-fidelity (62, 90) Cas9 variants can be

used to increase the specificity of binding. gRNA-independent off-target DNA edits are caused by nonspecific interaction of the deaminase with transiently accessible DNA bases; these events are more frequent with CBEs than with ABEs, but modifications in Cas9's deaminase domain or the use of alternative deaminases can diminish this type of off-target activity (27, 62, 123). Off-target RNA edits can also occur in a gRNA-independent manner; although this effect has been described with both types of base editors, RNA edits also appear to be more common for CBEs than for ABEs (27, 52, 62, 123). RNA edits occur in both protein-coding and noncoding sequences (41). Given the short half-life of RNA, these off-target effects are transient, but the potential functional consequences must be carefully evaluated before base-editing approaches are applied to humans in clinical trials (41). Notably, engineering of the TadA and rAPOBEC1 domains (41, 124) or the usage of alternative deaminases in CBEs (41, 122) resulted in base-editor variants with substantially lower levels of off-target RNA activity.

Three types of unwanted on-target events can occur with base-editing strategies: transversion mutations, bystander edits, and indels. Transversion mutations at the target nucleotide consist of A>non-G or C>non-T edits. These edits have been widely observed with CBEs, but ABEs give a purer product. Bystander edits consist of the deamination of nontargeted A or C bases close to the target base. The potential biological effects of bystander edits must be evaluated before clinical application. If the target sequence is in a protein-coding region, a bystander edit might lead to a nonsilent mutation that changes the protein's structure and/or function. It is noteworthy that silent mutations can disturb mRNA stability and translation (45). Bystander edits can be limited by base editors with a narrower editing window (87). Lastly, a Cas9 nickase may create a transient SSB on the opposite (nontargeted) strand, which can result in a DSB and thus the formation of indels. Importantly, on-target indels have been described mainly after CBE use and less so after ABE use.

CONCLUSIONS

Improvements in our knowledge of the genetic mechanisms involved in the physiopathology of SCD have driven the development of new, potentially curative gene therapies. The first gene therapy strategies featured gene addition via the LV-based transduction of HSCs and the subsequent expression of a therapeutic Hb. Although these strategies were successful in the treatment of SCD (99), they had some limitations in terms of efficacy and safety. First, their efficacy is limited when the vector copy number per cell is low and the therapeutic globin is therefore not sufficiently expressed (67). Second, the semirandom integration of LVs is associated with a genotoxic risk—a risk that is especially high in SCD because a high vector copy number is required for clinical benefit (67).

The discovery of the protective role of HbF (the complex regulation of which has been elucidated by studying genetic variants affecting its expression) allowed the development of LV-based genome-editing strategies for reactivation of the *HBG* genes (35). However, the long-term benefit and persistence of therapeutic levels of HbF expression remain to be investigated.

Genome-editing technologies have broadened the field of tailored medicine and constitute an excellent tool for increasing the concentration of HbF and reducing the production of HbS (the intra-RBC concentration is one of the main factors influencing the sickling rate) (29). Nevertheless, the vast majority of these approaches rely on the use of nucleases that can generate genotoxic DSBs. New research and clinical development projects will evaluate the efficacy and on-target and off-target safety of these approaches. The DSB-free base-editing strategies can overcome the potential genotoxicity caused by nucleases. However, these strategies can currently produce only a few types of base conversions and can also generate potential unwanted events at the on- and off-target sites. In theory, the recently developed prime-editing technology allows the generation

of precise insertions and deletions and all types of point mutations and does not require DSB generation or the delivery of a donor DNA template (7). For example, prime editing has been used to correct the SCD mutation precisely and efficiently in cell lines (7). Although this would convert the sickle *HBB* gene in its wild-type form, this approach will probably have to be optimized for the achievement of therapeutically relevant correction rates in primary cells.

Moving these strategies into routine care is a challenge at several levels. Product cost is a significant limitation for these gene therapy strategies but should be compared with the annual cost of years or decades of standard care for people with SCD, while also taking into account the post-treatment gain in quality of life. Most of these new genome-editing approaches are based on the use of CRISPR-Cas9 and derived tools and so avoid the need for LV production, which accounts for approximately 50% of the current cost of these gene therapies (22).

Lastly, future clinical trials in SCD should take into account the participants' various genetic backgrounds, in order to develop a tailored gene therapy with good long-term efficacy and limited toxicity.

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AUTHOR CONTRIBUTIONS

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