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Recent advancements in gene therapy for sickle cell disease and β-thalassemia

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β-hemoglobinopathies, including sickle cell disease (SCD) and β-thalassemia, are prevalent monogenic disorders causing abnormal hemoglobin structure or production that affect millions globally. Current available therapies for SCD and β-thalassemia are primarily symptomatic treatments and allogeneic hematopoietic stem cell transplant (HSCT). Allo-HSCT is the only curative treatment, which has limitations. Gene therapy using genetically modified hematopoietic stem cells (HSCs) holds promise to be an effective curative therapy. Recently approved *ex vivo* genetically modified HSC-based therapeutics (CASGEVY, LYFGENIA, ZYNTEGLO) have shown remarkable and durable therapeutic benefits for SCD and β-Thalassemia. In this review article, we discuss the current genetic approaches and innovative strategies to ensure safe and effective gene therapy for SCD and β-thalassemia and summarize findings from completed and ongoing clinical trials. We also discuss prospects and challenges of *in vivo* gene editing with CRISPR/Cas technology for SCD and beta-thalassemia that may simplify manufacturing and treatment process. *In vivo* gene therapy may minimize the risks associated with *ex vivo* gene therapy and may overcome multiple barriers associated with complex gene therapy products for wider patient access, especially in developing regions of the world where these diseases are highly prevalent.

KEYWORDS

sickle cell disease, beta-thalassemia, gene therapy, CRISPR/Cas (clustered regularly interspaced short palindromic repeats), fetal hemoglobin (HbF), beta-globinopathies, hematopoietic stem cells (HSC)

Introduction of sickle cell disease and β-thalassemia

Hemoglobinopathies, including sickle cell disease (SCD) and β-thalassemia, are common monogenic disorders affecting millions of people worldwide. The World Health Organization estimates that over 5% of the global population carries hemoglobinopathies, with nearly 300,000 – 400,000 children born annually with severe forms of these diseases (1, 2).

Hemoglobinopathies are caused by perturbed hemoglobin (Hb) biosynthesis, affecting the structure, function or production of Hb which is the oxygen-carrying protein in red blood cells (RBCs). The normal adult Hb molecule, known as Hemoglobin A (HbA), is composed of two α-globin chains and two β-globin chains (3). Hemoglobinopathies are broadly classified into two subgroups: a) Hemoglobinopathies with Hb structural aberrations that include SCD, Hemoglobin C (HbC), Hemoglobin D (HbD), Hemoglobin E (HbE), and Hemoglobin Constant Spring disease (HbCS), and b) Hemoglobinopathies with abnormal Hb production include thalassemia syndrome (α– and β–thalassemia). SCD is caused by a point mutation in β-globin (*HBB*) gene that substitutes Valine for Glutamic acid at codon 6 (β^{G6V}). G6V mutation results in production of sickle hemoglobin (HbS) which forms long polymers under poor oxygenation. HbS polymerization distorts the shape of RBC with a characteristic sickle shape (4). SCD syndrome is characterized by hemolysis, vaso-occlusion crises (VOC), and infections causing recurring episodes of acute pain, tissue damage, strokes, and potentially resulting in multi-organ damage or death in most severe cases (5). β-thalassemias are caused by mutations in *HBB* gene resulting in absence (beta-zero (β^0) Thalassemia) or quantitative reduction (beta-plus (β^+) thalassemia) in β-globin production (6). With a reduction in β-globin, the formation of functional Hb complex is impaired and normal development of RBC is affected, leading to anemia and associated health complications. β-thalassemia is caused by more than 200 mutations in the *HBB* gene and are classified in to major, intermedia, and minor forms depending on clinical severities with symptoms like severe anemia, bone deformities, and organ damage (7).

Until recently, treatment options for SCD and β-thalassemia were primarily limited to palliative symptomatic managements and allogeneic hematopoietic stem cell transplant (HSCT). However, challenges like unavailability of compatible donors and immunological risks associated with allogeneic HSCT necessitate more effective solutions. As the root cause of SCD and β-thalassemia lies in mutations in the *HBB* gene, employing genetic approaches targeting the mutant *HBB* gene present the most promising and effective approach. In 2023, the U.S. Food and Drug Administration (FDA) granted approval to two groundbreaking therapies for SCD patients: CASGEVY (exagamglogene autotemcel) and LYFGENIA (lovotibeglogene autotemcel). The approval of two SCD gene therapies represents a significant advancement, showcasing the role of innovative technologies in transforming medical treatment.

In the early embryonic development ε-globin is predominantly expressed in the yolk sac erythroblasts. During fetal development, fetal hemoglobin (HbF), containing γ-globin ($\alpha2\gamma2$) instead of β-globin, is predominantly expressed. γ-globin is produced by two closely related genes, *HBG1* and *HBG2*, which are silenced after birth. Postnatally, γ-globin is gradually replaced by β-globin (8). This switching of globin gene expression from ε -globin to γ-globin and then to β-globin, is governed through precise chromatin modifications, selective hypomethylation of globin gene promoter (9, 10) and expression of stage-specific transcription factors (11, 12).

The asymptomatic adult SCD patients have abnormally high levels of HbF, a condition known as hereditary persistence of fetal hemoglobin (HPFH) which disrupts the switching of *HBB* gene (8). With HPFH mutations, the elevated HbF in RBCs reduces the polymerization of sickle hemoglobin, resulting in minimal hemolysis and vaso-occlusive events in affected patients. Similarly, co-inheritance of genetic variants elevating HbF production has the strongest disease modifying effect in β-thalassemia with milder phenotype (13–15). Elevated γ-globin can pair with excess of α-chain, resulting in a functional HbF, thereby ameliorating the severity of β-thalassemia. Therefore, several HPFH related mutations have been identified as targets for gene-editing therapies to cure hemoglobinopathies, including *BCL11A*, the γ-globin transcriptional repressor, the *HBS1L-MYB* intergenic region, and the δ- and β-globin gene locus (16–18). Gene editing strategies for β-thalassemia and SCD aim to increase HbF levels by 30% to prevent α-chain precipitation and HbS polymerization, respectively. Achieving this threshold can significantly impact treatment efficacy (19).

Gene therapy for blood disorders entails isolating the patient's hematopoietic stem cells (HSCs), using vectors to introduce healthy genes *ex vivo*, and then reintroducing these modified cells into the bloodstream to produce healthy RBCs in the bone marrow (20). *Ex vivo* gene editing strategies encompass globin gene addition, β-globin gene correction, and HbF upregulation. For effective treatment, it's crucial to accurately target the genetic mutations, efficiently deliver gene-editing tools to HSCs, and minimize off-target effects to ensure the creation of healthy RBCs. The *in vivo* approach aiming to directly administrate genome editing reagents emerged as a promising methodology. In this review, we summarize the current state of gene therapy for β-hemoglobinopathies by highlighting recent advancements in pre-clinical research, clinical trials, and provide perspectives on the challenges in achieving desired clinical outcomes.

Genetic approaches for SCD and β-thalassemia

Genetic approaches for beta-globinopathies can broadly be classified in to three categories: a) β-globin gene addition to restore *HBB* function, b) genetic correction of causal mutation, and c) HbF upregulation (Figure 1). Each of these approaches are described in detail in the following sections.

β-globin gene addition

In this section, we discuss gene therapy approaches with functional β-globin gene addition. Earlier attempts to develop gene therapy approaches for SCD and β-Thalassemia co-evolved with the development of gamma-retroviral vector with the possibility of stable delivery of functional β-globin gene to hematopoietic stem and progenitor cells (HSPCs). In early 80s, Williams et al. demonstrated for the first time that genetic material

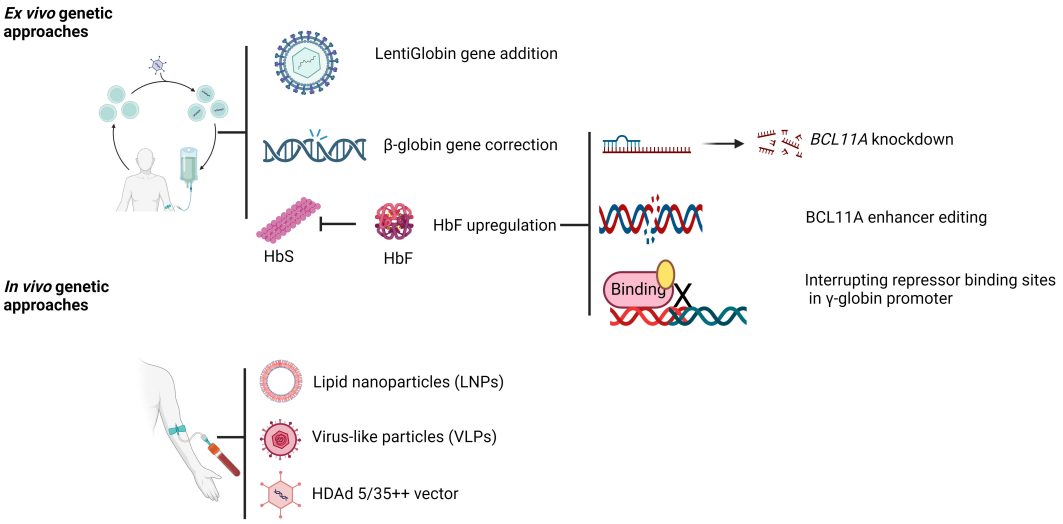

FIGURE 1
Gene therapy approaches for SCD and β-thalassemia. Created with BioRender.com.

can be stably transferred into murine HSPCs using a retroviral vector (21). Shortly thereafter stable production of human β-globin in mice reconstituted with human HSPCs *ex vivo* modified with retroviral vector encoding β-globin gene was demonstrated (22). However, in earlier studies, β-globin expression level was very low, position dependent, and dysregulated. Extensive characterization of β-globin gene locus led to the identification of distal regulatory elements located about 6 to 22 kb upstream of the ε-globin gene called the locus control region (LCR) (23–25), which is required for developmental stage regulated, erythroid-restricted, high level expression of globin gene. Initial attempts to incorporate LCR sub-fragments resulted in low viral titer, low β-globin expression and unstable vector. Through DNase hypersensitivity assay core regulatory regions (HS2, HS3, and HS4) within LCR were identified. Incorporation of core regulatory regions in retroviral vector generated high titer vector with elevated β-globin expression in MEL cells; however, the position-independent effect was lost due to shortening of the LCR, resulting in clonal variations in β-globin expression (26). With the development of HIV-based lentiviral vector (LV) (27), it became feasible to incorporate necessary elements of the complex β-globin gene (LCR HS elements, introns) to achieve erythroid-specific, high levels of β-globin expression. LV was very useful to achieve relatively high level of transduction in quiescent long-term hematopoietic stem cells as it can infect both dividing and non-dividing cells. The utility of such vector for gene therapy for β-globinopathies was demonstrated by successful use of the TNS9 β-globin LV in treating β-thalassemias intermedia and Cooley's anemia in mice (28). The TNS9 incorporates essential elements of LCR (a 3.2 kb region containing HS2, HS3, and HS4), together with an intron 2 internal deletion and the β-globin gene under endogenous β-globin promoter. The TNS9 LV demonstrated high transduction efficiency into murine HSPCs. It allowed long-term, erythroid-specific expression of β-globin resulting in on an average increase of 4-6 g/dL of Hb per vector copy at steady state in peripheral blood, and rescued β-thalassemia (intermedia and major) in murine models. The TNS9 β-globin LV (TNS9.3.55) was evaluated in a Phase 1 study in adults with β-thalassemia (NCT01639690). Four patients were treated with autologous CD34 + HSPCs *ex vivo* modified with TNS9.3.55 globin vector. 6-8-year follow-up of these patients indicate that gene marking within hematopoietic system was very stable but low, and were able to reduce transfusion requirements in two patients (29). However, transfusion independence was not achieved due to low transduction (VCN 0.15). The TNS9.3.55 vector or its variant has been studied in three different Phase 1/2 clinical studies. The LentiGlobin BB305 vector encodes anti-sickling $\beta^{A\text{-}T87Q}$-globin and contains both a β-globin promoter and the LCR for erythroid lineage-specific expression of $\beta^{A\text{-}T87Q}$-globin (30). LentiGlobin BB305 was evaluated in patients with transfusion dependent β-thalassemia (TDT) for safety and efficacy in two Phase 1/2 studies HGB-204 (NCT01745120) and HGB-205 (NCT02151526) (31) (Table 1). Treatment with autologous CD34+ cells transduced with the BB305 vector reduced or eliminated the need for RBC transfusions in 22 patients with severe β-thalassemia.

In another Phase 1/2 trial (TIGET-BTHAL, NCT02453477), a modified vector lacking HS4 LCR element (LV GLOBE) and encoding anti-sickling globin $\beta^{A\text{-}AS3}$ (described under SCD) was used to treat three adults and six children with β^0 or severe β^+ mutations (32). Three out of four pediatric patients discontinued RBC transfusion while transfusion frequency and volume requirement were reduced in the adults. Data from these clinical studies suggest that younger patients have better outcome from Lenti-globin gene therapy and a minimum transduction threshold of CD34 + HSPCs (VCN >0.5) is required to achieve a therapeutically meaningful outcome. Further optimization of this approach may provide a long-lasting therapeutic benefit for β-thalassemia.

Following favorable outcomes from Phase 1/2 study, two Phase 3 trials HGB-207 (Northstar-2, NCT02906202, non-β^0/β^0 genotypes) and HGB-212 (Northstar-3, NCT03207009, β^0/β^0, β^0/β^+ $^{IVS\text{-}I\text{-}110}$ or

TABLE 1 Gene therapy clinical trials for β-hemoglobinopathies.

Clinical Trial	Trial Code	Sponsor	Drug Product	Estimated enrollment	Location	Starting - completion
ARU-1801 γ-globin G16D LV	NCT02186418 Phase 1/2	Children's Hospital Medical Center, Cincinnati	Autologous CD34+ HSCs transduced ex-vivo with gamma-globin LVV	10 patients (SCD), ages 18-45.	Canada, Jamaica, Switzerland	07/2014 - 07/2037
P170006J	NCT03964792 Phase 1/2	Assistance Publique - Hôpitaux de Paris	Autologous CD34+ cells transduced with the GLOBE1 lentiviral vector expressing the βAS3 globin gene	6 patients (SCD), ages 12-20.	France	11/2019 - 01/2024
LentiGlobin BB305 anti-sickling β-globin (β^{A-T87Q}) LV	Northstar (HGB-205) NCT02151526 Phase 1/2	Bluebird bio	Autologous CD34+ cells transduced ex vivo with LVV BB305 that encodes the anti-sickling β^{A-T87Q}-globin expressed in the erythroid lineage	7 patients (β-thalassemia or SCD), ages 5-35.	France	07/2013 - 02/2019
	HGB-206 NCT02140554 Phase 1/2			50 patients (SCD), ages 12-50.	US	02/2015 - 01/2024
	HGB-210 NCT04293185 Phase 3			35 patients (SCD), ages 2-50.	US	02/2020 - 05/2027
	Long-term follow-up study (LTF-307) NCT04628585 Phase: Long-term follow-up			85 patients (SCD), ages 2-53.	France, US	10/2020 - 01/2038
LentiGlobin BB305 Drug Product	HGB-204 NCT01745120 Phase 1/2	Bluebird bio	Autologous CD34+ HSCs transduced with LentiGlobin BB305 LV encoding the human β^{A-T87Q}-globin gene.	19 patients (β-thalassemia major), ages 12-17.	US, Australia, Thailand	08/2013 - 02/2018
	NCT02633943 Phase 3			66 patients (β-thalassemia), ages 0-50	US, Australia, France, Germany, Greece, Italy, UK Thailand	01/2014 - 11/2035
	NCT02906202 Phase 3			24 patients (β-thalassemia), ages 0-50	US, France, Germany, Italy, UK Thailand	08/2016 - 03/2022
	HGB-212 NCT03207009 Phase 3			18 patients (β-thalassemia), ages 0-50.	US, France, Germany, Greece, Italy, UK	06/2017-11/2022
EudraCT	NCT02453477 Phase 1/2	IRCCS San Raffaele	autologous HSCs genetically modified with GLOBE LVV encoding for the human β-globin gene	10 patients (β-thalassemia), ages 3-64.	Italy	05/2015 - 08/2019
10-164	NCT01639690 Phase 1	Memorial Sloan Kettering Cancer Center	Autologous CD34+ HSPCs Transduced with TNS9.3.55 a LV encoding the Normal Human β-Globin Gene.	10 patients (β-thalassemia), ages ≥18.	US	07/2012 - 07/2024
Lenti/βAS3-FB anti-sickling β-globin (βAS3) LV	NCT02247843 Phase 1/2	Donald B. Kohn, M.D.	autologous peripheral blood CD34+ cells transduced ex vivo by the Lenti/G-βAS3-FB LV to express an anti-sickling (βAS3) gene.	6 patients (SCD), ages ≥18	US	12/2014 - 12/2025
BCH-BB694 anti-BCL11A shRNA LV	NCT03282656 Phase 1	David Williams	Autologous CD34+ HSCs transduced with the LVV containing a	10 patients (SCD), ages 3-40	US	02/2018 - 11/2024

(Continued)

TABLE 1 Continued

Clinical Trial	Trial Code	Sponsor	Drug Product	Estimated enrollment	Location	Starting - completion
			short-hairpin RNA targeting BCL11a.			
ST-400-01	NCT03432364 Phase 1/2	Sangamo Therapeutics	ST-400 Autologous Hematopoietic Stem Cell transplantation	5 patients (β-thalassemia), ages 18-40	US	03/2018 - 11/2022
2019-BRL-00CH1	NCT04211480 Phase 1/2	Bioray Laboratories	γ-globin Reactivated Autologous HSCs	6 patients (β-thalassemia), ages 5-15	China	10/2020 - 10/2023
CTX001-111	NCT03655678 Phase 1/2/3	Vertex Pharmaceuticals Incorporated	autologous CRISPR-Cas9 Modified CD34+ HSPCs using CTX001.	45 patients (β-thalassemia), ages 12-35	US, Canada, Germany, Italy, UK	09/2018 - 08/2024
VX21-CTX001-161	NCT05477563 Phase 3	CRISPR Therapeutics	autologous CD34+ hHSPCs modified with CRISPR-Cas9 at the erythroid lineage-specific enhancer of the BCL11A gene)	26 participants (β-thalassemia and SCD), ages 12-35	Germany, Italy, US	08/2022 - 02/2025
VX21-CTX001-151	NCT05329649 Phase 3	Vertex Pharmaceuticals Incorporated	autologous CRISPR-Cas9 modified CD34+ human hematopoietic stem and progenitor cells (hHSPCs) (CTX001).	15 Pediatric patients (SCD), ages 2 -11.	Germany, Italy, UK, US	05/2022 - 05/2026
CTX001-131 A Long-term Follow-up Study in Participants Who Received CTX001	NCT04208529 Phase 3	Vertex Pharmaceuticals Incorporated	autologous CRISPR-Cas9 Modified CD34+ HSPCs using CTX001.	160 patients (SCD or β-thalassemia), ages >2.	US, Canada, Germany, Italy, UK	01/2021 - 09/2039
ST-400 (BT) and BIVV003 BCL11A (BIVV003)-Targeted zinc finger disruption of BCL11A erythroid enhancer via NHEJ	PRECIZN-1 NCT03653247 Phase 1/2	Sangamo Therapeutics	CD34+HSPCs transfected ex vivo with ZFN mRNAs targeting BCL11A locus.	8 patients (SCD), ages 18-40	US	03/2019 - 01/2025
OTQ923/HIX763 Cas9 disruption of BCL11A erythroid enhancer via NHEJ or Cas9 disruption of BCL11A binding motif in γ-globin promoter via NHEJ	NCT04443907 Phase 1	Novartis Pharmaceuticals	Genome-edited HSPCs from patients With Severe SCD	5 patients (SCD), ages 2-17	US	08/2020 - 08/2025
EDIT-301 Cpf1 disruption of BCL11A binding motif in γ-globin promoter via NHEJ	RUBY trial, NCT04853576 Phase 1/2	Editas Medicine Inc	Autologous gene edited HSPCs	45 patients (SCD), ages 12-50	US, Canada	04/2021 - 08/ 2025
	EDIT-301 trial, NCT05444894 Phase 1/2		autologous gene edited HSPCs	9 patients (β-thalassemia), ages 18-35	US, Canada	04/2022 – 09/2025
GPH101 Hb β6: GTG->GAG via Cas9 HDR	NCT04819841 Phase 1/2	Kamau Therapeutics	Autologous CD34+ HSCs to Convert HbS to HbA	15 patients (SCD), ages, 12-40	US	11/2021 - 07/2027
BEACON	NCT05456880 Phase 1/2	Beam Therapeutics Inc.	autologous base edited CD34+ HSPCs	15 patients (SCD), ages 18-35	US	08/2022 - 02/2025

(Continued)

TABLE 1 Continued

Clinical Trial	Trial Code	Sponsor	Drug Product	Estimated enrollment	Location	Starting - completion
CASGEVY™ Gene editing Cas9 disruption of BCL11A erythroid enhancer via NHEJ	CLIMB-121 NCT03745287 Phase 1/2/3	Vertex Pharmaceuticals Inc.	autologous CD34+ HSPCs modified with CRISPR-Cas9 at the erythroid lineage-specific enhancer of the BCL11A gene	45 patients (SCD and β-thalassemia, ages 12-35)	US, Belgium, Canada, France, Germany, Italy, UK	11/2018 - 10/2024
CRISPR_SCD001 Hb β6: GTG->GAG via Cas9 HDR	NCT04774536 Phase 1/2	UCSF Benioff Children's Hospital Oakland	autologous sickle allele CRISPRCas9 modified CD34+ HSPCs in subjects with severe SCD.	9 patients (SCD), ages 12-35	US	06/2024 - 06/2025
BRL-101	NCT05577312	Bioray Laboratories	autologous CRISPR-Cas9 modified CD34+ human hematopoietic stem and progenitor cells (hHSPCs)	9 patients with Transfusion-Dependent β-Thalassemia	China	11/2022 - 09/2026
ET-01	NCT04925206	EdiGene (GuangZhou) Inc.	autologous CRISPR-Cas9 Modified CD34+ Human Hematopoietic Stem and Progenitor Cells (hHSPCs)	8 patients with With Transfusion Dependent β-Thalassaemia	China	08/2021 - 06/2024

$\beta^{+\ IVS\text{-}I\text{-}110}/\beta^{+\ IVS\text{-}I\text{-}110}$ genotype) were initiated to evaluate efficacy and safety of LentiGlobin BB305 in patients with TDT. In Northstar-2 study, 20 out of 22 patients had durable transfusion independence with a median HbA^{T87Q} level of 8.7 g/dL (range, 5.2 to 10.6) at 12-month post infusion and an average hemoglobin level of 11.7 g/dL (range, 9.5 to 12.8) during transfusion independence (33). Northstar-3 study evaluated the efficacy and safety of LentiGlobin BB305 in 18 patients, 88.9% of patients achieved transfusion independence at 24 months post-transplant with a mean HbA^{T87Q} level of 10.8 g/dL during transfusion independence (data source: clinicaltrials.gov). Based on the successful outcome from Phase 3 efficacy study, the European Commission granted BB305 (Proper name: betibeglogene autotemcel or beti-cel) conditional marketing authorization marketed as ZYNTEGLO™ gene therapy in 2019 for patients 12 years and older with TDT who do not have a β^0/β^0 genotype. ZYNTEGLO received the US FDA approval in 2022 for the treatment of adult and pediatric patients with β-thalassemia who require regular RBC transfusions. ZYNTEGLO is the first cell-based gene therapy marketed in the United States for the treatment of TDT. Preclinical and clinical studies have shown effectiveness of Lenti-globin gene addition approach for β-thalassemia. In patients with non- β^0/β^0 genotype transfusion independence was achieved in 12 out of 13 patients treated with Lenti-Globin. However, in patients with β^0/β^0 genotype or two copies of the IVS1-110 mutation (n=9), only three patients achieved transfusion independence while transfusion requirements were reduced for others, with a 73% reduction in the median annualized transfusion volume (31). This indicates that overall output of therapeutic β-globin together with balanced ratio of α/β-globin is critical to achieve transfusion independence in patients with β^0/β^0 genotype. To improve the efficacy of the Lenti-globin BB305 vector for the most severe β^0/β^0 genotype patients, a multiplex LV, LVβ-shα2 was developed (34) that allowed the coordinated expression of $\beta^{A\text{-}T87Q}$-globin and selective reduction in α2-globin mRNA expression without affecting α1-globin mRNA levels. LVβ-shα2 is 1.7-fold more potent than BB305, potentially offering greater therapeutic efficacy and reliability for severe β-thalassemia irrespective of genotype (34).

Unlike β-thalassemia, ectopic expression of correct β-globin gene is not sufficient to prevent sickling of RBCs. Any therapeutic strategy will also require reduction or inhibition of HbS polymerization to prevent sickling of RBCs and resulting vaso-occlusion. G6V mutation is exposed on the surface of β-globin and can interact with α-globin even in the presence of normal β-globin forming a HbS hybrid tetramer ($\alpha_2\ \beta^S\beta^A$). Several crucial observations led to the development of anti-sickling β-globin variants. Initial observation by Janet Watson showed that relatively few sickle cells were found in the circulation of newborn children with SCD (35) due to high level of HbF and its lack of sickling property. In fact, higher HbF level is positively correlated with increased life expectancy in SCD patients (36). HbF is the major genetic modulator of SCD clinical manifestations. Another line of evidence on the benefit of elevated HbF in SCD was provided by patients with compound heterozygosity for HbS and gene deletion HPFH with no symptoms of SCD or hemolytic anemia despite a very high level of HbS (37). In HPFH deletion, uniform distribution of HbF is observed among all erythrocytes and a level of 30% HbF is capable of preventing HbS polymerization (38). HPFH is a benign asymptomatic condition with continued HbF synthesis into adulthood. HbF is more potent inhibitor of HbS polymerization and very effective in preventing sickling of RBCs compared to HbA (39). The entrance of $\alpha_2\gamma\beta^S$ hybrid tetramer in to HbS polymer fibers occurs at a much lower extent than $\alpha_2\beta_2^S$ homotetramer or $\alpha_2\ \beta^S\beta^A$ hetrotetramer (40, 41) due to occurrence of glutamine (Q) at position 87 in γ-globin (instead of Threonine

(T) in β-globin) that does not favor critical intermolecular contact with $\beta 6^{V}$. This led to the development of β-globin with T87Q substitution with anti-sickling property. LV with T87Q substituted β-globin was able to correct SCD in two transgenic mouse models (42). Long-term expression of anti-sickling protein was detected in 99% of circulating RBCs with up to 52% accumulation of anti-sickling $\beta^{A\text{-}T87Q}$-globin of total hemoglobin. Similar to mouse models, stable expression of anti-sickling $\beta^{A\text{-}T87Q}$-globin was achieved in erythroid progeny derived from Lenti-globin T87Q vector transduced CD34+ cells from cord blood (43), healthy donors and adult SCD patients (44), suggesting that LV expressing β-globin with T87Q substitution can be used to treat SCD in human patients. Biochemical and structural characterization studies of HbS and other globin variants indicated that the residues 22, 80, and 87 of β chain play critical roles in stabilization of the deoxy-HbS polymer through intermolecular interaction (45). Such insight led to the development of another anti-sickling globin, harboring three mutations (β^{AS3} with amino acid substitutions at position 16 (G16D), 22 (E22A), and 87 (T87Q) with superior anti-sickling properties than HbF (46). E22A and T87Q disrupt axial and lateral contacts between HbS tetramers, preventing HbS polymerization, whereas G16D substitution increases the binding affinity for α-globin. Oxygen binding affinity of HbAS3 is comparable with HbF. When tested in a mouse model of SCD, Lenti-β^{AS3} transduced HSCs were able to correct SCD-associated pathology (47).

Both iterations of anti-sickling globin have been tested for safety and efficacy in clinical trials. The Lenti-Globin BB305 vector was studied in patients with TDT and SCD for its safety and efficacy in clinical trials HGB-205 (NCT02151526). Initially one SCD patient was treated with Lenti-Globin BB305 under this trial. BB305 modified cells remained persistent 15 months post-infusion with approximately 50% of anti-sickling $\beta^{A\text{-}T87Q}$-globin of total hemoglobin that resulted in no recurrence of sickle crises and correction of SCD hallmarks (48). In HGB-205 trial, 2 out of 3 SCD patients treated with BB305 transduced CD34+ cells achieved long-term clinical remission. In another patient, the need for transfusions was reduced (49).

A separate Phase 1/2 study (HGB-206, NCT02140554) evaluating safety and efficacy of Lenti-Globin BB305 (also known as bb1111) in approximately 50 adult and adolescent patients with severe SCD was conducted. Treatment process and outcome of this trial evolved over the study period. In the initial group (Group A), 7 patients were treated and modest level of HbA^{T87Q} level (≥0.46 g/dL) due to lower VCN (≥0.08 c/dg) was achieved 6 months post-infusion, which were insufficient for substantial clinical benefits. Through improved cell collection and manufacturing changes, higher VCN was achieved in next group (Group B) of patients (n=2) that resulted in higher VCN (≥0.53 c/dg and ≥2.14 c/dg), higher HbA^{T87Q} (≥2.69 g/dL and ≥6.40 g/dL), decreased hemolysis, and better clinical outcome (50). Notably, cases of acute myeloid leukemia (AML) (51) and myelodysplastic syndrome (MDS) (52) were observed in two patients treated with HGB-206, which were unlikely related to insertional oncogenesis. Though insertional mutagenesis was not the cause of AML or MDS cases observed with HGB-206, a recent published study observed an increased frequency of potential driver mutations associated with clonal hematopoiesis (such as DNMT3A and EZH2) in SCD patients (53). This suggests that pre-existing driver mutations of clonal hematopoiesis improve the fitness of HSCs post-gene therapy in SCD patients. Treatment of patients under Group C was initiated for pivotal evaluation of Lenti-Globin BB305 with changes in cell collection process (HSPC collection by plerixafor mobilization and apheresis) and increased cell dose. 36 patients were treated in Group C with modified cell collection and manufacturing process. While this study was ongoing, a Phase 3 study (HBG-210, NCT04293185) that also included pediatric patients, 12 to <18 years of age, was started to collect efficacy data. As of February 2023, 11 patients were treated in HGB-210 study. Combined data of 47 patients treated with BB305 showed median total Hb increased from 8.7 g/dL to 11.8 g/dL of which 40% or more was contributed by HbA^{T87Q}. Complete resolution of vaso-occulusive events were observed in more than 90% of evaluable patients (n=33) (54). Furthermore, correlative analysis of drug product quality attributes and the clinical outcome suggests that post-infusion transduction efficiency is positively correlated with the reduction in hemolysis. DP VCN in the myeloid lineage was the most predictive attribute of PB VCN. Post-infusion transduction efficiency in peripheral blood is a strong predicator of biological efficacy and successful treatment outcome with Lenti-Globin (55). Clinical data from HGB-206 and HGB-210 demonstrated that a single infusion of Lenti-Globin BB305-transduced autologous CD34+ cells sustains HbA^{T87Q} production and significantly reduces vaso-occlusive crises (54). Favorable outcomes from these studies led to the US FDA approval for the marketing of Lenti-Globin BB305 (under proper name: lovotibeglogene autotemcel or Lovo-cel, Trade name: LYFGENIA) for the treatment of patients 12 years of age or older with SCD and a history of vaso-occlusive events. Notably, LYFGENIA comes with a safety warning on the package insert as hematologic malignancy has occurred in two patients treated with LYFGENIA. The ongoing long-term follow-up study, LTF-307 (NCT04628585), will provide data on the long-term efficacy and safety of Lenti-Globin BB305 gene therapy for SCD.

The original Lenti-β^{AS3} vector contained LCR hypersensitive sites HS2, HS3, and HS4, produced poor viral titer, and showed low transduction efficiency (47). An optimized LV encoding $\beta\text{-}^{A\text{-}AS3}$ developed by removing HS4 sequence (GLOBE LV) allowed production of high titer virus, efficient transduction of Plerixafor-mobilized HSPCs, and showed efficient correction of SCD phenotype in SCD patient cells *ex vivo* (56). Lenti-vector GLOBE1 encoding anti-sickling globin $\beta\text{-}^{A\text{-}AS3}$ is evaluated for safety and efficacy in a Phase 1/2 study (DREPAGLOBE, NCT03964792). Data from 18 months follow-up of three patients showed variable efficacy. Two out of three patients clinically benefited from the treatment were no longer required transfusions, with HbAS3 levels correlated with their VCN. However, the 3rd patient did not show any benefit due to poor gene marking and rapid decline of genetically modified cells (57). Additional studies are required to assess the safety and efficacy of Lenti-vector GLOBE1.

γ-Globin is better than β-globin in preventing HbS polymerization and sickling of RBCs (58). LV expressing γ-Globin is also explored as therapeutic option for SCD in various pre-clinical

models (59–61). The γ-Globin expression vector sG^{b}G encodes γ-Globin under β-globin regulatory control elements. Study with sG^{b}G vector showed that a stable, long-term upregulation of HbF can be achieved post-nataly and SCD-associated pathologies can be ameliorated with an HbF level of over 10% (60). Using the same vector, long-term, stable expression of HbF expression (12 – 15%) was achieved in non-human primates (59). This vector is currently being evaluated in a Phase 1/2 study (NCT02186418) for its safety and efficacy. Papanikolaou and colleague have developed an LCR-less, γ-Globin encoding LV GGHI incorporating -117 HPFH point mutation and the HPFH-2 enhancer and showed increased production of γ-globin by 32.9% (62). GGHI vector was further optimized utilizing 3D enhancer element of the naturally occurring HPFH-1 deletion together with the β-globin gene 3' UTR (GGHI-mB-3D), which showed high and stable expression of HbF at low multiplicity of infection (63). Optimized GGHI-mB-3D vector showed significant increase in HbF level with concomitant decrease in HbS in SCD patient derived cells. Notably, GGHI and GGHI-mB-3D vectors are devoid of LCR present in Lenti-Globin vectors. LCR containing Lenti-Globin vectors are prone to genetic rearrangements and have lower viral titer (64, 65). LV-vector encoding γ-Globin may offer better safety and higher efficacy. However, safety and efficacy of such vectors need to be evaluated in clinical studies. Additional information on β-globin gene addition can be found in previously published reviews (66–69).

β-globin gene correction

Converting the SCD mutant codon GTG (encoding Valine) to the normal GAG (encoding Glutamic acid) in the β-globin gene is seen as an ideal approach for gene correction. Gene editing utilizes specific endonucleases, cellular repair mechanisms, and delivery methods for precise DNA changes (16). Site-specific DNA double-strand breaks (DSBs) created by engineered nucleases like ZFNs, TALENs, and CRISPR/Cas9 are repaired by cellular DNA repair machinery (such as nonhomologous end joining (NHEJ), homologous recombination (HR), homology-directed repair (HDR), mis-match repair (MMR) (70). NHEJ is the major repair pathway active throughout cell cycle (71). The erroneous NHEJ repair outcomes resulting from the incorporation of small insertion and deletion at the nuclease-induced DSB sites has been exploited for efficient gene disruption and has led to development of novel therapeutics (72). HDR, active in the late S or G2 phase, enables precise DNA repair or mutation correction using a homologous template (73). *HBB* gene correction in patient-derived HSCs followed by transplantation of HBB-corrected cells has the potential to provide long-term cure for SCD. Hoban et al. utilized ZFNs for correction of sickle mutation in the β-globin gene (74). Co-delivery of ZFN targeting β-globin gene together with homologous donor template (delivered as oligonucleotide or integrase-defective LV with wild-type sequence resulted in HDR-mediated correction of sickle mutation. Despite achieving high levels of gene correction in 10-20% of cells *in vitro*, the persistence of gene corrected human cells 16 weeks post transplantation was very low, suggesting that engagement of HDR pathway in long-term HSCs is infrequent (74), given HSCs are largely quiescent (75).

The CRISPR/Cas9 technology as an innovative genome editing tool not only simplified genome editing process but also proven to be highly effective across biological systems (76–80). Highly efficient ablation of genes in human HSCs with minimal off-target mutagenesis provided the framework for therapeutic genome editing in human HSCs and other primary cells (81). Delivery of Cas9 ribonucleoprotein (RNP) together with Adeno-associated virus (AAV) donor improved targeted gene knock-in efficiency by up to 12-fold (82). Of note, AAV is highly recombinogenic and exhibit a propensity of integration at the DSB sites in the genome, thus is a suitable HDR donor template carrier for targeted insertion (83). In addition, incorporation of chemical modifications in synthetic gRNA enhanced genome editing efficiency in human primary T cells and CD34+ HSPCs (84). Utilizing Cas9 protein complexed with chemically modified synthetic gRNA as RNP and AAV vector delivery of HDR template, Dever and colleague achieved efficient HDR at *HBB* locus with an average correction of 50% E6V mutation in SCD patient-derived CD34+ HSPCs (85). Significant reduction in cytotoxicity and off-target cleavage activity with the use of the RNP system was observed. Further optimization of this approach led to the successful correction of the *HBB* gene in SCD patient-derived HSCs. Stable production of HbA from the corrected HSCs was observed in a humanized globin-cluster SCD mouse model (85). Notably, robust HbA levels were achieved even with low chimerism levels, indicating effective gene correction and functional recovery of the HSCs (86). Another study employed a ribonucleoprotein (RNP) complex containing Cas9 protein and unmodified sgRNA, along with a single-stranded DNA oligonucleotide donor (ssODN), to correct the SCD mutation (87). The edited HSPCs showed a decrease in sickle hemoglobin RNA and protein levels, and an increase in wild-type hemoglobin upon differentiation into erythroblasts. When edited HSPCs were transplanted into immunocompromised mice, the corrected sequence at the SCD locus was retained for four months, showcasing the durability of the correction (87). Further optimization of this approach together with the use of a high-fidelity Cas9 variant led to efficient correction of >20% of *HBB* alleles (~30% cells with at least one corrected allele) in the long-term repopulating HSCs with minimal off-target effects (88). In addition, an enrichment of corrected β-globin allele was observed in erythroid lineage upon *in vivo* differentiation, suggesting selective survival advantage to erythroblasts carrying one or more corrected alleles. These studies demonstrated correcting SCD-causing mutation (E6V) in the *HBB* gene in patient-derived HSPCs using CRISPR/Cas9 could be useful in clinical settings.

Park et al. utilized a high-fidelity SpyCas9 variant alongside a single-stranded oligonucleotide template for correcting the sickle cell mutation in SCD patient-derived HSPCs from peripheral blood or bone marrow with 24.5 ± 7.6% efficiency without selection. Significant reduction in sickle cells was observed together with increased levels of normal hemoglobin (HbA) (25.3 ± 13.9%) (89). Using SpyFi Cas9 significantly reduced off-target effects including chromosomal rearrangement but retained on-target specificity. Gene corrected SCD HSPCs were able to engraft and persist for long-term in transplanted

mice. The rate of HDR remained unchanged compared to the pre-transplantation levels for peripheral blood derived CD34+ HSPCs from SCD patients. However, a reduction in gene corrected cells was observed in mice transplanted with gene corrected HSPCs obtained from bone marrow of SCD patients. In plerixafor-mobilized CD34+ HSPCs from both healthy donor and SCD patients, up to 60% allelic correction at *HBB* locus was achieved in a pre-clinical feasibility study at a clinical-scale manufacturing (90). In transplanted NSG mice, multilineage engraftment was observed with long-term persistence of 20% gene correction cells. Comprehensive long-term studies demonstrated no drug product relevant adverse effects, indicating the safety, effectiveness, and consistency of this approach (90). High levels of HDR are relatively challenging to achieve compared to gene deletion in primary cells. However, recently published studies indicate that a therapeutically meaningful *HBB* gene correction is achievable through process optimization (88, 90). *HBB* gene correction approach is currently being evaluated in ongoing Phase 1/2 studies for safety and efficacy (NCT04819841; RESTORE trial, and NCT04774536) for the treatment of SCD. Clinical studies with *HBB* gene corrected HSPCs will provide data on safety, efficacy, and durability, of this approach. Though majority of recently developed *HBB* gene correction approaches utilized CRISPR/Cas9, a GMP-compatible TALEN-mediated gene editing method using viral or non-viral donor template was shown to correct *HBB* gene, resulting in over 50% of normal globin expression in RBCs without inducing β-thalassemic phenotype (91).

Gene correction approach described above requires a donor template (either AAV, or ssODN) to execute gene repair. From therapeutic development point of view, it requires manufacturing and controls of an additional critical component - the donor template. In addition, there are safety issues with unintentional integration of donor template within genome other than intended β-globin site. Gene editing approaches independent of a donor template is highly desirable for therapeutic gene correction. Such approaches may have better safety and efficacy profile. To this end, novel gene editing tools were created by tethering various enzymatic domains to the mutated Cas9 variants which retain DNA binding activity but are devoid of DSB nuclease activity (nickase or enzymatically dead Cas9) (92). Base editors (BEs) contain active domain from a nucleotide deaminase enzyme (Cytidine or Adenine deaminases) fused with Cas9 nickase. Cytosine BEs (CBEs) contains catalytic domain from cytidine deaminases (APOBEC1) and Uracil glycosylase inhibitor (UGI) that execute direct conversion of C to U thereby imparting C to T (or G to A) substitution at the targeted site (93). Adenine BEs (ABEs) contain adenosine deaminase domain from tRNA-specific deaminase TadA engineered through directed evolution that convert A to G at the target site (94). Both ABEs and CBEs generate nucleotide substitution and have comparable genome editing efficiency to wild-type Cas9. However, BEs produce significantly lower DSB, genomic deletion/translocation, p53 activation and p53-dependent response, and inflammation pathways (95) compared to wild-type Cas9 (96, 97) and may have lower genotoxicities.

Using a custom ABE (ABE8e-NRCH), Newby and colleague converted the SCD allele (HBB^S) into a non-pathogenic Makassar β-globin (HBB^G) with high efficiency in HSPCs from SCD patients (98). 80% conversion of HBB^S to HBB^G was observed *ex vivo*. When transplanted into the mice, 68% of converted allele persisted long term and led to production of non-sickling RBSs. Compared to Lenti-globin approach, greater reduction in β^S was observed in erythroid cells. In contrast to Cas9, BE did not activate p53-dependent DNA damage response in HSPCs. In a humanized sickle cell mouse model, transplantation of BE-treated HSCs showed 80% of total globin contributed by Makassar β-globin. Persistence of converted alleles in secondary transplanted mice suggests efficient BE-mediated editing in long term repopulating HSCs. Using the structural information of Cas9 and TadA, Chu and colleague rationally designed a set of inlaid base editors (IBEs) with deaminase domain imbedded within Cas9 (99). Some of these IBEs exhibited shifted editing windows, greater editing efficiency with reduced DNA and RNA off-target editing activity and efficiently converted the pathogenic SCD mutation to a non-pathogenic, naturally occurring Makassar (HBB^G) variant. Given the precise editing without creating DSB, BEs/IBEs will likely lead to development of safe and effective autologous treatment for SCD.

By fusing an engineered reverse transcriptase (RT) domain to nickase Cas9 Liu and co-workers developed Prime Editors (PEs) (100). Together with prime editing guide RNA (pegRNA), which specifies target site specificity and contains desired complimentary sequence for genetic modification, PE can introduce targeted nucleotide insertions, deletions, and substitutions (transitions or transversions) independent of a donor repair template and without introducing DSB (101). BEs can only make six of the 12 possible point mutation and can introduce nucleotide substitutions within a target window. Same nucleotide occurring within target window are also modified by BE, introducing undesirable bystander editing within target window. BE activity is limited by presence of a protospacer adjacent motif (PAM) sequence at a specific distance range (typically 15 ± 2 nucleotide). By overcoming multiple limitations associated with other gene editing tools, PEs increases the scope of genome editing and can correct majority of known disease-causing genetic variants. Using an engineered pegRNA (epegRNA)((102) and an improved PE (103), Everette et al. corrected the sickle cell allele (HBB^S) to wild-type (HBB^A) in HSPCs from SCD patients with more than 20% correction frequency (104). When the PE-edited HSPCs were transplanted into mice, they successfully engrafted and produced normal, functional RBCs, effectively alleviating the symptoms of SCD in the animal model. PE HSPCs retained their differentiation potential, with 30-45% HbA in erythroid cells (104). Reticulocytes from mice transplanted with PE-treated HSPCs showed a significant reduction in sickling compared to untreated controls (37% vs 63%). *In vivo* prime editing of HSC in a sickle cell mouse model was recently reported (discussed below) (105).

Due to the genetic heterogeneity, developing β-globin gene correction approaches for β-thalassemia is comparatively challenging and will require specific guide RNAs and donor DNA templates tailored to unique mutation (6). Aberrant splicing of *HBB* observed with several of the most common *HBB* mutant alleles causes loss of β-globin expression. IVS1-110G>A (HBB:c.93-21G>A, rs35004220) is one of the most prevalent and severe β-thalassemia causing mutations (106) that generates a *de novo* splice

acceptor site in *HBB* intron-1. Aberrantly spliced *HBB* mRNA resulting from 110G>A mutation contains pre-mature stop codon that leads to truncated β-globin expression (107). IVS2-654C>T (HBB:c.316-197C>T, rs34451549) is commonly occurring *HBB* mutation in East Asia that creates a *de novo* splice donor site in *HBB* intron-2. Disrupting these aberrant splice sites or targeted degradation of aberrant mRNA may restore normal splicing of *HBB* gene and β-globin expression respectively. shRNA-targeted degradation of aberrant spliced mRNA with 110G>A restored β-globin levels in a novel cell model and primary HSPC (108). Several genome-editing based approaches have demonstrated that disruption of aberrant splice site resulting from 110G>A mutation restores normal splicing and β-globin expression in β-thalassemia patient-derived CD34+ HSPCs with very high efficiency (109–111). A universal approach of knocking-in a *HBB* cDNA in exon 1 of *HBB* gene was tested to correct different *HBB* gene mutations in two TDT patients-derived iPSC cell lines (6). HBB protein production was restored in the erythroid cells derived from corrected iPSCs. Though this study provided an universal approach for *HBB* gene correction, utility of this approach for the treatment of SCD and β-thalassemia is yet to be proven. As the severity of β-thalassemia often correlates with the number of inherited α-globin genes (*HBA1* and *HBA2*) and imbalance of α- and β-globin, deletion of the α-globin (*HBA1* or *HBA2*) gene together with targeted insertion of a β-globin transgene was shown to restore α/β globin imbalance in thalassemic HSPCs-derived erythroblasts (112, 113). This knock-out, knock-in approach could be developed as a novel therapeutic to restore globin homeostasis in β-thalassemia patients.

HbF upregulation

Elevated HbF level is positively correlated with milder SCD manifestation and a level of 30% HbF is capable of preventing HbS polymerization (38). HbF-containing RBCs are called F-cells which survive much longer than non-F cells (114). HbF level of >8.6% was associated with improved survival of SCD patients (36). Therefore, reactivation of the γ-globin genes (*HBG1/2*) is the key to increasing the production of HbF, which may alleviate the symptoms of severe β-globin disorders. The asymptomatic adult SCD patients have abnormally high levels of HbF, a condition known as hereditary persistence of fetal hemoglobin (HPFH), which disrupts the switching of globin gene (8). Several HPFH mutations were identified that elevated HbF in RBCs resulting in reduced polymerization of sickle hemoglobin, minimal hemolysis and vaso-occlusive events in affected patients. Elevated level of HbF also ameliorates β-thalassemia severity. Genome-wide association studies (GWAS) identified three quantitative trait loci (QTLs) (*BCL11A* at 2p15, *HBS1L-MYB* intergenic region at 6q23 and *XmnI-Gγ* at 11p16) containing single-nucleotide polymorphisms (SNPs) that resulted in elevated level of HbF (115–120). Single-nucleotide variants (SNVs) and small deletions were identified in the proximal promoter of the *HBG1* and *HBG2* gene that resulted in upregulation of HbF levels (121). Mechanistic studies with these variants, provided insights on globin gene regulation (11, 122–129) that could be exploited to develop therapeutic strategies for SCD and β-thalassemia by targeting the repressors of γ-globin gene or their binding sites in the promoter region of γ-globin gene. *BCL11A* is a principal repressor of γ-globin gene activity in adult RBCs and is responsible for more than half of the postnatal suppression of γ-globin gene expression (128, 130, 131). Targeting γ-globin gene repressors, specifically *BCL11A* and *ZBTB7A/LRF*, to enhance HbF production emerged as a promising therapeutic approach (18, 128, 130, 131). The *BCL11A* gene is a preferred target because it specifically influences γ-globin expression without impacting other essential genes (132). Strategies for upregulating HbF include knocking down the *BCL11A* gene, disrupting its enhancer, or interfering with BCL11A binding sites on the γ-globin promoter (described below).

Knocking down *BCL11A* gene

In primary adult erythroid cells, *BCL11A* binds to various sites in β-globin gene cluster. Suppressing *BCL11A* boosts γ-globin production, leading to a rise in HbF (131). However, *Bcl11a* is also essential for postnatal development, normal lymphopoiesis, and may act as a T-cell tumor suppressor (133, 134). BCL11A deletion is associated with persistent HbF and neurodevelopmental disorders (122). Ubiquitous knock-down of BCL11A may adversely affects the long-term engraftment of edited cells (135). Erythroid lineage-specific silencing of *BCL11A* circumvented engraftment impairment observed with ubiquitous knockdown and led to 90% reduction in BCL11A in erythroid lineage (135) and increased γ-chain expression with concomitant elevation in HbF levels. Transplantation of HSCs transduced with LV-LCR-shRNA^{miR}-BCL11A into Berkeley SCD mouse models demonstrated a significant alleviation of disease symptoms (135). A refined version of this vector (called as BCH-BB694) showed 3- to 5-fold induction of HbF without affecting growth, differentiation, and engraftment potential of genetically modified CD34+ HSPCs in a pre-clinical study (136). In the ongoing clinical study (NCT03282656), as of October 2020, 6 patients were treated with BCH-BB694 with a median follow-up for 18 months. All treated patients showed robust and stable HbF induction with 9.0 to 18.6 pg HbF per F-cell, resulting in reduced or absent SCD clinical manifestations (137). This study demonstrated a favorable risk-benefit profile of BCH-BB694 in patients with SCD. However, an increased frequency of driver mutations (mutations in *DNMT3A* and *EZH2*) of clonal hematopoiesis was observed at 3.5 to 5 years of follow-up in these patients (53). Following gene therapy, an increase in total number of mutations per HSPC was observed in four of the six patients compared to the baseline mutation burden. Although, there was no unique clonal expansions larger than 1% observed post-gene theapy, findings from this study suggest that gene therapy related procedure allows selection of HSCs with pre-existing mutations in genes associated with clonal hematopoiesis (such as *DNMT3A* and *EZH2*) which may "imporve" fitness of HSC post-gene therapy in SCD patients. Therefore, a long-term follow-up post-gene therapy is required to monitor clonal evolution and expansion of mutated HSCs. A recent study introduced a novel LV that co-expresses two short hairpin microRNAs (shmiRs)

targeting *BCL11A* and *ZNF410*. This dual-targeting strategy yielded up to a 70% reduction in targeted protein levels in erythroid cells and a synergistic increase in HbF levels by 10% compared to targeting *BCL11A* alone (138). When applied to erythroid cells from SCD patients, this approach markedly reduced the *in vitro* sickling phenotype, presenting a promising dual-target strategy for the treatment of β-hemoglobinopathies (138). These studies underscore the therapeutic potential of precise, lineage-specific genetic modifications which may offer a more refined approach to treating hemoglobinopathies.

Disrupting *BCL11A* enhancer

Common genetic variations associated with HbF level are located in the intron 2 of *BCL11A* with an erythroid-specific enhancer signature. Three erythroid-specific DNase Hypersensitive (DHS) sites named as DHS +62, +58, and +55 (based on their location) were identified in primary erythroid cell, which were not detected in other cell types (123). Furthermore, these sites were occupied by erythroid-specific transcription factors GATA1 and TAL1. Deletion of orthologous enhancer elements in mouse leukemia MEL cell line resulted in reduced expression of BCL11A and de-repression of γ-globin. Further interrogation of these sites through *in situ* saturating mutagenesis (124) using pooled sgRNA library led to the identification of +58 site as the most critical regulator of *BCL11A*. CRISPR-mediated deletion of +58 site in human CD34+ HSPCs led to the downregulation of BCL11A, upregulation of γ-globin, and increase in HbF in erythroid progeny derived from edited HSPCs. This suggests that disruption of critical sequence within +58 site within *BCL11A* enhancer with a single gRNA could be used to elevate HbF level as a therapeutic approach for β-globinopathies without impacting non-erythroid functions of *BCL11A* (such as lymphopoiesis) (133). Cas9: sgRNA ribonucleoprotein (RNP)-mediated disruption of GATA1 binding site at the +58 *BCL11A* erythroid-specific enhancer resulted in BCL11A downregulation and induction of HbF in erythroid cells differentiated from edited SCD and β-Thalassemia patient-derived HSPCs (139). Sickling of erythroid cells, differentiated from edited SCD-patient derived HSPCs, was prevented and globin chain homeostasis was restored in erythroid cells derived from edited β-Thalassemia patient-derived HSPCs. *BCL11A* enhancer edited HSCs persisted for more than 101 weeks and biallelic *BCL11A* enhancer editing resulted in robust HbF induction in rhesus monkey without any toxicity (140). Similar results were obtained with ZFN-mediated bi-allelic disruption of GATAA motif within erythroid-specific enhancer in human bone marrow derived CD34+ HSPCs (141). GATAA motif disruption resulted in upregulation of HbF without impairing HSPC functions or erythroid enucleation. However, biallelic disruption of *BCL11A* through exon 2 targeting led to erythroid enucleation impairment and poorer engraftment of edited HSPCs in mice (141). Another study reported erythroid-specific *BCL11A* enhancer editing in β-Thalassemia major patient-derived HSPCs using ZFN, showing *BCL11A* enhancer deletion resulted in increased γ-globin expression (132).

Encouraging results from per-clinical studies described above led to the translation of *BCL11A* enhancer editing approaches into the clinic. ZFN-mediated disruption of the GATA-binding region within erythroid-specific *BCL11A* enhancer is currently studied in a Phase 1/2 clinical trial, addressing safety and efficacy of this approach for TDT (ST-400, Thales trial, NCT03432364) (142). ST-400 is an ex vivo, ZFN-edited autologous HSC product for the treatment of TDT. At the time of interim reporting, 5 patients treated with ST-400 showed hematopoietic reconstitution. However, HbF level were not sustained (declined by 64% from peak level) to achieve transfusion independence. This decline in HbF level is presumably due to low levels of edited long-term HSCs in the ST-400. An analogous drug product SAR445136 (also known as BIVV003) is also being developed for SCD (NCT03653247, PRECIZN-1) (143, 144). Four patients treated with BIVV003 showed no SCD-related complications, with increased total hemoglobin and HbF along with improved clinical scores (143).

CRISPR/Cas9-mediated *BCL11A* enhancer deletion approach for SCD and β-Thalassemia (139) were evaluated for safety and efficacy under two clinical studies (CTX-001, NCT03655678; CLIMB THAL-111 and NCT03745287; CLIMB SCD-121). Initially, two patients — one with TDT and the other with SCD were treated with CRISPR-Cas9 edited autologous CD34+ HSPCs (CTX-001) (145). High levels of *BCL11A* enhancer editing were achieved in bone marrow and blood of both the patients with increased HbF. Transfusion independence and elimination of vaso-occlusive episodes were achieved in TDT and SCD patient respectively. Data from the pivotal CLIMB SCD-121 trial showed that a single dose of CTX-001 (Proper Name: Exagamglogene autotemcel or exa-cel) in first 31 patients aged 12 to 35 years with sever SCD led to upregulation of HbF >20% and all the treated patients were VOC-free (146). A phase 3 study was conducted and a total of 44 patients received exa-cel under CLIMB SCD-121 trial. Of the 30 patients with sufficient follwo-up, 29 were free from VOC for at least 12 consecutive months and all 30 patients were free from hospitalizations for VOC for at least 12 consecutive months (147). This study met its primary endpoint and both key secondary endpoints with 97% of patients free from VOC for at least 12 months and 100% were free from hospitalization. Exa-cel (Proprietary name: CASGEVY™) received FDA approval on December 8, 2023 for the treatment of SCD in patients 12 years of age or older with recurrent VOCs. exa-cel was also studied for its efficacy and safety in TDT patients in a Phase 3 trial (148). A total of 52 TDT patients received single infusion of exa-cel. Transfusion independence was achieved in 91% of patients and red cell transfusions were stopped at a mean of 35.2 ± 18.5 days after the exa-cel infusion. CASGEVY™ was approved by the FDA on January 16, 2024, for the treatment of patients aged 12 years and older with TDT. A long-term follow-up study continues to monitor hemoglobin levels, safety, potential secondary cancers, vaso-occlusive events, and patient-reported outcomes (CLIMB-131, NCT04208529). Several ongoing studies are evaluating CRISPR/Cas9-mediated *BCL11A* enhancer editing in autologous CD34+ HSPCs for SCD and TDT (NCT04211480, NCT05577312, NCT04211480, NCT04205435, NCT04925206) (149, 150) with encouraging results. Furthermore, editing the *BCL11A* enhancer at both +58 and +55 sites leads to disruption of alleles with high efficiency and specificity, resulting in targeted downregulation of

BCL11A in erythroid cells, and a significant increase in HbF production (151). Taken together, these studies underscore the efficacy and safety of CRISPR/Cas9-mediated targeting of *BCL11A* enhancer in treating β-hemoglobinopathies.

Newly developed CRISPR tools such as BEs and PEs were also used to edit *BCL11A* enhancer or BCL11A binding sites in HBG1/HBG2 promoter (described in the following section) with high efficiency. Utilizing ABE8e base editor, a recent study enhanced HbF production either by targeting the *BCL11A* enhancer or the *HBG* promoter in the CD34+ HSPCs from β-thalassemia patients (152). Additionally, ABE8e-SpRY, a modified version of ABE designed for extensive targeting range, was used to directly correct HbE and IVS II-654 mutations in CD34+ HSPCs from patients. Sustained therapeutic modifications were validated in self-renewing, repopulating human HSCs. These findings underscore the efficacy of ABE-mediated base editing in HSCs as a promising therapeutic approach for SCD and TDT (152).

Recent studies have identified additional strategies to modulate *BCL11A* gene function. The gene *HIC2* is a key regulator influencing hemoglobin switching, it represses *BCL11A* transcription by binding to erythroid *BCL11A* enhancers, thereby reducing chromatin accessibility and GATA1 transcription factor binding, leading to decreased enhancer activity. Overexpressing *HIC2* in SCD patient-derived HSPCs improved HBG1/2 mRNA levels and inhibited sickling (153).

Interrupting repressor binding sites in γ-globin promoter

HPHF is a benign condition with elevated HbF production into adulthood often caused by deletions or point mutations in the *HBB* gene or the promoter region of *HBG* genes. Targeting regions encompassing the δ- and β-globin genes in HSPCs from patients with SCD has significantly boosted HbF synthesis in adult erythroblasts, ameliorating the sickle cell phenotype (154). In SCD patients with HPFH, natural point mutations in the *HBG* promoter led to a shift from producing faulty adult β-globin to fetal γ-globin that reduces the severity of the disease. Recent studies identified more HPFH variants, including *GATA1, KLF1*, or *TAL1*, which serve as new binding sites to reactivate HbF for β-Hemoglobinopathies (155, 156). Reactivation of γ-globin gene through recreating HPFH mutations that disrupt repressor binding sites could be used therapeutically to mitigate β-hemoglobinopathies. In a study utilizing CRISPR-Cas9 technology, HPFH-like mutations were mimicked in the *HBG* promoters through the introduction of insertions and deletions (157). These modifications disrupted the repressor binding sites for *BCL11A* and *LRF*, with particular emphasis on the LRF-binding site in patient-derived HSPCs. This genetic editing enhanced γ-globin production and ameliorated the sickling phenotype in these cells. Notably, the HSPCs which were modified at the LRF-binding site exhibited effective gene editing and demonstrated efficient repopulation after being transplanted into a xenograft model (157).

Editing specific DNA sequences in the *HBG1/HBG2* gene promoters increased HbF production in erythroid cells, maintaining cell multipotency and lineage differentiation for extended periods without detectable off-target effects (158, 159). EDIT-301 is a gene editing product under investigation for the treatment of severe SCD and TDT. It consists of patient-derived $CD34^+$ HSPCs edited at the γ-globin gene promoters *HBG1/2*. RBCs derived from EDIT-301 cells demonstrate a sustained increase in HbF. These data support two clinical trials. The ongoing RUBY trial (NCT04853576) is evaluating the efficacy and safety of EDIT-301 in adult and adolescent participants with severe SCD. In this study, 4 patients received a single administration of EDIT-301 (160). Preliminary data demonstrated successful engraftment of edited cells, sustained increase in total Hb, HbF, and F cells. The ongoing EDIT-301 trial (NCT05444894) is to evaluate the safety, tolerability, and efficacy of EDIT-301 in adult participants with TDT.

OTQ923 is another gene therapy product comprised of CD34+ HSPCs that were edited using CRISPR-Cas9 targeting the *HBG1/2* promoters. Preclinical experiments showed sustained on-target editing without off-target mutations and elevated HbF levels in edited CD34+ HSPCs. A clinical study (NCT04443907) involving severe SCD patients assessed its safety and efficacy. Three study participants receiving OTQ923 experienced successful engraftment and a significant induction of HbF, resulting in a reduction of disease manifestations (161).

The heme-regulated eIF2α kinase (HRI, also known as EIF2AK1), regulates hemoglobin levels in SCD patients. Reducing HRI levels can increase HbF, and in turn, decrease the sickling of erythroid cells in culture, suggesting that HRI could be a potential target for increasing HbF levels and reducing the complications of SCD (162). *BCL11A* reduction is primarily accounted for the HbF increase following *HRI* depletion (162).

New regulatory elements have recently been identified as targets for enhancing HbF. A study showed that editing the -123 and -124 positions in the *HBG* promoter of erythroblasts from human CD34+ HSPCs led to a notable increase in HbF levels, surpassing the results from disrupting the *BCL11A* site. The introduction of HPFH-like mutations enhances γ-globin production, facilitated by the new binding site for the KLF1 protein, offering a potent method to augment HbF in patients (163). Another study identified a core-regulatory region known as PRR-bE1, an 11-kb core-regulatory region spanning from the putative repressor region to β-globin exon-1. Editing the PRR-bE1 region effectively shifted globin expression from β-globin to γ-globin and reversed β-hemoglobinopathy phenotypes (164).

A recent published study compared Cas9 and ABE and reported that compared to Cas9-generated indels at erythroid enhancer, ABE was more potent, with –175A>G causing the strongest induction of HbF (19). Utilizing a non-integrating, helper-dependent human CD46 targeting adenoviral vector (HDAd5/35++), Li and colleague delivered CRISPR/Cas9 (HDAd-HBG-CRISPR) in to HSC of β-YAC mice to disrupt a *BCL11A* binding site in the *HBG1/2* promoter that resulted in reactivation of γ-globin in red blood cells of adult mice (165). Further refining their approach, they developed BE vectors to target *BCL11A* binding site in the *HBG1/2* promoter, mimicking HPFH mutations (166). The BE effectively

reactivated γ-globin in human erythroid cells. *In vivo* experiments showed significant γ-globin+ erythrocyte levels and confirmed the targeted genetic modification's stability and safety, highlighting the potential of this base editing approach for treating β-hemoglobinopathies (166). Most importantly, use of BE resulted in marked reduction in intergenic *HBG1/2* deletion and detectable genotoxicity. A phase 1/2 trial (NCT05456880) is exploring the use of ABE to mimic HPFH variants in SCD patients. Furthermore, multiplex editing combining *BCL11A* enhancer deletion together with disruption of LRF or *BCL11A* binding sites within HBG promoter region (-115 and -197 sites) resulted in significantly higher levels of γ-globin expression in thalassemic CD34+ cells (167). Utilizing this dual editing approach, Psatha and colleague further showed successful editing in thalassemic CD34+ cells *in vivo* that led to induction of γ-globin and pancellular HbF expression in a thalessemic xenotransplantation model with their adenoviral vector platform (HD-Ad-dualCRISPR) (167). HPFH related mutations are clustered within -200 to – 115 nucleotides upstream of the *HBG1/2* transcriptional start sites. Using CBEs and ABEs, the dissection the -200 region of the *HBG1/2* promoter showed that creation of novel KLF1 activator binding sites in this region are the most potent inducer of γ-globin, resulting in higher levels of HbF compared to disruption of *BCL11A* binding site (163, 168).

Taken together, gene therapy for SCD and β-thalassemia have advanced notably, with strategies like Lenti-globin gene addition, β-globin gene correction, *BCL11A* manipulation, and base editing showing promising results, backed by clinical successes and regulatory approvals. Ongoing research is expected to improve the safety and efficacy of these gene therapy treatments.

In vivo gene editing in HSCs for β-globinopathies

Ex vivo gene therapy (GT) and gene editing (GE) approaches developed thus far are complex and expensive (169). *Ex vivo* manufacturing of HSPCs-based therapeutics requires CD34+ HSPCs mobilization, collection of mobilized HSPCs through apheresis, shipment of apheresis material to the manufacturing facility, *ex vivo* manipulation of mobilized CD34+ HSPCs, harvest and formulation of genetically modified cells, it's cryopreservation and shipment to the clinical site for infusion. *Ex vivo* manufacturing of HSPC products requires variety of resources, such as advanced manufacturing facility, complex logistics, and close coordination between doctors, patients, and manufacturers. *Ex vivo* culture of HSPCs can also result in loss of stemness and engraftment potential, which may reduce therapeutic efficacy (170, 171). In addition, successful transplantation of *ex vivo* modified HSPC products in patients requires myeloablative treatment, which is a high-risk procedure. HSCT remains a high-risk procedure with significant treatment related complications such as bacterial and fungal infections, toxicities, graft failure, safety issues arising from manufacturing process (insertional mutagenesis of lentiviral vectors, off-target editing, and myeloblative conditioning, and can result in death (172, 173). *In vivo* GE and GT via intravenous or intraosseous administration of GT vectors/GE reagents simplify the manufacturing process, and overcomes challenges associated with *ex vivo* manufacturing of HSPC-based products. Systemic *in vivo* delivery bypasses the need for conditioning and extensive care, and minimizes the risks associated with *ex vivo* methods like myeloablation, genotoxic effects. Unlike *ex vivo* methods, *in vivo* GT/GE offers a more accessible option for regions heavily burdened by β-hemoglobinopathies with shortage of advanced medical facilities and resources. Recently published reports demonstrated that therapeutically meaningful *in vivo* gene editing can be achieved (105, 174, 175). A recent study used a non-integrating, helper-dependent adenoviral (HDAd) vector (HDAd5/35++) with high affinity toward CD46 (a receptor that is uniformly expressed on primitive HSCs), to deliver PEs *in vivo* in a SCD mouse model (CD46/Townes mice) (105). HDAd5/35++ vector also allows selection of gene edited cells *in vivo* using a mutant O6-methylguanine DNA methyltransferase (MGMTP140K) gene that confers resistance to O6-BG/BCNU (O6-Benzylguanine/Carmustine). To overcome the physical barriers formed by the bone marrow stroma, granulocyte-colony stimulating factor (G-CSF)/plerixafor (AMD3100) were used to mobilize HSCs from bone marrow into peripheral blood. With a single intravenous injection of PE-expressing HDAd5/35++ vector, ~40% of HBB^{S} alleles were corrected in HSCs with minimal generation of insertion/deletion and undetectable off-target editing. With this approach 43% of HbS was replaced with HbA. Efficient induction of γ-globin was also achieved in non-human primates following *in vivo* transduction of CD34+ HSPCs with HDAd5/35++ vector expressing γ-globin (176). Following G-CSF/AMD3100-mobilization, infusion of HDAd5/35++ vector resulted in preferential transduction of CD34+ cells and $MGMT^{P140K}$-based *in vivo* selection of transduced cells resulted in up to 90% γ-globin+ RBCs, though a decline in γ-globin+ RBCs following selection was observed.

Non-viral delivery systems such as lipid nanoparticles (LNPs), virus-like particles (VLPs), and exosomes are also being studied as alternative to viral vectors to improve the safety and effectiveness of these gene therapies (174, 177). Systemic deliver via LNPs and VLPs are two commonly used non-viral-based delivery systems for *in vivo* delivery of GT/GE cargo (178, 179). LNPs are central to enhancing the delivery of genetic drugs with improved targeting capability and minimized side effects (180). For example, the LNPs targeting BM has shown the ability to genetically alter HSPCs *in vivo* in a humanized mouse model (175), providing a proof-of-concept for SCD treatment. A recently published report described targeted delivery of GE components to HSCs using anti-CD117 antibody conjugated LNP (174). anti-CD117/LNP were able to delivery mRNA cargos (Cre recombinase, Luciferase) to HSCs *in vivo* in mice. Cre-mediated recombination was detected in all the lineages in peripheral blood and bone marrow in a dose-dependent manner, suggesting targeting of HSCs. Using the same anti-CD117/LNP delivery platform, ex vivo delivery of ABEs to SCD HSPCs resulted in highly efficient (88%) conversion of HBB^{S} to $HBB^{G\text{-}Makassar}$ with

up to 91.7% increase in HBBG protein and a nearly complete absence of sickled RBCs in differentiated erythroid cells. These findings demonstrated that *in vivo* targeting of HSCs is achievable and may eventually be translated into clinics for the treatment of SCD and other monogenic blood disorders with a simple single intravenous infusion of targeted genetic medicines.

Outstanding challenges and future perspectives

Recent advances in gene therapy have revolutionized the treatment of SCD and β-thalassemia by targeting the underlying causal mutations. One time infusion of genetically modified cells may provide long-term therapeutic benefits and possibly cure SCD and β-thalassemia. Despite tremendous progress, several challenges remain for broader application of such therapies for SCD and β-thalassemia. Long-term safety and efficacy of gene therapy or gene editing approaches are primary concerns. Insertional oncogenesis from random integration of LV or editing at unintended sites in the genome could be detrimental to patients receiving such therapy. Lowering the VCN in the drug product and minimizing off-target editing by using improved Cas9 nuclease or limiting the duration of Cas9 expression will improve the safety profile of GT/GE products for SCD and β-thalassemia. High-fidelity CRISPR-Cas9 systems like HiFi Cas9, SpyCas9, SpCas9-HF1, eSpCas9, and HypaCas9 exhibit improved specificity (181). To achieve long-term therapeutic benefits, genetically modified CD34 + HSPCs must engraft and persist in the patients receiving such therapies. *Ex vivo* culturing and manipulation (LV transduction, gene editing) may lead to differentiation, activation of innate immune system, or activation of p53-dependent DNA damage pathways, which can adversely affect the engraftment potential of ex vivo modified CD34+ HSPCs. Improved culture conditions, better quality reagents and LV, and utilization of nucleases that do not induce DNA DSB may improve the quality of *ex vivo* modified CD34+ HSPCs resulting in better engraftment and persistence of these cells for long-lasting efficacy. Unlike Cas9, BEs and PEs do not generate DSBs and CD34+ cells modified with BE/PE have better engraftment potential than CD34+ cells modified with Cas9. In addition, use of milder conditioning regimen or antibody-mediated conditioning may improve the engraftment of *ex vivo* modified cells for better outcome.

Given the complexity of HSC-based gene therapy product manufacturing processes, scaling up the manufacturing is often challenging. Innovations in bioprocessing technologies and automation can help to improve product quality and reduce lot-to-lot variability. Modular and adaptable manufacturing processes may streamline the scalability and provide better control of critical variables that affect product quality. More effort is required to increase the accessibility of these genetic therapies to the resource limited regions of the world with the highest disease burden (182). Emerging *in vivo* HSPCs editing or targeted gene delivery to HSPCs would simplify the manufacturing and treatment process for wider applications.

Conclusive remarks

More than 50 years of basic research in vector biology, globin gene regulation, hemoglobin polymerization, coupled with newer technology such as CRISPR/Cas, and many years of pre-clinical and clinical research on SCD and β-thalassemia culminated with the US FDA approval of three HSC-based gene therapy products (ZYNTEGLO, LYFGENIA, and CASGEVY) for the treatment of SCD and β-thalassemia. SCD is often referred as poster child for gene therapy for monogenic disorders and along the way it helped to develop the framework for gene therapy and gene editing based therapeutics. Multiple approaches utilizing safer and effective gene editing tools (such as BEs and PEs) are currently being evaluated in pre-clinical and clinical studies with hope to develop safe, effective, and durable therapeutics, which may eventually cure SCD and β-thalassemia with a single, one-time infusion of genetically modified HSPCs. As these newer approaches correct SCD causal mutation, independent of a donor repair template and without generating DSB, correcting *HBB* gene mutation with BEs and PEs could be safer and more effective compared to wild-type Cas9. Recently published studies indicated that *in vivo HBB* gene editing is achievable in pre-clinical SCD model. Clinical translation of this approach would overcome several limitations associated with *ex vivo* manufacturing and transplantation related complications. *In vivo* editing of *HBB* will also simplify the treatment procedure and will likely make such therapy widely available for geographical areas where these diseases are prevalent with limited access to advanced healthcare infrastructure (5). Lessons learned with developing gene therapy products for β-globinopathies will also accelerate the development of such therapies for other monogenic blood disorders.

Author contributions

LL: Writing – original draft, Writing – review & editing. PM: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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