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Genetic Engineering in Hematopoietic Stem Cells for β-Hemoglobinopathies Treatment: Advances, Challenges, and Clinical Translation

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ABSTRACT

β-hemoglobinopathies rank among the most prevalent inherited blood disorders globally. Traditional management strategies are primarily palliative and often associated with significant challenges, including iron overload and limited long-term efficacy. Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially curative option for transfusion-dependent patients, but its broader applicability is constrained by factors that limit its use. Utilizing viral vectors and gene-editing tools, particularly CRISPR-Cas9 technology, researchers have developed therapies that target the root causes of these disorders. These innovative approaches have demonstrated substantial therapeutic potential, accompanied by favorable safety profiles, in clinical settings. Since the initial investigations, the genome editing tool has rapidly advanced for genetic abnormalities, particularly monogenic blood diseases, including β-hemoglobinopathies. This method suggests an approach with lower concerns in viral gene integration and insertional mutagenesis issues. This review comprehensively surveys the therapeutic strategies for β-thalassemia and sickle cell disease (SCD) currently in preclinical and clinical development, with a focus on the evolving treatment paradigm. Looking forward, critical research priorities include optimizing the efficiency and specificity of gene-editing platforms and pioneering novel delivery systems to guarantee both therapeutic efficacy and clinical safety.

Keywords: Gene therapy; CRISPR/Cas; β-thalassemia; Sickle cell anemia; Clinical trials

INTRODUCTION

β-Hemoglobinopathies

β-Thalassemia and sickle cell disease (SCD) are the most prevalent monogenic hereditary conditions with clinical implications[1,2]. A range of distinct β-globin gene mutations that either decrease or completely stop the synthesis of β-globin chains (the β^+ and β^0 genotypes, respectively) cause β-thalassemia. The excess of α-globin chain metabolites in the cells leads to hemolytic anemia,

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intramedullary erythroid precursor destruction, and inefficient erythropoiesis. Individuals with β-thalassemia major have severe anemia, persistent hemolysis, iron overload, hepatosplenomegaly, and other problems such as endocrine diseases and cardiopathies. These patients also require frequent transfusions to survive[2].

A broad spectrum of regions, including the Mediterranean, the North and Sub-Saharan Africa, the Middle East, the Indian subcontinent, and Southeast Asia, are affected by β-thalassemia[3]. The serious complications of falciparum malaria seem to be insulated heterozygotes for β-thalassemia. In the malaria-prone tropical and sub-tropical areas, natural selection has driven up and preserved their gene frequencies [4]. Despite this, as a consequence of recent population migration, β-thalassemia has extended to other nations, including North America and Europe, and has emerged as a significant public health concern in these regions [5].

An aberrant hemoglobin (Hb) variation (Sickle Hemoglobin (HbS), βGlu6Val) as a result of an A-to-T point mutation in hemoglobin subunit beta (HBB) causes Sickle cell disease. Because of this substitution, HbS is more likely to polymerize under low oxygen conditions, and these red blood cells (RBCs) acquire an inflexible sickle form instead of a typical doughnut-shaped form, which is a crucial step in the molecular pathophysiology of SCD [6].

Repeated episodes of sickling and unsickling lead to microvascular obstruction and short half-life of sickled RBCs. Young individuals with SCD are more likely to experience morbidity and death because of complications such as priapism, avascular necrosis, stroke, hypertension, chronic pulmonary impairment, and repeated veno-occlusive crises[7]. The number of babies born with SCD has increased by 13.7% between 2000 and 2021 [8]. Wealthy nations account for 10% of the world's 25 million SCD individuals, while Africa mostly endures the burden of SCD. Moreover, the highest death rate occurs in individuals around six months and three years old on this continent [9].

Autologous hematopoietic stem cell gene therapy (HSCT-GT) has shown therapeutic benefits for treating SCD and β-thalassemia. By utilizing a patient's own stem cells, this approach circumvents the complications and risks associated with allogeneic HSCT, such as graft-versus-host disease and the challenge of finding a compatible donor [10].

Molecular Mechanism of β-globin Genes Expression

The gene cluster of β and other β-like genes, including ε (Hemoglobin E (*HBE*)), Gγ (Hemoglobin Subunit Gamma-2 (*HBG2*)), Aγ (Hemoglobin Subunit Gamma-1(*HBG1*)), δ (Hemoglobin Subunit Delta (*HBD*)), and β (*HBB*), located on chromosome 11 (11p 15.15) in a sequential arrangement based on their developmental expression [11, 12]. Each of these five genes possesses its promoters at the 5' end and is regulated independently [10]. The locus control region (LCR) (16 kb), a *cis*-regulatory/enhancer element, is a substantial chromatin opening area that resides 40–60 kb upstream of β-globin genes. The LCR is composed of five DNase hypersensitive (HS) sites (named HS1 to HS5) that directly interact with the promoters of β-globin genes (looping pattern) and numerous transcription factors to control its expression and erythroid lineage differentiation [13, 14]. The formation of chromatin loop is mediated by a complex that includes GATA-Binding Factor 1 (GATA1), Krueppel-Like Factor 1 (KLF1), T-Cell Acute Lymphocytic Leukemia Protein 1 (TAL1), Nuclear Factor Erythroid 2 (NF-E2), LIM Domain Only 2 (LMO2), and additional co-factors such as Friend of GATA-1 (FOG), p300, and Specificity Protein 1 (sp1)[15,16].

Erythroid lineage-restricted Kruppel-like transcription factor (EKLF), a member of the KLF1 family, is identified as a crucial element in γ-globin genes switching [17]. EKLF is highly expressed in adult erythroid tissues and has a higher affinity for the β-globin promoter than its γ-globin, facilitating the activation of β-globin transcription [18]. Simultaneously, mutations in the EKLF-DNA binding region have been detected in individuals with β-thalassemia, thereby supporting the significance and practical applicability of KLF1 in controlling β-globin production [19, 20]. At the initial phases of erythroid differentiation, GATA-1, a zinc finger element, binds to the regulatory DNA sequences of globin genes and has a pivotal effect on different gene expressions. GATA-1 acts as an erythroid transcriptional activator

based on its activity in histone acetylation on β-LCR. Additionally, GATA-1 collaborates with NF-E2 in chromatin looping between the LCR and the γ-globin gene, a configuration essential for appropriate γ-globin expression [21-23].

Another crucial component of erythropoiesis is TAL1, the binding partner of GATA-1, which is necessary for globin gene expression in embryonic stem cells [24, 25]. In addition to GATA-1, TAL1 is essential for chromatin looping during γ-globin production [26]. Moreover, these components, along with the LMO2, form a complex with Ldb1, which promotes and maintains long-distance contact between erythroid promoters and β-LCR enhancer elements [27].

Hemoglobin Switching

γ-to-β Hb switching is the process by which fetal hemoglobin (HbF, α2γ2), which comprises less than 5% of total hemoglobin, starts to decline around six months after birth and reaches the adult level of less than 1% by two years of age [16].

KLF1, Myeloblastosis (MYB), stage selector protein (SSP), and nuclear receptors TR2/TR and COUP transcription factor 2 (COUP-TFII) contribute to Hb switching. The zinc-finger transcription factor B Cell Lymphoma 11A (BCL11A) is one of the main elements in suppressing the γ-globin expression in human cells [16, 28, 29].

Research indicates that BCL11A binds to the proximal and distal promoters of γ-globin to suppress its expression through interactions with GATA1, FOG1, SRY-box 6 (SOX6), DNA methyltransferase 1 (DNMT1), and the nucleosome remodeling/deacetylase complex (NuRD)[30, 31].

The transcription factor lymphoma/leukemia related factor (LRF)/ Zinc Finger and BTB Domain Containing 7 (ZBTB7A) have also been shown to suppress the fetal-like globin production in adult human erythroid cells. Independent of the HbF repressor BCL11A, LRF represses the Nucleosome Remodeling and Deacetylase (NuRD) repressor complex. Chromatin modifiers and remodeling factors (such as Euchromatic Histone-Lysine N-Methyltransferase (EHMT1/2), Chromodomain-Helicase-DNA-Binding Protein 4 (CHD4), DNA methyltransferase 1 (DNMT1), and lysine-specific demethylase (LSD116)), as well as theLin-28 Homolog B- Lethal-7 (LIN28B-let7) microRNA pathway, were also identified as suppressors [32-34].

Zinc Finger Protein 410 (ZNF410) enhances the transcription of CHD4, which encodes the NuRD complex and leads to preventing the γ-globin production (39). The findings further indicate that MYB binds to the promoter of the β-globin activator KLF1, transactivates the KLF1 production, and subsequently activates the γ-globin repressor GATA-1. Consequently, MYB has been identified as an effective γ-globin repressor [34, 35].

Conventional Treatments for β-thalassemia and Sickle Cell Disease

For many years, β-thalassemia and SCD have been alleviated using several medication approaches to mitigate their complications [1] (Figure 1).

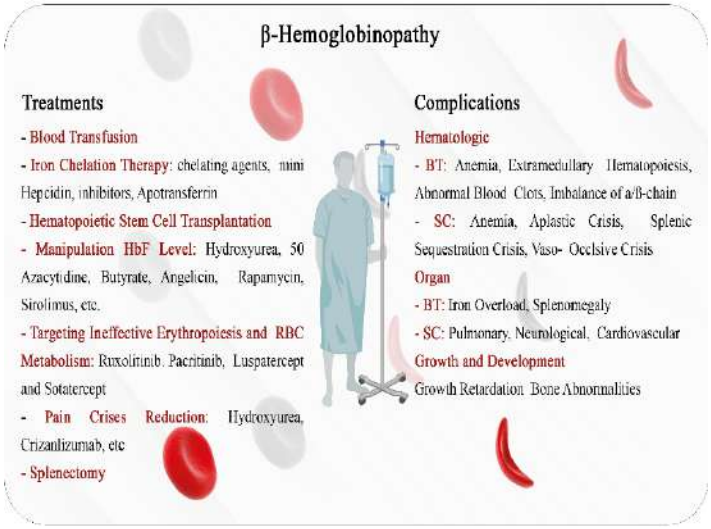

Figure 1. Comprehensive overview of complications and treatment approaches for β-hemoglobinopathies

Blood Transfusion

β-thalassemia and SCD patients may require periodic or regular blood transfusions throughout their lives to prevent inefficient erythropoiesis and reduce the risk of further pathophysiological complications. Iron overload, alloimmunization, and blood-associated infections are challenges related to blood transfusions. Although transfusion procedures have improved, some patients still encounter difficulties[36].

Iron Chelator Agents

Iron overload is a critical element in the pathophysiology of patients receiving blood transfusions. In these cases, deferoxamine mesylate has been a conventional iron-chelating treatment. Subsequently, deferiprone and deferasirox received orphan drug designations (ODD) in the United States (US) and the European Union (EU), respectively. Deferiprone and deferasirox are alternatives to deferoxamine due to their easier modes of administration [37].

Iron Regulation Pathway

Hepcidin, a hormone responsible for maintaining iron homeostasis, regulates dietary iron absorption and plasma iron content. Systemic iron excess through intestinal absorption in β-thalassemia patients came from the low hepcidin levels. Augmenting hepcidin by developing its hepcidin-like peptides, including mini-hepcidin (MH) and rusfertide, promotes ferroportin degradation[38]. Alternatively, the inhibition of TMPRSS6, a transmembrane serine protease that negatively regulates hepcidin, can increase hepcidin levels. SLN124 and sapablursen target TMPRSS6 production via hepatocyte-targeted siRNA and antisense oligonucleotide (ASO) methods [39, 40].

In β-thalassemia, patients have shown elevated transferrin saturation and the toxicity of excess iron lies in unchaperoned iron. Apotransferrin administration to β-thalassemia mice stabilized unstable plasma iron content, normalized RBC survivability, and enhanced hemoglobin levels while reducing reticulocytes, erythropoietin (EPO), and splenomegaly [41]. Another strategy involves altering the capacity of the transferrin receptor 2 (Tfr2) to regulate erythropoiesis. Animal models have shown that combining Tfr2 single-allele deletion and TMPRSS6-ASO leads to reduced splenomegaly and increased hemoglobin levels [42].

Several human clinical studies have reported the beneficial effects of ferroportin blockers. A small-molecule medication called Vamifeport (VIT-2763) is administered orally and works by binding to ferroportin to prevent cellular iron export and promote ferroportin internalization. In preclinical animals, Vamifeport improved β-thalassemia and caused iron restriction[43].

Targeting Ineffective Erythropoiesis and RBC Metabolism

β-thalassemia patients exhibit high levels of erythroid activity in their bone marrow; however, erythropoiesis fails due to the premature maturation of differentiating erythroid precursors. This leads to anemia and elevated erythropoietin (EPO) production under hypoxic conditions. Ineffective erythropoiesis is driven by several factors, including transforming growth factor-beta (TGF-β) signaling and the activin receptor type II (ActR-II) pathway. Luspatercept, a drug approved by the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) in 2020, inhibits overactive Suppressor of Mothers Against Decapentaplegic (SMAD) signaling in erythroid precursors and blocks activin binding to ActR-II. This results in enhanced erythroid maturation and increased red blood cell production[44, 45]. Alternatively, Janus kinase 2 (JAK2) is a key component of the EPO signaling pathway in erythroid progenitors. Elevated EPO levels in β-thalassemia mouse models are associated with increased JAK2 phosphorylation, leading to severely ineffective erythropoiesis and extramedullary hematopoiesis. Studies have shown that JAK2 inhibitors, such as ruxolitinib and paritinib, effectively reduce splenomegaly in β-thalassemia mice. JAK2 inhibitors may be particularly beneficial for non-transfusion-dependent thalassemia (NTDT) patients with splenomegaly[46]. RBC maturation depends heavily on intracellular adenosine triphosphate (ATP) and cyclic adenosine nucleotides. Lower ATP levels have been reported in the RBCs of patients with β-thalassemia. Mitapivat, an allosteric activator of pyruvate kinase in RBCs, has been shown to increase ATP levels and improve anemia in animal models of β-thalassemia. Furthermore, Mitapivat demonstrated improved hemoglobin levels, reduced hemolysis, and alleviation of ineffective erythropoiesis in an open-label phase II clinical trial involving individuals with NTDT α- and β-thalassemia[47,48].

Reducing α globin Synthesis

The accumulation of free, excess α-globin in erythroid precursor cells and its continuous synthesis are the primary pathophysiological mechanisms underlying ineffective erythropoiesis in β-thalassemia. Clinical studies have shown that the co-inheritance of α-thalassemia, which naturally reduces α-globin chain production, can improve the clinical condition of patients with β-thalassemia. Potential strategies to achieve tissue-specific, selective attenuation of α-globin levels to a therapeutic range include RNA interference (RNAi) using small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), epigenetic drug targeting to modify the chromatin structure of α-globin genes, and genome editing approaches to disrupt the expression of these genes[49].

Manipulating HbF Level

Understanding the molecular mechanisms regulating γ-globin gene expression led to the development of a new generation of agents [50].

The most commonly used HbF enhancer is hydroxyurea (HU), which has become the global standard treatment for SCD. Although HU is used as a treatment for transfusion-dependent thalassemia (TDT) and NTDT, its therapeutic benefits in β-thalassemia are less well-established. Sodium 2,2-dimethyl butyrate (HQK-1001) stimulates HbF expression and RBC production in both β-thalassemia and SCD [51, 52].

Tetrahydrouridine (THU), an inhibitor of cytidine deaminase, combined with decitabine (5-aza-2'-deoxycytidine), a hypomethylating agent that inhibits the chromatin-modifying enzyme DNMT1, forms the basis of EPI01 therapy. The EPI01 regulates the activity of genes such as *BCL11A*, which silences the β-globin gene in mature erythroid cells. Decitabine, a chemotherapeutic drug approved for treating myelodysplastic syndromes, has cytotoxic effects [53].

Sirolimus, a lipophilic macrolide with immunosuppressive properties (also known as rapamycin), has been approved for preventing organ rejection in kidney transplants [54]. Based on the administrating dose, sirolimus enhances HbF synthesis in erythroid progenitor cells. Another promising agent is benserazide hydrochloride. In preclinical animal studies, this DOPA decarboxylase inhibitor (used alongside levodopa to treat Parkinson's disease) was shown to increase HbF levels, likely by downregulating BCL11A, LSD1, and histone deacetylase 3 (HDAC3) [55].

Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation (HSCT) is a curative treatment for β-hemoglobinopathies. HSCT from a well-matched donor, including bone marrow or umbilical cord blood HLA-matched siblings or a matched unrelated donor, remains disease-free in almost 90% of patients, particularly in those aged < 14 years at the time of transplantation. This therapy relies on high-dose chemotherapy to eliminate β-hemoglobinopathy-producing cells in the marrow and replace them with healthy donor cells.

Although the expected costs associated with HSCT are lower than those associated with lifespan blood transfusion and iron chelation, there are always concerns regarding transplant-related challenges, such as finding a suitable donor, infections, rejection, acute and chronic graft-versus-host disease (GVHD), and toxicities such as veno-occlusive disease and hemorrhagic cystitis [56].

Gene therapy Approaches for Hemoglobinopathies

Gene therapies have the potential to serve as a curative approach for β-hemoglobinopathies. The goal of gene therapy in these diseases is to restore the ability of blood-forming stem cells to produce red blood cells with normal hemoglobin levels[57]. Two main directions have emerged in globin gene therapy: the first focuses on repairing the *HBB* gene, while the second aims to reactivate the *HBG* gene. These strategies are achieved through various methods, including viral vectors and gene editing tools[2,58]. (Table 1: List of some clinical trials in β-hemoglobinopathies gene therapy) placed at the end of the document.

Viral Vector Gene Therapy

Retroviral Vectors

Viral vectors were introduced as the first molecular method for effective and safe gene transfer into human somatic cells. There are two types of viral

vectors: integrating and non-integrating. Integrating vectors include retroviral and lentiviral vectors, which facilitate the long-term expression of the therapeutic gene product. Adenoviral vectors, on the other hand, function episomally, meaning they do not integrate into the host genome. Retroviruses are RNA viruses that undergo reverse transcription (the process of converting RNA into DNA) and integrate their genetic material into the genome of a host cell. These viral particles contain two copies of 7–10 kb single-stranded RNA (ssRNA) molecules. Researchers create the first transgenic mice with retroviral β-globin genes in 1986[59]. In several studies, LCR sequences have been integrated into retroviral vectors to achieve position-independent, erythroid-specific expression. Sadelain and colleagues identified core regulatory regions (HS2, HS3, and HS4) to produce high-titer retroviral vectors with effective β-globin mRNA-expressing cells[60]. However, retroviral vectors have shown several limitations, including low titers, poor gene transfer efficiency, unstable vector genomes, insufficient β-globin gene expression, and position-dependent expression outcomes [10].

Lentiviral Vectors

Lentiviral vectors (lentivirus HIV-1) offer distinct advantages over γ-retroviruses. Unlike γ-retroviruses, lentiviral vectors can facilitate gene delivery to non-dividing and post-mitotic cells, including HSCs, which are typically quiescent. Lentiviral vectors also enable shorter and simpler ex vivo culture conditions than γ-retroviral vectors. Additionally, lentiviral vectors can accommodate slightly longer transgenic sequences because the HIV-1 genome (~10 kb) is longer than murine γ-retroviruses (~8 kb). This expanded capacity allows lentiviral vectors to carry complex β-globin gene structures, including reverse orientation, LCR HS elements, and introns essential for high-level erythroid-specific expression. Moreover, the HIV-1 REV/RRE pathway enables improved nuclear export of intact vector genomes, facilitating more efficient delivery of functional transgenes [61].

A 133-bp deletion in the enhancer/promoter region of the U3 section of the viral long-terminal repeat (LTR) endows lentiviral vectors with "self-inactivating" (SIN) characteristics, making them safer than earlier retroviral vectors. Partial deletions in the LTR reduce the transactivation of nearby promoters at integration sites and eliminate viral promoter/enhancer activity. Consequently, unlike previous retroviral vectors, transcription of the provirus is driven not by endogenous LTR promoters but by an internal exogenous promoter, such as the phosphoglycerate kinase (PGK), cytomegalovirus (CMV), or the endogenous globin promoter for erythroid-specific expression[10,61]. The following vectors have played a significant role in improving the lentiviral therapy for β-hemoglobinopathies (Figure 2).

legends: (1) TNS9 contains an internal deletion within intron 2, a 3.2 kb LCR element comprising HS2, HS3, and HS4, along with an endogenous β-globin promoter that extends across the region, (2) In T87Q, the 87th threonine (T87) located within the hydrophobic region of the β-globin gene was replaced by a polar glutamine. This variant features a shortened intron 2 rather than its original TNS9 and includes 2.7 kb of LCR elements, (3) the GLOBE vector contains the β-globin promoter with only the HS2 and HS3 regions. It does not include the HS4 region, the β-globin 3' enhancer, or any insulator elements, (4) Lenti-βAS3 comprises a 265 bp β-globin promoter with 3.4 kb LCR elements developed in the Townes laboratory utilizing a more compact GLOBE LV backbone, (5) The BB305 vector incorporates the anti-sickling T87Q amino acid substitution with a 2.7 kb LCR region, (6) The sGbG contains the β-globin promoter with the HS2, HS3, and HS4 elements of the β-globin locus control region (LCR) and the γ-globin exons, (7) The GGHI-mB-3D LV contains the γ-globin gene bordered by distinct regulatory elements derived from the γ- and α-globin loci, and (8) The BCL11A shRNAmir lentiviral vector contains short 1.4 kb LCR elements, specifically HS2 and HS3.

Name/ID	Study Start Date/Sponsor	Indicate	Current Stage	Enrollment Status	Primary Outcomes	Toxicities
LG001 study[1]	Bluebird Bio, France September 2006	Transfusion-Dependent β-Thalassemia and Severe Sickle Cell Disease condition, β-globin restored autologous hematopoietic stem cells modified with HPV569 (Lentiviral Beta AT87Q-Globin Vector)	Phase 1/2	3 patients	Decline in both the volume and frequency of transfusion requirements	Delayed platelet recovery post-transplant
TNS9.3.55 NCT01639690	Memorial Sloan Kettering Cancer Center, USA July 2012	B-thalassemia major condition, β-globin restored autologous hematopoietic stem cells modified with TNS9.3.55 Lentiviral Vector	Phase 1	4 adult patients aged ≥18	None of the patients achieved transfusion independence with a median follow-up of 90 months	No SAEs or unexpected safety issues related to TNS9.3.55
HGB-204 NCT01745120	Bluebird Bio August 2013	β-thalassemia major condition, β-globin restored autologous hematopoietic stem cells modified with LentiGlobin BB305 (Lentiviral βA-T87Q-Globin Vector)	Phase 1/2	18 patients aged 12–35 years	Non-β^0/β^0 Genotype: 8 out of 10 patients achieved TI*, maintaining it for a median of 33 months β^0/β^0 Genotype: 2 out of 8 patients achieved TI. The remaining 6 patients experienced a median reduction in annual transfusion volume of 63%	No SAEs or unexpected safety issues related to HGB-204
HGB-205 NCT02151526	Bluebird Bio France June 2013	β-thalassemia and Sickle Cell Disease condition, β-globin restored autologous hematopoietic stem cells modified with LentiGlobin BB305 (Lentiviral βA-T87Q-Globin Vector)	Phase 1/2	7 patients were enrolled: 4 adolescents (ages 12–17) and 3 adults (ages 18–64).	2 patients with β^0/β^E achieved transfusion independence for 28 and 31 months 1 patient with a β^0/β^E remained transfusion-free for 9 months 1 with a severe β$^+$ was transfusion-free for 9 months	No LentiGlobin-related Grade ≥3 adverse events or serious adverse events were reported
NCT02186418	Cincinnati Children's Hospital Medical Center, USA July 2014	Sickle Cell Disease condition, transduced autologous hematopoietic stem cells with the γ-globin lentiviral vector (ARU-1801)	Phase 1/2	7 patients	Specific outcome measures included the level of therapeutic hemoglobin production, reduction in sickling events, and overall improvement in clinical symptoms	No SAEs or unexpected safety issues related to the study
HGB-206 NCT02140554	Bluebird Bio, USA February 2015	Sickle Cell Disease condition, β-globin restored autologous hematopoietic stem cells modified with bb1111 (LentiGlobin BB305)	Phase 1/2	44 patients	Complete elimination of severe VOEs$^\#$ between 6 and 24 months post-treatment Patients maintained median levels of gene therapy-derived HbA^T87Q contributing at least 40% of total hemoglobin at Month 6	1 non-serious adverse events of febrile neutropenia considered related to lovo-cel No serious adverse events were deemed related to lovo-cel

Name/ID	Study Start Date/Sponsor	Indicate	Current Stage	Enrollment Status	Primary Outcomes	Toxicities
TIGET-BTHAL NCT02453477 NCT03275051	**IRCCS San Raffaele, Italy May 2015**	**β-thalassemia condition, β-globin restored autologous hematopoietic stem cells modified with GLOBE lentiviral vector (OTL-300)**	**Phase 1/2**	**9 patients 3 adults (≥18 years) 3 adolescents (8–17 years) 3 young children (3–7 years)**	**Out of 4 treated children, 3 achieved transfusion independence within months post-treatment The remaining pediatric patient continued to require regular transfusions All 3 adults exhibited a significant reduction in transfusion needs (33–80%) but remained transfusion-dependent at last follow-up**	**No SAEs or unexpected safety issues related to the study**
HGB-207 NCT02906202	Bluebird Bio August 2016	β-thalassemia condition, β-globin restored autologous hematopoietic stem cells modified with LentiGlobin BB305 (Lentiviral βA-T87Q-Globin Vector)	Phase 3	23 patients (4 to 34 years)	Out of 19 evaluable patients, 17 (89%) achieved transfusion independence	3 instances of veno-occlusive liver disease. 2 instances of thrombocytopenia
HGB-212 NCT03207009	Bluebird Bio June 2017	β-thalassemia condition, β-globin restored autologous hematopoietic stem cells modified with LentiGlobin BB305(Lentiviral βA-T87Q-Globin Vector)	Phase 3	15 patients	≥60% reduction in red blood cell (RBC) transfusion volume (mL/kg) from Months 12 to 24 post-treatment	One SAE of thrombocytopenia was considered possibly related to study
NCT03282656 NCT05353647	David Williams, Boston Children's Hospital, USA February 2018 July 2022	Sickle Cell Disease condition, autologous hematopoietic stem cells transduced with the lentiviral vector containing a short-hairpin RNA targeting BCL11a	Phase 1/2	10 patients aged 3 to 40 years 25 patients aged 13 to 40 years	Specific outcomes were not detailed	Specific adverse events were not detailed No SAEs or unexpected safety issues related to the product
ST-400 NCT03432364	Sangamo Therapeutics March 2018	Transfusion Dependent β-thalassemia condition, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using ZFN	Phase 1/2	5 patients 18–36 years	β^0/β^0: 33% reduction in annualized PRBC units transfused since engraftment Homozygous for β^+ IVS-I-5 (G>C) mutation: Continued intermittent transfusions β^0/β^+ with IVS-II-654 (C>T) mutation: Initial transfusion-free period of 7 weeks; resumed intermittent transfusions thereafter	No SAEs or unexpected safety issues related to the product
CTX001 NCT03655678 NCT05356195 NCT03745287 NCT05329649	Vertex Pharmaceuticals, September 2018	β-thalassemia major, Sickle Cell Disease, Hemoglobinopathies conditions, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	Phase 2/3	CLIMB-Thal-111 (TDT): Up to 45 patients aged 18–35 CLIMB-SCD-121 (SCD): Up to 45 patients aged 18–35	All patients with ≥3 months of follow-up achieved transfusion independence With fetal hemoglobin levels ranged from 40.9% to 97.7% All patients with ≥3 months of follow-up remained VOC-free With fetal hemoglobin levels ranged from 39.6% to 49.6%	One TDT patient experienced SAEs related or possibly related to CTX001 No SAEs considered related to CTX001 in SCD patient

Name/ID	Study Start Date/Sponsor	Indicate	Current Stage	Enrollment Status	Primary Outcomes	Toxicities
CSL200 NCT04091737	**CSL Behring, USA October 2019**	**Severe Sickle Cell Disease condition, autologous hematopoietic stem cells transduced with Lentiviral Vector encoding human γ-GlobinG16D and Short-Hairpin RNA734) in adult**	**Phase 1**	**1 patient**	**Detailed results regarding efficacy are not available**	**Specific adverse events or toxicities were not detailed**
DREPAGLOBE NCT03964792	Assistance Publique - Hôpitaux de Paris, France November 2019	Sickle Cell Disease condition, β-globin restored autologous hematopoietic stem cells modified with GLOBE1 Lentiviral Vector (expressing the βAS3 Globin Gene)	Phase 1/2	6 patients 12 to 20 years	Specific outcomes were not detailed	Specific data on adverse effects or toxicities observed in the DREPAGLOBE trial have not been publicly disclosed
HGB-210 NCT04293185	Bluebird Bio, USA February 2020	Sickle Cell Disease condition, β-globin restored autologous hematopoietic stem cells modified with bb1111 (LentiGlobin BB305)	Phase 3	35 patients 2 to 50 years	Complete resolution of vaso-occlusive events (VOEs) between 6 and 18 months post-treatment	No SAEs or unexpected safety issues related to the lovo-cel
OTQ923 NCT04443907	Novartis Pharmaceuticals August 2020	Sickle Cell Disease condition, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	Phase 1	3 patients	Stable engraftment of gene-edited cells Increased HbF levels ranging from 19% to 26.8% of total hemoglobin Improved total hemoglobin levels post-infusion	No unexpected safety signals or treatment-related serious adverse events were reported
NCT04211480	Bioray Laboratories, China October 2020	β-thalassemia major condition, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	Phase 1/2	6 patients 5 to 15 years	Detailed safety outcomes are anticipated upon the release of comprehensive study results	Specific information regarding adverse effects or toxicities has not been publicly disclosed
NCT05745532	Shenzhen Hemogen, China December 2020	β-thalassemia major condition, β-globin restored autologous hematopoietic stem cells modified with LentiHBBT87Q	Early Phase 1	10 patients 8 to 16 years	Detailed safety outcomes are anticipated upon the release of comprehensive study results	Specific information regarding adverse effects or toxicities has not been publicly disclosed
BD211 NCT05015920	Shanghai BDgene, China April 2021	β-thalassemia major condition, autologous CD34⁺Stem Cells transduced with a Lentiviral vector encoding βA-T87Q-Globin	N/A	10 patients	Detailed safety outcomes are anticipated upon the release of comprehensive study results	Specific information regarding adverse effects or toxicities has not been publicly disclosed
EDIT-301 NCT04853576	Editas Medicine, USA May 2021	Sickle Cell Disease and Hemoglobinopathies conditions, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas12a	Phase 1/2	20 patients	All treated patients demonstrated successful neutrophil and platelet engraftment	No serious adverse events have been reported following EDIT-301 infusion
ET-01 NCT04925206	EdiGene (GuangZhou) Inc, China August 2021	Transfusion Dependent β-thalassemia condition, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	Phase 1	8 patients 12 to 35 years	In a related investigator-initiated trial involving a single patient, ET-01 demonstrated a safety profile consistent with autologous hematopoietic stem cell transplantation and myeloablative conditioning The patient achieved timely engraftment and became transfusion-independent for 15 months post-infusion	Specific information regarding adverse effects or toxicities has not been publicly disclosed
Nula-cel NCT04819841	Kamau Therapeutics, USA November 2021	Sickle Cell Disease condition, gene edited autologous hematopoietic stem cells, convert HbS to HbA using CRISPR-Cas9	Phase 1/2	15 patients 12 to 40 years	Following the discontinuation patient's blood counts improved, and she became transfusion-independent Notably, the hemoglobin profile revealed an unexpected predominance of fetal hemoglobin (HbF) (>78%) and a reduction of HbS to less than 5%, despite the therapy's design to restore adult hemoglobin (HbA) production	The first patient treated in the trial experienced prolonged pancytopenia, necessitating platelet and red blood cell transfusions

Name/ID	Study Start Date/Sponsor	Indicate	Current Stage	Enrollment Status	Primary Outcomes	Toxicities
NCT04205435	**Bioray Laboratories, China November 2021**	**β-thalassemia major condition, β-globin restored autologous hematopoietic stem cells by targeted CVS-654 mutation using CRISPR-Cas9**	**Phase 1/2**	**2 patients 5 to 15 years**	**Given the early termination and small sample size, comprehensive safety data may not have been collected or disclosed**	**No specific adverse effects or toxicities have been reported for this trial**
EDIT-301 NCT05444894	Editas Medicine, USA April 2022	Transfusion Dependent β-thalassemia condition, HbF reactivated autologous hematopoietic stem cells by targeted HBG1/2 promoter using CRISPR/Cas12a	Phase 1/2	2 patients 5 to 15 years	Given the early termination and small sample size, comprehensive safety data may not have been collected or disclosed	No specific adverse effects or toxicities have been reported for this trial
BEAM-101 NCT05456880	Beam Therapeutics, USA August 2022	Sickle Cell Disease condition, autologous base edited hematopoietic stem cells by targeted HBG1/2 promoter to increase HbF	Phase 1/2	30 patients	Patients experienced resolution of anemia post-treatment No VOCs were reported post-engraftment	No unexpected safety concerns or serious adverse events directly attributable to BEAM-101 have been reported to date
BRL-101 NCT05577312 NCT06287086	Bioray Laboratories, China November 2022 May 2024	β-thalassemia and Sickle Cell Disease condition, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	Phase 1	9 patients aged 3 to 35 years	Absolute peripheral blood neutrophil count of ≥ 0.5 × 10^9/L for three consecutive days within 42 days following BRL-101 infusion	Specific data on adverse effects or toxicities observed in participants have not been publicly disclosed
ET-01 NCT04390971	Institute of Hematology & Blood Diseases Hospital, China February 2023	Transfusion Dependent β-thalassemia condition, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	N/A	8 patients	Absolute peripheral blood neutrophil count of ≥ 0.5 × 10^9/L for three consecutive days within 42 days following ET-01 infusion	Specific data on adverse effects or toxicities observed in participants have not been publicly disclosed
LentiRed NCT05762510	First Affiliated Hospital of Guangxi Medical University, China February 2023	Transfusion Dependent β-thalassemia condition, β-globin restored autologous hematopoietic stem cells modified with LentiRed (GMCN-508B)	Early Phase 1	5 patients	While detailed results are pending, the study's design underscores a commitment to assessing both the safety and therapeutic potential of this innovative treatment	Specific data on adverse effects or toxicities observed in participants have not been publicly disclosed
VGB-Ex01 NCT06041620	Institute of Hematology & Blood Diseases Hospital, China August 2023	Transfusion Dependent β-thalassemia condition, HbF reactivated autologous hematopoietic stem cells by targeted HBG1/2 promoter using CRISPR/Cas12b	N/A	2 patients aged between 3 and 35 years	While detailed results are pending, the study's design underscores a commitment to assessing both the safety and therapeutic potential of this innovative treatment	Specific data on adverse effects or toxicities observed in participants have not been publicly disclosed
RM-001 ChiCTR2100052858 ChiCTR2100053406	Guangzhou Reforgene Medicine, China	Transfusion Dependent β-thalassemia condition, HbF reactivated autologous hematopoietic stem cells by targeted HBG1/2 promoter using CRISPR/Cas9	Phase 1	19 patients aged 6 to 35 years	All 19 patients (100%) discontinued transfusions within a median of 22 days post-infusion and maintained transfusion-free status for at least 6 months	No RM-001–related SAEs were reported

Name/ID	Study Start Date/Sponsor	Indicate	Current Stage	Enrollment Status	Primary Outcomes	Toxicities
NCT05442346	Bioray Laboratories, China December 2023	β-thalassemia major condition, HbF reactivated autologous hematopoietic stem cells using glycosylase base editors	N/A	The trial has been suspended due to a decision by the sponsor and no participants have been enrolled	The study was suspended before enrolling participants, and therefore, no clinical data are available to assess the safety or efficacy of the treatment	As the trial was suspended prior to enrolling any participants, there are no reported data on adverse effects or toxicities associated with the investigational treatment
KL003 NCT06280378	Kanglin Biotechnology, China February 2024	Transfusion Dependent β-thalassemia condition, β-globin restored autologous hematopoietic stem cells modified with KL003 (βA-T87Q-globin)	Phase 1/2	41 patients aged between 3 and 35 years	In earlier studies, KL003 demonstrated promising results, with reports indicating 100% efficacy in 17 patients, leading to rapid transfusion independence and engraftment	As the trial is ongoing and currently recruiting participants, detailed data on adverse effects or toxicities are not yet available
Exa-cel NCT05951205	Vertex Pharmaceuticals April 2024	Sickle Cell Disease β^S/β^C Genotype conditions, HbF reactivated autologous hematopoietic stem cells by targeted BCL11A using CRISPR-Cas9	Phase 3	44 patients with SCD 56 patients with severe genotypes (β^0/β^0 or β^0/β^0-like)	96.7% of SCD patients were free from severe VOCs for at least 12 consecutive months 94.2% of evaluable patients achieved transfusion independence for at least 12 consecutive months	No cases of malignancy or unexpected safety concerns have been reported to date
CRISPR_SCD001 NCT04774536	Mark Walters, MD-UCSF, USA June 2024	Sickle Cell Disease condition, HBB corrected autologous hematopoietic stem cells using CRISPR-Cas9	Phase 1/2	9 patients aged 12–35 years	N/A	As of now, detailed safety data are not publicly available, as the trial is in its early stages

1. Cavazzana, M. *et al.* Outcomes of Gene Therapy for Severe Sickle Disease and Beta-Thalassemia Major Via Transplantation of Autologous Hematopoietic Stem Cells Transduced Ex Vivo with a Lentiviral Beta AT87Q-Globin Vector. *Blood* **126**, 202–202 (2015).
* Transfusion Independence
\# Vaso-Occlusive Effects

Table 2: Most important Lentiviral vectors in β-hemoglobinopathies gene therapy

Name	Components	Advantages
TNS9	Promoter + intron + β-globin 3'enhancer + LCR elements (HS2, HS3, and HS4)	Express a high level of β-globin gene Anti-sickling feature
T87Q	Q87 + 2.7 kb LCR elements	Improve sickle cell disease
GLOBE	β-globin promoter+ HS2 and HS3 regions	Higher titer has been shown with removal of insulator elements sequences.
βAS3-globin	265 bp β-globin promoter + 3.4 kb LCR elements	More effective anti-sickling features
BB305	HPV569-derivative	Anti-sickling features
GGHI-mB-3D-globin	γ-globin gene + unique regulatory constituents from the γ- and α-globin loci	Anti-sickling and anti-thalassemia features Slight increase in HbF levels Enhancement in erythroid maturation
BCL11A shRNAmir	Short 1.4 kb LCR (HS2 and HS3) components	Suppress the regulatory gene BCL11A Anti-sickle cell disease features

TNS9

TNS9, the first operational therapeutic application of β-globin lentiviral vectors (LV), contained an internal deletion in intron 2, a 3.2 kb LCR element (HS2, HS3, and HS4), and an endogenous β-globin promoter. TNS9 demonstrated long-term, enhanced erythroid-specific expression and high gene transfer efficiency in mouse hematopoietic stem and progenitor cells (HSPCs)[62]. The first clinical trial in the United States on severe inherited globin disorders (NCT01639690) began in 2012, testing TNS9. Four patients showed low-level but persistent gene labeling, with two requiring fewer RBC transfusions [63].

T87Q

In the first developed anti-sickling variant (βA-T87Q), also known as T87Q, the Leboulch group engineered the LV that substitutes the hydrophobic threonine at position 87 (T87) with a polar glutamine. This variant also features truncated intron 2 and 2.7 kb LCR elements compared to the original TNS9. The Q87 amino acid substitution effectively prevents HbS polymerization, significantly reduces sickled RBCs and splenomegaly, and ultimately improves the sickle cell phenotype in an SCD animal model[62].

GLOBE

A phase I/II clinical trial for adult and pediatric patients with thalassemia major, sponsored by IRCCS San Raffaele in Italy, was initiated in 2015 (NCT02453477) based on the GLOBE vector. The GLOBE lentiviral vector expresses the wild-type β-globin transgene under the control of the β-globin promoter and includes only the HS2 and HS3 regions. The HS4 region, the β-globin 3' enhancer, and insulator elements were absent in this construct, resulting in a higher viral titer. Although the absence of an insulator in the GLOBE vector raises concerns about the potential activation of genes near the vector integration site, there is no evidence that the vector harbors transforming potential. Moreover, it has been effectively used to correct β-thalassemia in the cells of pediatric patients [63].

βAS3-globin

The Townes Laboratory introduced three amino acid changes into the β-globin gene to create a more effective anti-sickling variant known as βAS3-globin[64]. Lenti-βAS3, which incorporates a 265 bp β-globin promoter and 3.4 kb of LCR elements, was developed using a shorter GLOBE lentiviral vector backbone. It has been demonstrated that 82% of hemoglobin tetramers contain βAS3-globin subunits instead of the HBBS (β6V) subunit[65]. This finding supports the hypothesis that the G16D substitution increases the affinity for α-globin. Meanwhile, Ferrari et al. developed GLOBE as a treatment option for thalassemia. This vector, which carries a wild-type β-globin gene and a 2.6 kb LCR containing HS2 and HS3 elements, has recently completed clinical testing [66].

BB305

The HPV569 vector was modified to increase titer and transduction efficiency without compromising vector safety. The 5' LTR of the HPV569 vector was altered by replacing the wild-type HIV-1 U3 region with the strong constitutive CMV promoter to enhance the amount of RNA expressed in producer cells. This modification also eliminated the need for the Tat gene, reducing the plasmids required for lentiviral vector production and increasing vector yield. The resulting LentiGlobin BB305 lentiviral vector marked the development of a new and improved second-generation globin vector [67].

As a result, autologous gene therapy using LentiGlobin BB305 vector-transduced $CD34^+$ cells has emerged as a potential therapeutic option for individuals with non-β^0/β^0 severe β-thalassemia, showing positive outcomes without adverse events[68]. These findings led to the approval of Zynteglo, the first gene therapy for TDT, in 2019 [69].

In December 2023, the FDA approved LYFGENIA for patients aged 12 years and older with sickle cell disease SCD and a history of vaso-occlusive events. This therapy uses the BB305 lentiviral vector carrying the βA-T87Q-globin gene to produce HbA T87Q hemoglobin with anti-sickling properties and promising clinical results [70].

sGbG

In one study, the minimal β-globin lentiviral vector, designated as the sGbG vector, was developed based on the existing β-globin LV. It included the β-globin

promoter, HS2, HS3, and HS4 components of the β-globin LCR, along with the γ-globin exons. Using the Berkeley sickle cell disease animal model, treatment with this γ-globin LV combined with low-intensity conditioning demonstrated in vivo correction of the sickle phenotype and associated sickle cell-related pathologies. Furthermore, the study proposed that achieving an HbF/F-cell level greater than one-third of the total hemoglobin in sickle RBCs is sufficient to restore the sickle phenotype, based on measurements of HbF/F-cell levels in transduced mice using biotin surface labeling and intracellular HbF staining [71].

GGHI-mB-3D-globin

A notably distinct LV, GGHI-mB-3D-globin LV, is being investigated as a potential therapy for SCD and thalassemia by inducing HbF, building on the earlier GGHI LV. The newly modified GGHI-mB-3D LV is a β-globin LCR-free vector that carries the γ-globin gene and is flanked by unique regulatory elements from the γ- and α-globin loci. This vector has shown some improvement in SCD and thalassemia phenotypes in patients with differentiated HSPCs, along with a modest increase in HbF levels and partial restoration of erythroid maturation [72, 73].

BCL11A shRNAmir LV

Silencing BCL11A, one of the primary inhibitors of globin expression, promotes HbF synthesis, which in turn slows HbS polymerization and alleviates the symptoms of SCD[74].

MicroRNA-adapted short hairpin RNAs (shRNAmirs) were developed using a modified LV to suppress BCL11A in SCD. miRNA scaffolds allow the use of a polymerase II promoter, and the BCL11A shRNAmir LV contains short 1.4 kb LCR elements (HS2 and HS3), showing promise for erythroid-specific BCL11A knockdown [75, 76]. Clinical trials utilizing this in vivo gene therapy approach have demonstrated therapeutic benefits in SCD[77]. shRNAmir was also used to knock down ZNF410 expression. Compared to BCL11A shRNAmir alone, HSPCs from SCD patients treated with a vector carrying both ZNF410 and BCL11A shRNAmirs showed a 10% increase in HbF levels and a significant reduction in sickled cells [78].

Gene Editing Strategy

Over the past decade, direct gene editing has emerged as a viable alternative for genetically modifying cells. In theory, site-directed editing could reduce the risk of genotoxicity associated with the semi-random integration of viral vectors. Numerous studies have investigated target-specific endonucleases, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)-associated endonucleases such as CRISPR-associated protein 9 (Cas9), to optimize their use in gene therapy. CRISPR, a more convenient tool than its predecessors, has demonstrated effective gene editing in sickle cell disease and β-thalassemia[79].

ZFNs act by the DNA-binding domains of transcription factor zinc finger proteins (ZFPs) with the nuclease domain of the FokI restriction endonuclease. The binding specificity of the engineered zinc finger motifs directs ZFNs to selected genomic sites, enabling targeted cleavage at specific regions [80]. Using ZFNs, researchers have pursued gene therapy for β-thalassemia by introducing a mutation into the SOX6 binding site via lentiviral vectors to produce the γ-globin protein. Following erythroid differentiation, ZFN-treated cells exhibited γ-globin mRNA levels six times higher than untreated cells, and hemoglobin electrophoresis confirmed increased HbF expression[81]. ST-400 consists of autologous $CD34^+$ cells that efficiently produce HbF following precise ex vivo editing of the erythroid-specific enhancer of *BCL11A* using high-precision ZFNs. In this process, $CD34^+$ cells are transfected with mRNA-encoding ZFNs, which target binding sites flanking the GATA-binding region of the *BCL11A* erythroid-specific enhancer[82,83]. Although ZFNs have a simple structure and design, their editing specificity depends heavily on flanking genomic sequences beyond the target site, representing a substantial limitation that can lead to genetic instability and unintended DNA breaks[84].

TALENs operate similarly to ZFNs in their ability to target specific genomic regions. However, TALENs are more widely used in biological research because the DNA-binding domains of TALEs are less complex to construct [85]. In 2013, TALENs effectively addressed

the β-globin IVS2-654C>T mutation by repairing the β-globin gene in induced pluripotent stem cells (iPSCs) derived from β-thalassemia patients. Consequently, red blood cells derived from the corrected iPSCs exhibited higher β-globin gene expression than unedited controls. Moreover, studies using animal models and DNA sequencing confirmed the production of biallelic TALENs+/β654 mice by TALEN vectors targeting the human β-globin IVS2-654C>T mutation, successfully eliminating this specific mutation [86, 87].

CRISPR-Cas nucleases, originally part of the bacterial immune system, cleave phage and plasmid DNA. The CRISPR-Cas system detects target-specific CRISPR RNA (crRNA) and protospacer-adjacent motifs (PAMs) to guide RNA-directed cleavage of specific DNA sequences, allowing for precise cutting at designated genomic locations [88,89]. Compared to TALENs, Cas9 offers more genome stability following modification. Furthermore, CRISPR-Cas9 gene editing technology has led to the successful development and market introduction of gene editing therapies [90].

Conventional gene editing tools induce DNA double-strand breaks (DSBs) to stimulate the cell's endogenous repair machinery. Two main pathways can repair DSBs: homology-directed repair (HDR) and non-homologous end joining (NHEJ). NHEJ repairs DSBs by directly ligating the broken DNA ends; however, it is error-prone and often introduces insertions and/or deletions (indels) at the break site. Consequently, inducing NHEJ through site-specific DSBs has been used to disrupt genes via frameshift mutations. Alternatively, HDR uses a donor template to accurately repair the DSB by inserting the corrective sequence at the break site.

Utilizing electroporation editing reagents—such as recombinant Cas9 protein or mRNA with a single guide RNA (sgRNA)—delivered into HSPCs. The homologous donor template is introduced either as a single-stranded oligodeoxynucleotide (ssODN) or via a non-integrating viral vector, such as AAV6[91, 92](Table 3) (Figure 3).

Legends: (A) Gene addition: This approach aims to reinstate the lost functionality of a defective or absent gene by introducing a new gene into the impacted cells. (B) *HBB* gene correction via CRISPR tools: CRISPR/Cas9 induces a double-stranded break in the DNA, facilitating alterations in the genome. Prime editing involves the fusion of nCas9 with reverse transcriptase (RT) and an extended prime editing guide RNA (pegRNA), collectively known as a prime editor, to facilitate precise small insertions, deletions, and conversions between specific bases. Base editing enables the introduction of substitutions or base modifications without causing double-strand breaks. (C) HBF reactivation via CRISPR tools: The reactivation of HbF in adult hematopoietic cells holds significant clinical promise for individuals with harmful mutations in the β-globin gene, including those affected by β-thalassemia and SCD. Enhancing HbF production may offset the insufficient formation of β-globin chains seen in β-thalassemia and can also interfere with the polymerization of sickle hemoglobin in SCD.

CRISPR-Cas9 Gene Editing

In recent years, CRISPR-Cas technology has paved the way for precise gene editing in different conditions, from inherited monogenic disorders to cancer therapies. In the context of β-hemoglobinopathies, CRISPR is widely used to correct point mutations or to knock out specific regulatory elements to reactivate HbF [93].

HBF reactivation

Hereditary persistence of fetal hemoglobin (HPFH) is a genetic trait that mitigates the complications associated with β-hemoglobinopathies. The induction of single nucleotide variations or small deletions eliminates the γ-globin promoter-binding sites for the transcriptional repressors BCL11A or ZBTB7A, reactivating γ-globin expression[94,95]. Targeting the erythroid-specific enhancer of *BCL11A* using CRISPR-Cas9 has demonstrated remarkable success in clinical trials[96]. Subsequently, the FDA approved CASGEVY™, the first CRISPR-Cas9-based gene therapy product, in December 2023 and January 2024 for SCD and β-thalassemia, respectively. CASGEVY™ is a non-viral, ex vivo CRISPR/Cas9 gene-edited cell therapy indicated for patients aged ≥12 years with SCD or TDT.

Editas Therapeutics developed AsCas12a to modify the genomic sequences of the γ-globin genes

(*HBG1*/*HBG2*) promoters. EDIT-301 clinical trial (NCT04853576) reactivated γ-globin expression and enhanced HbF production in autologous hematopoietic stem cells. Preclinical studies of $CD34^+$ cells derived from SCD patients demonstrated ≥80% editing efficiency, robust HbF production, absence of off-target editing, and reduced sickling of erythroid progeny obtained from EDIT-301 treatment [97].

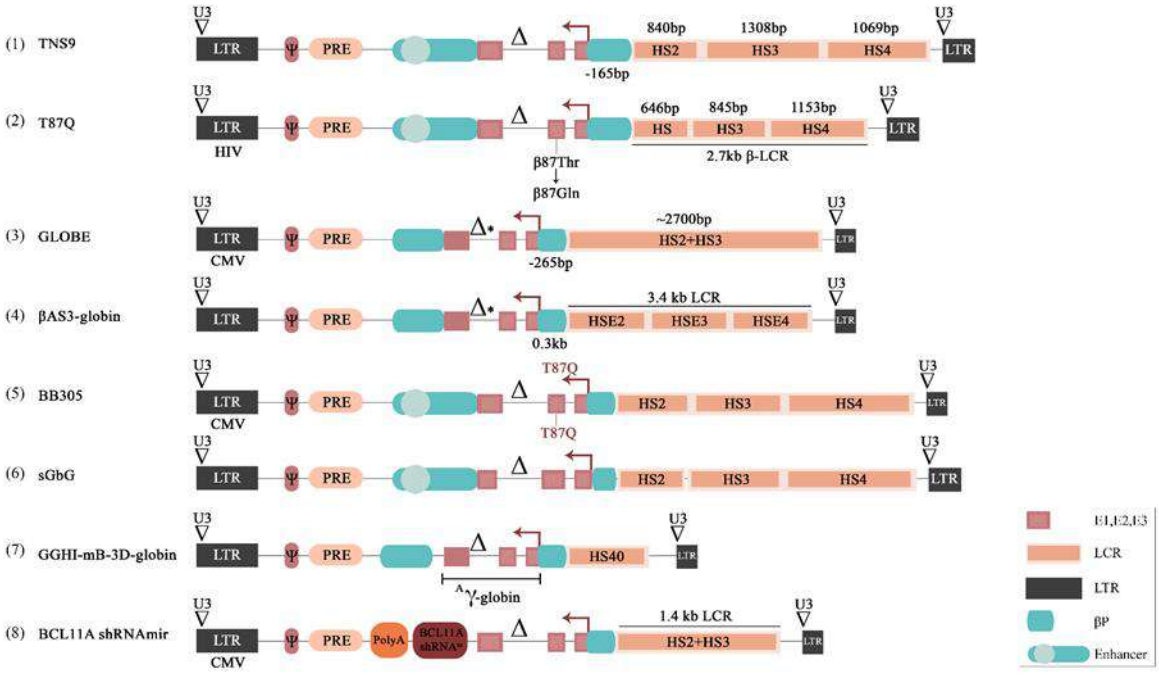

Figure 2. Schematic representation of lentiviral vectors for β-hemoglobinopathies

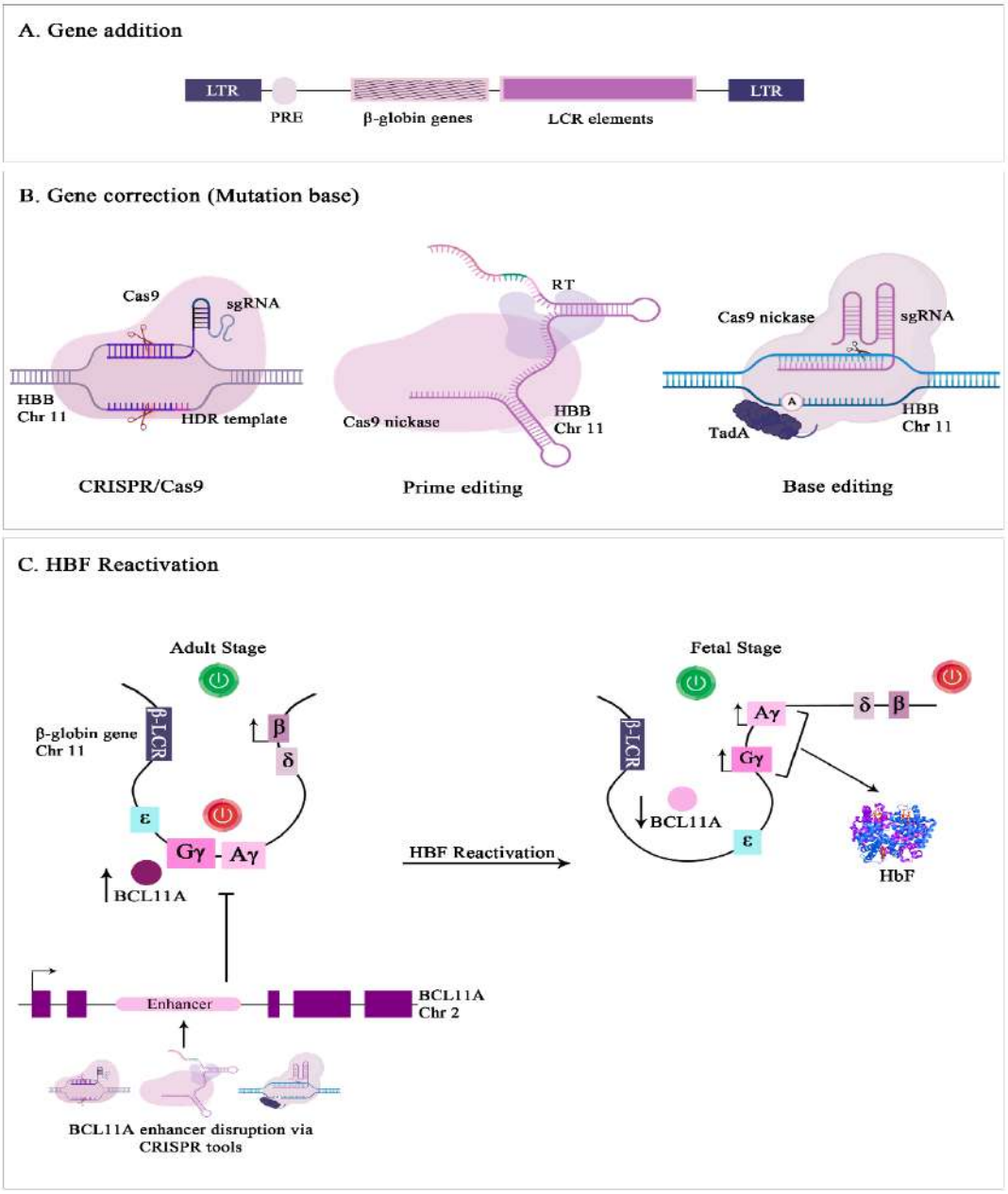

Figure 3. Gene editing strategies in β-hemoglobinopathies gene therapy

Table 3: Comparison of gene therapy tools in β-hemoglobinopathies treatment) placed at the end of the document

Characteristic	Lentiviral Vectors	CRISPR-Cas9	Prime Editing	Base Editing
Mechanism	Gene insertion lentiviral delivery system to integrate a healthy gene cassette into the genome.	Gene disruption for HBF reactivation, gene correction, or gene insertion. gRNA and Cas9, induces a double-strand break.	Precise gene correction to correct the mutation or HBF reactivation. Cas9 nickase, reverse transcriptase enzyme, pegRNA, without creating a double-strand break.	Precise gene correction to correct the mutation or HBF reactivation. Adenine Base Editors or Cytosine Base Editors fused to a CRISPR-Cas9 nickase, without creating a double-strand break.
Advantages	✓ Efficient and stable gene delivery ✓ Can target a wide range of cell types, including HSCs ✓ Potential for long-term therapeutic effects	✓ High efficiency and precision in gene editing ✓ Relatively simple and cost-effective design and delivery ✓ Can target multiple genes simultaneously	✓ Highly precise and flexible gene editing capabilities ✓ Reduced risk of unintended genomic alterations caused by DSB ✓ Can correct a wide range of genetic mutations	✓ Highly precise gene editing capabilities ✓ Reduced risk of unintended genomic alterations caused by DSB ✓ Can correct a wide range of genetic mutations
Limitations	✓Potential risk of insertional mutagenesis ✓Immune response to viral components ✓complex and costly	✓ Delivery challenges ✓ Off-target effects and potential for unintended genetic alterations ✓ Immune responses to Cas9	✓ Delivery challenges ✓ Lower editing efficiency ✓ Limited simultaneous editing of multiple loci ✓ Potential off-target effects	✓ Delivery challenges ✓ Lower editing efficiency ✓ Inability to correct SCD mutation ✓ Potential off-target effects

HBB correction

Further studies have employed homologous donors and HDR to correct the classical mutation in *HBB* that causes SCD [91]. CRISPR-Cas9 has demonstrated the potential to repair the IVS2-654 mutation in *HBB* and restore baseline gene expression in iPSCs [86]. In a trial, an adeno-associated virus 6 (AAV6) combined with Cas9 nuclease-initiated HDR was used to correct SCD mutations. However, following the development of transfusion-dependent pancytopenia in a patient, the study was discontinued. Notably, CRISPR-based correction of the SCD mutations was developed using non-viral delivery methods such as ssODN donors[98].

HBA deletion

In β-thalassemia, an excess of free α-globin leads to toxic precipitate formation that causes hemolysis of mature red blood cells and induces intramedullary apoptosis of ineffective erythroid precursors. In normal cells, there are two *cis* genes on chromosome 16 (*HBA1* and *HBA2*), resulting in four α-globin genes per cell (αα/αα). Clinical evidence indicates that the number of α-globin genes is closely correlated with the severity of β-thalassemia, and patients often show improvement when HBA deletions are present[99]. Reducing α-globin expression has emerged as a therapeutic approach for β-thalassemia to prevent the accumulation of undesirable precipitates in erythroid cells. Research has demonstrated that combining two strategies can be effective in managing β-thalassemia. The first approach involves the targeted insertion and expression of an *HBB* transgene using a lentiviral vector driven by the endogenous *HBA* promoter. The second approach creates a natural α-thalassemia phenotype (such as the $-\alpha^{3.7}$ deletion, -α/αα, or -α/-α) using CRISPR/Cas9 to reduce α-globin expression in β^{+} and β^{0} thalassemia cells. This dual strategy of

downregulating α-globin while upregulating β-globin may significantly improve disease management[100]. Moreover, Sachith et al. induce deletions employing the CRISPR/Cas9 tools in the α-globin enhancer region. Their findings showed a reduction in α-globin expression and an improvement in the imbalance of globin chains [101].

Base Editing

Different research groups developed numerous techniques for the reactivation of HBF. Despite the promising potential of CRISPR-Cas9, the unpredictably mixed insertion-deletion mutations (indels) caused by NHEJ have motivated researchers to develop modified nuclease editors[102]. Base editing utilizes the sgRNA-Cas9 system to deliver deaminase enzymes to the targeted site, allowing for the alteration of a single base (e.g., converting cytosine to thymine or adenine to guanine). The primary advantage of this innovative strategy is its ability to achieve precise and efficient gene editing, requiring only a single-strand nick, thereby potentially reducing the risks associated with the DSBs induced by Cas9 [103, 104]. Moreover, intermediate DSBs—which can lead to chromosomal aberrations and TP53-mediated DNA damage responses—are avoided with the base-editing approach [105, 106]. Currently, base editors are evaluated in clinical studies for β-thalassemia and sickle cell disease treatment (NCT05456880) [102].

Various studies employed base editors, including targeting γ-globin promoter variations and/or developing novel DNA-binding motifs for transcriptional activators to induce HPFH [94]. One approach involves using base editors to disrupt the +58 BCL11A erythroid enhancer or repressor-binding motifs within the γ-globin promoter[107]. Another strategy focuses on activating HbF by introducing novel transcription factor-binding sites into the γ-globin promoters[108,109]. Additionally, base editors can modify the tandemly organized, duplicated γ-globin genes, *HBG1* and *HBG2*, and prevent the deletion of the 4.9 kb intermediate DNA segment, which can result from Cas9-induced DSBs at both sites [110].

Notably, Liu's lab successfully generated three distinct γ-globin promoter HPFH variants using an adenine base editor (ABE) at positions -198, -175, and -113 bp upstream of the transcriptional start site. The highest induction of HbF was achieved with the -175A>G variant, which creates a novel TAL1 binding site that facilitates extended interaction with the LCR. Edited erythroid colonies homozygous for the -175A>G mutation produced 81 ± 7% HbF, compared to 17 ± 11% in the untreated group [103].

Using a customized adenine base editor (ABE8e-NRCH), Liu's group also converted the SCD allele (HBBS) into the non-pathogenic Makassar β-globin (HBBG) variant. They delivered mRNA encoding the base editor with a targeting guide RNA into the patient-derived HSPCs and achieved the HBBS-to-HBBG conversion with 80% efficiency [111].

Another study developed the adenine and cytosine base editors to engineer highly similar HBG proximal promoters without inducing substantial deletions. They identified a new regulatory region located at the -123 position. Base editing of the HBG promoter at positions -123 and -124 significantly enhanced HbF expression in erythroblasts generated from human $CD34^+$ HSPCs, surpassing the effects of disrupting the BCL11A binding site. Additionally, this study revealed that HPFH-like mutations (-123T>C and -124T>C) stimulated γ-globin expression by establishing a de novo binding site for KLF1. These findings highlighted potential new targets within the HBG promoter for the upregulation of HbF [112].

Hemoglobin E (HbE) β-thalassemia is also being explored as a target for base editing, as it accounts for approximately 50% of all severe thalassemia cases worldwide. A single mutation at codon 26 of the human *HBB* gene (GAG; glutamic acid → AAG; lysine; E26K) is responsible for HbE β-thalassemia, while a mutation causing severe β-thalassemia typically occurs on the other allele. In severe cases, mutations are inherited through compound heterozygosity. Using base editing, researchers have successfully corrected the HbE mutation in primary human $CD34^+$ cells, restoring it to the wild-type sequence or a typical hemoglobin variant (E26G), known as Hb Aubenas, with editing efficiencies exceeding 90% [113].

BEAM-101, a base editing therapy developed by Beam Therapeutics, utilizes *HBG1/2*-targeted guide RNAs and ABE8 (adenine base editor) mRNA. This approach induces a mutation that prevents the

binding of the BCL11A repressor to the *HBG1* and *HBG2* enhancers. In the BEACON study (NCT05456880), autologous HSPCs from three patients with sickle cell disease (HbSS, HbSβ^{o}, and HbSβ^{+}) are treated ex vivo with BEAM-101 to introduce the "British" HPFH mutation (a T•A to C•G mutation in the *HBG1* and *HBG2* enhancers) [114].

Prime Editing

Prime editing is an even more recent gene-editing technique that enables targeted insertions, deletions, and base-to-base replacements within the genome of living cells. This innovative method can introduce extended sequences, such as cDNA, without relying on DSBs or HDR [115, 116]. Prime editing precisely scans DNA for the target site using a pegRNA and employs a Cas9-fused reverse transcriptase enzyme to add a few targeted base pairs to the correction site. CRISPR-associated transposases (CASTs) and large serine recombinases (LSRs) further enhanced this process [117].

In an effort, an adenine base editor converted the pathogenic HBBS allele into the naturally occurring, non-pathogenic Makassar allele (HBBG) to correct the SCD mutation [111]. However, base editors couldn't reverse the SCD E6V substitution, as it requires a T·A-to-A·T transversion[98, 118]. Given the limitations of CRISPR and base editors, prime editing emerges as an optimal therapeutic approach for correcting the SCD mutation with minimal adverse genomic or cellular effects. Notably, Liu et al. demonstrated that prime editing can revert the SCD allele (HBBS) to the wild-type allele (HBBA) at frequencies ranging from 15% to 41% in HSPCs derived from SCD patients[119].

CONCLUSION

β-hemoglobinopathies can be treated with conventional medicines and allogeneic hematopoietic stem cell transplantation. However, these treatments are associated with various complications. Gene therapy has emerged as a potentially curative approach, with lentiviral vectors to deliver modified genes into hematopoietic stem cells. Some of these vectors received FDA authorization for clinical use in sickle cell disease and β-thalassemia.

The CRISPR/Cas9 system, an innovative gene-editing approach, has been advanced to double-strand break (DSB)-free techniques, such as base and prime editing, to reduce the probability of unwanted mutations and to develop high-fidelity Cas enzymes with lower off-target activity. This technology has been applied in clinical studies for hereditary and non-hereditary disorders, mitigating disease symptoms in β-hemoglobinopathies. Casgevy, the first CRISPR-based product approved by the FDA for sickle cell disease and transfusion-dependent β-thalassemia, has significantly improved patients' conditions. However, challenges remain, including long-term safety, off-target activity, cost, accessibility, and manufacturing. On the other hand, DNA damage and DNA DSBs can lead to genotoxic effects and off-target mutations. The transition to DSB-free CRISPR systems can help reduce mutational risk. Moreover, clonal expansion in a patient in the Graphite Bio trial is unclear, but developing ways to reduce genotoxicity of CRISPR-based therapies is crucial for safe clinical implementation. An enhanced understanding of base and prime editing technologies with further evidence of their therapeutic efficacy and safety will support broader acceptance of these innovative treatments. Future studies will focus on improving the effectiveness and specificity of gene-editing tools such as introducing the twin prime editing (twinPE) technic that enhances the capabilities of traditional prime editing by enabling precise and efficient modifications of larger DNA sequences without introducing double-stranded breaks (DSBs) in the DNA, developing novel delivery systems including in vivo nanoparticle delivery, and conducting extensive clinical trials to ensure efficacy and safety across diverse patient populations.

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Ethical Statements (Ethical Approval, Consent to participate, Consent to publish)

These declarations are not applicable

Abbreviation list
SCD Sickle Cell Disease
Hb Hemoglobin
β HbB Hemoglobin Subunit Beta
ε HbE Hemoglobin E
Gγ HbG2 Hemoglobin Subunit Gamma-2
Aγ HbG1 Hemoglobin Subunit Gamma-1
δ HbD Hemoglobin Subunit Delta
HbS Sickle Hemoglobin
RBCs Red Blood Cells
LCR Locus Control Region
HS Hypersensitive
GATA1 GATA-Binding Factor 1
KLF1 Krueppel-Like Factor 1
EKLF Erythroid Lineage-Restricted Kruppel-Like Transcription Factor
TAL1 T-Cell Acute Lymphocytic Leukemia Protein 1
NF-E2 Nuclear Factor Erythroid 2
LMO2 LIM Domain Only 2
FOG Friend of GATA-1
Sp1 Specificity Protein 1
Myb Myeloblastosis
SSP Stage Selector Protein
COUP-TFII COUP transcription factor 2
BCL11A B Cell Lymphoma 11A
SOX6 SRY-box 6
DNMT1 DNA Methyl Transferase 1
NuRD Nucleosome Remodeling and Deacetylase Complex
LRF Lymphoma/Leukemia Related Factor
ZBTB7A Zinc Finger and BTB Domain Containing 7A
NuRD Nucleosome Remodeling and Deacetylase
EHMT1/2 Euchromatic Histone-Lysine N-Methyltransferase
CHD4 Chromodomain-Helicase-DNA-Binding Protein 4
DNMT1 DNA Methyl Transferase 1
LIN28B Lin-28 Homolog B
Let-7 Lethal-7
ZNF410 Zinc Finger Protein 410
US United States
EU European Union
ODD Orphan Drug Designations
MH Mini-Hepcidin
TMPRSS6 Transmembrane Serine Protease
ASO Antisense Oligonucleotide
EPO Erythropoietin
Tfr2 Transferrin Receptor 2
VIT-2763 Vamifeport
TGF-β Transforming growth factor beta
ActR-II Activin Receptor-II
FDA Food and Drug Administration
EMA European Medicines Agency
SMAD Suppressor of Mothers against Decapentaplegic
ActR-II Activin receptor type II
JAK2 Janus Kinase 2
NTDT Non-Transfusion-Dependent Thalassemia
ATP Adenosine Triphosphate
TDT Transfusion-Dependent Thalassemia
RNAi RNA interference
HU Hydroxyurea
THU Tetrahydro Uridine
EPI01 EpiDestiny's therapy
DOPA Dihydroxyphenylalanine
HDAC3 Histone Deacetylase 3
LSD Lysine-Specific Demethylase
HSCT Hematopoietic stem cell transplantation
HSCT-GT Hematopoietic stem cell gene therapy
GVHD Graft-Versus-Host Disease
LV Lentiviral Vectors
LTR Long-Terminal Repeat
SIN Self-Inactivating
PGK Phosphoglycerate Kinase
CMV Cytomegalovirus
HSPCs Hematopoietic Stem and Progenitor Cells
ZFNs Zinc Finger Nucleases
TALENs Transcription Activator-Like Effector Nucleases
CRISPR Clustered Regularly Interspaced Short Palindromic Repeat
CAS9 CRISPR-associated protein 9
ZFPs Zinc Finger Proteins
iPSCs induced Pluripotent Stem Cells
DSBs Inducing Double-Strand Breaks
HDR Homology-Directed Repair
NHEJ Non-Homologous End-Joining
sgRNA a single guide RNA
HPFH Fetal Hemoglobin
ABE Adenine Base Editor

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