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A Review of CRISPR Cas9 for SCA: Treatment Strategies and Could Target β -globin Gene and BCL11A Gene using CRISPR Cas9 Prevent the Patient from Sickle Cell Anemia?

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A Review of CRISPR Cas9 for SCA: Treatment Strategies and Could Target β -globin Gene and BCL11A Gene using CRISPR Cas9 Prevent the Patient from Sickle Cell Anemia?

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Abstract

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BACKGROUND: Sickle cell anemia is a hereditary globin chain condition that leads to hemolysis and persistent organ damage. Chronic hemolytic anemia, severe acute and chronic pain, and end-organ destruction occur throughout the lifespan of sickle cell anemia. SCD is associated with a higher risk of mortality. Genome editing with CRISPR-associated regularly interspersed short palindromic repeats (CRISPR/Cas9) have therapeutic potential for sickle cell anemia thala.

AIM: This research aimed to see if using CRISPR/Cas9 to target β -globin gene is an effective therapeutic and if it has a long-term effect on Sickle Cell Anemia.

METHODS: The method used in this study summarizes the article by looking for keywords that have been determined in the title and abstract. The authors used official guidelines from Science Direct, PubMed, Google Scholar, and Journal Molecular Biology to select full-text articles published within the last decade, prioritizing searches within the past 10 years.

RESULTS: CRISPR/Cas9-mediated genome editing in clinical trials contributes to α -globin gene deletion correcting β -thalassemia through balanced α - and β -globin ratios and inhibiting disease progression.

CONCLUSION: HBB and BCL11A targeting by CRISPR/Cas9 deletion effectively inactivate BCL11A, a repressor of fetal hemoglobin production. However, further research is needed to determine its side effects and safety.

Introduction

Transfusion-dependent both beta-thalassemia (also known as TDT) and sickle cell disease (also known as SCD) is the most frequent forms of monogenic illness in the world. Each year, around 60,000 individuals are diagnosed with TDT, and 300,000 patients are diagnosed with SCD [1], [2], [3]. Both disorders are brought on by mutations in the gene for the beta component of hemoglobin (HBB). The mutations in HBB that cause TDT4 lead to decreased (+) or absence (0) production of β -globin, as well as an imbalance between the α -like and β -like globin chains of hemoglobin (e.g., β , γ , and δ), which results in inefficient erythropoiesis [4], [5], [6]. The production of sickle hemoglobin is caused by a point mutation in HBB that, at the sixth position of the

amino acid sequence, switches glutamic acid for valine. The polymerization of deoxygenated sickle hemoglobin results in erythrocyte deformation, hemolysis, anemia, excruciating vaso-occlusive events, permanent end-organ damage, and a decreased life expectancy [6], [7].

In patients who have TDT7, the primary treatment choices consist of transfusion and iron chelation, while in those who have SCD, the primary treatment options consist of pain management, transfusion, and hydroxyurea [8], [9]. However, neither treatment addresses the underlying cause of the disease nor fully ameliorates disease manifestations. Recently approved therapies, such as luspatercept and crizanlizumab, have reduced transfusion requirements in patients with TDT and the incidence of vaso-occlusive episodes in those with SCD, respectively. Both TDT and SCD can be cured with allogeneic bone marrow transplantation;

however, <20% of patients who are eligible have a related donor who has a human leukocyte antigen that is a match [10], [11]. Betibeglogene autotemcel is a gene-addition product that is based on lentiviral vectors. In the European Union, it has been approved for the treatment of patients with TDT who have non- β 0 mutations and who do not have a matched sibling donor [12], [13], [14]. In addition, it has been demonstrated that erythroid-specific suppression of BCL11A, which is being delivered by a lentiviral-encoded, microRNA-adapted short hairpin RNA molecule, may reactivate the beta-globin gene, and it is now in the early stages of clinical testing [7], [15], [16], [17], [18], [19].

Patients who have TDT or SCD and have elevated amounts of fetal hemoglobin (HbF), which is made up of two alpha chains and two gamma chains, have a reduced risk of developing complications and dying from their disease. The creation of HbF is developmentally controlled in such a way that the quantity of β -globin that is created in utero declines postnatally in conjunction with the formation of adult hemoglobin (HbA), which is composed of two alpha chains and two beta chains [20], [21], [22]. In most cases, neonates and newborns who have TDT or SCD do not exhibit any symptoms while their HbF levels are normal; nevertheless, symptoms often begin to manifest themselves within the 1st year of life, when the synthesis of HbF begins to decrease [23], [24]. Those patients who co-inherit the genetic persistence of HbF, a condition in which fetal expression continues into adulthood, and who have TDT or SCD have very little or no illness [7], [25].

Single-nucleotide polymorphisms, sometimes known as SNPs, have been discovered as being related with greater production of HbF in adults as a result of genome-wide association studies (GWAS) [26]. Some of these SNPs can be found in the BCL11A locus on chromosome 2, and they are linked to a reduced risk of TDT as well as SCD. SNPs that are associated with HbF are located in an erythroid-specific enhancer, downregulate BCL11A expression, and increase the expression of HbF [27]. BCL11A is a zinc finger-containing transcription factor that represses γ -globin expression and HbF in erythroid cells [7], [23], [26].

The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 nuclease system is a bacterial immune system that may break the DNA of bacteriophage or plasmids. This system enables the programmed targeting of insertions or deletions (indels) to a particular genomic DNA region [28], [29]. We used CRISPR-Cas9 gene editing techniques in hematopoietic stem and progenitor cells (HSPCs) at the erythroid-specific enhancer region of BCL11A to reduce BCL11A expression in erythroid-lineage cells. This allowed us to restore γ -globin synthesis and reactivate production of HbF [7], [30], [31].

Here, we describe the first two patients, one with SCD and the other with sickle cell trait, who were infused with CTX001 (autologous CRISPR-Cas9-edited CD34+ HSPCs that were genetically edited to reactivate the

production of HbF) and enrolled in CLIMB THAL-111 (for the patient with sickle cell trait) and CLIMB SCD-121 (for the patient with sickle cell trait) (for the patient with SCD) [7].

Methods

The methodology used is a literature review. The study sources consist of relevant journals from several databases Google Scholar, PubMed, Science Direct, and Journal Molecular Biology. The author searches with keywords and synonyms CRISPR Cas9 OR Gene editing OR Genome Editing OR Clustered Regular interspersed Short Palindromic Repeats-CRISPR-related AND SCA OR Sickle Cell Anemia AND TDT OR Transfusion-dependent β -thalassemia AND BCL11A gene OR B-cell lymphoma/leukemia 11A AND HBB gene OR HBB. The search was limited to publications from 2012 to 2022 to ensure the articles used were up to date. Language restrictions are also applied to limit the search to articles published only in English. All relevant articles were screened and analyzed for inclusion or exclusion from the literature review based on quality and relevance to the review topic, questions and objectives of the literature review. The title and abstract of each article were tailored to the research question simultaneously. The full text of the article is checked for availability if the title and abstract match. Finally, the researcher reads the entire article to see if it is relevant and valuable to the subject.

Results and Discussion

SCD

SCD is an inherited monogenic disorder characterized by a single substitution on chromosome where hydrophilic glutamic acid is replaced by hydrophobic valine in the sixth codon of the β -globin gene, leading to abnormal hemoglobin folding. The modified β -globin gene produces an abnormal hemoglobin S (HbS) which rapidly polymerizes in the deoxygenated state altering red blood cell (RBC) rheology and lifespan [32], [33]. HbS polymerization causes the characteristic sickle shape change and downstream effects of sickling that include anemia, vaso-occlusion, cell adhesion, and vasoconstriction [34].

β -thalassemia

Mutations that result in a single nucleotide substitution, modest deletions or insertions within the β -globin gene or its immediate flanking sequence, or,

in rare cases, massive deletions, cause β -thalassemia. These mutations cause a decrease in the formation of β -globin chains and HbA. More than 350 β -thalassemia mutations have been identified, and they are generally assigned a severity score, with β^+ indicating mild mutations that induce a relative reduction in β -globin chain synthesis and β^0 indicating severe mutations that might result in a complete absence of β -globin chain product. The degree of imbalance between the α -globin and β -globin chains is intimately related to the severity of anemia, the requirement for transfusions, and clinical morbidity in β -thalassemia. In erythroid cells, insufficient β -globin chain synthesis results in the formation of excess, unstable α -globin tetramers. Free α -globin protein is unstable and produces cytotoxic reactive oxidant species and cellular precipitates that impede red-cell precursor maturation and survival, resulting in inefficient erythropoiesis and early hemolysis of circulating red cells [33], [23].

Thus, people with severe β -thalassemia mutations in the homozygous or compound heterozygous state have more severe clinical signs, whereas patients with β -thalassemia coinherit have a milder disease. Overt illness may emerge in heterozygous people, who are generally asymptomatic, due to the coinheritance of extra-globin genes (duplications) as a result of the increased amount of free α -globin protein [35]. Furthermore, in β -thalassemia, red-cell precursors can detoxify and tolerate a small amount of free α -globin, which is stabilized by the molecular chaperone α -hemoglobin-stabilizing protein (AHSP) and removed by the ubiquitin-proteasome system and autophagy [23]. The severity of β -thalassemia is influenced by altered levels of AHSP expression [36] and the gene encoding the autophagy-activating Unc-51-like kinase 1 can both diminish autophagic clearance of α -globin in red-cell precursors and worsen disease severity [37]. The degree of imbalance between α -globin and β -globin chains can also be lowered by more effective β -globin chain and HbF synthesis after birth. Several genes contribute to the modification of the γ -globin chain response; some are encoded in the γ -globin gene cluster, while others are on separate chromosomes. BCL11A (a multi-zinc-finger transcriptional regulator) has been identified as a significant regulator of the changeover from fetal to HbA and HbF silencing in genome wide association studies analyzing common variation in HbF levels [38], [39]. BCL11A is thought to be controlled at the level of messenger RNA translation via the RNA-binding protein LIN28B and represses the genes encoding HbF [40], [41]. It has been demonstrated that genetic variations in the expression of BCL11A and sustained HbF production diminish the clinical severity of β -thalassemia [42], [43].

Hemoglobin-correlation with SCD and β -thalassemia

HbF; $\alpha_2\gamma_2$, a minor hemoglobin of normal adults, has major clinical significance for SCD. γ -Globin

is encoded in *HBG2* ($^G\gamma$) and *HBG1* ($^A\gamma$), nearly identical genes found in a developmentally regulated gene cluster on chromosome 11p15 (5'— ϵ — $^G\gamma$ — $^A\gamma$ — δ — β —3') [15].

In utero and during infancy, the abnormal HbS protein is produced at very low levels because the erythrocytes have not yet shifted from expression of the γ -globin gene (*HBG*), which encodes the developmentally regulated component of HbF, to expression of the *HBB* gene. Infants with SCD are typically free of clinical symptoms, due to the potent antisickling properties of HbF combined with the lower levels of HbS [15].

A high level of erythrocyte HbF comprising α - and γ -globins may ameliorate these manifestations by mitigating sickle hemoglobin polymerization and erythrocyte sickling. *BCL11A* is a repressor of γ -globin expression and HbF production in adult erythrocytes. Its down-regulation is a promising therapeutic strategy for induction of HbF [15].

A variety of clinical observations have shown that the severity of SCD and β -thalassemia is ameliorated through increased production of HbF. Children with SCD were noted to be asymptomatic until after infancy, which was postulated to be attributable to elevated HbF levels [23].

The switch from fetal to HbA

Shortly after the time of birth there is a switch from predominant expression of HbF to HbA, which is mediated by a transcriptional switch in definitive erythroid progenitors from g- to b-globin. Infants of diabetic mothers have a delayed fetal-to-HbA switch. A number of groups pursued the basis for common genetic variation in HbF levels using both targeted and GWAS [27], [44], [45], [46]. These studies resulted in the identification of three genomic loci harboring common variants that influenced HbF levels. This included a region of chromosome 2 within the *BCL11A* gene, a region intergenic between the genes *HBS1L* and *MYB* on chromosome 6, and variants within the b-globin locus on chromosome 11 [47]. *BCL11A* protein levels appeared to correlate with the developmental stage of expression, such that primitive and fetal liver definitive erythroid cells that expressed high levels of g-globin, had low or absent expression of the full-length forms of *BCL11A*. This result suggested that this gene product was acting as a repressor of the g-HBGs. To directly test this, knockdown of *BCL11A* using short-hairpin RNA (shRNA) approaches was performed in primary adult erythroid progenitors and g-globin expression could be robustly induced on such a knockdown [48], [49], [50]. It was additionally shown that *BCL11A* directly interacts with chromatin at the human b-globin locus in primary erythroid cells and that it appeared to act as part of a complex with the transcription factor *GATA1* and the NuRD chromatin remodeling and repressor complex [51]. Of interest, the

NuRD complex contains HDACs 1 and 2, which have been suggested to be the critical HDACs necessary for HbF silencing [52]. In addition, it has been suggested that the transcription factor SOX6 may cooperate with BCL11A to help silence the γ -HBGs in humans and it may be essential for binding the proximal promoter of these HBGs. We then go on to discuss how more recent molecular studies that have identified regulators, including BCL11A, MYB, and KLF1, hold great promise to develop targeted and more effective approaches for HbF induction [23], [53]

HbF: Why switch to hba?

Persistence of high levels of HbF has no clinical consequences in healthy individuals, coinheritance of HPFH with either of the two major β -hemoglobin disorders - SCD or β -thalassemia - alleviates their clinical severity.

BCL11A and ZBTB7A (also known as LRF) are two recently identified γ -globin gene repressors [35]. Two studies suggested that the expression of BCL11A appears to be controlled by the erythroid specific transcription factor KLF1 using independent and complimentary approaches. KLF1 was a direct positive transcriptional regulator of BCL11A expression [23]. Gene has an effect to HbF: BCL11A, KLF1, HBS1L, MYB, SOX6, and GWAS (Table 1) [23].

HBB gene

The HBB gene encodes a protein called beta-globin. Beta-globin is a component (subunit) of hemoglobin, a key protein present inside RBCs. In adults, hemoglobin is divided into four protein subunits: Two beta-globin subunits and two alpha-globin subunits provided by another gene called HBA. Each of those protein subunits is associated with an iron-containing molecule known as heme; each heme has an iron molecule in its center that can connect to one oxygen molecule.

Beta-plus (β^+) thalassemia is caused by HBB gene mutations that reduce beta-globin production. Beta-zero (β^0) thalassemia is caused by mutations that prohibit cells from generating any beta-globin. Problems with the components that make up hemoglobin, such as low amounts of beta-globin, limit, or abolish the molecule's synthesis. The regular growth of RBC is impaired by a deficiency of hemoglobin. A lack of mature RBC can lower the quantity of oxygen transported to tissues to levels below what is required to satisfy the body's energy requirements. A shortage of oxygen in the body's tissues can result in stunted development, organ damage, and other health complications linked with beta thalassemia.

The most common form of SCD is sickle cell anemia (also referred to as homozygous SCD or HbSS disease). This form is caused by a mutation in the HBB

gene, which results in the development of an aberrant variant of beta-globin known as HbS, or HbS. HbS replaces both beta-globin subunits in hemoglobin in this case. The HbS mutation alters a single protein building block (amino acid) in beta-globin. At position 6 in beta-globin, the amino acid glutamic acid is substituted with the amino acid valine, which is represented as Glu6Val or E6V.

BCL11A gene

BCL11A is a repressor of γ -globin expression and HbF production in adult erythrocytes [15]. The transcription factor BCL11A has been validated as a repressor of HbF levels in model systems. Down-regulation of BCL11A expression in primary adult erythroid cells leads to robust HbF expression. BCL11A is suppressed by an shRNA embedded in a microRNA architecture that harnesses the endogenous cellular machinery to produce a simultaneous increase in HbF protein and decrease in HbS protein [15]. Have a role in erythropoiesis, was first implicated as a potential modulator of HbF levels acts as a repressor of γ -HBGs expression [35].

Gene associated in B-thalassemia and SCA

Genetic factors are one of the causes of B-thalassemia and SCA. It has been identified by GWAS that dozens of genes influence B-thalassemia and SCA. Many genes involved in hematopoiesis, erythrocyte hemostasis, and iron-binding proteins were differentially expressed in both β -thalassemia and SCD, which might be expected since both disorders are hemolytic anemia's. Improving the ability of erythrocytes to deliver oxygen to tissues would also be a predictable response in both β -thalassemia and SCD, and upregulation of BPGM (bisphosphoglycerate mutase) is indeed seen in both cases [54]. There were 111 gene loci associated with B-thalassemia and SCA including ABCB6, ACSL6, ALPL, ANK1, ANKRD9, AQP9, RHGEF12, ARL4A, BCAM, BIRC2, BMP2K, BPGM, C14orf45, C5orf4, CA1, CA2, CCRL2, CISD2, CLCN3, CLEC4E, CLIC2, CTNNA1, CYP4F3, DAPK2, DCUN1D1, DNAJB4, DOCK5, DYRK3, E2F2, ELL2, ELOVL6, EMR3, ERMAP, FAM83A, FECH, FHDC1, GLCL, GLT1D1, GPR97, GYPA, HAL, HBA1, HBA2, HBB, HBE1, HBG1, HBM, HEPA, CAM2, HMBS, HRH2, HSPA13, IFI27, IFI44L, IL1R2, ISCA1, ITSN1, KANK2, KCNJ15, KCNJ2, KEL, KIAA1324, KLF1, KRT1, KRT23, MANSC1, MAP4K5, MARCH3, MARCH8, MGAM, MME, MMP9, MOSPD1, NFIX, NSUN3, OSBP2, PLVAP, PPME1, REPS2, RFESD, RNF14, RNF182, RSAD2, RUNDC3A, SFRP2, SLC14A1, SLC16A1, SLC2A1, SLC45A4, SLC7A5, SOX6, SPTA1, STEAP4, TAL1, TBC1D22B, TBCEL, TCEANC, TCP11L2, TFDP1, TMEM14B, TMEM158, TNFRSF10C, TRAK2, TREM1, TRIM10, UBXN10, USP12, XK, XPO7, YPEL4,

and ZNF23 [55]. AHSP (alpha hemoglobin stabilizing protein) and GATA1, a transcription factor that promotes AHSP expression, were among the genes differently expressed in thalassemia but not in SCD [56], [57]. AHSP inhibits globin aggregation during erythroid cell formation and is expected to attenuate pathogenic situations of globin excess, such as thalassemia. Because SCD is not connected with chain imbalance, it is apparent that increased AHSP expression is unneeded. Surprisingly, most of the genes that are differently expressed in thalassemia but not in SCD are implicated in inflammation and immunology. ADM, BCL3, BP1, DEFA3, IRAK3, JUN, LTF, LY96, SLC11A1, THBD, and TLR6 were among the genes activated in response to bacterial infections [55].

Stress erythropoiesis increases HBG1/2 expression in response to loss of HBB

Based on our multi-omics research, we conducted a series of studies to confirm the molecular concept that HBB deletion causes HBG1/2 expression to increase. Through DCFH probe and Trypan blue staining, we discovered that ROS concentration was approximately 1.5 times higher in HBB-KO cells than in WT cells and that cell viability was approximately 2.7 times lower in HBB-KO cells than in WT cells. Flow cytometry and capillary electrophoresis experiments demonstrated that the apoptotic rate in HBB-KO cells was higher than in WT cells. Cell cycle progression and cell proliferation were slower in HBB-KO cells, according to flow cytometry and CCK8 tests. RT-qPCR revealed that HIF1 expression was increased in HBB-KO cells compared to WT cells. In addition, we performed an erythroid differentiation experiment on WT and HBB-KO cells, collecting samples before (WT B; HBB-KO B) and after (WT A; HBB-KO A). The fluorescence intensity of CD71 and CD235a was higher in the HBB-KO B group than in the WT B group, indicating that the HBB-KO group had more erythroid cells than the WT group. The HBB-KO A cells were clearly separated into two groups, showing that some had undergone erythroid differentiation. RT-qPCR research revealed that after erythroid differentiation, γ -globin expression in the HBB-KO A group was approximately 160 times higher than in the WT A group. Other erythroid differentiation marker genes upregulated were KLF1, KLF3, FOG1, AHSP, NFE2, EPOR, and GFI1B, demonstrating that knocking down HBB in erythroid progenitor cells increased stress erythropoiesis. Thus, deletion of HBB in human erythroid progenitor cells resulted in increased ROS generation and oxidative stress, resulting in an increase in the rate of apoptosis. Cell cycle progression and proliferation were also inhibited in response to oxidative stress, and stress erythropoiesis was initiated, resulting in higher intracellular HBG1/2 expression levels. Furthermore, we examined previously published scRNA-seq data of CD34+-positive HSCs in healthy individuals, -thalassemia patients, and sickle anemia

patients. We discovered that the proportion of erythroid cells in CD34+-positive HSCs from patients with -thalassemia and sickle anemia (8% and 10%, respectively) was slightly greater than in normal CD34+-positive HSCs (7.5%). These data suggested that HSCs in individuals with -thalassemia and sickle anemia may have undergone partial erythroid differentiation, which is consistent with our molecular regulatory model [55], [58].

Current therapy

SCD therapies are focused on preventing HbS production or reducing the circulating amount of HbS [34], there are only two FDA approved medications to lessen disease severity, hydroxyurea (HU) (approved for adults in 1998; children in 2017) and L-glutamine (approved in 2018) [32]. Potential methods for gene therapy in SCD are multiple: First. Addition of therapeutic globin such as β -globin or β T87Q-globin to make HbA, or γ -globin to enhance HbF levels, second HbF induction by editing of globin regulatory elements or knockdown of HbF repressors, or third direct gene correction of the SCD mutation with programmable nucleases [32].

Hydroxyurea is well tolerated and effective in reducing the number of sickle cell-related complications in all ages of people with HbSS and HbSb0 thalassemia. HU, a once daily oral medication that is rapidly absorbed, alters the kinetics of erythropoiesis by inhibiting ribonucleotide reductase, which prevents cells from leaving the G1/S phase of the cell cycle. HU increases HbF levels, thereby decreasing HbS concentration within the erythrocyte and preventing HbS polymerization, thus decreasing erythrocyte sickling and subsequent hemolysis [34]. Pharmacological therapy with hydroxyurea may modulate disease severity through induction of HbF and reduction of HbS polymerization, but does not cure patients with SCD [35].

Deeper insights into the pathophysiology of SCD have led to the development of novel agents that target cellular adhesion, inflammation, oxidant injury, platelets and/or coagulation, vascular tone, and hemoglobin polymerization. L-glutamine, decreases RBC oxidant injury [37].

Three additional therapies, L-glutamine, crizanlizumab, and voxelotor, have been approved as adjunctive or second-line agents. In clinical trials, L-glutamine reduced hospitalization rates by 33% and mean length of stay from 11 to 7 days compared with placebo. Crizanlizumab reduced pain crises from 2.98 to 1.63 per year compared with placebo. Voxelotor increased hemoglobin by at least 1 g/dL, significantly more than placebo (51% vs. 7%). Hematopoietic stem cell transplant is the only curative therapy, but it is limited by donor availability, with best results seen in children with a matched sibling donor [38], treatment with L-glutamine led to a statistically significant reduction in the frequency of pain crises and rates of hospitalization [39].

Table 1: A summary of fetal hemoglobin regulators [23]

Regulator	Direction of modulation needed to increase HbF	Human genetic evidence supporting role in HbF regulation	Human or primate studies modulating factor involved in HbF regulation	Cell culture data supporting a role in HbF regulation	Evidence from mouse models suggesting a role in HbF regulation
BCL11A	↓	x		x	x
KLF1	↓	x		x	x
MYB	↓	x		x	
MicroRNAs 15a/16-1	↑	x		x	
SOX6	↓			x	
HDACs 1/2	↓		x	x	x
DNMT1	↓		x	x	x
TR2/TR4	↓ or ↑			x	x
COUP-TFII	↓			x	
FOP	↓			x	
NF-E4	↑			x	

KLF1: Kruppel-like factor 1; MYB: Myeloblastosis; SOX6: SRY-Box Transcription Factor 6; HDACs 1/2: Histone deacetylases 1 and 2; DNMT1: DNA methyltransferase 1; TR2/TR4: The human testicular receptor 2; COUP-TFII: COUP transcription factor 2; FOP: Fibrodysplasia ossificans progressiva; NF-E4: Nuclear Factor, Erythroid 4.

Blood transfusions are used for management of acute conditions and prevention of complications associated with SCD.⁵³ The main goal in transfusing individuals with SCD is to reduce the concentration of circulating HbS [40], [59].

Hematopoietic stem cell transplantation

Gene therapy strategies for SCD include replacement of the abnormal beta HbG, augmenting HbF production by manipulation of the gamma HbG, or reactivating silenced gamma HbGs [41].

SCD is characterized by hemolytic anemia, pain, and progressive organ damage. A high level of erythrocyte HbF comprising α - and γ -globins may ameliorate these manifestations by mitigating sickle hemoglobin polymerization and erythrocyte sickling. *BCL11A* is a repressor of γ -globin expression and HbF production in adult erythrocytes. Its down-regulation is a promising therapeutic strategy for induction of HbF [15].

Genome editing: The CRISPR/cas system as a tool for adaptive bacterial immunity

The CRISPR/Cas system provides adaptive immunity. A bacterial genome must have unique nucleic acid sequences if it is ever going to adapt to previously encountered attackers, like viruses. CRISPR stands for CRISPR and is a shortened way of saying CRISPR. Short exogenous nucleic acids derived from viruses or plasmids that integrate into the bacterial genome after the organism is challenged by exogenous genetic material are known as "repeating elements." Bacterial DNA fragments complementary to the viral or plasmid genetic material that invaded the host are transcribed into RNA. The CRISPR RNA is thought to be supplemented by previously encountered foreign DNA entering bacterial cells. Cas, a nuclease that silences foreign DNA by causing double-strand breaks in it, is the target of the complex [60].

Genome editing: The application of the CRISPR/cas system

The CRISPR/Cas system, developed in 2012 by Jennifer A. Doudna and Emmanuelle Charpentier,

was awarded the 2020 Nobel Prize in Chemistry for his discoveries in genome editing through programmable RNA [61]. When the CRISPR/Cas9 system of pyogenic *Streptococcus* was discovered, the use of the CRISPR/Cas system for genome editing exploded [62]. Genome editing with CRISPR/Cas9 requires only one Cas9 nuclease and one guide RNA (gRNA). Both will be discussed in more detail in the following paragraphs. One example of the current application is studying genetic diseases in cell and animal models to gain insight into pathophysiology and assess the impact of genetic variation. Adding mutations to treat diseases caused by specific mutations and diseases not primarily caused by genetic alterations are two potential therapeutic options [63].

To help cut foreign DNA material, the CRISPR/Cas system in bacteria requires two types of RNA called "crRNA" and "tracrRNA." The complexes are directed to specific DNA spots by guide sequences carried by crRNAs. For genome editing using the CRISPR/Cas system, a significant breakthrough occurred: Developing a synthetic gRNA that can act as a replacement for two bacterial RNA molecules [61]. Watson-Crick bases in the gRNA pair with the first 20 nucleotides of the target DNA sequence, guiding nucleases to specific genomic regions. The corresponding DNA sequence is referred to as a "protospacer." It must be close to the PAM, which interacts with nucleases (for example, the PAM sequence that interacts with *S. pyogenes* Cas9 is NGG, where "N" is any nucleotide and "G" is guanine) [62]. The CRISPR/Cas9 system can repair breaks in target-DNA and endogenous DNA using nuclease double-strand breaks, which has led to repair systems attempting to use a variety of approaches. However, this method is prone to errors and frequently results in random insertion or deletion of break sites. As a result, the reading frame changes, causing the gene to become defective [63] (Figure 1).

To make primary breaks in DNA, only the Cas9 nuclease is required. Many Cas9 nucleases have been discovered, and more Cas9 alternatives have been developed. Some researchers have used base editors by attaching Cas9 proteins to cytidine or adenosine deaminase enzymes that are not catalytically active [64]. Gene-regulatory RNAs (gRNAs) are essential for Cas9 and other base editors

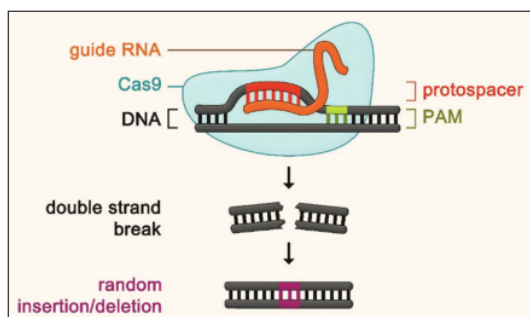


Figure 1: The genome-editing mechanism of regularly clustered short palindromic repeats (CRISPR)/CRISPR-associated (Cas) is outlined. Guide RNAs (gRNAs) are programmable RNAs that attach to specific DNA sequences known as "protospacers." Nucleases like Cas9 are attracted to DNA-gRNA complexes. The nuclease must attach itself to a specific DNA sequence near the protospacer, known as a "protospacer adjacent motif," to function (PAM). Nucleases cause double-strand breaks in DNA when these conditions are met. The body's own DNA repair mechanism then takes over. These methods are prone to errors and result in random insertions or deletions, rendering genes, and proteins useless. The catalytic domain of Cas9 is then deactivated and ligated to a base editor that produces precise single-nucleotide changes rather than double-strand breaks.

to function correctly. Instead of causing double-stranded breaks, base editors cause single-nucleotide changes within the protospacer, peaking at specific points in the protospacer DNA. When using adenine base editors, adenine (A) is deaminated to inosine (I) at the peak edit point (paired with thymine (T) on the other DNA strand). There was yet another snip of hair. Instead of T, Inosine (I) is attached to cytosine (C) through nick repair, and then G (G) is substituted for me. As a result, A-T becomes G-C, eliminating the need for a second break [65].

Even though the CRISPR-Cas system has a lot of potential for changing genomes, there are a lot of advantages and disadvantages to consider before using it in translational medicine (Table 2).

Table 2: The pros and cons of CRISPR [22]

Issues	Pros	Cons
gRNA design	The simple RNA: DNA base pairing of a gRNA makes it possible to design a gRNA that is highly specific to its target	In particular, the 2–5 pt PAM sequence must be present at 3' of the non-complementary strand.
Off target	Design of gRNAs for controlling the expression of CRISPR system components using powerful online software; availability of new high-fidelity variants	Large genomes frequently contain sequences that are highly homologous to the target site.
gRNA production	Any promoter can be used for efficient transcription and cleavage thanks to the design of RGR, an artificial ribozyme flanking gRNA (RGR)	Due to the lack of cell or tissue specificity, the RNA polymerase I promoter cannot be used for gRNA expression in conventional designs.
Multiple on-target mutations	Effective HDR-inducing strategies such as Cas9n, SpCas9-Gem, RS-1, and others	During the S, G1, and G2 phases, NHEJ is in charge of DNA repair.
Biallelic mutation	Biallelic mutations can be induced using inducible CRISPR, inducible knockout (K), and conditional KO.	At the one-cell stage of embryonic development, CRISPR-Cas9 may or may not cleave DNA.

Targeting the HBB gene with a CRISPR/Cas9 approach

We used the CRISPR/Cas9 method as previously described to achieve endogenous HBB gene editing. A plasmid expressing humanized SpCas9 protein (Addgene, Cambridge, MA, plasmid #41815) and a plasmid producing guide RNA (gR) were previously described [66].

We also employed pCas9-GFP (Addgene plasmid #447190), which coexpresses SpCas9 and GFP under the control of the CAG promoter. The gR-HBB-a selectively targeting HBB exon 1 was previously disclosed and is shown below [67]. The second gR-HBBUTR (5'-AAACTGGGGGATATTATGA-3') targets the HBB gene's 3' UTR. A donor vector supplying a needed DNA template for HDR is based on a previously published plasmid (Addgene, #31938), with additional DNA segments introduced by Gibson assembly [68]. In summary, it is made up of a left homology arm (560 bp), a right homology arm (880 bp), an HBB coding cDNA sequence followed by a P2A linker, a (e) GFP coding DNA segment, and a loxPflanked PGK-puromycin selection cassette. To prevent recutting by Cas9 complexed with gR-HBB-a, which detects the target DNA sequence comprising CTG (5'-GTCTGCCGTTACTGCCCTGT-3'), a synonymous substitution from CTG to TTA is inserted to the HBB cDNA in the donor vector. The donor vector's important DNA sequences (HBB-GFP-PGK-puroV2). We previously described gene targeting in multiple human iPSC lines. In brief, 2 million iPSCs were resuspended in 100 μ l P3 primary cell solution, combined with 10 μ g DNA comprising equal amounts of four plasmids (2.5 μ g for each of two guide RNAs, pCas9-GFP, and the donor vector), and electroporated using a 4D Nucleofector (Lonza, Allendale, NJ). PCR was used to confirm the locations of homologous recombination using primers L1-F and L1-R at the 50 terminal and L2-F and L2-R at the 30 terminal. The HDR-positive iPSC colonies were detected using genomic PCR with primers gDNA-75-F and gDNA363-R. Human iPSCs were transfected with the plasmid pCAGCre-IRES2-GFP (Addgene, #26646) as previously described to remove the loxP-flanked PGK-puromycin selection cassette. Following 3 days of transfection, GFP positive cells were picked by fluorescence-activated cell sorting on the FACSaria II (BD Biosciences, San Jose, CA). For clonal selection, the cells were plated at a low density. Individual clones were selected and tested for excision using genomic PCR primers L2-F and V4193-R. Sanger DNA sequencing verified the positive colonies with excision [66], [67].

Targeting the BCL11A Gene with a CRISPR/Cas9 approach

A modest CRISPR-Cas9-based genomic loss inside the BCL11A enhancer in the second intron might trigger adult-HbF reversal switching and HbF

production. Therefore, this strategy might be considered as an improved treatment genetic therapeutic approach for “hemoglobinopathies[69].”

We investigated generating targeted genomic deletions by CRISPR-Cas9 using pairs of sgRNAs. To induce genomic deletions, HEK293T cells were co-transfected using SpCas9-sgRNA expression vector including pMLM3636-gA, pMLM3636-gB, pMLM3636-gC, pMLM3636-gD and hCas9 expression vector as well as SaCas9-sgRNA expression vector including VVT1/mVVT1-gαI, VVT1/mVVT1-gαII and BPK2139. KU812, KG-1, and K562 cells were transfected using pMAK-gA + gC, pMAK-gA + gD, pMAK-gB + gC, pMAK-gB + gD as well as mVVT1-gαI/pSaCas9_GFP and mVVT1-gαII/pSaCas9_GFP. Transfection efficiency of HEK293T, K562, KG-1, and KU812 cell lines was approximately 84%, 70%, 64%, and 19%, respectively. KU812 cells were sorted 24 h post-electroporation by cell sorting with FACSria II and plated at limiting dilution. Genomic DNA was isolated from the transfected cells and deletion events analyzed using primer pairs flanking the sgRNA recognition sites. PCR products confirmed a 200bp genomic deletion for all tested combinations of sgRNAs. Heterozygote, homozygote, and non-deleted clones were identified by PCR on genomic DNA bulk population and edited single clones of K562 cell, bulk and edited clones of KU812 and KG-1 cells as well. These PCR products were gel purified and directly Sanger sequenced or cloned into pCR®-Blunt vector or cloned into the pBlueScriptccdB vector for sequencing. About 64% (16/25), 32% (8/25), 31.8% (7/22), and 18.5% (5/27) of single clones determined to be biallelic deleted clones for the combination of gA + gC, gA + gB, gB + gC, and gB + gD, respectively. The deletion frequencies of diverse combinations of sgRNA pairs were examined by PCR analyses of serially diluted amplicons in HEK293T cells using primer pairs flanking the targeted sequences.

Genomic segments were resolved by gel electrophoresis. The deletion frequency was 30%, 9%, 45%, 30%, and 9% for combination of sgRNAs gA + gC, gA + gD, gB + gC, gB + gD, and mVVT1/gαI+gαII, respectively, following co-transfection of two sgRNAs and Cas9, as computed using a PCR assay. The deletion frequency was 12%, and 2% for the combination of sgRNAs gA + gC and gA + gD into the K562 cells. Unpredictably, no deletion frequency was obtained for the combination of sgRNAs gB + gC and gB + gD into the K562 cells [69].

Reason using CRISPR as an effective therapy for SCD and β-thalassemia

With the development of CRISPR/Cas9 technology, autologous transplant of gene-edited hematopoietic stem cells may provide a cure for most patients with SCD. Despite the many challenges associated with translating gene-editing-based SCD treatment strategies to the clinic, such as the need for high editing efficiency and low off-target effects, there are several potential solutions to these problems. A quantitative understanding of the genotypic and phenotypic consequences of CRISPR/Cas9 edited SCD CD34+ cells is essential for safe clinical applications. Developing strategies for generating long-term, polyclonal HSCs that retain a high proportion of gene-edited cells after transplantation remains a challenge [70].

Genome editing is a revolutionary and possibly beneficial therapy method that can be used to treat thalassemia sufferers. It involves the use of targeted nucleases to fix mutations, in particular DNA sequences, and return the sequence to its natural wild-type state. CRISPR/Cas9 is a potent gene editing technique utilized in a variety of genetic engineering initiatives. It is an efficient and precise technique, which makes it appropriate

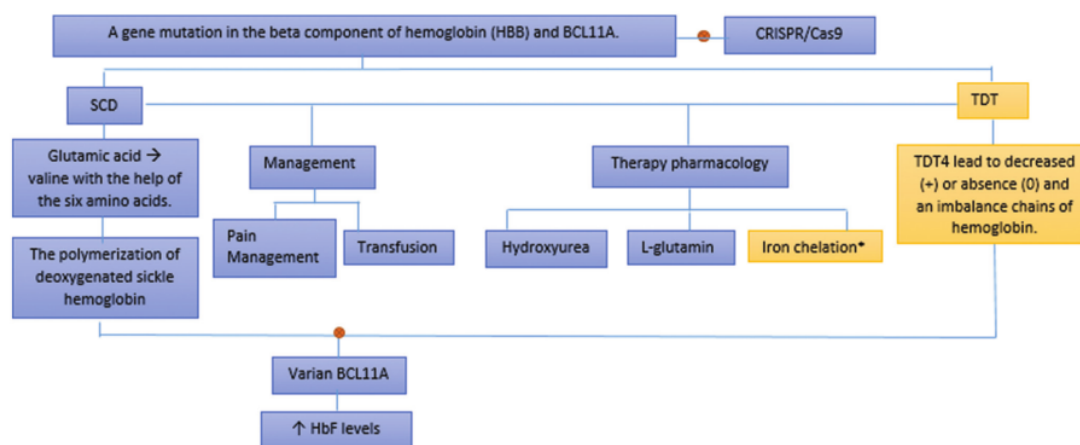


Figure 2: The framework of the deletion HBB and BCL11A (B-cell lymphoma/leukemia 11A) gene in SCA (sickle cell disease) and TDT (transfusion-dependent β-thalassemia). BCL11A: B-cell lymphoma/leukemia 11A; CRISPR/Cas9: Clustered Regular interspersed Short Palindromic Repeats-CRISPR-related; HBB: Hemoglobin Subunit Beta Gene; HbF: fetal hemoglobin; SCD: sickle cell disease; TDT: transfusion-dependent β-thalassemia. *Iron chelation is an adjunct therapy for TDT

for genome modification. CRISPR/Cas9 genome editing is a potent technology that can restore α -globin function without causing any negative effects. It is feasible to use CRISPR/Cas9 to reduce BCL11A expression, resulting in an increase in HbF production. Although these genome editing technologies are being evaluated *in vitro*, their full potential remains unknown. CRISPR/Cas9 may be used to precisely regulate transcription, modify the genome, and change epigenetics [71].

Furthermore, the CRISPR-Cas9 system provides numerous major benefits, including convenience of design and cloning, high genome editing accuracy, and the ability to multiplex, allowing simultaneous targeting of several loci [72], [73], [74].

Conclusion

TDT and SCD are the most frequent forms of monogenic illness in the world. Both disorders are brought on by mutations in the gene for the HBB. The mutations in HBB that cause TDT4 lead to decreased (+) or absence (0) production of β -globin, as well as an imbalance between the α -like and β -like globin chains of hemoglobin (e.g., β , γ , and δ). Therapy for both focuses solely on preventing the production of HbS or reducing the amount of HbS. Treatment options available and approved by the FDA are hydroxyurea and L-glutamine, with other options L-glutamine, crizanlizumab, and voxelotor as second-line therapy. In addition to the above treatment options, there is a blood transfusion treatment for certain cases. HBB deletion and BCL11A mutation using the CRISPR/Cas9 genome editing method can be a promising solution as a current therapy for SCD and TDT. Besides being faster, cheaper, and highly effective, this therapy is also believed to have great potential to provide therapy with permanent effects. It is hoped that further research will be carried out to determine the effectiveness of HBB and BCL11A removal therapy with CRISPR/Cas9. This method is clinically tested to determine the possible side effects of its use. So later, this therapy can be used as the main therapy for patients with SCD and TDT (Figure 2).

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