Advancing Gene Therapy in Sickle Cell Disease: Exploring the Potential of Prime Editing

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Abstract

Sickle cell disease (SCD) is a prevalent genetic disorder caused by a mutation in the *HBB* gene, leading to the production of abnormal hemoglobin that can block blood flow and cause severe pain. While conventional treatments for SCD are available, they have several limitations and are often not curative. As a result, gene therapy has emerged as a promising alternative for SCD treatment. CASGEVY, a gene therapy using CRISPR-Cas9 technology, has shown success in reducing symptoms of SCD by knocking out *BCL11A* and increasing fully functional, fetal hemoglobin. However, CASGEVY is prohibitively expensive and has some risks and inconveniences that limit its broader use. Prime editing, a newer gene editing technique, is considered more precise and potentially safer as it can be used to directly correct the *HBB* mutation. Additionally, unlike CASGEVY, prime editing could be used *in vivo* to correct the SCD mutation within the bone marrow. Recent studies have shown that prime editing can effectively correct the mutation both *ex vivo* and *in vivo*. Since *in vivo* prime editing could be more practical and affordable for people around the world, including in sub-Saharan Africa where SCD is most common, further research is needed to improve the efficiency and safety of prime editing to make it applicable to patients suffering from SCD globally. Here, we review the current application of CRISPR-Cas9 and prime editing for SCD treatment and explore further opportunities to enhance prime editing.

Introduction

Sickle cell disease (SCD) is a common inherited blood disorder that affects millions of people worldwide. Between 2000 and 2021, the global sickle cell birth rate increased by 13.7% to a rate of 382 per 100,000 live births, leading to the increase in the incidence of SCD from 453,000 to 515,000 infants per year[1]. SCD is a genetic disorder caused by a mutation in the *HBB* gene, which encodes the hemoglobin subunit β[2]. Hemoglobin (Hb) is a protein consisting of four subunits: two β-globin subunits and two α-globin subunits. Hb is located in red blood cells which are responsible for transporting oxygen to body tissues[3]. A single point mutation in the *HBB* gene, where a nucleotide substitution from adenine to thymine occurs, leads to the replacement of glutamic acid with valine in the 6th amino acid, forming sickle hemoglobin (HbS) (Figure 1). Under conditions of low oxygen, HbS polymerizes and builds hemoglobin polymers which result in the formation of abnormal sickled red blood cells[4]. The sickled red blood cells can easily aggregate with each other, obstructing blood flow in small vessels that can cause severe pain, known as vaso-occlusive crises (VOC)[5] (Figure 2).

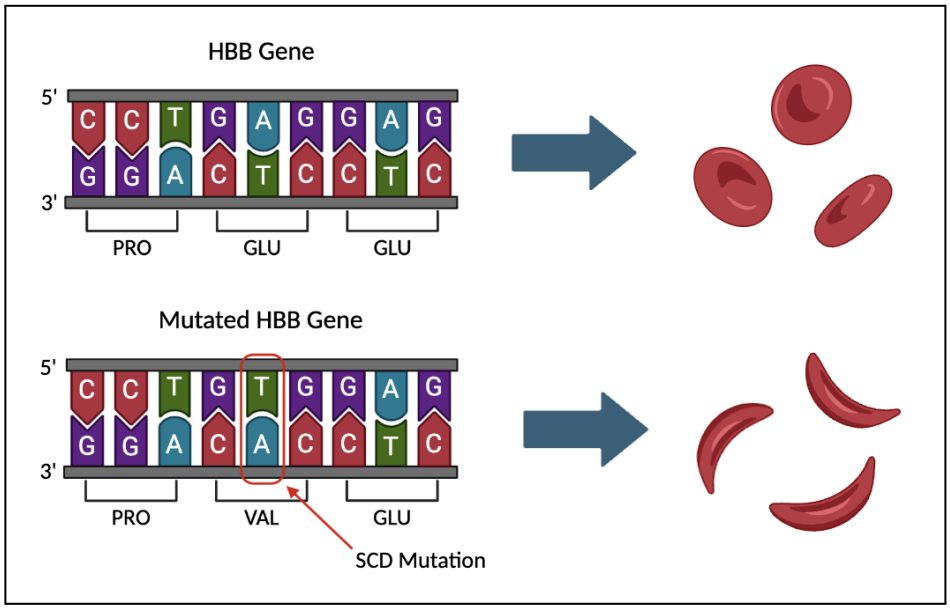

Figure 1. Genetic Mutation in the *HBB* Gene

- The original version of the *HBB* gene (top) has a specific DNA sequence which results in the formation of normal β-globin, contributing to the disk-shaped red blood cells. However, the mutated *HBB* gene (bottom) has a nucleotide substitution from adenine to thymine, which alters the amino acid sequence from glutamic acid to valine. The point mutation in the *HBB* gene forms an abnormal β-globin, thereby leading to the development of sickled red blood cells.

There are currently several treatments aimed at reducing the symptoms of SCD. These treatments include opioid medications to mitigate pain, blood transfusions, stem cell transplantations, and gene therapy[6]. While stem cell transplantation is potentially curative for SCD, it is restricted by the scarcity of matched donors and the risk of severe adverse effects, including pain episodes[7]. Therefore, many scientists are focusing on the potential of gene therapy as a cure for SCD.

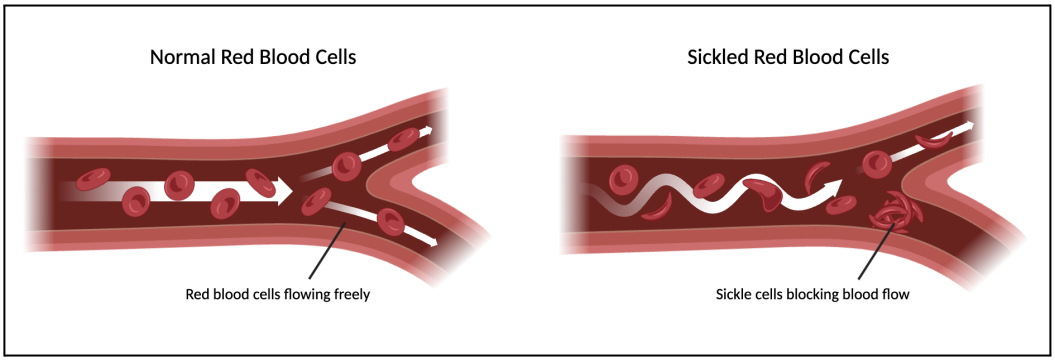

Figure 2. Effect of Sickled Cells on Blood Flow

- Normal red blood cells allow blood to flow freely through blood vessels (left). On the other hand, sickled red blood cells can easily accumulate and get stuck in small blood vessels, blocking blood flow and causing vaso-occlusive crises (right).

One gene therapy currently in use in the clinic is CASGEVY, which employs CRISPR-Cas9 technology[8]. CASGEVY disrupts the gene *BCL11A*, which prevents the production of fetal hemoglobin. CASGEVY edits the DNA in hematopoietic stem cells to reduce the activity of *BCL11A*, thereby increasing the production of fetal hemoglobin[9]. As fetal hemoglobin inhibits the sickling of red blood cells, the elevation of fetal hemoglobin levels can reduce the number of sickle cells in the blood, which can significantly alleviate severe crises of SCD, such as vaso-occlusive events[10]. In a clinical study, CASGEVY effectively reduced severe vaso-occlusive crises (VOC) in patients with SCD. In fact, 29 out of 31 patients (93.5%) had no severe VOC for at least 12 consecutive months after receiving the treatment[11]. Based on its successful outcomes, CASGEVY received approval from the FDA as one of the first gene therapies for SCD.

However, CASGEVY still contains risks and disadvantages. For instance, the treatment journey can take up to one year, although the duration may vary from person to person. Also, CASGEVY can provoke side effects, such as a decrease in platelets and white blood cell levels[11]. The reduction of platelets and white blood cells can raise the risk of infections and may cause abnormal bruising and bleeding. Other common side effects include mouth ulcers, nausea, musculoskeletal and abdominal pain, vomiting, febrile neutropenia, vomiting, headache, and itching[12].

While CASGEVY knocks out the *BCL11A* gene, the rapidly evolving field of gene editing offers improvements in the treatment of SCD by correcting the mutation in *HBB* itself. One way to do this is using prime editing, another gene editing technique. Prime editing is a targeted gene-editing technique that can perform insertions, deletions, and substitutions on specific DNA sequences[13]. Unlike CRISPR-Cas9, prime editing does not require DNA templates and does not generate double-stranded breaks in DNA (Table 1)[14]. This is a major advantage of prime editing as DNA double-stranded breaks can cause unintended mutations, genetic instability, and even cancer and other human diseases[15]. Prime editing works by using a prime editing guide RNA (pegRNA) and a prime editor (PE), which consists of Cas9 nickase fused with a reverse transcriptase (Figure 3). The pegRNA/Cas9 complex targets a specific sequence of nucleotide bases and edits it by reverse transcribing nucleotides from the pegRNA – replacing it with a new sequence. Then, using its intrinsic DNA mismatch repair mechanism, the cell corrects the mutation on both strands. Since prime editing is highly precise in targeting a specific base and editing it without causing a double-stranded break, it is regarded as an advanced technique in correcting disease-causing nucleotide mutations such as the SCD mutation. However, the prime editing technique is still in its nascent stage, requiring further developments to improve its editing efficiency and delivery strategies of prime editing systems into the target cells[16].

	CRISPR-Cas9	Prime Editing
Breaks	Double Stranded Break	Single Strand Break
RNA	guide RNA (gRNA)	prime editing guide RNA (pegRNA)
Cas9	Cas9 Endonuclease	Cas9 Nickase
Repair Mechanism	DNA template	Reverse Transcriptase

Table 1. Comparison of CRISPR-Cas9 and Prime Editing

- CRISPR uses a guide RNA and a Cas9 endonuclease to create a double-stranded break. On the other hand, prime editing uses a pegRNA and Cas9 nickase to generate a single-strand break. Also, CRISPR repairs a mutation using a DNA template, whereas prime editing uses a reverse transcriptase and a template embedded in the pegRNA.

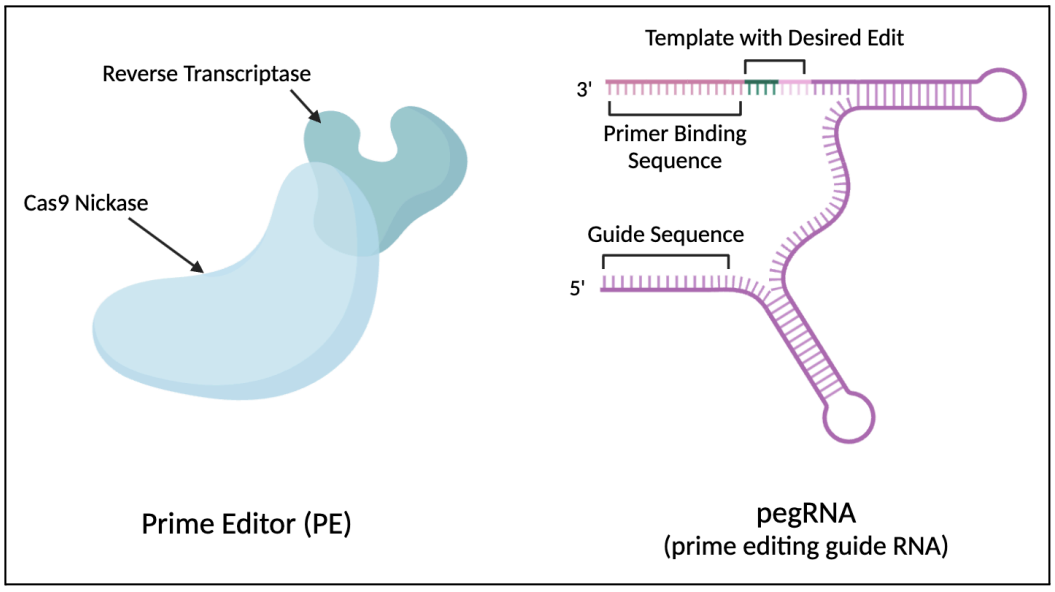

Figure 3. Components of Prime Editing

- Prime editing requires a prime editor (PE) and a prime editing guide RNA (pegRNA). The PE (left) includes Cas9 nickase fused with a reverse transcriptase. The pegRNA (right) contains a guide sequence at the 5' end, along with a primer binding sequence and a template with the desired edit at the 3' end.

Results

Current Approaches with Prime Editing

The prime editing technology consists of two components: a prime editor (PE) and a prime editing guide RNA (pegRNA). The prime editor (PE) contains a Cas9 nickase fused to a

reverse transcriptase, while the pegRNA carries a guide, a primer binding sequence, and a template sequence with the desired edit[17]. For prime editing to work, first, the Cas9 nickase of the PE generates a single-stranded break in the DNA strand with the target sequence. Next, the primer binding sequence helps the pegRNA bind to the nicked DNA strand, allowing the initiation of reverse transcription. Reverse transcription occurs with the template sequence from the pegRNA. Then, the newly generated sequence integrates with the original DNA strand, incorporating the edited sequence into both strands through the DNA repair mechanism (Figure 4)[18]. The editing efficiency of prime editing can differ from the design of elements that compose prime editing. Therefore, scientists are generating various versions of prime editing components in order to maximize editing efficiency. Some studies have shown promising results in increasing editing efficiency by testing different versions of prime editing components.

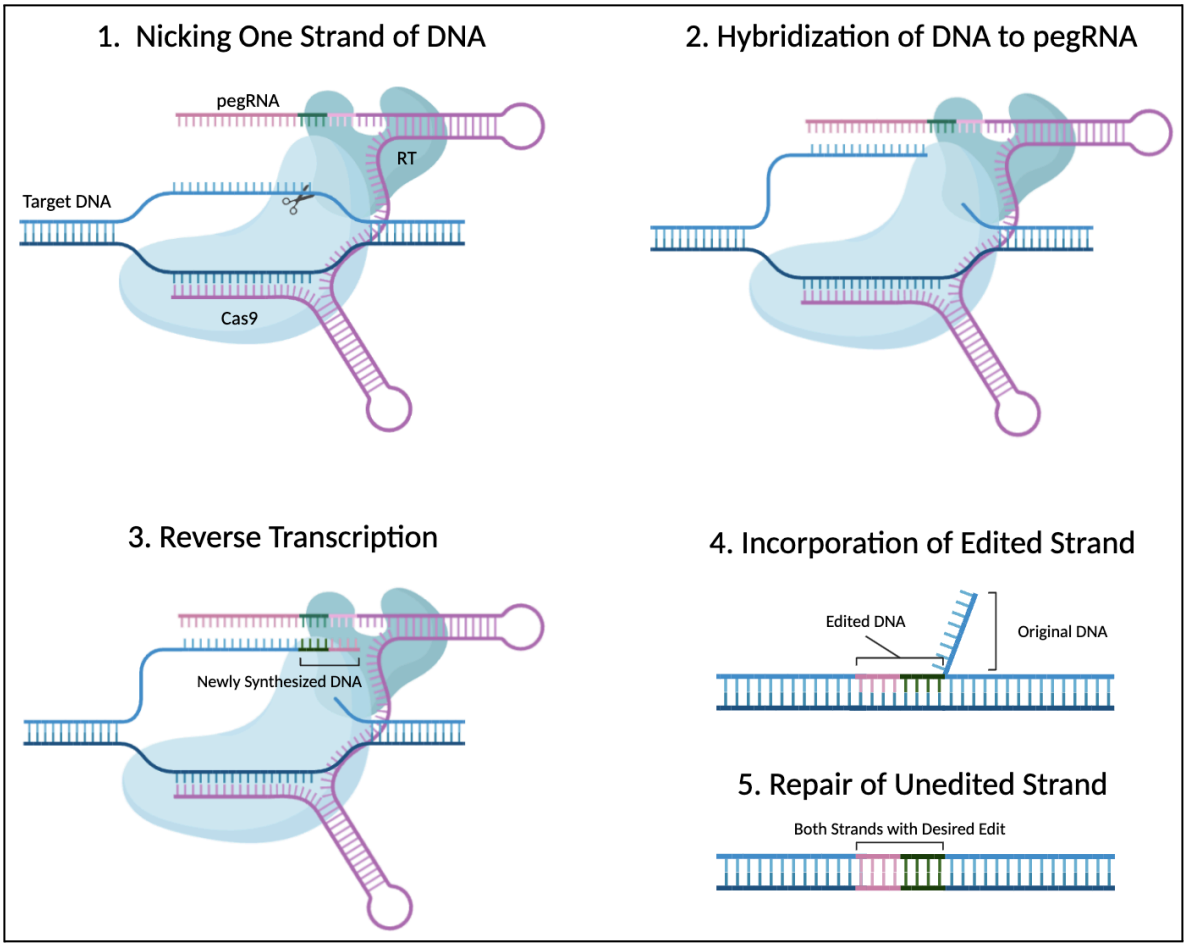

Figure 4. Process of Prime Editing

- The PE·pegRNA complex binds to the target DNA, and Cas9 nicks one strand. The DNA then binds to the primer binding site of the pegRNA. The RNA sequence is reverse transcribed, editing the target DNA with the newly transcribed DNA. Next, the edited DNA is incorporated into the DNA strand. Lastly, the unedited strand is repaired through the cell's intrinsic DNA repair mechanism.

One study published in *Nature Biomedical Engineering* by Everette et al. (2023) used *ex vivo* prime editing to correct a mutation in the *HBB* gene. *Ex vivo* prime editing is where hematopoietic stem cells (HSCs) are extracted from the bone marrow and gene-edited outside of the patient's body. In the study, researchers have developed different versions of PEmax, an improved prime editor (PE) (Table 2). After comparing four different versions of PEmax (PE2max, PE3max, PE4max, PE5max), they chose to utilize PE3max in further investigations of optimizing prime editing because of its highest editing efficiency. To determine the ability of PE3max edited HSPCs to repopulate when injected into the bone marrow, and reduce symptoms of SCD, they transplanted the edited HSCs in mice. They then collected the bone marrow of the mice for examination 17 weeks after injection when the majority of human cells have been expected to be obtained from bone-marrow-repopulating HSCs. Their strategy yielded an average of 42% prime editing levels in the edited HSCs of SCD patients 17 weeks after transplantation in mice. The red blood cells derived from repopulated HSCs demonstrated a significant reduction in sickling and an increase in normal hemoglobin levels[19]. The study using the *ex vivo* prime editing approach, yielded a moderately high percentage of edited *HBB* genes in HSCs.

	Engineered Protein	Dominant Negative MLH1	Nicking sgRNA
PE2max	✓		
PE3max	✓		✓
PE4max	✓	✓	
PE5max	✓	✓	✓

Table 2. Versions of PEmax

- PE2max, PE3max, PE4max, PE5max all include engineered prime editing proteins that were generated from the original prime editors to improve editing efficiency. The improved architecture includes a codon-optimized reverse transcriptase, additional nuclear localization sequences, and two mutations in the Cas9 which are associated with increased nuclease activity. PE4max and PE5max express the dominant-negative MLH1, which inhibits mismatch repair. PE3max and PE5max contain the nicking sgRNA.

Another study published in *Blood* by Li et al. (2023) used *in vivo* prime editing, where a vectorized prime editing system is directly injected into the bone marrow to correct the SCD mutation in hematopoietic stem cells (HSCs). The researchers' observations showed that in HSCs derived from mice, PE3, PE3max, and PE5max were able to correct the SCD mutation 5.4%, 18.0%, and 25.1% respectively *in vitro*. In human HSCs, their editing rates were 1.0%, 1.6%, and 3.4% respectively. When mice were treated with PE5max *in vivo*, the collected blood samples from mice 16 weeks after transplantation showed an average of 43% correction. *In vivo*

prime editing showed accuracy with <1% undesired insertions and deletions at the target sight. The study also showed significant reduction in the sickling of red blood cells[17].

Despite its potential to repair the SCD mutation, prime editing still has several challenges to overcome. The low editing efficiency of prime editing compared to other gene therapies and the requirement of mechanisms to inhibit DNA mismatch repair to enhance prime editing efficiency are some of the challenges faced today[21]. These difficulties likely limit prime editing from being used in therapeutic trials with SCD patients. According to ClinicalTrials.gov, out of 37 studies related to sickle cell disease with the treatment of gene therapy, none of them focus on prime editing. Instead, the majority attempts the use of CRISPR-Cas9 in gene-editing hematopoietic stem cells[22].

Applicability of Gene Therapy

Current clinical trials of gene therapies for SCD focus on the *ex vivo* approach using CRISPR-Cas9 due to its efficiency and safety compared to the *in vivo* approach, which is still in development[23]. In the *ex vivo* approach, hematopoietic stem cells (HSCs) are collected from patients for gene modification and are re-infused after myeloablative chemotherapy. High-dose chemotherapy is required to make space for the gene-edited HSC in the bone marrow[24]. Due to the complex process of the *ex vivo* approach combined with chemotherapy, specialized centers in developed nations with expertise in hematology and SCD are necessary for this treatment.

Among all the babies born each year with SCD worldwide, approximately 79% are born in sub-Saharan Africa[25]. Countries in sub-Saharan Africa are mostly low- and middle-income countries, and the treatment for SCD is poor and insufficient despite the high prevalence of SCD[26]. If untreated, patients with SCD have a high possibility of dying during childhood. The probability of early childhood death with SCD in sub-Saharan Africa where treatments are limited can be as high as 90%[27]. The World Health Organization (WHO) estimates that 70% of SCD deaths in Africa are preventable with simple interventions. These interventions include early identification using newborn screening, treatment of acute symptoms, and prophylaxis of infections[28]. Since gene therapies are estimated to cost $1.85 million USD in the base case, utilizing current gene therapies is an unrealistic strategy for worldwide application, especially in Africa where even basic treatments are often not possible[29].

In vivo prime editing may be the future of cost-effective gene therapy for SCD. Unlike *ex vivo* prime editing, *in vivo* omits the process of harvesting HSCs and modifying them in cell culture. Instead, the *in vivo* approach involves the direct injection of a vector with a gene-modifying agent, allowing gene editing to occur inside the bone marrow[23]. The systematic delivery of gene-modifying agents *in vivo* can also eliminate the need for chemotherapy. These differences would significantly reduce the cost of gene therapy. Due to its simplicity, *in vivo* prime editing is considered the future of gene therapy for patients with SCD in resource-poor countries where SCD is prevalent.

Proposed Prime Editing Strategy

Prime editing could potentially improve accessibility to SCD treatment. However, clinical trials are still needed to verify its ability to edit SCD mutation in humans. For broader application of prime editing towards SCD patients worldwide, a clinical trial of *in vivo* prime editing is a beneficial opportunity. In creating such a treatment for a clinical trial, we must come up with an *in vivo* prime editing strategy with the highest editing efficiency and the lowest unexpected drawbacks. In order to do so, I would like to propose some modifications to the existing prime editing strategy examined in the study published in *Blood* by Li et al., as their results were successful in editing the SCD mutation in HSCs of mice *in vivo*.

First, the study by Li. et al in *Blood* utilized PE5max as the prime editor for their examination. After comparing different versions of PEs, they noticed that PE5max had a significant advantage due to the addition of a dominant-negative MLH1 protein, which limits the cellular DNA mismatch repair mechanism, thereby increasing the prime editing efficiency. This system also includes nicking sgRNAs and PAM site disruption, two components associated with increased prime editing efficiency. Moreover, they used HDAd5/35++ vectors to deliver PEs to the HSCs in order to correct the SCD mutation. Instead of using a pegRNA, they used an engineered prime editing guide RNA (epegRNA) which has a stabilizing structure at the 3' end for improved pegRNA expression and editing activity[17].

Even though the study showed an average of 43% correction from sickle hemoglobin to normal hemoglobin in mice, there might be some possibilities of improvement that could increase editing efficiency. One option would be the inhibition of other mismatch repair (MMR) genes. In addition to reducing cellular mismatch repair through transient inhibition of MLH1 by using a dominant negative MLH1, we could inhibit other mismatch repair genes, such as the MSH2 gene[30]. To our knowledge, the combined inhibition of different MMR genes has been studied *in vitro* but not *in vivo*[31, 32]. Another option would be improving the nucleotide sequence of the epegRNA. Recent advances in machine learning have created pegRNA predictors that can provide prime editing efficiency predictions depending on the sequence of the pegRNA. One of the technologies we can use is the publically available PRIDICT tool[33]. On https://PRIDICT.it, selecting the *HBB* gene and the variant of *NM_000518.5(HBB):c.20A>T (p.Glu7Val)* provides the DNA sequence with the desired edit in the nucleotide sequence from adenine to thymine. Then, if we run prediction, PRIDICT provides various designs of epegRNAs and their expected editing percentage. For example, a epegRNA with a reverse transcription template (RTT) length of 11, a spacer sequence of *GATGGTGCATCTGACTCCTG*, and a primer binding site (PBS) length of 13 results in editing efficiency of 74.49% and 6.53% of unintended editing. Using this tool, we could come up with a new epegRNA that could derive the highest editing efficiency to correct the SCD mutation. We could also add the nucleotide sequence in the epegRNA responsible for PAM site inactivation. PAM site inactivation can increase the efficiency and accuracy of gene editing by introducing a silent mutation alongside the desired edit in the donor DNA, preventing the PE protein from re-editing the target site[34].

Conclusion

Sickle cell disease (SCD) is a widespread inherited blood disorder caused by a mutation in the *HBB* gene, affecting millions globally, with the birth rate of infants with SCD increasing over the years. SCD leads to the production of abnormal sickle hemoglobin (HbS) which sickle

under low oxygen conditions, leading to blockages in blood vessels and vaso-occlusive crises (VOCs).

Even though there are several traditional treatments for SCD, including pain management medications, blood transfusions, and stem cell transplantations, these treatments have limitations in application. Recently, gene therapy has shown promise in curing patients with SCD. CASGEVY, a gene therapy that uses the CRISPR-Cas9 technology, has shown its ability in reducing severe VOCs in SCD patients. However, due to the fact that the treatment of CASGEVY is expensive, lengthy, and carries risks of side effects, scientists are paying attention to the emerging technique of gene therapy: prime editing.

Prime editing is considered to be more precise than CRISPR-Cas9 technology and has the advantage of generating single-stranded DNA breaks instead of double-stranded DNA breaks, reducing the risk of unintended mutations. Unlike the CRISPR-Cas9 approach used in CASGEVY, prime editing offers the ability to correct the sickle cell mutation directly. Studies in both *ex vivo* and *in vivo* approaches of prime editing have shown significant editing efficiency and reduction in sickling. The *ex vivo* prime editing strategy yielded an average of 42% correction, while the *in vivo* prime editing strategy showed an average of 43% correction in hematopoietic stem cells (HSCs).

Considering the fact that SCD is most prevalent in the Sub-saharan Africa region, future research should particularly focus on improving the delivery mechanism and editing efficiencies for *in vivo* prime editing so that the treatment can be applicable in countries of low-resource and low-income settings. Therefore, further investigations should focus on the portability and safety of the *in vivo* prime editing approach.

Even though the proposed prime editing strategy is feasible, it has some limitations. Since mismatch repair (MMR) inhibition has not been fully studied *in vivo*, transient MMR inhibition could cause unintended consequences when applied *in vivo*. Additionally, the proposed epegRNA was obtained using machine learning technology and has not been validated *in vitro*; therefore, the epegRNA should be verified through further studies.

In summary, the successful advancement of prime editing will offer new hope to patients with SCD worldwide. This innovative approach could pave the way for a permanent cure, fundamentally altering the future of SCD.

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