Advancements and Challenges in Gene Therapy Approaches for Sickle Cell Disease: A Comprehensive Review

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Abstract: Sickle cell disease (SCD) is an autosomal recessive genetic blood disorder that occurs when both alleles of the HBB gene have mutations, leading to the production of abnormal haemoglobin (HbS). The presence of HbS causes red blood cells (RBCs) to take on the distinctive sickle-shaped form associated with the disease. This, in turn, leads to blockages in blood vessels, decreased blood circulation, and organs' damage. Traditional treatments such as blood transfusions and hydroxyurea offer relief but come with their own limitations and associated risks. Gene therapy has emerged as a promising paradigm shift in the quest to cure SCD, offering personalised solutions by targeting the genetic root of the disease.

This review article explores the principles and recent advancements in gene therapy for SCD. However, before gene therapy can become the main curative strategy for this disease, several challenges need to be overcome including the need for long-term safety and efficacy evaluations. Ongoing research and innovation hold the promise of enhanced treatments and the potential for a widely available gene therapy, ultimately improving the quality of life for individuals living with SCD.

Keywords: Sickle cell disease (SCD), HBB gene, haemoglobin S (HbS), gene therapy autologous haematopoietic stem cells (HSCs), gene addition, lentiviral vectors, gene editing, CRISPR/Cas9

Introduction

Molecular Basis of Sickle Cell Disease

Beyond Sickle cell disease, also known as sickle cell anemia, is the most prevalent autosomal recessive genetic blood disorder. It results from an inherited mutation in both alleles of the HBB gene (1). This monogenic disease is caused by a specific single base pair mutation (A=T) located within the sixth codon of the HBB gene (2). The HBB gene is responsible for encoding the β-globin subunit of haemoglobin A (HbA), which is the primary oxygen-carrying protein in adult red blood cells (RBCs). The missense mutation in the HBB gene results in the substitution of the hydrophilic amino acid glutamic acid with the hydrophobic amino acid valine. This change in amino acids leads to the misshaping of the β-globin chains within the HbA.

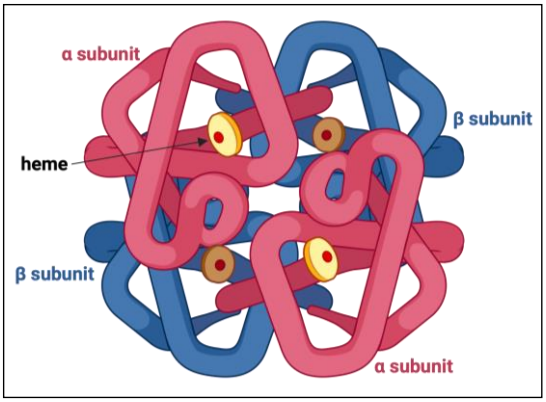

Figure 1: Haemoglobin A (HbA) protein is made up of four subunits: two α-globin subunits (pink) and two β-globin subunits (blue) each bound to a heme group (yellow). Image created using BioRender

Haemoglobin is composed of four subunits: two α-subunits and two β-subunits each bound to a heme group (**Figure 1**). Heme groups enable haemoglobin to bind to oxygen molecules by forming a stable and reversible association. Sickle cell haemoglobin (HbS) can perform the function of carrying out oxygen (which will be distributed throughout the body) perfectly well. However, when de-oxygenated, HbS molecules undergo anomalous hydrophobic interactions, leading to their aggregation into long, rigid structures within the RBCs (2). This eventually gives rise to the characteristic deformation of RBCs, causing them to assume a sickle-like shape (2) (**Figure 2**).

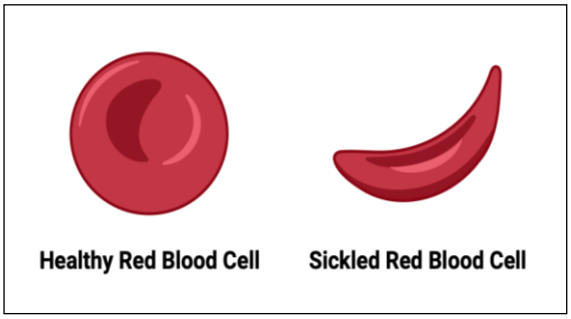

Figure 2: Healthy mature red blood cell (left) vs sickled de-oxygenated red blood cell (right). Image created using BioRender.

The sickle shape of these cells is problematic as it reduces their flexibility, making them more prone to getting stuck in small blood vessels. This, in turn, results in vascular blockages, decreased blood circulation, and premature cell death (1,3). Moreover, reduced blood circulation can lead to chronic organ damage, potentially culminating in conditions such as strokes, kidney failure, lung-related complications, and bone necrosis, ultimately contributing to premature mortality (4).

Heterozygote Advantage in Sickle Cell Disease

Sickle cell disease is the most prevalent monogenic disorder in the United States, affecting approximately 1 in every 500 African Americans. It is even more common in malaria-endemic regions, a phenomenon known as "heterozygote advantage" (5). Individuals with only one copy of the mutated HBB gene typically experience mild or no symptoms. In turn, research has shown that those with this heterozygous mutation are more resistant to severe malaria (5-7). This resistance occurs because HbS interferes with the Plasmodium parasite's life cycle within RBCs, providing a survival advantage for individuals carrying the heterozygous mutation in malaria-prone areas. Remarkably, approximately 1 in every 12 African Americans carries the autosomal recessive mutation in the HBB gene, resulting in roughly 300,000 infants being born with the condition each year (2,8).

Traditional Treatment Approaches

To date, the main therapeutic treatments for SCD remain blood transfusion and hydroxyurea (1). Blood transfusions aim to enhance oxygen-carrying capacity and reduce the ratio of HbS to HbA, thereby alleviating the complications associated with vascular occlusion (9). However, in many regions around the world, patients lack access to a secure and sustainable blood source. Even in countries where blood is accessible and economically viable, long-term transfusion therapy carries inherent risks, including alloimmunisation (an immune response to foreign cell antigens), iron overload (due to the body's inability to naturally break down excess iron), and potential risks of infections from contaminated blood (9). Overall, long-term transfusion therapy is associated with a significant burden on the patient, including the need for regular hospital attendance and the use of iron chelators to eliminate excess metal. Therefore, the approach to transfusion must balance these risks with the benefits, both in decisions regarding when to transfuse and in the practical aspects of how transfusions are administered.

Another common therapeutic approach for SCD is hydroxyurea, an oral medication renowned for its effectiveness in reducing the frequency and severity of various complications associated with the disease by elevating fetal haemoglobin levels (HbF) (10). Fetal haemoglobin differs from the adult haemoglobin by featuring two γ-globin subunits in place of β-globin subunits, thus effectively sidestepping the challenges stemming from the genetic mutation in the β-globin subunits. However, despite its proven efficiency in reducing the frequency of disease-related complications, its use requires careful monitoring to ensure that the dosage is tailored to the patient, as it can lead to side effects such as bone marrow suppression and a temporary decrease in blood counts.

Gene Therapy approach to cure Sickle Cell Disease

More recently, besides blood transfusions and hydroxyurea, other FDA-approved therapies like voxelotor, crizanlizumab, and L-glutamine have been used to diminish the frequency and severity of vaso-occlusive crises (1). However, the potential adverse effects associated with these therapies highlight the need for innovative interventions that can address the limitations of current treatments.

To date, the only cure for SCD is bone marrow transplant, which consists of the transplantation of healthy hematopoietic stem cells (HSCs) from a donor to the patient. However, this technique is associated with significant issues, including organ injury, infection, and graft-versus-host disease, which could eventually lead to death. Only about 10% of patients affected by the disease have a histocompatible sibling donor (11). Although it represents a viable curative option, before performing a bone marrow transplantation, the patient needs to undergo extensive testing and evaluation to determine eligibility and find a suitable donor (11,12).

Gene therapy offers a potential solution to the issues raised by bone marrow transplantation. By using autologous HSCs, scientists can either insert the functional gene or correct the genetic mutation and re-insert the cells back into the patient, thus overcoming potential immune complications and eradicating the disease at its core.

Gene Therapy: A Promising Paradigm Shift

Overview of Gene Therapy Principles

With a focus on patient-tailored medication, gene therapy approach holds an immense promise in revolutionising medical treatments by addressing the disease at the genetic level. This approach is grounded on the simple principle of replacing or correcting faulty genetic sequences causing a specific disease, to restore their functionality. Autologous HSC therapy has been actively pursued and holds a great promise for curing SCD (11). This technique consists of isolating the HSCs cells from either the bone marrow or the periphe-

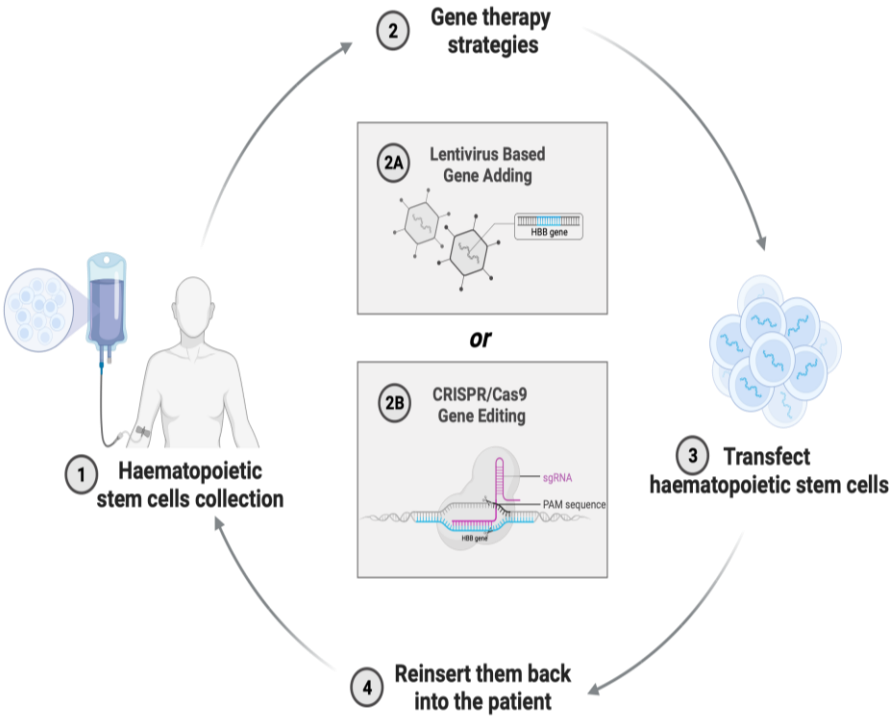

Figure 3: Schematic representation of gene therapy strategies for SCD. (1) Haematopoietic stem cells are collected from the patient affected by SCD. (2) Gene therapy strategies are implemented to either (2A) insert the functional HBB into a lentiviral vector or (2B) engineer CRISPR/Cas9 technology to correct the underlined gene mutation. (3) Haematopoietic stem cells are then transfected and (4) re-inserted back into the patient. Image created using BioRender.

ral blood and manipulating them *ex vivo* to either (i) insert the functional gene, (ii) correct the SCD mutation, or (iii) induce HbF expression. Once HSCs have been successfully transfected, the patient receives bone marrow conditioning with myeloablative agents, followed by infusion of the modified HSCs **(Figure 3)**.

Two Types of Gene Therapy for Sickle Cell Disease: Gene Addition and Gene Editing

Over the years, two different approaches have emerged in the field of gene therapy for SCD: gene addition and gene editing. Gene addition consists of introducing a functional copy of the β-globin gene into a patient's HSCs, usually using lentiviral vectors **(Figure 3 2A)**. This approach aims to replace defective haemoglobin with healthy haemoglobin, ultimately improving the quality and functionality of RBCs (11,13). On the other hand, gene editing seeks to provide a one-time treatment capable of either correcting the genetic mutation responsible for SCD or inducing the expression of HbF, using gene editing tools such as CRISPR/Cas9 **(Figure 3 2B)** (11,13).

Both approaches offer promising avenues for developing effective treatments and potential cures for SCD patients. However, there is a key distinction between the two methods. While gene addition does not integrate the functional gene into the genome - resulting in a transient curative strategy - gene editing has the potential to permanently correct the underlying genetic defect, offering a more robust curative solution. Ongoing research and clinical trials are continually enhancing our understanding of the effectiveness and safety of these therapeutic strategies.

Gene Addition: Lentiviral-Based Strategies

The transplant of genetically engineered autologous HSCs has emerged as a promising curative strategy for SCD. One approach consists of introducing the functional HBB gene inside the HSCs using viral vectors. Over the years, advancements in viral vectors manipulation have led to a transition from the use of γ-retroviral vectors to lentiviral vectors as the preferred approach (14). Initially, γ-retroviruses offered advantages such as stable integration, versatility in target cell types, and ease of vector manipulation. However, γ-retroviruses have been shown to come with limitations including limited transgene expression and risks of insertional mutagenesis (14,15). Lentiviral vectors have emerged as a promising alternative due to their ability to accommodate more complex DNA cassettes, a crucial factor for achieving high-level of β-globin expression (14). Unlike γ-retroviruses, lentiviral vectors can integrate into non-dividing HSCs, ensuring a safer and more stable integration profile. Safety modifications, including self-inactivation and removal of viral enhancer and promoter sequences, have been implemented to address concerns about insertional mutagenesis. Additionally, transgene expression has been improved by incorporating into the lentiviral vectors key transcriptional regulatory elements from the β-globin locus control region (16).

While gene addition strategies have marked significant progress in the development of gene therapy for SCD, they exhibit only partial effectiveness in alleviating the clinical manifestations of the disease (17). To tackle this challenge, a gene silencing approach has been employed. This method utilises a lentiviral vector that expresses a microRNA to silence the expression of the BCL11A gene, a crucial regulator of the gene encoding the γ-globin subunit in adulthood (18,19). Ongoing clinical trials are currently assessing the potential therapeutic benefits of reactivating HbF using lentivirus-based strategies (20). Nevertheless, it still remains the issue that even when combining gene addition with gene silencing, the formation of HbS cannot be completely prevented, ultimately leading to the premature degradation of RBCs (17,21).

A recent development involves the generation of a bifunctional lentiviral vector designed to express functional β-globin while concurrently employing a microRNA to specifically down-regulate sickling β-globin expression. This technique allows for the reduction of HbS levels and promotes the incorporation of functional β-globin into the haemoglobin molecule (21). The efficient transduction of autologous HSCs by this bifunctional lentiviral vector results in a significant expression of the functional β-globin and a reduction of the sickling β-globin transcripts within the erythroid progenitors and RBCs, ultimately resulting in the successful correction of the sickling phenotype (21). Overall, the integration of both gene addition and gene silencing strategies holds great promise for enhancing the effectiveness of existing lentiviral-based therapeutic methods. In particular, this approach pre-

sents an innovative treatment option ready for preclinical and clinical testing.

Gene Editing: Prime Editing and Base Editing Strategies

Genome editing allows for precise alterations to the human genome, with the goal of rectifying mutations that underlie genetic disorders (22). This process usually involves inducing DNA double-strand breaks (DSBs) using engineered designer nucleases, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or the CRISPR/Cas9 system. When DSBs occur under physiological conditions, they activate two primary repair pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR) (23). NHEJ is a rapid but error-prone mechanism, often resulting in insertions and deletions at the break site. Conversely, HDR is a slower but highly accurate DNA repair pathway that employs an introduced DNA fragment as a template to precisely rectify the occurred error. Because HDR requires a template strand, it is largely restricted to the S and G2 phases of the cell cycle; therefore, achieving gene targeting rates higher than 20% in predominantly inactive HSCs remains a challenge (24). Moreover, inducing DSBs is known to hold genotoxic potential, and mutations, loss of heterozygosity, and chromosome rearrangements can occur during DNA repair (25). To overcome this issue, alternative techniques where only one strand of DNA is cleaved have been developed, and they are mostly based on the CRISPR/Cas9 system (26).

One such technique is base editing, which allows for the introduction of single-nucleotide variants in the DNA or RNA of living cells. Base editors are composed of a Cas9 fused with a deaminase enzyme capable of precisely converting A to G or C to T at specific sites directed by single-guide RNA (sgRNA). In a 2021 study, successful base editing was implemented to rectify the SCD mutation in both patient blood-forming cells and a mouse model (27). While base editors cannot generate the required T-to-A change to restore the SCD mutation to its normal sequence, converting A to G - or T to C on the complementary DNA strand - generates a "haemoglobin Makassar," which consists of a rare benign haemoglobin variant found in healthy individuals (27).

Another emerging gene editing tool that allows for targeted small insertions, deletions, and base swapping - without the need for DSB - is the prime editing tool. In a recent study, prime editing has been used effectively to correct the HBB gene in HSCs collected from SCD patients (28). Correction rates ranged from 15% to 41% and exhibited successful engraftment, differentiation, and lineage maturation. Importantly, a genome-wide analysis revealed minimal off-target effects. These studies suggest the potential for a one-time treatment for SCD that mitigates the undesirable effects associated with DSB.

Challenges to overcome and future directions

Overall, lentiviral vectors have shown effective results and flexibility in incorporating various anti-sickling genes. Importantly, no vector-related clinical adverse events have been noted. Nonetheless, lentiviral vectors do carry a potential risk of insertional mutagenesis, although current data suggest this risk is relatively low in the treated patient population (14). Alternative approaches to gene adding, such as the CRISPR/Cas9 system, are in the early stages of clinical data collection, while others, like base editing and prime editing are just beginning clinical evaluation. A major concern posed by the use of CRISPR/Cas9 system, is the generation of off-target effects and potential genotoxicity. Evaluating the long-term effectiveness and safety of all these therapeutic strategies will require several decades of ongoing observation. In the meantime, it is likely that new approaches will continue to emerge, potentially offering even greater promise.

The ultimate approach for HSC-based gene editing to treat SCD would involve direct genome editing (*in vivo*), rather than the current method of isolating HSCs outside the body (*ex vivo*) with chemotherapy conditioning. If it becomes feasible to systematically administer gene editing agents and achieve efficient editing within HSCs *in vivo*, it would significantly broaden the application of this treatment and drastically reduce costs. The ultimate goal is to make gene therapy globally accessible, particularly for the majority of SCD patients who have limited resources (14).

Conclusion

The pursuit of a cure for SCD has witnessed remarkable advancements over the years, transitioning from traditional therapies like blood transfusion and hydroxyurea to innovative gene therapy approaches. While traditional treatments provide some relief, they come with limitations and risks, highlighting the need for more advanced interventions. Gene therapy has emerged as a promising paradigm shift in the quest to cure SCD. By targeting the genetic root of the disease, gene therapy aims to offer a patient-tailored solution. Ongoing research and clinical trials are shedding light on the effectiveness and safety of these innovative therapeutic strategies. However, despite the progress made in gene therapy, challenges persist, and long-term observations are required to assess the safety and efficacy of these approaches fully. The ultimate goal would be to transition from ex vivo gene editing, involving HSC isolation with chemotherapy conditioning, to in vivo editing, which could make gene therapy more accessible and cost-effective. With continued research and innovation, the vision of a globally accessible gene therapy for SCD may become a reality, providing lasting relief and improving the lives of countless individuals worldwide.

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Conflict of Interest statement

The author declares no conflict of interest.