CRISPR/Cas9 Genome Editing to Treat Sickle Cell Disease

By

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Declaration

It is hereby declared that

1. The thesis submitted is my/our own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I/We have acknowledged all main sources of help.

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Approval

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Ethics Statement

The study does not involve any kind of animal or human trial.

Abstract

The most prevalent inherited blood condition, Sickle Cell Disease (SCD) causes excruciating pain, permanent organ damage including liver, heart, and kidney also causes an untimely death. There is currently a dearth of treatment options for SCD. In order to lessen the severity of acute problems, only four medications have been licensed by the Food and Drug Administration (FDA). Hematopoietic stem cell transplantation, is the curative treatment for SCD, that is related to matched donor. Recent and significant advancements in genome editing techniques have shown promise as a curative option, with the potential to fix the mutation in patient-derived Hematopoietic Stem/Progenitor Cells (HSPCs), to increase Fetal Hemoglobin (HbF) expression to avoid sickling of Red Blood Cells (RBCs), and generate corrected induced Pluripotent Stem Cells (iPSCs). CRISPR/Cas9, which was discovered relatively recently, has not only changed the face of genome engineering, but also made it possible to use these ideas in the clinic. In this review, we provide an overview of CRISPR/Cas9 applications in genome engineering and discuss the advantages, disadvantages, how it is superior to other gene editing technologies such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) and potential of this technology as a treatment for SCD.

Keywords: SCD, CRISPR/Cas9, hematopoietic stem/progenitor cells, fetal hemoglobin, gene editing.

Dedication

Dedicated to my parents who could do anything for my happiness.

Acknowledgement

I would like to begin by thanking Almighty Allah for keeping me in good health and blessing me with the capability, strength and assistance needed to complete this project. However, this research paper would not have been completed without the support of several individuals and i would like to express my gratitude to all of them.

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List of Acronyms

AAV : Adeno-associated virus

Cas9: CRISPR associated protein 9

CRISPR: Clustered regularly interspaced short palindromic repeats

DSB: Double strand breaks

HPFH: Hereditary persistence of fetal Globin

HbA: Adult hemoglobin

HbS: Hemoglobin S

HbF: Fetal hemoglobin

HDR: Homology directed repair

INDELs: Insertions/deletions

iPSCs: Induced pluripotent stem cells

NHEJ: Non-homologous end-joining

OTEs: Off-target effects

PAM: Protospacer-adjacent motif

RBCs: Red blood cells

SCD: Sickle cell disease

TALENs: TAL-effector nucleases

ZFNs: Zinc finger nucleases

Chapter One

1.1 Introduction

Sickle cell disease (SCD) is a genetic disorder that affects 100 thousand individuals in USA and thousands of others worldwide. The Global Burden of Disease Study's systematic analysis estimates that 42 million population have this disease trait (i.e., they are carriers of the mutation), 3.1 million individuals have sickle cell disease, and 175,000 people die each year from complications associated with SCD(Sundd et al., 2019). A lifelong blood condition known as sickle cell disease is defined by red blood cells that take on an unusual, stiff sickle form. When hemoglobin in red blood cells clings or clumps together, it is known as sickling. As a result, the cell becomes brittle, inflexible, and crescent-shaped. Sickling decreases the cells' suppleness and raises the chance of several issues. Sickling is caused by a hemoglobin gene mutation (Ifeanyi & Ogechi, 2015). SCD refers to a collection of illnesses that have different clinical presentations but share the same pathophysiologic outcome resulting from a single monogenic mutation, whether inherited in homozygosity or in combination with another faulty beta-globin gene. The altered beta -globin gene generates aberrant hemoglobin S (HbS), that quickly polymerizes in the oxygen removing condition and changes the rheology and lifetime of red blood cells (RBCs). Stroke, acute pain, inflammation, chronic anemia, organ failure, repeated vaso occlusion, and early mortality are only a few clinical consequences brought on by this single gene substitution (Demirci et al., 2021).

Through the exact addition, deletion, correction, and interruption of particular sequences, gene editing tools like CRISPR/Cas9 can modify the disease-causing gene permanently. The aim of this research is to review how CRISPR/Cas9 can aid in SCD genome editing leading towards an effective treatment.

1.1.1 Epidemiology

People of African, Indian, and Arab ancestry are the groups most commonly affected by SCD. More than 300 thousand births each year take place in the Democratic Republic of the Congo and sub-Saharan Africa (SSA) bearing the burden. Frequency of HbS gene is very high in West African contries, where almost 30% people carry this gene. People from high SCD countries migrate into industrialized countries thus, the SCD increases in those countries too. Similar to France, the UK is projected to have around 14,000 people living with SCD, whereas populations from Africa are growing in Italy and Germany. It is currently believed that over 90% of persons born with SCD survive into middle-age in the United States,

France, and United Kingdom, whereas in SSA, 50–90% of people may die in very early years of their life. In low resource environments and countries where infant screening is not yet regarded as standard medical care, patients may perish away at a young age even before a diagnosis is definite. Without any diagnosis, care, education, proper medications like penicillin prophylaxis, and early treatment for infections, severe anemia , and multi-organ failure are among the leading causes of death (Inusa et al., 2019).

1.1.2 Pathophysiology of SCD

Hemoglobin, which is made up of four globin chains packed around individual hemoglobin molecules, is a significant protein of RBCs. Hemoglobin carries O_2 from the lungs to tissues, also carries CO_2 from the tissues to lungs. Approximately 97% of adult hemoglobin is HbA, which has 2α and 2β globin chains. Other haemoglobins are HbA2 and HbF. The HbF% quickly declines after 12 weeks of birth and HbA and HbA2 remain as the hemoglobin. The beta globin gene is found on chromosome 11p15.5 (Schnog et al., 2004). A single replacement of hydrophobic amino acid valine for glutamic acid on chromosome 11 results in SCD, a hereditary single gene illness that damages the β-globin gene's 6th codon.(Demirci et al., 2019). This β-globin gene is found on the arm of chromosome 11. When two mutant beta-globin subunits combine, hemoglobin S is produced. Red blood cells enlarge and become less flexible because there isn't a hydrophilic amino acid which is on sixth location of the hemoglobin sequence, which promotes non-covalent clumping of hemoglobin in very less oxygen environments (Ifeanyi & Ogechi, 2015).RBCs with HbS or HbS in combination with other faulty genes go through polymerization and harden when introduced to a oxygen less environment. Stiff RBCs can have an adverse effect on endothelial vessel wall integrity and blood flow because of their enhanced density and tendency for destruction of blood cells. Stiff RBCs cause hemolysis, vaso-occlusion. It also causes infarction and tissue ischaemia. Sickle RBCs have a half-life of just 10 to 20 days only. Healthy hemoglobin adapts to the carbon dioxide molecules it interacts with during deoxygenation, returning to its original shape upon release. HbS, on the other hand, has a tendency to polymerize into tactoids, these are gel-like substances. Whereas Hb are crystals, and hard, insoluble threads (Inusa et al., 2019). The main feature of the pathophysiology of this disease is the loss of RBSc flexibility. Normal RBCs are quite elastic, they can bend and flow via capillaries. In sickle cell disease, low levels of oxygen in blood encourages the sickling of RBCs, and frequent sickling weaken

and destroy the red cell. Thus, when the oxygen pressure returns to normal, these cells do not reform (Ifeanyi & Ogechi, 2015).

Figure 1demonstrates a single gene mutation that causes faulty hemoglobin that, when subjected to deoxygenation (on the right side), polymerizes (upper right) to produce sickle cells. Vaso-occlusion might take place (Inusa et al., 2019).

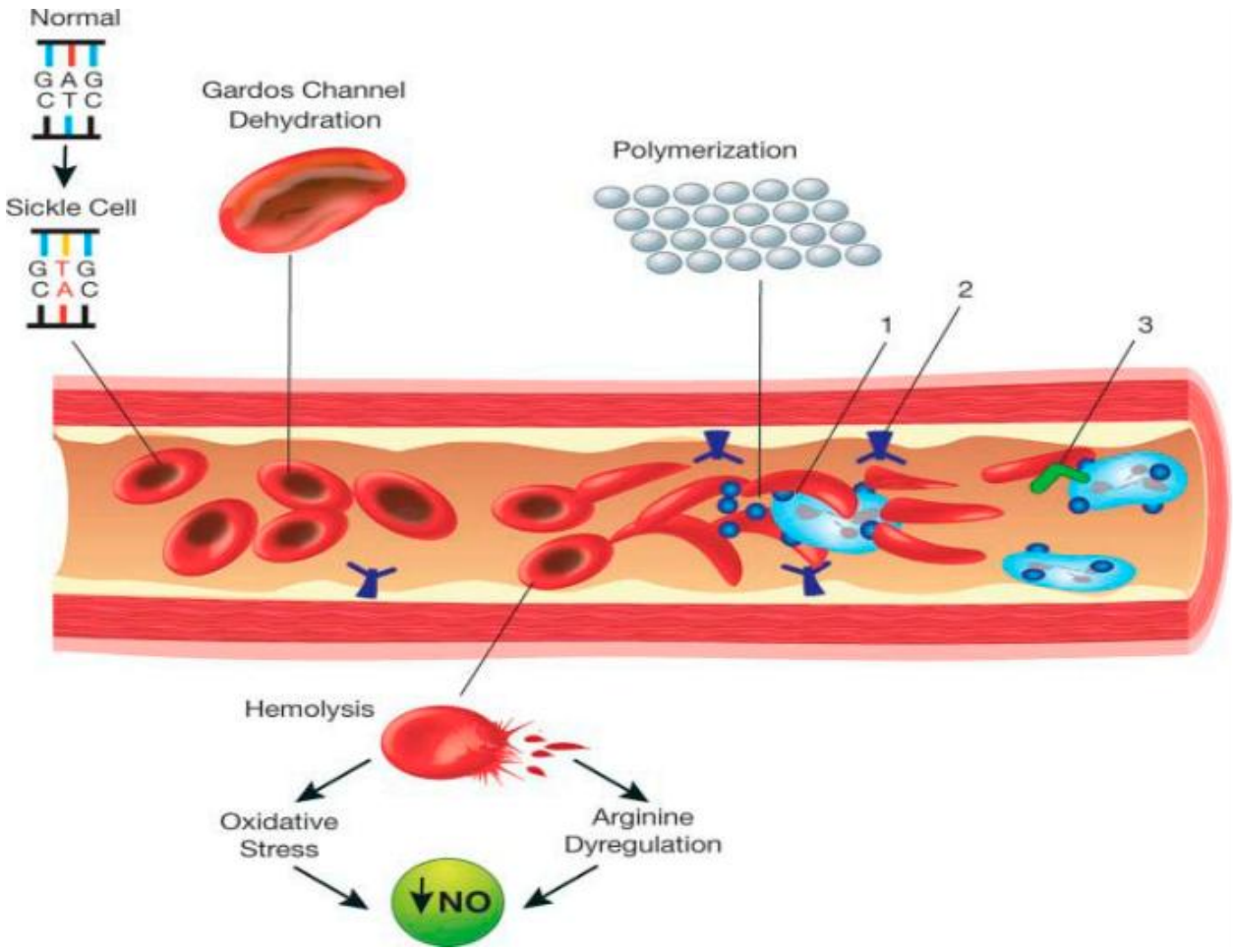

Figure 1: Sickling of RBCs

(Inusa et al., 2019)

Table 1: Comparison between healthy hemoglobin & sickle cell hemoglobin

(Schnog et al., 2004)

Healthy Hemoglobin	Sickle Cell Hemoglobin
Hemoglobin is the oxygen carrying pigment in the blood which is a main protein in red blood cell	HbS is the very common form of abnormal form of hemoglobin and basis of Sickle cell anemia
Contain glutamate on position 6 on the beta chain's surface.	Valine replaces glutamate on the same 6th position
RBC becomes biconcave in shape	HbS makes RBC crescent shape
Types of hemoglobin: Hemoglobin A, Hemoglobin A2, Hemoglobin F	Hemoglobin S
Allows RBCs to flow freely throughout the veins	Sickle RBCs to become sticky at the branching points of the veins

1.1.4 Classification of SCD

SCD is a hereditary homozygous HbS condition; the proportion changes depending on the individual's country of origin. The second most prevalent kind of SCD in Western Africa is HbSC, also known as the combination of HbS and HbC. This condition is particularly common in Burkina Faso and Mali, as well as the coastal countries. Based on the hereditary damage on the thalassaemia element, the clinical manifestations of a sickle-thalassaemia genotype (HbS/o or HbS/+) may be mild or equally serious to those of homozygous SCD (HbS/HbS) (Inusa et al., 2019).

1.1.5 Genetics of Sickle Cell Anemia

Black people in Africa and nations with a history of slave trading are those that suffer from SCD at the highest rates. The β gene has separately appeared as a novel mutation in different areas of Africa and Asia. These haplotypes, which may be distinguished by differences in β globin gene group, are called after the regions where they were initially identified (Senegal, Bantu, Benin, Cameroon, and Arab/Indian haplotypes). SCD, or homozygous SCD, is brought on by the inheritance of two β genes (HbSS). Other genotypes that result in SCD include double heterozygous situations, where the β gene is inherited alongside other defective genes, or mutations that cause reduced production of β globin genes (-thalassemias). A β gene mutation in HbC causes glutamic acid to replace lysine. The HbSC genotype, followed by HbS -thalassemia, is the most prevalent double heterozygous condition.Those who have the sickle cell trait (HbAS), there is only one β mutation, are typically asymptomatic (Schnog et al., 2004). The p-globin gene has a known mutation that changes a single nucleotide (A to T), causing valine to replace glutamate at position 6. As opposed to typical adult HbA, hemoglobin S having the mutation is known as HbS. The GAG to GTG codon mutation, which is the cause of the genetic condition, has only affected one nucleotide. Usually, the second, third, or fourth structure of hemoglobin is unaffected by this mutation. The polymerization of the HbS itself is done, what it does permit when there is little oxygen present.The deoxyhemoglobin variant of hemoglobin displays a hydrophobic area between the E and F helices of the protein (Ifeanyi & Ogechi., 2015).

1.2 Genome Editing

The rapid advancement of highly effective sequencing technology enables researchers to make great progress in genetic analysis in a very short amount of time. Finding the molecular mechanism by which certain genes affect phenotypes is currently a major challenge for researchers. Shutting down or over-expressing a gene in living creatures is a fine technique to clarify the role of a gene, which was previously difficult and time-consuming. The past few decades have seen a new method known as "genome editing" become popular in research on transgenic species, and gene therapy. An designed, highly specific nuclease serves as the foundation for genome editing. It may alter the genomes of biological organisms at precise sites consisting of a DNA-binding domain and a DNA cutting domain that is not sequence dependent. Ensuing the mechanism of cellular DNA repair results in the mutations at the

target loci, such as desirable entries, deletions, or replacements. Genome editing may one day be used to completely eradicate disease via the interruption of genes, and repair the mutations(Zhang et al., 2014).

1.3 CRISPR/Cas9

The term "CRISPR-Cas9" refers to the "Clustered Regularly Interspaced Short Palindromic Repeats-Cas CRISPR-associated protein 9". Gene editing technique CRISPR hold enormous promise to treat cancer, infectious illnesses, and genetic disorders. This technology provides a foundation for genome editing so that numerous diseases can be researched and studied (Barman et al., 2020). It was discovered that CRISPR/Cas is an acquired defense mechanism in opposition to pathogens in archaebacteria via DNA recognition by CRISPR RNA (crRNA) and enzymatic DNA cleavage by Cas nucleases (Zhang et al., 2014). Prokaryotes, which include bacteria and archaea, have a wide range of defense systems to fend off intruders from outside their own species, particularly viruses. There are two forms of immunity present in prokaryotic organisms. The second line of defense, adaptive immunity provides immunogenicity and retains immunogenic memory for protection in future encounters. Prokaryotes participate in the first line of defense in innate immunity by immediately identifying foreign intruders. As a result of the CRISPR-Cas structure's ability to identify foreign mobile genetic elements (MGEs) and subsequently eliminate them, archaeal and bacterial genomes exhibit adaptive immunity. During a second encounter, the CRISPR-Cas organization gives bacteria immunogenic memory to help them defend themselves against foreign invaders (Barman et al., 2020). CRISPR/Cas is present in almost 90% of the sorted archaea genomes and about 40% of the clustered bacterial genomes (Zhang et al., 2014).

1.3.1 Classification of CRISPR–Cas system

Six types (I-VI), 27 subtypes, and two types of classes make up the structure of CRISPR-Cas.It is known that the genomes of a significant portion of bacteria and archaea (including all hyperthermophiles) include Class 1 CRISPR-Cas systems, whereas bacteria contain Class 2 systems but do not contain hyperthermophiles(Barman et al., 2020).

Depending on the nuclease effector's characteristics, Systems of Types I, III, and IV belong to Class 1, which consists of systems with multi-subunit Cas protein effector complexes;

systems of Types II, V, and VI belong to Class 2, which consists of systems with single protein effector modules. Nuclease effector proteins are essential for the interference process. Viruses of types I, II, and V are eliminated, while RNA viruses are the focus of Type VI.. However, type III targets the CRISPR-Cas system with both DNA and RNA, whereas type IV's target has not yet been identified (Barman et al., 2020).

1.3.2 CRISPR-CAS locus

CRISPR loci were found to be almost palindromic and were first discovered in an intergenic region upstream of the iap gene (gene causing alkaline phosphatase isozyme conversion) of Escherichia coli. A set of repeats flank the "spacer" sequence in CRISPR loci, and this spacer sequence corresponds to the sequences in virus, plasmid, or other pathogen genomic elements. Prokaryotes' acquired immune system, the CRISPR-Cas system, provides resistance to invading genetic elements. The repeat sequences in the CRISPR array are separated by spacers. Spacers have distinctive regions extracted from foreign DNA, offering immunity to foreign DNA elements particular to a given sequence. Such repeat-spacer units are typically found close to a cluster of Cas genes (Figure 2)(Zhang et al., 2014).

New spacers can also be inserted throughout infection in order for the CRISPR locus to function as a memory after a future interaction with similar attackers. Average number of repeat-spacer units is 65, however this number can range from a few to several hundred. It has been discovered that Chloroflexus sp., a thermophilic bacterium, has the highest concentration of repeat-spacer units across any of its 4 CRISPR loci. Varying loci of same genome may include repetitive sequences of different lengths. According to recent research, spacer sequences are between 17 and 84 nucleotides (nt) long while repeat sequences range between 18 and 50 nt long. A 20 nt long gRNA is located next to an upstream 3 nt region, this is known as PAM, it is a component of an intrusive foreign factor but this is not the fragment of CRISPR locus (Gupta et al., 2019).

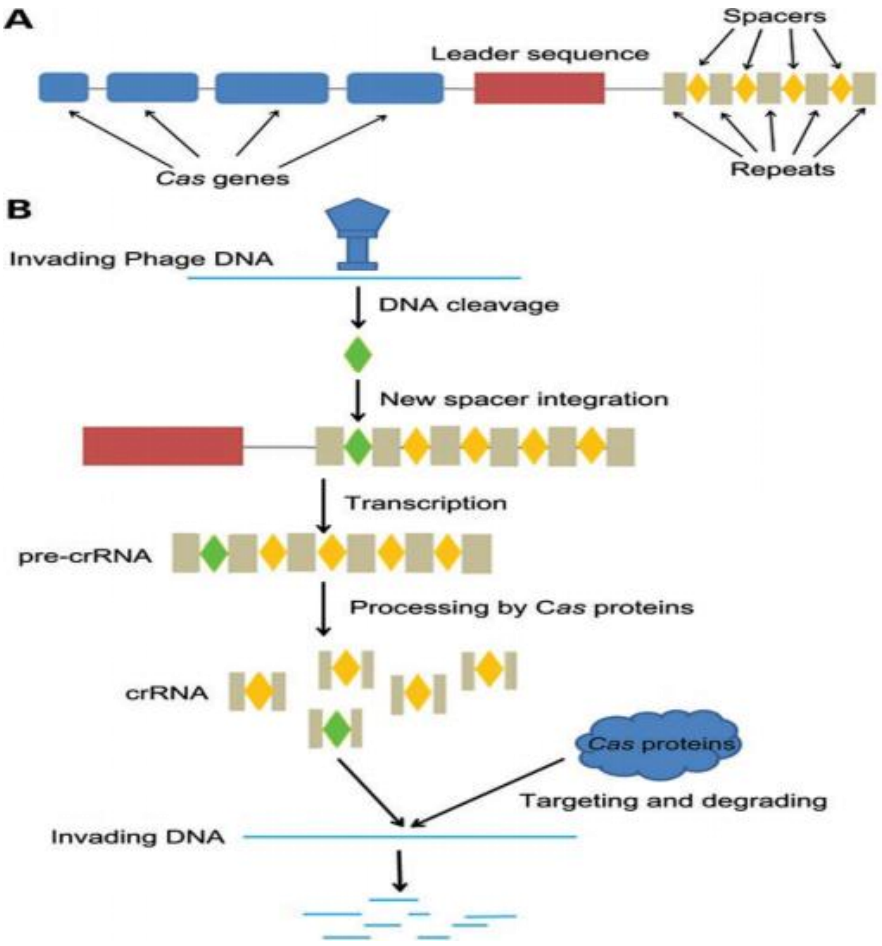

Figure 2: Overview of the bacterial immune system using CRISPR/Cas

(Zhang et al., 2014)

1.3.3 CRISPR/CAS9 Genome modification

A collection of conserved repeating sequences called spacers are inserted between the sequences in the CRISPR locus. In the CRISPR/Cas system, Cas nuclease breaks invading foreign DNA to manageably short DNA fragments, which later inserted as spacers into the host's CRISPR locus. The spacers served as transcriptional templates to produce crRNA in response to virus and bacteriophage infections; this RNA tells Cas to cleave the target DNA sequences of invading organisms and phages (Zhang et al., 2014). There have been several Cas protein families identified, and they all play crucial roles in the synthesis of crRNA, the integration of spacers, and the cleavage of invading DNA. The Cas9 protein, which possesses both a RuvC-like nuclease domain and the HNH nuclease domain, is all that is necessary for

the type II CRISPR/Cas system. After attaching to the target site, Cas9's HNH nuclease domain and RuvClike nuclease domain, respectively, cleave the DNA single-strand matching crRNA and alternate strand, causing double-strand breaks (DSBs) at the target site. CRISPR/Cas9-mediated genome editing causes DSBs, and cellular DNA repair follows(Zhang et al., 2014).

The trans-activating crRNA is a separate RNA molecule that the pre-crRNA first creates a base pair with at its 3′ end (tracrRNA). After dividing the RNA duplex into its two strands, This twofold pre-crRNA-tracrRNA complex is fragmented by the RNA-specific ribonuclease III (RNase III). A tiny, broken pair of crRNA and tracrRNA is utilized as a guide RNA (gRNA), which acts as a probe to recognize its appropriate foreign DNA sequence. DNA cleavage is initiated by the crRNA-tracrRNA duplex, which binds to and activates Cas9 endonuclease. An further tertiary Cas9-crRNA-tracrRNA complex is formed when the active Cas9 endonuclease interacts with the crRNA-tracrRNA recognition complex. When the 5′ end sequence of the gRNA (or the crRNA sequence) identifies and nucleotide bases to the protospacer sequence of the foreign DNA, the Cas9-crRNAtracrRNA complex readily detects and slices the foreign DNA strands. Cas9's two nuclease domains, RuvC and HNH, cause a DNA double strand break by introducing a nick into the target DNA's complementary and non-complementary strands, respectively. The protospacer adjacent motif (PAM) of the target DNA is commonly found to be three bases upstream of the region of cleavage. There are several different CRISPR/Cas9 variants that can detect target sites with 2-4 nt PAM sequences and 20–24 nt sequences matching planned gRNA. CRISPR/Cas9 may hypothetically be used to choose a particular DNA sequence of 22–29 (nt), which is uncommon in most genes. Recent research, however, demonstrated that CRISPR/Cas9 was sensitive to the amount, distribution, and position of mismatches and was very tolerant to base pair mismatch here between corresponding target sequences of the gRNA. Thus, this CRISPR/Cas9 system of Streptococcus pyogenes seems to be tolerant of a maximum of six base pair mutations at target loci (Zhang et al., 2014).

Figure 3 depicts the CRISPR/Cas9 pathway conceptually. sgRNA, a single guide RNA, and Cas9, a protein that contains the nucleases RuvC and HNH. Trans-activating crRNA (tracrRNA), combine to form sgRNA. The spacer region is another term for the crRNA region.The target DNA sequence protospacer, which is 20 bp long, and the spacer component of the crRNA are complementary. A brief segment called a PAM follows the target DNA

sequence right away. Cas9 nuclease enzyme produces a double strand break at the target spot when sgRNA binds to the complementary target DNA sequence (Jo et al., 2015).

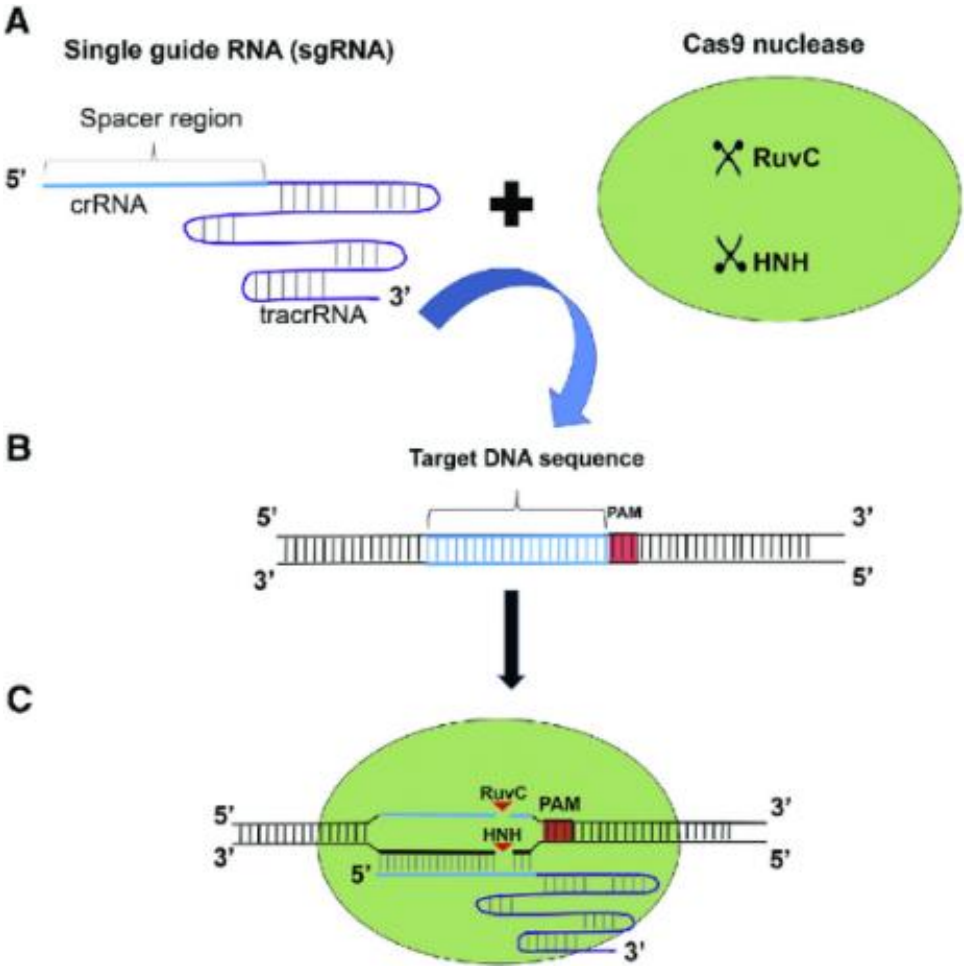

Figure 3: CRISPR/Cas9 mechanism

(Jo et al., 2015)

1.4 Aim of the Project

The aim of this project is to review how CRISPR Cas9 can aid in SCD genome editing leading towards an effective means of treatment.

1.5 Objective of the project

- Identify how CRISPR increases the HbF production and causes HPFH mutation in our genes.
- How point mutation occurs in both in-vivo and in-vitro and how off targeting affects our desired results.
- Investigate whether CRISPR is superior compared to other gene editing technology to treat SCD.

Chapter Two

Methodology

Relevant literature was selected, analysed and summarized for this review work.The information and data for this review were compiled from relevant articles. To gather the journals connected to this topic, an electronic search has been done.After scrutinizing data from the selected recent articles an outline was created to present the information as the requirement of the project objectives. According to the aim of the work, it was important to explore about SCD and its pathophysiology, CRISPR/Ca9 and its mechanism of action and the significant implication of CRISPR to treat SCD.

In order to gather as much essential information as possible regarding CRISPR/Cas9genome editing to treat sickle cell disease, a thorough search of several journals, review articles and research papers from official websites and research databases was carried out. Utilizing well-known and reliable sources including PubMed, Google Scholar, SCOPUS and ScienceDirect, the articles for this review study was collected.Relevant literatures were gathered using appropriate important keywords, such as CRISPR/Cas9, sickle cell disease, recent gene editing technology, TALENs and ZFNs. Selected articles were connected to SCD, CRISPR/Cas9 and recent gene editing technology. 73 articles have been assessed based on the title and keyword content. Then, 45 papers were reduced after reading the abstracts. 33 papers were selected after going through the entire text, which made up this review paper. Mendeley software was used for accurate and fair referencing in order to show respect for the writer’s original works.

Chapter Three

3.1 Association of CRISPR-Cas9 with SCD

Genome editing is advantageous because it produces insertions or deletions that are protective or the permanent correction or eradication of a dangerous mutation. Ideally, at a specific genomic location, programmed nucleases produce DSBs, which are then fixed by enlisting DNA repair mechanisms through non-homologous end-joining (NHEJ) or homology directed repair (HDR) (using homologous sequences found in sister chromatids, homologous chromosomes, or extrachromosomal donor DNA sequence provided for correction purposes) (Demirci et al., 2019).

In CRISPR/Cas9, an RNA (guide RNA) sequence identifies the effector Cas protein, which is then aimed to the targeted DNA region of importance. This technology changed genome editing procedures and increased the likelihood of applying genome editing techniques in a clinical context because it is simple to use, efficient, and reasonably priced. Once transported into target cells, CRISPR/Cas9 mediated DSBs activate DNA repair mechanisms (Jinek et al., 2012). If HDR is activated, this mechanism either results in INDELs, which results in the distruction of a gene's function, or it uses homology strands to repair the DNA break. CRISPR/Cas9 technology can be used to specifically fix the mutation of SCD by editing the chromosomal areas that regulate fetal hemoglobin expression, albeit there are still problems with this approach's effectiveness, safety, and delivery (Wiedenheft et al., 2012).

Currently, two methods are being studied for genetically altering autologous hematopoietic stem cells (HSCs): (1) production of healthy adult hemoglobin (HbA) or an HbA variant can be achieve by direct causative point mutation repair using CRISPR/Cas9 or by the inclusion of an anti-sickling variant. 2) HbF expression can be elevated by adding new genes, altering HbF regulatory elements, or silencing HbF repressors (Demirci et al., 2018). DSBs are the foundation of traditional CRISPR-Cas9 editing, which is then followed by cellular repairs of chromosomal breaks via either NHEJ or HDR. While damaged DNA ends in NHEJ are tied up with no consideration for homology, a donor template is provided in HDR, making it a precise repair mechanism that can introduce or repair necessary mutations. Induce HbF expression was focused at very beginning for clinical trials of gene editing in SCD than direct sickle mutation correction as expressed HbF does not require HDR and in HSCs it is tough to achieve HDR (Frangoul et al., 2021) .

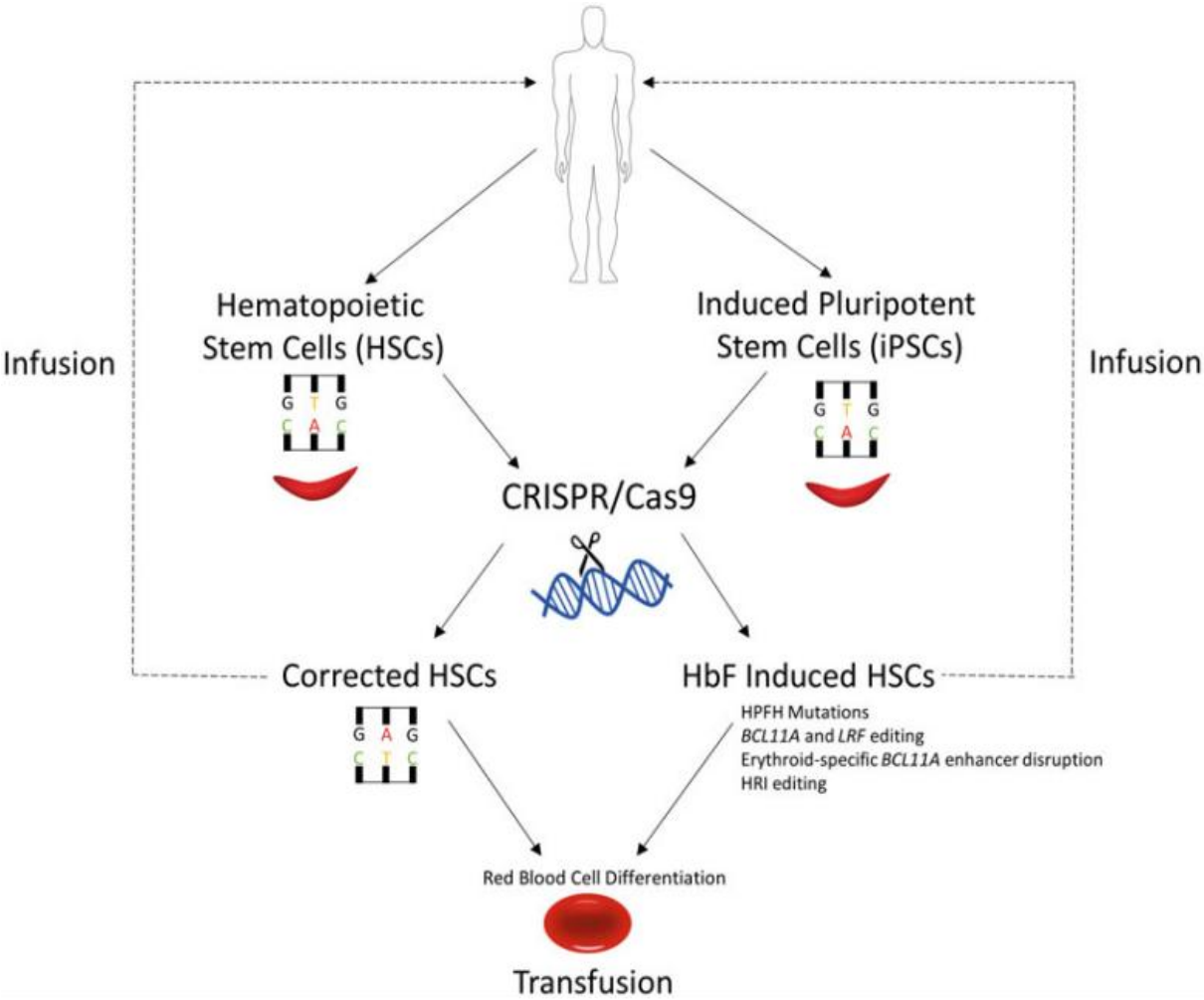

Figure 4: Applications of CRISPR/Cas9 for SCD.

(Demirci et al., 2019)

3.2 HbF Induction

Right after the first trimester of gestation HbF becomes the predominant globin type and it is restored by HbA after a few months of infants birth. Both HbA and HbF are conserved on chromosome 11.The transition from HbF to HbA is mainly regulated by the locus control region (LCR), a potent upstream enhancer that binds to each globin promoter to trigger their production. HbF is not completely inhibited after switching to HbA, however it is not distributed equally among RBCs. When there is no genetic basis for its persistence in all

RBCs, In some cells, called F-cells, HbF is very low, whereas in others it is highly concentrated(Demirci et al., 2018).

Babies do not exhibit SCD difficulties for a a time frame that has been predetermined because the infant's blood has high amounts of HbF, researchers currently focused to boosting HbF levels in a grown person. The results further reinforced the crucial role of eleveted HbF for SCD protection, showing that silent SCD patients with high HbF due to co inheriting hereditary persistence of fetal hemoglobin (HPFH) mutations (Forget, 1998). Major deletions or minor deletions/single nucleotide polymorphisms (SNPs) in the beta-globin promoter or HbF-regulating quantitative trait loci (QTL) are examples of these alterations in the -globin gene (Demirci et al., 2019) .

3.3 Preclinical studies for ex vivo HSPCs

Ex vivo gene editing of human HSPCs was used in the majority of the preclinical research, then these are transplanted into immunocompromised mouse models. Since the ability to permanently alter HSCs is a requirement for an autologous HSCT to be durable, this is done to evaluate the gene-edited HSCs' capability for long-term engraftment (Park & Bao, 2021).

3.3.1 Cell Culture

Numerous origins, including cord blood, bone marrow, can be used to isolate human CD34+ HSPCs. Most genome editing experiments used CD34+ HSPCs from peripheral blood. Since cytokine exposure in post isolation culture had been very effective to boost the editing effectiveness,Just before gene editing, isolated CD34+ cells were grown in pre-stimulation medium for a number of days. It has been demonstrated that less cell density culture settings and the use of a hematopoietic stem cell self-renewal agonist, for example UM171 and StemRegenin 1 (SR1), can promote the development of gene-edited HSPCs as indicated by greater engraftment levels in immunocompromised mouse. These edited CD34+ can grow on erythroid differentiation-supporting medium for globin measurement by HPLC (Charlesworth et al., 2018).

3.3.2 Reagent delivery

The CRISPR/Cas9 method from Spy Cas9 was used in the majority of research. The 20-nt guide sequences in SpyCas9's gRNAs typically include a 5′ -NGG-3′ PAM essential. The effectiveness of gene editing in HSPCs were greatly enhanced throughout time by the invention and improved the CRISPR/Cas9 editing reagent and its transport strategy. Initial efforts employed a plasmid DNA-based method for Cas9 and gRNA appearance, however this had limited editing effectiveness and severe toxicity. gRNA and Cas9 delivery as a pre-complexed ribonucleoprotein (RNP) is tolerated in CD34+ HSPCs through inducing a DNA damage response (DDR), despite the fact that every editing components triggered immunological, and apoptotic responses (Park & Bao, 2021).Straight distribution of RNPs to HSPCs through electroporation is frequently recommended because it permits the RNP to get into the cell nucleus fast and begin cutting the genome right away. For good editing effectiveness and selectivity with less cytotoxicity in CD34+ HSPCs, genome editing experiments used RNP. Chemical modifications to gRNAs improved genome editing effectiveness even further while lowering toxicity in CD34+ HSPCs. In HSPCs, high-fidelity SpyCas9 variants have less off-target activities when maintaining on-target activities comparable to those of wild-type SpyCas9 (Lomova et al., 2019).

3.3.3 Transplantation study

Two commonly employed xenotransplant hosts are NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) and NOD,B6.SCID Il2r / KitW41/W41 (NBSGW) for multilineage engraftment of human hematopoietic cells. For the NSG strain to exhibit a higher human chimerism, sublethal myeloablative irradiation is necessary. HSCs that have been engrafted are put through in vitro erythroid differentiation after engraftment to test globin expression and editing frequency in the erythroid lineages as NSG does not support human erythropoiesis. An NSG-derivative strain called NBSGW can sustain the engraftment of human HSCs without the need for preconditioning radiation. NBSGW encourages erythroid engraftment in addition to myeloid and lymphoid engraftment. Although the frequent usage of these models, it is challenging to predict how cells would act in a clinical environment from a xenotransplant model. In order to precisely mimic stem cells in the matter of the timings of hematological recovery, and cross sensitivity amongst cytokines, researchers developed a nonhuman primate (NHP) model. Ex vivo gene editing using a very clinically relevant large-

animal paradigm provides a chance to track long engraftment and hemoglobin profile, helping gene editing treatment to the clinic (Humbert et al., 2019).

3.4 HbF Induction by BCL11A gene editing

A promising strategy for treating SCD is to reactivate HbF by targeting particular genes. Numerous genetic loci that cause HPFH have been found through genome-wide association studies (GWAS), and several transcription factors have also been linked to HbF muting. Main regulator of HbF is BCL11A, which also directly inhibits the HBG promoter and associating with other DNA binding factors at different sites throughout the beta-globin locus to limit fetal hemoglobin expression. As a result, HbF reactivation triggered by the interruption of BCL11A binding motifs suggests a good target for therapeutic gene editing to treat SCD (Liu et al., 2018).

3.4.1 BCL11A gene disruption

BCL11A plays a variety of functions in distinct hematopoietic pathways, and gene disparity in BCL11A is extremely detrimental. By focusing on the centre of the BCL11A enhancer in HSPCs, several researchers have identified BCL11A erythroid booster as a candidate for inducing HbF and providing a basis for more specific to erythroid therapeutic genome editing. BCL11A expression and HbF suppression depend on a GATA1 motif that functions as the heart of an enhancer. While protecting BCL11A expression and function in non-erythroid situations, specific gRNAs that disrupt the enhancer significantly increase HbF induction in primary erythroid precursors (Canver et al., 2015). Roughly the same HbF reactivation were observed with BCL11A enhancer disruption compared to BCL11A coding deletion, but BCL11A was still able to promote HSC function, including maturation, reconstruction, and long-term engraftment prospects. By disrupting the GATA1 binding site at the +58 BCL11A erythroid promoter using CRISPR/Cas9 technology, highly effective corrective gene editing can be achieved in HSPCs. Thus,This led to therapeutic activation of fetal γ-globin in engrafting SCD HSCs and erythroid-specific decrease of BCL11A expression occurred.The highest concentrations of HbF induction were seen in erythroids with a high rate of indels when gRNA effectively cleaved the +58 erythroid enhancer of BCL11A. Biallelic alterations at the cleavage location led to a substantial

induction of γ-globin, as per clonal study of the erythroid heirs of CD34+ HSPCs altered at the BCL11A enhancer. To investigate the effects of BCL11A enhancer editing on lengthy engrafting HSCs, edited human SCD CD34+ HSPCs were implanted into immunocompromised NBSGW mouse. NBSGW facilitated gene editing of renewing HSCs with comparable levels of human myeloid, and erythroid engraftment of already corrected cells compared to cells that are not edited. It is clear from the persistence of indels at the BCL11A enhancer following secondary transplant that altering the BCL11A enhancer has no negative effects on stem cell function. The ability of BCL11A enhancer modified cells to sustain proposes that NHEJ-mediated gene disruption may be more effective than HDR- or Microhomology-mediated end joining-based gene editing techniques (MMEJ). This is because NHEJ primarily occurs in HSCs and is active throughout the cell cycle. This research proved that CRISPR/Cas9 editing of the BCL11A enhancer is an effective therapeutic method for producing a long-lasting therapeutic amount of HbF induction in engrafting HSCs (Wu et al., 2019).

3.5 HPFH Mutations

Direct binding of the key fetal hemoglobin gene repressors BCL11A and Leukemia/Lymphoma-Related Factor (LRF) to the HbG promoter occurs at locations 115 and 200 base pairs upstream of the transcription start site, respectively. Numerous HbF production was generated by CRISPR/Cas9 moderate distruction of LRF- or BCL11A-binding sites in the HBG promoters. 13 nucleotide (nt) HPFH deletion that naturally occurs in the HBG promoter as a potential selection of editing. Since the Cas9 cleavage site is bordered by an 8-nt tandem repeat, the 13-nt removal is similar to the normal mutation that prevails among other indels after CRISPR/Cas9 editing. Modified progenitors generate RBCs with elevated HbF levels which was adequate to alter the sickling in vitro thanks to repetitions that enhance MMEJ repair (Traxler et al., 2016).

3.5.1 HBG base edit

In order to boost HBG expression, a recent study showed that base editing, which caused a mono nucleotide changes at the BCL11A region on the HBG promoter, was enough. Since base editing does not result in additional DSB induced destruction to the genome, it may have greater clinical uses than DSB-inducing technologies like CRISPR/Cas9. To date, there have

been no studies conducted to evaluate the efficacy of this method for engrafting HSCs(Wang et al., 2020).

3.6 Correction of mutation using DNA donor template

This method corrects point mutation by HDR by delivering the CRISPR gRNA/Cas9 RNP complex targeting both HBB and DNA donor template into HSPCs that are separated from the patients, as illustrated in Figure 5. Numerous viral-based vector approaches, including as integrase-deficient lentiviral systems (IDLVs), adenovirus 5/35 serotype (Ad5/35), and adeno-associated viruses (AAVs), have been studied in HSPCs for donor template administration. Very less amount of vector incorporation into the host genome and the reduced risk of associated gene mutations and genotoxicity make AAV superior to other viral vectors in this regard. Various studies using RNP in conjunction with single-stranded oligodeoxynucleotides (ssODNs) exhibit effective targeted incorporation at the HBB locus in CD34+ HSPCs. Several researches have employed rAAV6 and ssODNs donors because of safety concerns and effective HDR mediated by these donors. The mean levels of HbA were higher in differentiated erythroblasts that are made from gene-edited cells, and the sickle cell phenotype was diminished (Dever et al., 2016).

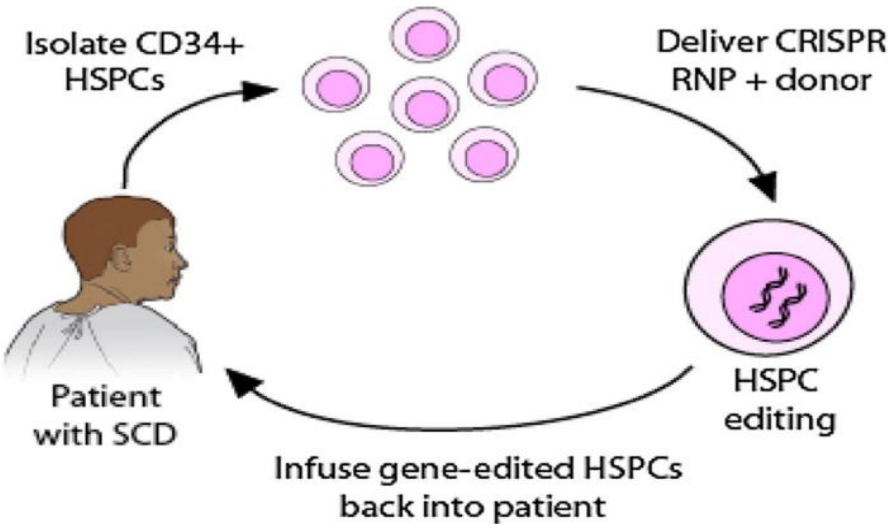

Figure 5: Sickle cell disease treatment based on genome editing

(Park & Bao, 2021)

3.7 In vivo gene editing

There are many drawbacks of ex-vivo editing, such as lethal myeloablation, expense and complexity of ex vivo cell manipulation, may be avoided by in vivo gene editing. Additionally, as SCD is endemic in impoverished nations like Sub-Saharan Africa, the ease of in vivo techniques may make it possible to apply this treatment to those people there. In vivo gene-editing therapy to be effective, the gene-editing machinery has to be delivered precisely to the target tissues and then expressed constitutively. It is important to address the difficulties associated with in vivo editing, such as how to achieve strong expression while optimizing delivery to target cells and limiting delivery to off target cells (Germino-Watnick et al., 2022).

There have been initiatives to create non-integrating adenovirus-based in vivo HSC transduction/selection technologies. In-vivo CRISPR/Cas9 HBG-promoter editing has been carried out in mice that are YAC/CD46 transgenic. The HDAd-HBGCRISPR/mgmt human CD46-targeting adenovirus vector expresses CRISPR/Cas9, which reactivate γ-globin by targeting the HBG promoter. CRISPR/Cas9 vehicles can be introduced either systemically or locally when in vivo gene editing occurs. CRISPR/Cas9 vehicles are delivered systemically when they are injected intravenously into the body, circulated throughout, extravasate from blood arteries, travel into the tissue spaces, and then reach target cells. Direct injection of editing cargo into the tissue spaces, known as local delivery, reduces dissemination into off-target tissue. In the target tissue, it causes a heterogeneous distribution (Germino-Watnick et al., 2022).

In vivo editing to treat the SCDs has numerous difficulties. High editing efficacy and high in vivo process efficiency are needed in SCD HSCs, and off-target cell editing is a feasible factor. Even though in vivo delivery using viral vectors can be extremely effective, it has the potential to cause Cas9/gRNA to express uncontrollably, producing genotoxicity and induce immune response. However, techniques for in-vivo administration that don't include viruses that often suffer from low delivery efficiency, extensive biodistribution, and a high number of injections needed to achieve the desired effect. Systemic versus local injection comparisons are also important in the search for the optimal delivery strategy. It is unclear how many in vivo gene-edited HSCs are necessary to cure SCD because the unmodified HSCs would continue to produce sickle cells (A. Li et al., 2020).

3.8 Miscellaneous Medication

There are only a few treatment opportunities for SCD. FDA has only approved four drugs (hydroxyurea, L-glutamine, crizanlizumab-tmca, and voxelotor) to treat this severe condition. Only a hematopoietic stem cell transplant (HSCT) from a related donor can cure sickle cell disease but itz is only accessible to about 15% of patients. When HSCT is performed on matched but distant donors or haploidentical donors, morbidity and death are dramatically increased. Additionally, there are significant risks and consequences associated with the treatment and without changes to current regimens, this therapy is not safe for general use (Park & Bao, 2021).

In case of gene therapy, there is no need for a matched donor. Other than CRISPR, there are two gene editing methods available that are TAL-effector nucleases (TALENs) and zinc finger nucleases (ZFNs) (Tasan et al., 2016).

3.8.1 Zinc Finger Nucleases (ZFNs)

The very first programmable nucleases employed in gene editing were ZFNs. ZFNs are artificial proteins which mainly have two functional domains. 1) A FOKI endonuclease domain 2) DNA binding domain (DBD) which is modeled after eukaryotic zinc finger transcription factors. Each of the 3 to 6 zinc finger proteins found in tandem repeats in DBD can detect 3 base pairs (bp). Zinc finger proteins have been developed to detect the majority of the three bp codons. Therefore, a required sequence of 9–18 bp can easily be detected by each ZFN monomer. The recognition location for each monomer can be altered by exchanging the zinc finger proteins in ZFNs. The DNA cleavage domain, endonuclease FokI, is linked to the DNA binding domain to introduce a DSB to a particular region. FokI works only after dimerization occurs. It explains why both ZFNs cooperate to induce DSBs. In order to enable FokI dimerization, the dimer identifies a total of 18–36 bp of codons and attaches to the DNA in a tail to tail configuration with a spacer between every monomer's binding sites. In the space between two ZFN binding sites, the DSB is induced. Research in which ZFNs were utilized to treat sickle mutation in patient-derived iPSCs provided evidence for ZFNs' therapeutic potential in SCD. It was previously highly challenging to fix the sickle mutation in human iPSCs, but the use of site-specific nucleases made it possible (Sebastiano et al., 2011).

3.8.2 Transcription Activator-Like Effector Nucleases (TALENs)

TALENs are also an artificial endonuclease. ZFNs and TALENs both attach to the target DNA sequence and act as heterodimers to cause DNA DSB at a particular site. A DBD linked to a FokI nuclease domain makes up a TALEN monomer.The plant pathogenic bacteria Xanthomonas produces transcription activator-like effectors (TALEs), which are injected into plant cells and function as transcriptional activators in the nucleus. TALEs are the source of the DBD. A tandem repeat of 33–35 amino acids makes up the DBD. TALENs are typically made with 14–31 repetitions. The amino acid residues 12 and 13, also known as repeat variable direct (RVD) peptide repeats, are particularly important; these are the only places where these essentially identical repetitions diverge. A single nucleotide is recognized by each repeat, and the RVDs ensure the base selectivity. TALENs also make use of the DNA cleavage domain of FokI nuclease, but a pair of TALEN is necessary for the creation of DSB. There are many TALEN production platforms, all of which rely on the repetitions being assembled in a specific order (Liang et al., 2014).

Following TALENs' discovery, their clinical application for SCD was also investigated. In a preliminary investigation, a set of TALENs with excellent efficiency and optimal architecture were created to cause a DSB at the HBB locus mutation. There was no need for a 5'T at the target locations, as is typically the case for TALEN binding, thanks to the enhanced TALEN architecture. This discovery expands the pool of potential locations that TALENs may target, providing more flexibility. Following this work, the endogenous HBB locus was targeted with an effectiveness of 60% in drug-resistant clones using patient-derived human iPSCs, demonstrating an increase in gene targeting efficiency in comparison to the ZFN investigations (Sun & Zhao, 2014).

Table 2: Comparison among three gene editing technology (CRISPR/Cas9, TALEN & ZFN)

(Tasan et al., 2016)

	CRISPR/Cas9	Transcription activator-like effector nucleases (TALENs)	Zinc finger nucleases (ZFNs)

Component	Cas9 endonuclease and gRNA	Based on TALE proteins,The FokI nuclease is associated with the DNA binding motif.	Several zinc finger proteins make up the The DNA-binding domain of the FokI nuclease is catalytically active.
Target recognition	DNA-RNA interference	DNA and protein interference	DNA and protein interference
Recognition site	Target sequence is 20 base pairs long, while the PAM sequence (for Streptococcus pyogenes Cas9) is 3 base pairs long.	Generally 30 to 40 bp for each TALEN pair.	Generally 18 to 36 bp for each ZFN pair.
Construction simplicity	It is significantly easier than the other two as it only needs the conventional cloning of an oligonucleotide that has been generated.	Despite requiring specialized cloning techniques and a bank of sequences coding for DNA binding domain subdomains, reduced complexity compared to ZFNs.	It might call for intensive protein engineering.
Modularity	Extremely modular	Extremely modular	Low modularity because nearby zinc finger proteins may interfere with one another's ability to bind

Chapter Four

4.1 Results

While lentiviral gene-addition experiments have been running since the early 2000s, gene-editing research has only just gained momentum. While the majority work to modify the BCL11A enhancer to boost HbF expression, some work to fix the erroneous β-globin gene. The first clinical trial, which is being supported by Vertex Pharmaceuticals, began in 2018 and is employing the medication CTX001. It is being utilized to treat Transfusion-dependent -thalassemia (TDT) and SCD (NCT03655678, NCT03745287) and is made out of CD34+ cells that have been engineered with CRISPR/Cas9 and mobilized with plerixafor . The BCL11A erythroid-specific HbF enhancer is the target of CTX001 (Germino-Watnick et al., 2022).

In preclinical study we can see, edited CD34+ HSPCs were extracted, edited, and developed toward the erythroid lineage, resulting in a mean increase in fetal hemoglobin of 29.0 ± 10.8%, compared to a decrease of 10.5 ± 5.2% in the reference cells that were left unaltered. In a clinical study we saw, hemoglobin levels in SCD patients (NCT03745287-33 year old female) increased without transfusion from 7.2 g per deciliter at baseline to 10 g per deciliter at 3 months and 12 g per dl on 15th month. HbF levels were 9.1% and HbS levels were 74.1% at the start. At three months, HbF level was 37.2% and HbS level was 32.6%. HbF and HbS levels rose to 43.2% and 52.3% during the 15th month. F-cell expression was 99.9% at month 5 and remained almost 100% until month 15 of her most recent research visit. Before clinical trials she had severe vaso occlusion episodes.whereas,For 16.6 months after receiving CTX001, she had no vaso-occlusive incidents(Frangoul et al., 2021).

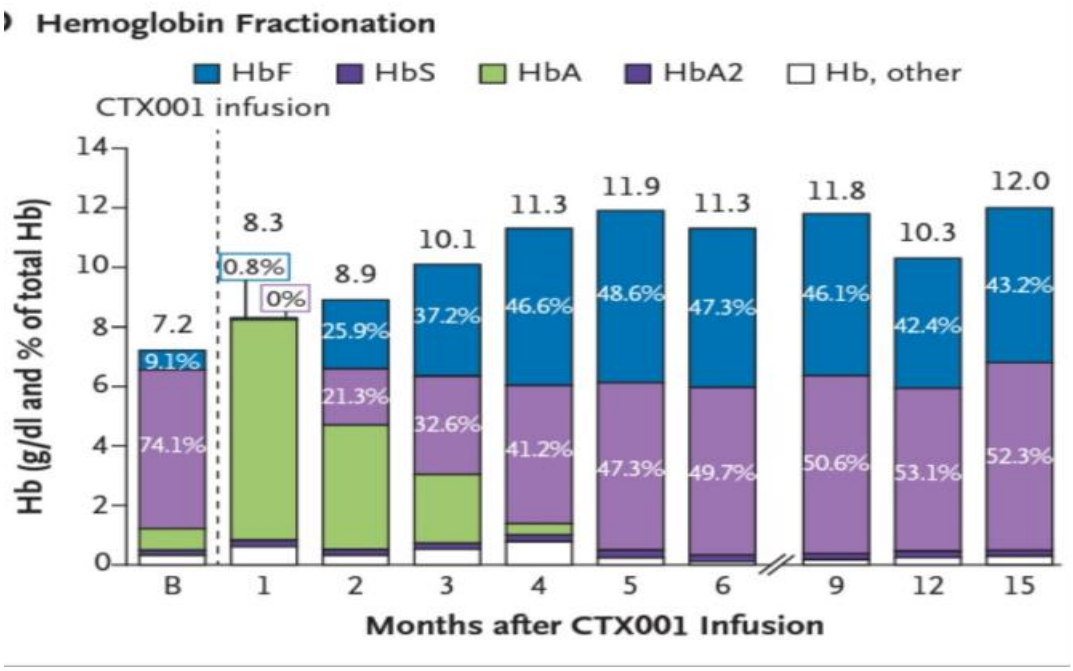

Figure 6: Comparison among HbF, HbS & HbA

(Frangoul et al., 2021)

To determine Several sets of TALENs and gRNAs (hence referred to as CRISPR 1-6) were developed to cleave at exon 1 of human -globin to test the efficiency of applying these tools for gene correction at the human beta-globin locus. Samples that had been exposed to TALEN, β-globin cleavage varied between 10% and 15%. From CRISPRs 1 to 6, β-globin cleavage varied from 17 to 39%, and all gRNAs displayed high rates of cleavage at β-globin (Hoban et al., 2016).

Chapter Five

5.1 Discussion

The most popular editing method for SCD is CRISPR since it has been demonstrated to have greater efficiency and it is simpler to use for target mutations compared to ZFNs and TALENS. Researchers have corrected mutant HBB both ex vivo and in vivo using CRISPR/Cas9. High rates of allelic disruption were consistently provided by the majority of the CRISPR gRNAs examined. On the other hand, TALENs allelic disruption is much lower than CRISPR/Cas9 gRNA (Hoban et al., 2016).

Although programmable nucleases are only supposed to identify a single site, they occasionally also detect other undesired or "off-target" sites. Targeting sequences with high similarity to other sections of the genome may present particular challenges due to this off-target recognition. Off-target mutations may be a significant issue in human applications because just a single unusual mutation may have unanticipated effects, and perhaps even remote dangers must be considered. For instance, ZFNs may be cytotoxic and may have a significant off-target rate despite being utilized in numerous research.According to one study, TALENs are a preferable choice since they have fewer carcinogenic and off-target effects. The fact that TALENs often have longer recognition sites could be one of the causes. Similar to ZFNs, TALEN specificity depends on the ideal distance between monomers. TALENs can still cause off-target alterations, although their careful design can reduce off-targets (Cornu et al., 2008).

Due to their shorter recognition site and lack of obligatory heterodimers, CRISPRs are also quite challenging technology. As the CRISPR gRNA identification site is only 20 bp long and only the seed sequence 8–12 bp near the PAM is essential for precision, finding particular targets recognized by the system can be challenging. Cas9 from S. pyogenes is thought to identify a PAM sequence of 5'-NGG-3', but it also can identify 5'-NAG-3', which may be another cause of unintended mutations. It has been reported in a systematic analysis that the Off-target effects are more common with CRISPR/Cas9 than with TALENs. However, it was demonstrated that gRNA optimization might boost specificity by at least 5,000 times. Another method of boosting specificity involved using fusions of Cas9 and FokI raise the bar for DSB by requiring the binding of two CRISPRs instead of one. The specificity of two recently developed Cas9 variants, eSpCas9 and SpCas9-HF1, was also much improved (Fu et al., 2014).

To conclude, CRISPR/Cas9 has higher off-target recognition than TALENs. But it has a higher cleavage rate than ZFNs and TALENS. Thus, CRISPR is a superior method than other two gene editing tools.

5.1.1 Limitation and challenges of CRISPR

As genome editing tools like CRISPR/Cas9 become more accessible, autologous transplantation of modified hematopoietic stem cells may one day heal the vast majority of SCD patients. There are, however, other challenges that must be surmounted prior the genome editing based SCD therapy approach can be implemented in the clinic. These include the need for high editing effectivity and reduced off-target repercussions(Park & Bao, 2021).

In gene therapy, the off-target function of Cas9 nuclease raises considerable concerns since it might impair normal gene function and lead to genomic instability through significant chromosomal rearrangements, which could have unpredictable adverse consequences. Furthermore, off-target cleavages have the ability to build up over time because of the long-term production of Cas9 nuclease in treated cells through plasmid and viral vectors. Off-target effects are still there despite the fact that it has been shown that off-target editing can be greatly reduced by employing high-fidelity Cas9 and delivering gRNA and Cas9 as RNP. Determine the system's off-target or chronic toxicity effects, especially for therapeutic trials of CRISPR-Cas9, a reliable, quick, high-throughput technique is required (Fu et al., 2013).

As it is, ex vivo gene editing technologies have their limitations. The proportion of HSCs among the CD34+ cells recovered from SCD patients is low. Extracting HSCs from the bone marrow is a rather invasive process. Additional chemo-related side effects for patients undergoing myeloablative chemotherapy include infections as well as low blood levels. The pluripotency and engraftment potential of HSCs are lost during in vitro culture and gene editing. In addition, providing an ex vivo gene-editing based therapy to patients may be impossible due to the prohibitive cost, which is driven by the need for highly laboratories and the technical expertise necessary. As in vivo therapeutic administration has the potential to be less invasive and cost-effective, it could overcome the limitations of ex vivo gene-editing and increase its availability in resource-poor regions. Establishing high in vivo transport efficiency and high editing efficacy are just two of the many challenges facing the development of in vivo gene editing as a feasible clinical technique. Even though in vivo gene-editing method are still in the early stages of development, the collaboration between

the NIH and the Bill and Melinda Gates Foundation to encourage the research of a therapeutic in vivo gene therapy method for SCD would significantly speed up technological advancement and innovation (Park & Bao, 2021).

Chapter Six

6.1 Conclusion

Although SCD was first identified over a century back, there is still no definite therapy for all patients due to a shortage of appropriate donors for curative HSCT. SCD is a monogenic condition that makes CRISPR/Cas9 one of the best choices for treatment because it is affordable, simple to use, and extremely successful. Proof-of-concept tests have shown that CRISPR/Cas9 may be efficiently used to correct the SCD mutation or promote HbF synthesis in ex vivo cell culture conditions and mice models. However, there are still safety concerns due to the indeterminate off-target effect and sub therapeutic efficacy. Ex vivo optimization studies and research into the method's safety in larger animal models should be conducted. The future of CRISPR/Cas9 for SCD will likely be determined by the results of the ongoing clinical trials investigating its potential. The program has potential, but it is not yet feasible for widespread adoption, especially in developing countries like Africa where SCD is common. To increase usage and guarantee a safer, efficient, cost-effective manufacturing procedures for Cas9 proteins and clinical grade guide RNAs should be created.However, several problems must be solved before gene therapy may be deemed a safe, effective treatment option and long-term therapeutic approach, the idea of curing SCD is getting closer to being a reality (Demirci et al., 2019).

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