Utilizing CRISPR/Cas9 to treat Sickle Cell Anemia (SCA)

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Abstract

Sickle Cell Anemia (SCA) is a hemopathology hereditary disorder with no known cure. It is characterized by vaso-occlusive events, hemolytic anemia and in extreme cases, or improper or late treatment, even premature death. CRISPR/Cas9 is a genome editor which is widely utilized in biomedical research as a tool to treat hereditary disorders at the source, the genome. This literature review discusses the various ways in which the CRISPR/Cas9 could be useful in the search of a potential cure for SCA and other Sickle Cell Diseases (SCDs).

Keywords: CRISPR/Cas9, Sickle Cell Anemia, Sickle Cell Disease, Genome engineering, Genome editing.

INTRODUCTION

Sickle Cell Disease (SCD) is a genetic disorder distinguished by sporadic vaso-occlusive events, chronic hemolytic anemia, end-organ injury, and premature death (Platt et al., 1991; Platt et al., 1994). It is a monogenic disorder that afflicts millions of people worldwide (Pauling et al., 1949). The A69T point mutation in the hemoglobin beta chain (HBB) gene that encodes the beta globin subunit of hemoglobin-A (HbA) in red blood cells (RBCs), is the primary cause of SCD. This mutation in HBB leads to the formation of abnormal hemoglobin-S (HbS). In SCD patients, RBCs express HbS and do not express HbA due to the inheritance of two HbS alleles and no HbA alleles. When HbS proteins are in high concentration within the RBC, they aggregate, or stick together, which creates undue stress on the cell. The aggregation of HbS causes the contortion of RBCs making them sickle shaped and inflexible. These sickle shaped blood cells can get stuck in small vessel walls and clog them, which in turn leads decreased blood flow and oxygen levels (Serjeant, 2010; Rees et al., 2010).

There are many types of SCDs, the most common ones being Sickle Cell Anemia (SCA/HbSS), Sickle Cell Trait (HbAS), and Hb Beta-Thalassemia. SCA is the most severe SCD, due to the inheritance of two defective HBB alleles, whereas other SCDs are heterozygous, inheriting one normal HBB allele and one sickle cell HBB allele. This homozygous defective inheritance results in symptoms such as anemia, which is where RBCs typically die within 10-20 days when normal RBCs typically need to be replaced every 120 days (Eadie et al., 1955). This constant need to replace the defective RBCs results in a shortage of RBCs which contributes to the other symptoms of SCA such as severe pain in the chest, abdomen and joints, delayed growth, and frequent infections. Treatment for SCDs are minimal, with the only curative therapy for SCD being hematopoietic stem cell transplantation, typically from a related donor (Walters et al., 2001; Mentzer et al., 1994).

Genome engineering is a powerful tool for research and applications in translational and clinical medicine. The evolution of the clustered regularly interspaced short palindromic repeats (CRISPR) series has revolutionized gene editing (Jansen et al., 2002; Mojica et al., 2005). The CRISPR associated nuclease-9 (Cas9) facilitates efficient genome editing through the creation of targeted double-strand breaks through endonuclease activity, and has proven to be efficient in almost any organism and cell type (Torres-Ruiz R et al., 2017; Chen et al., 2022).

Genome editing is advantageous for putative clinical treatments as mutations that are unfavorable may be removed or corrected, or even protective insertions and deletions can be integrated to ameliorate gene defects (Demirci et al., 2019).

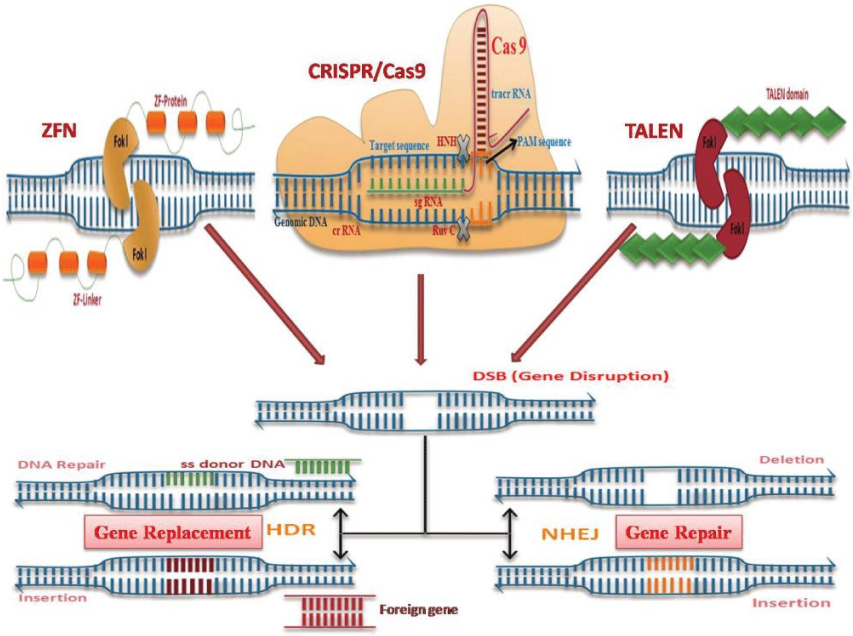

Figure 1. Genome editing by ZFN, CRISPR-Cas9 and TALEN. Repair of ZFN-fok1, TALEN domain and Cas9·sgRNA-induced DSBs possible by either NHEJ or by HDR pathways (Pandey et al., 2017).

CRISPR/Cas9 functions by creating double strand breaks (DSB) at a specific genomic locus that is selected by the guide RNA (gRNA) loaded into the programmable nuclease (Cas9), which is then followed by the activation of DNA repair mechanisms utilizing either non-homologous end-joining (NHEJ) or homology directed repair (HDR) to repair the DSB site (Fig. 1) (Demirci et al., 2019; Pandey et al., 2017). NHEJ is the most prevalent repair mechanism employed, which is unfavorable in the context of gene editing as the repair of the DSB through NHEJ results in insertions and deletions terminal to the edit site, as NHEJ is the error-prone repair mechanism due to its priority to repair DNA quickly over accuracy (Holt et al., 2010). However, to overcome this preferred mechanism of DSB repair in CRISPR/Cas9 editing, a donor template with the correct edit can be provided, in conjunction to the CRISPR/Cas9 machinery, to switch the preferred repair mechanism to HDR, which is known to be relatively error free, which is preferred in the context of gene editing (Salsman and Dellaire, 2017).

There are many advantages to utilizing CRISPR/Cas9 to edit the genome to treat diseases. It has a high efficiency, is cost effective, and is programmable to the base pair to result in the desired corrected sequence. These pros to CRISPR/Cas9 editing have improved the prospects for the use of genome editing in a clinical setting. By this method, the SCD mutation correction can be targeted; yet setbacks are present surrounding the actual safety and delivery of the genome editor (Demirci et al., 2019; Doudna et al., 2012; Wiedenheft et al., 2012; Jinek et al., 2012).

However, this uncertainty has pushed forward alternative genome editing techniques to treat SCD such as the use of transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) in conjunction to CRISPR/Cas9. Preliminary studies have reported that by using this method SCD could be treated by editing the beta globin gene. Here the mutations are cleaved in a site-specific way, recruiting homologous donor templates to replace or edit altered DNA with the correctly sequenced DNA donor. 18% of gene modification was sufficient to edit the sickle mutation and permit production of HbA, comprising 7.3% of the total hemoglobin, with the high rates being around 12.6% (Ribeil et al., 2017).

While the use of CRISPR/Cas9 to treat SCD is in its infancy, there are many promising *in vivo* and *ex vivo* studies that indicate this method is a feasible treatment for SCD. Therefore, the scope of this literature review is to cover the usage of the CRISPR/Cas9 as a treatment for SCDs, specifically SCA, discussing the current approaches, trials, and success.

Utilizing CRISPR/Cas9 *in vivo* and *ex vivo* to treat SCA

Mutations in the HBB gene which cause SCA and beta-thalassemia, results in an unsuitable level of normal beta-globin chains of hemoglobin (Weatherall, 2011). The correction of the globin locus by nucleases, which are aimed at curing hemoglobinopathies permanently, shows a new approach. Defective genes that result in diseases, such as SCA, can be corrected by two approaches in gene therapy: *in vivo* and *ex vivo* therapies. In *in vivo* therapy, the genome editing reagents like donor templates and programmable nucleases are directly transfused into the human body. In *ex vivo* therapy, the tissues of a cell type that are being targeted are removed from the human body and edited using a programmable nuclease in cell culture, then the edited cells are reintroduced back into the host's body, hence decreasing the complications of rejection by the host's immune system. (Fig 2) Both methods have their advantages and disadvantages, and they are used according to the disease that is to be treated (Kim et al., 2017; Voit et al., 2014).

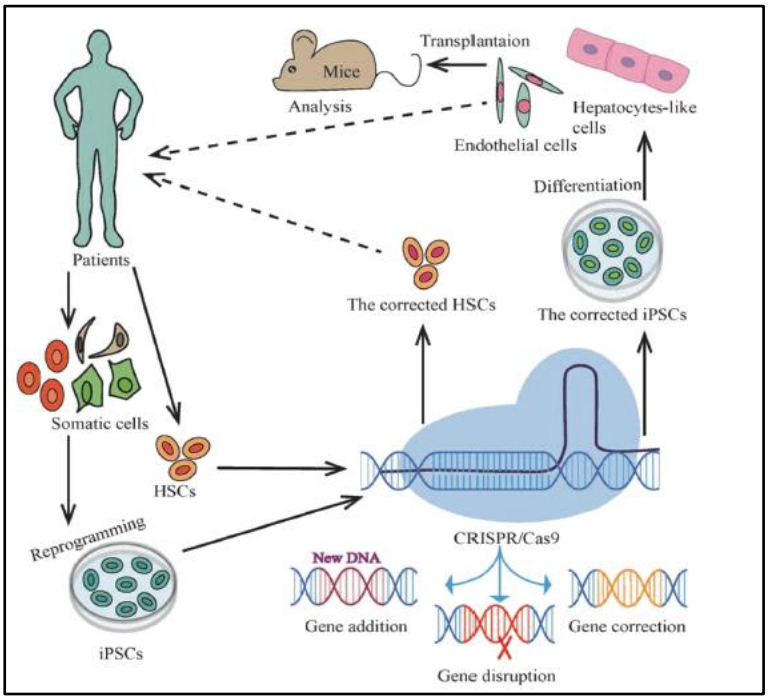

Figure 2. Workflow of *ex vivo* gene editing in hereditary hematological disorders using CRISPR/ Cas9-mediated therapeutic. Somatic and HSC cells are extracted from the patient, somatic cells are dedifferentiated into pluripotent cells. The HSCs and iPSCs are then transfected with the CRISPR/Cas9 machinery and cultured and subsequently validated to ensure the correct gene edit was made. These correctly edited cells are then transplanted back into mice for validation and humans for treatment. (Chen et al., 2022).

There are also alternatives to these *in vivo* and *ex vivo* therapies that have arose due to the quickly evolving CRISPR-based genome engineering technology. These alternatives include editing the genome in unicellular embryos by direct injection of CRISPR/Cas9 machinery, which has demonstrated to be successful in murine and primate models (Li et al., 2013; Chen et al., 2015). Another alternative being the direct delivery of CRISPR/Cas9 machinery to specific cell types/tissues, which bypasses the need for germline-modified mutant strains, which is especially of interest for diseases that only are prevalent in certain cell types, such as SCA. (Torres-Ruiz et al., 2017). However, these alternatives are in their infancy and there is not enough research on the topic to make firm conclusions on their efficacy in comparison to more canonical CRISPR/Cas9 editing methods. Therefore, for this review, the scope will focus on the most robustly supported methods regarding CRISPR/Cas9 editing and SCA, the canonical *in vivo* and *ex vivo* therapies.

In vivo therapies

Base editors, such as CRISPR/Cas9, when used to introduce targeted genomic modifications in animal and cellular models, have been generally successful (Molla et al., 2019). Given the ability of base editing to accurately induce base transition mutations at single-nucleotide resolution, it has been recruited to create new animal and cellular models of genetic syndromes and cancer. The outlook of using base editing to revert mutations which are causing diseases is even more encouraging (Doudna, 2020). To give an example, to rescue animal models of sickle cell disease, base editing has already been utilized. The hematological parameters were nearly normal and splenic pathology was reduced in mice that received base-edited haematopoietic stem and progenitor cells (HSPCs) than the ones that received unedited cells. The p53 activation and larger deletions detected subsequent to Cas9 nuclease treatment were avoided by providing a donor template to human HSPCs for repair (Newby et al., 2021).

While there has been significant head-way to utilizing CRISPR/Cas9 as a treatment for SCA, *in vivo* gene engineering still has a lot of challenges. Both high editing efficiency and high delivery *in vivo* in SCA hematopoietic stem cells (HSCs) are needed, and off-target editing could be a potential concern. Furthermore, effective delivery of the CRISPR/Cas9 machinery is a huge obstacle. Viral vector based *in vivo* delivery of the gene editing machinery can be very efficient, but it could result in uncontrollable expression of Cas9, which may activate an immune response and cause genotoxicity. In contrast, non-viral vectors may have larger biodistribution with low efficacy and may require frequent injections for a high delivery efficiency. We must also consider the comparison to systemic delivery and local injection to figure out the best plan for delivery. (Li A et al., 2020; Charlesworth et al., 2019; Wagner et al., 2019; Tong S et al., 2019).

Ex vivo therapies

There have been several *ex* vivo studies regarding genetically treating SCA through methods other than CRISPR/Cas9. For example, TALENs have been programmed to target the beta-globin locus by presence of a full size complementary DNA (cDNA) knock in. This is followed by HDR-mediated repair and ZFN function to rectify the SCA related point mutation in CD34+ hematopoietic stem progenitor cells. For hemoglobinopathies, there is an added benefit to *ex vivo* treatment as extracted patient induced Pluripotent Stem Cells (iPSCs) can be differentiated into hematopoietic stem cells which can then be inserted into the patients again by autologous transplantation, decreasing the risk of transplantation rejection (Hanna et al., 2007). This has already been done with all ZFNs, TALENs, CRISPRs in SCA and beta-thalassemia (Voit et al., 2014). The β-globin locus was cleaved by a pair of ZFNs, which along with a homologous donor template was able to induce HDR at high levels in progenitor cells (Hoban et al., 2015).

In a study by Huang et al. (2015), one allele of the SCD HBB gene in human iPSCs was corrected by utilizing an gRNA homologous to the HBB locus, Cas9 for endonuclease activity, and providing a donor DNA template of the correct HBB sequence to repair the DSBs induced by Cas9 in a HDR manner. The outcome of this study resulted in normal HBB proteins being expressed in the RBCs after hematopoietic differentiation of gene-corrected iPSCs. In case of disease modeling and upcoming gene therapies, these findings are an important development in genome editing of patient-specific iPSCs and possibly other stem cell types for generating gene-edited and functionally repaired or enhanced cells (Huang et al., 2015).

While *in vivo* and *ex vivo* methods can vary widely, delivery of the genetic editing components is still a challenge for both. While viral and non-viral delivery methods can be used for both *in vivo* and *ex vivo* approaches, non-viral delivery is preferred as in this method, the donor nucleotides and proteins are present transiently in cells, and therefore are projected to lessen the frequency of cell toxicity and also off-target or unwanted effects comparative to viral systems. (Behr et al., 2021) However, current literature on efficacies of delivery methods of CRISPR/Cas9 machinery is minimal at best and requires further exploration to determine which delivery method would be most suitable for human treatment.

Discussion

While the field of genetic editing to treat genetic conditions is rapidly evolving, there are still many hurdles scientists must encounter to push these studies forward into clinical trials. One major challenge of gene editing is delivery to target cells with respect to specificity and effectiveness. Viral and non-viral delivery methods are both being evaluated at the moment for introducing Cas9 into target cells either *in vivo* or *ex vivo*. Both immune reactions and off-target deliveries are possible depending on the delivery mode and time taken for the nuclease expression (Li et al., 2020). Another obstacle is the low ratio of HDR to NHEJ in long-term reconstituting HSCs, currently hindering the clinical translation of the corrected SCD mutation using the corrective donor template due to its unsatisfactory efficacy. It is important that technologies such as CRISPR/Cas9 do not process forward into clinical trials until these kinks have been "ironed out" as faulty editing could possibly trigger and induce beta-thalassemia major, minor or intermediated due to the off-target cutting of HBB by Cas9 not being thoroughly evaluated (Park and Bao, 2021). Therefore, before the clinical applications of CRISPR/Cas9 in human patients can proceed, safety and effectiveness of the system must be thoroughly validated and accounted for. The efficiency and specificity of genome-editing tools can be ameliorated by targeting modifying nucleases, DSB repair pathways, and changing the mode of delivery.

One possible way of achieving this aim of efficiency and specificity is to enhance the targeting specificity of the Cas9. What is important for high specificity is the cautious design of the single guide RNA (sgRNA), as well as tight control of the quantity and duration of sgRNA and Cas9 (Hsu et al., 2013). Furthermore, the target DNA site with 140-fold greater specificity than the wild-type protein in the cells of humans can be recognized by a fusion protein of catalytically inactive Cas9 and FokI nuclease (Guilinger et al., 2014).

Nevertheless, the high rate of off-target effects generated by CRISPR/Cas9 has been its most prominent concern since its discovery. However, new evidence suggests that off-target effects might have been specific to different cell types and mostly dependent on the proper functionality of the cell DSB repair machinery (Torres-Ruiz et al., 2017). Regardless, it is clear that there is a need for further robust regulation of CRISPR/Cas9 usages in humans, and all research must be done as conscientiously as possible to ensure steady progress regarding the treatment of SCA and other genetic disorders with genetic editing technologies.

Methods

For this systematic literature review, credible literature obtained from databases such as Google Scholar, PubMed, NCBI, BioRXIV, and more were curated to compile the work that has been done by various research groups regarding CRISPR/Cas9 utilization for SCA treatment. The keywords 'sickle cell anemia', 'CRISPR/Cas9', 'CRISPR/Cas9 *in vivo* applications',

‘CRISPR/Cas9 *in vitro* applications’ and ‘CRISPR/Cas9 and sickle cell anemia’ were utilized to curate literature.

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