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CRISPR, ZFNS, and TALENS for Hemoglobinopathies an Analysis

Venya Khare

Abstract: Sickle Cell Disease is one of the most common genetic disorders in the United States and is incredibly prevalent throughout Africa and the Middle East. By 2050, the annual number of newborns with Sickle Cell Disease is projected to increase by 33%. A similar story can be told about Beta-Thalassemia: another hemoglobinopathy that has no standard treatment. The future of treating hemoglobinopathies looks bleak and more research must be done to prevent fatalities and the lifelong problems associated with it now. Sickle Cell Disease and Beta Thalassemia have one defining similarity: they are both monogenic disorders. This unique characteristic of having a single gene variation allows them to be the ideal candidate for one of the newest breakthroughs in biotechnology: target genome editing. As of now, there are three major competitors in the field, Zinc Finger Nuclease (ZFN), Transcription Activator-like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) target genome editing. Here I summarize the possibilities target genome editing provides in terms of research and a potential treatment for both Sickle Cell disease and Beta Thalassemia with a focus on comparing the three target genome editing technologies.

I. INTRODUCTION

Hemoglobinopathies are genetic disorders caused by changes in structure or differences in hemoglobin expression (1,2). Normally, hemoglobin cells are red blood cells that carry oxygen throughout one's body. However, this mutation causes proteins to form in the blood cells that force a rigid sickled shape. Not as flexible as normal hemoglobin cells are, they now form blockages in the arteries. Sickle Cell Disease is due to hemoglobin, beta point-mutation of Adenine to Thymine at codon six. This results in a replacement of the amino acid glutamic acid with valine. Commonly associated with sickle cell disease is anemia, which means a low number of red blood cells. This often results in stunted growth, slow developments, or intense fatigue. The now sickle-shaped blood cells get stuck in small blood vessels. Thus, depriving tissues and organs of oxygen-rich blood and organ damage whose severity depends on a case-by-case basis (1). Beta Thalassemia is another blood disorder that reduces the production of hemoglobin and thus leads to reduced oxygen in several parts of the body sometimes resulting in anemia. It is caused by point mutations, change of single nucleotide in DNA, or deletions in the beta-globin gene on chromosome 11, leading to reduced (beta+) or absent (beta0) synthesis of the beta chains of hemoglobin (Hb). Patients with beta-thalassemia consequently are more prone to developing abnormal blood clots. There are several variations of beta-thalassemia: Beta Thalassemia major or Cooley's anemia, Beta-thalassemia intermedia, and Beta thalassemia minor. Minor beta-thalassemia consists of one gene damage. Beta thalassemia major consists of two damaged genes and those with Beta-thalassemia major have a severe expression of the disorder, usually resulting in regular blood transfusions and continuous medical care. Intermedia thalassemia, however, has a scale of different levels of severity and symptoms depending on the patients (1,3). Quite often patients with beta-thalassemia minor have mild symptoms or are asymptomatic and quite often do not know that they have the condition. Sickle Cell Disease and Beta Thalassemia remain the two most common forms of hemoglobinopathies in the world (1). Sickle cell disease is one of the most common inherited blood disorders in the world. It is genetically inherited in more than two million people in the United States alone. Beta thalassemia is similarly common around the world (6). Like sickle cell disease, it is much more common in the Indian subcontinent, Middle East, Africa, and the Mediterranean. By 2050, the annual number of newborns with Sickle Cell Disease is projected to increase by 33%, the future of hemoglobinopathy looks bleak and more research must be done to prevent fatalities and the lifelong problems associated with it now (1). Currently, the known remedies have high correlations with negative side effects and with numerous constraints. For instance, graft failures associated with transplants cause severe fatalities while blood transfusions are bound by risks of bloodborne pathogens, immune responses to foreign antigens, hemolysis as well as several others. The severity of these results can go from barely noticeable to fatal. As a result, allogeneic hematopoietic stem cell transplantation is the best desirable therapy for both diseases despite its risks regarding the patient's immune system attacking the transplanted stem cells causing transplant failure due to graft versus host disease(1). With the number of risks associated with the current treatments, it is important to look at alternative strategies; One of the most hopeful being target genome editing.

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Target Genome Editing has become a powerful tool for studying and correcting gene function/mutations. Forms of target genome editing include small brakes placed on the target DNA based upon sequencing of the DNA. As of now, the three primary methods of target genome editing encompass Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). Although they all have their disadvantages and advantages, CRISPR is the optimal choice for most target genome editing. Zinc Finger Nucleases are built through the synthesis of a DNA-binding domain and a DNA-cleavage domain. They complete target genome editing by creating double-stranded breaks at exact locations These Zinc Finger Motifs are built to attach to exact DNA nucleotide sequences and then cleave with dsb or double stranded breaks. Similar to Zinc Finger Nucleases, TALENS cleaves the genome using DNA binding motifs. TALENS are restriction enzymes that are made by fusing transcription activator-like effector (TALE) and a DNA nuclease domain (7). Lastly, the CRISPR system, also the most preferred form of gene editing, cleavages the DNA by delivering Cas9 nuclease and guide RNA into a cell's genome, then carrying out the gene-editing using either HDR or NHEJ methods (8).

II. ZINC FINGERS

The structure of Zinc Finger Nucleases (ZFNs) specifically allows for ZFNS to inhibit two critical characteristics crucial to successful genome editing: DNA binding specificity and flexibility. The DNA-binding domain contains three to six zinc finger repeats. Each of these repeats recognizes three base pairs to result in a total of nine to eighteen base pairs. In other words, Cys2-His2 fingers each recognize about three base pairs. Therefore, a target site of about 15 pairs would require Cys2-His2 fingers. Then the DNA cleavage domain looks after the actual double-stranded break created by the process and repairs it using the natural cell repair process (7). The entirety of this process works at incredibly high frequencies (8).

Zinc Finger Nucleases were discovered in 1985 and were the first genome editing tool to be discovered. Thus, naturally, the first studies and analysis of target genome editing to combat hemoglobinopathies were conducted using zfns (9). With this knowledge, using ZFN for human induced pluripotent stem cells was studied. Human-induced pluripotent stem cells allowed scientists to use a virtually infinite number of cells that could be then used for autologous transplantation and other techniques for various diseases (10,11). The high efficiency found in these results proved to be significant as methods till then included low efficiency and residual sequences (12,13). Hope to utilize zinc finger technology for sickle cell disease began when Zinc Finger Nucleases were used to correct the sickle mutation in patient-derived induced pluripotent stem cells (iPSCs) (14). In this study, ZFN technology was used to correct the E6V mutation in the beta-globin gene in two patients with sickle cell anemia (14). A drug-resistance cassette was also included in the donor construct to enhance the identification and accuracy of the process (14,15). With this method, around 9.8 percent of targeting efficiency was found. The benefits of studies performed by Sebastiano et. al. included minimum off-target mutagenesis at potential off-target sites (14,15). However, a loxP site is still left behind in the genome in this study (14). Another similar study was conducted that confirmed not only the potential, but the feasibility of target genome editing in iPSCs from SCD patients using zinc finger technology. Here two muted Beta-alleles were used with a gene targeting plasmid, drug-resistant gene cassette, and ZFN technology aimed at homologous recombination at the Beta(s) locus (16). Although one HbS allele was successfully corrected, target efficiency was relatively low at 1 in 300 drug-resistant clones, possibly due to the HBB gene being silent in iPSCS. Therefore, making it more difficult to target (15,16).

In general, the use of HSCs for genetically inherited diseases is preferred over iPSCs as they can transition into normal red blood cells after the engraftment (1,17). A fairly recent study revolving around this included CD34(+) hematopoietic stem and progenitor cells and delivery of a homologous donor template (through either a DNA oligonucleotide or integrase-defective lentiviral vector). They concluded finding minimum off-target modification, fairly efficient targeted cleavage efficiency from 35% to 65%, modified cells were able to be engrafted into mice in vivo and in vitro, and were able to produce cells from multiple lineages (18). It is important to note that human cells resulted in significantly lower gene correction levels (18).

Newer research in regards to zinc finger nuclease technology for sickle cell disease and beta-thalassemia revolves around high fetal hemoglobin (HbF levels) usually through y-globin expression silencers. Higher fetal hemoglobin levels have directly correlated with lower severity and mortality rates in Sickle Cell Disease (19). BCL11A is an NbF level regulator as it represses HbF levels in adult erythroid cells (20). In this study, zinc finger nuclease technology is being used to reactivate HbF expression using autologous hematopoietic stem and progenitor cells (21). BCL11A ESE editing resulted in a 'three-fold HbF increase', hence reducing the sickling of the blood cells (21). Another attempt at reactivating the y-globin expression was made by creating a mutation in the binding domain region of SOX6 (22).

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III. TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES

Transcription Activator-Like Effector Nucleases (TALENs) are artificially engineered restriction enzymes engineered to cut specific sequences of DNA. They are made by fusing Transcription activator-like Effector (TAL effector) DNA binding domain to a DNA cleavage domain. Transcription Activator Like effectors are proteins produced by the Xanthomonas bacteria to help infect other plant species. TALEs aids by recognizing plant DNA sequences through a central repeat domain consisting of around 34 amino acid repeats. The 12th and 13th amino acids are known to vary (referred to as the Repeat Variable Diresidue (RVD)): Their variance correlation with specific nucleotide recognition (7). Their versatility and ease of constructing such proteins allow them to be a highly utilized tool in target genome editing. When fused with a nuclease, DNA can be recognized and cut at specific locations. TALEN technology induces double-strand breaks (DSB) to edit genomes. Cells then respond to these cuts with their repair mechanism: Non-Homologous End Joining (NHEJ) and Homology Directed Repair (HDR). Non-homologous end joins DNA from either side of the double-stranded break. The latter, Homology Driven Repair repairs the enzyme using a template. It can often insert foreign DNA at times (7).

One of the first experiments utilizing TALENs for sickle cell disease revolved around creating a double-stranded break near the mutation in the HBB locus. This study was immediately followed by another that proved a 60% improvement in gene targeting efficiency in drug-resistant clones (15,23,24). This efficiency proved to be higher than most reported in zinc finger nuclease technology studies.

IV. CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS

Discovered in the late 1900s, Clustered Regularly Interspaced Short Palindromic Repeats were found as a natural genome editing tool recognized through a repetitive pattern in the DNA sequence in Escherichia coli's genome. The E. Coli would secure bits of infecting viruses and create CRISPR arrays which would allow the bacteria to create defense mechanisms against such a virus. Thus, later when the virus attacked again the bacteria would debilitate the virus (25, 26). Since then scientists have mirrored this natural mechanism in bacteria to suit humans by creating a piece of RNA that complements the target sequence and binding it to a Cas enzyme to have it be sent to the genome. Then the Cas9-RNA complex cuts the DNA strands where required and using either NHEJ (Non-homologous end joining) or using HDR (Homology directed repair) edits the DNA to the scientist's liking (25).

CRISPR is undoubtedly the most researched and valuable target genome editing tool due to its efficiency and simplicity. In one of the first studies conducted studying CRISPR CAS cutting efficiency at the HBB locus in human iPSCs cells, efficiency rates surpassed Zinc Finger technology and TALENs (15,27). This study used sgRNA and Cas9 to correct one allele of the sickle cell disease mutation in the HBB gene using a donor DNA template (18). More studies comparing traditional target genome editing tools with CRISPR showed a continued increase in efficiency of cutting at the HBB locus in the CRISPR Cas system (28). However, with these benefits, the CRISPR Cas system has also shown heightened off-target activity. A study exhibited CRISPR systems that were targeting the human hemoglobin β and C-C chemokine receptor type 5 genes displayed off-target activity that resulted in mass chromosomal deletions and several insertions, deletions, and point mutations (29). Even with these concerns, other tests have determined CRISPR Cas 9 when used to correct a Sickle Cell mutation resulted in high efficiency which surpassed clinical minimum, something its alternative Zinc Finger Technology was unable to produce (30). A study specifically testing beta-thalassemia found using a combination of CRISPR Cas technologies and iPSC technologies in beta-thalassemia in mice proved to have successfully corrected on point mutations correlated with beta-thalassemia in mice (31). This study also analyzed if iPSCs could differentiate into HSCs and the tumor buildup in mice as a reaction to the implantation of the mutated stem cells. The findings indicated they did differentiate into HSCs or red blood cells, maintained normal karyotypes, and retained their pluripotency (31). There was no evidence of tumor buildup post-implantation and as a result, it was concluded this could be a potentially safe strategy for beta-thalassemia curation (32).

V. DISCUSSION

To begin, let's create a quick comparison between the three target genome editing tools discussed throughout this paper. Zinc Fingers and TALENs are each made from DNA binding domains based on Zinc Finger and TALEN proteins respectively and fused to a Fokl nuclease. The major components of the CRISPR system are gRNA and a Cas system (Cas9 for our purposes) endonuclease. Hence, the basis of ZFN and TALENs is a protein-DNA reaction and the basis of CRISPR is the RNA-DNA interaction. The longest recognition site is in the TALENs system, averaging from around 30-40 bp. ZFN has a relatively small coding sequence. However, ZFN struggles in terms of modularity due to the fingers' interaction with one another, while TALENs and CRISPR maintain relatively high modularity. CRISPR Cas 9 is by far the simplest of the three target gene editing tools (18).

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Most concerns regarding targeting genome editing for the treatment of hemoglobinopathies can be put in three major topics: off-target effects, delivery methods, and efficiency. These comparisons result in a complex analysis of what remains the preferred tool.

Off-target effects are when the nuclease recognizes an additional unintended site. This can result in an insertion, deletion, mutation that is not intended by the researcher. Here is where TALENs has the biggest advantage; its long sequence. This long sequence ensures specificity and minimum off-target effects. The shorter sequences, like those in Zinc Finger Nucleases and CRISPR, are much more vulnerable to off-target effects. However, it is important to note that the long sequence TALENs use is also a big disadvantage, which will be discussed later. Amongst the three, CRISPR seems to have the most concerns. Since the coding sequence is only about 20bp long, the unwanted DNA sections could be recognized by the CRISPR gRNA (33). This is something that was seen earlier when a study found many off-target effects (insertions, deletions, and point mutations) caused by the CRISPR technology. As a result, it caused major chromosomal deletion (29). Another common explanation for this occurrence is due to the fact that it has been found that a CRISPR system can recognize 5'-NGG-3' or 5'-NAG-3' (32). This has been seen in many experiments comparing the three programmable nucleases (34). Attempts have been made to increase CRISPR's specificity. They include using Cas9 variants, nickases, or Cas9-FokI fusions. A good example is two cas9 variants that proved to improve the specificity of the system by much: SpCas9 and SpCas9-HF1 (1–4).

TALENs harbor the largest disadvantage when it comes to delivery being the largest gene editing tool, limiting their delivery potential. While delivery vectors such as the adeno-associated virus vectors which can hold their counterparts, Zinc Finger Nucleases, with ease. More research must be done in order to make TALENs a remotely possible method of genome editing for hemoglobinopathies as this remains one of their most immense complications. There have been some advances in CRISPR delivery methods, however, there too must be further research so that the larger size of the Cas9 enzyme doesn't cause potential issues in delivery and usage.

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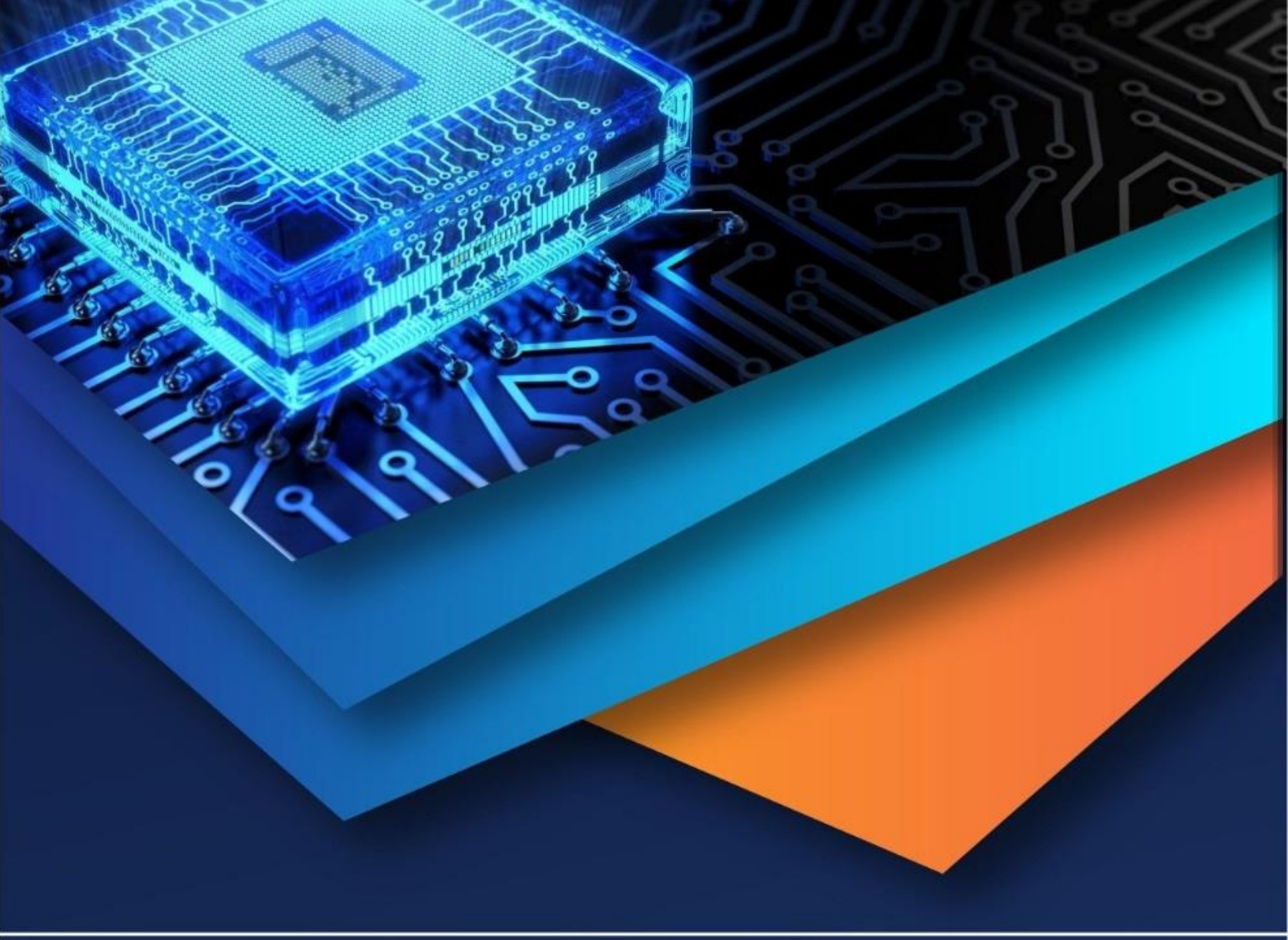

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