Different Gene Therapies Fortreatment of Hemophilia A

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Abstract. According to US Center for Disease Control and Prevention, there are 12 per 100000 males born with hemophilia A in US, and there are 400 male babies born with hemophilia A per year. Hemophilia A is type of disease that is inherited with X-linked recessive that have mutation in Factor VII gene. Recently, there are types of gene therapies success in hepatic vivo gene transferring. In this review, this paper will discuss three types of gene therapies for hemophilia A which are Zinc Finger Nucleases (ZFNs) and Transcription Activator Factors (TALENs) and CRISPER Cas9. ZFNs using the zinc finger DNA binding array in conjunction with FoKI cleavage nucleases domain to target and cleave to trigger the DNA repairing. In TALENs researchers induced pluripotent stem cells (iPSCs) derived by TALENickases to target the FVIII site. The latest research is using CRISPR Cas9 as a gene editing method to treat hemophilia A. The researchers used this method to repair the mutated gene so that the FVIII factor could be expressed normally. The paper also explains the current methods and process of delivery in gene therapy and shows the pros and cons of each. In conclusion, this paper express our prospects for future research about gene therapies.

Keywords: Gene therapy, Crisper-cas9, TALENs, ZFNs, Hemophilia A.

1. Introduction

Hemophilia is a type of disease that is X-liked recessive, including hemophilia A and hemophilia B. Hemophilia A refers to the genetic disorder caused by the insufficiency of FVIII, a clotting factor. According to the WFH (World Federation of Hemophilia), there are approximately 400,000 patients with hemophilia worldwide and around 210000 patients with hemophilia A. With the right therapeutic products and care, patients can lead relatively healthy lives. However, the reality is that about three quarters of people with hemophilia have little or no treatment due to lack of access. Patients are exposed to spontaneous hemorrhage represented by bleeding from the joints and have difficulty in clotting after receive trauma. The main treatment for hemophilia A is direct supply the clotting factors. However, this method has major drawbacks, requiring high frequency and is very expensive. With the advancement of technology, gene therapy is becoming an important focus of researchers. Gene therapy can lead to complete healing. When the technology is available, gene therapy can eliminate many of the problems associated with traditional therapies, such as the short supply of blood and the potential dangers associated with blood transfusions like getting HIV. Gene therapy can also protect the health of the patient's offspring. This article will introduce zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENS), and CRISPR Cas9, presenting the progress of them and the challenges they face.

2. Gene therapies

2.1. Zinc finger nucleases (ZFNs)

ZFNs is first chimeric nucleuses that has been used. ZFNs consists of zinc finger DNA binding array and FokI cleavage nuclease domain which is a type of synthesis protein. The zinc finger DNA binding array has the same target sequence as the target site, and the FokI cleavage nuclease domain has a higher fold that increases the activity of cleavage that shown by Chandrasekaran laboratory [1]. The function of ZFNs is to target the specific site and break the DNA double strand to trigger the repair through error-prone non-homologous end joining (NHEJ) and homology direct repair (HDR)

[2]. These repairs of DNA could fix the original problem. ZFNs is an approach that targets and cleaves the specific target site, and this is the advantage compared with other gene therapies, as other gene therapies have multiple DNA targets that have a higher risk of off-target and cytotoxicity [3]. ZFNs is used to treat hemophilia A which is an inversion of the gene intron and exon 1 or intron and exon 22.

To be specific, the homologous gene is first deleted to isolate the clonal population. The high sequence resembles homologous sequence at intron 1 or intron 22, corresponding with NAHR and leading to an inversion of sequence. Thus, to treat hemophilia A caused by intron 1 or 22 inversion, the researchers need to invert the segments and change them into the correct orientation so that they insert back into patient cells. In intron 22 and exron 22 reversion, the two separate target sites have 186 kilobase pairs (kbp), which are located on chromosome 18. The overall steps for intron 1 or 22 inversion treatment with zinc finger nucleases are the same other than the chromosome location, target site location, and segment length for these two types of causes that lead to inversion of the FVIII gene.

By using ZFNs to treat the intron 1 homolog inversion. Researchers use HEK 293 cells as the in vivo model that replaces the hemophilia A mutant cells. The reason for using the HEK 293 cell is that it has the same genetic sequence as a mutant cell, and it is the most direct approach compare with test in ex vivo. The ZFNs Lee et al. used is Z10 to target the junctions on intron homolog 2 and intron homolog 1, which have 140-kb-long segments. Meanwhile, the piece of gene that length 140-kb should include the promoter and exon 1. The two homologous regions are then oriented in the opposite direction. The researchers Fyodor D. et. Al wants to assemble two ZFNs to cut the double strand, so they apply the engineering motif. To stable the gene in HEK 193 cell they use the stop codon and frame-ship. As researchers investigate, ZFNs could correct 20% of chromatids meanwhile it last for long period [3]. The ZFNs is the new generation compare with expression cassette and adenovirus vectors [4].

The PCR is used to test if the inversion is correct with the same content and direction. Using the T7 endonuclease assay (T7E1) to identify the specific target site the inversion DNA is found to have PCR amplification, and the activity of NHEJ reveals the repair. Researchers then used digital analysis to observe the inversion frequency and obtained the isolating colonies by limiting dilution. Moreover, no additional mutation was observed after the therapy [5]. To be more specific about the methods, the ZFN-encoding plasmid is transfected into HEK 293 cells.However, if there is persistent expression of ZFNs, there are adverse consequences make take place such as off-target mutation and cytotoxicity. Also, ZFNs' efficiency is low, and the high cost of selecting it makes it not practical, which led to the introduction of TALENs, which are easier to design and put into practice [6,7].

2.2. Transcription activator factors (TALENs)

TALENs is similar to ZFNs that is type of protein used to cleavage to stimulate the NHEJ and DNA repair. Using the iPSCs to make the FVIII animal model and iPSC cell line. The reason for using induced pluripotent stem cells is that they are an animal model that can be used in vitro and are useful for gene correction [8]. In this study, researchers used mice as an animal model [9]. Researchers derive the induced pluripotent stem cells (iPSCs) from patients who get hemophilia A's urine and use viruses transfected into HEK 293T cells to differentiate into hepatocyte cells [6]. In this circumstance, using other approaches is not recognized as a way to decrease the episodes of bleeding. The iPSCs are derived by TALENickase to target rDNA's FVIII site in order to find a stable harbor for gene therapy, which means aiming to find a location with high recombination activity during mitosis and meiosis, which indicates that HR is efficient and effective [10]. In addition, the rDNA is safe; after identification, nucleases cleave the rDNA. As the NHEJ is the repair pathway, scientists would use the TALEnickases to induce the nicked DNA and trigger the HDR [10].

The stem cell is centrifuged on the side of TALENickase, then using agarose gel electrophoreses to pick the resistant clones. Scientists use membrane markers to mark the F8 gene, then transplant the iPSCs to the given endothelial cells. The membrane markers enable them to detect the endothelial

cell. the iPSCs and endothelial cells in vitro by using a non-integrating episomal vector. Using the techniques of ZFNs, TALENs, and CRISPER to correct the gene and transplant it back to mice Scientists first inverse the template and then correct the templet orientation input via the Yamanka factor via the episomal vector or Sendai virus [11].

TALENs is a type of gene therapy that has higher efficiency compared with ZFNs. Yong Wu et al. state that "TALENs are generated to distinguish the specific genomic sequences and induce DSB , which can stimuli the targeted gene correct greatly."

The TALENs technique enables us to reverse the gene and correct it into the correct orientation for the WT situation in the iPSC model. Firstly, breaking the already known DNA sequencing double strand by endonuclease, which is a type of enzyme that cleaves the DNA or RNA phosphodiester bond, with the target site TALENs is used to recognize the specific genomic sequence for manipulation. Then the process would activate a natural DNA repair like on the NHEJ or homologous recombination on a donor's DNA template to correct the nick. The number of NHEJ changes could reveal the activity of cleavage. HR is a method of targeting the gene site. There are two sites in hemophilia A that face reversion.

First, the paper would discuss exon 22 and intron 22 in FVIII reversion [10]. As the inversion of intron 22 leads to a 0.6 Mb inversion, it cleaves and separates the FVIIII into two segments that have opposite transcription orientation [1]. Two pairs of TALENs that are L1R1 and L2R2, cleave the targeting junction of exon 22 and intron 22. TALENs are expressed in HEK293R cells. Using the PCR to sequence the DNA and amplify it, proliferate to test the activity before repairing. Using PCR, integrate at 5 and 3'. Sanger sequencing is used to sequence the PCR product, and a positive result is identified by Southern blot [11]. Yong Wu et al. report that nine mutations are deleted. The researchers use the online bioinformatics tool 13 to test the off-target T7E1 to perform gene targeting to analyze potential targets and off-targets. Also using the Southern blot analysis to test the positive or negative PCR colonies the efficiency for targeting junctions in Yong Wu et al.'s experiments is 62.5% and 52.9% [11].

In the second type of inversion, which occurs at the intron 1 homolog. The HEK293T cells contain the test TALENs (TALEN 01) that were tested by the T7E1 [12]. The inversion segment is 140 kb in iPSCs and is shown by a hemophilia model in mice. First, electroporate the TALENS plasmids and clone them. In other words, plasmids encode TALENs. Isolating the colony with a specific primer and detecting the inversion of genes PCR is also used to multiple the number and determine the gene sequence to test the inversion of the genotype. Through error-free non-allelic homologous recombination (NAHR), Taken induces only one DSB. But there is still the potential that two DSBs are in both homologs 1 and 2, which are joined by NHEJ. As a result, the absence of mRNA and the existence of proteins reflect the expression of FVIII in endodermal and endothelial cells [12]. As a result, the research has mutation efficiency of 33% with no off-target site by T7E1[9].

In compare with ZFNs which is first generation of nucleases, TALENs have higher efficiency and easier to manufacture.

2.3. CRISPR Cas9

Previously, researchers used custom-made ZFNs and TALENs to artificially reverse the staining region between WT induced pluripotent stem cells (iPSCs) to mimic the staining regions between the mutated genes [9]. Although ZNFs show excellent results, it is difficult to achieve zinc finger structural domains with high specificity and affinity.Because of this drawback, ZFNs are not widely used.

For these kinds of reasons, researchers focus on the next generation of tools of gene therapy, the CRISPR-Cas9. In 2012, the researchers found for the first time that a synthetic crRNA was able to hybridize with its matching DNA sequence, which allowed Streptococcus pyogenes Cas9 to catalyze and then lead to break at the site of a specific DNA fragment. At the same time, this group of scientists simplified the entire Cas9 system with a specific gRNA [13]. The simplest application of CRISPR Cas9 is to allow Cas9 to catalyse double-strand breaks at the PAM site so that cells can repair as well

as edit DNA through non-homologous end joining (NHEJ)[14]. Homology-directed repair (HDR) can also occur within the cell. DNA carrying sequences homologous to those in the cleaved region will act as a template in the process of recombination. In short, CRISPR Cas9 ensures a more accurate and complex modification of genes. These are basis of gene therapy for hemophilia A.

It was shown that endothelial cells differentiated from inversion-corrected iPSCs were able to express the FVIII gene, which is missing in hemophilia A, in vitro. In the process, the researchers used an appropriate CRISPR Cas9 designed to target specific gene sequences to repair the original chromosomal inversion. This avoided to some extent the probability of off- target effects occurring [9]. In 2019, in this potential gene therapy for hemophilia A study, researchers inserted the deleted B domain in the FVIII gene into human elongation factor 1 alpha (EF1α) via HDR directed by CRISPR Cas9. After this operation, the expression of iPSCs in the FVIII gene was restored to normal. The genetically modified endothelial cells were able to express the FVIII gene normally and produce active FVIII protein. For the first time, scientists have demonstrated that the FVIII locus is a appropriate site for the integration of the normal FVIII gene by using CRISPR Cas9 and that FVIII expression can be restored via the EF1α promoter in endothelial cells which are differentiated from genetically corrected iPSCs from hemophilia A patients, leading to a therapeutic effect in hemophilia A [15]. In preclinical studies of haemophilia A, the use of dual AAVs as gene vectors for the treatment of haemophilia A appears to be efficacious. A AAV contained Staphylococcus pyogenes Cas9 (SpCas9) and a AAV contained human B-domain missing FVIII-guided RNA are used to integrate human FVIII gene into the albumin locus. As a result, the mice's livers did produce FVIII. In follow-up observations of the mice, the researchers also found that the improvement in the haemophilia A persisted for at least seven months, with no apparent off-target effects or signs of toxicity found in the liver [16]. In 2022, scientists extracted highly concentrated CD157+ groups of cells with significant levels of FVIII activity in CRISPR Cas9-edited FVIII- corrected iPSCs. At the same time, the researchers found that highly concentrated CD157+ groups of cells transplanted with a high dose of these cells in hemophilia A mice. The cells also survived in the mice for about three months [2]. These studies demonstrate the feasibility as well as the development of CRISPR Cas9 for the treatment of hemophilia A.

Researchers are faced with the problem of how to efficiently and safely get the genes that need to be changed to the right place. There are several delivery methods were tried to be used. AAV is a method that has been studied for a long time and is now widely used. As a member of the viral delivery method, it possesses less virulence and therefore it causes fewer immune responses [13]. However, it associates with some serious disadvantages. As a vector that carries the gene, AAV, although it has passed clinical trials, about 30% of hemophilia A patients have inhibitors for FVIII, which is produced within the first 75 days of treatment for 95% of patients [17]. There is no complete solution to the problem. Recent studies have demonstrated the possibility of using lipid nanoparticle (LNP) as vectors. The researchers used LNP to deliver Cas9 mRNA to mice along with single guide RNA targeted to antithrombin (AT). This method results in the improvement in thrombin generation, no active off-targets, liv-induced toxicity, and immune response occur which indicate that this approach is safe in the test of mouse. In the process of using LNP as a vector, CRISPR Cas9 reduced the off- targets effect because of its transient expression. LNP helps to improve the accuracy when delivering AT factors using CRISPR Cas9, inhibiting the expression of pathogenic TTR (transthyretin) by 90%. At the same time, improved clotting problems were observed in hemophiliacs. For those patients who do not have access to long-term treatment, the use of LNP, when dosed, can also have long-term therapeutic effects. In mouse experiments, AT expression was inhibited by approximately 40% after just three consecutive doses [11].

In addition to these wide used transmissions, researchers are actively exploring the use of non-viral transmission. The first is electroporation, which leaves small holes in the surface of the cell through the impact of an electric current. Then, DNA or mRNA can be transported into the cell through the interaction between the charges. The advantage of this method is its low dependence on cell type. Regardless of the type of cell, the transport can be accomplished with an electric current

that leaves small holes in the surface. But the use of electroporation is accompanied by toxicity due to the high voltage required [18]. Lipid-mediated delivery is also a non-viral delivery method. It works by delivering DNA or mRNA wrapped in a mixture of cationic lipids and nucleic acids into cells. The cost of this approach is low which makes it more favorable for large-scale use in the treatment of mass-type diseases. In addition, the off- target effects of lipid- mediated delivery are low [19]. One of the drawbacks of this method is the high dependence on cell type because of its lipid outer layer, which also leads to it demonstrating very low transport efficiency in unsuitable cells [15]. In addition to these methods, researchers are actively exploring research into other ways to increase the potency of treating hemophilia A by CRISPR Cas9 gene editing.

The available experimental data demonstrate the feasibility of gene editing and the promise of the research. But in order to meet clinical standards, it will require researchers to continuously improve the molecules used to reduce off-target effects and other potential risks.

3. Conclusion

This review discussed three main therapies of Hemophilia A that cause by mutation of Factor VIII in gene, and this paper mainly discuss the Int 1h and Int 22h inversion of gene sequence leading to hemophilia A. In conclusion, the ZFNs is the first generation of using nuclease to make gene therapy that last long for two decades, and TALEN is second generation, the CRISPER-Cas 9 is the newest developed technology that they all use the gene sequence and repair the chromosome inversion, meanwhile avoiding the off-target occurred. However, scientists are focus on put this technology into practical. They all cleave the two double DNA strands and PiggyBac use transposon technology therefore there are homologous recombination then isolated the clones. Finally transplanted into endothelial progenitor cells. The inversion of int 1h is 140-kbp that mimic the wrong sequence repair and the second one is 600-kbp that inversion 3 homologous using the ZFNs and TALENs. CRISPER-Cas 9 use the guide RNA to engineer the nucleases and change the orientation in vitro that induced the DSB. Finally isolate the colonies and sequence the DNA and use the electroporator transfect RGEN to the iPSCs. These developments of technology shown increasing in efficiency, decrease in off target, less cost and easier to manufacture the nucleuses.

Although there is cutting-edge technology like CRISPR Cas9 and some technologies that been apply for several years, they are still a lot of aspect people need to conquer. The use of the CRISPR/Cas9 system in vivo faces many challenges, such as the occurrence of off-target modifications, the possibility of causing autoimmune diseases, the identification of appropriate delivery techniques, and finally, ethical issues. This paper look forward to further developments that will refine the use of CRISPR Cas9 as a technology. This is not just an advancement of gene therapy for hemophilia A, but for gene therapy as a whole. Through it, more lives will be saved. In the meantime, as CRISPR Cas9 is developed, so will the tools to support it. More research results will be published gradually. For TALENs the efficiency is improved compare ZFNs have higher cost than TALENs Hoping that in future researchers could enhance the efficiency and lower the cost of gene therapies, thus, leading to wider usage.

Authors Contribution

All the authors contributed equally, and their names were listed in alphabetical order.

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