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Review Article

A REVIEW ON GENE EDITING TECHNOLOGIES AND THEIR APPLICATIONS

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Abstract: Disorders involving disruption in functioning of a specific gene has led to the increasing concern about development of gene therapies. The use of gene silencing methods like RNA interference has been used for the treatment of cancer, HIV, hepatitis A, hepatitis B, and many more diseases, but due to continuous requirement of siRNA, its efficacy has always been questioned. To develop this field further, the concept of gene editing has been brought into light, where correction and deletion of mutant gene, and even the addition of genes has been done to obtain the therapeutic results. This gene editing technology uses the artificially induced DNA double stranded break to fuel the cellular repair machinery. The nucleases used to target the mutated DNA are mega nucleases, zinc finger nucleases (ZFNs), TALENs, and CRISPR/Cas9. These nucleases have been used for diseases such as haemophilia A, haemophilia B, cancer, HIV, SCID, hurler's disease, cystic fibrosis and many more.

Although, these nucleases have been highly appreciated for their results, their side effects are still under improvement. The major side effect is off-target activity and selection of appropriate nuclease delivery method. Apart from this, the cost of developing therapeutic for some of these diseases is very high due to their rare occurrence. In this review, we briefly give an introduction to the gene editing technologies and their applications in animals as well as plants. We also discuss about their limitations and side effects and finally conclude with their future prospective.

1. INTRODUCTION

Nucleases can be engineered to target a particular locus on chromosome in control manner to obtain desirable output. These engineered nucleases utilize the cellular repair mechanism. When there is initiation of a DNA double strand break (DSBs), the repair machinery is activated. The repair mechanisms are of two types. Non-homologous end-joining (NHEJ) and homologous recombination (HR) repair [1]. Both the systems have their own importance. NHEJ mediated mechanism causes variable length insertions and deletions, known as indels. The length of indel introduced can vary among nucleases. The indel caused by TALEN and ZFN is larger than CRISPR [2]. Due to these indels, the NHEJ pathway is error prone and causes the gene to shift out of frame [3]. This pathway is favoured when there is need to

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knock out or delete a gene as in HIV treatment. When two pairs of TALENs were targeted in cell, they led to chromosomal deletions, inversions and translocation through NHEJ pathway [4]. On the other hand, the HR repair mechanism, which is an important process to repair DSBs in somatic cells and in meiotically dividing cells for the exchange of genetic material between parental chromosomes is used when there is need for gene correction and gene insertion [5]. The HR mechanism is precise and controllable unlike NHEJ. The HDR repair pathway, occurs during S and G2 phases of the cell cycle and requires template of a homologous DNA for a double stranded break to occur [6]. Generally, in a cell NHEJ pathway is involved but it can be down regulated to favour HR by down regulating enzymes involved in NHEJ mechanism.HR can also be favoured by introducing single stranded nicks instead of DSBs [2]. For HR repair pathway, donor DNA is delivered to obtain desired nucleotide sequence at the site of DSBs [1, 3]. The plasmid DNA and Single stranded oligonucleotide (ssODNs) have been used in clinical trials. The 80 bp ssODNs have been observed as efficient donor templates. With CRISPR/Cas9 nucleases, these ssODNs have shown precision in their activity of about 90%. The 10% inefficiency is due to blunt ends produced by cas9 which reduces the availability of complementary “acceptor” ends [3, 7].

Other areas where nuclease engineering is extensively used are:

- For developing mammalian cell lines so as to study diseases affecting them and design the therapeutics accordingly.
- They have also been used in plant engineering in different ways. CRISPR/Cas9 has been used to modify multiple alleles in hexaploid bread wheat so as to develop resistance against powdery mildew. In the same way nucleases have also been used to infect the pathogenic or viral genes to stop the infection.
- Engineered nucleases have also found use in synthetic biology to produce desirable bacterial strains and metabolic pathways. *Actinomycetales* are industrially significant bacterial species to produce secondary metabolites.
- Genomics has utilized nuclease engineering technology to map the working of cells. This has led to the better understanding of infection mechanism of viruses and how cells control the immune response against them. Also, CRISPR has given us new insights regarding noncoding functional genes through which we can study the human evolution.
- TALEN and CRISPR/Cas9 systems can also be used to regulate gene expression by addition of gene activator or repressor protein. For example, a VP64 domain bound with TALEN DNA binding domain can activate cellular gene expression mechanism and a SRDX

can act as repressor. This concept can be used for therapeutic purpose by regulating the expression of genes related to diseases. Apart from regulating genes associated with disease, targeted transcriptional regulation can also be used to impede viral reproduction [3, 8].

These wide applications of engineered nucleases have made them one of the most extensively studied tools of genetic engineering. In the year 2011, *Nature Methods* chose these engineered nucleases as method of the year due to the wide potential they behold in therapeutics [9].

2. MEGANUCLEASES: these are the naturally occurring homing endonucleases that are re-engineered to bind the specific DNA targets. These Meganucleases are designed to recognize DNA base pairs which are range from 12 to 42 base pairs in length [3, 10]. Popular families of Meganucleases are LAGLIDADG, His-Cis box, GIY-YIG, and HNH families. The family of LAGLIDADG homing endonucleases is the largest class that has been used [3, 5]. These enzymes are known as genetic parasites. When they recognize a specific allele they make a double stranded break which creates a four base 3' overhang on that allele. After making a DSB they transfer their protein coding sequence by homologous recombination into that allele [2]. These enzymes are known for their DNA specificity and have the smallest size of all the nucleases which make them easy to deliver with any delivery method [3]. Although, these Meganucleases have great binding specificity, the DNA recognition domain and enzymatic domain of homing endonucleases are difficult to separate, also the engineering of these proteins possesses limitation to these nucleases. To overcome the above limitations, Meganucleases have been combined with TALENs and ZFNs, to increase both binding affinity and target specificity. I-CreI and I-SceI are the most commonly used Meganucleases [3].

3. ZFN: zinc finger nucleases are the most advanced and the most studied form of nucleases which have reached the clinical trials of HIV [2]. Cys_2-His_2 is the most commonly used DNA binding zinc finger protein (ZFP) which when bound to Fok1 nuclease forms zinc finger nuclease (ZFN). These zinc fingers form ββα configuration of about 30 amino acids. The α helical region of the protein structure, also known as "recognition helix" recognizes about 3-4 base pairs using six amino acid in the DNA major groove [8, 3]. A zinc finger module is formed by linking 3-6 finger modules together which together recognize 9-18 base pairs. Fok1 activates as a nuclease after dimerization and hence two zinc finger module arrays are designed to bind nine to eighteen nucleotides on opposite strand of the target sequence [11]. To enhance the working of ZFNs, many architectural models for its construct

have been proposed. One of the model, having many zinc finger domains which recognize distinct DNA triplets have been fused together in tandem by using canonical linker peptide to recognize a wide variety of DNA sequences. This form of ZFN assembly is known as "modular assembly". Another method of assembly is Oligomerized pool engineering (OPEN) in which proteins are selected from a randomized library. Although this method is labour intensive, it takes into account context dependent interactions between adjacent zinc finger domains [3, 12].

These ZFNs hold high potential to correct, add or disrupt a particular gene. They have also been used in vaccination therapy by disrupting TAP2 gene in hiPSCs to produce large amount of antigen presenting cells [13]. Despite of having many advantages of ZFNs, they possess some limitations too. First of all, their construction is challenging. The creation of zinc finger domain that can recognize all DNA triplets, including that of 5'CNN3' and 5'TNN3' type is difficult. They also have lower affinity for AT rich regions [12]. Clearly, ZFNs lack target flexibility. Apart from lacking target flexibility, ZFNs also show off-target mutations. There has been research going on to improve their working. One such approach is by delivering them into cells as proteins. As ZFN proteins are cell-permeable, this approach will limit their off-target activity. Another method of improving ZFN activity is by modifying Fok1 to create nicks instead of double strand breaks. This will mediate HDR repair pathway and will minimize off-target mutagenesis due to NHEJ [8].

4. TALEN: It refers to Transcription activator-like Effector Nucleases. These are the effectors discovered in plant pathogen *Xanthomonas* which affect plants such as rice and cotton. As the name describes, these effectors activate the transcription of target genes. They enter cell system through type III secretion mechanism. The whole TALEN assembly comprises of: a central DNA binding domain, an acidi transcription activation domain (AD) at C terminal, nuclear localization signals (NLS), and secretion and translocation signal in the N terminal. The DNA binding region consists of 33-35 amino acid tandem repeat sequence which are nearly identical. Each repeat is engineered to recognize one specific nucleotide. The difference in these tandem repeats occur at only 12 and 13 position which are known as repeat variable di-residues (RVD), and it is only these RVDs that recognize the specific nucleotide sequence and bind to them. These tandem repeats are followed by a 20 amino acid sequence, which is known as half repeat. The RVDs which recognize nucleotides A, C, G, T are: NI (Asn Ile), HD (His Asp), NN (Asn, Asn)/ NK (Asn Lys) and NG (Asn Gly) respectively. The working of TALEN is same as that of ZFN. Each TALE repeat is bound

with a Fok1 nuclease region. And as Fok1 is activated after dimerization, a pair of TALEN is required to produce a cut at the target sequence [1, 4, 11]. When the TALE is delivered in the cell, they are translocated to the nucleus and bind to the target DNA and then mimics host transcription factors to reprogram gene expression of the host [10]. After binding of the TALEN region to the target DNA sequence, the Fok1 region cleaves at the spacer region which is usually 14-18 bp long. The binding specificity of TALEN is determined by repeat number, which determines the length of target and the sequence of RVD, which directly binds to the nucleotide sequence. The only structural limitation with TALEN is that, the recognition code should always be preceded by a thymine nucleotide [1, 4] Fig1 .

This TALEN structure can further be improved by adding more functional domains, such as that of repressors, nucleases, activators, recombinases, nickases, integrases, methylases and others [4]. As compared to ZFNs, TALENs provide a greater design flexibility by one to one bp binding rather than triplet binding like that in ZFN [12]. Due to minimal cytotoxicity and off target mutations, TALEN is becoming a highly favoured engineered nuclease [11]. The cellectis bioresearch company in Paris, has used this technology to develop an engineered T cell with knocked out endogenous T cell receptor, known as universal chimeric antigen receptors, to recognize the CD19 antigen on the surface of tumor cell and eradicate them. This technology has received successful clinical trials, but work on off-target activity still needs to be done [14].

This TALEN technology beholds many limitations along with its advantages. First of all, its construction of assembling many tandem repeats is challenging. This technology is expensive and hence cannot be adopted customarily. To ease the process, many assembly platforms have been proposed for TALEN construction. There are three platforms which have been used for TALEN assembly. (1) Standard cloning assembly, (2) golden gate assembly, and (3) solid phase assembly method [1, 4].

The golden gate assembly method was invented by *Voytas Laboratory* and is the most widely accepted method for designing TALEN because, it is simple, fast and widely used. In this method, type II restriction enzymes are used to create multiple sticky ends and after that, digestion and ligation are carried out in same mixture to give at least 10 TALE repeats in one reaction. Apart from golden gate assembly method, other methods like REAL, FLASH (fast ligation based automatable solid phase high through output), ICA (iterative capped assembly), Chip and LIC (Ligation independent cloning) methods are also used [4].

Other limitation includes, big size of TALEN because of which their delivery with a vector is problematic. The tandem repeats are unstable, which further increase the problem of packaging in a delivery vector. Adeno-associated virus has shown successful results with TALEN due to their, less immunogenicity and non-pathogenicity [15].

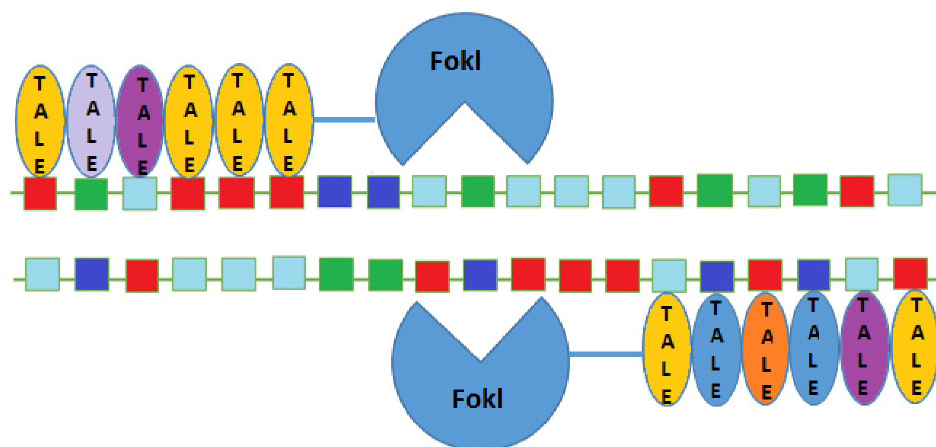

Figure 1: MECHANISM OF TALEN. EACH TALE PROTEIN RECOGNIZES SINGLE NUCLEOTIDE AND BINDS TO IT. THERE ARE DIFFERENT TALE FOR DIFFERENT NUCLEOTIDE

5. CRISPR/Cas9: Clustered, regularly interspaced, short palindromic repeats/ CRISPR associated protein is a RNA-guided DNA endonuclease system of adaptive immunity in bacteria and archaea which is used against invading plasmids and viruses. This system works by incorporating the DNA of past invaded pathogen as spacer in CRISPR genomic loci. This genome is then transcribed to form CRISPR RNA or crRNA. The crRNA contains a protospacer region, complementary to the target DNA. This crRNA hybridizes with trans-activated crRNA (tracrRNA). The hybrid of tracrRNA and crRNA guides the cas9 to the target site[13]. This CAS protein has helicase and nuclease activity which creates double stranded break at the target site [9]. Later on these two crRNA and tracrRNA were fused into one single form, known as guide RNA (gRNA) in which few alterations in the sequence were done to recognize desired target sites. CRISPR binds to the target nucleotides through Watson-crick base pairing model [3]. For binding of CRISPR system to the target site, an adjacent sequence of nucleotides called as protospacer adjacent motif (PAM) needs to be present at 3’ end of the target site. And this is the only sequence limitation for CRISPR system [16]. After binding of the CRISPR system, the DNA double helix is unwinded to bind it with the sgRNA and after this the cas9 performs its action. Once the double strand

break has been created the DNA repair machinery is activated leading to the mutation which knockout the unrequired piece of DNA (Fig 2).

The bioinformatics analysis of CRISPR/Cas9 systems isolated from different organisms, showed us that, these systems can be subdivided into three main types, and 10 subtypes. Type II-A CRISPR/Cas9 system, isolated from *S. pyogenes*is the most widely used [9].Like other engineered nucleases, this system also holds some obvious advantages and limitations.

Its advantages are:-

i. It is not labour intensive as it requires only small alterations in gRNA to target any sequence of nucleotide. Also, the cas9 is a readily available moiety, this system is cost effective and easy to construct.

ii. Cas9 can be easily converted into nickases to induce HDR repair pathway rather than NHEJ pathway to reduce the off-target effects [11].

iii. Due to its small size, the delivery of multiple gRNA can be done to target multiple targets in a genome at the same time.

iv. Due to low toxicity of Cas9 protein, CRISPR method outclassed TALEN in terms of gene disruption, bi-allelic targeting and gene knockout in clinical trials of targeting genomic sites in hPSCs [13].

Its limitations are:-

i. As described above, the presence of PAM is essential for the working CRISPR system at the 3' send of target site [2, 3, 11].

ii. Even a single mismatch 13-14 nucleotide from 3' terminal crRNA sequence can abolish the cleavage activity of the CRISPR system. Hence the engineered gRNA needs to be highly specific.

iii. Under certain circumstances, mismatches occur, CRISPR systems are active under that condition too, which means that those mismatches are ignored. And due to these mismatched crRNA and target DNA interactions, off-target activity at different genomic loci is observed [11].

Several strategies have been tested so as to improve the specificity and fidelity of CRISPR system. The length of base pairing was increased to increase the interactions between gRNA and target site. This approach failed to give desirable results. After this, the double nicking strategy was tested to reduce the off-target mutations. In this strategy, the Cas9 protein which induces double stranded breaks was re-engineered to work as a nickases. Single stranded nicks are repaired by high fidelity base excision repair (BER). So while approach induces

double stranded breaks at target loci, it induces only single stranded nicks at off-target sites and these nicks are repaired by HDR pathway without any indel formation and hence reducing off-target mutations [11, 17, 18]. This system has also been used to promote gene transcription by deactivating cas9 and joining the transcription activators instead. Also, by fusing a deaminase enzyme with system, it can also be used to mutate a single specific base of DNA. Apart from above two, fluorescent proteins have also been added to this system, to study the location of specific gene.

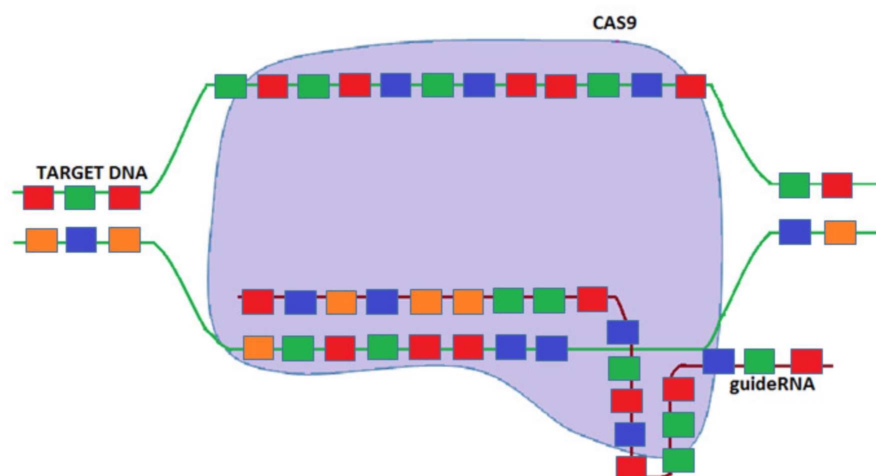

Figure 2: THE CRISPR/CAS9 SYSTEM ALONG WITH guideRNA. THE CAS FIRST RECOGNIZES THE PAM SEQUENCE AFTER THAT IT UNWINDS THE DNA SO THAT guideRNA CAN BIND TO IT. AFTER THAT CAS9, WHICH IS A NUCLEASE MAKES THE CUT AT APPROPRIATE PLACE REQUIRED AND SUBSEQUENTLY THE WHOLE MACHINERY MOVES OUT FOR DNA REPAIR TO TAKE PLACE.

6. Evaluation of off-target activity: All these gene editing platforms, have a common disadvantage of off-target activity. To overcome this limitation, many tactics have been developed. The most palpable approach is the change in design. Nevertheless, the desired output was still not obtained with these nugatory structural changes. Therefore, nucleases delivery at genomic safe harbour (GSH) sites has been proposed. Due to integration of foreign DNA, these safe harbours are at negligible risk of insertional mutagenesis. The GSH could be any site which are distant from genomic regulatory sequence (extragenic site), or intragenic sites (within a gene), whose interruption is tolerable. The selection criteria for extragenic sites are:-

I. It should not be a part of gene transcription unit.

II. It should be located >50kbs away from the 5' end of gene.

III. It should be located >300kb away from cancer related genes.

IV. It should be located >300kb away from microRNA

V. It should be located away from highly conserved and non-coding RNA regions.

As work is going on to reduce the off-target effects, appropriate methods should be developed to evaluate these effects too. Deep sequencing method was applied to study indels and translocations created. But this method was not cost efficient for low frequency events and the sequencing itself has error rates [2].

Further improvements were made, and a web based bioinformatics tool, PROGNOS (Predicted report of genome-wide nuclease off-target sites was developed. This tool can provide potential target sites for ZFN and TALEN in whole genome, and once the target site is identified, this program can provide a list of potential off-target sites which experimental based tools failed to obtain. Apart from this, another program known as COSMID (CRISPR Off-target sites with mismatches, insertions and deletions) was developed to study potential off-target sites developed by mismatches between gRNA and DNA sequence [19].

7. Genome editing technologies in plants: These sequence specific nucleases have been used in plants like *Arabidopsis thaliana, Nicotiana benthamiana,* as well as rice, sorghum, wheat, corn, soybean, wheat, corn, tobacco, potato, petunia, sweet orange, liver worth and poplar to prepare antiviral defences and to alter metabolic pathways to get desired function [20]. ZFN has been used to disrupt ABI-4 gene of *Arabidopsis* to regulate the expression of ABA gene which resulted in increased stress tolerance to higher glucose concentration. TALEN has been used to knock out locus for mildew resistance for developing powdery mildew impervious bread wheat. With the help of SSN, integration of genes is also possible to modify specific phenotypic traits. Endochitinase gene of tobacco has been modified with ZFN through integration of phosphinothricin phosphotransferase herbicide resistance gene [21]. Through CRISPR-Cas9 hygromycin resistance gene in soybean and bialaphos resistance gene can be integrated into maize. Phytic acid, which is a major storage for phosphorus and mineral cations in crops is poorly digested by monogastric animals has been targeted in maize seeds through TALEN and CRISPR system [10].

Viral inhibition is another area of application for plants. Artificial zinc finger proteins (AZP) have been used to target intergenic region (IR) of Beet severe curly top virus (BSCTV), belonging to *Geminiviridae* family. The replication of BSCTV was reduced by targeting this IR in *Arabidopsis thaliana*, as it has binding site which is recognized by replication initiator protein which initiates viral replication. Apart from this AZP has also been used to block replication initiator protein of *tomato yellow leaf curl virus* (TYLCV) and promoter sequence

in *rice tungro bacilliform virus* (RTBV). TALE has been used develop resistance against *Begomovirus*[10].A knockdown of eIF(iso)4E gene by CRISPR/Cas9 system confers resistance against cucumber vein yellowing virus (CVYV), zucchini yellow mosaic virus (ZYMV) and papaya ringspot virus type W(PRSV-W) in cucumber. These genetically engineered nucleases have been successfully applied to *Geminiviridae, Nanoviridae, Caulimoriviridae, Metaviridae,* and *Pseudoviridae* plant virus families [20].

8. Future of gene editing technologies and conclusion: These engineered nucleases are still in their infancy stage and need lot of attention to develop. Each nuclease has its own private limitations. For example, the presence of PAM sequence on the target gene is a major restriction for these nucleases. New system of CRISPR needs to be developed to generate target flexibility. The CRISPR-Cpf1 system is reported to make a 42nt gRNA instead of 100nt like that of Cas9 system [5].

Delivery and safety are the central challenges concerning with these engineered nucleases. DNA independent delivery (ex-vivo delivery) for plant gene editing is extensively explored, but the in vivo delivery through viral vectors is more popular because of its less toxicity. Lentivirus has been used for CRISPR system and lentivirus system is used for TALEN system. Delivery through non-integrating viral vectors is a future yet to be explored. Off-target effects are observed which can be lethal if not monitored properly. The immune response after the administration of these engineered nucleases needs to be studied properly. The sensitivity of nucleases towards the target needs to be increased [22, 23].

Genetically engineered nucleases have brought about revolution in the field of medicine, epigenome editing and agriculture. Apart from application in agriculture as discussed above, GETs have found immense use in therapeutics. CCR5 and CXC4 co-receptors have been targeted to bring the functional cure of HIV [15]. Similarly, various attempts have been made for diseases like cystic fibrosis, bone marrow disorders (beta-thalassemia, and sickle cell anaemia), cancer, respiratory disorders, haemophilia and many more. But, due to its off-target effects, need for appropriate delivery mechanisms, exploration of new target sites, and architectural problems there is still lot of space for improvements [6].

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