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The Principles and Application of Gene Editing as a Tool in Research, Industry and Health Biotechnology

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Abstract: ***The discovery of zinc finger nucleases (ZNFs) and transcription activator-like effector nucleases (TALENs) has revolutionized the area of biological research, these engineered chimeric nucleases are protein based systems consisting of programmable sequence-specific DNA binding sites linked to a non specific DNA cleavage domain, capable of introducing double stranded breaks (DBS) or single strand nick (SSN) in organisms' genomic sites, thereby activating DNA damage response pathways homologous recombination (HR) or non-homologous end joining (NHEJ). These error prone DNA repair pathways, in the presence of donor sequences can introduce insertion/ deletion mutations (indels) to a targeted genomic site in various industrial or biomedically important cell types however, the recent development of clustered regularly interspaced short palindromic repeats (CRISPR) technology has redefined targeted genome editing owing to its simplicity and efficiency, adopted from bacterial and archaeal acquired immune system, CRISPR/Cas9 system is a complex of customizable single guide RNA fused to Cas9 nuclease which uses simple base pairing rules between the guide RNA and DNA at the target genomic site to mediate cleavage of complementary target-DNA sequences adjacent to protospacer adjacent motifs (PAM). Introduction of DBS or SSN by CRISPR/Cas9 system also induces the indigenous error-prone DNA repair pathways HR and NHEJ, thereby facilitating indels mutations or gene knockout, mutant Cas9 can mediate gene regulation. Although engineering of ZFNs has been challenging and the specificity of CRISPR/Cas9 across wide range of genomes has not been well established, the application of these genome editing tools promises a great future in research, industry and health biotechnology.***

Keywords: Zinc finger nucleases (ZNFs) Transcription activator-like effector nucleases (TALENs) Clustered regularly interspaced short palindromic repeats (CRISPR) Homologous recombination (HR) Non-homologous end joining (NHEJ)

1. Introduction

Genome editing encompasses manipulations of organisms' DNA by altering, removing or addition of nucleotides to the genome, the process is accomplished through the application of engineered nucleases that can induce double stranded breaks (DSB) or single strand nick in an organisms' DNA, these sites are altered and then repaired by homologous recombination or non-homologous end joining (Allele Biotech, 2018).

The early techniques employed in introducing double stranded breaks (DSB) on specific genomic sites were the use of zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), chimeric protein-based systems with programmable sequence specific DNA-binding modules linked to a non-specific DNA cleavage domain (Gaj et al, 2013; Sander & Joung, 2014).

Recently, clustered regularly interspaced short palindromic repeats, (CRISPR) technology, which is based on bacterial nucleases called CRISPR-associated protein 9 (Cas9) was developed; it utilizes the adaptive bacterial and archaeal immune systems with the aid of CRISPR loci (Kamburova et al, 2017). This method of gene editing is more precise, unique and flexible in that, RNA is the moiety that directs the nuclease to a target DNA sequence using simple DNA-RNA base pairing rules between the target DNA and guide RNA, unlike ZFNs and TALENs which uses protein-DNA interaction for targeting (Sander & Joung, 2013; Kamburova et al, 2017). This type of targeted genomic editing using customized nucleases facilitates easy introduction of targeted gene deletion, insertion or precise DNA sequence changes in a wide range of organisms and cell types (Sander & Joung, 2013).

The crucial step towards achieving a successful targeted genome editing in all these technologies is the introduction of nuclease induced double stranded break (DSB) at the target genomic loci which can be repaired by one of the pathways, homology-directed repair and non-homologous end joining, which occur in nearly all organisms (Sander & Joung, 2014). The later pathway is error prone and can lead to introduction of insertion/deletion mutations known as indels (Sander & joung, 2014). This paper is aimed at reviewing the basic principles and application of ZFNs and CRISPR/Cas9 systems as tools in modern Biotechnology industries.

2. Molecular Structure and Mechanisms of genome editing tools

2.1 Zinc Finger Nucleases (ZFNs)

Zinc Finger Nucleases (ZFNs) are fusions of nonspecific DNA cleavage domain from Fok1 restriction endonuclease at the C-terminus with artificial Cys2-His2 zinc finger proteins at the N-terminus joined by a specific linker, each subunit consist of about 30 amino acid residues in a conserved ββα configuration, with amino acids present on the surface of the α-helix which typically interact with three (3) base pairs by inserting the α-helix of the protein into the major groove of the DNA double helix, thereby allowing the amino acids interaction (Gaj et al, 2013; Kamburova et al, 2017).

A functional ZFNs must dimerized in order to cleave DNA, therefore a pair of ZNFs each containing at least 3 subunits is required to target non palindromic genomic sites, also in order to dimerized and cleave DNA the two individual ZFNs must bind opposite strands of the DNA with their C-termini certain distance apart (Szczepek et al, 2007; Kathomen &

Joung, 2008; Maeder et al, 2008). The ZNFs when dimerized can induce targeted DNA double stranded breaks (DSBs) that stimulate DNA damage response pathway (Kathomen & Joung, 2008; Gaj et al, 2013; Kamburova et al, 2017).

Repair of the DSBs on the target gene can be achieved by one of the following DNA damage response pathways; non-homologous end joining (NHEJ) or in the presence of a donor DNA sequence, homologous recombination (HR) become favored, NHEJ is error prone and can therefore result in the introduction of insertion /deletion, and subsequent expression of a truncated or non-functional proteins (Kathomen & Joung, 2008).

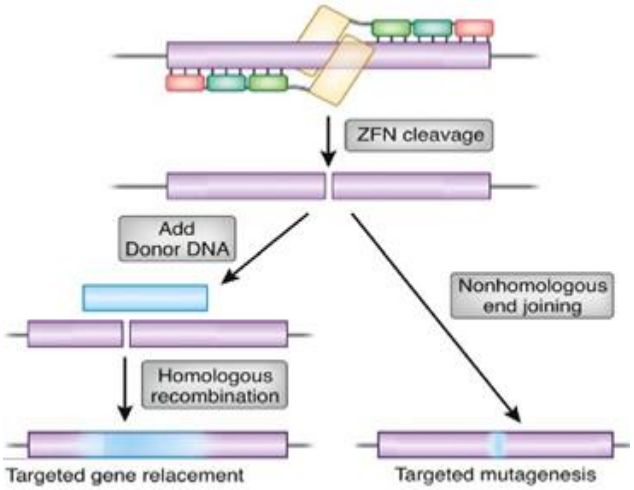

Figure 1: Showing a pair of zinc finger, each with 3 zinc fingers binding to target DNA, the arrows showing the introduction of double stranded break at Fok1 domain and subsequent repair by HR & NHEJ

Zinc finger domains have been developed usually to recognize nearly all the 64 possible codons, preselected zinc finger motifs from zinc finger library can be linked together in a tandem to target DNA sequences that contains a series of these DNA triplets (Gaj et al, 2013). The versatility and binding specificity of ZFNs is owing to the ability to reprogram or customize the DNA binding domain forming a tandem of 3-4 specific Cys2-His2 zinc finger, to recognize virtually any genomic sequence (Gaj et al, 2013; Sander & Joung, 2014).

3. Mechanism of ZFNs activity

The customized ZFNs can be delivered into the cell containing the target gene, the DNA binding domain locate the target genome site and bind via protein-DNA interaction and form a dimer, after dimerization, ZFNs is activated and cuts the DNA at the spacer sequence separating the target sequence into two halves, making a double-strand break (DSB). Attempt to repair the DSB by the indigenous DNA repair system can result in gene disruption, gene correction or gene addition as represented in steps b, c and d in the figure below.

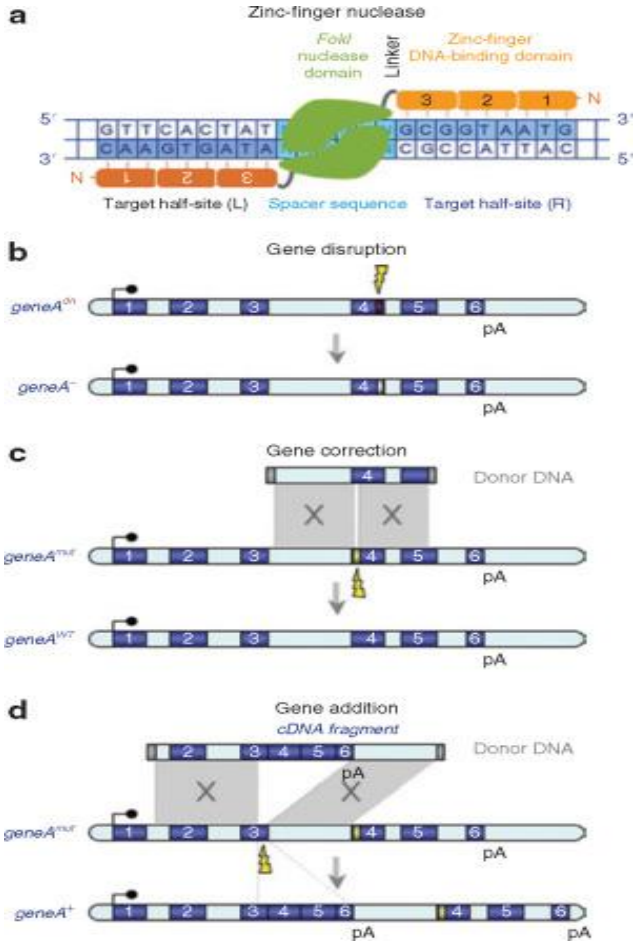

Figure 2: Zinc finger mediated genome editing showing gene disruption, gene correction and gene addition at different instances b, c and d. (Kathomen & Joung, 2008)

Gene disruption (fig. 2 b); A double strand break introduced into the target dominant sequence (geneA^{dn}) by ZFNs is repaired by error prone non-homologous end joining (NHEJ) pathway, insertion/deletions (indels) that occur disrupts the coding sequence (geneA^{-}) and render the function of the expressed protein faulty (Kathomen & Joung, 2008).

Gene correction (fig. 2 c); To restore genetic aberration directly in a mutant genome (geneA^{mut}), a donor DNA which contains the wild type sequences homologous to the mutant gene is transduced into the target cell, ZFNs induced DSB stimulates homologous recombination (HR) between the donor DNA and mutant gene (geneA^{mut}), to produce a corrected locus (geneA^{wt}) (Kathomen & Joung, 2008).

Gene addition (fig. 2 d); In order to restore the phenotype of a cell carrying a genetic disorder (geneA^{mut}), a partial cDNA flanked by sequences homologous to the defective gene, is placed in the targeting vector, a ZFNs induced DSB stimulates homologous recombination (HR) between the targeting vector and the mutant gene. The gene is thereafter controlled by cell's indigenous promoter and expressed as a construct (geneA^{+}) (Kathomen & Joung, 2008).

Creation of enhanced ZNFs; Specificity of ZFNs was optimized and refined by the design of Sharkey, ZFNs containing variant Fok1 cleavage domain with >15 folds increased activity and creation of ZFNickase which makes

only single strand DNA breaks and induce only homology directed repair (HDR) without the activation of mutagenic NHEJ pathway, to improve performance and site specificity of ZFNs (Gaj et al, 2013).

Application of ZFNs in therapy

The use of site specific nucleases have revolutionized gene therapy arena by allowing the introduction of targeted modifications in different model of organisms common to biological research, for instance ZFN-induced homology directed repair (HDR) has been used to directly repair the gene mutations associated with X-linked severe combined immune deficiency (SCID), hemophilia B, sickle cell disease and α1-antitrypsin deficiency (Kathomen & Joung, 2008; Gaj et al, 2013), also, ZFNs have also been used to repair the gene mutations associated with Parkinson's disease (Carroll D, 2008; Gaj et al, 2013).

Targeted gene knockout using ZFN-induced non-homologous end joining (NHEJ) mediated repair has been shown to effectively combat HIV/AIDS, ZFNs have been used to confer HIV-1 resistance by silencing the HIV co-receptor C-C-chemokine receptor type-5 (CCR5) in primary T-cells and hemopoietic stem/progenitor cells (Gaj et al, 2013). In a recent development, ZFN-mediated targeted integration of anti-HIV restriction factors into the CCR5 locus has led to the establishment of T-cells with near-complete resistance to R5 and X4 strains of HIV (Carroll D, 2008; Gaj et al, 2013).

The role of ZFNs in genome editing has been tremendous in other areas, for instance in Agriculture, food crops were improved where ZFNs was used to develop herbicide resistant *Zea mays* by insertion of the gene into a targeted sites in *Zea mays* genome (Kamburova et al, 2017), ZFNs was also used for the targeted modification of an indigenous malate dehydrogenase (MDH) gene in plants, the plants with modified MDH gene subsequently showed high yield (Kamburova et al, 2017). Beside the nuclease activities, the DNA binding domain of ZFNs can be combined with different effector domains to cause changes in genomic structure and functions of organisms like transcriptional activators and repressors, recombinases, transposases, DNA/Histone- methyltransferases and histone acetyltransferases (Gaj et al, 2013).

However, despite the tremendous impacts of ZFNs in genome editing, the technology is faced with challenges such as targeting a complex genome with multiple copies of sequences that are homologous to the target genome (Gaj et al, 2013). Attempts to increase the number of zinc fingers in order to boost the specificity of ZFNs can lead to off-target binding and offsite cleavage (Carroll D, 2008), since too many zinc fingers in a complex genome increases its likelihood of binding to parts of the genome outsides the target area (Gaj et al, 2013), such off-target cleavage may lead to generation of enough double stranded breaks (DSB) to overwhelm the repair mechanism and consequently lead to chromosomal rearrangement, toxicity or apoptosis (Carroll D, 2008; Gaj et al, 2013). Moreover, robust design of engineered zinc finger arrays presented some challenges because of the need to account for context-dependent effects between individual zinc finger domains in an array (Sander & Joung, 2014).

2.2 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 system

CRISPR/Cas9 is a bacterial immune system against phage infection and plasmid transfer in nature, turned into a sophisticated RNA-guided DNA targeted gene editing tool (Jiang &Doudna, 2017). The system is made up of DNA endonuclease, Cas9 and a short piece of RNA that guides the enzyme to the target genome site (Hara et al, 2016).

Once the complex reaches its destination, the guide RNA base pairs with the complementary sequence of the target DNA and induce double stranded breaks (DSBs) which like ZFNs-induced DBS, activates DNA damage repair pathways HR or NHEJ (Rath et al, 2015; Hara et al, 2016). CRISPR/Cas9 technology tends to be advantageous over ZNFs in that it is unique, flexible owing to the ease of changing the gRNA, simple and easy to perform, efficient, versatile and can be used to target several genome sites simultaneously, so is widely adopted as a better tool for genome editing (Rath et al, 2017).

Structure and Mechanism CRISPR/Cas9 mediated Genome Editing

Native CRISPR is a cluster of DNA sequences in a bacterial genome containing snippets of DNA from viruses which may have previously attacked that bacteria, these snippets are accumulated as repeats of sequences (CRISPR) over time of different attacks and used as "memory" to identify and destroy DNA from the same or similar viruses in subsequent attacks (Szczepek et al, 2007; Jiang & Doudna, 2017). Transcripts from CRISPR repeat arrays are transcribed into CRISPR RNAs (crRNAs), each containing "protospacer ", a variable sequence copied from the phage and part of the CRISPR repeat (Sander & Joung, 2014).

Broadly about six (I-XI) different types of CRISPR/Cas systems have been identified recently based on composition and mode of action (Jiang & Doudna, 2017) however, type II system from *Streptococcus pyogenes* has been adopted as the most characterized, effective and precise tool for gene editing in the recent years (Sander & Joung, 2014; Rath et al 2015), and is therefore referred to in this context.

The type II CRISPR system consist of a single guide RNA (sgRNA), a fusion of crRNA and transactivating CRISPR RNA (tracrRNA), these two RNAs of about 20 nucleotides forms a complex with Cas9 nuclease, 5' end of the sgRNA directs the Cas9 to specific genome site which is recognized by the presence of protospacer adjacent motifs (PAM) at 3' end, and base pairs via the standard RNA-DNA complementarity base pairing rule Sander & Joung, 2014). Once achieved, the base pairing activates Cas9 nuclease to make a single double-stranded break (DSB) 3 nucleotides from the NGG PAM sequences on the target site and induce the DNA repair pathways HR or NHEJ in eukaryotes (Rath et al, 2015; Jiang & Doudna, 2017), in the same manner as described previously for ZFNs.

Application of CRISPR/Cas9 system

In order to perform gene editing, CRISPR/Cas9 complex containing engineered targeted-sgRNA is delivered into the cell harboring gene of interest along with a donor DNA template in form of plasmids or using Lentiviral vectors, binding of the complex to the target genome site introduces DSB by Cas9 nuclease and activate HDR pathway in the presence of a donor DNA template to bring about the desired targeted gene correction, or mutagenic NHEJ pathway to yield small indels or inversions between DSBs to cause mutation **(Fig.3A)** (Sander & Joung, 2014; Reis et al, 2014; Rath et al, 2015).

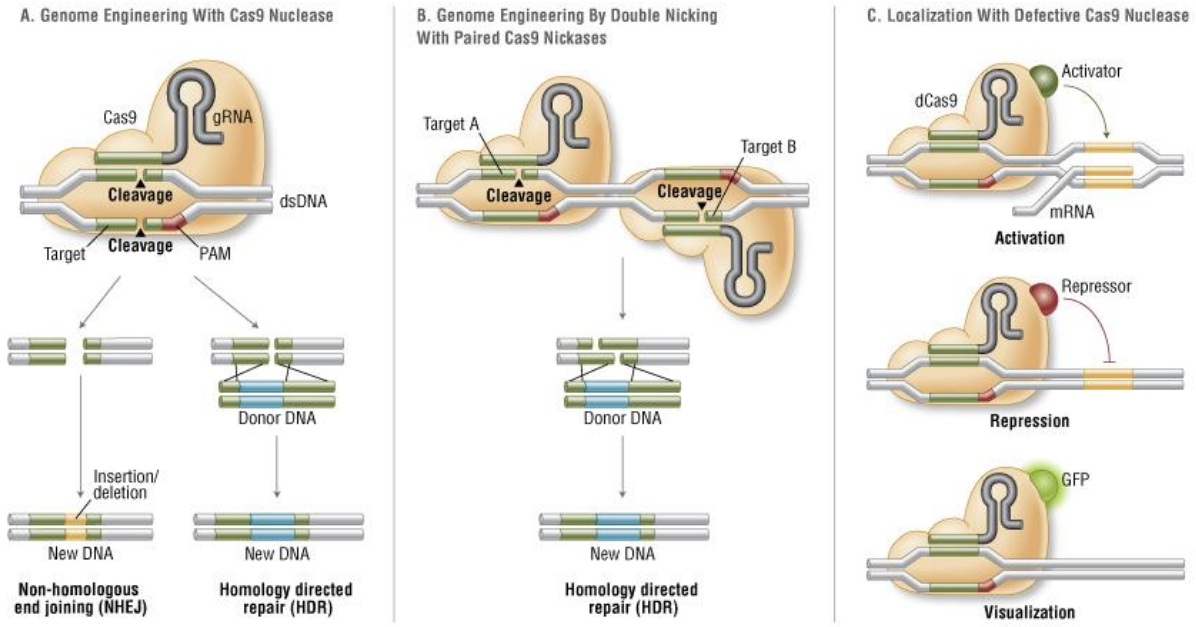

Figure 3: Showing the application CRISPR/Cas9 system in genome editing involving gene correction (fig. 3 A), gene alteration (fig. 3B) and gene regulation (fig. 3C) (Reis et al, 2014)

Mutant Cas9 (Cas9nickase) have been designed to enhance the specificity CRISPR/Cas9 system, it can make a site specific single stranded nick, in which case two sgRNAs are used to introduce a staggered DSB and in the presence of donor DNA induces only HDR pathway **(Fig.3B)** (Reis et al, 2014).

CRISPR/Cas9 systems have also been developed for programmable indigenous gene regulations, where fusion of mutant dCas9 without catalytic domain, with transcriptional activators or repressors can be guided to interfere with RNA polymerase binding at promoter regions to effect gene activation or silencing **(Fig.3C)** (Reis et al, 2014; Rath et al, 2015).

In therapy, CRISPR/Cas9 system has been applied in developing antimicrobial Cas9 to target specific strains of bacteria that showed antibiotic resistance or high level of virulence, also the system has also been demonstrated to repair *cftr* gene in cultured human cells from cystic fibrosis patients (Rath et al, 2017). Cas9 system has been used to cure dominant cataract disorder and Duchenne muscular syndrome by altering genes in mouse germ-line, it holds potential to cure HIV/AIDS and Hepatitis B (Rath et al, 2017) in the same manner used for ZFNs described earlier. In Agriculture, CRISPR/Cas9 technology has been applied in crop improvement where food crops genomes were edited to produce high yield, diseases and pests resistant, and environmental stress resistant varieties (Hilscher et al, 2017).

In industries, starter cultures for improved cheese production has been marketed by Danisco, a company producing starter cultures containing bacteria that have CRISPRs which confer resistance to phage that can cause potential production damage (Rath et al, 2017). In research areas, scientists have been able to investigate the role of individual genes in the functioning of the individual cells and the organism through the introduction of targeted modification to various genomic elements (Rath et al, 2017). The major limitation being attributed to CRISPR/Cas9 system is the need for protospacer adjacent motifs (PAM) sequence at the targeted genome site for any binding between sgRNA and the GOI to occur (Hilscher et al, 2017).

4. Conclusion

Genome editing platforms have revolutionized biotechnology and genetic engineering, emergence of ZFNs and CRISPR/Cas9 technologies as genome editing and engineering nucleases (GEEN) has transformed biological research, targeted editing of genomes in living organisms not only permitted the improvement of productivity and quality of gene products, but also allowed the investigation and unprecedented understanding of the fundamental basis of biological systems, provide solutions to many pending issues in functional genomics, gene therapy and molecular medicine.

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