TARGETED GENOME EDITING: APPROCHES AND APPLICATIONS

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Abstract

The work was an investigation on the genome editing techniques and its applications in various fields. This article reviews the foundational ad fundamental techniques of genome editing such as Zinc- Finger nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated protein 9 (Cas9). It discuss several achievements in genome editing that were made possible by these tools and also the pros and cons of the technique.

Key words : Genome editing, ZFN, TALEN, CRISPR/Cas

Introduction

Targeted Genome editing is the precise manipulation of gene sequences in their natural chromosomal context and addition of transgenes to specific genomic loci. (Pablo Perez- Pinera *et al*., 2013) It is a type of genetic engineering, with the help of specially engineered nucleases by which DNA is inserted, deleted or replaced in the genome of a living cell . These nucleases create site-specific double-strand breaks at desired locations in the genome. The induced double-strand breaks are repaired by the cell's DNA repair pathway through non-homologous end-joining or homologous recombination. This will result in targeted mutations or 'edits'. Genome editing enables the investigators to directly manipulate virtually any gene in the genomes of a diverse range of organisms.

Objectives

- To find out the recent tools used in the field.
- To excel the radical steps in the field of genome editing.
- To analyse the pros and cons due to the editing of genetic material.

Methodology

It is a descriptive study based on the secondary data sources such as various publications and books.

Tools used in genome editing

The tools used in genome editing are different nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and RNA-guided engineered nucleases (RGENs) derived from the bacterial clustered regularly interspaced short palindromic repeat (CRISPR)-Cas (CRISPR-associated) system - enable targeted genetic modifications in cultured cells, as well as in whole animals and plants. However, these nucleases differ in several respects, including their composition, targetable sites, specificities and mutation signatures, among other characteristics. (Kim H. & Kim J. S., 2014)

1) Zinc Finger Nucleases (ZFNs)

Various ZnF motifs will bind DNA, usually in a sequence-specific manner. DNA-binding ZnF proteins are involved in transcriptional processes. The Cys_2-His_2 Zinc-finger domain is among the most common types of DNA-binding motifs found in eukaryotes and represents the second most frequently encoded protein domain in the human genome. An individual zinc-finger consists of approximately 30 amino acids in a conserved ββα configuration. Several amino acids on the surface of the α-helix typically contact three base pairs in the major groove of DNA, with varying levels of selectivity. Zinc-finger proteins can be engineered to make customized DNA-binding proteins. Zinc Finger Nuclease is such an engineered zinc-finger protein.

Each Zinc-Finger Nuclease (ZFN) consists of two functional domains; A DNA-binding domain and a DNA-cleaving domain containing the nuclease domain of Fok 1. The specificity if the ZFN is achieved by manipulating the amino acid sequence in the DNA binding domain. When the DNA-binding and DNA- cleaving domains are fused together, a highly-specific pair of 'genomic scissors' are created. ZFN target sites on the DNA consist of two zinc-finger binding sites separated by a 5-7 base pair spacer sequence. The spacer sequence is the one recognized and cleaved by the endonuclease Fok 1.

ZFN system binds to the target DNA as a dimer. The FOK 1 nuclease cleaves the DNA in between the two ZFNs. The induced double-strand breaks are repaired by the cell's DNA repair pathway through non-homologous end-joining or homologous recombination. This will result in targeted mutations or 'edits'.

According to Urnov F D *et al*., 2010 targeted genome cleavage by engineered, sequence-specific zinc finger nucleases followed by gene modification is now established in human cells and a number of model organisms, thus it open the door to a range of new experimental and therapeutic possibilities.

2) Transcription activators – Like Effector-based Nucleases (TALEN)

Transcription activator-like effector (TALEN) proteins are produced by bacteria in the genus *Xanthomonas*, which are widely distributed plant pathogens. Natural TAL bind to specific sequences of host DNA, altering the infected plants's gene expression in ways that help the bacterium to multiply in the host plant. The natural TAL effector proteins have two distinct domains: an effector domain and an extraordinarily specific DNA-binding domain. DNA- binding domain consists of a variable number of amino acids and recognizes a single DNA base pair. The DNA recognition occurs via 2 hypervariable amino acid residues at positions 12 and 13 within each repeat, called repeat-variable di-residues (RVDs).

Transcription activator-like effector based nuclease (TALEN) systems are a fusion of the DNA binding domain of TALEN derived from the *Xanthomonas sp.* and a restriction endonuclease Fok 1. The structure of the DNA-binding domain can be manipulated to produce a protein domain that can bind specifically to any desired DNA sequence in the genome. The TALEN systems are therefore chimeric proteins capable of precisely targeted DNA manipulation. TALEN system binds to the target DNA as a dimer. The Fok 1 nuclease cleaves the DNA in between the two TALENs. The induced double-strand breaks are repaired by the cell's DNA repair pathway through non-homologous end-joining or homologous recombination. This will result in targeted mutations or 'edits'.

3. CRISPR/Cas System

Bacterial CRISPR system and its functions:

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is an important component of the bacterial immune system that allows bacteria to remember and destroy phages. When phage DNA enters the bacterial cells, a Cas complex recognizes it and cleaves it into small fragments. These repeats were initially discovered in the 1980s in *E. coli*, but their function wasn't confirmed until 2007 by Barrangou and colleagues, who demonstrated that *S. thermophilus* can acquire resistance against a bacteriophage by integrating a genome fragment of an infectious virus into its CRISPR locus.

Three types of CRISPR mechanisms have been identified, of which type 2 is the most studied. In this case, invading DNA from phages is cut into small fragments and incorporated into a CRISPR locus along with a series of short repeats (around 20 bps). The loci are transcribed and transcripts are then processed to generate small RNAs (crRNA- CRISPR RNA), which are used to guide endonuclease (Cas 9) that target invading DNA based on sequence complementarity. Makarova KS *et al*., 2011 designed a computational pipeline for the discovery of novel class 2 variants and they used it to identify six new CRISPR-Cas subtypes.

To achieve site-specific DNA recognition and cleavage, Cas 9 must be complexed with both a crRNA(CRISPR RNA) and a separate trans-activating crRNA or trRNA. The tracrRNA is required for crRNA maturation from a primary transcript encoding multiple pre-crRNAs. The endonuclease activity of Cas9 also requires that a short conserved sequence (2-5 nucleotides) known as protospacer-associated motif (PAM), follows immediately 3'- of the crRNA complementary sequence of the invading phage DNA. PAM helps the bacterium to recognize its own DNA from the phage DNA.

Structure of CRISPR system modified for genome editing:

The simplicity of the type 2 CRISPR nuclease, with only three required components (Cas9, crRNA and trRNA) makes this system useful for genome editing. This potential was realized in 2012 by the Doudna and Charpentier labs. Basically the CRISPER/Cas9 genome editing systems has the following components

- Non-specific endonuclease (Cas9 or Cpf1) to cut the genome end.
- Guide RNA (gRNA) or single guide RNA(sgRNA) to guide the endonuclease to a user-defined cut site on the DNA.

Non-specific endonuclease: The modified CRISPER/Cas system uses only Cas9 endonuclease (or a variant form called Cpf The endonuclease has all the components necessary to, (i) bind to Guide RNA, (ii) bind to Target DNA in the presence of a Guide RNA, (iii) Cleave Target DNA Resulting in a Double –Strand Break(DSB).

Guide RNA (gRNA) or single guide RNA(sgRNA): In the native CRISPR/Cas system, Cas9 is guided to its target sites by two types of RNAs; the crRNA which defines the genomic target for Cas9, and the tracrRNA which acts as a scaffold linking the crRNA to Cas9 and facilitates processing of mature crRNAs from pre-crRNAs derived from CRISPER-mediated genome editing, these two small RNAs have been joined into one RNA sequence known as the guide RNA (gRNA) or single-guide RNA(sgRNA). The gRNA contains both the 20 nucleotide target sequence to direct Cas9 to a specific genomic locus and the scaffolding sequence necessary for Cas9 binding.

i. To date, three different variants of the Cas9 nuclease have been adopted in genome-editing.
ii. The first is wild type Cas9, which can site-specifically cleave double-stranded DNA, resulting in the formation of double strand break (DSB). Double strand break activates the DNA repair machinery. Double strand break can be repaired by the cellular non-homologous end joining(NHEJ) pathway and may result in insertions and deletions. This will disrupt the targeted locus. Alternatively, if a DNA template which is homologous to the targeted locus is supplied, the DSB may be repaired by the homology-directed repair (HDR) pathway allowing for precise replacement of DNA sequences.
iii. Another system uses a mutant Cas9 called Cas9D10A. The mutant Cas9 only has nickase activity. Therefore it cannot make double strand break. It cleaves only one DNA strand (nick) and hence does not activate non-homologous end joining (NHEJ) pathway. But, if a homologous repair template DNA is provided, homology-directed repair (HDR) pathway is activated and causes precise editing of the target DNA.
iv. The third variant is a Cas9 without nuclease activity or nickase activity (Eg: dCas9). But the DNA binding activity is retained. Therefore, this variant can be used to specifically target any region of the genome without cleavage. By fusing with various effector domains, dCas9 can be used either as a gene silencing or activation tool. Furthermore, it can be

used as a visualization tool. For example, dCas9 fused to Green Fluorescent Protein (GFP) to visualize repetitive DNA sequences with a single gRNA.

The CRISPR/Cas system offers several advantages over the ZNF and TALEN mutagenesis strategies.

- Target design simplicity-Because the target specificity relies on RNA complex formation and not protein/DNA recognition, gRNAs can be designed easily and cheaply to target nearly any sequence in the genome specifically.
- Efficiency- the system is super-efficient. Modifications can be introduced by directly injecting RNAs encoding the Cas protein and gRNA into the cell whose genome needs editing.
- Multiplexed mutations- Mutations can be introduced in multiple genes at the same time by injecting them with multiple gRNAs.

Genome Editing: Pros and Cons

1) **CRISPR Could Correct The Genetic Errors That Cause Disease**

Medics hope that on going works in this field will make it possible to treat acquired diseases such as cancer and AIDS completely and also gives a hope that, it will be able to cure many monogenic hereditary diseases such as cystic fibrosis, Huntington's disease, muscular dystrophy etc.

CRISPR has wide application in the field of cancer treatments. CRISPR-Cas9 should be the preferred approach in deciphering the complex components of gene expression leading to any cancer like ovarian cancer, acute myeloid leukemia, cervical cancer, breast cancer, renal cell carcinoma, hepatocellular carcinoma, colorectal cancer, urinary bladder cancer, brain cancer etc. (Zubair Ahmed Ratan *et al*., 2018). In a study on genome editing on HIV co-receptors conducted by Zhepeng Liu *et al*., 2017 they modified the cancerous cells into the normal ones. Researchers have proven that it is possible to use CRISPR tool in human lung cells derived from patients with cystic fibrosis and fix the most common mutation behind the disease which are located in a gene called CFTR. Similarly research in mice has shown CRISPR technology could be used to fix the genetic mutations behind the muscular dystrophy.

Genome editing has potential for the targeted correction of germline mutations. According to Ma Hong *et al*., 2017 about "The correction of pathogenic gene mutation in human embryo". They corrected the heterozygous *MYBPC3* mutation in human pre-implantation embryos with precise CRISPR–Cas9-based targeting accuracy and high homology-directed repair efficiency by activating an endogenous, germline-specific DNA repair response. They induced double-strand breaks (DSBs) at the mutant paternal allele and were predominantly repaired using the homologous wild-type maternal gene instead of a synthetic DNA template. By modulating the cell cycle stage at which the DSB was induced, they were able to avoid mosaicism in cleaving embryos and achieve a high yield of homozygous embryos carrying the wild-type *MYBPC3* gene without evidence of off-target mutations.

2) **CRISPR Can Eliminate the Microbes That Cause Disease**

For example, powerful gene editing techniques could be used to eradicate the mosquito species *Aedes aegypti*, a major carrier of many diseases like dengue, chikungunya, yellow fever and Zika virus. Researchers from the University of California, developed mosquitoes whose germlines express the Cas9 enzyme in a more stable way. The result is a yellow, three-eyed, wingless mosquito, made possible through disruptions in the insect's cuticle, wing, and eye development. These transgenic mosquitoes are now more susceptible to the use of CRISPR-Cas9 to facilitate edits that could lead to the eventual eradication of the species and can eliminate the organisms that cause dieseases. (Kistler KE *et al*., 2015)

3) **CRISPR Could Resurrect Species**

Concept of de-extinction is well known in the scientific community. Attempts to revive the woolly mammoth or some genetically modified version of it, have been going on for even longer. Japanese scientists have been trying to clone a woolly mammoth for more than 15 years, an effort has tracked since 2011. George Church's lab at Harvard University's Wyss Institute reported their first successes in editing living elephant cells so that they contain gene sequences from the elephant's recently extinct relative, the woolly mammoth. Using a CRISPR (clustered regularly interspaced short palindromic repeats)-Cas 9 approach, Church's team replaced 14 loci in the elephant genome with the mammoth version of those sequences. Although they have not yet created a mammoth, the works are still going on. (Beth Shaprio., 2015)

4) **CRISPR Could Create New, Healthier Foods(in agriculture)**

Conventional plant breeding is unlikely to meet increasing food demands and other environmental challenges. By contrast, CRISPR technology is erasing barriers to genome editing and could revolutionize plant breeding**.** (Gao, Caixia., 2018). Genome editing in crops can leads to the production of breed resilient, high yield plants to combat famine.

5) **CRISPR Could Eradicate The Planet's Most Dangerous Pest**

At the Imperial College London in 2016, a team of researchers used CRISPR to target female reproduction of the type of mosquito that carries malaria through a gene drive system that influenced female-sterility traits into being more likely to be inherited. They identified three genes (*AGAP005958*, *AGAP011377* and *AGAP007280*) that confer a recessive female-sterility phenotype upon disruption, and inserted into each locus CRISPR-Cas9 gene drive constructs designed to target and edit each gene. For each targeted locus they observed a strong gene drive at the molecular level, with transmission rates to progeny of 91.4 to 99.6%. **(**Hammond, Andrew *et al*., 2016)

Challenges:

- Genome editing has the power to alter DNA; the source code of life itself. It may bring many ethical questions and concerns. The current trend in the advancement depicts the fact that we are not far away from the "designer humans". Ethicists fear that this could lead to "designer humans", which in turn violates the natural selection.
- Off-targeted genome editing may result in unintended genetic modification.
- Understanding and improving specificity of editing tools.
- Resurrection of new species may imbalance the biotic – abiotic relationships and may result in ecological problems.
- Misuse of this technique might create lethal conditions that could destroy even the human civilization itself.

Conclusion

The enormous excitement surrounding the technique genome editing needs to be coupled with strategic planning and enabling regulatory processes to ensure successful development of the classes like agriculture, medicine etc. Recent progress in the field is finally providing optimism for additional successes in the near future. The accelerating pace of technological advances and broad range of basic science and clinical applications, makes the road ahead undoubtedly an exciting one.

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