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CRISPR-Cas9 – EDITOR OF DNA SEQUENCE IN ANY GENOME

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Clustered regularly interspaced palindromic repeats (CRISPR - Cas9) is a unique technology that enables geneticists and medical researchers to edit parts of the genome by removing, adding or altering sections of the DNA sequence. It makes it possible to correct errors in the genome and turn on or off genes in cells and organisms quickly, cheaply and with relative ease. Clustered regularly interspaced palindromic repeats (CRISPR) refers to sequences in the bacterial genome. They afford protection against invading viruses, when combined with a series of CRISPR-associated (Cas) proteins. Cas9, one of the associated proteins, is an endonuclease that cuts both strands of DNA. Cas9 is directed to its target by a section of RNA. This can be synthesised as a single strand called a synthetic single guide RNA (sgRNA); the section of RNA which binds to the genomic DNA is 18–20 nucleotides. In order to cut, a specific sequence of DNA of between 2 and 5 nucleotides (the exact sequence depends upon the bacteria which produces the Cas9) must lie at the 3’ end of the guide RNA: this is called the protospacer adjacent motif (PAM). Repair after the DNA cut may occur via two pathways: non-homologous end joining, typically leading to a random insertion/deletion of DNA, or homology directed repair where a homologous piece of DNA is used as a repair template. It is the latter which allows precise genome editing: the homologous section of DNA with the required sequence change may be delivered with the Cas9 nuclease and sgRNA, theoretically allowing changes as precise as a single base-pair.

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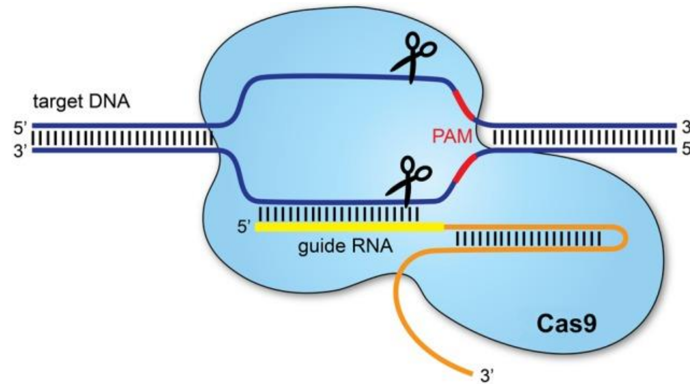

OVERVIEW OF CRISPR/Cas9

CRISPR/Cas9 is a gene-editing technology which involves two essential components:

- A guide RNA to match a desired target gene, and
- Cas9 (CRISPR-associated protein 9) - an endonuclease which causes a double-stranded DNA break, allowing modifications to the genome (Fig. 1).

Genome editing Vs CRISPR-Cas9

Genome editing (also called gene editing) is a group of technologies that give scientists the ability to change an organism's DNA. These technologies allow genetic material to be added, removed, or altered at particular locations in the genome. Several approaches to genome editing have been developed. A well-known one is called CRISPR-Cas9, which is short for clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9. The CRISPR-Cas9 system has generated a lot of excitement in the scientific community because it is faster, cheaper, more accurate, and more efficient than other genome editing methods.

CRISPR-Cas9 was adapted from a naturally occurring genome editing system that bacteria use as an immune defense. When infected with viruses, bacteria capture small pieces of the viruses' DNA and insert them into their own DNA in a particular pattern to create segments known as CRISPR arrays. The CRISPR arrays allow the bacteria to "remember" the viruses (or closely related ones). If the viruses attack again, the bacteria produce RNA segments from the CRISPR arrays that recognize and attach to specific regions of the viruses'

DNA. The bacteria then use Cas9 or a similar enzyme to cut the DNA apart, which disables the virus.

Researchers adapted this immune defense system to edit DNA. They create a small piece of RNA with a short "guide" sequence that attaches (binds) to a specific target sequence in a cell's DNA, much like the RNA segments bacteria produce from the CRISPR array. This guide RNA also attaches to the Cas9 enzyme. When introduced into cells, the guide RNA recognizes the intended DNA sequence, and the Cas9 enzyme cuts the DNA at the targeted location, mirroring the process in bacteria. Although Cas9 is the enzyme that is used most often, other enzymes (for example Cpf1) can also be used. Once the DNA is cut, researchers use the cell's own DNA repair machinery to add or delete pieces of genetic material, or to make changes to the DNA by replacing an existing segment with a customized DNA sequence.

CRISPR-Cas 9 Vs Human Genome

Genome editing is of great interest in the prevention and treatment of human diseases. Currently, genome editing is used in cells and animal models in research labs to understand diseases. Scientists are still working to determine whether this approach is safe and effective for use in people. It is being explored in research and clinical trials for a wide variety of diseases, including single-gene disorders such as cystic fibrosis, haemophilia, and sickle cell disease. It also holds promise for the treatment and prevention of more complex diseases, such as cancer, heart disease, mental illness, and human immunodeficiency virus (HIV) infection.

Ethical concerns arise when genome editing, using technologies such as CRISPR-Cas9, is used to alter human genomes. Most of the changes introduced with genome editing are limited to somatic cells, which are cells other than egg and sperm cells (germline cells). These changes are isolated to only certain tissues and are not passed from one generation to the next. However, changes made to genes in egg or sperm cells or to the genes of an embryo could be passed to future generations. Germline cell and embryo genome editing bring up a number of ethical challenges, including whether it would be permissible to use this technology to enhance normal human traits (such as height or intelligence). Based on concerns about ethics and safety, germline cell and embryo genome editing are currently illegal in the United States and many other countries.

Genome Editing Methods

Scientists have had the knowledge and ability to edit genomes for many years, but CRISPR technology has brought major improvements to the speed, cost, accuracy, and efficiency of genome editing. The history of genome editing technologies shows the remarkable progress in this field and also relays the critical role that basic science research plays in the development of research tools and potential disease treatments.

Homologous recombination

The earliest method scientists used to edit genomes in living cells was homologous recombination. **Homologous recombination** is the exchange (recombination) of genetic information between two similar (homologous) strands of DNA (Capecchi 2005). Scientists began developing this technique in the late 1970s following observations that yeast, like other organisms, can carry out homologous recombination naturally.

To perform homologous recombination in the laboratory, one must generate and isolate DNA fragments bearing genome sequences similar to the portion of the genome that is to be edited. These isolated fragments can be injected into individual cells or taken up by cells using special chemicals. Once inside a cell, these DNA fragments can then recombine with the cell's DNA to replace the targeted portion of the genome.

This type of homologous recombination is limited by the fact that it is extremely inefficient in most cell types. This technique can have as low as a one-in-a-million probability of successful editing. Another weakness of homologous recombination is that it is inaccurate and has a high rate of error when the injected DNA fragments insert into an unintended part of the genome, causing what are known as off-target edits (Vasquez *et al.,* 2001).

Zinc-finger nucleases (ZFN)

In the 1990s researchers started using **zinc-finger nucleases (ZFN)** to improve the specificity of genome editing and reduce off-target edits. The structures of ZFNs are engineered from naturally-occurring proteins that were discovered in eukaryotic organisms. Scientists can engineer these proteins to bind to specific DNA sequences in the genome and cut DNA. Once bound to their target DNA sequence, the ZFNs cut the genome at the

specified location, allowing scientists to either delete the target DNA sequence or replace it with a new DNA sequence via homologous recombination.

Although ZFNs improved the success rate of genome editing to about 10 percent, it is difficult and time-consuming to design, construct, and produce successful zinc finger proteins, and a new ZFN must be engineered for each new target DNA sequence.

Transcription activator-like effector nucleases (TALENs)

In 2009, a new class of proteins called **Transcription Activator-Like Effector Nucleases (TALENs)** arrived to the genome editing scene. Similar to ZFNs, transcription activator-like effector nucleases (TALENs) are engineered from proteins found in nature and are capable of binding to specific DNA sequences.

While TALENs and ZFNs are comparable in terms of how efficiently they can create edits to the genome, TALENs bear the advantage of greater simplicity. It is much easier to engineer TALENs than it is to synthesize ZFNs (Joung and Sander 2013).

***Clustered regularly interspaced short palindromic repeats* (CRISPR)**

Though ZFN and TALEN technology increase the specificity and efficiency of genome editing, they are relatively expensive and complicated to use in the lab. Each edit would require the construction of a new ZFN or TALEN protein, and engineering proteins can be a difficult process that is prone to error. This is one reason why CRISPR is a game-changing technology; unlike its predecessors, CRISPR is a simple technology with little assembly required.

CRISPR associated DNA sequences were first observed in bacteria in the early 1990s, but it was not until the 2000s that the scientific community understood its ability to recognize specific genome sequences and cut them via the Cas9 protein, a protein that works with CRISPR and that has DNA-cutting abilities. In nature, CRISPR is used by bacteria as an immune system to kill invading viruses, but it has now been adapted for use in the lab (Lander 2016).

With CRISPR, researchers create a short RNA template that matches a target DNA sequence in the genome. Creating synthetic RNA sequences is much easier than engineering proteins as is those required for ZFNs and TALENs. Strands of RNA and DNA can bind to each other when they have matching sequences. The RNA portion of the CRISPR, called a

guide RNA, directs Cas9 enzyme to the targeted DNA sequence. Cas9 cuts the genome at this location to make the edit. CRISPR can make deletions in the genome and/or be engineered to insert new DNA sequences. One group of scientists found that CRISPR is six times more efficient than ZFNs or TALENs in creating targeted mutations to the genome (Varshney 2015). Large-scale genomics projects that once took many years and tens of thousands of dollars can now be completed at a small fraction of time and price.

CRISPR - Mechanism

The CRISPR molecule is made up of short palindromic DNA sequences that are repeated along the molecule and are regularly-spaced. Between these sequences are "spacers", foreign DNA sequences from organisms that have previously attacked the bacteria. The CRISPR molecule also includes CRISPR-associated genes, or Cas genes. These encode proteins that unwind DNA, and cut DNA, called helicases and nucleases, respectively (Jinek *et al.,* 2012).

The CRISPR immune system protects the bacteria from repeated virus attacks through three steps:

1. **Adaptation** – When DNA from a virus invades the bacteria, the viral DNA is processed into short segments and is made into a new spacer between the repeats. These will serve as genetic memory of previous infections.

2. **Production of CRISPR RNA** – The CRISPR sequence undergoes transcription, including spacers and Cas genes, creating a single-stranded RNA. The resulting single-stranded RNA is called CRISPR RNA, which contains copies of the invading viral DNA sequence in its spacers.

3. **Targeting** – The CRISPR RNAs will identify viral DNA and guide the CRISPR-associated proteins to them. The protein then cleaves and destroys the targeted viral material.

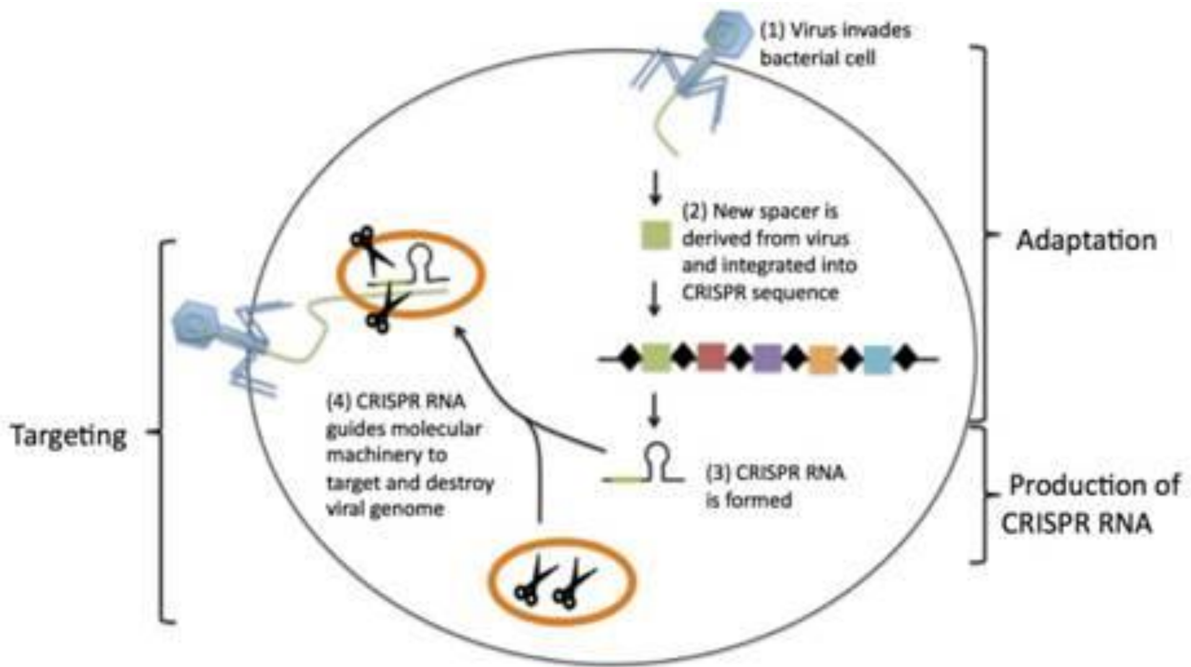

Fig. 2. The steps of CRISPR-mediated immunity (Jinek *et al.,* 2012)

Scientists make use of the CRISPR-Cas9 systems' recognition of specific DNA sequences and apply it in the process of development of improved crops. Instead of viral DNA as spacers, scientists design their own sequences, based on their specific gene of interest. If a gene's sequence known, it can be easily used in CRISPR. It will then act just like a spacer for the system and guide the Cas9 protein to a DNA matching sequence.

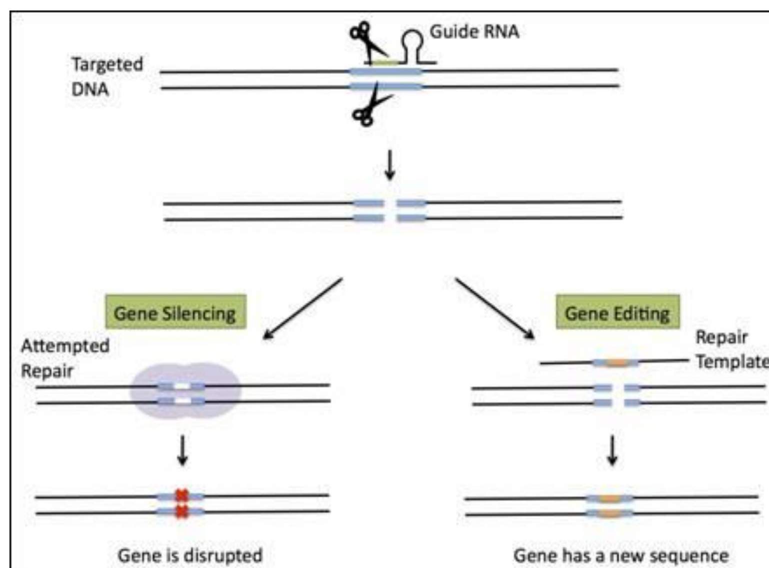

Fig. 3. Mechanism of Gene Editing of CRISPR/Cas96

CRISPR-Cas9 Allows Researchers to Perform the Following

Gene Knock-Out

Gene silencing using CRISPR starts with the use of a single guide RNA (sgRNA) to target genes and initiate a double stranded break using the Cas9 endonuclease. These breaks are then repaired by innate DNA repair mechanisms, the non-homologous end-joining (NHEJ). However, NHEJ is error-prone and results in genomic deletions or insertions, which then translates into permanent silencing of the target gene (Cong *et al.,* 2013).

DNA-Free Gene Editing

CRISPR can be used for DNA-free gene editing without the use of DNA vectors, requiring only RNA or protein components. A DNA-free gene editing system can be a good choice to avoid the possibility of undesirable genetic alterations due to the plasmid DNA integrating at the cut site or random vector integrations (Cong *et al.,* 2013).

Gene Insertions or “Knock-ins”

The CRISPR-induced double-strand break can also be used to create a gene “knock-ins” by exploiting the cells’ homology-directed repair. The precise insertion of a donor template can alter the coding region of a gene. Previous studies have demonstrated that single-stranded DNA can be used to create precise insertions using CRISPR-Cas9 system (AddGene 2014).

Transient Gene Silencing

By modifying the Cas9 protein so it cannot cut DNA, transient gene silencing or transcriptional repression can also be done. The modified Cas9, led by a guide RNA, targets the promoter region of a gene and reduces transcriptional activity and gene expression. Transient activation or upregulation of specific genes can be effectively done (Cong *et al.,* 2013).

Applications in Agriculture

Researchers have found that the CRISPR - Cas9 system can be applied to nearly every organism. Early studies using CRISPR - Cas9 for gene editing have focused on crops important for agriculture. It was realized early on that the system could be used in crops to improve traits, such as yield, plant architecture, plant aesthetics, and disease tolerance.

Rice

CRISPR has been used to edit the genome of rice. The team of Ying Wang from Syngenta Biotechnology China designed several CRISPR sgRNAs and successfully deleted fragments of the *dense and erect panicle1* (*DEP1*) gene in the Indica rice line IR58025B. Improvements in yield-related traits, such as dense and erect panicles and reduced plant height, were observed in the mutant plants produced (Wang *et al.,* 2017).

Soybean

A team of researchers from the Chinese Academy of Agricultural Sciences led by Yupeng Cai also used the CRISPR-Cas9 system to induce mutations on *GmFT2a*, an integrator in the photoperiod flowering pathway of soybean. The developed soybean plants showed late flowering, resulting in increased vegetative size. The mutation was also found to be stably inherited in the following generation (Cai *et al.,* 2017).

Vegetables

Researchers from Beijing Key Laboratory of Vegetable Germplasm Improvement, led by Shouwei Tian used CRISPR-Cas9 to target *ClPDS*, the phytoene desaturase in watermelon, to achieve the albino phenotype. All genome-edited watermelons harbored mutations in *ClPDS* and showed full or mosaic albino phenotype. This study served as a proof of concept of using the CRISPR-Cas9 system in watermelon breeding (Tian *et al.,* 2016).

Citrus

Researchers from the Chinese Academy of Agricultural Sciences and National Center for Citrus Variety Improvement and Southwest University have also developed citrus plants resistant to citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), a serious disease of citrus, through CRISPR-Cas9. The team targeted the promoter of the *CsLOB1* gene, which promotes canker development in citrus. The developed lines showed enhanced resistance to citrus canker compared to wild types (Peng *et al.,* 2017).

Tomato

Cold Spring Harbor Laboratory, together with various research institutions, also used CRISPR-Cas9 to generate mutations in the flowering suppressor *SELF-PRUNING5G* (*SP5G*)

in tomato to manipulate photoperiod response. The mutations brought about by CRISPR-Cas9 caused rapid flowering and enhanced the compact growth habit of field tomatoes, resulting in a quick burst of flower production and early yield (Soyk *et al.,* 2017).

Humans

Researchers from the University of California also used CRISPR for studies in gene therapy. Using CRISPR-Cas9, they corrected mutations associated with the genetic disease, β-thalassemia by creating induced pluripotent stem cells (iPSCs) from the β-thalassemia patients. The team used CRISPR-Cas9 to correct mutations in the human hemoglobin beta (HBB) in patient iPSCs, resulting in gene-corrected iPSCs with restored expression of the HBB gene, which can be used for gene therapy (Xie *et al.,* 2014).

US scientists are also studying the use of CRISPR for treating the Human Immunodeficiency Virus (HIV). They used CRISPR to edit the HIV genome out of immune cells, called T cells, from an HIV patient. Scientists found that CRISPR can prompt the HIV virus to mutate. However, more studies are still needed before CRISPR can be used to treat HIV.

Limitations

CRISPR/Cas is an extremely powerful tool, but it has important limitations as follows.

- difficult to deliver the CRISPR/Cas material to mature cells in large numbers, which remains a problem for many clinical applications. Viral vectors are the most common delivery method.
- not 100% efficient, so even the cells that take in CRISPR/Cas may not have genome editing activity.
- not 100% accurate, and “off-target” edits, while rare, may have severe consequences, particularly in clinical applications.

Conclusion

CRISPR has played a huge part in the increase in genome editing studies in recent years. The system has broad applications in plant and animal improvement, as well as in the medical field. As a relatively young technique, various discoveries and innovations for its efficient use in wider applications are in the offing.

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