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CRISPR-Cas9: A new approach to genome editing and clinical treatment

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Abstract
The development of clustered short palindromic repeats within the CRISPR/Cas system for genome editing has opened up new possibilities for direct targeting and modification of genome sequences in all eukaryotic cells. This opens up new perspectives in the field of biotechnology and new treatment options for human diseases. The year 2012 was of crucial importance, as at that time CRIPSR-Cas9 was introduced in an interesting way, which gave hope to patients with inherited diseases, as more competent work was now being done to improve various strategies and models of the CRISPR-Cas system. The CRISPR-Cas9 genome editing system, its applications for treatment and delivery, and future prospects are summarized in this review. Although there are still concerns about the efficiency and safety of this method, its success cannot be denied, nor can the fact that genome editing in this way will soon be used in clinical practice, making the work and the treatment itself much easier.

Keywords: CRISPR-Cas9, Genome editing, Clinical treatment, *In vivo*, *Ex vivo*

1. Introduction

Genome editing, also known as gene editing, is a set of techniques that let scientists change an organism's DNA. [1]. This gives them the ability to add, remove or change genetic material at specific locations. There are several ways to edit the genome, the best known being CRISPR-Cas9 (clustered short palindromic repeats) [2]. The name CRISPR is derived from the clustered, regularly spaced short palindromic repeating sequence that serves as an adaptive immune system to help bacteria protect themselves from foreign DNA [3]. The system uses the Cas9 protein and a CRISPR locus in the genome. As a genomic locus for tandem repeat sequences, CRISPR is well-known. Both small transcoded CRISPR RNA sequences and CRISPR RNA sequences for non-coding RNA elements are represented by a combination of Cas9 genes at these loci. These two sequences together produce a guide RNA that is responsible for determining the way the sequence is separated in the nucleic acid, and it achieves this with the help of the Protospacer Adjacent Motif (PAM), a 5'-NGG sequence [4]. The protospacer region is a place where double-stranded DNA splits. The Cas9 protein is an endonuclease that binds to CRISPR loci and is essential for causing double-strand breaks (DSBs) at the site of target RNA [5]. Targeted genome editing uses nuclease-mediated DNA cleavage with customized nucleases to create desired endogenous modifications like gene disruption, addition, or correction at specific genomic sites [3]. According to Doudna J.A. & Charpentier E. [6], the CRISPR-Cas9 system has resulted in significant breakthroughs in biology, primarily due to its accessibility and simplicity. It has attracted a lot of attention from many people due to its speed, precision, cheapness, and more efficient operation compared to other genome editing methods [1]. It arose from a natural system used to edit the genome that bacteria need for immune defense [7]. Because of this, the so-called CRISPR arrays are formed when bacteria are infected with viruses and insert small portions of the virus's DNA into their own DNA. These sequences are crucial

because they enable bacteria to remember viruses and, in the event that they are retrieved, produce RNA segments from the aforementioned CRISPR sequences and bind to specific regions of the virus's DNA. After that, the bacterium uses Cas9 to cleave the DNA and thus prevent the virus from developing [8]. This system has attracted the attention of a lot of scientists because it has a huge advantage in editing the genome. As a result, they have begun to see it as a powerful treatment for diseases caused by genome mutation. According to Zhan T et al. [9] The primary objective of CRISPR-Cas9 therapy in the treatment of cancer is to remove the malignant mutation and then replace it with normal DNA sequences.

1.1. History of CRISPR-Cas9 system

The CRISPR sequence was discovered in the genome of *E. coli* in 1987 by researchers who discovered regularly arranged repetitions of unknown functions [10]. Two bioinformatics studies were discovered in 2002, one of which showed the presence of conserved operons that encoded a new DNA repair system [11] known as Cas genes and the other of which represented a link between the Cas genes and CRISPR arrays [11], they came to the conclusion that the CRISPR arrays might be a component of the immunity utilized by the corresponding phage after they observed that the sequences that lie between the CRISPR repeats correspond to the sequences that are found in the genomes of the phages. In research with *Streptococcus thermophilus*, multiple spacers were found to match phage sequences. As a result, a CRISPR-related protein with the HNH domain for DNA cleavage (Cas9) was identified [12]. The findings demonstrated that CRISPR-Cas is an adaptive immune system based on microbiology. The initial hypothesis [13] was that these systems used a mechanism like RNAi to interfere, but evidence showed that DNA, not RNA, was the target. A subsequent study revealed that Cas9-mediated interference was highly dependent on the protospacer adjacent motif (PAM), a short sequence motif at the end of CRISPR targets [14]. The Cas9 protein is the only one required for DNA cleavage, and it was demonstrated in 2010 that *S. thermophilus* Cas9 is driven by crRNA to create blunt double-stranded breaks (DSBs) in DNA upstream of PAM [15].TracrRNA, a small RNA that is associated with the CRISPR array, was discovered in 2011. TracrRNA is responsible for the formation of duplexes on pre-crRNA with direct repeat sequences, which are necessary to produce mature crRNA, as well as for interfering with Cas9 [16]. This year, another study found that the CRISPR-Cas locus could be expressed in E. coli, facilitating interference with plasmid DNA [17].We came to the conclusion that the natural Cas9 system's nuclease complex consists of three components—Cas9, crRNA, and tracrRNA—and that a suitable PAM must be followed by the target DNA site [18] and later for the inoculation of the *Thermophilus virus* alongside the *S virus*, which can further be used in the dairy industry for the production of cheese and yogurt [19]. Over time, this system became more adapted for use. It has also been suggested that it could be used to prevent antibiotic resistance, target DNA destruction, and imprint genes in microorganisms.

1.2. Development of CRISPR-Cas9 for genome editing

As the genome undergoes new modifications, new opportunities arise for expanding our knowledge of biology and human health, as well as for developing novel treatments for serious illnesses. Homologous recombination was used to insert genes into mice in 1987 [20], which was an extremely large development, particularly on the grounds that CRISPR was distributed without precedent for that very year, yet it worked out that the productivity in warm blooded creatures was extremely low beyond undeveloped foundational microorganisms. According to Urnov FD. Et al. [21], all of this encouraged the development of targeted nucleases (TALE, meganucleases, and zinc finger nucleases) that are adaptable in recognizing a specific DNA sequence and have the capacity to generate DSBs at specific loci, greatly facilitating genome editing. Due to the fact that these technologies had a limited capacity, it was difficult to reprogramme them in practice, reducing their influence significantly. Zhang et al, [22] worked with zinc finger nucleases and began developing TALE for use in mammalian cells with his assistant Le Chong [23]. After hearing Michael Gilmore talking about his research on *Enterococcus*, claiming that Enterococcus has a new class of nucleases, called CRISPR-Cas system. After reading and researching a lot, he realized that reprogramming CRISPR-Cas was much simpler than reprogramming TALE, so he decided to focus on modifying Cas9 to edit genomes in eukaryotic cells. According to Garneau C et al. [15], it was already known that Cas9 can cleave its own DNA in bacterial cells when guided by crRNA. According to a large body of research, the natural Cas9 system's nuclease complex consists of three components (Cas9, crRNA, and tracrRNA) [24]. Because of this, Zhang attempted to obtain an answer to the question of whether a human cell culture system can be used to make

Cas9 capable of editing the genome in eukaryotic cells. As a result, he was able to create a genome-editing three-component CRISPR-Cas9 system made up of Cas9, crRNA, and tracrRNA while working with eukaryotic cells. His method was primarily based on CRISPR literature syntheses that demonstrated the necessity of these three components for bacterial function [23]. Jinek et al. [25] published a comprehensive analysis of the mechanism of Cas9 in vitro following the experiments and their findings. According to Garneau C. [15], they discovered that the purified Cas9-crRNA-tracrRNA complex of *S. pyogenes* is responsible for DNA cleavage 3 bp upstream of the target site's PAM. This finding is consistent with previous in vivo results with *S. thermophilus*. Next, they discovered that Cas9-crRNA and tracrRNA complexes highly rely on tracrRNA and crRNA for target cleavage [25]. In addition, tracrRNA truncation demonstrated that the Cas9-crRNA-tracrRNA complex can extend robust double-stranded RNA-guided DNA cleavage in vitro with only a short tracrRNA fragment [26]. Further, this study discovered that the Cas9-crRNA-tracrRNA complex stops DNA cleavage because of single-base mutations in the PAM and the 3' region of the leader sequences, but not because of a single-base mismatch close to the 5' region of the leader RNA. The biochemical analysis yielded comparable findings three months later [26]. Based on the mentioned information, we can conclude that this work provided an incomplete picture of the mechanism of Cas9 for *in vitro* cleavage and did not identify the requirements for tracrRNA for Cas9 function because it demonstrated that the study purified Cas9-crRNA complexes from bacteria without analyzing the components of the complex. Since this work was done completely *in vitro*, it didn't uncover the basic parts for accomplishing hearty genome altering in cells. Therefore, researchers questioned whether Cas9 could be used in eukaryotic cells. Jinek et al. established the basis for the molecular mechanism by which CRISPR-Cas9 will mediate genome editing with the assistance of his assistants after a series of analyses and obtained results.

1.3. CRISPR-based genome editing mechanisms

An RNA-guided endonuclease system is the name given to the CRISPR-Cas system, which consists of Cas enzymes and an RNA guide (Fig.1A) [27].

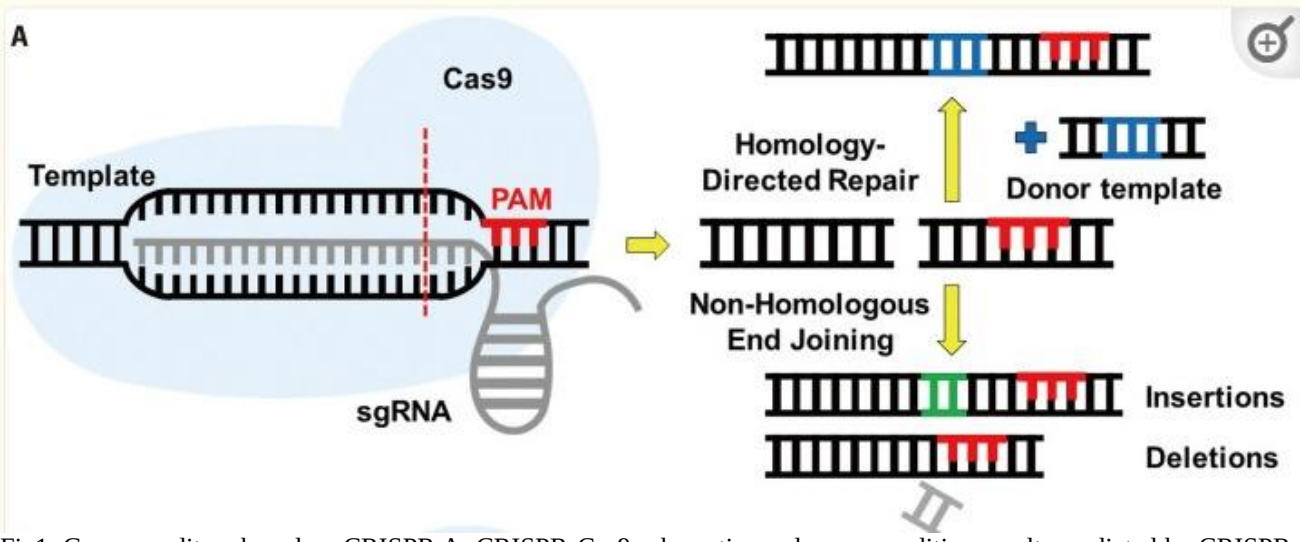

Fig1. Genome editors based on CRISPR.A: CRISPR-Cas9 schematics and genome editing results mediated by CRISPR-Cas9 [27]

According to Kim H. [28], the Cas enzyme is responsible for modifying the template by causing a double strand break at a specific location. On the other hand, the guide RNA is in charge of delivering the Cas enzyme to the target sequence using Watson-Crick's base pairing method. According to Kim H. [28], crRNA and tracrRNA are made by single-stranded guide RNA (sgRNA), which can be easily designed, making the system programmable. The Cas enzyme that initiates its activity can recognize a brief sequence known as the Protospacer Adjacent Motif (PAM) that follows the protospacer. The PAM is connected to the location where the double chain breaks, and in the case of Cas9, it typically occurs three bases upstream of the PAM (Fig. 1A) [3]. The CRISP-Cas system is responsible for the repair of double-stranded breaks, which is when genome editing takes place.

Repair is carried out in two ways: a repair directed by homology and a repair directed by non-homologous end-joining (NHEJ), both of which are common in mammalian cells (Fig.1A) [3]. A donor template that is homologous to the context sequence is required for this homology-directed repair strategy. This donor template further integrates into the location of the double-strand break, allowing for precise genome editing. The CRISPR-Cas system has significantly increased the efficiency of the genome editing process, which takes a significant amount of time. The precise corrections and insertions of the mutation or gene of interest that can be accomplished with homology-directed repair are all contingent on the co-delivery of the donor template. Their effectiveness is very low, and the process only occurs in dividing cells. However, NHEJ repairs double-strand breaks in a stochastic manner, resulting in tiny insertions or deletions at the double-strand break site. The repair, which frequently results in frame-shift mutations and a loss of target gene function, has varying effects. Although this strategy is unable to produce precise mutations, it has the advantage of not requiring a donor template, being able to be found in both dividing and non-dividing cells, and generally being more effective than the first one.

1.3.1. The development of Cas9 variants

Numerous Cas9 variants were discovered or created when the CRISPR-Cas9 system was first introduced. The first was found in a variety of bacterial species. SpCas9, a protein derived from Streptococcus pyogenes [29] was used for genome editing for the first time. Later, Streptococcus aureus, Neisseria meningitides, Campylobacter jejuni were discovered, which differ in protein size and can recognize various PAM sequences. Additionally, various types of Cas enzymes have been discovered, allowing for the modification of various templates. Cas9 and Cas12 are used to cut the DNA sequence, while the Cas13 enzyme is used for the RNA sequence [30]. There are also high-fidelity variants (eSpCas9, SpCas9-HF1, evoCas9 and HypaCas9) that are responsible for increasing the specificity, but also the clinical safety of genome editing results. There are also variants that recognize different types of Pam, which are responsible for expanding the range of targeting of Cas9 [35].

1.4. Advancing biological research

Because of their simplicity and accessibility, all tools based on CRISPR-u are widely used in the life sciences. Technologies based on CRISPR have had an impact on the progress of biological studies by creating plant, animal and cell models [36]. These types of technologies have reduced the work and time required to modify genomes in eukaryotic organisms and cell lines [37]. They also made a large number of organisms genetically susceptible, such as parasites, microorganisms, wasps, and crustaceans. This method made it possible to study numerous biological processes in their natural context, which is important for the advancement of biological studies in all fields. By replicating the genetic variants that are found in human patients, CRISPR-based technologies are used to create individualized animal and cell models. These models extraordinarily worked with the comprehension of the sub-atomic pathology of countless human infections, as the need might have arisen for treatment.

1.4.1. Presenting new opportunities for agricultural and plant science

CRISPR technology also affected the biology of plants, where it made changes in their breeding and thus reduced the time for creating genotypes. With the help of this method, it is possible to produce a homozygous knockout mutant in one generation [41]. Because it is difficult to use conventional methods of genetic manipulation, the Cas enzyme's inherent ability to edit polyploid genomes (like wheat's) is very helpful. Until today, gene knockout mediated by CRISPR is widely represented in numerous agricultural crops (barley, rice, soybeans, wheat, tomatoes), with the effort of which they are trying to construct these plants in order to achieve a number of features, such as resistance to drought, to pathogens, increased yields, as well as shortened time until ripening itself [42]. According to Bewg WP [43], this strategy has also been successful in woody plants—plants that have traditionally been out of reach for targeted gene changes. The number of plant biology-specific technology tools based on CRIPSR is growing. Cas12a has been shown to be very effective in plants [44] and Cas13a has been used to interfere with RNA viruses, which has created a new way to give immunity [45]. CRISPR-mediated genome editing has resulted in the creation of numerous crop strains, including gluten-reduced wheat, higher-yielding tomatoes, and decaffeinated coffee.

1.5. Promoting health for people

According to Cox DB [46], the capacity to precisely alter the genome as well as edit DNA and RNA holds enormous promise for enhancing human health and providing a platform that can adapt to any genetic disorder. A set of effective Cas enzymes and tools with delivery modalities, which can be combined to provide an answer to the specific challenges of individual diseases, will be required to realize this type's potential. There have been a lot of editing tools made so far, many of which are used in clinical trials. However, there are still problems that need to be solved before CRISPR-based therapies can be developed.

1.5.1. Applications for the elimination of pathogens caused by bacteria and viruses

By producing novel antibacterial agents, CRISPR-Cas systems are designed to improve human health in a variety of ways. By generating programmable sequence-specific antimicrobial agents, they can be used to selectively treat target bacteria when packaged into phages [47]. This system can be used to treat viral infections, according to several studies. In a cell model of HIV, it has been demonstrated that Cas9 can not only target integrated HIV virus copies but also prevent HIV infection [48]. Endogenous retroviruses can be eliminated from pigs using CRISPR-based technologies, resulting in animals that could be a good source of organs for transplants into human patients [49].

1.5.2. Applications for the detection of viruses and bacteria

The creation of CRIPSR-based diagnostics is another method for improving human health. Sensitive genotyping, the detection and monitoring of infectious diseases, and the detection of target molecules of interest at low levels all benefit from these systems [50]. In addition, this diagnostic can be utilized in industrial and agricultural settings to safeguard food safety and stop the spread of contaminating agents. The detection system based on CRIPSR is growing quickly. All CRIPSR-based assays can be quickly optimized for a variety of diagnostic applications outside of the laboratory, and they have the potential to provide needy regions of the world with quick and easy detection tools [51].

1.5.3. Strategies for CRISPR-based therapy

A wide variety of treatment approaches are included in CRISPR-based therapies. Due to the fact that their DNA will not be transmitted during reproduction, use is restricted to somatic cells in the body. RNA-targeting approaches, on the other hand, do not have the capability to permanently alter the genome but offer the potential for transient and renewable treatments. DNA-based therapies, on the other hand, offer one-time treatments with curative results. A versatile tool for developing a new generation of therapeutic options to improve human health are strategies that jointly target DNA and RNA. The three types of CRISPR-based strategies used to treat diseases are as follows: treatment of diseases that are caused by one person (hemophilia, sickle cell disease); preventing and treating common illnesses by introducing beneficial natural genetic variants that are already present in the human population and have the potential to protect against them; and disease treatment through the introduction of novel cell types used to achieve therapeutic benefit [52]. Each of these three categories can benefit from CRISPR-based technologies. The simplest method for gaining function, particularly in the case of pathogenic mutations, is gene inactivation. Base editing at the DNA/RNA level, which provides a safer method for repairing pathogenic mutations [53], which typically disrupt regulatory regions or cause truncated or abnormal protein variants, is one promising treatment option. The possibility of RNA editing opens up new treatment options. When regulated cells have an advantage in selection against unregulated cells, gene insertion strategies can be used in disorders where there is a high probability that a small increase in the regulated genotype will have an excessive phenotypic impact [54]. Ex vivo and in vivo methods are both available. In terms of editing efficiency and safety, ex vivo methods are superior, but they are restricted to a subset of cells that can be manipulated in the laboratory and then transplanted [55]. In contrast, in vivo methods can be used on a wider variety of tissues, but there is a risk of going off-target [52].

1.6. Transmission of the CRISPR system

In order to successfully edit a genome, the CRIPSR system must be delivered to the therapeutic target [56]. Ex vivo and in vivo strategies are the two types of delivery strategies, and their characteristics vary depending on the therapeutic approach. The ex vivo strategy involves editing the genome in isolated cells taken from the patient. The engineered cells have the potential to grow and return to the patient. Using plasmid vectors, ribonucleo-protein complexes, and viral vectors, this procedure can be carried out in cells. One of the many benefits of this method is that the delivery occurs at the cellular level. The fact that the CRISPR system is not introduced into the body is crucial because it reduces the likelihood of causing unneeded changes to non-targeted tissues. The majority of the time, cells derived from the patient are used, but it can be challenging to isolate, grow, and expand these cells, which limits the variety of conditions that can be treated. Only a small percentage of these kinds of cells are functionally incorporated into the relevant tissue when they are reintroduced into patients. Then again, there is the in vivo strategy, which happens inside human tissues, and is relevant to many sicknesses. Nanoparticles and viral vectors are typically used to deliver the CRIPSR system in this setting. Due to the possibility of physical barriers and immune responses from the host, this approach is significantly more complicated than the ex vivo strategy and will result in a lower rate of planned genome editing. According to Xu Y [57], when viral vectors and non-viral delivery systems were compared, it was discovered that non-viral systems have many more advantages, including greater flexibility in cargo size, the ability to target only specific organs, and a lower immune response [57]. Numerous variants of this delivery method are being examined for potential applications in the delivery of CRISPR systems [58]. The use of lipid nanoparticles to deliver CRISPR-Cas9 for the treatment of human diseases has demonstrated the significance of biomaterial science in clinical applications, according to recent research [59]. They hope for safe and effective therapy in the near future, despite the fact that much more research is required to fully comprehend this field.

1.7. Future directions

Because many genetic disorders are caused by point mutations that can be fixed by new types of genome editing that have a very low level of unintended genomic changes, it will be very interesting to see the application of basic and major editors for human trials in the coming years. Due to the encouraging results of animal studies and the efforts of researchers to improve editing efficiency, it is anticipated that clinical trials utilizing the CRISPR method will see a significant increase.

Scientists work on new possibilities for treating ordinary, non-Mendelian disorders using CRISPR. By focusing on genes that are important to the disease's pathophysiology, they try to stop multifactorial diseases from happening. Although the path is long and complicated, it is thought that focusing on the right genes, which affect pathological pathways could help treat chronic diseases for which there aren't many treatments or a good drug at the moment.

2. Conclusion

The results of many studies showed that genome editing systems have made a significant contribution to the development of therapeutic approaches for a variety of diseases. The CRIPSR/Cas9 system was most notable for its ability to disrupt the target gene. This system provides another means of controlling non-coding regions of the disease genome and will be used to search for new drug targets. The prevention and treatment of human diseases will benefit greatly from this genome editing technique. Scientists are making great efforts to demonstrate the safety and efficacy of this method for human application. A wide range of diseases form the basis for research, including single gene disorders such as cystic fibrosis, sickle cell anemia and hemophilia, as well as more complex diseases such as cancer, mental illness, HIV infection and heart disease.

There are numerous applications for CRISPR-based technologies that have the potential to fundamentally change disease treatment and improve human health. It can be concluded that the extensive research in genome editing and CRISPR is bringing us closer to drugs that can be used in human genetic diseases. Although there are major concerns about the efficiency and safety of this system, it cannot be denied that this genome editing system will be the most important and prevalent in clinical practice. The current state of research has greatly improved the efficiency and reduced the toxicity during the administration process, which brings the application of this type of genome editing technology closer to the clinic.

It is believed that this system has great potential for the development and progression of diseases, as it will enable new therapies, but also contribute to the development of life sciences. In time, the word "im" will be dropped from the word "impossible" because this path will certainly become possible.

Declaration of competing interest

The authors declare that they have no known financial or non-financial competing interests in any material discussed in this paper.

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