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CRISPR Cas9: Revolutionising the Future of Genome Editing

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ABSTRACT

CRISPR-Cas9 is one of the most revolutionary genome editing tools in the field of molecular biology, biotechnology and precision medicine, given its simplicity, efficiency and unprecedented accuracy. The CRISPR-Cas9 system, an adaptive immune system of bacteria, uses a single-guide RNA (sgRNA) to guide the Cas9 endonuclease to specific DNA sequences to enable the precise double-stranded DNA cleavage which is repaired by cellular mechanisms to introduce targeted genetic modifications. This article explores the key factors and molecular mechanism of CRISPR-Cas9, including recognition, cleavage, and DNA repair. It also explores the present delivery systems, such as viral or non-viral delivery, that enable effective genome editing in a variety of biological systems. Moreover, the article discusses various other applications of CRISPR-Cas9, especially in agriculture and functional genomics. However, some problems exist in Good Manufacturing Practices, such as off-target mutation, delivery problems, immunogenicity, and ethical concerns in human genome editing, which are still significant obstacles. Both technological development and responsible regulation are required to ensure that CRISPR-Cas9 is applied safely, accurately, and clinically in the future to realize its potential for biomedical and agricultural innovation.

Keywords: CRISPR Cas9, gRNA, mechanism, applications, challenges

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Introduction

Gene therapy is an alternative therapeutic strategy for a variety of conditions, including blood disorders, ocular disease, cancer, and nervous system disorders. Gene editing techniques have facilitated the ability of researchers to specifically and selectively target and modify the eukaryotic cell genome, making it a valuable tool for gene therapy. Genome editing techniques involve breaking the DNA double-strand (DSBs) sequence at specific locations in the genome, which can be repaired using different cellular DNA repair mechanisms, such as endogenous homology-directed repair (HDR), microhomology-mediated end joining (MMEJ), classical non-homologous end joining (cNHEJ), and single-strand annealing (SSA) pathways. Gene editing tools, including zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR)–Cas-associated nucleases, can be employed for gene therapy purposes [1]. Clustered regularly interspaced short palindromic repeats (CRISPR) were first discovered as an adaptive immune system effector in prokaryotes [2]. CRISPR comprises a group of small DNA sequences found in the genomes of prokaryotes that were acquired from previous infections by bacteriophages. It offers a defense mechanism for prokaryotes to fight against reinfection by similar bacteriophages. Subsequent development of this technology into a gene-editing tool in eukaryotic cells enabled the application of gene editing for human diseases [2]. CRISPR-Cas9 technology has revolutionized the field of genome editing, offering precise and efficient methods to modify genetic material [3]. The CRISPR-Cas9 system was first harnessed for genome editing by Jennifer Doudna and Emmanuelle Charpentier, who demonstrated its potential in a seminal study published in *Science* in 2014 [5] . They showed that the Cas9 nuclease could be guided by a synthetic single-guide RNA (sg RNA) to introduce targeted DNA cleavage *in vitro*. This groundbreaking work laid the foundation for the rapid advancement of CRISPR-Cas9 technology [5]. CRISPRs were first identified in 1987 upstream of the iap gene, responsible for alkaline phosphatase isoenzyme conversion in *Escherichia coli* [6].

CRISPR-Cas9 is a powerful gene-editing tool that harnesses the bacterial immune system's ability to target and cleave specific DNA sequences [3]. Before the discovery of CRISPR/Cas-9, scientists relied on two gene-editing techniques using restriction enzymes, zinc finger nucleases (ZFN), and Transcription activator-like effector nucleases (TALENs). ZFN has a zinc finger DNA-binding domain used to bind a specific target DNA sequence and a restriction endonuclease domain used to cleave the DNA at the target site. TALENs are also composed of a DNA-binding domain and a restriction domain like ZFN, but their DNA-binding domain has more potential target sequences than the ZFN gene-editing tool. In both cases, the difficulty of protein engineering, being expensive and time-consuming, was the major challenge for researchers and manufacturers [10]. The development of a reliable and efficient method of a gene-editing tool in living cells has been a long-standing goal for biomedical researchers. After figuring out the CRISPR mechanism in prokaryotes, scientists understood that it could have beneficial use in humans, plants, and other microbes. It was in 2012 that Doudna, J, and Charpentier, E discovered CRISPR/Cas-9 could be used to edit any desired DNA by just providing the right template. Since then, CRISPR/Cas-9 has become the most effective, efficient, and accurate method of genome editing in all living cells and is utilized in many applied disciplines [10]. CRISPR-Cas9 technology has revolutionized genome editing, providing researchers with a versatile and efficient tool for genetic modification. Its applications in biomedical research, Agriculture, bioinformatics, and biotechnology hold immense promise for addressing various challenges and improving human lives. The aim of this article was to comprehensively understand the mechanism of CRISPR-Cas9 and explore its applications and future prospects, as well as the ethical and safety considerations associated with its use [3].

Components of CRISPR/Cas9:

CRISPR/Cas9 is a simple two-component system that consists of two RNAs (CRISPR RNA or crRNA, Tracer RNA or tracrRNA) or guide RNA (gRNA) and a single protein called Cas9 [3]. The Cas-9 protein, the first Cas protein used in genome editing, was extracted from *Streptococcus pyogenes* (SpCas-9) [10]. It is a large (1368 amino acids) multi-domain DNA endonuclease responsible for cleaving the target DNA to form a double-stranded break and is called a genetic scissor. Cas-9 consists of two regions, called the recognition (REC) lobe and the nuclease (NUC) lobe. The REC lobe consists of REC1 and REC2 domains responsible for binding guide RNA, whereas the NUC lobe is composed of RuvC (UV-sensitive gene product C), HNH (Histidine Asparagine Histidine), and Protospacer Adjacent Motif (PAM) interacting domains. The RuvC and HNH domains are used to cut each single-stranded DNA molecule, while the PAM-interacting domain confers PAM specificity and is responsible for initiating binding to target DNA [10]. Guide RNA is made up of two parts, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA is an 18–20 base pair in length that specifies the target DNA by pairing with the target sequence, whereas tracrRNA is a long stretch of loops that serve as a binding scaffold for Cas-9 nuclease [10]. In prokaryotes, the guide RNA is used to target viral DNA, but in the gene-editing tool, it can be synthetically designed by combining crRNA and tracrRNA to form a single guide RNA (sg RNA) in order to target almost any gene sequence supposed to be edited [10]. The system utilizes a short RNA molecule called single guide RNA (sg RNA) to guide the Cas9 nuclease to the target DNA sequence, resulting in a double-stranded break. This break can then be repaired through cellular repair mechanisms, allowing for the introduction of desired genetic modifications. The simplicity, cost-effectiveness, and high efficiency of CRISPR-Cas9 have made it a game-changer in the field of genetic engineering [3].

Mechanism of CRISPR Cas9 Genome editing:

The mechanism of CRISPR/Cas-9 genome editing can be generally divided into three steps: recognition, cleavage, and repair. The designed sg RNA directs Cas-9 and recognizes the target sequence in the gene of interest through its 5′crRNA complementary base-pair component. The Cas-9 protein remains inactive in the absence of sg RNA. The Cas-9 nuclease makes double-stranded breaks (DSBs) at a site 3 base pairs upstream of the PAM. PAM sequence is a short (2–5 base-pair length) conserved DNA sequence downstream of the cut site, and its size varies depending on the bacterial species. The most commonly used nuclease in the genome-editing tool, Cas-9 protein recognizes the PAM sequence at 5′-NGG-3′ (N can be any nucleotide base). Once Cas-9 has found a target site with the appropriate PAM, it triggers local DNA melting followed by the formation of an RNA-DNA hybrid, but the mechanism by which the Cas-9 enzyme melts the target DNA sequence is not clearly understood yet. Then, the Cas-9 protein is activated for DNA cleavage. HNH domain cleaves the complementary strand, while the RuvC domain cleaves the non-complementary strand of target DNA to produce predominantly blunt-ended DSBs. Finally, the DSB is repaired by the host cellular machinery [10].

Common CRISPR/Cas9 delivery strategies:

The delivery of the CRISPR/Cas9 system for efficient gene editing is challenging. The Cas9 protein has a molecular weight of approximately 160 k Da [11], and after forming an RNP complex, the long phosphate backbone of the sg RNA imparts a net negative charge to the complex [12]. Both of these properties make it difficult for the Cas9 RNP to cross the cell membrane. Moreover, once inside cells, both the Cas9 protein and sg RNA must survive the degradation processes in the cell and translocate into the nucleus to enable gene editing. Therefore, choosing an appropriate delivery strategy for the CRISPR/Cas9 system is critical to achieving efficient and precise gene editing. If it is to be used in clinical settings, the safety profile must also be considered to avoid or minimize insertional mutagenesis. The delivery strategies for CRISPR/Cas9 can be broadly classified into viral or non-viral approaches, depending on whether viral transduction is used. The non-viral approach includes various physical and chemical delivery strategies. Each of these methods has its own pros and cons that should be considered for each gene editing application [2].

Viral Vectors:

Adeno-associated viruses (AAVs) are a common viral vector used for gene delivery. The unique properties of AAVs (i.e., replication-defective, non-integrating into the genome, and low immunogenicity in humans) have triggered huge interest in their potential as delivery vehicles, especially for in vivo applications [13]. CRISPR/Cas9 can be delivered by AAVs in two ways: First, AAVs can serve as a vehicle to deliver Cas9, sg RNAs, and/or donor templates into cells by transduction [14]. However, AAV vectors are limited by their low cloning capacity (< 4.7 kb) [2].

Lentiviruses (LVs) are another viral vector used for CRISPR/Cas9 delivery. LV vectors have a more generous cloning capacity (< 8 kb) than do AAV vectors, which enables the cloning of both Cas9 and sg RNA into a single LV vector. The production of LVs is also less laborious than that of AAVs. The LV transduction process is highly efficient in a wide variety of cell types in both dividing and non-dividing cells [15]. These advantages indicate that LV vectors are an optimal vehicle for delivery *in vitro* and *ex vivo* [15].

Non-Viral Physical Methods:

Microinjection is the physical method of injecting Cas9 and sg RNAs directly into cells with a microscope and needle. Because the needle pierces through the cell membrane to directly deliver the cargoes into the nucleus, the molecular weight of Cas9, which is usually an issue in viral vector-mediated delivery due to its limited cloning capacity, is not an obstacle in microinjection [2]. Electroporation is a popular physical delivery method. It applies pulses of electrical current to stimulate the transient opening of pores in cell membranes, permitting the delivery of cargoes into cells. Electroporation is commonly used in in vitro and ex vivo gene editing because it efficiently delivers cargoes into a wide variety of cell types [2].

Applications:

In Agriculture: Crop improvement is a major focus of CRISPR-Cas9 technology in agriculture. The ability to precisely edit plant genomes allows for the enhancement of desirable traits, such as disease resistance, yield, nutritional content, and stress tolerance. For example, in a study by Shan *et al.*, CRISPR-Cas9 was used to successfully target the susceptibility gene in rice, leading to enhanced resistance against bacterial blight. CRISPR-Cas9 has also shown promise in accelerating the breeding process. Traditional breeding methods often require multiple generations to achieve desired traits. With CRISPRCas9, targeted genetic modifications can be introduced directly into the germline of plants, significantly reducing the breeding timeline. In a study by Li *et al*., CRISPR-Cas9 was utilized to enhance rice grain yield by precisely editing genes associated with grain size regulation. CRISPR-Cas9 technology has the potential to address global food security challenges. By modifying crop genomes, it is possible to develop crops that are more resistant to pests, diseases, and environmental stresses, thereby increasing productivity and reducing yield losses. This technology also offers opportunities for the production of nutritionally fortified crops, which can help combat malnutrition and improve human health [3].

In Medicine: One of the key applications of CRISPR-Cas9 in medicine is in the study of gene function and disease mechanisms. By selectively modifying genes in human cells or model organisms, researchers can gain insights into the underlying causes of genetic diseases. For example, in a study by Shalem *et al.*, CRISPR-Cas9 was employed to systematically knock out each gene in the human genome, leading to the identification of genes essential for cancer cell survival. CRISPR-Cas9 also holds promise for the development of gene therapies [9]. It offers the potential to correct disease-causing mutations directly in the genomes of patient cells. In addition, CRISPR-Cas9 technology makes it possible to correct a mutation associated with a genetic blood disorder, β-thalassemia, in patient-derived stem cells. CRISPR-Cas9 technology has opened up new avenues for cancer research and therapy. It allows for the targeted disruption of genes involved in cancer progression or the introduction of specific modifications to sensitize cancer cells to existing treatments. In a study, CRISPR-Cas9 was used to knock out a gene involved in chemotherapy resistance, enhancing the effectiveness of the treatment in cancer cells [3].

Challenges & Ethical concerns:

Initiating the CRISPR-Cas9 system in genetics poses significant challenges, particularly ethical concerns that may result in strict penalties for manipulating genetic material. A key dilemma involves editing the human germline, as these irreversible changes can affect future generations. The potential to select traits in embryos raises issues of inequality, discrimination, and broader moral questions. Another challenge of the CRISPR-Cas9 system is the off-target phenomenon, which can lead to unintended effects and harm. The risk of off-target effects increases with more than three mismatches between the PAM and sg RNA, potentially leading to mutations and immune responses. Strategies to reduce off-target effects include optimizing sg RNA, utilizing different Cas proteins (such as Cas3 and Cas12), modifying Cas9's nuclease properties, employing Acr, and employing non-viral vectors, such as nanoparticles. These methods help mitigate off-target adverse effects [7]. One of the challenges of the CRISPR-Cas9 system is immunogenicity. The Cas9 protein and sg RNAs can trigger immune reactions, primarily if derived from microbial or viral sources. Additionally, viral delivery methods and nanoparticles may pose immunogenic and inflammatory risks [8].

The ethical challenges of CRISPR include concerns about human genome editing, the creation of designer babies, and negative impacts on ecosystems. Additionally, risks from low accuracy and unintended mutations are also present [4].

Conclusion:

CRISPR-Cas9 has changed the game of genome editing by providing an effective, flexible, and very precise way to genetically modify organisms. Its simplicity, affordability, and wide range of applications have sped up research in molecular biology, agriculture, biotechnology, and clinical medicine as compared to previous genome-editing methods. This work has reviewed the structure, mechanism, delivery and main application of CRISPR-Cas9, as well as the challenges currently hindering wide therapeutic use. Despite the notable advancements in editing precision and off-target effects reduction, the distribution efficiency, immunological responses, long-term safety, and ethical implications are still the crucial factors for clinical and agriculture applications. The accuracy and safety of this method are anticipated to be enhanced by ongoing developments in modified Cas proteins variants, better guide RNA design, and next-generation genome-editing techniques. With continued interdisciplinary research, robust ethical concerns, and well-defined regulations, CRISPR-Cas9 holds the promise of being a key player in precision medicine, sustainable agriculture, and the creation of new therapeutic strategies for complex genetic diseases and other global health challenges.

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