CRISPR Development and Application

Xiaohan Ma*

Department of College of Life Science and Technology, School of Xinjiang University, The Xinjiang Uygur Autonomous Region, China

*Corresponding author: gtxwsjsjrsk@lc.shandong.cn

Abstract. Gene editing technology rapidly develop, addressing diseases that cannot be treated with conventional medical methods, nowadays, gene modification has become a hotspot of current research. A variety of methods utilizing DNA damage repair mechanisms to achieve targeted gene editing have gradually emerged. The CRISPR, which has been continuously optimized and improved since its development, has surpassed the previous two generations and become the third generation technology with more practical value., CRISPR technology compared to the previous two generations of technology have significant advancements in terms of application scope, specificity, and accuracy. CRISPR technology originates from bacteria themselves. As an acquired immune system of bacteria, it is used to identify intruding gene fragments and degrade them.The main content of this article includes the mechanism of CRISPR, functioning in bacteria and current popular classification methods for CRISPR systems. Focus on introducing, the delivery mode of CRISPR system in practical applications, like AAV, AdV or LV-based methods. And limitations of the current delivery mechanism and current development trends, that is to address the immunogenicity issues caused by viral vectors, researchers are continuously developing non-viral vectors. They have been made progress in many directions. It also introduces the current limitations of CRISPR/Cas9 itself. Especially addressing the issue of its high miss rate and the improvements made by scientists on this at present, such as developing high-fidelity variants of CRISPR/Cas9. In modern times, researchers have developed various artificial Cas9 variants that have similar functions to wild-type Cas9 and possess higher practical value.

Keywords: CRISPR/Cas9; Delivery Mechanisms; Variants of Cas9.

1. Introduction

Recently, gene editing technology has little by little attract attention , due to its ability to modify specific loci in genes, alter gene sequences, and achieve effects such as gene knockin, knockout, upregulation, or downregulation within the genome. This enables the modification of genes, allowing for potential treatments for diseases. At specific sites in the genome, DNA double strands are induced to break (DNA double-strand breaks, DSBs), and subsequently, the cell's DNA damage repair mechanism begins to operate, such as mismatch repair (MMR) [1], excision repair (ER) [2], and photoreactivation repair (PHR) [3]. The working principle of gene targeted editing technology is as follows. Under the influence of these repair systems, new bases may be randomly inserted or deleted at the DSB break sites. Then, it can regulate the expression of target genes, thereby achieving gene specific editing.

When this technology was just noticed by people , Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs) were recognized in 2011 by Nature Methods for their significant achievements in efficient gene editing technology. These tools expanded the range of gene editing, making it possible to edit not only lower organisms but also higher organisms. However, despite the breakthrough in targeted editing made possible by ZFNs, the technology also has limitations. For instance, if the zinc finger protein library is insufficient, it cannot target different gene sequences. While TALENs are simpler in design and have higher specificity, they can exhibit cytotoxicity [4].

CRISPR/Cas9 gene editing technology is a new generation of technology that has been improved over the previous two generations. Compare the first two generations, transcription activator-like effector nuclease and Zinc finger nuclease, CRISPR/Cas9 gene editing technology consists only of

CRISPR that integrates exogenous DNA fragments and Cas9 that plays a role in cutting.It has simple structure and operation process， and it can precise editing at specific sites.

In 1987, Japanese scientists Y.Ishino [5] ,among others first discovered CRISPRS sequence in K12 Escherichia coli. Even from the current perspective it isn't attract attentions， but with continuous research, the results are becoming increasingly abundant .

In 2002, Mojica and Jansen [6] named this sequence as regularly clustered interspaced short palindromic repeats (CRISPR). In 2005, Research found that CRISPR is not originate the cell itself . But it comes from DNA sequence of invaded bacteriophage or plasmid . And CRISPR-Cas is the immune mechanism evolved by bacteria to resist invasion of exogenous genes [7].

This article primarily focuses on CRISPR/Cas9, providing a detailed explanation of its mechanism and applications.

2. Mechanism of the CRISPR System

CRISPR comes from bacteria, which is an adaptive immune system, that have important and irreplaceable influence in protecting against bacteriophage and foreign plasmid invasion. Together with Cas proteins (CRISPR-associated proteins), the Crispr/cas9 system can play a great role in gene editing technology. In bacteria, CRISPR sequences consist of a range of identical direct repeats separated by different spacer sequences. These sequences are transcribed and processed into CRISPR RNAs (crRNAs), which guide Cas-expressed proteins such as nucleases, helicases, and polymerases to recognize and degrade invading genetic material.

The main process can be seen as connected by three processes, which interact with each other and jointly complete the effect:

The first stage occurs when the foreign DNA of phage or plasmid invades the bacterial cell. After a bacteriophage infection, the system detects and identifies the foreign genes. The Cas1 protein, which has DNA endonuclease activity, cuts the foreign DNA into fragments. These cleaved fragments are then recognized by Cas proteins with nuclease activity and interposed it into the CRISPR locus between the leader sequence and the first repeat sequence. Simultaneously, as the new sequence is inserted, the repeat sequence is duplicated, forming a new repeat-spacer (R-S) structure. Through this method, the CRISPR system can obtain exogenous spacer sequences that are not its own.The newly acquired spacer sequence is of foreign origin, and to distinguish it from the host's own sequences, the same repeats distinguish foreign spacers.

In the way, the information from the foreign gene sequence is stored within the CRISPR system. The foreign sequences in bacteriophages or plasmids that are highly homologous to the stored sequence are referred to as "protospacers." The conserved sequences at both ends of the protospacer are named Protospacer Adjacent Motifs (PAMs), and they form the structural basis for bacterial adaptive immunity. PAM sequences serve as target sites during the cleavage of foreign gene sequences and also make great contributions in protecting the host's own genome. Since PAM sequences differ between bacterial species, and even between strains within the same species, the CRISPR system can effectively avoid cutting the host's own sequences when cleaving foreign DNA.

The second stage occurs when a gene, highly homologous to the protospacer sequence, from a bacteriophage or foreign plasmid, re-enters the bacterial cell. At this point, the previously low transcription level of the CRISPR locus rapidly increases. This resulted in the transcription of CRISPR precursor RNA (pre crrna).The pre-crRNA undergoes various processing steps to form mature crRNA, which has the ability to bind with Cas proteins. Once mature, the crRNA binds with the Cas proteins.

The third stage, and the most critical in this system. At this stage, the ribonucleoprotein complex is formed by the combination of crRNA and CAS protein. This complicated binds to the foreign DNA fragments. The crRNA within the complex uses the PAM sequence to recognize and locate the corresponding foreign DNA fragment. The Cas protein, with its nuclease activity, then cleaves and degrades the foreign gene fragment.

This RNA, formed from the transcription and processing of a spacer sequence and a portion of the repeat sequence, guides the nuclease activity of Cas proteins in recognizing and degrading foreign DNA through various pathways.

Research has shown that the ability of crRNA to pair and bind with the foreign gene is the critical and basic requirements for the achievement of the interference process [8-10].

3. Classification of the CRISPR System

Crispr/cas system has more than one classification standard. It is not standardized due to the diverse types and functions of Cas proteins. Some scientists propose that the classification should be based on the degree of conservation of Cas sequences, dividing the system into core Cas genes, subtype-specific Cas genes, and Repeat-Associated Mysterious Proteins (RAMPs) with unknown functions [11]. Others suggest classifying the CRISPR/Cas system based on the evolutionary relationships between conserved Cas proteins and the composition of Cas operons, resulting in three main types: Type I, Type II, and Type III systems [12].

The Type I CRISPR system is primarily found in bacteria and archaea and contains six Cas proteins. The core protein is Cas3. Multiple Cas proteins work together with mature crRNA to form the CASCADE (CRISPR-associated complex for antiviral defense) complex. In this system, the Cas3 protein acts as a nuclease, cleaving foreign genes under the guidance of crRNA. Therefore, in the Type I system, Cas-associated proteins are related to both the maturation of pre-crRNA and the recognition of foreign genes.

The type III CRISPR system was found in bacteria. The core protein is Cas10. Cas10 has RNA binding and digesting activity and performs functions similar to those of the CASCADE complex found in the Type I system. Currently, two subtypes of the Type III system have been discovered: Type III-A, which interferes with mRNA, and Type III-B, which targets DNA. Like the Type I system, the Cas-associated proteins in Type III are involved in the maturation of pre-crRNA and the recognition of foreign genes.

The Type II CRISPR/Cas system is also turn up exclusively in bacteria and is the most widely applied version today. The core protein in this system is Cas9, which not only degrades foreign gene sequences but also become an integral part in the maturation of crRNA. Under the action of RNase III, pre-crRNA is processed into mature crRNA. After forming a double stranded RNA dimer with tracrrna, it breaks down and connects with cas9 protein. The complex thus formed has the ability to accurately edit foreign genes. SpCas9 is often used in experiments now. It is found in Streptococcus pyogenes, and SaCas9, from Staphylococcus aureus [13].

4. Delivery Mechanisms of the CRISPR/Cas System

There are three primary categories for delivering the CRISPR/Cas system in vivo: (1) Delivery of plasmids that can express the Cas9 protein and single guide RNA (sgRNA). (2) Delivery of mRNA that encodes the Cas9 protein and sgRNA. (3) The ribonucleoprotein complex (RNP) formed by cas9 protein and sgRNA is directly transferred. The first method requires the intracellular transcription and translation process, which can be time-consuming. The second method, while easier for mRNA delivery, faces issues with mRNA instability and degradation. The third method, however, provides a transient gene-editing approach with rapid delivery, high editing efficiency, minimal off-target effects, and low cytotoxicity [14].

The gene-editing delivery using the RNP complex has seen significant advancements in recent years. Physical delivery methods, such as electroporation and microinjection, while achieving high transfection efficiency, are not suitable for therapeutic applications in vivo. Viral vector delivery methods, including adenovirus vector（AdV）, adeno-associated virus (AAV),bacteriophages and lentivirus vectors(LV), has been applied in many aspects.

AAV compared to other viral vectors has less immunogenic . It is the most widely used viral vector in crispr/cas9 delivery. However, due to its high requirement for the size of cas9, up to 4.7kb of protein can be encapsulated, The size of spcas9 frequently-used streptococcus pyogenes Cas9 (SpCas9) limits its role. For this characteristic of AAV, variants of smaller CAS proteins were developed. In 2015, Ran et al performed experiments in six smaller cas9 orthologs, screen out Staphylococcus aureus Cas9 (SaCas9) [15]. Its length is shortened by more than 1KB. After SaCas9 and single guide RNA expression cassette packaged into a single AAV vector and injected into the liver of mice. It was found that the content of PCSK9 and total cholesterol in serum was significantly reduced again demonstrating that sacas9 mediated gene editing has similar gene editing ability to spcas[9].

but come with high risks of gene mutations and immune responses. The development of non-viral delivery systems for RNP delivery is crucial to meet the therapeutic needs of CRISPR-based treatments in vivo.Cationic lipid and lipoid carrier can load nucleic acids through electrostatic interactions. Nowadays, they have been widely used in delivery mechanism of microRNA、siRNA and shRNA. It also make a contribution in crispr/cas9 system delivery.

In 2018, Wen et al used biodegradable black phosphorus nanosheets (BPS) as carriers [16]. This vector can be used to deliver ribonucleoprotein (RNP) complexes. They used BP as a delivery platform to load the Cas9 ribonucleoprotein (Cas9N3), which has three nuclear localization signals (NLS) at its C-terminus. The Cas9N3-BP complex enters the cell through membrane penetration and endocytosis. Subsequently, the BP undergoes biodegradation-associated endosomal escape, releasing the Cas9N3 complex into the cytoplasm. This provided a novel BP-based delivery mechanism for gene editing.

In 2020, Wei et al engineered ionizable lipid nanoparticles (LNPs) by incorporating permanently cationic lipids to encapsulate and maintain the activity of the RNPs [17]. This enabled efficient delivery of RNPs into cells for editing tissues in vivo.

In 2021, Yao et al inserted RNA aptamers into the sgRNA and fused aptamer-binding proteins (ABPs) to both ends of CD63, a tetraspanin protein enriched in exosomes [18]. This created a three-component complex comprising CD63, sgRNA, and ABPs, which facilitated the packaging of RNPs into exosomes. This method allowed for the delivery of RNPs targeting multiple gene loci, enabling multiplex gene editing. in the human body.

5. Off target effects of CRISPR

Stack up with the preceding two gene editing technologies, CRISPR/Cas9 not only has the ability to edit specific sites with high precision, but it also exhibits a high degree of specificity, making it widely applicable in fields such as bioengineering and medicine. Through continued research and deeper understanding of the bacterial CRISPR system, scientists have uncovered its mechanism for defending against foreign gene invasion in bacteria and adapted this capability for gene editing, enabling the simultaneous editing of multiple genes. Today, structural modifications of the Cas9 protein have already been achieved. For example, the inactive Cas9 (dead Cas9, dCas9) is a Cas9 mutant that lacks nuclease activity but can serve as an RNA-guided DNA-binding domain, binding to specific DNA sequences [19, 20]. When dCas9 is fused with epigenetic effector proteins, it can add or remove epigenetic markers at specific sites, allowing researchers to study how epigenetics regulate gene expression [21, 22].

However, CRISPR/Cas9 also has its limitations. For instance, the cleavage site must be located upstream of a PAM sequence, restricting the choice of target sites. The recognition sequence is relatively short, limiting its ability to target longer sequences. Additionally, the off-target rate cannot be fully controlled, leading to potential mutations in non-target sequences. In order to reduce the off-target effect, scientists have found a strategy to reduce the concentration of sgRNA and cas9, though the effectiveness of this approach is still under debate, with studies suggesting that while it reduces both target and off-target mutations, it may also decrease the overall editing efficiency. Another

strategy is to modify or shorten the sgRNA, such as truncating its 3' end, adding two GG nucleotides at the 5' end, or shortening it overall, which can also help reduce off-target mutations [23].

In 2014, Fu et al used tools to modify or truncate sgRNA, such as truncating its 3 'end, adding two GGS at the 5' end or truncating it, which can also reduce the generation of off target mutations [24].

In 2017, Hsin-Kai et al suggested that gene-editing technologies relying on double-strand breaks (DSBs) were limited in their clinical applications due to off-target effects [25]. To solve this problem, they improved the CRISPR system, modifying it to activate target genes without inducing DSBs. Under these conditions, they successfully utilized transgene-mediated epigenetic remodeling of endogenous target genes. Their research demonstrated the use of CRISPR to activate endogenous genes, establishing a novel pathway for targeted epigenetic regulation.

In 2019, researchers at the City University of Hong Kong designed a novel SaCas9 variant called SaCas9-HF [26]. This engineered variant exhibited high specificity in genome-wide activity within human cells and does not reduce its original efficiency of targeting target genes. In tests at fifteen endogenous human loci, no off-target activity was detected at nine sites, and minimal off-target activity was observed at six sites. Additionally, its targeting efficiency remained comparable to wild-type SaCas9. Moreover, SaCas9-HF significantly reduced off-target activity at four known off-target loci compared to the wild-type version. They further developed a KKH-SaCas9 variant, which recognized a broader range of PAM sequences. The resulting KKH-HF variant significantly reduced off-target activity and increased the on-target editing ratio, providing an engineered variant that could replace wild-type SaCas9 for broader gene-editing applications while reducing off-target rates.

In 2020, Liu et al lay down a "caged RNA" tactics, where Cas9 could combined in DNA but was unable to cleave it until activated by light [27]. This approach, called "very fast CRISPR" (vfCRISPR), operated at submicrometer and second-scale resolutions. This synchronous cutting method enhances the DNA repair mechanism. Because after Cas9 induced DSB, the cells immediately responded in a very short period of time. Afterwards, the DNA has been repaired, but MRE11 remains bound. Experimental results show that after DNA damage, the phosphorylation rate of H2AX rapidly increases, reaching up to 30 megabases.Their study demonstrated that vfCRISPR enables high-resolution studies of DNA repair in spatial, temporal, and genomic contexts, allowing for precise gene editing within seconds.

6. Recent Developments in CRISPR

In 2013, Cong et al demonstrated that in human and mouse cells，the SpCas9 nuclease ,which in the CRISPR/Cas9 system, could accurately cut target sites in the endogenous genome. They also showed that multiple foreign sequences could be encoded into a single CRISPR/Cas system, thereby enabling editing of multiple target sites within the genome [28].

In 2017, Zetsch et al have detection that cpf1 can be used for simultaneous multi-gene editing. It has the ability to process its own CRISPR RNA (crRNA). In this way, it is feasible to edit four genes in cells at the same time. In the brain of mice, it is also possible to edit three genes at the same time [29].

In 2018, Crispr-cas9 system that found and extracted in Streptococcus pyogenes , had already been applied in various scenarios. Lei et al developed an efficient crispr-cpf1 system. In addition, by inactivating cpf1 (ddcpf1) by DNase, they developed an integrated crispri system. This system can achieve multiple gene suppression by using only a single crrna.Their experimental results showed that the applicability of fncpf1 and spcas9 was quite different among the Streptomyces strains they used in the experiment [30].

In 2020, Zhang et al combined oligonucleotide recombination with CRISPR/Cas9 targeting technology, applying it for rapid site-directed mutagenesis in cloning pathways. They directly transferred foreign genes into host cells for expression and identified several key factors influencing mutagenesis [31].

In 2022, Ameruoso et al used CRISPR-Cas-based synthetic gene regulators to activate silent biosynthetic gene clusters (BGCs). They not only improved CRISPR interference (CRISPRi) but also created a CRISPR activation (CRISPRa) system, enabling precise and efficient gene repression or activation in Streptomyces [32].

7. Conclusion

Crispr-cas9 make a contribution in the bacterial immune system It is a system for cleaving foreign genes in phages and plasmids. Nowadays, CRISPR is widely used in clinical medicine biology and other fields. CRISPR has made great contributions in editing biological genes and treating gene diseases. This article explains the immune mechanism of CRISPR system in bacteria, it also introduces the most widely used classification method of CRISPR system at present. Then it explains the deficiency of high off target rate of CRISPR and the research achievements made in recent years to improve the targeting, as well as the improvements made in non viral delivery mechanism recently. Through various methods, researchers in this field have improved the targeting rate of the CRISPR/Cas9 system,that is, the ability to specifically target the target site, high miss rate is still the biggest challenge currently facing. Researchers are currently working to develop various high fidelity CAS variants,Such as spcas9-hf1, hificas9.They were also used a series of optimization methods were also used. Recently developed machine learning tools can assist in predicting post DBS outcomes. This pair in canonical crispr-cas9 editing Scientists cannot fully grasp the results of DBS is a great progress. At present, this technology has not been applied in vivo. Maybe it is a direction of CRISPR development combined with artificial intelligence in the future. This demonstrates the cross domain nature of CRISPR Technology. The wide application of CRISPR will also promote its development, May be used for precise editing of specific nucleotides and generation of new genetic information. It may also be combined with machine learning, live cell imaging technology. CRISPR technology has been applied in clinic in the past by scientist, they provided a large amount of basic data.When the safety and reliability of CRISPR are fully assured, it may be used to prevent cardiovascular and cerebrovascular diseases in the future. Through the precise regulation of genes, people can keep healthy.

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