Overview of CRISPR/Cas Gene Editing System and Its Carrier System

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Abstract. The CRISPR/Cas9 system, which is currently extensively employed in gene editing-related engineering, is a flexible immune system in bacteria and archaea that reacts to viruses, bacteriophages, and foreign DNA. Cas proteins can cut foreign DNA into small molecule fragments, which are then integrated into CRISPR arrays; When the relevant DNA invades again, it can be accurately identified and cut off. The discovery of the CRISPR system is a major breakthrough in biology because it operates more efficiently and concisely than the previous two generations of technology, providing a powerful tool for gene therapy and diagnosis, as well as fine medicine. With the iteration and evolution of CRISPR/Cas systems, the first problem today is not only a better system, but also how to accurately and efficiently deliver this system to the target cell or tissue. Today's mature carrier technology is roughly divided into viral and non-viral vectors, viral vectors are more easily absorbed and accepted by cells due to viral characteristics, but their problems are limited viral capacity, difficult large-scale production, immunogenicity and random insertion mutations. Non-viral vectors are now developing extremely rapidly, using a variety of materials including lipids, polymers, peptides and inorganic nanoparticles. This article mainly introduces the molecular mechanism of today's CRISPR/Cas system, introduces today's mainstream gene vector technology and proposes some improvement measures, and finally discusses the current limitations of this technology and the future development direction.

Keywords: CRISPR/Cas gene editing, viral and non-viral carrier, CRISPR/Cas delivery.

1. Introduction

The CRISPR/Cas system was first discovered in bacteria, primarily to fight phage infections and foreign DNA (Fig. 1). Due to the cleavage function of the Cas enzyme, there is great potential for gene editing. Due to its versatility and convenience, the system is currently widely used in many fields, including medical, biomodification, genetic research, and more.

The working schematic of CRISPR/Cas system: The CRISPR/Cas system mainly includes the synthesis of crRNA and RNA binding and cleavage guided by crRNA.

1.1. Biological synthesis of crRNA and Cas proteins

CrRNA, also known as CRISPRRNA, is located upstream of the first repetitive sequence in the CRISPR region, which serves as a promoter to initiate transcription of subsequent CRISPR sequences. Cas protein, a nucleic acid cleavage protein in this system, is formed after the transcription and translation of Cas genes, and can form the CASCADE complex.

1.2. RNA synthesis and cleavage

Firstly, the crRNA and tracrRNA in the CRISPR/Cas system form chimeric RNA molecules, namely unidirectional RNA, or sgRNA. Under the mediation of sgRNA, Cas9 protein (CASCADE complex) can be cleaved at specific sequences to form DNA double strand breaks, and finally gene directed editing is achieved through DABR.

Compared to the two predecessors TALEN and ZFN technologies, CRISPR/Cas technology breaks away from the tedious process of synthesizing and assembling protein modules with specific DNA recognition ability. Its characteristic of mediated by sgRNA greatly simplifies the related design process. Although there are still problems such as excessive dependence on PAM regions at the cleavage site and high risk of miss under sgRNA guidance, CRISPR/Cas is still a gene editing system

with great potential, Currently, relevant application experiments have been conducted in multiple fields.

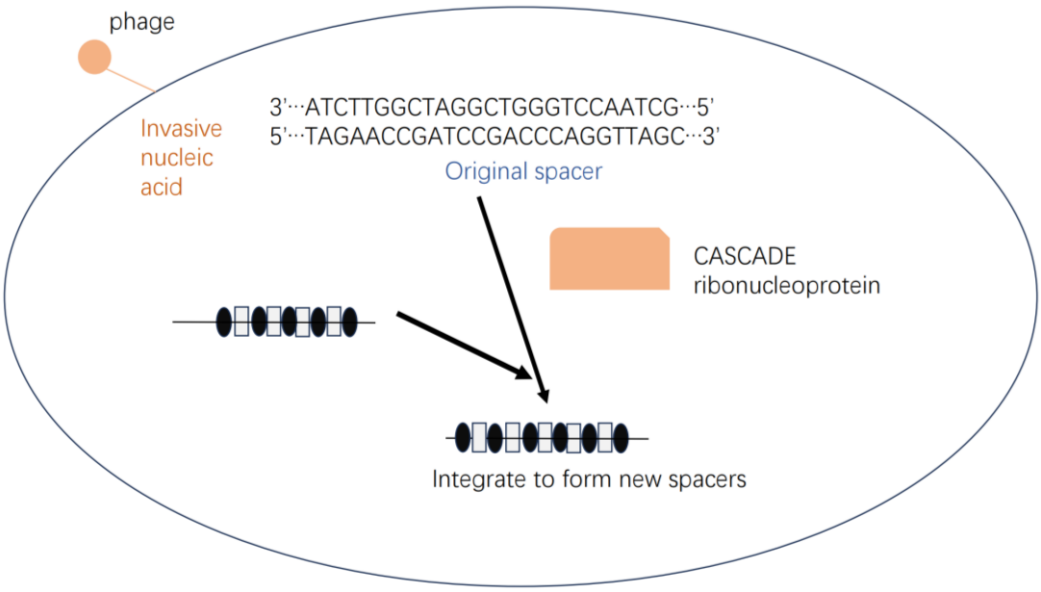


Figure 1. CRISPR antiviral operation mechanism.

2. Classification of CRISPR system

The two classifications of CRISPR/Cas systems that are most prevalent now are Class 1 CRISPR, which includes I, III, and IV, and Class 2, which includes II, V, and VI. The number of protein effectors is the primary distinction between Class 1 and Type 2 systems. Class 2 systems, such as Cas9, Cas12, Cas13, and Cas14, only need a single protein effector to accomplish the target cleavage, in contrast to Class 1 systems, which require multi-protein effector complexes. Therefore, the more complex and difficult to reproduce type 1 systems on the system are not as versatile as the type 2 systems with simpler structure and easier to produce, so most of the type 2 CRISPR systems used in the field of medical biology today are class 2 CRISPR systems [1].

2.1. The CRISPR/Cas 9 system

As the most fancy gene editing technique, CRISPR/Cas9 system is mostly employed to edit the genetic makeup of serval kinds of cells. Cas9 has a nucleic acid leaf with RuvC, HHNH, and PI domains and a recognition leaf with a longbridge helix, a Rec1 domain, and a Rec2 domain, which is a DNA-targeted recognition site [2]. It mainly uses endonuclease to cleave double-stranded RNA under the guidance of CrRNA, which will form genetic damage, and then initiate DNA repair through homologous directed repair or non-homologous end ligation, and provide artificial homologous templates to control mutation changes. Today, many researchers have also created advanced systems (such as the DEAD-Cas9 system, base editing system, Cas9 variant system, and Specifically, PAM sequences on the genome's targeting range, non-target effects, low efficiency, and low specificity are some of the system constraints that must be addressed.

2.2. The CRISPR/Cas12 system

The Cas12 family currently includes Cas12a and Cas12b systems. Cas12a contains two RuvC nuclease domains that can cut both RNA and DNA. A single RNA-guided Cas12a system requires a short T-dependent PAM, such as 5'-YTN-3', 5'-TTN-3', or 5'-TTTN-3' (the middle T is more important than the first T) to achieve efficient target DNA cleavage. Cas12a has a variety of advantages, such as the formation of Cas12a is very far from the PAM site, NHEJ-induced nucleotide insertion or deletion does not lead to the change of PAM sequence, and can repeatedly identify and

cut the target gene; Cas12a can cut single-stranded DNA (ssDNA) indiscriminately; On the other hand, Cas9 is toxic to Corynebacterium glutamate and cyanobacteria, while Cas12a does not. Another Cas protein, Cas12b (also known as Cpf1C2c1), only exerts properties at high temperatures, mainly because it requires RNA for guided delivery, which has led to great difficulties in its development. The updated Cas12b is more selective for target sequences in cell culture tests and has been improved to increase its activity at human body temperature (37°C).

2.3. The CRISPR/Cas 13 system

CRISPR/Cas13 is a new RNA-targeting enzyme that has just been discovered in recent years. There are currently four subtypes of members of the Cas 13 family, which are Cas13a (C2c2), Cas13b (C2c4), Cas13c (C2c7 and Cas13days.

The DNase domain of Cas12a/Cas12b/Cas9 is absent from Cas13, which serves as a prototype protein for type VI. Instead, two HEPN domains are located on the outer surface of Cas13. Cas13a is also a double ribonuclease that cleaves the pre-crRNA to promote its maturation

The Cas13 system works by the fact that when crRNA recognizes the target RNA, a special mechanism emerges that non-specifically cleaves other single-stranded RNAs, known as the "collateral effect", but its molecular mechanism has not yet been interpreted, and it has not shown this activity in eukaryotic species.

Several problems with the current CRISPR/Cas13 system include: (1) RNA editing of small RNA targets (<22nt) is limited because crRNAs take long enough to recognize; (2) possible off-target activity of CRISPR/Cas13; Finally, single-stranded RNA cleavage is likely to have negative effects within eukaryotic cells.

2.4. The CRISPR/Cas 14 system

The CRISPR/Cas14 system contains a RuvC nuclease domain about one-third the size of Cas9, which is found in an archaeal line. Compared to Cas12a, the enzyme domain of Cas14 is more conserved and more specific for single-stranded DNA, enabling single-stranded DNA cleavage without the need for restriction sequences. Its application potential in the field of biotechnology is huge. Improvements to Cas14 have the potential to improve CRISPR diagnostic systems currently under development for the rapid diagnosis of infectious diseases, genetic mutations and tumors [3].

3. CRISPR/Cas transport carriers

As an intracellular molecular manipulation system, how to smoothly deliver CRISPR/Cas to target cells is also a major challenge today. Currently, transferring the entire CRISPR/Cas system into the cell interior mainly depends on two key aspects; The first is the method of forming the CRISPR/Cas system, and the second is the method of transmitting the CRISPR/Cas system.

There are currently three mainstream methods for the former: 1. The first method is to directly transmit relevant sgRNA and mRNA, with the advantage of being able to translate directly in the cytoplasm and start working immediately. However, the disadvantage is that the instability and easy degradation of RNA itself make it difficult to maintain a stable working state, greatly shortening the duration of gene editing; The second method is to transfer plasmid DNA (PDNA) encoding Cas protein and sgRNA. Plasmid DNA is more stable than RNA, but it must enter the nucleus for editing, so editing efficiency is relatively low; The third type is the ENP complex that transmits Cas9/sgRNA, with the advantage that it directly bypasses the transcription and translation processes and can directly perform gene editing, reducing the miss rate. However, due to the molecular size of the Cas enzyme itself, the transmission efficiency is limited.

For the latter, the application environment and selection methods are more complex and diverse. The choice of formation and transmission methods will affect the time and miss probability required before editing is completed, and different forms of CRISPR/Cas systems will also affect the corresponding transmission methods. Nowadays, mainstream delivery systems are divided into viral

and non-viral types (Fig. 2). The advantage of viral vectors is that they can easily deliver the system into target cells, but the disadvantage is that the viral load is small and antigenicity makes it difficult to be utilized multiple times; There are various non-viral methods, including lipid based, polymer based, peptide based, and inorganic nanocarriers. However, most of these carriers face issues of ion toxicity and difficulty in accurately entering target cells for absorption. In addition, there are purely physical delivery methods, such as electroporation, micro injection, and fluid dynamic injection, which can effectively avoid the issues of ion toxicity and difficulty in transmembrane transportation of macromolecules by directly injecting into external concentrated cells. This method is reliable and stable, but lacks specificity and scalability, and has significant limitations in usage scenarios.

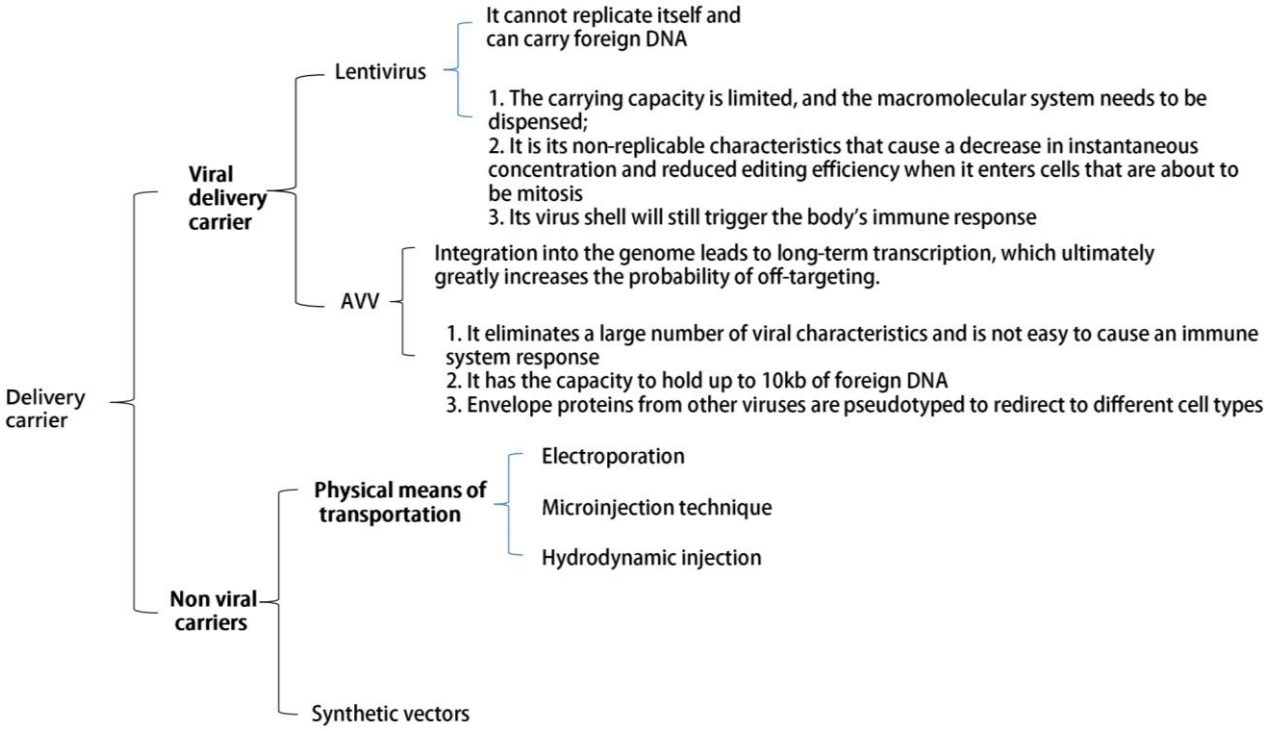


Figure 2. CRISPR/Cas carrier system

3.1. Viral delivery carrier

Viruses, due to their unique way of reproduction, are excellent intracellular molecular transport carriers, so research using viruses as drug carriers is also very popular. Here, we will take the most popular adeno-associated virus (AVV) vectors and lentivirus vectors as examples to briefly describe the current development status of virus vectors [4].

3.1.1. AVV (adeno-associated virus)

As a relatively mature carrier, AVV is also the main research object at present. Its inability to replicate on its own and the ability to carry exogenous DNA enable targeted drug delivery without causing physiological diseases. However, the first problem is that the carrying capacity is limited, and the macromolecular system needs to be repackaged; Secondly, its non replicable nature leads to an instantaneous decrease in concentration and editing efficiency when it enters cells that are about to undergo mitosis; Finally, the virus shell can still trigger the body's immune response, but this problem can be alleviated or even solved through protein shell technology [5].

3.1.2. Lentivirus

The lentivirus vector is constructed by deleting most of the components of the parent lentivirus. The most common parent is HIV-1, which has multiple advantages and can be falsely identified with other viral proteins. It eliminates a large number of viral characteristics and is less likely to cause immune system reactions. It has a capacity to accommodate up to 10kb of exogenous DNA. Lentiviral

vectors have primarily been employed up to this point for in vitro gene transfer, particularly in stem cells from the bloodstream and T cells, and have been used in two FDA-approved CAR-T treatments. To reroute to other cell types, slow virus vectors may be pseudotyped with envelope protein sequences from other viruses. Though integrating vectors is advantageous for gene therapy of mitotic cells, the issue is that due to possible genotoxicity and immune response issues, and its inevitable integration into the host cell genome, over time expression of related enzymes after transportation greatly increases the likelihood of off target mutations.

3.2. Non viral carriers

3.2.1. Lipid based carriers

For gene transfection, lipid nanoparticle (LNP)-based delivery techniques are frequently employed. Cationic phospholipids, which combine with negative ion DNA complexes and are then ingested by cells, are often present in these systems. However, the traditional lipid carrier has low carrying capacity and insufficient editing efficiency, and it is in urgent need of improvement. In addition to the use of special cations to maintain lipid carrier integrity, there is also a more specific lipid carrier such as extracellular vesicles (EVs). It mainly includes exosomes, microvesicles and apoptotic bodies. Compared with other types of carriers, EVS is mostly produced by biocells, and it can effectively avoid toxicity and immune response problems through the body's own cells. At the same time, because EVS itself will also participate in various reaction regulation in organisms, it also has a certain directional transport capacity, and it also solves the off-target problem that is often common in transcarriers. Specific carriers can be made based on protein differences in EVS secreted by different cells. At present, the main problem of EVs is the complexity of the manufacturing process and the difficulty of purification, but despite this. EVs also now show unique potential in the treatment of malignant tumors. And because of the unique characteristics of EVs, the system can be further improved through positive feedback from genetic engineering in the future. Although traditional lipid carriers are difficult to use for system transport, lipid modifications can still be useful in other vector constructions in the future [6].

3.2.2. Polymer based carriers

Polymers can be combined with the CRISPR/Cas9 system to form highly versatile molecular complexes, further forming functional units to circumvent targeting, cell uptake, and nuclear endosomal escape. Cation polymer carriers have greater chemical diversity and functional potential than cationic lipids, enable the design of more flexible structures, and may be directly compounded with CRISPR/Cas9 to improve their transport characteristics. Polymer materials such as polyethyleneimine (PEI) and chitosan are currently under development. Polymers may also be included in cationic LNP-based systems to transport CRISPR/Cas9 cargo and improve in vivo stability.

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3.2.4. Carriers based on various types of exosomes

At present, exosomes include such as endogenous exosome and heterozygous exosomes. The endogenous exosome comes from tumor cells in vivo, which has a unique targeting ability for some cancer cells in vivo, which can carry part of the CRISPR/Cas9 system to accumulate inside cancer cells, and finally accelerate its withering by changing some antidote sensitivity of cancer cells.

Because the carrying capacity of endogenous exosomes is too low, in order to be able to transport more and more complex systems, there is a technology to mix exosomes and liposomes, and the two will form a double-layer structure under a certain effect. The current heterozygous exosome capacity has been effectively increased to carry large vectors such as CRISPR/Cas9 [7].

3.2.5. Carrier based on nanometallic particles

In order to find a better CRISPR/Cas system carrier, inorganic nanoparticles have been put on the agenda, currently divided into metal nanoparticles, metal-organic frameworks, carbon nanotubes and mesoporous silica nanoparticles. Due to chemical inertness, the gold nanoparticles do not trigger the host immune system, and because of the characteristics of gold-sulfur bonds, gold nanoions are easily crosslinked with various sulfhydryl molecules to form various hydrophilic compounds [8].

Today, gold nanoparticles have been successfully used in many in vitro, in vitro and in vivo experiments. Both the size of gold nanoparticles (AUNP) and the intracellular retention time affect their delivery efficiency. Today, carriers for gold nanoions include in vitro CRISPR/Cas9 PDNA delivery systems based on colloidal gold nanoparticles and a class of cationic arginine functionalized gold nanoparticles, mainly used to treat difficult-to-transform hematopoietic stem/progenitor cells.

3.2.6. Physical means of transportation

The advantage of physical transportation is that it does not need to consider the properties and efficiency of the transporter itself. It is simple and convenient to destroy the receptor cell and its nuclear membrane through physical means. Although the current physical drug delivery methods are already in the laboratory, their scalability and technical limitations make it difficult to widely expand. The current mainstream methods include electroporation, micro injection, and fluid dynamics injection. Electroporation is the process of stimulating the formation and opening of nuclear pores on the cell membrane by an electric current, thereby allowing the CRISPR/Cas system to enter [9]. However, it is difficult to use in vivo and can easily cause cell death and loss of cell stem cells without prior cell separation. Microinjection technology is relatively common, with the advantage of being able to deliver any form of CRISPR/Cas system, but its injection characteristics result in extremely high labor costs (requiring injection of cells one by one) and intensive technical requirements, Currently, it is still limited to animal experiments. Finally, there is fluid dynamics injection. Contrary to the previous two, fluid dynamics injection mainly involves injecting a large amount of solution mixed with the CRISPR/Cas system into the body, changing the fluid dynamic pressure and causing changes in cell permeability. This method can deliver the CRISPR/Cas system into various tissues including the heart, liver, kidneys, and more. However, its fluid power requirements make it unsuitable for external use and pose a significant burden on the body, which can easily cause symptoms such as heart failure, liver dilation, and elevated blood pressure.

4. Summary

In this article, we briefly introduce the operating principle of the CRISPR/Cas system, which exhibits strong potential in gene editing by cutting and importing specific gene sequences. On the other hand, it summarizes the advantages and disadvantages of current mainstream delivery systems. Viral carriers have natural advantages in vivo applications due to their properties, but their carrying capacity and immunogenicity limit their further development; Various nano carriers have diverse forms, complex properties, and diverse application scenarios; Although physical methods are stable enough, their high labor costs and insufficient development space are also major drawbacks [10].

CRISPR/Cas is hailed as a revolutionary breakthrough in gene therapy, and compared to previous generations of gene editing technology, the system is more efficient and stable. Due to the lack of a specific and efficient drug delivery system, the application of this system is subject to multiple limitations. Nowadays, carrier systems are complex and diverse, and although most carriers are based on nanoparticles, their advantages and disadvantages are not the same. Either the production process is complex and difficult to mass produce, or the usage environment is limited, or its own properties lead to various potential problems, but different carriers also have unique advantages. Viral carriers have strong guidance and binding abilities, stable physical techniques, and non-viral carriers are easy to transform.

Although most current treatment methods are still based on in vitro methods, more technological breakthroughs have also emerged. In recent years, the emergence of tissue specific and selective organ targeted delivery has provided an effective solution to the problem of carrier specific selection

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