Research status of CRISPR/Cas9 delivery system

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Abstract. Deriving from the adaptive immune system of prokaryotes, the emerging gene-editing technology CRISPR/Cas9, has become the most popular and powerful tool for the manipulation of genes at the molecular level. It has the advantages of easy construction, high efficiency along with good specificity. CRISPR/Cas9 system has been extensively applied to model constructions, clinical treatment and mechanisms of tumorigenesis. As a number of strategies for CRISPR/Cas9 system have been developed, this review systematically summarizes the commonly applied methods along with discussions on their advantages and limitations and different scenarios for specific applications. The basic delivery forms include plasmid DNA with sgRNA, mRNA with sgRNA and a complex of Cas9 protein and sgRNA, ribonucleoprotein (RNP). For the methods of delivery into cells, there are viral vectors and non-viral vectors, which further consist of physical and chemical methods. While the efficiency and rate of successful transfection vary in different strategies, they commonly suffer from off-target effects, mutagenesis, cytotoxicity and immunogenicity to different extents.

Key words: CRISPR/Cas9, delivery, gene editing

1. Introduction

Clustered regularly interspaced short palindromic repeats (CRISPR) is the system which provide prokaryotes with immunity against viruses, by storing DNA fragments of the invaders in spacers between palindromic repeats and generate Cas9 protein to recognize and cleave the foreign genetic element containing the fragment. It serves as the basis for a genome-editing tool which enables permanent gene modification in a specific, targeted order.

In prokaryotic cells, the CRISPR/Cas9 system recognizes the target sequence of invader through the guidance of crRNA and tracrRNA. The crRNA and tracrRNA are transcribed from the CRISPR sequence separately and combine together to form a gRNA on the complementary sections. The gRNA then associates with the Cas9 endonuclease and form a ribonucleotide complex, in which it locates the specific sequence on the genome of bacteriophage for accurate cleavage. In applications of CRISPR/Cas9 system in gene-editing, a synthesized single guide RNA (sgRNA) replaces the crRNA and tracrRNA. When designed sgRNA and Cas9 protein are expressed simultaneously, it guides the Cas9 protein towards the specific sequence for induction of double-strand break at the target point.

Nowadays, delivery forms of CRISPR/Cas9 systems can be subdivided into three levels based on plasmid DNA, mRNA and ribonucleoprotein. Common physical systems for delivery of CRISPR/Cas9 systems include electroporation, microinjection, and tail vein hypertension. Viral vectors in CRISPR/Cas9 systems include adenovirus (AAV), lentivirus (LV), and bacteriophages. Along with the rapid development of nanotechnology in recent years, the non-viral vectors of CRISPR/Cas9 systems have also effectively realized the delivery to cells and tissues *in vivo* and *in vitro*, and nanocarriers have gradually become potential tools in applications of gene therapy in CRISPR/Cas9 systems, and emerging new strategies have also made the delivery of CRISPR/Cas9 more mature.

2. Delivery forms for CRISPR/Cas9 systems

2.1. Plasmid DNA

Plasmids designed for DNA delivery of Cas9 endonuclease and sgRNA has been the most popularly applicated method of CRISPR/Cas9 system delivery in researches. The use of plasmids provides a major benefit of its high efficiency and great ease in design [1]. Furthermore, higher stability is shown in CRISPR-Cas9 system based on plasmid DNA, which enables simpler and less consuming purification processes [2].It has a wide range of application, and serves as the only choice for a number of studies on translation control. However, the transfection efficiency of plasmid DNA highly relies on the mitotic activities of cells, which becomes a barrier of transfection of non-dividing cells. Another limitation of it is that it could trigger immune responses in eukaryotic cells [3]. Since plasmid DNA is recognized as foreign in the cytoplasm, it may become strongly immunogenic and get hydrolyzed before entering the nucleus.

2.2. mRNA

The method of delivering CRISPR Cas9 protein and sgRNA directly into cells has shown advantages in other aspects. While it is necessary for plasmid DNA to enter the nucleus for successful transfection, mRNA exerts effect once entered the cytoplasm. This makes it desirable in applications in hard-to-transfect cells. As it does not integrate itself into the host genome, the use of mRNA eliminates potential risks of gene mutations due to random insertions as well. Since mRNA only lasts for a short period, it is an ideal form of delivery in a number of applications, especially studies of transient gene-editing [4]. Additionally, the rate of protein expression is far rapider comparing to plasmid DNA, while the rate is manageable through modification of the concentration of mRNA [5]. The main drawback of mRNA transfection is its poor stability, and as for plasmid DNA, mRNA could generate an immune response as well, which considerably limits the period of expression.

2.3. Ribonucleoprotein

As a third alternative, direct delivery of ribonucleoprotein complexes (RNP) as combinations of Cas9 protein and sgRNA has been most extensively researched during the recent several years. RNP offers numerous advantages over the traditional method of plasmid delivery. First, it performs the promptest cleavage once enter the nucleus, as the processes of transcription and translation are not required [6]. Besides, it is able to avoid the potential mutagenesis caused by unexpected insertions while creating long-term transgene expression. Another main benefit of RNP delivery is the alleviation of off-target effects. As RNP degrades rapidly in the cytoplasm, it can minimize the possibility of occasional cleavage of a similar sequence, while reducing the immune responses [7]. Due to the great number of advantages, RNP has become the most promising platform of CRISPR/Cas9 system with a high utility.

Table 1. Characteristics of different delivery forms for CRISPR/Cas9 system

CRISPR/Cas9 platforms	Advantages	Disadvantages
CRISPR/Cas9 plasmid	Easy preparation Broad range of application	High dependence on mitotic activities of cell Can be immunogenic in eukaryotic cells More off-target effects
Cas9 mRNA and sgRNA	Rapid protein expression Controllable rate of protein expression	Unstable, easily degraded by nuclease Can be immunogenic in eukaryotic cells
Cas9 protein and sgRNA	Extreme efficiency of editing Reduced off-target effect Low immunogenicity	Time-consuming and complex purification of Cas9 protein [8] Protease sensitivity

3. Viral vector for CRISPR/Cas9 systems

Over the past few years, viral vectors of the CRISPR/Cas9 system have been powerful tools widely used in *in vivo* due to their excellent transfection efficiency and stable transgene expressions. Common viral vectors include adenoviral vectors (AdV), adeno-associated virus (AdV) and lentiviral vectors (LV).

3.1. AAV

AAV, adeno-associated virus is a small-size, non-enveloped linear single-stranded virus belonging to the Dependoparvovirus genus. It is known to be the most promising viral vector which has a wide range of application. One of the advantages of AAV vector is its significant lack of pathogenicity, and tend to cause low immune response [9]. It requires a co-infector in order to replicate due to its replication-defensive feature as well. And AAV vector is able to transfect both diving and non-dividing cells. These features made it a competitive candidate for gene therapy. As the major percentage of naturally exist AAV is non-integrating, a small percentage are able to integrate their genome to human genome, at the chromosome 19. It shows the possibility of long-term expression, and the specific site of integration further prevent the mutation caused by random insertions [10]. However, the transgene of the majority would remain as episomes in the cell and get removed eventually. This removal is slower than adenovirus due to its lower pathogenicity. Another benefit of AAV in human gene therapy is its varied natural tropisms towards specific cell types that can be achieved by different capsid serotypes. For instance, AAV2 has presented tropisms toward skeletal muscles, neurons, vascular smooth muscle cells and hepatocytes, and AAV5 exhibits tropisms toward astrocytes and vascular epithelial cells. However, there is an unneglectable disadvantage of AAV, as its size becomes the greatest limitation in gene delivery [11]. The length of the DNA of AAV is only 4.7kb in length, which critically limits the length of gene that can be packaged in. As a combination of a spCas9 and sgRNA occupies ~4.2 kb, there is much less space left for regulatory elements.

3.2. AdV

Adenovirus is a non-enveloped virus with a dsDNA genome. It has an outstanding packaging capacity up to 7.5 kb as well as a superior efficiency of gene delivery. Since adenovirus is a non-integrating virus, its genome remains as an episome in the cell instead of being integrated into the host genome, which contributes to the minimization of risks of undesired mutations as results of random insertions.Due to its properties, adenoviral vectors are often used in production of short-term transgene expression, and unpreferred in researches where long-term gene expression is studied [11]. The major risk of using adenovirus is its high immunogenicity. According to past researches, they tend to cause strong immune and inflammatory responses in animal models, which becomes a significant limitation to its clinical applicability [12].

3.3. LV

Lentivirus, a single-stranded RNA virus belonging to the retroviridae family is one of the most common in use viral vectors. Comparing to other retroviruses, the most outstanding advantage of using lentivirus is its ability to penetrate through the nuclear membrane, which enables it to infect both dividing and non-dividing cells [11]. Once entered the cell, it stably integrates its genome to the host genome. Two enzymes are expressed in the cell to carry out this process. A reversed transcriptase converts the single-stranded RNA into a complementary DNA, and as the DNA migrates into the nucleus, an integrase catalyzes the integration of the viral DNA [13]. The Cas9 protein is then able to be transcribed and translated after they leaves the cell. Hence stable, long term transgene expression can be provided, as it persists after cell division. Due to its low pathogenicity, lentivirus does not trigger the production of immunogenetic proteins in a large scale [11].

4. Non-viral vectors for CRISPR/Cas9 systems

The non-viral delivery methods are generally lower in cost and take less time in production, while offering reduced immunogenicity. In comparison to non-viral vector, viral vector has a major advantage of transfection efficiency, due to its accuracy of introducing extracellular genetic material into the cells.

4.1. Liposomes

Lipid-mediated transfection is one of the most popular techniques in laboratories, since it is convenient and effective for transferring nucleic acids into suspension and adherent culture cells. In a nucleic acid molecule, the bonds between phosphate groups and oxygen atoms causes it to carry a negative charge. And this allows the phosphate backbone to form complexes with cations. Therefore, nucleic acid molecules can form complexes named liposome with cationic lipids as they carry positive charges. The liposome can then merge with the cell membrane and enter the cell via endocytosis [14]. Transfection efficiency varies depending on the reagent and the cell type. Liposomes have also shown a great potential in delivering RNPs, which successfully performed gene modification in up to 80% human cells [15].

4.2. Calcium phosphate-DNA coprecipitate transfection

Calcium phosphate-DNA coprecipitate transfection is another popular modal of CRISPR-Cas9 delivery, it is an economic technique, as another major advantage is its low cytotoxicity. It is widely applied the research of transient and stable transgene expression. At the start of the preparation of the experiment, DNA is directly mixed with a CaCl2 solution. The phosphate groups of DNA which carry negative charges would bind with the calcium cations though electrostatic interaction. The mixture is then added dropwise into a PBS (phosphate buffer saline), a compound of Na2HPO4 and KH2PO4, and left at room temperature for 12-48 hours to incubate, and format nanoparticle coprecipitates that contain condensed DNA molecules[16]. When added into cells, the nanoparticles adhere to the surface of cell membranes and can therefore enter via endocytosis. The vesicles containing DNA burst in the reaction with lysosome, releasing DNA into the cytoplasm, while the calcium phosphate covering prevents DNA from degradation by the nuclease. 1-5% of DNA entered the cell are able to enter the nucleus, and only 1% would succeed to integrate themselves with the host genome [17].Despite its high applicability to eucaryotic cells, calcium phosphate transfection has a poor repeatability and is highly unstable because of the extremely precision required for the pH condition. The efficiency is greatly sensitive to pH changes, and a small change could result in the failure of experiment.

5. Conclusion

The emergence of CRISPR/Cas9 has brought a revolution to genome editing, providing researchers a powerful and swift tool to edit the genes of a broad range of organisms. The forms of delivery of CRISPR/Cas9 system, consisting of the sgRNA and Cas9 protein, can be classified into 3 types, including plasmid DNA, mRNA and ribonucleoprotein (RNP). In the current phase, plasmid DNA remains as the most extensively used platform. However, due to its significant advantages, especially the ability to reduce off-target effects, RNP has received an increasing amount of attention in recent years. As for the delivery methods of CRISPR/Cas9 system, viral vectors are commonly applied due to their high efficiency of transfection and accuracy towards target cells in *in vivo* gene modification. In comparison, the non-viral vectors provide unlimited packaging capacity and able to be produced in large scales, through which they present more advantages despite a low efficiency and transiency of gene expression.

The reliability of delivery has always been a vital factor which impacts the rate of success and efficiency for gene-editing. For CRISPR/Cas9 system, the off-target effects have always been one of its major limitations, and the development of RNP platform seems to provide an effective solution.

As the currently existing strategies of delivery still encounter issues including low efficiency, cytotoxicity, insertional mutagenesis and immunogenicity, there has not yet been a dominating method. And it is obvious that great potential has remained undiscovered in this field, which would deserve further exploration in the future.

References

[1] Ran, F. A., Hsu, P. D., Wright, J., et al. Genome engineering using the CRISPR-Cas9 system [J]. Nature protocols, 2013, 8(11), 2281–2308.

[2] Liu, M. A. A Comparison of Plasmid DNA and mRNA as Vaccine Technologies [J]. Vaccines, 2019, 7(2), 37.

[3] Terenin, I. M., Dmitriev, S. E., Shatsky, I. N.. Pros and cons of pDNA and mRNA transfection to study mRNA translation in mammalian cells [J]. Gene, 2016, 578(1), 1–6.

[4] Liu, C., Zhang, L., Liu, H., et al. Delivery strategies of the CRISPR-Cas9 gene-editing system for therapeutic applications [J]. Journal of controlled release : official journal of the Controlled Release Society, 2017, 266, 17–26.

[5] Xiao, Z. Y., Chen, X. J., Xu, R. X. Research progress on RNA transfection dendritic cell vaccine for glioma [J].Western Medicine, 2015, 27(11):1759-1761.

[6] Zhang, S., Shen, J., Li, D., et al. Strategies in the delivery of Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing [J]. Theranostics, 2021, 11(2), 614–648.

[7] Kim, S., Kim, D., Cho, S. W., et al. Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins [J]. Genome research, 2014, 24(6), 1012–1019.

[8] Lin, Y., Wagner, E., Lächelt, U. Non-viral delivery of the CRISPR/Cas system: DNA versus RNA versus RNP [J]. Biomaterials Science, 2022, 10(5), 1166–1192.

[9] Vance, M. A. , Mitchell, A., Samulski, R. J. (2015). AAV Biology, Infectivity and Therapeutic Use from Bench to Clinic. In (Ed.), Gene Therapy - Principles and Challenges. IntechOpen.

[10] Rajapaksha, I., Angus, P., Herath, C. (2018). Adeno-Associated Virus (AAV)-Mediated Gene Therapy for Disorders of Inherited and Non-Inherited Origin. In (Ed.), In Vivo and Ex Vivo Gene Therapy for Inherited and Non-Inherited Disorders. IntechOpen.

[11] Chen P., Zhang L. J., Li, N. Research progress on adeno-associated viral vectors [J]. Chinese Journal of Biologics, 2022, 35(04):500-507.

[12] Liu, Y., Du, L. F. Research progress on adenovirus vectors [J]. Medical Review, 2015, 21(24):4438-4440.

[13] García-Vallejo, F., Domínguez, M. C. (2012). Systemic Approach to the Genome Integration Process of Human Lentivirus. In (Ed.), Bioinformatics. IntechOpen.

[14] Pan, X. H., Wu, Z. H., Qi, X. L. Research status and application progress of CRISPR/Cas9 delivery system [J]. Journal of China Pharmaceutical University, 2020, 51(01):10-18.

[15] Zuris, J. A., Thompson, D. B., Shu, Y., et al. Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo [J]. Nature biotechnology, 2015, 33(1), 73–80.

[16] Jiang, M., Chen, G. High Ca2+-phosphate transfection efficiency in low-density neuronal cultures [J]. Nature Protocols, 2006, 1(2), 695–700.