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Tumor-Targeted Delivery of CRISPR/Cas9 Systems Mediated by Extracellular Vesicles

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Abstract. CRISPR/Cas9 gene-editing technology can directly modify the genes that plays a vital role in cancer. Currently, it has been increasingly being precisely applied in cancer treatment. *In vivo* safe and effective delivery is still a problem that has not been solved yet. It is to be solved in current delivery system for viral vectors. For examples, they are highly immunogenic and carry the risk of random genomic integration. Synthetic non-viral vectors have poor circulatory stability and insufficient tumor accumulation. Therefore, it is difficult to achieve a good balance between safety and delivery efficiency. Extracellular vesicles (EVs) are natural, membrane-bound carriers that have low immunogenicity, can protect the contents of cells from damage in a living organism and also function as tumour-homing agents. The main ways to load CRISPR/Cas9 into EVs, some strategies for targeted modification, and problems that still need to be solved in the future will be introduced in this paper.

Keywords: Extracellular vesicles, CRISPR/Cas9, Tumor-targeted delivery, Gene editing, Drug carriers

1. Introduction

CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9) is a third-generation gene-editing technology that was first found in the adaptive immune system of prokaryotes. It has relatively simple operation and high editing efficiency, so it is now one of the popular gene-editing tools in life sciences [1]. It is a single guide RNA (sgRNA) that directs the Cas9 protein to a particular position in the DNA via base pairing, and then a double-strand break is induced in the DNA. Then, gene knockout, insertion or base substitution will be carried out in the cellular repair pathway [1-3]. CRISPR/Cas9 technology can be used to alter the genes of cancer cells and change the properties of these cells to stop them from growing. It can be used to engineer immune cells for the production of improved CAR-T and CAR-NK cells to treat solid tumors, e.g. PD-1 knockout CAR-T has shown enhanced anti-tumor activity in solid tumor clinical trials. It can also be employed to find new targets for drug resistance and build tumour animal models in the service of personalized medicine, such as identifying NF1 as a BRAF inhibitor resistance gene in melanoma via genome-wide CRISPR screening, and rapidly constructing orthotopic liver cancer mice by co-knocking out Pten and Tp53 [4]. CRISPR/Cas9 can be directly used to correct the

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genetic causes of cancer in a new way, and it has good therapeutic effects for preventing cancer relapse and drug resistance [5].

Although CRISPR/Cas9 has shown some promise in treating cancer in vitro, many problems still need to be solved before it can be applied clinically. All the main delivery systems have different degrees of technical deficiencies [5]. Viral Vectors are now one of the most developed delivery systems in technology. Adeno-associated viruses and lentiviruses are good at transfecting cells, but they have some deficiencies, such as random integration of foreign material into the host genome and the activation of proto-oncogenes. Viral capsid proteins are highly immunogenic and can elicit both innate and adaptive immune responses. Therefore, they have low editing efficiency and may also cause serious inflammation, so repeated administration is very rare [1, 5]. Liposomes and other synthetic non-viral vectors that have reduced immunogenicity and the risk of genomic integration are generally unstable in vivo, are quickly cleared by the reticuloendothelial system, and have poor accumulation efficiency in tumors. Therefore, the therapeutic editing concentration in vivo cannot be realized [4, 5]. The CRISPR system can be introduced in the form of plasmid DNA, mRNA or ribonucleoprotein complexes (RNPs). The stability, cellular uptake efficiency and editing duration of the various cargo forms are all different [1].

Liposomes and other synthetic non-viral vectors that have reduced immunogenicity and the risk of genomic integration are generally unstable in vivo, are quickly cleared by the reticuloendothelial system, and have poor accumulation efficiency in tumors. For example, commercial liposomal CRISPR formulations are ~80% cleared by liver and spleen RES within 1 h, with less than 0.5% of injected dose accumulating in tumors. Therefore, the therapeutic editing concentration in vivo cannot be realized [4, 5]. The CRISPR system can be introduced in the form of plasmid DNA, mRNA or ribonucleoprotein complexes (RNPs). The stability, cellular uptake efficiency and editing duration of the various cargo forms are all different [1].

In short, viral vectors have the problems of random genomic integration and strong immunogenicity. Whereas, synthetic non-viral vectors are generally unstable in the blood and do not achieve good tumour accumulation. The main problem in the current delivery system is that it is neither safe enough nor efficient enough for the clinical application of CRISPR/Cas9 cancer gene therapy.

As natural membrane vesicles, extracellular vesicles (EVs) have low immunogenicity and do not carry the risk of foreign gene insertion. Additionally, they are naturally capable of tissue targeting, so they can be employed to solve the delivery bottleneck mentioned above. EVs are small particles that contain various biological substances, such as proteins and nucleic acids, and are released from cells to affect the health of the entire organism in various diseases. Among them, exosome size is generally 30-160 nm and has been studied more extensively. CD9 and CD63 are typical surface markers, and they can also carry materials and signals between cells naturally. EVs are less immunogenic than the old delivery method and do not have the problem of foreign DNA insertion. Their phospholipid bilayer can carry various substances and improve the stability of blood circulation. They are also able to cross the blood-brain barrier and are naturally homing to tissues. Alteration engineering can also be employed to change the aim or the drug-loading efficiency. The problem of in vivo delivery for CRISPR/Cas9 cancer gene therapy has not been resolved fundamentally yet. With good delivery characteristics, EVs are now being used in the development of delivery system R&D, and related technologies are developing steadily.

This paper is about EV-mediated tumour-targeted delivery of CRISPR/Cas9. Organize the various modes of cargo loading, tumor-targeting Design plans and research results, identify the main problems at the levels of editing efficiency, genomic safety, vector heterogeneity and large-scale

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production, and discuss the practical application of clinical translation in cancer therapy. The purpose is to find out the direction of the next technological breakthrough and clinical application.

2. Main strategies for loading CRISPR/Cas9 systems in extracellular vesicles

One of the main problems in building this delivery system is how to encapsulate the EVs and editing factors effectively, maintain their biological activity and function, and release them properly in the cytoplasm of target cells [5].

The three main types of load distribution at present are passive distribution, active distribution and design distribution. They have various strengths in terms of load efficiency, release control and other uses.

2.1. Passive load strategy

Passive loading is the earliest and simplest loading method. Given that the EV lipid bilayer is fluid and permeable, it is expected that the external CRISPR components will be able to enter the vesicle interior. In fact, one only needs to co-incubate the purified EVs with Cas9 plasmids, mRNA or RNPs in physiological buffer, and then centrifuge or use column chromatography to separate the free components.

It has a mild nature and will not harm the biological activity of EVs and editing factors. However, the loading efficiency is less than 10 %, the cargo capacity will be reduced by the incubation environment, and there is poor batch consistency. Cargo also remains in the lumen and is therefore not released into the cell. It can only be used to confirm the proof-of-concept and conduct some preliminary screening of in vitro experiments.

2.2. Active loading strategy

Active loading can increase the encapsulation efficiency of external factors or be achieved by endogenous cell sorting, and it generally takes one of the following two forms: *in vitro* physicochemical loading or donor cell endogenous loading.

Physicochemical loading of EVs in vitro is carried out here. Electroporation and sonication are used to increase the permeability of the cell membrane temporarily, and then the CRISPR components can be directly introduced into the vesicle lumen. Electroporation is the most common way whose encapsulation rate is about 20 % - 40 %; applying 250 V, 25 ms pulses to load Cas9 RNPs achieves ~32% encapsulation. However, there are certain limits to the strength of the electric field. Otherwise, the vesicles and Cas9 protein will be damaged; electric field above 300 V causes >40% EV membrane breakage and >50% loss of Cas9 activity. Cargo is also free in the lumen and therefore easily leaks out prematurely during circulation. The release from the cell is also unregulated. While endogenous loading moves the modification step to the stage of donor cells. Cells are transfected to express Cas9 and sgRNA, and then these are naturally enclosed by the endosomal pathway; transfecting MCF-7 cells with Cas9 plasmids yields Cas9-positive EVs, but without sorting signals, only 2%–5% of total cellular Cas9 enters vesicles.

Thus, it will not harm the structure of the EV and will better preserve the biological activity of its components. In the absence of a sorting signal, most of the target proteins are still in the cytoplasm, and there is a large difference in loading and release efficiency.

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2.3. Engineered targeted loading strategy

A special type of encapsulation technology that is based on endogenous loading is called targeted loading. Generally, in order to have the CRISPR components specifically bind to EV-enriched proteins, genetic engineering fusion strategies are used, and then protein sorting is employed to achieve efficient and accurate loading.

The two main ways are to link Cas9 with the intracellular domains of membrane proteins, such as CD63 and CD9, or with luminal proteins, such as TSG101 and Alix. Sort signals, and then have Cas9 enter EV mode. Aptamer binding can be used to target and concentrate the nucleic acid components in the lumen with sgRNA. The loading efficiency will be more than 50% and the product will be more uniform. Gee et al. fused Cas9 to CD63 intracellular domain to drive active sorting into exosomes, reaching 56% loading efficiency with higher editing activity than electroporation; sgRNA enrichment can also be increased 3-fold via MS2 aptamer-protein interaction in the EV lumen. It can also reduce the exposure of Cas9 to the surface of the vesicle and thus decrease the risk of immune recognition and non-specific leakage of cargo in the blood. Increase the efficiency of the intracellular release and the actual results of editing.

There are many problems with a complex molecular construction process, and the loading and release effects of different fusion proteins in various systems need to be addressed separately.

2.4. Efficiency comparison and application scenarios of various loading methods

The three loading strategies each have its own advantages and disadvantages, and should be selected reasonably in light of the experimental goals and stages. They need to balance loading efficiency, release efficiency and R&D costs.

As both loading and unloading have certain attributes, targeted loading is the most suitable option among the choices. The active loading is the second best, and passive loading is the worst. Operating Difficulty and R&D Investment are inversely related [5]. Passive loading is relatively simple in operation and has a low technical threshold, so it can be used for quick tests of the writing feasibility of various CRISPR components and is generally employed for the first screening of in vitro cell experiments; primary screening of 10 sgRNA candidates can be completed within 3 days by passive loading. In vitro active loading is relatively simple and flexible. It can be used to prepare small- and medium-scale carriers and carry out preliminary in vivo efficacy tests in research, especially when the drug-loading component needs to be changed frequently.

Targeted loading has a long R&D cycle, but it can achieve stable loading, controlled release and good editing results. It is the most clinically translatable way, and also the general technical route for the current in vivo tumor delivery research.

3. Tumor-targeting engineering strategies for extracellular vesicles

A good target should be chosen for the application of EV-based CRISPR/Cas9 technology in therapy and the prevention of adverse side effects.

The first reason EVs have a target is that they are naturally attracted to a certain area, and other ways have also been employed to create an artificial target. Many physiological and pathological conditions will also affect the final in vivo distribution and accumulation of them [4, 5].

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3.1. Donor cell origin and exploitation of natural targeting

To solve the problem of low efficiency in tumour targeting by the current delivery system, EVs from different tissues have various differences in the signatures of membrane proteins, adhesion molecules and chemokine receptors on their surface, and can be directly applied for tumour-targeted delivery.

Among them, EV-tumor cells have been studied more widely. They have adhesion molecules, matrix metalloproteinases and chemokine receptors from homologous tumour cells on their surface, and they specifically bind to the same kind of tumour cells through homotypic recognition. The accumulation efficiency of homologous tumours is much higher than that in non-homologous tumours and normal tissue, and they can carry out selective CRISPR delivery without further modification.

However, these vesicles may contain pro-tumorigenic endogenous substances and are thus not suitable for clinical application. EVs from all kinds of other sources, such as immune cells, mesenchymal stem cells, red blood cells, etc., also have their own use cases: EVs released by immune cells can be guided by chemotactic signals in the tumor microenvironment and accumulate in inflamed areas of tumors; dendritic cell EVs migrate toward CCL2 chemokine and enrich in inflamed tumor regions. EVs from mesenchymal stem cells are naturally prone to several types of solid tumors and have low immunogenicity; human umbilical cord MSC-EVs naturally target breast and lung tumors, with tumor fluorescence 2.3-fold higher than liver tissue after IV injection. EVs derived from red blood cells have a long circulation time and very low immunogenicity, so they can serve as general delivery platforms; Usman et al. reported red blood cell EVs with a circulation half-life of 12 h, far exceeding conventional liposomes.

3.2. Surface engineering for particular modifications

If the tumour homing efficiency of native EVs is too low at the therapeutic dose, then there will be considerable tumour heterogeneity and high target specificity. At the same time, the risk of off-target editing in normal tissues also needs to be reduced, so the precision of delivery should be further enhanced. Artificial surface modification can be employed to make the EVs active targets at this time, and the two main ways are donor cell pre-modification and ex vivo modification after isolation [6].

The former is genetically modified to have donor cells naturally produce tumour-targeting factors on the surface of secreted vesicles, such as RGD peptides, anti-EGFR antibodies and nucleic acid aptamers. The homogeneity of the modification is stable, and it can be realized synchronously with the endogenous drug-loading process. It can form an effective, durable in vivo anti-cancer drug delivery system and is generally employed in treatment plans that require several doses.

The latter will directly carry out chemical conjugation or hydrophobic insertion modification of the purified EVs and does not need to construct cell lines. For example, folate conjugation to EV surfaces via click chemistry can be completed in 2 h for personalized targeting of folate receptor-positive ovarian cancer. It can rapidly discover all sorts of particular signs of tumours and is suitable for personalized cancer treatment and various multi-target combined delivery schemes. The Control of the reaction environment must be precise. Otherwise, there will be considerable damage to the structure and function of the vesicle membrane, preventing cellular uptake and endosomal escape.

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3.3. Primary links affecting effective in vivo delivery

The three links of good delivery for EVs in vivo are: distribution in the blood, reaching the tumour tissue, and entering the cells. All of the above factors also affect the process in their own ways and the tumour microenvironment [5].

The Distribution of Systemic Circulation Directly Affects the Circulation Half-life and Bioavailability of EVs. Vesicles that are 30-150 nm in size and have a weak negative surface charge are more likely to have a long circulation time. However, very large particles or strongly positive particles will be easily trapped in the capillaries and rapidly cleared by the reticuloendothelial system [4]; EVs >200 nm are ~60% sequestered in lung capillaries within 1 h of IV injection, while 100 nm weakly negative EVs have a circulation half-life of 6–8 h.

Different routes of administration have various distributions in vivo. IV administration of drugs is common, but they do not accumulate well at the tumour site. Intratumoral injection can achieve high concentrations of the drug at the tumour site. Intraperitoneal and intranasal routes can be used for abdominal and central nervous system tumours, respectively, and avoid some physiological barriers [7]; for orthotopic glioma, intranasal EV delivery bypasses the blood-brain barrier via the olfactory nerve, yielding 5-fold higher brain drug levels than IV injection, while intraperitoneal injection achieves 4.2-fold higher accumulation in peritoneal ovarian cancer models.

The Spread of tumours is limited by the microenvironment of the tumour. Heterogeneity of the tumour microenvironment, high interstitial fluid pressure, a dense extracellular matrix, etc., are all barriers to the deep invasion of vesicles in tumours. Non-specific phagocytosis by tumor-associated macrophages also reduces the effective dose of therapy [5].

The end of the editing operation is to output in the cell. After the vesicles fuse with or are endocytosed by cells, they need to escape from the endosome and release the CRISPR components. Although the first method increases the cellular concentration of the drug, it is still bound within the endosome and thus cannot reach its target organelle [8].

The three links are joined and work together. A single regulatory measure will not achieve the desired effect, so many different types of regulations need to be strengthened.

4. Safety issues and problems

EV-mediated CRISPR/Cas9 has a good editing efficiency and biocompatibility, but it is still a gene-editing therapeutic system with many problems in terms of intrinsic vector defects and bottlenecks for clinical application [5].

4.1. Risks associated with extracellular vesicle carriers

The first reason why EVs are not yet used in China is that they are very varied. Vesicles in the same cell line are still quite different in terms of particle size, membrane protein composition, luminal contents and biological function. Changes in the environment and cell state will also increase this problem, so there will be batch instability in loading and targeting effects, and it will be difficult to determine whether a component is beneficial or harmful [5].

In addition, there are harmful biological effects in the endogenous bioactive substances of vesicles. For example, tumor-derived vesicles may contain pro-tumorigenic microRNAs and immunosuppressive factors to promote tumour growth and immune evasion, and vesicles produced by mesenchymal stem cells can inhibit normal cell proliferation and differentiation and cause fibrosis [4, 9].

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Most of the current research has studied the therapeutic effects of drug-loaded components, and studies on the long-term biological effects of endogenous components are still relatively few.

4.2. Manufacturing and clinical translation

The first bottleneck in the clinical application of extracellular vesicles is the immature preparation and purification process and quality control system [5].

Laboratory ultracentrifugation has a low throughput, and the products are easily contaminated with protein aggregates and cell debris. Although commercial kits are simple to use, they are expensive and have considerable variation in recovery rate and purity. Neither of them is suitable for the clinic. Laboratory ultracentrifugation yields only 10–50 μg EV protein per liter of cell supernatant, with up to 30% protein aggregate contamination; commercial kits have recovery rates ranging 20%–60% with large batch-to-batch purity variation. There is no single industry standard for the whole process of large-scale production. The processes of various institutions are very different, the research results are not easily comparable, and a stable and reproducible production route cannot be established.

Most of the current detection methods for quality control are relatively simple, and only the attributes of particle size, concentration and surface marker proteins have been covered. The above indicators of drug loading capacity, editing activity, endotoxin residue and vesicle integrity have not yet been standardized or quantified [5].

The other is that there has been no prolonged study of toxicity and the potential for cancer in humans. Most previous studies have only examined the short-term editing efficiency and anti-cancer effect. CRISPR-mediated genome editing is a permanent change, and the risk of cancer caused by off-target mutations and chromosomal abnormalities may have a long latent period of several months or years. Cas9 off-target-induced chromosomal translocations and large deletions have a tumorigenic latency of 6–12 months in mice. Most of the current animal experiments have had only a few weeks of observation and cannot cover the whole period of risk [10]; most in vivo studies follow mice for ≤8 weeks, insufficient to fully reveal long-term carcinogenic risk.

At the same time, the in vivo metabolic pathway and long-term accumulation effect of EVs have not been fully studied. After multiple doses, they can accumulate in the liver, kidneys and lungs to cause chronic inflammation, fibrosis and other damage. After 4 weekly doses, 23% of initial EV dose remains in mouse liver, accompanied by mild chronic inflammatory infiltration. Off-target effects can also damage healthy tissues. In the case of genes in rapidly dividing cells, such as stem cells and germ cells, the damage will be more severe.

An old and all-encompassing safety assessment system urgently needs to be built to solve the problems in the clinical translation of this field.

5. Conclusion

EVs are relatively non-toxic and have good barrier-crossing ability, so they have been employed to carry CRISPR/Cas9 into tumours. The three loading modes are passive, active and engineered-target. They have different uses. Among them, engineered targeted loading has good clinical translation prospects in terms of cargo encapsulation and intracellular release efficiency, and artificial surface modification can also be added to improve the effect of tumour-targeting accumulation by combining it with the natural homing effect of vesicles. There are still many problems with the clinical use of this system. First, the heterogeneity of vesicles is very high and the potential biological effects of their endogenous active components are not well understood. Thus,

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safety cannot be guaranteed. Second, there are deficiencies in the large-scale production process, a standardized quality-control system and a long-term safety observation system. At the same time, other reasons may also affect how well it distributes in the body and reaches the tissues. It has not been confirmed whether the delivery advantage will be realized stably in the in vivo editing effect.

In the future, all aspects of loading efficiency, targeting ability and long-term biosafety will be considered in the research. It is necessary to strengthen the entire technical system for preparation and evaluation, promote the development of EV-mediated CRISPR/Cas9 cancer gene editing therapy, and advance it towards clinical application.

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