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Extracellular Vesicles as Promising Vectors for CRISPR/Cas9 Delivery: Advantages, Challenges, and Clinical Implications

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ABSTRACT

The CRISPR/Cas system, with its capacity to modify DNA sequences and thus change cellular and organ traits, holds promise for gene study and therapeutic interventions. However, the absence of reliable and efficient delivery mechanisms curtails its clinical usage. Extracellular vesicles (EVs) present an appealing avenue for transporting CRISPR/Cas9. They offer multiple benefits over other delivery vehicles, especially in safety, protection, carrying capacity, tissue penetration, targeting precision, and adaptability. As a result, there's a growing trend in employing EVs for in vivo delivery of CRISPR/Cas9. This article provides an overview of the strengths and weaknesses associated with various CRISPR/Cas9 delivery mechanisms and vehicles. It underscores the unique attributes of EVs as carriers, delving into their inherent features, physiological and pathological roles, safety profile, and targeting proficiency. The piece also delves into the origins of EVs, methods for their isolation, and techniques for packing CRISPR/Cas9 within them. In its conclusion, the article charts a path forward, emphasizing the pivotal areas of focus for optimizing EVs as carriers in CRISPR/Cas9's clinical utilization, namely the safety, storage potential, uniformity, production rate, and precise targeting of EVs.

Keywords: CRISPR/Cas9, Extracellular vesicles; Delivery, Biomedical applications

1 INTRODUCTION

During the 1970s, the emergence of genetic engineering brought about a significant advancement in genome editing technology. This breakthrough provided a potent tool for precisely modifying DNA sequences [1]. Up until now, genome editing technology has opened up new possibilities in the field of biomedical research, including the study of genes, understanding biological processes, and developing treatments for diseases. Restriction enzymes were used in 1971 to successfully manipulate specific DNA fragments, marking the early application of genome editing tools. The development of genome editing tools has progressed through the integration of endonucleases and locators. Examples of these tools include zinc finger nucleases, transcription activator-like effector nucleases, and the CRISPR/Cas systems [2]. The CRISPR/Cas systems have particularly revolutionized the field of genome editing. Originally discovered during the investigation of defensive mechanisms in bacteria and archaea, CRISPR/Cas can be likened to an adaptive immune system that protects against foreign nucleic acids [3]. Once foreign nucleic acids invade, the received nucleic acid fragments undergo integration and processing, ultimately producing CRISPR RNA (crRNA). This crRNA can then bind to foreign nucleic acid sequences through complementary pairing and direct the Cas protein. Subsequently, a complex is formed between the Cas protein, other RNA molecules, and the crRNA, which ultimately leads to the cleavage of DNA (Fig.1) [4]. The CRISPR/Cas9 system is widely utilized in genome editing due to its simplicity and the ability of a single Cas9 protein to cleave nucleic acid segments. Among the various CRISPR/Cas systems discovered, the type II system is

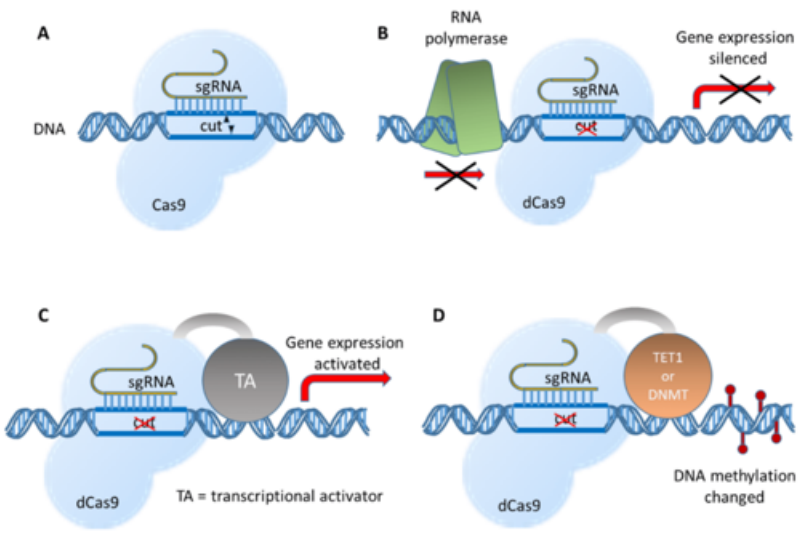

Fig. 1. Overview of the foundational CRISPR/Cas9 mechanism and its evolved fusion enzyme variants. (A) At its core, the CRISPR/Cas9 system creates a double-strand disruption near the sgRNA's attachment point. (B) An altered Cas9 protein, lacking DNA severing capability (termed dead Cas9 or dCas9), retains its DNA binding function at the specific site guided by sgRNA. This binding impedes the movement of the RNA polymerase, leading to transcription suppression. (C) When merged with transcriptional enhancers, targeted sites can activate or amplify gene expression. (D) By connecting dCas9 with histone modifying agents or DNA methylation enzymes, precise epigenetic modifications can be introduced (10).

particularly favored. In practical applications, a synthetic guide RNA (sgRNA) is created by combining crRNA and trans-activating crRNA (tracrRNA). This sgRNA has the capability to bind specifically and direct Cas9 to cleave DNA. The CRISPR/Cas9 system is commonly employed in research fields such as basic biology, biomedicine, and agriculture [5].

To carry out genome editing using CRISPR/Cas9, it is important to address the method of delivery. In order to perform genome editing with CRISPR/Cas9, it is crucial to consider the approach for delivering the technology. Typically, the methods for delivering CRISPR/Cas9 can be divided into two main categories: viral vectors and nonviral vectors [6]. Viral vectors have been shown to greatly enhance the delivery and gene editing efficiency of CRISPR/Cas9 [7]. Unfortunately, the use of viral vectors is limited due to various factors such as safety concerns, high expenses, complex procedures, and limited capacity for packaging [8]. Nonviral vectors primarily rely on physical techniques and synthetic chemical and natural substances, including microinjection, electroporation, liposomes, nanoparticles, and gold nanoparticles [9]. However, the use of nonviral vectors is hindered by their cytotoxicity, limited ability to penetrate cells, and lack of precise targeting capabilities, despite their advantages of simplicity, cost-effectiveness, and versatility [10].

Therefore, it is important to address the challenges of safety, efficiency, stability, targeting, penetrability, and biocompatibility in the delivery of CRISPR/Cas9. Additionally, new methods for delivering genes should be explored. One potential option is extracellular vesicles (EVs), which are produced by different cells and have a structure consisting of a phospholipid bilayer. Initially, EVs were considered to be waste material from cells [11]. It is challenging to isolate pure EVs due to their overlapping size, biomarkers, and composition, even though they can be categorized into exosomes, microvesicles, and apoptotic bodies based on their size and how they are generated [12]. Hence, in this review, the terms exosomes, microvesicles, and apoptotic bodies will be collectively referred to as EVs due to the difficulties in distinguishing them based on size, biomarkers, and composition. Recent advancements in EV research have revealed their crucial role as mediators in intercellular communication [13]. The potential significance of EVs has been unveiled in the realm of diagnostic biomarkers and therapy due to their involvement in a range of physiological and pathological processes, such as immune response regulation, tissue repair, and cell growth [7]. The transport capabilities of EVs are valuable in the field of diagnostic biomarkers and therapy as they can efficiently carry lipids, proteins, and various types of nucleic acids including mRNA, miRNA, lncRNA, circular RNA, ribosomal RNA, tRNA, and DNA fragments . Furthermore, recent research has demonstrated that EVs can serve as carriers for biomolecules, particularly in gene delivery platforms [8]. This review examines and compares the delivery methods and vectors used in the CRISPR/Cas9 system. It highlights the advantages of using EVs as vectors. The review also discusses the sources and isolation strategies of EVs, as well as the delivery methods and loading techniques for the CRISPR/Cas9 system. The applications of this system in both in vitro and in vivo settings are also explored. Overall, the use of

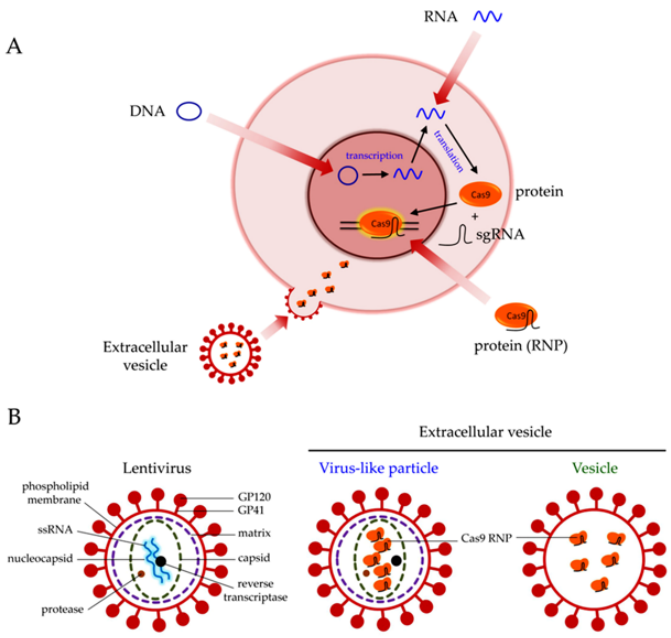

Fig. 2. Delivery of Cas9 Ribonucleoproteins (RNPs) through Extracellular Vesicles. (A) Cas9 can be introduced as DNA, mRNA, or in protein form. When delivered as a protein, Cas9 acts immediately upon reaching the nucleus. Extracellular vesicles introduce the pre-loaded Cas9 RNPs into cells, facilitating effective gene modification. (B) Comparing the structures of a lentivirus, a virus-like entity, and a vesicle [6].

EVs to deliver the CRISPR/Cas9 system shows promise for potential clinical disease treatments (Fig.2).

2 CRISPR/CAS9 DELIVERY

The cell membrane poses a challenge for the gRNA and Cas9 protein due to their large molecular weight and negative charge, making it hard for them to pass through [8]. In order to carry out gene editing using the CRISPR/Cas9 system in a laboratory setting, it is necessary to transport the sgRNA and Cas9 protein into cells. This is important to prevent their degradation and enable them to enter the nucleus for gene editing purposes [14]. Moreover, in the intricate in vivo surroundings, the CRISPR/Cas9 system could potentially undergo degradation and be counteracted by various physical, chemical, and biological barriers as well as the immune system [7]. In addition, within the complex environment of a living organism, the CRISPR/Cas9 system may face challenges such as degradation and opposition from various barriers, including physical, chemical, and biological factors, as well as the immune system. Hence, it is crucial to carefully consider the form in which the CRISPR/Cas9 system is delivered in order to enhance its effectiveness, safety, and precision. Generally, the delivery form can be categorized as DNA, RNA, or ribonucleoprotein complexes (RNPs) [8]. The ribonucleoprotein complex (RNP) refers to the combination of sgRNA and Cas9 protein that is created in a laboratory setting. The introduction of the CRISPR/Cas9 system into cells involves the use of plasmids containing DNA sequences for gRNA and Cas9 protein [8]. This method of delivery is both affordable and easy to use. However, the process of transcription and translation within cells is required to generate gRNA and Cas9 protein, which leads to longer working time and a higher risk of off-target effects [15].

On the other hand, this procedure, which is challenging to regulate, may pose potential hazards for DNA sequences integrated into the genome. However, the efficiency of editing can be effectively reduced by delivering the CRISPR/Cas9 system in its RNA form, as transcription is not required [16]. Unfortunately, the effectiveness of gene editing can be diminished by using RNA as it is prone to degradation [17]. The utilization of RNA in gene editing can reduce its efficacy due to its susceptibility to degradation. Consequently, the delivery of the RNP form of the CRISPR/Cas9 system directly into the nucleus enhances the efficiency of gene editing and reduces the occurrence of off-target effects compared to using DNA and RNA forms of the CRISPR/Cas9 system. The presence of RNPs in cells is temporary, which has advantages in treating genetic and infectious disorders. However, transporting RNPs is more expensive compared to DNA and RNA [8].

In laboratory settings, the focus is primarily on the effectiveness of gene editing and the occurrence of unintended effects, which is a simpler process compared to in living organisms. However, when it comes to gene editing using CRISPR/Cas9 in living organisms, there are several crucial factors that need to meet specific criteria: 1) ensuring

safety in terms of delivery products and strategies; 2) overcoming physiological and pathological barriers such as dense tissue characteristics, acidic and enzymatic microenvironments, as well as blood-embryo and blood-brain barriers; 3) effectively targeting specific tissues or organs; and 4) achieving accurate gene editing by enhancing efficiency and minimizing damage to the genome [7]. Different methods can be categorized as either viral or nonviral vectors for delivering the CRISPR/Cas9 system [18]. To transport the CRISPR/Cas9 system, different methods can be categorized as either viral or nonviral vectors [19].

Various viral vectors, such as adeno-associated viruses (AAVs), adenoviruses (AVs), lentiviruses (LVs), and baculoviruses, have been utilized to transport gene editing systems [6]. These viral vectors are capable of effectively transporting the CRISPR/Cas9 system into cells, overcoming physiological barriers and the cell membrane, and demonstrating superior transfection and gene editing capabilities [20]. Nevertheless, particularly in live organisms, viruses possess certain inherent qualities that give rise to safety concerns. These include the potential to trigger immune responses due to their immunogenicity and the possibility of their genetic material integrating haphazardly into the genomes of host organisms [21]. In contrast to other viral vectors, AAV is considered to be a safer option due to its inability to reproduce, lack of integration into host genomes, and minimal immune response when used in living organisms [22]. However, the effectiveness of delivering the CRISPR/Cas9 system using AAV may be hindered by the limited space available for packaging [23].

In vitro, traditional nonviral physical methods such as electroporation and microinjection have been utilized for delivering the CRISPR/Cas9 system. Electroporation relies on the use of electric currents to generate temporary pores in cell membranes, through which the CRISPR/Cas9 system can be introduced into cells [6]. Electroporation is a cost-effective and convenient method with high efficiency and scalability. However, the process is characterized by variable conditions and can pose risks to cell viability. Microinjection, which involves the use of microscopes and needles to inject RNPs into zygotes for creating small animal models, has limitations due to its high cost, intricate procedure, low efficiency, and inability to perform in vivo editing [24].

Lipid-based synthetic chemical materials have been commonly utilized for transporting the CRISPR/Cas9 system [9].

Various commercial liposomes have been created, specifically designed to deliver the CRISPR/Cas9 system in the form of DNA, RNA, and RNP [7]. Liposomes can naturally form lipid bilayers due to their hydrophobic tail and hydrophilic head. . Liposomes can be utilized to encapsulate or combine with the CRISPR/Cas9 system, in its DNA, RNA, and RNP formats. This results in the formation of complexes, which can then merge with cell membranes, facilitating their entry into cells . Liposomes offer a safer approach compared to viral vectors as they rely on endocytosis for their delivery mechanism [7]. In contrast, liposomes are a gentler alternative to electroporation when it comes to cell delivery. As a result, liposomes possess favorable qualities in terms of their inherent structure, biocompatibility, low immunogenicity, and low toxicity [6]. The CRISPR/Cas9 system can be transported in vivo and in vitro using gold nanoparticles. The benefit of using gold nanoparticles is that they are biologically inactive, minimizing the likelihood of triggering an immune response. In summary, each form and strategy for delivering the CRISPR/Cas9 system has its own advantages and disadvantages. The appropriate forms and strategies are chosen based on the specific research goals and methods.

3 POTENTIAL OF EVS AS VECTORS

In brief, EVs can be produced and released by both eukaryotes and prokaryotes, enabling the transfer of biomacromolecules (such as nucleic acids, proteins, lipids, glycoproteins, etc.) to facilitate information exchange [13]. EVs have been found to play a role in a range of biological processes and diseases in living organisms, such as the immune system, viral pathogenicity, liver disease, cardiovascular disease, tumors, lung disease, and central nervous system disorders . As a result, EVs have the potential to offer valuable information about the biological condition of bodily fluids, leading to improved disease diagnosis. Additionally, the ability of EVs to transport and deliver therapeutic cargoes suggests their potential use in therapeutic applications [25]. Due to their capacity to carry different biomolecules, ability to specifically target cells, regulation of immune responses, and ease of manipulation, EVs have the potential to serve as an excellent carrier for delivering therapeutic agents . In addition, the advantageous properties of EVs are increasingly being utilized in the delivery of CRISPR/Cas9, offering potential for in vivo gene editing [26].

3.1 Innate characteristics of EVs

EVs are produced by various cell types. Based on how they are formed, EVs can be categorized as exosomes, microvesicles, and apoptotic bodies . Exosomes are typically released when the plasma membrane combines with multivesicular bodies (MVBs), which are created through the inward budding of the endosome. On the other hand, microvesicles and apoptotic bodies are formed when the plasma membrane buds outward, depending on various circumstances. As a result, the characteristics of the membrane of EVs are determined by the way they are formed, which involves the presence of a phospholipid bilayer and different proteins that are either embedded in the membrane or attached to it [12]. These features are beneficial to the targeting and uptake of EVs. EVs offer benefits in terms of safeguarding and storage capacity when it

comes to delivering cargo. Naked nucleic acids and proteins, which are large in size and have varying charges and hydrophilicity, face challenges when attempting to pass through cell membranes [7]. Therefore, EVs were used to encapsulate and transport naked nucleic acids and proteins, providing both delivery and protective capabilities. Additionally, the structure of the EV membrane serves as an effective shield against serum endonucleases and the immune system, safeguarding these molecules. EVs have the ability to transport various substances such as saccharides, nucleic acids, proteins, and lipids, setting them apart from other delivery methods [13]. EVs possess the unique capability to transport a range of substances, including saccharides, nucleic acids, proteins, and lipids, which distinguishes them from other delivery methods. EVs naturally encapsulate various functional biomacromolecules such as nucleic acids and proteins, which are important for cell communication, disease development, and therapeutic purposes [27]. The transportation of cargoes in living organisms can be hindered by various natural barriers, including dense tissue, acidic and enzymatic microenvironments, and vascular endothelial cell barriers [7]. EVs have the ability to efficiently traverse natural biological barriers and transport cargoes due to their high biocompatibility and natural generation [27]. The presence of dense tumor tissue poses a significant obstacle to the effective delivery of nanomedicine, thus restricting its potential application. The delivery of substances into tumor tissue can be achieved by liposomes. However, the effectiveness of liposomes is primarily influenced by factors such as size, charge, cargo type, and tumor type, which restricts their potential application [28]. Tumor cell-derived EVs have been found to easily penetrate tumors. In a study by Sánchez et al., it was demonstrated that by overexpressing specific surface proteins on EVs (CD147, tetraspanin Tspan8 or CD44) and certain miRNAs (miRNA-494, miRNA-542-3p, and miRNA-21-5p), the expression of metalloproteinases increased, leading to degradation of the extracellular matrix and improved EV penetration [29]. Kim et al. achieved successful gene editing in tumor tissue by effectively utilizing tumor-derived EVs that carried the CRISPR/Cas9 system and were able to penetrate into the tumor [30]. The intestinal mucosa has the ability to impede the uptake of various delivery vectors that are used to transport cargoes [7]. The research findings indicated that smaller size and negative charge of these vectors significantly improved their ability to penetrate [31]. Due to their small size, negative charge, and stable structure, milk-derived EVs can be readily absorbed by the intestine. In the meantime, EVs originating from epithelial cells also have the ability to cross through the intestine. EVs have the potential to safeguard biomacromolecules, such as curcumin and different RNA types, from being degraded by digestive juices [7]. The blood-brain barrier (BBB) is a vital natural defense mechanism that can selectively permit certain small molecules to pass through the endothelial membrane while blocking the entry of harmful substances or toxins. As a protective mechanism, the BBB hinders the delivery of drugs and other biomacromolecules into the brain. However, EVs can overcome this barrier by encapsulating drugs and biomacromolecules, and their ability to penetrate depends on the size and composition of the EVs [7]. Morad et al. demonstrated the successful traversal of tumor-derived EVs across the BBB in live organisms, primarily through the process of endocytosis. During this mechanism, EVs reduced the expression of Rab7 in endothelial cells, facilitating their transportation [32].

3.2 Physiological and pathological functions of EVs

EVs are widely distributed and generated in the body, allowing them to transport a diverse range of biomacromolecules in an organized manner. This process plays a regulatory role in numerous physiological processes [27]. EVs, produced by B cells, have the ability to display antigens through MHC proteins on their surface, particularly in relation to immune regulation. . Recent research has been dedicated to investigating the role of EVs in immune regulation within tumors. Recent reports suggest that EVs produced by tumor cells can either promote cancer progression or have anti-tumor effects through their immunoregulatory capabilities .Hoshino and colleagues demonstrated that EVs derived from tumor cells can fuse with resident cells in specific organs, such as fibroblasts and epithelial cells, during tumor metastasis. This fusion process involves the interaction of exosomal integrins, which can trigger Src phosphorylation and the expression of proinflammatory S100 proteins. These events promote tumor metastasis [33]. However, various research studies have shown that EVs originating from tumor cells have the ability to transport tumor antigens. This in turn can enhance tumor immunity by activating CD8(+) and CD4(+) T cells, which are capable of eliminating tumor cells and impeding tumor advancement.

Moreover, EVs have the capacity to control numerous interactions between cells that are associated with processes such as cell division (proliferation) and cell death (apoptosis), as well as tissue healing (angiogenesis) and renewal [27]. In addition, platelet-derived EVs play a role in controlling blood clotting, cell growth and repair, and tissue regeneration due to their content of various coagulants, growth factors, inflammatory factors, cytokines, RNA, and lipids [34]. Kim et al. showed that lipids present in EVs released by platelets can induce the growth of human umbilical vein endothelial cells (HUVECs) [35]. In a separate study, Zhang and colleagues demonstrated that EVs derived from bone marrow mesenchymal stem cells (BM-MSCs) were able to enhance angiogenesis and promote bone repair in rats with femoral nonunion. These EVs were able to be internalized by HUVECs, leading to increased proliferation of HUVECs in laboratory settings [36].

EVs also play a role in the development of tumors and chronic and infectious diseases [27]. In tumor research, EVs derived from tumor cells, stromal cells, and immune cells contain various DNA, RNA, proteins, and other molecules. These EVs contribute to the progression of tumors, drug resistance, metastasis, and immune responses. EVs derived from tumor cells have the ability to influence the outcomes of both the tumor cells that produce them and the surrounding tumor and stromal cells. Raimondo and colleagues showed that EVs originating from chronic myeloid leukemia can enhance the growth of tumor cells by triggering pathways that prevent cell death [37]. Nedawi et al. discovered that in glioma, only a small fraction of tumor cells expressing EGFRvIII could generate EGFRvIII-carrying EVs to neighboring tumor cells, which could activate the anchorage-independent growth of neighboring tumor cells through an activation of the AKT pathway [38]. Additionally, Antonyak and colleagues demonstrated that EVs derived from MDA-MB-231 and U87 glioma cells could endow normal fibroblasts and epithelial cells with various tumor cell traits [39].

Recent research indicates that EVs play a role in various chronic and infectious conditions, including pulmonary fibrosis (PF), cardiovascular diseases, and viral infections. PF, a long-term lung condition, is marked by a rise in fibroblasts and the extracellular matrix. While the exact cause of PF remains unclear, there's evidence linking EVs to its occurrence [40].

Yao and colleagues showed that when miR-328 is overexpressed, EVs from M2 macrophages enhance fibroblast growth in laboratory conditions and accelerate the development of pulmonary fibrosis (PF) in live organisms [41]. The specific roles of EVs in atherothrombosis depend on their types, contents, and the cells they originate from. Generally, EVs are crucial at every stage of atherosclerosis, encompassing the start of lesions, the growth of plaques, their rupture, and thrombosis. Research on RNA viruses reveals that EVs coming from infected cells can carry a range of large biological molecules to influence processes related to the infection and the body's cellular responses.

3.3 *Safety of EVs*

EVs have unique properties, including their natural origin and minimal immune response and toxicity, which distinguish them from viral vectors (AAVs, AVs, and LVs) and chemical materials (liposomes and synthetic nanoparticles) [12].

Kamerkar et al. demonstrated that EVs have distinct advantages over liposomes in terms of their ability to evade phagocytosis and their effectiveness in inhibiting tumor growth without triggering an immune response [42].

Nevertheless, when considering the use of EVs as therapeutic tools or carriers in living organisms, it is crucial to carefully assess their safety by examining factors such as the origin of the EVs, the conditions under which they were cultured, and the methods used to isolate them. The characteristics of EV membrane and cargoes, which can impact the immune response, cell transformation, and cell invasion, are determined by the type of cell used as a source for EV generation (12). MSCs are a frequently utilized cell source for generating EVs. Several research studies have demonstrated the minimal immune response to EVs derived from MSCs when tested in living organisms [43].

Mendt et al. demonstrated that in vivo, there were no noticeable signs of toxicity or immune reactions when mice were repeatedly given MSC-derived EVs [43]. Additionally, Zhu et al. showed that when HEK293T cell-derived EVs were administered repeatedly to mice, there were no observed immune reactions or signs of toxicity [44]. Nevertheless, there is controversy surrounding the safety of tumor cell-derived EVs due to their composition of various tumor-associated biomacromolecules that have the potential to impact tumor development [8]. Various research studies have indicated that tumor cell-originated EVs possess the ability to hinder tumor growth in animal models of melanoma, liver tumors, and colon carcinoma. This effect is achieved by triggering an immune response dependent on T-cells. On the other hand, EVs derived from tumor cells have been found to promote the growth and spread of tumors, as well as prevent cell death, thereby aiding in the progression of cancer [8]. However, it is important to consider the source of EVs in order to ensure their safety for use. Additionally, the potential toxicity and lack of immune response of EVs should be examined in various situations where they are applied.

Currently, transformed cell lines are primarily used to produce EVs [8]. In order to meet the clinical requirement for producing a large quantity of high-quality EVs, the conditions for culturing cells that generate EVs should adhere to good manufacturing practice (GMP) standards. This is essential to ensure the uniform phenotype of EVs across different batches [45, 46]. The elements of cell culture conditions, such as the components of the medium (including factors and serum), pH level, and the type of cell culture (adherent or suspension), can have an impact on the structure of the membrane and the contents carried by EVs [47]. Moreover, the methods used for isolating EVs also have an impact on the quality and safety of the EVs, which will be explored in more detail at a later point.

3.4 *Targeting ability of EVs*

Regarding the delivery methods of biomacromolecules and drugs in vivo, particularly in CRISPR/Cas9 gene editing systems, the limited availability of tissue-specific vectors hinders their broader use in clinical applications [18] . EVs possess the capability to encapsulate and safeguard cargoes, as well as achieve targeting towards organs, tissues, and specific cells. Typically, various molecules play a

Table 1. The CRISPR-Cas9 system delivered by EVs. UC: ultracentrifugation; SEC: size exclusion chromatography.

EV Source	Delivery Form of CRISPR/Cas9	Loading Methods of CRISPR/Cas9	Targeting Ability	Applications	Ref
HEK293T cells	RNPs	Endogenous loading	None targeting ability	Editing cells in vitro and in vivo	[48]
LX-2 cells	RNPs	Electroporation	The liver tissue	Treatment of acute liver injury, chronic liver fibrosis and hepatocellular carcinoma	[18]
SKOV3 cells and HEK293 cells	Plasmids	Electroporation	The tumor of SKOV3 xenograft mice	Treatment of ovarian tumor	[30]
HEK293T cells	RNPs	Sonication	The tumor of HepG2 xenograft mice	Treatment of liver tumor	[49]
Anti-CD19-CAR-HEK293T cells	Plasmids	Electroporation	The tumor of Raji xenograft mice	Treatment of B-cell malignancies	[50]
HEK293T cells	RNPs	Endogenous loading	None targeting ability	Editing cells in vitro	[51]
HEK293 cells	RNPs	Endogenous loading	None targeting ability	Treatment of liver damage	[52]
Red blood cells	mRNA	Electroporation	None targeting ability	Editing cells in vitro	[53]
HEK293FT cells	Plasmids	Transfection kit	None targeting ability	Editing cells in vitro	[54]
HEK293T cells	RNPs	Endogenous loading	None targeting ability	Editing cells in vitro	[55]
HEK293T cells and MSCs	Plasmids	Transfection kit	None targeting ability	Treatment of pancreatic tumor	[56]
Halobacterium	Plasmids	Incubation	None targeting ability	Editing cells in vitro	[57]
HEK293T cells	RNPs	Endogenous loading	None targeting ability	Editing cells in vitro	[58]
Expi293F cells	RNPs	Endogenous loading	None targeting ability	Editing cells in vitro	[59]
A549 cells and B16-F10 cells	RNPs	Endogenous loading	None targeting ability	Editing cells in vitro and in vivo	[60]
Serum	RNPs	Transfection kit	None targeting ability	Treatment of Duchenne muscular dystrophy	[61]

role in directing EVs towards specific cells, thereby enhancing their targeting ability [26]. As a result, the inherent capacity of EVs to target specific cells is influenced by the type of cell. The composition of glycans, proteins, and lipids within the membrane of EVs dictates the particular organs, tissues, and cells that EVs can effectively target [12]. Hoshino et al. discovered that EVs derived from tumor cells in the lung, liver, and brain exhibited a preference for uptake by specific cell types: lung fibroblasts, Kupffer cells, and brain endothelial cells, respectively. Additionally, the study demonstrated that the EV integrins $\alpha 6\beta 4$ and $\alpha 6\beta 1$ were linked to targeting the lung, while the integrin $\alpha v\beta 5$ was associated with targeting the liver. In a study conducted by Kim et al., it was found that EVs derived from SKOV3 cells showed a specific accumulation in ovarian tumors in vivo, unlike EVs derived from HEK293 cells. This selective accumulation can be attributed to cell tropism [30]. In a study conducted by Wan et al., it was demonstrated that EVs derived from hepatic stellate cells had the ability to specifically target the liver, while no EVs were found in other organs such as the heart, spleen, lung, and kidney [18]. Certain cell types provide EVs with a distinct ability to target specific areas. Nevertheless, when using EVs in vivo, the main challenges remain the inadequate and unreliable targeting ability, as well as the off-target effects [62]. Recent research suggests that modifying the membranes of EVs with ligands may enhance their ability to target specific areas [12]. In essence, there are two main approaches to modifying EVs: 1) directly altering the EV membrane and 2) modifying the source cells to produce modified EVs. For instance, Zhuang et al. demonstrated the direct modification of EVs derived from HEK293T cells by attaching valency-controlled tetrahedral DNA nanostructures (TDNs) conjugated with DNA aptamers. These modified EVs were able to specifically target the liver by anchoring to cholesterol [49]. Xu et al. utilized a different approach by creating EVs with a chimeric antigen receptor (CAR) that specifically targeted CD19 on B-cell malignancies. These EVs were derived from HEK293T cells transfected with anti-CD19CAR LVs, allowing them to effectively target and treat these types of cancers in vivo [50].

4 EVS FOR DELIVERY OF CRISPR/CAS9 SYSTEM

The CRISPR-Cas9 system has become increasingly effective in treating genetic disorders and tumors, including transthyretin amyloidosis, which is currently undergoing phase 1 clinical trials. As previously mentioned, Evs have gained attention as promising vectors for delivering the CRISPR-Cas9 system and other biomacromolecules due to their biocompatibility, low immunogenicity, capacity, protection ability, modification potential, and targeting ability. In this section, we will focus on the delivery of the CRISPR/Cas9 system using EVs. This will include discussing the source of EVs, strategies for isolating EVs, the form in which the CRISPR/Cas9 system is delivered by EVs, methods for loading the CRISPR/Cas9 system into EVs, and experimental applications of this delivery approach Table 1.

4.1 EV sources

As mentioned earlier, the origin of EVs plays a crucial role in determining their membrane composition and cargo content, which in turn can impact the safety and targeting capabilities of EVs [12]. Hence, researchers have been investigating the use of EVs as a means of delivering the CRISPR/Cas9 system. EV sources can be categorized into four main groups: 1) noncancerous cells; 2) cancerous cells; 3) bacteria; and 4) non-cellular origins. Among noncancerous cells, the HEK293 and HEK293T human embryonic kidney cell lines are commonly utilized due to their high EV production, minimal endogenous cargo, and ease of cultivation for generating EVs for CRISPR/Cas9 delivery [51]. In addition, these cells can be easily altered to generate customized EVs. For instance, by introducing Cas9-sgRNA plasmids into HEK293 and HEK293T cells, EVs containing RNPs can be produced to enhance gene editing effectiveness. Similarly, anti-CD19 CAR-HEK293T cells can generate anti-CD19 CAR EVs to enhance tissue targeting capabilities [50]. Moreover, the ability of HEK293 and HEK293T cell-derived EVs to specifically target tissues is currently limited. In a study conducted by Lainšček et al., it was observed that when injected intravenously into mice, EVs derived from HEK293 cells were quickly distributed throughout various organs including the heart, lung, spleen, kidney, and brain. Notably, a significant accumulation of these EVs was observed in the liver. Even after 24 hours of circulation in the bloodstream, the EVs were still primarily found in the liver, with no distribution observed in the heart [52]. In addition, the CRISPR/Cas9 system can be transported by hepatic stellate cells and red blood cells through EVs. The EVs derived from hepatic stellate cells were proven to be safe and capable of targeting the liver [18]. Usman et al. demonstrated that red blood cells, which lack nuclear and mitochondrial DNA but are readily available in large quantities, can effectively deliver CRISPR/Cas9 for gene editing purposes while ensuring safety in clinical applications [53]. Furthermore, mesenchymal stem cell (MSC)-derived EVs have the ability to transport the CRISPR/Cas9 system for gene editing [56].

In addition, EVs derived from MSCs can be utilized to carry the CRISPR/Cas9 system for gene editing purposes, and this approach extends to tumor cells like SKOV3, A549, and B16-F10 cells [60]. The origin of tumor cells raises concerns about the potential toxicity and immune response of EVs. However, Kim et al. demonstrated that EVs derived from SKOV3 cells caused only a minor release of TNF-α and INF-α in human peripheral blood mononuclear cells, compared to lipopolysaccharide or CpG oligodeoxynucleotides. Furthermore, these EVs exhibited a cell-specific targeting ability towards SKOV3 xenografts in vivo. In addition, Ye et al. showed that EVs derived from A549 and B16-F10 cells had a tendency to accumulate primarily in the liver during in vivo gene editing. Notably, B16-F10 cell-derived EVs contained ITGβ5, a protein linked to liver targeting.

Furthermore, the CRISPR/Cas9 system has also been delivered using EVs derived from bacteria like Halobacterium [57]. Liu et al. proposed the concept of utilizing bacteria as manufacturing units to produce EVs containing RNPs and targeted ligands, which offers a cost-effective and efficient approach [63]. Furthermore, Majeau et al. found that serum-derived EVs, which are not derived from cells, can effectively transport the CRISPR/Cas9 system. These EVs have the advantage of being non-immunogenic and easily accessible. In their study, they observed that both intravenous and intramuscular administration of serum-derived EVs did not lead to any negative reactions such as inflammation in mice within a 7-day period [61].

4.2 Isolation of EVs

The isolation of EVs is a challenging task due to their small size, low density, and mixture with other substances such as lipoprotein complexes, cell fragments, and protein aggregates. However, controlling the quality, safety, purity, and yield of EVs is crucial for their clinical applications in vivo. Furthermore, the differentiation between various types of EVs, such as exosomes, microvesicles, and apoptotic bodies, becomes increasingly problematic because they share similar size and morphological features [64]. The different methods used to isolate EVs can impact their purity, yield, and integrity [65]. Therefore, it is important to develop standardized and appropriate methods for isolating EVs in order to ensure their purity, yield, and integrity. In general, there are four main categories of isolation strategies based on different principles of separation: 1) ultracentrifugation, which separates EVs based on their density; 2) ultrafiltration and size exclusion chromatography, which separate EVs based on their size; 3) polymer precipitation, which separates EVs based on their solubility; and 4) immunoaffinity magnetic beads, which separate EVs based on their immunoaffinity. Ultracentrifugation is currently considered the gold standard method for EV isolation and is the most commonly used strategy, accounting for nearly half of all EV isolations [66]. UC is a widely used method for isolating EVs due to its ability to separate them based on their density and size, effectively removing cells, fragments, and contaminants. It offers several benefits such as being easy to use, cost-effective, and suitable for large-scale isolation [64]. On the other hand, the process of UC is time-consuming, has a low capacity for processing samples, cannot eliminate nucleic acids, lipoproteins, and proteins, and results in inconsistent purity levels of EVs [27]. Furthermore, microfluidic techniques have been employed to separate extracellular EVs in addition to the aforementioned methods. The University of Florida (UF) and the Southeastern Conference (SEC) both employ a common technique known as size exclusion chromatography (SEC). The inclusion of porous beads in the procedure results in slower elution of smaller particles compared to larger particles. This is beneficial for preserving the purity of EVs, but it also raises the

possibility of EVs becoming mixed with similarly sized impurities [65].

The technique of polymer precipitation is utilized to extract components, like EVs, that have limited solubility [64]. At present, the polymer precipitation method incorporates various reagents such as protamine, acetate, and polyethylene glycol (PEG). PEG has been widely used in EV separation due to its affordability, gentleness, and simplicity, yet it has led to a decrease in EV purity. By leveraging their immunoaffinity, immunoaffinity magnetic beads can efficiently isolate EVs with exceptional purity. Nonetheless, this method is expensive and results in limited quantities of isolated EVs [65].In addition to the methods mentioned earlier, microfluidic techniques have also been employed for the separation of EVs [67]. Numerous kits for EV isolation have been created, which are now widely available for commercial use as electric vehicles have advanced.

Currently, the use of EVs for delivering the CRISPR/Cas9 system is predominantly achieved through UC. Furthermore, Lin et al. employed a solution consisting of 500 $\times$ 10^{-3} M NaCl and 12% PEG 6000 to isolate EVs. Majeau and colleagues purified serum EVs using a size exclusion column (SEC qEV10/70 nm) and subsequently refined them through UF.

4.3 Loading methods for delivery of the CRISPR/Cas9

The efficiency of gene editing is influenced by the method of delivery of the CRISPR/Cas9 system. RNPs, which do not require transcription and translation, result in faster and more efficient gene editing with fewer off-target effects compared to DNA and RNA forms of the CRISPR/Cas9 system. However, RNPs are more costly. Recent research has shown that EVs can be used to transport and deliver the DNA, RNA, and RNP components of the CRISPR/Cas9 system for gene editing both in laboratory settings and in living organisms. One example is the use of EVs derived from tumor cells to transport CRISPR/Cas9 plasmids and inhibit the activity of poly (ADP-ribose) polymerase-1 (PARP-1). Furthermore, in order to enhance the capabilities of EVs, Lin et al. devised hybrid nanoparticles composed of a combination of EVs and liposomes. Usman et al. demonstrated that EVs derived from red blood cells possess the ability to transport CRISPR/Cas9 mRNA for genomic editing purposes. Moreover, the effectiveness of mRNA transport was observed to exceed that of plasmids. Furthermore, EVs derived from HEK293T cells were found to be capable of transporting CRISPR/Cas9 RNPs for gene editing purposes. Conversely, the form in which the CRISPR/Cas9 system is delivered can influence how it is loaded. The effective packaging of the CRISPR/Cas9 system into EVs is essential for gene editing. Unlike other carriers, EVs are anticipated to remain undamaged after loading with the CRISPR/Cas9 system. There are two primary techniques for loading the CRISPR/Cas9 system:

1. Exogenous loading: This directly introduces the CRISPR/Cas9 system into EVs using methods such as electroporation, incubation, transfection, and sonication.

2. Endogenous loading: This involves altering cells so they generate EVs that already contain the CRISPR/Cas9 system.

At present, exogenous loading techniques, particularly electroporation and transfection kits, are employed to introduce CRISPR/Cas9 plasmids and mRNA into EVs. Both exogenous (like electroporation, sonication, and transfection) and endogenous techniques can be utilized to load CRISPR/Cas9 RNPs into Evs [18]. Loading large molecular weight cargoes, like CRISPR/Cas9 RNPs, as endogenous cargoes, presents ongoing challenges that are currently being explored. Hence, numerous studies have focused on enhancing the enrichment of RNPs in EVs by leveraging specific interactions between modified RNPs and EVs. Wang and colleagues carried out studies to produce EVs integrated with the arrestin domain containing protein 1 (ARRDC1), which facilitates interactions with proteins possessing WW-domains. As a result, they linked Cas9 to WW domains, leading to an increased concentration of Cas9 in EVs without affecting its activity or functionality. On the other hand, Yao and his team demonstrated that the concentration of RNPs in EVs can be boosted by leveraging the interaction between RNA aptamers and aptamer-binding proteins (ABPs). Furthermore, the addition of a myristoyl group to Cas9 proved advantageous in terms of its accumulation in EVs, resulting in enhanced enrichment of RNPs within the EVs. The enrichment of RNPs in EVs can also be achieved using techniques that involve light-induced protein heterodimerization within the EVs. Apart from the aforementioned loading methods, the CRISPR/Cas9 system has the potential to be loaded into EVs through the anthrax lethal toxin. This toxin is composed of protective antigen (PA) and lethal factor (LF). PA can create a channel to recruit and transfer LF. Using this transportation system, foreign proteins fused with the N-terminus of LF can be conveyed into cells, including the Cas9 protein [68]. Simultaneously, LF is transported not only into the cytosol but also into extracellular vesicles (EVs), which have the capability to encapsulate a diverse range of cargo, including LFn-DTA, siRNA, ASOs, and the Cas9 protein [69].

4.4 The application of CRISPR/Cas9 loaded- EVs

Due to several benefits offered by EVs, such as safety, protection, capacity, penetration ability, targeting ability, and potential for modification, the delivery of the CRISPR/Cas9 system in vivo using EVs is preferred over its use in vitro [70]. Currently, the application of EVs

loaded with the CRISPR/Cas9 system has been employed in therapeutic approaches for tumors and various other diseases.

4.4.1 Malignant tumor treatment

Conventional methods of treating tumors, such as surgery, chemotherapy, and radiotherapy, only provide successful outcomes for a limited number of patients and can result in severe side effects and unfavorable prognosis. The utilization of CRISPR/Cas9 technology offers a promising outlook for tumor therapy [71]. PARP-1 has emerged as a potential focus of cancer treatment, particularly for breast and ovarian tumors, due to its association with the DNA damage response [72]. Kim et al. demonstrated that by administering CRISPR/Cas9-loaded EVs intravenously to SKOV3 xenograft mice, they successfully suppressed PARP-1 activity, resulting in a decrease in tumor size and weight. In addition, the suppression of PARP-1 activity led to enhanced sensitivity to cisplatin treatment. The presence of the oncogenic mutant KrasG12D can promote the growth of pancreatic tumors through Ras signaling [73]. McAndrews and colleagues showed that EVs derived from MSCs have the ability to transport and deliver the CRISPR/Cas9 plasmid. This plasmid is used to disable the mutant KrasG12D oncogenic gene in KPC689 cells, which are derived from a pancreatic tumor in mice. The experiment successfully demonstrated that this treatment could effectively inhibit tumor growth in both subcutaneous and orthotopic KPC689 graft mice. The viability and migration of HepG2 cells can be suppressed by inhibiting the WNT10B gene through the use of siRNA, which is highly expressed in liver tumor tissue and cells [74]. Zhuang and colleagues created EVs that were modified with TDNs, allowing them to target and accumulate in HepG2 cells and liver tumor organoids in laboratory settings. In addition, they used EVs to deliver CRISPR/Cas9 RNPs to HepG2 xenograft mice, which effectively reduced the expression of WNT10B and consequently suppressed tumor growth [75]. The growth of hepatocellular carcinoma relies heavily on the activity of K (lysine) acetyltransferase 5 (KAT5). Hence, inhibiting KAT5 can effectively hinder tumor growth [75]. Wan and colleagues demonstrated that EVs derived from LX-2 cells are capable of encapsulating and delivering CRISPR/Cas9 RNPs. These RNPs are used to suppress KAT5 expression in an orthotopic Huh-7 xenograft mouse model. This treatment resulted in a reduction of KAT5 expression, which effectively inhibited tumor growth and extended the survival of the mice [18]. Overexpression of the MYC oncogene has been observed in approximately 30% of human tumors, including Burkitt's lymphoma and other hematological malignancies. Xu and colleagues created EVs that were engineered with an anti-CD19 CAR modification. These EVs had the ability to specifically accumulate in tumor tissue and transport the CRISPR/Cas9 plasmid into subcutaneous Raji xenograft mice. This resulted in a MYC mutation, which triggered apoptosis. As a result, the tumor volume decreased when compared to the control group.

4.5 Benign disease treatment

The use of CRISPR/Cas9 technology has emerged as a promising approach to treat genetic disorders and various diseases. This technology enables the inactivation or correction of genes responsible for causing these conditions. Lainšček and colleagues demonstrated that the Hgf gene could be enhanced using CRISPR/Cas9 technology to specifically target the first exon in laboratory settings. They also utilized EVs loaded with CRISPR/Cas9 plasmids to deliver them to a mouse model with liver damage induced by alpha-naphthylisothiocyanate. This resulted in elevated levels of HGF, promoting liver regeneration, and reduced levels of ALT, bile acids, bilirubin, and cholesterol. The development of liver cancer is greatly influenced by the function of K (lysine) acetyltransferase 5 (KAT5). As a result, impeding the activity of KAT5 can effectively impede the growth of tumors [76]. Wan and colleagues demonstrated that by using EVs loaded with CRISPR/Cas9 RNPs to target the expression of PUMA protein in a mouse model with liver injury induced by APAP, they were able to significantly decrease the levels of PUMA protein, AST and ALT, the number of cells undergoing apoptosis and necrosis, and the presence of hyperemia. This treatment also resulted in prolonged survival of the mice [18]. The presence of Cyclin E1 (CcnE1) has been found to promote the proliferation of hepatic stellate cells and is associated with the development of liver fibrogenesis. By using siRNA to inhibit CcnE1, it is possible to effectively hinder the occurrence and progression of fibrosis [77]. In the murine model of chronic liver fibrosis induced by CCl4, treatment with EVs containing CRISPR/Cas9 RNPs that specifically target CcnE1 resulted in a significant decrease in the expression of CcnE1 and α-SMA protein. Moreover, the progression of liver fibrosis was significantly attenuated, indicating that this treatment effectively inhibits the initiation and development of fibrosis in the liver [18]. Duchenne muscular dystrophy (DMD) is a prevalent genetic disorder characterized by reduced expression of the dystrophin protein. This condition is caused by multiple mutations in the Dmd gene and is among the most widespread genetic diseases worldwide [77]. In a study by Majeau et al., it was shown that mdx mice with a nonsense mutation in exon 23 of the Dmd gene were treated with EVs containing CRISPR/Cas9 RNPs. This treatment resulted in the successful deletion of exons 23 and 24 in comparison to the control group. As a result, the expression of the dystrophin protein was effectively restored in the muscles. Additionally, the Dmd genes in hDMD/mdx mice, which express the human Dmd gene, could also be successfully modified.

5 CONCLUSIONS AND FUTURE DIRECTIONS

The CRISPR/Cas9 gene editing system allows for precise modification of DNA sequences, enabling changes in cellular and organ characteristics. This system holds great potential for advancing gene mechanistic research and the treatment of various diseases. As an illustration, CRISPR/Cas9 in vivo editing is being utilized in human clinical trials to treat genetic diseases like transthyretin amyloidosis (NCT04601051) and Leber congenital amaurosis (NCT03872479). The delivery mechanisms for this treatment consist of proprietary LNPs and AAVs [78]. When it comes to treating clinical diseases, it is crucial to deliver the CRISPR/Cas9 system accurately and safely to the specific cells and organs that require gene editing. Therefore, there is an urgent requirement for suitable vectors in order to facilitate the clinical application of this technology. Currently, human clinical trials are assessing the use of therapeutic cargoes carried by EVs for disease treatment. For instance, in the treatment of ischemic stroke (NCT03384433), EVs derived from MSCs that were transfected with miR-124 have been utilized. Similarly, in the treatment of pancreatic cancer (NCT03608631), MSC-derived EVs loaded with miRNA targeting mutant KRAS have been employed [79]. Nevertheless, there haven't been any clinical trials conducted on the use of EVs as carriers for delivering CRISPR/Cas9. However, due to their favorable biocompatibility, capacity, and physiological characteristics, EVs are increasingly being considered as an ideal "vehicle" equipped with GPS to transport the CRISPR/Cas9 system for precise targeted delivery, in comparison to viral and other vectors [26]. This review covers various aspects related to the delivery of the CRISPR/Cas9 system using EVs. It discusses topics such as the types of vectors used and the delivery methods for the CRISPR/Cas9 system. Additionally, it highlights the characteristics of EVs as vectors, including their innate properties, physiological functions, safety, and targeting capabilities. The review also explores the delivery of the CRISPR/Cas9 system using EVs, covering areas such as EV sources, isolation strategies, methods of loading the CRISPR/Cas9 system, and various applications.

In an ideal scenario, EVs would encapsulate CRISPR/Cas9 RNPs effectively and target specific cells or tissues, providing a gene editing method with optimal efficacy and safety. Several research pieces have showcased EVs' capability to transport and release the CRISPR/Cas9 system for gene modifications. Yet, certain obstacles remain, such as ensuring the safety, capacity, consistency, output, and targeting abilities of EVs. A key concern stems from the biogenesis of EVs, as they house biomacromolecules that might alter cell physiological functions. Specifically, while tumor-derived EVs can target tumors, they also carry molecules linked to tumor growth and spread, requiring meticulous examination. This underscores the need for further R&D to verify the safety and origins of EVs.

The encapsulation capabilities of EVs and how efficiently they load CRISPR/Cas9 RNPs are pivotal for successful delivery and gene editing. Merging EVs with other materials and refining the interaction between altered RNPs and EVs hold promise in boosting EVs' capacity. Moreover, the reliability and yield of EVs are essential for their clinical application. Consistency across batches and ample EV production are critical. The trustworthiness of the EV source, paired with effective separation and purification techniques, ultimately influences the quality and volume of EVs produced. Therefore, it's vital to strengthen the stability of EV sources and formulate standardized procedures for their separation and purification. Further, EVs' capability to specifically target cells to execute precise gene editing using CRISPR/Cas9 in living beings is essential. The existing targeting potential of EVs, based on cell tropism, has its limitations. Efforts to enhance this are underway, including cell alterations to yield modified EVs equipped with ligands or the direct modification of EVs with these ligands, hinting at future potential. Introducing innovative CRISPR technologies, like base editors, primer editors, and RNA-focused Cas13, can amplify the safety, precision, and efficacy of genetic alterations. As such, forthcoming advancements in EVs could set the stage for applying genome editing in addressing a range of illnesses.

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