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Chapter

Extracellular Vesicles: "Stealth Transport Aircrafts" for Drugs

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Abstract

Extracellular vesicles (EVs) can deliver many types of drugs with their natural source material transport properties, inherent long-term blood circulation capabilities and excellent biocompatibility, and have great potential in the field of drug carrier. Modification of the content and surface of EVs according to the purpose of treatment has become a research focus to improve the drug load and the targeting of EVs. EVs can maximize the stability of the drugs, prevent immune clearance and achieve accurate delivery. Therefore, EVs can be described as " stealth transport aircrafts " for drugs. This chapter will respectively introduce the application of natural EVs as cell substitutes in cell therapy and engineered EVs as carriers of nucleic acids, proteins, small molecule drugs and therapeutic viral particles in disease treatment. It will also explain the drug loading and modification strategies of EVs, the source and characteristics of EVs. In addition, the commercialization progress of EVs drugs will be mentioned here, and the problems in their applications will be discussed in conjunction with the application of EVs in the treatment of COVID-19.

Keywords: extracellular vesicles, exosomes, drug carrier, drug loading, targeting modification

1. Introduction

Extracellular vesicles (EVs) are a collective term for tiny vesicles with a phospholipid bilayer structure that are actively secreted by cells. Almost all known cell types can be secreted. The two main categories of EVs are exosomes and microvesicles (**Table 1**). Exosomes (30-150 nm in diameter) are intraluminal vesicles, formed by the invagination of the multivesicular endosome membrane, and are released into the extracellular space after the multivesicular endosomes fuse with the cell membrane [1]. Microvesicles (50–1,000 nm in diameter) are a group of highly heterogeneous EVs characterized in that their origin and secretion are budding through the plasma membrane [1]. Considering the complexity of identifying its biogenesis, the size of the vesicle is the most widely used parameter for classifying EV types,

Vesicle	Size (nm)	Density (g/mL)	Origin	Markers
Exosomes	30-150	1.13-1.18	Endosomes	Tetraspanins, Alix, TSG101
Microvesicles	50-1000	1.16-1.19	Plasma membrane	Intergrins, Selectins, CD40

Table 1.
The main characteristics of EVs [1, 2].

and on this basis they are described as small EVs or medium and large EVs. In this article, unless otherwise specified, the term "EVs" generally refers to small EVs.

In recent years, people's understanding of the biogenesis, composition, function and mechanism of EVs has continued to deepen [3–5]. Their application in clinical treatment has also attracted more and more attention. One of the most useful properties of EVs is their ability to cross barriers, such as the plasma membrane and blood/brain barrier. This makes them very suitable for delivering therapeutic molecules. With their natural source material transport properties, inherent long-term blood circulation capabilities and excellent biocompatibility, EVs can deliver a variety of chemical drugs, proteins, nucleic acids, gene drugs and other drugs. They have great potential in the field of drug carriers. CD47 is the ligand for signal regulatory protein alpha (SIRPα), and CD47-SIRPα binding initiates the 'don't eat me' signal that inhibits phagocytosis. Therefore, CD47 on EVs prevents them from being engulfed by immune cells [6]. EVs are more efficient than their synthetic analog liposomes. The application of EVs as drug delivery carriers is like putting a "stealth coat" on the drug molecules, which can maximize the stability of the drugs, reduce the immune system's clearance of them, and make "precise delivery" possible. Therefore, EVs can be described as "stealth transport aircrafts" for drugs. EVs therapy has shown great application prospects from oncology to regenerative medicine.

2. Therapeutic application of natural EVs as cell substitutes

A number of studies have shown that EVs derived from mesenchymal stem cells (MSCs) can be used for stem cell replacement therapy [7–21]. In most cases, it is not clear which component of the unmodified EVs exerts curative effects. The researchers' operations are only the separation and purification of EVs produced by therapeutic cells. The curative effects are based on the biological functions of the donor cells, such as the regulation of the immune environment, the repair of damaged cells and the promotion of angiogenesis.

At present, the most extensive research is the attempt to use stem cell-derived EVs for disease treatment. The main application ranges are to repair and regenerate tissues and organs. Such researches involve central nervous system diseases [7, 8], cardiovascular diseases [9–12] and other organ damage repair and regeneration [13–21].

2.1 EVs derived from stem cells and the treatment of central nervous system diseases

In the treatment of central nervous system disease, there is a blood-brain barrier, which often results in that drugs can not reach the diseased site and work well. Stem cells have been gradually used in the treatment of central nervous system diseases in recent years. A large number of research results have been obtained [22, 23]. However, there are still potential risks faced by direct stem cell transplantation, such as tumorigenicity, infection, transplant failure, graft versus host disease, hemorrhagic cystitis, and long-term complications [24].

The application of stem cell EVs avoids a variety of potential risks of direct stem cell transplantation. EVs have low immunogenicity and are easy to preserve and transport, showing unique advantages as a "cell-free stem cell therapy technology". Spinal cord injury (SCI) is one of the deadliest diseases. The complex inhibitory microenvironment needs to be fully mitigated. EVs derived from MSCs have the function of microenvironmental regulation. Studies have established innovative implantation strategies using human MSC-derived EVs immobilized in

peptide-modified adhesive hydrogels (Exo-pGel) [7]. Exo-pGel plays an important role in nerve recovery and urinary tissue protection by effectively reducing inflammation and oxidation [7]. In addition, small extracellular vesiclesderived from embryonic stem cells (ESC-sEVs) can significantly reduce the time-related aging of hippocampal neural stem cells (H-NSCs) through intravenous injection into vascular dementia (VD) rats. ESC-sEVs can restore the damaged proliferation and neuronal differentiation ability, and reverse cognitive impairment. The application of ESC-sEVs may be a new cell-free treatment tool for VD and other diseases related to aging [8].

2.2 EVs derived from stem cells and the treatment of cardiovascular diseases

Stem cells can be induced to differentiate into cardiomyocytes. Early studies believed that the transplanted stem cells can differentiate into heart cells and necrotic cells in the body to repair damaged myocardium and maintain heart function [25]. At present, a large number of preclinical studies have found that EVs derived from transplanted stem cells also have the function of myocardial repair [26, 27]. EVs mainly promote myocardial regeneration by activating cardiac precursor cells, promoting the survival and proliferation of cardiomyocytes, inhibiting their apoptosis, promoting cardiac angiogenesis, reducing infarct size and tissue fibrosis, and regulating inflammation. Extracellular vesicles secreted by cardiovascular precursor cells (hCVPC-EVs) derived from human pluripotent stem cells (hPSCs) play a role in protecting the heart in myocardial infarction by improving cardiomyocyte survival and angiogenesis [9]. Mouse ESC-derived EVs promote angiogenesis, cardiomyocyte survival and proliferation, reduce cardiac fibrosis, and improve cardiac function by carrying miR-294-3p [10]. IPSC-derived EVs inhibit cardiomyocyte apoptosis through miR-21 and miR-210 loaded, and also have a cardioprotective effect [11]. Exosomes produced by immature bone marrow-derived macrophages (BMDM-exo) contain anti-inflammatory microRNA-99a/146b/378a. They can reduce the necrotic lesions of atherosclerosis [12].

2.3 EVs derived from stem cells and the damage repair and regeneration of other organs

With the continuous discovery of the repair and regeneration effects of stem cell EVs in brain tissue and cardiovascular tissues and organs, the application of stem cell EVs in the repair and regeneration of other tissues has also made a lot of progresses.

MSC-derived EVs reduce radiation-induced lung injury through miRNA-214-3p [13]. Replacing autologous cells with EVs derived from hair follicle papillary cell spheres can promote hair growth [14]. Human umbilical cord mesenchymal stem cell-derived exosomes (UMSC-Exo) can inhibit pyrolysis and repair muscle ischemic injury by releasing circular RNA circHIPK3 [15]. Hertwig's EVs derived from epithelial root sheath cells promote the regeneration of dentin plasma tissue [16]. Exosomes from neural progenitor cells retain photoreceptor cells during retinal degeneration (RD) by inactivating microglia. This suggests that NPC-exos and its contents may be the mechanism of stem cell therapy to treat RD [17].

Aging is the process of cell and tissue dysfunction. Small extracellular vesicles (sEVs) isolated from primary fibroblasts from young human donors can improve certain biomarkers of cellular senescence from elderly and Hutchinson-Gilford progeria donors. Studies have shown that sEVs have GST activity to improve aging-related tissue damage [18]. In obesity diseases, EVs derived from adipocytes, as new adipokines, are related to the body's metabolic homeostasis. EVs released

from brown adipose tissue or adipose stem cells can help control the remodeling of white adipose tissue, making it brown and maintaining metabolic homeostasis. EVs have been considered as new regulators of diseases such as insulin resistance, diabetes and non-alcoholic fatty liver. The results provide new treatment strategies for obesity and metabolic diseases [19].

In addition, some reports suggest that some EVs derived from mesenchymal stem cells contain some tumor suppressor molecules. For example, it has been reported that miR-206 in exosomes derived from bone marrow mesenchymal stem cells could inhibit the progression of osteosarcoma by targeting TRA2B [20]. The exosomes derived from human umbilical cord mesenchymal stem cells deliver miRNA-375 to delay the progression of esophageal squamous cell carcinoma [21]. However, although EVs contain these small RNAs that have been reported to exert anti-cancer effects, they also contain a large number of growth factors and pro-angiogenesis factors. When these substances are transported to tumor cells by EVs, can EVs derived from MSCs still exert a tumor suppressor effect? This needs more research to prove.

At present, cell replacement therapy based on the characteristics of donor cells has been studied earlier and more frequently in the field of EVs. There is also a clearer understanding of the components that play a major role. With the continuous increase of clinical needs, people began to try to modify the surfaces and contents of EVs to adapt to more disease treatments.

3. Application of engineered EVs as carriers of nucleic acid drugs in disease treatment

Although natural EVs have been used for cell replacement therapy based on their source and achieved good results, their therapeutic range is far from meeting the current treatment needs. One of the most important therapeutic areas is the treatment of malignant tumors. The secretion ability of EVs in malignant tumor itself is enhanced and contributes to tumor progression. Considering that MSC-derived EVs generally contain high levels of growth factors and pro-angiogenic factors, most natural EVs are not suitable for tumor therapy, except that EVs derived from antigen-presenting cells can be used as tumor vaccines to activate anti-tumor immune responses [28]. Based on the biological characteristics of EVs, it has become the focus of researchers and biopharmaceutical companies to transform EVs as carriers of multiple drugs.

Most diseases have characteristic down-regulation of small RNA expression. Small RNA is the main content of extracellular vesicles, the most abundant and the most easily carried component. Therefore, EVs can be used to carry and deliver small RNA and other gene therapy systems. This section will discuss the progress of engineered EVs to deliver nucleic acid drugs and the strategies of drug loading and targeting.

3.1 Research progress of engineered EVs to deliver nucleic acid drugs

There are three main problems in the development of nucleic acid drugs: the instability of nucleic acid molecules in the body, potential side effects and difficulties in drug delivery systems. The most important one is the development of delivery systems. Because a good drug delivery system can improve drug stability and target cell absorption efficiency, and can reduce its side effects. At present, the commonly used delivery vehicles in the field of nucleic acid drugs are mainly adeno-associated virus (AAV) and liposomal nanoparticles (LNPs). A small number of companies also use lentivirus (LV) and exosomes as delivery vehicles.

The packaging capacity of AAV is small (≤5kb). AAV will be used more than once in patients for therapeutic purposes and the second use will cause the body to produce a strong immune response. The safety of LNPs is relatively high, and the carrier capacity and delivery efficiency can meet the current demand for drug carriers. However, the organ selectivity of LNPs is still relatively limited. The main delivery area is concentrated in the liver, and the delivery efficiency to other tissues and organs is relatively low.

EVs are now candidate carriers for nucleic acid drugs by virtue of their advantages. The red blood cell extracellular vesicles (RBCEVs) have a large loading capacity (≤11kb), can be loaded with many types (including DNA, mRNA, antisense oligonucleotides, siRNA and other nucleic acid types), and contain very little nucleic acid. The advantages make them high-quality natural blank nucleic acid carriers. RBCEVs can be delivered to many different organs and tissues. In mouse experiments, the delivery effects of lung, liver, kidney, bone tissue, immune cells, etc. are all obvious [29]. Moreover, the raw materials used to produce RBCEVs are mainly blood from type O blood donors. This means large quantities of raw materials are readily available, and costs are controllable. Carmine Therapeutics focuses on the research and development of nucleic acid delivery technology using RBCEVs as carriers.

In addition, researchers are also committed to modifying the surfaces of EVs to improve their targeting. Many results show that this strategy can indeed improve the therapeutic effect of engineered EVs [30–33].

The researchers combined ligand-coupled superparamagnetic nanoparticles with specific membrane proteins of blood exosomes to achieve the separation, purification and tumor targeting of exosomes [30]. The chemotherapy drug doxorubicin (Dox) and the cholesterol-modified single-stranded miRNA-21 inhibitor (chol-miR21i) were co-loaded onto the exosomes. Then the cationic endolysin peptide was absorbed on the negatively charged membrane surface of exosomes to promote the cytoplasmic release of the packaged cargo (**Figure 1**). The research results showed that these effectively released drugs and RNA simultaneously interfered with nuclear DNA activity and down-regulated the expression of oncogenes, thereby significantly inhibiting tumor growth and reducing side effects [30].

Chimeric antigen receptors (CAR) are cell surface receptors that recognize specific proteins (antigens). Tumor cells express their specific antigens. Modification of EVs surfaces with CAR targeting tumor antigens enables EVs to target tumors for

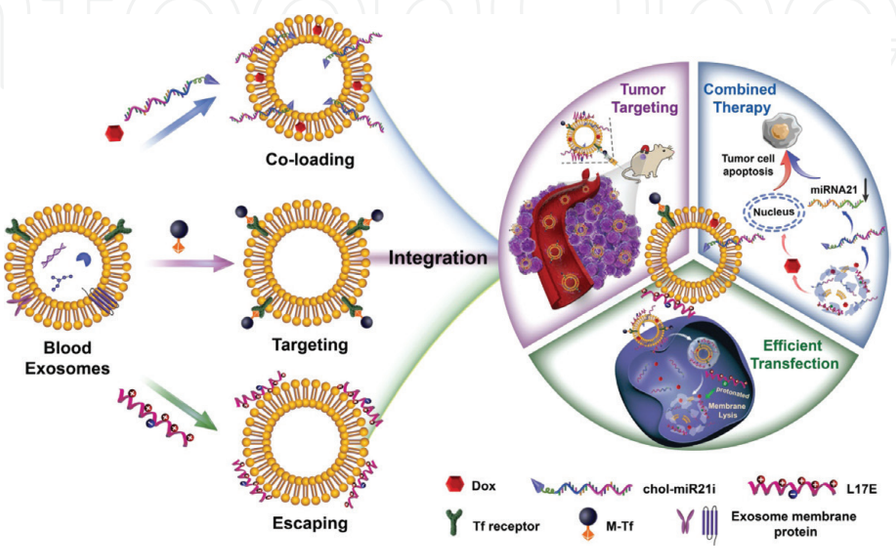

Figure 1.
Schematic representation of engineered blood exosomes for effective gene/chemo combined antitumor therapy [30].

drug delivery. Modified EVs with CAR can serve as a biosafety delivery platform for the CRISPR/Cas9 system to improve their tumor selectivity. Compared with unmodified EVs, CAR-EVs accumulate rapidly in tumors and effectively release the CRISPR/Cas9 system targeting MYC oncogenes in vitro and in vivo [31].

Rabies virus glycoprotein (RVG) is neurogenic. At present, it has become the most active guide molecule for brain targeted drugs. Lysosomal-associated membrane glycoprotein 2b (Lamp2b) is the membrane surface protein of EVs. RVG fused with Lamp2b is located on the surface of the EV to achieve brain targeting. Engineered Lamp2b-RVG-circSCMH1-extracellular vesicles (Lamp2b-RVG-circSCMH1-EVs) can selectively deliver circSCMH1 to the brain. The treatment can improve the functional recovery of mice and monkeys after stroke [32].

In addition, EVs without modification for targeting have also shown certain curative effects. The miR-214 inhibitor was transfected into HEK293T cells. Their exosomes Exo-anti-214 can reverse the resistance of gastric cancer to DDP [33]. HEK293T cells were transfected with HGF siRNA and their exosomes were harvested. In vivo and in vitro experiments have shown that exosomes loaded with HGF siRNA can inhibit the proliferation and migration of cancer cells and vascular cells [33].

3.2 Methods of loading nucleic acid drugs into engineered EVs

Methods of loading nucleic acids into EVs include: chemical reagent transfection, electroporation transfection, transfection of donor cells, protein and characteristic sequence targeting methods. The application scope and advantages and disadvantages of different methods are shown in **Table 2**.

The use of proteins that can bind to specific RNA sequences (**Figure 2**) or the conservative sequences of Exosome-enriched RNAs (eRNAs) to achieve active packaging is a promising direction. The researchers used the specificity of protein binding to the RNA sequence to develop EXOtic devices for mRNA delivery [38]. Archaeal ribosomal protein L7Ae specifically binds to the C/Dbox RNA structure [40–42]. Based on this, L7Ae was conjugated to the C-terminus of CD63. C/D box

Methods	Application scope	Merit and demerit	References
Chemical reagent transfection	Broad-spectrum.	Easy to operate, but EVs should be purified before and after transfection.	[34]
Electroporation transfection	The most commonly used method, but not for miRNA, shRNA, mRNA containing chemical modification.	Easy to operate, but EVs should be purified before and after transfection.	[35]
Transfection of donor cells	Broad spectrum, but not for biotoxic molecules.	Purify EVs after transfection, but the effect of the transfected molecule on the donor cell should be taken into account (e.g. biotoxicity).	[33, 36, 37]
Protein and characteristic sequence targeting	mRNA and miRNA.	High specificity of loading, but the therapeutic molecules will be modified. Whether this will affect the efficacy remains to be determined.	[38, 39]

Table 2.
Methods of loading nucleic acid drugs into engineered EVs.

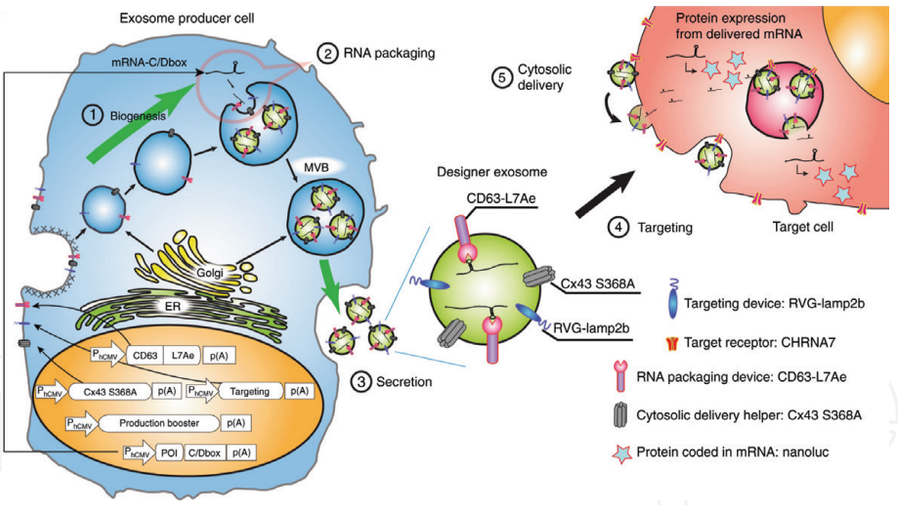

Figure 2.
EXOtic devices for mRNA delivery. A schematic illustration of the EXOtic devices [38].

was inserted into the 3′-untranslated region (3′-UTR) of the reporter gene. Therefore, the mRNA encoding the reporter protein could be well incorporated into exosomes via the interaction between L7Ae and the C/D box in the 3′-UTR. Exosomes containing the RNA packaging device (CD63-L7Ae), targeting module (RVG-Lamp2b to target CHRNA7), cytosolic delivery helper (Cx43 S368A) and mRNA (nluc-C/Dbox) were efficiently produced from exosome producer cells by the exosome production booster. The engineered exosomes were delivered to target cells and the mRNA was delivered into the target cell cytosol with the help of the cytosolic delivery helper. Finally, protein encoded in the mRNA was expressed in the target cells [38] (**Figure 2**). In the future, researchers need to obtain more specific RNA sequence binding proteins and conserved sequences of eRNAs through bioinformatics analysis.

4. Application of engineered EVs as protein transporters in disease treatment

The lack of protein and malfunction are important causes of many diseases. For example, the occurrence of malignant tumors is related to the lack of certain tumor suppressor factors and malfunctions. Therefore, increasing the corresponding protein level is one of the ways to treat diseases. Considering the risk of genome changes, researchers aim to deliver therapeutic protein molecules to the lesion through effective drug delivery vehicles. This section will introduce the use of EVs to transport protein molecules for the prevention and treatment of tumors, immune diseases, cardiovascular diseases, atherosclerosis, myocardial infarction and other diseases.

4.1 Research progress of engineered EVs as protein transporters for disease treatment

Compared with the previous small molecule compound drugs, protein drugs have the characteristics of high activity, strong specificity, low toxicity, clear biological functions, and are beneficial to clinical application. However, protein drugs are unstable in the internal and external environments, and may undergo a variety of complex chemical degradation and physical changes, such as aggregation, precipitation, racemization, hydrolysis, and deamidation. Protein drugs have short half-life, high clearance rate, large molecular weight, poor permeability, susceptibility to the

destruction of enzymes, bacteria and body fluids in the receptor, and low bioavailability of non-injection administration. These problems greatly limit the use of protein drugs. Although researchers have improved the stability and absorption efficiency of protein drugs through methods such as PEG modification, microsphere sustained release, and liposome embedding, they still look forward to the emergence of better drug carriers. The application of EVs has brought dawn to this field.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a promising anticancer agent. Delivery of TRAIL through EVs can efficiently induce cancer cell apoptosis. When combined with dinaciclib, they inhibit the growth of drug-resistant tumors [43]. Immunosuppressive drugs must be taken after organ transplantation, but long-term use of these drugs increases the risk of infection and other serious diseases. Using bioengineering methods, researchers prepared exosome-like nanovesicles (NV) displaying the dual target cargo of PD-L1/CTLA-4. These NVs enhanced the PD-L1/PD-1 and CTLA-4/CD80 immunosuppressive pathways and could be used as prospective immunosuppressive agents in organ transplantation [44]. Using extracellular nanovesicles to package CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping can avoid off-target mutagenesis and immunogenicity. And this method can achieve effective genome editing in a variety of cell types that are difficult to transfect, including human induced pluripotent stem cells (iPS), neurons and myoblasts [45]. Catalase could be loaded into exosomes by incubating at room temperature, saponins penetrating the membrane, repeated freezing and thawing and mechanical extrusion for the treatment of Parkinson's disease (PD) [46].

Surface modification of EVs carrying protein drugs can greatly improve their targeting. In the study of stroke, nerve growth factor (NGF) exerts various neuroprotective functions after ischemia. NGF was loaded into EVs with RVG peptide modification on the surface. Through systemic administration, NGF was effectively delivered to the ischemic cortex. The delivered NGF reduced inflammation by remodeling microglia polarization, promoted cell survival, and increased the number of neuroblast marker doublecortin-positive cells. The results of the study indicated the potential therapeutic effect of NGF@Exo (RVG) on stroke [47]. In addition, integrin αVβ5 exhibits tropism for the liver while integrin α6Vβ4 and integrin α6β1 target lung [48, 49]. The iRGD specifically recognizes αV integrins on the surface of tumor cells [50]. RVG and c(RGDyK) peptides target brain tissue [51]. Klotho protein has the property of binding to circulating endothelial progenitor cells (EPCs) [52]. And chimeric antigen receptor (CAR) targeting specific tumor antigens and so on. These guiding molecules are utilized either by fusion with EVs membrane surface proteins (such as Lamp2b, VSVG, CD63, and other transmembrane proteins, etc.), or by chemical cross-linking on the surface of EVs to achieve the EVs targeting modification. Liu et al. summarized the surface modification strategies to improve the targeting of EVs (**Figure 3**) [53]. In addition, EVs derived from antigen-presenting cells with tumor antigens can be used as tumor vaccines to activate anti-tumor immune responses.

4.2 Methods of loading protein drugs into EVs

How to load protein drugs into EVs? There are currently the following strategies:

4.2.1 Expression of therapeutic protein in donor cells

Transfect donor cells with plasmids carrying the gene of interest. The cell will synthesize the target protein. These proteins are then secreted into EVs through a

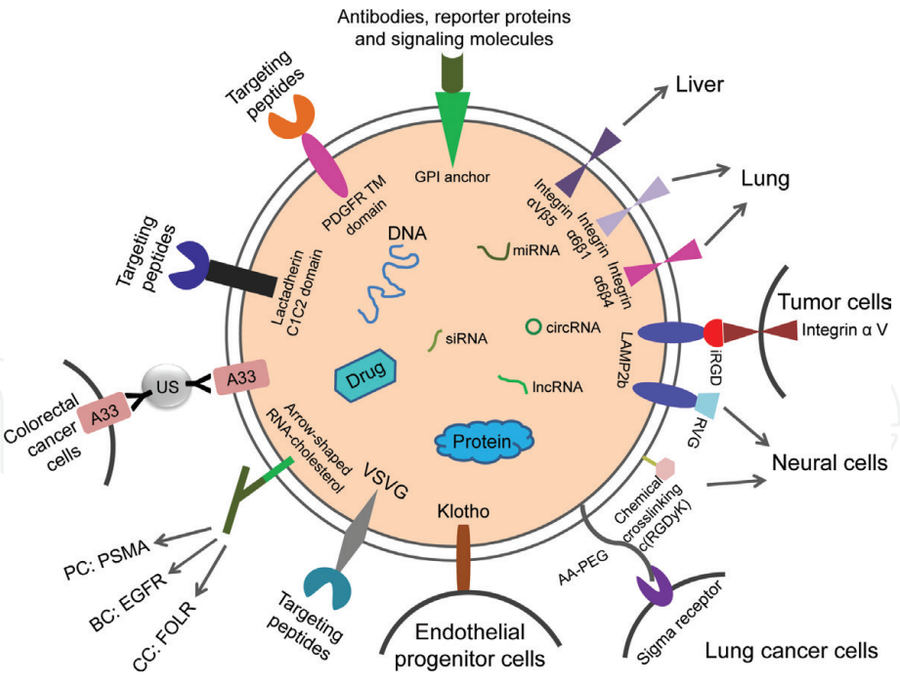

Figure 3.
Design strategies for therapeutic exosome targeting [53].

natural packaging process. Subsequent separation and purification of EVs in the cell culture supernatant is sufficient [54]. Although this method seems simple and easy to implement, cytotoxicity, mixed interactions and impaired biological responses will provide great obstacles to the production of EVs. And the loading efficiency of the target protein is relatively low. Therefore, researchers have carried out various attempts to specifically load target proteins into EVs. For example, the fusion of therapeutic proteins with the constituent proteins of EVs and the specific modification of therapeutic proteins.

4.2.2 Fusion of therapeutic protein with the constituent proteins of EVs

The therapeutic proteins are fused with the constituent proteins of EVs. Then they will be distributed into EVs mediated by the constituent proteins. This method can improve the specificity of protein loading into EVs. The fused constituent proteins of EVs that have been tried include: CD63, Nef [55], vesicular stomatitis virus glycoprotein (VSVG) [56], apolipoprotein E (ApoE) [57], lysosome-associated membrane glycoprotein 2 (LAMP2B) [58], etc.

In addition, based on the idea of fusion proteins, researchers have developed a conditional loading method called "exosomes for protein loading via optically reversible protein-protein interaction (EXPLORs)" [59]. The principle is to couple the exosomal membrane protein CD9 with CIBN, and CRY 2 with the therapeutic protein. After light excitation, CIBN and CRY2 interact, and the therapeutic protein can be loaded into EVs through "photoreversible protein-protein interaction" [59].

All in all, the fusion expression of therapeutic proteins with the constituent proteins of EVs can indeed increase the enrichment level of therapeutic proteins in EVs. However, whether the fusion protein affects the uptake and function of the therapeutic protein by the recipient cells needs to be verified. Therefore, exploring the fusion of peptides that can play a sorting role with therapeutic proteins and minimize the impact on protein functions will become one of the research hotspots in the field of engineered EVs.

4.2.3 Specific modification of therapeutic protein

Currently, known protein modifications that can target therapeutic proteins into EVs mainly include two types. One is ubiquitination modification. The fusion of ubiquitin to the C-terminus of therapeutic protein can make the concentration of the fused therapeutic protein in EVs increased by nearly 10 times [60]. The other is to fuse the N-terminus of the therapeutic protein with a palmitoylated or myristoylated peptide, which can further increase the therapeutic protein in EVs [61]. However, it is still unknown whether the modification of proteins, especially ubiquitination, will cause the degradation of the therapeutic protein by the proteasome and affect its function in the recipient cell.

4.2.4 Combined with mechanical methods to produce small vesicles containing therapeutic proteins

Expression of therapeutic protein in donor cells, combined with mechanical methods that pass through different pores, can produce small vesicles containing the therapeutic proteins [46, 62]. In addition, there are methods such as incubation at room temperature, permeabilization with saponin, freeze-thaw cycles and sonication, [46]. There are two main problems with engineered EVs obtained by mechanical methods. One is that the technical requirements for the separation and purification of EVs are relatively high. The second is the maintenance of the integrity and biological activity of EVs. The composition of EVs actively produced by cells is different from the composition of mechanically produced EVs. The difference may affect the efficacy of engineered EVs. In the future application research of EVs, these two problems need to be solved and proved urgently.

So, what are the possible development directions for the existing cytotoxicity and the interaction of biological functions? The expression of tumor suppressor protein molecules may cause cytotoxicity to donor cells, which is not conducive to the production of EVs. If an inducible expression system is established, the coding DNA containing the inducible promoter is introduced into the donor cell to make the donor cell produce EVs containing the coding DNA, which will avoid cytotoxicity to the donor cell. Then prepare EVs containing small molecules that induce DNA expression. The two types of EVs can be used in combination to express tumor suppressor molecules in target cells. It can play a therapeutic role without affecting the production efficiency of EVs. The dual targeting of the two EVs will greatly reduce the impact of engineered EVs on non-targeted tissues. Because single-component EVs are randomly engulfed by cells and will not affect the cells. This may become one of the follow-up development directions in this field.

5. Application of engineered EVs as carriers of small compounds in disease treatment

Chemotherapeutics and traditional Chinese medicine ingredients with anticancer effects are often used in the clinical treatment of a variety of malignant tumors. However, their toxic, side effects and short half-life limit their efficacy. The packaging and transportation with EVs will improve the targeting of chemotherapeutic drugs, increase the uptake efficiency of tumor cells, promote drug stability, reduce the use concentration, and reduce toxic side effects on other organs and normal tissues [63].

The hydrophobic drug curcumin could be packaged into exosomes by direct mixing for tumor treatment [64]. Paclitaxel (PTX) was loaded into EVs secreted by

macrophages by different methods such as room temperature incubation, electroporation and sonication. Studies have found that ultrasound treatment increases the load of EVs on drug molecules and the sustained release [65]. Small compounds can also be naturally secreted into EVs by incubating with donor cells. Incubation with paclitaxel make mesenchymal stromal cells produce microvesicles containing paclitaxel [66]. Injecting methotrexate-containing plasma membrane microvesicles (MTX-TMP) from apoptotic human tumor cells into the bile duct lumen of extrahepatic CCA patients could mobilize and activate neutrophils, and relieve the bile duct obstruction in 25% of patients, almost no adverse reactions [67].

At present, small molecule drugs are often loaded into EVs by passive loading methods, such as direct mixing, incubation, ultrasonic treatment, vortexing, saponin permeation, repeated freezing and thawing, and mechanical extrusion. The disadvantages of these methods have always existed, that is, the loss and quality reduction of EVs caused by multiple purifications. In addition, long-term in vitro processing and the physical and chemical properties of drug molecules will also affect the biological activity and stability of EVs. Therefore, before EVs can be widely used in treatment, the storage methods and stability factors of EVs are also worthy of research.

6. Application of engineered EVs as virus carriers in gene therapy

Why are EVs a "stealth cap" for drugs? Because we know viruses to use them exactly like this. In nature, viruses "hijack" EVs to secrete and infect other cells. This method helps to provide a "cover" for the virus to prevent the virus from being cleared by the immune system or neutralized by antibodies, such as the infection process of HAV, HBV and HCV.

In gene therapy, currently widely used adeno-associated virus (AAV), oncolytic adenovirus (OAV) and lentivirus (LV) mediated gene therapy can cause the body's immune response. After the same kind of AAV is used once, the body will produce a strong immune response, making the second injection ineffective. If EVs encapsulate viral particles to mediate their delivery, perhaps the therapeutic effect will be better.

Studies have shown that AAV isolated from conditioned media could bind to exosomes (exo-AAV) [68]. Compared with conventional AAV, exo-AAV was more resistant to neutralizing antibodies. After systemic injection in mice, compared with conventional AAV, exo-AAV delivered genes to the brain more efficiently at low vector doses. Importantly, no cytotoxicity was found in exo-AAV transduced cells. This may make exo-AAV widely used as a neuroscience research tool [68]. Compared with non-targeted modified EV-AAV, the expression of brain-targeting peptides on the surfaces of EVs can significantly enhance transduction [69].

In gene therapy of ophthalmic diseases, transferring genes to the retina is challenging. Because it requires a carrier system to overcome physical and biochemical barriers to enter and spread throughout the retinal tissue. After the exo-AAV was injected into the vitreous cavity (IVT), it was found that the expression of exo-AAV was better than the traditional AAV. Exo-AAV exhibited a deeper penetration in the retina, effectively reaching the inner core and outer plexus, and to a lesser extent the outer nuclear layer. Exo-AAV is a reliable mouse retina gene delivery tool. Its simplicity of production and isolation makes it widely used in basic eye research [70].

Due to the low efficiency of gene delivery to the inner ear sensory hair cells. AAV is not so advanced in the field of gene therapy for hearing impairment. Studies have shown that Exo-AAV1-GFP is more effective than traditional AAV1-GFP, whether

injected in mouse cochlear explants in vitro or directly injected into the cochlea in vivo. Exo-AAV was not toxic in the body. Exo-AAV1 gene therapy partially rescued the hearing in a mouse model of hereditary deafness. It was a powerful hair cell gene delivery system that could be used for gene therapy of deafness [71].

Oncolytic viruses show unique anti-cancer mechanisms in cancer treatment. Chemotherapeutic drugs combined with oncolytic viruses showed stronger cytotoxicity and oncolytic effects. Someone has studied the systemic delivery of oncolytic adenovirus and paclitaxel encapsulated by EVs. The results have shown that this combination therapy enhanced anticancer effects in lung cancer models both in vitro and in vivo. EVs play a key role in the effective transmission of oncolytic viruses and chemotherapeutic drugs [72].

7. Sources of EVs that can be used for drug delivery

EVs currently used for therapeutic research are mainly derived from the following sources: mesenchymal stem cells (MSCs), dendritic cells (DCs), tumor cells, red blood cells, macrophages and plants. EVs from different sources have different biological characteristics. Materials should be selected according to the purpose of treatment. The characteristics, advantages and disadvantages of EVs from different sources will be described below.

7.1 Mesenchymal stem cells

The MSCs involved in the study of EVs include adipose-derived MSCs, bone marrow MSCs, progenitor cells from different tissues, and so on. MSCs can be extracted from the patient's bone marrow, fat, or other tissues. EVs derived from MSCs are very attractive. Because they have anti-inflammatory, anti-apoptotic and anti-microbial capability, and promote angiogenesis and the repair and regeneration of damaged tissues. As mentioned above, EVs derived from MSCs have been widely used in the treatment of central nervous system diseases, cardiovascular diseases, bone and cartilage damage repair and regeneration, wound repair, and other organ damage repair and regeneration [7–21].

7.2 Dendritic cells

One potential source of therapeutic EVs is immature dendritic cells (imDCs). EVs secreted by imDCs lack surface markers such as CD40, CD86, MHC-I and MHC-II. Therefore, they have low immunogenicity. They can be isolated from CD34+ cells from the patient's peripheral blood. It is one of the preferred sources of therapeutic EVs.

7.3 Tumor cells

The use of EVs derived from tumor cells to deliver drugs and vaccines for immunotherapy is very promising. Tumor EVs can deliver antigens to dendritic cells, thereby inducing T cell-mediated immune responses to tumor cells [73]. As tumor-derived EVs specifically express Tetraspanins, they can target different tissues. This makes it possible to use tumor-derived EVs for tumor-targeting and selective drug delivery [74]. However, tumor-derived EVs also have many potential risks. Due to the presence of Tetraspanins, Urokinase plasminogen activator, Cathepsin D, Vimentin and other molecules derived from the surface of tumor cells [75, 76], they may promote tumor proliferation and metastasis, and Immunosuppressive effect [77–79].

7.4 Red blood cells

Blood EVs mainly secreted by reticulocytes (RTC) are a potential source of safe and sufficient EVs. Because they integrate various membrane proteins including Transferrin (Tf) receptors, but they do not have any immune and cancer stimulating activity [30]. Red blood cell EVs (RBCEV) also have the following advantages: large load; low self-nucleic acid content (red blood cells without nucleus and mitochondria); they can be delivered to a variety of different organs and tissues; large quantities of raw materials and easily available (the raw materials for producing RBCEVs are mainly O-type Blood of blood donors). Using blood EVs as carriers can efficiently target tumors to co-deliver chemotherapeutics and nucleic acid drugs. Significant tumor growth inhibitory effects were observed in tumor-bearing mice. There were no obvious side effects [30].

7.5 Macrophages

Macrophages are an important immune cell in the antigen-presenting cell family. EVs derived from immune cells can mimic immune cells to target tumor cells. Macrophage EVs can transfer miRNAs or proteins to tumor cells, mediate tumor cell resistance to chemotherapy, promote cell invasion and other regulatory effects. Therefore, in the study of tumor treatment of EVs, in addition to using the targeting properties of macrophages-derived EVs, the influence of their contents must also be considered. It has been reported that the contents of EVs derived from macrophages can be removed. Then the EVs were used to carry chemotherapeutic drugs to achieve targeted therapy of triple-negative breast cancer [80].

7.6 Plant-derived EVs

Based on reliable sources and safety, fruits and plants have been used as alternative sources for the isolation of EVs for clinical use [81]. Plant-derived EVs have similar structural characteristics to animal cell-derived EVs. EVs from different plant sources have the physiological functions of the plant from which they are derived. For example, lemon-derived EVs have certain anti-cancer effects. Some researchers have tried to isolate lemon-derived EVs (LDEVs) for the treatment of gastric cancer. LDEVs caused s-phase arrest of gastric cancer cell cycle and induced cell apoptosis. LDEVs could be retained in the organs of the gastrointestinal tract and had strong anti-tumor activity against gastric cancer [82]. The isolated plant EVs can also be used after being engineered. Some researchers isolated EVs from grapefruit, modified the EVs in a targeted manner, and then loaded the anti-tumor drugs doxorubicin and curcumin. These modified EVs could target inflammatory tumors and have anti-inflammatory effects in mouse models [83].

Plant-derived EVs have a wide range of sources, are safe and non-toxic, have low immunogenicity, low cost, and are edible. They have great clinical application potential as edible chemotherapeutic drug carriers.

8. Commercialization progress and potential problems of EVs

8.1 Progress in the commercialization of EVs

So far, no EVs drugs have entered the clinic. Codiak BioSciences, a leading company in the development of engineered EVs as a new type of biopharmaceutical, uses its proprietary engEx platform to engineer EVs with different characteristics, load them

with various types of therapeutic molecules and change their orientation, so that they can reach specific cellular targets. Recently, Evox Therapeutics Ltd. and Eli Lilly and Co. reached a cooperation agreement to apply its exosome technology to the system to deliver RNA interference and antisense oligonucleotide drugs to the central nervous system, treating five unspecified Neurological diseases. Carmine Therapeutics is also a gene therapy company based on EVs, established in 2019. Carmine's REGENT technology platform focuses on using red blood cell extracellular vesicles (RBCEV) as drug delivery vehicles. Mantra Bio also joined the emerging group of exosome drug development companies. With the deepening of research, more and more companies will join the field of EVs treatment.

8.2 EVs treatment and COVID-19

The Severe Acute Respiratory Syndrome (which first appeared in December 2019) related to the new coronavirus (COVID-19) has rapidly developed into a pandemic, and the morbidity and mortality rates are increasing worldwide. COVID-19 respiratory tract infection is characterized by an imbalanced immune response, leading to an increased possibility of severe respiratory disease and multiple organ disease.

Because EVs derived from MSCs have anti-inflammatory, anti-apoptotic and anti-microbial capability, promote angiogenesis and the repair and regeneration of damaged tissues. In related lung disease models, including acute lung injury and sepsis, systemic administration of MSC-EVs preparations can modulate immune responses. In a mouse model of pneumonia induced by Escherichia coli, it was found that MSC-EVs administration could enhance the phagocytosis of bacteria. In the pig model, MSC-EVs could reduce influenza virus-induced acute lung injury by inhibiting influenza virus replication. In other disease models, the disease alleviation effect of MSC-EVs on the inflammatory immune response has also been observed. It is speculated that they may also have anti-COVID-19 efficacy. In cell therapy research for COVID-19, some registered clinical trials have turned their targets to EVs in the conditioned medium of MSCs. MSC-EVs can be administered intravenously (ChiCTR2000030484) or by inhalation (NCT04276987, ChiCTR2000030261).

However, before using MSC-EVs for COVID-19 patients, many other issues should be considered, such as the huge heterogeneity of MSC-EVs composition and source. In fact, comparing MSC-EVs harvested from the conditioned medium of bone marrow MSCs derived from different donors, there are significant differences in cytokine content and different therapeutic effects. In addition to immune regulation, MSC-EVs can also control other biological processes and may cause unpredictable side effects. For example, increasing the risk of thrombosis.

In short, in order to reduce the risk of potential life-threatening side effects, International Society for Extracellular Vesicles (ISEV) and International Society for Cell and Gene Therapy (ISCT) strongly require that the clinical data from reasonable clinical trial should be carefully weighed. The EV preparations with good characteristics and produced under strict GMP conditions and appropriate regulatory supervision could be used. Any application of EVs should be carefully evaluated [84].

8.3 Potential problems in the industrialization of EVs

The potential application of EVs in new diagnostic and therapeutic strategies has attracted increasing attention. However, due to the inherent complex biogenesis of EVs and their huge heterogeneity in size, composition and source, the research of

EVs still faces huge challenges. It is necessary to establish a standardized method to solve the heterogeneity of EVs and the analysis of pre-processing and analysis of sources of variability in the study of EVs. The quality standards, extraction specifications and especially the stability of preparation conditions for therapeutic EVs also need to be clarified.

In addition, the diversity and uncertainty of EVs content are also issues that need to be considered in the application. Before metastasis, malignant tumor cells use EVs to modify the microenvironment of the organ targeted by cancer metastasis, making it a suitable "soil" for tumor cell growth. The contents of EVs secreted by most tumor cells play a role in promoting tumor metastasis and progression. As mentioned earlier in this article, macrophage EVs can transfer miRNAs and proteins to tumor cells, mediate tumor cell resistance to chemotherapy, promote cell invasion and other regulatory effects. Therefore, if EVs from such sources are used as drug carriers, it is particularly important to first remove the adverse effects of their contents.

9. Conclusion

As an important medium of intercellular communication, EVs play an important physiological function and are also involved in the occurrence and development of a variety of diseases. In recent years, there have been numerous studies on the treatment of related diseases with EVs from different cell sources, and EV has shown its unique advantages in drug transportation. EVs are similar to natural liposomes, which can enhance the function of EVs to treat specific diseases through targeting modification and delivery of functional active substances and other technical modifications according to the characteristics of different diseases. EVs with improved function have shown obvious advantages in the treatment of tumors and difficult diseases of central nervous system. However, the clinical application of EVs technology is still in its infancy, and the challenges it faces are accompanied by the possibility of numerous new discoveries and new technologies. We expect that with the continuous in-depth research, EVs as a new drug carrier in the treatment of a variety of diseases will bring more and greater surprises.

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