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REVIEW

Advances in Therapeutic Applications of Extracellular Vesicles

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Abstract: Extracellular vesicles (EVs) are nanoscale bilayer phospholipid membrane vesicles released by cells. Contained large molecules such as nucleic acid, protein, and lipid, EVs are an integral part of cell communication. The contents of EVs vary based on the cell source and play an important role in both pathological and physiological conditions. EVs can be used as drugs or targets in disease treatment, and changes in the contents of EVs can indicate the progression of diseases. In recent years, with the continuous exploration of the structure, characteristics, and functions of EVs, the potential of engineered EVs for drug delivery and therapy being constantly explored. This review provides a brief overview of the structure, characteristics and functions of EVs, summarizes the advanced application of EVs and outlook on the prospect of it. It is our hope that this review will increase understanding of the current development of medical applications of EVs and help us overcome future challenges.

Keywords: extracellular vesicle, nanotherapeutic, disease diagnosis, engineered exosome, advances applications

Introduction

Extracellular vesicles (EVs) were originally described as a general term for all extracellular particles of unknown origin.[1] Although evidence of their existence has been available for more than 80 years, it is only in recent decades that research into their production, function, and potential application has begun to emerge. Early studies on EVs received little attention from scientists, and EVs were generally regarded as a waste product of cell metabolism.[2] In 1987, Rose M. Johnstone defined a material produced by the release pathway of polyvesicles after fusion with the cell membrane as exosomes. At the time, exosomes were believed to be just a type of "garbage can".[3,4] Later, a breakthrough was made in the study of the outer membrane of cells. Scientists found that the function of exosomes was not limited to the transport of metabolic waste. For example, exosomes have enzyme activities that promote blood clotting and antigen presentation, and there is interaction between bacteria-derived exosomes and human intestinal cells.[5,6] Among them, exosomes, play the most important role in anti-tumor immunity. Dendritic cell-derived exosomes (Dex) can carry cancer cell antigens and be utilized for tumor treatment, which has been verified by clinical experiments.[7] Since then, the field of EVs has gradually gained the attention in the fields of cell biology and biotechnology, especially has experienced rapid development in the past 20 years.[8] In 2018, the International Extracellular Vesicle Association (ISEV) published the Minimal Information for Studies of Extracellular Vesicles (MISEV 2018) to better isolate and classify different types of EVs. It provides a reference and research paradigm for EVs research.[9]

In recent years, there has been an increasing clarity in understanding the relationship between the components of EVs from different cell sources and their biological functions. Meanwhile, advancements in click chemistry and biological orthogonal technology have led to a surge in application studies that focus on EV components and biological characteristics. The applications can be roughly divided into the following three classes based on their basis: 1.

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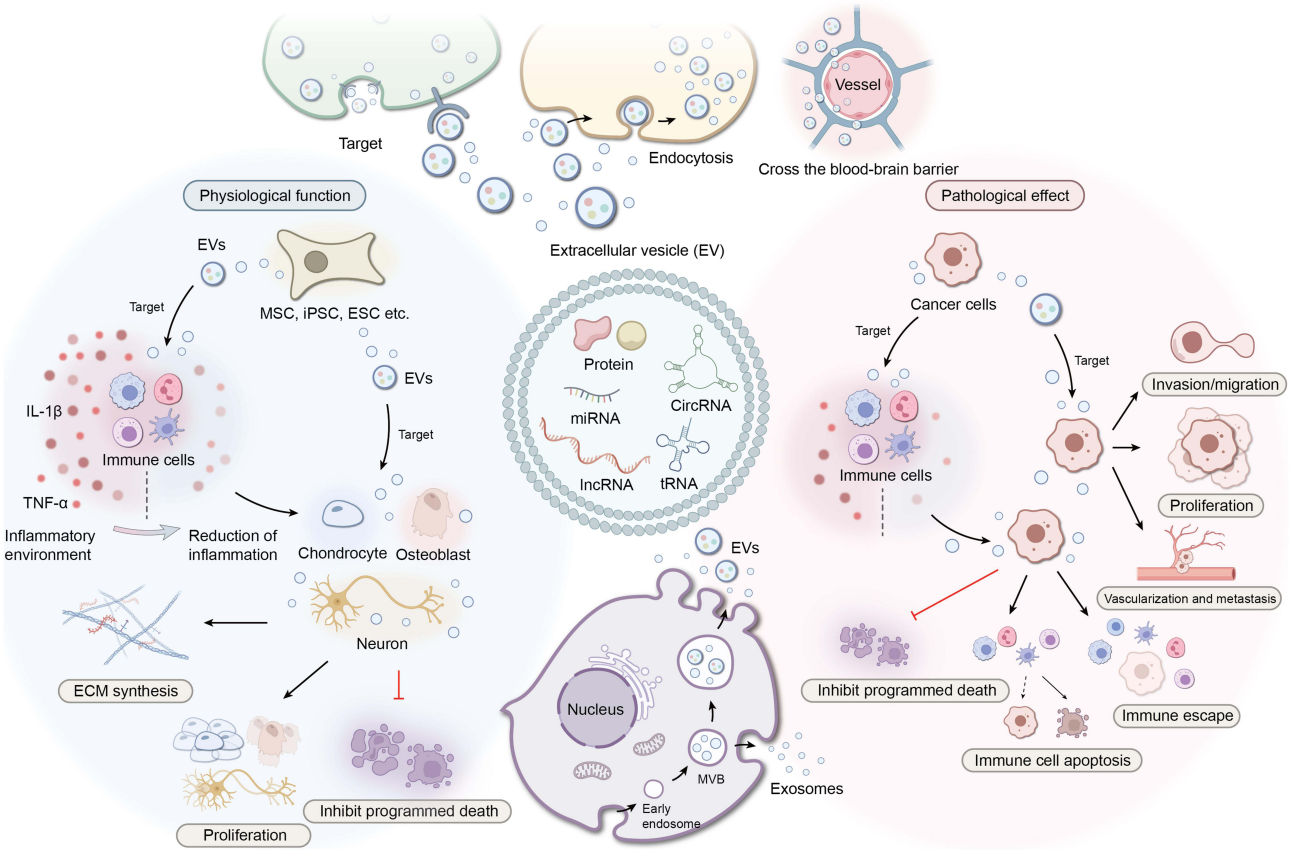

Figure 1 Brief overview of the structure, characteristics and functions of EVs.

Application based on EVs' biological function (pure application, treatment or blocking). 2. Applications based on EVs' biological properties (membrane structure, engineered exosomes). 3. Applications based on the relationship between EVs and diseases (diagnostic application).

In this review, the characteristics and functions of EVs are briefly described. And the main cutting-edge applications of EVs in disease therapy, engineering, and diagnosis in recent years are reviewed.

Characteristics of Extracellular Vesicles

Structure

EVs are nanoscale bilayer phospholipid membrane vesicles covered by membranes. There are lipid rafts on the vesicles that restrict the membrane fluidity. The vesicles contain large molecules such as nucleic acid, protein, and lipid.[10,11] EVs are released by most cells type and can be isolated from various body fluids, including blood, cerebrospinal fluid, tears.[12,13]

EVs are diverse and can be divided into three subtypes based on their cell origin, biogenesis, size and function to receptors: Exosomes (50–150nm EVs from the endocytic pathway); Microvesicles (MVs) (200–800 nm EVs from plasma membrane budding); Apoptotic bodies (diameter > 1μm EVs from migratory and apoptotic cells).[14–16] Most current research has focused on the first two types of EVs.

EVs contain proteins that are related to membrane transport, such as attached proteins, Rab GTPases, flotillins, and multivesicular body (MVB)-production proteins. Exosomes also contain various nucleic acids, including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), which perform as regulators of gene expression and could be used as biomarkers.[17] Lipid components in exosomes contain sphingomyelin (SM), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylacid (PA), ceramides, and cholesterin.[18] The bilayer phospholipid membrane of EVs contain

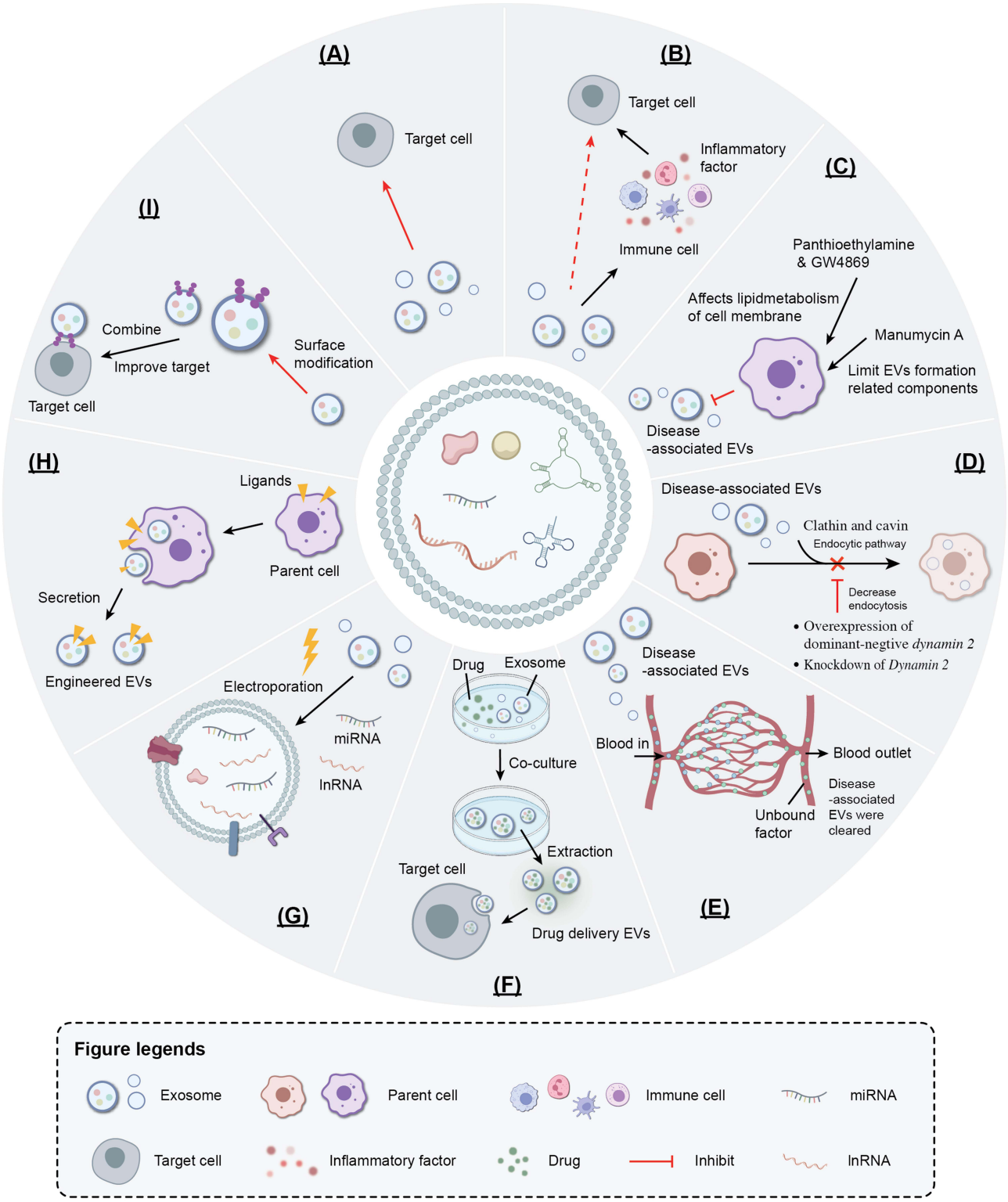

Figure 2 Advanced application of EVs. A. Direct therapeutic B. Indirect therapeutic C. Block EVs associated with diseases D. Block the uptake of EVs associated with diseases E. Physical elimination of EVs associated with diseases F. Engineered EVs: drug delivery G. Engineered EVs: Internal modification H. Engineered EVs: parent cell surface modification I. Engineered EVs: EVs surface modification.

phosphatidylcholine and sphingolipin in the outer layer and PS and phosphatidylethanolamine (PE) in the inner layer. The lipid in the outer layer helps protect EVs from hydrolysis by enzymes in different body fluids and under different potential of hydrogen (PH) values.[19]

Recent studies have shown that components of exosomes are heterogeneous and depended on the source cells that secrete them.[11,20] For example, a proteomic analysis showed that exosomes secreted by breast cancer epithelial and mesenchymal cells contain completely different proteins and nucleic acids.[21] The cholesterin and phospholipids in exosomes secreted by cancer cells and non-cancer cells are also significantly different.[22]

Biological Genesis Mechanism

EVs have the following mechanism: the formation of exosomes begins with endocytosis on the cell membrane surface, and early endosomes are formed through inward budding. As these early endosomes mature into late endosomes, they then form multiple intraluminal vesicles (ILVs). During this process, RNA, DNA, and lipids are actively and selectively incorporated into the ILVs.[23] The ILVs then sprout inside the cell and form the MVB. Most MVBs fuses with the lysosome, resulting in degradation of their contents. Under the regulation of Rab27a and Rab27b enzymes, a few MVBs that contain CD63, lysosomal-associated membrane protein 1 (LAMP1), and LAMP2 are exocytosed through fusion with the cell membrane or degradation pathways, and their contents are wrapped in vesicles and released outside the cell to form exosomes.[24,25]

The biological genesis mechanism of ILVs and MVB are driven by the endosomal sorting complex required for transport (ESCRT). This is a multiprotein complex consisting of thirty proteins.[26] The primary function of ESCRT is to classify specific cargo into ILVs, which serve as precursors to exosomes.[27] The process of MVBs fusing with the cell membrane and releasing exosomes into body fluids is mainly dependent on the Rab family and the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family. The SNARE and Rabs control the germination, transport, and localization of intracellular vesicles on the plasma membrane.[24,28–30] MVs are formed through outward budding of the plasma membrane and finally breaking off under the action of ARF6, RhoA, and Rab22A.[31,32]

Physiological Function

EVs are an integral part of cell communication. Cells release EVs into the extracellular space to participate in cell biological functions, and they hold an important status in pathophysiological conditions.[33] EVs interact with receptor cells through three primary mechanisms of entry into cells: 1. Binding to surface receptors triggers a signaling cascade that complements classical paracrine signaling of secreted soluble factors;[34] 2. Endocytosis, phagocytosis or macropinocytosis;[35,36] 3. Fusion with cells to deliver substances directly to the cytoplasmic membrane and cytoplasmic matrix, delivering large amounts of functional biomolecules to neighboring cells.[37]

Under physiological conditions, EVs play a crucial role in transmitting intercellular signals that regulate the function of other cells and maintain physiological homeostasis, such as angiogenesis, cell migration, and immune regulation.[38] For instance, exosomes can influence angiogenesis by regulating the expression of miR-424, miR-31-5p, and miR-21-3p.[39–41] Additionally, exosomes secreted by cells can induce the migration of parent cells. Developments in imaging and labeling techniques have made it possible to capture of exosome secretion and internal trafficking events in both parent and recipient cells. A study have demonstrated the role of EVs in cell migration through the use of a novel bicolor reporter. The results showed that exosomes are secreted at the front end of migrating cells, and the cells move along the exosome trail with intense pathfinding behavior.[42]

Exosomes are also involved in immunomodulation, with their regulatory mechanisms primarily involving direct action on target cells to initiate downstream signals, as well as miRNA-mediated regulation.[43] There are Fas Ligand (FasL) and Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) expressed by placental tissue and exosomes, respectively. These signaling molecules trigger T cell apoptosis through contact with target cells, thus maintaining normal pregnancy.[44] Another study found that the main component of placental-exosome miRNA, C19MCmiRNA, can inhibit the proliferation of viruses and induce self-phagocytosis, endowing the fetus with antiviral protection.[45] Dex can migrate to tumors or present antigens to T cells, inducing an immune response.[46] For example, Dex

can improve the tumor microenvironment (TME) of hepatocellular carcinoma (HCC), leading to the increase of immune-stimulating cytokines and the infiltration of CD8 cytotoxic T cell, and the decrease of immunosuppressive cytokines and regulatory T-cell (Tregs).[47] As more studies are conducted, more functions of EVs are being discovered, suggesting that they may play a more crucial role in normal physiological activities than previously thought.

Pathological Effect

In the pathological process, EVs can enhance tumor cell invasion, cardiovascular disease, and pathogen infections.[48–52] Exosomes derived from tumor cells act as signal sensors or messengers, regulating communication between tissues and cells. This regulation contributes to the migration and growth of tumor cells, modification of the microenvironment, metastasis, and invasion.[53–55] Cancer cells may secrete different types of exosomes, depending on their genetic background and the stage of cancer. Primary tumors may contain heterogeneous populations of cancer cells with different invasive abilities, and communication between these populations can occur through the potential pathway of EVs. This creates ideal conditions for cancer cell proliferation and invasion, and enhances the invasion of low-grade cancer cell populations through the influence of high-grade cancer cells, promoting the invasion and metastasis of the tumor.[54,56] The cancer pre-metastatic niche is an important link in cancer metastasis. Studies have shown that in colorectal cancer, cancer cells transfer metastasis-promoting miRNA (miR-25-3p) to endothelial cells through exosomes, targeting KLF2 and KLF4 to promote endothelial angiogenesis and increase vascular permeability.[49] The creation of TME around cancer cells is closely related to the EVs produced by cancer cells. These EVs can recruit fibroblasts through the release of signals, promoting the transformation of mesenchymal stem cells into myofibroblasts and the formation of endothelial cells into blood vessels. All these behaviors promote the creation of TME.[57,58] EVs are essential to the immunological escape mechanism of tumor cells. The interaction of PD-L1 (CD274)/PD-1 (PDCD1) can inhibit the T cell response in TME, and peripheral blood T cells can be stimulated to produce IL-10 to mediate immunosuppression. PD-L1 is a critical checkpoint molecule that suppresses the immune response and accelerates tumor growth by allowing tumor cells to evade detection by the immune system.[59] In the TME, cancer cells release exosomes that can increase the expression of PD-L1 around the cell population, leading to T cell apoptosis and inhibition of the immune response.[60] The TME also contains cancer-associated fibroblasts (CAFs), which play a key role in tumors growth. MicroRNA-92 in the exosomes of breast cancer CAFs bind to target gene LATS2 in cancer cells, increasing PD-L1 expression and T cell apoptosis.[61] Moreover, human lung tumor cells produce epithelial cell adhesion molecule^{+} (EpCAM^{+}) exosomes that can regulate the phenotype of immune cells in the TME, leading to the preference of M2 phenotype in TME-infiltrating macrophages, which is associated with tumor progression and survival.[62] T-cell immunity is reduced in patients with chronic lymphocytic leukemia (CLL). Leukemia cells can also promote disease development through paracrine exosome transport, with recent studies showing that CLL-induced myeloid-derived suppressor cells (MDSCs) secrete miR-155 exosomes that bind to T-cells and inhibit Treg activity, allowing the leukemia to escape immune system attack and spread.[63] In airway inflammation, EVs secreted by the airway are involved in the pathogenesis of allergic airway inflammation under allergen exposure, and EVs in bronchoalveolar lavage fluid of asthmatic patients induce significantly higher levels of IL-8 in bronchial epithelial cells.[64] In the pathological condition of atherosclerosis, smooth muscle cells of the cardiovascular tissue release EVs containing calcification-promoting cargo, which become risk factors for plaque formation when released into the extracellular matrix.[50,52] Macrophages stimulated by lipopolysaccharide during septicemia release more EVs containing proinflammatory cytokines, and this increase in cytokines in EVs is associated with the progression of myocardial dysfunction.[65] Exosomes are also involved in the transmission of some viruses. After the induction of Epstein-Barr virus, exosomes contain the viral BGLF2 protein and infect the uninfected receptor cells. In this process, exosomes can avoid the body's immune surveillance and attack.[48] Related studies between exosomes and viruses have found that exosomes carrying RNA virus components can promote viral infection, and viruses may transfer proteins and miRNAs through exosomes. Some RNA viruses, such as human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV), can rely on the ESCRT pathway that generates exosomes and use the double membrane of exosomes as acquired cysts to neutralize host antibodies and escape host immunity.[66] In terms of neurodegeneration, exosomes containing substances such as interferon and tumor necrosis factor (TNF) can destroy the blood-brain barrier and release their contents, leading to a decrease of the organization and tightness between the blood-brain barrier and

brain endothelial cells. Exosomes also cause the migration of white blood cells, leading to detachment of the myelin sheath and multiple sclerosis.[67]

Many research studies have been published on the role of EVs in cancer, trauma, autoimmune diseases, infectious diseases, and cardiovascular disease . In conclusion, it has become a new therapeutic strategy that promoting beneficial EVs and inhibiting the release of pathogenic EVs to mitigate the negative effects of disease. Above all, the structure, features, and functions of EVs are summarized in this review (Figure 1).

EVs as Drug or Target in Therapeutic Application

As previously stated, researchers are investigating the role of EVs in pathophysiological conditions to enhance the positive stimulatory effect of EVs for a direct therapeutic effect. Current research focuses on mesenchymal stem cells, which are considered the "bank" of regenerative medicine due to their ability to evade the immune system, self-regenerate, and differentiate into various cell types with greater specificity than differentiated cells.[68–70] However, safety concerns have been raised regarding stem cell therapy, including ethical issues and the risk of teratoma formation with embryonic stem cell therapy, genomic instability and tumorigenesis with pluripotent stem cell therapy, and promoting tumor growth, metastasis, and differentiation into non-target tissues with mesenchymal stem cell therapy.[71–75] Encouragingly, recent evidence suggests that stem cells may exhibit beneficial effects through the EVs they release, playing a role similar to stem cells in tissue regeneration and damage repair.[69,76] Furthermore, the secretions of stem cell-drived EVs are more controllable compared to stem cells, thus avoiding the risk of tumorigenesis.[77]

Direct/Indirect Therapeutic

Current studies have shown that MSCs-drived EVs can promote tissue regeneration and regulate the immune response, which is expected to be an important part of cell-free therapy.[78–80] Nerve injury repair is always a difficult problem in tissue regeneration. Studies of nerve injury repair have shown that R28 retinal cells can endocytose MSC-EVs via the heparin sulfate proteoglycans (HSPG) receptor-mediated pathway on the cell surface. By competitively blocking the HSPG binding site on EVs, endocytosis of MSC-EVs in R28 retinal cells occurs by disrupting cell membrane cholesterol or dose-dependently blocking heparin, reducing neuroinflammation, and promoting retinal functional recovery.[81] Another study found that embryonic stem cells (ESCs)-drived EVs induced a significant increase in regulatory Tregs after stroke, possibly due to the enrichment of ESC-sEVTGF-β, Smad2, and Smad4 proteins in stem cell-EVs. These proteins can be delivered to activate the TGF-β/Smad pathway in CD4 T cells, inducing Treg amplification, regulating neuroinflammation, and promoting nerve recovery.[80] In studies of intervertebral disc degeneration, MSCs-derived exosomes inhibit reactive oxygen species (ROS) production and NLRP3 inflammasome activation while reducing nucleus pulposus cell apoptosis. Moreover, MSC-EVs can inhibit the expression of catabolic enzymes (MMP3, MMP13) and enhance anabolism (Col2a1, SOX). These findings suggest that MSC-EVs can alleviate the destruction of extracellular matrix in the nucleus pulposus and contribute to the alleviation of intervertebral disc degeneration.[82] The therapeutic effect of exosomes can also be achieved through the regulation of macrophages. In a study with osteoarthritis (OA), intraarticular injection of bone marrow stem cells (BMSCs)-derived exosomes can significantly downregulated inflammatory cytokines IL-1β, TNF-a, and IL-6 while promoting the expression of the anti-inflammatory cytokines IL-10 and TGF-1. By the way, BMSC-derived exosomes also reduced synovitis cell infiltration, decreased damage to articular cartilage, and delayed the progression of OA.[83] Exosomes can promote the transformation of macrophages from M1 macrophages to M2 macrophages in the synovium of joint and inhibit chondrocyte hypertrophy and degeneration caused by M1 macrophages.[84] Parturition is an important process of human reproduction and development. During parturition, human umbilical cord mesenchymal stem cell (UMSC)-derived exosomes can release HIPK3. As miR-421/FOXO3a is a direct target of circular IPK3, UMSC- derived exosomes can regulate miR-421 and increase the expression of FOXO3a, thereby inhibiting phosphorylation and release of IL-1β and IL-18, and protecting the process of human parturition.[85] In addition to the parturition process, heart health is also critical. In a mice model of myocardial infarction, EVs derived from cardiosphere-derived cells (CDCs) were enriched with miR-146a, which can improve cardiac function by enhancing cardiomyocyte viability and preventing oxidative stress.[86]

Block EVs Associated with Diseases

Although there may be therapeutic effects of EVs, it is important to note that EVs are also involved in disease progression. Therefore, current research is focusing on how to identify and block major EV subgroups that are associated with specific diseases, without affecting those EVs that perform important physiological functions. In tumor therapy, current research is investigating how to regulate the release of cancer cell-derived exosomes, block the uptake of specific exosomes by recipient cells, and remove exosomes from body fluids.[87] Researchers have found that in cancer cells overexpressed with the Rab27a gene, the enrichment of immunoactivating molecules, such as Hsp70 or Hsp90, in exosomes can induce a stronger anti-tumor immune response and inhibit tumor invasion.[88] In addition to gene regulation, other pathways have also been explored for exosome regulation. For example, calcitonin inhibits EV release and causes the accumulation of docetaxel and methotrexate within tumor cells, resulting in a significant reduction in tumor cell proliferation.[89,90] Ras enzyme, ESCRT, and hnRNP H1 all play a key role in exosome release. Manumycin A can inhibit these substances, thereby inhibiting exosomes secretion.[91–93]

Compounds associated with lipid metabolism include pantethine and GW4869. Pantethine affects the membrane lipid bilayer recombination process in MV formation by reducing lipid metabolism and total cholesterol levels, which inhibits the release of MV from the membrane.[94,95] For example, in the brain syndrome model of Plasmodium bergi Anka-infected mice, the disulfur group in the molecular structure of pantethine inhibits EV release from infected cells, thus preventing the occurrence of cerebral malaria.[96] GW4869 is a dihydroimidazolamide compound that affects lipid metabolism, and can be used as a non-competitive inhibitor of membrane lipid neutral sphingomyelinase (nSMase) to indirectly inhibit exosome release. In melanoma studies, B16BL6 melanoma cells treated with GW4869 showed significant growth inhibition compared to the control group, which may be related to the inhibition of GW4869 on exosome secretion, while also inhibiting the autosecretory regulatory proliferation of mouse B16BL6 cells.[97] Moreover, the drug has been shown to reverse ischemic preconditioning (IPC)-mediated heart damage, improve postoperative cognitive dysfunction caused by miR-182-5p in exosomes, and reduce the survival rate of pancreatic cancer (PC) cells.[87,98–102]

Block the Uptake of EVs Associated with Diseases

Inhibiting exosome uptake is a potential method of regulating exosome activity. Cells take up exosomes through pathways such as macrocytosis and phagocytosis.[103–105] Current studies have shown that treating exosomes with heparin and protease K can significantly reduce their uptake and internalization by cancer cells. This indicates that heparan sulfate (HS) proteoglycans and exosome surface proteins play a role in exosome uptake.[106–108] Furthermore, exosome uptake is related to clathrin and cavin endocytosis pathways, and knocking down dynamin2 or overexpressing a dominant-negative form of dynamin2 can inhibit exosome uptake.[104]

Physical Elimination of EVs Associated with Diseases

Another successful strategy for treating cancer is the physical elimination of exosomes that are secreted by cancer cells. As previously mentioned, cellular communication is mainly through the inclusion of exosomes and uptake by endocytosis, which is particularly significant in tumor cell communication.[109,110] Therefore, the use of a hemofiltration system that target cancer cell exosomes by specifically targeting the human epidermal growth factor receptor 2 (HER2) on their surface could play an important role in blocking communication between cancer cells and inhibiting cancer cell invasion.[111,112]

Even though the above studies have proven that the regulation of detrimental EVs can be used reliably as therapeutic methods, further studies on their effects on EV release in healthy cells are still needed (Table 1). Precise delivery of EV inhibitors to cancer cells is required to minimize the impact on normal tissue cells. Ultimately, the suitability of exosomes as therapeutic agents must be determined by the benefit-to-risk ratio.

EVs as Disease Marker in Diagnostic Application

Solid biopsy is considered the gold standard for pathological diagnosis. In clinical practice, it is necessary to perform solid biopsy to determine the histology and staging of tumor, as these pathological results serve as the basis for cancer

Table 1 Examples of Exosomes for Therapeutic Applications

Direct/indirect Therapeutic	Disease	Pathways	Effect	References
MSCs-drived EVs	Retinal ischemia	R28 retinal cells can endocytose MSC-EVs via the HSPG receptor-mediated pathway	Reduce neuroinflammation and promote retinal functional recovery	[81]
ESCs-drived EVs	Stroke	Activate the TGF-β/Smad pathway in CD4 T cells	Induced Treg amplification, regulating neuroinflammation, and promoting nerve recovery	[80]
MSCs-derived exosomes	IVDD	Inhibit ROS production and NLRP3 inflammasome activation	Reduce nucleus pulposus cell apoptosis	[82]
BMSCs-derived exosomes	OA	Downregulated inflammatory cytokines IL-1β, TNF-a, and IL-6 while promoting the expression of the anti-inflammatory cytokines IL-10 and TGF-1.	Reduced synovitis cell infiltration, decreased damage to articular cartilage, and delayed the progression of OA	[83]
BMSCs-derived exosomes	OA	miR-135b in BMSCs-Exo promoted M2 polarization of SMs through targeting MAPK6, thus improving cartilage damage.	Reduce OA-induced upregulation of pro-inflammatory factors in rat's serum and damage in cartilage tissues	[84]
UMSC-derived exosomes	Phosphorylation	Regulate miR-421 and increase the expression of FOXO3a	Inhibit phosphorylation and release of IL-1β and IL-18, and protecting the process of human parturition	[85]
CDCS-derived EVs	MI	Enhancing cardiomyocyte viability and preventing oxidative stress.	Improve cardiac function	[86]
Block EVs Associated with Diseases	**Disease**	**Pathways**	**Effect**	**References**
Calcitonin	PC	Inhibits EV release and causes the accumulation of docetaxel and methotrexate within tumor cells	Significant reduction in tumor cell proliferation	[89]
Manumycin A	PC and HCC	Inhibit Ras enzyme, ESCRT, and hnRNP H1	Inhibit EVs associated with diseases secretion	[91–93]
Pantethine	PbA	Affects the membrane lipid bilayer recombination process in MV formation by reducing lipid metabolism and total cholesterol levels	Prevent the occurrence of cerebral malaria	[96]
GW4869	Melanoma	Affects lipid metabolism, and can be used as a non-competitive inhibitor of membrane lipid nSMase to indirectly inhibit exosome release.	Inhibit the autosecretory regulatory proliferation of mouse B16BL6 cells	[97]
Block the Uptake of EVs Associated with Diseases	**Disease**	**Pathways**	**Effect**	**References**
Heparin and protease K	OC, GBM and melanoma	Reduce exosome uptake and internalization by cancer cells.	Inhibit tumor invasion	[106–108]
Knock down dynamin2 or overexpress a dominant-negative form of dynamin2	Leukemia	Exosome uptake is related to clathrin and cavin endocytosis pathways	Inhibit tumor invasion	[104]
Physical Elimination of EVs Associated with Diseases	**Disease**	**Pathways**	**Effect**	**References**
Physical elimination	BC	Target cancer cell exosomes by specifically targeting the HER2 on their surface	Block communication between cancer cells and inhibit cancer cell invasion	[111,112]

Abbreviations: MSCs, mesenchymal stem cells; EVs, extracellular vesicles; HSPG, heparin sulfate proteoglycans; ESCs, embryonic stem cells; Treg, regulatory T cells; IVDD, intervertebral disc degeneration; ROS, reactive oxygen species; BMSCs, bone marrow stem cells; OA, osteoarthritis; M2, macrophages 2; UMSCs, umbilical cord mesenchymal stem cells; CDCs, cardiosphere derived cells; MI, myocardial infarction; PC, prostate cancer; HCC, hepatocellular carcinoma; ESCRT, endosomal sorting complex required for transport; hnRNP H1, heterogeneous nuclear ribonucleoprotein H1; PbA, Plasmodium berghei strain ANKA; nSMase, neutral sphingomyelinase; OC, ovarian cancer; GBM, glioblastoma multiforme; BC, breast carcinoma; HER2, human epidermal growth factor receptor 2.

diagnosis and treatment.[113] Early detection of tumor markers is beneficial for early cancer diagnosis and plays an important role in the early detection and treatment, precision medicine, efficacy monitoring, and prognosis prediction. However, solid biopsies are invasive and pose a risk of bleeding and infection. Additionally, the small tissue samples obtained from solid biopsies have limited ability to effectively monitor dynamic tumor progression due to the heterogeneity of tumors both spatially and temporally. Morever, invasive detection methods may also increase the risk of metastasis and negatively impact survival and prognosis.[114,115] To address the limitations of solid biopsies, liquid biopsies are being promoted as a complement to solid biopsies. These minimally invasive liquid biopsies allow for real-time cancer diagnosis and monitoring, leading to early detection and reduced cancer mortality.[11] Exosomes, which are widely distributed and highly stable, are attractive as liquid biopsies that can reflect the overall status of tumors in real time.[11,116] Thus, the identification of highly effective liquid biopsy biomarkers holds promise for the non-invasive diagnosis of cancer. Currently, the diagnosis of central nervous system diseases, cancer, and cardiovascular diseases is a critical area of research and application for EVs.[117,118]

Protein

Specific proteins can be found in exosomes, and these specific proteins can be used as a basis for fluid diagnosis. Studies have shown that these proteins are valuable in detecting neuron-associated mutations in serum exosomes. For instance, research on exosomes in the plasma of patients with Parkinson's disease (PD) has shown that the concentrations of astrocytes and oligodendrocyte derived-exosomes, as well as apolipoprotein A1 in the serum of patients with early PD, are correlated with disease progression and consistent with the severity of the disease.[119,120] In addition, plasma exosome detection also plays a role in the identification of other diseases. HSP90 levels in SOD1G93A astrocytes and peripheral blood mononuclear cells were found to be lower in amyotrophic lateral sclerosis (ALS) mice than in other atrophic diseases, suggesting that ALS patients can be distinguished from spinal and bulbar muscular atrophy (SBMA) by measuring HSP90 in plasma EVs.[121] These exosomes also contain specific proteins that can be used in the diagnosis of tumors. For instance, exosomes contain lectin galactoside-binding protein (LGALS3BP), which contributes to the proliferation and migration of endometrial cancer (EC) cells by activating the PI3K/AKT/VEGFA signaling pathway in vitro and in vivo. Therefore, elevated exosome levels, including LGALS3BP, provide a new perspective for the diagnosis of EC.[122]

Different types of RNA play an important role in the development of diseases. When these RNAs are contained in exosomes and secreted into body fluids, they can serve as diagnostic tools for disease detection.

MiRNA

The miRNAs contained in exosomes can serve as biomarkers for diagnosis and prognosis, providing unique insights and a more dynamic perspective on the progression and therapeutic response of various diseases. For example, in the prediction of heart failure, significant changes in the exosome contents of miR-425, miR-744, and miR-92b-5p were detected earlier than conventional biomarkers, suggesting that monitoring exosomal contents may enable earlier detection of heart failure. Moreover, the expression level of these miRNAs increases with the severity of the disease.[123–125] Currently, several studies have found associations between exosomal miRNAs and cardiovascular diseases. For instance, exosome-miR-21-3p, exosome-miR-132, and exosome-miRNA-200 are associated with cardiac hypertrophy, while exosome-miR-106A and miR-24 are associated with aortic aneurysm. In acute myocardial infarction (AMI) patients, the plasma levels of miR-122-5p, miR-17-5p, miR-126-5p, and miR-145-3p were significantly increased.[126–134] These findings suggest that the discovery of miRNA in exosomes has the potential to contribute to the early diagnosis of cardiovascular diseases. In the diagnosis of cancer, exosomal miRNAs have been found to be valuable biomarkers for early detection. Low alpha-fetoprotein HCC enters the extracellular environment through the release of miR-21-5p by exosomes to participate in the tumorgenesis process, making the exosome miR-21-5p a biomarker for early diagnosis.[135] Exosomes containing miR-145, miR-155, and miR-382 were overexpressed in breast cancer cells, while the expression of miR-148a was down-regulated.[136,137] These specific miRNAs have the potential to enable the early diagnosis of breast cancer. In the diagnostic application of metabolic diseases, acromegaly is an endocrine and metabolic disease caused by growth hormone secreting pituitary adenoma (GHPA). Studies have found that miR-21-5p contained in exosomes secreted by GHPA activates osteoblasts through the GH/IGF1 pathway and leads to osteogenesis, suggesting that exosome-derived miR-21-5p may be a candidate biomarker for acromegaly.[138,139]

CircRNA

CircRNA, a recently discovered non-coding RNA, has gained attention for its potential as a reliable diagnostic marker. CircRNA is highly stable and specific, and is not easily affected by the external environment, particularly when present in exosomes.[140] In a study comparing the circRNA profiles of breast cancer patients and benign patients, significant differences in expression patterns were found between the cancer patients and the control group. Nine specific circRNAs (circ_0002190, circ_0007177, circ_0000642, circ_0001439, circ_0001417, circ_0005552, circ_0001073, circ_0000267, and circ_0006404) were identified as potential circRNA markers for breast cancer.[141] In a study of plasma exosomes in patients with HCC, circ_0051443 was found to mediate the upregulation of Bri1-associated kinase 1 (BAK1) by competing with miR-331-3p, promoting apoptosis, and preventing the inhibition of HCC proliferation. The level of circ_0051443 in plasma exosomes of HCC patients was significantly lower than that in healthy individuals.[142,143] Similarly, in the early diagnosis of gastric cancer, exosome circNRIP1 has been found to promote the proliferation, migration, and invasion abilities of gastric cancer cells in vivo through the AKT1/mTOR pathway and can be detected early by liquid detection.[144] Furthermore, highly expressed circGAPVD1 in plasma-derived exosomes of colorectal cancer (CRC) patients may become a novel diagnostic biomarker for CRC.[145] These findings suggest that circRNA in exosomes holds great potential as a diagnostic marker for various diseases, and further research is needed to explore its clinical applications.

lncRNA

Lately, lncRNAs have gained recognition for their potential as effective molecular markers for early cancer detection and as effective therapeutic targets for early cancer treatment. By separating exosomes from patients with early gastric cancer and healthy individuals, researchers have screened characteristic exosome lncRNAs as potential biomarkers for early gastric cancer through performing exosome lncRNA sequencing and diagnostic ability analysis.[146] Exosome lncRNAs also play a role in the diagnosis of diabetic retinopathy (DR). According to research, exosome lncRNAs DLX6-AS1 and PRINS have a high diagnostic value in evaluating DR in the general population. The expression of exosome lncRNA PRINS is also involved in the prediction and diagnosis of female DR as a unique biomarker.[147] In an atrial fibrillation (AF) diagnosis study, the authors analyzed the serum exosome lncRNAs of AF patients and the normal population and discovered that lncRNA LOC107986997 in serum exosomes was closely correlated with AF expression, potentially making it a sensitive and specific diagnostic biomarker for AF.[148] Furthermore, lncRNA-Linc00662, lncRNA-CHASERR, and lncRNA-PCA3 were significantly elevated in the extracellular vesicles isolated from the urine of prostate cancer (PC) patients.[149] Plasma and urinary EVs as a potential sources of RNA biomarkers for prostate cancer in liquid biopsies.

tRNA

Exosomes tRNA is a regulatory factor involved in cell proliferation and differentiation that is specifically expressed in various diseases, including breast cancer, liver cancer, and the thymus, making tRNA a potentially effective biomarker. RNA sequencing technology has been used to analyze differences in the levels of tRNA-derived small RNAs (tRFs) in exosomes. The levels of four tRNAs (tRNA-ValTAC-3, tRNA-GlyTCC-5, tRNA-ValAAC-5, and tRNA-GluCTC-5) were significantly increased in the plasma exosomes of patients with liver cancer.[150] However, the levels of tRF-Leu-TAA -005, tRF-Asn-GTT-010, tRF-Ala-AGC-036, tRF-Lys-CTT-049 and tRF-Trp-CCA-057 were significantly downregulated in patients with non-small cell lung cancer (NSCLC).[151] Moreover, the levels of tRF0-Ile-AAT-01 and tiRNA0-Lys-CTT -01 contained in urinary exosomes of lupus nephritis (LN) patients were upregulated, which could be used to distinguish systemic lupus erythematosus (SLE) patients with or without LN. The levels of tRF3-Ile-AAT-1 and tiRNA5-Lys-CTT-1 derived from urinary exosomes were higher in SLE patients with mild and moderate-to-severe activity.[152] These studies highlight the potential of tRFs in plasma exosomes as a novel molecular marker for tumor and LN diagnosis.[150,152]

Exosomes, which are associated with cancer invasion and malignancy, are commonly used in liquid biopsies as highly potential markers for cancer diagnosis. However, exosome analysis requires complicated pretreatment and is challenging to perform quickly. If a method with high sensitivity and simple detection of exosomes in body fluids can be developed, it could be an effective disease detection method. A newly developed nanomembrane system, iTEARS, can rapidly obtain high yield and purity exosomes from tears, allowing clinicians to diagnose diseases and disorders more quickly

and effectively.[153] The new system, iTEARS, separates exosomes in just five minutes by using an oscillating pressure flow to filter a small amounts of tear across a nanoporous membrane to reduce clogging. Proteins from exosomes are labeled with fluorescent probes, which are then transferred to other instruments for further analysis. Based on the proteomic analysis of extracted proteins, researchers were able to distinguish between healthy people and patients with dry eye disease. Similarly, they analyzed miRNAs in tear exosomes in patients with and without DR. The analysis results showed that miR-145-5p, miR-214-3p, miR-218-5p, and miR-9-5p were disordered in the development of DR, which verified that iTEARS can also help track the progress of the disease. Similarly, other researchers have developed a multifunctional system, MORPH (mechanical metamaterial operating at a critical point for hyper-responsive analysis), that leverages the advanced behavior of mechanical metamaterials for nanoscale molecular analysis of EVs in the ascites of cancer patients. This system uses a mechanical metamaterial based on a double-response hydrogel as a shape-shifting chiral interferometer. The researchers used amplitude and kinetic analyses of MORPH to perform molecular analyses of entire exosomes, characterizing the biomarker composition of these nanoscale vesicles. This system enables sensitive quantification and differentiation of vesicle mixtures with different biomarker distributions.[154]

In conclusion, exosomes and their components are promising biological markers for the diagnosis of a variety of diseases, opening up a new avenue for early screening, diagnosis, and treatment of diseases, particularly in cases where tissue samples are not available (Table 2). However, the road to biomarker validation has not been smooth, and there is a need for improved liquid biopsy tools for cancer detection and monitoring. In the process of conducting research on

Table 2 Examples of Exosomal Contents for Clinical Diagnostic Applications

Protein(s)	**Disease**	**Body Fluid**	**Correlation**	**References**
ApoA1	PD	Blood serum	Up-occurrence	[119]
HSP90	ALS	Blood plasma	Up-occurrence	[121]
LGALS3BP	EC	Blood plasma	Up-occurrence	[122]
miRNA(s)	**Disease**	**Body Fluid**	**Correlation**	**References**
miR-425, miR-744, and miR-92b-5p	HF	Blood serum	miR-425, miR-744, miR-92b-5p-up-occurrence	[123,125]
miR-21-3p, miR-132, and miR-200	MH	Blood serum	Up-occurrence	[128,130]
miR-24, miR-106a	AAA	Blood serum	miR-106a-up-occurrence, miR-24-up-remission	[131,132]
miR-122-5p, miR-17-5p, miR-126-5p, and miR-145-3p	AMI	Blood serum	Up-occurrence	[133,134]
miR-21-5p	Low-AFP HCC	Blood plasma	Up-occurrence	[135]
miR-145, miR-155, miR-382, and miR-148a	BC	Blood serum	miR-145, miR-155, miR-382-up-occurrence, miR-148a-down–occurrence	[136,137]
miR-21-5p	Acromegaly	Blood serum	Up-occurrence	[139]
CircRNA(s)	**Disease**	**Body Fluid**	**Correlation**	**References**
Circ_0051443	HCC	Blood plasma	Down-occurrence	[143]
Circ_NRIP1	GC	Blood plasma	Up-occurrence	[144]
Circ_GAPVD1	CRC	Blood plasma	Up-occurrence	[145]
lncRNA(s)	**Disease**	**Body fluid**	**Correlation**	**References**
lncRNA-DLX6-AS1 and lncRNA-PRINS	DR	Blood plasma	Up-occurrence	[147]
lncRNA-LOC107986997	AF	Blood serum	Up-occurrence	[148]
lncRNA-Linc00662, lncRNA -CHASERR and lncRNA-PCA3	PC	Urine	Up-occurrence	[149]

(*Continued*)

Table 2 (Continued).

tRNA(s)	Disease	Body Fluid	Correlation	References
tRNA-ValTAC-3, tRNA-GlyTCC-5, tRNA-ValAAC-5, and tRNA-GluCTC-5	HCC	Blood plasma	Up-occurrence	[150]
tRF-Leu-TAA-005, tRF-Asn-GTT-010, tRF-Ala-AGC-036, tRF-Lys-CTT-049, and tRF-Trp-CCA-057	NSCLC	Blood plasma	Down-occurrence	[151]
tRF3-Ile-AAT-1 and tiRNA5-Lys-CTT-1	LN	Urine	Up-occurrence	[152]

Abbreviations: miRNA, microRNA; CircRNAs, circular RNAs; lncRNAs, long noncoding RNAs; tRNAs, transfer RNAs; ApoA1, apolipoprotein A1; PD, parkinson's disease; HSP90, heat shock protein 90; ALS, amyotrophic lateral sclerosis; LGALS3BP, lectin galactoside-binding soluble 3 binding protein; EC, endometrial cancer; HF, heart failure; MH, myocardial hypertrophy; AAA, abdominal aortic aneurysm; AMI, acute myocardial infarction; Low-AFP HCC, low-alpha fetoprotein hepatocellular carcinoma; BC, breast cancer; GC, gastric carcinoma; CRC, colorectal cancer; DR, diabetic retinopathy; AF, atrial fibrillation; PC, prostate cancer; NSCLC, non-small cell lung cancer; LN, lupus nephritis.

exosomes, a series of steps such as extraction, purification, identification, staining, and sequencing are generally required. The amount of exosomes extracted and the purity level are critical to the success of the experiment. More evidence is required to support the transition from the research phase to clinical application. Nonetheless, the potential of exosomes as biomarkers should not be ignored.

Engineered EVs in Targeted Therapy and Drug Delivery

Exosomes have garnered widespread attention due to their exceptional biocompatibility, safety, stability, and ability to cross the blood-brain barrier, particularly as drug carriers.[155–157] Moreover, exosomes can be modified to reach the target cell effectively, encapsulating the therapeutic components within the exosome and facilitating their uptake by the cell to play a therapeutic regulatory role. However, it is essential to ensure that the methods used to modify exosomes do not significantly alter their structure and function.

As described above, EVs are cell-derived lipid membrane structures that transport various active biomolecules between cells, thereby altering the physiology of recipient cells.[158] The uptake of EVs by receptor cells is partially selective, and the specific proteins, lipids, glycans, and overall negative charge can affect their targeting to specific organs and ensure minimal non-specific interactions. This becomes the basis for targeted drug delivery.[23,25,159–162] The superior tissue-targeting ability of exosomes has been identified in studies of parent and target cells. For example, during T-cell immunosynapse, microRNA-loaded exosomes are unidirectionally transferred from T cells to antigen presenting cells.[163] Another important reason for using EVs as drug delivery vehicles is their low response to the immune system during delivery, as demonstrated by the low toxicity and low immune response observed in vivo trials of EV therapeutics.

Engineered EVs: Drug Delivery

Exosomes can carry various therapeutic drugs, such as small molecule chemicals, proteins, and nucleic acids. By encapsulating small molecule chemical drugs in exosomes, the limitations of low solubility, high toxicity, and poor specificity can be avoided. This approach enables the drugs to easily enter the tumor and have a therapeutic effect. For instance, erastin is added to exosomes derived from human lung fibroblasts-1, which were labeled with folate. These exosomes are transported to folate receptor-rich MDA-MB-231 cells in triple-negative breast cancer for targeted antitumor effects, mitigating the nephrotoxic effects of erastin.[164] Macrophage-derived exosomes loaded with the chemotherapeutic drug doxorubicin have also been used to treat triple-negative breast cancer. This loading approach can avoid the disadvantages of doxorubicin's high clearance rate and high toxicity in vivo, providing a new way for hydrophobic chemotherapy drug delivery that targets tumor cells more accurately.[165] In the study about ischemic stroke treatment, the researchers prepared an engineered exosome loaded with brain-derived neurotrophic factor (BDNF) from human neural stem cells (HNSCs). The results showed that BDNF-hNSC-Exo engineered exosomes led to a reduction in infarct volume and improvement in neurological function.[166] In addition, a study found that foreign exosomes can effectively deliver the NF-kB inhibitor (SR-IκBα) to the fetus in a mouse model, slowing fetal immune cell migration and delaying preterm birth. This method of loading drugs into exosomes can achieve sustained efficacy and prolong pregnancy, significantly improving fetal viability.[167]

Engineered EVs: Internal Modification

For naturally isolated exosomes, EVs can be further engineered to enhance their stability and biological activity, as well as their binding ability to target cells. At present, there are two methods of modifying exosomes: internal and surface modification. In internal modification studies, many researchers have explored the active binding mechanism of exosomes after separation and purification. Active binding involves temporarily disrupting the cell membrane to allow drug entry, followed by recovery of the exosome membrane after drug diffusion.[168–172] Transfection is the most widely used method for introducing RNA into exosomes. This is achieved by incubating exosomes with drugs and surfactants that form membrane pores, enabling transfection of RNA. Transfection has a good loading efficiency and molecular stability. For example, transfection techniques have been used to introduce head and neck squamous cell carcinoma-related regulatory miRNAs into EVs, leading to observed inhibition or promotion of tumor growth after targeted binding of the engineered exosomes to tumor cells.[173] However, transfection changes the genetic material contained in EVs, which may impair the ability of EVs to deliver stably expressed siRNA and even raise potential biosafety problems.[174,175] Electroporation, on the other hand, uses electric fields to create temporary hydrophilic pores in the phospholipid membrane of exosomes and load hydrophilic biomaterials. This technology is widely applicable and has been used to successfully deliver Adriamycin (for increased targeting) and doxorubicin (an antitumor agent) into MSC-EXO to TUBO breast cancer cell lines, reducing tumor growth rates, while free Adriamycin and non-targeted doxorubicin exosomes had no effect.[176] In the study of liver cancer cells, the migration and proliferation of cancer cells were significantly inhibited after the miR-26a-loaded exosomes enter cancer cells through the endocytic pathway.[177] However, electroporation may lead to morphological changes and aggregation in exosomes, which is not conducive to the storage and stability of EVs in vivo.[178–180]

Engineered EVs: Parent Cell Surface Modification

The biological distribution of exosomes, their ability to target specific cells, and their therapeutic potential are critical considerations. Therefore, modifying the surface of exosome can achieve the desired properties and improve their ability to target cells. This can be accomplished through the genetic engineering of the parental cells. Parent cells are genetically modified by inserting the required ligands, which then secrete exosomes with the inserted properties on their surfaces. In the field of immunotherapy, cytokine release syndrome induced by chimeric antigen receptor (CAR) T cell therapy is is a significant concern.[181,182] In preclinical models of cytokine release syndrome, the non-expression of the PD-1 protein in CAR-containing exosomes prevents the influence of PD-L1 on the tumor cell membrane surface. These exosomes from CAR T cells were found to express high levels of cytotoxic molecules without causing cytokine release syndrome, suggesting that they may be useful in future therapies targeting tumors.[181]

Engineered EVs: EVs Surface Modification

Similarly, in order to achieve more specific delivery to target cells, isolated exosomes can be also directly modified to achieve more selective targeting of target tissues or cells by mixing specific ligands on their surfaces.[183] For instance, EVs secreted by adipose-derived mesenchymal stem cells can rebind with fluorinated peptide dendritic polymer (FPG3) to form exo@FPG3. Results showed that surface modification of the EVS with FPG3 improved the cellular uptake and biological activity of the extracellular vesicles. Exo@FPG3 significantly enhanced the in vitro angiogenesis and migration of human umbilical vein endothelial cells (HUVECs).[184] In other studies, a HER2-targeting peptide was fused to the N-terminus of Lamp2 on the exosome surface, along with the green fluorescent protein (GFP). This engineered exosome delivers 5-FU and miR-21i to HCT-116 with greater accuracy, enabling treatment of colorectal cancer while also tracking drug uptake and metabolism.[185–187] Synthetic multivalent antibodies retargeted exosomes/SMART-Exos have also been designed. By adding antibodies to the T cell membrane surface protein CD3 and EGFR antibodies to the breast cancer cell membrane proteins to exosomes, SMART-Exos were able to specifically enhance the immune response of T cells to breast cancer cells and inhibit tumor growth.[188] External regulation can also enhance the targeting effect. A study showed that after intravenous injection of IOPN-containing MSC-EXO, an external magnetic field controlled the biological distribution of iron oxide nanoparticles (IOPN) in the body, allowing the nanoparticles to

navigate to the target area and increasing the concentration at the location site by 5.1 times.[189] A Phase 1 trial of Exo-ASO-STAT6 is underway, bringing us closer to practical clinical trials. Exo-ASO-STAT6 is a novel engineered exosome drug candidate loaded with antisense oligonucleotides (ASO) for the STAT6 (signal transducer and activator of transcription 6) transcription factor. It can selectively target tumor-associated macrophages and precisely interfere with STAT6 signaling, reprogramming TAMs into a pro-inflammatory M1 phenotype for anti-tumor immunity.[190] To increase the accumulation of drugs in the lesion site of the target disease and avoid rapid elimination of EVs, EVs were coated with polyethylene glycol, expressed with CD47, or combined with albumin binding domains (ABDs). These engineering modifications prolonged the presence of EVs in vivo and improved drug distribution.[191–197]

In general, engineered exosomes are designed to retain their inherent characteristics, such as strong stability, minimal immune response, robust targeting ability, and excellent barrier penetration ability. By loading additional designed molecules, engineered exosomes can become more ideal vectors for genetic material and drug delivery (Table 3). However, as research on engineered exosomes continues to evolve, some scholars have expressed concerns. Compared

Table 3 Engineered Exosome-Based Drug Delivery Systems

Engineer Method (Drug Delivery)	**Disease**	**Exosome Source**	**Character**	**References**
Erastin-loaded exosomes labeled with FA to target TNBC cells with overexpression of FA receptors	TNBC	HFL-1	Actively and selectively target MDA-MB-231 cells and efficiently induce ferroptosis in tumor cells	[161]
Exosomes loaded with DOX	TNBC	Macrophage cell	Avoids the disadvantages of easy clearance and high toxicity in vivo and targets tumor cells more precisely	[162]
Exosomes loaded with BDNF	Ischemic stroke	HNSC	BDNF-hNSC-Exo resulted in reduced infarct volume and improved neurological function	[163]
Exosomes have been bioengineered to carry the inhibitor of NF-κB	Premature birth	HEK293T cells	Slow the migration of fetal immune cells and delayed preterm birth	[164]
Engineer Method (EVs Internal Modification)	**Disease**	**Exosome Source**	**Character**	**References**
HNSCC is associated with regulation of miRNA transfection into Evs	HNSCC	WSU-HN6 cells	Transfection of different miRNAs regulates the promotion and promotion of tumor growth	[170]
DOX successfully loaded into exosomes by electroporation	TUBO breast cancer cell line	Mesenchymal stem cells	The type of exosome reduced the tumor growth rate compared to free DOX and non-targeted DOX loaded exosomes	[173]
The exosomes were loaded with a batch of fluorescently labeled miR-26a by electroporation	LC	Human 293T cells	Engineered exosomes containing miR-2a are effectively endocytosed by HepG2 cells. Subsequently, up-regulated miR-2a in HepG2 cytoplasm inhibits the migration and proliferation rate of cancer cells	[174]
Engineered M (Parent Cell Surface Modification)	**Disease**	**Exosome Source**	**Character**	**References**
The CAR is loaded onto the surface of the T cell	BC	CAR-T cells	Exosomes expressed by CAR T cells showed a good antitumor effect, and the exosomes containing CAR did not express PD-1 protein to avoid the influence of PD-L1 on tumor cell membrane	[178]
Engineered Method (EVs Surface Modification)	**Disease**	**Exosome Source**	**Character**	**References**
EVs secreted by adipose-derived mesenchymal stem cells can rebind with FPG3 to form exo@FPG3	Vascular injury	ADSC	Achieve HUVECs specific targeting	[181]

(*Continued*)

Table 3 (Continued).

Her2-targeting peptides were fused to the N terminus of LAMP2 on the exosome surface and fused with green fluorescent GFP	RC	THLG-293T or LG-293T cells	More accurate delivery of 5-FU and miR-21i to CRC cell HCT-116 to achieve the purpose of treating CRC and tracking drug uptake and metabolism	[184]
Antibodies to T cell membrane surface protein CD3 and EGFR antibodies to breast cancer cell membrane protein were added to exosomess	BC	Expi293F cell	SMART-Exos can specifically enhance the immune response of T cells to BC cells and inhibit tumor growth	[185]
IOPN are loaded onto the exosome surface	AIS	SMCs	Exosomes can navigate to the target area under the guidance of the external magnetic field, which increases the concentration of the location by 5.1 times	[186]
Exosomes surface loaded with ASO targeting STAT6 transcription factors	TAMs	HEK	TAMs were reprogrammed into a proinflammatory M1 phenotype to play an anti-tumor role	[187]
EVs surfaces were loaded with polyethylene glycol, expressed with CD47, or combined with albumin binding domains	The clearance rate of EVs in vivo is higher	HEK-293T, N2A, or CDCs	Prolong its circulation time in the body	[190,192,193]

Abbreviations: FA, folate; TNBC, triple negative breast cancer; HFL-1, human lung fibroblasts-1; DOX, doxorubicin; BDNF, brain-derived neurotrophic factor; HNSCs, human neural stem cells; BBB, blood-brain barrier; NF-κB, nuclear factor kappa-B; HNSCC, head and neck squamous cell carcinoma; LC, liver cancer; CAR, chimeric antigen receptor; PD-1, programmed death-1; PD-L1, programmed cell death 1 ligand 1; BC, breast cancer; FPG3, fluorinated peptide dendrimers; ADSC, adipose-derived mesenchymal stem cells; HUVECs, human umbilical vein endothelial cells; GFP, green fluorescence protein; RC, rectal cancer; 5-FU, 5-fluorouracil; CRC, colorectal cancer; EGFR, epidermal growth factor receptor; IOPN, iron oxide nanoparticles; AIS, acute ischemic stroke; ASO, antisense oligonucleotides; STAT6, signal transducer and activator of transcription 6; TAMs, tumor-associated macrophages; HEK, human embryonic kidney; M1, macrophage1; CD, cluster of differentiation; N2A, Neuro2A; CDCs, cardiosphere-derived cells.

to the manufacturing processes for protein and synthetic drug delivery systems, small changes in the production of EVs can have significant effects on product quality and activity. Due to their small size and complex composition and structure, detecting and evaluating changes in EV product quality is more challenging. Furthermore, processes for EV manufacturing are considered less stable than those for cell and antibody production.[198]

To improve the controllability of exosome production, comprehensive characterization, product analysis, and quality control of EVs are needed.[27,199] Comprehensive characterization of EVs includes characterization of exosome proteomes, which can be used to identify amplified signaling pathways indicating cancer, as well as observation of tumor stromal responses and responses to cancer therapies.[200] To characterize EVs, NP-TRFIA techniques use transmembrane proteins and biotinylated antibodies to capture EVs directly from urine and cellular supernatants for detection and characterization. This technique has several advantages, including a simple separation procedure, high signal-to-noise ratio, identification and evaluation of tumor-related proteins on the surface of EVs, and the potential for comprehensive and rapid characterization of exosomes.[201] Evaluating the efficacy and safety of EVs requires determining their characteristics and purity, and measures must be taken to ensure that the final product meets previously identified key quality attributes. This poses significant analytical challenges, such as measurements of size and concentration, exclusion of contaminants, identification of functional markers, and evaluation of drug loads to assess therapeutic activity of each vesicle. Among the separation analysis methods, the size exclusion chromatography (SEC) technique may be the best method to isolate exosomes from proteins. SEC has the characteristics of high purity and high efficiency, and can improve plasma protein contamination to achieve the formation and function of complete exosomes from plasma.[202,203] It can also be used in combination with other techniques to increase exosome purity.[204] Dual mode chromatography (DMC) has been successfully used to reduce contamination of lipoprotein particles (LPP) in plasma exotics.[205] However, SEC techniques cannot distinguish between exosomes and MVs of the same size, and cannot avoid biological target degeneration.[206] Therefore, SEC combined with ultrafiltration may be the best separation method.[207–209] Although SEC is currently an excellent candidate for exosome isolation, more direct comparative studies with other separation methods are needed as evidence.[209] In addition to improving exosome separation technique, a new exosome production technique called the exosome analog technique has been developed.[210,211] This exosome-mimetics (EMs) technology mainly produces vesicles that retain the main characteristics of exosomes and eliminates the chemical process required to attach the targeted ligand to the surface of vesicles. It has the characteristics of low production time and high yield, and it is also a potential research direction to promote exosomes for practical clinical applications in the future.[212–214]

Despite these potentially worrisome factors, most animal studies conducted so far have shown positive results for EVs, making them a promising component for future cell-free therapies. However, before these therapies can be widely used, individual EV agents must be assessed for factors such as immunogenicity, biocompatibility, liposomal carrier, and biological process. To improve the experimental design, the research should be based on more complex in vitro model, such as three-dimensional cell tissue model. Researchers should also more adequately evaluate exosome organ distribution and content expression in animal models, and conduct studies on repeated administration under various regimens for further clinical examination.[215,216] A clinically oriented approach to using EVs involves transferring patient cells into culture and isolating vesicles for re-administration to the patient. However, there are still issues that need to be addressed, especially for acute diseases, such as infections or cardiovascular events.[217] The use of autologous EVs is feasible in cases where genetic compatibility needs to be maintained or where autogenous EVs are readily available.[218,219] Nonetheless, most current applications prefer non-autologous EVs, mainly due to their well-established safety profiles and rigorous validation. For example, current MSC-EVs for regenerative medicine and EVs from dendritic cells for vaccine delivery are undergoing validation, and these vesicles have shown favorable safety profiles in several Phase I clinical trials.[220,221]

Conclusions and Prospects

Because of its diverse and pleiotropic effects on physiology and pathology, EVs have been increasingly studied as intermediates of intercellular communication. To date, EVs have been successfully used in various preclinical studies for disease treatment, engineering, and disease diagnosis (Figure 2). However, it is critical to review and summarize the these results and ongoing challenges.[222,223] Although current studies showing impressive results, the complex process of EV sample analysis still needs to be standardized for robust and repeatable results across laboratories. Further work is needed to be done before these studies have practical clinical applications, including controlling costs of EVs, improving isolation methods, comprehensively characterizing EVs, and exploring the underlying pathophysiological mechanisms to avoid any uncontrolled harm to patients. Despite the promising results in clinical trials of EVs, which show good efficacy and safety, caution is still required due to the complexity of EVs. The prospects for the clinical application of exosomes are broad, and we believe that the current research and applications are just the tip of the iceberg. With continued research, the challenges described in this paper will be addressed, eventually leading to the practical application of EVs in the treatment and diagnosis of diseases, bringing tangible benefits to patients.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; All authors took part in drafting, revising or critically reviewing the article; All authors gave final approval of the version to be published; All authors have agreed on the journal to which the article has been submitted; and all authors agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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