

Advances in Exosome-Based Drug Delivery and Tumor Targeting: From Tissue Distribution to Intracellular Fate

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Juntang Shao^{1,2}
Jennica Zaro³
Yuxian Shen^{1,2} 

¹Department of Pharmacology, Anhui Medical University School of Basic Medicine, Hefei 230032, People's Republic of China; ²Biopharmaceutical Research Institute, Anhui Medical University, Hefei, People's Republic of China; ³Department of Pharmacology and Pharmaceutical Sciences, University of Southern California School of Pharmacy, Los Angeles, CA 90089-9121, USA

Abstract: Exosomes or small extracellular vesicles are considered a new generation of bioinspired-nanoscale drug delivery system (DDS). Endogenous exosomes function as signalosomes since they convey signals via ligands or adhesion molecules located on the exosomal membrane, or packaged inside the exosome. Recently, exosome membrane modification, therapeutic payloads encapsulation, and modulation of in vivo disposition of exosomes have been extensively investigated, among which significant advances have been made to optimize exosome-mediated delivery to solid tumors. Exosomes, specifically tumor cell-derived exosomes, are presumed to have tumor-preferential delivery due to the homotypic features. However, quality attributes that dictate the tissue distribution, cell type-selective uptake, and intracellular payload release of the administered exosomes, as well as the spatiotemporal information regarding exosome fate in vivo, remain to be further investigated. This review summarizes recent advances in developing exosomes as drug delivery platforms with a focus on tumor targeting. The pharmacokinetic features of naive exosomes and factors influencing their intracellular fate are summarized. Recent strategies to improve tumor targeting of exosomes are also reviewed in the context of the biological features of tumor and tumor microenvironment (TME). Selected approaches to augment tumor tissue deposition of exosomes, as well as methods to enhance intracellular payload delivery, are summarized with emphasis on the underlying mechanisms (eg, passive or active targeting, endosomal escape, etc.). In conclusion, this review highlights recently reported tumor-targeting strategies of exosome-based drug delivery, and it's in the hope that multiple approaches might be employed in a synergistic combination in the development of exosome-based cancer therapy.

Keywords: exosome, tumor-targeting, PK, drug delivery, nanovesicles

Introduction

Extracellular vesicles (EVs) are lipid bilayer-enclosed membranous structures secreted by cells under physiological conditions or following biological cues.¹ EVs comprise heterogeneous populations with various sizes and subcellular origin of membranes. Since a consensus regarding specific markers of EV subtypes has not emerged, the term “exosome” in this review refers to small EVs with a size range of 40–160 nm with putative membrane origin from endosomal pathways.² Exosomes are initially formed as intraluminal vesicles (ILV) in late endosomes through inward invagination. Subsequently, ILV form multivesicular bodies (MVB) through endosomal-sorting complexes required for transport (ESCRT)-dependent or

Correspondence: Yuxian Shen; Juntang Shao
Department of Pharmacology, Anhui Medical University School of Basic Medicine, 81 Meishan Road, Hefei 230032, People's Republic of China
Tel +86 65161133
Email shenyx@ahmu.edu.cn; shaojuntang@ahmu.edu.cn

ESCRT-independent processes.³ The term “exosome” was coined in the 1980s in the first report that exosomes were secreted upon MVB fusion with the plasma membrane during reticulocyte maturation.⁴ Since then, the physiological functions of exosomes as well as their roles in disease progression have been appreciated.⁵ Exosomes permit contact-free substance exchange between cells and act as signaling vesicles in autocrine, endocrine, juxtacrine, paracrine, or distal modes of communication.⁶ It has been shown that exosomes are involved in various biological processes including viral infection, immune responses, mammalian development, and reproduction.⁷ Interestingly, exosomes may exert opposite functions depending on the producer cells and the biological context. For example, leukocyte-derived exosomes containing IFN α limit virus replication, whereas exosomes released by infected cells promote infection through exosome-mediated viral transfer.⁸ The purpose of physiological secretion of exosomes and the mechanisms that compel their release remain largely unknown and require further in-depth investigation. On the other hand, research interests have extended to the value of exosomes in the diagnosis of cancer, degenerative and metabolic diseases, which are evaluated by many ongoing clinical investigations.⁹

Exosomes are also characterized as signalosomes since they convey signals via ligands or adhesion molecules expressed on the exosomal membrane, or packaged as cargo inside the exosome.¹⁰ Therefore, exosomes mediate signal transduction either by membranous interactions with the plasma membrane of recipient cells, or by payload release following cell uptake of exosomes.¹¹ This exosome-mediated communication has several unique features. First, exosomal membrane derived from MVB contains combinations of ligands that can simultaneously engage with multiple cell-surface receptors, providing a network of information in a way that highly simulates direct cell–cell contact.¹² Second, binding of exosomes to recipient cells results in ‘new’ cell membrane bearing surface molecules from donor cells. Therefore, targeted cells acquire new properties of adhesion or cell recruitment.¹³ Third, exosomes provide an excellent platform for intracellular delivery of genes or proteins to reprogram targeted cells. For example, mesenchymal stem cell (MSC)-derived exosomes loaded with an anti-microRNA oligonucleotide can effectively silence the targeted microRNA in glioblastoma multiforme cells.¹⁴ By taking advantages of those aforementioned features, exosomes open up opportunities for the

development of safer and more effective drug delivery tools. Compared with liposomes or other synthetic polymer-based drug carriers, exosomes contain transmembrane and membrane-anchored proteins that enhance endocytosis and therefore, promote payload delivery.¹⁵ Another advantage of exosomes is due to their bio-originated membrane, which is inert to protein corona formation.¹⁶ Moreover, there is ample flexibility in exosomal surface modification by genetic manipulation of donor cells to accommodate multiplexed purposes. For instance, exosomes derived from CD47-overexpressed human foreskin fibroblasts have been shown to decrease phagocytosis-mediated clearance by monocytes and macrophages, as well as enhance cell uptake by cancer cells.¹⁷

This review summarizes recent advances in developing exosomes as drug delivery platforms with a focus on tumor targeting. Properties influencing PK of naive exosomes, as well as modifications to overcome tissue and cellular barriers for tumor targeting, are reviewed and summarized. In this review, modifications of exosomes to augment the enhanced permeability and retention (EPR) effect is reviewed in the passive targeting part. Strategies involve utilizing affinity ligands or targeting moieties on the surface of exosomes for tumor microenvironment (TME) normalization, increased retention at the tumor site, and uptake by the cancer cells are discussed in the active targeting section. The ligands are selected to bind to overexpressed proteins on cancer cell surfaces or to target specific cell populations in TME. The tissue-selective homing property of exosomes is influenced by the producing cells. Therefore, the other classification of active or passive targeting exosomes is according to whether the modification is by alteration of exosome-producing cells’ transcriptome.¹⁸ As for exosome biogenesis, content, and functions under physiological or pathological conditions, readers are referred to excellent reviews for detailed information.^{19–21} In addition, technologies in cargo loading, surface modification, and up-scale manufacture of exosome have been comprehensively reviewed in previous publications.^{22–25}

Exosomes and Exosome-Inspired Drug Delivery

Great efforts have been invested to optimize exosomal membrane properties and to improve the manufacturing process of exosomes or exosome mimetics. Here we categorized exosome-based drug delivery tools into three

subgroups according to the degree of human manipulation, ie, how “natural” they are compared with exosomes released from cells (Table 1). The most well-studied exosomes are isolated from cell culture conditioned medium. Common protein components found in exosomes include cytoskeletal (eg, actin), cytosolic (eg, GAPDH), heat shock (eg, HSP90), antigen presentation (MHC-I, -II), and membrane proteins (eg, CD9, CD63), as well as proteins involved in vesicle trafficking (eg, Tsg101).³ In addition, exosomes derived from different cell types may exhibit unique protein profiles, which are associated with intrinsic functions of donor cells and are also impacted by cell conditions. Therefore, the origins of producer cells will be summarized below in order to provide information about choosing the cell/cell lines to fulfil different study objectives. Furthermore, efforts in developing exosome mimetics to overcome technical obstacles, such as low

loading efficiency or poor yield, are also discussed. On a supplementary note, milk- or plant-derived exosomes have shown potentials as drug carriers for oral delivery with minimal biosafety concerns.^{26,27}

Exosomes from Culture Conditioned Media

Exosomes can be isolated from conditioned cell culture media using various methods, including sequential or gradient ultracentrifugation, filtration, polymer precipitation, size exclusion-chromatograph, and immunoaffinity capture.²⁸ Various properties of exosomes are affected by the isolation method, including the yield, size distribution, surface charge, and protein content. However, standard operation procedures in exosome preparation, including isolation and purification, have not been established. Selection of one specific method or combination of

Table 1 Exosome-Based Delivery Vesicles and Features

Cell-Secreted		Exosome Features	Ref.
Immune cells	DCs	- induce potent cellular immune responses - serve as cancer vaccine to boost NK cell activity in non-small cell lung cancer patient	123
	imDCs	- contain miR-682 and promote immune tolerance via suppression of Rho-associated protein kinase - reduced immunogenicity as drug carrier	124
	NK cells	- contain killer proteins (granzyme and perforin) to exert cytotoxicity to tumor cells - tumor-specific accumulation of NK-EXOs	125
	effector CAR-T cells	- express a high level of cytotoxic molecules and inhibit tumor growth - do not express Programmed cell Death protein 1 (PD1) compared with CAR-T cells	46
Cancer cells or cell lines		targeting homotypic tumors	103
MCSs		- contain tissue regenerative growth factors for degenerative diseases - allogeneic host to provide “off-the-shelf” cell-derived product	126 127
HEK293T		- ideal host cell for membrane modification through genetic manipulation - immune inert	93
Exosome mimetics		Exosome features	
liposome-exosome hybrid		- increased exosome yield - mitigate functional loss of cargo during loading	128
Cell extrusion		- increased exosome yield - exosome-mimetics from primary hepatocytes aid liver regeneration	51,129
Food-derived		Exosome features	
milk edible plants		- cross gastrointestinal tract via the neonatal Fc receptor - desirable nanoparticle morphology, environmentally safe and non-hazardous - eminent potential for industrial pharmaceutical production	116 130

Abbreviations: DCs, dendritic cells; imDCs, immature dendritic cells; NK cells, natural killer cells; NK-EXOs, NK cell-derived exosomes; CAR-T cells, chimeric antigen receptor T cells; MCSs, mesenchymal stem cells.

methods depends on the nature of the samples as well as the study objectives. Various different cell lines have been utilized for exosome isolation (Table 1). For example, Tian et al used mouse immature dendritic cells (imDCs) to produce exosomes with surface expression of iRGD peptide, which navigated exosomes towards α_v integrin expressing cancer cells.²⁹ ImDCs were chosen as the donor cells to reduce immunogenicity and toxicity of exosomes, considering that imDCs are immune tolerogenic cells with low ratios of co-stimulatory to inhibitory signal.³⁰ On the other hand, exosomes released from antigen-pulsed DCs boost immune responses against cancer and might serve as a cell-free nanoscale vaccine in cancer immunotherapy.³¹ Natural killer (NK) cells have also been utilized to produce exosomes with antitumor effects, by means of the encapsulation of cellular perforin and granzymes during EV biogenesis.^{32,33} Apart from immune cells, MSCs are a popular candidate for EV production, and exosomes are considered as a vital venue for the secretion of trophic factors, by which MSCs aid tissue repair and regeneration.³⁴ In vivo studies have demonstrated that MSCs-derived exosomes can facilitate spinal cord repair after injury,³⁵ mitigate joint inflammation in osteoarthritis,³⁶ and improve cardiac functions in ischemia-refusion injury.³⁷ Moreover, MSCs possess large ex vivo expansion capacity and are immunosuppressive and, therefore, are compatible with mass production of exosomes for both autologous and allogenic clinical applications.³⁸

Human embryonic kidney (HEK293) cells have wide applications in biopharmaceutical manufacturing, and represent one of the few mammalian cell lines to produce therapeutic biologics accepted by FDA.³⁹ In addition, HEK293 cells are amenable to various transfection methods and permit genetic manipulation to engineer the exosome surface or to load cargos during exosome biogenesis.⁴⁰ Previous studies have also reported that exosomes generated from HEK293 are immunologically inert and do not trigger inflammatory responses in vivo.⁴¹

Cancer cell lines were presumed to be efficient exosome producers since cancer cells overexpressed Rab GTPases Rab27a and Rab27b, which promote exosome secretion by facilitating multivesicular endosome (MVE) docking at the plasma membrane.⁴² In addition, exosomes' tropism toward their cell origin could be utilized for cancer targeting. As exemplified in studies from Kim et al,⁴³ ovarian cancer cell line SKOV3-derived exosomes displayed enhanced tumor accumulation in SKOV3

xenografted mice compared with HEK293-derived exosomes. However, there have been concerns about directly using tumor cell-produced naïve exosomes as drug carriers for several reasons. First of all, the pharmacokinetic profile of unmodified exosomes is less ideal in regards of their short plasma half-life and low targeting efficiency. Unmodified tumor exosomes exhibit a short half-life of less than 5 minutes after IV bolus in mice, and rapid clearance, which is partly due to complement opsonization in the innate immune system.⁴⁴ Minimal tumor accumulation is observed as well, though intratumor-injected exosomes remain associated with tumors to a significantly greater extent compared with liposomes. In addition, cancer cells by nature release exosomes with surface expression of programmed death-ligand 1 (PD-L1), an important form of soluble PD-L1 that mediates immunosuppression to aid tumor growth.⁴⁵ Therefore, measurement of exosomal PD-L1 expression is necessary before applying those unmodified exosomes as drug carriers, especially in combination with PD1- or PD-L1-blocking immunotherapy.⁴⁶ In addition to membrane features of exosomes, the landscape of the encapsulated protein or gene materials is disparate.⁴⁷ For example, miRNAs in exosomes derived from PC3 cells, but not from HEK293, can modulate the integrin pathway and induce macrophage M2 polarization. On the other hand, miRNAs in HEK293 exosomes are involved in cell migration through cadherin signaling.⁴⁸ Therefore, the endogenous content within exosomes needs stringent investigation and risk-to-benefit assessment before choosing a cell line as the producer cell of exosomes for drug delivery.

Exosome-Mimetics

Naturally released exosomes inspire the development of exosome-mimetic nanovesicles (EMNVs). Even though exosome secretion is considered as highly efficient with a secretion rate of about 100 exosome particles/cell/hour based on single-cell assays,⁴⁹ the yield of exosome isolation using culture conditioned is limited, with the commonly observed yield of <0.1 exosome particles/cell/hour.⁵⁰ One straightforward approach to overcome the issue for large-scale production is to generate EMNVs by serially extruding cells through micro-sized filters (Table 1). Using this method, the yield is boosted over 100-fold, while EMNVs keep the biological functions similar as naïve exosomes.

EMNVs derived from different cell lines have been used to treat various diseases. For example, EMNVs

manufactured using primary hepatocytes promote liver regeneration in mice with partial hepatectomy (PH).⁵¹ Pancreatic β -cell-derived EMNVs induce bone marrow (BM) cells to differentiate into insulin-producing cells, serving as potential therapeutics for diabetes.⁵² In addition, payloads such as anticancer drug doxorubicin could be encapsulated simultaneously along with serial extrusion of cells to further improve treatment of cancer.⁵³ However, EMNVs have mixed membrane components from both plasma membrane and intracellular organelles, whereas the exosome membrane is primarily originated from ILV in endosomal pathways.³ Additionally, certain lipid species, such as sphingolipids, cholesterol, and phospholipid phosphatidylserine, are more enriched in exosomes compared with that in their donor cells.⁵⁴ Therefore, one open question is whether the altered membrane composition would impact in vivo PK/PD behavior of EMNVs compared with naive exosomes, especially after long-term application. This concern also applies to nanoparticles cloaked with plasma membrane.⁵⁵

Exosome-liposome hybrid nanoparticles are developed to overcome the low encapsulation of large size nucleic acids, such as plasmid. Lin et al⁵⁶ have used liposome as an intermittent carrier for Cas9 plasmids with a minimal size of 5–6 kb. Afterwards, liposome is incubated with exosomes to form hybrid NVs and enables plasmid loading by vesicles fusion. However, hybrid exosomes display cytotoxicity similar as liposomes (Lipofectamine) and, therefore, they should be further investigated using liposomes with less toxicity.

Pharmacokinetic Characteristics and Tumor Distribution of Naive Exosomes

Tissue distribution of exosomes, similar to other nanovesicles, is dictated by particle size, surface charge, and the composition of lipid bilayers.⁵⁷ Quantitative distribution studies using radioactive tracers have shown that the majority of unmodified exosomes is distributed to the liver, lung, and spleen after IV administration.⁵⁸ Clearance of exosome is primarily mediated by the mononuclear phagocyte system (MPS).⁵⁹ Exosomes carry a slightly negative charge on the surface, and are expected to reduce MPS-mediated clearance compared with positively-charged NPs.⁶⁰ However, phosphatidylserine, a negatively charged lipid, is involved in macrophage uptake of exosomes as part of exosome

clearance.⁶¹ On the other hand, surface expression of glycosylphosphatidylinositol (GPI)-anchored CD55 and CD59 have been shown to protect exosomes from complement-mediated lysis.⁶² On a special note, endogenous exosomes present in blood circulation also impact the in vivo fate of administered exosomes. For instance, a pre-IV injection of peripheral blood-derived exosomes reduces the hepatic accumulation of subsequently injected grapefruit-derived exosomes. Furthermore, tissue distribution of grapefruit-derived exosomes is redirected to the lung and tumor. In the study, endogenous exosomes inhibited macrophage uptake of exogenous exosomes by activating the CD36-mediated pathway, therefore serving as a competitor in hepatic clearance of exosomes.⁶³ In addition to the circulated level of endogenous exosomes, the doses of administered exosomes could also alter the tissue distribution. It has been observed that increasing the dose level of exosome (400 μ g) leads to massive lung deposition, while a lower dose of exosome (60 μ g) is predominantly distributed to the liver and spleen.⁴⁴

As for tumoral distribution, exosomes falling within the size range of 100–200 nm are anticipated to exhibit the enhanced permeability and retention (EPR effect) in tumors.⁶⁴ Moreover, exosomes are well known for their homotropic feature, therefore offering an additional mechanism to enable tumor targeting when utilizing tumor cell generated exosomes as delivery vectors. The expression pattern of integrin has been shown as one determining factor in exosome organotropism. For example, exosomal integrins $\alpha_6\beta_4$ and $\alpha_6\beta_1$ were associated with lung metastasis, while exosomal integrin $\alpha_v\beta_5$ was linked to liver metastasis.⁶⁵ In addition, glycans exposed on exosomes also impact their tissue distribution as well as cell targeting. For example, glycosaminoglycans exposed on glioblastoma exosomes direct the targeting of exosomes to CCR8-positive glioblastoma cells via a triple interaction with CCR8 ligand CCL18 as the bridging molecule.⁶⁶ The mildly acidic pH in the tumor microenvironment (TME) also boosts exosome uptake by cancer cells. This targeting effect is attributed to high levels of sphingomyelin/ganglioside GM3 (N-acetyl neuraminylgalactosyl glucosylceramide) in the exosomal membrane. GM3 becomes positively charged in a rich acidic environment, resulting in better exosome-cell fusion than that at physiological pH 7.4.⁶⁷

Strategies to Improve Tumor Targeting of Exosome

The homotypic targeting of tumor cell-derived exosomes has been demonstrated using *in vitro* accumulation studies.⁶⁸ However, the tumor targeting shows discrepancies *in vivo* across studies. Qiao et al⁶⁹ reported that HT1080 cell-derived exosomes homed to their origin cells, resulting in enhanced tumor accumulation in HT1080 cell-xenografted mice compared with HeLa-derived exosomes. On the other hand, Smyth et al⁴⁴ found that exosomes released by 4T1, MCF-7, and PC3 cells displayed minimal tumor accumulation after IV injection, which may be partly due to the rapid blood clearance. *In vivo* blockade of scavenger receptor-A (SR-A) with dextran sulfate dramatically decreased monocytes/macrophage-mediated hepatic clearance of exosomes in mice and, therefore, enhanced tumor accumulation of exosomes by 5-fold.⁷⁰ Those results suggest that optimization of exosomes is necessary to achieve efficient tumor targeting. The following sections focus on strategies to improve tumor targeting of exosome-based delivery vectors, where passive or active targeting mechanisms are discussed separately (Figure 1).

Boosting EPR in Passive Tumor Targeting

Passive tumor targeting of nanoparticles (NPs) is a result of the EPR effect, described as the increased permeability of tumor vasculature and ineffective lymphatic drainage. However, only a small fraction (less than 1%) of NPs accumulate even in high-EPR xenografted tumors.⁷¹ The targeting efficiency of NPs is compromised due to uneven permeability of the heterogeneous size of endothelial gaps, dense interstitial tumor matrix, and inadequate blood perfusion.⁷² In addition, studies indicated that actively targeted NPs must first reach the target to take advantage of this increased affinity and avidity to cancer cells.⁷³ Efficient passive targeting is considered a prerequisite for systemically administered NPs designed to target tumor specific-cells. Approaches to enhance EPR includes prolonging the circulation time of NPs as well as boosting their extravasation.⁷⁴ Exosomes are primarily cleared via phagocytosis and endocytosis by macrophages in MPS.⁵⁹ Overexpression of antiphagocytic factors on exosomes, such as CD47, protects exosomes from MPS-mediated clearance, thereby significantly increasing their circulation time of exosomes by 3-fold.⁷⁵ Stealth strategies,

such as surface PEGylation, also reduce the clearance of exosomes^{76,77} with the tradeoff of potential immunogenicity.⁷⁸ In some cases, the reduced clearance resulted in improved tumor retention, which was attributed to passive accumulation of the PEGylated exosomes via EPR.^{76,77} In other cases, however, PEGylation of exosomes does not lead to increased tumor retention.⁷⁹ Although tumoral blood vessels are commonly described as leaky and are compatible with extravasation of NPs, targeting surface molecules expressed on endothelial cells can potentiate tumor penetration. For example, one study showed that exosomes modified with iRGD (CRGDKGPDC) bind specifically to α_v integrin which is highly expressed on endothelial cells of tumoral vessels,⁸⁰ leading to an increased tumoral accumulation in breast cancer cell xenografted mice.²⁹

Active Tumor Targeting

The downside of passive tumor targeting is the lack of ligand-selective binding for specific tissues or organs. In addition, once exosomes are distributed to the desired tissue or organ, manipulation of exosomes to increase cell-specific uptake is another challenge. In this section, various approaches to augment active tumor targeting of exosomes are reviewed from two aspects, including TME normalization and selective uptake by cancer cells (Table 2). These approaches aim to overcome sequential hurdles for efficient tumor targeted delivery.⁸¹

Tumor Microenvironment Normalization

The pathophysiology of TME is regulated by angiogenesis, fibrotic signaling, and hypoxia, which limit the intratumor infiltration of NPs following extravasation.⁸² In TME with an inflamed immune phenotype, the cancer-immunity cycle of antitumor immune response is disrupted partly due to T cell exhaustion.⁸³ A novel application is to display synthetic bispecific antibodies on exosomal membranes. For example, one study utilized anti-EGFR scFv, which targets cancer cells, and anti-CD3 scFv, which redirects and activates cytotoxic T cells.⁸⁴ The bispecific antibodies were displayed on exosomal membranes by fusing scFv antibodies with human platelet-derived growth factor receptor (PDGFR) transmembrane domain (TMD). The dual targeted exosomes normalized the immunosuppressed TME

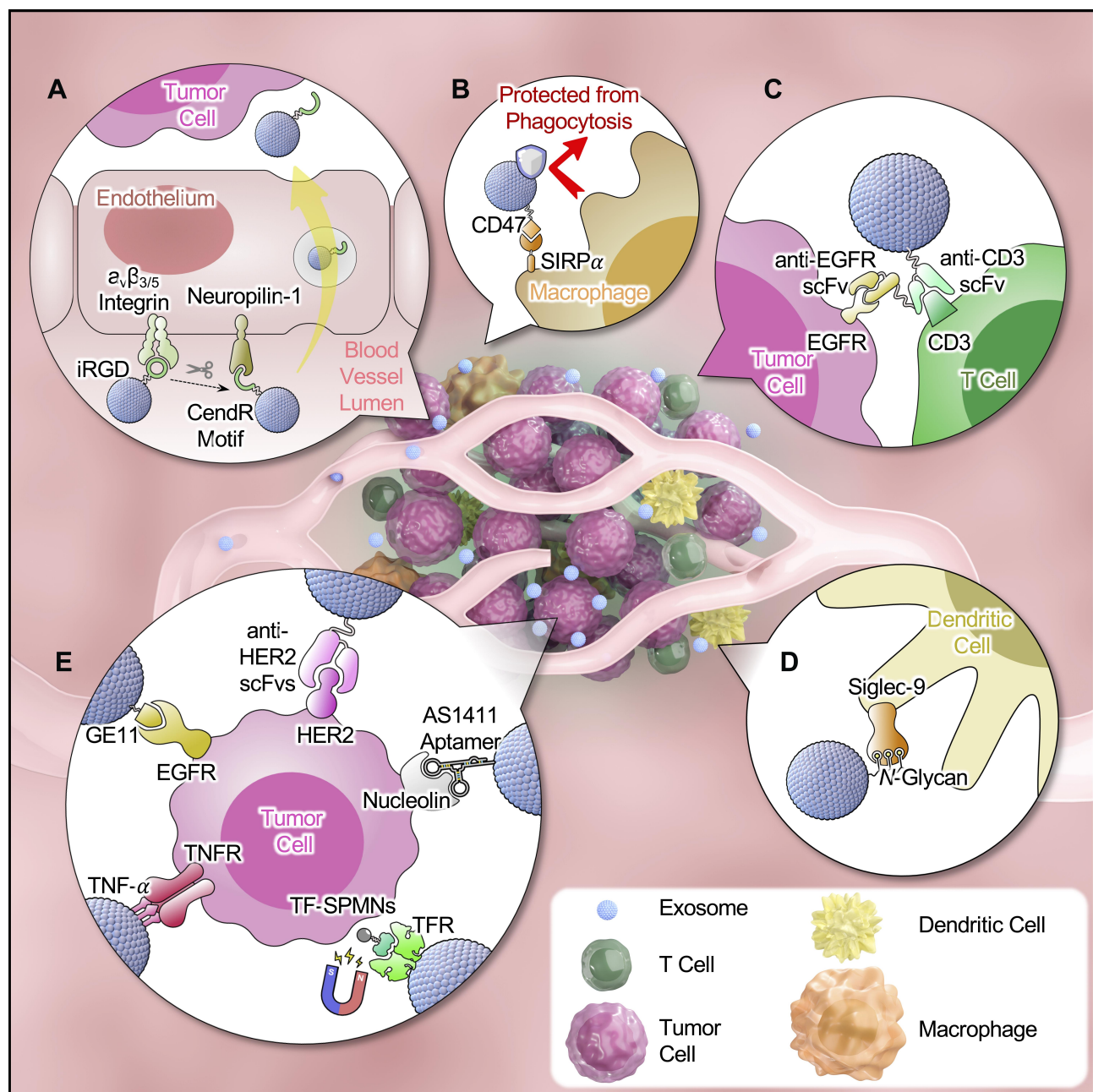


Figure 1 Graphical illustration of tumor targeting strategies of exosome-based delivery. In passive targeting, exosomes pass through the leaky vascular walls and accumulate at the tumor site by the enhanced permeability and retention (EPR) effect. The EPR effect is boosted by (A) increasing endothelial penetration of tumor vasculature by surface modification of iRGD peptide,⁸⁰ and (B) prolonging circulation time of exosomes by overexpressing antiphagocytic factors.¹⁷ In addition, reshaping tumor microenvironment (TME) by targeting specific immune cells could enhance anti-tumor immune responses for cancer immunotherapy. For example, (C) exosome expressing in-tandem scFvs (anti-EGFR and anti-CD3) redirect cytotoxic T cells to attack tumor cells;⁸⁴ and (D) glycan-modified exosomes displayed increased uptake by DC cells and augment DC-mediated immune responses.⁸⁶ Active targeting can be achieved (E) by using specific targeting moiety to bind to the receptors on tumor cells or by modification to synergize external stimuli-guided tumor targeting.^{93,95,101,102,105}

by facilitating cancer recognition by T cells, a principle similar to bispecific T cell engagers (BiTEs).⁸⁵ Compared with free bispecific antibodies, exosomes with genetically displayed antibody molecules showed significantly increased antibody avidity and more potent cytotoxicity in vitro.¹³ Another approach is to boost

dendritic cell (DC)-mediated immune activation by glycan modification on exosomes. For example, glioblastoma-derived exosomes after de-sialylation and insertion of Lewis antigen, which contains fucosylated structures bound to DC-specific adhesion molecule CD209, resulted in a 4-fold increase of DC uptake and serves

Table 2 Targeting Ligands Used in Preclinical Studies for Tumor Targeting

Ligand Type	Examples	Effect	Parent Cell	Payload ^a	Preparation Method ^b	Size ^c (nm)	Ref.
Antibody and fragments	anti-SSTR2 mAb	target somatostatin receptor 2-overexpressed neuroendocrine cancer	HEK293 cell	Rom	UF	125	¹³¹
	anti-EGFR nanobodies	target cancer cells overexpressing EGFR	Neuro2A cells	NA	UF and SEC	100	⁹⁸
	in-tandem scFvs against CD3 and HER2	redirect and activate cytotoxic T cells to attack HER2-expressing breast cancer cells	Expi293 cells	NA	UC	109	¹³
	anti-EGFR CAR, anti-HER2 CAR	exosomes containing cytotoxic molecules and inhibit tumor growth	effector CAR-T cells	NA	UC and IMS	85	⁴⁶
Proteins or ligands	ITGβ4	suppress lung cancer cell proliferation and migration	MDA-MB-231 cells	miRNA-126	PureExo [®] exosome isolationKit	30 - 120	¹³²
	IL3	sensitize IL-3R-expressing CML cells to imatinib	HEK293T cells	BCR-ABL siRNA	UC	30 - 60	⁴⁰
	biotin and avidin	target cancer cells overexpressing lectins	HUVECs	Dox	microfluidic chip with IC	<150	¹³³
Peptides	GE11 peptide	target EGFR-overexpressing cancer cells	HEK293 cells	miRNA	UC	100	⁹³
	R8 peptide, K4 peptide	stimulate tumor cell uptake of exosomes	HeLa cells	saporin	UC	160, 197	^{134, 106}
Nucleic acid-based ligands	AS1411 aptamer	target tumor cells overexpressing nucleolin	MDA-MB-231 cells	NA	microfluidic sonication	181	¹⁰¹
	Sgc8 aptamer	target protein tyrosine kinase 7-positive cancer cells	imDCs	Dox	UC	111	¹³⁵
Small molecules	Folic acid	bind folate receptors on cancer cells	milk exosome	PTX, DTX	UC	40 - 100	¹³⁶
	Sialic acid	bind lectin receptors on HeLa cells	MSCs	NA	UC	178	¹³⁷

Notes: ^aThe abbreviations in the column of Payload stands for romidepsin (Rom), doxorubicin (Dox), paclitaxel (PTX) and docetaxel (DTX). ^bThe abbreviations in the column of Preparation method stands for ultrafiltration (UF), ultracentrifugation (UC), and size-exclusion chromatography (SEC), immunomagnetic separation (IMS) and immunocapture (IC). ^cThe sizes of exosomes were determined using either DLS (dynamic light scattering) or NTA (nanoparticle tracking analysis). The mean values of exosome diameters or size ranges were cited wherever one was reported.

as a potential anticancer vaccine.⁸⁶ Since TME normalization using exosomes is still an emerging field, strategies utilized by other NPs that either depend on the physiochemical characteristics (eg, redox pressure, pH) or on targeting specific cell populations in TME could be leveraged by exosomes. For instance, NPs modified with α -peptide and M2 pep fusion peptide exhibited preferential delivery to M2 TAM over tissue resident macrophages by binding to scavenger receptor B type 1 (SR-B1) on M2 macrophages.⁸⁷ Surface conjugation of methotrexate as both a ligand and a toxin on NPs enables targeting and depletion of ovarian TAMs with high expression of folate receptor-2.⁸⁸

Cancer Cell Selectivity

The primary cell type responsible for exosome uptake after tissue distribution may vary depending on organ as well as the origin of exosomes. For example, IV infused cholangiocyte-derived exosomes are preferentially taken up by hepatic stellate cells (HSCs) and HSC-derived fibroblasts in mouse liver.⁸⁹ Kupffer cells (KCs) take up the bulk part of exosomes released by pancreatic ductal adenocarcinomas (PDACs).⁹⁰ The underlying mechanisms of the cell type-specific targeting of exosomes remain to be elucidated. By nature, phosphatidylserine (PS) and carbohydrate moieties on exosomal membranes are involved in the recognition and uptake of exosomes by macrophages.

For example, sialic acid on exosomes is recognized by CD169 on macrophages *in vivo*.⁹¹ This characteristic should therefore be considered in the design of targeted delivery systems, since exosomes taken up by macrophages such as KCs might be largely subject to hepatic clearance. One example is that surface modification of exosomes with cationized pullulan enhanced hepatocyte-specific uptake of exosome by binding to asialoglycoprotein receptors on hepatocytes. As a result, the anti-inflammatory effect of MSC-derived exosomes was potentiated in a concanavalin A-induced liver injury model.⁹² Moreover, a growing list of cell-targeting peptides, such as GE11 peptide targeting EGFR on solid tumor cells,⁹³ and ischemic myocardium targeting peptide (IMTP) with unknown receptors to target ischemic myocardium,⁹⁴ have been studied for use in modifying the exosomal surface. In addition, antibody or antibody fragments serve as versatile alternatives to active tumor targeting in exosomal surface modification. Compared with natural ligands or homing peptides, antibodies can be raised to target any antigens and, therefore, offer more freedom in target selection, in addition to an exquisite specificity due to higher binding affinity. The binding affinity of antibody or antibody fragments along with the target expression levels on cancer cell collectively determine the selectivity of exosomes. For example, high-affinity anti-Her2-scFv ($K_D \leq 1$ nM) and a high expression level of Her2 ($\geq 10^6$ copies per cell) on cancer cells are optimally required to enable selective cell uptake of exosomes in Her2-positive cells.⁹⁵

On a special note, the impact of fusion partners employed to display targeting moieties (peptide or antibody fragment) on the exosomal surface should not be neglected. There are many reports about the impact of membrane-inserted fusion proteins on the targeting effect of an exosome. For examples, membrane display by fusion with lysosomal associated membrane protein 2b (Lamp2b) may cause ligand loss since it leads to peptide cleavage during exosome biogenesis.⁹⁶ Lactadherin, a membrane-associated protein,⁹⁷ and its peptide domains are located close to the membrane surface and not freely accessible to interact with cell receptors. Thus, incorporation of the C1C2 domain of lactadherin might interfere with cell uptake of exosomes.⁹⁵ Furthermore, glycosylphosphatidylinositol (GPI) anchor signal peptides from decay-accelerating factor (DAF) renders an insufficient density of displayed ligands and, consequently, the increase of cell uptake of the modified exosomes is limited due to lack of

receptor clustering to enable subsequent exosome internalization.⁹⁸

Chimeric antigen receptor T (CAR-T) cell-based immunotherapy has emerged as a promising new treatment with unprecedented results for hematological malignancies.⁹⁹ Exosomes released from CAR-T cells also carry CAR on the exosomal surface. Interestingly, PD1 was not detected on CAR exosome surfaces compared with their parental cells. Therefore, CAR exosomes are compatible with recombinant PD-L1 immunotherapy without antagonizing the anti-tumor effects.⁴⁶ Non-peptide targeting ligands, such as aptamers, have also been used to modify exosome surface. Aptamers are folded nucleic acid strands capable of binding to different target molecules with high programmability and low immunogenicity.¹⁰⁰ In one study, cholesterol modified aptamer AS1411 conferred exosome enhanced tumor targeting in an MDA-MB-231-xenografted mouse model due to specific binding of AS1411 aptamers to nucleolin on breast cancer cells.¹⁰¹

Surface modification of exosomes also allows for 2-in-1 or all-in-1 strategies to achieve tumor specific-killing. The synergistic tumor-killing effect has been demonstrated in the study of engineered Cas9 vector-encapsulated exosomes with the surface expression of tumor necrosis factor (TNF α). In this case, TNF α not only served as a targeting moiety to bind cancer cells overexpressing TNF receptors, but also triggered cancer cell necroptosis by activating TNF receptors. The downstream signaling pathways of TNF α for survival or apoptosis are inactivated by cancer-selective expression of Cas9.¹⁰² In addition, to take advantage of the intrinsic feature of cancer cells, exosomes have also been modified to synergize external stimuli-guided targeting, such as the encapsulation of sinoporphyrin sensitizer for focused ultrasound-guided delivery,¹⁰³ or with conjugation with superparamagnetic nanoparticles (SPMNs) in external magnetic field-driven targeting.^{104,105}

Exosomes as Delivery Tools to Overcome Biological Barriers

The antitumor effects of exosomes with therapeutic payloads also depend on the ability to deliver cargo to the intracellular action site (Figure 2). Approaches to enhance intracellular delivery for exosomes with encapsulated cargo are discussed, as the cell membrane is an existing barrier to the anti-tumor efficacy.⁸¹ In addition, the ability of exosomes to cross biological barriers, including the blood-brain barrier (BBB) and the gastrointestinal (GI)

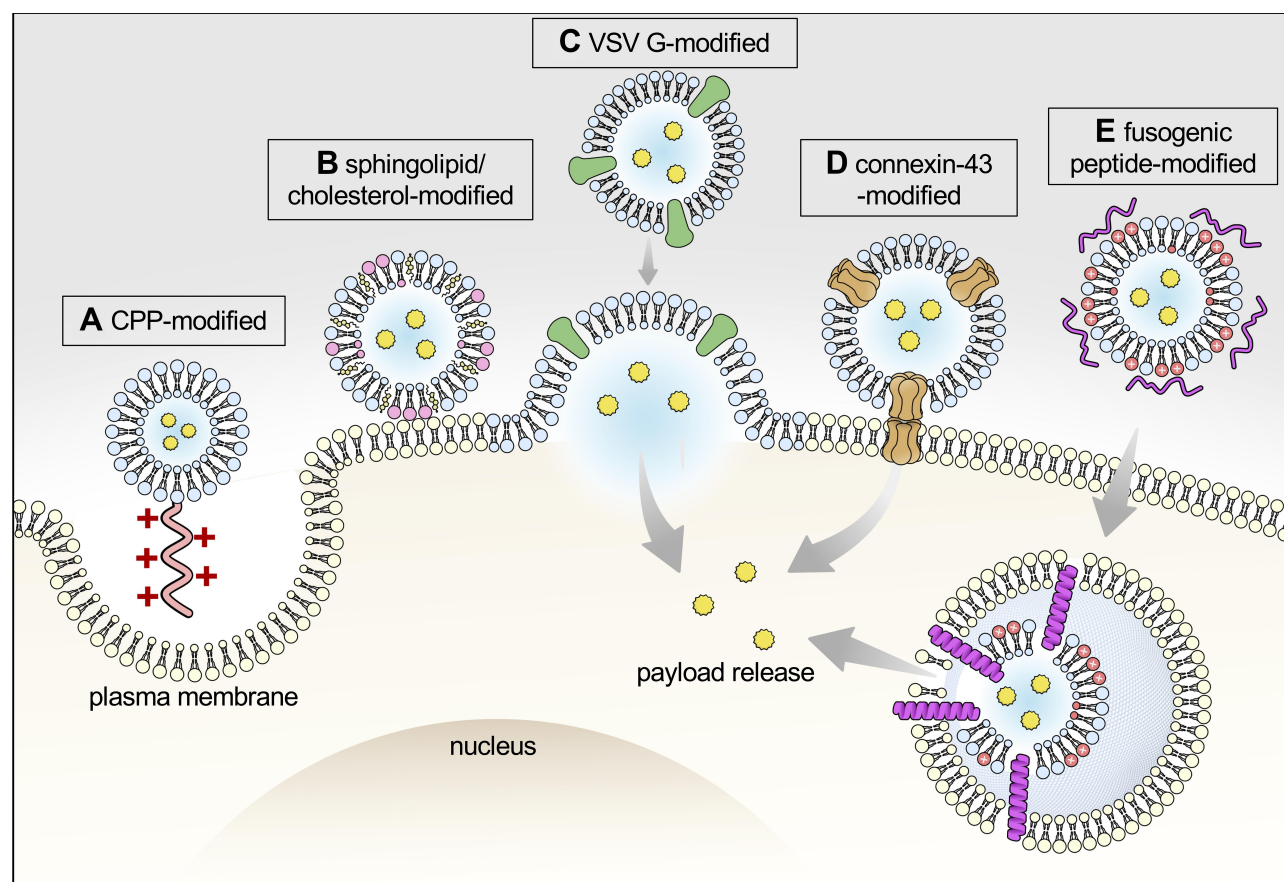


Figure 2 Graphical illustration of approaches to enhance intracellular payload delivery. Cell uptake of exosomes is mediated by several means, including endocytosis, phagocytosis, and direct fusion with plasma membrane. Intracellular payload delivery can be enhanced by several approaches, including: **(A)** modifying exosomes with cell-penetrating peptides (eg, arginine-rich peptide) to stimulate cell micropinocytosis; Promoting exosome-cell fusion by **(B)** increasing membrane rigidity of exosome via enriched sphingolipid and cholesterol;⁶⁷ or by **(C)** expressing VSV-G on exosome to aid membrane fusion.¹⁰⁷ **(D)** Exosomes expressing connexin-43 that forms hexameric channels to allow exosome to dock to gap junction pore on plasma membrane and, therefore, enable direct cytoplasmic transfer of cargo.¹⁰⁸ In addition, **(E)** integrating fusogenic peptide (eg, GALA peptide) facilitates exosome escape of payload before lysosomal degradation.¹⁰⁹

tract, inspire growing areas of research interests utilizing exosomes for drug delivery.

Enhanced Intracellular Delivery

Cells can take up exosomes by various means including direct membrane fusion, pinocytosis, and endocytosis.¹⁹ In order to facilitate exosome entry into cells, one approach is to equip exosomes with cell-penetrating peptides (CPPs), such as arginine-rich CPPs, which enhance exosome internalization by stimulating cell micropinocytosis.¹⁰⁶ Another approach is by increasing the chance of membrane fusion. Biophysical studies suggest that increasing membrane rigidity by enriching sphingolipid and cholesterol improves fusion efficiency between exosomes and the recipient cells.⁶⁷ In addition, exosomes with a surface display of G protein of vascular stomatitis virus (VSV-G) exhibit increased intracellular delivery of payloads by promoting exosome-cell membrane fusion.¹⁰⁷ Integration of exosomes

with connexin 43 (Cx43), a membrane protein that assembles to form hexameric channels, allows exosomes to dock at the gap junction pore embedded in the plasma membrane of recipient cells, and therefore provides another route for direct cytoplasmic transfer of exosome payload.¹⁰⁸ As for internalized exosomes following the endosomal traffic route, the ability of the cargo to escape before lysosomal degradation is another challenge. Exosomes complexed with cationic lipids together with a pH-sensitive fusogenic peptide, GALA, increased exosome-endosome fusion and, therefore, aided in the cytosolic release of cargo.¹⁰⁹ Currently, specific mechanisms for exosome internalization by cells remain to be elucidated. The inclusion of targeting moieties on exosomal membrane also impact the uptake of exosomes, as well as exosome distribution among cell organelles. For example, cell uptake of HEK293-derived naive exosomes is similar to the uptake of cholera toxin subunit B (CtxB), a marker for lipid raft-associated cell uptake.¹¹⁰ On the other hand,

exosomes with a surface display of anti-Her2 ScFv increased overall cell uptake, with a greater extent of colocalization with transferrin and dextran, markers for clathrin-mediated endocytosis and micropinocytosis, respectively.⁹⁵ The uptake mechanism also differs depending on recipient cells. The observed contributing fractions by caveolae-dependent endocytosis of exosomes vary among cell lines, which is partly subject to caveolin-1 expression levels.¹¹¹ As the research community is starting to appreciate the diversity of exosomes in terms of size and originated cell organelles, trafficking routes other than the putative endosome destination, such as the nucleus, have also been described.¹¹² Thus, exosomes may serve as potential tools to confer cell organelle-targeted delivery once the intracellular fates of exosomes are fully elucidated.

BBB

The BBB prevents the entry of toxic compounds into the brain and, consequently, restricts the delivery of most drugs for treatment of brain-associated diseases. Circulating exosomes are able to cross the BBB. Therefore, exosomes are considered as promising tools for drug delivery to the central nervous system (CNS) (Table 3). Exosomes derived from brain endothelial bEND.3 cells are able to deliver anticancer drugs across the BBB in zebrafish embryos due to the homotypic effect of exosomes.¹¹³ Another study suggests that unmodified exosomes from macrophages are sufficient to confer BBB penetration in the context of brain inflammation, where the expression level of adhesion molecule 1

(ICAM-1) on endothelial cells increased the interaction with lymphocyte function-associated antigen 1 (LFA-1) on macrophages-derived exosome membranes.¹¹⁴ Exosomes modified with rabies viral glycoprotein (RVG) peptide, a CNS-specific peptide bound to acetylcholine receptor, also showed the ability to cross the BBB in vivo with minimal liver accumulation.¹¹⁵

GI Tract

Oral drug delivery is still a challenging task for many therapeutic biologics. Exosomes derived from milk or edible plants are able to cross intestinal barriers and, therefore, are considered as potential drug vectors for oral delivery.^{116,117} An interesting finding is the discrepancy of vector effect; milk exosomes were mainly distributed into the liver and spleen, whereas the encapsulated miRNA accumulated in the spleen and brain. The authors speculated that the distinct tissue distribution of payload was subject to a relayed substance transfer from foreign exosomes to endogenous exosomes in the endosomal traffic routes in cells, therefore allowing the cargo re-distribution to the brain.¹¹⁸

Conclusion

There are currently over 100 exosome-related clinical trials registered at Clinicaltrials.gov with projects spanning from early Phase I to Phase II. For trials to investigate exosomes as therapeutics (Table 4), the most commonly used producer cells are MSCs and immune cells with indications for neurodegenerative disease, inflammation, and cancers. MSC-

Table 3 Exosomes Displaying BBB Penetration

Exosome Type	Examples (Approach or Ligand/Target)	Parent Cell	Payload	Disease Model	Ref.
Naïve exosome	intranasal administration	EL-4	curcumin	brain inflammation	138
		Raw 264.7	caltase	Parkinson's disease	139
	LFA-1/ICAM-1	Raw 264.7	BDNF	LPS-induced brain inflammation	114
Modified exosomes with targeting peptide	RVG peptide/ acetylcholine receptor	DCs	BACE1 siRNA	Alzheimer's disease	115
		HEK293	opioid receptor Mu siRNA	Morphine relapse	140
	cyclic RGD peptide/ integrin $\alpha\beta_3$	mesenchymal stromal cell	curcumin	Cerebral ischemia /transient middle cerebral artery occlusion (MCAO) mice	141
	RGE peptide/ Neuropilin-1	Raw 264.7	curcumin	orthotopic glioma models	142

Table 4 Exosomes in Clinical Trials

Derived Sources	Payload	Indications	Phases	Sponsor	ClinicalTrials.gov Identifier
Allogenic adipose MSCs	NA	Severe COVID-19 Pneumonia	Phase I	Ruijin Hospital	NCT04276987
Allogenic MSCs	miR-124	Acute Ischemic Stroke	Phase I/2	Isfahan University of Medical Sciences	NCT03384433
Adipose derived stem cells	NA	Periodontitis	Phase I	Beni-Suef University	NCT04270006
Autologous plasma	NA	Cutaneous ulcers	Phase I	Kumamoto University	NCT02565264
Plant	curcumin	Colon Cancer	Phase I	University of Louisville	NCT01294072
Allogenic COVID-19 Specific T Cell	NA	Corona Virus Infection	Phase I	TC Erciyes University	NCT04389385
Umbilical MSCs	NA	Dry Eye	Phase I/2	Sun Yat-sen University	NCT04213248
Umbilical MSCs	NA	Type I Diabetes Mellitus	Phase I	General Committee of Teaching Hospitals and Institutes, Egypt	NCT02138331
MSCs	KRAS G12D siRNA	Metastatic Pancreatic Adenocarcinoma	Phase I	M.D. Anderson Cancer Center	NCT03608631
MSCs	NA	Macular Holes	Phase I	Tianjin Medical University	NCT03437759
Grape	NA	Head and Neck Cancer	Phase I	University of Louisville	NCT01668849
Allogenic Adipose MSCs	NA	Alzheimer's Disease	Phase I/2	Ruijin Hospital	NCT04388982
Allogenic MSCs	NA	Dystrophic Epidermolysis Bullosa	Phase I/2	Aegle Therapeutics	NCT04173650

Abbreviation: MSCs, mesenchymal stem cells.

derived exosomes with KRAS G12D siRNA for treating subjects with metastatic pancreas cancer is in phase I studies. Compared with liposomes, exosomes offer several unique advantages, including reduced immunogenicity,¹¹⁹ enhanced tissue penetration,¹²⁰ and exosomes enable endogenous and site-specific cargo loading during exosome biogenesis.¹²¹ However, there are still challenges in exosome manufacturing, such as scaling-up the production yield and control of purity, especially considering the size overlap between exosomes and other extracellular vesicles (eg, microvesicles) or manufacture contamination (eg, lipoprotein aggregates).¹²² As for exosome-based targeted delivery, a comprehensive understanding of determinant attributes leading to selective-cell uptake of exosomes and factors influencing their intracellular disposition is still lacking. In addition, elucidation of mechanisms driving the homotypic tropism of exosomes, as well as to deciphering the relative contribution in exosome targeting when combining with the

aforementioned targeting approaches, will bring great value for the rational design of exosome-based delivery tools. Lastly, extensive research is required to optimize the targeting of exosomes as signalosomes and drug delivery vehicles, as they will also resolve issues encountered by cell-based therapy, and may provide a path forward as an “all-in-one” cell-free therapeutic modality.

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Disclosure

The authors report no conflicts of interest in this work.

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