

Extracellular Vesicles – Advanced Nanocarriers in Cancer Therapy: Progress and Achievements

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Abstract: Extracellular vesicles (EVs) are a class of cell-derived, lipid bilayer membrane composed vesicles, and some of them such as exosomes and ectosomes have been proven, playing remarkable roles in transmitting intercellular information, and being involved in each property of cell physiological activities. Nowadays, EVs are considered as potential nanocarriers which could partially resolve the problems of current chemotherapy because of their distinctive advantages. As endogenous membrane encompassed vesicles with nanosize, EVs are able to pass through the natural barriers with prolonged circulation time in vivo and have intrinsic cell targeting properties, they are less toxic, and less immunogenic. Recently, studies focusing on EV-based drug delivery system for cancer therapy have exploded dramatically. This review aims to outline the current applications of EVs as potential nanosized drug carriers in cancer therapy. Firstly, the characteristics and biofunctions of each EV subtype are described. Then the variety of therapeutic cargoes, the loading methods, and the targeting strategy of engineered EVs are emphatically introduced. Thereafter the pros and cons of EVs applied as therapeutic carriers, as well as the future prospects in this field, are discussed.

Keywords: Extracellular vesicles, exosomes, nano-sized carriers, tumor, drug delivery system

Introduction

Cancer is one of the most destructive diseases with a high recurrence and mortality rate and has become the greatest health problem worldwide.¹ The global cancer situation is dire due to belated diagnosis, drug resistance, metastasis, and poor prognosis.² So far chemotherapy is one of the predominant treatments of cancer therapy, which has made numerous progressions and prolonged tens of millions of lives. However, there are still inherent limitations of chemotherapy, including low efficacy, nonspecific distribution, and other undesirable adverse effects.³⁻⁵ Hence, methodological improvements, such as developing a targeted, efficient, and safe drug delivery system (DDS) applied in chemotherapy are urgently required.

The key challenge impacting the chemotherapy effect is how to deliver specific drugs to the tumor site and alleviate the side effects to normal tissue and cells. Efforts have been made to solve this problem such as preparing nanosized drug carriers, which are artificial nanoparticles (NPs) embedded with various drugs, including chemotherapeutic drugs, therapeutic proteins or nucleic acids, to deliver the drugs to disease sites.⁶ Several types of artificial NPs like liposomes, micelles, dendrimer, carbon nanotube, polymeric nanoparticles, magnetic nanoparticles, etc have been made and used in delivering various anticancer drugs.⁷⁻¹¹ Compared to traditional administration methods, such as oral administration or intravenous administration of free drug, NPs

are able to deliver high doses of therapeutic drugs into the tumor site while bypassing healthy cells because of their enhanced permeability and retention (EPR) effect.¹² However, there are inevitable disadvantages of artificial NPs which limited their clinical application. For example, some metal NPs show embryo toxicity after administration;¹³ artificial NPs have nonuniform particle size and are prone to form agglomerates which can be eliminated rapidly by the mononuclear phagocyte system (MPS), as well as their nonspecificity targeting effort in vivo.^{2,14,15} Thereupon, endogenous membrane vesicles with nanoscale have received increasing attention. Extracellular vesicles (EVs) are cell-derived lipid bilayer composed vesicles released by almost all living cells. Although EVs were previously considered as cellular trash, they actually are natural information carriers and play important roles in intercellular communication via shuttling biofunctional cargoes between cells. In addition to the physical properties of NP, EVs have additional advantages such as excellent biodegradability, biocompatibility as well as sequence programmability, and have showed ideal prospects as drug delivery carriers in cancer therapy.¹⁴

Herein, we review recent progress and achievements of using EVs as nanosized carriers for drug delivery in cancer therapeutics and highlight the challenges and research

directions of this field for further promoting their application.

General Concept of EVs

Extracellular vesicle is the generic term to describe cell released membrane vesicles, which covers different types. Currently, according to the guidelines of the International Society for Extracellular Vesicles (ISEV), there are four members in the EV family, which are exosomes, microparticles (or microvesicles, ectosomes), oncosomes, and apoptotic bodies^{16,17} (Figure 1). Exosomes are the smallest EVs with a diameter between 30 and 100 nm, which are formed by intraluminal vesicles (ILVs). Then ILVs inward budding of endosomal membrane and form multivesicular endosomes (MVEs). After maturation, exosomes are secreted via fusion of MVEs with the cell surface.¹⁸ Microvesicles represent a group of medium-size membrane vesicles which are 100 nm to 1000 nm in diameter, including shed membrane vesicles, microparticles and ectosomes. They are generated by outward budding and blebbing from the plasma membrane.¹⁹ Oncosomes are used to describe a class of tumor-derived microvesicles that are between 1 and 10 μm in size and could propagate oncogenic information to other cells and tissues in the tumor microenvironment.^{20–22} Apoptotic bodies are special kinds of vesicles with sizes

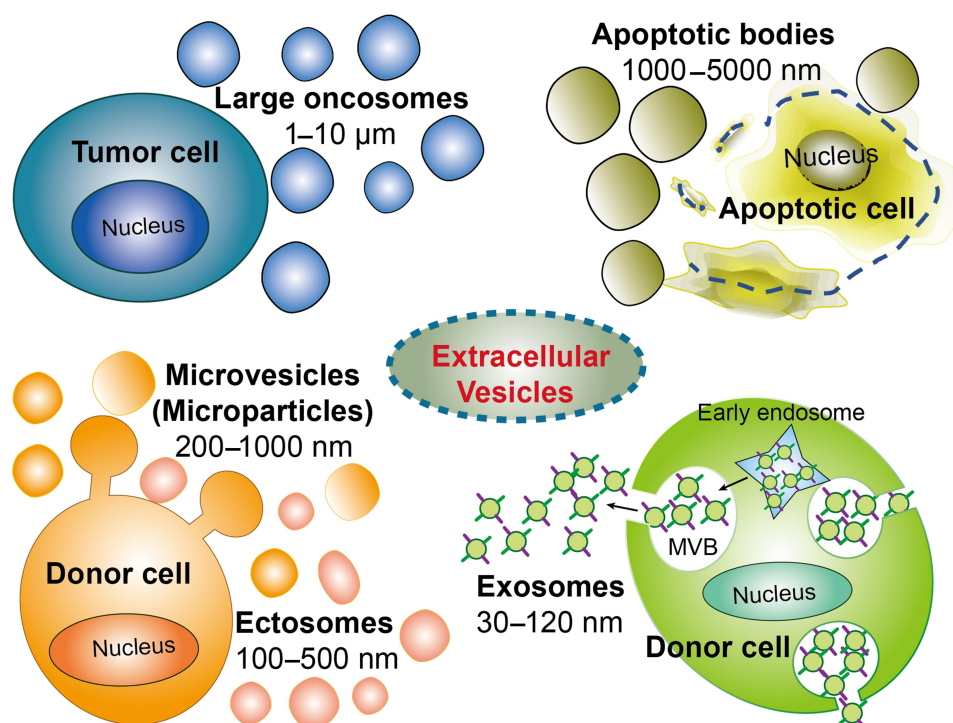


Figure 1 Schematic representation of the EV biogenesis. EVs family has more than four members, they are exosomes (30–120 nm), microparticles (or microvesicles, 200–1000 nm), ectosomes, 100–500 nm), large oncosomes (1–10 μm), and apoptotic bodies (1–5 μm).

ranging from 1 μm to 5 μm , which are formed by disassembled apoptotic cells. They contain information and substances from dying cells and are capable of delivering cellular materials to healthy recipient cells.²³

Each type of EV differs in their origin, composition and size, thus presenting heterogeneous properties. Exosomes are the most well studied EVs and found in many kinds of biological fluids, including peripheral blood, cerebrospinal fluid, amniotic fluid, urine, milk, bronchoalveolar lavage fluid, malignant effusions and cell culture medium.²⁴ Exosomes and ectosomes are found enriched in specific lipids, nucleic acids and proteins, after being released into the extracellular environment, they can transfer information from the originating cells to recipient cells and induce phenotypic modulation in recipient cells.^{25,26} Different kinds of RNAs including mRNA, microRNAs (miRNAs), lncRNA, tRNA and circRNA have been found in exosomes (Figure 2), and most exosomal RNAs play important roles in regulating bioactivity of recipient cells.²⁷ Compared to RNA, people still know little about exosomal DNA.²⁸ Sansone et al reported that DNA fragments originated from either nucleus or mitochondria could be packaged into exosomes by an unknown pathway.²⁹ As containing specific mutation of parental cells, exosomal DNA could emerge as a diagnostic marker for disease.²⁸ However, new

evidence indicated there might be no DNA in exosomes. Jeppesen et al reassessed the composition of exosomes and reported that the double-stranded DNA (dsDNA) associated histones were present in the nonvesicular (NV) components rather than in small EVs.³⁰ Therefore, it is still unclear whether there is DNA in exosomes or not, and if there is, the function of exosomal DNA is still waiting to be illuminated.

It is very important to recognize each type of exosomal marker correctly. The potential classical biomarkers for exosomes are major histocompatibility complex (MHC), flotillin-1, CD9, CD63, CD81, HSP70, Alix and TSG101, etc (Figure 2).³¹ However, some of them like MHC, flotillin-1 and HSP70 have been found presenting in other types of EVs as well.²⁶ To draw the precise biomarker profiles for each type of EV is still a tough challenge. Therefore, researchers were recommended to provide at least three positive protein markers and one negative marker, and better cooperate with electron microscope data to confirm the subtype of EV which they studied.¹⁷ In fact, compared to artificial NPs, EVs contain plenty of proteins on or embedded in their membrane, which give them special advantages in delivering drugs in vivo. For example, CD47, an enriched protein in fibroblast-derived EVs can interact with its receptor (SIRP α) on macrophages and release a “do not eat me” signal, therefore

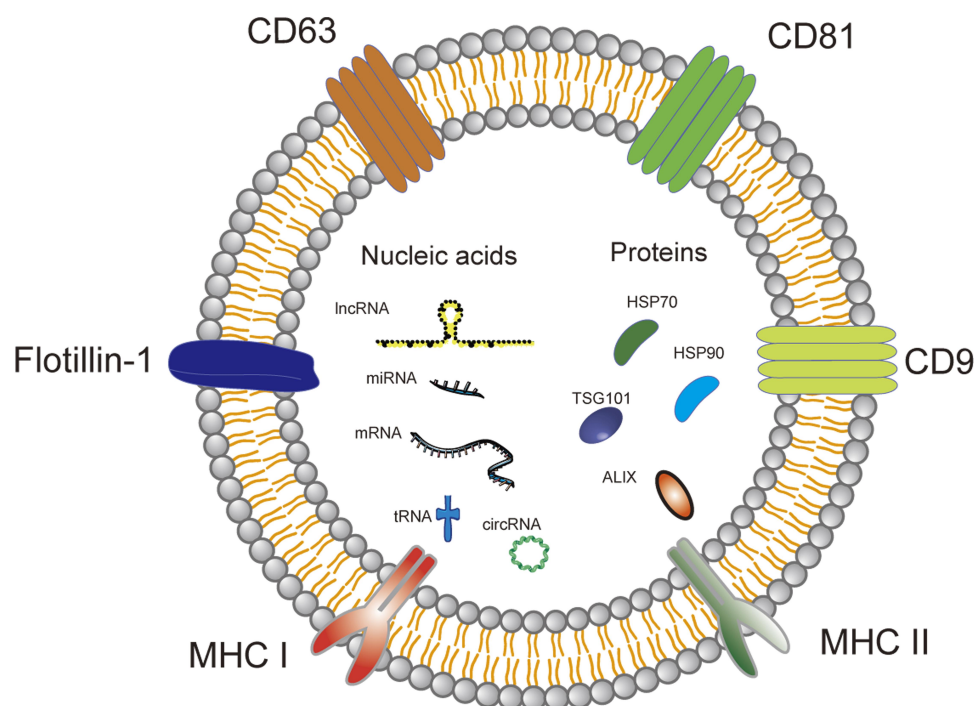


Figure 2 The composition and typical surface markers of exosomes. Exosomes are extracellular vesicles composed of bilayers of phospholipids, riched in proteins (HSP70, HSP90, TSG101 and ALIX) and nucleic acids (mRNA, miRNAs, lncRNA, tRNA and circRNA). There are some common proteins on the surface of exosome such as CD9, CD63, CD81, MHC and flotillin-1.

help fibroblast-derived EVs escaping from MPS clearance.³² Furthermore, the surface adhesion proteins and vector ligands on EVs such as tetraspanins, integrins, CD11b and CD18 receptors, make EVs prone to being taken up by target cells.^{33,34} Meanwhile, the specific origin of exosomes could boost them homing to their derived cells or tissue.³⁵ Thus, EVs have the natural advantages in protecting their cargoes, reducing clearance by the MPS and effectively increasing delivery to target tissues with reduced immunogenicity.^{15,36} Since the isolation, purification and identification of EVs have been well described in recent articles,^{37–39} here we focus our review on strategies for loading and modifying EVs to target delivering drugs. In addition, the term of EV is used throughout for convenience and general narration, but for the specific EV subsets such as exosomes or microvesicles, we will follow the original statement.

Methods for Loading EVs with Therapeutic Cargoes

EVs have tight and ordered phospholipid bilayers, destruction of EV integrity may lead to their elimination by the MPS, thus to load EVs efficiently without disrupting their membranes is very important. The current strategies can

be typically classified as endocellular loading methods and extracellular loading methods (Figure 3).

Endocellular loading methods mean incubating or transfecting donor cells with the therapeutic cargo, and then the cargo is enriched in donor cells and packed into EVs via endogenous cargo-selecting machinery during EV generation.^{40–44} Subsequently, the EVs with therapeutic cargo entrapped are isolated from culture medium of donor cells. This method mainly is applied to load nucleic acid drugs like miRNAs, small interfering RNAs (siRNAs) and small molecule drugs. Nevertheless, the loading efficiency of incubation or transfection are still unsatisfactory, on top of that the cargo or transfection reagents themselves might be toxic to donor cells. The methods to load cargo into EVs directly in extracellular environment are called extracellular loading methods, which include using transfection reagents, extrusion, electroporation, saponin treatment, sonication and freeze–thaw method.⁴⁵ Extracellular loading methods which mainly make transitory apertures on EVs, such as sonication, extrusion, and saponin, generally showed greater drug loading efficiency than endocellular loading methods,^{46–48} however, they require additional purification steps that may disrupt EV structural integrity.⁴⁹ The appropriate loading method should be considered carefully according to the

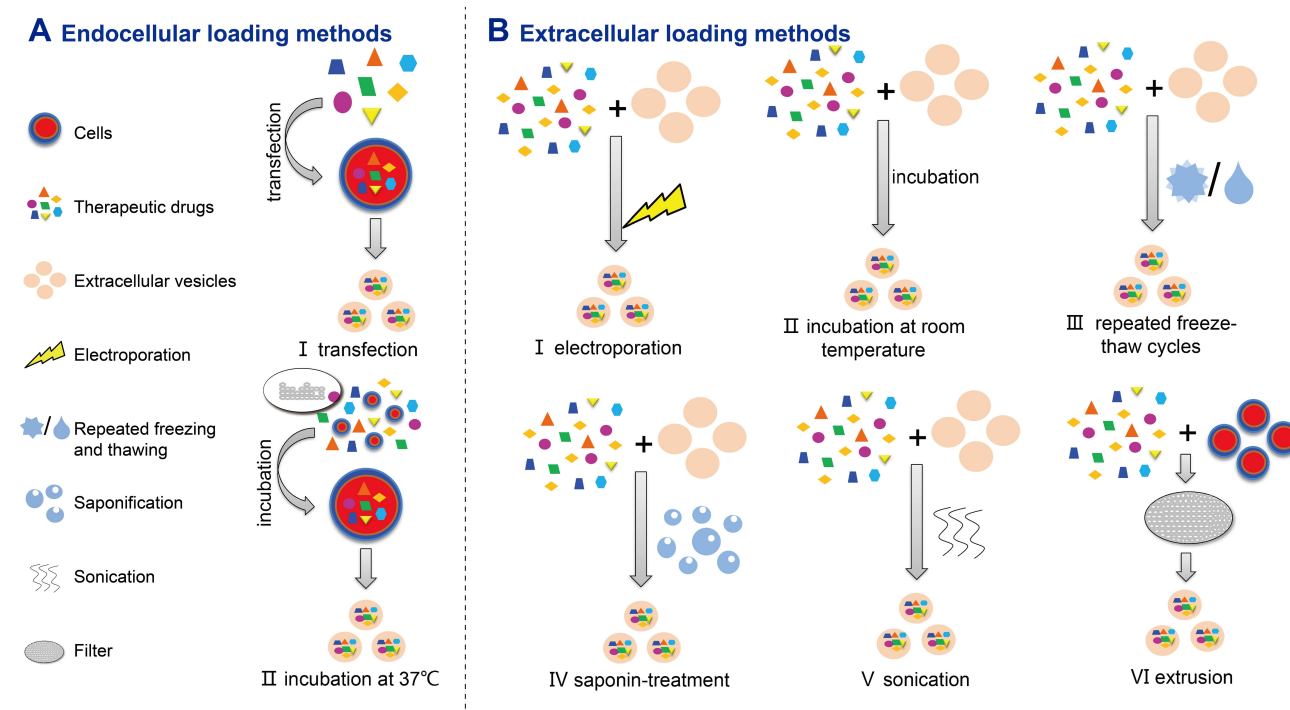


Figure 3 Schematic diagram of EV loading methods. **(A)** Loading of cells before EV isolation (endocellular loading): (I) transfection and (II) incubation; **(B)** loading of EVs after isolation (exocellular loading): (I) electroporation, (II) incubation at room temperature, (III) repeated freeze–thaw cycles, (IV) saponin treatment, (V) sonication, (VI) extrusion.

physical and chemical properties of the specific cargo. For hydrophobic agents, such as paclitaxel and curcumin, efficient loading can be achieved through simple co-incubation of EVs and drugs at room temperature.^{50,51} For hydrophilic drugs, the lipid bilayer will restrain their passive penetration into EVs.⁵² Hence, it is necessary to make transitory apertures on EV membranes to promote the hydrophilic drugs entering into the EVs. Recently, Haney et al studied the efficiency of loading catalase into EVs by different strategies. Their results confirmed that sonication is the most efficient method for loading proteins into EVs. The loading efficiency of these methods increased as follow: incubation, freeze–thaw cycles, extrusion, and sonication.⁴⁷ However each method has its advantages and disadvantages. For instance, using transfection reagent can improve the loading efficiency, but additional complicated purification processes are needed. Electroporation is another typically used method, which involves making a transient electrical field to generate pores on the EV lipid bilayer membrane. But electroporation needs the special equipment and testing optimum working conditions before experiment. The study focused on the horizontal comparison of drug loading efficiency between endocellular and extracellular methods, which is still rare. There is also an urgent need to develop a method that can detect the loading capacity of EVs precisely and conveniently to monitor delivery efficiency of EVs.

Delivery of Nucleic Acid Cargo by EVs

EVs have been considered as endogenous delivery carriers in delivering nucleic acids to cells. Thereafter, many of the studies have used EVs to deliver nucleic acid drugs. siRNAs are a class of double-stranded RNAs, which could interfere with the expression of specific genes by causing mRNA excision or restraining mRNA translation.⁵³ However, siRNA is unstable in circulation, and easy to eliminate as well as difficult to enter target cells.⁵⁴ Therefore, a specific delivery carrier is needed to bring siRNA to the disease site and then alleviate adverse effects.⁵⁴ EVs were first documented and engineered to deliver siRNA between cells in 2011. In this pioneering work, Alvarez-Erviti et al designed an experiment to express a neuron-targeting protein which is rabies virus glycoprotein (RVG)-derived peptide on the surface of exosomes derived from mouse dendritic cells. Then the siRNA of *BACE1* gene was loaded into these engineered exosomes. By injecting these exosomes into the bloodstream of mice, siRNA was specifically delivered to target cells in mice brains and effectively knocked down *BACE1*.³⁵ In another representative study, by using electroporation strategy,

fibroblast-derived exosomes were loaded with nucleic acid cargo targeting KRAS^{G12D}, a common oncogenic in pancreatic cancer. These nucleic acid cargos could be delivered into tumors in pancreatic ductal adenocarcinoma (PDAC) mice by exosomes. This study further confirmed exosomes have superior ability to deliver either siRNA or shRNA and suppress tumor growth in vivo.³² miRNA is a type of noncoding RNA with a length of 18 to 22 nucleotides, which has been proved to play a critical role in regulating gene expression by combining with the 3'UTR of target mRNA sequences to restrain mRNA translation or induce degradation.⁵⁵ The miRNAs that negatively regulate oncogene expression are potential tumor suppressors.⁵⁵ Theoretically, nanosized carriers designed for delivering siRNA can be easily introduced to miRNAs because of their similar physicochemical properties. In a recent study, Liu et al used miR-128-3p expression lentivirus to transfect normal intestinal FHC cells and got miR-128-3p packaging FHC cell exosomes. These exosomes could efficiently deliver miR-128-3p to oxaliplatin-resistant colorectal cancer (CRC) cells, suppress epithelial-mesenchymal transition (EMT) and increase intracellular oxaliplatin accumulation by suppressing the expression of Bmi1, an E-cadherin and ATP-dependent glutathione S-conjugate export pump, and a drug transporter MRP5.⁵⁶ In another study, Baldari et al constructed a lentiviral vector which expressed miR-125b linked with a specific ExoMotif sequence tag that was used to target miRNA into EVs. By transfecting mesenchymal stromal/medicinal signaling cells (ASCs) derived from human adipose tissue, miR-125b was efficiently loaded into ASC EVs, and specifically reduced cell proliferation in vitro by modulating a series of miR-125b target genes in hepatocellular carcinomas.⁵⁷ Similar studies emerged, by using lentiviral vector transfected into the donor cells as well, Zhang et al prepared miR-101 packaging EVs, which were able to inhibit osteosarcoma cell invasion and migration in vitro as well as inhibited osteosarcoma metastasis in vivo.⁵⁸

In addition to delivery of nucleic acid directly, EVs were employed as an advanced tool for genomic editing. CRISPR-Cas9 is a novel implement to accurately incise DNA sequences with the aid of a single guide RNA (sgRNA), which is complementary to the target gene.⁵⁹ In Lin et al's study, exosomes carrying sgRNA were prepared by transfecting sgRNA expressing vector into HEK293 FT cells. Results showed that this kind of nanoparticles could deliver CRISPR/Cas9 system to mesenchymal stem cells (MSCs) and regulate the target gene expression.⁶⁰ In Kanada et al's study, microvesicles derived from breast cancer cell lines 4T1 were used as the delivery vehicles. The authors cloned the gene of

a thymidine kinase (TK)/nitroreductase (NTR) fusion protein into a minicircle (MC) DNA vector. Through transfecting this MC DNA vector to 4T1 cell, they obtained the MC DNA vector loaded microvesicles. These microvesicles effectively delivered TK-NTR-encoding vector into the tumor tissue of breast cancer xenograft mice, and showed effective prodrug conversion and antitumor effect.⁶¹ Most studies focused on using EVs to deliver nucleic acid cargo via endocellular loading methods. However, although some specific sequence motifs of RNA appear to promote preferential EV sorting, such as the sequence motifs including “ACCAGCCU”, “CAGUGAGC”, “UAAUCCCA”, or zipcode-like 25-nt sequence with a “CTGCC” code domain,^{62,63} it is still unclear whether cells can selectively package target nucleic acid into EVs or not, and it is ambiguous how much transfected nucleic acid cargoes can be loaded into EVs. Moreover, lentiviral vector is the most popular transfection tool in these studies, and it should be very carefully evaluated whether the lentiviral vectors themselves have potential biosecurity risk before being used in vivo.

Delivery of Protein Cargo by EVs

Because proteins are relatively big in size and prone to loss activity, it is difficult to load any proteins into EVs directly. In an early study, cargo protein was overexpressed in donor cells to make them accumulate in EVs. Lee et al first overexpressed MHC class II (MHC-II) molecules in the murine melanoma cell line by transducing the Class II trans activator (*CIITA*) gene. The exosomes derived from transfected cells packaged a high amount of MHC-II and tumor antigen TRP2, which could significantly inhibit tumor growth by activating the immune response of DCs in vitro and inducing antitumor immune responses including promoting splenocyte proliferation and activation as well as IL-2 secretion in tumor-bearing mice.⁶⁴ In a subsequent study, a protein cargo, protein-cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT), was overexpressed in donor cells (HEK-293T cells) by Mizrak et al. Then the microvesicles (MVs) carrying CD-UPRT-EGFP mRNA and protein were isolated from donor cells and intratumorally injected into tumor-bearing mice. These MVs were capable of transferring their cargo and achieving a high level expression of functional protein in recipient cells, and further inhibiting schwannoma tumor growth in vivo.⁶⁵ Based on this method, Sterzenbach et al developed a novel approach of effectively packing protein into EVs. By labeling with a WW tag that leads to late-domain

(L-domain) pathway dependent recognition by the L-domain-containing protein Ndfip1, and resulting in cargo protein ubiquitination and loading into exosomes, Cre recombinase was ubiquitinated and subsequently loaded into EVs via transfecting corresponding vector to donor cells. The WW-Cre embed EVs could be taken up by recipient neurons in brain regions in the mouse model, indicating the potential usage of engineered EVs to deliver biologically active proteins across the BBB and treat brain tumor.⁶⁶ A few studies loaded protein cargo via simply co-incubation therapeutic protein with donor cells. In Barok et al's work, trastuzumab emtansine (T-DM1), an antibody drug, was used to treat the donor cells, HER2-positive tumor cells. Then the exosomes with T-DM1 embed could be obtained from HER2-positive tumor cells. These exosomes were able to deliver T-DM1 to other cancer cells and reduce their viability.⁶⁷ In general, before applying EVs to packaging protein cargo, some issues should be carefully considered. Firstly, what is the upper limit of the protein size that can be loaded into EVs, and how to load macromolecular cargo into EVs effectively? Moreover, whether the transmembrane proteins expressed on EVs for targeting or therapy purposes can be folded and modified correctly to maintain their biological activity. Noguchi et al found that the biological activity of proteins encapsulated in the EVs was highly affected by lyophilization. Thus, the proper preservation method of EVs carried proteins needs to be further considered.⁶⁸ Finally, whether the engineering and modification processes on donor cells would affect the morphology and composition of EVs remain unclear.

Delivery of Chemotherapy Drugs by EVs

Conventional chemotherapy drugs such as paclitaxel (PTX) and doxorubicin (DOX) have been most studied since the EV-based drug delivery strategy was established. PTX is a powerful antimitotic drug that has been approved by the Food and Drug Administration (FDA) to treat solid tumors. Due to its low aqueous solubility, PTX needs to be dissolved by Cremophor EL and ethanol before being used in patients.⁶⁹ In study from Kim et al, PTX was loaded into exosomes to treat multiple drug resistant (MDR) cancer. Through sonication treatment, PTX was loaded into exosomes derived from RAW 264.7 cells. These PTX-exosomes have shown ideal antitumor effects in several tumor cells and produced strong antineoplastic effect in lung metastases in mice models.⁷⁰ Then the same research team further improved the EV delivery system in the

following study. By mixing RAW 264.7 exosomes with aminoethylanisamide-polyethylene glycol (AA-PEG) and PTX under sonication condition, AA-PEG-exoPTX was prepared. As AA-PEG can target the sigma receptor, which is overexpressed by lung cancer cells, thus AA-PEG-exoPTX could accurately deliver PTX to lung cancer cells and show superior antimetastatic efficacy in murine pulmonary metastasis mice model.⁷¹

DOX, a powerful topoisomerase II inhibitor, is another popularly used chemotherapy drug. Unfortunately, DOX-based therapy has severe side effects like cardiotoxicity, nephrotoxicity, and neurotoxicity, hence further improvement on its target ability is needed urgently. In a study from Wei et al, exosomes derived from bone marrow MSCs (BM-MSCs) with tumor-homing features were isolated and mixed with DOX-HCl in a specific buffer (desalinizing with triethylamine), and then dialyzed overnight. The prepared Exo-DOX, is able to enhance cellular uptake efficiency and shows antitumor effects on osteosarcoma MG63 cell lines, but low cytotoxicity on myocardial cells.⁷² In another work, an exosome delivery strategy was developed to treat triple-negative breast cancer. A disintegrin and metalloproteinase 15 (A15) is the protein containing Arg-Gly-Asp (RGD) motif, which is a widely expressed membrane protein on tumor cells and involved in tumor progression and suppression. Gong et al reported that phorbol 12-myristate 13-acetate (PMA) stimulating could effectively increase the content of A15 in exosomes derived from THP-1 cells. By mixing DOX with A15 exosomes in triethylamine solution overnight, the DOX packaged A15-exo (A15-Exo/DOX) were obtained. Then the authors further co-incubated cholesterol-modified miR-159 (Cho-miR-159) with A15-Exo/DOX to form a co-delivery system. This co-delivery system showed therapeutic effects on triple-negative breast cancer both in vitro and in vivo.⁷³ Several nano materials for drug loading such as liposomes, chitosan and inorganic NPs have been employed to deliver chemotherapy drugs to tumors. As successor vehicles, EVs have become the new focus in this field. However, the origin of EVs might affect their uptake efficiency by recipient cells,⁷⁴ because EVs have an inherent homing property. Consequently, when delivering drugs to tumors in vivo, the donor cells should be carefully selected because EVs derived from normal cells may induce undesired drug accumulation in normal tissue. Thus, if the exosomes derived from heterologous normal cells can be used in chemotherapy drug delivery to avoid the drug

accumulating in normal tissue becomes an interesting question waiting to be answered.

EVs Engineering Strategies

In addition, to further enhance the efficiency of loading cargo into EVs and/or targeting delivery to the tumor site, many strategies have been established to engineer EVs by genetically engineering donor cells and/or modifying EVs directly via nongenetic engineering methods.

Indirect EV Engineering via Donor Cell Modification

Through genetically engineering donor cells, EVs can be modified including expressing new protein on their membrane or increasing the packaging efficiency of therapeutic protein cargo loading by specific localized peptide. In a pioneering work, a transmembrane domain of platelet-derived growth factor receptor was fused to the GE11 peptide which can specifically bind to EGFR on HEK293 cells, and then GE11 can be located on the exosomes of HEK293 cells. These engineered HKE293 cells were further transfected with synthetic let-7a miRNA. Through interacting between GE11 and EGFR expressed on tumors, this let-7a carrying exosomes was able to target tumor cells and deliver let-7a efficiently. This delivery system showed hypothetical effect on treating EGFR-expressing breast cancer in a xenograft breast cancer mouse model.⁷⁵ Also via targeting EGFR, Kooijmans et al anchored glycosylphosphatidylinositol (GPI) signal peptides with anti-EGFR nanobodies. By transfected donor cells with the vectors encoding fusion protein, anti-EGFR nanobodies enriched EVs were obtained, which could efficiently target EGFR-expressing tumor cells.⁷⁶ In addition to EGFR, Her2 is another commonly used tumor-associated membrane protein in related studies. Gomari et al expressed ankyrin repeated proteins (DARPs), a specific ligand against Her2 in MSCs, subsequently MSCs exosomes with Her2 ligand were isolated and loaded with DOX by electroporation. Through treating Her2+ and Her2- tumor cell lines respectively, these DOX-loaded exosomes showed better uptake efficiency by Her2+ target cells compared to the other one, which highlighted their target delivery ability.⁷⁷ In addition to utilizing guide peptides, cargo protein was fused with the protein associated with EV biogenesis to enhance the EVs loading efficiency in some studies. For example, by fusing the fragment of interleukin-3 (IL-3) ligand with EV marker protein

Lamp2b, Bellavia et al improved the loading efficiency of IL-3 ligand into EVs of HEK 293T cells. These IL-3 ligand-expressed EVs (IL3-L EVs) which carried imatinib or BCR-ABL siRNA, could target chronic myelogenous leukemia (CML) cells and inhibit cancer cell growth in vitro and in vivo.⁷⁸ As a tetraspanin, CD63 is a classical EV marker protein that plays a role in the sorting of EV cargos. And apolipoprotein A1 (Apo-A1) is a target of the scavenger receptor class B type 1 (SR-B1) receptor, which is highly expressed on the liver cancer cells surface. Liang et al transfected HEK 293T cells with a fusion protein which constructed both *CD63* and *Apo-A1* genes to obtain engineered EVs. These EVs, which were loaded with miR-26a via electroporation, could be taken up efficiently by HepG2 cells and decrease their proliferation and migration in vitro.⁷⁹ For tracking purposes, the fluorescent dyes that specifically label lipids such as PKH67, PKH26, and near-infrared lipophilic dye were used to label exosomes in many studies.^{80–83} However, it is difficult to eliminate the interference from the debris of plasma membrane and organelle by using these fluorescent dyes to label EVs as well because it is still unknown if fluorescent dye affects the size and

structure of EVs. Therefore, the improved strategy is to integrate the luminescent proteins such as eGFP, tdTomato and luciferase with EV membrane proteins to obtain luminous EVs in EV membrane to tracing uptake of EVs in vitro and biodistribution of EVs in vivo in some studies.^{83–85} The peptides that have been used in recent studies for anchoring cargo protein to EV surfaces or targeting tumor cells are summarized in Table 1.

At least two studies are devoted to controlling cargo protein anchored on the EVs membrane or released into the internal space as required. Yim et al developed a method to load cargo proteins into EVs during EV biogenesis. Two specific proteins, CRY-interacting basic-helix-loop-helix 1 (CIB1) and photoreceptor cryptochrome 2 (CRY2) were used in this study. Both of these proteins were from *Arabidopsis thaliana* and can regulate floral initiation through blue light-dependent phosphorylation. There are reversible protein–protein interactions that can be controlled by blue light between the two proteins. Using this module, a truncated CIB1 was conjugated to CD9 (an EV-associated tetraspanin protein), while cargo protein was conjugated to CRY2. Along with blue light turning on or turning off, the two proteins combined or

Table 1 The Guide Peptide and Targeting Proteins Employed in Studies

No	Guide Peptide and Protein on EVs Surface	Protein for Targeting to the Tumor Site	Donor Cells	Loaded Drugs	Recipient Cells and Target Site	Reference
1	The domain of platelet-derived growth factor receptor	GEII peptide (amino-acid sequence YHWYGYTPQNV)	HEK293 cells	let-7a miRNA	EGFR expressed breast cancer cells	75
2	Glycosylphosphatidylinositol (GPI)	Anti-EGFR nanobodies	Neuro2A cells HeLa cells A431 cells	NA	EGFR-expressing tumor cells	76
3	Ankyrin repeated proteins (DARPs)	Her2	MSCs	DOX	Her2+ and Her2- tumor cell lines	77
4	L-domain motifs on Ndfip1 with WW tag	Cre recombinase	Stable mT/mG reporter MEF line	NA	Brain tumor	66
5	Lamp2b	Interleukin-3 receptor	HEK293T	Imatinib mesylate	Chronic myelogenous leukemia cell	78
6	CD63	Apo-A1 sequence	293T	miR-26a	Scavenger receptor class B type I-expressing liver cancer cells (HepG2)	79

Abbreviations: HEK293 cells, human embryonic kidney cell line 293; MSCs, mesenchymal stem cells; DOX, doxorubicin.

separated, and the cargo protein could be controlled to anchor on the EV membrane or be released into the intraluminal space.⁸⁶ Based on similar concepts, Pi et al developed RNA nanotechnology that could control placing of RNA nanoparticles on the outer surface of the EV membrane, or loading into the EV lumen, for cargo loading purposes. In their strategy, membrane-anchoring cholesterol was placed at the arrowtail or arrowhead of the RNA aptamer, resulting in anchoring the RNA nanoparticles to the EV outer surface or loading the RNA into the EVs, respectively. These ligand-displaying EVs could specifically deliver siRNA to target cells and effectively inhibit tumor growth in three cancer models.⁸⁷

Direct EV Modification

Although the authors claimed they got the modified EVs by engineering the donor cells, the specific modification efficiency is still unclear. Direct modification strategy provides another simple and convenient way to engineer EVs. Smyth et al reported a strategy that can conjugate biomolecules to the EV surface directly via the click chemistry method, which is a copper-catalyzed azide-alkyne cycloaddition. In this study, alkyne groups were conjugated to EVs through a copper-catalyzed azide-alkyne cycloaddition. This process neither changed EV size nor affected its adherence or internalization by recipient cells. In addition, this method could bioconjugate the small molecules and macromolecules onto the surface of EVs successfully with several advantages, including fast high specificity and compatibility with aqueous buffers.⁸⁸ However, whether this method can be introduced to conjugate proteins such as receptors, ligands, or antibodies to EVs and keep their therapeutic activity is still to be revealed. A modified method that aimed to extend the *in vivo* circulation time of EVs was also developed. In the work from Kooijmans et al, EVs modified with EGFR conjugated to polyethylene glycol (PEG) were prepared. By mixing micelles with EVs of neuro2A cells, a temperature-dependent transfer process of nano-PEG-lipids to the EV membranes was achieved. This modification process did not affect EV morphology, size distribution, and protein composition but prolonged circulation times of the EVs in an animal model, possibly increasing EV accumulation in targeted tissues and improving cargo delivery efficiency.⁸⁹ However, there is an opposite view that EVs carrying PEGylation might trigger an anti-PEG IgM response, and lead to rapid clearance from the circulation.⁹⁰ In another study, Zhu et al mixed EV suspensions with special micelles formed by the

reaction of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]-c(RGDyK) (DSPE-PEG2000-cRGDyK) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer to obtain cRGD-modified EVs. These EVs could effectively deliver the antitumor drug PTX to glioblastoma cells.⁹¹ A33 is a uniformly expressed antigen in CRC. Li et al coated surface of carboxyl superparamagnetic iron oxide nanoparticles with A33 antibodies, which can bind to the A33 antigen on the exosomes derived from A33-positive CRC cells to prepare A33Ab carrying exosomes. Through multiple mixing steps in a special buffer system, DOX was loaded into this exosome to form A33Ab-US-Exo/DOX complex (A33 antibodies-nanoparticles-exosomes). This complex could be efficiently taken up by A33-positive colorectal cancer cells and exerted high binding affinity and antiproliferative effects *in vitro* and suppress tumor growth and extend the survival in the CRC mice model with lower cardiotoxicity.⁹² Endocytosis has been considered as the main pathway for the cellular uptake of EVs, and the macropinocytosis of the recipient cells will reorganize the actin, ruffle the plasma membrane, and engulf the large volumes of extracellular fluid.⁹³ Therefore, the activation of macropinocytosis will greatly help the cellular uptake of EVs. Nakase et al reported that it was helpful to functionalize EVs by enhancing the macropinocytosis induction for improving EV-based intracellular delivery of therapeutic molecules. In their studies, they demonstrated some useful methodologies for achieving enhanced cellular EV uptake by the epidermal growth factor (EGF) receptor, oncogenic K-Ras, pH-sensitive fusogenic peptide, and arginine-rich cell-penetrating peptides (CPPs).^{93–97}

Along with the rapid development of EV-based drug delivery in cancer treatment, various strategies have been constantly developed. However, it is still immature to apply EV-based DDS to clinical application. Based on a few clinical trials, therapeutic EVs only showed safety and feasibility in non-small cell lung cancer (NSCLC) patients (ClinicalTrials.gov identifier: NCT01159288) or a limited beneficial clinical response in lung cancer patients with malignant pleural effusion.⁹⁸ The major hindrance of applying an EV delivery system clinically includes the relative low yield of available EV carriers and unsatisfactory drug loading efficiency. To address these challenges, exosomes originated from a novel source could be tested in future.

Novel EVs in Antitumor Drug Delivery

Natural Product Derived EVs for Antitumor Drug Delivery

Natural derived EVs such as EVs derived from milk, fruit, and vegetables, etc may be the potential resources of available EVs in subsequent utilization to obtain a large number of EVs is to use milk as a very common food ingredient with large and stable source. The study of Aqil et al¹⁰⁰ proved that exosomes derived from bovine milk have properties of biocompatibility, low-cost, accessibility, stability in acidic environment and lack of toxicity when used as drug carriers.⁹⁹ In their subsequent work, exosomes isolated from raw milk were loaded with siRNA against specific genes including VEGF, EGFR, AKT, MAPK and KRAS by electroporation or chemical transfection. Results showed that these milk exosomes carrying siRNA can be taken up and showed the silent rate of target gene from near 20% to over 80% in different tumor cell lines. Among them, exosomes carrying siKRAS^{G12S} showed significant antitumor effect on lung tumor bearing mice.¹⁰⁰ Li et al reported that ginger derived exosome-like nanovesicles (GDENs) displayed similar size, density, and morphology to human derived exosomes. These ginger oriented nanovesicles have folic acid (FA) on their surface and can be targeted to deliver to KB cancer cells. By using exosomes transfection reagent, GDENs were mixed with arrow-tail RNA nanoparticle to prepare GDENs carrying the siRNA targeting *BIRC5* (baculoviral inhibitor with apoptosis repeat-containing 5) gene. These exosome-like nanovesicles from ginger were able to knockdown the target gene efficiently in vitro and inhibit tumor growth on a xenograft model by intravenous administration.¹⁰¹ Not limited to laboratory study, EVs derived from plants have been introduced into clinical trials. The researchers from the James Graham Brown Cancer Center used fruit-derived EVs to deliver curcumin to treat cancer patients. Curcumin has powerful inhibiting effect on colon cancer cell lines, however, because of low solubility, poor stability and is prone to be metabolized, oral curcumin has showed only limited bioavailability even at very high doses. Through using fruit-derived EVs transmitting, most of the major obstacles of curcumin application could be solved, including increased solubility, bioavailability, and stability. In this trial, the effect of curcumin on cellular metabolism, immune modulation, and phospholipid profiles of normal and malignant colon cells from colon cancer patients was characterized. Because it is still an ongoing work, the conclusion needs to be further

clarified (NCT01294072). Furthermore, some plant originated exosomes, such as lemon,¹⁰² grape,¹⁰³ and carrot¹⁰⁴ have been used in treating diseases. Because of the natural superiority, including numerous, they are inexpensive and easy to be obtained, exosomes derived from milk or plants are the potential candidates as drug delivery vehicles in cancer therapy.

EVs-mimetic Nanovesicles for Antitumor Drug Delivery

Other novel strategies to solve the main obstacles of lacking enough quantity of EVs are the prepared EV mimics. In a pioneering work, Jang et al developed a platform to prepare bioinspired exosome-mimetic nanovesicles rapidly and conveniently. The monocytes or macrophages were broken down by using a serial extrusion through filters with diminishing pore sizes (10, 5, and 1 μ m) in the DOX contained buffer to prepare DOX carrying nanovesicles (NVs). These DOX loaded NVs showed similar antitumor activity as DOX loaded exosomes and have better performance than DOX-loaded liposomes in vivo.⁴⁶ In a further study, Zhu et al prepared generous amounts of EVs like nanovesicles by extruding NK cells through filters with progressively smaller pore size. These NK cell derived EV mimetics have significant antitumor effect in vitro and in vivo.¹⁰⁵ By utilizing mechanical forces as well, a microfluidic device was fabricated by Jo et al to prepare EV mimetic nanovesicles. This microfluidic device mainly composed by microchannels, when cells flowed through slits in microchannels, they were stretched and generated nanovesicles due to their elongated shape caused by shear stress. These nanovesicles incorporated membrane proteins and were similar to exosomes in shape and content, and they were able to deliver RNA to target cells.¹⁰⁶ Some other devices based on squeezing cells by using mechanical force have also been developed recently,^{107–109} which can generate a great quantity of exosome mimetic nanovesicles and they are valuable for applications in drug delivery. However, further study is needed to investigate whether these devices of prepared nanovesicles contain organelle or cell fragments, and whether these by-products would induce undesirable inflammatory response in vivo.

EVs-liposome Hybrid Nanovesicles for Antitumor Drug Delivery

Liposomes are artificial vesicles composed of a lipid bilayer being widely used as nanocarriers in clinical trials.^{110–112} As the pioneer in nanobased DDS, liposomes

have been certified to reduce the side effects relative to free drugs.¹¹³ However, this kind of DDS has critical limitations: first, the chemical solvents and additives during the liposome generation procedure may cause toxicity for clinical use; second, the membrane of the liposome lacked biofunctional proteins which can initiate the signal transduction.¹⁰⁷ EVs have exhibited better cell targeting ability and low toxicity, therefore they were considered a promising nanocarrier in DDS.^{35,114} Despite being alternatives to conventional DDS, EVs still face drawbacks in low yield and the drug loading capacity limitation.¹¹⁵ Therefore, a novel hybrid approach was established recently. In this hybrid system, EVs with targeting ability and biocompatibility were combined with liposome production and drug loading. As in an aforementioned study, both fibroblast-derived exosomes and liposomes were loaded with nucleic acid cargo targeting KRASG12D, showing that this hybrid nanocarrier has better antitumor effect than liposomes.³² In another representative study, exosomes were incubated with a mixture of liposomes and pEGFP-C1 plasmids for 12 h at 37°C to prepare hybrid nanovesicles, which could overcome the volumetric restriction.⁶⁰ Other novel hybrid methods have been developed subsequently. Piffoux et al fused cell line-derived EVs and liposomes by using PEG. This strategy could efficiently enrich EVs with exogenous hydrophilic and lipophilic compounds while maintaining their intrinsic content and biological properties.¹¹⁶

Discussion

As emerging nanocarriers, EVs have many advantages in targeting delivery of drugs to tumor site; however, this DDS is still at an initial stage. How to choose appropriate EVs for drug delivery is the first challenge waiting to be solved. For instance, tumor cell derived EVs contain oncogenic drivers that may induce unexpected side effects in antitumor therapy. Substantial evidence demonstrated that EVs derived from MSCs or immunocytes may be ideal candidates. MSCs are well known to produce large amounts of EVs¹¹⁷ and show pleiotropic biological activity in treating multiple diseases, including cancer.^{118–122} Hence, MSC-derived EVs are a considerable option for drug delivery carriers in future studies. The donor cells used in the mentioned drug delivery studies are summarized in Table 2. Other potential candidates for clinical application are EVs derived from immunocytes. The protein composition of EVs depends on the donor cell type, somehow reflecting the specific functions of their parent cell. For instance, EVs derived from B cells carrying B cell

receptors (BCR),¹²³ T cells secrete EVs bearing T cell receptor (TCR), Src-like tyrosine kinases and adhesion molecules,¹²⁴ as well as DC-derived EVs containing the DC-associated proteins.¹²⁵ These specific immunoreceptors drive these EVs to target tumors exclusively, which make them excellent carriers for antitumor drug delivery studies. It is particularly worth mentioning that EVs derived from natural killer (NK) cells express NK-associated receptors, such as FASL, NKG2D, and cytotoxic proteins including perforin and granzyme.^{126,127} NK cells are one of the most essential antitumor immune cells, which could recognize and kill tumor cells without prior immunization and subsequently trigger the adaptive immune responses via secretion of various chemokines and cytokines.¹²⁸ NK cells have been widely used in clinics to treat cancers.^{129–131} Recent evidence indicated that NK cell-derived EVs have a cytotoxic effect on tumor cells and perform an antineoplastic immune response by activating other immune cells.¹³² Therefore, NK cell-derived EVs are ideal carriers to deliver antitumor drugs and have natural antitumor activity simultaneously in cancer therapy.¹³³ However, some obstacles of using immunocytes derived EV-delivery antitumor drugs should be considered. There are still biosafety risks remaining when using EVs derived from immune cell lines, but EVs derived from neither autologous nor allogenic primary immune cells may not meet the required quantity. A promising solution is preparing EVs like engineered nanovesicles carrying immunocyte-associated proteins or receptors. For instance, by incubating NK cell exosomes (NKEXO) with biomimetic core-shell nanoparticles (NNs) carrying let-7a mimics, Wang et al developed a NN/NKEXO cocktail DDS, and these cocktail nanoparticles have targeting and antitumor capabilities both in vitro and in vivo.¹³⁴ It is worth trying to ascertain whether these approaches could be utilized to acquire enough quantities of EV-like vesicles for clinical therapy. Furthermore, more novel trials have been reported including enhancing the release of bioactive EVs by interfering with the endolysosomal trafficking,¹³⁵ culturing cells at low pH conditions to increase the release of EVs¹³⁶ and developing a 3-D culture system with tangential flow filtration to enhance the yield and improve the activity of EVs.¹³⁷

Furthermore, there are other issues should be considered seriously in developing the EV-based DDS in future. First, what are the optimum sizes and the types of EV for delivering cargo to specific tissue? The size distribution of EVs depended on the extraction method, for instance, ultracentrifugation-isolated EVs are generally bigger than the EVs extracted by the ExoQuick precipitation method.¹³⁸ There

Table 2 The Donor Cells Used in the Mentioned Drug Delivering Studies

Donor Cells/Type	Species	Cargo	Target	Experiment Type	Reference
HEK-293T cells /normal cell line	Human	Protein-cytosine deaminase (CD) fused to uracil phosphoribosyltransferase (UPRT)	HEI-193FC cells (schwannoma tumors cell line)	In vitro In vivo	65
		Truncated CIB1 protein conjugated to CD9	Cerebral cortex tissues of mice	In vitro In vivo	86
Colorectal cancer (CRC) cells /tumor cell line		A33 antibodies, doxorubicin (DOX)	A33-positive CRC cells	In vitro In vivo	92
Foreskin fibroblast (BJ) cells/primary cell		SiRNA and shRNA of Kras ^{G12D}	Panc-1 cells (pancreatic cancer cell line)	In vivo	32
Fetal normal colonic cell /normal cell line		miR-128-3p	HCT116OxR and HT29OxR cells oxaliplatin-resistant cell lines ()	In vitro In vivo	56
Adipose tissue-derived mesenchymal stromal /primary cell		miR-125b	Hepatocellular carcinoma cell	In vivo	57
		miR-101	Osteosarcoma cells	In vitro In vivo	58
HER2-positive or negative tumor cells/tumor cell line		Antibody-drug trastuzumab emtansine (T-DM1)	HER2-positive or negative tumor cells	In vitro	7
THP-1 cell/Tumor cell line		Disintegrin and metalloproteinase 15 (A15), DOX, cholesterol-modified miR-159	MDA-MB-231 cells (triple-negative breast cancer cell line)	In vitro In vivo	73
H9 cell (embryonic stem cells)/normal cell line		Paclitaxel (PTX)	Human tumor cell lines (U87, U251, A549, HepG2, B16, MDA-MB-231, and DU145)	In vitro In vivo	91
HEK293 FT cells/normal cell line		CRISPR/Cas9 system	Mesenchymal stem cells	In vitro	60
Dendritic cells/primary cell	Murine	siRNA of BACE1	C2C12 cells, mice	In vitro and in vivo	35
Breast cancer cell 4T1/ tumor cell line		Thymidine kinase (TK)/nitroreductase (NTR) fusion protein DNA vector	Tumor tissue	In vivo	61
Melanoma cell B16F1/ tumor cell line		MHC-II, tumor antigen TRP2	DC and other immunocyte of mouse,	In vivo	64
Mouse embryonic fibroblasts (MEF)/primary cell		Cre recombinase with WW tag	LN18 glioblastoma cells; mice brain	In vitro In vivo	66
RAW 264.7 cells/Tumor cell line		PTX	Multiple drug resistance (MDR) tumor cells	In vitro in vivo	70
		PTX, aminoethylanisamide-polyethylene glycol (AA-PEG)	Lung cancer cells	In vitro In vivo	71
Mouse bone marrow MSC/ primary cell		DOX-HCl	Osteosarcoma MG63 cell	In vitro	72

is evidence that the size of EVs could affect the interaction between EVs and recipient cells; smaller EVs can be taken up more by recipient cells and induce higher response to the “EV cargo”.¹³⁸ In the case of NPs, 40–50 nm is the optimum diameter range with the greatest cell uptake rate.¹³⁹ In

regard to EVs, what is the optimal size for EV uptake by recipient cells is a question worth investigating.

Second, during EV isolation, the impurities that have similar sizes or sedimentation coefficients with EVs, such as debris from the cell membrane, large heteroproteins, or

organelles, could not be excluded. These impurities may disturb the effect of the EV-based drug delivery. Recently, the recommended method for EV isolation has been published in the Minimal Information for Studies of Extracellular Vesicles 2018 guidelines.¹⁷ Subsequent studies should follow the standard protocol to obtain EVs with stable quality and make the drug delivery study scalable and reproducible. Furthermore, more economical and convenient methods for EV isolation are still urgently needed.

Third, it is known that there are at least three ways for EVs to interact with recipient cells, including engulfment by recipient cells, fusing EVs with the cytoplasmic membrane of recipient cells and releasing their intraluminal contents, and transmitting signaling molecules and inducing signaling cascades through interactions between the receptor and ligand located on the cytomembranes of recipient cells and EVs.¹⁹ Which is the most efficient way for EVs to affect recipient cells and delivering their cargo, including the cargo loaded into EVs or linked on their outer membrane to recipient cells, is still to be deciphered?

Last but not least, although studies have proven that intraperitoneal injection of EVs into experimental animals

cannot generate both toxicity and immune response,¹⁴⁰ several crucial issues should be settled before the EV-based DDS is translated to clinical therapeutics. Evidence showed that in animals, unmodified EVs would preferentially accumulate in liver, kidney, and spleen and are easily eliminated by bile excretion, renal filtration, or phagocytosis in the reticuloendothelial system, leading to very low concentrations of exogenous EVs in target tissues.¹⁴¹ Moreover, the results obtained from animal models cannot be directly applied to humans because of the differences in physiological structure and immune response between human and animals. Finally, as an endogenous mediator for intercellular communication *in vivo*, EVs play a very important role in every aspect of the biological process. Whether there are any potential risks that the exogenous EVs disturb the normal function of endogenous EVs by competition or some unknown mechanism should be assessed carefully.

Conclusion

The EVs based drug delivering system is being developed. Various cargoes such as nucleic acid, proteins, and chemotherapeutics have been loaded into EVs and tested in

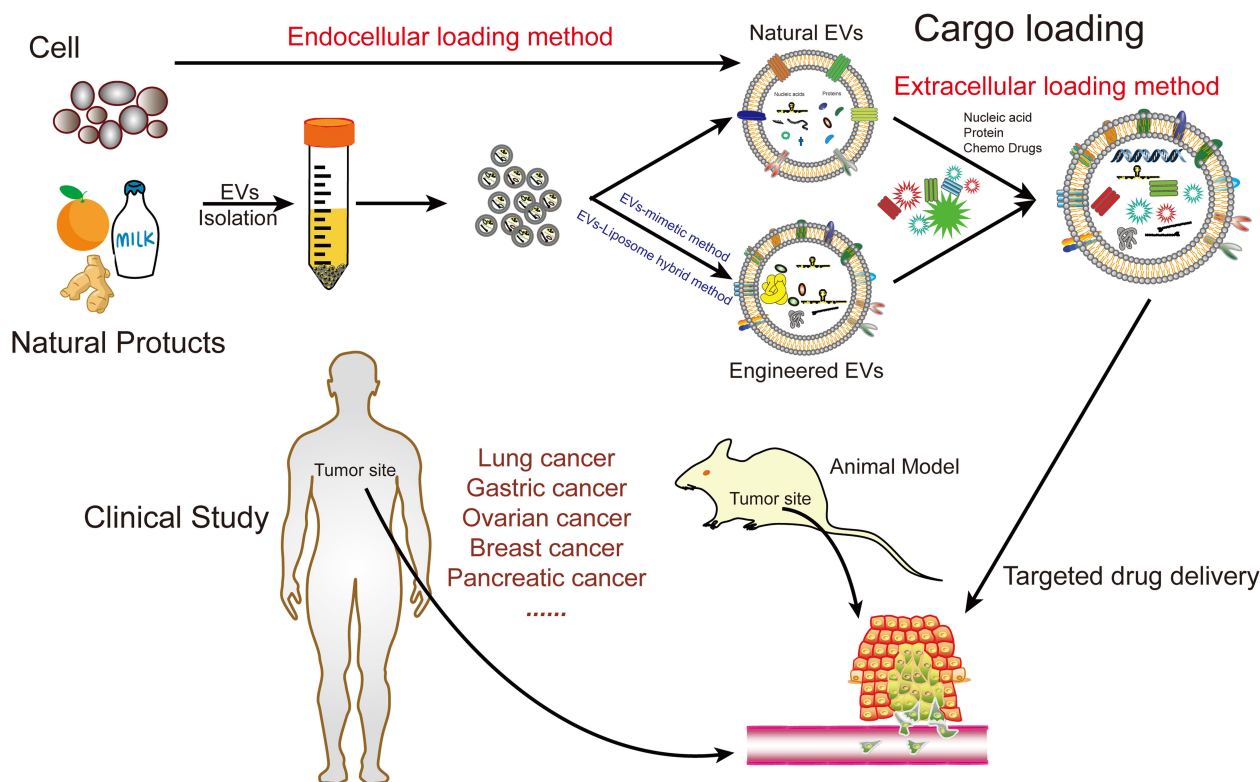


Figure 4 EVs as targeted transport nanocarriers in cancer therapy.

multifarious in vitro and in vivo antitumor models as well as clinical treatment of cancer (Figure 4). Several key points, including increased loading efficiency, increased targeting efficiency, increased circulation time in vivo, reduced side effects, and obtaining stable and abundant EVs are still the main development directions which deserve the researchers in this field to devote their vigor in.

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Disclosure

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