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Review

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Extracellular vesicles (EVs)' journey in recipient cells: from recognition to cargo release

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Abstract: Extracellular vesicles (EVs) are nano-sized bilayer vesicles that are shed or secreted by virtually every cell type. A variety of biomolecules, including proteins, lipids, coding and non-coding RNAs, and mitochondrial DNA, can be selectively encapsulated into EVs and delivered to nearby and distant recipient cells, leading to alterations in the recipient cells, suggesting that EVs play an important role in intercellular communication. EVs play effective roles in physiology and pathology and could be used as diagnostic and therapeutic tools. At present, although the mechanisms of exosome biogenesis and secretion in donor cells are well understood, the molecular mechanism of EV recognition and uptake by recipient cells is still unclear. This review summarizes the current understanding of the molecular mechanisms of EVs' biological journey in recipient cells, from recognition to uptake and cargo release. Furthermore, we highlight how EVs escape endolysosomal degradation after uptake and thus release cargo, which is crucial for studies applying EVs as drug-targeted delivery vehicles. Knowledge of the cellular processes that govern EV uptake is important to shed light on the functions of EVs as well as on related clinical applications.

Key words: Extracellular vesicle (EV); Exosome; Endocytosis; Uptake; Release

1 Introduction

In 2018, the International Society for Extracellular Vesicles (ISEVs) proposed to use "extracellular vesicles" (EVs) as a generic term for particles naturally released from cells (Théry et al., 2018). Currently, at least three major subgroups of EVs have been defined based on their biogenesis: (a) apoptotic bodies, 50 nm–5 μm; (b) microvesicles, 50 nm–1 μm; and (c) exosomes, 30–150 nm (György et al., 2011; el Andaloussi et al., 2013; Yáñez-Mó et al., 2015; Deb et al., 2021; van Niel et al., 2022). Apoptotic bodies and microvesicles bud directly from the plasma membrane, while the release of exosomes involves the formation of multivesicular bodies (MVBs). MVBs are formed by the fusion of intracellular vesicles with early endosomes (EEs), and these MVBs fuse with the plasma membrane to release intraluminal vesicles (ILVs) into the extracellular environment to form exosomes (van Niel et al., 2022). It is worth noting that current EVs' separation strategies allow for the classification of EVs based on physical characteristics (size, density), different biochemical components, and surface charges, but not based on their biogenesis sites (Claridge et al., 2021). As a result, some researchers rely on the general term "EVs" to avoid misunderstandings or incorrect definitions. The ISEV is also suggesting in their 2018 guidelines Minimal Information for Studies of Extracellular Vesicles (MISEV) the use of the term "EVs" instead of "exosomes" and "microvesicles." Here, in order to have a better understanding, we use the original term ("exosomes," "microvesicles," or "EVs") in the cited literature when quoting the corresponding literature.

EVs are widely found in body fluids and can be released by almost all types of cells in the human body (Wiklander et al., 2019). They are rich in bioactive molecules such as proteins, lipids, and nucleic acids, and play an important role in intercellular communication as well as the regulation of a variety of biological processes (Cheng and Hill, 2022). For example,

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in Parkinson's disease, exosomes translocate toxic α-synuclein oligomers to immature neurons and into the extracellular environment, inducing oligomerization of α-synuclein in normal neurons (Li KL et al., 2022). Wang et al. (2022) found that EVs derived from malignant ascites promoted the invasion and migration of ovarian cancer by transferring microRNA (miR)-1246 and miR-1290. Therefore, therapeutic interventions could be developed to alleviate disease progression by reducing circulating EV load or blocking crucial components of EVs (el Andaloussi et al., 2013). In addition, the cargo carried by EVs is a promising biomarker. For example, miR-21, miR-222, and miR-124-3p in serum exosomes may be molecular biomarkers for use in early tumor progression assessment during the post-surgical treatment of high-grade gliomas (Olioso et al., 2021). Tao et al. (2021) suggested that EVs-carried adipocyte enhancer-binding protein 1 (*AEBP1*) mRNA could be used as a novel and effective biomarker for the diagnosis of diabetic kidney disease. Wang X et al. (2021) found that plasma exosomal miR-363-5p has great potential for non-invasive lymph node staging and prognosis prediction in breast cancer.

Recent research has focused on EVs as targeted delivery vehicles for therapeutic molecules. EVs have several advantages that make them ideal for drug delivery: (a) their ability to utilize endogenous cellular mechanisms to produce and sort the desired cargo; (b) their ability to efficiently cross biological barriers, such as the blood–brain barrier, while maintaining their structural integrity; and (c) their ability to reduce the toxic effects of drugs (Elsharkasy et al., 2020; Chitti et al., 2022). It was found that loading doxorubicin onto exosomes effectively increased the uptake efficiency of doxorubicin compared to free doxorubicin, thereby improving survival in mice (Li et al., 2018). Recently, Zhou et al. (2022) prepared hybrid lipid nanovesicles by fusing hepatocellular carcinoma cell-derived EV membranes with phospholipids. They found that these vesicles demonstrated "homing" targeting to parent cells and could bypass the endosomal degradation pathway, thus improving the delivery efficiency of small interfering RNA (siRNA).

Given the important role of EVs in normal physiological processes and diseases and their therapeutic potential, there is a need to better understand the biology of EVs. Several mechanisms have been described to explain the biogenesis, composition, and release of EVs from donor cells (van Niel et al., 2018, 2022). However, the mechanisms of EV binding, entering, or releasing the cargo in recipient cells are still not fully understood. Here, we will focus on these three steps based on current studies (Fig. 1).

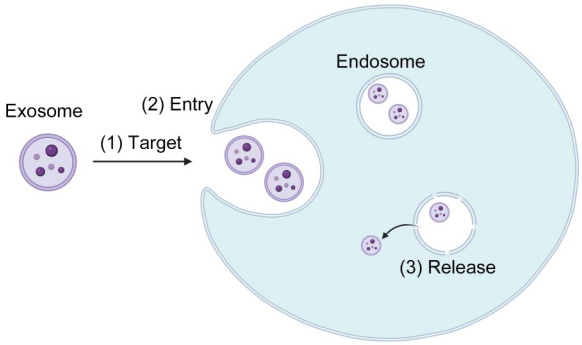

Fig. 1 Diagram of exosome uptake patterns. It is divided into the following three main steps: (1) exosome targets the recipient cell; (2) exosomes are uptaken by the recipient cell and enter into the cell; and (3) exosomes are released into the recipient cell. This figure was created with BioRender.com.

2 Recognition

The interaction between EVs and recipient cell surface components plays an important role in the recognition of, and binding to, recipient cells of EVs, thereby facilitating subsequent uptake. Here, we mainly review the components that have been shown to be involved in the recognition and uptake of EVs (Table 1).

2.1 Tetraspanin

Tetraspanin is a conserved transmembrane protein that has the ability to bind to different transmembrane receptors and form tetraspanin-enriched microdomains among themselves as a platform for regulating the avidity of adhesion receptors at the plasma membrane and linking receptors to intracellular or recycling routes (Yáñez-Mó et al., 2009). Among them, cluster of differentiation 9 (CD9), CD63, and CD81 are abundantly expressed on the surface of EVs and are commonly used as EV biomarkers (Andreu and Yáñez-Mó, 2014; Mizenko et al., 2021). There is increasing evidence that tetraspanin not only affects the biogenesis and composition of EVs but is also involved in the docking of EVs to recipient cells (Hazawa et al.,

Table 1 Components involved in the identification and uptake of extracellular vesicles (EVs)

Surface recognition molecules (EVs or exosomes)		Donor cells	Recipient cells	Target molecules on the surface of recipient cells	Function	Action mechanism	Potential application	Reference
Molecular type	Specific molecule							
Tetraspanins	Cluster of differentiation 9 (CD9)	Melanoma cells (FEMX-I, A375, and C8161) and mesenchymal stromal cells	Melanoma cells (FEMX-I cells)	Unknown	Participates in the uptake of EVs by recipient cells	Unknown	Divalent CD9 Ab could cross-link CD9 proteins associated with host cells and EVs to stimulate the endocytosis on EVs which may favor tissue/organ repair.	Santos et al., 2019
		Colorectal carcinoma cells (COLO-320)	Colorectal carcinoma cells (COLO-320)	Unknown	Results in reduced adhesion and uptake of exosomes by recipient cells	CD9 inhibits the interaction between the ligand ADAM17 on exosomes and the receptor integrin α5β1 on cells.	CD9 may be a promising therapeutic target to inhibit peritoneal carcinogenesis in colorectal cancer.	Cardeñes et al., 2021
	CD81	Human cervical cancer cells (HeLa)	Liver carcinoma cells (Huh-7) and gastric carcinoma cells (NCI-N87)	Laminin	Promotes the binding and uptake of EVs by recipient cells	CD81 interacts with laminin.	Taking advantage of the feature that laminin-binding CD81 enhances cellular uptake of EVs, recombinant expression of CD81 on the surface of EVs enables EV-mediated specific delivery to various cells and tissues.	Vogt et al., 2021
	Tetraspanin-8 (TSPAN8)/CD151	Human breast cancer cells (T-47D)	Human breast cancer cells (T-47D) and human fetal lung fibroblasts (MRC-5)	Unknown	Promotes small EV (sEV) attachment to recipient cells and uptake in vivo	TSPAN8/CD151 increases the attachment efficiency and reduces the evasion frequency.	TSPAN8-sEV promotes cancer cell motility and invasion and may serve as an important direct or indirect therapeutic target.	Wang T et al., 2021

To be continued

Table 1 (continued)

Surface recognition molecules (EVs or exosomes)		Donor cells	Recipient cells	Target molecules on the surface of recipient cells	Function	Action mechanism	Potential application	Reference
Molecular type	Specific molecule							
Integrin	Integrin α2β1	Carcinoma-associated fibroblasts (CAF-A1 and A2)	Lung fibroblasts (isolated from the lung tissues of C5BL/6 J mice)	Unknown	Mediates the uptake of EVs by recipient cells	Unknown	Plasma EV integrin β1 can be used as a candidate biomarker for predicting the prognosis of salivary adenoid cystic carcinoma metastasis and directing anti-metastatic therapies.	Kong et al., 2019
	Lymphocyte function-associated antigene-1 (LFA-1)	Macrophages (Raw 264.7)	Human cerebral microvascular endothelial cells (hCMEC/D3)	Intercellular adhesion molecule-1 (ICAM-1)	Mediates the uptake of exosomes by recipient cells	LFA-1 interacts with ICAM-1.		Yuan et al., 2017
Laminin	Laminin α5, laminin β1, and laminin γ1	Human ovarian cancer cells (A2780)	Omental macrophages	Integrin αvβ5	Promotes the uptake of exosomes by recipient cells	Laminin α5, laminin β1, and laminin γ1 interact with integrin αvβ5.	Laminin plays an important role in tumor progression by remodeling the tumor microenvironment in exosomes generated from E26 transformation specific-1 (ETS1)-overexpressing ovarian cancer cells, which may contribute to the treatment of omental metastasis in ovarian cancer.	Li HY et al., 2022
	Laminin γ2	Oral squamous cell carcinoma cells (LN1-1)	Human dermal lymphatic endothelial cells	Integrin α3	Mediates the uptake of EVs by recipient cells	Laminin γ2 interacts with integrin α3.	The uptake of laminin γ2-enriched EVs by lens epithelial cells (LECs) enhanced in vitro lymphangiogenesis, so EV-borne laminin-332 may be a viable biomarker for oral squamous cell carcinoma (OSCC).	Wang et al., 2019
Proteoglycan	Syndecan-4	Human breast cancer cells (BT-549)	Human glioblastoma cells (LN229)	Fetuin-A	Promotes the uptake of exosomes by recipient cells	Syndecan-4 interacts with fetuin-A.		Ochieng et al., 2018

To be continued

Table 1 (continued)

Surface recognition molecules (EVs or exosomes)		Donor cells	Recipient cells	Target molecules on the surface of recipient cells	Function	Action mechanism	Potential application	Reference
Molecular type	Specific molecule							
Fibronectin (FN)	Unknown	Human myeloma cells (CAG)	Human myeloma cells (RPMI-8226)	Heparin sulfate proteoglycan (HSPG)	Participates in the interaction between recipient cells and exosomes	FN binds to the surface of exosomes via HSPG and acts as a ligand to the receptor HSPG on the surface of the recipient cells.		Purushothaman et al., 2016
Sialic acid	Unknown	Human adipose-derived mesenchymal stem cells	Human cervical cancer cells (HeLa)	Siglecs	Mediates the uptake of exosomes by recipient cells	Sialic acid interacts with siglecs.		Shimoda et al., 2017
Carbohydrate	Mannose and glucosamine	Macrophages (Raw 264.7)	hCMEC/D3	C-type lectin	Mediates the uptake of exosomes by recipient cells	Mannose and glucosamine interact with C-type lectin.		Yuan et al., 2017
Chemokine	C-C motif chemokine ligand 2 (CCL2)	Breast cancer cells (EO771)	C-C-motif receptor 2 (CCR2) immune cells	CCR2	Alters exosome biodistribution and cell lineage-specific uptake	CCL2 binds to the surface of exosomes via the glycosaminoglycan (GAG) side chains of proteoglycans, and CCL2-modified exosomes are targeted to a subpopulation of cells expressing CCR2.		Lima et al., 2021
	CCL18	Glioblastoma cells (GBM8)	Glioblastoma cells (GBM8)	CCR8	Mediates the specific uptake of EVs	CCL18 acts as a “connectokine” between glycans decorated on the EV membrane and the cellular receptor CCR8.	The mechanism of EV uptake involving a chemokine receptor and its natural ligand acting as a connecting adaptor opens the door to new anticancer approaches based on chemokine-receptor targeting of tumor EV crosstalk.	Berenguer et al., 2018

To be continued

Table 1 (continued)

Surface recognition molecules (EVs or exosomes)		Donor cells	Recipient cells	Target molecules on the surface of recipient cells	Function	Action mechanism	Potential application	Reference
Molecular type	Specific molecule							
Adhesion molecules	Activated leukocyte cell adhesion molecule (ALCAM)/ CD166	Human ovarian adenocarcinoma cells (SKOV-3), colorectal carcinoma cells (COLO-320), and human peritoneal mesothelial cells (LP9/ TERT-1)	Human ovarian adenocarcinoma cells (SKOV-3) and colorectal carcinoma cells (COLO-320)	ALCAM/CD166	Supports the interaction of EVs with recipient cells and participates in subsequent EV uptake	ALCAM/CD166 interacts with ALCAM/CD166.	The ALCAM/CD166 may be potentially exploited to block the peritoneal metastasis cascade promoted by EVs in colorectal cancer and ovarian cancer patients.	Cardeñes et al., 2022
Postsynaptic density-95, Drosophila discs-large, and zona occludens-1 (PDZ) proteins	Unknown	HEK293T cells	Human breast cancer cells (MCF-7)	Syndecan-binding protein (SYNTENIN1), Golgi reassembly stacking protein 2 (GORASP2), and mitochondrial serine peptidase 2 (HTRA2)	Promotes the uptake of exosomes by recipient cells	Unknown		Castro-Cruz et al., 2023
	Unknown	HEK293T cells	Human breast cancer cells (MCF-7)	LNX1	Inhibits the uptake of exosomes by recipient cells	Unknown		Castro-Cruz et al., 2023
Interferon-induced transmembrane protein (IFITM)	Unknown	Fibroblast	Colorectal carcinoma cells	IFITM1	Inhibits the uptake of exosomes by recipient cells	Unknown	The *Apc* mutation-induced IFITM1 expression and the higher level of IFITM1 in CRC cells raise the possibility that normal colon epithelial cells may be targeted even more efficiently by EVs.	Kelemen et al., 2021
Others	A disintegrin and metalloproteinase 17 (ADAM17)	Colorectal carcinoma cells (COLO-320)	Colorectal carcinoma cells (COLO-320)	Integrin α5β1	Mediates the binding and uptake of exosomes by recipient cells	ADAM17 interacts with integrin α5β1.	α5β1 and ADAM17 are promising therapeutic targets to inhibit peritoneal carcinogenesis in colorectal cancer.	Cardeñes et al., 2021

2014; Rappa et al., 2017; Santos et al., 2019; Cardeñes et al., 2021; Wang T et al., 2021; Nigri et al., 2022). Several studies have highlighted the importance of CD9 in EV internalization. The silencing of CD9 in cells or EVs significantly inhibited the uptake of EVs by recipient cells, and treatment with a monovalent Fab fragment of the anti-CD9 monoclonal antibody also reduced the internalization of EVs (Santos et al., 2019). Interestingly, divalent CD9 antibody treatment enhanced EV uptake, possibly because divalent CD9 antibodies could cross-link CD9 proteins associated with host cells and EVs and consequently stimulate the endocytosis of EVs (Santos et al., 2019). Nigri et al. (2022) also indicated that CD9 on the cell surface played a key role in the internalization of cancer-associated fibroblasts-derived annexin A6-positive EVs in pancreatic ductal carcinoma cells. In contrast, Cardeñes et al. (2021) discovered that integrin α5β1 on colorectal cancer (CRC) cells or peritoneal mesothelial cells (PMCs) interacts with exosome ligand a disintegrin and metalloproteinase 17 (ADAM17) to mediate the binding and uptake of cancer-derived exosomes, a process that is negatively regulated by exosome surface CD9. A recent study showed that tetraspanin-8 (TSPAN8) promoted the adhesion of small EV (sEV) to parent cells and human fetal lung fibroblasts in vitro by increasing the attachment efficiency and reducing the evasion frequency (Wang T et al., 2021). Meanwhile, the results of in vivo experiments revealed that TSPAN8 promoted the uptake of sEV by cells in the lung, liver, and spleen (Wang T et al., 2021). Hazawa et al. (2014) also suggested that exosomes selectively bind to the radiation-induced formation of integrin (CD29)/tetraspanin (CD81) complexes on the cell surface, increasing the uptake of exosomes by mesenchymal stem cells.

2.2 Integrin

Integrin is a transmembrane heterodimer, formed by the noncovalent bonding of alpha and beta subunits, that mediates cell adhesion and migration, intercellular communication, and intracellular signaling activation (Shen et al., 2021). Recent studies have shown that integrins play a significant role in the binding and uptake of EVs (Carney et al., 2017; Shentu et al., 2017; Wang et al., 2019; Altei et al., 2020; Fuentes et al., 2020; Yoon et al., 2020; Cardeñes et al., 2021; Li et al., 2021). On the one hand, recipient cells relied on their surface integrins for EV binding and uptake. For example, Kuroda et al. (2019) treated human cerebral microvascular endothelial cells (hCMEC/D3) with a mixture of anti-integrin α5 and anti-integrin αV antibodies and found a reduced uptake of exosomes compared to controls. Integrin β3 on the surface of breast cancer cells interacted with heparin sulfate proteoglycan (HSPG) to promote the extracellular recognition and the intracellular endocytosis of EVs through activating focal adhesion kinase (FAK) (Fuentes et al., 2020). Integrin α5β1 on CRC cells (and PMCs) interacted with the ligand ADAM17 on CRC cell-derived exosomes to mediate exosome binding and uptake (Cardeñes et al., 2021). Oral squamous cell carcinoma (OSCC) cells secreted laminin γ2-enriched EVs, which acted as ligands to bind integrin α3 on the surface of lymphatic endothelial cells (LECs) to regulate EV uptake (Wang et al., 2019). Similarly, the interaction of laminin and integrin αvβ5 promotes the uptake of exosomes derived from E26 transformation specific-1 (*ETS1*)-overexpressing ovarian cancer cells by omental macrophages (Li HY et al., 2022). Interestingly, gastric epithelial cell-derived exosomes were only internalized into gastric epithelial cells and gastric cancer cells, and this gastric-specific uptake of gastric-derived exosomes was dependent on integrins α6 and αX in both gastric cells and exosomes (Yoon et al., 2020). On the other hand, integrins expressed on EVs mediate their interaction with recipient cells. Feng et al. (2022) treated placental EVs with integrin-blocking peptides, RGD and YIGSR, and found that the uptake of EVs by the recipient cells was significantly reduced. Kong et al. (2019) demonstrated that the use of TC I-15, an inhibitor of integrin α2β1, resulted in a dose-dependent inhibition of the uptake of carcinoma-associated fibroblasts-derived EVs by lung fibroblasts. The binding of sEV to breast epithelial cells also depended on exosomal integrin αvβ3 via forming integrin–extracellular matrix (ECM) complexes that might further interact with cellular integrin receptors (Altei et al., 2020). Yuan et al. (2017) found that the integrins lymphocyte function-associated antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) were expressed in macrophage exosomes and endothelial cells, respectively, and anti-ICAM-1 or anti-LFA-1 antibodies and their combinations inhibited the uptake of exosomes. It was suggested that the interaction of LFA-1 and ICAM-1 plays an important role in

the uptake of exosomes from macrophages by brain endothelial cells.

2.3 Proteoglycans

Proteoglycans (PGs) are composed of a "core" protein and one or more covalently linked glycosaminoglycan (GAG) side chains (Esko et al., 2009). Six major types of GAGs are currently identified in mammals: chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparan sulfate (HS), heparin (Hep), and hyaluronic acid (HA) (Walimbe and Panitch, 2020). Among them, HSPG and chondroitin sulfate proteoglycan (CSPG) have been shown to be involved in the recognition and uptake of EVs (Christianson et al., 2013; Nangami et al., 2014; Chen and Brigstock, 2016; Purushothaman et al., 2016; Osawa et al., 2017; Ochieng et al., 2018; Choi et al., 2021; Lima et al., 2021). Studies have shown that HS plays a dual role in exosome–cell interaction; HS on exosomes captures fibronectin (FN) and, on target cells, it acts as a receptor for FN, which is essential for the interactions between cells and exosomes (Purushothaman et al., 2016). Besides FN, histones can also serve as ligands by which HSPG can mediate the uptake of exosomes (Nangami et al., 2014; Ochieng et al., 2018). Notably, breast cancer cell exosomes bound the tumor microenvironmental cytokine C-C motif chemokine ligand 2 (CCL2) via PGs and were specifically absorbed by subpopulations of cells expressing the CCL2 receptor, CCR2, leading to exosome accumulation in specific cell subpopulations and organs (Lima et al., 2021). Similarly, glioblastoma cell-derived EVs were specifically internalized into CCR8-positive cells through the binding of PGs to CCL18 (Berenguer et al., 2018).

2.4 Glycans

Glycans are complex carbohydrates with many important biological functions, including protein folding, cell adhesion, cell differentiation and proliferation, and signal transduction (Varki, 2017). Recent studies have shown that glycans are involved in the biogenesis, cellular recognition, and uptake of EVs (Costa, 2017; Williams et al., 2018). Several studies have demonstrated that surface glycan modifications perform an instrumental role in mediating the targeting and uptake of EVs, which may help in the evaluation and development of new EV-based therapies (Hung and Leonard, 2015; Dusoswa et al., 2019; Lin et al., 2020; Matsuki et al., 2021). Nishida-Aoki et al. (2020) reported that the removal of *N*- and/or *O*-glycosylation on the surface of the brain-metastatic breast cancer cell subline BMD2a-derived EVs enhanced EV uptake by human umbilical vein endothelial endothelial cells (HUVECs) in vitro, and the removal of *O*-glycosylation instead of *N*-glycosylation significantly increased EVs in the lung and slightly increased EVs in the spleen and brain in vivo, suggesting that surface glycosylation has inhibitory effects on cellular uptake. Similarly, the removal of *N*-glycans from murine melanoma cell-derived sEVs enhanced the cellular uptake of peritoneal macrophages (Yamamoto et al., 2021). However, de la Torre-Escudero et al. (2019) found that treating EVs with glycosidase blocked the uptake of fasciola hepatica EVs by macrophages, which suggested that de-glycosylation of the EVs blocked their uptake by macrophages. Clos-Sansalvador et al. (2022) showed that treatment with glycosidase peptide-*N*-glycosidase F (PNGase-F) to remove the surface *N*-glycan of EVs derived from immortalized mesenchymal stromal cells significantly reduced EV uptake by HUVECs, suggesting that *N*-glycoylation is critical for EV uptake. There appears to be no consensus on how glycosylation affects EV internalization, but EVs' surface glycans do act as significant players in EV uptake.

2.5 Lectins

Lectins are a class of proteins with characteristic carbohydrate recognition domains that bind to specific glycosylated structures. They participate in several different biological processes, including glycoprotein transport, cell adhesion, and signaling (van Breedam et al., 2014). In fact, some literature indicates that they may also be involved in the absorption of EVs (Hao et al., 2007; Barrès et al., 2010; Bonjoch et al., 2017; Shimoda et al., 2017; Yuan et al., 2017). According to one study, adding sialic acid-binding immunoglobulin-like lectins (siglecs) antibodies or preincubating siglecs-expressing human cervical cancer HeLa cells with sialic acid in vitro reduced the uptake of adipose-derived mesenchymal stem cell (ADSC) exosomes, whereas in vivo experiments revealed that ADSC exosomes were selectively internalized into siglecs-positive cells, implying that the interaction between sialic acid on exosomes and siglecs on the cell

surface was essential for the cellular uptake of ADSC exosomes (Shimoda et al., 2017). The binding of C-type lectin receptor to carbohydrates is calcium-dependent. Yuan et al. (2017) found that a group of carbohydrates, calcium chelators, and antibodies to the C-type lectin receptor all reduced the accumulation of macrophage exosomes in hCMEC/D3 cells. They concluded that the binding of the C-type lectin receptor in hCMEC/D3 cells to mannose/glucosamine on exosomes partially mediates the uptake of macrophage exosomes. Meanwhile, free lectins may inhibit the internalization of EVs. For example, Bonjoch et al. (2017) reported that, in the case of pancreatic ductal adenocarcinoma, regenerating islet-derived 3β released from healthy pancreatic tissue surrounding the tumor inhibited EV internalization through binding of the lectin structural domain to glycoproteins on the surface of THP-1 macrophages-derived EVs. The disruption of the signaling mediated by EVs prevents the phenotypic switch in macrophages induced by EVs, inhibits the increased cell migration of cancer cells, and reverses a number of metabolomic changes promoted by EVs.

2.6 PDZ domain-containing proteins

Postsynaptic density-95, Drosophila discs-large, and zona occludens-1 (PDZ) proteins are soluble cytoplasmic adapter proteins that function as transient scaffolding structures to assemble multiprotein signaling complexes by virtue of highly conserved modules (Romero et al., 2011). A recent study found that PDZ proteins regulate the uptake of EVs. Castro-Cruz et al. (2023) investigated the effect of PDZ protein depletion on the uptake of mCherryCD63-positive EVs by MCF-7 cells and found that PDZ proteins could increase (syndecan-binding protein (SDCBP, also known as SYNTENIN1), Golgi reassembly stacking protein 2 (GORASP2), and mitochondrial serine peptidase 2 (HTRA2)) or decrease (LNX1) CD63-positive cellular uptake of EVs, suggesting that PDZ proteins are involved in the uptake of EVs through regulating the levels of HS at the cell surface.

2.7 Interferon-induced transmembrane proteins

Interferon-induced transmembrane proteins (IFITMs) are a kind of small-molecule transmembrane protein induced by interferon (Ren et al., 2020). IFITMs are found in the plasma membrane and could block viral replication by preventing viral–host membrane fusion subsequent to viral binding and endocytosis (Perreira et al., 2013). The research on EV uptake of different CRC subpopulations suggested that CRC subpopulations with a high IFITM1 level on the cell surface (IFITM1^{high}) took up significantly fewer EVs than IFITM1^{low} subpopulations, while these subpopulations have the same EV release intensity. Of note, deleting IFITM1 leads to a marked increase in EV uptake in CRC organoid cells, proving that IFITM1 is involved in the process of EV recognition and uptake (Kelemen et al., 2021).

In conclusion, the above components do play a role in the recognition and uptake of EVs, but this recognition seems to have no strict specificity because there is not yet a particular EV subtype that targets specific recipient cells. In addition, we have to admit that the current study seems to mix the general view of EV recognition and uptake with regard to these important molecules, and cannot really identify whether they play a role in the recognition or uptake step of EVs. However, we still find some hints suggesting that these molecules might provide valuable clues to improving existing drug delivery systems. For example, Vázquez-Ríos et al. (2019) achieved a higher efficiency using integrin α6β4-functionalized liposomes for delivery.

3 Uptake

When EVs attach to the cell surface, they can trigger signaling through the interaction of ligands on their surface with receptors expressed on the cell, or they can be internalized by the recipient cell through endocytosis or direct fusion with the plasma membrane (Gurung et al., 2021). If EV attachment is not linked to uptake, the EVs may again detach from the cell surface (Fuentes et al., 2020). Numerous studies have shown that cells incubated at 4 °C significantly reduce their ability to take up EVs (He et al., 2019; Fuentes et al., 2020; Murdica et al., 2020; Cerezo-Magaña et al., 2021), suggesting that uptake is an energy-dependent process, such as endocytosis, which is the mechanism most commonly studied today. There is also some evidence that plasma membrane fusion is involved in the process of EV internalization (Chen et al., 2016; Guo et al., 2020). The following are several pathways by which exosomes can enter recipient cells.

3.1 Endocytosis

3.1.1 Clathrin-mediated endocytosis

Clathrin-mediated endocytosis (CME), named for its need for clathrin, is involved in processes such as the neurotransmission, signal transduction, and regulation of plasma membrane activity (McMahon and Boucrot, 2011). CME occurs through a series of processes involving clathrin coat formation, cargo loading, clathrin coat- and actin-mediated membrane bending, dynamin-mediated membrane fission, and auxilin- and heat shock cognate 70 (HSC70)-dependent uncoating (McMahon and Boucrot, 2011; Kaksonen and Roux, 2018; Smith and Smith, 2022).

Many studies have demonstrated the close association of CME with EV uptake. Yuan et al. (2017) reported that macrophage exosomes were co-localized with transferrin (a marker used to study CME) and anti-clathrin heavy chain (CLTC) antibodies in endothelial cells, suggesting that clathrin participated in the internalization of exosomes. It has been demonstrated that the knockdown of CLTC (Tian et al., 2014; Yao et al., 2018; Wan et al., 2020; Tu et al., 2021) and μ2 (the subunit of clathrin adaptor complex AP2) (Tian et al., 2014) significantly inhibits EV uptake by recipient cells, highlighting the role of CME in EV uptake. Chlorpromazine, an inhibitor of clathrin-coated pit assembly, could inhibit EV uptake by a variety of cells, such as endothelial cells (Banizs et al., 2018), hepatocellular carcinoma cells (Yao et al., 2018), colon cancer cells (Bajic et al., 2020), and ovarian cancer cells (Escrevente et al., 2011). It is known that incubation with potassium depletion or hypertonic medium (hypertonic sucrose) is an effective method for blocking clathrin-coated pits (Hansen et al., 1993). The internalization of exosomes was inhibited by potassium depletion buffer (Tian et al., 2014) and hypertonic sucrose (Yuan et al., 2017), further supporting the importance of CME in mediating EV absorption. Pitstop2 was discovered to inhibit CME by blocking the contact of the ligand with the terminal structural domain of clathrin (von Kleist et al., 2011), resulting in reduced uptake of EVs by pancreatic cancer cells (Sun et al., 2022), colon cancer cells (Horibe et al., 2018), and trophoblast cells (Holder et al., 2016).

Dynamin represents an important component involved in CME. The chemical suppression of dynasore (a dynamin inhibitor) (Yao et al., 2018; Bajic et al., 2020; Kanno et al., 2020; Tu et al., 2021) and short hairpin RNA (shRNA)-mediated dynamin knockdown (Tian et al., 2014; Tu et al., 2021) both induced a significant reduction of EV accumulation in recipient cells, suggesting that dynamin mediated EV internalization. It should be pointed out that dynamin also engages in other endocytic pathways, such as caveolin-dependent endocytosis. Therefore, dynamin-dependent endocytosis must be combined with the results of additional inhibition experiments to determine which endocytic pathway mediates the uptake of EVs.

3.1.2 Lipid raft-mediated endocytosis

Lipid rafts are defined as small (10–200 nm), heterogeneous, highly dynamic, cholesterol- and sphingolipid-enriched domains that discriminate cellular processes (Pike, 2006), and perform important roles in signaling, viral infection, and membrane transport (Simons and Gerl, 2010). Svensson et al. (2013) observed that the lipid raft marker cholera toxin subunit B (CtxB) co-localizes with exosomes in HUVEC and glioblastoma cells, implying a possible role for lipid rafts in EV uptake. Cholesterol is a critical component of lipid rafts, so the commonly used inhibitors of lipid raft-mediated endocytosis currently target cholesterol. Simvastatin, an inhibitor of the cholesterol biosynthesis rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, has been shown to dose-dependently inhibit EV uptake by HeLa cells (Costa Verdera et al., 2017), glioblastoma cells (Svensson et al., 2013), and HUVECs (Svensson et al., 2013; He et al., 2019). Methyl-β-cyclodextrin (MβCD) depletes cholesterol from the plasma membrane by forming soluble complexes with cholesterol, resulting in the reduced uptake of exosomes by ovarian cancer cells (Escrevente et al., 2011) and peripheral blood mononuclear cells (Chen et al., 2022). A series of studies showed that treatment with the cholesterol chelators filipin and nystatin effectively blocked EV uptake by recipient cells (Svensson et al., 2013; Hazan-Halevy et al., 2015; Yuan et al., 2017; Banizs et al., 2018; Emam et al., 2018; Rai and Johnson, 2019), further confirming the involvement of lipid raft-mediated endocytosis in EV internalization.

Endocytosis in the lipid raft region usually occurs in two pathways: (a) the formation of endocytic vesicles in lipid rafts are stabilized by enriching certain proteins, such as caveolae-dependent endocytosis

and flotillin-dependent endocytosis; (b) vesicles are formed by small GTases, such as GTPase regulator associated with focal adhesion kinase-1 (GRAF1)-dependent endocytosis, ADP ribosylation factor 6 (Arf6)-dependent endocytosis, and RhoA-dependent endocytosis (El-Sayed and Harashima, 2013).

Many authors have described how the EVs are taken up via lipid raft-mediated endocytosis as caveolae- or flotillin-dependent. The inhibitor of caveolae-dependent endocytosis, genistein, caused reduced EV internalization in epithelial cells (Rai and Johnson, 2019), HeLa cells (Costa Verdera et al., 2017), and myeloma cells (Tu et al., 2021), suggesting that caveolae plays at least a partial role in EV absorption. It is known that dynamin can be recruited into the caveolar membrane, leading to caveolae fission by contracting its neck (Kiss, 2012). It was found that the treatment of epithelial cells with genistein, dynamin inhibitory peptide (DIP), and filipin III blocked EV internalization (Rai and Johnson, 2019). In another study, the treatment with inhibitors of lipid raft-mediated endocytosis, nystatin and simvastatin, and the dynamin inhibitor dynasore alone, but not a specific siRNA against caveolin-1 or clathrin, significantly inhibited exosomes being internalized into Jeko-1 cells (Hazan-Halevy et al., 2015), further confirming the involvement of caveolae in EV uptake.

Caveolin-1, a major component of caveolae, inserts into the inner leaflet of the plasma membrane with its hairpin structural domain and has a specific amino acid sequence at the N-terminal end that binds to lipid rafts (Kiss and Botos, 2009). However, although caveolin-1 is integral to the formation of caveolae, the role of caveolin-1 in caveolae-mediated endocytosis is controversial. Tu et al. (2021) showed that the knockdown of caveolin-1 reduced the accumulation of sEV in multiple myeloma cells. Similarly, the knockdown of caveolin-1 in HeLa cells resulted in significant inhibition of EV uptake (Costa Verdera et al., 2017). In contrast, Svensson et al. (2013) reported that both HeLa cells and glioblastoma cells, stably transfected to overexpress caveolin-1, exhibited significantly reduced exosome uptake, and further studies revealed the mechanism by which caveolin-1 negatively regulates the endocytic uptake of exosomes by inhibiting extracellular signal-regulated kinase 1/2 (ERK1/2) signaling activation; whether the caveolin-1 facilitates EV internalization remains unresolved.

Flotillin-dependent endocytosis is similar to that mediated by caveolae, but the endocytic vesicles are enriched in flotillin rather than in caveolin (El-Sayed and Harashima, 2013). Flotillin-1 and flotillin-2 are ubiquitously expressed and highly conserved membrane-associated proteins, which are considered to be scaffold proteins of lipid rafts (Liu et al., 2018), and exist in plasma membrane domains distinct from caveolae (Doherty and McMahon, 2009). A study demonstrates that the knockdown of flotillin-1 in HeLa cells significantly inhibited EV internalization (Costa Verdera et al., 2017). Likewise, flotillin-1 knockdown resulted in a significant reduction in sEV uptake in multiple myeloma cells (Tu et al., 2021). Emam et al. (2018) reported that anti-flotillin-1 antibody treatment reduced exosome accumulation in melanoma cells and colon cancer cells. These results suggest that EV uptake may occur through a lipid raft-dependent pathway requiring flotillin.

3.1.3 Macropinocytosis

Macropinocytosis is a liquid-phase endocytosis that mediates the non-selective uptake of solute molecules, nutrients and antigens. It typically relies on growth factor-activated receptor tyrosine kinases (RTKs) to initiate signaling cascades that induce actin cytoskeletal rearrangement to trigger plasma membrane protrusions. Lamellipodia probably fold back on the plasma membrane to form a cave-like invagination, while the protrusions in the circular ruffles bind to each other, and they both eventually close the macropinosome by way of membrane fission (Mercer and Helenius, 2009; Lim and Gleeson, 2011).

Cytochalasin D (an inhibitor of actin polymerization) was found to inhibit EV uptake in microglia (Fitzner et al., 2011), multiple myeloma cells (Tu et al., 2021), colon cancer cells (Emam et al., 2018), and ovarian cancer cells (Escrevente et al., 2011), suggesting that endocytic pathways requiring cytoskeletal remodeling, such as macropinocytosis, may be involved in EV internalization. The significant increase in induced liquid-phase uptake is characteristic of macropinocytosis. The incubation of recipient cells with EVs significantly enhanced the uptake of dextran, a liquid-phase marker of macropinocytosis (Costa Verdera et al., 2017; Yao et al., 2018), indicating that EVs can be internalized through macropinocytosis.

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a Rho GTPase responsible for triggering membrane ruffles during macropinocytosis (Mercer and Helenius, 2009). 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA) and amiloride, both inhibitors of Na/H^+ exchanger, significantly inhibited EV internalization into multiple myeloma cells (Tu et al., 2021), hepatocellular carcinoma cells (Yao et al., 2018), and ovarian cancer cells (Escrevente et al., 2011) by lowering submembrane pH to block Rac1 signaling. Fitzner et al. (2011) reported that treatment with NSC23766 (a specific Rac inhibitor) resulted in impaired EV uptake in microglia, and another study found that siRNA-mediated knockdown of Rac1 inhibited EV uptake in HeLa cells (Costa Verdera et al., 2017), further confirming the role of macropinocytosis in EV uptake.

Phosphatidylinositol 3-kinase (PI3K) is required for stimulating membrane ruffles and closing macropinosomes (Mercer and Helenius, 2009); treatment of recipient cells with the PI3K inhibitors LY294002 (Tian et al., 2014) and wortmannin (Costa Verdera et al., 2017; Tu et al., 2021; Chen et al., 2022) revealed significant inhibition of EV uptake. P21-activated kinase 1 (PAK1) and protein kinase C (PKC) are two serine/threonine kinases required for macropinocytosis (Mercer and Helenius, 2009). Yao et al. (2018) demonstrated that exosome entry into hepatocellular carcinoma cells was remarkably inhibited by the PAK inhibitor 3 (IPA-3) and the PKC inhibitor rottlerin, and Costa Verdera et al. (2017) reported that knockdown of PAK1 in HeLa cells significantly reduced the internalization of EVs.

3.1.4 Phagocytosis

Phagocytosis is the cellular process by which cells internalize particles larger than 0.5 μm (Hallett, 2020). It is initiated by the interaction of specific receptors on the cell surface with ligands on the particle surface, and particles are internalized via an actin-based mechanism (Allen and Aderem, 1996).

Phagocytosis is generally thought to engulf larger particles. However, there is evidence to support that EVs can be internalized by phagocytosis (Feng et al., 2010; Harischandra et al., 2018; Ogese et al., 2019; Tabak et al., 2021). The co-localization of gold-labeled exosomes and phagocytosis tracers (polystyrene carboxylate-modified latex beads) was found in cells (Feng et al., 2010), suggesting that phagocytosis plays a role in the uptake of exosomes.

Because actin polymerization is required for phagosome formation (Freeman and Grinstein, 2014), inhibitors of actin polymerization are extensively utilized in the literature to research phagocytosis. Several investigations indicated that treating recipient cells with actin polymerization inhibitors (cytochalasin B/D and latrunculin A/B) dramatically inhibited EV internalization, suggesting that endocytic pathways involving actin polymerization are implicated in EV internalization and that EVs may be internalized by phagocytosis (Feng et al., 2010; Ogese et al., 2019). PI3K is an essential regulator of phagosome closure (May and Machesky, 2001). Feng et al. (2010) indicated that the PI3K inhibitors wortmannin and LY294002 decreased exocytosis uptake by macrophages in a dose-dependent manner. Tabak et al. (2021) discovered that wortmannin treatment of trabecular meshwork cells resulted in a substantial reduction in EV uptake, suggesting that EV uptake may occur through a mechanism involving phagocytosis.

Annexin-V (AnV) has a high affinity for phosphatidylserine (PS). Matsumoto et al. (2017) discovered a substantial decrease in the absorption of PKH67-labeled exosomes by macrophages treated with AnV, suggesting that the PS on the exosomes was involved in the internalization process. According to Feng et al. (2010), incubating macrophages with T-cell immunoglobulin and mucin domain-4 (TIM-4) (a PS receptor) antibodies partially suppressed the internalization of exosomes, suggesting that TIM-4 may be one of the receptors that macrophages use to recognize and phagocytose exosomes. We speculate that macrophages internalize exosomes through phagocytosis, possibly through the interaction of TIM-4 with PS.

3.2 Plasma membrane fusion

Some evidence supports the suggestion that EVs enter the recipient cell by direct fusion with the cell membrane (del Conde et al., 2005; Parolini et al., 2009; Montecalvo et al., 2012; Chen et al., 2016; Guo et al., 2020), resulting in direct cargo release into the cytoplasm (Weston et al., 2019). The fusion of lipid bilayers in an aqueous environment occurs in several steps. First, two lipid bilayers approach each other until they are <10 nm apart, at which point the lipids of the outer leaflet interact to form an intermediate destabilized membrane stalk. The stalk then expands, causing the distal monolayer to bend, resulting in the formation

of a membrane fusion pore, followed by the formation of the syncytium (Jahn et al., 2003; Petrany and Millay, 2019).

Fluorescence lipid quenching is frequently used to track the occurrence of membrane fusion. R18 is a lipophilic fluorescent dye that, at high doses, experiences fluorescence self-quenching. However, the surface density of the fluorophores integrated into the lipid membranes decreases when the R18-labeled lipid membrane fuses with other lipid membranes, alleviating R18 quenching and allowing a fluorescent signal to be released (Montecalvo et al., 2012). In light of this, unlabeled melanoma cells were added to R18-labeled exosomes and fluorescence was monitored within 30 min; the rapidly rising fluorescent signal (within 10 min) suggests that melanoma cells take up exosomes via membrane fusion (Parolini et al., 2009). Similarly, the addition of unlabeled bone marrow-derived dendritic cells to R18-labeled exosomes results in a continuous rise in fluorescence over a short period of time (Montecalvo et al., 2012). del Conde et al. (2005) monitored membrane fusion utilizing fluorescence signal transfer between two fluorescently labeled (Rh and nitrobenzoxadiazole (NBD)) phospholipids, and the increased NBD fluorescence in activated platelets indicated that monocyte-derived microvesicles underwent membrane fusion with activated platelets. Furthermore, botulinum toxin type A (BONT/A), a pharmacological agent that inhibits soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated membrane fusion, was reported to limit the absorption efficiency of EVs loaded with doxorubicin by tumor cells (Guo et al., 2020). These findings imply that plasma membrane fusion plays a role in EV uptake.

3.3 Influencing factors

Numerous studies have revealed that several factors impact EV absorption by recipient cells. On the one hand, EV uptake is related to the recipient cells themselves. Horibe et al. (2018) compared the ability of three different cell types to absorb exosomes and discovered that, regardless of the donor cell type, exosome uptake increased in the order of colon cancer cell HCT116, lung adenocarcinoma cells, and colon cancer (COLO205) cells. This finding suggests that the exosome uptake capacity is closely related to the recipient cell type rather than being dependent on the donor cell type. Lázaro-Ibáñez et al. (2017) treated prostate cancer (PC-3) cells with EVs and discovered that the EV signal was substantially higher in G2/M phase than in other cell cycle phases, suggesting that the cell cycle affects EV uptake.

On the other hand, external elements such as radiation, pH, and oxygen concentration are also significant. It has been demonstrated that radiation stimulates exosome absorption and enhances cell adhesion to exosomes by inducing the co-localization of CD29 and CD81 on the cell surface (Hazawa et al., 2014). Mutschelknaus et al. (2016) also indicated enhanced uptake of exosomes by irradiated recipient cells compared to that of non-irradiated recipient cells. Nakase et al. (2021) found that EVs secreted under low-pH cell culture conditions during serum starvation showed higher internalization in living cells than EVs secreted under neutral cell culture conditions. This might be owing to the increased zeta potential of EV membranes caused by low pH, which enhanced the interaction of EV membranes with negatively charged target cell plasma membranes. Parolini et al. (2009) investigated the uptake and fusion of exosomes under different pH conditions. They discovered that the fusion activity and the uptake by parent cells of the exosomes released under pH 6.0 culture conditions were higher than those of the exosomes released under pH 7.4 culture conditions. In addition, the storage pH of exosomes also affects their internalization. The researchers found that storage at pH 4 and 10 resulted in greater cellular uptake of exosomes than storage at pH 7 (Cheng et al., 2019). In conclusion, the effect of pH on exosome uptake deserves further study. Xue et al. (2018) found that exosomes produced under hypoxic conditions were more readily absorbed by HUVECs. Similarly, the treatment of HUVECs with glioma cell-derived exosomes isolated under hypoxic conditions (Hypo-Exos) or normoxic conditions (Nor-Exos) revealed a significant increase in the internalization of Hypo-Exos compared to that of Nor-Exos. This may be related to the upregulation of Connexin 43 in Hypo-Exos by hypoxia, as the uptake of exosomes by HUVECs was effectively blocked by either using the Connexin 43 inhibitor Gap27 or knocking down Connexin 43 (Yang et al., 2022). Moreover, hypoxia was found to induce an acute and transient increase in EV uptake in human high-grade gliomas (HGG) cells and mouse HGG cells, and there was

significant co-localization of internalized EVs and CtxB in hypoxic cells, suggesting that the hypoxic induction of EV uptake involves lipid raft-mediated endocytosis (Cerezo-Magaña et al., 2021).

In a word, endocytosis appears to be the main mechanism of EVs' entry into recipient cells, such as CME, lipid raft-mediated endocytosis, macropinocytosis, and phagocytosis. Significantly, most of the current studies on the mechanisms of EV uptake have been conducted by blocking specific pathways with inhibitors or knocking down key components that play a role in endocytosis. The use of inhibitors has made an important contribution to the understanding of EV uptake, but one problem that may undermine the use of inhibitors for endocytosis is their potentially poor specificity. For example, as suggested by Willox et al. (2014), Pitstop 2 inhibition of CME was due to non-specificity and needed to be used with caution and should not be used to summarize any function of the N-terminal structural domain of Clathrin. Dynamin-2 has been found to be involved in both CME and phagocytosis (El-Sayed and Harashima, 2013), so its inhibition cannot readily distinguish between these two pathways. Studies have shown that PI3K is important for both macropinocytosis and phagocytosis (Feng et al., 2010; Tu et al., 2021), which cannot be distinguished by using PI3K inhibitors, but experiments combined with the inhibition of liquid phase uptake can distinguish between the two pathways well. Therefore, we suggest that these inhibitors should be used judiciously.

4 Release or delivery of EV cargo

Despite the complexity of the endocytic pathways, these internalized vesicles fuse with endosomes and follow the classical endosomal pathway. The plasma membrane buds inward to form EEs, where cargo sorting takes place. Cargo can be recycled directly to the cell surface via a fast recycling route or returned to the cell surface via a slow recycling route with the aid of recycling endosomes (REs). Alternatively, EEs' growth and maturation could lead to the trans-Golgi network (TGN) or late endosomes (LEs). Through the TGN and entry into the secretory pathway (referred to as "retrograde transport"), cargo can also be recycled back to the cell surface. LEs could undergo homotypic fusion reactions, enlarge and acquire more luminal vesicles, form MVBs, and eventually fuse with lysoso-mes to form endolysosomes, leading to the degradation of EV contents (Cullen and Steinberg, 2018; Khan and Steeg, 2021).

However, EV cargo could bypass degradation. Joshi et al. (2020) treated HEK293T cells expressing mCherry-tagged anti-green fluorescent protein (GFP) fluobody with GFP-CD63 EVs, leading to the co-localization of GFP, mCherry, and LAMP1 (a marker of LE/MVB and lysosomes) in the cells, revealing that internalized EVs may undergo fusion with endosomes and/or lysosome, thus resulting in the exposure of the EV cargo to the cytoplasm. In another study, the treatment of unlabeled HepG2 cells with R18-labeled exosomes did not capture the membrane fusion dequenching signal until approximately 45 min had passed, suggesting that membrane fusion occurs at a site within the cell, not within the cell membrane. Further studies showed that the dequenching signal for membrane fusion was co-localized with the LE/MVB marker CFP-RAB7 and the ILV marker CFP-CD63, but not the EE marker CFP-RAB5, suggesting that exosome membrane fusion occurs in LE/MVB. The dequenching signal R18 of exosomes was partially co-located with the signal of the LE-specific anionic lipid lysobisphosphatidic acid (LBPA). Anti-LBPA antibody pretreatment significantly inhibited membrane fusion and led to the increase of co-location between exosomes and lysosomes, indicating that some exosome cargo might escape from LEs through LBPA-dependent membrane fusion to avoid lysosome degradation (Yao et al., 2018). Furthermore, Costafreda et al. (2020) found that the exosomes are endocytosed via PS binding to the immunoglobulin variable (IgV) domain of hepatitis A virus cellular receptor 1 (HAVCR1) at the cell surface, followed by the translocation of exosomes to LEs enriched in N-terminal Niemann-Pick C1 (NPC1). HAVCR1 and NPC1 are anchored to the LE-delimiting membranes by their transmembrane domains, and to the outer leaflet of exosomes by the IgV HAVCR1 bound to PS and the N-terminal domain (NTD) NPC1 bound to cholesterol, which brings the exosomal membrane and the LE-delimiting membrane into close proximity, forming a hemifusion, resulting in the viral RNA contained in the lumen of the exosomes passing through the fusion pore into the cytoplasm (Costafreda et al., 2020).

Galactose lectin was used to demonstrate alterations in endosomal membrane permeability. It was found that the treatment of hippocampal neurons

expressing mCherry-tagged galectin with brain exosomes from rTg4510 mice expressing human four-repeat tau resulted in the production of galactose lectin spots; the spots were also co-localized with internalized exosomes, suggesting that exosomes allow the escape of exosomal cargo from endolysosomes into the cytoplasm by inducing endolysosomal permeabilization (Polanco et al., 2021).

The ER is now recognized as the nucleation site for translation in general. Heusermann et al. (2016) found that exosome-containing endosomes are targeted for translocation to the endoplasmic reticulum (ER), potentially permitting efficient access of exosomal microRNA (miRNA) and messenger RNA (mRNA) cargoes into the RNA interference (RNAi) and translation machinery, enabling cargo release.

In addition to releasing the cargo directly into the cytoplasm of the recipient cell, the EV cargo can also be transported into the nucleus during fusion of the EVs with the late endosomal membrane. It has been shown that some of the EVs arriving at the LEs enter and/or dock to the nuclear envelope invaginations (NEIs) and are tethered to the outer nuclear membrane (ONM) with the aid of a tripartite protein complex composed of vesicle-associated membrane protein (VAMP)-associated protein A (VAP-A), oxysterol-binding protein (OSBP)-associated protein 3 (ORP3), and LE-associated small GTPase Rab7. After docking of the LEs to the ONM, fusion of the EVs with the endosomal membrane may expose the EV contents near the nuclear pore, thus enabling nuclear transfer of the EV cargo (Santos et al., 2018; Corbeil et al., 2020).

Exosomes can also be re-secreted by hijacking the secretory endosomal pathway of cells, which may lead to more distant effects, thereby increasing the pathogenic potential and range of action of exosomes. PKH67-positive exogenous exosomes were internalized by neurons in the Ch1 compartment and co-localized with endosomes expressing mCherry-CD9. Subsequently, mCherry-CD9- and PKH67-dual positive endosomes were internalized by neurons in the Ch2 compartment via axonal migration, indicating that endosomes containing exogenous exosomes can fuse with endosomes containing endogenous intraluminal nanovesicles and were secreted together (Polanco et al., 2018).

Despite more than 80 years of research on EVs, there is still a lack of clarity about the mechanisms by which EVs release their cargoes, which could be critical for the development of EVs as drug delivery vectors or for studying how to block the release of cargoes in order to prevent or slow down the onset of disease. Therefore, much work remains to be undertaken to more fully understand cargo transport and the release of EVs.

5 Conclusions and future directions

The EVs could transfer cargo from donor to recipient cells to induce phenotypic changes and thereby realize cell-to-cell communication. However, the complex biological journey of EVs in the recipient cells remains ill-defined. In this review, we summarize the most up-to-date knowledge about the entire process of EVs, including the binding to the recipient cells, the uptake, and the release of cargo after uptake (Fig. 2). Firstly, the binding of EVs by recipient cells is mainly dependent on ligand–receptor interactions on the cell and EVs' surfaces. It has been demonstrated that tetraspanin, integrins, proteoglycans, and lectins are involved in EV recognition, binding, and even uptake, but the results of some of these studies are still somewhat controversial, and the specific mechanisms are unclear and need to be further explored. Secondly, we focus on the mechanism of EV uptake by cells. As we described earlier, the most used approach for studying EV uptake is currently the use of endocytosis inhibitors. However, no inhibitor of the endocytosis pathway has absolute specificity for the pathway of interest. The lack of absolute specificity does not mean that pharmacological inhibitors should be excluded from probing endocytosis pathways. Some of them are still useful tools, especially when used in combination with modern molecular biology methods. For example, for differentiating pathways that are difficult to distinguish using endocytosis inhibitors, more specific inhibition can be achieved using siRNAs or shRNAs to knock out key components that play a role in endocytosis. We also expect that future work will identify more highly selective inhibitors of endocytosis that can be used for experimental and clinical applications. Finally, we also summarize several fates of the EVs found so far, after uptake. One fate is that the uptaken EVs eventually fuse with the lysosome and are degraded, and the other fate is that the EVs escape lysosomal degradation and eventually release their cargo into the nucleus or cytoplasm for further action.

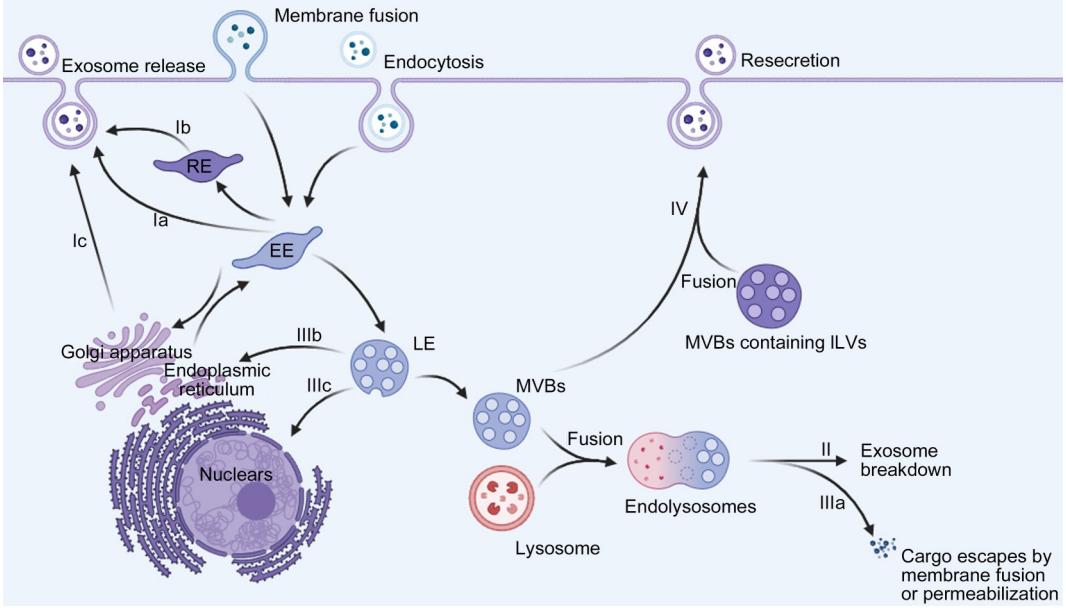

Fig. 2 Exosome uptake and post-uptake fate. Exosomes are uptaken by the recipient cell via membrane fusion or endocytosis and then enter the early endosomes (EEs). EEs can return to the plasma membrane via the fast recycling route (Ia) or the slow recycling route (Ib). EEs could also grow, and maturation leads to trans-Golgi network (TGN) or late endosomes (LEs). The extracellular vesicles (EVs) could pass through the TGN and enter the secretory pathway, then also returning to the plasma membrane (Ic). LEs further form multivesicular body (MVB), which fuses with lysosomes to form endolysosomes. Some exosomes are degraded in endolysosomes (II), and others are able to bypass degradation: (1) membrane fusion of exosomes with endosomes and/or lysosomes, resulting in exposure of exosomal cargo to the cytoplasm (IIIa); (2) fusion of endosomes containing exosomes with lysosomes leads to permeability, which is used by exosomal cargo to enter the cytoplasm (IIIa); (3) LEs target the endoplasmic reticulum (ER), and exosome contents can also be released (IIIb); (4) LEs move to the perinuclear areas of the cell where they undergo fusion with each other and may expose the EV contents near the nuclear pore to lead to nuclear transport of EV cargo (IIIc). Exosomes can also be re-secreted by hijacking the secretory endosome pathway of the cell: endosomes containing exogenous exosomes fuse with intraluminal vesicle (ILV)-containing endosomes and are secreted together (IV). Created with BioRender.com. RE: recycling endosomes.

In conclusion, we have given an overview of the whole process of EV identification, uptake, and post-uptake fate based on the current studies with the aim to have a better understanding of the biology of EVs.

A growing body of research has identified numerous advantages of EVs over traditional synthetic carriers, opening up new frontiers for modern drug delivery. Despite ongoing research, the clinical translation of EV-related therapies still faces many challenges. Here, we focus on the impact of the process of EV recognition uptake and subsequent cargo release on EVs as drug delivery carriers. The lack of specific recognition sites may lead to an insufficient ability of EVs to target receptor cells and the low efficiency of intracellular delivery, and there is an urgent need to develop protocols to improve the targeting function of EVs. In addition, there is a critical issue of the endosomal escape pathway of cargoes in EVs, as this step seems to be the main bottleneck preventing the efficient functional delivery of cargoes. Notably, a recent study found that nanovesicles co-functionalized with fusion proxies and tumor-targeting fragments act as drug carriers to efficiently and selectively bind to the target, and trigger membrane fusion, enabling both endolysosomal escape and cytoplasmic drug delivery and improving the therapeutic efficacy of the drugs acting on cytoplasmic targets (Wang et al., 2023). Given the great potential of EVs for drug delivery, knowledge about EV identification, uptake, and cargo release is crucial for EVs to become promising and powerful tools for nanomedicine and precision medicine.

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Author contributions

Lingxiang MAO and Huayuan XIANG conceived, designed, discussed the work, and revised the manuscript. Chenxuan BAO and Qiaoqiao CHEN edited the manuscript. Qing GAO and Qianqian GAO prepared Figs. 1 and 2. Nan WANG prepared Table 1. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Huayuan XIANG, Chenxuan BAO, Qiaoqiao CHEN, Qing GAO, Nan WANG, Qianqian GAO, and Lingxiang MAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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