Review

Molecular Cancer Research

Internalization of Exosomes through Receptor-Mediated Endocytosis

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Abstract

The tumor microenvironment is replete with factors secreted and internalized by surrounding cells. Exosomes are nanosized, protein-embedded, membrane-bound vesicles that are released in greater quantities from cancer than normal cells and taken up by a variety of cell types. These vesicles contain proteins and genetic material from the cell of origin and in the case of tumor-derived exosomes, oncoproteins and oncogenes. With increasing understanding of the role exosomes play in basic biology, a more clear view of the potential exosomes are seen to have in cancer therapeutics emerges. However, certain essential aspects of exosome function, such as the uptake mechanisms, are still unknown. Various methods of cell–exosome interaction have been proposed, but this review focuses on the protein–protein interactions that facilitate receptor-mediated endocytosis, a broadly used mechanism by a variety of cells.

Introduction

Extracellular vesicles (EV) play an integrative role in basic biological processes, such as cell-to-cell communication, but have recently gained widespread attention for their potential role in pathology. Supporting evidence exists for exosome involvement in cardiovascular disease, oncology, autoimmune syndromes, neurodegenerative disorders such as Alzheimer's and Parkinson's diseases, HIV, tuberculosis, and more (1). Extracellular vesicles are classified on the basis of their cellular origin, biological function, size, and most commonly by their biogenesis (2). On the basis of their formative processes, there are three main classes: microvesicles, apoptotic bodies, and exosomes. Microvesicles originate from the plasma membrane as a result of outward budding and fission of membrane vesicles from the cell surface (3). Apoptotic bodies result from the blebbing of the plasma membrane during apoptosis (2). Exosomes, the focus of this article, derive from intracellular inward budding of the limiting membrane of endocytic compartments that form multivesicular bodies (MVB), which release these vesicles in the form of exosomes (4, 5). Exosomes are a type of extracellular spherical shaped membrane-bound vesicle with a diameter size ranging between 30 and 150 nm (6, 7). Studies revealed that exosomes are shed from various kinds of cells and can be isolated from virtually all biological fluids (4, 5). Currently, exosomes are being explored as biomarkers for different cancers and diseases as noninvasive techniques for diagnosis (8, 9). Exosomes elicit various functions in cancer progression such as inducing angiogenesis (10–12), resistance to therapy in their cell of origin by sending the drugs outside these targeted cells (13), and conferring chemoresistance to their target/receiving cells (14). Dendritic cell–derived exosomes (DEX) have been shown to possess an additional function of antigen presentation (15, 16) due to the presence of MHC class II and other immunologically important molecules such as MHC class I, CD80, and CD86 (17). In addition, exosomes are reservoirs for biomarkers such as proteins (6, 8, 9, 18, 19), mRNAs, miRNAs (11, 20–22), lipids (23), and more recently DNA (24, 25). In addition, they play a role in niche preparation for metastasis (26, 27) and immune suppression (28–30). Because of their lipid membrane bilayer, exosomes are endowed with a protective ability for their cargo, and so are thought to play a role in cell-to-cell communication (31, 32). These processes make exosomes excellent candidates for therapeutic targets.

Accessing the vast therapeutic potential of exosomes is dependent on a fuller understanding of the vesicular–cellular protein interactions underlying exosomal function. Targeting exosomes containing protumorigenic messages (33) or modifying their contents and characteristics (34) to hinder the further spread or development of the tumor burden is one of many proposed therapeutic methods. Another promising therapy would be to utilize the biological functions of exosomes to deliver cancer drugs and therapies (2, 35–38). For example, nanoparticle drug delivery is a rapidly burgeoning area of inquiry that capitalizes on the endogenous functions of extracellular particles by applying these to manufactured vesicles (39–41). Each of these potential therapies relies on a clear understanding of exosome internalization by recipient cells.

Several mechanisms of uptake have been proposed for exosomes and are well reviewed in the literature (42–45). Evidence indicates that exosomes can be internalized by way of fusion (46–48) and/or endocytosis (43, 49). Fusion of exosomes with the plasma membrane has been described by several groups. As seen below, it is often cell-type or environment-dependent. Montecalvo and colleagues showed that with dendritic cells,

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exosomes bind to the plasma membrane, delivering their contents through the fusion or hemi-fusion of the two membranes (47). Platelets have also been identified as structures to which monocyte-derived microvesicles deliver their contents by fusion. Activated platelets fused with the microvesicle membrane more rapidly than unstimulated ones, and reduced platelet activity was observed when annexin V inhibited the fusion process (50).

The conditions to which the recipient cells are subjected can also affect the mechanism of uptake. Membrane fusion requires interacting bilayer destabilization and overcoming high activation energy barriers (51). On the basis of this primary role of lipids in the fusion process (46), the fluidity and rigidity of the membrane caused by changing temperatures may direct the mechanism of internalization. The fusion process causes a "lipid interdigitation" that occurs more readily in the presence of high amounts of fusogenic lipids such as phosphatidic acid and bis(monoacylglycero) phosphate (BMP), both of which are present in exosomes. BMP's fusogenic properties are most potent at a low pH (52, 53). Acidic environments, as found within a tumor or in metastatic sites, as well as increasing temperatures, improve efficiency of exosomal fusion to melanoma cancer cells (46). Whether these conditions dictate fusion of exosomes preferentially over endocytosis remains to be evaluated.

In addition to fusion, various types of endocytosis have been identified as mechanisms of intercellular transport of exosomal contents such as macropinocytosis (54–56), phagocytosis (57), clathrin-mediated (55, 58), caveolin-dependent (59), lipid raft–dependent (60, 61), and clathrin-/caveolin-independent (62) endocytosis. While these processes have unique aspects, there is some functional overlap between them. Macropinocytosis is a form of endocytosis that consists of membrane ruffles forming intracellular vesicles to internalize large amounts of extracellular fluid, as seen by several antigen-presenting cells that sample the immediate environment (63). This varies from other forms of endocytosis in its formation of separate and distinct intracellular vesicles (macropinosomes) and the nonspecific internalization of materials. Research has identified macropinocytosis of exosomes by microglia (56), human epidermoid carcinoma–derived A431 cells stimulated by EGFR, and by the pancreatic cancer MiaPaCa-2 cell line (54). Macropinocytosis is not selective in which molecules are internalized from the extracellular environment, and so uptake may be dictated simply by proximity to the cells and not targeted by the exosome specifically. However, it has been shown that some exosomes naturally induce macropinocytosis internalization (64) and others, through manipulation of exosomal content, can selectively activate this mechanism increase uptake (40). Phagocytosis is a much more common method of taking up exosomes, especially with phagocytic cells of the immune system. Feng and colleagues showed that two leukemia cell lines, K562 and MT4, solely utilized phagocytosis for exosome internalization (57). Phagocytosis depends on specific receptors and mechanisms that are present primarily in specialized cells. These cells envelope the exosomes in phagosomes, eventually directing the cargo toward the lysosome (65).

Four other general categories of endocytosis focus on specific cellular proteins that facilitate the uptake of particles. Clathrin and caveolin are both cytosolic proteins that form specific pits with which to internalize various substances (66). The exact reasons why and when a cell uses clathrin, caveolin, or neither, is still incompletely understood but particle size and cell type seem to play a role (60, 67). Caveolin-dependent endocytosis is important in albumin uptake, cholesterol transport, and intracellular signaling. Because of the small size of the caveolae, its endocytosed material tends to be smaller than 60 nm (66). Clathrin-dependent mechanisms, however, can internalize particles up to 120 nm. The size restrictions may indicate, with further investigation into which uptake mechanism is utilized by which cells, a possible functional difference between vesicle sizes within the current exosome size range. The clathrin-dependent process is involved in many different cell types and functions ranging from vesicle recycling in the neuronal synapse to organ development and ion homeostasis (66). Many of the common, well-known endocytosis receptors utilize clathrin-coated pits, such as low-density lipoprotein receptor (LDLR) and transferrin receptor (TfR). One of the most commonly used ways to determine which of these mechanisms is in operation is through inhibitory drugs or knocking down certain key players. Dynamin, a GTPase, facilitates the fission of the intracellular clathrin-coated vesicle (66, 68). Dynasore, an inhibitor of dynamin, has been utilized to effectively block endocytosis of extracellular vesicles and establish clathrin-mediated endocytosis as a mechanism of uptake for these vesicles (43, 56, 58). Following siRNA downregulation of caveolin-1 (the primary protein involved in caveolae-dependent endocytosis), exosome internalization was significantly reduced in B cells (59). Inhibitory drugs have also been useful in the determination of a third mechanism, lipid raft–mediated endocytosis. The lipid raft is a small portion of the plasma membrane–rich in sterols and sphingolipids, which facilitates various cellular processes (69). Use of methyl-β-cyclodextrin (MβCD), which alters the cholesterol content of the membrane and disrupts lipid rafts, has been seen by several groups to impair exosomal internalization (60, 70, 71). While lipid raft–dependent endocytosis is the primary clathrin- and caveolae-independent mechanism, other pathways and independent interactions have been described in the internalization of exosomes (62, 69). Endocytosis is the primary method of exosomal delivery of its contents, but research is still needed to understand what determines the specific mechanism whether it is cell type, exosome type, or condition specific.

Research into internalization mechanisms has shown that experimental manipulation of the exosomal membrane protein profile, such as stripping the membrane, affects the uptake of exosomes (58, 72). On the basis of that understanding, receptor-mediated endocytosis (RME) is another proposed mechanism of uptake. While RME traditionally is associated with clathrin-mediated endocytosis, the receptor/ligand interaction facilitating uptake has also been linked to several other endocytosis categories. Its overall dependence on receptors offers an excellent source of potential targets for therapeutic manipulation. Potential therapies can use these receptors in two ways: receptors can be targets to prevent uptake of oncogenic exosomes, or manufactured drug–containing nanoparticles can be designed with an overexpression of ligands for these specific receptors. While RME is a common mechanism of uptake, identification of the receptors involved particularly with extracellular vesicles is still in progress. The significance of RME is connected to its ability to closely monitor the internalization of extracellular materials. It is dependent upon

a ligand binding to a specific receptor resulting in the engulfment of the bound complex. Ligands are proteins that bind specifically to a receptor to initiate signaling or to influence recipient cell function. Some of the well described receptor–ligand complexes include low-density lipoprotein (LDL) and its receptor (LDLR), or transferrin (Tf) and transferrin receptor (TfR; refs. 73–75). These complexes enter the cell and are either degraded in the lysosome or recycled to the surface. LDL/LDLR complex is endocytosed and ends up in the lysosome, which allows for LDL degradation into free cholesterol for cellular function. TfR, on the other hand, releases its iron cargo in the endosome and then is recycled back to the surface with the Tf and receptor intact. As illustrated here, receptor and ligand fates differ based on the receptor and mechanism of endocytosis (76).

Many proteins have been identified as participants in the endocytosis of exosomes (Table 1). Similar protein–protein interactions significantly contribute to recognition and endocytosis of molecules important for cellular activities pertaining to the uptake of viruses (44, 77, 78), liposomes (79, 80), and nanoparticles (81, 82). One of the many ways viruses induce internalization by the host is through apoptotic mimicry, which involves externally expressing phosphatidylserine that binds to cellular T-cell immunoglobulin mucin (TIM) receptors (78). Liposome uptake by several C-type lectin receptors is enhanced by altering the carbohydrate membrane profile (80). Indicative of the therapeutic potential of this field of research, manufactured nanoparticles have identified certain proteins, such as apolipoproteins, essential to uptake (82). Many of these proteins have been identified on exosomes, but further research is needed to clarify their role in exosomal uptake (83). Those that have been linked to vesicle internalization are outlined in Table 2 and described below.

Lectins

Lectins are a population of soluble and membrane-bound receptors that recognize and bind glycan moieties (Fig. 1). This large protein family participates in a wide variety of functions that facilitate cell-to-cell communication, including adhesion and intracellular trafficking (84). There are three classes of lectins, the transmembrane C-type lectins and selectins, the transmembrane Siglecs (that bind sialic acid), and the cytosolic galectins (galactoside binding). All three classes have been linked to exosomes, and two have specifically been identified as mediators of exosome uptake. The first class, the selectins, is found on immune cells and endothelial cells and is involved particularly with cell adhesion (84). While the mechanism of uptake has yet to be directly ascribed to the p-selectin CD62, it has been shown on platelet-derived extracellular vesicles (44, 85), and p-selectins are endocytosed with the aid of the cytosolic protein Numb3 (86), allowing for the hypothesis that p-selectins may play a role in exosome uptake. P-selectin on platelets has also been shown to bind to its ligand p-selectin glycoprotein ligand-1 (PSGL-1) on microvesicles; however, this facilitates fusion delivery instead of endocytosis (50). Using antibodies to cellular c-type lectin receptors, as well as calcium

Table 1. Receptor–ligand complexes facilitating exosomal internalization

Receptor	Receptor location	Ligand	Ligand location	References
Direct				
C-type lectin receptor	Dendritic cell, brain endothelial cell	C-type lectin	Macrophage exosome (exo)	87, 88
CD169 (Siglec)	Splenic and lymph node macrophages	A2,3-linked sialic acid	B-cell–derived exosomes	89
Siglec-3 (CD33)	Cervical cancer and antigen-presenting cells	Sialic acids	Mesenchymal stem cell–derived exo	90
	Macrophage	Galectin 5	Reticulocyte derived exo	58
Cadherin 11	Osteoblast exosomes		Prostate cancer cells	103
LFA-1	Macrophage exo, dendritic cells	ICAM-1	Brain endothelial cell, dendritic exo	87, 88, 104–107
	Pancreatic adenocarcinoma (rat) exosomes	CD11b	Spleen and lymph node leukocytes	72
	Pancreatic adenocarcinoma (rat) exosomes	CD11c	Spleen and lymph node leukocytes	72
	Pancreatic adenocarcinoma (rat) exosomes	CD44	Spleen and lymph node leukocytes	72
CD106	Endothelial cells	CD49d	Spleen and lymph node leukocytes	72, 112
	Pancreatic adenocarcinoma (rat) exosomes	CD54	Spleen and lymph node leukocytes	72
Integrin $\alpha_6\beta_4$	Lung fibroblasts		Breast cancer exosomes	110
Integrin $\alpha_v\beta_5$	Liver macrophages		Pancreatic exosomes	110
CD62	Platelet exo	CD62L	Spleen cells	72, 85
CD9	Pancreatic adenocarcinoma exo		Spleen, lymph node, peritoneal exudate cells	72
CD81	Pancreatic adenocarcinoma		Spleen, lymph node, peritoneal exudate cells	72
Tspan8	Pancreatic adenocarcinoma exo		Endothelial cell	112, 113
HSPG	Glioblastoma multiforme cells	Fibronectin	Myeloma exosomes	111, 122, 127
TIM1/TIM4	Phagocytic cells and endothelial cells	Phosphatidylserine	Dendritic cell exosome, mouse melanoma cell exosomes, squamous cell carcinoma	54, 97, 128, 129, 132, 134
Indirect				
EGFR	Epidermoid carcinoma cells, pancreatic carcinoma cells	EGF	HeLa cell exosomes	54, 139

Table 2. Protein–protein interactions involved in exosomal uptake

Receptor	Ligand	Receptor location	General function	References
Lectins				
C-type/Selectin	PSGL-1	Immune, endothelial cells, platelet-derived EVs	Cell adhesion, inflammation	50, 84, 87, 88
Siglecs	α-2,3-linked sialic acid	Leukocytes, stromal cells	Cell adhesion, signaling	89, 90
Galectins	Glycans	Nasopharyngeal carcinoma EV, dendritic EV, reticulocyte EV	Cell adhesion, signaling	58, 91, 92, 95, 96
Adhesion molecules				
Cadherins	Cadherins	Epithelium, placenta, neural, muscle, kidney	Cell adhesion	99, 103
Selectins	PSGL-1	Immune, endothelial, platelet derived EV	Cell adhesion, inflammation	44, 50, 84–86
Mucins	Galectin3	Epithelial	Cell signaling, maintain barriers	99–102
Integrins	Fibronectin, collagen, laminin	Ubiquitous	Intracellular signaling, cell adhesion, migration	72, 98, 109–111
Immunoglobulin (Ig)(ICAM-1)	Various	Immune cells, phagocytic cells, endothelial cells, platelets	Facilitate immune response	87, 88, 97, 104–107
HSPG	Fibronectin	Ubiquitous	Endocytosis, adhesion, migration, growth factor, binding, coreceptor	44, 111, 114–127
TIM family	Phosphatidylserine (PS)	T cells, dendritic cells, B cells, mast cells, NK cells, some endothelial cells	Regulate immune responses, phagocytosis, antigen presentation, recognize apoptotic cells	57, 97, 128–136
EGFR	EGF and TGFα	Ubiquitous	Intracellular signaling leading to DNA synthesis, cell proliferation, adhesion, and migration	54, 133, 137, 139

chelators and a panel of carbohydrates, two groups have identified these receptors as integral in the uptake of dendritic cell–derived and macrophage-derived exosomes (Fig. 1; refs. 87, 88). The interaction of the selectins and c-type lectins with exosomes seems to be an emerging area of research into the intercellular communication that enhances immune cell–antigen recognition and movement. Further studies with these receptors in exosomal uptake could advance the field in increasing immune cell involvement in cancer and immunotherapy methods.

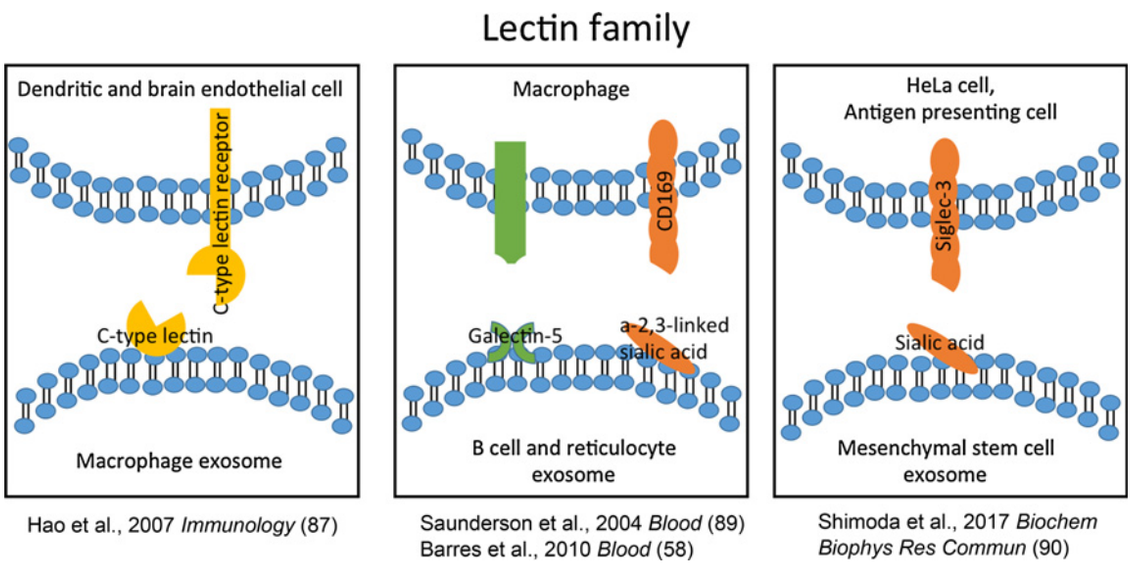


Figure 1.
Lectin family members have been shown to play a role in exosome internalization. Lectin family members have been identified on various cellular membranes as well as on exosomal membranes. C-type lectin receptor has been identified on both dendritic cells and brain endothelial cells and interacts with c-type lectin to internalize macrophage-derived exosomes (87). Galectin 5 on reticulocytes is involved in uptake by macrophages (58). Siglecs, another lectin subcategory of proteins, are seen responding to exosomes with the interaction of CD169 on macrophages and B-cell exosomal α-2,3-linked sialic acid (89) and Siglec-3 on HeLa cells or APCs and sialic acid on stem cell–derived exosomes (90).

Saunderson and colleagues described the dependence of B-cell and dendritic cell–derived exosome internalization on CD169, a transmembrane Siglec family member expressed on leukocytes and stromal cells. Alpha 2,3–linked sialic acid is the primary ligand for CD169 and it has been found to be enriched on B-cell–derived exosomes (Fig. 1; ref. 89). Siglecs are sialic acid–binding immunoglobulin-like lectins that are cell-type specific and primarily function in cell adhesion and signaling (84). Siglec-3 (CD33) on HeLa cells and antigen-presenting cells has also been shown to mediate the uptake of exosomes, as antibody blocking and competition with sialic acid decrease uptake of adipose-derived stem cell exosomes (Fig. 1; ref. 90). This second class of lectins has been described most frequently in cell-to-cell interactions in the immune system, but as seen above, functions in vesicular endocytosis as well.

A third class, the cytosolic galectins, is responsible for interpreting the results of glycosylation into changes in function, and so participates in a variety of cellular pathways. Galectins are small proteins that bind to galactose- and N-acetyllactosamine–based motifs and are widely conserved across species. These proteins have the unique ability to slow receptor internalization by dimerization and cross-linking (84). Galectins are now being targeted by chemotherapeutics due to the prevalence of their mutations in cancer cells (91). In addition, they have been linked to exosome uptake by target cells. Several galectins have been identified on exosomes, such as galectin-9 on exosomes from nasopharyngeal carcinoma cells and others (58, 92), and galectin-3 on dendritic cell–derived exosomes (93). Galectin-9 interacts with T-cell transmembrane, immunoglobulin, and mucin 1 (TIM1), a membrane receptor that plays a key role in exosomal uptake with phosphatidylserine (PS), as commonly seen with phagocytic cells (94). While galectin-3 has yet to be shown to influence exosome uptake, its adhesion properties have been established in relation to neural growth and is a required receptor for clathrin-independent internalization of CD44, an important surface glycoprotein for cell adhesion and migration (44, 95, 96). Barrès and colleagues showed how internalization of exosomes derived from reticulocytes is influenced by the presence and concentration of galectin-5 (Fig. 1; ref. 58). In this study, a membrane dye, PKH67, was used to show internalization of exosomes containing surface galectin-5. In the presence of unstained exosomes or purified protein, however, uptake was decreased. Endocytosis, especially as mediated by receptors, is dependent on the recipient cell's ability to interact with the extracellular environment. Receptors, like the lectins, designed to directly bind a variety of proteins are ideal for the internalization of extracellular vesicles that may present various surface ligands. In addition, the high incidence of some lectins in malignant tissues also increases the importance this receptor family plays in the interaction of cancer-spreading exosomes and the tumor microenvironment. Targeting these proteins by either an antagonist drug or a competitive nanoparticle could reduce the available receptors, decreasing the cellular uptake of oncoprotein-containing exosomes. The Zöller laboratory has proposed the idea of creating nanoparticles that can "outsmart" or outcompete endogenous exosomes (34), which would be able to effectively utilize such ubiquitous receptors as targets.

Adhesion molecules

Because of their role in cell-to-cell and cell-to-extracellular environment interactions, adhesion molecules are in a prime position to play an integral role in receptor-mediated endocytosis of exosomes. Cell adhesion molecules (CAM) consist of five classes including cadherins, immunoglobulins, selectins (also part of the lectin family), mucins, and integrins. Calcium dependence and specific interactions with cells and extracellular matrix are some of the main differences between the various classes (97). Several different adhesion molecules have been identified on exosomes such as intercellular adhesion molecule-1 (ICAM-1), CD11 integrins, milk fat globule-EGF factor 8 (MFG-E8) (98), epithelial cell adhesion molecule (Epcam), mucin13 (99), and mucin-1 (muc-1; refs. 100–102), which could potentially be tied to uptake mechanisms by recipient cells. When pretreated with an antibody to cadherin-11, exosomes from osteoblasts are less likely to be taken up by prostate cancer cells (103).The immunoglobulin ICAM-1 and its receptor, leukocyte function–associated antigen-1 (LFA-1), function primarily in the interaction between leukocytes and endothelial cells. Abnormal expression is linked to several pathologies, including cancer (104). ICAM-1 and LFA-1 play an important role in dendritic cell–derived exosome function as well as facilitate uptake of macrophage exosomes in the brain (Fig. 2; refs. 87, 88, 105, 106). Engineered nanovesicles have also shown that ICAM-1 and LFA-1 are crucial players in uptake by human umbilical vein endothelial cells (HUVEC; ref. 107). While many of these adhesion molecules have not yet been directly linked to internalization mechanisms, the significance of this receptor family is illustrated in a 2006 study done by Miksa and colleagues They found that in sepsis, deficient phagocytosis of apoptotic bodies is tied to decreased MFG-E8, but when exosomes containing this protein are introduced, phagocytosis increases and sepsis is attenuated (108). This finding illustrates the significance of increasing the understanding of exosomal uptake mechanisms for the development or manipulation of vesicles as therapeutics.

The integrin protein profile that has been linked to exosomes is generally involved in the interactions between extracellular material and fibroblasts, as well as in initiating intracellular signaling (109). However, unique integrin profiles have been linked to targeted cells for the uptake of specific exosomes. Many of these studies show that integrin receptors are located on the exosome and interact with ligands on the targeted cell (Fig. 2; ref. 110). For example, integrin $\alpha_6\beta_4$ on breast cancer exosomes and integrin $\alpha_v\beta_5$ on pancreatic cancer exosomes showed an essential role in the uptake of exosomes by lung fibroblasts and liver macrophages, respectively. These both indicate an integral role exosomes can play in the development of lung and liver metastasis (110). Chen and colleagues, has shown that integrins $\alpha v\beta 3$ and $\alpha 5\beta 1$ play a key role in exosome attachment to hepatic stellate cells furthering liver fibrosis development. This group showed delivery of miRNAs after attachment, indicating that these receptors play a role in delivery of exosomal contents, but whether by fusion or endocytosis is still undetermined (111). Furthermore, rat pancreatic adenocarcinoma–derived exosomes were shown, through antibody blocking and flow cytometry analysis, to be taken up by leukocytes in a CD11b (spleen and peritoneal exudate cells), CD11c (spleen and lymph node cells), CD44 (spleen and lymph node cells), CD49d (lymph node cells), CD54 (spleen, lymph node, and peritoneal exudate cells), and CD62L (spleen and lymph node cells) dependent manner. As assessed by

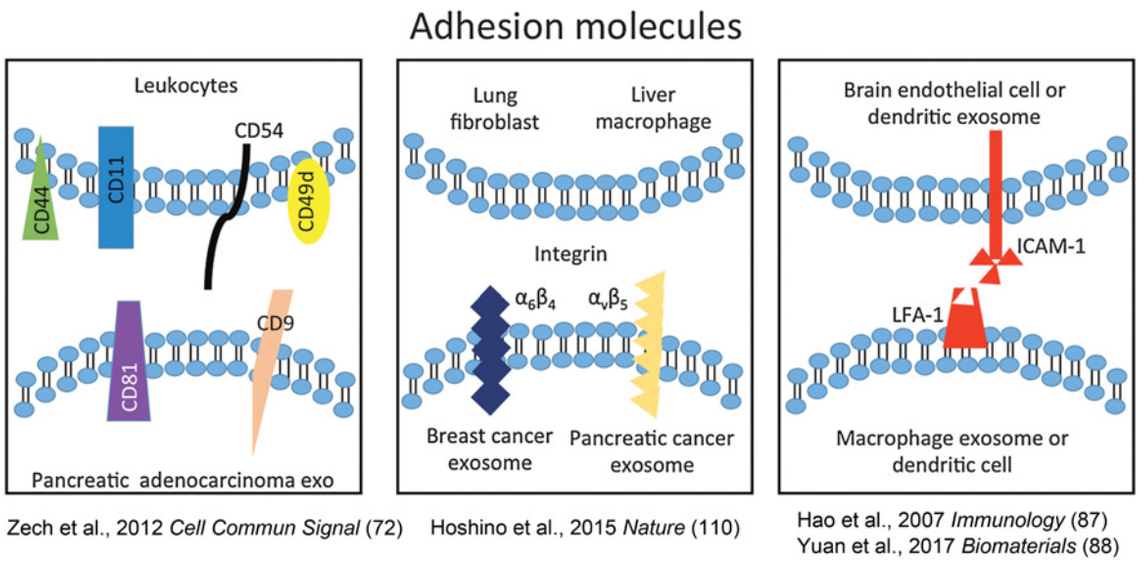


Figure 2.

Cellular adhesion molecules play an important role in anchoring and internalizing exosomes. Various leukocytes are involved in the exosome interactions and CD44, CD11, CD54, CD49d, are all important to internalization. Tetraspanins CD81 and CD9 on the exosome surface facilitate this interaction (72). Integrins are important facilitators of cell-to-cell interaction and have been identified with exosome uptake in lung fibroblasts and liver macrophages (110). ICAM-1 and its ligand LFA-1 are widely used receptors to internalize exosomes (87, 88).

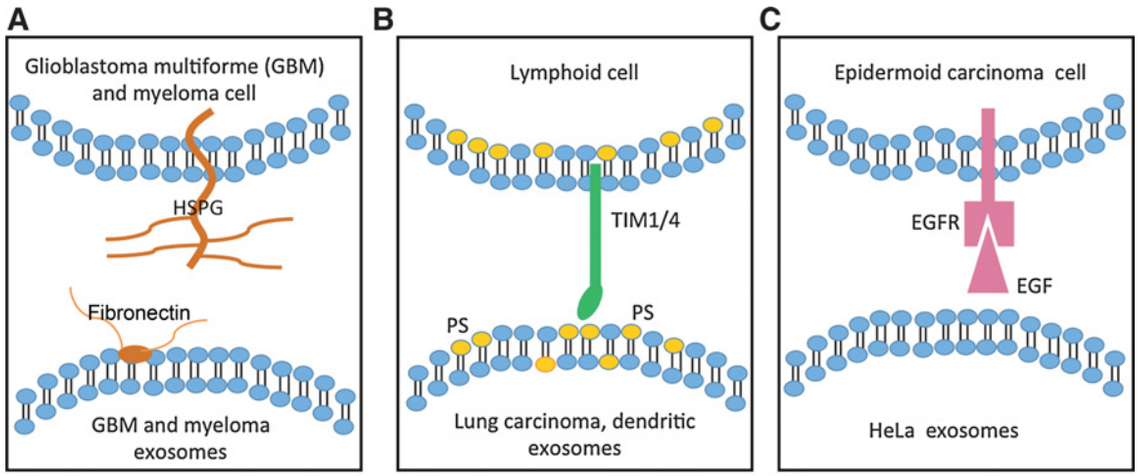
antibody blockade, the availability of these ligands on various leukocytes dictated the degree of internalization. Subsequent blocking of common exosomal tetraspanins such as CD81 and CD9 on the exosome inhibited uptake by each of the groups (peritoneal exudate cells were only CD81 dependent; Fig. 2; ref. 72). Other groups have provided evidence that supports the role of additional tetraspanins in exosomal integration into target cells, such as tetraspanin 8 (Tspan8; refs. 112, 113). Exosomal proteins therefore are equally responsible for the endocytosis process as are those found on the cell membrane (Fig. 2).

Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPG) are ubiquitous glycoproteins involved in a wide variety of cellular functions. These proteoglycans are promiscuous receptors, binding a variety of ligands through their heparan sulfate (HS) chains (114). Of seven major functions ascribed to these glycoproteins by Sarrazin and colleagues, two can be directly tied to extracellular vesicles and their interaction with cells. First, they facilitate extracellular interactions, including attachment and motility. This function of HSPGs has been tied to exosomal binding and content delivery to hepatic stellate cells (111). Second, they play an integral role in endocytosis for delivery of ligands. Because the HS chains can bind different proteins, several ligands capitalize on the endocytosis function of the HSPGs and enter the cell attached to this receptor. Mahley and colleagues, described the HSPG as a "co-endocytosis receptor," which internalized structures by transferring ligands to other receptors or by forming a complex that takes the ligand into the cell. However, they also show that it can act as an independent receptor in ligand uptake (115). Work by Wittrup and colleagues, describes the endocytosis of HSPG along with its ligand heparan sulfate, establishing it as a receptor/ligand complex that can internalize and not simply bind to exosomes (116). Further support of the potential endocytic function of HSPG with exosomes is illustrated generally in cells by its well-established promiscuity in ligand binding (117–120) as well as variability in endocytic mechanism (117, 121). Christiansen and colleagues, has connected this receptor to exosome function by showing the dependency of U-87 MG (glioblastoma multiforme cell line) exosomal uptake on HSPG (Fig. 3A). Both syndecans and glypicans, members of the HSPG family have been identified on exosomes, but neither participate in internalization (122). Syndecans are involved instead in the biogenesis of exosomes (123). Location of HSPG, therefore, is important to its influence on exosomes. Cellular HSPG, and not exosomal HSPG, is operative in internalization, but blocking the cellular HSPG does not completely abolish uptake indicating it is not the only functioning mechanism (122). This phenomenon is supported by the Mulcahy review illustrating the various entry mechanisms exosomes utilize (43). Heparin, a drug that interacts with HSPG internalization, is effective at reducing exosomal uptake (124, 125) and has been specifically shown to be effective on the recipient cell rather than the exosome itself (126). In addition, the existence of possible ligands, such as fibronectin, on the exosome surface that interact with the HSPGs supports the role for HSPG-dependent exosome internalization (127). Furthermore, evidence shows that many viruses, such as HIV, hijack the HSPG endocytosis pathway, supporting the hypothesis that this mechanism may also be occurring with extracellular vesicles that are similar in size to viruses such as exosomes (44, 114). While there is still a paucity of evidence of HSPG directly internalizing exosomes, as seen above, the understanding of it as an endocytic receptor and its presence in relation to exosomes is becoming clearer.

T-cell immunoglobulin and mucins

Endocytosis of debris and apoptotic cells is an important part of cellular homeostasis and is performed by phagocytic



Christianson et al., 2013 *Proc Natl Acad Sci U S A* (122)
Purushothaman et al., 2016 *J Biol Chem* (127)

Baglio et al., 2016 *Proc Natl Acad Sci U S A* (132)
Sims et al., 2017 *Int J Nanomedicine* (134)

Nakase et al., 2015 *Sci Rep* (39)

Figure 3.

Other receptor–ligand interactions are important to exosome–cellular interactions. **A,** Heparan sulfate proteoglycans bind to fibronectin on exosomes from different cell types to facilitate uptake (122, 127). **B,** Externally facing PS allows exosomes to be recognized and internalized by antigen-presenting cells and phagocytes, often by way of TIM receptors (132, 134). **C,** Cellular EGFR when binding its ligand indirectly increases exosome internalization (39).

cells. One of the key signals that identify an apoptotic body from a healthy cell/vesicle is the presence of PS on the extracellular side of the plasma membrane. This lipid, which is usually facing the cytoplasm, is recognized by various receptors on phagocytes and immune cells, some of which belong to the TIM family (128). The reversed PS is a shared characteristic with extracellular vesicles, especially exosomes, and was correlated with exosomal uptake by Morelli and colleagues in 2004 (98). In 2007, Miyanishi and colleagues proposed that TIM1 and TIM4 are the cell receptors responsible for uptake through binding exosomal PS (Fig. 3B; ref. 129). Matsumoto and colleagues explained that the negative surface charge created by external PS facilitates uptake by macrophages (130). This receptor/ligand complex seems to predominate in phagocytic cells (57, 128, 130–132) and may not be a common endocytic process for all cells to internalize exosomes. However, there is evidence of exosome uptake by endothelial cells being reduced by the blocking of PS with Annexin V (133). Furthermore, recent evidence suggests that the TIM4-PS complex plays an essential role in exosome-mediated uptake of HIV-1 and other viruses (78, 134). As mentioned in a previous paragraph, the TIM family has additional members that bind to ligands found on exosomes, such as TIM1 or TIM3 with galectin 9 (94, 135). Overall the TIM family plays an important immunologic role recognizing and internalizing phosphatidylserine, which is most often indicative of cell death and debris. Blocking TIM4 decreases apoptotic body clearance (128, 129) and absence of this receptor can result in altered immune cell function, including development of autoimmunity and hypersensitive lymphocytes (136). Mimicking the apoptotic body with surface PS, exosomes are able to exploit this mechanism and introduce their unique contents to immune cells. Altered immune cell function after exposure to tumor-derived exosomes (29) may play a role in a tumor's ability to evade immune detection or response, or can alternatively enhance the immune response (16) opening targets for future therapeutics.

While the above receptors illustrate receptor–ligand binding that result in direct endocytosis of exosomes, there are additional receptor/ligand interactions that indirectly result in the internalization of exosomes. Macropinocytosis and phagocytosis nonspecifically envelop extracellular material, which results in exosome uptake (54). The following receptors play a role in the indirect internalization of these vesicles.

EGFR is an important player in several intracellular signaling pathways and mutations of this receptor are common in many cancers (137). Nakase and colleagues found that in the presence of increased EGF, exosomal uptake is enhanced; however, it is done so indirectly (Fig. 3C). EGFR/EGF binding stimulates micropinocytosis, which corresponded with increased amounts of exosomes internalized by Mia PaCa-2 pancreatic adenocarcinoma cells (54). While this group showed an indirect role the EGF/EGFR complex plays in enhancing macropinocytic uptake of exosomes, both EGFR and EGF have been identified on exosomal surfaces, indicating a potential direct role (138). Kooijmans and colleagues also showed that cellular EGFR can be utilized by exosomes for uptake when they incorporated glycosylphosphatidylinositol (GPI)-anchored EGFR nanobodies on vesicles. However, they noted that sufficient binding to cause receptor clustering was required for EGFR internalization (139). Receptor clustering and the dependence of receptor internalization on this process have also been described with other receptors such as TfR (77, 140). Nakase and colleagues also describe a similar indirect receptor-mediated endocytosis with chemokine receptor $CXCR_4$ and stromal cell–derived factor 1α (SDF-1α; ref. 54). This receptor has been identified on exosomes from platelets

Table 3. Exosome-identified ligands/receptors

Receptor	Ligand	Extracellular vesicle origin	References
MFG-E8	Phosphatidylserine and/or $\alpha_v\beta_{3/5}$	Dendritic cells	98, 108
EpCam	Unknown	Colon carcinoma cells	99
Unknown	Mucin13	Colon carcinoma cells	99
Muc-1	Galectin3	Epithelial cells, breast cancer cells, pancreatic ductal adenocarcinoma cells	107–109
Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$	Unknown	Hepatic stellate cells	110
$CXCR_4$	SDF-1α	Platelets and T cells	54, 141, 142
TNFR1, TNFR2	Unknown	Dendritic cell	143
TfR1, TfR2	Transferrin	Leukemia and hepatoblastoma cells	73, 74
LDLR	LDL	Prostate cancer, colon cancer, ovarian cancer, hepatocellular cancer	138, 144–146

and T cells (141, 142). These complexes show the importance of the receptor/ligand complex not only in direct endocytosis of the exosome as a ligand, but as an indirect recipient of macropinocytosis (54, 56).

In addition to the detailed receptor/ligand complexes, other receptor-mediated endocytosis ligands and receptors have been separately identified, but still need to be evaluated for their role in exosomal uptake (Table 3). Obregon and colleagues, has identified the presence of both TNFR 1 and 2 in exosomes derived from dendritic cells (143). Other common endocytosis receptors such as TfR (73, 74) and LDLR (138, 144–146) have also been identified on exosomes from various cell lines and may play a role in their internalization.

Conclusion

The current understanding in the field of extracellular uptake remains an unfinished puzzle. Many different mechanisms of uptake have been identified and important proteins have been linked to internalization, but the understanding of which mechanism works, when, and with which cells is still unclear. It appears that exosomes may utilize several different mechanisms of uptake in the same cell and at different times. In addition, more research needs to be conducted on how the mechanism of uptake affects the phenotypic changes undergone by the recipient cell. But whether it is cell-cycle–dependent, cell type, or simple opportunistic mechanisms of the extracellular vesicles, the understanding of how this internalization occurs is yet to be determined.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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