Review

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Ectosomes and exosomes: shedding the confusion between extracellular vesicles

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Long- and short-distance communication can take multiple forms. Among them are exosomes and ectosomes, extracellular vesicles (EVs) released from the cell to deliver signals to target cells. While most of our understanding of how these vesicles are assembled and work comes from mechanistic studies performed on exosomes, recent studies have begun to shift their focus to ectosomes. Unlike exosomes, which are released on the exocytosis of multivesicular bodies (MVBs), ectosomes are ubiquitous vesicles assembled at and released from the plasma membrane. Here we review the similarities and differences between these two classes of vesicle, suggesting that, despite their considerable differences, the functions of ectosomes may be largely analogous to those of exosomes. Both vesicles appear to be promising targets in the diagnosis and therapy of diseases, especially cancer.

EVs: what's in a name?

The past few decades have seen the development of a new chapter in membrane dynamics: the release of specific EVs (see Glossary) from the cell. Originally considered artifacts or fragments of degenerated or dead cells, these vesicles have since been recognized as sources of communication between cells, possibly of short and/or long distance. The two classes of EV are often named exosomes [intraluminal vesicles (ILVs) when not yet released to the extracellular space] and ectosomes. It should be acknowledged, however, that these vesicles, especially those of the latter class, are often given other names, including shedding vesicles, microvesicles, exosome-like vesicles, nanoparticles, microparticles, and oncosomes. Often scientists do not choose the term exosome or ectosome but rather one of the names previously employed in the nomenclature for the single type of vesicle they research. The term EVs has been recommended to be used for the two classes of vesicle when

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Glossary

Back fusion: the fusion of vesicles with their membrane of origin, as described for ILVs, which can back fuse with the MVB limiting membrane.
Ectosome: an extracellular vesicle generated by outward budding from the plasma membrane followed by pinching off and release to the extracellular space.
Endocytosis: a process by which cells internalize endosomes containing nutrients and particles and recycle part of the membrane to the cell surface.
Endosomal sorting complex required for transport (ESCRT): machinery including four major complexes, ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, comprising several subunits. ESCRTs are involved in the sorting of proteins and remodeling of membranes. In addition, the ESCRTs that mediate budding are involved in the pinching off of ILVs and ectosomes.
Endosome: intracellular membrane-bound organelle generated by endocytosis and classified as early or late according to the time spent in the cytoplasm after their generation.
EV fusion with cells: fusion initiated by interaction of the external faces of the membranes. It is mediated, not by complexes of SNARE proteins, which are associated with the cytoplasmic faces of membranes, but by fusogens such as syncytin-1.
Exocytosis: the fusion of intracellular vesicles and other organelles with the plasma membrane, mediated by the establishment of specific SNARE complexes comprising one vesicle protein (vSNARE, such as VAMP7) and two plasma membrane proteins (pSNAREs). Fusion may be followed by the release of the organelle's contents to the extracellular space.
Exosome: an extracellular vesicle released on exocytosis of MVBs filled with ILVs.
Extracellular vesicles (EVs): mixtures of ectosomes and exosomes released to the extracellular space.
G proteins: GTPase enzymes that hydrolyze GTP to GMP to release energy and participate in specific processes.
Intraluminal vesicle (ILV): a vesicle formed by budding of the limiting membrane of late endosomes that become MVBs. On exocytosis of MVBs, ILVs are released as exosomes.
Lysosome: a membrane-bound organelle that can fuse with endosomes and other organelles. Fusion is followed by degradation of the molecules and structures released to the lumen.
Macropinocytosis: a process of internalization in which ruffles of plasma membrane engulf the extracellular milieu. The process occurs spontaneously and is guided neither by ligands nor by the contact of the internalizing particles.
Multivesicular body (MVB): a late endosome engulfed with ILVs.
Navigation of EVs: the transport, release, and accumulation of EVs in the major fluids of the body.
Phagocytosis: a process by which cells engulf one or more large particles (such as bacteria, apoptotic bodies, or large ectosomes). The plasma membrane wraps tightly around the particle and then internalizes it into the cytoplasmic phagocytic vacuole.
Rabs: small G proteins of the Ras superfamily involved in the regulation of several cellular processes, from cytoskeletal architecture to membrane budding and fusion.
Soluble NSF attachment protein receptor (SNARE): a family of proteins associated with the cytoplasmic face of membranes. Their complexes mediate the fusion of various organelles with the plasma membrane or other organelles.

Table 1. Exosomes and ectosomes: major similarities and differences

Characteristic	Exosomes[a]	Ectosomes
Assembly and release		
Membrane of assembly	MVB	Plasma membrane
Diameter	50–100 nm	100–350 nm[b]
Ceramide	Large	+
ESCRT machinery	+[c]	+
Protein/membrane anchorage	Small	Large
Piggyback of luminal proteins/RNAs	Small	+
ESCRT-III/Vps4 in pinch off	+	+
MVB exocytosis	Large	
Timing of release	Delayed	Seconds
Interaction in extracellular space and with target cells		
Rapid dissolution	+	+
Rolling and membrane fusion	+	+
Rolling and endocytosis	+	+
Navigation of EV mixtures	+	+
Markers[d]	CD63, CD61	TyA, C1q
Diagnosis and therapy	+	+

[a]Under 'Exosomes', vesicles both before (ILVs) and after MVB exocytosis are noted.

[b]The 350 nm given for the large diameter of ectosomes is a mean of the values in the literature, varying from 200 to 500 nm.

[c]+, mechanism/response present; difference in size represents differential intensity.

[d]Here only two markers are indicated. Uncertainty about others is due to the differences and changes known to occur between different cells and in the same cell under different conditions.

taken together [1]. Here we use the exosome and ectosome nomenclature for the two individual classes of vesicle and EVs for their mixture.

The ectosome nomenclature derives from the definition of ectocytosis introduced in 1991 by Stein and Luzio [2] to describe the shedding of vesicles from the plasma membrane of stimulated neutrophils. Ectosomes are now recognized as EVs generated by all cells. However, ectosome shedding can be variable, with some cells exhibiting high rates and other cells low rates of release [3]. By contrast, exosomes were first described in the 1980s during work on the maturation of reticulocytes [4–6]. These vesicles are generated by the inward budding of small domains in the membrane of late endosomes on their way to becoming MVBs and are later released on exocytosis of the latter organelles. Despite differences in their site and membrane of origin, the two EV classes function similarly when released (Table 1). In particular, they play critical roles in cell physiology and pathology by binding and fusing to their cell targets before and after navigating throughout the body [7–16]. Navigating EVs are now employed as tools in various types of diagnosis and therapy (Box 1). Here we review the steps that both classes take to assemble, release, and act on target cells, revealing the similarities and differences between these two classes of EV.

Biogenesis of exosomes and ectosomes

Exosomes and ectosomes are assembled by similar mechanisms whereby their components are sorted in their membrane of origin into small domains that undergo

Box 1. EVs in physiology, pathology, and advanced medicine

EVs are now recognized to play critical roles in almost all physiological events occurring in tissues and organs. Most intensely investigated are their roles in immunology, specifically: their modulation in lymphocytes and macrophages; in the heart and vessels, with the stimulation of coagulation, angiogenesis, and thrombosis; in the central nervous system, with the integration of neurons and various glial cell types (astrocytes, oligodendrocytes, microglia) in the control of synaptic function, neuronal plasticity, neuronal–glial communication, and myelination; and in bones, with changes of specific enzymes. Interestingly, a mechanism involved in many of these processes is the exchange of RNAs occurring among cells via EV trafficking [21,36,43,85–89].

EVs have been investigated in numerous diseases, including inflammation, diabetes, and cardiovascular and neurodegenerative diseases. EVs play a role through receptor activation and cell-to-cell transfer of biological information (enzymes, reactive oxygen species, genetic information) [56,85–89]. Various oncogenes have been identified within EVs. Moreover, RNAs have been shown to confer proneoplastic effects [33,90]. Important differences have been observed between EVs produced by normal and cancer cells. Vesicles from colon cancer and from other cancer cells were reported to induce rapid tissue growth. Unsurprisingly, other extracellular vesicles could also carry tumor suppressors. Taken together, these observations suggest that EVs transfer factors to induce recipient cells to either promote or suppress cancer-related phenotypes [33,56,63]. In addition to cancer initiation, EVs could promote processes favorable to cancer progression such as angiogenesis, nerve regeneration, and the 'education' of prometastatic sites [90–95]. Since most pathological studies have been performed on EV mixtures, it is unknown whether, and to what extent, exosomes and ectosomes are both active in the pathogenesis of cancer. Nevertheless, future therapies should consider the role played by EVs in promoting this pathogenesis [21,88–90].

Finally, there is great interest in the possible role of EVs in advanced medicine, whereby EVs can be used for the identification of novel biomarkers [96–100], the improvement of diagnosis [99,100], and the targeted delivery of drug and human gene therapies [100–102]. At present many of these studies are strictly focused on operative tasks. However, future interest could be expanded to include the mechanisms by which EVs act in pathology and medicine, which could contribute to the progress of basic science regarding exosomes, ectosomes, and their target cells.

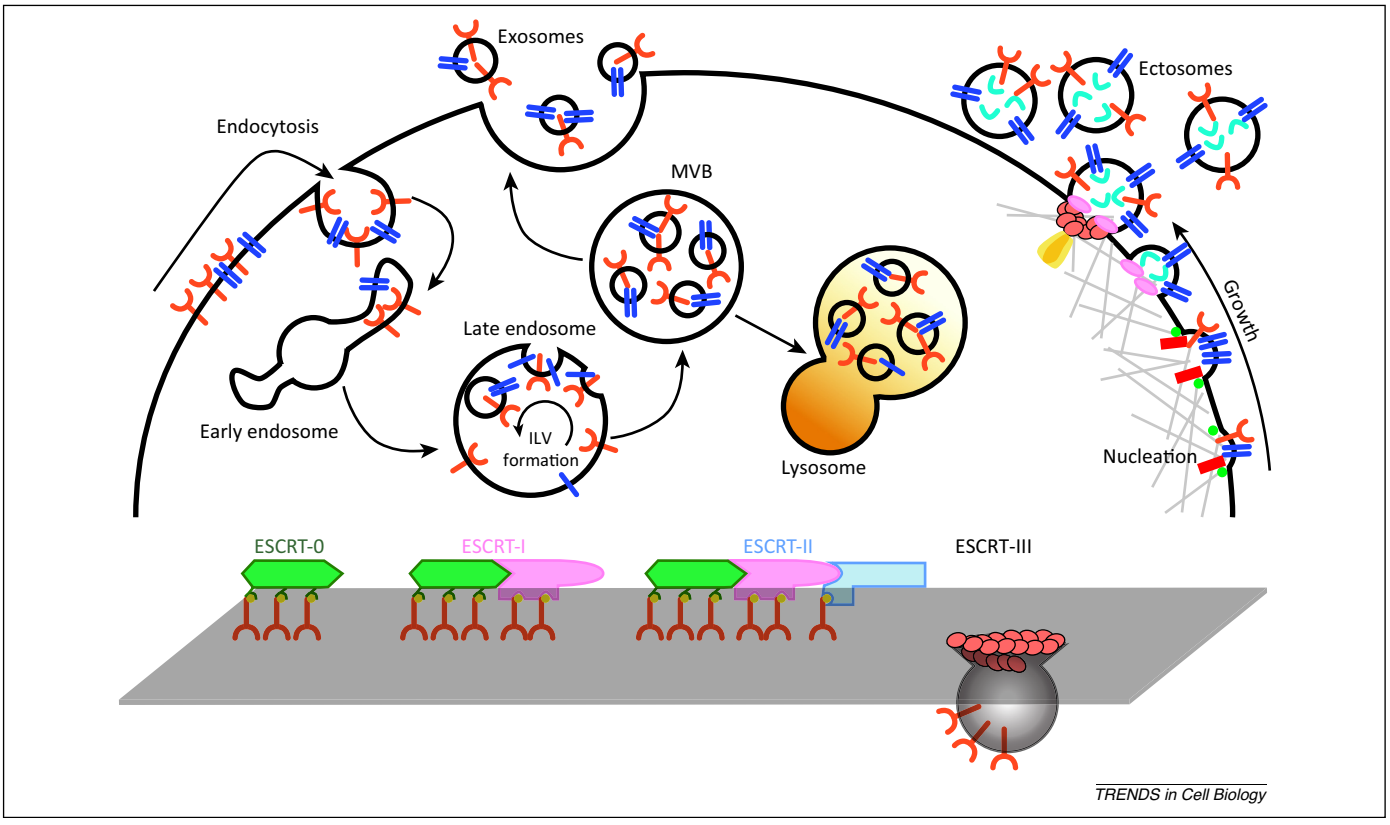

Figure 1. Exosome and ectosome biogenesis. Exosome biogenesis and intracellular life are depicted on the left. Transmembrane proteins (red) are endocytosed and trafficked to early endosomes. Once sorted to late endosomes, the endosomal sorting complex required for transport (ESCRT)-0 complex recruits ubiquitinated proteins, while ESCRT-I and -II mediate the budding of intraluminal vesicles (ILVs). The multivesicular body (MVB) can either follow a degradation pathway fusing with lysosomes (orange) or proceed to release the ILVs as exosomes to the extracellular space by an exocytic step. A summary of the ESCRT cycle as determined from exosome studies is shown at the bottom. Ectosome assembly is illustrated on the right. During nucleation at the plasma membrane, transmembrane proteins (blue) are clustered in discrete membrane domains that promote outward membrane budding. Tetraspanins and other proteins abundant at the domain may have a role by promoting the sorting of other components. Lipidic anchors (myristoylation, palmitoylation) of proteins (green) accumulate proteins in the lumen as well as contributing to membrane curvature. Additional mechanisms of ectosome formation include the Ca^{2+}-activated scramblases (red rectangles), which randomize the distribution of lipids between the two faces of the plasma membrane. The cytoskeleton (light gray) becomes looser, while cytosolic proteins and RNA molecules (aquamarine) are sorted to ectosomes. Recruited TSG101, a member of the ESCRT-I complex, mediates mobilization to the plasma membrane of ESCRT-III (salmon subunit), which promotes the assembly of a spiral. The specific ATPase VPS4 (orange) mediates the disassembly of the spiral by pulling its end.

budding and then pinching off. In the final step, the two classes of EV are released to the extracellular space by different processes (Figure 1).

Vesicle assembly and budding

The assembly of both ILVs and ectosomes comprises the accumulation of components in small membrane domains that undergo budding while in direct continuity with the membrane of origin (Figure 1). However, the initial processes that lead to the generation of exosomes and ectosomes appear largely different. Exosomes derive from the formation of the MVB, which is a late endosome loaded with ILVs. The primary role of many MVBs is as an intermediate in a general degradative lysosomal pathway. These organelles undergo fusion with lysosomes resulting in the discharge and digestion of their ILVs in the lysosome lumen. The process depends on surface proteins (such as HD-PTP, the HOP complex, and the GTPase Rab7) and on the assembly of a membrane-fusion soluble NSF attachment protein receptor (SNARE) system including VAMP7, Vti1b, syntaxin 8, and syntaxin 7 [17–20]. By contrast, the formation of exosomes relies on the fusion of MVBs with the plasma membrane. On fusion, the MVB limiting membrane and ILVs diverge. The limiting membrane integrates with the endosomal recycling system, while the ILVs are released as exosomes to the extracellular space [20–22].

On their way to becoming MVBs, the small membrane domains become enriched with various sorted molecules including lipids such as cholesterol (high concentration) [20,23], lysobisphosphatidic acid (an unconventional phospholipid, not detected elsewhere in the cell) [20,23–26], and sphingomyelins along with their product ceramide, which is known to play a role in budding [20,27]. A critical aspect of the process is the accumulation and processing of ubiquitinated proteins that are transferred by the cytosolic endosomal sorting complex required for transport (ESCRT) machinery from the plasma membrane and endosomes to the budding ILVs (Figure 1) [20,28–31]. The four ESCRT complexes, each comprising several subunits, play distinct roles: ESCRT-0 binds and sequesters the ubiquitinated proteins; ESCRT- I, in concert with ESCRT-II, initiates local budding of the endosomal membrane; and ESCRT-III, through its interaction with the enzyme HD-PTP, participates in protein deubiquitination [20,28–32] (Figure 1). In addition, ESCRT-III allows the subsequent pinching off of ILVs to become exosomes. The interaction of ESCRT subunits, with their associated protein Alix, its

homologs, and others, also allows the accumulation of luminal cargo [20,32]. Recent studies have revealed that ESCRT complex subunits and associated proteins may vary in expression and/or function. These and other heterogeneities among exosomes have been observed in various cell types, including cancer cells [21,31–35].

Compared with the assembly of ILVs, the initial process that leads to the assembly of ectosomes appears largely different (Figure 1). Since ILVs have no direct connection with the plasma membrane, their biogenesis is in contrast with that of ectosomes, which are assembled by the regulated outward budding of small plasma membrane domains [7–9,36]. Similarly to lipids contributing to ILV budding, cholesterol, sphingomyelin, and its product ceramide [9,10,37] are segregated during the formation of ectosomes. Other phospholipids, such as unsaturated phosphatidylcholine and phosphatidylethanolamine, are not abundant in the plasma membrane domains [37]. While some ectosome membrane proteins (tetraspanins and integrins, receptor agonists) are present in exosomes, others are exclusive to ectosomes (Table 1). The latter proteins are not always the same for each cell type: matrix metalloproteinases (MMPs), particularly MT1-MMP, are enriched in the ectosomes of many cancers; the membrane glycoproteins GP1b and GPIIb/IIa and P-selectin are abundant in platelets but not in megakaryocytes; and the integrin Mac-1 is found in neutrophils [36].

Furthermore, the accumulation of cargo proteins in the ectosome lumen, which is often larger than that of ILVs, occurs by various mechanisms. The ESCRT machinery was initially considered important only for MVBs. However, ESCRTs play important roles in other processes involving membrane sorting, budding, and fission, such as cytokinesis and virus budding [20,38,39], which are specific to the plasma membrane. Recent evidence demonstrates that at least some ESCRT subunits participate in the assembly and budding of ectosomes (Figure 1). ESCRT-I subunit TSG101, when relocated to the plasma membrane, interacts with Alix and another accessory protein, ARRDC1, on ectosomes. Moreover, ESCRT-III is critical for the pinching off and release of ectosomes [40]. In addition to ESCRT, the assembly of ectosome luminal cargo requires the binding of cytoplasmic proteins to the plasma membrane. Such binding is based, not on specific motifs or features of the proteins involved, but on their plasma membrane anchors (myristoylation, palmitoylation, and others) and high-order polymerization, which concentrate them to the small plasma membrane domains of ectosome budding [41,42]. Interestingly, the anchored proteins of ectosomes appear to be specific for the plasma membrane. Therefore, they cannot participate in ILV assembly at MVBs [41,42]. Additional proteins that accumulate in the ectosome lumen are sustained by direct interactions with the anchored complexes [42]. These interactions appear to be critical for the establishment of proteomic networks and clusters in the ectosome lumen. In addition, the lumen contains molecules common to exosomes: cytoskeletal proteins, heat-shock proteins, and numerous enzymes, together with various RNAs such as mRNAs, siRNAs, and long noncoding RNAs [36,41–43].

Vesicle pinch off and release

After budding, the vesicles must pinch off the membrane to be released into the MVB lumen or extracellular space; however, the mechanisms by which exosomes and ectosomes achieve release into the extracellular space are significantly different (Figure 1). Among the molecules likely to be active in the process are: ceramide, which accumulates in the membrane of both types of vesicle [27,37]; the Ca^{2+}-dependent scramblase, which favors phospholipid exchange between the two leaflets and thus increases the membrane curvature and favors pinching off; annexin-2 [44]; chromosome segregation 1-like protein [45]; and hyaluronan synthase 3 [46]. An additional mechanism of vesicle pinching off is similar to other forms of membrane scission and requires ESCRT complexes and their associated proteins, particularly the AAA-ATPase Vps4, which is critical for protein complex disassembly [47]. Several models have been proposed to interpret the interaction of ESCRT-III with the cytoplasmic surface of ILVs and ectosome domains: purse strings [48], polymer-driven membrane buckling [49], and dome-based membrane scission [50]. An additional model [51], conceived from the results of experiments in which the process of ESCRT-III filament generation had been reduced, demonstrates the critical role of these filaments in the segregation of endocytic membrane domains destined to become ILVs. This stage is followed by the rearrangement of the ESCRT-III filaments into conical spirals that induce the membrane domains to bud. Finally, constriction by the Vps4 ATPase of the ring of ESCRT-III spirals at the budding neck leads to membrane scission, with ensuing pinching off of the vesicle [51–53] (Figure 1). This model is likely to also be plausible for the pinching off of ectosomes. At least part of the energy necessary for the membrane dynamics originates from the remodeling of the cortical actin cytoskeleton [54,55], which is governed by small G proteins of the Ras family: Arf6, Cdc42, and various Rabs [55–58].

On pinching off, the two classes of vesicle diverge. ILVs (50–100 nm in diameter) remain trapped within MVBs for a considerable time, resulting in the delayed release of exosomes into the extracellular space. In some MVBs, the ILVs fuse to each other, creating larger, pleiomorphic structures. In addition, some ILVs undergo back fusion, which is the process of reintegrating their membrane with the limiting MVB membrane and discharging their cargo back into the cell cytoplasm [20]. This process is likely to impact the number of exosomes released into the extracellular space. Exocytic fusion of MVBs with the plasma membrane is required for the release of exosomes. Currently the mechanisms governing this process remain largely unknown. A few small GTPases, such as Rab11, Rab27, and especially Rab35, with its activators TBC1D10A–C, have been shown to be involved [59–61]. As with other types of membrane fusion, this process is mediated by a SNARE complex that might include VAMP7, the vSNARE associated with most endosomes [19,21]. The constitutive nature of this process is suggested by the observation that the release of exosomes is often assayed tens of minutes after the application of stimulation [62–64].

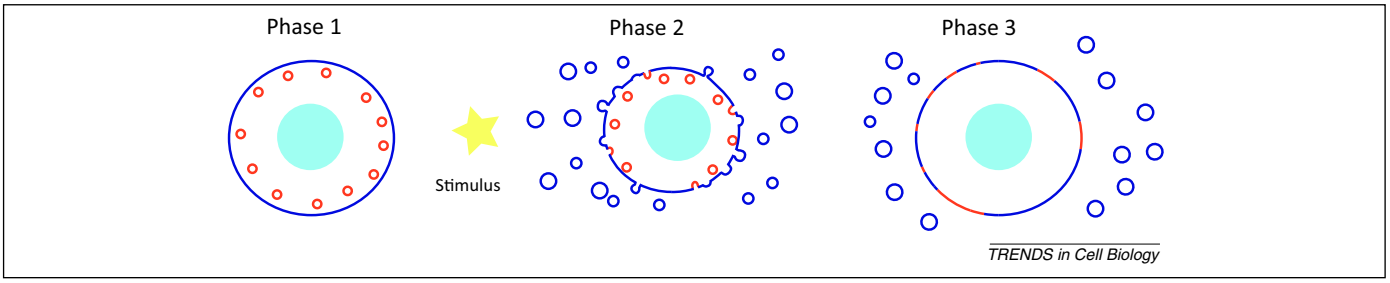

Figure 2. Ectosome release matched by the exocytosis of intracellular vesicles. The three panels illustrate an ectocytic release response and a compensatory exocytic process. Phase 1 shows the steady state of a resting cell competent for strong ectosome release. Phase 2 shows the same cell a few tens of seconds after the application of an effective stimulus (e.g., ATP). Many ectosomes have been, and many others are being, released, thereby inducing the concomitant loss of segregated cytoplasm and its delimiting plasma membrane, with ensuing shrinkage of the cell. Phase 3 shows a late phase. The ectosome release is finished. The shrinkage has induced the exocytosis of intracellular vesicles (red) to compensate for the loss of plasma membrane.

In contrast to exosomes, the release of ectosomes does not require exocytosis (Table 1 and Figure 1). On pinching off, these larger vesicles (100–350 nm in diameter) are released into the extracellular space at a high rate. In cells where the process is greatest (dendritic cells, macrophages, microglia), the release is visible within a few seconds after stimulation by ATP from the P_2X_7 receptor and proceeds for several minutes, accompanied by retraction and the rearrangement of the cell's shape [9,12,36,42,65,66]. An increase in the concentration of free Ca^{2+} acts as a cytosolic second messenger to sustain the release of ectosomes [36,42,65–67]. However, the activation of protein kinase C by phorbol esters has also been shown to induce strong ectosome release responses [36,67].

Membrane traffic

The biogenesis and release of EVs requires the trafficking of membranes throughout the cytoplasm and back to the cell surface for release into the extracellular space. The shedding of ectosomes results in the removal of small portions of the plasma membrane, while exosome shedding occurs with the generation of ILVs and is compensated for by the homotypic fusion of MVBs with other endosomes [20]. The surface traffic concerns the limiting membrane of exocytosed MVBs. In the case of both exosomes and ectosomes, important measures are taken by cells to maintain the plasma membrane equilibrium. Studies of other traffic events demonstrate that significant expansion or reduction of the cell surface requires compensation by opposite changes in the area [68].

When shedding is slow, as observed for exosomes, shedding is compensated for by the trafficking of endosomes, maintained in equilibrium at the surface. By contrast, stimulation can induce high rates of shedding of ectosomes, which can rapidly affect cell volume. Here the plasma membrane area is first reduced and then progressively re-established [9,36] by expansion of the cytoplasm. At least in some cases, this expansion is associated with stimulated heterophilic fusion (exocytosis) of intracellular vesicles [67] (Figure 2). These exocytoses can occur in proximity to ectosome release or at different sites, where they contribute not only to the expansion of the plasma membrane but also to the compositional differences among plasma membrane areas [36]. The changes in membrane trafficking that occur during the biogenesis of ILVs and ectosomes are therefore profoundly different.

Rapid dissolution, navigation, and cell fusion of EVs

EVs communicate with other cells by either releasing their segregated agents shortly on discharge or by directly contacting target cells over short or long distances (Figure 3) [20,36,63]. The consequences of this transfer can be significant.

On release, some EVs do not remain intact and can break down and expel their contents into the extracellular space (Figure 3, left). The released segregated agents, including interleukin-1β (IL-1β), tissue factors, and various growth factors such as transforming growth factor beta (TGFβ), bind their receptors in adjacent cells and activate rapid responses [42,69–71]. The EVs that are abundant and maintain their structure longer may undergo long-term navigation in major fluids, including blood serum, lymph, and cerebrospinal fluid, impacting the fate of target cells (Figure 3) [13–16]. For example, EVs present in blood serum are released mostly, but not exclusively, by platelets, monocytes, red blood cells, granulocytes, and endothelial cells to regulate various functions such as coagulation [72]. Other vesicles have also been shown to reach lymph and blood from the extracellular space of tissues [73]. Further, navigating EVs promote the degradation of the extracellular matrix by their transmembrane proteinases, a function critical for the circulation of macrophages and for tissue invasion by cancer cells [36,56]. Their roles in regulating disease processes has sparked interest in them as possible markers of numerous diseases and as potential transporters of cargos of drugs addressed to critical cell targets (Box 1).

On release, EVs do not interact with any cell, but show a preference for certain target cells. For example, vesicles shed from platelets interact with macrophages and endothelial cells but not with neutrophils [74]. Moreover, ectosomes of a different origin can induce different effects in a single cell type. For example, after engaging with macrophages, ectosomes shed from platelets downregulate while those from neutrophils activate multiple signaling pathways [75,76]. Information about the various proteins exposed on the surface of exosomes, ectosomes, and their target cells that account for these heterogeneous responses remains largely unknown, but this is currently an active area of investigation. Further interactions of EVs with their cells of origin and with other cells of the same tissue remain to be clarified.

Although the specific mechanisms that permit EVs to act on one cell over another remain undefined, their

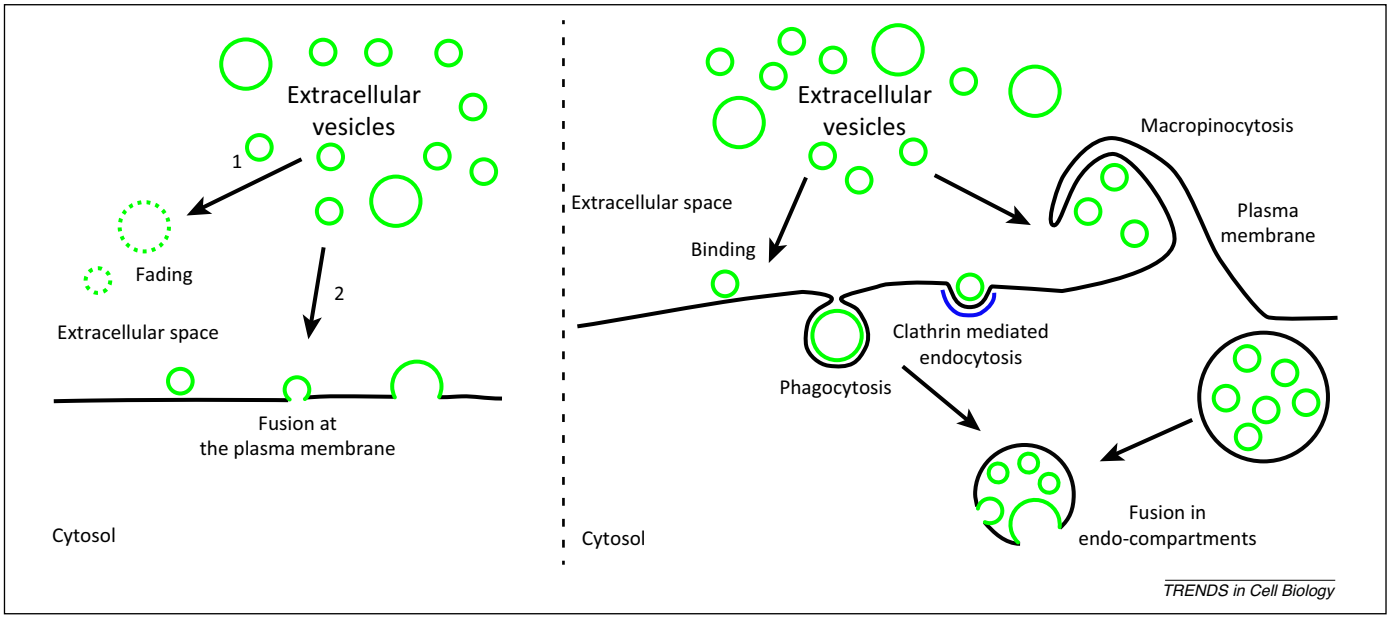

Figure 3. Extracellular vesicle (EV) interactions with target cells. Various forms of interaction are described in the two panels. The left panel shows EVs either disassembled in the extracellular space (releasing the active factors contained in their lumen) (fading, 1) or establishing specific binding with the plasma membrane of their cell targets (2). This stage can last many seconds, with the EVs rolling on the cell surface, until direct fusion starts and the luminal material is released into the cytosol of the target cells. The right panel illustrates the uptake of entire EVs by target cells, which occurs by various forms of endocytosis (here the clathrin-mediated form), by macropinocytosis, and by phagocytosis. Within target cells many EVs end up within MVBs. Some then fuse their membrane with the MVB limiting membrane by a process known as back fusion, corresponding to the opposite of intraluminal vesicle (ILV) generation.

general interaction with cells make it clear that EVs operate as signal transduction platforms inducing the stimulation or inhibition of their targets. On contact, exosomes and ectosomes roll over the cell surface. Soon after their rolling and binding phases, the vesicles stop, most likely by binding cell surface proteins and receptors, and proceed to transfer materials inside the cells (Table 1 and Figure 3). In many cases the EVs discharge their contents to the target cell cytoplasm by directly fusing with the plasma membrane (Figure 3, left). The mechanisms of fusion of EVs and plasma membranes, which are profoundly different from the processes sustained by SNARE complexes [9,77], might resemble the fusion of retroviruses with their targets or cytokinesis of striated muscle, bone, placenta, and other tissues. Fusion is triggered by the receptor binding of specific surface proteins, the so-called fusogens. So far only one family of mammalian fusogens has been identified, the syncytin-1 family, discovered in the placenta and recognized in various cells, including several cancer cells [78–81]. Here the membranes merge on the binding of syncytin-1 to its receptor ASCT2, a neutral amino acid transporter [80]. In some cells, syncytin-1 has been shown to also operate in the fusion of exosomes [82,83]. Whether the same fusogen works in the fusion of ectosomes is unknown.

Uptake of EVs can also occur without membrane fusion through a type of endocytosis (Figure 3, right). Exosomes and small ectosomes fit within clathrin vesicles, whereas the larger ectosomes employ other processes of internalization such as macropinocytosis and phagocytosis [84]. On uptake into the endosome network, the EVs might ultimately fuse with the organelle membrane (back fusion) and discharge their contents into the cytoplasm [20] (Figure 3, right). Alternatively, the vesicles could be retained within the endosome lumen or fuse with lysosomes and be completely digested.

Box 2. Purification of EVs and isolation of exosomes and ectosomes

The need for adequate procedures to purify and isolate EVs is widely recognized in the community. EV-containing preparations from cell cultures, cell suspensions, tissues, and fluids (navigating EVs) inevitably contain contaminants such as fragments of apoptotic cells, protein aggregates, protein oligomers, lipoproteins, or proteins associated with the EV surface [14,15]. To ensure their complete purification, EVs are pre-cleaned and filtered before being processed, most often by procedures based on centrifugation (differential centrifugation, sucrose gradients, or buoyant density gradients). Alternative procedures are also employed based on immunoaffinity beads, gel filtration, or flow field–flow fractionation [14–16]. Techniques for EV characterization, such as resistive pulse sensing, nanoparticle tracking analysis, and small-angle X-ray scattering, are now being investigated [34,16,103]. The potential for future development appears promising; however, current technical standardizations are not yet fully established [15]. Therefore, results with purified exosomes and ectosomes that have been reported so far need to be considered with caution. Isolation of the two classes of EV remains problematic. Since exosomes and ectosomes both display unique markers (see Table 1 in main text), their isolation should be feasible by the various techniques mentioned. However, the physical differences between the two EV classes are subtle. While preparations enriched in one of the EVs can be further purified, attempts to separate mixtures of similar percentages have failed. This failure is most notable in studies that isolated navigating EV preparations for the investigation of protein, lipid, and RNA composition, which reference the mixture and not single classes [14–16].

A major interest of many studies is not the isolation but the analysis of the multiple EV components, especially proteins that first need to be isolated. At present, the various procedures employed for isolation include SDS-PAGE, 2D liquid chromatography of peptides, and isoelectric focusing. Once isolated, the peptides prepared from the proteins can be identified by high-throughput mass spectrometry [15]. Several hundred proteins from EVs, identified by previous studies together with lipids and RNAs, can be found in the free web-based EVpedia database (http://evpedia.info).

Despite advances in our understanding EV release and navigation, the procedures for isolating EVs have led some to question the exact mechanism by which the two classes of EV perform their functions. While some studies have been conducted on vesicle preparations comprising almost exclusively either exosomes or ectosomes [9,21,40,65,67,75,85], other studies, despite using procedures believed to be for the isolation of a single class, obtained only mixtures of EVs. The results obtained by this approach need to be considered with caution. Finally, other studies, conducted especially on navigating vesicles, are usually attributed, most probably correctly, to EV mixtures [14,15] (Box 2).

Concluding remarks

In this review we have focused primarily on recent progress in our knowledge about exosomes and ectosomes. The present state and advances of such knowledge in the next few years will continue to make clear their relevance for the progress of research and the treatment of diseases. EVs play unique roles in cell communication. It should be emphasized that their membranous shell prevents degradation of their contents, which comprise primarily soluble factors, proteins, and RNAs. This protection makes possible long-duration and long-distance actions. During these events, EVs induce a type of functional expansion in the cell, expanding the confines of their transcriptome, proteome, and lipidome from the plasma membrane to the extracellular space they reach. Here we anticipate some future directions: the development of better procedures to isolate EVs and to distinguish ectosomes and exosomes, both in the tissue environment and in body fluids (Box 2); the identification of various families of active molecules exposed at the surface of exosomes and ectosomes; and the recognition of specific properties of target cell populations and subpopulations. Studies of the interactions of EVs with their cells of origin and with those of the adjacent tissue will provide explanations for the horizontal transfer of materials and information among cells; for example, DNA, RNA, signaling cascades, and gene expression [36,43,85–89]. In addition, EVs play critical roles in disease as well as diagnostic approaches. Approaches to efficiently deliver hydrophilic and large drug compounds and siRNAs to test early detection, therapy responses, and follow-up of diagnoses should continue (Box 1). It can be concluded that the two classes of EV, although partially different from each other (Table 1), constitute a single, unconventional communication system that has opened new paths to our understanding of cellular physiology and pathology, together with opportunities for the development of advanced medicine.

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