

Review

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Exosomes: the ideal nanovectors for biodelivery

Abstract: Nanomedicine aims to exploit the improved and often novel physical, chemical, and biological properties of materials at the nanometric scale, possibly with the highest level of biomimetism, an approach that simulates what occurs in nature. Although extracellularly released vesicles include both microvesicles (MVs) and exosomes, only exosomes have the size that may be considered suitable for potential use in nanomedicine. In fact, recent reports have shown that exosomes are able to interact with target cells within an organ or at a distance using different mechanisms. Much is yet to be understood about exosomes, and currently, we are looking at the visible top of an iceberg, with most of what we have to understand on these nanovesicles still under the sea. In fact, we know that exosomes released by normal cells always trigger positive effects, whereas those released by cells in pathological condition, such as tumor or infected cells, may induce undesired, dangerous, and mostly unknown effects, but we cannot exclude the possibility that exosomes may also be detrimental for the body in normal conditions. However, whether we consider extracellular vesicles as a whole, thus including MVs, it appears that even in normal conditions, extracellular vesicles may lead to unwanted effects, depending on gender and age. This review aims to critically emphasize existing data in the literature that support the possible roles of exosomes in both diagnostic and therapeutic scopes.

Keywords: biodelivery; biomimetism; diagnosis; exosomes; extracellular vesicles; microvesicles; nanomedicine; therapy.

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Introduction: exosomes' nature

Most, if not all, cell types release their vesicles in the extracellular microenvironment. In general, the extracellular vesicle population comprises different types of small vesicles including microvesicles (MVs) and exosomes. Both exosomes and MVs are membrane-bound vesicles that differ in biogenesis and biophysical properties, such as size and surface protein markers, and in biological roles, such as cell-cell communication, maintenance of normal physiological process and disease pathology (Lee et al., 2012). However, this review wants to focus mostly on exosomes as potential nanocarriers for molecules and potentially useful in future strategies of nanomedicine.

From a morphological point of view, exosomes are nanovesicles ranging from 50 to 150 nm released from a variety of cells. The cells release exosomes in a constitutive manner and after stimulation or overstimulation. However, it appears conceivable that, while the exosomes are released by viable cells, the performance status of the exosome-releasing cells may greatly differ. For instance, although the peripheral blood mononuclear cells, monocytes, and B cells normally release high amount of exosomes, the exosome levels may vary both quantitatively and qualitatively after stimulation. On the contrary, resting T cells do not release a detectable amount of exosomes, whereas they release exosomes only after overstimulation, which suggests that exosome release by T cells is not to be considered a real 'physiological' function. Exosomes do not derive from a plasma membrane shedding, but they are secreted by peculiar intracellular structures called multivesicular bodies (MVBs), which

are probably derived from a cascade of multifusion phenomena between internal vesicles, including early and late endosomes, lysosomes, and other structures that vary depending on the cellular source. Exosomes constitutively express tetraspanins (CD63, CD9, CD81), endosomal and lysosomal markers (Rab5, LAMP), and heat shock proteins (HSP 70). These nanovesicles are totally distinct from apoptotic microparticles, which bear markers such as CD31 or annexin V (Katzmann et al., 2002; Stoorvogel et al., 2002; Raiborg et al., 2003; Fevrier et al., 2004; Keller et al., 2006; Simons and Raposo, 2009; Record et al., 2011). It is worth mentioning that exosomes produced from a single cell can in fact comprise a heterogeneous population of vesicles that may feature different markers composition and probably different functions.

At the beginning (some decades ago), exosomes were considered a sort of 'nano-waste bin' of the cells having the role of removing unwanted or unneeded membrane or cytosolic or even nuclear material as an integrated machinery to the lysosomal system (Katzmann et al., 2002; Stoorvogel et al., 2002; Fevrier et al., 2004). However, more recent data support the role of exosomes in both the maintenance of the normal body homeostasis and the pathophysiology of many diseases. Exosomes are released not only by a variety of cells (hematopoietic, endothelial, epithelial, Schwann and neuronal cells, adipocytes, and fibroblast) but also by cells in different conditions of proliferation and differentiation. Extracellular vesicles are normally released within the organs and tissues, and they are detectable in urine,

epididymal fluid, amniotic liquid, ascites, bronchoalveolar lavage fluid, synovial fluid, and breast milk. Moreover, they are also spilled over into the bloodstream, making them detectable in the peripheral blood as well.

A crucial issue in future investigations on exosomes is to better distinguish between normal and pathological exosome cargo in terms of proteins, lipids, and nucleic acids. In fact, with this review, we provide evidence that would help in distinguishing the molecular composition and content of exosomes in either normal or pathological condition. Moreover, we review all the evidence suggesting the future applications of exosomes as nanovectors for both diagnostic and therapeutic use.

Proteins

Exosome protein composition includes both an ubiquitary and a cell type-specific protein and covers numerous biological functions. Table 1 shows a summary of more representative exosome proteins produced by different cellular source, such as B lymphocytes (Wubbolts et al., 2003), mast cells (Skokos et al., 2002), reticulocytes (Carayon et al., 2011), dendritic cells (Segura et al., 2005), intestinal epithelial cells (van Niel et al., 2001), and tumor cells (Iero et al., 2008). A complete database of exosomal proteins can be found at ExoCarta (exocarta.ludwig.edu.au/) (Mathivanan et al., 2012).

Type/biological function	Exosome protein composition
Adhesion molecules	$\alpha 3$, $\alpha 4$, $\beta 1$, $\beta 2$, 11a, 11b, 11c, integrins, CD63, CD9, CD37, CD53, CD81, CD82 tetraspanins
Antigen presentation	cadherin 1, ICAM-1, MFG-E8, thrombospondin 1, plexin A1, C1 neuropilin 1, dectin 1, 2
Cytoskeletal/structural proteins	MHC class I, II, CD86
Enzymes	Actin, ezrin, radixin, moesin, tubulin, keratin, cofilin, vinculin, F-actin capping protein β -subunit, vimentin, talin, coronin, advillin, fascin 1, CAP1, actinin, myosin heavy chain IX, gelsolin, lumican
Heat shock proteins/chaperones	Enolase 1, pyruvate kinase, lactate dehydrogenase, glutathione S-transferase, phosphogluconate dehydrogenase, phosphoglycerate mutase 1, ATP citrate lyase, fatty acid synthase, M2 pyruvate kinase, GAPDH, aldose reductase, thioredoxine peroxidase, ATPase Na^+/K^+ transporting, cytosolic malate dehydrogenase
Lipid raft proteins	Hsc70, Hsc71, Hsp73, Hsp71, Hsp84/90, cyclophilin A, B, H2-M, invariant chain
Membrane trafficking, transport, and fusion	Flotillin 1, CD55, CD59, stomatin, nicastrin
MVB formation	Annexin 1, 2, 4, 5, 6, 7, 11, RAB 1, 2, 4, 5, 6, 7, 10, 11, 13, 14, 15, SNAP23, RAP1B/RABGD1, syntaxin 7, syntaxin binding protein 3, dynamin 2, P-1, Arp 2/3, clathrin heavy chain, ARFs
Signal transduction	Alix, Tsg101, ubiquitin, clathrin
Transcription/protein synthesis	ERK2, Fyn, SH2 phosphatase 1, FRL, RhoA, RhoC, Rho GDI, RAP1B, Grb3-3, H-ras, SHPS-1, STAT-1, syntenin, catenin $\text{E}\alpha$, β -catenin, phospholipase C, C-src tyrosine kinase, CBL/LCK, $\text{Gi}2\alpha/14\text{-}3\text{-}3$, $\text{Gi}3\alpha$, $\text{Gs}\alpha$
Transmembrane molecules	EEF1a1, EEF2, ADP ribosomal proteins L18, L6, S18, histone 1, 2, 3
	LAMP 1, 2, transferrin receptor, CD13, CD26, CD98, PGRL, P-selectin ATPase channel, A33 antigen

Table 1 Summary of more representative exosome proteins produced by different cellular source (B lymphocytes, mast cells, reticulocytes, dendritic cells, intestinal epithelial cells, and tumor cells, etc.) assembled according to major biological function.

Most of the exosomal proteins that have been identified derive from the endocytic compartment or the plasma membrane of cellular source and very rarely from other internal compartments such as Golgi, nucleus, endoplasmic reticulum, and mitochondria. In fact, typical exosomal proteins are tetraspannins, lysosomal markers, and proteins involved in trafficking and membrane fusion. Other proteins are part of the protein cargo of exosomes, such as cell adhesion molecules, cytoskeletal molecules, heat shock proteins and chaperones, metabolic enzymes, and proteins referring to exosomes biogenesis or MVB biogenesis, signaling proteins, and proteins involved in transcription and protein synthesis. Although the presence of some common exosomal proteins can be related to their biogenesis and affinity sorting with other ubiquitous exosomal proteins and lipids, the functional significance of certain proteins persistently found on exosomes of different origins is not completely understood. Markers commonly used to characterize a composition of exosomes may have varying distribution between cell types. For instance, the transferring receptor is absent in exosomes derived from B cells (Raposo et al., 1996), whereas it is present in erythrocyte-derived exosomes (Olver and Vidal, 2007). Given that exosomes may be released by almost all the cells of the body compartments, many other examples of a cellular source-specific exosome-associated proteins may be provided, including, but not limited to, cell adhesion molecules (such as L1 on neuronal or EpCam on epithelial-derived exosomes), MHC molecules on dendritic cells and macrophage-derived exosomes, CD41 predominant on platelet-derived vesicles, and so on. Moreover, in many disease conditions, exosomes can acquire proteins

that may define the cellular source, thus providing a novel biomarker of the disease. For example, in cancer, CD63 and caveolin 1 were significantly increased in melanoma patients as compared with healthy donors (Logozzi et al., 2009). Additional reports showed that extracellular vesicles may express other tumor markers, such as MelanA/Mart-1 in melanoma-derived exosomes, carcinoembryonic antigen in exosomes from colon carcinoma, and HER2 exosomes from breast cancer cells (Andre et al., 2002). In kidney disease, the presence of water channel aquaporin 2 protein associated with exosomes in urine is considered a marker of kidney damage (Pisitkun et al., 2004; Takata et al., 2008).

Lipids

Exosomes show a peculiar lipid organization and composition (Figure 1). High levels of both sphingomyelins and cholesterol, without substantial differences in cellular source, have been reported (Subra et al., 2007). Exosomes also contain phosphatidylcholine and phosphatidylethanolamines, with some differences on the cellular source, particularly in terms of saturated fatty acid content (Laulagnier et al., 2004a,b). This enrichment in saturated phospholipids confers a high level of stability to the exosomes due to the high level of intrinsic rigidity of these molecules. In fact, *in vivo* exosomes remain perfectly functional in peripheral compartments (Luketic et al., 2007). Moreover, many reports clearly suggest the presence of lipid raft-like domains in exosome membranes due to the high content

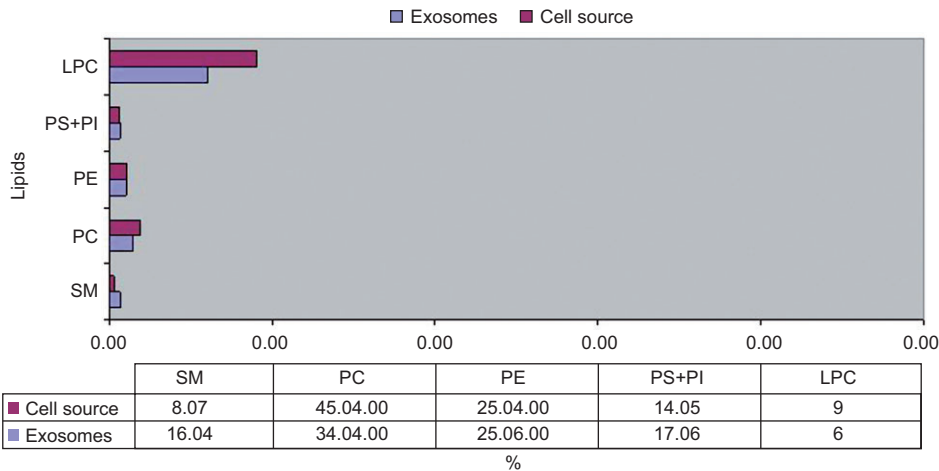


Figure 1 Comparison of lipid composition of exosomes and the corresponding cellular source. Values are expressed in percentage of total lipids. LPC, lysophosphatidylcholines; PS+PI, phosphatidylserines and phosphatidylinositols; PE, phosphatidylethanolamines; PC, phosphatidylcholines; SM, sphingomyelins.

of lipid raft-associated proteins (flotillin and stomatin) (de Gassart et al., 2003; Fevrier et al., 2004) and also by the contemporary finding in exosomes of all membrane lipids rafts (i.e., cholesterol, sphingomyelin, glycolipid GM3, and glycerophospholipids with long and saturated fatty acyl chains) (Wubbolts et al., 2003; Fevrier et al., 2004; Subra et al., 2007). Consistently, proteomic analyses have revealed the presence of a phospholipid scramblase, which is involved in mixing the phospholipids between the two membrane leaflets (Subra et al., 2007). Purified exosomes from Olineu cells (mouse, myelinating cells of the central nervous system) are enriched in ceramide, and the release of exosomes was reduced by the inhibition of neutral sphingomyelinase (Trajkovic et al., 2008). Noteworthy, some of the lipids that are found enriched in exosomes, in particular in correlation with their cellular source, are considered markers of some pathological conditions (such as ceramide in bladder cancer).

Nucleic acids

Exosomes contain nucleic acids (Table 2), but it is generally accepted that, unlike larger MVs, exosomes do not contain DNA. However, some contradictory reports exist pointing out that the presence of DNA might be dependent on the source of analyzed exosomes. For instance, it has been claimed that glioblastoma and astrocytoma cell-released exosomes might carry mitochondrial DNA (Guescini et al., 2010). Since the first report demonstrating the presence of mRNA and microRNA in exosome preparations (Valadi et al., 2007), thousands of different mRNAs may be identified in exosomes derived from murine and human cells. Interestingly, some hundreds of gene transcripts were exclusively detectable in MVs/exosomes and not in the cellular source of exosomes (Hu et al., 2012; Lässer, 2012; Ramachandran and Palanisamy, 2012). This suggests that specific selection mechanism/s operate during the intracellular sorting of RNAs into exosomes (Valadi et al., 2007; Taylor and Gercel-Taylor, 2008; Rabinowits et al., 2009; Mittelbrunn et al., 2011). Exosomes were also enriched in about 120–150 microRNA (Valadi et al., 2007). MicroRNAs are small, 22–25 nucleotides in length, noncoding RNAs that interfere with RNA post-transcriptionally by binding to the 3' untranslated regions of their target mRNAs to degrade it or suppress or stimulate translation. MicroRNAs have pleiotropic effects that are particularly involved in stem cell differentiation, differentiation and organogenesis, hematopoiesis, exocytosis, and tumorigenesis. Overall, the idea of the diversity

of RNA molecules shuttled by exosomes comes from latest reported ExoCarta entries (Mathivanan et al., 2012) comprising 2375 mRNA and 1630 miRNA molecules. However, this manually curated database is likely to contain only a small fraction of all the RNAs that can be found in exosomes from different sources because the database is limited by the number and focus of performed exosome genomics studies so far. The exosome-associated mRNA and microRNA are functional, being translated or biologically active in the recipient cell, thus affecting its phenotype. This notion strongly supports a key role of exosomes as vehicle for RNA. It has been shown that placental-specific miRNAs, released *via* exosomes, can be exported from the placental trophoblast into the maternal circulation, where they can be found abundantly. MicroRNAs have been found within exosomes isolated from human saliva (Michael et al., 2010). These exosomes may transfer their genetic information to human oral keratinocytes, thus modifying gene expression in the cell. Lastly, exosomes released by tumor cells may transport molecules, including RNAs, with the ability to increase tumorigenesis but possibly useful as biomarkers as well (Skog et al., 2008). Although it is not what could be defined as a normal condition, most healthy individuals in the Western countries are EBV positive. In exosomes purified from EBV-transformed lymphoblastoid B cells (LCL), the presence of miRNA has been detected, among which BHRF1 and BART EBV miRNAs are abundantly represented in EBV-associated tumors (Vallhov et al., 2011; Verweij et al., 2011). LCL exosomes were capable of transferring EBV miRNA in recipient monocyte-derived dendritic cells, and the EBV miRNA BHRF1 or EBV miRNA BART permitted the repression of their specific target mRNAs, CXCL11 and LMP1, respectively. An interesting finding was the discovery of EBV miRNA in non-B-cell populations of PBMC recovered from asymptomatic HIV patients, whereas EBV DNA was exclusively restricted to B-cell population, suggesting an miRNA transfer between these cells *in vivo*.

All the above studies agree with the fact that specific microRNAs, proteins, or lipids found in exosomes could represent a basis on which future biomarkers for the diagnosis and prognosis of diverse human pathologies can be developed.

Exosome as biomarkers of pathologies

Exosomes have been detected in most biological fluids and, due to their specific protein, lipid and RNA content,

Biological function	Exosome shuttled RNAs	Source	Reference
Transcription regulation, regulation of expression of protein kinases and other enzymes, translation regulation	Type: mRNA 1300 mRNAs including 47 gene products (HMG1, MEF20, RPL7, SP1, EIF2AK2, PRKG1, ASNA, BRAF, PN4, SOD1, EIF5A, GHR, etc.)	Mast cell exosomes <i>in vitro</i>	Valadi et al., 2007
Stem cells differentiation control, differentiation, and organogenesis, hematopoiesis, exocytosis, tumorigenesis	Type: miRNA 120 microRNAs (e.g., Let-7, miR-1, 181, 17, 18, 19a, 20, 19-b1, 92-1)	Mast cell exosomes <i>in vitro</i>	Valadi et al., 2007
Transcription and replication regulation, cell signaling, regulation of expression of different enzymes, cell differentiation	Type: mRNA Gene products (Topol, Ccgp2, KRIT1, Amy2a1, 2FP, 38PC, PHD finger protein 6, Sox15)	Mast cell exosomes <i>in vitro</i>	Eldh et al., 2010
Communication in upper airways, source of biomarkers of upper airways diseases	Type: miRNA	Human nasal secretions	Lässer et al., 2011
Regulation of cell growth and manipulation of tumor environment	Type: miRNA EBV miRNAs	Nasopharyngeal carcinoma cells harboring latent EBV	Meckes et al., 2010
Role in cardiovascular diseases and disease biomarkers	Type: miRNA Circulating muscle-specific miRNAs (e.g., MiR-133a)	Activated H9c2 cardiomyoblasts and serum of myocardial infarction mouse model	Kuwabara et al., 2011
Cell processes, physiological processes, growth regulation, reproduction, biological regulation	Type: mRNA 509 mRNA transcripts including gene products (annexins, moesin, IL-6, EEF2, keratin 6A, OS9)	Human saliva	Palanisamy et al., 2010
Abnormalities in saliva production, peripheral inflammatory response in salivary gland, pathogenesis of salivary gland tumors	Type: miRNA miRNA species highly expressed in normal (27) individuals and patients with Sjogren syndrome (21)	Human saliva	Michael et al., 2010
Angiogenesis, cell proliferation, immune response, cell migration, histone modification, retroviral RNA sequences	Type: mRNA Overall 27 000 transcripts 4700 exclusively present in exosomes	Primary glioblastoma cell cultures	Skog et al., 2008
Oncogenic mutations	Type: mRNA EGFR VIII mutant variant	Serum exosomes from glioblastoma patients	Skog et al., 2008
Tumor-specific miRNAs involved in tumorigenesis	Type: miRNA Tumor-specific miRNAs (e.g., miR-21, let7a, miR-16)	Primary glioblastoma cell cultures and serum exosomes from the same	Skog et al., 2008
Cellular differentiation, metabolic pathways, immune regulation	Type: miRNA 104 significantly expressed miRNAs including mir-223, 448, 191, 146, 16, 26a, 222, 24, 126, 32, etc.	Human plasma	Hunter et al., 2008
Angiogenic effects, activation and proliferation of endothelial cells	Type: mRNA/miRNA	Pancreatic tumor cells	Nazarenko et al., 2010
Tumor-specific miRNAs involved in tumorigenesis	Type: miRNA 218 mature miRNAs out of which 8 specifically enriched in exosome fraction (miR-21, 141, 200a, 200b, 200c, 203, 205, 214)	Ovary tumor cells	Taylor and Gercel-Taylor, 2008
Local protein translation, protein synthesis	Type: mRNA Gene products for engrailed 1, CAM kinase α , AMPA receptor subunits, TEF 1A and 2	Postsynaptic exosomes	Smalheiser, 2009

(Table 2 continued)

Biological function	Exosome shuttled RNAs	Source	Reference
TNF-mediated signaling, unknown effects on maternal tissues	Type: miRNA Placenta-specific miRNAs (e.g., miR-517a)	Human chorionic with cells	Luo et al., 2009
Effective silencing of target gene	Type: siRNA Exogeneously administered siRNA for MAP1	Peripheral blood	Wahlgren et al., 2012
Effective downregulation of BACE1 and decrease of β amyloid levels in mouse brain	Type: siRNA Exogeneously administered siRNA for BACE1 (exosomes were engineered to express LAMP2)	Immature dendritic cells	Alvarez-Erviti et al., 2011

Table 2 Summary of findings on type of mRNAs/miRNAs assembled to major source and suggested biological function.

are now considered as potential biomarkers for the early detection of many pathological conditions. In fact, exosomes are detected in the plasma of both healthy individuals and patients affected by a variety of diseases, including cancer (Koga et al., 2005; Logozzi et al., 2009). Comparable to the exosome protein contents, very often, the microRNAs detected in tumor cell-released exosomes are similar to those detected within the tumor mass (e.g., lung tumors), suggesting that the microRNAs in circulating exosomes are representative of those expressed in the tumor, opening a perspective for the early detection of the disease using the analysis of circulating exosomes as a screening tool. Glioblastoma tumor cells release exosomes containing mRNA and microRNA, with former being translated in brain microvascular endothelial-recipient cells after internalization (Skog et al., 2008). Notably, as previously mentioned, the exosomal RNA repertoire does not simply mirror the parent tissue transcriptome, featuring and concentrating nucleic acids that are not or are poorly expressed by the producing cells, thus providing a source of novel biomarkers that can hardly be detected in the tissue of origin. This suggests that exosomes are much more than just a mechanism of elimination of cell dregs. Also, exosomes secreted in other easy accessible biofluids, such as human saliva, contain mRNA that may serve as disease biomarkers (Palanisamy et al., 2010). Exosomes are detectable in amniotic fluids as well, suggesting the potential use of exosomes as biomarkers for prenatal diagnosis (Keller et al., 2007).

In particular, exosomes detectable in urine represent appealing markers for diseases of the genitourinary tract, including prostate cancer. For instance, the presence of specific transcripts for PCA3 and for a specific product resulting from a chromosomal rearrangement, the TMPRSS2:ERG fusion in urine exosomes from prostate cancer patients has been described (Nilsson et al., 2009).

Exosomes also express aquaporin 1 and 2, which may represent useful markers of renal ischemia/reperfusion injury and antidiuretic hormone action, respectively (Takata et al., 2008). Thus, exosomes could constitute a source of multiple markers of malignancy that could provide clinically useful information (Logozzi et al., 2009).

Interaction of exosomes with cells

Exosomes may interact with cells with at least two mechanisms: ligand-to-receptor interaction (i.e., death ligands and receptors: FasL/Fas or TRAIL/TRAIL-R) and exosome fusion with the target cell membrane with the subsequent uptake of the exosome content by the cell (Figure 2A and B).

Exosomes as extracellular vector for triggering intracellular pathways

Current proteomic analysis of MVs/exosomes indicates that a range of 350–400 different proteins are present in these extracellular vesicles (Katzmann et al., 2002; Stoorvogel et al., 2002; Raiborg et al., 2003; Fevrier et al., 2004; Keller et al., 2006; Simons and Raposo, 2009; Mathivanan et al., 2010, 2012). Moreover, data support the specialized function of exosomes as a vectorized signaling device that appears more efficient than a soluble agonist. The reason contributing to this preferential action of exosomes as signaling devices may include both proteins and lipids. In fact, exosomes contain peripheral membrane proteins such as MHC I and II (Katzmann et al., 2002; Stoorvogel et al., 2002; Raiborg et al., 2003; Fevrier et al., 2004; Keller et al., 2006; Simons and Raposo, 2009), transferring

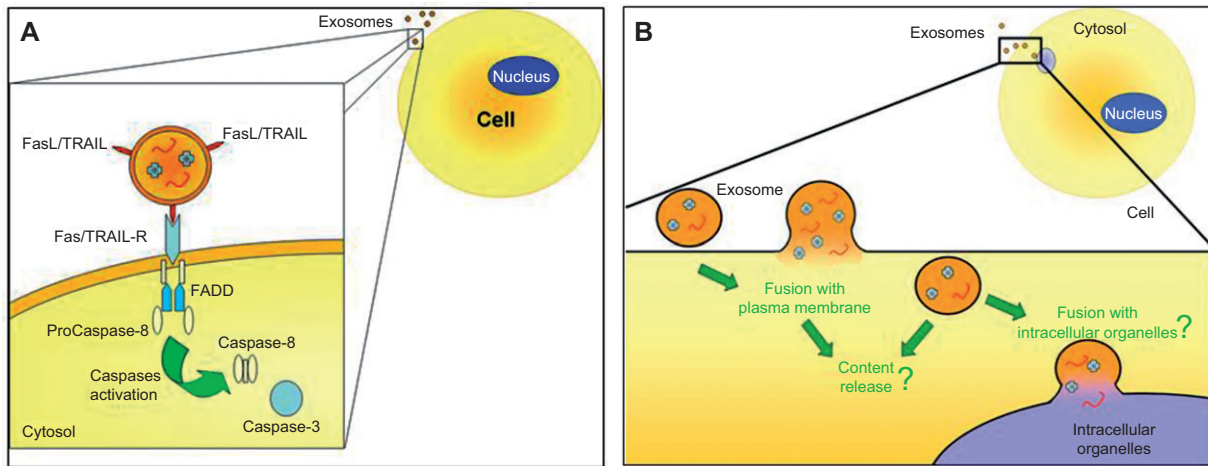


Figure 2 Exosome interaction with cells.

(A) A ligand to receptor interaction and signal transduction. (B) Exosome fusion with the target cell membrane and subsequent uptake of the exosome content by the cell.

receptors (Calzolari et al., 2006), and tetraspanins (Nazarenko et al., 2010), which are active in the downstream signaling pathways of target cells, triggering, for instance, integrins and calcium signaling (Clayton et al., 2004), MAPK activation (Calzolari et al., 2006), or NKG2D signaling (Clayton and Tabi, 2005; Viaud et al., 2009). However, it appears extremely conceivable that the enrichment in signaling molecules is not sufficient in allowing the hyperfunction on exosomes. In fact, exosomes also contain active lipolytic moieties such as phospholipases leading to the formation of bioactive lipid mediators (fatty acids and prostaglandins), which can interact with peripheral G-protein-coupled receptors and nuclear receptors in target cells (Subra et al., 2010). Lastly, exosome-to-cell interaction and exosome capture at the cell surface is highly facilitated by the reciprocal expression of intercellular adhesion molecules, such as ICAM-1 and LFA-1 (Segura et al., 2007; Nolte-’t Hoen et al., 2009; Simons and Raposo, 2009). Moreover, exosomes express other adhesion molecules such as VLA-4 (reticulocytes and B-cell exosomes) and $\beta 2$ integrin (dendritic cell exosomes), which potentially mediate adhesive interactions with a multitude of ligands, including cell surfaces and/or extracellular matrix components. A diminution of exosome uptake by dendritic cells was observed *in vivo* by the inhibition of avb3 integrins, CD11a and its ligands CD54, antibodies directed against the tetraspanins CD9 and CD81, or a soluble analogue of phosphatidylserine (PS) (Morelli et al., 2004). Among the other factors that may contribute to the specific function of exosomes as extracellular devices are exosome phospholipids. Although these are randomly distributed between the two membrane leaflets, due to exosome membrane curvature,

about two-thirds of the phospholipids are exposed to the outer layer of the membrane (Laulagnier et al., 2004a,b). This accounts for an apparent enrichment of cholesterol and the exposure of PS at outer membrane leaflet. Thus, multiple PS-binding proteins on recipient cells are susceptible to bind exosomes, and this includes several classes of scavenger receptors, integrins, CD14 complement, and PS receptors (Zakharova et al., 2007). Thus, exosomes’ adhesion to the recipient cell membrane is mediated through both lipids and ligand-receptor interactions.

A direct and clear example of the functional role of exosome expression of ligands for membrane receptors is given by ligands for death receptors. In fact, tumor cell-released exosomes express ligands for death receptors, including FasL and TRAIL. These ligands are perfectly functional in inducing death receptor-mediated cell death, even when expressed on circulating exosomes. Intriguingly, the FasL- and TRAIL-bearing exosomes released by malignant tumor cells may kill those lymphocytes that should kill them instead, while being unable to trigger cell death in the exosome-releasing tumor cells themselves (Andreola et al., 2002; Huber et al., 2005). Moreover, tumor exosomes are able to control the antitumor immune response by triggering the formation of myeloid suppressor cells (MDSCs; Valenti et al., 2006). This suggested the participation of tumor exosomes to the tumor immune escape (Iero et al., 2008).

Exosome uptake

In the preceding section, we have provided evidence and discussed the role of exosomes in triggering an

intracellular signaling pathway through a simple interaction with plasma membrane molecules. However, exosomes may undergo a quick internalization, in turn leading to different cascades of intracellular events. Exosome internalization/uptake by target cells appears to depend upon the type of recipient cells. Some evidence suggests that the internalization of exosomes by recipient cells occurs through phagocytosis (Morelli et al., 2004; Valadi et al., 2007; Skog et al., 2008) and that the extent of exosome uptake may depend upon the phagocytic capabilities of the recipient cell (Feng et al., 2010). Moreover, macropinocytosis may represent an alternative way through which exosomes may transfer their content between two different cell types (Fitzner et al., 2011).

However, recent reports provide evidence for another mechanism for exosome uptake, one that is mediated by a fusion between the exosome and the cell membranes. In fact, the delivery of exosome content can occur either by (i) fusion between exosome and the plasma membrane of the cell (Parolini et al., 2009), leading to the release of exosome contents into the cell cytoplasm, or (ii) internalization and degradation of the exosome membrane inside the cell (Thery et al., 2009). The internalization of exosome content by its fusion with the plasma membrane might be limited to acidic pH conditions such as that found inside a tumor (Parolini et al., 2009). Membrane fusion requires a similar fluidity between the two membranes supposed to fuse. The exosome and plasma membrane display the same fluidity at pH 5.0 (Laulagnier et al., 2004a,b) but not at neutral pH, which makes the membrane more rigid, therefore precluding fusion with another membrane (Subra et al., 2007). It is worth noting that the pH value of MVBs is about 5.0, and the fusion of the ILVs present in the MVB back to the MVB-limiting membrane has been reported to occur (van der Goot and Gruenberg, 2006). The internalization of exosomes appears to be a general phenomenon. It has been argued, however, that many works reporting internalization actually monitored the fate of exosome aggregates because exosomes spontaneously aggregate *in vitro*.

However, we do not have so much information on the fate of exosomes after their intracellular uptake. In the mast cell line RBL-2H3, exosome internalization occurs after a lag time of about 15–20 min and thereafter accumulated in the late endosomes/MVB of target cells after a sustained pulse (Subra et al., 2007). This observation indicates that the MVB compartment features a dual function of being a producer of exosomes and a receiver of exogenous exosomes. In each case, a different subset of MVB would be involved because exosome release is sectorized in the cell (Raiborg et al., 2003) and appears not to involve

all the MVB. In that respect, two distinct MVB pathways, one for lysosomal targeting and the other for exosome secretion, have been characterized (Buschow et al., 2009).

Exosome-mediated paracrine propagation within tissues and compartments: nanoregulators of the body pathophysiology

One can conceive that exogenous exosomes being internalized into a resting cell could in turn be released upon the activation of this cell, randomly reaching another recipient cell. Therefore, the simplest fate of exosomes coming from an initial producer cell is to propagate from cell to cell, i.e., in a paracrine manner within a tissue or a compartment. However, the presence of exosomes in the plasma of both healthy individuals and patients with various diseases suggests that exosomes may serve as vectors for transferring information to tissues and organs far from the place of production. This indicates that exosomes may well diffuse normal, abnormal, or aberrant messages to cells very close to their original source and at a distance as well by crossing the thickness of a tissue. This in turn suggests that exosomes may have a key role as a nanodevice belonging to an integrated network involved in multiple pathophysiologicals. The current knowledge seems to propose exosomes as key regulators of normal functions of the body. We know, for instance, that exosomes exert a very important role in reticulocyte differentiation. Reticulocytes normally release a mess of exosomes in the bloodstream (Blanc and Vidal, 2010). Although it appears conceivable that most of these exosomes undergo clearance to avoid unwanted events, the exact role of the reticulocyte-derived exosomes in the body is virtually unknown. B lymphocytes release antigen-presenting exosomes (Raposo et al., 1996), and dendritic cell-derived exosomes bear molecules involved in direct T-cell activation, including the MHC I and II complex, costimulatory molecules such as CD40, CD80, CD86, and heat shock proteins, conferring them a role in antigen presentation. This evidence has triggered numerous investigations to assess exosome potency to modulate the immune response. Moreover, exosomes derived from murine bone marrow dendritic cells loaded with tumor peptides display antitumor activity *in vivo*, both toward the murine mastocytoma P815 and the murine mammary carcinoma TS/A tumor models (Zitvogel et al., 1998). It was also observed that T cells were primed by exosomes in an antigen-specific manner, and an antitumor activity

mediated by cytotoxic lymphocytes was revealed in treated animals (Wolfers et al., 2001). In addition, exosomes can be viewed as an amplification process for dendritic cell-mediated T-cytotoxic responses (Andre et al., 2004). In fact, immature DCs are able to release amazing amounts of HLA class I- and II-bearing exosomes. Mast cell exosomes also participate in the immune response because they have the capacity to stimulate B- and T-cell proliferation and cytokine production such as IL-2 and IFN γ (Skokos et al., 2001). It was shown that peptide-loaded exosomes from mast cells elicit an efficient antibody production *in vivo* after a subcutaneous injection in mice. Mast cell-derived exosomes can trigger phenotypic and functional maturation of dendritic cells.

However, while the physiological role of exosomes is in fact poorly known, exosomes have been detected and investigated in association to many human diseases.

In fact, HIV-1-infected dendritic cells, monocytes, macrophages, and lymphocytes can produce both exosomes and HIV virions (Chertova et al., 2006). However, they have similar density, requiring separation by immunocapture or appropriate gradient density separation. Meanwhile, exosomes and HIV virions have similar mechanisms for entering into the endocytic compartment (Izquierdo-Useros et al., 2009). Moreover, HIV virions are partly generated and incorporated into the MVB. In fact, about 10% proteins are identical between HIV virions and exosomes issued from the same cell type (Chertova et al., 2006). These data support pioneer investigation showing that HIV-1 virions are released by infected cells in a polarized manner, co-localizing with intercellular adhesion molecules (Fais et al., 1995). In fact, HIV-1 virions bud from the infected cells carrying cellular proteins as well (Capobianchi et al., 1994). However, what is extremely intriguing is that HIV-1 exosomes released in a polarized manner in intercellular spaces allow a cell-to-cell infection without spreading free virions in the extracellular milieu. This represents a very efficient mechanism of infection that hides the virus from the immune system. This mechanism, on the one hand, could explain how viruses such as HIV can pass from one cell to another without being visible in the extracellular microenvironment. On the other hand, HIV-1-containing exosomes may be spilled over into the bloodstream, thus infecting other cells and organs, including the brain, given the relative ease with which exosomes pass the blood-brain barrier.

Exosomes may also indirectly favor infection by opportunistic pathogens, such as *Mycobacterium avium*, in HIV-positive individuals. *M. avium* express glycopeptidolipids (GLPs) as a major cell wall constituent, and *M. avium*-infected macrophages release exosomes containing

GLPs, in turn allowing the transfer of GLPs from infected to uninfected macrophages. Infected macrophages release exosomes that incorporate bacterial molecule pathogen-associated molecular patterns, which act as TLR ligands and lead to uninfected macrophages activation. Thus, exosomes from infected macrophages can stimulate a pro-inflammatory response (TNF- α and RANTES secretion) in resting macrophages (Bhatnagar and Schorey, 2007).

Opposite to the potent immune stimulatory properties of exosomes released from immunocompetent cells, many studies have demonstrated that tumor cell-derived exosomes display detrimental effects on the immune response against tumors, leading to suppressive pathways hampering the immune defenses in patients (Escudier et al., 2005). Part of this effect may be mediated by the action of death receptor ligands that are fully expressed and functional on exosomes released by human malignant tumor cells. In fact, exosomes secreted from tumor cell lines or tumor cells coming from patients can induce T-cell apoptosis *in vitro* through the expression of death ligands such as CD95 ligand (FASL) and TRAIL (Andreola et al., 2002; Huber et al., 2005). Moreover, it has been shown that exosomes may trigger the proliferation of MDSCs, which are potent inhibitors of the antitumor immune response (Valenti et al., 2006). MDSCs are found in large number in lymphoid organs, blood, and tumor tissues in cancer patients, acting as immature myeloid cells. These cells express myeloid marker stimulatory molecules (CD14 and CD11b) and are devoid of co-stimulatory molecules (HLA DR, CD80, CD86). They spontaneously secrete TGF β and have suppressive activity on activated T lymphocytes because they can inhibit T-cell proliferation and cytolytic functions. It was shown that melanoma and colorectal carcinoma-derived exosomes altered the monocyte differentiation into dendritic cells, leading to the generation of myeloid suppressive cells (Valenti et al., 2006). Consequently, tumor exosomes may contribute to tumor growth and participate in tumor evasion. Furthermore, it was demonstrated that MDSC-mediated promotion of tumor progression was dependent on the TGF β present on exosomes and on the lipidic mediator prostaglandin E2 transported by tumor exosomes (Xiang et al., 2009). The angiogenic properties displayed by tumor exosomes have also been documented. In a model of endothelial spheroid generated in matrigel with an endothelial cell line capable of spontaneously aggregating in spheroids, it was demonstrated that melanoma (B16) exosomes could promote angiogenesis, establishing the communication network between cells (Hood et al., 2009). Given that hypoxic cells are thought to acquire a metastatic character, a quantitative proteomic analysis of carcinoma exosomes under

hypoxia has been performed, which revealed the presence of proteins involved in metastasis and angiogenesis (Park et al., 2010). It was observed that mRNA found in glioblastoma exosomes could be translated in recipient cells such as endothelial cells yielding proteins with angiogenic properties and stimulating tubule formation (Skog et al., 2008). Also, transcripts related to cell cycle were evidenced in colorectal exosomes, concordant with their capacity to modulate the endothelial cell cycle and proliferation, underlying their involvement in angiogenesis (Hong et al., 2009).

Lastly, on the one hand, exosomes released by tumor cells is greatly enhanced by an acidic condition (Parolini et al., 2009), which does not allow the survival of normal cells (Lugini et al., 2006). On the other hand, exosomes released by tumor cells in acidic conditions showed an increased fusogenic activity with cell plasma membrane, probably due to the different lipid composition induced by the low microenvironment pH (Parolini et al., 2009).

A recently set-up method allowing the characterization and quantification of exosomes in either cell culture supernatant or human plasma has shown that patients with advance-stage melanoma have increased levels of plasmatic exosomes containing caveolin 1, a protein involved in tumor cell vesicle trafficking (Logozzi et al., 2009) and highly expressed in tumor exosomes (Parolini et al., 2009). It is conceivable that the tumor microenvironment conditions, including low pH (Fais et al., 2007; Fais, 2010), may be at least in part responsible for the increased exosome production and spillover in the bloodstream. These data make exosome detection in the human body fluids as one of the most important future clinical approach in the screening, diagnosis, and follow-up of tumor patients.

Some pathological proteins responsible for known neurodegenerative conditions, such as prions in Creutzfeldt-Jacob disease and amyloid precursor protein (APP) derivatives involved in Alzheimer disease, exploit the mechanism of exosome-mediated secretion. Prion disease leads to the accumulation of the abnormally folded prion protein in the central nervous system, thus leading to scrapie. Alzheimer disease is characterized by a continuous loss of neurons and the extracellular accumulation of insoluble amyloid fibrils of abnormally cleaved APP in the brain. Exosomes carry prions (Fevrier et al., 2004), and with their capacity to easily pass the blood-brain barrier, exosomes may well deliver prions produced elsewhere in the body into the brain, thus triggering the pathological process leading to the Creutzfeldt-Jacob disease. Moreover, exosomes could be considered the ideal candidates as a vector mediating horizontal spreading of

cell-free amyloid oligomers and therefore the nanoshuttles for transmission of prion-related diseases (Tasaki et al., 2010).

Therefore, the normal exosome biogenesis pathway can be 'hijacked' by viruses and bacteria in cells and by abnormal proteins responsible for central nervous system diseases.

Exosomes as therapeutic tool

The reviewed features and roles provided the background and rationale for the use of exosomes in cancer immunotherapy. Exosome-mediated immunotherapy could be referred to as a type of cellular therapy because they are biological products. However, they are more convenient to handle than a cell because they are stable vesicles that keep their biological activities for at least 2 years at -80°C . After storage, there is no need to expand them, and they can be used directly, either alone or in combination with other pharmacological agents. In addition, they maintain the antigen presentation within a lymph node two times longer than an antigen-presenting cell, which indicates that they potentiate the immune response (Luketic et al., 2007). The only limitations are that they must be autologous (i.e., prepared from and used on the same patient) and that the yield of the tumor antigen-loaded exosomes prepared from dendritic cells features large variations between individuals.

The first clinical trials using immature DC-derived exosomes (Dex) as a cell-free vaccine against advanced melanoma and lung cancer-bearing patients failed to detect vaccine-specific T-cell responses but observed potent Dex-related NK cell activation (Viaud et al., 2010), suggesting that Dex can also stimulate the innate immune response. After injection in patients of Dex loaded with antigenic peptides from human melanoma during the phase I clinical trials, an increased number of NK cells was observed and NKG2D expression was restored in the NK cells and $\text{CD}8^{+}$ T cells in some patients (Escudier et al., 2005).

More recently, it has been demonstrated that resting and activated NK cells, freshly isolated from the blood of healthy donors, release exosomes expressing both typical protein markers of NK cells and killer proteins (i.e., Fas ligand and perforin molecules). These 'killer' nanovesicles display cytotoxic activity against several tumor cell lines and activated, not resting, immune cells. Consistently, exosomes purified from the plasma of healthy donors express NK cell markers, including $\text{CD}56^{+}$ and perforin,

and exert cytotoxic activity against different human tumor target cells and activated immune cells as well (Lugini et al., 2012). These data suggest, on the one hand, a physiological role of NK cell-derived exosomes in controlling the immune response. On the other hand, a potential role of NK cell-derived exosomes in future antitumor strategies seems conceivable as well.

Another study reported that exosomes can deliver anti-inflammatory agents, such as curcumin, a natural polyphenol, to activated myeloid cells *in vivo* (Sun et al., 2010). This study has shown that curcumin delivered by exosomes is more stable and reaches higher plasmatic concentration in the blood than the curcumin alone. Moreover, this study provides evidence that exosomes increase the target specificity of curcumin directly to the inflammatory sites and with elevated effectiveness-toxicity ratio. These results have clearly proposed these vesicles as an ideal carrier for drugs and are not limited to target only inflammation-related diseases.

In a further study, exosomes containing curcumin or a Stat3 inhibitor (JSI124) were administered *via* an intranasal route to a lipopolysaccharide-induced brain inflammation model, an experimental autoimmune encephalitis, or a GL26 brain tumor model. These exosomes were delivered noninvasively to microglia cells, and both protected against the inflammatory process and/or inhibited the development of brain tumors. As a direct effect, exosomes containing curcumin or JSI124 were selectively taken up by microglial cells, which were in turn induced to die by apoptosis. This study has provided evidence that drug-containing exosomes may be used for the treatment of brain-related diseases of different nature (Zhuang et al., 2011).

More recently, adenoviral vectors associated with exosomes (vexosomes) have shown higher transduction efficiency as compared with conventionally purified AAV vectors (Maguire et al., 2012), suggesting that vexosomes may improve gene delivery.

Other recent studies on the potential role of exosomes as an ideal vector for therapeutic use focus on miRNA. In 2007, a major advance in the understanding of exosome biology was made when exosomes from human and mouse mast cells were found to be natural carriers of nucleic acids, including over 1300 mRNA and 121 non-coding miRNA. Furthermore, it was established that the RNA content of exosomes did not strictly reflect that of the parent cell, suggesting the existence of actively selective pathways involved in the loading of exosomes with RNA within the parent cell. As an example, GW182 and AGO2, two important components of the RNA-induced silencing complex, were shown to associate with MVB and were

suggested to be involved in miRNA sorting into exosomes (Valadi et al., 2007).

Exosomes can be also considered a promising treatment for a variety of diseases (van Balkom et al., 2011). In fact, mesenchymal stem cell-derived exosomes display cardioprotective effects by reducing cardiac infarct size after experimental ischemia-reperfusion (Lai et al., 2010). Comparable results have been provided with cardiomyocyte progenitor cell-derived exosomes (Vrijssen et al., 2010) and in experimental stem cell therapy of acute kidney injury (Bruno et al., 2009).

However, a groundbreaking experiment showing that exosomes may be used for delivery was performed by Alvarez-Erviti et al. (2011), and they explored the ability of these nanovesicles to transfer nucleic acids to cells in a very specific manner. In this study, this result was achieved using ‘self’ exosomes loaded with chemically modified siRNAs and displaying specific targeting molecules. Thus, the target cells (dendritic cells) were engineered to express LAMP-2b fused to the central nervous system-specific rabies viral glycoprotein (RVG) peptide that specifically binds to the acetylcholine receptor. Exosomes were loaded with exogenous siRNA by electroporation and, after *in vitro* experiments, were injected into mice together with a series of control exosome preparations. The injection of RVG exosomes resulted in a significant knockdown of GAPDH mRNA in the brain regions expressing the target of the RVG ligand-nicotinic acetylcholine receptors. These results not only showed the therapeutic potential of RVG exosome technology for new therapeutic approaches against neurodegenerative diseases but also strongly supported the use of implemented and innovative exosome technologies for targeting therapies for a number of diseases.

However, at present, the few examples of exosome technology as a therapeutic approach are not extremely encouraging. In fact, in the clinical trials undertaken so far, the amount of immunocompetent exosomes produced *ex vivo* by dendritic cells originating from patients with melanoma was highly variable and constituted a limiting step in the immunotherapy approach. Therefore, understanding in depth the biogenesis and release mechanisms of exosomes in cells is essential. However, decreasing the amount of tumor exosomes released by modifying the pH of MVB with amiloride enhances the efficacy of chemotherapeutic agents (Chalmin et al., 2010). This is also supported by experiments performed with another class of anti-acidic molecules directed against proton pumps (Parolini et al., 2009). Thus, inhibiting the release of tumor exosomes would be a therapeutic strategy to prevent tumor growth and metastasis. In this respect,

proton exchanger inhibitors may well represent a pharmacological class of agents to block tumor exosome release. It is of great interest to identify other drugs that are potentially useful in exosome release inhibition using a large-scale systematic screening of a wide spectrum of drugs that inhibit tumor exosome release.

However, a general treatment avoiding exosome release in cancer patients might have side effects because the release of immunocompetent exosomes, which can enhance tumor recognition, by the immune system (Zitvogel et al., 1998) would be inhibited as well. Therefore, an optimal treatment would require differential therapeutic targets between tumor and immunocompetent cells. One can consider that an efficient cancer treatment would trigger an enhanced immunocompetent exosome biogenesis and/or release while inhibiting tumor exosome production. Therefore, drugs used to cure cancers should be screened in terms of their effect on the ratio between immunocompetent MHC-bearing exosomes and tumor immunosuppressive-containing molecule exosomes, both *in vitro* (in cell cultures) and *in vivo* (in serum from patients), to bring new perspectives on the efficacy of the various treatments. Notably, the activity and production of exosomes might be improved by manipulating

the biosynthesis of a fusogenic lipid (Laulagnier et al., 2004a,b; Scott et al., 2009).

However, the modification of the numerous exosome molecules (proteins, lipids, mRNA/miRNA) appears to be the most promising therapeutic strategy for both increasing the efficacy of existing molecules and reducing the side effects, which are due to the enhanced specific delivery of a given drug into the disease site. Another way is to interfere with the exosome formation by modifying the molecular content of typical exosome compartments such as MVBs.

In summary, exosomes contain protein and lipid determinants that allow them to interact with target cells, thus avoiding their dilution in the intercellular space. This ‘vectorized’ signaling appears more efficient than soluble agonists that can be diluted in the extracellular medium. Moreover, it appears conceivable that molecules of various origins may be more stable and functional when expressed on a membrane rather than in a free soluble state. Overall, exosomes appear as a ‘multisignaling device’ that can signal target cells at the cell periphery or bring information to the cytosol and translation machinery and possibly to the nucleus as well.

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