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INTRODUCTION

Introduction to Extracellular Vesicles: Biogenesis, RNA Cargo Selection, Content, Release, and Uptake

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Abstract Extracellular vesicles are a heterogeneous group of membrane-limited vesicles loaded with various proteins, lipids, and nucleic acids. Release of extracellular vesicles from its cell of origin occurs either through the outward budding of the plasma membrane or through the inward budding of the endosomal membrane, resulting in the formation of multivesicular bodies, which release vesicles upon fusion with the plasma membrane. The release of vesicles can facilitate intercellular communication by contact with or by internalization of contents, either by fusion with the plasma membrane or by endocytosis into "recipient" cells. Although the interest in extracellular vesicle research is increasing, there are still no real standards in place to separate or classify the different types of vesicles. This review provides an introduction into this expanding and complex field of research focusing on the biogenesis, nucleic acid cargo loading, content, release, and uptake of extracellular vesicles.

Keywords Extracellular vesicles · Exosomes · Microvesicles · Biogenesis · RNA · Cargo selection

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Introduction

Extracellular vesicles (EVs) are a heterogeneous family of membrane-limited vesicles originating from the endosome or plasma membrane. Pan and Johnstone (1983) were among the first to describe EVs. Initially, it was shown that the release of EVs was part of a disposal mechanism to discard unwanted materials from cells. Subsequent research has shown that the release of EVs is also an important mediator of intercellular communication that is involved in normal physiological process as well as in pathological progression (Frühbeis et al. 2012, 2013; Marcilla et al. 2012; Luga et al. 2012; Regev-Rudzki et al. 2013; Barteneva et al. 2013).

EVs are currently classified based on their mode of release or size. EVs can be released by "donor" cells either through the outward budding of the plasma membrane, termed shedding microvesicles (MVs) or ectosomes (Minciacchi et al. 2015). Another release process involves the inward budding of the endosomal membrane, resulting in the formation of multivesicular bodies (MVBs), with exosomes released by fusion of the outer MVB membrane to the plasma membrane (Théry et al. 2009; Denzer et al. 2000). Vesicles may also be released from nanotubular structures extending from the plasma membrane (Rilla et al. 2013, 2014). In addition to the differences in the mode of release, the size of the vesicles is also used for characterization. Although different scales are used, MVs range from 50 to 10,000 nm, and exosomes are smaller with a diameter of 30 to 150 nm (György et al. 2011; Baietti et al. 2012; Colombo et al. 2013). Overall EVs comprise a wide variety of vesicles ranging from 30 to 1000 nm in size with a variety of cargos, and the different types of vesicles overlap in their size distribution. It must be emphasized that there is some controversy on

nomenclature and sizes of the different types of vesicles (Gould and Raposo 2013; Witwer et al. 2013); however, basic requirements of criteria for EVs have been established (Lötvall et al. 2014). So far no real standards have been set to classify the different types of vesicles, so one should be careful with the use of size alone in defining different types of vesicles. In the future the mode of biogenesis, means of isolation and cargo may turn out to be far more important criteria. Given how the different isolation methods may influence the nature of EVs, methods should be compared in order to develop a gold standard for the different protocols and measurements (Momen-Heravi et al. 2012). To be able to compare results, it must be stressed that publications on EVs need to clarify their isolation methods in detail, and in general term, EVs should be used unless there are specific markers defined to classify the different types of vesicles.

So far, extensive evidence on all these different types of vesicles indicates that EVs are a key player in the intercellular communication between cells, along with secretion of small soluble molecules (the secretome) and cell–cell contact (Raposo and Stoorvogel 2013; Cocucci et al. 2009). Once released the EVs can be internalized via endocytosis or membrane fusion, releasing their contents into "recipient" cells (Mulcahy et al. 2014). Recent studies have shown that these EVs contain various proteins, sugars, lipids, and a wide variety of genetic materials, such as DNA, mRNA, and non-coding (nc)RNAs with the content protected from proteases and nucleases in the extracellular space by the limiting membrane (Henderson and Azorsa 2012; Théry et al. 2002). EVs have the potential to deliver combinatorial information to multiple cells in their tissue microenvironment and throughout the body (Baj-Krzyworzeka et al. 2006; Ratajczak et al. 2006; Skog et al. 2008).

This review provides an introduction into the world of EVs, focusing primarily subtypes labeled as exosomes and MVs, and discusses basics of the biogenesis, nucleic acid cargo loading, content, release, and uptake of these vesicles. Thus, it provides the necessary background for interpretation of the articles in this Special Issue on the role of EVs in the neurobiology and diseases of the nervous system.

The Ins and Outs of EVs

Vesicle Biogenesis

As EVs have traditionally been classified based on differences in biogenesis, we will focus on the different molecular mechanisms resulting in either the release of vesicles upon the fusion of the MVBs with the plasma membrane or the release via the outward budding and fission of the plasma membrane (Akers et al. 2013).

Exosome Biogenesis

Exosomes are derived from the endosomal system, and are formed as intraluminal vesicles (ILVs) in the MVBs. This network of ILVs is used to degrade, recycle or exocytose proteins, lipids, and nucleic acids. Within the endosomal system or endocytic pathway, the endosomes are divided into different compartments—early endosomes, late endosomes, and recycling endosomes (Grant and Donaldson 2009). Endosomes form by invagination of the plasma membrane. The early endosomes can fuse with endocytic vesicles, at which point the content is destined for degradation, recycling or secretion. Contents to be recycled are sorted into recycling endosomes (Morelli et al. 2004). The remaining early endosomes transform into late endosomes (Stoorvogel et al. 1991). The late endosomes accumulate ILVs formed by inward budding of the endosomal membrane. During this process, cytosolic proteins, nucleic acids, and lipids are sorted into these small vesicles. Late endosomes containing a multitude of small vesicles are termed MVBs. These MVBs can either fuse with the lysosome if the content is fated for degradation or fuse with the cellular membrane releasing the ILVs as exosomes into the extracellular space (Grant and Donaldson 2009).

The formation of the ILVs within MVBs is the start of the biogenesis of exosomes. ILV formation requires two distinct processes. First, the endosome membrane is reorganized such that it becomes highly enriched for tetraspanins (Pols and Klumperman 2009). The two tetraspanins that are thought to play a critical role in exosome formation are CD9 and CD63. Second, the endosomal sorting complexes required for transport (ESCRTs) are recruited to the site of ILV formation (Wollert and Hurley 2010; Colombo et al. 2013). Four different ESCRTs have been identified, ESCRT 0, I, II, and III (Henne et al. 2011). ESCRT 0 recognizes ubiquitinated proteins on the outside of the endosomal membrane (Raiborg and Stenmark 2009). ESCRT I and II are recruited to cytosolic side of the early endosomes via various stimuli. For example, ESCRT recruitment is stimulated by the presence of phosphatidylinositol 3-phosphate (PIP3), the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS), the ubiquitination of the cytosolic tail of endocytosed proteins and/or the curved membrane topology (Tamai et al. 2010; Shields et al. 2009; Razi and Futter 2006; Katzmann et al. 2001; Bache et al. 2003; Fernandez-Borja et al. 1999). It has been suggested that ESCRT I and II are the initiators and drivers of the intraluminal membrane budding, whereas ESCRT III completes this process (Babst et al. 2002; Henne et al. 2013). In short, ESCRT I binds the

ubiquitinated cargo on the endosomes, upon which ESCRT II is activated (Katzmann et al. 2001). In turn, ESCRT III is recruited through programmed cell death 6 interacting protein (PDCD6IP or ALIX), and associates with the tumor susceptibility gene 101 (TSG101) as part of the ESCRT I complex (Matsuo et al. 2004; Baietti et al. 2012). To conclude, ALIX serves as intermediate between the association between ESCRT I and ESCRT III, as it binds the TSG101 component of ESCRT I and the charged MVB protein 4A (CHMP4A) components of ESCRT III (McCullough et al. 2008). This process is finalized through the sequestration of MVB proteins and recruitment of a deubiquitinating enzyme, which removes the ubiquitin tag from the cargo proteins prior to sorting them into the ILVs. Finally, ESCRT-III is disassembled for recycling by AAA-ATPase suppressor-of-potassium-transport-growth-defect-1 protein (SKD1) (Bishop and Woodman 2001; Benedetto et al. 2006) (Fig. 1a).

Recent evidence has revealed an alternative ESCRT pathway, the syndecan-syntenin-ALIX pathway, where heparanase, syndecan heparan sulfate proteoglycans, ADP ribosylation factor 6 (ARF6), phospholipase D2 (PLD2), and syntenin mediate exosome biogenesis, including vesicle formation and loading of proteins (Baietti et al. 2012). The formation of ILVs is facilitated by the interaction of syntenin with ALIX and depends on the availability of heparan sulfate, syndecans, ALIX, and ESCRTs (Baietti et al. 2012). Heparanase stimulates release of exosomes containing syntenin-1, syndecan, and CD63 and facilitates endosomal membrane budding and the biogenesis of exosomes by enzymatic digestion of heparan sulfate chains on syndecans (Roucourt et al. 2015). This mode of ILV budding is controlled by ARF6 and PLD2 (Ghossoub et al. 2014) (Fig. 1b).

Although the ESCRT pathway is generally thought to be the main driver of exosomal biogenesis, different studies have shown the existence of ESCRT-independent exosome biogenesis. For example, inactivation of the ESCRTs does not inhibit the formation of MVBs (Stuffers et al. 2009). Other mechanisms of exosome biogenesis could operate in parallel to the ESCRT pathway and vary depending on the cell type and vesicle content. Trajkovic and colleagues (2008) found that the formation of ILVs through the inward budding of the limiting membrane of the MVBs required sphingolipid ceramide. This lipid could facilitate the membrane invagination of ILVs through its cone-shaped structure. The release of exosomes could even be reduced after the inhibition of neutral sphingomyelinase (nSMase), a protein responsible for the production of ceramide. Adding to this observation is the abundance of ceramide and its derivatives in exosomes, as well as the presence of proteolipoprotein (PLP), CD63, CD81, and TSG101 (Wubbolts et al. 2003; Brouwers et al. 2013). However, in some cell types, e.g., human melanoma cells depletion of nSMase does not inhibit the formation of MVB or exosome release (Fig. 1b). To summarize, the difference in exosome content relates, at least in part, to the various machineries involved in exosome biogenesis (Fig. 1).

Exosome Release

Release of exosomes into the extracellular space is facilitated by the fusion of the MVB limiting membrane with the plasma membrane. Similar to the different mechanisms

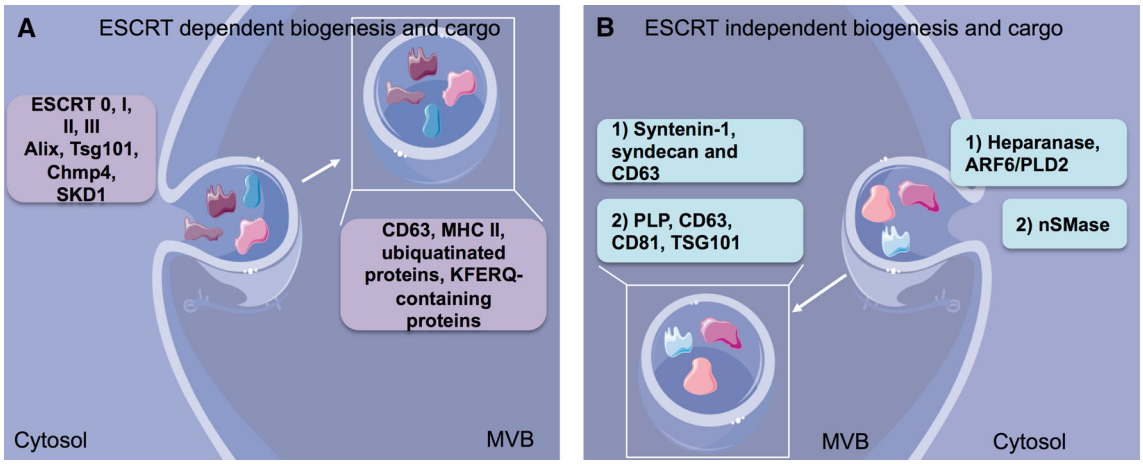

Fig. 1 Molecular mechanisms of ESCRT-dependent and -independent MVB biogenesis. Multiple biogenesis machineries have been described for generating ILVs in MVBs. **a** ESCRT-dependent MVB biogenesis requires the ESCRT protein and ESCRT-associated proteins (ALIX, TSG101, Chmp4, and SKD1) to form MVBs containing CD63, MHC II, ubiquitinated proteins and KFERQ-containing proteins. **b** Three ESCRT-independent pathways are controlled by different proteins: *1* heparanase and ARF6/PLD2, associated with the presence of syntenin-1, syndecan, and CD63 in exosomes; *2* nSMase, in which the exosomes are enriched with PLP, CD63, CD81, and TSG101 [Components in image derived from Servier Medical Art Powerpoint image bank (Servier 2016)]

proposed for the biogenesis of exosomes, a variety of mechanisms have also been proposed for the release of exosomes. A number of Rab GTPases, including RAB11 and RAB35, or RAB27A and RAB27B, are recognized to play an important role. Release of exosomes through fusion of the MVB with the plasma membrane facilitated by RAB11 and RAB35 was first found when screening with dominate-negative Rab GTPase mutants (Savina et al. 2003; Hsu et al. 2010). The exosomes release via this mechanism are enriched in proteins, such as flotillin and other cell-specific proteins, including Wnt, PLP, and the transferrin receptor (TfR) (Laulagnier et al. 2004) (Fig. 2a). The exosomes released via RAB27A/B are linked to late endosomal and secretory compartments and are enriched in late endosomal proteins (e.g., CD63, ALIX, and TSG101) (Stenmark 2009; Ostrowski et al. 2010). Interestingly, a different Rab GTPase, RAB7, is involved in the release of exosomes containing ALIX and syntenin by breast tumor cells (Baietti et al. 2012), although depletion of RAB7 does not affect exosome release in some other cells, e.g., HeLa cells (Ostrowski et al. 2010). In addition, some exosomes may be released through budding from the plasma membrane independently of Rab GTPases. For example, diacylglycerol kinase α (DGKα) has been shown to negatively control release of LAMP1/CD63 positive exosomes containing Fas-ligands (Alonso et al. 2007). But since DGKα is a negative regulator of MVB formation, the inhibition of exosome release could also result from decreased exosome generation (Alonso et al. 2011, 2007). SNARE proteins have been implicated in the membrane fusion of two organelles (Rao et al. 2004; Logan et al. 2006; Puri and Roche 2008; Tiwari et al. 2008). One of the SNARE proteins, the vesicle-associated membrane protein 7 (VAMP7), has been described to stimulate the release of acetylcholinesterase-containing exosomes (Fader et al. 2009). To conclude, exosomes can be generated and released from different subtypes of endosomes by various mechanisms and harbor different cargos as a function of cell type and probably physiologic state (Fig. 2a).

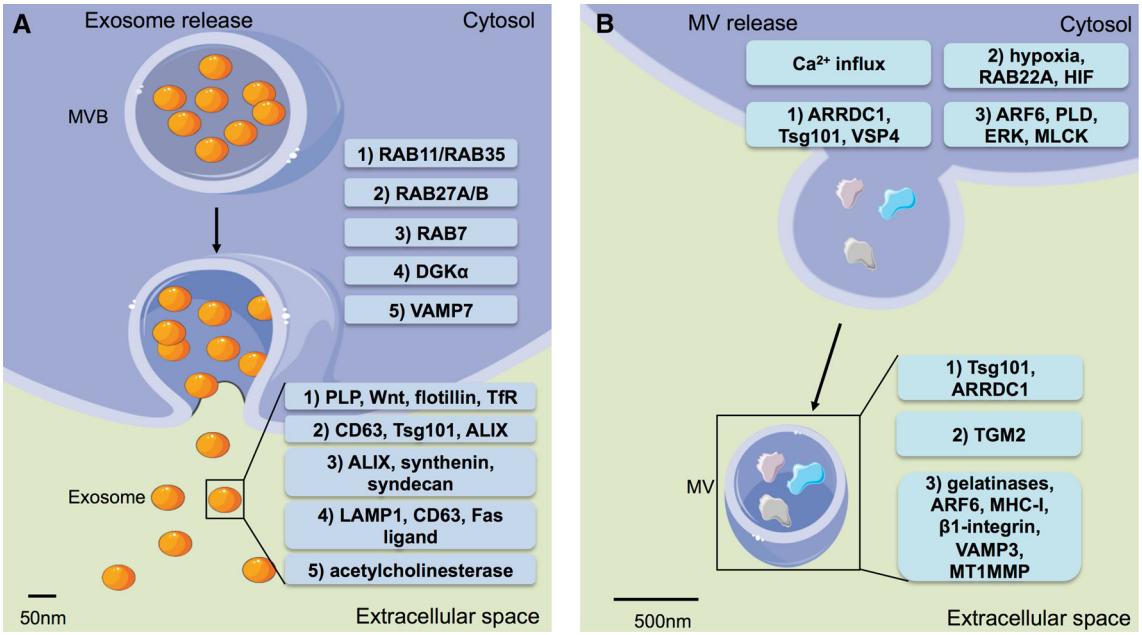

Fig. 2 Molecular machineries of EV release. **a** Proteins involved in controlling the fusion of MVBs with the outer membrane to the plasma membrane, resulting in release of exosomes. Five different machineries have been described so far; *1* RAB11 and RAB35 facilitate the fusion of MVBs to the plasma membrane, releasing exosomes containing PLP, Wnt, flotillin, and TfR; *2* RAB27A and RAB27B promote release of exosomes loaded with CD63, TSG101, and ALIX; *3* RAB7-dependent release yields release of exosomes harboring ALIX, synthenin, and syndecan; *4* DGKα protein is implicated in release of exosomes carrying LAMP1, CD63, and Fas ligand; and *5* VAMP7 regulates the membrane fusion associated with release of acetylcholinesterase-containing exosomes release. **b** EV released via the outward budding and fission of the plasma membrane controlled by different proteins and extracellular signaling results in release of MVs with a distinct protein profile. Three pathways have been described including markers found in released MVs: **a** ARRDC1, TSG101, and VSP4 are responsible for the shedding of MVs containing TSG101 and ARRDC1; **b** hypoxia following expression of RAB22A via HIF, characterizes the secretion of EVs carrying TGM2; and **c** the ARF6, PLD, ERK, and MLCK cascade induces release of EVs containing gelatinases, ARF6, MHC-I, β1-integrin, VAMP3, and MT1MMP. [Components in image derived from Servier Medical Art Powerpoint image bank (Servier 2016)]

Microvesicle Biogenesis and Release

The biogenesis of the MVs is far less defined as compared to exosomes. Biogenesis and release of MVs has been investigated in several cellular model systems. Different mechanisms are found to be responsible for the shedding of MVs. In general, these types of vesicles appear to be formed though the outward budding and fission of the plasma membrane (Fig. 2b). A combination of factors will result in the formation of MVs such as the redistribution of phospholipids, including the repositioning of phosphatidylserine to the outer leaflet, and contraction of the actin-myosin machinery (Akers et al. 2013). In detail, ADP-ribosylation factor 6 (ARF6) initiates a cascade that activates phospholipase D (PLD). Next, the extracellular signal-regulated kinase (ERK) is recruited to the plasma membrane, where it phosphorylates and activates the myosin light chain kinase (MLCK). Finally, the phosphorylation and activation of the myosin light chain by MLCK trigger the release of the MVs. These MVs have been described as being specifically loaded with ARF6, MHC-I, β1-integrin, VAMP3, and MT1MMP (Muralidharan-Chari et al. 2009). Interestingly, a recent study provided evidence for the recruitment of the ESCRT-I subunit TSG101 to the plasma membrane through its binding to a tetrapeptide protein within the Arrestin 1 domain–containing protein 1 (ARRDC1), resulting in the release of MVs containing TSG101, ARRDC1, and other cellular proteins (Nabhan et al. 2012; Tauro et al. 2012). The formation of these MVs required VPS4 ATPase with E3 ligase WWP2 interacting and ubiquitinating ARRDC1 (Nabhan et al. 2012). Furthermore, external factors can induce MV release. For example, the influx of calcium induces the redistribution of the phospholipids resulting in increased release of MVs (Bucki et al. 1998; Pasquet et al. 1996). In addition, hypoxia is been shown to promote MV release via HIF-dependent expression of RAB22A (Wang et al. 2014). Again, the different mechanisms underlining the release of MV from the plasma membrane can be distinguished based on the content of the released MVs (Fig. 2b). Some of these mechanisms are similar to those described for extracellular budding of virus particles, such as retroviruses (Gould et al. 2003), and, in fact, a substantial portion of EVs released from cancer cells are retrovirus-like particles (Akers et al. 2013; Balaj et al. 2011).

Contents of the Different Types of Vesicles

The contents of vesicles vary with respect to mode of biogenesis, cell type, and physiologic conditions. In general, all EVs are loaded with various proteins, lipids, and nucleic acids. The loading of the different types of cargo can be specific per vesicle and cell type. Extensive research has been carried out to characterize the content of EVs. This has resulted in the assembly of different databases collecting the datasets from the many EV studies. Three different databases are publicly accessible: Exocarta, Vesiclepedia, and EVpedia (Kim et al. 2013; Kalra et al. 2012; Mathivanan and Simpson 2009; Simpson et al. 2012; Mathivanan et al. 2012). All databases include the protein, nucleic acid, and lipid content together with the isolation and purification procedures used to generate the data. Here we give a broad overview of various cargos within EVs, with an emphasis on the nucleic acid content.

Protein Content

Comprehensive research has been done on the protein cargo of EVs, profiling the contents of different-sized vesicles produced by various cell types (Conde-Vancells et al. 2008; Demory Beckler et al. 2013; Gonzalez-Begne et al. 2009; Graner et al. 2009; Théry et al. 2001; Turiák et al. 2011). However, due to the variations in isolation techniques and the different cell types and culture conditions used to analyze the protein content, it is difficult to give a conclusive view of the protein composition of the different types of vesicles. Commonly found proteins in EVs are those associated with the mechanisms responsible for biogenesis, including proteins associated with the endosomal pathway. For example, components of the ESCRTs are enriched in the vesicle fraction, e.g., ALIX, TSG101. Additionally, proteins responsible for EV formation and release, such as RAB27A, RAB11B, and ARF6, are also commonly found. Moreover, EVs contain different types of tetraspanins, including CD63, CD81, and CD9, as well as proteins involved in signal transduction (EGFR), antigen presentation (MHC I and MHC II) and other transmembrane proteins (LAMP1, TfR). In general, proteins associated with the endoplasmic reticulum, Golgi, and nucleus are not found in EVs (Théry et al. 2001), but there have been reports of transcription factors inside EVs, e.g., Notch, Wnt, which are normally found in the nucleus (Kalra et al. 2012). Some research has gone into discovering principles of how proteins can be loaded into vesicles, which involves association with the plasma membrane as an oligomeric complex (Yang and Gould 2013). As mentioned before, the discrepancies between the different datasets and the different techniques used to analyze the content of the vesicles calls for standardization of isolation and analysis techniques to clarify the protein composition of the different EV subtypes, as well as the signals which enrich proteins in the EVs.

Lipid Content

In addition to the proteins within EVs, the lipid composition has been extensively studied in various settings (Van Blitterswijk et al. 1982; Carayon et al. 2011; Llorente et al. 2013). In general, the lipid composition share common features with the cells of origin. Although further investigation has shown that some lipids can be specifically associated with different types of EVs. Lipids enriched in EV include sphingomyelin, cholesterol, ganglioside GM3, disaturated lipids, phosphatidylserine, and ceramide (Llorente et al. 2013). In contrast, phosphatidylcholine and diacyl-glycerol are decreased compared to the lipid membrane composition of the cell of origin (Laulagnier et al. 2004). More examples of lipid enrichment are found in vesicles derived from the MVBs which contain more phosphatidylserine facing the extracellular environment as compared to the cellular plasma membrane, a feature that may facilitate their internalization by recipient cells (Subra et al. 2007; Fitzner et al. 2011). Although the lipid composition of MVs is highly similar to that of their donor cell, vesicles are unique in being enriched for polyunsaturated glycerophosphoserine and phosphatidylserine (Bicalho et al. 2013; Zaborowski et al. 2015). Overall, the membrane composition of both MVs and exosomes contains more phosphatidylserine as compared to the cellular plasma membrane composition. But the differences in lipid composition between the different types of vesicles reflect the biogenesis of the different types of EVs, either originating from the MVBs or the plasma membrane.

Nucleic Acid Content

A diverse composition of genetic material is found in EVs. In a small number of cases, DNA has been found, including genomic and mitochondrial DNA (Guescini et al. 2010; Balaj et al. 2011; Waldenström et al. 2012). But overall, EVs are primarily enriched with small RNAs, with many derived from ribosomal 18S and 28S rRNAs and tRNAs. Using various techniques, including next-generation sequencing, an abundance of small RNAs have been characterized. In addition to the commonly known RNA species, such as mRNAs, miRNAs, and rRNAs, long and short non-coding RNA, tRNA fragments, piwi-interacting RNA, vault RNA, and Y RNA have been found in EVs (Crescitelli et al. 2013; Cheng et al. 2013; Huang et al. 2013; Ogawa et al. 2013; Xiao et al. 2012; Nolte'T Hoen et al. 2012; Li et al. 2013) (Fig. 3a). Most of the RNA in EVs is around 200 nucleotides in length with a much smaller portion extending out to 4 kb (Batagov and Kurochkin 2013). So, although there appear to be some intact mRNA and long ncRNAs, most are probably fragmented, both in exosomes and MVs (Fig. 3a, b). Interestingly, circular RNAs are also enriched and stable in EVs (Li et al. 2015). Packaging of RNA within the lipid bilayer membrane is thought to protect it from RNase digestion once released into the extracellular environment (Fig. 3b). Alternatively, different RNA species can also be stably associated with ribonucleoproteins (RNPs), such as argonaute 2 (AGO2), or high- and low-density lipoproteins (HDLs and LDLs), which can be associated with the EVs or included with the EV fraction depending on the isolation procedure (Arroyo et al. 2011; Vickers et al. 2011; Vickers and Remaley 2012).

How Does RNA Get Packaged into EVs?

One of the surprising aspects of EV content has been that it has a somewhat different profile as compared to the RNA content of the cells from which it is derived (Skog et al. 2008; Pigati et al. 2010; Guduric-Fuchs et al. 2012; Jenjaroenpun et al. 2013). Since the discovery that specific RNAs are enriched in EVs, the search for selective loading mechanisms has been underway (Fig. 3c). In general, the RNA cargo of the EVs reflects the levels and types of cytoplasmic content, and is based on the biogenesis of the EVs and type and physiologic state of the cells releasing them. As such, it has been shown that miRNA are sorted to EVs by cell-activation-dependent changes of miRNA target levels in the cell of origin. This was found by overexpressing miRNA target sequences, resulting in relative miRNA enrichment in P-bodies and depletion from MVBs and EVs. Conversely, overexpression of a miRNA enriches it in EVs (de Jong et al. 2012; Squadrito et al. 2014). In depth analysis of the contents of EVs shows that specific mRNA fragments are enriched, especially the 3′UTR fragment of mRNAs (Batagov and Kurochkin 2013). In addition, specific sequences were found to be associated with loading into EVs. For example, a sequence motif within the 3′UTR of a number of mRNAs enriched in EVs may act as a "zipcode" that targets mRNAs into EVs. One potential zipcode consists of a 25 nucleotide sequence which contains a short CTGCC core domain on a stem-loop structure and carries a miR-1289 binding site (Bolukbasi et al. 2012). Interaction with this miRNA with the zipcode increases loading of RNAs containing this sequence.

Different mechanisms have been proposed for the cargo selection of miRNAs into EVs. First, a four nucleotide motif (GGAG) has been found to be enriched in miRNAs in exosomes and an interaction between this motif and the ribonucleoprotein (hnRNPA2B1) appears to be involved in loading these miRNAs into MVBs (Villarroya-Beltri et al. 2013). This RNP is also involved in the RNA transport in oligodendrocytes and must undergo a post-translational

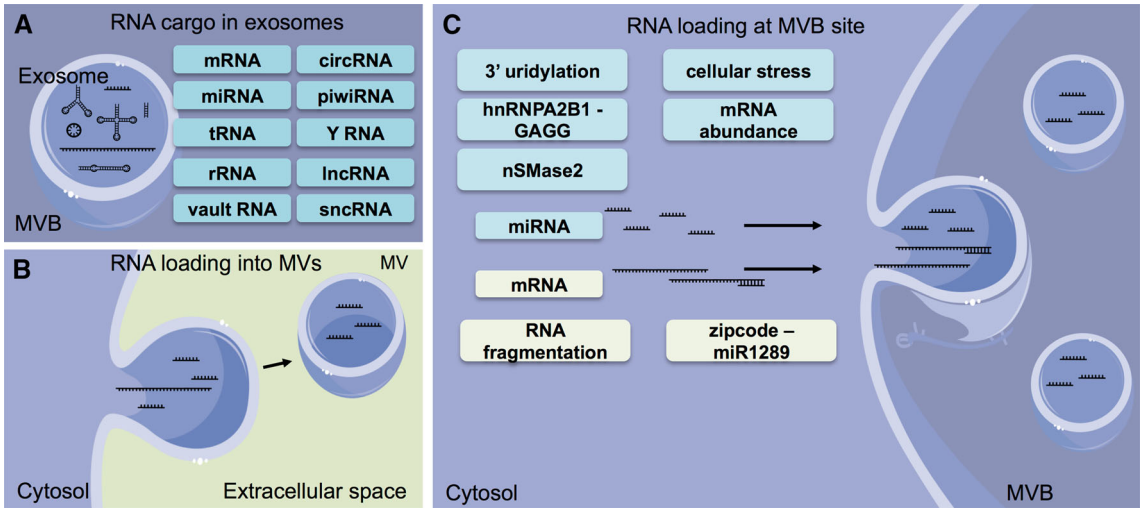

Fig. 3 RNA loading mechanisms and RNA species found in EVs. **a** A graphical representation of the different RNA species found in EVs including mRNA, miRNA, tRNA, rRNA, vault RNA, circRNA, Y RNA, lncRNA, and sncRNA. **b** Packaging of RNA within the lipid bilayer membrane is thought to protect it from RNase digestion once released into the extracellular environment. **c** Different mechanisms of mRNA and miRNA loading into EV as shown in the left panel include: the enrichment of miRNA in EVs due to cellular stress; hnRNPA2B1 binding to GAGG motif present miRNA; 3′ end uridylation of miRNAs/increasing nSMase2 activity resulting in miRNA loading; the abundance of miRNA target mRNA transcripts in the cell and the binding of miRNA to lipids associated with EVs. Loading of mRNA or mRNA fragments is based on the presence of zipcode sequence and association with miR1289. [Components in image derived from Servier Medical Art Powerpoint image bank (Servier 2016)]

modification (sumoylation) prior to loading of miRNAs into EVs (Munro et al. 1999). Notably, miR-1289, the binding site for which is found in the zipcode for mRNA (Bolukbasi et al. 2012), contains the hexanucleotide motif found to be enriched in the miRNAs present in EVs (Villarroya-Beltri et al. 2013). Second, posttranscriptional modifications of miRNAs, in the form of 3′ end uridylation, appears to contribute to direct miRNA sorting into EVs whereas 3′-end adenylated miRNA isoforms are relatively enriched in cells (Koppers-Lalic et al. 2014). Third, overexpression of nSMase2 increased extracellular amounts of miRNAs. Since this protein is also involved in MVB biogenesis, this increase in miRNA in EVs could be due to an increase in exosome production (Kosaka et al. 2010). Fourth, AGO2, a protein associated with the RISC complex involved in RNA silencing, is thought to control the loading of miRNA into EVs (Guduric-Fuchs et al. 2012) and RNPs (Arroyo et al. 2011). Knockout of AGO2 decreases the amount of specific miRNAs in EVs, which are normally enriched in this fraction. Although the role of AGO2 in miRNA loading remains unclear, some studies report the absence of this protein in exosomes, but only found localized at the site of MVBs and endosomes inside cells (Gibbings et al. 2009), while others report AGO2 to be present in EVs (Li et al. 2012) (Fig. 3c). The variations on the loading mechanisms of mRNA and miRNA show the complexity of EV research and the probable differences among EV content and vesicle subtypes among cells.

Uptake of EVs

So far, it has been proposed that the cells internalize EVs either by fusion with the plasma membrane or via endocytosis (Mulcahy et al. 2014). Uptake via endocytosis can be categorized into the different types of endocytotic processes, including clathrin-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, macropinocytosis, and phagocytosis. The uptake mode of EVs may be dependent on the type of cell and its physiologic state, and whether ligands on the surface of the EV recognize receptors on the surface of the cell or vice versa. Different mechanisms of internalization have been described for different cell types. For example, clathrin-dependent endocytosis or phagocytosis in neurons, macropinocytosis by microglia, phagocytosis or receptor-mediated endocytosis by dendritic cells, caveolin-mediated endocytosis in epithelial cells, and cholesterol- and lipid raft-dependent endocytosis in tumor cells (Morelli et al. 2004; Barrès et al. 2010; Feng et al. 2010; Fitzner et al. 2011; Montecalvo et al. 2012; Frühbeis et al. 2013; Nanbo et al. 2013; Svensson et al. 2013). An important factor in EV uptake is heparin sulfate proteoglycans (HSPGs) of the plasma membrane of the recipient cell as blocking this with heparin reduces the uptake of EVs in cell culture (Atai et al. 2013; Christianson et al. 2013). Decreased EV uptake was also achieved by blocking the scavenger receptor type B-1 (SR-B1) with a synthetic nanoparticle mimic of HDL

(HDL NP) that binds SR-B1, resulting in the removal of cholesterol (Plebanek et al. 2015). Lastly, membrane fusion of EVs with the plasma membranes could serve as method of uptake; this requires low pH conditions which are found within tumors. The cellular uptake and cytosolic release of EV contents is enhanced by combining a pH-sensitive fusogenic peptide to promote the fusion of endosomal and EV membranes inside cells, thus releasing the EV content into the cytosol (Parolini et al. 2009; Nakase and Futaki 2015).

The mode of EV interaction with and/or entry into cells determines their functional effects. The EV membrane surface can trigger signaling through interaction with receptors/ligands on the cell surface without EV entry as, for example with EGFRvIII (Al-Nedawi et al. 2008); Notch and RHEB (Patel et al. 2015); and IFN-γ/STAT (Cossetti et al. 2014). In many cases functionality of the EV contents depends on entry into the cytoplasm, and potentially even into the nucleus. Direct entry into the cytoplasm can be achieved by fusion of EVs to the plasma membrane of the recipient cells, but some form or endocytosis seems to be the most common mode of entry (Mulcahy et al. 2014). If the EVs enter by endocytosis, their cargo must exit that inherently degradative pathway, as endosomes mature into lysosomes, or be ejected out again through the MVB-plasma membrane fusion pathway. There must be a way through this maze, as so far, the functional transfer of nucleic acids has been described both in culture as well as in vivo (e.g., Pegtel et al. 2010; Ridder et al. 2014; Lai et al. 2015). The mechanism of effective transfer out of the endosomal compartment is still unclear. This process has been visualized using fluorescent probes labeling EVs in tumor and dendritic cells (Parolini et al. 2009; Montecalvo et al. 2012). A different approach utilized luciferin-loaded EVs internalization into cytosol containing luciferase which allowed monitoring of the fate of the cargo (Abrami et al. 2013). To conclude, different cell types are able to take up EV using various mechanisms resulting in either functional transfer of cargo or degradation of the EV content. The fate may be determined by cell-specific ligands/receptors that "direct the conversation."

Conclusion

The field of EVs is expanding rapidly. Although a lot of new exciting findings and applications for EVs are being published, the need for general consensus on the mode of isolation, classification and contents of different EV subtypes remains to be determined. By developing more selective isolation techniques, it should be possible to distinguish between the different subpopulations of vesicles and define their biogenesis, cargo, and function more precisely. Once accomplished, future research can focus on new methods to manipulate the biogenesis, content loading, composition, release, and interaction as a means not only for understanding the "language" of EVs, but also for the development of novel therapeutic strategies.

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Compliance with ethical standards

Conflict of interest The authors declared that there is no conflict of interest.

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