Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles

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Annu. Rev. Cell Dev. Biol. 2014. 30:255–89

First published online as a Review in Advance on August 21, 2014

The *Annual Review of Cell and Developmental Biology* is online at cellbio.annualreviews.org

This article's doi: 10.1146/annurev-cellbio-101512-122326

Keywords

microvesicles, microparticles, ectosomes, multivesicular bodies, endosomes, cell-cell communication

Abstract

In the 1980s, exosomes were described as vesicles of endosomal origin secreted from reticulocytes. Interest increased around these extracellular vesicles, as they appeared to participate in several cellular processes. Exosomes bear proteins, lipids, and RNAs, mediating intercellular communication between different cell types in the body, and thus affecting normal and pathological conditions. Only recently, scientists acknowledged the difficulty of separating exosomes from other types of extracellular vesicles, which precludes a clear attribution of a particular function to the different types of secreted vesicles. To shed light into this complex but expanding field of science, this review focuses on the definition of exosomes and other secreted extracellular vesicles. Their biogenesis, their secretion, and their subsequent fate are discussed, as their functions rely on these important processes.

Contents

INTRODUCTION

The secretion of extracellular vesicles (EVs) (i.e., membrane vesicles containing cytosol from the secreting cells enclosed in a lipid bilayer) is a process that appears to be conserved throughout evolution (Raposo & Stoorvogel 2013): Cells from different organisms, including all eukaryotes (from amoebae, *Caenorhabditis elegans*, and parasites to mammals) but also prokaryotic cells, have been demonstrated to release vesicles into the extracellular environment. In pluricellular organisms, EVs have been isolated from diverse bodily fluids, including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile, and semen. The origin, nature, and features of these vesicles are diverse, and many different names have been used in the literature, referring to their size [prefix micro or nano: microparticles, microvesicles (MVs), nanovesicles, nanoparticles], their cell or tissue of origin (prostasomes, oncosomes), their proposed functions (calcifying matrix vesicles, argosomes, tolerosomes), or simply their presence outside the cells (prefix exo or ecto: ectosomes, exosomes, exovesicles, exosome-like vesicles). Although the nomenclature is still

a matter of debate (Gould & Raposo 2013), the terms ectosome, shedding vesicle, microparticle, and MV generally refer to 150–1,000-nm vesicles released by budding from the plasma membrane (PM). The term exosome was initially used to name vesicles ranging from 40 to 1,000 nm released by a variety of cultured cells and carrying 5′-nucleotidase activity (Trams et al. 1981). However, this term was adopted in the late 1980s for small (30–100-nm) vesicles of endosomal origin that are released during reticulocyte differentiation as a consequence of the fusion of multivesicular endosomes or multivesicular bodies (MVBs) with the PM (Johnstone et al. 1987). A decade later, B lymphocytes and dendritic cells (DCs) were shown to release similar vesicles of endosomal origin (Raposo et al. 1996, Zitvogel et al. 1998). Many different cell types of hematopoietic and nonhematopoietic origin have now been shown to release exosomal vesicles. Most of the studies using cultured cells have hinted at the biogenetic origin of the secreted vesicles (i.e., their endosomal origin). However, most cells can probably release both PM- and endosome-derived vesicles. Thus, although in many studies EVs were named exosomes and were assumed to correspond to intraluminal vesicles (ILVs) of MVBs, solid evidence for their origin is often lacking, because diverse complementary methods are required, and such evidence is sometimes difficult to obtain. For example, fusion of MVBs with the cell surface is a very dynamic process that is often difficult to catch using electron microscopy (EM).

One major, ongoing challenge is to define methods that will allow us to discriminate between exosomes and MVs. It may turn out to be impossible to discern them on the basis of intrinsic properties, such as size, structure, buoyant density, or protein composition, and the community is seeking novel methods of isolation and purification. In addition, to further understand the origin of the different populations of vesicles and to unravel their physiological relevance, a better knowledge of the mechanisms of biogenesis and secretion is also required. Although several studies have now started to address these mechanisms, especially based on knowledge acquired for the formation of MVBs or budding of retroviruses, we are still at an early stage of being able to consistently interfere with and modulate the secretion of EVs.

In this review, we highlight and discuss the current state of EV cell biology, with a special focus on endosome-derived exosomes. We discuss the experimental limitations that must be resolved, the current state of the art in the mechanisms involved in their formation and secretion, and the challenges of a tremendously expanding field with many applications in human health. Because they represent the vast majority of the studies published so far, and because of space constraints, we essentially discuss EVs produced by higher eukaryotic cells and organisms.

THE DISCOVERY OF EXOSOMES, OTHER EXTRACELLULAR VESICLES, AND THEIR FUNCTIONS

It has been reported for decades that membrane-enclosed vesicles are present outside cells in solid tissues, such as cartilage (Anderson 1969), or in biological fluids, such as blood (Crawford 1971) or semen (Stegmayr & Ronquist 1982), and that mammalian cells (Trams et al. 1981), especially tumor cells (Dvorak et al. 1981) and platelets (George et al. 1982), shed membrane vesicles or microparticles. These EVs were assumed to be released by outward budding of the cells' PM (**Figure 1*a***). In the early 1980s, however, a more complex EV secretion pathway was described, in which vesicles initially formed intracellularly within so-called multivesicular endosomes or MVBs were subsequently secreted (Harding et al. 1983, Pan & Johnstone 1983) (**Figure 1*a***). Since then, the term exosome, proposed for these EVs of endosomal origin (Johnstone et al. 1987), has seen a rise in popularity, with increasing numbers of articles choosing this term to designate EVs. Of note, however, this term is now often used in a less restrictive manner than Rose Johnstone's original definition (Gould & Raposo 2013).

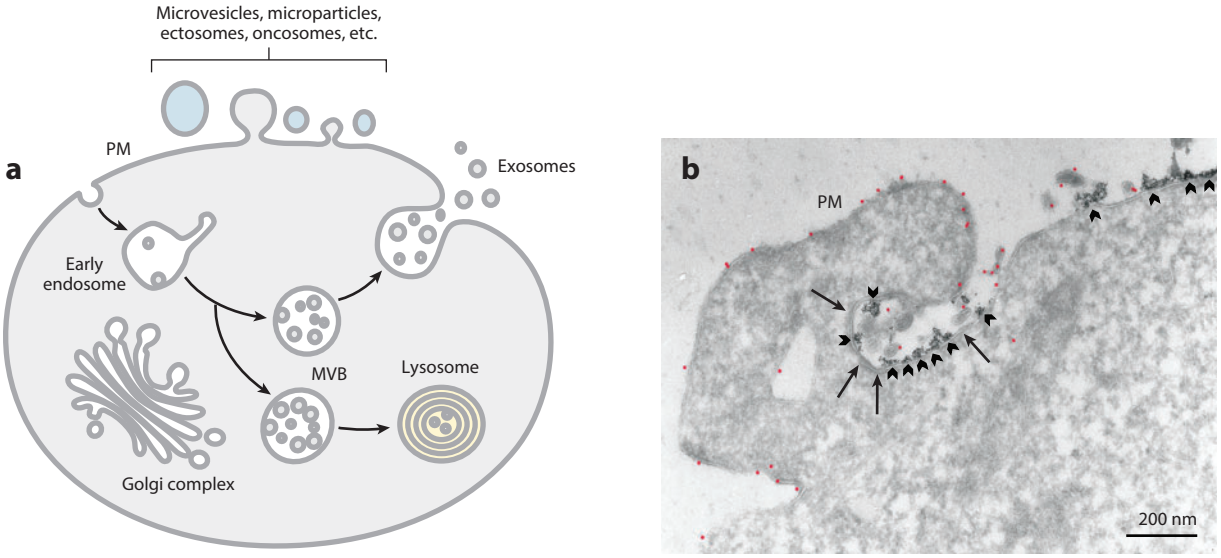

Figure 1

Extracellular vesicles (EVs) of different intracellular origins can be released by eukaryotic cells. (*a*) Schematic representation of the different types of membrane vesicles released by eukaryotic cells, either by direct budding from the plasma membrane (PM) or by fusion of internal multivesicular compartments (MVB) with the PM. (*b*) Electron microscopy image of fusion of a MVB with the PM (*arrows*) in an Epstein-Barr virus–transformed B cell. BSA-gold (*small particles*) was internalized into MVB (clusters of BSA-gold are indicated by arrowheads); large gold particles (pseudocolored in red) label MHC class II at the cell PM and on internal vesicles of the MVB.

The Reticulocyte Unravels a Novel Pathway of Secretion

While studying the maturation of reticulocytes into erythrocytes, which can be monitored by the loss of the transferrin receptor (TfR) originally found in the PM, the groups of Stahl and Johnstone unraveled the mechanism of TfR release into the extracellular medium. Using transferrin bound to gold particles (Harding et al. 1983), or anti-TfR antibodies (Pan et al. 1985), the authors used EM to follow the fate of the endocytosed receptor during its trafficking in the cell and subsequent release. They observed the TfR in multivesicular endosomes and found that most of the gold staining corresponding to the receptor was associated with the small internal bodies of approximately 50 nm in diameter, which were released upon fusion of the endosomes with the PM of the cells. These and the following studies suggested that this novel form of secretion was the way that PM components (such as the TfR and enzymatic activities) were discarded from maturing reticulocytes (Johnstone et al. 1987). In 1987, the term exosome was first used to describe small membrane vesicles formed by vesiculation of intracellular endosomes and released by exocytosis (Johnstone et al. 1987).

Exosomes/Extracellular Vesicles in the Immune System

In the following years, exosomes were marginally studied, mostly in reports related to the differentiation of red blood cells, and exosomes were merely viewed as a means for cells to dispose of unwanted components. Two publications by our groups in the late 1990s sparked a renewed interest in the field of exosome biology, because they suggested that exosomes might be important mediators of intercellular communication (Raposo et al. 1996, Zitvogel et al. 1998). In 1996, we showed that Epstein-Barr virus (EBV)-transformed B cell lines secreted exosomes enriched in

major histocompatibility complex (MHC) class II molecules (Raposo et al. 1996). The endosomal origin of the secreted vesicles was demonstrated by the observation by EM of fusion profiles with the PM of multivesicular MHC class II–containing compartments, which also contained a previously internalized tracer (BSA gold) (**Figure 1*b***). Furthermore, surface biotinylation of the cells showed that the protein composition of the secreted exosomes was different from that of the PM, thus ruling out the possibility that the vesicles were produced by shedding of the PM. Importantly, in both human and murine models, exosomes released by B lymphocytes have the capacity to stimulate specific $CD4^+$ T cell clones in vitro, suggesting a possible role of exosomes as vehicles for MHC class II–peptide complexes between immune system cells. Zitvogel et al. (1998) took these findings one step further by demonstrating the release of exosomes by human DCs and the ability of tumor peptide–pulsed DC-derived exosomes to suppress the growth of established tumors in vivo. These potential roles as mediators of immune responses, and the suggestion of a possible use of exosomes as immunotherapeutic agents, has led to a myriad of articles related to the immune function of exosomes in vitro and in vivo (previously reviewed by us in Bobrie et al. 2011 and Chaput & Théry 2011).

At the same time, microparticles or MVs released by platelets, monocytes, or neutrophils were also studied, mainly for their role in blood coagulation (Satta et al. 1994, Sims et al. 1989) or binding of opsonized bacteria (Hess et al. 1999). In the late 1990s, however, a function in intercellular communication was suggested by observed effects of microparticles on lipid metabolism (Sims et al. 1989), on the release of inflammatory mediators (Gasser & Schifferli 2004, Mesri & Altieri 1998), or on survival and proliferation (Baj-Krzyworzeka et al. 2002) of immune or endothelial cells exposed to them. However, none of these studies proposed a role of these PM-derived EVs in the induction of antigen-specific immune responses.

Exosomes/Extracellular Vesicles and Cancer

Since then, many different cell types have been reported to secrete exosomes in vitro, based on the presence of MVBs and the enrichment of MVB components in the secreted vesicles, including epithelial cells (Van Niel et al. 2001), neurons (Fauré et al. 2006), Schwann cells (Fevrier et al. 2004), and tumor cells (Wolfers et al. 2001), among others. Furthermore, EVs containing endosomal proteins, hence likely including heterogeneous types of EVs but at least also exosomes, have been purified from numerous bodily fluids (reviewed in Raposo & Stoorvogel 2013), thus proving that exosomes are also secreted in vivo.

Shedding of membrane-enclosed vesicles by both normal and neoplastic cells and the presence of such vesicles in ascites fluids were also reported in the early 1980s (Taylor et al. 1983b, Trams et al. 1981, Van Blitterswijk et al. 1982). At that time, direct shedding from the PM was the only mechanism considered for secretion of these vesicles (Poutsiaka et al. 1985, Trams et al. 1981), but after demonstration that at least some tumor-derived EVs could originate from MVBs (Wolfers et al. 2001), the search for endosomal proteins in tumor-derived vesicles, and the use of the term exosomes, began to spread in the literature (Taylor & Gercel-Taylor 2005), in parallel with a growing body of literature on other tumor-derived EVs (Al-Nedawi et al. 2008, Muralidharan-Chari et al. 2009, Skog et al. 2008). Tumors clearly secrete EVs, but the relative proportion of EVs corresponding to exosomes or to PM-derived vesicles cannot be determined from the experimental results provided, and it probably varies depending on the tumor cell analyzed. Also, despite general statements written in many reviews on tumor EVs, it is still not clear from the literature whether tumors secrete more EVs than nontumoral cells do.

The functions attributed to tumor-derived EVs have also grown exponentially since their initial description. A role in preventing immune responses was proposed as early as 1985, with

the observed inhibition of MHC class II expression by macrophages in the presence of melanoma MVs (Poutsiaka et al. 1985). Since then, various anti-immune effects of tumor-derived exosomes or EVs have been described, through inhibition of effector or activation of inhibitor immune cells (reviewed in Bobrie et al. 2011, Filipazzi et al. 2012, Zhang et al. 2012). Conversely, EV-mediated transfer of tumor antigens to DCs for efficient induction of antitumor immune responses has also been described (Wolfers et al. 2001), and the immune consequences of EV secretion by tumors in vivo is thus still not fully understood. Recently, by inhibiting exosome secretion in two tumor cell lines (Bobrie et al. 2012b), we could show, for one of these tumors, that in vivo secretion of exosomes, by participating in the recruitment of protumoral neutrophils, was instrumental in promoting local development. For the other tumor, however, its ability to secrete exosomes in vivo did not affect its development, thus highlighting the variability of possible functions of tumor exosomes, which strongly depend on the local microenvironment generated by the tumor itself.

Tumor exosomes have been proposed recently to participate in metastatic dissemination of tumor cells by educating bone marrow progenitor cells and promoting their migration to the future sites of metastasis (Peinado et al. 2012), by directly seeding tumor-draining lymph nodes before further migration of tumor cells themselves (Hood et al. 2011), or by increasing local motility of tumor cells via a complex interplay with surrounding fibroblasts (Luga et al. 2012). Tumors also secrete large, PM-derived EVs bearing matrix metalloproteinases (Ginestra et al. 1997, Muralidharan-Chari et al. 2009), which could help migration of tumor cells within a solid tissue. Tumor cells can also spread their intrinsic oncogenic portential to surrounding cells via EVs, as shown for an oncogenic variant of epidermal growth factor (EGF) receptor in glioblastoma cells (Al-Nedawi et al. 2008). Finally, tumor-derived EVs also display proangiogenic activities, mediated by interaction with endothelial cells (Al-Nedawi et al. 2009, Sheldon et al. 2010). Whether these functions really take place upon in vivo secretion of EVs remains unclear.

Exosomes/Extracellular Vesicles and RNA

A breakthrough in the field was made in 2007 when it was discovered that exosomes carry nucleic acids, namely mRNA and miRNA (Valadi et al. 2007). Strikingly, when mouse exosomes were fed to human cells, selected mouse proteins, which did not exist as proteins but did exist as mRNA in the mouse exosomes, were detected in these cells, suggesting that mRNA shuttled via exosomes had been translated. The presence of mRNA in EVs called MVs, and their influence on gene expression in recipient cells, was also reported in tumor cell– and stem cell–derived large EVs (Baj-Krzyworzeka et al. 2006, Ratajczak et al. 2006, Skog et al. 2008). Identification of microRNAs was also confirmed in glioblastoma and blood cell–derived mixed EVs (Hunter et al. 2008, Skog et al. 2008).

From then on, a new perspective on the possible roles of exosomes or other EVs emerged as vectors of genetic information able to modify the range of genes expressed within recipient cells. Finally, the recent use of next-generation sequencing tools has even expanded the range of genetic materials associated with EVs, including other noncoding RNA endowed with potential regulatory capacities on the genomes of target cells (Nolte-'t Hoen et al. 2012).

DEFINITION OF EXOSOMES AND OTHER EXTRACELLULAR VESICLES

Owing to their endosomal origin, exosomes display hallmarks of the internal vesicles of MVBs, called ILVs, and some crucial characteristics should be described to determine if EVs correspond to exosomes.

As seen by EM, the diameter of ILVs ranges from 30 to 100 nm; consequently, the diameter of isolated exosomes observed whole mounted after fixation and contrasting should be in this size range, or possibly slightly larger (maximum 150 nm), when observed in a close-to-native state by cryo-EM or nanoparticle tracking analysis (see Size and Morphology, below). EVs budding from the cell's PM, or resulting from fragmentation of dying cells, do not display this size restriction and can thus be as large as 1 μm or a few micrometers or as small as, or even smaller than, exosomes. The molecular composition of exosomes may also be closer to the composition of endosomes than to the composition of the PM, whereas the opposite may apply for PM-derived EVs. Finally, the existence of MVBs in close apposition and possibly fusion with the PM should be documented by EM in the secreting cells.

However, all these criteria are difficult to obtain on a routine basis and, further, will not demonstrate that 100% of the EVs recovered from a tissue culture supernatant or a biological fluid indeed represent exosomes. Therefore, a somehow less strict use of this nomenclature has developed in the past few years. We describe here some of the currently used criteria for EV definition.

Methods of Isolation

The protocol initially developed to purify reticulocyte exosomes from tissue culture conditioned medium (Johnstone et al. 1987) was then used to purify these vesicles from antigen-presenting cells (Raposo et al. 1996, Zitvogel et al. 1998), as described in detail by Théry et al. (2006). This protocol is based on differential centrifugation, whereby the smallest vesicles (including exosomes) are sedimented by ultracentrifugation at 100,000 × *g*. Before ultracentrifugation, larger vesicles were eliminated by successive centrifugations at increasing speeds to sediment these vesicles without artificially creating small vesicles from large ones by direct high-speed centrifugation. Several variants of this method are used nowadays that can involve higher-speed ultracentrifugation [e.g., 140,000 × *g* (Baietti et al. 2012)] and/or include different steps before final ultracentrifugation, such as filtration to eliminate debris and vesicles larger than 220 nm (Théry et al. 2006) or size-exclusion chromatography to recover entities larger than 50,000 kDa and thus eliminate soluble proteins (Taylor et al. 1983a). Similarly, most protocols used to purify larger EVs also involve centrifugation, generally at lower speed, i.e., from 10,000 × *g* (Muralidharan-Chari et al. 2009) to 50,000 × *g* (Baj-Krzyworzeka et al. 2006).

In any case, ultracentrifugation only allows enrichment in subtypes of EVs or exosomes and is not a proper purification, because different vesicles of similar size as well as protein aggregates can cosediment at 100,000 × *g*. One way to separate membrane-enclosed vesicles from aggregates of proteins is to allow vesicles to float into a sucrose gradient (Escola et al. 1998, Raposo et al. 1996): Protein aggregates sediment through sucrose, whereas lipid-containing vesicles float upward to a position of equilibrium buoyant density. A variant of this approach has been used to purify EVs of clinical grade for therapeutic use (Lamparski et al. 2002) by combining filtration/concentration through a 500-kDa membrane and ultracentrifugation through a D_20/sucrose cushion to retain membrane vesicles.

Recently, commercially available methods claiming fast and simple exosome-purification procedures without ultracentrifugation have been advertised by various companies. Either (presumably) polymer-based precipitation or immunocapture by antibody-coated beads is used in these kits. The former should precipitate a wider, and the latter conversely a more restricted, range of vesicles than that precipitated by ultracentrifugation. Therefore, a thorough comparison process is still needed to validate these new tools and determine what kinds of vesicles they precipitate.

Because none of these methods is perfect, efforts to develop new technologies are currently under way, but none has reached worldwide use yet. In the meantime, scientists working in the field

and coordinated within the International Society for Extracellular Vesicles have recently published a first position paper to propose standardization procedures for collecting biological fluids and processing them for EV purification and to highlight the currently known possible caveats of these procedures (Witwer et al. 2013). We recommend that scientists entering the field read this paper, and the other position articles that will be published regularly as the field evolves, in the Society's journal (*Journal of Extracellular Vesicles*, **www.journalofextracellularvesicles.net**).

Regardless of the protocol used, each technique must be validated for any given cell type—or biological fluid, as a source of exosomes—to confirm the identity of the purified vesicles. This requires the use of a combination of several methods to determine their morphological, biochemical, and physical features.

Size and Morphology

Because they fall below the resolution threshold of optical microscopy, transmission EM (TEM) has been so far the preferred technique for direct observation of the size and morphology of exosomes (Raposo et al. 1996). Analyzed as whole-mounted vesicles deposited on EM grids and fixed and contrasted/embedded in a mixture of uranyl acetate and methylcellulose (Raposo et al. 1996), exosomes display a cup-shaped appearance. Although this feature has been commonly considered in the past 10 years as a demonstration of the exosomal nature of vesicles, this morphological appearance is an artifact of the fixation/contrast step that induces shrinking of subcellular structures: Exosomes observed by cryo-EM (a technique in which samples are vitrified in liquid ethane to prevent the formation of ice crystals that can alter the ultrastructure of cells and membranes) have a round shape (Conde-Vancells et al. 2008, Raposo & Stoorvogel 2013). Other EM techniques more recently used in preparations of EVs include TEM of sectioned membrane pellets of fixed EV (Crescitelli et al. 2013) and atomic force microscopy, a variant of scanning EM (Sharma et al. 2011), in which a mechanical probe measures the size and structure of individual EVs in their native state. When analyzed in a quantitative manner, i.e., by measuring hundreds of individual elements in each sample, rather than showing one image of a single vesicle, all these EM studies have highlighted the heterogeneity of EVs, especially when recovered using low-speed centrifugation (M. Colombo, J. Kowal & C. Théry, unpublished observations; Crescitelli et al. 2013), from biological fluids (Aalberts et al. 2012, Sharma et al. 2011), or from primary DCs in which some dying cells were also present (Colombo et al. 2013). By contrast, exosomes obtained by high-speed centrifugation of a conditioned medium of homogenous, healthy tumor cell lines are less heterogeneous and contain mainly vesicles 30–150 nm in diameter (Baietti et al. 2012, Colombo et al. 2013).

Larger vesicles, by contrast, can be visualized by fluorescence microscopy, and shedding from cells of vesicles of 0.5 μm to a few micrometers in diameter has been observed by video microscopy (Di Vizio et al. 2012, MacKenzie et al. 2001, Muralidharan-Chari et al. 2009). Although fluorescence microscopy is also used by some groups to show vesicles smaller than 100 nm, exosomes, or viruses, either directly on glass slides or after internalization by target cells, given the 200-nm resolution limit of classical optical microscopes, the objects thus observed are not individual vesicles but rather are aggregated or concentrated EVs, or even aggregates of the antibodies or fluorophores used to label exosomes. The recent advances of superresolution microscopy will eventually lead to new technologies to analyze both small and large vesicles, but they are not yet in widespread use to be available as a basic characterization tool of EVs.

A device allowing Nanoparticle Tracking Analysis (NTA) has been designed to measure the size distribution and concentration of nanoparticles (Dragovic et al. 2011). It tracks the movement of laser-illuminated individual particles under Brownian motion and then calculates their diameter using statistical methods. This method has the advantage of being a fast and simple way of analyzing

large numbers of particles simultaneously, and at a relatively cheap price (as compared with sophisticated fluorescence or electron microscopes). However, the method does not differentiate a vesicle from a protein aggregate of similar size. So far, most articles using this technique to analyze exosomes show a major population of particles approximately 100 (±20) nm in diameter, with fewer larger ones, consistent with the sizes observed by TEM of tumor cells' EVs (Baietti et al. 2012, Colombo et al. 2013), whereas EV pellets of lower-speed centrifugation show a larger range of diameters (J. Kowal & C. Théry, unpublished observations). However, because optimal parameters to visualize small and large particles are not identical and are still determined manually by the user, the reproducibility of this technique in different laboratories is still not optimal. A detailed protocol on the best method to use this device for EV analysis has been published recently (Gardiner et al. 2013), which will hopefully increase the reliability of quantifications and size-distribution results.

Physical Features

As stated above (see Methods of Isolation), one of the most defining characteristics of membrane-enclosed vesicles is their ability to float in density gradients. The actual density of the various types of EVs is, however, not as clearly established as we would have proposed a few years ago (Théry et al. 2009).

Using this technique, different groups described exosomes as equilibrating at densities ranging from 1.13 to 1.19 g/ml in sucrose (reviewed in Théry et al. 2009). In most studies, vesicles recovered in all these fractions were pooled for further analysis. But the more recent literature shows that such a large range of densities in fact reflects the heterogeneity of vesicles obtained by ultracentrifugation and suggests that these different fractions should be analyzed separately. Indeed, careful examination of the distribution of different protein markers shows, for instance, that HSC70 (HSPA8) and HSP70 (HSPA1A/B), flotillin-1, and milk fat globule-EGF-factor VIII (MFGE8, also called lactadherin) equilibrate at slightly different densities than does ALIX (PDCD6IP) or CD9 (Baietti et al. 2012, Bobrie et al. 2012a, Fruhbeis et al. 2013, Tauro et al. 2012). In addition, a recent observation initially made on prostasomes (Aalberts et al. 2012), but then confirmed on tumor-derived EVs (Bobrie et al. 2012a, Palma et al. 2012), shows that some vesicles recovered in the high-speed pellet, especially those rich in tetraspanins, take more time than others to reach their equilibrium density during centrifugation in a sucrose gradient. Hence, they remain in the high-density fractions (above 1.19 g/ml) after a classical overnight centrifugation of the gradient. Therefore, subtypes of EVs can be separated by performing differential buoyant velocity centrifugation, in which the samples are centrifuged into a sucrose gradient for different lengths of time (Palma et al. 2012). Finally, short-term sedimentation (instead of flotation to equilibrium) of EVs in iodixanol-based (OptiPrep™) gradients has been proposed to efficiently separate myeloid cell–derived vesicles from HIV virions (Cantin et al. 2008).

Concerning the large EVs sedimented at speeds lower than 50,000 × *g*, we are not aware of published studies analyzing their density after equilibrium sedimentation. Our recent unpublished analysis of EVs recovered from human DCs shows that both 10,000 × *g* and 100,000 × *g* sedimented membrane vesicles equilibrate in sucrose gradients at the originally proposed density of exosomes (i.e., 1.13 to 1.19 g/ml), but among the 4–5 gradient fractions encompassing this range, the medium-speed pellet is prominently recovered in one of slightly higher density than the high-speed pellet (J. Kowal & C. Théry, unpublished observations). These results suggest that small and large EVs, and subtypes of small EVs, probably display different densities, but a refined definition of these actual densities is still called for.

Other physical parameters of EVs, such as light scatter, which is correlated to size but also to geometry and composition, can be measured by flow cytometry. Flow cytometry has been used for

decades to quantify and analyze surface markers on circulating microparticles (Nieuwland et al. 1997); however, most routinely used flow cytometers do not properly distinguish between noise and beads (or vesicles) of sizes below 300 nm and do not separate beads with size differences lower than 200 nm (Lacroix et al. 2013). Thus, flow cytometry analyses have not so far taken into account the small EVs. Recently, by combining the use of a new generation flow cytometer (with manually optimized settings to allow detection of the smallest particles in the forward scatter channel), fluorescent labeling of vesicles by lipid dyes (to discriminate these vesicles from noise signals using the fluorescence channel), and equilibrium sedimentation in sucrose gradients (to eliminate non-vesicle-bound aggregates of lipid dye) (van der Vlist et al. 2012b), the heterogeneity of small vesicles released by mixed DC–T cell culture was again highlighted (van der Vlist et al. 2012a). The ongoing developments of high-sensitivity flow cytometers should allow direct analysis of individual vesicles as small as exosomes (100 nm and below) in the coming years, but they still require optimization.

Biochemical Features: Composition

Most studies of biochemical composition of EVs involved analysis of bulk populations of vesicles obtained by differential ultracentrifugation, which, as stressed above, most often provides a mixed population of EVs. In some studies, exosomes were further purified by immunoisolation (Tauro et al. 2012, Wubbolts et al. 2003), which may eliminate a subpopulation of vesicles. Thus, the actual composition of each subtype of EV or exosome is unknown. We summarize here the current state of the literature and indicate where efforts have been made or are in progress to identify specific molecular markers of different subtypes of EVs.

Proteins. The protein content of exosomes or shed membrane vesicles has been studied extensively since their initial description. Techniques allowing antibody-based detection of specific proteins (western blotting, immuno-EM) were first used, but the development of proteomic analysis techniques in the 1990s soon allowed large-scale identification of nonpredetermined proteins in EV preparations. We were the first to use trypsin digestion and peptide mass mapping on exosomes (i.e., 100,000 × *g* pellet) obtained from mouse DC cultures (Théry et al. 1999, 2001), but we were soon followed by numerous similar studies performed on exosomes recovered from other cell types or purified from various bodily fluids. The results of these and many other studies on mammalian exosomes were assembled in a database named Exocarta (Mathivanan et al. 2012). Exocarta was recently incorporated into a more comprehensive database named Vesiclepedia, which also includes data from other types of EVs (**http://microvesicles.org**) (Kalra et al. 2012) and is continuously updated with the help of the scientific community researching EVs. Another database including studies of nonmammalian EVs of all sizes has also been established recently (**http://evpedia.info**) (Kim et al. 2013). Both databases include data not only on proteins but also on nucleic acids and lipids, as well as on the purification procedures used. Their continuous updating makes them a crucial tool to improve comprehension of EV complexity.

Initial proteomic studies showed that exosomes contain a specific subset of cellular proteins, some of which depend on the cell type that secretes them, whereas others are found in most exosomes regardless of cell type. The latter include proteins from endosomes, the PM, and the cytosol, whereas proteins from the nucleus, mitochondria, endoplasmic reticulum, and the Golgi complex are mostly absent. These observations highlight the specificity of formation of these vesicles and show that they represent a specific subcellular compartment and not a random array of cell fragments. They led us to propose a schematic representation of a canonical exosome (Chaput & Théry 2011; Théry et al. 2001, 2009) as a lipid-enclosed vesicle exposing at its surface

the extracellular domain of transmembrane proteins and containing various types of cytosolic proteins (**Figure 2**). However, we propose this figure as a global scheme of EVs rather than of exosomes specifically. Indeed, more recent proteomic analyses of other EVs (Turiák et al. 2011) (J. Kowal & C. Théry, unpublished observations) show a large overlap of protein expression with those listed above in exosomes, suggesting that proteins specifically expressed in exosomes as opposed to PM-derived vesicles remain to be identified. In addition, some of these proteins are most probably not equally present in all subtypes of vesicles copurified in the samples analyzed so far. The recent development of quantitative or semiquantitative proteomic analysis tools first led to the proposal of such heterogeneous protein composition within the 100,000 × *g* pellet (Tauro et al. 2012, 2013a). Upon further purification of tumor-derived pellets by OptiPrep™ density gradient, or upon immunoaffinity capture of vesicles expressing the adhesion molecule EpCAM, Tauro et al. observed a strong enrichment of some proteins (e.g., TSG101, CHMP2A, RAB11B) but not others (e.g., ALIX, CHMP4B, RAB11A, RAB5), suggesting that the latter are more ubiquitous in all EVs. Immunoaffinity purification also strongly enriched CD63 and CD81 but not CD9 in EVs, and we recently observed that secretion of CD9 or the phosphatidylserine (PS)-binding protein MFGE8/lactadherin was not reduced as much as secretion of CD63 upon inhibition of RAB27A-dependent exosome secretion in tumor cells (see Functions of Rab GTPases in Exosome Secretion, below) (Bobrie et al. 2012a), confirming that CD9 (and MFGE8) is probably more ubiquitous than other tetraspanins in EVs. Finally, through careful quantification of the relative proportion of CD63- or MHC class II–bearing vesicles in 100,000 × *g* pellets from HeLa-CIITA and DCs, we recently observed a stronger enrichment of CD63 in the smallest vesicles (<50 nm) (Colombo et al. 2013), whereas MHC II molecules were more abundant in larger vesicles (>100 nm), again showing the concomitant presence of different subtypes of vesicles. A refinement of analysis and purification techniques in coming years should allow us to clarify the protein composition of each EV subtype, which is certainly important to further understand their function.

Lipids. Fewer studies have analyzed the lipid composition of exosomes (Laulagnier et al. 2004b, Llorente et al. 2013, Trajkovic et al. 2008, Wubbolts et al. 2003). When comparing secreted vesicles with the total cell membranes, three out of four of these studies observed enrichment of sphingomyelin, PS, and cholesterol, and generally of saturated fatty acids. In addition, the ganglioside GM3 (Llorente et al. 2013, Wubbolts et al. 2003) and ceramide or its derivatives (Laulagnier et al. 2005, Llorente et al. 2013, Trajkovic et al. 2008) were enriched in exosomes, whereas lysobisphosphatidic acid (LBPA), a lipid thought to be present in ILVs (Matsuo et al. 2004), was not enriched (Laulagnier et al. 2004b, Wubbolts et al. 2003). A recent analysis of two subpopulations of EVs purified from semen (prostasomes) confirmed high sphingomyelin, cholesterol, and PS content but also showed different relative levels of sphingomyelin and hexosylceramide in the two different prostasomes (Brouwers et al. 2013). These results thus show a specific lipid composition of EVs, with some features (e.g., sphingomyelin and cholesterol) reminiscent of detergent-resistant subdomains of the PM called lipid rafts (Ikonen 2001). This observation is consistent with the presence in EVs of lipid raft–associated proteins, GPI-anchored proteins and flotillins, and the observed resistance of B cell exosomes to detergents (Wubbolts et al. 2003). Whether this means that ILVs form inside MVBs as lipid rafts or that PM-derived lipid raft domains are released from the cells simultaneously with MVB-derived vesicles is still unclear; a recent article strengthens the first hypothesis by showing that lipid rafts endocytosed from the PM are segregated into ILVs and released in exosomes (Tan et al. 2013).

Of note, exposure of PS on microparticles, and consequent binding of annexin-V, has been used to characterize these membrane vesicles (Dachary-Prigent et al. 1993). Although a lower level of PS was initially reported on exosomes as compared with PM-derived MVs (Heijnen et al.

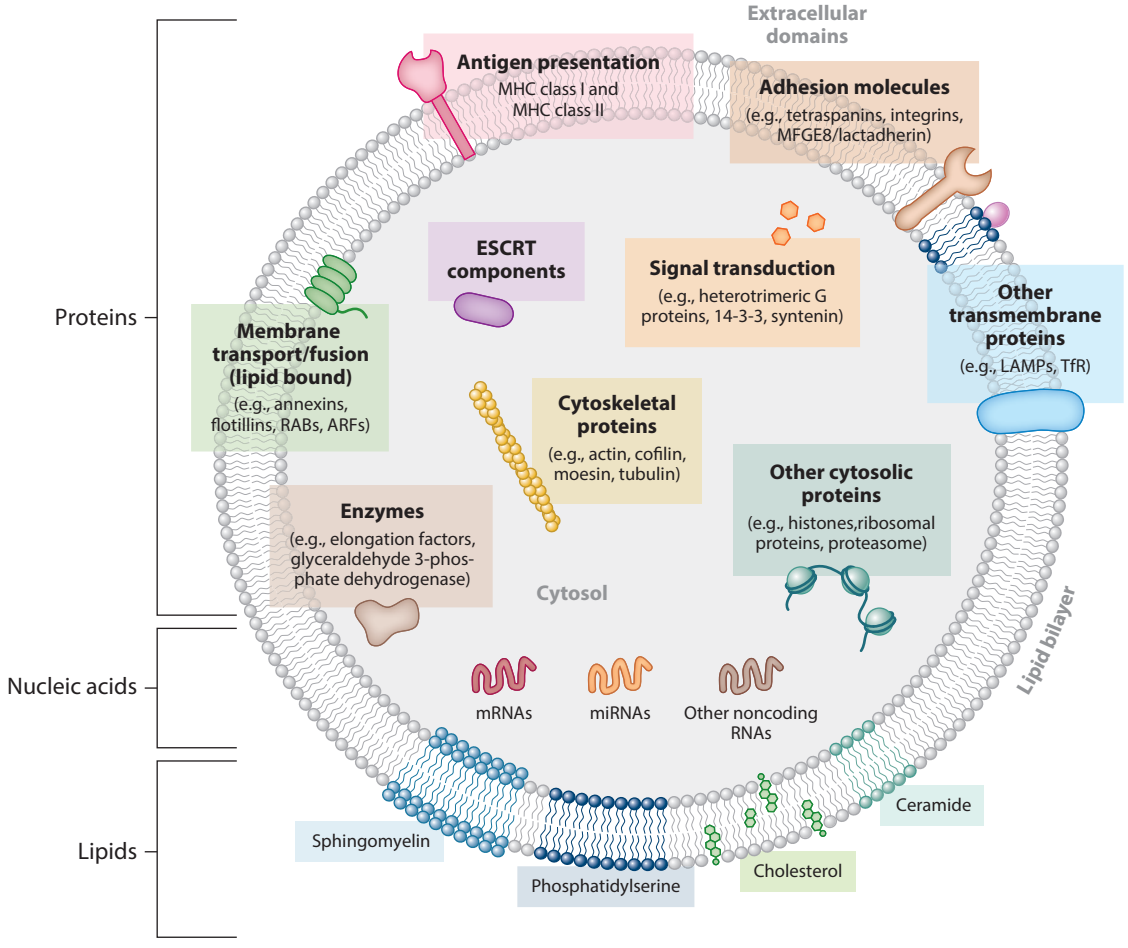

Figure 2

Overall composition of extracellular vesicles (EVs). Schematic representation of the composition (families of proteins, lipids, and nucleic acids) and membrane orientation of EVs. Examples of tetraspanins commonly found in EVs include CD63, CD81, and CD9. Note that each listed component may in fact be present in some subtypes of EVs and not in others. For instance, histones and proteasome and ribosome components are probably secreted in large plasma membrane–derived EVs and/or apoptotic vesicles rather than exosomes. Abbreviations: ARF, ADP ribosylation factor; ESCRT, endosomal sorting complex required for transport; LAMP, lysosome-associated membrane protein; MHC, major histocompatibility complex; MFGE8, milk fat globule–epidermal growth factor-factor VIII; RAB, Ras-related proteins in brain; TfR, transferrin receptor.

1999), the recurrent description of PS enrichment on exosomes suggests that they also expose this phospholipid, which, in live cells, is confined to the inner leaflet of the PM. The absence in secreted EVs of flippase, the enzyme that actively generates asymmetrical distribution of phospholipids in the PM of live cells, is probably responsible for PS exposure on secreted vesicles (Hugel et al. 2005).

Interestingly, the lipid composition of reticulocyte-derived exosomes is overall similar to that of the producing cells (Johnstone et al. 1987), with no particular enrichment in PS/phosphatidylinositol or sphingomyelin (Carayon et al. 2011). But this composition, especially the enrichment in ceramide, changes over the course of reticulocyte maturation into red blood cells, suggesting a modification of the intracellular mechanisms of exosome biogenesis (Carayon

et al. 2011) (see Mechanisms of Exosome Biogenesis, below). These studies collectively show that exosomes differ from the secreting cells in terms of lipid composition and point to a mechanism allowing sorting of these specific lipid species into the vesicles.

Nucleic acids. After the first description of nucleic acids in exosomes secreted by mast cells (Valadi et al. 2007), numerous groups have analyzed the presence of genetic material in EVs. Most studies thus describe small RNA, including mRNA, and miRNA of various sizes, with low or undetectable levels of ribosomal 18S and 28S RNA (on bioanalyzer) in purified EVs. A recent, careful comparison of RNA sizes in EVs sedimented at intermediate or low speed, or from apoptotic cells (Crescitelli et al. 2013), shows rRNA present specifically in the apoptotic cell–derived materials and also, in one out of three cell lines, in large MVs. This suggests that the presence of dead cells during tissue culture probably accounts for the contaminating presence of rRNA (Miranda et al. 2010) and possibly mtDNA (Guescini et al. 2010) in some EV preparations.

As expected from the inside-in membrane orientation of all secreted EVs (i.e., cytosol inside and extracellular domains facing outside) (**Figure 2**), in most studies, mRNA are contained inside EVs, as shown by their resistance to RNAse digestion (Valadi et al. 2007). However, some groups instead use RNAse digestion to show that the functional activity of their EVs requires RNA (Deregibus et al. 2007). Like for the unexpected observation of a cytosolic RAB5 protein at the surface of EVs (Logozzi et al. 2009), our preferred interpretation of such results is that nucleic acids originating from lysis of dead cells stick nonspecifically to EVs before purification, or that direct high-speed centrifugation of large vesicles induces breaking of these EVs into smaller outside-in vesicles. However, we cannot fully exclude the possible existence of an unknown molecular mechanism leading to natural formation of outside-in EVs, or to outside translocation of some specific EV components.

One of the most interesting outcomes of miRNA discovery in exosomes is the recent understanding that they are exported outside cells and can affect gene expression in distant cells. Such a functional transfer has been demonstrated clearly in vitro in three situations in which the intra-EV level of miRNA was not artificially increased by overexpression of the miRNA in the secreting cells: B-EBV-derived exosomes containing EBV-miRNA (Pegtel et al. 2010), murine DC-derived exosomes with miR451 (Montecalvo et al. 2012), and miR-223 in PM-derived MVs released by monocytes exposed to granulocyte-macrophage colony-stimulating factor (CSF2) (Ismail et al. 2013). In these studies, the authors showed the inhibition of expression of a reporter gene, target of the studied miRNA, in recipient cells that did not express themselves the EV-enclosed miRNA. However, whether the amount of EV used for these in vitro experiments can be achieved in a physiological situation is still unclear.

Of note, following the discovery of RNAs in exosomes (and EVs), other forms of extracellular miRNA have been reported: miRNAs have now been described in blood circulation as complexes with Ago2 protein (Arroyo et al. 2011) or with high-density lipoproteins (Vickers et al. 2011). The relative quantitative and functional importance of all these types of secreted miRNA still must be determined, but their description constitutes an important early-twenty-first-century discovery.

From the time of their initial description in EVs (Ratajczak et al. 2006, Skog et al. 2008, Valadi et al. 2007), we have known that mRNA were not randomly secreted in exosomes, because different sequences were either preferentially secreted or, conversely, retained inside the cells. A bioinformatics analysis of specifically exported RNA sequences recently unraveled a putative RNA export sequence (Batagov et al. 2011), but its actual export function has not been experimentally confirmed. Similarly, extensive comparison of intracellular and extracellular miRNA has now shown a selection of specific sequences of miRNA for extracellular export as well (Montecalvo et al. 2012). One more level of subtlety comes from the observation that different types of extracellular

miRNA carriers seem to transport different subsets of miRNA sequences (Palma et al. 2012, Wang et al. 2010), suggesting the existence of different mechanisms of RNA cargo selection. A recent study identified a specific sequence within miRNA, which binds to a sumoylated ribonucleoprotein to induce their targeting to EVs in T cells (Villarroya-Beltri et al. 2013). Whether these findings will be confirmed in other EVs from other sources will be an interesting future development.

Finally, next-generation sequencing techniques have now been used to characterize all small RNA present in mixed EVs released by DC/T lymphocyte cocultures (Nolte-'t Hoen et al. 2012), prion-infected neuronal cells (Bellingham et al. 2012), saliva (Ogawa et al. 2013), or semen (Vojtech et al. 2014). Several small noncoding RNA were thus found, the most abundant including vault-RNA, Y-RNA, and selected tRNA, and many of these exosomal RNA were enriched relative to cellular RNA, indicating a specific release of certain species via EVs. Except in the most recent study, EVs were recovered by ultracentrifugation at 100,000 × *g* not followed by buoyant density separation and possibly contained other extracellular RNA species (e.g., RNA associated to protein aggregates). Future analysis of membrane-enclosed extracellular RNA will become possible with increased sensitivities or decreased costs of deep sequencing.

Changes in extracellular vesicle composition. The overall composition of exosomes or EVs we have so far described is representative of mixed populations. In the past few years, numerous studies have reported changes in EV composition induced by modifications of the culture conditions, which can mimic different extracellular environments or different physiological or differentiation states of the secreting cells. We can give only a few examples of such studies; more of them are reviewed by Kucharzewska & Belting (2013) and in several recent articles published in the *Journal of Extracellular Vesicles*. For instance, inflammatory signals (e.g., LPS, TNFα, IFNγ) strongly affect the protein and/or RNA composition of EVs released by dendritic (Segura et al. 2005), endothelial (de Jong et al. 2012), or mesenchymal stem cells (Kilpinen et al. 2013). Hypoxia, a pathological situation observed in the core of large tumors or upon vascular injury, modifies the protein and RNA composition of EVs released by endothelial (de Jong et al. 2012) and tumor (Kucharzewska et al. 2013) cells. Expression of an oncogenic form of either *KRAS* (Demory Beckler et al. 2013) or *HRAS* (Tauro et al. 2013b) deeply changes the composition of the secreted EVs. The lipid composition of secreted vesicles is also altered when tumor cells are cultured in an acidic environment, which mimics the deep core of tumors (Parolini et al. 2009). It will be important in the future to determine whether these changes in overall composition reflect changes in the type of EVs secreted [especially because *HRAS* overexpression in tumor cells has also been shown to induce budding of MVs from the PM (Liao et al. 2012)], or rather in the intracellular targeting of the analyzed components to these EVs.

THE BIOGENESIS OF EXOSOMES

The Formation of MVBs

The endocytic pathway consists of highly dynamic membrane compartments involved in the internalization of extracellular ligands or cellular components, their recycling to the PM, and/or their degradation (Gould & Lippincott-Schwartz 2009, Klumperman & Raposo 2014). Early endosomes mature into late endosomes (Stoorvogel et al. 1991), and during this process, they accumulate ILVs in their lumen. Because of their morphological features, they are generally referred to as multivesicular endosomes or MVBs. The ILVs that are formed by inward budding of the early endosomal membrane sequester proteins, lipids, and cytosol that are specifically sorted. In most cells, the main fate of MVBs is to fuse with lysosomes, acidic compartments that

contain lysosomal hydrolases, ensuring the degradation of their content. However, organelles with hallmarks of MVBs, bearing the tetraspanin CD63, lysosomal-associated membrane proteins LAMP1 and LAMP2, and other molecules that are generally present in late endosomes (for example, MHC class II in antigen-presenting cells), can also fuse with the PM, releasing their content into the extracellular milieu (Jaiswal et al. 2002, Raposo et al. 1996). Interestingly, in reticulocytes, MVBs that fuse with the PM bear markers of early, rather than late, endosomes, such as RAB4 or RAB5 (Vidal & Stahl 1993). These observations suggest that different subpopulations of MVBs coexist simultaneously in cells, with some being destined for the degradation pathway, whereas others are fated for exocytosis.

Cells host different populations of MVBs. That cells can host different subpopulations of MVBs is supported by ultrastructural observations showing morphologically distinct MVBs on the basis of the size and appearance of the ILVs that they host in their lumen (**Figure 1*b***). Strengthening these observations, in EBV-transformed B cell lines (Mobius et al. 2003), cholesterol-positive and -negative MVBs coexist, and interestingly, most of the cholesterol-containing MVBs appeared to fuse with the cell surface in an exocytic manner, in agreement with the finding that exosomes are enriched in cholesterol. In HeLa cells, at least two distinct populations of MVBs have been described after stimulation with the EGF (White et al. 2006). The EGF-receptor reaches CD63-positive endosomes, whereas another subset of MVBs contain LBPA and CD63 but no EGF-receptor. The MVBs containing LBPA likely are fated for degradation, because exosomes are not enriched in LBPA (Wubbolts et al. 2003). In epithelial cells, morphologically different MVBs have been observed at the apical and basolateral sides of the cells. Likewise, the comparison of immature DCs and DCs undergoing cognate interactions with T cells revealed the presence of different MVBs in these cells (Buschow et al. 2009). In immature cells, ubiquitinated MHC class II molecules are sorted into MVBs mainly fated for lysosomal degradation. In the presence of antigen-specific T cells, DCs host a population of smaller MHC class II–CD9-containing MVBs that fuse with the PM to release exosomes that accumulate at the surface of T cells.

Mechanisms of intraluminal vesicle formation in MVBs. The best-described mechanism for formation of MVBs and ILVs is driven by the endosomal sorting complex required for transport (ESCRT), which is composed of approximately thirty proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins (VPS4, VTA1, ALIX also called PDCD6IP) conserved from yeast to mammals (Hanson & Cashikar 2012) (**Figure 3**). The ESCRT-0 complex recognizes and sequesters ubiquitinated transmembrane proteins in the endosomal membrane, whereas the ESCRT-I and -II complexes appear to be responsible for membrane deformation into buds with sorted cargo, and ESCRT-III components subsequently drive vesicle scission (Hanson & Cashikar 2012). ESCRT-0 consists of HRS (hepatocyte growth factor–regulated tyrosine kinase substrate, official gene symbol *HGS*) that recognizes the monoubiquitinated cargo proteins and associates with STAM (signal transducing adaptor molecule, the other ESCRT-0 component) and Eps15 and clathrin (two non-ESCRT proteins). HRS recruits TSG101 of the ESCRT-I complex, and ESCRT-I is then involved in the recruitment of ESCRT-III via ESCRT-II or ALIX. Finally, the dissociation and recycling of the ESCRT machinery require interaction with the AAA-ATPase VPS4. The mechanisms of inclusion of soluble cytosolic proteins into ILVs are still not very well understood, but a role for HSC70 has been proposed recently (Sahu et al. 2011): The chaperone binds to soluble cytosolic proteins containing a KFERQ sequence and to PS on the MVB outer membrane and thus enters ILVs formed in a TSG101- and VPS4-dependent manner.

However, some evidence suggests that MVBs and ILVs can form in absence of ESCRT function (**Figure 3**). The concomitant inactivation of four proteins of the four different ESCRT complexes

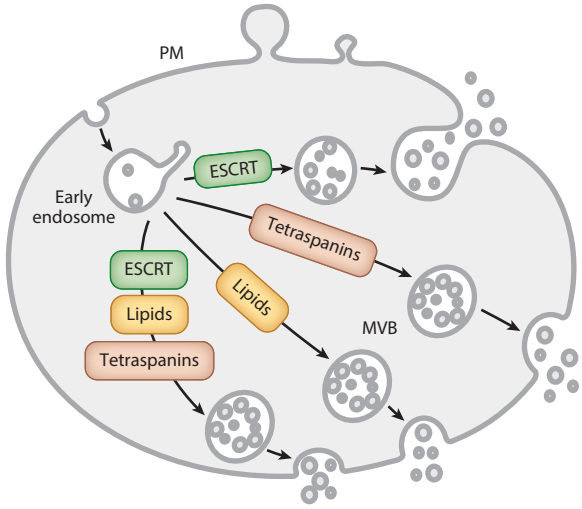

Figure 3

Molecular machineries of exosome/extracellular vesicle (EV) biogenesis. Multiple machineries are involved in biogenesis of intraluminal vesicles of multivesicular bodies (MVBs) and thus of exosomes/EVs. Endosomal sorting complex required for transport (ESCRT) components, lipids, and tetraspanins have been described, but whether each acts in different MVBs, or if they can simultaneously act on the same MVB, is not known. Abbreviation: PM, plasma membrane.

does not abolish MVB formation (Stuffers et al. 2009). Moreover, in melanocytic cells, the sorting of premelanosomal protein PMEL to the ILVs of MVBs does not require ubiquitination or ESCRT-0, ESCRT-II (Theos et al. 2006), and ESCRT-III components (S. Simoes, I. Hurbain, C. Delevoye, N.M. Peterson, G. Van Niel, H. Stenmark & G. Raposo, unpublished observations). Its sorting requires the tetraspanin CD63 (Van Niel et al. 2011), which accumulates in ILVs even in the absence of ESCRT function (Stuffers et al. 2009). Consistently, CD63 was recently shown to be instrumental in formation of small (<40 nm) ILVs, independently of HRS, in MVBs of HeLa cells (Edgar et al. 2014). Finally, two lipid metabolism enzymes have been shown to generate lipids in the limiting membrane of MVBs, which induce inward budding and thus formation of ILVs in an ESCRT-independent manner: neutral sphingomyelinase (nSMase) allowing hydrolysis of sphingomyelin into ceramide (Trajkovic et al. 2008), and phospholipase D2 allowing hydrolysis of phosphatidylcholine into phosphatidic acid (Ghossoub et al. 2014). Therefore, MVBs and their ILVs can be formed by both ESCRT-dependent and -independent mechanisms certainly related to the cargo that is sorted within a given cell (Carayon et al. 2011). We now discuss the consequences of these different mechanisms on exosome biogenesis.

Mechanisms of Exosome Biogenesis

ESCRT-dependent mechanisms. A function for ESCRT proteins in exosome biogenesis was inferred initially from proteomic studies showing the presence, in exosomes from different cell types, of TSG101 and ALIX (Théry et al. 2001). ALIX was also related to exosome biogenesis in reticulocytes, where its binding to the cytoplasmic domain of TfR was proposed to compete with binding to HSC70 and promote TfR sorting onto ILVs (Géminard et al. 2004). More recently (Baietti et al. 2012), tumor cell exosomes were shown to contain syndecan, syntenin, and ALIX. Overexpression of syntenin induced an increase in the ALIX-dependent release of exosomes

(as evidenced by an increase in exosomal markers CD63 and HSP70), whereas the downregulation of syndecan, syntenin, or ALIX impaired exosome release. The biogenesis of syndecan-, syntenin-, and ALIX-containing exosomes was dependent on ESCRT-II, ESCRT-III, and VPS4 function, as well as on clustering of syndecan. These data support a role of ALIX in exosome biogenesis and exosomal sorting of syndecans via an interaction with syntenin.

Three studies linked the ESCRT-0 protein HRS (gene name *HGS*) to exosome secretion by showing reduced exosome release in HRS-deficient DCs (Tamai et al. 2010) or HGS-depleted HEK293 cells (Gross et al. 2012) and tumor cells (Hoshino et al. 2013). In DCs, this decrease was observed only after incubation with an antigen and not in a steady-state situation, thus suggesting different mechanisms of exosome secretion in different cellular physiological states (Tamai et al. 2010).

To obtain a more comprehensive overview on the role of individual ESCRT proteins in exosome biogenesis and secretion, we recently performed an RNA interference screen targeting 23 individual ESCRT components in HeLa cells (Colombo et al. 2013). We defined exosomes as EVs simultaneously bearing two MVB-enriched proteins: CD63 and MHC class II. We could thus conclusively demonstrate a role of some of these components in the secretion of exosomes: Silencing of two ESCRT-0 genes (*HGS*, *STAM1*) or the ESCRT-I gene *TSG101* reduced their secretion, and the remaining secreted EVs carried less CD63 and MHC class II, whereas silencing of *VPS4B* increased their secretion without modifying their composition. In HeLa, depletion of ALIX by silencing *PDCD6IP* increased the overall level of MHC class II expression in the cells, and consequently in the released EVs, without obviously affecting the level of total EV secretion, whereas in primary DCs, the same silencing decreased EV secretion in half of the donors (Colombo et al. 2013). Comparison of our results in HeLa and DCs, and other analyses of several ESCRT proteins in exosome secretion by MCF7 breast tumor cells (Baietti et al. 2012), thus shows some common mechanisms (decrease of exosome secretion by TSG101 depletion) but also discrepancies: inhibition (Baietti et al. 2012) versus increase (Colombo et al. 2013) of exosome secretion induced by VPS4B depletion and inhibition of exosome release by ALIX depletion in MCF7 and possibly in DCs but not in HeLa cells. A recent study in a muscle cell line showed increased release of PM-derived EVs containing HSC70 but decrease of CD63 secretion upon ALIX depletion (Romancino et al. 2013), whereas in an oligodendroglial cell line (Trajkovic et al. 2008), none of the tested ESCRT components (TSG101, ALIX, VPS4) were involved in exosome-dependent release of the GPI-anchored proteolipid protein (PLP), and in RPE-1, depletion of ALIX or TSG101 impaired release of anthrax toxin–containing but not flotillin-containing exosomes (Abrami et al. 2013). This again highlights the molecular and mechanistic heterogeneity of the types of EVs secreted by different cells.

Interestingly, the relationship between ESCRT-dependent formation of exosomes and their cargo load has not yet been clearly determined. MHC class II molecules display a ubiquitination sequence that allows their incorporation into ILVs (Van Niel et al. 2006), but a mutant MHC class II β-chain lacking the ubiquitination site is still recovered in exosomes through incorporation into detergent-resistant membranes containing CD9 (Buschow et al. 2009). We observed, however, decreased amounts of CD63 and MHC class II on EVs recovered from *TSG101*- or *STAM1*-knockdown HeLa cells (Colombo et al. 2013), suggesting that TSG101 and STAM1 participate in transmembrane cargo inclusion in EVs. Another possible mechanism involves the chaperone HSC70, whose binding to the cytosolic tail of the TfR has been shown to allow targeting of this transmembrane protein to exosomes (Géminard et al. 2004). For soluble cargoes, ubiquitinated (Buschow et al. 2005) and KFERQ-containing proteins (Sahu et al. 2011) are abundant in exosomes. The machinery that drives ubiquitinated proteins into exosomes is not known, whereas

HSC70 may target KFERQ-containing proteins to exosomes, although these observations have not been confirmed by others.

ESCRT-independent mechanisms. That exosome biogenesis could occur via an ESCRT-independent mechanism was demonstrated initially in an oligodendroglial cell line (Trajkovic et al. 2008). Inhibition of nSMase (enzymes that hydrolyse sphingomyelin to ceramide) with GW4869 decreased PLP-bearing exosome release. Thus, sorting of PLP into ILVs is ESCRT independent and requires the synthesis of ceramide. Reduced secretion of more classical exosomal proteins (CD63, CD81, or TSG101) and/or miRNA was also observed upon GW4869 treatment of embryonic kidney (HEK293) (Chairoungdua et al. 2010, Kosaka et al. 2010) or tumor cell lines (Dreux et al. 2012, Hoshino et al. 2013). In primary cells, however, GW4869 treatment induces cell death, which prevents reliable analysis of exosome secretion (neurons: R. Sadoul, personal communication), or, when used at a concentration that does not induce death, increases release of EVs of all sizes (DCs: J. Kowal & C. Théry, unpublished observations).

In human melanoma cells, by contrast, the depletion of neutral sphingomyelinases impairs neither MVB biogenesis (Van Niel et al. 2011) nor exosome secretion (G. Van Niel & G. Raposo, unpublished data); instead, a CD63-dependent mechanism is required for ILV/exosome formation. CD63 is instrumental in targeting the EBV-encoded LMP1 protein to ILVs and allowing its subsequent release in exosomes (Verweij et al. 2011). In HEK293 cells, CD9 or CD82 (but not CD63) overexpression was shown to induce secretion of β-catenin by exosomes, which were still generated through a ceramide-dependent mechanism (Chairoungdua et al. 2010). In a rat pancreatic adenocarcinoma cell line, expression of the tetraspanin Tspan8 led to modifications in the exosome content in mRNA and transmembrane proteins (VCAM-1, α4 integrin) (Nazarenko et al. 2010). CD81-enriched domains have been proposed recently as sorting platforms for exosomal proteins (Perez-Hernandez et al. 2013) and may certainly account for ESCRT-independent sorting of some cargoes and the formation of a population of ILVs: Proteins that are known to interact with certain tetraspanins were found in exosomes via mass spectrometry, and in CD81-deficient animals, exosomes were found to be devoid of CD81-interacting molecules, which are normally loaded onto exosomes.

Finally, a small integral membrane protein of lysosomes and late endosomes, called SIMPLE, was recently shown to be secreted in association with exosomes, and fibroblasts expressing its mutant form found in Charcot-Marie-Tooth disease patients, CMT1C, secreted less CD63- and ALIX-containing exosomes, whereas flotillin secretion was unaffected (Zhu et al. 2013). How SIMPLE regulates exosome secretion, and whether it is through binding to TSG101 or to Nedd4 type-3 ubiquitin ligase, two proteins for which SIMPLE contains a binding domain, was not elucidated in this study. The latter interaction is potentially relevant to exosome biogenesis and secretion, because a transmembrane protein able to bind Nedd4, Nedd-family interacting protein 1, has been shown to promote exosome secretion and targeting of cytosolic proteins, such as the PTEN tumor suppressor, into these exosomes (Putz et al. 2012).

THE SECRETION OF EXOSOMES OR OTHER EXTRACELLULAR VESICLES

Constitutive or Regulated Secretion?

Some tumor cells spontaneously release large PM-derived EVs, termed oncosomes (Di Vizio et al. 2012, Muralidharan-Chari et al. 2009), that display metalloproteinases with proinvasive properties. However, release of PM-derived EVs is more commonly induced by stimuli leading

to a rise in intracellular calcium and cytoskeleton remodeling (Pasquet et al. 1996). Thus, calcium ionophores directly trigger MV release, as well as extracellular signals, such as formyl-Met-Leu-Phe on neutrophils (Hess et al. 1999); ATP binding to $P2X_7$ receptors on myeloid cells (Bianco et al. 2005, MacKenzie et al. 2001, Pizzirani et al. 2007); and simple feeding of tumor cells with fresh fetal calf serum (FCS)-containing medium (Ginestra et al. 1997).

Treatment with Ca^{2+} ionophores has also been used in the literature to increase secretion of exosomes by the erythroleukemia cell line K562 (Savina et al. 2003), oligodendroglial cells (Krämer-Albers et al. 2007), DCs (Montecalvo et al. 2012), and mast cells (Raposo et al. 1997, Valadi et al. 2007). Again, the proportion of EVs coming from intracellular compartments, versus the cell surface, after such treatments probably varies with the cell type.

In contrast with PM-derived EVs, exosome secretion is generally analyzed at the steady state (i.e., in the absence of a stimulus known to trigger this secretion). However, it is difficult to exclude the possibility that some unsuspected signals trigger or modify this secretion, because fresh culture medium is generally fed to the cells one or two days before exosome collection. In particular when, to avoid co-purification of FCS-derived with cell-derived exosomes, cells are abruptly changed to FCS-free medium, stress induced by this abrupt starvation probably results in altered quantitative and/or qualitative EV secretion.

In cells that spontaneously secrete exosomes, some clear exosome-stimulating conditions have also been described, although the intracellular signals involved are not known. For instance, exosome secretion by murine DCs is increased by cognate interactions with antigen-specific $CD4^+$ T lymphocytes (Buschow et al. 2009). The secretion of exosomes by rat cortical neurons can be stimulated by depolarization of the cells (Fauré et al. 2006) or stimulation by neurotransmitters (Lachenal et al. 2011), a signal that also promotes exosome secretion by oligodendrocytes (Fruhbeis et al. 2013). γ-Irradiation-induced DNA damage can also promote EV secretion by tumor cells or fibroblasts through the activation of the p53-regulated protein TSAP6 (Lespagnol et al. 2008, Yu et al. 2006). In these studies, however, whether the vesicles recovered were MVB-derived exosomes or other EVs was not clearly determined. More recently, silencing of papilloma virus E6/E7 oncogenes in HeLa cells was clearly shown to induce senescence and a concomitant increase of p53 and TSAP6 expression, as well as a large increase in secretion of EVs, including those bearing endosomal markers (CD63, TSG101) (Honegger et al. 2013). Finally, some cells, such as B or T lymphocytes, secrete very little EVs at the steady state, but MVB-derived exosome secretion is strongly enhanced by activation through interactions with T cells (Blanchard et al. 2002, Mittelbrunn et al. 2011) or the B cell receptor (Muntasell et al. 2007).

Functions of Rab GTPases in Exosome Secretion

Rab proteins (reviewed in Stenmark 2009) are essential regulators of intracellular vesicle transport between different compartments: Rabs can be involved in either vesicle budding, mobility through interaction with the cytoskeleton, or tethering to the membrane of an acceptor compartment. The Rab family is composed of more than 60 GTPases, each of which is preferentially associated with one intracellular compartment.

RAB11 was the first Rab reported to be involved in exosome secretion: Its inhibition by overexpression of a dominant negative mutant in the erythroleukemia cell line K562 decreased secretion of TfR and HSC70-containing exosomes (Savina et al. 2002). Later, parallel screening strategies highlighted two new Rabs in exosome secretion: RAB27 and RAB35. In a murine oligodendroglial cell line (Hsu et al. 2010), inhibiting Rabs by overexpressing a library of Rab GTPase–activating proteins revealed RAB35 as necessary for PLP-bearing exosome secretion. RAB35-dependent exosome secretion was confirmed by another group in primary oligodendrocytes (Fruhbeis et al.

2013). Simultaneously, our small hairpin RNA (shRNA)-based screen in HeLa cells expressing MHC class II molecules showed that silencing of RAB2B, RAB5A, RAB9A, and most efficiently RAB27A and RAB27B decreased secretion of exosomes bearing CD63, CD81, and MHC class II (Ostrowski et al. 2010). RAB35 (Hsu et al. 2010) and RAB27A/B (Ostrowski et al. 2010) were shown to allow docking of MVBs to the PM.

Interestingly, these different observations were individually confirmed in different cell types. In *Drosophila* S2 cells, RAB11 depletion by dsRNAs inhibited secretion of small EVs bearing wingless or Evi (Beckett et al. 2013, Koles et al. 2012), whereas neither RAB27 nor RAB35 had an effect. In retinal epithelial cells, either RAB11 or RAB35 depletion reduced secretion of flotillin- and anthrax toxin–bearing vesicles, whereas RAB27A/B did not play a role (Abrami et al. 2013). In tumor cell lines of various origins (melanoma, breast, head and neck or prostate carcinoma), RAB27A silencing decreased the secretion of small EVs collected by ultracentrifugation (Bobrie et al. 2012b, Hoshino et al. 2013, Peinado et al. 2012, Webber et al. 2014). The human breast carcinoma cell line MDA-MB-231, by contrast, did not display reduced exosome secretion upon RAB27A depletion (A. Bobrie & C. Théry, unpublished observation), but codepletion of RAB27A and RAB27B did decrease the number of secreted EVs (Zheng et al. 2012). Importantly, by analyzing several protein markers associated to exosomes in a mouse mammary carcinoma cell line (Bobrie et al. 2012a), we observed that secretion of the CD9 tetraspanin and the PS-bound MFGE8 was not decreased upon RAB27A depletion, as opposed to secretion of the MVB-associated CD63, ALIX, or TSG101.

According to the literature (Stenmark 2009), RAB11 and RAB35 associate mainly to recycling and early sorting endosomes, respectively, and RAB27A/B to late endosomal and secretory compartments (often called lysosome-related organelles). Our current hypothesis to reconcile these apparent discrepant observations is that different types of small EVs are collectively purified by the classically used protocols (**Figure 4**). Some of them are secreted via a RAB27-dependent mechanism from late endosomes: They are enriched in late endosomal proteins (e.g., CD63, ALIX, and TSG101). Others are secreted from early or recycling endosomes, which fuse with the PM thanks to RAB35 or RAB11 and possibly contain flotillin and other cell-specific proteins (Wnt-associated, PLP, TfR). Of note, RAB7, a late-endosome associated Rab, is involved in secretion of ALIX- and syntenin-containing exosomes by MCF7 tumors (Baietti et al. 2012), whereas its depletion does not affect exosome secretion in HeLa cells (Ostrowski et al. 2010). Thus different subtypes of late endosomes can generate exosomes. In addition, some secreted vesicles probably also form through direct budding from the PM, and the requirement for Rab GTPase activity in such a secretion is not known.

A Role for SNAREs and Other Components of the Fusion Machinery

SNARE proteins [for soluble *N*-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors, reviewed in Zylbersztejn & Galli 2011] form complexes with SNAPs between two membranes and mediate membrane fusion between the two organelles. Ca^{2+}-regulated fusion of secretory lysosomes with the PM has been shown to involve SNAP-23 (at the PM) and lysosomal VAMP8 in mastocytes (Puri & Roche 2008, Tiwari et al. 2008), or VAMP7 in epithelial cells (Rao et al. 2004) or neutrophils (Logan et al. 2006), but the SNAREs involved in the fusion of MVBs with the PM to release exosomes have so far been little studied. VAMP7 has been reported to promote exocytosis of MVBs to release acetylcholinesterase-containing EVs in the human leukemia K562 cell line (Fader et al. 2009). By contrast, VAMP7 inhibition in MDCK cells (Proux-Gillardeaux et al. 2007) was shown to inhibit lysosomal secretion, as in other epithelial cells (Rao et al. 2004), but not secretion of HSP70-bearing EVs.

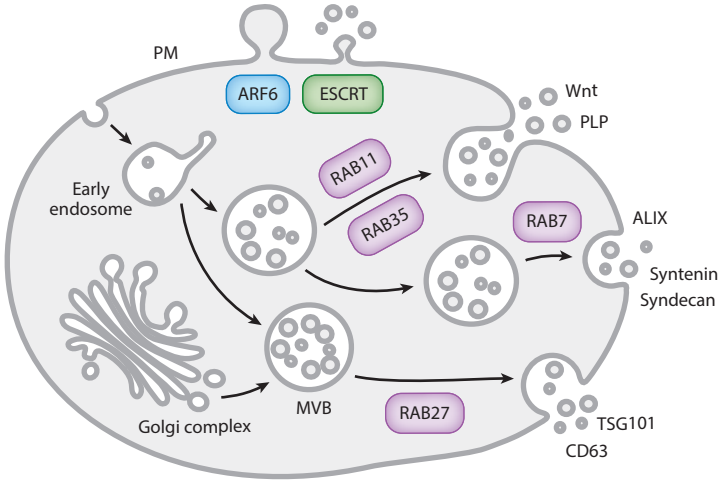

Figure 4

Molecular machineries of exosome/extracellular vesicle (EV) secretion. Multiple secretion machineries of EVs have been described. For multivesicular body (MVB)-dependent secretion, proteins of the Ras-related proteins in brain (RAB) family, RAB11, RAB35, RAB7, and RAB27, have been shown to promote exosome secretion and may act on different MVBs along the endocytic pathway. For plasma membrane (PM)-derived EVs, ADP-ribosylation factor 6 (ARF6), but also some components of the endosomal sorting complex required for transport complex (ESCRT) family (including ALIX and TSG101, but not ESCRT-0 components), have been shown to regulate outward budding. Abbreviations: ALIX, ALG2-interacting protein X; PLP, proteolipid protein; TSG101, tumor susceptibility gene 101; Wnt, Wingless/Int-1 family of proteins.

Independent screenings performed in *Drosophila* identified Ykt6, a SNARE involved in endoplasmic reticulum–to-Golgi complex transport but also observed in MVB (Meiringer et al. 2008), as required for the secretion of Wnt-bearing exosomes (Gross et al. 2012), or syntaxin1a (STX1 in mammals), as required for secretion of Evi-bearing exosomes (Koles et al. 2012). However, in our model of MHC class II–expressing HeLa cells, downregulation of YKT6 or STX1 rather increased exosome secretion (M. Colombo & C. Théry, unpublished observations).

Thus, in different cell types, distinct SNARE complexes may be involved in the fusion of a given organelle. Alternatively, different SNARE complexes could mediate the fusion of different subpopulations of MVBs within a single cell type; thus, the downregulation of one SNARE protein might affect the secretion of only a particular subpopulation of exosomes.

A Potential Role for Other Molecules in Exosome Secretion

Several other intracellular effectors may be involved in exosome release, although it is unknown if their role is related to the biogenesis of ILVs in MVBs or to their secretion.

Overexpression of diacyl glycerol kinase α (DGKα) in T cells inhibits activation-induced secretion of CD63/LAMP1-positive exosomes bearing Fas-ligand (Alonso et al. 2007), and its inhibition leads to increased exosome release. Further analyses suggested that DGKα acts as a negative regulator of the formation of MVBs (Alonso et al. 2011), which then explains the decrease in exosome secretion when the protein is overexpressed, although the authors use LBPA to label MVBs, and as mentioned above (see section on Lipids) LBPA is not particularly enriched in exosomes (Wubbolts et al. 2003) and is likely to be present rather in organelles fated for lysosomal degradation. During HIV-1 viral release in human cell lines, in addition to increasing

viral production and release, citron kinase (a RhoA effector) enhanced the release of vesicles bearing HSC70, CD82, and LAMP1 (Loomis et al. 2006), suggesting that citron kinase is involved in the exocytosis of late endosomal compartments.

Finally, genetic approaches in the worm *C. elegans* have proposed the V0 subunit of V-ATPase as a possible mediator of the fusion of MVBs with the PM. Using EM, Liegeois et al. (2006) noted that cuticle mutants appeared to specifically accumulate MVBs at the PM and identified the V0 subunit as the mutated gene responsible for this phenotype. This suggests that the V0-ATPase is required for the fusion of MVBs at the apical epidermal PM, leading to the production of the cuticle. Because several isoforms exist in mammalian cells, it is still unclear whether the V0 ATPase is involved in exosome secretion.

Biogenesis and Release of Plasma Membrane–Derived Vesicles

Oligomerization of cell surface receptors, for instance by antibody-mediated crosslinking, promotes release of early endosome–derived EVs from reticulocytes (Vidal et al. 1997) or PM-derived EVs from T lymphocytes (Fang et al. 2007). Interestingly, in the latter cells, although EVs did bud from the PM, their formation involved the TSG101 and VPS4 components of the ESCRT machinery, which led the authors to call these EVs exosomes (Booth et al. 2006) (**Figure 4**). To induce budding of EVs at the PM, TSG101 must interact with a PXAP sequence present in the gag protein of retroviruses (Booth et al. 2006), but also in the endogenous protein arrestin domain–containing protein 1 (ARRDC1) (Nabhan et al. 2012). Localization of gag or ARRDC1 at the PM directs EV outward budding there, instead of into MVBs. Of note, MVB-independent functions of the ESCRT machinery, such as budding of PM-derived vesicles, membrane fission during cytokinesis (Henne et al. 2011), or plasma membrane repair (Jimenez et al. 2014), do not involve the ESCRT-0 proteins HRS and STAM. Therefore, a requirement for ESCRT-0 proteins in EV secretion (as shown in Colombo et al. 2013, Gross et al. 2012, Hoshino et al. 2013, Tamai et al. 2010) strongly supports the MVB origin, hence exosomal nature, of these EVs.

In platelets, a strong rise in intracellular calcium induces the activation of the protease calpain, which cleaves cytoskeletal proteins and thus remodels the cytoskeleton (Pasquet et al. 1996). This, together with a loss of membrane asymmetry owing to modulation of the activities of flippase, floppase, and scramblase, leads to outward budding of EVs from the PM (see review, Hugel et al. 2005). Depolymerization of the actin cytoskeleton by overexpression of the small GTPase ARF6 (Muralidharan-Chari et al. 2009) and/or loss of the actin-nucleating protein diaphanous related protein 3 (Di Vizio et al. 2009) has also been shown to allow efficient production of large PM-derived oncosomes from tumor cells (**Figure 4**), and these features are correlated with the acquisition of an amoeboid shape and higher migration ability of the cells. ARF6, however, has also recently been implicated in secretion of MVB-derived exosomes by a tumor cell line, via activation of phospholipase D2 leading to generation of phosphatidic acid at the limiting membrane of MVBs (Ghossoub et al. 2014). Consistently, overexpression of phospholipase D2 induces an increase in exosome release by mast cells upon degranulation (Laulagnier et al. 2004a). Sphingomyelinases are other lipid-metabolism enzymes involved in budding of vesicles: Acid sphingomyelinase originating from lysosomes was shown to allow the release of large vesicles from the PM of astrocytes upon $P2X_7$ receptor triggering (Bianco et al. 2009), whereas neutral sphingomyelinase in MVBs is required for ILV formation and exosome secretion by oligodendrocytes (Trajkovic et al. 2008). Thus, like for ESCRT, the same machineries can play similar roles (i.e., vesicle budding away from the cytosol) either at the PM or in intracellular compartments.

It was recently found that overexpression of hyaluronan synthase 3, a feature of chondrosarcoma and mesothelial cells, induced the formation of long microvilli coated with hyaluronan, which were

released into the culture medium as MVs (Rilla et al. 2013). Finally, a role for a RAB protein, RAB22A, has been recently shown in MV shedding by tumor cells under hypoxia (Wang et al. 2014). Of note, bacteria also release MVs from their membranes; however, for lack of space, we do not discuss the mechanisms of this secretion, which are poorly defined.

THE EXTRACELLULAR FATE OF EXOSOMES AND OTHER EXTRACELLULAR VESICLES

Targeting of Exosomes/Extracellular Vesicles to Recipient Cells

The concept of EVs as messengers in intercellular communication implies that EVs secreted by one cell interact with a recipient cell to induce changes in its physiology. The discovery of how single exosomes interact with target cells has been held back by the difficulty of visualizing exosomes using optical microscopy (the resolution limit being 200 nm). Nonetheless, numerous studies have analyzed bulk interaction of exosomes or EVs with recipient cells by fluorescence microscopy, flow cytometry, or functional transfer of surface molecules. Because it is impossible to determine whether the interactions observed involve aggregates of exosomes, which could be as large as oncosomes, or single vesicles, we collectively refer to EVs without distinguishing between the EV subtypes.

A first step in binding of EVs to the cell surface should involve specific receptors on both EVs and the PM. Several classical ligand/receptor pairs have been described in these interactions, each one probably specific to a given cellular source of EVs and a given recipient cell type. For instance, we can cite a specific role of LFA-1 on DCs to capture ICAM-1-bearing DC-EVs (Segura et al. 2005), or a partial inhibition of DC-EV capture by DCs by antibodies blocking either LFA-1, ICAM-1, αv or β3 integrins, or CD9 or CD81 tetraspanins (Morelli et al. 2004). MFGE8 (which binds to PS at the surface of EVs) is a ligand for αvβ3 and αvβ5 integrins and has been shown to allow capture of apoptotic cells by macrophages expressing these integrins (Hanayama et al. 2002), and possibly also of DC-EVs by DCs (Morelli et al. 2004); however, other PS-binding receptors, such as TIM4 on phagocytes (Miyanishi et al. 2007), also allow capture of EVs, which could explain the efficient capture by DCs of MFGE8-deficient EVs (Veron et al. 2005). EVs from a tumor cell line expressing the tetraspanin Tspan8 are enriched in α4 integrin and probably bind to ICAM1 on endothelial and pancreatic cells (Rana et al. 2012). The lectin receptor Siglec (or CD169) mediates in vivo capture of splenocyte EVs bearing α2,3-sialic acid (Saunderson et al. 2014), tumor cells recognize heparan sulfate proteoglycans on tumor-EVs (Christianson et al. 2013), and macrophages use galectin-5 at the surface of erythrocyte EVs for capture (Barres et al. 2010). These are only a few examples from a long list of published studies, and we apologize for not quoting work from several colleagues.

The specificity of targeting EVs to particular recipient cells is probably conferred by the surface ligands present on both. In several studies, DCs and macrophages were designed as the major recipients of EVs, but this observation is probably linked to their strong phagocytic capacity (Feng et al. 2010, Mallegol et al. 2007). However, other cells can capture EVs; for example, activated T cells bind DC-derived EVs through LFA-1 (Nolte-'t Hoen et al. 2009). α4 Integrin expression in Tspan8-expressing tumor cells redirects their secreted EVs to endothelial and lymph node stromal cell lines, whereas fibroblasts are the major target of non-α4-bearing EVs (Rana et al. 2012). A cardiomyocytic cell line captures mesenchymal-derived EVs (Chen et al. 2010). DC-derived EVs engineered to present a surface receptor containing an amino acid sequence recognized by receptors specifically expressed on either neuronal or muscle cells were readily bound to a neuron or muscle cell line, respectively, in vitro (Alvarez-Erviti et al. 2011).

Fate of Exosomes/Extracellular Vesicles Within Recipient Cells

In some cases, binding of EVs to recipient cells might be sufficient to induce changes in the physiological state of the recipient cells, for instance during the presentation of MHC-peptide complexes on the surface of antigen-presenting cell-derived EVs to antigen-specific T cells (Denzer et al. 2000, Segura et al. 2007).

In other cases, the content of EVs must be transferred inside the recipient cell. Depending on the recipient cells, internalization has been described to occur through dynamin-, PI3-kinase-, and actin polymerization–dependent phagocytosis in macrophages (Barres et al. 2010, Feng et al. 2010); receptor-mediated endocytosis (Morelli et al. 2004) or phagocytosis in acidic compartments (Montecalvo et al. 2012) in DCs; dynamin-dependent but receptor-independent macropinocytosis in microglia (Fitzner et al. 2011); dynamin- and actin polymerization–dependent phagocytosis but also clathrin-dependent endocytosis in neurons (Fruhbeis et al. 2013); clathrin-independent but cholesterol- and lipid raft–dependent endocytosis in endothelial and some tumor cells (Svensson et al. 2013); or caveolae-dependent endocytosis in epithelial cells (Nanbo et al. 2013). Of note, the behavior of recipient cells facing large or small particles is different, and this applies to large versus small EVs: Large EVs (or aggregates of small ones) probably induce phagocytosis, whereas individual small EVs can be internalized by nonphagocytic processes.

Once endocytosed or phagocytosed, EVs can be degraded and their components used by the cells for their own physiology. But for the content of EVs to gain access to the cytosol of the recipient cell, which is necessary, for instance, for gene silencing or expression induced in the recipient cell by nucleic acids contained inside the EVs, a fusion step with either the PM or the limiting membrane of endocytic compartments must take place. Such fusion has been demonstrated in two articles using the self-quenched fluorescent R18 lipid probe, which upon dilution increases its fluorescence (Montecalvo et al. 2012, Parolini et al. 2009). Increased fluorescence upon mixing of R18-labeled EVs with recipient tumor cells or DCs thus demonstrated fusion of the EV membrane with the much larger membrane of recipient cells. In tumor cells, fusion was enhanced under an acidic pH (Parolini et al. 2009), which is representative of what occurs in the tumor mass or possibly inside recipient cells in late endosomes or phagosomes. In DCs, the authors also demonstrated the release of an internal component of EVs in the cytosol of recipient cells by incubating luciferin-loaded exosomes with DCs expressing the enzyme luciferase and showing light emission by the resident luciferase enzyme in the presence of its substrate. The recently described long-term protection of a toxin inside ILVs, and its slow release into the cytosol by back-fusion with the limiting MVB membrane of the ILVs inside the infected cells, or of exosomes once transferred to recipient cells (Abrami et al. 2013), also functionally demonstrates that membrane fusion can occur between EVs and recipient cells.

Fate of Exosomes/Extracellular Vesicles In Vivo

It is difficult to translate the results obtained in vitro to the in vivo situation; thus, it is difficult to determine what happens to EVs once they are secreted by a given cell within an organ. EVs are found in various biological fluids, thus demonstrating their secretion in vivo. The presence in blood of EVs bearing markers not only of the cells present in blood vessels (endothelial and immune cells) but also, in the case of cancer patients, of a distant tumor shows that EVs secreted within a solid tumor are not entirely captured in situ by surrounding cells, or the extracellular matrix but can instead cross through the endothelium and gain access to blood circulation. EV half-life in blood circulation is difficult to determine. Studies showing the abundant presence on exosomes from B cells or DCs of molecules inhibiting destruction by the complement system (Clayton et al. 2003) led to the idea that EVs should remain stable for a long time. But this

was not confirmed by direct analysis of the half-life of EVs in blood after intravenous injection: Two recent studies using tumor-derived EVs exposing a membrane-bound luciferase fused to the C1C2-domain of MFGE8/lactadherin (Takahashi et al. 2013), or biotinylated splenocyte-derived EVs (Saunderson et al. 2014), calculated a half-life of only two minutes. In both studies, several hours after they had disappeared from the circulation, EVs were recovered in spleen, and melanoma-derived EVs also accumulated in lung, liver, and bone marrow—organs thought to be preferred sites of metastasis (Peinado et al. 2012, Takahashi et al. 2013). Interestingly, EVs from a pancreatic adenocarcinoma cell line were recovered in pancreas, lung, and kidney more than in spleen and liver (Rana et al. 2011), suggesting different organ targeting in vivo of EVs with different cellular origins. Organ targeting could be modified by changing the tetraspanin network expressed on the adenocarcinoma-derived EVs (Rana et al. 2011), but also by introducing a cell-specific ligand inside an EV transmembrane protein: DC-derived EVs could thus be redirected to the brain (Alvarez-Erviti et al. 2011). EVs can also travel through the lymphatic system and thus reach lymph nodes, as shown after subcutaneous injection of melanoma EVs (Hood et al. 2011). Whether EVs secreted in vivo in a solid tissue will preferentially reach the lymphatic vessels and lymph nodes, where they can affect immune responses, or the blood vessels and other organs is not known yet.

CONCLUSION

As we have highlighted, although clinical trials using EVs are already under way (Viaud et al. 2011), there are still many unknowns in the field. In particular, one asset would certainly be the development of more accurate methods of isolation of different subpopulations of vesicles to which an origin and function can be attributed. Once such limitations are overcome, new methods to manipulate their biogenesis, composition, secretion, and interaction could be of use not only for understanding their function but also for the development of novel therapeutic strategies. The recent interest of scientists and physicians in this field of research has been stimulating. An EV and exosomal type of intercellular communication is certainly not limited to the functions exemplified in this review. Not only mammalian cells but also diverse microbial pathogens, such as Gram-negative bacteria, eukaryotic parasites of the kinetoplast lineage, and opportunistic fungal pathogens (Barteneva et al. 2013, Silverman & Reiner 2011), exploit such a membrane-based process of secretion (exosomes and MVs) for vehiculating components. Recent findings with *Leishmania* (Silverman et al. 2010), *Trichomonas* (Twu et al. 2013), parasitic helminths (*Fasciola*, *Echinostoma*) (Marcilla et al. 2012), and *Plasmodium* (Martin-Jaular et al. 2011, Regev-Rudzki et al. 2013) have shown release by the parasites (or by parasite-infected red blood cells in the case of *Plasmodium*) of EVs that influenced the behavior of host cells and promoted parasite infection or survival. These new findings linking EVs to infection biology have important implications for the design of research strategies aimed at the development of novel therapeutics, biomarkers, and vaccines in diverse areas.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Some parts of the PhD thesis of M.C. were used to write this review. Work performed in the authors' groups was funded by Institut National de la Santé et de la Recherche Médicale (INSERM),

Centre National de la Recherche Scientifique (CNRS), Institut Curie, the Agence Nationale de la Recherche (grant numbers ANR-09-BLANC-0263 to C.T. and G.R. and ANR-10-IDEX-0001-02 PSL* and ANR-11-LABX-0043 to C.T.), the Fondation de France [Annual Award 2010] to C.T., the Institut National du Cancer [2008-PL-BIO] to C.T. and G.R., and Fondation ARC [SL220100601359] to G.R., [SL220120605293] to C.T., and [DOC20110603085] to M.C. We thank J. Kowal and M. Tkach for critical reading of the manuscript. We apologize to numerous colleagues for being unable to extensively quote their work.

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