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Ceramide and exosomes: a novel target in cancer biology and therapy

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Abstract

Exosomes are secreted extracellular vesicles (EVs) that carry micro RNAs and other factors to reprogram cancer cells and tissues affected by cancer. Exosomes are exchanged between cancer cells and other tissues, often to prepare a premetastatic niche, escape immune surveillance, or spread multidrug resistance. Only a few studies investigated the function of lipids in exosomes, although their lipid composition is different from that of the secreting cells. Ceramide is one of the lipids critical for exosome formation and it is also enriched in these EVs. New research suggests that lipids in the exosomal membrane may organize and transmit “mobile rafts” that turn exosomes into extracellular signalosomes spreading activation of cell signaling pathways in oncogenesis and metastasis. Ceramide may modulate the function of mobile rafts and their effect on these cell signaling pathways. The critical role of lipids and in particular, ceramide for formation, secretion, and function of exosomes may lead to a radically new understanding of cancer biology and therapy.

Got exosomes: What’s (really) in your prep?

Exosomes are a type of extracellular vesicles (EVs) that are formed as intraluminal vesicles in multivesicular endosomes (MVEs). The MVEs fuse with the plasma membrane and release these vesicles to the extracellular space (Fig. 1A). It is assumed that there are two different pathways leading to exosome formation, endosomal sorting complex required for transport (ESCRT)-dependent and independent (Colombo et al., 2013; Colombo, Raposo, & Thery, 2014; Hurley, 2015; Hurley & Odorizzi, 2012; Juan & Furthauer, 2017; Kowal, Tkach, & Thery, 2014; Marsh & van Meer, 2008; Stoorvogel, 2015; Trajkovic et al., 2008; Villarroya-Beltri, Baixauli, Gutierrez-Vazquez, Sanchez-Madrid, & Mittelbrunn, 2014). ESCRT proteins such as Alix 1 and TSG 101 form a neck-shaped complex with other proteins such as vacuolar sorting associated protein 4 (VPS4) initiating budding of exosomes at the MVE membrane. ESCRT-independent exosome formation relies on ceramide generation by neutral sphingomyelinase 2 (nSMase2), a key cell signaling enzyme (Fig. 1A). However, this distinction may not be as strict as previously thought and the same cell type can secrete different types of exosomes. Exosomes are distinct from microvesicles, another type of EVs that are released by blebbing off the plasma membrane. These two types of EVs are different not only in size, function, and cargo, but also in the intrinsic signals leading to

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their secretion (for comprehensive reviews on EV formation and secretion, see (Desrochers, Antonyak, & Cerione, 2016; Edgar, 2016; Hyenne, Lefebvre, & Goetz, 2017; Kowal et al., 2014; Raposo & Stoorvogel, 2013; Stoorvogel, 2015; Tkach, Kowal, & Thery, 2018; Tkach & Thery, 2016; van Niel, D'Angelo, & Raposo, 2018)). Exosomes carry RNA (micro RNA (miRNA), long-noncoding RNA (lncRNA), messenger RNA (mRNA), mitochondrial DNA, signaling proteins, enzymes, metabolites, and lipids from the donor to a recipient cell (Choi, Kim, Kim, & Gho, 2013; Kai, Dittmar, & Sen, 2017; Kinoshita, Yip, Spence, & Liu, 2017; Record, Carayon, Poirot, & Silvente-Poirot, 2014; Salehi & Sharifi, 2018; H. G. Zhang & Grizzle, 2014)) (Fig. 1). The recipient cell takes up exosomes by endocytosis, pinocytosis, or simply fusing the exosomal with the plasma membrane (Abels & Breakefield, 2016; Costa Verdera, Gitz-Francois, Schiffelers, & Vader, 2017; Horibe, Tanahashi, Kawauchi, Murakami, & Rikitake, 2018; H. Jiang, Li, Li, & Xia, 2015; McKelvey, Powell, Ashton, Morris, & McCracken, 2015; Tian et al., 2013; Tian et al., 2014). A multitude of different effects of exosomes was reported. In cancer, exosomes reprogram tissue to allow growth of metastases, act as decoys to escape the immune system, or spread factors conferring resistance to drugs used in chemotherapy (Chiarugi & Cirri, 2016; Desrochers et al., 2016; Dorsam, Reiners, & von Strandmann, 2018; Dreyer & Baur, 2016; Kahlert & Kalluri, 2013; Kalluri, 2016; Ruivo, Adem, Silva, & Melo, 2017; S, Mager, Breakefield, & Wood, 2013; M. Silva & Melo, 2015; Steinbichler, Dudas, Riechelmann, & Skvortsova, 2017; Weidle, Birzele, Kollmorgen, & Ruger, 2017; H. G. Zhang & Grizzle, 2014; Zhao et al., 2017). Exosomes secreted by tumor cells can be found in the blood and urine, which makes analysis of exosomal content attractive for early cancer diagnosis in "liquid biopsy" (Kai et al., 2017; Minciacchi, Freeman, & Di Vizio, 2015; Minciacchi, Zijlstra, Rubin, & Di Vizio, 2017; A. Sharma, Khatun, & Shiras, 2016; Yokoi, Yoshioka, & Ochiya, 2015; Yoshioka et al., 2014; W. Zhang et al., 2017). Since exosomes are the physiological equivalent to liposomes they can be used as vector to deliver miRNAs and drugs, or for cell-free vaccination in cancer therapy (Pitt et al., 2014; Schorey & Bhatnagar, 2008; Viaud et al., 2010). Our own research showed that stimulating exosome secretion from breast cancer stem-like cells can break their resistance to chemotherapy (Kong, He, et al., 2015). Others have reported that exosomes from stem cells can be used for "cell-free stem cell therapy" (Phinney & Pittenger, 2017; Rani, Ryan, Griffin, & Ritter, 2015). However, most of the *in vitro* studies rely on the preparation of exosomes from medium of a (large) donor cell culture to incubate a (smaller) recipient culture. Despite the exploding number of publications on exosomes, only a few studies strived to answer a simple but fundamental biochemical question: what is the stoichiometry between the effector molecule and the effect? In other words, is the number of exosomes in a given tissue really sufficient to recapitulate *in vivo* the effects seen *in vitro*? The simple answer to this question is: no one really knows since the *in vivo* function of exosomes is largely unclear and difficult to test.

The difficulty in testing the function of exosomes stems from the dirty secret of their preparation: exosomes are intrinsically inhomogeneous. Most exosome preparations rely on two methods based on centrifugation: differential ultracentrifugation and low speed-centrifugation using a polymer matrix such as polyethylene glycol (PEG) (for reviews on exosome preparation, see (P. N. Brown & Yin, 2017; Carpintero-Fernandez, Fafian-Labora, & O'Loghlen, 2017; Coumans et al., 2017; Helwa et al., 2017; P. Li, Kaslan, Lee, Yao, &

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Gao, 2017; Taylor & Shah, 2015; Xu, Greening, Zhu, Takahashi, & Simpson, 2016; Zeringer, Barta, Li, & Vlassov, 2015). These methods do not completely remove other vesicular and particulate contaminants such as microvesicles, lipoproteins, or simply cell debris. This is not as much of a problem for *in vitro* studies when harvesting exosomes secreted by cultured cells into the medium supernatant. Provided that exosome- and lipoprotein-free serum is used for cultivation of cells, main contaminants are cell debris (including apoptotic bodies) and microvesicles. The bulk of these contaminants can be removed by pre-centrifugation at 20,000×g or filtration of the cell culture medium at 0.2 μm prior to ultracentrifugation at 110,000×g. Microvesicles are still a concern since their size varies broadly around 1 μm, while exosomes constitute a population of vesicles with a narrow size range from 50–150 nm. Density gradient ultracentrifugation improves exosome purity, however, densities of lipoproteins and different vesicle population are too close for being efficiently separated. Exosome purity is also a concern when using polymers for dehydration leading to precipitation of exosomes at low speed centrifugation, since these methods do not distinguish between different vesicle populations. Therefore, exosomes and microvesicles are often combined under the term of EVs, with some of the functions such as delivery of effector molecules being shared between these two vesicle populations (for reviews comparing exosomes with microvesicles, see (Lee, El Andaloussi, & Wood, 2012; P. Li et al., 2017; Minciacchi et al., 2015; Raposo & Stoorvogel, 2013)). The problem of purity is even more serious when it comes to preparation of exosomes from tissues and bodily fluids such as blood, urine, or cerebrospinal fluid (CSF). Tissue dissociation using proteases and gentle mechanical disruption can introduce artifacts by releasing intracellular vesicles into the preparation or contaminating the sample with endogenous and exogenously added proteases that associate with exosomes. While exosome preparation from serum is still easier than that from solid tissue, vesicles can be contaminated with lipoprotein particles, which limits interpretability of “liquid biopsies”. Immunoprecipitation using antibodies against exosome surface proteins can alleviate this problem and even separate exosomes derived from different cell types. However, the yield is usually lower than with centrifugation techniques and often compromises a quantitative analysis of exosome function (Brett et al., 2017; Momen-Heravi et al., 2013; Nakai et al., 2016; Szatanek, Baran, Siedlar, & Baj-Krzyworzeka, 2015). Recently, great strides were made in the field of exosome isolation introducing new techniques such as ultrafiltration and microfluidics-based separation. Although each newer technique has its own advantage, they also pose distinct limitation, keeping the isolation of pure and homogenous exosomes a challenging task (M. He & Zeng, 2016; Momen-Heravi et al., 2013; Pietrowska et al., 2017; S. Sharma, Scholz-Romero, Rice, & Salomon, 2018; Szatanek et al., 2015; Xu, Simpson, & Greening, 2017). To date, there is no reliable method for efficient purification or sorting of exosomes from tissues, regardless of being soft or more solid, tumor or healthy tissues.

Methods used in our lab and that of other groups largely rely on a hit-or-miss approach. The purity of a particular exosome preparation is not predictable but has to be rigorously tested after the preparation was accomplished. Apart from mistakenly analyzing exosomes that originate from the tissue’s blood supply, main contaminants in exosome preparations from tissues, including tumors, are vesicles released from damaged cells. The post-preparation quality control is critical and encompasses an array of physical tests, detection of exosomal

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marker proteins, and loss-of-function assays. Must-have physical tests include laser light scattering microscopy, electron microscopy, or tunable resistive pulse sensing to visualize and quantify exosomes (Akers et al., 2016; Coscia et al., 2016; Koritzinsky, Street, Star, & Yuen, 2017; Maas, Broekman, & de Vrij, 2017; Vogel et al., 2016). We use nanoparticle tracking analysis (NTA) based on laser light scattering microscopy to reliably quantify the number and size distribution of exosomes and other vesicles in our preparation (Coumans et al., 2017; Dinkins, Enasko, et al., 2016; Helwa et al., 2017; Kong, He, et al., 2015; Koritzinsky et al., 2017; Szatanek et al., 2017; Tkach et al., 2018). For a preparation with high purity, the number of exosomes should match up with the quantity of marker proteins such as Alix1, TSG101, Flotillin, or CD63 as detectable in immunoblots. Finally, loss-of-function assays will help exclude other sources of bioactive molecules in the exosomes preparation. These assays include UV irradiation (to destroy RNA in exosomes) or mild detergent treatment, which either obliterates the effect of exosomes (if based on RNA) or exosomes themselves (Coumans et al., 2017; Kinoshita et al., 2017; Osteikoetxea et al., 2015). Our group has specialized on pre-purification loss-of-function assays by preventing formation or secretion of exosomes *in vitro* and *in vivo* (Dinkins, Dasgupta, Wang, Zhu, & Bieberich, 2014; Dinkins, Enasko, et al., 2016; Kong, Hardin, et al., 2015; G. Wang et al., 2012). To date, inhibition or genetic deficiency of nSMase2 is a method widely used to prevent exosome secretion in various cell types and tissues (Chairoungdua, Smith, Pochard, Hull, & Caplan, 2010; Dinkins et al., 2014; Dinkins, Enasko, et al., 2016; Dinkins, Wang, & Bieberich, 2016; Goldkorn, Chung, & Filosto, 2013; Guo, Bellingham, & Hill, 2015; Kong, He, et al., 2015; Kosaka et al., 2010; Marsh & van Meer, 2008; Menck et al., 2017; Shamseddine, Airola, & Hannun, 2015; Tan et al., 2013; Trajkovic et al., 2008; Yuyama, Sun, Mitsutake, & Igarashi, 2012). nSMase2 converts sphingomyelin into ceramide, a reaction shown to be instrumental for exosome secretion *in vitro* (Trajkovic et al., 2008) (Fig. 1A). Our laboratory showed that inhibition and genetic deficiency of nSMase2 also suppresses exosome secretion *in vivo* (Dinkins et al., 2014; Dinkins, Enasko, et al., 2016; Dinkins, Wang, et al., 2016). These discoveries and the critical role of ceramide for the function of exosomes, particularly in cancer, will be discussed in the next sections of this review.

Why ceramide in exosomes? It’s all in the numbers.

The answer to the initial question of just how much of an effector molecule needs to be in exosomes to actually affect the recipient cell has remained controversial. Exosomes are not the only form of transferring bioactive molecules. Prior to the massive upswing of exosome research, most of the intercellular communication was thought to be managed by exchange of humoral factors such growth factors, cytokines, hormones and other secreted smaller and larger molecules. The importance of these factors does not vanish with the growing importance of exosomes. One has to realize that exosomes simply add to the secretome by stabilizing and combining certain signaling molecules such as miRNAs for long-distance communication and targeted delivery (for review on secretome, including EVs, see (Sinha et al., 2018; van der Pol, Boing, Harrison, Sturk, & Nieuwland, 2012; Vizoso, Eiro, Cid, Schneider, & Perez-Fernandez, 2017)). Enrichment of specific miRNAs in exosomes may be suitable for early diagnosis in cancer, but it still needs to be tested if the amount of miRNA

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transferred in exosomes is really sufficient to reprogram tissue for metastasis or fulfill other functions of exosomes found *in vitro*. If looking for signaling molecules that are transferred in functionally significant quantities one may want to shift focus away from RNA and include other signaling molecules transported by exosomes. But first, one will need to do the numbers to assess the contribution of RNA and other factors to cell signaling in cancer.

Despite the excitement emerging from the function of RNA in reprogramming tissue, their content in exosomes appears to be very low. According to a study from 2014, the abundance of miRNA is less than 1 molecule/100 exosomes (Chevillet et al., 2014). In a more recent study by our collaborators and us, very low abundance (copy number) of two specific miRNAs (miRNA-16 and 451) was confirmed using different methods for exosome preparation (Helwa et al., 2017). However, these numbers were based on the assumption that miRNAs were evenly distributed over the entire exosome population. Our study showed that based on an average miRNA length of 22 nucleotides (double-stranded), an average molecular mass of 640 g/mol base pair and recovery of about 25 ng miRNA/5×10^{11} exosomes isolated from 5 ml serum, there is about one-to-two molecules of miRNA in a single exosome (Helwa et al., 2017). With respect to miRNA, the concentration of a specific miRNA/cell needs to reach a threshold to efficiently silence its target gene expression (B. D. Brown et al., 2007; Mukherji et al., 2011). Titration experiments show that the typical concentrations are in the range of several hundred to more than 1000 copies of a specific miRNA/cell, which is similar to the threshold levels for gene silencing (Bissels et al., 2009). Provided that a specific miRNA copy is less than 1 in 100 exosomes, one will need at least 10^4 to 10^5 exosomes taken up by a single cell to reach a concentration comparable to endogenous miRNAs. At an average size of 100 nm/exosome and 10 μm/cell (surface ratio $1:10^4$), a single cell will have to take up exosomes equivalent to 1–10 times of its own cell surface to reach an efficient copy number of a specific miRNA. The 2014 study provided a potential solution to this dilemma. Since the copy number of miRNA was quantified as an average over the entire exosome population, “low occupancy/high abundance” exosomes may be rare (≤1/100), but could deliver ≥10–100 copies of a specific miRNA/exosome (Chevillet et al., 2014). In this case, taking up the equivalent of ≤1/10 of the cell surface could be sufficient to reach the efficient copy number/cell. This does not appear unreasonable since some cells such as macrophages can pinocytose an equivalent of the cell surface in 33 min (Chevillet et al., 2014). However, this calculation implies two additional predicaments: 1) The concentration of a specific miRNA is massively enriched in exosomes (≥10–100-fold), requiring an efficient sorting mechanism. This assumption is supported by a recent study that identified a set of exosomal miRNAs abundant in exosomes derived from human neural stem cells with at least 10 copies/exosome (Stevanato, Thanabalasundaram, Vysokov, & Sinden, 2016). Upon function transfer analysis, the group showed that exosomal miRNA can reach the molecular machinery of miRNA-suppressed gene repression at a physiologically relevant level. 2) The recipient cell is specifically targeted by “low occupancy/high abundance” exosomes. Cell culture supernatants of 10^7 donor cells (equivalent to a confluent 100 mm dish with 10 ml medium) can yield about 10^{11} exosomes after overnight incubation (equivalent to number of exosomes in 0.5–1 ml serum (Helwa et al., 2017)). At 1% abundance of a specific miRNA copy, one will have to collect 10^{12} exosomes to achieve 1000 copies miRNA/cell transferred to 10^7 recipient cells, which

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corresponds to conditioned medium from about 10 dishes (or exosomes from 5–10 ml serum). However, if many identical copies are packaged into few exosomes, the “harvest” of 10^7 cells may be enough to transfer a sufficient number of miRNA copies to 10^6 cells. Therefore, one of the main reasons for exosomal miRNA being found to reprogram cells may simply rely on using a large amount of donor cells (or serum exosomes) to incubate a much smaller number of recipient cells. Another reason may result from mistaking the effect of miRNA with that of other more abundant cell signaling molecules transferred by exosomes.

In addition to miRNA, proteins transferred by exosomes were discussed to affect cancer cells (for reviews and exemplary studies on proteomics and protein function in exosomes see (Azmi, Bao, & Sarkar, 2013; Choi et al., 2013; Greening, Xu, Gopal, Rai, & Simpson, 2017; Intasqui, Bertolla, & Sadi, 2018; Jakobsen et al., 2015; A. Li, Zhang, Zheng, Liu, & Chen, 2017; Pietrowska et al., 2017; Pocsfalvi et al., 2016; Sandfeld-Paulsen, Aggerholm-Pedersen, et al., 2016; Sandfeld-Paulsen, Jakobsen, et al., 2016; Sinha et al., 2018; Tanase et al., 2017; Taylor, Zacharias, & Gercel-Taylor, 2011; van Niel et al., 2018; Weidle et al., 2017)) (Fig. 1). For example, exosomal fibronectin can bind to integrin and enhance cell adhesion and prepare a premetastatic niche for fibrosarcoma cells (Sung, Ketova, Hoshino, Zijlstra, & Weaver, 2015; Tkach & Thery, 2016). The effect of exosomes on preparing a premetastic niche will be discussed in more detail in the next section of this review. Among exosomal proteins, enzymes are particularly attractive since it only takes a few molecules to amplify their activity in the target cells. Matrix metalloproteinases (MMPs) are transferred by exosomes and facilitate cancer cell invasion (Hakulinen, Sankkila, Sugiyama, Lehti, & Keski-Oja, 2008; Sanderson, Bandari, & Vlodavsky, 2017; Shay, Lynch, & Fingleton, 2015). However, while stoichiometry is most likely the greatest barrier in understanding the effect of exosomal miRNAs, topology is the problem with most exosomal proteins. Unless they are bound to the exosomal surface such as MT1-MMP, the majority of proteins discussed to promote neoplasia and metastasis are encaged inside of exosomes (Fig. 1). Hence, it is difficult to reconcile the activity of proteins modifying the extracellular matrix (ECM) with topological enclosure inside of exosomes. Solutions such as slow or induced disintegration of the exosomal membrane for controlled release and activation of exosomal enzymes are conceivable, but membrane disintegration is in stark contrast to the presumed stability of exosomes when discussing other functions of exosomes such as miRNA transfer (Kumeda et al., 2017). Perhaps, there are functionally specialized exosomes that may be prone to release of their contents, while others remain stable and are meant for long-distance transport of bioactive molecules. It was suggested that sphingomyelin is one of the lipids stabilizing the exosomal membrane (Record et al., 2014). Secreted sphingomyelinases may convert sphingomyelin into ceramide and potentially destabilize the exosomal membrane, thereby releasing proteins and other factors enclosed in exosomes. This speculation brings us the next class of molecules that is far less explored, albeit it may offer several explanations for yet unresolved obstacles in understanding exosomes: lipids.

The few in-depth analyses of exosomal lipids have shown that the lipid composition of the exosomal membrane is distinct from that of most cellular membranes in the donor cells (Record et al., 2014). For starters, a simple geometrical correlation between surface and volume of a vesicle implies that the smaller the vesicle the larger the surface in comparison

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to volume. Hence, it is not surprising that many lipids appear to be enriched in the exosomal membrane when normalizing lipid to protein content, the latter being mainly correlated with volume. Apparent lipid enrichment due to this geometrical correlation may thus not ignite much excitement, but one should also realize that it implies a major function of exosomes as vector for autocrine and paracrine membrane and lipid transport and exchange between cells. Independent of geometry, there is specific enrichment when comparing the molar proportions of lipids in the exosomal to other cellular membranes. This difference is one of the most important features that distinguishes exosomes from microvesicles, the lipid composition of which is very similar to that of the parental plasma membrane (Record et al., 2014). A recent lipidomics analysis using liquid chromatography tandem mass spectrometry (LC-MS/MS) showed that the molar proportions of cholesterol, glycosphingolipids, and sphingomyelin are enriched by up to 3-fold compared with lipids abundant in other membranes such as phosphatidylcholine, the proportion of which is reduced in exosomes (Record et al., 2014). Lysobisphosphatidic acid (LBPA), an endosomal membrane lipid also known as bis-monoacylglycerophosphate (MBP) is exclusively contained in exosomes and was shown to partake in ESCRT-dependent exosome formation and exosomal membrane stability (Record et al., 2014; Tkach & Thery, 2016). However, in contrast to the proposed functions of exosomal miRNA and proteins in cancer, the role of most exosomal lipids has remained unclear.

The incredible journey of a lipid: rafting the extracellular space on exosomes

As discussed in the previous section, ceramide is a sphingolipid that is critical for exosome formation and/or secretion. While it is not understood how ceramide may contribute to the inward budding of vesicles at the MVE, facilitating or inducing membrane curvature may be one of its tasks (for biophysical properties of ceramide in membrane curvature, see (Burgert et al., 2017; Goni & Alonso, 2006; Goni, Contreras, Montes, Sot, & Alonso, 2005; Pinto, Silva, Futerman, & Prieto, 2011; L. C. Silva et al., 2012). Our studies showed that exosomes contain mainly two ceramide species, C18:0 (about 70% of total ceramide) and C24:1 ceramide, while isolated intracellular vesicles also contain C16:0 ceramide (Q. He et al., 2014; G. Wang et al., 2012). In synthetic vesicles, mixtures of C18:0 and C24:1 ceramide lead to in-plane phase separation at ≥1 % ceramide and up-regulation of medium-chain ceramides (such as C16:0 and C18:0 ceramide) favors negative curvature (inward budding) of the membrane (Carrer, Hartel, Monaco, & Maggio, 2003; Carrer & Maggio, 1999; Castro, Prieto, & Silva, 2014; Goni & Alonso, 2006; Goni et al., 2005; Pinto et al., 2011). Interaction of very long chain sphingolipids in the outer leaflet with phosphatidylserine in the inner leaflet was suggested to facilitate the enrichment of cholesterol in the exosomal membrane, probably to increase its stability and/or prevent phagocytosis through binding to phospatidylserine exposed to Annexin V type receptors on macrophages (Skotland, Sandvig, & Llorente, 2017). Anisotropic demixing of membrane lipids is a necessary condition for curvature, suggesting that formation of ceramide microdomains and their inward budding may critically contribute to the intraluminal formation of exosomes in MVEs. Using an anti-ceramide antibody developed in our laboratory and TEM, we showed that enrichment of ceramide is common to several intraluminal membranes in MVEs, including exosomes and

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multilamellar vesicles (Fig. 2). Therefore, it appears reasonable to presume that whatever the function of ceramide in exosomes, it is the composition of lipids that is critical. Hence, instead of looking at enrichment of individual lipids, one should rather look at ratios. Lipidomics analyses showed that the molar proportion of ceramide was enriched by 1.3–3-fold when compared to that of membranes in the donor cells, which was also found in our studies on exosomes secreted by astrocytes (Record et al., 2014; G. Wang et al., 2012). However, when comparing the proportion of ceramide to cholesterol, we and others found that ceramide was >3-fold more enriched than cholesterol (Record et al., 2014). Based on this observation, the function of ceramide in exosomes may not just rely on its enrichment, but its composition and proportion in comparison to other lipids such as cholesterol.

There are only a few studies showing that uptake of exosomes actually increases the lipid content of the recipient cell (Record et al., 2014). More likely is the localized alteration of the membrane lipid proportion by exosomal lipids (e.g., after fusion with the cell membrane or endocytotic uptake). As discussed in the previous section, membrane lipids are not homogeneously distributed, but they are anisotropically organized in suprastructures such as lipid rafts, microdomains, or platforms. These terms are often used interchangeably, the reader interested in further details is referred to excellent reviews on this topic (Bieberich, 2008; Gulbins & Kolesnick, 2003; Lingwood & Simons, 2010; Lopez, 2015; Ma, Hinde, & Gaus, 2015; Mollinedo & Gajate, 2015; Owen, Magenau, Williamson, & Gaus, 2012; Simons & Gerl, 2010; Simons & Ikonen, 1997; Simons & Sampaio, 2011; Staubach & Hanisch, 2011; Y. Zhang, Li, Becker, & Gulbins, 2009). In brief, the “classical” raft is about 50–200 nm in diameter, stabilized by complexes between cholesterol and sphingomyelin, and enriched in glycosphingolipids such as ganglioside GM1 (“Raft” in Fig. 1B). The main function of suprastructures is to regulate proteins associated with rafts (“raft-associated protein” or RAP in Fig. 1B), such as receptors or caveolins, often modified by attachment to membrane anchors such as glycosylphosphatidylinositol (GPI) or palmitoyl residues (Busija, Patel, & Insel, 2017; Lajoie & Nabi, 2010; Simons & Toomre, 2000). As the result, lipid rafts can activate cell signaling pathways or induce endocytosis. In exosome biology, evidence was provided that parts or the entire exosomal membrane is derived from endocytosed lipid rafts (Skotland et al., 2017; Staubach & Hanisch, 2011; Tan et al., 2013) (Fig. 1A). Not yet proven, but very likely is enrichment of exosomal lipids in endosomes of the recipient cell after endocytic or pinocytotic uptake (Fig. 1C). Also exciting is the idea that exosomes may actually transfer lipid rafts from the donor to the recipient cell (Fig. 1B). Most recently, research in our and our collaborators’ laboratories has shown that ceramide-rich platforms (CRPs) are 50–200 nm in size and contain ≥ 20 ceramide molecules (Burgert et al., 2017). CRPs are formed when sphingomyelinases convert sphingomyelin into ceramide (Fig. 1A). As discussed, ceramide is ≥3-fold enriched over cholesterol in exosomes, suggesting that exosomes could serve as mobile ceramide-rich lipid rafts or CRPs (Fig. 1B). The surface ratio between a sphere and a circle of equal diameter is 2, suggesting that the surface of a single 100 nm-sized exosome covers a plasma membrane (approximated as a plane) area of about 200 nm diameter, quite within a size range of larger CRPs. Therefore, it does not appear unreasonable to suggest that one of the functions of exosomes is to exchange lipid rafts between cells.

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The main concept underlying these “mobile rafts” is that lipid rafts formed in donor cells are transported via exosomes to the recipient cell, thereby inducing the same cell signaling pathway as activated in the donor cells (Fig. 1). This mechanism is analogous to signalosomes in neurons, vesicles that form after receptor activation in synapse and transport the activated receptor to the cell body (Cosker, Courchesne, & Segal, 2008; Cosker & Segal, 2014). Hence, the function of mobile lipid rafts in regulating cancer cell signaling pathways is broadcasted to distant cells in a tumor or even to metastases. Experimental evidence for mobile rafts is provided by testing if a particular receptor or cell signaling pathway activation in recipient cells is similar to donor cells when exposed to donor-derived exosomes. Even though the overall lipid composition of the recipient cells due to uptake or fusion with exosomes does not change, the suprastructure of the plasma membrane or other cellular membranes may adopt the lipid raft organization of the donor cells (Fig. 1A and C). There are several areas in cancer biology that may benefit from broadcasting cell signaling events via mobile rafts in exosomes: 1) metabolic or morphogenetic reprogramming in a tumor in response to scarcity of resources such as oxygen or glucose; 2) preparation of a premetastatic niche; 3) communication with the immune system to escape surveillance, and 4) acquisition and spreading of resistance to drugs or other tumor suppressive signals. In all of these instances, donor and recipient cells may be of the same or different cell types (e.g., cancer to cancer cell, cancer to tissue cell, tissue to cancer cell, or cancer to immune cell and vice versa), and the raft-activated cell signaling pathway may induce the same or a different response in donor and recipient cells. In principle, the range of possible combinations in donor and recipient cells and responses to mobile rafts is similar to the potential of miRNA-mediated programming through exosomes. However, since lipids in the exosomal membrane are abundant and instrumental to the activity of mobile rafts the effect of exosomal lipids on recipient cells may outperform or at least complement that of miRNAs for reasons discussed in the previous section.

Exosomes in cancer: target tissue biohacking and hijacking of the immune system

In metabolic and morphogenetic reprogramming, cancer cells are often found to switch from mitochondrial to cytosolic generation of adenosine triphosphate (ATP) by limiting glucose consumption to aerobic glycolysis without invoking oxidative phosphorylation in mitochondria (Gatenby & Gillies, 2004; Liberti & Locasale, 2016). This so-called Warburg effect appears reasonable since oxygen becomes a scarce resource in a growing tumor. In a recent study, it was shown that patient-derived, cancer-associated fibroblasts secrete exosomes that suppress oxygen consumption in pancreatic and prostate cell culture by 80% within 24 h (Rabinowitz & Coller, 2016; Zhao et al., 2016). While the authors of the study hypothesize that miRNA-mediated silencing of oxidative metabolism genes and nutrients delivered by exosomes are accountable for this rapid effect, it was noted that the amount of exosomes and timing do not reconcile with this hypothesis (Rabinowitz & Coller, 2016). However, since lactic acid production is a consequence of aerobic glycolysis and low/acidic pH stimulates exosomes secretion (Parolini et al., 2009), it is possible that a positive feed-back rapidly increases the amount of exosomes in the metastatic niche. As an alternative (or complement) to miRNA, mobile rafts transported by exosomes could regulate metabolism in

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cancer cells. The possibility of reprogramming cancer cell metabolism by mobile rafts has not been discussed yet, but it is known that cell signaling pathways promoting glucose uptake and metabolism, particularly the PI3K/Akt axis, are affected by lipid rafts and ceramide (Mollinedo & Gajate, 2015; Powell, Hajduch, Kular, & Hundal, 2003; Zhou, Summers, Birnbaum, & Pittman, 1998). For example, insulin and insulin-like growth factor 1 (IGF-1) receptors are embedded into lipid rafts and can activate PI3K/Akt to stimulate glucose uptake and fuel glycolysis (Mollinedo & Gajate, 2015). Exosomes from prostate cancer cells were found to spread raft-associated proteins (RAPs, Fig. 1B) such as IGF-1 receptors (DeRita et al., 2017). We showed that the PI3K/Akt activator atypical protein kinase C (aPKC) is a ceramide-associated protein (CAP) transported by exosomes, probably when bound to CRPs in mobile rafts (Fig. 1B) (G. Wang et al., 2012). On the other hand, ceramide can inhibit PI3K/Akt cell signaling and when delivered in nanoliposomes, counteract the Warburg effect in cancer (Y. Jiang et al., 2011; Ryland et al., 2013; Tagaram et al., 2011). It is reasonable to speculate that different populations of exosomes – for example, depending on their ceramide content - may reprogram metabolism of cancer cells in distinct ways. Likewise, exosomes and EVs can reprogram tissues to allow tumor growth, particularly by inducing morphogenetic changes in endothelial cells and stem cells. Exosome-induced angiogenesis and capillary morphogenesis contribute to blood supply to tumors, which is an established research field addressing EV cancer biology (for reviews, see (Grange et al., 2011; Mostefai, Andriantsitohaina, & Martinez, 2008)). As with metabolic reprogramming, almost all the studies focus on effects mediated by miRNAs. However, it is well known that endothelial growth factor (EGF)-receptors are activated by lipid rafts and transported by EVs (Al-Nedawi, Meehan, Kerbel, Allison, & Rak, 2009; Pike, 2005). Secretion of sphingosine-1-phosphate (S1P), a ceramide derivative, is instrumental in promoting angiogenesis during tumor growth (Mizugishi et al., 2005; Nagahashi et al., 2012; Ogretmen, 2018; Pyne, El Buri, Adams, & Pyne, 2017; Spiegel & Kolesnick, 2002; Spiegel & Milstien, 2003; Takabe, Paugh, Milstien, & Spiegel, 2008; Takabe & Spiegel, 2014). It was found that stable complexes of S1P with G-protein coupled receptors (GPCRs) are critical for formation of exosomes in MVEs through constitutive activation of Rho GTPases and stabilization of F-actin (Kajimoto et al., 2018). However, it has not been determined if these stable complexes are formed on exosomes that could then function as extracellular signalosomes. In colon cancer, *enterobacteria* trigger secretion of mucosa-derived exosomes that contain S1P and prostaglandin E2 (PGE2) (Z. Deng et al., 2015), an eicosanoid promoting cancer cell proliferation and angiogenesis. PGE2 in mucosa-derived exosomes has also been found to suppress liver-derived natural killer T (NKT) cells (Z. B. Deng et al., 2013), a part of cancer exosome-immune cell crosstalk discussed in the next paragraph. While it is known that the PGE2-EP4 receptor complex is activated in lipid rafts (Lin et al., 2017), it is currently unknown if this or other ligand-receptor complexes are embedded in mobile rafts and secreted via exosomes that function as extracellular signalosomes. The reason for this lack of knowledge is the technical difficulty to combine exosome preparation with the isolation and analysis of lipid rafts. The very few studies on this topic have shown that detergent-insoluble membranes isolated from exosomes – the next best equivalent to lipid rafts – contain a multitude of RAPs, including proteins regulating hepatocyte growth factor and EGF receptor cell signaling pathways in cancer cells (Ji et al., 2013).

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By far, most of the studies on the effect of exosomes in cancer focus on their role in reprogramming tissue to generate a premetastatic niche and the cancer-to-immune cell crosstalk. As with other research testing the function of exosomes, most of these studies invoke miRNA and do not explore the function of exosomal lipids or mobile lipid rafts (Kai et al., 2017; Kinoshita et al., 2017; Salehi & Sharifi, 2018). miRNAs exchanged by exosomes between cancer cells promote cell growth (miRNA-155) modulate epithelial-mesenchymal-transition (EMT, miRNA-23a, miRNA-191), contribute to breakdown of ECM (miRNA-29 family), and induce migration (miRNA-21). Hence, the function of cancer exosomes is comparable to biohacking by genetic and epigenetic reprogramming of target tissue for metastasis. Up to recently, analysis of lipids in cancer metastasis was mainly focused on autocrine and paracrine signals mediated by S1P or lysophosphatidic acid (LPA), two moderately soluble lipids promoting cell growth and migration by targeting GPCRs and downstream PI3K/Akt and mammalian target of rapamycin (mTOR) cell signaling pathways (Radeff-Huang, Seasholtz, Matteo, & Brown, 2004; Ye, Ishii, Kingsbury, & Chun, 2002). The function of lipid rafts was invoked in facilitating GPCR activation by these lipids. However, only a few studies have investigated if EVs or exosomes exchange GPCRs between cells (Isola & Chen, 2016; Ye et al., 2002). Recently, ectosomes, EVs budding off cilia, were reported to accumulate GPCRs, but a role of ectosome-bound GPCRs in cancer has not been described yet (Isola & Chen, 2016; Nager et al., 2017; Soetedjo & Jin, 2014). One study found that exosomes can transfer the A2A adenosine receptor (A2AR) from A2AR expressing to non-expressing cells with functional recovery of this GPCR, which suggests that exosomal transfer of receptors endows the recipient cell with the ability to respond to the same signals as the donor cell (Clayton, Al-Taei, Webber, Mason, & Tabi, 2011; Isola & Chen, 2016) (Fig. 1).

Apart from spurring cell growth and migration, exosomes were invoked in preparing a premetastatic niche (for reviews, see (Nogues, Benito-Martin, Hergueta-Redondo, & Peinado, 2017; Zhao et al., 2017)). Initial mechanisms activated by exosomes are similar to those facilitating evasion from the primary tumor, particularly ECM breakdown and migration, but in reverse sequence. In the previous paragraph, we discussed MMPs transported by exosomes to degrade the ECM for cancer cell invasion. In addition to opening a route for invasion, cancer cells need to stop migrating and settle into the premetastatic niche. A study on human breast cancer MDA-MB-231 cells settling into a premetastatic niche in the liver showed that exosomal communication can be bi-directional. Upon priming of HepN liver cells with cancer cell-derived exosomes, liver cells secrete exosomes that turn down migratory gene expression and induce mesenchymal-to-epithelial reverse transition (MET) in cancer cells (Dioufa, Clark, Ma, Beckwitt, & Wells, 2017). As the result of this bi-directional communication, HepN-derived exosomes enhance cancer cell seeding, but also suppress cell outgrowth, which renders cancer cells temporarily dormant once settled into their niche. The authors show that the behavior of the two cell types was due to exosome-induced changes in the miRNA composition, although it was not clear how many exosomes from both cell types were required to induce this behavior. Interestingly, the study also reported up-regulation of E-cadherin and ZO-1 in the dormant cancer cells, two junctional cell adherence proteins associated with lipid rafts and promoting MET (Bruewer et al., 2003; Nusrat et al., 2000) CD44 is another exosome-associated transmembrane receptor

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glycoprotein the interaction of which with the ECM is regulated by lipid rafts (Murai, 2015). CD44 is upregulated in cancer stem cells, regulates adhesion and metastasis, and it is a well-known metastatic marker in exosomes (Senbanjo & Chellaiah, 2017). It was found to be delivered from ovarian cancer cells to mesothelial cells where it upregulates degradation of the ECM by inducing gene expression of MMPs to promote cancer cell invasion (Nakamura et al., 2017). A specific involvement of changes in the miRNA profile, either induced by CD44 or due to RNA transported by exosomes was not noted. Since CD44 and tetraspanins are RAPs that are abundant in exosomes it remains to be elucidated if they are also functional in mobile rafts (Rappa, Mercapide, Anzanello, Pope, & Lorico, 2013; Ronquist, Ronquist, Larsson, & Carlsson, 2010).

Crosstalk between cancer and immune cells is an area of exosome biology with great translational potential. As with vaccinations, immunizations, and other fields of immune biology involving the generation of antibodies, exosomes can be engineered to utilize the physiological potential of (re)programming the immune system to fight cancer cells. Therefore, we will first discuss mechanisms by which cancer cells use exosomes to escape immune surveillance and then touch on the potential to hijack these mechanisms and restore vigilance of immune cells toward cancer. Not surprisingly, the majority of these studies has focused on miRNA-mediated and immunoinhibitory protein-mediated reprogramming of immune cells, which is comprehensively reviewed in the following articles (Czernek & Duchler, 2017; Eichmuller, Osen, Mandelboim, & Seliger, 2017; Whiteside, 2017a, 2017b). However, it is known that antigen recognition and activation of immune cells critically relies on membrane lipids, which provides the unique opportunity to utilize lipids and mobile rafts in exosomes to manipulate the immune system. In the immune response to antigens, dendritic cells, B-cells, or macrophages act as antigen-presenting cells (APC) that process antigens and present them to lymphocytes such as T-cells and NKTs (Balato, Unutmaz, & Gaspari, 2009; Chaplin, 2010). This presentation activates T-cells and leads to the production of antibodies against the antigen or phagocytosis. The activation relies on a complex between antigen peptide fragments bound to major histocompatibility complex (MHC) class I and II proteins on the APC surface and T-cell receptors (TCRs) on lymphocytes. This complex is also known as “immunological synapse” or supramolecular activation cluster (SMAC) and it is critically dependent on glycolipid-associated and lipid raft-associated receptors in the APC and T-cell membrane (Dustin, 2014; Dustin & Baldari, 2017; Huang & Sauer, 2010). Macrophages (Daudi cells) were found to sort MHC proteins into exosomes via lipid rafts, although it was not investigated what the function of these exosomes is once taken up by other cells (de Gassart, Geminard, Fevrier, Raposo, & Vidal, 2003). Lipid rafts were also shown to facilitate uptake of oncogenic viruses such as Epstein Barr Virus (EBV) and its association with exosomes to escape proteolytic degradation prior to exosomal spreading of the virus by B-lymphocytes (Verweij et al., 2011). It is unknown, however, if specific lipids in mobile rafts contribute to the immunosuppressive function of cancer cell-derived exosomes. In nanomedicine, a promising field is the use of raft associated-lipids in engineered exosomes or nanovesicles to activate T-cells (Fais et al., 2016). Dendritic cells are among the cells with the highest output of exosomes and can be genetically manipulated to produce exosomes with specific RNA content. Recently, tumor-derived exosomes were laced with alpha-galactosylceramide, a glycolipid activating the

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immune synapse, to enhance T-cell activation by dendritic cells, a promising strategy to use engineered exosomes for vaccination against cancer cells (Liu et al., 2017). Alpha-galactosylceramide binds to Cd1b and Cd1d, a lipid raft-associated MHC-like receptor on APCs to trigger TCR-mediated activation of T-cells (Lang, Maltsev, Besra, & Lang, 2004). It is not known if mobile rafts in exosomes can be used to enhance this process, or exosomes secreted by tumor cells interrupt T-cell activation by interfering with activation of the immune synapse by lipid rafts.

Another area in exosome biology with great translational potential is understanding and utilizing exosome-regulated multi-drug resistance (MDR) of cancer cells. There are several mechanisms by which exosomes or more generalized, EVs modulate MDR: 1) spreading of MDR from resistant to non-resistant cancer cells, 2) loss of factors increasing MDR; 3) loss of factors suppressing MDR, and 4) acquisition of factors suppressing MDR. One may speculate that mechanism 1) and 2), as well as 3) and 4) are complementary in that the secretion of a particular factor may be a loss for the donor and gain for the recipient cell. As a shared consequence of these distinct mechanisms, circulating EVs contain miRNAs or proteins critical for MDR and can be analyzed for cancer diagnosis and targeted chemotherapy in personalized nanomedicine (for reviews, see (Batrakova & Kim, 2016; Bell, Kirk, Hiltbrunner, Gabrielsson, & Bultema, 2016; Fais et al., 2016; Mirzaei, Sahebkar, Jaafari, Goodarzi, & Mirzaei, 2017; A. Sharma, 2017; A. Sharma et al., 2016; J. Wang, Zheng, & Zhao, 2016)). EV-mediated spreading of MDR is mostly attributed to transport of RNAs or proteins mediating export or inactivation of chemotherapeutic drugs, particularly ABC transport proteins and MDR-1/P-glycoprotein (Lopes-Rodrigues et al., 2016; Lopes-Rodrigues et al., 2013; Torreggiani, Roncuzzi, Perut, Zini, & Baldini, 2016). Our laboratory has found that induction of exosome secretion by activating ceramide generation through drug-mediated interference with sterol metabolism increases sensitivity of breast cancer stem-like cells to doxorubicin, a widely used drug in breast cancer therapy (Kong, He, et al., 2015; Spassieva & Bieberich, 2011). Increase of drug sensitivity was achieved by treating human breast cancer MDA-MB-231 cells with the farnesoid × receptor (F×R) antagonist guggulsterone and the retinoid × receptor (R×R) agonist bexarotene, and it was directly related to ceramide levels and secretion of breast cancer resistance protein (BCRP)/ABCG2 associated with exosomes. While this or similar mechanism of restoring drug sensitivity via EV-mediated secretion of MDR proteins (MRPs) has only been reported in a few studies, it provides the opportunity to break MDR by stimulating ceramide generation. On the other hand, restoring drug sensitivity and spreading of MRPs to other cancer cells may be prone to a delicate tradeoff and it remains to be elucidated whether this mechanism can be utilized in cancer therapy.

Conclusions

While miRNA was targeted in exosome biology, the limited amount of RNA in exosomes makes it challenging to understand or assess their proposed impact. Instead, other signaling factors such as proteins and lipids abundant in exosomes may be better suited to explain many functions of exosomes in cancer. In particular, we have focused on lipid rafts in exosomes (mobile rafts) and the role of ceramide in cell signaling pathways activated in donor and recipient cells. Ceramide is unique in that its generation is required for exosome

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formation and secretion and that it is a component of lipid rafts regulating cell signaling pathways for reprogramming of cancer cells and their microenvironment. This provides the intriguing opportunity of broadcasting and manipulating cell signaling events via mobile rafts in extracellular signalosomes that are regulated by ceramide. While the idea of mobile rafts and exosomes as vectors is novel and not yet explored, it is likely to generate a new target for cancer diagnostics and therapeutics.

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Abbreviations:

APC	antigen-presenting cell
ATP	adenosine triphosphate
CRP	ceramide-rich platform
ECM	extracellular matrix
EGF	endothelial growth factor
EMT	epithelial-to-mesenchymal transition
ESCRT	endosomal sorting complexes required for transport
EV	extracellular vesicle
GPI	glycosylphosphatidylinositol
GPCR	G-protein coupled receptor
LBPA	lysobisphosphatidic acid
LC-MS/MS	liquid chromatography tandem mass spectrometry
lnc RNA	long non-coding RNA
LPA	lysophosphatidic acid
MDR	multidrug resistance
MET	mesenchymal-to-epithelial reverse transition
MHC	major histocompatibility complex
miRNA	micro RNA
MMP	matrix metalloproteinases
MVE	multivesicular endosome

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nSMase2	neutral sphingomyelinase 2
NKT	natural killer T cell
NTA	nanoparticle tracking analysis
PGE2	prostaglandin E2
PI3K	phosphatidyl inositol 3-kinase
RAP	raft-associated protein
S1P	sphingosine-1-phosphate
TCR	T-cell receptor
TEM	transmission electron microscopy

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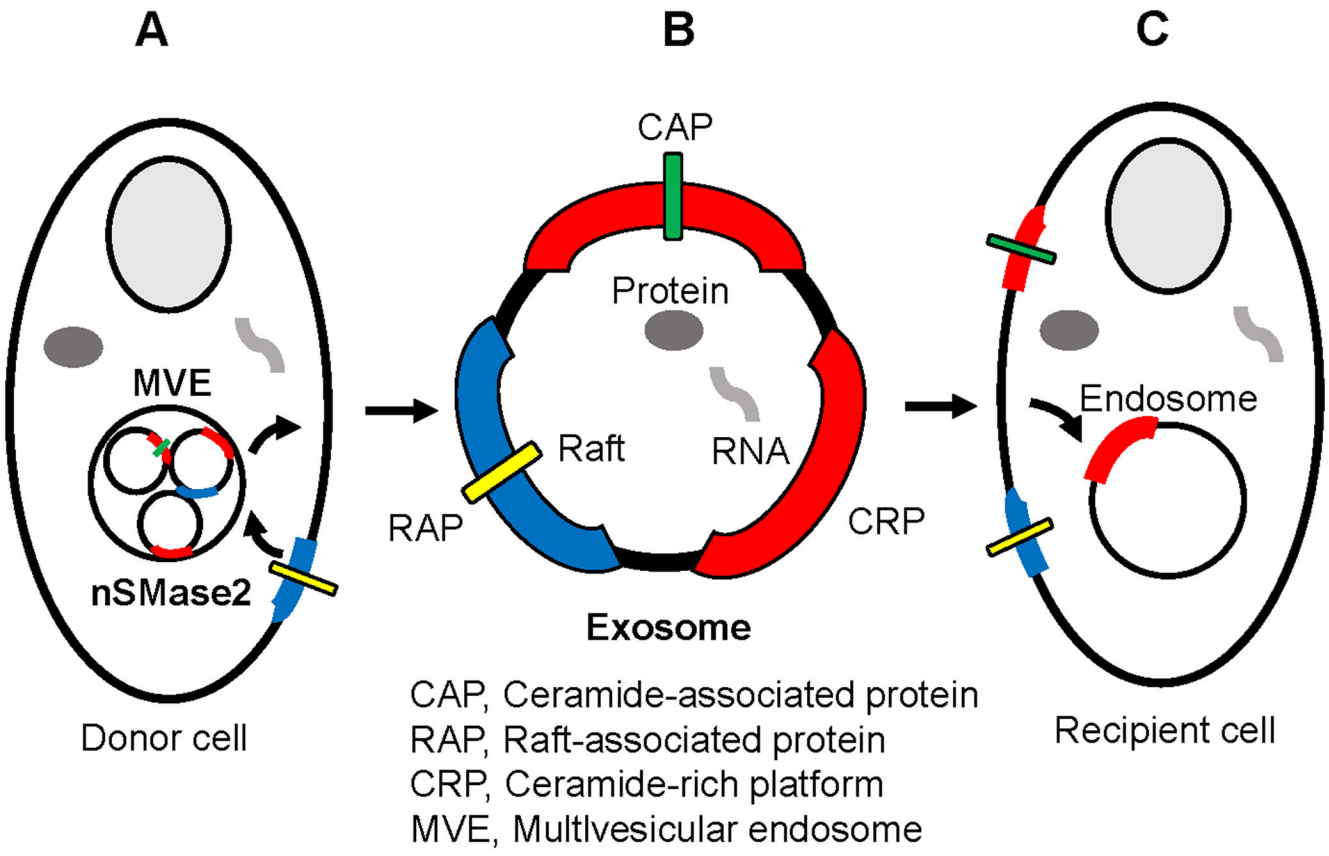

Figure 1.

Ceramide in exosome formation and the concept of mobile rafts. A. nSMase2 generates ceramide that induces formation of ceramide-enriched intraluminal vesicles in MVEs. These vesicles can contain endocytosed lipid rafts and are secreted as exosomes after fusion of MVEs with the plasma membrane of the donor cell. In addition to lipids, RNA and protein is packaged into exosomes. B. Exosomes can contain mobile rafts with raft-associated protein (RAP) and ceramide-rich platforms (CRPs) with ceramide-associated protein (CAP). C. Exosomes are taken up by recipient cells via endocytosis, pinocytosis, or fusion with the plasma membrane. Uptake leads to incorporation of lipid rafts or CRPs into the plasma membrane or the endosome. RNA and proteins inside of exosomes are released to the cytosol. MVE, multivesicular endosome; nSMase2, neutral sphingomyelinase 2.

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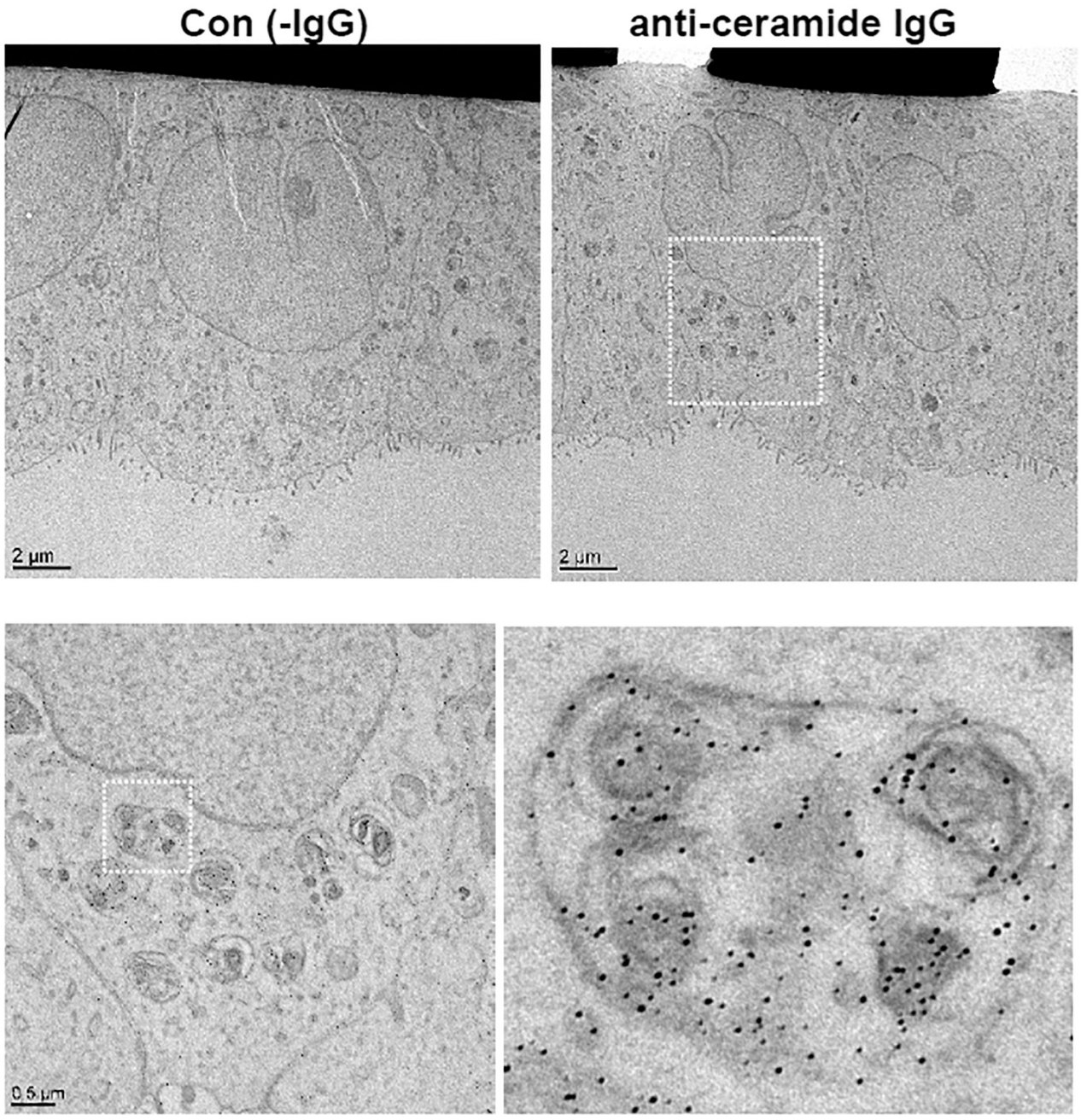

Figure 2.

TEM of MVEs in MDCK cells. Anti-ceramide IgG was used for immunogold labeling. MVEs contain many ceramide-enriched multi- and unilamellar luminal vesicles. Unilammellar vesicles are presumed to be secreted as exosomes. MVE, multivesicular endosome; MDCK, Madin Darby Canine Kidney; TEM, transmission electron microscopy.