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Biogenesis and function of extracellular vesicles in cancer

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Abstract

Extracellular vesicles (EVs) are heterogeneous multi-signal messengers that support cancer growth and dissemination by mediating the tumor-stroma crosstalk. Exosomes are a subtype of EVs that originate from the limiting membrane of late endosomes, and as such contain information linked to both the intrinsic cell 'state' and the extracellular signals cells received from their environment. Resolving the signals affecting exosome biogenesis, cargo sorting and release will increase our understanding of tumorigenesis. In this review we highlight key cell biological processes that couple exosome biogenesis to cargo sorting in cancer cells. Moreover, we discuss how the bidirectional communication between tumor and non-malignant cells affect cancer growth and metastatic behavior.

Keywords: Extracellular vesicles, exosome biogenesis, cargo sorting, tumor microenvironment, tumor-stroma communication

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Abbreviations

EVs: extracellular vesicles

MVB: multivesicular body

ILVs: intraluminal Vesicles

nSMase2: neutral sphingomyelinase 2

ESCRT: endosomal sorting complex required for transport

ARF6: ADP ribosylation factor 6

PLD2: phospholipase D2

SNARE: soluble N-ethylmaleimide-sensitive component attachment protein receptor

RBP: RNA-binding protein

gDNA: genomic DNA

mtDNA: mitochondrial DNA

MDSC: myeloid-derived suppressor cell

PRR: pattern recognition receptor

MSC: mesenchymal stem cell

ECM: extracellular matrix

PMN: pre-metastatic niche

MIF: macrophage inhibitory factor

MM: multiple myeloma

DAMP: danger-associated molecular patterns

ISG: interferon-stimulated gene

1. Introduction

Cells can communicate with each other by releasing extracellular vesicles (EVs), which interact with and modify the behavior of target cells at close or distant sites. Based on their origin, EVs can be divided into subgroups of 'small' vesicles generated within endosomal compartments called "exosomes", and vesicles that bud directly from the plasma membrane. Regardless their origin, all EVs enclose or expose on their surface a multitude of biomolecules including RNA, lipids, proteins and possibly DNA. However, the mechanisms underlying the biogenesis of different EV subtypes and the sorting of these molecules are extremely difficult to define as EVs are generally analyzed in bulk and contaminated with protein, lipid and viral-like particles.

As critical mediators of cell-to-cell communication, EVs support cancer progression by mediating the crosstalk between tumor and stromal cells in their direct environment. However, EVs from cancer cells as well as from healthy tissues also seem to have systemic properties (Peinado, et al., 2012; Pucci, et al., 2016; Thomou, et al., 2017; Zomer, et al., 2015). Since exosomes derive from major sorting hubs at the limiting membrane of endosomes, they are at the center of a signaling network that connects external stimuli with tumor cell intrinsic features. Consistent with this notion, the difference in composition between exosomes and other EV subtypes and between EVs and parental cells indicates the existence of regulated sorting mechanisms determining the phenotypic and functional properties of these vesicles.

In this review we describe the key findings on the mechanisms underlying exosome biogenesis and cargo sorting. We discuss how these processes are deregulated in

cancer and consequently change the functional properties of exosomes. However, because most of the current purification protocols and functional studies do not accurately discriminate between exosomes and plasma membrane-derived vesicles their respective role(s) in cancer remain elusive. We highlight the in vivo studies that establish EVs as crucial mediators in the tumor-to-stroma, stroma-to-tumor and tumor-to-tumor communication and discuss to what extent their effect can be attributed to the functional transfer of defined cargo molecules.

2. Extracellular vesicle biogenesis

2.1 Exosome biogenesis

Intraluminal vesicle budding and MVB formation

Exosomes are nanosized (30-100 nm) vesicles formed by inward budding of the limiting membrane of multivesicular bodies (MVBs), leading to the generation of intraluminal vesicles (ILVs). Upon early to late endosome maturation, MVBs can fuse with the plasma membrane releasing the enclosed ILVs (then referred to as exosomes) in the extracellular space (Figure 1). Because cancer exosome subsets critically contribute to cancer development and progression, understanding the pathways implicated in their biogenesis might provide new options for therapeutic intervention.

Multiple mechanisms for MVB biogenesis have been described. A critical role for lipid raft microdomains in MVB formation was established in 2008 by Trajkovic and colleagues. The authors demonstrated that ceramide, generated from sphingomyelin

hydrolysis by neutral sphingomyelinase 2 (nSMase2), induces negative membrane curvature via its cone-shaped structure leading to ILV budding into MVBs (Trajkovic, et al., 2008). Subsequent studies into the genesis of endosome-derived vesicles revealed that, apart from the lipid composition and physical characteristics of endosome membrane microdomains, exosomes biogenesis is closely connected to the sorting of cargo molecules.

The Endosomal Sorting Complex Required for Transport (ESCRT) machinery is the most extensively described pathway of MVB biogenesis, responsible for the sorting of ubiquitinated proteins into ILVs (Henne, Buchkovich, & Emr, 2011). This process is initiated by ESCRT-0, which recognizes and retains ubiquitinated proteins in the late endosomal membrane. After initial involution of the limiting membrane into the MVB lumen triggered by ESCRT-I/II, ESCRT-III forms a spiral-shaped structure that constricts the budding neck and the ATPase VPS4 drives membrane scission. Despite extensive research into the role of ESCRT in MVB biogenesis, whether MVBs generated by ubiquitin-dependent mechanisms are mainly destined to lysosome for cargo degradation or can fuse with the plasma membrane to release exosomes is still unclear (Christ, Raiborg, Wenzel, Campsteijn, & Stenmark, 2017; Raiborg & Stenmark, 2009; Schoneberg, Lee, Iwasa, & Hurley, 2017).

Sorting of proteins into ILVs can also occur independently of ubiquitination. In 2012 Baietti et al. demonstrated a key role for the heparan sulfate proteoglycan syndecan in ESCRT III-mediated exosome formation (Baietti, et al., 2012). The authors showed that interaction between syndecan and ESCRT is mediated by the small cytosolic adapter protein syntenin, which connects syndecan to the ESCRT-III-associated

protein ALIX. Further research into the syndecan-syntenin-ALIX exosome biogenesis pathway uncovered additional layers of regulation. Trimming of the heparan sulphate chains by heparanase triggers syndecan clustering which stimulates syntenin-ALIX-ESCRT-mediated sorting and exosome production (Roucourt, Meeussen, Bao, Zimmermann, & David, 2015; Thompson, Purushothaman, Ramani, Vlodavsky, & Sanderson, 2013). Interestingly, heparanase stimulates selective cargo sorting, determining CD63, but not CD81, flotillin and CD9 incorporation into exosomes. Moreover, syntenin-mediated CD63 ILV budding is specifically controlled by the small GTPase ADP ribosylation factor 6 (ARF6) and its effector protein phospholipase D2 (PLD2) (Ghossoub, et al., 2014).

Altogether, these observations raise one of the major questions in the exosome field, which is whether different sorting mechanisms determine the incorporation of specific molecules into exosomes or distinct vesicle subpopulations carrying different cargo exists. Thus, interfering with specific sorting mechanisms might influence the composition or the subtypes of EVs released by cancer cells, and impact on the tumor-stroma communication in the tumor microenvironment.

MVB fate and fusion with the plasma membrane

Upon maturation, MVBs can either fuse with the plasma membrane to secrete exosomes or degrade their cargo by fusing with lysosomes. A high rate of exosome secretion from transformed cells suggests that the balance between these two processes in cancer is shifted towards exosomal cargo release (Riches, Campbell,

Borger, & Powis, 2014; Wei, et al., 2017). This type of shift may also occur in non-transformed cells such as antigen-presenting cells, which are known to release large amounts of exosomes upon stimulation (Muntasell, Berger, & Roche, 2007). However, what distinguishes 'secretory MVBs' from 'degradative MVBs' is poorly understood. A first clue came from a recent study by Villarroya-Beltri and colleagues who elegantly demonstrated that ISGylation of the ESCRT-I component Tsg101 decreases exosome release by promoting the fusion of MVBs with lysosomes, suggesting that posttranslational modifications of cargo proteins might influence MVB fate (Villarroya-Beltri, et al., 2016). Additionally, the fate of MVBs can change in response to altered cellular conditions such as starvation, which induces MVB degradation by fusion with autophagosomes resulting in decreased exosome release (Fader, Sanchez, Furlan, & Colombo, 2008) (Figure 1).

MVBs that are destined for exocytosis are transported to the plasma membrane along microtubules by the molecular motor kinesin. Interestingly, anterograde trafficking of late endosomes is mediated by multiple kinesin isoforms, and can be regulated by Arl8- (Guardia, Farias, Jia, Pu, & Bonifacino, 2016; Pu, et al., 2015) as well as RAB7-dependent protein complexes (Raiborg, et al., 2015). The multiple late endosomal transport mechanisms might be subject to differential regulation or control the trafficking of distinct MVB subtypes. Alternatively, the mechanisms responsible for secretory MVB transport might be cell type-specific, as demonstrated for the RABs that regulate MVB docking to the plasma membrane. RAB35 mediates MVB docking in oligodendroglial cells (Hsu, et al., 2010), whereas RAB27 controls this process in several cancer cell lines both *in vitro* (D. Hoshino, et al., 2013; Ostrowski,

et al., 2010) and *in vivo* (Peinado, et al., 2012), possibly by stabilizing the branched actin filaments that form the MVB docking site (Sinha, et al., 2016).

After transport and docking to the plasma membrane, secretory MVBs couple to the SNARE (soluble N-ethylmaleimide-sensitive component attachment protein receptor) membrane fusion machinery. Humans have multiple SNARE proteins that localize to different intracellular membranes and mediate the fusion of cellular compartments by forming distinct SNARE complexes (Hong & Lev, 2014; Sudhof & Rothman, 2009). A crucial step in exosome release is the recruitment of exocytic membrane SNAREs to the docked MVB, and a study by Hyenne et al. suggests a role for small GTPases such as RAL-1 in this process (Hyenne, et al., 2015).

Over the last decade, several SNARE proteins, such as YKT6, Syntaxin-1a and Syntaxin-5 have been implicated in exosome release from different cell types and organisms (Gross, Chaudhary, Bartscherer, & Boutros, 2012; Hyenne, et al., 2015; Koles, et al., 2012; Ruiz-Martinez, et al., 2016). Moreover, we and others demonstrated a role for the SNARE proteins SNAP23, Syntaxin-4 and VAMP7 in the release of exosomes from tumor cells (Fader, Sanchez, Mestre, & Colombo, 2009; Verweij, et al., 2018; Wei, et al., 2017). These proteins form an exocytic SNARE-complex that has previously been reported to promote invadopodia formation and tumor cell invasion (Williams, McNeilly, & Coppolino, 2014), processes that indeed have been linked to cancer exosome release (D. Hoshino, et al., 2013). SNARE complex formation and membrane fusion is tightly controlled by multiple regulatory mechanisms (Snyder, Kelly, & Woodbury, 2006; Sudhof & Rothman, 2009). In the

context of tumor exosome release, Hoshino and colleagues demonstrated a role for the calcium-sensing SNARE interacting protein synaptotagmin-7 (D. Hoshino, et al., 2013). Furthermore, SNARE activity is partly controlled by their phosphorylation profile, which influences their localization or the interaction with SNARE partners (Snyder, et al., 2006). Indeed, recent independent studies revealed that phosphorylation of SNAP23 promotes exosome secretion (Verweij, et al., 2018; Wei, et al., 2017). Interestingly, SNAP23 phosphorylation at Ser95 by PKM2 mediates constitutive release in tumor cells, whereas, as we recently demonstrated, phosphorylation of Ser110 triggers exosome release in response to histamine stimulation (Verweij, et al., 2018; Wei, et al., 2017). These findings are consistent with the idea that multiple regulatory mechanisms control SNARE activity through phosphorylation of different residues and support a role for the microenvironment in the regulation of exosome release.

Finally, when MVBs fuse with the plasma membrane, the exosomes are released into the extracellular space where they interact with the extracellular matrix, influence cells in the microenvironment, but can also enter the circulation via lymph or blood. Importantly, several studies report that in certain cell-types a proportion of exosomes remains attached to the cell surface, where they might function as signaling platforms for juxtacrine communication (Edgar, Manna, Nishimura, Banting, & Robinson, 2016; Mobius, et al., 2002; Verweij, et al., 2018).

Altogether, the studies into exosome release mechanisms indicate that it is a tightly controlled process that is regulated at multiple levels between ILV budding and MVB

fusion. This realization implies that exosomes are unlikely merely an alternative method for 'waste' disposal, but suggests that exosomes play a physiological role. Accordingly, the regulation of exosome release in vivo is likely a dynamic process where cells adapt the magnitude of release for each exosome subpopulation in response to defined external and internal stimuli. It is important to note that current methods to study exosome biogenesis mechanisms are based on the isolation of EVs from culture supernatant, which requires long-term cell culture (24-72 hours), thus making it impossible to capture the dynamics of exosome release. To overcome this limitation we recently developed tetraspanin-based reporters that can be used to study exosome release in real time by visualizing the fusion of MVBs with the plasma membrane (Verweij, et al., 2018). Using this method we revealed a role for G protein-coupled receptor signaling in the regulation of exosome secretion. Future studies using these reporters could provide further knowledge on how dynamic changes in the tumor microenvironment might contribute to cancer exosome release.

2.2 Microvesicle biogenesis

In addition to the MVB-derived exosomes, extracellular vesicles can directly bud and pinch of from the plasma membrane. These vesicles are often generically referred to as microvesicles although they are extremely heterogeneous in size, ranging from exosome-like EVs of 50nm to EVs as large as 10µm. Consistent with their heterogeneity, microvesicles can be generated via multiple distinct mechanisms that partially overlap with those involved in exosome biogenesis. For instance, the ESCRT machinery is involved in the production of nano-sized vesicles that are enriched in

cell surface proteins, reflecting their plasma membrane origin (Nabhan, Hu, Oh, Cohen, & Lu, 2012; Q. Wang & Lu, 2017). Furthermore, similar to the role of nSMase2 in ILV formation, acid sphingomyelinase induces ceramide-dependent microvesicle production, indicating a general role for sphingomyelinases in the biogenesis of MVB- and plasma membrane-derived EVs (Bianco, et al., 2009).

Another mechanism of microvesicle biogenesis is linked to non-apoptotic plasma membrane blebs that are frequently observed in highly aggressive cancer cells. These blebs continuously expand and retract at the cell surface and contribute to cell motility via a currently unknown mechanism (Fackler & Grosse, 2008). In addition, these blebs can be released as microvesicles by actin cytoskeleton rearrangements at the vesicle budding neck that result in membrane scission (B. Li, Antonyak, Zhang, & Cerione, 2012; Muralidharan-Chari, et al., 2009; Schlienger, Campbell, & Claing, 2014; Sedgwick, Clancy, Olivia Balmert, & D'Souza-Schorey, 2015). During migration *in vivo*, tumor cells can adopt a so-called amoeboid phenotype that is associated with extensive plasma membrane blebbing and microvesicle release, suggesting a role for this type of EV biogenesis during cancer invasion and metastasis (Clancy, et al., 2015; J. Kim, et al., 2014; Paul, Mistriotis, & Konstantopoulos, 2017; Sedgwick, et al., 2015).

3. Extracellular vesicle cargo

The functional properties of EVs in the tumor microenvironment are dictated by their cargo and by their release and uptake dynamics. Identifying the components responsible for the pro-tumorigenic effects of cancer EVs and the pathways leading

to their incorporation into vesicles is currently one of the major challenges in the field.

3.1 Protein cargo

Recent studies suggest that proteins are incorporated into EVs by interacting with components of the EV biogenesis machinery (Baietti, et al., 2012; Guix, et al., 2017; S. B. Kim, et al., 2017). Similarly, the association of membrane proteins with tetraspanins, either by direct interaction or by entrapment in tetraspanin-enriched microdomains facilitates their sorting into EVs (Mazurov, Barbashova, & Filatov, 2013; Nazarenko, et al., 2010; Perez-Hernandez, et al., 2013; van Niel, et al., 2011; Verweij, et al., 2011). As tumor exosomes carry mediators of tumorigenesis such as oncoproteins, growth factors and immunomodulatory molecules (Chalmin, et al., 2010; Clayton, Mitchell, Court, Mason, & Tabi, 2007; Peinado, et al., 2012), defining the protein-protein interaction network responsible for the loading of specific proteins into vesicles might provide options to impair the EV oncogenic function.

3.2 RNA cargo

In 2006, Ratajczak et al were first to propose horizontal transfer of RNA between donor and recipient cells (Ratajczak, et al., 2006). Subsequently, Valadi and Skog independently demonstrated that mRNA molecules transported by EVs can be actually translated into protein, providing evidence of virus-independent horizontal transfer of genetic material between cells (Skog, et al., 2008; Valadi, et al., 2007). This finding opened the way to intense efforts to address the functionality of EV-associated RNA molecules in vivo. Elegant studies using reporter systems under the control of the CRE recombinase demonstrated that vesicles-enclosed CRE mRNA can

be transferred *in vivo* over long distances (Ridder, et al., 2014; Ridder, et al., 2015; Zomer, et al., 2015). Using this methodology in combination with intravital imaging, Zomer et al. showed that EV released by aggressive tumor cells can induce the acquisition of malignant traits into less aggressive ones, suggesting a role for EV-RNA in this process (Zomer, et al., 2015).

Apart from mRNA, exosomes are highly enriched in small non-coding RNA species, suggesting that they function in large part through gene regulation (Baglio, et al., 2015; Koppers-Lalic, et al., 2014; Nolte-'t Hoen, et al., 2012; van Balkom, Eisele, Pegtel, Bervoets, & Verhaar, 2015). The first proof for the functional transfer of small RNAs via exosomes was provided in 2010, when using viral miRNAs endogenously produced in Epstein Barr virus-infected B cells Pegtel et al demonstrated that small RNAs are secreted via exosomes and can regulate gene expression in recipient dendritic cells (Pegtel, et al., 2010). While certain EV-associated miRNAs have a demonstrated role in cancer (Le, et al., 2014; Zhou, et al., 2014), conclusive evidence that their transfer via EVs is responsible for promoting cancer progression *in vivo* is still lacking. Indeed, it is difficult to discriminate EV-mediated miRNA transfer from endogenous miRNA expression in recipient cells. A compelling demonstration of EV-mediated miRNA transfer in vivo was recently provided by Thomou and colleagues. Using an adipose tissue-specific Dicer knockout mouse model, the authors demonstrated that brown fat is a major source of EV-associated circulating miRNAs. Transplantation of normal adipose tissue into the engineered mice not only restored the circulating levels of EV miRNAs, but could regulate gene expression in distant

organs, an effect that could be reproduced administering normal serum EVs (Thomou, et al., 2017).

A number of defined RNA binding proteins (RBP) have been suggested to mediate RNA sorting into exosomes (Lu, et al., 2017; Mukherjee, et al., 2016; Santangelo, et al., 2016; Shurtleff, Temoche-Diaz, Karfilis, Ri, & Schekman, 2016; Teng, et al., 2017; Villarroya-Beltri, et al., 2013), yet how these proteins connect to the endosomal system is far from resolved, and the stoichiometry between a given RBP and RNA molecules has not been established. In fact, two independent studies showed that certain pol III transcripts are sorted into exosomes after uncoupling from their binding partners (Baglio, et al., 2016; Nabet, et al., 2017). Thus there are presumably multiple sorting mechanisms functioning in parallel.

The localization of AGO2 and other components of the RNA-induced silencing complex in close proximity of MVBs suggests their involvement in exosomal sorting of miRNAs (Gibbings, Ciaudo, Erhardt, & Voinnet, 2009). However, because AGO2 has not been consistently found in EVs, its role in miRNA sorting remains controversial (McKenzie, et al., 2016; Melo, et al., 2014; Shurtleff, et al., 2016; Van Deun, et al., 2014), in particular since AGO is a stable protein found outside of vesicles in human plasma (Arroyo, et al., 2011). A debated study from Melo et al. proposes that breast cancer derived exosomes possess the protein machinery to process pre-miRNAs into mature miRNAs. Interestingly, the authors report that mature miRNAs generated within these cancer exosomes influence the transcriptome of non-malignant target cells promoting transformation (Melo, et al., 2014). Though highly provocative, this study awaits independent confirmation.

Although suggested by independent studies (Koppers-Lalic, et al., 2014; Mittelbrunn, et al., 2011; Squadrito, et al., 2014), whether miRNAs are selectively incorporated into vesicles and which regulatory mechanisms control RNA sorting into vesicles or retainment into the cell of origin are still open questions. In 2014 two interesting studies provided new insights into this subject. Squadrito et al. proposed that the relative abundance of target mRNAs in the cytoplasm can influence miRNA sorting into exosomes. In the presence of high expression levels of the target transcript, the mRNA-miRNA interaction can prevent miRNA incorporation into EVs (Squadrito, et al., 2014). On a different note, Koppers-Lalic et al. demonstrated that post-transcriptional modifications of miRNAs known as non-templated nucleotide additions, are associated with enrichment of miRNAs in EVs (3'-urydilation) or retainment within the cell of origin (3'-adenylation), although the underlying mechanisms are still unclear (Koppers-Lalic, et al., 2014). Interestingly, high levels of EV-associated 3'-uridilated miRNA isoforms seem to be present in plasma vesicles obtained from cancer patients compared to their normal counterparts (Pegtel, unpublished observation).

3.3 DNA cargo

Several studies have reported the presence of fragmented genomic DNA (gDNA) (Balaj, et al., 2011; Kahlert, et al., 2014; Thakur, et al., 2014), mitochondrial DNA (mtDNA) (Guescini, Genedani, Stocchi, & Agnati, 2010; Sansone, et al., 2017) and even parasitic DNA (Sisquella, et al., 2017) in EVs derived from cell culture medium and plasma. Although the mechanisms by which DNA can be incorporated in EVs

remain to be elucidated, the fact that the EV gDNA fragments are equally distributed over the whole genome suggests a random process (Kahlert, et al., 2014; Thakur, et al., 2014). However, because all studies rely on ultracentrifugation as exosome purification method, evidence that DNA is actively sorted into exosomes remains elusive, as co-isolation of contaminating plasma membrane-derived vesicles, protein complexes and apoptotic bodies is difficult to completely rule out. One possibility is that the EV-associated DNA is present on the surface of the EVs wrapped in nucleosomes, which are approximately 10nm in size. Indeed, while most protocols include DNAse treatment of purified EV preparations to degrade unprotected DNA, surface-bound DNA molecules could be partially protected by binding to proteins.

Also the physiological significance of DNA transfer via EVs is unclear. Recent studies demonstrated that upon DNA damage, fragments of genomic DNA can be exported to the cytoplasm, where they can trigger cytoplasmic DNA sensors leading to cellular senescence or apopotosis (Lan, Londono, Bouley, Rooney, & Hacohen, 2014). Interestingly, secretion of DNA fragments via EVs prevents activation of cytoplasmic DNA sensors, thereby contributing to cellular homeostasis (Takahashi, et al., 2017). This aspect might be particularly relevant in cancer, where elevated levels of DNA damage might require efficient removal of cytoplasmic DNA via EVs. Consistent with this hypothesis, cancer cells seem to display enhanced EV production.

4. Deregulation of EV release in cancer

A large body of evidence suggests that cancer cells release higher amounts of EVs compared to non-malignant cells, which makes the EV biogenesis machinery or components thereof attractive targets for anticancer therapy. Indeed, overexpression of ESCRT components as well as syntenin and heparanase has been observed in various tumors (Koliopanos, et al., 2001; Koo, et al., 2002; R. T. Liu, et al., 2002; Oh, Stanton, West, Todd, & Wagner, 2007; Toyoshima, et al., 2007; Vlodavsky, et al., 1999). In addition, hyperactivation of RalB in pancreatic carcinoma and colorectal cancer, overexpression of YKT6 in non small-cell lung cancer and elevated Rho-ROCK signaling observed in various tumor types might enhance EV production in tumor cells (Lim, et al., 2006; Martin & Der, 2012; Morgan-Fisher, Wewer, & Yoneda, 2013; Ruiz-Martinez, et al., 2016).

Enhanced EV release in cancer cells can be determined by both cell-intrinsic and environmental signals. Activation of oncogenic signaling pathways, for instance by EGFRvIII and H-RAS^{V12} (Al-Nedawi, et al., 2008; Lee, et al., 2014; Takasugi, et al., 2017), can increase EV production in cancer cells. Moreover, it was recently shown that the proto-oncogene SRC specifically stimulates the syntenin exosome biogenesis pathway by phosphorylating the cytosolic domain of syndecan and syntenin (Imjeti, et al., 2017). Enhanced cancer EV release can also depend on the (de)regulation of the membrane fusion machinery. For instance, overexpression of PKM2, a glycolytic enzyme implicated in the Warburg effect, enhances EV secretion by phosphorylating the tSNARE SNAP23 (Wei, et al., 2017).

Apart from cell-autonomous mechanisms, microenvironmental conditions such as hypoxia can contribute to the regulation of both microvesicle and exosome release from cancer cells (King, Michael, & Gleadle, 2012; L. Li, et al., 2016; T. Wang, et al., 2014). Interestingly, while hypoxia-induced microvesicle production requires HIF1a-dependent expression of RAB22 (T. Wang, et al., 2014), enhanced exosome release mainly involves the phosphorylation of the Akt-substrate PRAS40 (Guo, et al., 2017), which provides the opportunity to specifically evaluate the contribution of different hypoxia-induced EV subtypes to cancer progression.

Besides displaying elevated EV release, cancer cells release vesicles that differ in protein and RNA content from normal cell EVs (Griffiths, Cormier, Clayton, & Doucette, 2017; Lobb, et al., 2017; Melo, et al., 2014; Tuzesi, et al., 2017). Also in this case the deregulation can be the result of activated oncogenic signaling (Cha, et al., 2015; Demory Beckler, et al., 2013) or altered microenvironmental conditions, such as hypoxia (Ramteke, et al., 2015; Tadokoro, Umezu, Ohyashiki, Hirano, & Ohyashiki, 2013). Interestingly, several independent groups have reported the enrichment of tumor suppressor miRNAs in EVs from various tumor cell types (Lawson, et al., 2017; Ostenfeld, et al., 2014; Rashed, et al., 2017). Consistently, blocking tumor EV release results in cellular accumulation of suppressor miRNAs and a decrease in tumorigenic properties (Ostenfeld, et al., 2014; Rashed, et al., 2017), suggesting that EV release might also function as a disposal mechanism for unwanted tumor suppressor miRNAs.

Deregulation of cancer EV release and cargo critically affect the crosstalk between tumor and stromal in the tumor microenvironment and even at distant (pre-) metastatic sites. In the following section we will discuss the consequences of such alterations on cancer development and progression.

5. Function of EVs in cancer

5.1 Tumor-to-stroma communication

Tumor-stroma communication within the tumor microenvironment

Whether the sorting of 'modified' cargo into cancer EVs reflects the rapid turnover of abundant cancer cell components or a regulated process for intercellular communication is still an open question. However, cancer EVs profoundly alter the behavior of local or recruited stromal cells, which results in the generation of a tumor-promoting niche supporting tumor angiogenesis, immunosuppression and the acquisition of malignant traits by cancer cells (Figure 2).

Endothelial cells are critical stromal components susceptible to manipulation by cancer EVs. Al-Nedawi et al. demonstrated that the horizontal transfer of the oncogenic constitutively active EGFRvIII via EVs not only can transmit oncogenic activity among cancer cell subsets (Al-Nedawi, et al., 2008), but activates autocrine VEGF signaling in endothelial cells stimulating tumor angiogenesis (Al-Nedawi, Meehan, Kerbel, Allison, & Rak, 2009). Apart from transferring oncoproteins, cancer EVs can trigger specific cellular responses by mimicking the status of the parental cancer cells. For instance, cancer EVs produced under hypoxic conditions are highly

enriched in hypoxia-regulated RNA and proteins that induce endothelial cell function and vascular permeability (Al-Nedawi, et al., 2009; Y. L. Hsu, et al., 2017; Kucharzewska, et al., 2013; Umezu, et al., 2014).

Although cancer EVs are carriers of tumor-antigens that could in principle mediate antitumor immunity (Robbins & Morelli, 2014; Wolfers, et al., 2001), extensive evidence suggests that they mainly act by suppressing the immune response. Independent studies demonstrated that tumor-derived EVs can inhibit proliferation and activation of CD8+ cells while promoting the expansion of regulatory T cells (Clayton, et al., 2007; Wieckowski, et al., 2009) through mechanisms that have not yet been defined. In addition to their effect on the adaptive immune system, cancer EVs can 'educate' innate immune components towards a pro-tumorigenic phenotype (Chalmin, et al., 2010; Haderk, et al., 2017; Shinohara, et al., 2017). For instance, Hsp72 on the surface of cancer EVs activates myeloid-derived suppressor cell (MDSC) by inducing the IL-6/Stat3 signaling pathway through a TLR2-dependent mechanism (Chalmin, et al., 2010). Moreover, EV-associated miRNAs have been reported to polarize tumor-associated macrophages towards a pro-tumorigenic M2 phenotype (Y.-L. Hsu, et al., 2017; Shinohara, et al., 2017). Interestingly, a recent study by Haderk and coworkers showed that CLL EV-enclosed Y RNA induces the expression of the immune checkpoint protein PD-L1 as well as the production of pro-tumorigenic cytokines in monocytes by triggering TLR7/8 (Haderk, et al., 2017). However, since Y RNAs are found abundantly in many types of cancer exosomes but also in exosomes from healthy cells, urine and plasma, additional factors must play a role. Nevertheless, this study adds to the existing evidence that exogenous EV-protected

non-coding RNAs can act through mechanisms independent from gene regulation, by activating pattern recognition receptors (PRR) triggering an innate immune response (Baglio, et al., 2016; Fabbri, et al., 2012; Nabet, et al., 2017).

The pro-tumorigenic effects of cancer EVs can also be mediated by cells of mesenchymal origin. Webber and colleagues demonstrated that EVs released by prostate cancer cells trigger the differentiation of myofibroblastic cells inducing angiogenesis and accelerating tumor growth in vivo. These effects were mediated by a membrane-associated form of TGFβ on the surface of the vesicles and could not be reproduced by exposing stromal cells to the soluble TGFβ counterpart (J. Webber, Steadman, Mason, Tabi, & Clayton, 2010; J. P. Webber, et al., 2015). Consistent with this finding, we recently demonstrated that EVs released by malignant bone tumor cells carry high levels of membrane-associated TGFβ, which induces IL-6 release in mesenchymal stem cells (MSCs) (Baglio, et al., 2017). Importantly, injection of EV-educated MSCs in mice bearing bone tumors was associated with STAT3 activation and increased metastatic dissemination, suggesting that the inflammatory loop initiated by cancer EVs within the tumor microenvironment enhances the metastatic behavior of tumor cells. In accordance with the observations by Webber and colleagues, soluble TGFβ failed to induce a prometastatic phenotype in MSC. We conclude that EV-bound growth factors might have different or highly enhanced biological activities compared to their soluble counterparts, as suggested by a recent independent study (Feng, et al., 2017). Whether this is dependent on a different conformation acquired by the growth factor when bound to the EV surface, or on the presence of co-stimulatory signals needs further investigation.

Effects of tumor EVs on extracellular matrix

Apart from their effects on local or recruited tumor-associated stromal cells, EVs contribute to cancer progression by acting on the extracellular matrix (ECM). Sung et al. reported that tumor exosomes enhance directional cell movement through tissue by secreting an exosome-bound form of fibronectin. The release of fibronectin-rich exosomes at the leading edge of migrating cells promotes the formation of focal adhesions, which stabilize leading edge protrusions and increase migration speed in an autocrine fashion (Sung, Ketova, Hoshino, Zijlstra, & Weaver, 2015). The same group later demonstrated that tumor exosomes not only enhance cell migration speed, but also promote directional movement towards a chemotactic gradient, although the exact mechanisms remain unclear (Sung & Weaver, 2017). Importantly, tumor exosomes contribute to invasive cell behavior by stimulating the formation and function of invadopodia. The branched actin filaments in invadopodia are important docking and fusion sites for MVBs (Sinha, et al., 2016), and exosome secretion not only increases invadopodia formation and stability, but also promotes matrix degeneration by transporting the proteinase MT1-MMP to the plasma membrane (D. Hoshino, et al., 2013).

EVs in pre-metastatic niche formation

The tumor-promoting activity of tumor-derived EVs is not restricted to the tumor microenvironment. Rather, cancer EVs enter the circulation and reach distant organs, where they can generate favorable environmental conditions enabling the outgrowth of disseminated tumor cells. This process, known as pre-metastatic niche (PMN) formation, requires a series of predefined steps involving induction of

vascular leakiness, alteration of stromal components and immune suppression (Peinado, et al., 2017).

The first evidence for the EV involvement in pre-metastatic niche formation was provided by Peinado and colleagues (Peinado, et al., 2012). The authors demonstrated that EVs from highly metastatic melanoma cells enhance metastasis formation by educating bone marrow progenitor to a prometastatic phenotype through the horizontal transfer of Met. This concept was further explored by Costa-Silva et al, who showed that pancreatic cancer EVs initiate an intercellular signaling cascade promoting pre-metastatic niche formation in the liver (Costa-Silva, et al., 2015). The authors demonstrated that high levels of macrophage inhibitory factor (MIF) within PDAC-EVs induce TGFβ production in Kupffer cells leading to the activation of hepatic stellate cells and subsequent fibronectin production. The resulting fibrotic liver environment attracts bone marrow-derived macrophages, promoting metastasis formation.

Soon after these elaborate studies, the same group demonstrated that EVs released from metastatic cells not only preferentially target and reprogram stromal cells in their predicted destinations, but also can drive organ-specific metastatic outgrowth. Intriguingly, the authors found that EVs from lung-tropic metastatic breast cancer cells could redirect metastasis formation of bone-tropic tumor cells to the lung (A. Hoshino, et al., 2015). They concluded that this effect was dependent on the integrin profile of the vesicles, which dictates EV organotropism and promotes an inflammatory response in recipient cells. While this fascinating study strengthen the

role of cancer EVs in the pre-metastatic niche formation, whether the organ-specific tropism of cancer EVs is solely determined by their integrin repertoire remains an open question. Moreover, future studies should be conducted to clarify the relative contribution of EV-mediated signaling and cancer cell-intrinsic factors to the organotropic behavior of malignant cells (A. Hoshino, et al., 2015).

Not only cancer EV-associated proteins, but also EV-enclosed small RNAs can contribute to the generation of the pre-metastatic niche through distinct mechanisms (Fong, et al., 2015; Tominaga, et al., 2015; Zhou, et al., 2014). MiR-105 secreted by breast cancer cells induces vascular leakiness in distant organs by targeting the tight junction protein ZO-1 (Zhou, et al., 2014) while miR-122 inhibits glucose uptake by stromal cells in the PMN, thus increasing glucose availability and metastatic outgrowth (Fong, et al., 2015). Furthermore, non-coding small nuclear RNAs transferred by cancer EVs can trigger TLR3 signaling in lung epithelial cells, which results in neutrophil recruitment and lung metastatic niche formation (Y. Liu, et al., 2016).

While these studies indicate a critical role for cancer EVs in the sequence of events preceding the arrival of cancer cells to the future metastatic sites, the physiological relevance of these findings awaits confirmation. Indeed, these studies have been mainly conducted by exposing stromal cells to arbitrary amounts of cancer EVs or by directly injecting tumor vesicles in the circulation. In physiological conditions the amount of cancer EVs entering the circulation and reaching defined (pre-metastatic) organs might be limited for instance by internalization by phagocytic cells. In line

with this notion, Pucci and colleagues demonstrated that melanoma EVs are trapped by a barrier of subcapsular sinus macrophages in the tumor-draining lymph nodes, which prevents their dissemination (Pucci, et al., 2016). Importantly, the authors show that cancer progression as well as certain anticancer treatments can disrupt the macrophage barrier, thereby allowing cancer EVs to enter the lymph node cortex and trigger a tumor-promoting humoral immunity. This study demonstrates that many context-dependent factors may influence the systemic function of tumor EVs in vivo, and highlights the need for preclinical models that faithfully reproduce the dynamics of EV release, tropism and stroma education.

5.2 Stroma-to-tumor communication

Early studies on EV-mediated interaction in cancer described the tumor-stroma communication mostly as a unidirectional process where tumor EVs alter the behavior of stromal cells. This concept was reconsidered in 2012, when a provocative study by Luga and colleagues showed that fibroblast-derived vesicles can be modified and recycled by cancer cells to activate an autocrine Wnt-planar cell polarity signaling enhancing tumor cell motility and metastasis (Luga, et al., 2012).

Since then, a number of studies demonstrated that stromal EVs control multiple aspects of cancer progression including the acquisition of a more aggressive phenotype (Nabet, et al., 2017; Roccaro, et al., 2013; Yang, et al., 2011), the development of therapy resistance (Boelens, et al., 2014; Nabet, et al., 2017; Sansone, et al., 2017) and the outgrowth of disseminated cancer cells (Zhang, et al., 2015) (Figure 2). Importantly, the pro-tumorigenic function of stromal EVs is often

dictated by the exposure of the producing cells to cancer-derived signals. For instance, polarization of tumor-associated macrophages towards the M2 phenotype determines the secretion of EV-associated oncogenic miRNAs regulating breast cancer cell invasiveness (Yang, et al., 2011). Interestingly, Roccaro and colleagues showed that EVs released by mesenchymal stem cells can have opposite effects on MM progression (Roccaro, et al., 2013). While multiple myeloma (MM)-MSC EVs promote tumor growth, EVs from normal bone marrow-derived MSCs inhibit cancer cell proliferation, demonstrating that the tumor microenvironment can completely subvert the function of stromal EVs. The non-tumorigenic nature of naïve MSC EVs makes them an attractive option for cancer therapy. Indeed, MSC EVs are natural, non-immunogenic delivery system that could be used for transferring therapeutics to tumors, and have been already used in the clinic to treat graft versus host disease (Kordelas, et al., 2014). Moreover, as these vesicles retain the surface expression profile of the MSCs (H. S. Kim, et al., 2012), they might share with their parental cells a similar tropism for tumor sites (Baglio, Pegtel, & Baldini, 2012).

The tumor-promoting function of EVs is often mediated by EV-associated RNA molecules, which can act as danger-associated molecular patterns (DAMP) promoting inflammation (Baglio, et al., 2016; Boelens, et al., 2014; Haderk, et al., 2017; Nabet, et al., 2017). Boelens et al. demonstrated that induction of interferon-stimulated genes (ISGs) by stromal EV RNAs can induce resistance to chemo- and radiation therapy in triple negative breast cancer cells (Boelens, et al., 2014). However, the mechanisms regulating endogenous RNA recognition or evasion from PRRs remained unclear. This question was addressed by two recent studies (Baglio,

et al., 2016; Nabet, et al., 2017). We and others independently demonstrated that within the producing cell pol III transcripts are mainly associated with their RNA-binding partners that shield them from recognition by intracellular sensors. Under specific conditions leading to the alteration of the RNA-RBP stoichiometry, naked RNA molecules can be incorporated into exosomes and, upon delivery into recipient cells, activate PRR and ISG expression. Nabet and colleagues showed that this mechanism is strongly implicated in the tumor-stroma communication promoting cancer progression (Nabet, et al., 2017). Specifically, the authors demonstrated that stroma activation by tumor cells increases the transcription of the non-coding RNA RN7SL1, which is then sorted in its naked form into stromal exosomes. The antiviral immune response triggered by unshielded RN7SL1 upon delivery in cancer cells enhances tumor growth, metastasis formation and therapy resistance, thereby linking stromal activation to EV-mediated inflammation and cancer progression.

Strengthening the role of EV-associated RNA in stroma-to-tumor communication, an impressive study by Zhang et al. revealed that EV-mediated transfer of astrocyte-derived miRNAs support the outgrowth of breast cancer brain metastases (Zhang, et al., 2015). The authors show that both mouse and human breast cancer cells reversibly lose the expression of the tumor suppressor gene PTEN when disseminating to the brain, but not to other metastatic sites. They then combine a series of elegant in vivo approaches, including astrocyte-specific miRNA depletion and adenoviral-mediated inhibition of EV release, to demonstrate that the transfer of astrocyte-derived miR-19a is responsible for the reversible PTEN downregulation leading to metastatic outgrowth. This study establishes a role for stromal EVs in the

co-adaptation between tumor cell and host environment leading to the development of organ-specific metastasis. It would be interesting to investigate whether the astrocyte-EV content changes in response to cancer-released signals and how this would influence the outgrowth of brain metastasis. Moreover, future research should be aimed to understand whether this model can be extended to the formation of metastatic lesion in other organs.

Finally, a novel form of EV communication based on the transfer of mitochondrial DNA has been recently implicated in the development of drug resistance (Sansone, et al., 2017). Sansone and colleagues found that cancer-associated fibroblast derived EVs, enclosing the full mitochondrial genome, restore defects in oxidative phosphorylation of hormone therapy-induced dormant breast cancer stem cells, leading to escape from dormancy and development of hormone therapy-resistance (Sansone, et al., 2017). This original work raises multiple questions: which EV subclass is responsible for the transfer of the mitochondrial genome? How is mitochondrial DNA incorporated into EVs? Most importantly, how can the mitochondrial DNA be functionally transferred into the correct subcellular location and restore the metabolic activity in recipient cells? Although many aspects need to be clarified, this study reveals a novel modality of metabolic communication in cancer.

5.3 Tumor-to-tumor communication

The intra-tumor heterogeneity characterizing the majority of neoplastic lesions represents a major challenge in cancer, since it increases the chances of existence of individual tumor cells or clones with highly malignant or therapy resistant phenotypes. In this context, the EV-mediated transfer of cancer-derived signals between different tumor cell subpopulations can have important functional consequences.

Key examples of this concept were provided by Al-Nedawi et al., who demonstrated that transfer of the oncogenic EGFRvIII receptor via glioblastoma EVs results in the activation of oncogenic signaling in recipient tumor cells (Al-Nedawi, et al., 2008), and by Zomer and colleagues, who showed that EVs from aggressive breast cancer cells increase the metastatic potential of less-malignant tumor cells possibly by functional RNA transfer (Zomer, et al., 2015). Moreover, this modality of tumor-to-tumor communication is particularly relevant in the presence of specific microenvironmental conditions such as hypoxia. Indeed, EVs from hypoxic tumor cells can induce an EMT switch and promote invasion and metastasis of recipient normoxic tumor cells (L. Li, et al., 2016).

Finally, recent studies by Ricklefs and Godlewski revealed that the EV-mediated communication between distinct glioblastoma stem cell subsets results in the generation of cell populations with intermediate phenotypes, indicating that cancer EVs not only transfer malignant traits between cancer cell subpopulations, but can also propagate tumor heterogeneity (Godlewski, et al., 2017; Ricklefs, et al., 2016).

6. Concluding remarks

It is now fully established that EVs and exosomes have functional properties in vivo that can be applied in clinical settings. The role of exosomes, as defined by their intracellular endosomal origin, remains unclear as in vivo tools to follow exosomes release and biogenesis are still lacking. The only way to conclusively identify a role for exosomes in cancer is by interfering with their biogenesis, cargo-loading and/or function. This is not easy since, as we described in this review, multiple mechanisms exist that presumably act in parallel, and the nature and dynamics of exosome subpopulations are far from understood. How to block one exosome pathway? While much has been learnt from in vitro characterization and administration of EVs to (tumor bearing) mice, specific inhibition of exosome biogenesis in cancer cells will provide defining answers into the actual role of cancer released exosomes in oncogenesis and progression.

7. Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Schematic representation of EV biogenesis pathway. While microvesicles bud directly from the plasma membrane, exosomes are generated within MVB subpopulations that upon maturation fuse with the plasma membrane. Alternative MVB pathways include fusion with lysosomes or with autophagosomes, although little is known about the mechanisms determining MVB fate. MVB fusion with the plasma membrane is a tightly regulated multistep process that includes MVB trafficking along microtubules, docking at the plasma membrane and SNARE-mediated fusion.

Figure 2. Function of EVs in the tumor-to-stroma, stroma-to-tumor and tumor-to-tumor communication fueling cancer progression. EVs released by cancer cells can act within the tumor microenvironment by educating different types of stromal cells to a proangiogenic, prometastatic and immune suppressive phenotype. In addition, cancer EVs participate in the pre-metastatic niche formation, by altering the behavior of bone marrow-derived progenitors or resident specialized cells. Also stromal cell-derived EVs can strongly influence cancer progression, promoting cancer cell growth and invasive behavior, outgrowth of cancer cells at the metastatic site and development of therapy resistance. Finally, cancer EVs can transfer malignant traits between different tumor cell subpopulations and propagate tumor heterogeneity.

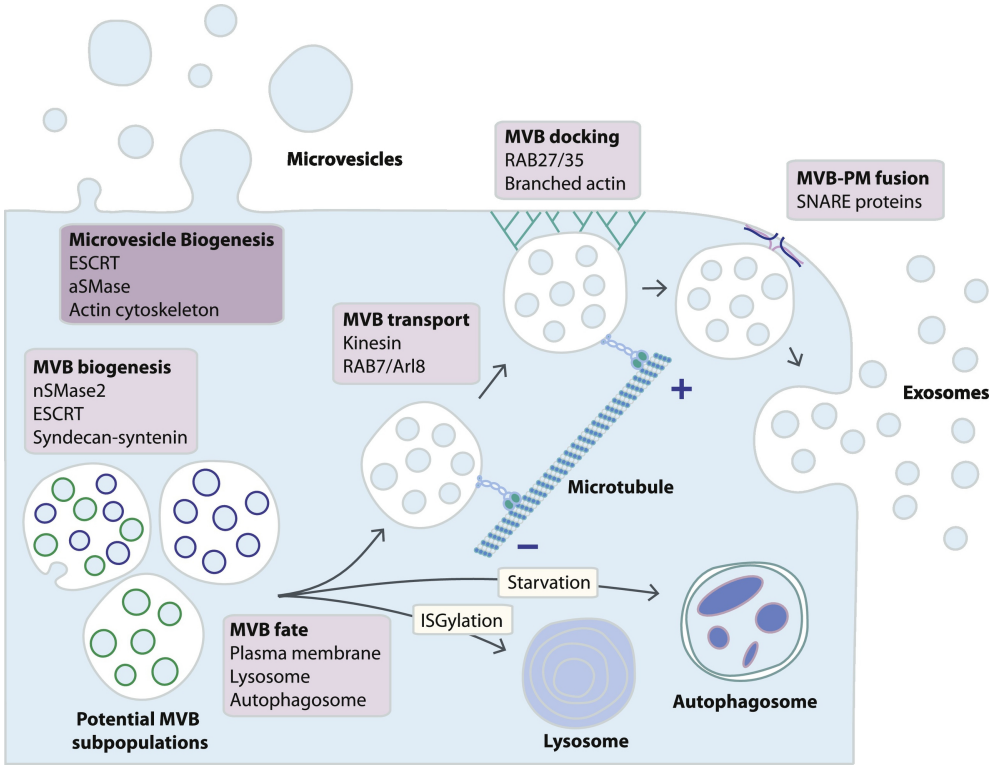
Microvesicles
MVB docking
RAB27/35
Branched actin
MVB-PM fusion
SNARE proteins
Microvesicle Biogenesis
ESCRT
aSMase
Actin cytoskeleton
MVB transport
Kinesin
RAB7/Arl8
MVB biogenesis
nSMase2
ESCRT
Syndecan-syntenin
Exosomes
Microtubule
Starvation
ISGylation
MVB fate
Plasma membrane
Lysosome
Autophagosome
Potential MVB subpopulations
Lysosome
Autophagosome

Figure 1

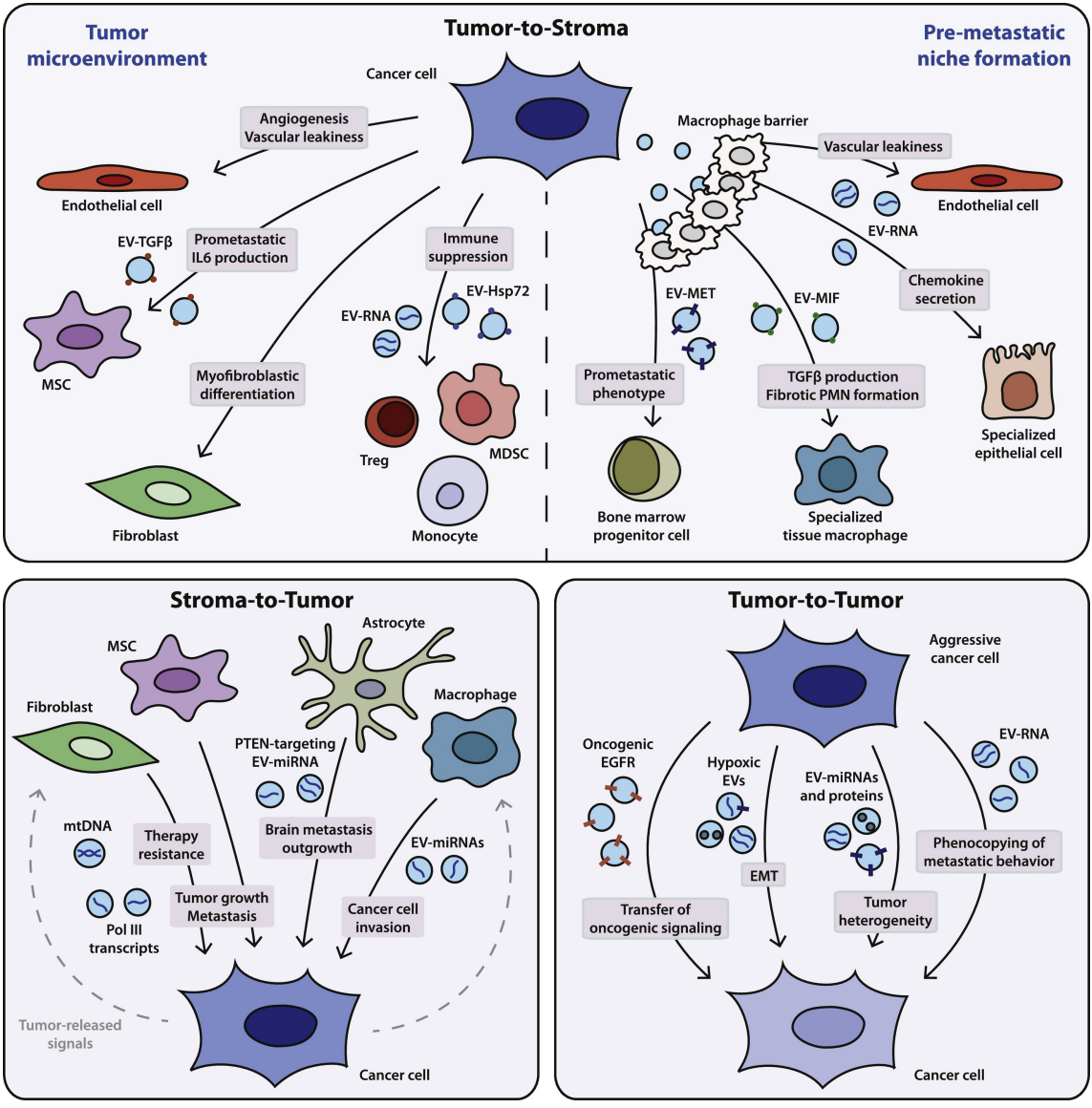
Tumor microenvironment
Tumor-to-Stroma
Pre-metastatic niche formation
Cancer cell
Angiogenesis
Vascular leakiness
Endothelial cell
EV-TGFβ
Prometastatic IL6 production
MSC
Myofibroblastic differentiation
Fibroblast
Immune suppression
EV-RNA
EV-Hsp72
Treg
MDSC
Monocyte
Macrophage barrier
Vascular leakiness
Endothelial cell
EV-RNA
Chemokine secretion
EV-MET
EV-MIF
Prometastatic phenotype
TGFβ production
Fibrotic PMN formation
Specialized epithelial cell
Bone marrow progenitor cell
Specialized tissue macrophage
Stroma-to-Tumor
MSC
Astrocyte
Fibroblast
Macrophage
PTEN-targeting EV-miRNA
mtDNA
Therapy resistance
Brain metastasis outgrowth
EV-miRNAs
Pol III transcripts
Tumor growth Metastasis
Cancer cell invasion
Tumor-released signals
Cancer cell
Tumor-to-Tumor
Aggressive cancer cell
Oncogenic EGFR
Hypoxic EVs
EV-miRNAs and proteins
EV-RNA
EMT
Phenocopying of metastatic behavior
Transfer of oncogenic signaling
Tumor heterogeneity
Cancer cell

Figure 2