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Review

Exosome-Mediated Metastasis: Communication from a Distance

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Metastasis, a critical phase of tumor progression, remains a primary challenge in treating cancer and a major cause of cancer mortality. Cell-cell communication via extracellular vesicles (exosomes and microvesicles) between primary tumor cells and the microenvironment of distant organs is crucial for pre-metastatic niche (PMN) formation and metastasis. Here, we review work on the contribution of exosome cargo to cancer progression, the role of exosomes in PMN establishment, and the function of exosomes in organotropic metastasis. We also describe the clinical utility of exosomes.

Introduction: Tumor-Derived Exosomes

Exosomes are nano-sized vesicles (30–150 nm diameter) that are secreted by most cells. They are enclosed by a lipid bilayer and carry various biomolecules, including proteins, glycans, lipids, metabolites, RNA, and DNA (Mathieu et al., 2019). When exosomes are taken up by other cells, these cargoes are transferred and influence the phenotype of recipient cells. As such, exosomes are appreciated as essential mediators of cell-cell communication.

Exosome biogenesis originates in the endocytic pathway (D'Souza-Schorey and Schorey, 2018; Mathieu et al., 2019; van Niel et al., 2018). It begins with invagination of endosomal limiting membranes, leading to the formation of intraluminal vesicles (ILVs) contained within the endosome. This resulting compartment, termed a multivesicular body (MVB), fuses with the plasma membrane, culminating in the extracellular release of ILVs as exosomes. Exosome formation requires the coordinated efforts of several protein networks in the cell. Among these are (1) Rab GTPase proteins, which control endosomal trafficking; (2) endosomal sorting complexes required for transport (ESCRT), which consists of multiple protein complexes that regulate ILV formation; (3) tetraspanins, which are transmembrane proteins that induce membrane curvatures enabling vesicle formation; and (4) various lipid-modifying enzymes such as sphingomyelinase, which generates ceramides that promote vesicle formation. Notably, many of these factors interact directly with exosome cargo, indicating that vesicle formation is an intricately regulated process that is tightly coupled to the selective sequestration of substrates destined for exosomal secretion. In addition to classically described exosomes, there is also heterogeneity within the exosome population (Kowal et al., 2016), and subsets can be classified into three groups: Exo-Large (90–120 nm), Exo-Small (60–80 nm), and the membrane-less exomere (<50 nm) (Zhang et al., 2018a). Each of these subtypes exhibits unique traits with regard to their macromolecular composition, further highlighting the specificity and selectivity of exosome biogenesis mechanisms. Since exomeres are not membrane bound and therefore better resemble a particle rather than a vesicle (Zhang et al., 2018a), exosomes can be considered to consist of not only small extracellular vesicles (EVs) but also other intracellularly derived secreted complexes of proteins, nucleic acids, and lipids.

Alongside exosomes, cells produce other types of EVs, including microvesicles (MVs) that form by direct plasma membrane budding and are considered to be larger than exosomes, ranging in size from 100 to 1,000 nm (Mathieu et al., 2019; van Niel et al., 2018). Hence, two defining distinctions between exosomes and MVs include the site of biogenesis and size. Like exosomes, MVs carry a variety of bioactive factors including proteins and nucleic acids. The initial process of MV budding relies on actin polymerization and occurs at plasma membrane lipid domains enriched for ceramides, cholesterol, and extracellular facing phosphatidylserine, which collectively promote membrane curvature and budding (Antonyak and Cerione, 2014; D'Souza-Schorey and Schorey, 2018). Scission of MVs from the cell surface is regulated by the small GTPase ADP-ribosylation factor 6 (ARF6)-induced actomyosin contractility (Muralidharan-Chari et al., 2009), and ESCRTs have also been implicated in this final phase of MV shedding (Nabhan et al., 2012). ARF6 can also regulate ILV budding into MVBs during exosome biogenesis (Ghossoub et al., 2014). Thus, in addition to the currently defined distinctions in cellular origin, size, and particular aspects of biogenesis, MV formation and exosome biogenesis employ common protein machineries.

Intercellular communication is a key feature of tumor progression and metastasis. Remarkably, in addition to local signaling within the primary tumor microenvironment, tumors also signal over long distances to sites of future metastases to promote formation of a hospitable, pre-metastatic niche (PMN) that will foster growth of disseminated tumor cells upon their arrival (Peinado et al., 2017). Because they transport and transfer bioactive molecules, exosomes are currently of immense interest for their ability to regulate metastasis (Costa-Silva et al., 2015; Hoshino et al., 2015; Peinado et al., 2012). Indeed, reduction of exosome secretion via depletion of Rab27a in tumor cells (Bobrie et al., 2012; Peinado et al., 2012) or pharmacological inhibition of exosomal uptake at sites of future metastases (Ortiz et al., 2019) was sufficient to impair PMN formation and decrease spontaneous

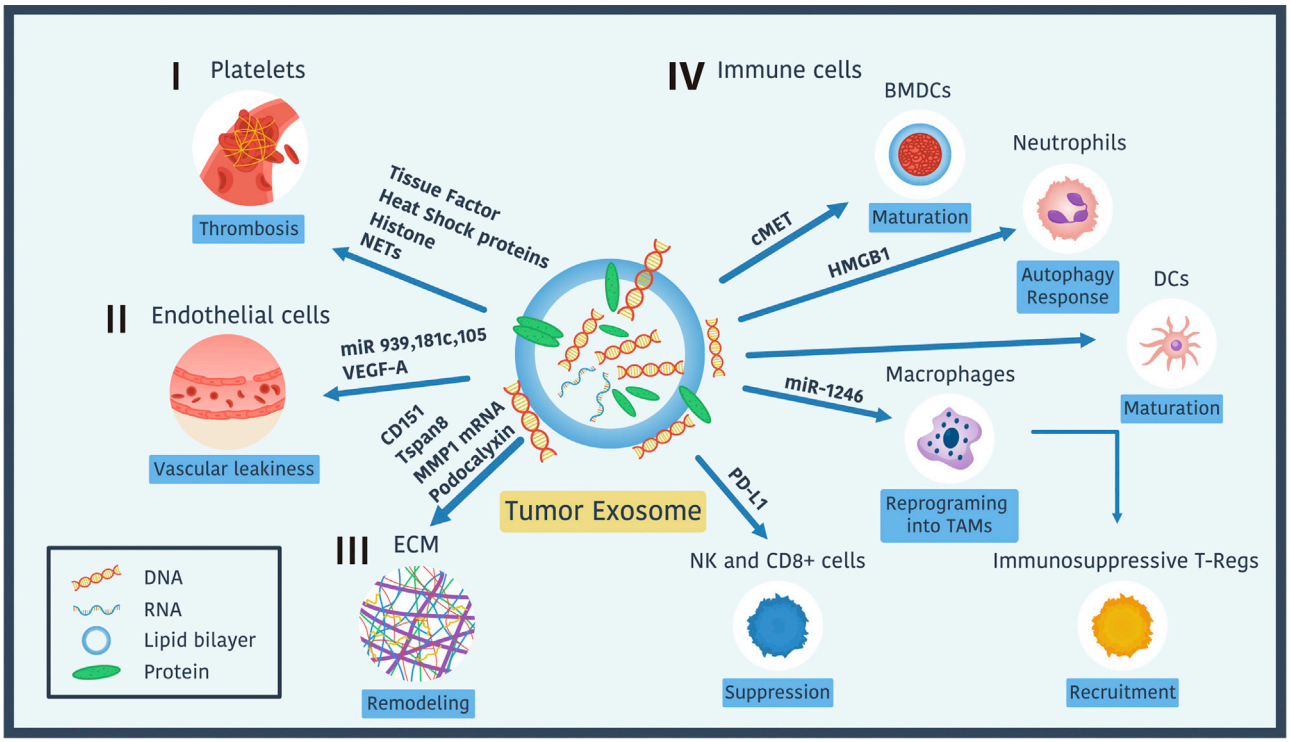

Figure 1. Cancer-Derived Exosome Content and Mechanisms for PMN Establishment

Exosomes are secreted vesicles that are encapsulated by a lipid bilayer and contain various biomolecules such as protein (membrane bound or encapsulated within the vesicle), RNA (coding mRNA or various non-coding RNAs), DNA (dsDNA and ssDNA), as well as glycans. Exosomes modify endothelial cells to promote vascular leakiness, and they alter the ECM and induce thrombosis. To enable metastasis, exosomes contribute to immune regulation by suppressing anti-cancer immunity and stimulating pro-tumorigenic processes. Finally, exosomes contribute to CAF activation, which in turn secrete exosomes that promote cancer cell migration and invasion.

metastasis in tumor-bearing mice. It is also notable that MVs participate in cellular cross talk during cancer; one of the earliest studies described how MVs from metastatic melanoma cells enhance lung colonization of less aggressive, non-metastatic melanoma cells (Poste and Nicolson, 1980). Further work on MVs has mainly highlighted the ability of MVs to support primary tumor growth and survival (Antonyak and Cerione, 2014; Lee et al., 2011; Muralidharan-Chari et al., 2010). Here, we focus on exosomes because, based on their historically defined size criteria and cellular origin and the fact that they include secreted particles that are not true vesicles, they have been described to be the principal EV population mediating long-range signaling during PMN formation and metastasis. We review progress in our understanding of the role of exosomes in cancer metastasis by highlighting the contribution of specific exosome cargos to cancer progression, the role of exosomes in PMN development, and the clinical application of exosomes (Figure 1).

Packed with Tumor Information

Protein Packaging

Cancer exosomes contain various proteins; some are shared between different cell types, whereas others are uniquely packaged, reflecting the cell of origin. Cancer exosomes express an array of proteins, including oncogenic proteins, integrins, and signaling molecules (Choi et al., 2015). Analysis of exosomes from cancer cells uncovered that exosomal packaging of proteins varied between different cancer types and between cancers of different metastatic potential but with a similar origin. Proteomic characterization of EVs from a panel of 60 cancer cell lines representing nine different types of cancer from the National Cancer Institute (NCI-60) found that only 213 proteins were shared among EVs from these cancers, whereas overall, more than 6,000 proteins were unique (Hurwitz et al., 2016). The shared proteins represented factors involved in biogenesis, while the unique proteins reflected the cell of origin and thus were proposed as biomarkers. Other studies provided a comprehensive proteomic analysis of pancreatic cancer (PaC) exosomes. Characterization of exosome proteomes from human PaC and non-malignant human pancreatic epithelial cell lines found 362 proteins specifically expressed in PaC exosomes with known roles in PMN regulation and tumor cell proliferation, invasion, and metastasis (Emmanouilidi et al., 2019). Similarly, over 4,000 proteins were found to be expressed in exosomes derived from two PaC cell lines with varying degrees of metastatic potential. However, 79 of these were differentially expressed, and proteins found in exosomes from the more highly metastatic cell line had roles in adhesion, invasion, growth, metabolism, and metastasis (Yu et al., 2017). Likewise, exosomes from the metastatic mouse melanoma cell line, B16F10, had higher levels of cMet compared to the less aggressive mouse melanoma variant, B16F1 (Peinado et al., 2012). In these studies, exosomes from the more aggressive cell lines were more metastatic compared to exosomes from the less metastatic variants,

suggesting these unique patterns of exosomal protein expression influence metastatic progression.

Differential and selective protein packaging also occurs between cancers of different origins and with specific metastatic tropisms. Packaging of distinct integrins in exosomes from different cancer types had a role in determining the organs that take up tumor exosomes. α6β4 and α6β1 integrins were most abundant in breast cancer (BC) exosomes that metastasize to the lung (lung tropic), while αvβ5 integrin was enriched in exosomes from liver metastatic (liver tropic) PaC exosomes. Interestingly, this exosomal integrin expression pattern did not represent cellular integrin expression, suggesting specific packaging pathways promote preferential sorting of these integrins into exosomes. Specifically, α6β4 exhibited higher expression in exosomes from lung metastatic cells compared to the expression level in the cells themselves. Furthermore, despite liver metastatic cells having higher α6β4 expression than lung metastatic cells, the exosomes from lung tropic cells packaged more α6β4 than exosomes from liver tropic cells. The pattern of uptake displayed by these exosomes reflected the metastatic organotropism of tumor cells and depended on these integrins, indicating tumor-secreted exosomal integrins have a crucial role in determining where tumors metastasize, and they can serve as biomarkers for predicting organotropic metastasis (Hoshino et al., 2015). Likewise, in gastric cancer (GC), integrin αvβ6 was transferred between tumor cells via exosomes, enhancing adhesion and migration of recipient cells (Fedele et al., 2015). Moreover, in prostate cancer (PrC) patients, integrin αvβ6 was highly expressed in peripheral blood mononuclear cells. αvβ6 integrin inhibited STAT1/MX1/2 signaling in cancer cells and their exosomes and reprogramed monocytes to an M2 tumor-supportive phenotype when transferred via exosomes (Lu et al., 2018).

Epidermal growth factor receptor (EGFR) expressed on cancer cells has a known role in tumorigenesis, and EGFR signaling mediated by EVs was described to also support tumor progression and metastasis. Early studies on the role of secreted EGFR during primary tumorigenesis showed that horizontal transfer of oncogenic EGFR by tumor cell-derived MVs to other tumor cells or endothelial cells (ECs) enhanced growth and survival of cancer cells in glioma (Al-Nedawi et al., 2008) and induced angiogenesis in human squamous cell carcinoma (Al-Nedawi et al., 2009), respectively. GC exosomes also expressed functionally active EGFR, which can be delivered to the plasma membrane of liver stromal cells. EGFR translocated from the membrane, activated HGF, which binds to cMet on the cancer cell, and facilitated seeding and proliferation of metastatic cells (Zhang et al., 2017). EGFR ligand can also be transferred by exosomes and promote metastasis. Amphiregulin, an EGFR ligand, was found in human BC and colon cancer (CoC) exosomes and increased invasiveness of surrounding cancer cells (Higginbotham et al., 2011). In CoC, both EGFR and Amphiregulin were expressed in patient plasma exosomes (Higginbotham et al., 2016).

The expression of podoplanin (PDPN), a transmembrane glycoprotein, was elevated in cancer. Cells expressing high PDPN secreted more exosomes that contained proteins involved in cell adhesion, cytoskeletal remodeling, signal transduction, intracellular trafficking, and EV biogenesis. PDPN itself was expressed on exosomal membranes, suggesting that glycoproteins have an important but poorly understood role in regulating exosome biogenesis and cargo selection (Carrasco-Ramírez et al., 2016; Zhang et al., 2018a).

Heterogeneity between exosome subsets from the same cells was also shown. The three subsets of exosomes, Exo-Large, Exo-Small, and exomeres each selectively packaged unique proteins, which are associated with different cellular pathways and different organelles. While exomeres were significantly enriched in proteins related to metabolism and glycan biology, the larger exosomes highly expressed annexins, ESCRTs, integrins, and signaling pathway molecules. Rab proteins that are essential for exosome release and supported primary tumor growth and metastasis were mainly expressed in Exo-Large and Exo-Small but not exomeres (Peinado et al., 2012; Zhang et al., 2018a). These patterns of protein expression suggest each subtype has a distinct role in cancer.

Nucleic Acid Parcels

Exosomes also contain various RNAs. Most work showed that microRNAs (miRs) and non-coding RNAs were the predominant RNA species transported by exosomes; however, the presence of mRNA, rRNA, and tRNA was also reported (Wei et al., 2017). Different exosome subsets contained different amounts of RNA, but, in general, larger vesicles contained more RNA (Zhang et al., 2018a). Exosomal RNA mediated communication between cells, including educating distinct cells in the tumor microenvironment, and demonstrated potential as cancer biomarkers (Skog et al., 2008; Valadi et al., 2007; Wei et al., 2017) (Table 1). For example, in PrC, exosomal miRs induced fibroblast activation, migration, angiogenesis, and osteoblast differentiation, which promoted the bone PMN (Sánchez et al., 2016). Exosomal miR-1245 from CoC cells reprogramed macrophages to tumor-associated macrophages (TAMs) with high transforming growth factor β (TGF-β) expression that enabled tumor growth and metastasis (Cooks et al., 2018). Exosome miRs promoted the metastatic niche (MN), as well. In the brain, exosomes from astrocytes transferred miR-19a to BC cells, causing a reduction in PTEN expression, which led to secretion of CCL2 and recruitment of myeloid cells, enhanced proliferation, and eventually increased brain metastasis (Zhang et al., 2015). These studies illustrate the diverse roles of exosomal miRs in regulating cancer progression.

Although exosomal miRs have been the best studied, the function of other exosomal RNAs was also interrogated. Transfer of MMP1 mRNA by ovarian cancer (OvC) exosomes to mesothelial cells *in vitro* and *in vivo* induced destruction of the peritoneal mesothelium barrier and promoted cancer spread (Yokoi et al., 2017). Another mechanism of RNA damage-associated molecular pattern (DAMP) transfer by cancer exosomes was recently revealed. Under non-pathological conditions, RN7SL1 was normally shielded in the cytoplasm of fibroblasts by the RNA binding protein SRP9/14. However, in BC, tumor cells activated the Notch-Myc pathway in cancer fibroblasts, which deployed unshielded RN7SL1 in exosomes. The unshielded RN7SL1 activated RIG-I and resulted in an inflammatory response when transferred to immune cells and induced tumor growth and invasion when transferred to BC cells (Nabet et al., 2017).

RNA transfer via exosomes also serves to remove tumor suppressive molecules from cancer cells. In CoC, tumor-suppressive miRs were highly packaged into exosomes, while oncogenic miRs were upregulated in the cell compared to exosomes; this

Table 1. Exosome Cargo and Its Role in Cell-Cell Communication

Molecule	Cell of Origin	Effect	Reference
Proteins			
EGFR	GC	increased localization and proliferation of metastatic cells	Zhang et al., 2017
Podoplanin	melanoma	increased cell adhesion, cytoskeletal remodeling, and lymphatic vessel formation	Carrasco-Ramírez et al., 2016
Integrins α6β4, α6β1, αvβ5	BC, PaC	determined organotropism of metastasis	Hoshino et al., 2015
Integrin αvβ6	GC	enhanced adhesion and migration	Fedele et al., 2015
	GC	reprogramed monocytes to M2 monocytes	Lu et al., 2018
VEGF-A	GBM	induced angiogenesis and permeabilization of brain endothelial cells	Treps et al., 2017
cMET	melanoma	promoted a pro-metastatic phenotype and mobilization of BMDCs to PMNs	Peinado et al., 2012
TF	BC	increased TF activity in recipient cells	Lima et al., 2013
	BC	promoted plasma clotting and platelet aggregation	Gomes et al., 2017
HMGB1	GC	induced neutrophil's autophagy response and cell migration	Zhang et al., 2018b
CD151, Tspan8	PDAC	promoted ECM degradation	Yue et al., 2015
Podocalyxin	non-small cell lung cancer	modulated integrin trafficking in fibroblasts, increased tumor cell migration and invasion	Novo et al., 2018
IRF-2	CRC	induced VEGF-C in LN macrophages resulting in lymphangiogenesis	Sun et al., 2019
CD97	GC	increased metastasis	Liu et al., 2016a
CXCR4	hepatocarcinoma	promoted LEC proliferation and metastasis	Li et al., 2018
L-plastin	BC	induced osteolysis	Tiedemann et al., 2019
MIF	PDAC	promoted fibronectin secretion and metastasis	Costa-Silva et al., 2015
Amphiregulin	NSCLC	induced osteoclastogenesis and metastasis	Taverna et al., 2017
RNA			
miR-1245	CoC	reprogramed macrophages to TAMs, promoted tumor growth and metastasis	Cooks et al., 2018
miR-155, miR-210	melanoma	induced metabolic changes	La Shu et al., 2018
miR-19a	astrocytes	reduced PTEN expression in BC, increased metastasis	Zhang et al., 2015
miR-939	BC	downregulated VE-cadherin, increased HUVEC permeability	Di Modica et al., 2017
miR-181c	BC	impaired BBB, increased metastasis to the brain	Tominaga et al., 2015
miR-105	BC	downregulated ZO-1, inducing vascular leakiness and metastasis	Zhou et al., 2014
miR-221	CAF exosomes	promoted cancer stem cell proliferation	Sansone et al., 2017a
miR-221	bone marrow mesenchymal stromal cells	promoted GC cell migration, invasion, and adhesion to the matrix	Ma et al., 2017
miR-221	cervical squamous cell carcinoma	promoted migration and lymphangiogenesis	Zhou et al., 2019
miR-21	CRC	induced liver macrophage polarization and metastasis	Shao et al., 2018
miR-141-3p	PrC	promoted osteoblast activity, tumor growth, and metastasis	Ye et al., 2017
miR-940	PrC	induced an extensive osteoblastic lesion	Hashimoto et al., 2018
miR-192	lung adenocarcinoma	reduced bone osteolytic lesions, preventing metastatic angiogenesis	Valencia et al., 2014.
miR-520	brain tumor	decreased TF and reduced fibrin	D'Asti et al., 2016
MMP1 mRNA	ovarian cancer	interaction with peritoneal mesothelium barrier, promoted metastasis	Yokoi et al., 2017

exosome-mediated rebalancing of cellular miRs favoring pro-tumorigenic pathways promoted primary tumor progression (Teng et al., 2017).

DNA was first discovered in exosomes as single-stranded DNA (ssDNA) (Balaj et al., 2011). Later, double-stranded DNA (dsDNA) was found to be the major form of DNA within exosomes

and was located on the surface of and inside of the vesicle (Thakur et al., 2014). Exosomal DNA (exoDNA) originated from both the nucleus and the mitochondria (mtDNA) and represented the whole genome without a bias toward a particular sequence or DNA structure (Kahlert et al., 2014; Sansone et al., 2017b; Thakur et al., 2014). DNA packaging into exosomes was significantly higher in cancer-derived exosomes compared to exosomes from non-cancer cells (Thakur et al., 2014). Moreover, DNA was found in all exosome subsets, but the distribution varied between cancer cell lines (Zhang et al., 2018a). While the mechanism of exoDNA packaging is undefined, it was shown that mouse embryonic fibroblasts (MEFs) utilized exosomes to remove excess cytoplasmic DNA (cytDNA), which may otherwise induce senescence-like cell-cycle arrest or apoptosis (Takahashi et al., 2017). Given the high level of genomic instability and the accumulation of cytDNA in cancer cells (Bakhoum et al., 2018), it is plausible that cancer cells secrete more exoDNA to prevent senescence and apoptosis, ensuring their survival and proliferative potential.

While it is clear that cancer-derived exosomes contain more DNA, the functional effect of exoDNA uptake is unknown. The role of tumor-derived exoDNA was studied mainly in the context of chemotherapy or irradiation treatments. For example, treatment of mice bearing BC tumors with topotecan or irradiation induced secretion of immunostimulatory DNA, leading to an antitumor response by promoting dendritic cell (DC) maturation and $CD8^+$ T cell activation (Diamond et al., 2018; Kitai et al., 2017). Additionally, treatment of CoC cells with irinotecan induced release of DNA-containing exosomes. When this exoDNA was taken up by intestinal innate immune cells, it activated the inflammasome, inducing IL-1β and IL-18 secretion and severe gastrointestinal tract toxicity (Lian et al., 2017). Finally, many cancers were characterized by a loss of mtDNA, leading to increased dependency on anaerobic metabolism and dormancy. In a hormone therapy-sensitive BC model, cancer-associated fibroblasts (CAFs) packaged high amounts of mtDNA in their exosomes. Dormant cancer stem cells (CSCs) that acquired this mtDNA exited metabolic quiescence and contributed to the transformation into hormone therapy-resistant disease (Sansone et al., 2017b).

As mentioned earlier, exoDNA represents the entire genome; hence, any mutation is likely to be found in exoDNA. Thus, exoDNA from patient plasma could be useful in the early detection of cancer-specific mutations. Indeed, several reports showed that PaC circulating exosomes could serve as a liquid biopsy for cancer mutations in genes such as *KRAS* and *TP53* (Allenson et al., 2017; Bernard et al., 2019; Möhrmann et al., 2018; Yang et al., 2017). Moreover, increased mutation allelic frequency in the exoDNA pool correlated with poor prognosis and survival (Bernard et al., 2019; Möhrmann et al., 2018).

Exosomes Engineer PMNs: From Their Foundation to Final Construction

The PMN results from changes that occur in a distant organ in preparation for seeding and growth of circulating tumor cells (CTCs) into overt metastases. In the PMN model, the decision regarding the organ in which metastatic lesions will grow is not random but is a predetermined process initiated and orchestrated by primary tumor-secreted factors (Kaplan et al., 2005). Furthermore, recent mathematical modeling showed that two factors were crucial for PMN formation and metastasis: (1) development of metastasis-promoting mutations and (2) a suitable environment. Without the latter, invasion was not possible because of competitive elimination and a lack of potential niche sites (Qian and Akçay, 2018).

To prepare the environment, the primary tumor secretes factors that collectively constitute the tumor cell secretome and includes cytokines and exosomes. These secreted factors target specific organs to induce changes that will create a welcoming environment for CTCs. Although the relative contribution of each factor has not been directly compared, studies in mouse melanoma (Kaplan et al., 2005; Peinado et al., 2012) and rat PaC (Jung et al., 2009) have shown *in vivo* treatment with exosomes isolated from conditioned media of these cells is sufficient to recapitulate the PMN induction observed with conditioned medium treatment. Here, we focus specifically on how exosomes regulate PMN formation, but we direct the reader to other reviews on PMN formation that cover the important role of additional secreted factors in this process (Liu and Cao, 2016; Peinado et al., 2017). It will certainly be critical for future work to better dissect how tumor secretome components collaborate to regulate the PMN. These PMN changes include thrombosis, vascular leakiness, bone marrow (BM) immune cell infiltration, and extracellular matrix (ECM) and stroma modulation (Figure 1).

Thrombosis: The Clot Thickens

Cancer patients have an increased risk for thrombosis, a major cause of poor prognosis (Geddings and Mackman, 2013; Gil-Bernabé et al., 2013; Hisada and Mackman, 2017; Khorana, 2010). The first evidence linking EVs to thrombosis demonstrated procoagulant activity in microparticles shed from a guinea pig hepatocarcinoma cell line and a mouse BC cell line (Dvorak et al., 1981). Since then, several studies demonstrated that tumor-derived EVs exhibited procoagulant properties that may contribute to cancer-associated thrombosis, which frequently correlates with metastasis. Enrichment of tissue factor (TF), a transmembrane receptor and initiator of blood coagulation, in tumor-derived exosomes and MVs was associated with increased thrombosis. TF binds coagulation proteins to initiate a cascade of events that result in formation of a fibrin clot and platelet activation. In the MDA-MB-231 BC model, exosomes and MVs were enriched in TF and accelerated coagulation compared to EVs from non-metastatic MCF7 BC cells, suggesting a TF-related aggressive phenotype. Transfer of TF via MDA-MB-231 MVs to MCF7 cells significantly increased TF activity in MCF7 cells (Lima et al., 2013). MDA-MB-231 exosomes promoted plasma clotting and indirect platelet aggregation through TF-dependent thrombin generation, but they also interacted directly with platelets and activated them independently of TF (Gomes et al., 2017). Moreover, exosomal miR-520 from medulloblastoma cell lines and pediatric embryonal brain tumors directly targeted and decreased TF, reducing fibrin clots (D'Asti et al., 2016).

Exosomes also induce thrombosis through other factors or by indirectly affecting clotting. Melanoma exosomes and MVs were enriched in histones and heat-shock proteins, which can enhance thrombin generation in platelet plasma (Muhsin-Sharafaldine et al., 2016). Activated platelet MVs (PMVs) also supported cancer cell proliferation and metastasis upon transfer to lung cancer cells by promoting MAPK and PI3K-Akt signaling,

upregulation of MMP expression, and adhesion of cancer cells to the endothelium and fibrinogen (Janowska-Wieczorek et al., 2005), suggesting these clotting-related pathways also support other aspects of cancer progression. Indeed, repeated treatment of oral squamous carcinoma cells with heparin, an anticoagulant drug, inhibited metastatic phenotypes *in vitro* and reduced tumor growth *in vivo* (Sento et al., 2016). Furthermore, exosomes induced the formation of neutrophil extracellular traps (NETs), a process that enhances cancer-associated thrombosis. NETs induce platelet aggregation and degradation of coagulation inhibitors. *In vivo*, 4T1 mouse BC exosomes expressing TF induced NET formation and accelerated thrombosis. A similar induction of NETs and thrombosis was observed in 4T1 tumor-bearing animals, suggesting exosomes are a critical tumor-secreted factor involved in NET-dependent establishment of thrombosis (Leal et al., 2017).

Vascular Disruption and Leakiness

The vascular EC layer provides a physical barrier to fluids, proteins, and cells. ECs are connected by adherens and tight junctions, which maintain vascular barrier function. To access distant tissues, cancer cell-secreted soluble factors and exosomes were capable of impairing EC junctions, which in turn led to increased vascular permeability for further vesicle and cellular entry into the tissue parenchyma (García-Román and Zentella-Dehesa, 2013; Hoshino et al., 2015; Peinado et al., 2012). In BC, multiple studies showed that exosomal miRs induced vascular permeability. MiR-939 was highly expressed in BC-patient tumors and was transferred by exosomes to ECs, resulting in downregulation of vascular endothelial (VE)-cadherin, an EC-specific adherens junction protein, increasing vascular permeability *in vitro* (Di Modica et al., 2017). In mice, exosomal miR-181c destabilized the blood brain barrier (BBB) by downregulating the actin regulator, PDPK1, leading to abnormal actin localization in ECs. This perturbed binding of actin to tight junction proteins and increased vascular permeability and brain metastasis (Tominaga et al., 2015). Similarly, exosomal miR-105 destroyed tight junctions by downregulating another tight junction protein, ZO-1, inducing vascular leakiness in lungs and liver and promoting metastasis. MiR-105 is detected in the circulation of patients at the pre-metastatic stage, and its levels in blood and tumors were associated with metastatic progression in early-stage BC (Zhou et al., 2014). In patients with glioblastoma multiforme (GBM), exosomes from GBM stem-like cells contained high levels of functional VEGF-A, which induced angiogenesis and permeabilization of brain ECs *in vitro* (Treps et al., 2017). Collectively, these studies demonstrate how exosomes exploit various mechanisms to promote vascular leakiness at multiple metastatic sites in different cancer types.

Immune Function Blockade

Antitumor immunity mediated by immune cells such as natural killer (NK) cells and T cells that attack tumor cells is a natural defense against cancer. To overcome this barrier, tumor cells engage immunosuppressive mechanisms at metastatic sites, which involves recruitment of other immune cells that can suppress these antitumor responses (Grivennikov et al., 2010). Recruitment of immune cells is a hallmark of PMN establishment, and these cells have been shown to have immunosuppressive abilities. In particular, a key initiating event in PMN formation involved expansion of hematopoietic stem and progenitor cells in the BM and mobilization of these cells to PMNs (Kaplan et al., 2005) where they were reprogrammed into immunosuppressive myeloid lineages that block T cell-mediated antitumor immunity (Giles et al., 2016). Interestingly, exosomes induced recruitment of BM-derived cells (BMDCs) to lung PMNs (Peinado et al., 2012), and they also promoted accumulation of myeloid-derived suppressor cells and directly suppressed T cells and NK cells in lungs and liver of mice lacking tumors (Wen et al., 2016). Mature DCs also played important roles in antitumor immunity by presenting tumor antigens, but melanoma-derived exosomes impaired DC maturation in lymph nodes (LNs) (Maus et al., 2017). Because LN metastasis is critical for the progression of melanoma to systemic metastatic disease, these results illustrate the importance of exosome-mediated immune suppression throughout the development of metastasis.

Molecularly, diverse mechanisms underlie exosome-mediated immune modulation. B16F10 melanoma exosomes transferred activated cMet to BMDCs to promote a pro-metastatic phenotype and mobilization to PMNs (Peinado et al., 2012). Melanoma exosomes also mediated immune suppression through exosomal PD-L1 to suppress $CD8^+$ T cell function and facilitate tumor growth. Importantly, the level of circulating exosomal PD-L1 positively correlated with metastasis in melanoma patients, suggesting an important role for this mechanism of exosome-mediated immune suppression in promoting metastasis (Chen et al., 2018; Haderk et al., 2017; Monypenny et al., 2018). B16F10 melanoma exosomes contained small nuclear RNA that activated TLR3 expression in lung epithelial cells, which was essential for neutrophil recruitment to the lung for PMN formation (Liu et al., 2016b). In a GC model, exosomes expressing high mobility group box-1 (HMGB1) activated neutrophils by binding to neutrophil TLR4 receptors (Zhang et al., 2018b). Although the effect of neutrophil activation on PMN formation was not investigated in this study, these neutrophils may also influence PMNs, as in melanoma. In a PaC model, exosomes expressed many tumor-specific antigens. When secreted into plasma, these exosomes bound autoantibodies, creating a decoy for complement machinery and preventing an immune response against the cancer cells themselves (Capello et al., 2019). Conversely, exosomes derived from non-metastatic cancer cells promoted the expansion, recruitment, and differentiation of TRAIL-positive tumor-reactive macrophages, which kill and phagocytize tumor cells, contributing to diminished metastasis (Plebanek et al., 2017).

Educating the Neighborhood

As tumors form, they modify the stroma through soluble factors, exosomes, and direct cell-cell interaction. Moreover, BMDCs and fibroblasts in distant organs are a common target of primary tumor exosomes. In addition to promoting fibroblast activation, exosomes also induced extracellular acidification, an essential step in PMN establishment, by metabolically reprogramming human fibroblasts *in vitro* (La Shu et al., 2018). Multiple myeloma (MM) cells modified BMDCs to create a tumor-supportive environment. The modified BMDCs then secreted exosomes that induced MM tumor growth and promoted dissemination of tumor cells (Roccaro et al., 2013; Wang et al., 2014). Similarly, miR-221 from BMDC exosomes promoted GC cell migration, invasion, and matrix adhesion (Ma et al., 2017). Melanoma exosomes were shown to potentiate their own uptake by blocking

cholesterol 25-hydroxylase (CH25H), an oxysterol that inhibited exosome uptake, in normal cells; this was mediated by exosome-induced downregulation of IFNAR1 in BM cells and fibroblasts and promoted PMN formation (Ortiz et al., 2019).

Exosomes from primary tumor CAFs also impact metastasis. In BC models, CAF exosomes increased motility, migration, and invasiveness of cancer cells, which depended on Wnt signaling (Chen et al., 2017; Luga et al., 2012). Moreover, transfer of CAF-derived exosomal miRs promoted anchorage-independent cell growth, invasiveness, and bone metastasis of BC cells (Donnarumma et al., 2017; Sansone et al., 2017a).

Altered Extracellular Matrix

During PMN establishment, the ECM is altered by reorganization of pre-existing molecules or by new ECM deposition. These changes were promoted by many factors, including soluble factors, immune cells, and exosomes to create a permissive environment for CTC seeding and growth (Peinado et al., 2017). Cancer-derived exosomes modulated the ECM, and exosome-induced fibronectin deposition was described for both liver and lung PMNs. In liver, PaC exosomes carrying macrophage migration inhibitory factor (MIF) promoted TGF-β secretion from Kupffer cells, which induced fibronectin production by stellate cells (Costa-Silva et al., 2015). Similarly, uptake of BC exosomes by lung fibroblasts promoted their activation and fibronectin secretion (Hoshino et al., 2015). In addition, exosomal small nuclear RNA enhanced MMP9 and fibronectin expression in lung PMNs, thus promoting neutrophil recruitment (Liu et al., 2016b). In a rat PaC model, depletion of CD151 and Tspan8, which belong to the tetraspanin protein family, from exosomes impaired exosome-mediated PMN formation due to defects in ECM degradation, leading to decreased metastasis (Yue et al., 2015). PrC cells that were exposed to hypoxic conditions secreted exosomes enriched in cell-cell junction remodeling enzymes, leading to increased motility, invasiveness, and stemness of PrC cells targeted by these exosomes (Ramteke et al., 2015). EVs derived from highly metastatic OvC cells carried MMP1 mRNA that was transferred to mesothelial cells, where MMP1 expression and secretion were in turn upregulated to promote cancer progression (Yokoi et al., 2017). Finally, non-small cell lung cancer (NSCLC) cells modulated the expression of podocalyxin in exosomes, which in turn impacted integrin trafficking in fibroblasts and created a microenvironment supportive of tumor cell migration and invasion by introducing tumor-promoting ECM components (Novo et al., 2018).

Mystery of Organotropic Metastasis

Although the concept of organotropism, which describes the propensity for certain tumors to metastasize to specific organs, was initially proposed by Stephen Paget more than 120 years ago, the mechanisms underlying this aspect of metastasis remain elusive. Recent work on exosome-mediated metastasis has partially solved this mystery. Tumor-derived exosomes expressing particular integrin patterns, namely α6β1, α6β4, αvβ5, and αvβ3, that associated with ECM molecules, such as laminin and fibronectin, and certain cell types in target organs, partially dictated future PMNs at lung, liver, and brain organotropic sites (Hoshino et al., 2015). Furthermore, additional exosome adhesion molecules and other exosome components found on the vesicle surface, such as lipids, may also mediate selective adhesion and cell fusion of exosomes at sites of future metastases to contribute to organotropism. Studies on the role of exosomes in promoting metastasis also revealed that not all niches are alike, with lung, liver, bone, and LN PMNs displaying unique features. Critically, systemic transfer of EVs from tumors to metastatic sites such as lung and LN was visualized *in vivo*, providing direct proof for the ability of tumor-derived EVs to signal over long ranges (Zomer et al., 2015). This ability of exosomes to induce distinct microenvironmental changes at distant, future sites of metastasis substantiate their role in governing organotropic metastasis.

Lung, Liver, Bone, and Brain: Stationary Niches

Studies of lung metastasis have illuminated defining principles of exosome-mediated PMN formation, including recruitment of immune cells, education of resident cells, and stromal alterations. An initial description of PMN formation demonstrated that BMDCs expressing vascular endothelial growth factor receptor 1 (VEGFR1) homed to the lungs before the arrival of cancer cells. These cells interacted with the local stroma and generated receptive sites for future metastatic cells (Kaplan et al., 2005). Follow-up work demonstrated that primary tumor-derived exosomes were key mediators of this process. Melanoma exosomes expressing cMet were directly taken up by BMDCs, promoting their infiltration into the lungs where they contributed to the PMN characterized by vascular leakiness and promoted metastasis (Peinado et al., 2012). In addition to migratory immune cell recruitment to the lungs, exosomes directly targeted lungs to induce vascular leakiness in a BC model and modulated resident lung fibroblasts and epithelial cells in BC and melanoma (Hoshino et al., 2015; Liu et al., 2016b). This uptake of exosomes required expression of laminin-binding integrins α6β4 and α6β1 in exosomes, and exosomal α6β4 also supported fibroblast activation by promoting *S100* gene expression and Src signaling pathways (Hoshino et al., 2015). In epithelial cells, small nuclear RNA carried by melanoma exosomes activated the TLR3 receptor, resulting in recruitment of pro-metastatic neutrophils to the lung PMN (Liu et al., 2016b). Additional work showed that BC exosomes targeted ECs and downregulated cell-cell junction proteins to promote vascular leakiness (Zhou et al., 2014). Upregulation of pSTAT3 and p38MAPK-NF-κB signaling pathways in the lung stroma by Annexin II (Anx II) in BC exosomes was also associated with enhanced lung metastasis (Maji et al., 2017). Altogether, these studies of lung PMN formation show that exosomes take a multifaceted approach to PMN induction by targeting different cell types, inducing multiple stromal modifications, and activating various pro-metastatic signaling processes, all which coalesce to create an environment conducive to tumor cell colonization.

Liver metastasis is more common than primary liver tumors and is found in many types of cancers, especially of the gastrointestinal tract, breast, lung, and pancreas, and is associated with poor survival. In contrast to lung PMN formation, exosomes from PaC expressing integrin αvβ5 favored liver organotropism by binding to fibronectin-rich ECM in the liver and were uptaken mainly by mature, resident macrophages termed Kupffer cells to promote liver metastasis (Costa-Silva et al., 2015; Hoshino et al., 2015). This exosome uptake by Kupffer cells resulted in liver metastatic enhancement through subsequent activation of liver stellate cells, which secreted fibronectin to promote recruitment of BM-derived macrophages, and it required exosomal MIF

(Costa-Silva et al., 2015). Additional work supported these findings, demonstrating that PaC exosomes were not only taken up by Kupffer cells but also persisted in other macrophage populations that organized into pre-metastatic clusters and promoted metastasis (Pfeiler et al., 2019). Similarly, macrophages took up colorectal cancer (CRC) exosomes carrying miR-21, which bound to TLR7 and induced liver macrophage polarization. The activated macrophages then secreted inflammatory cytokines such as IL-6 and S100A family members, which supported liver metastasis (Shao et al., 2018). Our current understanding of liver PMN formation highlights macrophages as key players responsible for receiving and relaying tumor exosome messages for promoting liver organotropic metastasis.

Bone is a preferred metastatic site for many solid tumors and occurs in later stages of cancer. While in lung and liver metastasis, exosomes exert their effect through immune cells and stromal cells; in the bone, they mainly modulate local stromal cells, osteoclasts, and osteoblasts. Bone metastatic lesions are classified as osteolytic, which induce bone breakdown, or as osteoblastic, which increase bone production. They can also result from a disruption in the balance between osteoclast and osteoblast functions. In NSCLC, an osteolytic cancer, plasma exosomes highly expressed Amphiregulin, which bound to and continuously activated EGFR in pre-osteoclasts. This led to increased expression of proteolytic enzymes, osteoclastogenesis, and metastasis through osteoclast activation via RANKL upregulation (Taverna et al., 2017). L-plastin, an actin binding protein involved in cell invasion, was transferred via MDA-MB-231 exosomes to osteoclasts and induced osteolysis through RANK *in vivo* (Tiedemann et al., 2019). In MM, exosomes modulated pre-osteoclast migration and osteoclast differentiation, characterized by elevated osteoclast markers through activation of the CXCR4 pathway (Raimondi et al., 2015). In contrast, exosomes from an osteoblastic PrC were transferred to osteoclast progenitor cells, causing decreased proliferation and differentiation of osteoclast precursor cells, as well as a reduction in osteoclast differentiation markers (Karlsson et al., 2016). Additionally, exosomal miR-141-3p from PrC promoted osteoblast activity, tumor growth, and metastasis through decreased expression of DLC1 (Ye et al., 2017). Moreover, exosomal miR-940 was highly expressed in exosomes from osteoblastic cancers. Exosomal miR-940 transfer to mesenchymal stem cells *in vitro* promoted osteogenic metastasis (Hashimoto et al., 2018).

Brain metastasis is poorly understood, and the role of tumor-derived exosomes in mediating brain PMN formation and metastasis is unclear. Importantly, brain tropic exosomes expressing $\alpha v\beta 3$ integrin, but not other integrins that favor liver and lung organotropism, were shown to fuse with brain ECs (Hoshino et al., 2015). Different studies demonstrated that exosomes from BC induced brain metastasis by impairing the cell-cell junction protein ZO-1 in ECs, which led to increased BBB permeability (Zhou et al., 2014) and by activating p-STAT3 and phospho-p38-NF-κB in the brain stroma via exosomal Anx II (Maji et al., 2017). In another study, astrocyte-derived exosomes induced an oncogenic switch in tumor cells, which increased brain metastasis (Zhang et al., 2015). Although common paradigms of exosome-mediated metastasis have emerged for other organs, much remains to be learned about the brain. There is an unmet clinical need that justifies further investigation into the contribution of exosomes to brain metastasis, particularly for patients with small cell lung cancer, BC, and melanoma.

Immune Mediators: Traveling Niches

Tumor-secreted factors, including exosomes, play key roles in metastasis by influencing the immune system and modulating lymphangiogenesis. Additionally, metastasis in lymphatic organs occurs in many types of cancer and often serves as a transitory site that precedes further metastatic dissemination to other organs. One of the earliest characterizations of exosome-mediated metastasis showed that B16F10 melanoma exosomes traffic to sentinel LNs and facilitate PMN formation by increasing expression of genes related to angiogenesis, ECM modulation, and recruitment and growth of tumor cells (Hood et al., 2011). More recent work on melanoma showed that B16F10 exosomes were uptaken in non-draining LNs (ndLNs) by subscapular sinus (SCS) 169^+ macrophages. Normally, SCS 169^+ macrophages were tumor suppressive and blocked exosome dissemination by serving as a barrier. However, during tumor progression, this barrier was disrupted, allowing exosomes to enter the LN cortex and interact with B cells, which enhanced tumor growth (Pucci et al., 2016). Interestingly, Exo-Large exosomes from B16F10 cells were highly uptaken by LN compared to other exosome subsets, indicating they may have a role in LN metastasis and interaction with the immune system (Zhang et al., 2018a). In cervical squamous cell carcinoma, exosomes expressed high levels of miR-221-3p. Trafficking of miR-221-3p to lymphatic ECs (LECs) promoted migration and lymphangiogenesis through downregulation of vasohibin-1, a known inhibitor of lymphangiogenesis, *in vitro* and promoted LN metastasis in mice (Zhou et al., 2019). Exosomes from MDA-MB-231 BC cells promoted primary tumor growth and lymph metastasis by stimulating macrophage polarization to M2 tumor-supporting macrophages in the axillary LN (Piao et al., 2018). In CRC, exosomal IRF-2 induced VEGFC secretion by sentinel LN macrophages, which resulted in lymphangiogenesis and metastasis (Sun et al., 2019). CD97 is overexpressed in most GCs and is associated with tumor cell differentiation and aggressiveness. *In vitro*, exosomes from lymph tropic GC cells, which highly expressed CD97, enhanced cell proliferation and invasion. In mice, intra-footpad injection of CD97-expressing exosomes elevated expression of CD55, CD44v6, CD31, EpCam, CD151, and CD97 expression in the LN and enhanced metastasis, suggesting that CD97 supports PMN formation via interaction with other membrane receptors (Liu et al., 2016a). In hepatocellular carcinoma, SDF-1α/CXCR4 is important for invasion and migration. Transfer of exosomal CXCR4 from highly metastatic hepatocarcinoma (Hca-F) to less invasive hepatocarcinoma (Hca-P) enhanced migration and invasion of Hca-P by inducing MMP-9, MMP-2, and VEGF-C expression. Furthermore, exosomal CXCR4 from Hca-F cells promoted LEC proliferation and lymphatic tube formation (Li et al., 2018). These studies show that not only are exosomes critical in conditioning final metastatic sites but that they also mediate the ability of lymphatic sites to serve as weigh stations for tumor cells en route to their final destinations.

Clinical Implications

Because of the emerging importance of exosomes in cancer biology, many studies demonstrated the clinical relevance of

exosomes. In this section, we describe how exosomes mediate drug resistance and can be used in liquid biopsy for early detection or for therapy.

Drug Resistance by Exosomes

Drug resistance is a major hurdle in cancer therapy. Interestingly, exosomes originating from either tumor cells or CAFs can mediate chemotherapy and radiotherapy (RT) resistance. Originally, it was shown that drug-resistant cells could transfer resistance to drug-sensitive cells through membrane microparticles *in vitro* (Bebawy et al., 2009). Subsequently, work showing exosomal proteins and small RNAs, including long non-coding RNAs (lncRs) and miRs, impacted drug resistance provided mechanistic insight into this phenomenon. In OvC cells, paclitaxel induced miR-443 expression, which induced senescence in neighboring cells when transferred via exosomes, leading to resistance (Weiner-Gorzel et al., 2015). Similarly, CAFs and cancer-associated adipocytes secreted exosomal miR-21, which can be taken up by OvC cells. MiR-21 reduced the expression of APAF1, resulting in chemotherapy resistance and decreased apoptosis of OvC cells (Au Yeung et al., 2016). M2-polarized macrophage-derived exosomes reduced sensitivity to cisplatin, and miR-21 within these exosomes suppressed apoptosis and enhanced PI3K/AKT signaling in GC tumor cells (Zheng et al., 2017). CAF exosomes from PaC also contributed to therapy resistance; treatment of CAFs with gemcitabine increased the secretion of exosomes enriched in Snail and miR-146a. Together, these factors facilitated the survival of CAFs and tumor cells (Richards et al., 2017). In renal cell carcinoma, lncARSR was packaged into exosomes, thus transferring resistance to sensitive cells (Qu et al., 2016). The lncR UCA1 was increased in both tamoxifen-resistant BC cells and their exosomes. When tamoxifen-sensitive cells were treated with lncUCA1-enriched exosomes, they developed tamoxifen resistance and exhibited reduced apoptosis (Xu et al., 2016). Mesenchymal stem cell-derived exosomes are transferred to myeloma cells and confer proteasome inhibitor resistance via exosomal lncPSMA3-AS1 (Xu et al., 2019).

Exosomes can also mediate drug resistance by inducing the proliferation of CSCs. Stromal exosomes transferred 5′-triphosphate RNA to activate antiviral RIG-I dependent response in BC cells. This activation induced the expansion of CSCs, which mediated clinical resistance to chemotherapy and RT in basal-like BCs (Boelens et al., 2014). CAF vesicles mediated miR-221 transfer to BC cells, which led to the expansion of CSCs and resulted in hormone therapy resistance (Sansone et al., 2017a). Finally, exosomes mediated drug resistance independently of their RNA content. When myeloma cells were exposed to commonly utilized anti-myeloma drugs, exosome secretion was enhanced. These exosomes were enriched in heparanase, which remodeled the ECM and altered tumor and host cell behaviors leading to chemotherapy resistance (Bandari et al., 2018).

Therapeutic Deliverables

Exosomes are emerging as promising drug delivery agents because of their natural, nontoxic, and biodegradable characteristics and their ability to cross various biological barriers, including the BBB. One of the first demonstrations for this potential capability of exosomes came from work in which exosomes were engineered to target the central nervous system (CNS) by expression of Lamp2-RVG on the exosome surface. These exosomes were loaded with siRNA that yielded a specific depletion of target genes in the CNS upon systemic administration (Alvarez-Erviti et al., 2011). The retina is also a formidable biological barrier, and here, adenovirus encapsulated within exosomes (AAV2) showed improved ability to transduce the retina compared to conventional AAV alone (Wassmer et al., 2017). In cancer models, PaC patient exosomes were engineered to have enhanced ability to be taken up by recipient cells while also carrying siRNA therapeutics targeting oncogenic *KRAS*. When PaC tumor-bearing mice were treated with these exosomes, primary tumor growth was decreased, demonstrating the efficacy of this innovative approach (Kamerkar et al., 2017). Recently, the use of non-modified exosomes derived from pro-inflammatory immune cells was explored as a cancer therapy. Exosomes derived from M1 polarized antitumor macrophages displayed a tropism toward LN and were uptaken by local macrophages and DCs. The exosomes induced the release of pro-inflammatory cytokines leading to tumor growth inhibition and proved to be a more potent immunopotentiator than CpG, an immunostimulatory synthetic DNA oligodeoxynucleotide that contains CpG motifs to mimic the DNA of viral or bacterial infections, which is thus a powerful stimulator of the immune response (Cheng et al., 2017). Similarly, NK-derived exosomes induced apoptosis of B16F10 cells upon treatment *in vitro* and inhibited tumor growth *in vivo* (Zhu et al., 2017). Although these advancements in the use of exosomes for drug delivery hold promise for improving therapy, challenges remain. It will be necessary to identify and optimize exosome deliverables to promote maximal uptake at primary tumors and at metastatic sites.

The growing interest in exosomes as a therapeutic tool for cancer and other diseases has led to the development of exosome-like particles, which are often completely synthetic and, hence, suitable for pharmaceutical purposes. Furthermore, nanoparticle specificity could be modified for the target. For example, specificity was achieved by introducing a protein to the vesicle surface and was further increased through combinations of ligands (Peiris et al., 2018). Nanoparticles were also susceptible to siRNA loading by electroporation to induce a specific knockdown (Lunavat et al., 2016). Finally, exosome-like particles were shown to target metastatic sites; PEGylated liposome nanoparticles accumulated within lung metastatic sites less than 1 mm in size (Goldman et al., 2017). This pioneering approach might combine the therapeutic potential of exosomes with pharmaceutical practicality.

Exosomes Join the Liquid Biopsy Ranks

As exosomes represent their cell of origin, contain information in the form of biomolecules, and are secreted into the bloodstream, they are ideal candidates for non-invasive liquid biopsy and early detection. Thus, recent work has sought to identify the right biomarker or combination of biomarkers for each disease. In melanoma patients, increased expression of the immune checkpoint proteins PD-1 and CD28 in exosomes derived from T cells and DCs was found to be predictive of improved treatment response (Tucci et al., 2018). Additional work showed the ability to use miRs as a biomarker for different disease types. In acute myeloid leukemia, relapse remains a critical issue and is usually a result of minimal residual disease (MRD) following treatment;

however, early detection of MRD can dramatically prevent relapse. Combined expression of exosomal miR-150, -155, and -1246 was significantly different between serum of patients and healthy individuals, suggesting these markers can serve in early detection of MRD (Hornick et al., 2015). Exosomal miR-210 and miR-1233 expression levels were able to distinguish patients from healthy donors in clear cell renal cell carcinoma (Zhang et al., 2016). An increase in serum exosomal miR-19b-3p and miR-106a was found to be predictive of GC and to differentiate patients from healthy donors (Wang et al., 2017), and miR-7641 predicted CRC tumors (Chen et al., 2019b). Exosomal miR-223-3p expression was low in plasma exosomes of healthy donors, but its expression increased with BC disease progression (Yoshikawa et al., 2018). Finally, in PaC, high expression of exosomal miR-4525, miR-451a, and miR-21 was associated with recurrence and worse prognosis (Kawamura et al., 2019; Takahasi et al., 2018). Interestingly, DNA can also serve as a prognostic tool; in PaC patients, bulk exoDNA amounts and the frequency of specific exoDNA mutations were shown to be predictive of disease prognostics (Bernard et al., 2019; Yang et al., 2017).

A primary obstacle in implementing exosomes as a reliable liquid biopsy tool is establishing an optimal isolation method. Ultracentrifugation (UC) is the classical and most commonly used method of exosome isolation. While UC is robust and reproducible for tissue culture, biofluids are messy, and it is difficult to achieve a clean exosome preparation. Moreover, UC pools all exosome subpopulations together. Asymmetric flow field-flow fractionation (AF4) is a promising tool that can overcome the latter and provides rapid and reproducible results, but it requires specialized expertise in operating the instrument and analyzing the data it generates and large starting material (Zhang and Lyden, 2019). Other exosome isolation methods include microfluidic devices (MDs), sucrose gradients (SGs), size exclusion chromatography (SEC), and affinity-based exosome isolation kits (AfBs) (Chen et al., 2019a; Li et al., 2017). Though all of these methods have advantages, they lack robustness (MDs, SGs, and SEC) or specificity (AfBs). Therefore, finding a method that will be robust, reproducible, specific, and available for general use in the clinic is crucial for exosomes to take a leading position as a diagnostic tool.

Looking Forward

The work discussed here highlights how exosomes are crucial determinants of PMN development and metastasis in multiple cancers (Figure 1); however, several outstanding issues remain. Going forward, it will be necessary to establish a comprehensive understanding of exosome biology, particularly regarding mechanisms of distinct protein, RNA, and DNA packaging and which specific exosome biogenesis pathways are active in cancer cells. Because cancer exosomes contain unique cargo, deciphering these processes and how selective they are for cancer cells may uncover tumor-specific pathways for therapeutic targeting. Interestingly, recent work identified numerous compounds that inhibit both ESCRT-dependent and -independent exosome secretion, suggesting these drugs should be further tested for their ability to prevent *in vivo* cancer metastasis (Datta et al., 2018). Furthermore, inhibition of Rab22a was shown to decrease exosome secretion and impair metastatic phenotypes of BC cells (Sun et al., 2018), indicating that similar to Rab27a, targeting of Rab22a may also prevent *in vivo* PMN formation and metastasis. Finally, further investigation into the relationships between different exosome populations and their cellular origins may uncover additional therapeutic opportunities.

It is also not completely understood how exosomes reprogram recipient cells in PMNs and how durable the PMN is in the absence of exosomes. Studies so far suggest that continued exosomal transfer of proteins and miRs may be necessary for PMN maintenance, but whether more stable modifications due to epigenetic or genetic changes may also occur is less studied. Interestingly, recent work showing that pharmacological blockade of exosomal uptake is sufficient to revert the PMN and inhibit metastasis in melanoma suggests phenotypic plasticity is a feature of the PMN that can be exploited for therapy (Ortiz et al., 2019). However, because of the diversity of exosome-mediated mechanisms of PMN formation, it is possible that the PMN may be less reversible in other cancer models, so these findings should be validated in other contexts. Nevertheless, as detailed here, our current knowledge highlights an array of exosome-dependent pathways that are ripe for therapeutic targeting to treat metastasis. In addition to directly targeting exosomes for therapy, exosome-targeted therapies should also be considered for their potential ability to augment the potency of existing treatments, such as immunotherapy.

Many unanswered questions regarding the role of exosomes in regulating established metastases also remain. (1) Does the primary tumor continue supporting the established MN? (2) Are MN-derived exosomes distinct from primary tumor exosomes? (3) Do exosomes from the MN induce further changes in their immediate environment or educate PMNs in other distant organs? (4) Do MN exosomes return to the primary tumor? Investigating these areas will enhance our understanding of exosome-dependent metastasis for optimized use of exosomes as biomarkers of metastatic disease and therapeutic targets.

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