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Tumor-derived nanoseeds condition the soil for metastatic organotropism

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Abstract

Primary tumors secrete a variety of factors to turn distant microenvironments into favorable and fertile 'soil' for subsequent metastases. Among these 'seeding' factors that initiate pre-metastatic niche (PMN) formation, tumor-derived extracellular vesicles (EVs) are of particular interest as tumor EVs can direct organotropism depending on their surface integrin profiles. In addition, EVs also contain versatile, bioactive cargo, which include proteins, metabolites, lipids, RNA, and DNA fragments. The cargo incorporated into EVs is collectively shed from cancer cells and cancer-associated stromal cells. Increased understanding of how tumor EVs promote PMN establishment and detection of EVs in bodily fluids highlight how tumor EVs could serve as potential diagnostic and prognostic biomarkers, as well as provide a therapeutic target for metastasis prevention. This review focuses on tumor-derived EVs and how they direct organotropism and subsequently modulate stromal and immune microenvironments at distal sites to facilitate PMN formation. We also outline the progress made thus far towards clinical applications of tumor EVs.

Keywords

Tumor-derived EVs; Organotropism; Pre-metastatic niche reprogramming; Biomarker; Therapy

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Introduction

More than a century ago, Stephen Paget proposed the “seed and soil” hypothesis to explain the nonrandom pattern of cancer metastasis [1]. Paget’ s theory was strengthened when Isaiah Fidler provided the first experimental evidence for organotropic metastasis in the 1970’s. Fidler demonstrated that metastatic dissemination was not determined by nonspecific arrest of circulating tumor cells in the capillary bed of the first organ encountered, but rather it was dictated by both the properties of tumor cells (“the seed”) and distant organ environments (“the soil”) [2, 3]. Since then, much emphasis has centered on tumor cell biology while the impact of distant host tissues in metastasis remained elusive. However, in 2005, Lyden and colleagues discovered that tumors induced the formation of a pre-metastatic niche (PMN) prior to the arrival of metastatic cells. They showed that primary tumor-secreted factors promote vascular leakiness and upregulate the synthesis of extracellular matrix (ECM) components, including fibronection, in specific distal organ sites which in turn recruited VEGFR1 and VLA-4 (a fibronectin receptor) positive bone marrow-derived haematopoietic progenitor cells[4] to these sites. The changes in local stromal cells and the recruited bone marrow cells lead to the upregulation of pro-inflammatory mediators, including S100 proteins, creating an immune suppressive microenvironment and immune priveledged niche for incoming tumor cells.

In contrast to Paget’s theory where distant organs were thought to be inherently receptive to metastatic cells, Lyden’s further studies demonstrated that in addition to tumor-secreted soluble factors, including growth factors and chemokines[4, 5], tumor-derived extracellular vesicles (EVs)[6-8] also actively remodel these distal organs to support metastatic seeding.

Tumor-derived nano-sized EV subpopulations termed exosomes, contain a variety of proteins, lipids, RNA, and DNA, that are derived from the tumor cells and tumor-associated stromal cells. The heterogenous small EV subpopulations and their specific “cargoes” are not only responsible for creating the PMN but also for directing organotropic seeding of disseminated metastatic cells. Recent technical advances have allowed for high resolution separation of exosomes into large exosome vesicles (90-120 nm), small exosome vesicles (60-80 nm), and non-membranous exomeres (~35 nm) and has facilitated our understanding of molecular composition, biodistribution, and functions of the EV subtypes [9, 10]. The increased mechanistic understanding of tumor EV-mediated PMN formation and EV biogenesis have revealed potential opportunities to predict and prevent metastasis. For example, EVs may serve as surrogate biomarkers for the early detection of metastasis, and blocking EV biogenesis, secretion, and uptake may inhibit PMN formation to prevent metastasis.

This review will focus on how tumor-derived EVs facilitate PMN formation and direct organotropism and provide a brief summary of selective EV cargo molecules that support this process. In addition, therapeutic and diagnostic applications of tumor-derived EVs for the prevention and treatment of metastasis are also discussed.

EV cargos

EVs cargo consists of bioactive molecules, including proteins, glycans, lipids, and nucleic acids. While EV cargos generally reflect the biosynthetic status of the cell of origin, there are cases when EVs show an enrichment for specific bioactive molecules. For instance, ~35% of the entire spectrum of proteins produced in pancreatic ductal adenocarcinoma (PDAC) cells are present in EVs, which are associated with critical cancer pathways [11]. However, some proteins, lipids, and miRNA can be enriched in EVs as compared to the cell of origin. For example, EGFRvIII oncoprotein[12] and 11 miRNAs[13] are more highly concentrated in EVs than in the glioma cells of origin. While mechanisms detailing the trafficking of cellular molecules into EVs have been proposed[14], the underlying mechanism of cargo selection remains largely unknown.

Proteins are well-studied EV cargo and proteomic technologies have provided clues to EV biogenesis, targeting, and function. Notable EV protein classes include transmembrane proteins, such as tetraspanins, signaling receptors, and integrins, as well as intraluminal proteins, including heat shock proteins (HSPs), cytoskeletal proteins, endosomal sorting complex required for transport (ESCRT) proteins, RNA-binding proteins, and ribonucleoproteins [15]. Twenty-two consistently abundant proteins in EVs were recently identified from 14 cell lines irrespective of the isolation method, with most being transmembrane proteins and GTPases[16]. Secretory proteins such as growth factors and cytokines are normally exported through the classical endoplasmic reticulum (ER)/Golgi-dependent pathway[17, 18]. Whether these these secretory proteins can be packaged into EVs as an unconventional secretion pathway [19] remains controversial since proteomic detection of cytokines, chemokines, or growth factors is rarely reported. Meanwhile HSPs (e.g., HSPA8, HSP90AB1), tetraspanins (e.g., CD9), ESCRT proteins (e.g., TSG101), cytoskeletal proteins (e.g., ACTB, moesin), and GTPases (e.g., RAP1B) are common among EVs and, therefore, can serve as pan-EV markers. Other proteins, such as versican, tenascin C, and thrombospondin 2, are specific tumor tissue EV markers. Among various EV protein families, integrins are of particular interest due to their crucial role in guiding cellular tropism of EVs and their use as biomarkers of metastatic organotropism[8]. In contrast to integrins, some proteins can block EV uptake. For example, CD47, an anti-phagocytic signal, is ubiquitously expressed on tumor cell-derived EVs and prevents EV uptake by immune cells to prolong systemic EV circulation[20]. The enrichment of cancer-specific proteins in tumor-derived EVs provides a wealth of novel biomarkers for diagnosis and prognosis of various cancers. A number of EV protein biomarkers have proven clinical utility, including elevated levels of melanoma-specific protein TYRP2, VLA-4, HSP70, and the oncoprotein MET found in circulating EVs from melanoma patients [6]. Exploration of EV biomarkers specific to different cancer types can be a future direction to pursue due to the potential application to increase the yield of tumor-specific material and decrease unwanted background in downstream analyses.

Most types of EVs, including exosomes, microvesicles, and apoptotic bodies, are surrounded by a lipid bilayer originating from the endosome or cell membrane. As another example of cargo selection, EVs have been demonstrated to exhibit 2–3-fold enrichment of cholesterol, sphingomyelin, glycosphingolipids, and phosphatidylserine, but package lower

concentrations of phosphatidylcholine and phosphatidylinositol compared to lipids in cellular membranes. The lipid composition of EVs also varies based on cell of origin[21]. EVs isolated from melanoma and osteosarcoma were enriched in multiple saturated and unsaturated fatty acid as compared to melanocytes and osteoblasts. The tumor EV fatty acid cargo, particularly palmitic acid, induces tumor necrosis factor alpha (TNFα) secretion by Kupffer cells, which generates a proinflammatory microenvironment and promotes fatty liver formation [22]. In another lipidomic comparison of EVs derived from high- and low-metastatic triple-negative breast cancer cells, increased accumulation of unsaturated diacylglycerols was found within EVs from high-metastatic cells, which is associated with enhanced angiogenesis[23]. In addition to membrane-bound vesicle structures, Lyden and colleagues discovered a minuscule (~35nm) non-membranous EV subpopulation termed “exomere”, which contains less lipid content than exosomes [10]. Exomeres, as compared to exosomes, selectively package metabolic-associated proteins and enzymes. Significant variations in the relative levels of ceramide, triglyceride, lysophosphatidylglycerol, glycosphingolipid, and mitochondrion-specific lipids between exomeres versus exosomes were detected across different cancer cell-types.

In EVs, lipid and protein cargo are heavily glycosylated and reflect the glycosylation patterns of host cells [24]. Glycosylation profiles differ markedly between normal and tumor or metastatic cells, and characterization of glycan structure and glycosylation sites in EV cargo, might also identify new biomarkers for cancer diagnosis and prognosis. For example, EVs derived from a metastatic colorectal cancer (CRC) cell line showed increased O-GlcNAcylation of cadherin and ATPase superfamilies of proteins compared to EVs derived from non-metastatic cells [25]. In contrast, increased amounts of bisecting GlcNAc branches on integrin β1 in breast cancer or cancer cell-derived EVs interferes with interactions with galectin-3 and FAK/AKT signaling activation to block carcinogenesis and metastasis[26].

Distinct glycolipids on tumor cell-derived EVs also interfere with biological processes in recipient cells. For example, the disialoganglioside G_{D3}, a specific sialic acid-containing glycosphingolipid, is enriched in EVs secreted from melanoma and ovarian cancer cells and inhibits T cell activation and stimulates migration of normal cells [27, 28]. As glycosylated components are recognized by the ESCRT [29], manipulation of protein glycosylation via chemical or genetic engineering has been exploited to facilitate selective cargo sorting into EVs[30, 31]. A recent study overexpressing α (1,6) fucosyltranferase, an oncogenic-associated glycotransferase, in prostate cancer cells resulted in an increased abundance of proteins associated with metastasis in EVs derived from engineered cancer cells[32]. Glycosylation also impacts recognition and uptake of EVs by recipient cells and biodistribution. Surface glycosylation has been widely reported to suppress EV uptake[33-35]. Specifically, removal of N- and/or O-glycosylation in breast cancer cell-derived EVs enhances their uptake by endothelial cells[35]. O-deglycosylation of breast cancer cell-derived EVs enhanced EV accumulation in lungs, whereas N-deglycosylation did not alter biodistribution. Since N-glycosylation represents the majority of the cellular glycome, researchers developed nanosomes coated with N-glycans derived from various cancer cells as a model to study functional roles of surface N-glycosylation of tumor derived EVs and the impact on systemic dissemination and organotropic biodistribution[36].

High levels of mannose-type N-glycans on nanosomes leads to rapid clearance by C-type lectin-expressing tissue-resident dendritic cells (DCs) and macrophages. In contrast, reduced levels of mannose-type N-glycans and increased fraction of Neu5 Ac-terminated N-glycans prolong *in vivo* circulation and extrahepatic distribution.

Nucleic acid EV cargos can transfer genetic information to cells and alter the recipient cell's gene expression and phenotype. The most widely studied nucleic acid in cargos of EVs is RNA. In EVs, RNA subtypes include mRNAs and various non-coding RNAs (ncRNAs), such as small non-coding RNAs, miRNAs, transfer RNA fragments, small nuclear RNA (snRNA), small nucleolar RNA, PIWI-interacting RNA, long non-coding RNA (lncRNA), ribosomal RNA, mitochondrial RNA, and circular RNA (circRNA) . The small ncRNAs have a peak size of 200bp but EV RNAs can extend to 5kb or more [37].

miRNAs are the most abundant small ncRNAs in EVs and inhibit translation of target mRNA in recipient cells. Various miRNAs within tumor-derived EVs have been identified which promote PMN formation. For example, breast cancer cell-derived EV miR-200b-3p[38] and miR-21[39] are taken up by alveolar epithelial type II cells and osteoclasts and facilitate PMN establishment in lung and bone, respectively. MiR-105 is another breast cancer-derived EV miRNA that disrupts endothelial tight junctions, thus promoting metastases in the lung and brain[40]. Interestingly, a liver-specific miRNA, miR-122-5p, is highly enriched in EVs derived from lung cancer cells, which promotes migration and epithelial-mesenchymal transition (EMT) in liver epithelial cells to create a liver PMN [41]. Other tumor-derived EV miRNAs involved in PMN generation include hepatocellular carcinoma (HCC)-derived EV miR-1247-3p for lung metastasis [42] and colorectal cancer-derived EV miR-934 for liver metastasis[43]. snRNA is another small ncRNA subtype enriched in lung tumor-derived EVs and induces chemokine release from lung alveolar epithelium via Toll-like receptor 3 (TLR3) to recruit neutrophils to support lung PMN formation [44].

LncRNAs are ncRNAs longer than 200bp that possess diverse regulatory functions, including negative regulation of miRNAs by serving as "miRNA sponges", marking of mRNAs for degradation and transcriptional regulation of genes. Intriguingly, some lncRNAs, with relatively low cellular expression levels, such as HOX transcript antisense intergenic RNA (HOTAIR), are highly enriched in EVs[45], indicating an indispensable role of EV-transferred lncRNAs in biological processes. Elevated levels of HOTAIR in tumor EVs can facilitate metastases by blocking expression of the HoxD10 tumor suppressor gene which inhibits breast cancer cell migration and metastasis [46]. In addition, HOTAIR mediated loss of HoxD10 could lead to increased angiogenesis, although this has not been directly investigated [47]. A number of studies have demonstrated increased cancer cell proliferation, migration, and invasion upon transfer of EV lncRNA [48-52]. A large number of changes in lncRNA expression were observed in lung fibroblasts treated with breast cancer EVs, which resulted in increased proliferation and migration of fibroblasts[53]. While the impact of cancer-derived EV lncRNA on phenotypic changes in fibroblasts remains to be determined and awaits testing *in vivo*, this suggests cancer -derived EV lncRNAs could promote PMN formation.

In addition, some protein-coding RNAs, such as mRNA and circRNA, have been detected in tumor-derived EVs and expressed in EV recipient cells. Ovarian cancer cells secrete EVs containing mRNA encoding matrix metalloproteinase (MMP)1 and EV uptake in mesothelial cells leads to expression of MMP1 resulting in destruction of the peritoneal mesothelial barrier to allow tumor spread [54]. Similarly, circRNA encoding ubiquitin-like with PHD and ring finger domain 1 (UHRF1), a critical protein for regulating DNA methylation, is overexpressed in various cancer types, including HCC and was detected in HCC-derived EVs[55]. Upon EV uptake by NK cells, circUHRF1 is expressed and induces NK cell exhaustion by suppressing interferon (IFN)γ and TNFα secretion.

RNA packaging into EVs involves multiple mechanisms, including specific RNA sequence motifs and secondary configurations, differential affinity for membrane lipids, association with RNA-binding proteins (RBP), and other sorting signals such as ubiquitylation, sumoylation, phosphorylation, and uridylation RNA and RBP modifications [37].

EV-associated DNA was recently discovered within the past decade[56] and has not been as extensively investigated. Single-stranded DNA[57], double-stranded DNA (dsDNA)[58, 59], and mitochondrial DNA (mtDNA)[60, 61] have repeatedly been detected in EVs with the DNA located both within and on the surface of EVs. The predominant form of EV DNA is external dsDNA greater than 2.5kb in size, as indicated by >50% reduction in EV DNA post dsDNase digestion[58]. While the mechanism of DNA packaging into EVs remains largely unexplored, a recent study showed that dsDNA inclusion into tumor-derived microvesicles is regulated by activation of a small GTPase, ADP-ribosylation factor 6 [62]. EV-associated DNAs are derived from both nucleus and mitochondria and can reflect the genome mutational status of parental cells[58, 59]. This also raises the potential for using EV DNAs as biomarkers for cancer diagnosis and prognosis. As most cell-free DNA (cfDNAs) have been reported to associate with EVs [63, 64] and EV DNAs are relatively intact compared with non-vesicular cfDNA (~130bp in size), it is not surprising that EV DNAs show superior sensitivity and specificity compared to non-vesicular cfDNA in identifying mutations in patients with early-stage non-small-cell lung cancer [65]. In limited studies with EV DNAs derived from cancer cells, immune modulation was triggered by pathways downstream of EV DNAs in the recipient cell [56].Using chemotherapy-treated or irradiated tumor cells secreted EVs enriched with dsDNA, the cytosolic DNA damage receptor cyclic GMP-AMP synthase (cGAS) in recipient DCs was activated followed by subsequent recruitment and activation of stimulator of interferon genes (STING) resulting in cytokine release and anti-tumor immune responses[66, 67]. In addition, horizontal gene transfer and transcription of exogenous EV DNAs into recipient cells has also sparked much interest[56]. Donor cell-derived EV-genomic DNA localized to the nucleus of recipient HEK293 cells. Leukemia K562 cells secrete EVs containing the unique K562 genes, BCR/ABL hybrid gene involved in the pathogenesis of chronic myeloid leukemia. Uptake of K562-derived EVs by HEK293 cells induced the expression of BCR/ABL gene at both the mRNA and protein level[68]. The horizontal gene transfer of EV-associated DNAs is not limited to genomic DNA. Cancer-associated fibroblasts package mtDNA into EVs, and the subsequent uptake by hormone therapy-resistant breast cancer cells contributed to upregulation of mitochondrial genes necessary for oxidative phosphorylation[61]. Whether or how tumor-derived EV DNAs also contribute to PMN formation remains to be explored.

Metastatic organotropism

Metastatic organotropism refers to the ability of certain tumor types to disseminate to and colonize specific distant organs, such as lungs, liver, brain, and bone[69]. Although organotropism was first described by Stephen Paget well over a century ago, regulation of this crucial aspect of metastasis has largely remained a mystery. The poor understanding of the drivers of organotropism underlies the lack of effective predictive measures and therapies for metastasis. However, recent work has implicated tumor-derived EVs as critical determinants of metastatic organotropism. EV-dependent organotropism relies on the enrichment of specific molecules in organotropic cancer cell-derived EVs, as well as on reprogramming cell types unique to the various organ niches[7, 8, 44, 70]. Notably, EV-mediated organotropism depends on integrin adhesion molecules on the surface of tumor cell EVs, which bind specific extracellular matrices in metastatic organs. This adhesion enables uptake of EVs by cells at metastatic sites for PMN reprogramming.

Studies of lung and liver metastasis have been particularly insightful in revealing the important role of EV integrins in directing organotropic metastasis, illuminating how distinct EV integrin repertoires select for the specific extracellular matrices of these organs[8]. EVs derived from lung-tropic breast cancer cells have increased levels of integrins α6β1 and α6β4 compared to EVs from bone-tropic and brain-tropic breast cancer cells or liver-tropic pancreatic cancer cells. Importantly, these two integrins bind to the laminin-rich extracellular matrix of the lung microenvironment to promote targeting of breast cancer-derived EVs to the lung. Remarkably, education of mice with EVs derived from lung-tropic breast cancer cells was sufficient to confer lung metastatic outgrowth of bone-tropic breast cancer cells. By contrast, EVs from liver-tropic metastatic cancer cells carry αvβ5, which binds fibronectin in the liver, favoring hepatic uptake of EVs secreted by liver-tropic cancer cells.

Other EV integrins may also be essential for metastasis to additional organ sites. For instance, integrin α5 packaged into breast cancer derived EVs enabled breast cancer metastasis to bone by creating an osteogenic PMN following osteoblast uptake of integrin α5 EVs[71]. EVs secreted by brain-tropic metastatic breast cancer cells package integrins α2, α3, β1 and β3, suggesting a potential role in organtropic brain metastasis[8]. Many other integrins have been implicated in organotropic metastasis, indicating their loading into EVs may similarly promote EV-dependent PMN formation and organotropic metastasis[72]. Notably, EVs secreted by various cancers have been shown to participate in the preparation of a favorable lymphatic niche for tumor cell metastasis[73]. Tumor cell integrins that are critical for lymph node metastasis, namely integrin α4β1, may also support EV-mediated education of lymph nodes during cancer progression[72].

An additional key aspect of organotropism involves the uptake of EVs by particular cell types within each organ that contribute to generation of favorable niches for metastatic outgrowth. In lungs, EVs from lung-tropic cancer cells are uptaken by fibroblasts and epithelial cells[8, 44]. As a result, fibroblasts in the lung stromal niche become activated to express PMN factors, including fibronectin and S100 molecules. Fibroblast activation is mediated by integrin β4 which is enriched in lung-tropic EVs compared to other EVs[8]. Additionally, EV-educated type II alveolar epithelial cells in lungs increase secretion of

chemokines, namely CXCL1, CXCL2, CXCL5, and CXCL12, that attract pro-metastatic neutrophils to the PMN[44]. In the liver, pancreatic cancer cell EVs are taken up by liver-resident macrophages, known as Kupffer cells, which then secrete TGFβ to initiate a cascade of intercellular crosstalk resulting in activation of hepatic stellate cells, increased fibronectin deposition, and recruitment of pro-metastatic bone marrow-derived cells[7]. Organotropic brain metastasis is also regulated by the uptake of EVs by cells unique to the brain microenvironment, including microglia and brain endothelial cells to influence the brain vascular niche[70]. Cell migration-inducing and hyaluronan-binding protein (CEMIP), is distinctly enriched in EVs from brain-tropic cells and mediates the EV-dependent formation of a metastasis supporting vascular niche in the brain.

Reprogramming PMNs

Reprogramming PMN-associated stromal cells

A key step in PMN formation is reprogramming endothelial cells to increase adhesion, vascular permeability, angiogenesis, and lymphangiogenesis, which all facilitate adhesion, extravasation, and colonization of tumor cells at secondary organs. Vascular endothelial growth factor (VEGF) was the first reported molecule promoting PMN formation via induction of angiogenesis as well as mobilization and recruitment of $VEGFR1^+$ hematopoietic progenitors to the distant site[4]. In addition to being secreted as soluble factors, VEGF isoforms such as VEGF-A, VEGF-C and $VEGF_{90k}$ have been detected in EVs isolated from glioblastoma (GBM), pancreatic ductal adenocarcinoma (PDAC) and breast cancer cells, and increase vascular and/or lymphatic endothelial cell proliferation and permeability *in vitro*[74-76]. As EVs in circulating blood of GBM patients posess elevated levels of VEGF-A compared to healthy donors[74], the $VEGF^+$EVs could enhance vascular permeability and angiogenesis both locally and at a distance. Compared to normal melanocytes or poorly metastatic melanoma cell-derived EVs, nerve growth factor receptor (NGFR) is markedly upregulated in small EVs secreted by highly metastatic melanoma cells [77]. The EV-associated NGFR induces lymphangiogenic gene expression and VEGFR-3 phosphorylation, ERK and nuclear factor-κB (NFκB) activation, as well as intracellular adhesion molecule (ICAM)-1 expression in lymphatic endothelial cells (LECs), promoting endothelial cell proliferation and tumor cell adhesion. Annexin II, a $Ca2^+$-dependent phospholipid-binding protein associated with the plasma membrane and endosomal system, is one of the most highly expressed proteins in EVs that promotes breast cancer metastasis to the lung and brain via tissue plasminogen activator-dependent angiogenesis and activation of macrophages and proinflammatory signaling at the secondary organ[78]. Amphoterin-induced gene and open reading frame 2 (AMIGO2) is a novel cell adhesion molecule that can be transferred from tumor cells to the surface of endothelial cells via EVs. Hepatic sinusoidal endothelial uptake of AMGIO2-containing EVs derived from cancer cells, resulted in greater adhesion by gastric cancer cells which may facilitate liver metastasis [79]. Unsaturated diacylglycerols in EVs from highly metastatic breast cancer cells also promote angiogenesis and metastasis by activation of protein kinase D signaling in endothelial cells[23]. Several tumor cell-derived EV miRNAs and lncRNAs, such as miR-221-3p[80] and ELNAT1[81] correlate with lymphangiogenesis and lymphatic metastasis. EV-mediated ELNAT1, in particular, correlated with lymph node metastasis and poor prognosis in bladder

cancer patients[81]. Other EV-associated ncRNAs (e.g., miR-23a[82], CCAT2[83]) suppress the tight junction protein ZO-1, upregulate VEGF-A expression, and inhibit apoptosis of endothelial cells, thereby enhancing vascular permeability and angiogenesis in the tumor microenvironment. Whether these EV-associated ncRNAs similarly promote PMN formation at distant sites remains to be determined.

As mentioned above, activated fibroblasts are another subset of heterogenous stromal cells that are largely reprogrammed from resident tissue fibroblasts to facilitate PMN formation. Activated fibroblasts secrete ECM molecules (e.g., fibronectin (FN)) and MMPs, as well as transforming growth factor β (TGFβ), S100 calcium binding protein A4 (S100A4), interleukin (IL)-6, C-C motif chemokine ligand (CCL)2, and stromal cell-derived factor 1 (SDF1), which attract bone marrow-derived haematopoietic progenitor cells and forge a proangiogenic and antiapoptotic microenvironment permissive to incoming tumor cells[76]. Various tumor-derived EV factors have been linked to fibroblast activation and heterogeneity in distal tissues [84]. For instance, the primary CRC-derived integrin beta-like 1 (ITGBL1)-enriched EVs can activate lung fibroblasts and hepatic stellate cells (HSCs), the predominant resident fibroblasts in the liver, via TNFα-induced protein 3 (TNFAIP3)-mediated NFκB signaling[85]. The activated fibroblasts, in turn, produce high levels of pro-inflammatory cytokines, such as IL-6 and IL-8, and promote stemness, migration, and EMT of CRC cells. A similar integrin beta 1 (ITGB1)-NFκB signaling-mediated activation of lung fibroblasts was observed with miR-1247-3p-enriched EVs isolated from highly-metastatic HCC cells[42]. By directly targeting fibroblast β-1,4-galactosyltransferases III (B4GALT3), a glycosylation protein that inhibits ITGB1 activation and stability, exosomal miR-1247-3p promotes the formation of both intrahepatic metastasis niches and lung PMNs by primary HCC[42]. The correlation between nidogen 1 (NID1) in tumor cell-derived EVs or circulating EVs from HCC with metastatic potential, was recently identified. EV-NID1 not only destabilizes the vascular architecture and promotes angiogenesis in the lung, but also activates pulmonary fibroblasts to secrete soluble TNFR1, which facilitates HCC cell growth, mobility, and colonization into the lung[86]. A robust increase of tissue transglutaminase-2 (TG2), an ECM-crosslinking enzyme upregulated in metastatic cells, was detected in EVs derived from metastatic breast cancer cells. TG2 promotes FN dimerization to a fibrillar form on the EV surface, which in turn educates pulmonary fibroblasts to form a niche suitable for metastatic colonization[87]. In addition to tumor cells, cancer-associated fibroblasts (CAFs) at the primary tumor site also secrete EVs that may be more potent in inducing ECM remodeling of the PMN than tumor cell-derived EVs. For example, as compared to salivary adenoid cystic carcinoma (SACC) cell-derived EVs, EVs produced by SACC CAFs promote faster and more pronounced upregulation of known ECM-related PMN markers in the lung, including FN, MMP9, and lysyl oxidase (LOX), via activation of TGFβ signaling in lung fibroblasts[88].

Tumor-derived EVs can also directly orchestrate ECM architectural changes to facilitate PMN formation. Tumor EVs contain ECM molecules (ie., FN, tenascin-C [TnC]) and ECM-remodeling regulators (e.g., MMPs, LOXs, transglutaminases). EV secretion is required for appropriate extracellular deposition of TnC, an ECM glycoprotein that regulates cell adhesion to other ECM components, by several tumor cells and associated fibroblasts in breast cancer and PDAC[89]. EV TnC not only fosters distant ECM fiber nucleation, but it

also promotes invasiveness of incoming tumor cells by activating WNT/β-catenin and NFκB signaling[90, 91]. Several studies also demonstrated enzymatically functional EV-associated MMPs including MMP2, MMP9, and MMP14, as well as other proteinases in EVs shed from ovarian cancer, fibrosarcoma, and melanoma. These proteases degrade and remodel existing ECM at distant sites to create favorable paths for metastatic cells to enter the PMN[92, 93].

Reprogramming PMN-associated immune profiles

Initiation of proinflammatory and immunosuppressive signaling at distant sites by tumor-derived EVs has been widely reported across multiple cancer types[6, 94-98]. The majority of EVs show a similar size and structure to liposomes, which are known to undergo rapid accumulation and clearance by the reticuloendothelial system. The innate immune system, particularly macrophages, engulf a large fraction of circulating EVs secreted from the primary tumor and undergo immunological reprogramming. Alterations in macrophage polarization by tumor-derived EVs are well documented[99-104]. For example, EVs secreted by metastatic osteosarcoma cells direct alveolar macrophages to adopt a pro-tumor phenotype by inducing the release of immunosuppressive factors IL-10, TGFβ2, and CCL2, which in turn decrease phagocytosis and efferocytosis of tumor cells[101]. Uptake of PDAC-derived EVs enriched for macrophage migration inhibitory factor (MIF) by liver-resident Kupffer cells induced TGFβ secretion, which subsequently stimulated hepatic stellate cell (HSC) activation, FN deposition, and recruitment of immunosuppressive myeloid cells to generate the liver PMN [7]. Moreover, tumor cell-derived EVs can induce metabolic reprogramming of macrophages. Lung cancer cell-derived EVs altered glycolytic metabolism in tissue resident interstitial macrophages and upregulated their expression of the immune checkpoint programmed death ligand-1 (PD-L1), which curtails effector T cell-mediated anti-tumor immune responses in the pre-metastatic lung and draining lymph node[105].

In addition to tissue resident macrophages, tumor EVs can directly educate and mobilize various bone marrow-derived cells to promote metastatic progression. Metastatic melanoma, for example, horizontally transfers MET, an oncogenic receptor tyrosine kinase, to bone marrow progenitor cells via EVs, which reprograms recipient bone marrow cells to a pro-vasculogenic phenotype and increases lung and bone metastases[6]. EVs derived from several cancer types, including acute myeloid leukemia (AML)[106, 107], esophageal squamous cell carcinoma[108], breast carcinoma[109], and glioma[110] promote conversion of immature bone marrow progenitor cells or bone marrow-derived monocytes to myeloid-derived suppressor cells (MDSCs), leading to T and natural killer cell (NK) dysfunction. Moreover, the immunosuppressive potential of MDSCs can be further augmented by tumor-derived EVs that are enriched with heat-shock proteins, including HSP86[96], HSP70[111] and HSP72[112], and miRNAs, including miR-21[96, 113], miR-10a[114], miR-29a, miR-92a[115], miR-107[116], miR-155[117], miR-1246[110]. Not only do tumor EVs guide the differentiation of bone marrow cells towards immuno-evasive phenotypes, but also lung and breast cancer cell EVs block the differentiation of bone marrow progenitor cells into DCs[118, 119] and can inhibit maturation and migration of DCs[119], thereby obstructing tumor antigen presentation.

Neutrophils, the most abundant innate immune cells, are also stimulated by tumor-derived EVs and are instrumental in driving PMN formation. Neutrophil extracellular traps (NETs) are web-like structures composed of chromatin DNA filaments coated with granule proteins and released by activated neutrophils. NETs promote vascular permeability, ECM remodeling, EMT, proliferation, migration, invasion, dormant cell awakening and can prevent NK cell- and effector T cell-mediated tumor cell killing, and serve as an emerging PMN hallmark [120-126]. EVs released by breast cancer cells induce NETs in neutrophils pretreated with granulocyte colony-stimulating factor[127]. *In vitro* treatment of neutrophils with metastatic melanoma cell-derived EVs also induces NET formation and prolongs neutrophil life span to promote a pro-tumorigenic phenotype marked by diminished phagocytic and cytotoxic activity and increased chemotaxis [128]. Gastric and colorectal cancer-derived EVs also enhanced neutrophil survival and polarization towards an N2 pro-tumorigenic phenotype [129, 130]. In addition to these *in vitro* studies, breast cancer cell-derived EVs have shown systemic effects on NET induction[127] and further support the contribution of tumor EVs in NET formation at the PMN.

NK and T cell-mediated cytotoxic immunity are pivotal sentinels of anti-tumor immune surveillance. Compared to T cells, NK cells respond early to malignant cells to counteract tumor progression and metastasis independent of additional activation. Nevertheless, cancer cells exploit various strategies, including tumor-derived EVs, to impair NK cell recruitment, proliferation, and survival, as well as cytotoxic function[131]. For instance, exposure of NK cells to AML EVs resulted in ligand-mediated downregulation of C-X-C motif chemokine receptor 3 (CXCR3), a main chemokine receptor responsible for NK cell recruitment[132] and decreased NK cell migration[133]. Similarly, EVs derived from mesothelioma, breast cancer, and melanoma block IL-2-induced NK cell proliferation by downregulating IL-2 receptor (IL-2R) or blocking IL-2R downstream pathways[134, 135]. Chronic exposure of NK cells to ligands of NKG2D, the predominant activating receptor on NK cells, induces NK cell tolerance by downregulating NKG2D expression[136, 137]. Interestingly, NKG2D ligands (NKG2DLs), such as MHC class I-related chain (MIC) A and MICB molecules are shed into EVs from $NKG2DL^+$ tumor cells[138]. NK cells treated with these NKG2DL-enriched EVs display significantly reduced NKG2D and marked reduction in cytotoxic activity[138-140]. In addition to NKG2DLs, the high level of membrane-associated TGFβ carried by EVs isolated from tumor cells, including AML, renal cell carcinoma, mesothelioma, oral cancer, and PDAC, induce NK cell dysfunction by diminishing NKG2D levels[98, 133, 139, 141] and upregulating the expression of NKG2A (the main inhibitory receptor on NK cells) [142], which results in cancer progression. NK cells in PMNs generally show reduced maturation and cytotoxic effector functions[143]. However, the range of dynamic interactions between NK cells with tumor-derived EVs and the subsequent phenotype changes of NK cells within PMNs remain to be explored.

In addition, tumor-derived EVs also impact adaptive immune responses to favor metastatic progression. CD8+T cells are the dominant adaptive immune cell that conduct tumor-specific cytotoxicity. Interactions of tumor-derived EVs and $CD8^+$ T cells have been extensively studied, and the EV-bearing immune checkpoint molecules, such as TNF and TNF receptor (TNFR) superfamily and PD-L1, contribute to escape from T cell-mediated immune surveillance. Tumor EVs enriched with Fas ligand (FasL, a member of the

TNFR superfamily) and TNF-related apoptosis-inducing ligand (TRAIL, a member the TNF family of death factors) suppressed proliferation and promoted apoptosis in $CD8^+$ T cells in melanoma[144-146], PDAC[147], CRC[148], oral cancer[149, 150], head and neck cancer[151-153], and ovarian cancer[154]. Moreover, FasL-bearing EVs can polarize other Fas-expressing macrophages to maintain an immunosuppressive environment[155]. PD-L1, a B7 family ligand, is a coinhibitory signal regulating T cell responses via binding to PD-1. PD-L1 is frequently enriched in EVs secreted from various cancer types[156-163]. Apart from inducing local and systemic immunosuppression by suppressing T cell proliferation, mobility, cytotoxic function, and cytokine release, tumor-derived $PD\text{-}L1^+$ EVs can also compromise anti-PD-1/PD-F1 monoclonal antibody therapies[156, 164]. Other checkpoint molecules that have been detected on tumor-derived EVs or circulating EVs from cancer patient plasma include cytotoxic T-lymphocyte associated protein 4 (CTLA4)[155], as well as transmembrane, immunoglobulin, and mucin (TIM)3 and galectin-9 (the ligand for TIM3)[165], Interestingly, high levels of plasma EV TIM3 from non-small cell lung cancer (NSCLC) patients are associated with lymph node metastasis[165].

Adenosine is an ATP derivative that potently diminishes anti-tumor activities of $CD4^+$ and $CD8^+$ T cells and NK cells. Adenosine is generated by stepwise dephosphorylation of extracellular ATP via ectonucleotidases, including CD39 (ectonucleoside triphosphate diphosphohydrolase-1) and CD73 (5′-nucleotidase)[166]. CD39 and CD73 are present in EVs from breast, prostate, and colorectal cancers and glioblastoma, and contribute to ATP hydrolysis to adenosine and subsequent T cell dysfunction and inhibition of clonal expansion [167, 168]. Horizontal transfer of tumor cell-derived EV CD39/CD73 to NK and T cells leads to *in situ* deposition of adenosine and autocrine adenosine signaling-mediated loss of function[168, 169]. Other tumor-derived EV proteins that suppress T cell proliferation and activity include arginase I (ARGI) and TnC. Ovarian cancer-derived $ARGI^+$ EVs enzymatically deplete L-arginine, which is essential for T cell function, and inhibit $CD4^+$ and $CD8^+$ T cells and accelerate tumor progression[170]. TnC^+ EVs from glioblastoma inhibit T cell proliferation via interaction with α5β1 and αvβ6 integrins on T lymphocytes and suppress mammalian target of rapamycin (mTOR) signaling[171].

Tumor-derived EVs also expand immunosuppressive T regulatory cells (Tregs) to further restrict cytotoxic T cell function. Several studies have demonstrated that TGFβ-enriched tumor EVs skew expansion or induce phenotypic alteration of T cells towards Tregs[134, 172, 173]. Interestingly, following exposure to tumor cell EVs, transcriptional analysis in different T cell subsets showed that Tregs were more sensitive to EV treatment than other T cells, such as $CD8^+$ T cells[174]. Together, these findings imply a central role for Tregs in mediating tumor EV-induced immunosuppression.

Numerous EV-associated ncRNAs are also implicated in the suppression of effector T cells and NK cells and induction of Treg cells. For example, CRC-derived EVs contain miR-424 that induced resistance to immune checkpoint blockade (ICB) therapy by disrupting the CD28-CD80/86 costimulatory pathway in T cells and DCs[175]. Metastatic CRC cells release EVs enriched for lncRNA-SNHG10 that activates TGFβ signaling by targeting inhibin subunit beta C (INHBC) and impairs proliferation and cytotoxicity in NK cells [176]. SNHG16 is another lncRNA transmitted via breast cancer EVs to γδT cells to

induce a $CD73^+$ Treg phenotype via sponging miR-16-5p and activating the TGFβ/SMAD5 pathway[177].

Antibody producing B cells are another major component of adaptive immunity but the role of B cells in cancer progression is controversial. There are several studies linking B cell infiltration to a favorable clinical outcome in patients with NSCLC, ovarian cancer, and breast cancer[178-180] and increased response to ICB therapy in those with melanoma[181], mediated by anti-tumoral antibody secretion, antigen presentation, and T cell activation. On the other hand, tumor-promoting humoral immunity facilitates tumor development and metastasis via engagement of Fc receptors on myeloid cells [182], antagonizing anti-tumor antibodies [183] and activating tumor antigens, such as HSPA4[184]. B cell subsets, such as B regulatory cells[185-187], can also inhibit T cell or NK cell-mediated anti-tumor response and induce resistance to cancer therapies. Interestingly, tumor EVs can interact with B cells within the lymph node cortex when the subcapsular sinus macrophage barrier is compromised during tumor progression or by therapeutic agents. This fosters plasma cell amplification and tumor-promoting autoantibody production and generates a PMN in the lymph node [188].

Clinical application

Diagnosis and prognosis

The most immediate clinical application of EVs in cancer are as diagnostic and prognostic biomarkers. Several ongoing clinical trials have focused on circulating EVs to identify new EV cancer biomarkers to predict early metastatic progression (Table 1). A number of pre-clinical studies have verified the essential role of tumor-derived EVs in establishing a distant PMN, and a recent comprehensive proteomic analysis of human tissue explant-derived EVs has confirmed the existence of unique damage-associated molecular patterns, including S100A4, S100A13, basigin, and galectin-9, packaged in EVs from tumor tissues but not non-tumor tissues. These damage-associated proteins potentiate induction of pro-inflammatory PMNs that support future metastasis[189]. Notably, the study demonstrated that proteomes of plasma-derived EVs from cancer patients reflect alterations in distant organs and the immune system and thus have the potential to serve as PMN biomarkers. For example, liver-derived selenoprotein P is frequently found in plasma-derived EVs from lung cancer patients, but not in plasma EVs in healthy donors nor is it present in EVs shed by lung cancer tissues. Thus, the presence of selenoprotein reflects specific alterations in liver function induced by the primary lung tumor and represents a liver PMN marker[189].

Clinical observations also support the critical role of EV integrins in directing organotropism. Increased integrin β_4 on plasma EVs was detected in breast cancer patients with lung metastases compared to patients with only primary breast tumors or those with liver metastases. High integrin α5 expression in primary breast tumors correlates with the presence of disseminated tumor cells in bone marrow in breast cancer patients[190]. In contrast to patients with lung or bone metastases, plasma EVs from breast cancer patients with liver metastases contained upregulated integrin α_v[8]. Similarly, plasma EV-integrin α6A is a diagnostic marker that predicts PDAC recurrence and metastasis much earlier (e.g., one month after surgery) compared to conventional cancer antigen markers CEA

and CA19-9[191]. Establishing which and how EV integrins direct metastatic targeting in specific organs in various cancer types could yield a panel of biomarkers for early prediction of metastasis occurrence at different sites.

ncRNAs, especially miRNAs, are the most widely studied EV cargo from cancer patient liquid biopsies and exhibit great potential for predicting metastasis. For example, circulating exosomal miR-25-3p is differentially expressed in healthy donors, CRC patients without metastases, and CRC patients with metastases[192]. Exosomal miR-25-3p targets VEGFR2 and tight junction proteins in endothelial cells to promote vascular permeability and angiogenesis at the distant pre-metastatic site. Therefore, quantitative evaluation of miR-25-3p levels in circulating EVs may serve as an early indicater of CRC patients at risk for metastasis and inform the subsequent treatment course. While most clinical studies focused on individual EV-associated miRNAs, using a panel of different EV miRNAS could increase prognostic accuracy in patients likely to develop metastasis. This approach has been supported by applying a panel of plasma EV miRNAs to distinguish lung cancer patients with or without metastasis[193].

Treatment

Therapeutic targeting of EVs and EV cargo could also provide an approach to impair metastasis, for which there are no specific treatments. Specifically, pathways of EV biogenesis and uptake may be prime targets for such intervention. Exosome biogenesis occurs via the multivesicular body (MVB) endosomal pathway, where inward budding of the endosome membrane leads to accumulation of intralumenal vesicles (ILVs) contained within the endosome[194, 195]. The MVB then traffics to the plasma membrane where it fuses and ILVs are released from the cell as exosomes. Alternatively, vesicles known as ectosomes and of similar size to exosomes can bud directly off the plasma membrane. The MVB pathway of biogenesis has been extensively studied and several essential regulatory factors have been identified including ESCRTs, Alix, syntenin, and lipids, such as ceramide, that participate in ILV formation, and proteins that control trafficking and plasma membrane fusion of MVBs, such as Rab27, Rab35, Ral GTPases, and SNAREs[196]. Ectosome biogenesis pathways are less studied, but involve key proteins, such as ARF6, ARRDC1, and ESCRTs[197]. Uptake of EVs may occur via direct fusion with recipient cell plasma membranes or through endocytosis[194, 196].

Functional studies on the impact of EV biogenesis and uptake on metastasis indicate that inhibiting these pathways can reduce metastatic burden. Depletion of Rab27[6] and Ral GTPases[198] diminished lung metastasis in mouse models of melanoma and breast cancer, respectively. Furthermore, treatment of melanoma-bearing mice with the drug reserpine blocked EV uptake and attenuated lung metastasis[199]. Interestingly, screening efforts to identify existing compounds that inhibit biogenesis have found several small molecule candidates that could be repurposed for therapeutic targeting of EV-dependent metastasis[200-202]. These drugs, which include manumycin A, tipifarnib, neticonazole, climbazole, ketoconazole, triadimenol, and simvastatin, could decrease the levels of biogenesis and reduce EV production, but their effect on *in vivo* cancer metastasis requires further study. Importantly, as EVs have developmental and physiological functions in non

tumor cells, targeting biogenesis will require identifying potential cancer-specific pathways of EV formation.

Inhibiting EV uptake by recipient cells may be an additional therapeutic approach. In support, the anti-hypertensive drug, reserpine, was shown to block uptake of melanoma EVs, consequently diminishing PMN formation and impairing metastasis to lungs[199]. Organ-specific blockade of uptake could prevent unwanted side effects associated with systemic inhibition of EV uptake and perhaps be achieved by targeting EV integrins driving organotropic metastasis, such as α6β4 in lung-tropic EVs[8].

Conclusions and Perspective

The work discussed here emphasizes the critical role of circulating tumor-derived EVs in mediating communication between the primary tumor and distant organs (Figure 1). EVs direct the organotropic delivery of bioactive materials shed from primary tumors to recipient stromal and immune cells within the distant pre-metastatic site. Upon arriving at the distant organ, tumor-derived EVs modulate the function and phenotype of recipient cells via a broad array of pathways and mediators. Tumor EVs can initiate proinflammatory and immunosuppressive signaling as well as remodel the ECM to increase adhesiveness of incoming tumor cells. EVs can also promote angiogenesis and vascular permeability, thus allowing tumor cells to enter a nutrient-rich PMNs. Collectively tumor-derived EVs can reprogram hostile or neutral microenvironments into favorable “soil” for metastatic tumor cells to colonize and grow. Our knowledge of these EV-mediated events suggests several possibilities to improve diagnosis or treatment of metastatic disease. Blocking tumor-derived EV release and/or uptake by recipient cells could prevent the creation of the PMN and ultimately metastasis. More specifically, further investigation into the mechanisms of cargo loading into EVs, intracellular pathways supporting EV biogenesis as well as signaling events upon uptake of EVs in recipient cells, could identify unique targets. In addition, profiling of bioactive EV cargos in biological fluids is a non-invasive manner to evaluate their prognostic significance in cancer patients. Several practical issues remain, including the shed and elimination rates and whether the concentration of tumor-derived EVs in the circulation is sufficient to detect tumor EVs using current technologies. Recently, EVs from as little as 10 μL of patient plasma were successfully characterized and reflected EV phenotypic changes in patients with melanoma in response to treatment[203]. An asymmetric flow field-flow fractionation technique was developed to understand the heterogeneity of EVs with high resolution [10]. These technical advances will allow us to establish criteria for use of optimal EV subpopulations for therapeutics and prognostics and accelerate the clinical translation of the role of tumor EV-mediated PMN formation and metastatic progression.

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Data Availability

No data was used for the research described in the article.

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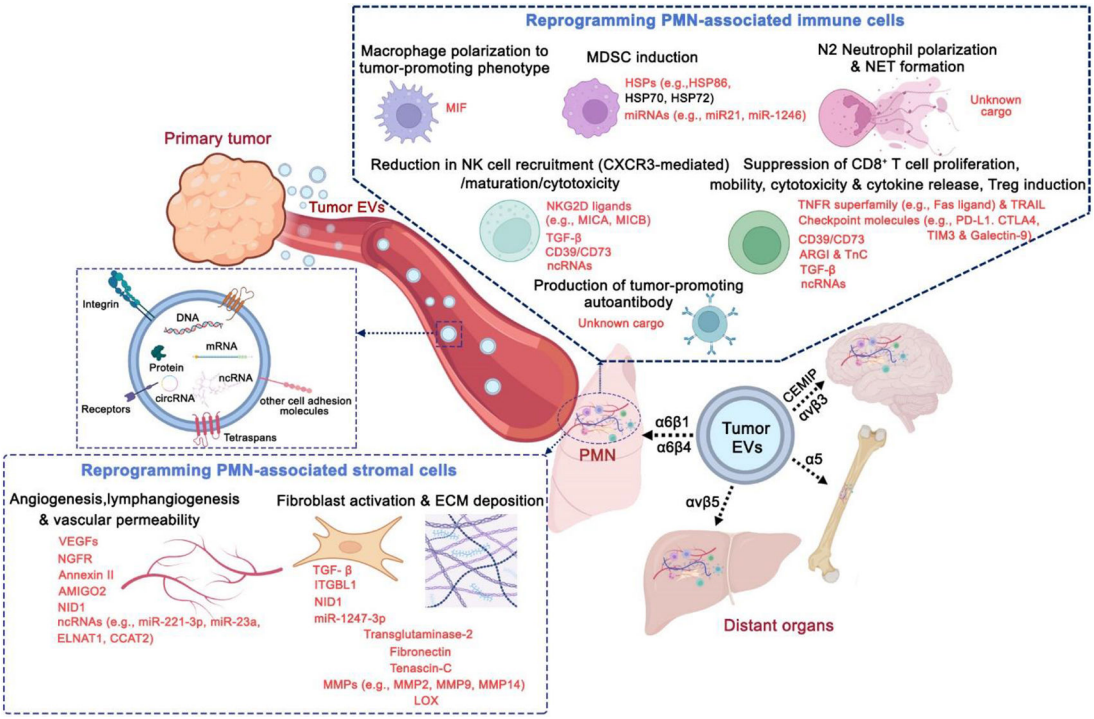

Figure 1.

Tumor EVs prime pre-metastatic niches (PMNs) by modulating stromal and immune cells at distal sites. Tumor EVs contain distinct integrins and cell adhesion molecules that determine their organotropism. Specific EV cargos attributed to stromal and immune microenvironment modulation at PMNs are highlighted in red.

Table 1

Summary of tumor EV-mediated reprogramming of stromal microenvironment in PMN

Stromal cell being targeted	PMN site	EV cargo	Reprogramming mechanism	Source tumor
Endothelial cells	Not defined	VEGF	Angiogenesis and endothelial permeability	GBM, breast cancer
	Lymph nodes, liver			PDAC
	Lymph node	NGFR	Angiogenesis and tumor cell adhesion	Melanoma
	Lung, brain	Annexin II	Tissue plasminogen activator-dependent angiogenesis	Breast cancer
	Liver	AMIGO2	Tumor cell adhesion	Gastric cancer
	Not defined	Unsaturated diacylglycerols	Angiogenesis via protein kinase D signaling	Breast cancer
	lymph node	miR-221-3p, ELNAT1	Lymphangiogenesis	Bladder cancer
Lung fibroblasts	Lung	TG2	Fibroblast activation via FN dimerization	Breast cancer
Endothelial cells, lung fibroblasts		NID1	Angiogenesis, fibroblast activation	HCC
Lung fibroblasts		miR-1247-3p	Fibroblast activation via inhibition of ITGB1 activation and stability	
Lung fibroblasts, hepatic stellate cells	Lung, liver	ITGBL1	fibroblast activation via TNFAIP3-mediated NFκB signaling	CRC
ECM	Not defined	TnC	Foster ECM fiber nucleation, promote invasiveness of tumor cells via WNT/β-catenin and NFκB signaling	PDAC, breast cancer
		MMPs (MMP2, MMP9, MMP14)	Degrade and remodel existing ECM	Ovarian cancer, fibrosarcoma, melanoma

Table 2

Summary of tumor EV-mediated reprogramming of immune microenvironment in PMN

Immune cell being targeted	PMN site	EV cargo	Reprogramming mechanism	Source tumor
Macrophages	Lung, brain	Annexin II	Activate proinflammatory signaling	Breast cancer
	Lung	Not defined	Secrete immunosuppressive factor release, decrease phagocytosis and efferocytosis	osteosarcoma cells
	Lymph node		Alter glycolytic metabolism, upregulate PD-L1 expression	Lung cancer
Kupffer cells	Liver	MIF	TGFβ secretion	PDAC
Hematopoietic progenitors	Lung, liver	VEGF	Mobilization and recruitment of hematopoietic progenitors to distant sites	Lung cancer, melanoma
Bone marrow derived cells	Lung, bone	MET	Pro-vasculogenic phenotype differentiation	Melanoma
	Not defined	MUC1, palmitoylated proteins	MDSC differentiation	AML
	Lymph node	miR-21	Monocytic MDSC differentiation	Esophageal squamous cell carcinoma, lung cancer, glioma
	Not defined	miR-10, miR-29a, miR-92a	MDSC differentiation	Glioma
		miR-107		Gastric cancer
		miR-155		Chronic lymphocytic leukemia
	Lung	miR-9, miR-181a		Breast cancer
	Not defined	miR-1246		GBM
		HSP86		Melanoma
		HSP70		Breast cancer, lung cancer, ovarian cancer
	Lung	HSP72		CRC, Lung cancer
	Not defined	Not defined	Induce immuno-evasive phenotype differentiation, block DC differentiation, maturation and migration	Lung cancer, breast cancer
Neutrophils			NET formation	Breast cancer
			NET formation, promote life span	Melanoma
		HMGB1	Promote life span and N2 polarization	Gastric cancer
		RNAs (long interspersed nuclear elements, short interspersed nuclear elements, and long terminal repeats)	Sustain survival and polarization towards a pro-tumorigenic phenotype	CRC
NK		Not defined	Reduce NK recruitment and migration by CXCR3 downregulation	AML
		TGFβ1	Impair NK proliferation by downregulation of IL-2R	Mesothelioma, prostate cancer
			NK dysfunction by downregulation of NKG2D	PDAC, AML, mesothelioma, prostate cancer, renal cell carcinoma

Immune cell being targeted	PMN site	EV cargo	Reprogramming mechanism	Source tumor
			NK dysfunction by downregulation of NKG2D and upregulation of NKG2A	Oral cancer
Treg			Skew expansion or induce phenotypic alteration of T cells towards Tregs	CRC
NK	Lung	Not defined	Block IL-2-mediated NK proliferation	Breast cancer
	Not defined	NKG2DLs (MICA/B)	NK tolerance by downregulation of NKG2D	Liver cancer, CRC, melanoma, mesothelioma, prostate cancer
	Liver	lncRNA-SNHG10	Impaire NK proliferation and toxicity by activation of TGFβ signaling	CRC
$CD8^+$ T	Not defined	FasL, APO2L/ TRAIL	Suppress $CD8^+$ T cell proliferation and promote apoptosis	Melanoma, PDAC, Ovarian cancer
	Liver	FasL, TRAIL		CRC
	Lymph node	FasL		Oral cancer, head and neck cancer
	Not defined	PD-L1	Suppress T cell proliferation, mobility, cytotoxicity and cytokine release	Melanoma, lung cancer, gastric cancer, prostate cancer, CRC, GBM, head and neck cancer, breast cancer
$CD4^+$ T, $CD8^+$ T, NK		FasL, TRAIL, CTLA4	Suppresse CD8+ T cell proliferation and cytokine release and CD4+ T /NK cell activation and promote macrophage differentiation into M2 phenotype	GBM
$CD4^+$ T, $CD8^+$ T	Lymph node	TIM3, galectin-9	Promote T cell exhaustion	Lung cancer
	Not defined	ARGI	T cell dysfunction by L-arginine depletion	Ovarian cancer
T cell, NK		CD39, CD73	T/NK cell dysfunction and inhibit clonoal expansion through adenosine production	Bladder cancer, CRC, prostate cancer, breast cancer, mesothelioma, GBM
T cell		TnC	Inhibit T cell proliferation	GBM
γδT cell	Lymph node	SNHG16	Induce a $CD73^+$ Treg phenotype	Breast cancer
B cell		Not defined	Promote plasma cell amplification and tumor-promoting autoantibody production	Melanoma

Table 3

Ongoing clinical trials involving EVs for the prediction of metastatic progression in cancer patients (data collected from http://clinicaltrials.gov, accessed in Dec 2022).

Clinical Trial	Cancer Type	Identifier
Contents of circulating extracellular vesicles: biomarkers in colorectal cancer patients	CRC	NCT04523389
Development of novel imaging and laboratory biomarkers to monitor the liver pre-metastatic niche and guide treatment of colon cancer: a pilot study		NCT03432806
Diagnostic and prognostic values of EUS-FNA specimens and circulating exosomal small RNA in patients with pancreatic cancer	PDAC	NCT04636788
Circulating exosomes as potential prognostic and predictive biomarkers in advanced gastric cancer patients: a prospective observational study (“EXO-PPP Study”)	Gastric cancer	NCT01779583
Exosomes-derived ncRNAs as biomarkers in cholangiocarcinoma patients	Cholangiocarcinoma	NCT03102268
Non-coding RNA in the exosome of the epithelia ovarian cancer	Epithelia ovarian cancer	NCT03738319
A prospective study of predicting prognosis and recurrence of thyroid cancer via new biomarkers, urinary exosomal thyroglobulin and galectin-3	Thyroid cancer	NCT03488134
Study of exosomes in monitoring patients with sarcoma (EXOSARC)	Sarcoma	NCT03800121
A pilot study of circulating exosome RNA as diagnostic and prognostic markers in lung metastases of primary high-grade osteosarcoma	Osteosarcoma	NCT03108677
Feasibility of exosome analysis in cerebrospinal fluid during the diagnostic workup of metastatic meningitis from breast cancer	Breast cancer	NCT05286684
Interest of circulating tumor DNA in digestive and gynecologic/breast Cancer	Breast cancer/ Digestive cancer/ Gynecologic cancer	NCT04530890
Validating the miR Scientific Sentinel™ platform (Sentinel PCC4 assay) in men undergoing core needle biopsy due to suspicion of prostate cancer for distinguishing between no cancer, low-, intermediate- and high-risk prostate cancer	Prostate cancer	NCT04100811
Quantification and purification of circulating prostasomes as diagnostic tool for prostate cancer detection		NCT03694483
A prospective, randomized blinded, shared decision impact trial of the ExoDx Prostate (IntelliScore), EPI test, in men presenting for initial biopsy.		NCT03235687
To investigate the diagnostic accuracy of exosomal microRNA in predicting the aggressiveness of prostate cancer in Chinese patients		NCT03911999
Identification and characterization of predictive factors of onset of bone metastases in cancer patients	Not specified	NCT03895216