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Exosomal proteins: Key players mediating pre-metastatic niche formation and clinical implications (Review)

MEI WANG[1], XINXIN ZHAO[1], FENG HUANG[2], LIN WANG[1], JIAYING HUANG[1], ZHENG GONG[1] and WANJUN YU[1]

[1]Key Laboratory of Medical Science and Laboratory Medicine of Jiangsu Province, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013; [2]Department of Clinical Laboratory, The First People's Hospital of Kunshan Affiliated to Jiangsu University, Suzhou, Jiangsu 215300, P.R. China

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Abstract. Tumor metastasis is a destructive characteristic of malignant tumors and the fundamental reason why malignant tumors are difficult to cure. The concept of a pre-metastatic niche (PMN) provides a novel way to elucidate the molecular mechanism of tumor metastasis. At present, the PMN has been considered as a critical determinant priming distal sites for metastasis. Accumulating evidence has suggested that exosomes are cellular communicators serving a pivotal role in mediating tumor cell metastasis by establishing the PMN. Among exosomal cargos, non-coding RNAs and proteins are two commonly studied components; however, the latter has received less attention. The present review aimed to summarize the findings regarding cargo proteins selectively loaded in malignant tumor-derived exosomes. Metastasis-associated proteins have been demonstrated to be selectively enriched in malignant tumor-derived exosomes. Exosomal proteins promote PMN formation to mediate the site-specific metastasis of tumor cells by inducing lymphangiogenesis, angiogenesis and permeability, educating stromal cells, remodeling the extracellular matrix, and suppressing the antitumor immune response. These exosomal proteins have great potential in predicting organ-directed metastasis and prognosis, as well as in cancer therapy.

Contents

Correspondence to: Dr Mei Wang, Key Laboratory of Medical Science and Laboratory Medicine of Jiangsu Province, School of Medicine, Jiangsu University, 301 Xuefu Road, Zhenjiang, Jiangsu 212013, P.R. China
E-mail: wangmei8417@163.com

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1. Introduction

Tumor metastasis is the primary cause of cancer morbidity and mortality. It is responsible for ~90% of cancer-associated deaths (1). Three theories have served a role in understanding the mechanism of metastasis. In 1928, Stevens and Ewing (2) proposed the 'anatomical mechanism theory' or 'metastatic fluid dynamics theory', which states that the fluid mechanics and blood vessels are involved in tumor metastasis. Fidler (3) noted that tumor cells derived from a tumor site could metastasize to a specific site, and the pre-metastatic microenvironment was formed even before the tumor cells reached the site. In 1976, Bross and Blumenson (4) proposed the 'metastatic waterfall theory', which states that tumor metastasis is complex, dynamic and a continuous biological process. According to the 'seed-soil' theory proposed by Paget (5) in 1989, tumor metastasis does not occur randomly. Tumor metastasis occurs when a favorable interaction occurs between metastatic tumor cells (the 'seed') and target organ microenvironment (the 'soil') (5). These three theories have aided the design of experiments and understanding of the basics of tumor metastasis. However, these three theories do not explain how the primary tumor drives target organ selection and metastasis. Previous studies have demonstrated that the primary tumor induces changes in the target organ microenvironment prior to metastasis (6-8).

The concept of a 'pre-metastatic niche (PMN)' was proposed in 2005 (9). According to this concept, the target tissue microenvironment undergoes a series of molecular and cellular changes to facilitate the proliferation of metastatic tumor cells (10). Therefore, metastasis depends not only on the ability of tumor cells to detach from the primary tumors and travel to distant organs, but also on the microenvironment of the target organ (11). In other words, a series of steps are involved in target organ metastasis. Furthermore, studies have demonstrated that the formation of the PMN is the rate-limiting

step (11-13). Therefore, the PMN theory fills the gap and helps understand how primary tumors drive target organ selection and metastasis. The PMN provides a favorable environment for colonization, survival and proliferation of tumor cells (11,13).

Exosomes contain various bioactive substances, such as proteins and nucleic acids, that serve a role in cell-to-cell communication (14). A previous study revealed that exosomes act as signals and trigger the formation of a pre-metastatic microenvironment (15). The majority of previous studies have focused on the role of exosomal non-coding RNAs in metastasis. To the best of our knowledge, the role of proteins in regulating PMN formation is not yet fully understood. Therefore, the present review focused on the role of exosomal proteins in PMN formation, and evaluated the potential of using these proteins for cancer detection and therapy.

2. Biological characteristics of exosomes

Discovery and biosynthesis. The term 'exosome' was first used in 1981. It was defined as a substance with 5'nuclease activity that is released from tumor cell lines (16). In 1987, Johnstone *et al* (17) studied the differentiation of reticulocytes and reported that exosomes were formed by the invagination of the plasma membrane. The 'redundant' plasma membrane released from cells was named 'exosome' (17,18). Exosomes are extracellular vesicles with a diameter of 40-150 nm and can be secreted from a variety of cells under physiological and pathological conditions (19). They are commonly found in body fluids, such as amniotic fluid, ascites, saliva, serum, plasma, breast milk, urine and cerebrospinal fluid (20). Therefore, they can serve as a biomarker for tumor diagnosis and for monitoring treatment (21,22). Extracellular vesicles can be divided into two main categories: Ectosomes and exosomes. Ectosomes are vesicles generated by the direct outward budding of the plasma membrane and have a diameter of 50-1,000 nm (23,24). Exosomes are intraluminal vesicles (ILVs) with a diameter of 40-160 nm formed through the fusion of multivesicular bodies (MVBs) to the plasma membrane and exocytosis (23). The formation of exosomes is a complicated process (23). The endogenous pathway can be divided into the following steps (Fig. 1): The cell membrane sags inward to form early sorting endosomes; the early sorting endosomes mature into late sorting endosomes; inward invagination of the late sorting endosomal limiting membrane leads to vesicles accumulation in the cavity, thereby generating MVBs; the ILVs are released from MVBs; the membranes of ILVs are fused with the cell membrane, and the internal vesicles that are discharged are exosomes (25).

Composition of exosome cargo and its delivery mode. The composition of exosomes is relatively complex and includes proteins, lipids, DNA, various types of RNAs, metabolites and glycoconjugates (26,27). According to the statistics released by Exocarta, 41,860 proteins, 1,116 lipid structures and 7,540 RNAs have been identified in exosomes (28). At present, increasing high-throughput detection shows that tumor cells and serum/plasma-derived exosomes of patients with cancer contain different amounts of cargo compared with corresponding cells in healthy individuals (29,30).

It has been revealed that the composition of exosomes serves a role in cellular communication (23). The cargo is delivered between cells in three ways: i) Through antigen presentation and receptor-ligand interaction, thereby activating specific signaling pathways and releasing protein and RNA contents into target cells; ii) through a direct fusion of exosome membrane proteins with the cell membrane of the target cell, so that the contents are released into the cytoplasm of the target cell; and iii) through content transport between cells via internalization mechanisms, such as endocytosis by target cells, phagocytosis or receptor-mediated endocytosis (31,32).

3. Metastasis-associated proteins enriched in exosomes

Exosomes contain a variety of biologically active molecules (e.g., microRNA, long non-coding RNA, mRNA, protein and lipids) (26,27). Proteins are the most abundant component of exosomal cargo (33). The differential expression of exosomal proteins between cancer and normal cells has been intensively investigated (30). Exosomes derived from malignant cancer cells have a distinctive oncogenic protein profile and selectively enriched metastasis-associated proteins (34). A few studies have directly investigated whether metastasis-associated proteins are present in exosomes derived from tumor cell lines (35,36). Metastasis-promoting lysyl oxidase-like 4 has been identified in hepatocellular carcinoma (HCC) cell exosomes (35). These exosomes, when transferred to cancer cells and endothelial cells (ECs), could promote cancer cell migration and angiogenesis (35). It has been considered that the ability of tumor exosomes to promote tumor metastasis is associated with the metastatic ability of cancer cells (36). Previous studies screened the protein profiles of exosomes derived from different cancer cell phenotypes (37-39). A comparison of exosomal proteins in mouse breast cancer cells with different metastatic abilities revealed that highly metastatic cancer cell exosomes were enriched in migration-, invasion- and angiogenesis-promoting proteins (37). Additionally, membrane proteins serving a crucial role in guiding target organ metastasis were detected in exosomes derived from metastatic cancer cells (37). Metastatic prostate cancer cells deliver integrin (ITG) $\alpha v\beta 3$ into the target cells via exosomes and increase their migration capacity (38). Furthermore, screening the protein profiles of exosomes helped analyze the differential phosphorylation status of the proteins, such as Annexin A2 and filamin-B (40). Weeraphan *et al* (40) performed phosphoproteomics and identified 43 differentially expressed phosphoproteins between the highly invasive cholangiocarcinoma cells and their corresponding control cells. Among these phosphoproteins, heat shock protein 90 (HSP90) was finally validated and associated with tumor metastasis (40).

A fluid biopsy is an important minimal invasive approach for monitoring cancer and diagnosis. Body fluids, including plasma, serum, urine and saliva, from patients with cancer have been used to isolate exosomes, which were further subjected to proteomics and compared with their corresponding controls, to explore the potential role of exosomal protein cargo for cancer detection (30). Plasma exosomes from healthy donors were used to treat cancer cell lines (41). Exosomes enhance cancer cell migration and invasion *in vitro* and dissemination *in vivo* (41). Metastasis-promoting proteins involved in the activation of focal adhesion kinase signaling have been

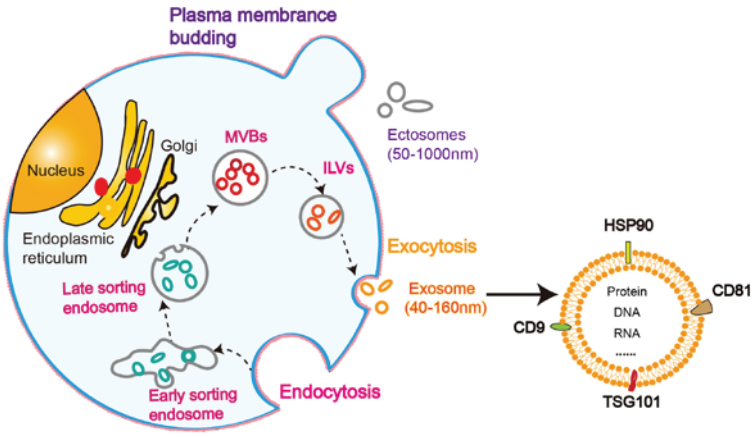

Figure 1. Exosome biosynthesis. Extracellular vesicles are divided into ectosomes and exosomes. Ectosomes are released through plasma membrane budding and have a size range of 50-1,000 nm. The production of exosomes involves two invaginations of the plasma membrane and the formation of intracellular MVBs. Finally, ILVs are secreted by MVBs fusing to the plasma membrane and exocytosis, forming exosomes with a diameter of 40-160 nm. The first invagination causes the merging of the ESE with a pre-existing ESE in certain cases. Additionally, the endoplasmic reticulum and Golgi are beneficial to the formation of ESEs. The ESE transits into LSE, and finally, MVBs are generated through inward invagination of the endosome restriction membrane (the second invagination). Exosomes loaded with different cargos, including DNA, RNA and proteins, are released when MVBs fuse with the plasma membrane. Membrane-associated proteins, such as CD81, TSG101, CD9 and HSP90, are used as specific markers of exosomes. MVBs, multivesicular bodies; ILVs, intraluminal vesicles; ESE, early sorting endosome; LSE, late sorting endosome; TSG101, tumor susceptibility 101.

identified on the surface of cancer cell exosomes (41). The proteome profiles of serum exosomes from patients with papillary thyroid cancer (PTC) with or without lymph node metastases (LNM) were compared, revealing that a total of 697 proteins, including several well-known and tumor metastasis-associated proteins, were selectively present in patients with PTC and LNM (42). Furthermore, 238 proteins were identified as the core urinary exosome proteome by analyzing the intersection of proteins from four urinary exosome proteome databases (43). Functional analysis of these proteins revealed that they were involved in biological processes, including regulation of cell movement and migration (43). A total of 86 proteins were identified by exploring the proteome profile of saliva and serum exosomes from patients with lung cancer. They were unique to lung cancer and associated with cancer progression (44). Exosomes secreted by both invasive breast cancer cells and glioma cells contain HSP90α, which increases cancer cell motility by converting plasminogen into plasmin (45).

It is well known that tumor stromal cells serve a pivotal role in tumor progression (46). Fibroblasts are the most abundant cell type in the tumor stroma. It has been demonstrated that the proteins present in exosomes derived from cancer-associated fibroblasts (CAFs) are also involved in tumor metastasis (47). Shimoda *et al* (48) generated tissue inhibitors of metalloproteinases (Timp)-deficient fibroblasts, which acquired the phenotype of CAFs. A disintegrin and metalloproteinase domain (ADAM) 10, present in the exosomes derived from Timp-deficient fibroblasts, is crucial for fibroblasts promoting breast cancer motility (48). Chen *et al* (49) reported that Wnt10b is densely packed into exosomes derived from p85α-deficient breast CAFs and mediates epithelial-to-mesenchymal transition of cancer cells. Based on the available evidence, it is clear that, in exosomes derived from body fluids of patients with cancer, cancer cells and cancer-associated stromal cells, metastasis-associated proteins are selectively enriched (Table I).

4. Exosomal proteins promote PMN formation

Studies have demonstrated that the integrins present in exosomes derived from tumor cells are involved in the formation of the PMN in target organs (39). Secretory integrins can be used as a predictor of metastasis (39). These findings emphasize the critical role of exosomal proteins in the formation of PMN in target organs and metastasis. Exosomal proteins are involved in the following important events, including lymphangiogenesis, angiogenesis and permeability, stromal cell education and extracellular matrix (ECM) remodeling and immunosuppression, which can contribute to PMN formation.

Lymphangiogenesis. LNM has been demonstrated to be an independent prognostic indicator of various types of cancer, including oral squamous cell carcinoma and cancer (50,51). Lymphatic vessels act as the initial route for tumor cell dissemination to the lymphatic system (52-54). Studies have demonstrated that LNM is a critical step for the systemic spread of tumor cells (51) by providing abundant blood vessels for tumor cells entering the blood circulation in lymph nodes (55). Furthermore, lymphangiogenesis in distant organs enhances the dissemination of tumor cells to these organs (51). Therefore, lymphangiogenesis is an important event involved in PMN; however, to the best of our knowledge, its underlying mechanism is not yet fully understood.

Recent studies have demonstrated that exosomal proteins serve a role in lymphangiogenesis (56,57). Lymphatic endothelial cells (LECs) are essential for lymphangiogenesis (58). Interferon regulatory factor 2, which is present in colorectal cancer cell exosomes, induces the secretion of vascular endothelial growth factor (VEGF) C by macrophages, thereby promoting LEC proliferation and lymphatic network formation required for sentinel LNM (56). Exosomal C-X-C chemokine receptor type 4 derived from mouse hepatocarcinoma Hca-F cells with high metastatic ability enhances LEC proliferation and lymphatic tube formation (57). Pancreatic

Table I. Metastasis-associated proteins enriched in exosomes.

First author, year	Sample type	Tumor	Protein	(Refs.)
Gangoda *et al*, 2017	Cancer cells with different malignant potentials	Breast cancer	Alix/TSG101	(37)
Emmanouilidi *et al*, 2019		Pancreatic cancer	ST14/LAMP1	(34)
Nakamura K *et al*, 2017		Ovarian cancer	CD44	(98)
Weeraphan *et al*, 2019		Cholangiocarcinoma	HSP90	(40)
Singh *et al*, 2016		Prostate cancer	ITGαvβ3	(38)
Zhang *et al*, 2019		Ovarian cancer	LBP/FGG	(99)
Ji *et al*, 2013		Colorectal cancer	MET/S100A8/S100A9/TNC	(100)
McCready *et al*, 2010		Breast cancer cells and glioma cells	HSP90α	(45)
	Cancer cells without specified phenotypes			
Liang *et al*, 2013		Ovarian cancer	EPCAM	(101)
Li *et al*, 2019		Hepatocellular carcinoma	LOXL4	(35)
DeRita *et al*, 2017		Prostate cancer	c-Src/IGF-IR/GRK/FAK	(102)
Li *et al*, 2011		NSCLC	LRG-1	(103)
Lu *et al*, 2017		Pancreatic cancer	Tetraspanins	(104)
Chen *et al*, 2019		Colorectal cancer	CCL2	(90)
	Body fluid			
Luo *et al*, 2018	Serum	Papillary thyroid cancer	SRC/TLN1/ITGB2/CAPNS1	(42)
Chen *et al*, 2017		Colorectal cancer	FN1/HSP90/MMP9	(105)
Zhang *et al*, 2019	Plasma	Ovarian cancer	FGA/GSN	(99)
Shtam *et al*, 2019		Breast cancer	ECM1/FGA/VTN/FN1/ THBS1/TLN1	(41)
Sun *et al*, 2017	Saliva	Lung cancer	AZU1/CD81/SLPI/DPP4	(44)
Erozenci *et al*, 2019	Urine	Prostate cancer, bladder cancer	FABP5/ EPS8L2/Mucin4	(43)
Greening *et al*, 2016	Stromal cells	Malignant mesothelioma	FN1/ITLN1/MAMDC2/ PDGFD/GBP1	(106)
Shimoda *et al*, 2014		Breast cancer	ADAM10	(48)
Luga *et al*, 2012		Breast cancer	CD81	(107)
Chen *et al*, 2017		Breast cancer	Wnt10b	(49)

Alix, apoptosis inducing factor 6 interacting protein; TSG101, tumor susceptibility gene 101; ST14, tumor suppressor 14; LAMP1, lysosomal associated membrane protein 1; ITGαvβ3, integrin αvβ3; HSP90, heat shock protein 90; LBP, lipopolysaccharide binding protein; FGG, fibrinogen gamma chain; MET, S100A8, S100 calcium binding protein A8; S100A9, S100 calcium binding protein A9; TNC, tenascin C; HSP90α, heat shock protein 90α; EPCAM, epithelial cell adhesion molecule; LOXL4, lysyl oxidase like protein 4; IGF-IR, insulin-like growth factor-Ireceptor; GRK, G protein-coupled receptor kinase; PRKD1, protein kinase D1; FAK, focal adhesion kinase; LRG1, leucine-rich α-2-glycoprotein; CCL2, C-C motif chemokine ligand 2; TLN1, Tlin1; ITGB2, integrin β2; CAPNS1, calpain, small subunit 1; FN, fibronectin1; MMP, matrix metalloproteinase 9; FGA, fibrinogen α1; GSN, gelsolin; ECM1, extracellular matrix protein 1; VTN, vitronectin; THBS1, thrombospondin 1; AZU1, azurocidin 1; SLPI, Antileukoproteinase; DPP4, dipeptidyl peptidase IV; FABP5, fatty acid binding protein5; EPS8L2, epidermal growth factor receptor kinase substrate 8-like protein 2; ITLN1, intelectin 1; MAMDC2, MAM domain-containing protein; PDGFD, platelet derived growth factor D; GBP1, G protein binding protein; ADAM10, ADAM metallopeptidase domain 10; NSCLC, non-small cell lung cancer.

ductal adenocarcinoma (PDAC) exosomes enhance lymphangiogenesis and tumor cell dissemination via the delivery of VEGFC into LECs (59). These findings suggest that LECs are important for exosomal protein-mediated lymphangiogenesis. Additionally, exosomal CD97 derived from SGC-7901 gastric cancer cells with high lymphatic metastatic potential increases the LNM capability of cancer cells with weak metastasis potential (60). This is accompanied by the induction of PMN-associated molecule expression in lymph nodes (60). Although there is no direct experimental evidence

for lymphangiogenesis in this study (60), the levels of proteins, such as transforming growth factor-β induced protein, which is an important lymphangiogenesis-promoting factor (61), are increased. Representative exosomal proteins modulating lymphangiogenesis are presented in Fig. 2A.

Angiogenesis and permeability. The generation of new blood vessels and increased vascular permeability are key steps in disseminating tumor cells. They provide tumor cells with the necessary nutrients and oxygen, and also increase vascular permeability, facilitating tumor cell intravasation and extravasation (62). Therefore, angiogenesis and increased vascular leaking have been defined as one of the characteristics of PMN (11). Studies have demonstrated the involvement of exosomal proteins in these two pathological processes (63,64).

Annexin II loaded in exosomes derived from malignant breast cancer cells promotes angiogenesis *in vitro* as shown by EC migration, invasion and tube formation assays, and *in vivo* as observed in a Matrigel plug assay (63). Additionally, exosomes collected from metastatic breast cancer cell lines with knockdown of annexin II have a reduced capacity in guiding targeted metastasis (63). Thrombospondin-1-enriched exosomes derived from metastatic breast cancer cells suppress the expression of tight junction proteins, including vascular endothelial cadherin (VE-cadherin) and zona occluden-1, leading to the increased leakage of vessels and enhanced transendothelial migration (64). The aforementioned two studies (63,64) explored the unilateral role of exosomal proteins in angiogenesis and permeability.

Studies have demonstrated that a few exosomal proteins serve a regulatory role in both angiogenesis and permeability (64-66). Exosomes from highly metastatic ovarian cancer cells contain soluble E-cadherin (sE-cad) (67). Exosomal sE-cad interacts with VE-cadherin to activate β-catenin and NF-κB signaling in human umbilical vein ECs (HUVECs), thereby promoting HUVEC migration, tube formation and permeability *in vitro* (67). Furthermore, sE-cad promotes angiogenesis *in vivo* and is positively associated with ascites formation and dissemination of tumor cells (67). Exosomal epiregulin derived from salivary adenoid cystic carcinoma cells increases the migration of human pulmonary microvascular ECs, tube formation and permeability *in vitro* and *in vivo*, leading to lung metastasis (65). In addition to the proteins enriched in exosomes, reduced levels of C-type lectin domain family 3 member B in exosomes derived from HCC cells promotes EC migration and invasion and increases VEGF release from HCC cells and ECs by activating adenosine 5'-monophosphate-activated protein kinase signaling to synergistically induce angiogenesis (66).

Cell migration-inducing and hyaluronan-binding protein (CEMIP) is specifically present in brain metastatic cancer cell exosomes (68). The delivery of CEMIP$^+$ exosomes into brain endothelial and microglial cells promotes EC branching and induces the expression of several pro-inflammatory cytokines that facilitate brain vascular remodeling and metastasis (68). Hypoxia is one of the main driving forces of tumor angiogenesis. It can stimulate the release of exosomes containing high levels of pro-angiogenesis proteins by tumor cells (69). Additionally, treatment of renal carcinoma cells with hypoxia-mimicking agent cobalt chloride results in elevated exosomal carbonic anhydrase 9 levels in HUVECs, and promotes HUVEC migration, tube formation and MMP2 expression (70). In addition to hypoxia serving as an inducer of pro-angiogenesis proteins in exosomes, exosomal Wnt5a from melanoma cells could increase the exosomal content of IL6, VEGF and MMP2, which confers increased pro-angiogenic function to malignant melanoma cells as observed by blood vessel formation assays (71). Representative exosomal proteins involved in angiogenesis and permeability are presented in Fig. 2B.

Stromal cell education and ECM remodeling. When cancer cells metastasize to distant target organs, they have to adapt to the new environment. The main components of these distant organ microenvironments are stromal cells and the ECM. Stromal cell education and ECM remodeling in target organs determine whether tumor cells can successfully colonize and metastasize (72,73).

Exosomes derived from different site-specific tropic cancer cells could be internalized by distinct resident stromal cells (39). Lung-tropic exosomes are mainly taken up by S100 calcium-binding protein A (S100A) 4-fibroblasts and surfactant protein C-epithelial cells in the lungs, while liver-tropic exosomes are mostly internalized by Kupffer cells in the liver (39). Brain-tropic exosomes are preferentially taken up by CD31-ECs in the brain (39). Specific integrins packaged into these exosomes induce distinct stromal cells to express S100 genes, which facilitate tumor metastasis (39). Prostate cancer exosomes selectively deliver pyruvate kinase M2 (PKM2) to bone marrow stromal cells (BMSCs) and educated BMSCs by inducing the secretion of C-X-C motif chemokine ligand 12 (CXCL12), which promotes cancer cell seeding and growth in the bone marrow to form bone metastasis (74). The exosomal transfer of MET proto-oncogene, receptor tyrosine kinase from melanoma cells with high metastatic ability to bone marrow-derived progenitor cells induces the acquisition of a pro-vasculogenic phenotype, which may further elicit cancer cell metastasis (75).

In addition to directly affecting tumor cells, the educated stromal cells are responsible for ECM remodeling, triggering a cascade effect and promoting PMN formation (74-77). Macrophage migration inhibitory factor (MIF) expressing PDAC-derived exosomes induces the secretion of TGF-β by Kupffer cells, which subsequently upregulates fibronectin generation in hepatic stellate cells (76). Such a fibrotic microenvironment induces the infiltration of bone marrow-derived macrophages that promote liver metastasis (76). Exosomal bone morphogenetic protein (BMP) derived from gastric cancer cells induces pericyte transition into CAFs by increasing the secretion of CAF markers (α-smooth muscle actin and fibroblast activation protein) in pericytes (77). The two markers are important for cancer cell motility (78). CAFs promote malignant tumor progression by regulating multiple cellular events, such as angiogenesis, ECM remodeling and metabolism (79). Exosomal BMP is an important signaling molecule that confers pericytes with CAF-like functions during cancer progression (77). Additionally, tumor-associated leukocytes induce breast cancer cells to express fibronectin and generate fibronectin-positive exosomes (80). The uptake of these exosomes by cancer cells produces pro-inflammatory cytokines

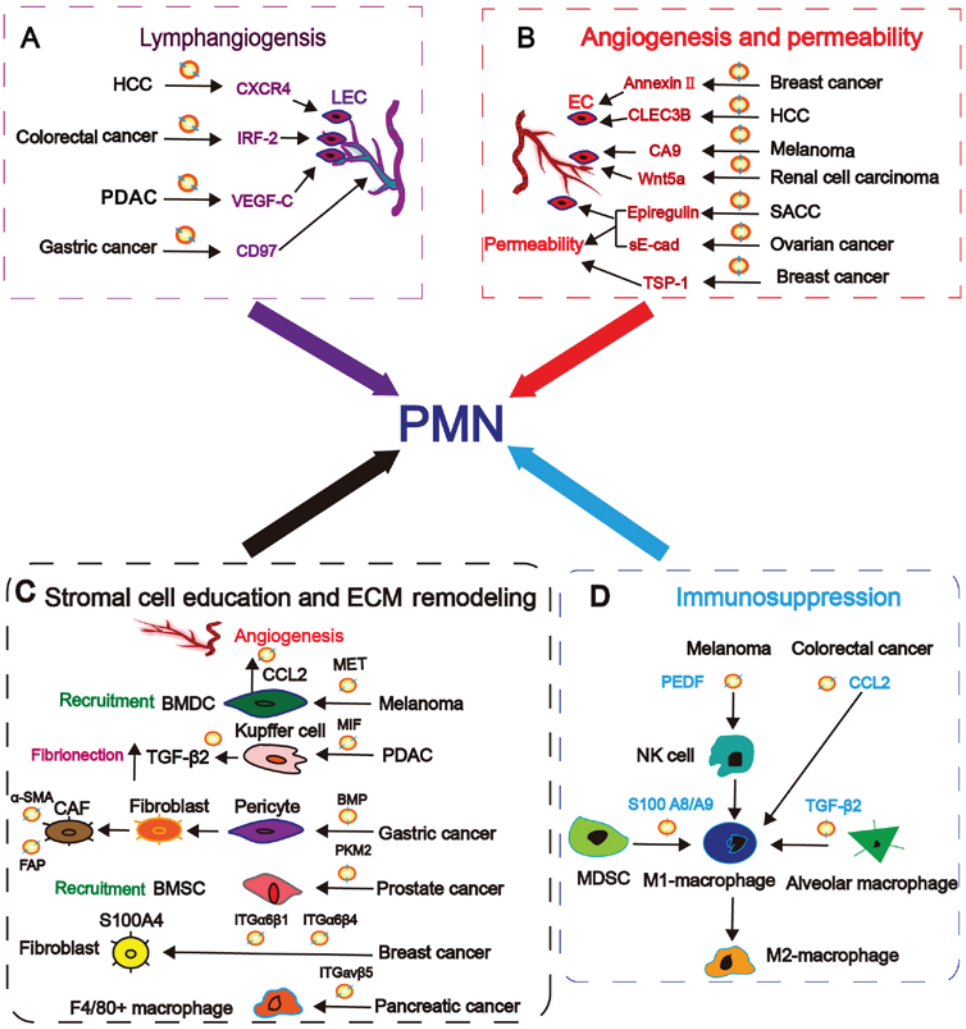

Figure 2. Exosomal proteins regulate PMN formation by inducing lymphangiogenesis, angiogenesis and permeability, stromal cell education and ECM remodeling, and immunosuppression. (A) Lymphangiogenesis. Colorectal cancer cell exosomal IRF-2, mouse Hca-F cell exosomal CXCR4 and PDAC exosomal VEGF-C act on lymphatic endothelial cells to induce lymphangiogenesis, whereas gastric cancer cell exosomal CD97 directly induces lymphangiogenesis. (B) Angiogenesis and permeability. Breast cancer-exosomal Annexin II, HCC exosomal CLEC3B, renal cell carcinoma exosomal CA9, and melanoma exosomal Wnt5a promote angiogenesis by regulating endothelial cells. Breast cancer exosomal TSP-1 induces vascular permeability by inhibiting the expression of tight junction proteins. Both exosomal epiregulin derived from SACC cells and exosomal sE-cad derived from ovarian cancer cells induce angiogenesis and permeability. (C) Stromal cell education and ECM remodeling. Melanoma cell exosomal MET converts BMDCs into the pro-vasculogenic phenotype by increasing the secretion of CCL2. Exosomal PKM2 derived from prostate cancer cells educates BMSCs to secrete CXCL12, leading to PMN formation in bones. PDAC-exosomal MIF induces Kupffer cells to secrete TGF-β2, which increases fibronectin levels and promotes ECM remodeling. Pancreatic cancer-exosomal ITGαvβ5 interacts with F4/80$^+$ macrophages in the liver with high levels of fibronectin, facilitating ECM remodeling. However, breast cancer exosomes packaged with ITGα6β4 and ITGα6β1 interact with S100A4-fibroblasts in lung tissues with high levels of laminin, which is also beneficial to ECM remodeling. Gastric cancer cell exosomal BMP converts pericytes into CAFs, which secrete a series of proteins, including α-SMA and FAP, thereby inducing ECM remodeling. (D) Immunosuppression. Melanoma cell exosomal PEDF recruits NK cells to the PMN, leading to the polarization of M1 macrophages, while MDSC exosomal S100A8/A9, colorectal cancer cell exosomal CCL2 and alveolar macrophage exosomal TGF-β2 induce the conversion of M1-type macrophages into M2-type macrophages. PMN, pre-metastatic niche; IRF-2, interferon regulatory factor 2; CXCR4, C-X-C chemokine receptor type 4; PDAC, pancreatic ductal adenocarcinoma; HCC, hepatocellular carcinoma; CLEC3B, C-type lectin domain family 3 member B; CA9, carbonic anhydrase 9; SACC, salivary adenoid cystic carcinoma; TSP-1, thrombospondin-1; sE-cad, soluble E-cadherin; MET, MET proto-oncogene, receptor tyrosine kinase; BMDCs, bone marrow-derived progenitor cells; ECM, extracellular matrix; PKM2, pyruvate kinase M2; BMSC, bone marrow stromal cells; CXCL12, C-X-C motif chemokine ligand 12; MIF, migration inhibitory factor; TGF-β2, transforming growth factor β2; ITG, integrin; S100A, S100 calcium-binding protein A; BMP, bone morphogenetic protein; CAF, cancer-associated fibroblast; α-SMA, smooth muscle actin; FAP, fibroblast activation protein; PEDF, pigment epithelium-derived factor; NK, natural killer; MDSC, myeloid-derived suppressor cell; CCL2, C-C motif chemokine ligand 2; LEC, lymphatic endothelial cell.

and MMP9, which further promotes cancer cell invasion (80). Furthermore, exosomal proteins could directly remodel the ECM by altering the related protein and protease levels in target sites. Under hypoxia, prostate cancer cell exosomes selectively increase MMP activity and expression, and promote fibronectin and collagen IV expression at the PMN (81). Additionally, the differential expression of proteins in exosomes has been screened and analyzed before and after inducing hypoxia, and the results suggested that exosomal protein-mediated ECM remodeling served an important role in the formation of the PMN (81). ITGα6β4 and ITGα6β1 enriched in lung-tropic exosomes interact with S100A4-fibroblasts in the laminin-rich lung tissues, whereas liver-tropic exosomes packaged with ITGαvβ5 interact with F4/80$^+$ macrophages in fibronectin-rich liver tissues (39). Despite no direct evidence indicating how ECM remodeling is regulated, the altered composition of the

ECM is likely caused by exosomal integrins, further suggesting that the interaction between integrin and the ECM determines the target site for metastasis (Fig. 2C) (33).

Immunosuppression. Exosomes released by tumors and stromal cells serve an important role in modulating the host immune response and creating an immunosuppressive microenvironment (82). In 2003, researchers revealed that the nonclassical human leucocyte antigen-G class I molecule, an immunosuppressive molecule, could be loaded into exosomes to help cancer cell escape from immunosurveillance (83). At present, immunosuppression is deemed as another important characteristic of the PMN (11). The immunosuppressed microenvironment shaped by exosomal proteins in primary lesions has been investigated (84). However, few studies have focused on the roles of exosomal protein-mediated immunosuppression in secondary metastasis sites. By contrast, exosomal proteins modulate the immunosuppressive microenvironment at the PMN mainly by recruiting immunosuppressive cells and polarizing the tumor-promoting phenotypes of immune cells (11,85-88).

Recruiting immunosuppressive cells is an important aspect contributing to the permissive microenvironment at the PMN (11). Myeloid-derived suppressor cells (MDSCs) are classic immunosuppressive cells recruited to the PMN (85). MDSC recruitment triggered by exosomal proteins at the PMN has been reported in two studies (86,87). S100A8/A9 enriched in MDSC-derived exosomes at the PMN could enhance chemotactic activity and further recruitment of MDSCs (87). Macrophages have the M1 phenotype with antitumor activity and M2 phenotype with tumor-promoting activity (89). Their phenotypic shift has been extensively studied in the formation of the immunosuppressed PMN (88,90). Colorectal cancer cell exosomal C-C motif chemokine ligand 2 (CCL2) recruits macrophages and confers the M2 phenotype to them in liver PMN (90). Exosomes derived from highly metastatic osteosarcoma cells exhibit an enhanced ability to induce alveolar macrophage secretion of TGF-β2 and transition into tumor-promoting M2 phenotype, thus suppressing their ability to kill tumor cells (88). However, this study (88) did not reveal which component in the exosomes mediates the immunosuppression regulation and this should be investigated in the future.

The role of exosomal protein-mediated immunosuppression in the PMN has been studied extensively. However, Plebanek *et al* (91) revealed that pigmented epithelium-derived factor, an outer surface protein of exosomes derived from less metastatic melanoma cells, could expand Ly6C^{low} patrolling monocytes (PMo) in the bone marrow, recruit natural killer cells, induce the differentiation of PMo into macrophages and M1 polarization, thus enhancing the capacity of PMo to kill tumor cells in lung tissues and suppressing lung metastasis (91). The contradictory aspect of exosomal proteins in immune response regulation increases the complexity of the PMN, indicating that the diversity of exosomal proteins in immune regulation should be further clarified (Fig. 2D).

5. Exosomal proteins involved in PMN formation for cancer prognosis prediction and therapy

Exosomal proteins have been demonstrated to serve an essential role in PMN formation and target organ metastasis. Therefore, it is important to know whether they have potential clinical applications. A review of the current literature revealed that most studies also paid attention to the potential prognostic and therapeutic implications of exosomal proteins. A study by Hoshino *et al* (39) was the first to uncover the role and molecular mechanism of exosomal proteins in organ-specific metastasis by inducing PMN. It was determined that different integrins were associated with site-specific metastasis, and integrins were specifically knocked down or integrin binding was blocked using peptides, and it was observed that integrins could efficiently suppress the arrival of exosomes to target organs and internalization of exosomes by stromal cells, and reduce metastasis. Notably, Hoshino *et al* (39) identified the upregulation of specific lung metastasis-associated ITGβ4 expression in exosomes derived from plasma of patients with lung metastasis. Patients whose plasma exosome contained a higher amount of ITGβ4 developed lung metastasis (39). The detection of binding partner ITGαV and liver metastasis-associated ITGβ5 in exosomes derived from plasma of patients with liver metastasis revealed similar results (39). MIF was critical for PDAC-derived exosomes creating fibrotic PMN in the liver (76). The knockdown of MIF blocked a series of events primed by PDAC exosomes involved in liver PMN formation and reduced liver metastasis (76). The plasma exosomal MIF level was increased in patients with PDAC with disease progression compared with those with no evidence of disease, indicating that exosomal MIF could be used as a prognostic marker in patients with PDAC (76). CEMIP is specifically enriched in exosomes of cancer cells with brain-metastatic ability and remodels brain vascular metastasis (68). Knockdown of CEMIP markedly reduces brain metastasis (68). Its content in exosomes derived from primary tumor cells could be used to predict the risk of brain metastasis and survival for patients with cancer (68). Prostate cancer cell exosomes deliver PKM2 into BMSCs and induce the secretion of CXCL12 for bone-specific metastasis (74). Targeted inhibition of BMSC education eliminates exosome-mediated bone metastasis (74). Higher levels of PKM2 in serum exosomes are associated with metastases (74). In addition to being a potential circulating biomarker for cancer detection, exosomal S100A8/A9 mediates MDSCs contributing to the immune-suppressive niche for metastasis and has been explored as an imaging marker for PMN formation (87). These findings suggested that proteins in exosomes involved in regulating PMN formation could be used as therapeutic targets and predictors of target organ metastasis.

The majority of studies have focused on exosomal protein cargo-mediated PMN formation. Lin *et al* (92) investigated the upstream regulators of pro-metastatic exosome generation and determined that aspartate β-hydroxylase (ASPH) specifically guides exosomal protein content (MMPs and ADAMs) assembly. Using an ASPH inhibitor could efficiently block its pro-metastatic effect *in vitro* and *in vivo* (92). Lobos-Gonzalez *et al* (93) revealed that knockdown of antisense non-coding mitochondrial RNA altered the protein cargo in breast cancer cell exosomes, which abrogated the regulatory role of exosomes in PMN formation and suppressed cancer metastasis. Chairoungdua *et al* (94) demonstrated that CD82 and CD9, as suppressors of tumor metastasis, inhibit tumor intracellular Wnt/β-catenin signaling by increasing

Table II. Potential of exosomal proteins involved in pre-metastatic niche formation for prediction and therapy of site-specific metastasis.

First author, year	Protein	Function	Clinical implication	(Refs.)
Hoshino *et al,* 2015	ITGβ4	Adhesion	Lung metastasis	(39)
Hoshino *et al,* 2015	ITGβ5	Adhesion	Lung metastasis	(39)
Costa-Silva *et al,* 2015	MIF	Antitumor immune response	Liver metastasis	(76)
Rodrigues G *et al,* 2019	CEMIP	Mediates depolymerization of hyaluronic acid	Brain metastasis	(68)
Dai *et al,* 2019	PKM2	Participate in glycolysis	Bone metastasis	(74)
Eisenblaetter *et al,* 2017	S100A8/A9	Remodeling immune-suppressive niche	Lung metastasis	(87)
Lin *et al,* 2019	ASPH	Participate in calcium homeostasis	Lung metastasis	(92)
Chen *et al,* 2019	CCL2	Inhibiting chemokine ligand	Liver metastasis	(90)
Zhao *et al,* 2020	S100A4	Metastasis-promoting protein	Therapeutic target of Lung metastasis	(95)
Qian *et al,* 2011	CCL2	Chemokines	Lung metastasis	(108)
Wang *et al,* 2019	Tspan8/CD44v6	Biomarkers of Pancreatic cancer-initiating cell	Dissemination in peritoneal cavity, lymph node, spleen, bone marrow, liver and lung	(109)
Jung *et al,* 2009	CD44v6	Mediating cell-cell and cell-matrix interactions	Lymph node and lung metastasis	(110)
Melo *et al,* 2015	Glypican-1	A cell surface proteoglycan acting an oncogenic role in cancer progression	A potential early biomarker for pancreas cancer detection	(111)
Liu *et al,* 2016	CD97	Adhesion	Lymph node metastasis	(60)
You *et al,* 2015	MMP13	Promoting nasopharyngeal cancer cell metastasis and angiogenesis	A potential therapeutic target	(112)
Dai *et al,* 2019	CLEC3B	Suppressing HCC metastasis and angiogenesis	An independent prognostic factor and potential therapeutic target	(66)
Yang *et al,* 2017	Epiregulin	Promoting SACC metastasis, angiogenesis and permeability	Lung metastasis	(65)

ITGβ4, integrin β4; ITGβ5, integrin β5; MIF, macrophage migration inhibitory factor; CEMIP, cell migration induced hyaluronic acid binding protein; PKM2, pyruvate kinase 2; S100A4 and S100A8/A9, members of the S100 protein family; ASPH, aspartate β hydroxylase; CCL2, C-C motif chemokine ligand 2; CD44v6, cd44v6 subtype; MMP13, matrix metalloproteinase 13; CLEC3B, C-type lectin domain family 3 member B.

the export of β-catenin into exosomes. Based on the universality of the intercellular transfer of exosomal proteins, the therapeutic effect of the upregulation of CD82 and CD9 may be limited.

Gene knockout can effectively inhibit the pro-metastatic effect of proteins in exosomes (90,95). Chen *et al* (90) explored the therapeutic effect of traditional Chinese medicine in targeting a pro-metastatic protein in exosomes. It was revealed that Dahuang Zhechong Pill suppressed CCL2 expression in colon cancer cell exosomes and subsequently reduced macrophage infiltration and transition to the M1 phenotype and ECM remodeling in distant organs (liver), thus impairing PMN formation for liver metastasis (90). The aforementioned studies (39,68,74,76,87,90,92-94) suggested that the factors mediating metastasis-promoting protein cargo assembly and the cargo proteins can be potential therapeutic targets to inhibit metastasis. Representative exosomal proteins that can serve as potential biomarkers for metastasis detection, prediction and therapy are listed in Table II.

6. Conclusions

Tumor metastasis, particularly distant metastasis, has been widely acknowledged as the main cause of cancer-associated mortality and the bottleneck of tumor cure. In the last decades, scientists have paid attention to this field and have performed numerous studies, aiming to elucidate the molecular mechanism of tumor metastasis. Based on their findings, several classical metastasis-related hypotheses have been developed, emphasizing the interaction between

'seeds' and 'soil' and establishing the concept of the PMN (9). Tumor cell exosome-loaded integrins have been revealed to serve a decisive role in directing tumor cells to form secondary metastasis by reshaping permissive PMN in target organs (39). These findings suggest the following: i) Distant metastasis is not random but directional; ii) PMN formation in secondary organs is crucial for organ-specific metastasis of tumor cells; and iii) tumor cell-derived exosomes are important mediators that trigger and establish the PMN in target metastatic organs. Subsequently, studies analyzed the role and mechanism of action of tumor cell exosomes in PMN formation in different tumors with organ-specific metastases. They further demonstrated that exosomes serve a critical role in dictating PMN formation by affecting a number of aspects, and different exosomal cargos are involved in this process.

Due to the complexity of exosomal cargo, different studies have focused on different cargos. At present, non-coding RNAs and proteins are the two most extensively studied components encapsulated by exosomes mediating PMN formation; however, the former have received more attention. Non-coding RNAs are of numerous types, among which microRNAs and long non-coding RNAs in exosomes have been under intensive investigation and have been demonstrated to serve vital roles in PMN formation (96,97). Non-coding RNAs perform their biological functions through complex and diverse regulatory mechanisms, and their downstream proteins are the real effector molecules. By contrast, the function of proteins is much clearer than that of non-coding RNAs. Furthermore, high-throughput proteomics and protein function annotation have demonstrated that metastatic tumor cell exosomes contain metastasis-promoting PMN formation-related proteins, thus providing a basis for studying the key role of exosomal proteins in PMN formation. Therefore, understanding exosomal protein-mediated PMN formation in directed metastasis is an important direction in the field of tumor metastasis research. The synergistic effects of exosomal proteins and other molecules within exosomes should be evaluated, since this can facilitate an improved understanding of the underlying mechanisms of tumor metastasis. It could provide information on potential effective molecular targets for the prediction and treatment of secondary organ-specific metastasis. Furthermore, it is worth noting that exosomal proteins from stromal cells have been demonstrated to be involved in PMN formation. Investigations should not be restricted to tumor cell-derived exosomes. In other words, the role of exosomal proteins from other cells in PMN formation should not be ignored in the future.

In summary, metastasis-associated proteins have been demonstrated to be enriched in exosomes. However, few studies have been conducted to explore the role of exosomal proteins in the PMN. The findings demonstrated that exosomal proteins promote PMN formation and mediate site-specific metastasis of tumor cells by inducing lymphangiogenesis, angiogenesis and permeability, educating stromal cells, remodeling the ECM, and suppressing the antitumor immune response. Exosomal proteins mediating PMN formation have great potential in predicting organ-directed metastasis and prognosis, as well as in cancer therapy.

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Authors' contributions

MW and XZ collected the related papers, drafted the manuscript, initiated the study, revised and finalized the manuscript. FH, LW, JH, ZG and WY participated in the design of the review. MW and XZ were responsible for confirming the authenticity of the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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