Minireview

Regulation of lymphangiogenesis by extracellular vesicles in cancer metastasis

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Impact statement

This review article summarizes and highlights the most up-to-date information regarding extracellular vesicle-mediated lymphangiogenesis and its impact on cancer metastasis. Although the exploration of biogenesis and the biological functions of extracellular vesicles are still in its infancy, many previously believed concepts are revisited and/or revised. More and more studies reveal that extracellular vesicles are versatile and efficient communicating carriers that carry numerous kinds of messengers. Understanding how cancer cells utilize this extracellular vesicle-mediated lymphangiogenesis for dissemination may shed light on developing novel preventive or therapeutic strategies in the battlefield against cancer.

Abstract

Metastasis is not only one of the hallmarks of cancer but, unfortunately, it also is the most accurate biomarker for poor prognosis. Cancer cells metastasize through two different but eventually merged routes, the vasculature and lymphatic systems. The processes of cancer metastasis through blood vessel have been extensively studied and are well documented in the literature. In contrast, metastasis through the lymphatic system is less studied. Most people believe that cancer cells metastasize through lymphatic vessel are passive because the lymphatic system is thought to be a sewage draining system that collects whatever appears in the tissue fluid. It was recently found that cancer cells disseminated from lymphatic vessels are protected from being destroyed by our body's defense system. Furthermore, some cancer cells or cancer-associated immune cells secrete lymphangiogenic factors to recruit lymphatic vessel infiltration to the tumor region, a process known as lymphangiogenesis. To ensure the efficiency of lymphangiogenesis, the lymphangiogenic mediators are carried or packed by nanometer-sized particles named extracellular vesicles. Extracellular vesicles are lipid bilayer particles released from eventually every single cell, including bacterium, with diameters ranging from 30 nm (exosome) to several micrometers (apoptotic body). Components carried by extracellular vesicles include but are not limited to DNA, RNA, protein, fatty acid, and other metabolites. Recent studies suggest that cancer cells not only secrete more extracellular vesicles but also upload critical mediators required for lymphatic metastasis onto extracellular vesicles. This review will summarize recent advances in cancer lymphatic metastasis and how cancer cells regulate this process via extracellular vesicle-dependent lymphangiogenesis.

Keywords: Lymphangiogenesis, lymph node metastasis, exosome, extracellular vesicles

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Lymphatic metastasis and lymphangiogenesis in cancer progression

Cancer metastases account for up to 90% death in at least 2/3 solid tumors.[1] The process of metastasis is considered inefficient, which requires sequential biological processes from initiation to completion. Typically, cancer cells may first acquire abilities to detach, migrate, and invade into the basement membrane and extracellular matrix (ECM). Following local invasion, cancer cells intravasate endothelial barriers and enter the circulation, where they survive and escape immune surveillance, and then adhere to the endothelium of capillaries of the target organ. After extravasation, they have to implant and grow at secondary sites.

Through an alternate route, solid tumors frequently metastasize through the lymphatic system. T1 breast cancer has 18–29% rate of sentinel lymph node (LN) metastasis,[2] and 67% of LN metastasis was observed in pancreatic cancer.[3] Lymphatic systems are a network of organs, tissues, and vessels that have vital functions including removal of interstitial fluid from tissue, transport of

immune cells, and absorbance of fatty acid from digestive systems. Lymphatic and blood vessels work closely as lymphatics remove fluids that leak out of blood vessels in tissues and return them back to the bloodstream. Compared to the structure of blood vessels surrounded by pericytes and smooth muscle cells, lymphatics capillaries are closed-end layers of endothelium. The lack of a complete basement membrane (only lose junctions and anchoring filaments) around lymphatic capillaries makes the entry of interstitial macromolecules to the lymph relatively easy. As interstitial pressure is higher in the tumor microenvironment and due to the loosely and permeable structure of lymphatic capillaries, initial lymphatics are considered as a more accessible route for tumor cells to escape from the primary site.[4] Fluorescent imaging has been performed to visualize tumor cells moving in the lymphatic vessels in mouse model,[5] demonstrating the trafficking of cancer cells by the lymphatics. After passing through a series of lymph nodes and thoracic duct, tumor cells can enter blood circulation via subclavian vein and further spread to distant organs. The presence of tumor cells in lymph nodes does not merely indicate higher risk of distant metastasis and poor prognosis; some recent studies demonstrated that traveling by lymphatic system provides survival advantages for tumor cells, and lymph node metastases can be the source of distant (lung) metastasis. Implanted cancer cells that express photoconvertible protein were traced in an animal model of mammary carcinoma that a fraction of cancer cells were able to colonize the lung via invading lymph node blood vessels and entering the blood circulation.[6] Another study in melanoma showed that metastasis through lymph nodes provides a survival advantage for cancer cells. Cancer cells showed decreased oxidative stress in the lymph where oleic acid protects them against ferroptosis (an iron dependent programmed cell death), increasing their ability to survive for subsequent migration through the blood.[7]

In addition to enter the lymphatic system via pre-existing lymphatic vessels by a passive way, more evidence suggests that tumor cells utilize active mechanisms to escape from the primary site. Some studies demonstrated that C-C chemokine receptor (CCR) expressed on tumor cells mediates tumor cell homing toward the lymph nodes and distant organs. Furthermore, tumor cells can secrete growth factors to promote the formation of new lymphatic vessels (lymphangiogenesis). In both animal models and clinical observation of breast cancer, tumor-associated lymphangiogenesis correlates with increased lymphatic vessel density, lymph node metastasis, and distant metastasis.[8] In most adult tissues, lymphatic vessels are able to expand when stimulated by inflammatory conditions.[9] Tumor microenvironment is considered chronic inflammation and a range of lymphangiogenic factors or cytokines can be produced by tumor cells or immune cells. Family members of vascular endothelial growth factors (VEGF), fibroblast growth factors (FGF), platelet-derived growth factors (PDGFs), and more are all able to induce lymphangiogenesis in different tumor models.

Both intratumoral and peritumoral lymphangiogenesis have been observed. The formation of lymphatic vessels within tumors is not frequently seen and may not be functional, while lymphatic vessels in the tumor margins are enlarged, permeable, and have drainage capacity.[10] Besides primary tumor, lymphangiogenesis can also be induced in sentinel lymph nodes and was found to be a strong predictor of further lymph node metastasis.[11] It has been observed that VEGF-C expressing in the skin induced sentinel lymph node lymphangiogenesis before the onset of metastasis in an animal model of chemical-induced skin carcinogenesis,[12] suggesting primary tumors induce sentinel lymph node lymphangiogenesis to prepare for future metastasis. Furthermore, lymphangiogenesis also occurs at the distant metastatic site. In melanoma patients, high lymphatic vessel density in the metastasized lung correlates with poor prognosis.[13] In a transgenic mouse model with inducible VEGF-C expression in the lung, greater lymphangiogenesis and the growth of metastases in the lungs and in other organs were observed in experimental melanoma and breast cancer model.[13] In addition to facilitating tumor metastasis, increased tumor-associated lymphangiogenesis may also modulate immune response as the major function of lymphatic system is to transport tumor antigens by dendritic cells to lymph nodes, presenting to T-lymphocytes and initiate adaptive immune response.[14] Therefore, lymphangiogenesis can enhance the efficacy of immunotherapy in some tumor models. However, since tumors can produce immunosuppressive molecules to attenuate anti-tumor immunity, lymphangiogenesis may still serve as a pro-metastatic function in cancer progression (Figure 1). Together, lymphangiogenesis facilitates the spread of primary tumors to tumor-draining lymph nodes and promotes further dissemination and metastasis formation in distant organs.

Regulation of lymphangiogenesis in cancer

A set of genes have been identified to be indispensable for lymphatic vessel development, which includes the processes of sprouting, maturation, and separation of lymphatic vessels from the blood vasculature.[15] The development of lymphatic vessels starts with a subpopulation of endothelial cells of cardinal veins (which express high level of VEGFR3) commit to the lymphatic lineage and the expression of VEGFR3 become restricted to lymphatic endothelial cells (LECs) as the system develop. VEGFR-3 is activated by VEGF-C and VEGF-D, while homozygous deletion in mice showed the requirement of VEGF-C, not VEGF-D, in lymphatic development.[16,17] Therefore, among many growth factors which have been demonstrated to induce lymphangiogenesis, VEGF-C is considered the major direct player since it can activate corresponding receptors (VEGFR3) expressed on LECs, resulting in the activation of downstream MAPK and AKT signaling to promote proliferation and survival of LECs.[18] Besides VEGFR, transmembrane receptor integrins are also involved in the transduction of pro-lymphangiogenic signal. Integrin is a family of cell surface receptors for extracellular matrix (ECM) proteins that activate signaling pathways by clustering with focal

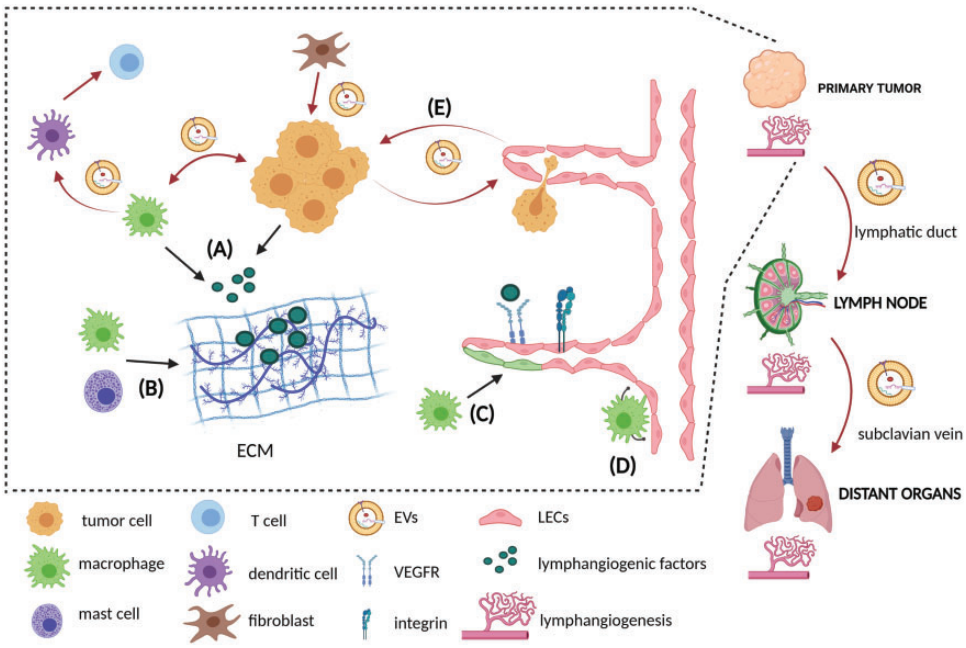

Figure 1. Schematic overview of potential mechanisms involved in lymphangiogenesis and metastasis. (a) Tumor cells and macrophages secrete various lymphangiogenic factors (such as VEGF-C), which binds to VEGFR3 of lymphatic endothelial cells (LECs) and activates downstream signaling. (b) Macrophages and mast cells secrete enzymes (such as MMP) to remodel the extracellular matrix (ECM), releasing or activating ECM bound lymphangiogenic factors. (c) Macrophages can be the progenitors of LECs and being incorporated into the lymphatics. (d) Direct interaction of macrophages with LECs and activates integrin signaling. (e) Tumor-secreted extracellular vesicles (EVs) mediate lymphangiogenesis via delivery of RNAs or protein cargos to the LECs. EVs may be transported by lymph fluid to draining lymph node and remodels the microenvironment (lymphangiogenesis and immune modulation). EVs can be further delivered to distant organs, setting up the metastatic niche. EVs from tumor cells can also polarize macrophages. Macrophages produced EVs transfers antigen to dendritic cells and enhances T cell response. Tumor cells also receive EVs secreted from other cell types (such as fibroblasts and LECs), enhancing metastatic ability of cancer cells. (A color version of this figure is available in the online journal.)

adhesion kinases and adaptor proteins. While the main ligands for integrins are ECM proteins, it was found that VEGF-C and VEGF-D can also bind to integrins $\alpha 9\beta 1$ and stimulate downstream ERK and paxillin phosphorylation in cells lacking VEGFR3.[19] In endothelial cells which express VEGFR3, integrins $\alpha 9\beta 1$ promotes VEGF-C-stimulated cell migration.[19] In a mouse model of breast cancer, blocking integrin $\alpha 4\beta 1$ suppressed VEGF-C and VEGF-A-induced lymphangiogenesis and lymph node metastasis.[20] Therefore, integrins can transduce and amplify growth factor-mediated lymphangiogenesis while distant profiles of integrins may be involved in different cancer models.[21]

In the tumor microenvironment, tumor cells or immune cells are considered the primary source of lymphangiogenic factors. In terms of VEGF-C, the increased expression in cancer cells may be resulted from multiple mechanisms, such as transcription factors, cytokines, or post-translational regulation.[22] Loss of dual-specificity phosphatase 2 (DUSP2), the negative regulator of MEK/ERK signaling, significantly increases VEGF-C secretion and promotes lymphangiogenesis and lymphovascular invasion of pancreatic cancer.[23] Downregulation of circular RNAs (circNFIB1) promotes lymphangiogenesis and LN metastasis via upregulating PIK3/AKT signaling and VEGF-C expression in pancreatic cancer.[24] Macrophages can promote lymphangiogenesis via multiple mechanisms. First, similar to cancer cells, tumor-associated macrophages (TAM) promote lymphangiogenesis via the secretion of a variety of cytokines and growth factors. In many types of cancers, such as breast, pancreatic, and gastric cancer, VEGF-C-expressing TAMs correlate with increased tumor lymphangiogenesis, lymphovascular invasion, and incidence of lymph node metastasis.[25] The secretion of VEGF-C in macrophages can be induced by TNF-α in cancer model.[26] Cell-autonomous contribution of macrophages has been identified that macrophage-derived lymphatic endothelial progenitor cells are integrated into lymphatic vessels.[27] Stimulation of lymphangiogenesis by macrophages can also be mediated via matrix remodeling and growth factor release. The production of matrix metalloproteinase (MMPs) or urokinase plasminogen activator by macrophages remodels ECM and activates growth factors, altering the affinity of growth factors to the corresponding receptors. A subset of TAMs which express podoplanin was identified to bind to LEC-derived galectin 8 and promotes the activation of integrin $\beta 1$, stimulating lymphatic sprouting in syngeneic 4T1 breast tumors model.[28]

The role of mast cells in cancer progression is controversial, while recent evidence implicates mast cells regulate blood and lymphatic vessel formation. In gastric and breast cancer, mast cell density correlates with lymph node metastasis and patient survival.[29] Mechanistically, tryptase derived from mast cells acts as an agonist of the proteinase-activated receptor-2 in stimulating vascular endothelial cell proliferation or releasing lymphangiogenic factors bound to the ECM.[29]

Regardless of different sources, secreted growth factors have been demonstrated as the critical regulator of lymphangiogenesis in the past. Interestingly, emerging evidence shows that tumor cells-derived RNA or cytoplasmic proteins can also regulate lymphangiogenesis conveyed by extracellular vesicles (EVs). Growth factors associated with the surface of EVs also show enhanced or distinct functions as compared to the free form. The role of EVs in tumor progression has received extensive attention which also provides alternative mechanisms that lymphangiogenesis can be regulated. The general understanding of EVs and the current findings in regulating lymphangiogenesis are discussed in the following sections.

Extracellular vesicles in cancer progression

Endorsed by International Society for Extracellular Vesicles (ISEV), extracellular vesicle (EV) is the generic term representing nanometer-sized lipid bilayer particles released from but not limited to bacteria, archaea, and almost all eukaryotic cells. EV was initially thought to be the way of cells to get rid of unneeded materials; however, more and more studies showed EV is important for bidirectional communication in a tightly regulated manner. It is considered that cellular status, such as transformation, stimulation, or stress will result in different types of EV production and cargo loading. Besides the increase in EV secretion, large scale analysis also demonstrated different contents of EV in normal and cancer cells. As tumor progresses, limited nutrients and oxygen result in hypoxic condition and further enhanced the release of EV of cancer cells compared to normoxic cancer cells.[30] Hypoxic tumor cells secreted EV which contains microRNA to polarize macrophages, further promoting malignant behaviors of cancer cells and established the vicious cycle.[31] On the other hand, tumor cell behavior can also be affected by EVs from other cells in the microenvironment. Fibroblasts-secreted EV regulates breast cancer cell migration and metastasis via tetraspanin CD81.[32] EV-microRNA from TAMs can enhance drug resistance or anti-apoptosis of gastric cancer cells.[33] In addition, EVs mediates crosstalk among immune cells that macrophage-EVs transfer antigen to dendritic cells and enhance T cell response;[34] dendritic cells-derived EVs transfer major histocompatibility complex to regulate T cell function.[33] The role of EVs in cancer progression is an emerging field of study. However, due to the complexity of EV, detailed mechanisms are still largely unknown. Herein, we concisely summarize current understanding of EV biogenesis and function. We further review current findings regarding the role of EV in lymphangiogenesis, focusing on cancer model.

Biogenesis and classification of EV

Extracellular vesicles originate from the endosomal compartment or cellular plasma membrane; both biogenesis pathways include protein sorting, transport, release, and recycling. Exosomes, usually 30–150 nm in diameter, are derived from late endosomes. Invagination of late endosome generates intraluminal vesicles within multivesicular bodies (MVBs). As demonstrated by siRNA knockdown experiments, endosomal sorting complex required for transport (ESCRT) machinery and proteins of the tetraspanin family are important for endosomal sorting, intraluminal vesicles budding, and MVB formation.[35] Upon maturation, MVBs are either sent to the lysosome for degradation or fuse with plasma membrane for EV release into the extracellular space. The fate of MVBs and the release of EVs are affected by cellular condition. DNA damaging agent[36] and hypoxia[37] were shown to promote EV release of cancer cells, implying the function of EV in eliminating waste products. In fibroblast cells, inhibition of EV secretion leads to accumulation of nuclear DNA and provokes DNA damage response,[38] suggesting the function of EV in maintaining cellular homeostasis.

EVs originated from the outward budding of plasma membrane are called microvesicles (MVs), ranging from 100 to 1000 nm in diameter. Current evidence suggests MVs secretion involves cytoskeleton and motor proteins, while the detailed mechanism is still not fully understood. Compared to exosomes, MV population is heterogeneous and transmembrane proteins such as integrins and selectins are commonly used markers. Apoptotic body (AP) also belongs to a type of EVs, which is the by-product of apoptosis due to membrane blebbing. APs are 1–5 μm in size and are regulated by rho-associated kinase and myosin ATPase activity.[39] In general, the composition of EVs is highly correlated with the biogenesis path. However, there is no specific marker that can clearly distinguish different types of EVs. In general, ESCRT-related proteins (ALIX, TSG101) and tetraspanin proteins (CD63, CD9, CD81) are more enriched in exosomes, while cytosolic (HSP90 and HSP70) and plasma membrane proteins (Integrins and Annexins) can be incorporated with outward budding MVs.

The unique characteristics of EV

In contrast to traditional knowledge that cells secrete some proteins through the secretory pathway to mediate cell-to-cell communication, EVs-carried biomolecules (nucleic acids, proteins, lipids, and metabolites) are thought to be the major mediators that regulate biological functions of recipient cells over a short and long distance. It has been demonstrated that EVs from pancreatic cancer cells prepare a pre-metastatic niche via alteration of Kupffer cells in the liver, activating hepatic stellate cells, and enhancing macrophages recruitment.[40] For metastatic melanoma, secreted EVs educate bone marrow progenitors and create a vasculogenic pre-metastatic site.[41] As DNA or RNA degrading enzymes are abundant in the biofluids, EVs can be utilized by tumor cells to transfer functional molecules with protection from degradation. Transcriptome profiling of melanoma cells found small RNA (miRNA) was predominantly contained in exosomes compared to that in MV or apoptotic bodies,[42] suggesting a mechanism that controls the loading of RNA into exosomes. Although the mechanism of RNA loading into EVs is not fully understood, EV-associated miRNA participates in many steps of cancer development, from tumor initiation, regulation of angiogenesis, immune modulation to malignancy promotion.[43] Similar to the

function within cells, EV-associated miRNAs control gene expression by regulating mRNA turnover in the recipient cells. EV-DNA has emerged as a molecular marker for the detection of specific mutations and variation. Higher percentage of *KRAS* mutations was detected in EV-DNA of pancreatic cancer patients than cell free DNA,[44] implying the enrichment of genetic information provided by EV-DNA, which may be due to the protection of DNA from blood DNase degradation. The amount of EV-DNA is higher from tumor cells (melanoma, breast, prostate) than nontransformed normal fibroblast cells[45] and different contents of DNA are found in EV subtypes in prostate cancer cells.[46,47] Shedding of genetic material via EV not only reflects the status of cells but also has a biological function. Mitochondrial DNA was found packed in EVs of cancer-associated fibroblast, which promotes breast cancer cells to become resistant to hormone therapy via restoration of oxidative phosphorylation.[48] It was found that EV-carried proteins are modified differently and with higher stability. For example, $VEGF_{189}$ was found to be associated on the surface of EV via heparin binding and this EV-VEGF is substantially more stable than recombinant $VEGF_{189}$ when incubated in human plasma at 37°C.[49] *In vitro* tube formation assay and *in vivo* ovarian xenograft model demonstrated that heparin-bound EV-VEGF could not be neutralized by anti-angiogenesis inhibitor Bevacizumab.[49] In addition, a unique 90 kDa form of VEGF, generated by the crosslinking of VEGF165, was found associated with MVs via interaction with chaperone HSP90. MV-VEGF90K was able to activate VEGF receptor while has weakened affinity for Bevacizumab, therefore causing the poor response of breast cancer to Bevacizumab.[50]

EVs may exert cell or organ-specific function via specific protein or surface modification. Integrin profile of EV derived from pancreatic cancer cells determines the organotropism that $\alpha_6\beta_4$ and $\alpha_6\beta_1$ are associated with lung metastasis and $\alpha v\beta 5$ is linked to liver metastasis.[51] Surface modification of EVs, such as glycosylation, determines cellular recognition and efficient uptake of EVs by recipient cells. It has been observed in mice that glycosidase treatment induced an accumulation of EV in the lungs, while neuraminidase-treated EVs distributed better at the axillary lymph nodes.[52] Specific glycosylation patterns were found in different cancer cells. Glycosylation profile of breast cancer cells showed that both cellular and EVs proteins have different glycosylation patterns in brain-metastatic cells compared to the parental or lymph-node metastatic lines.[53] Removal of surface O-glycosylation enhanced EVs accumulation to lungs and increased endothelial uptake of EV from the brain-metastatic subline.[53] Altogether, different lipid raft content and surface modification of EVs may facilitate their delivery to specific cell types. With the protection within lipid bilayers, internal cargo can be efficiently delivered over long distance and cross tissue barriers.

Regulation of lymphangiogenesis via EV

In the past, secreted factors were considered as the central regulators of lymphangiogenesis. The emerging roles of EV in cancer biology provide versatile potential mechanisms that lymphangiogenesis can be modulated. Compared to lymphangiogenesis, tumor angiogenesis, the growth of blood vessels, has been extensively studied and the effects of tumor derived EV on angiogenesis and anti-angiogenesis drug resistance have been reviewed.[54] Herein, we reviewed and summarized current available studies about the regulation of lymphangiogenesis via EV in cancer models.

EV-RNA in lymphangiogenesis

Messenger RNA and non-coding RNA, including microRNAs (miRNAs), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs), all have been detected in EVs,[55] while short RNA (<200 bp) was found as the predominant type of RNA in EVs. miRNA is about 22 nucleotides small noncoding RNA which mediates post-transcriptional repression via pairing to 3′ untranslated region of mRNA. miR-221-3p was identified from clinical specimens of cervical squamous cell carcinoma of which expression correlates with lymphangiogenesis (LYVE-1 expression) and lymph node metastasis.[56] miR-221-3p is enriched in cervical squamous cell carcinoma-secreted EVs and its overexpression promotes EV-mediated LEC tube formation *in vitro* and lymph node metastasis in the popliteal lymph node metastasis model. VASH, the negative regulator of lymphangiogenesis, was identified as the target of miR-221-3p. Molecular mechanism revealed that miR-221-3p-VASH axis activates ERK/AKT pathway and promotes lymphangiogenesis independent of VEGF-C.

LncRNAs exceed 200 nucleotides in length and are generally nuclear localized where they regulate chromosome architecture via recruitment of chromatin modifiers. The lncRNA, lymph node metastasis-associated transcript 2 (LNMAT2), was found overexpressed in bladder cancer with LN metastasis.[57] EV from *LNMAT2*-overexpressing cancer cells promotes lymphangiogenesis *in vitro* and *in vivo*. RNA-binding protein hnRNPA2B1 was identified being bound by *LNMAT2*, by which was loaded into bladder cancer-secreted EVs. EV-*LNMAT2* recruited hnRNPA2B1 and epigenetically upregulated prospero homeobox 1 (PROX1) promoter via increasing the H3K4 trimethylation in the LECs. As PROX1 is a critical transcription factor for lymphatic specification and budding in embryogenesis, this study also highlights a VEGF-C independent mechanism regulated by EV from cancer cells.[57] In the cytoplasm, lncRNA regulates mRNA stability or translation. Being as a sponge for miRNAs is one of the mechanisms by which lncRNA diminishes miRNA functions. In another study, regulation of lymphangiogenesis via the interaction of lncRNA and miRNA was demonstrated. In hepatocellular carcinoma cell (HCC), lncRNA HANR directly interacts with miR-296 and downregulates its expression.[58] Since EV-miR-296 targets EAG1 in LECs and inhibits VEGF expression and lymphangiogenesis, this study provides a mechanism that increased lncRNAs expression in cancer cells decreases miRNA, de-represses the target genes of EV-miRNA, and thus promotes lymphangiogenesis.

CircRNAs are formed by back splicing and act as miRNA or RNA binding protein sponges, and thus regulate the expression of parental genes.[59] Without typical 5′ cap and

3′ poly(A) tail, circRNAs cannot be degraded easily and are relatively stable compared to linear RNA. CircRNAs have been detected in cancer-derived EV. Recently, it has been reported that EV-circ-IARS secreted by pancreatic cancer cells regulates the permeability of vascular endothelial monolayer via increased RhoA activity and F-actin expression.[60] However, whether tumor-derived EV-circRNA regulates LECs has not been demonstrated yet.

EV–protein in lymphangiogenesis

Mass spectrometry-based proteomic analyses have led to identifying proteins involved in EV biogenesis, sorting, and different cargos.[61] EV-associated proteins can be inside, outside, or transmembrane, and mediate recipient cells in different ways.

The effect of EV-transcription factor on sentinel lymph node has been demonstrated in colon cancer model. Transcription factor IRF-2 was identified highly expressed in EV from serum of colon cancer patients with LN metastasis. EV-IRF-2 from colon cancer cells was uptaken by macrophages and promoted the secretion of VEGF-C, resulting in LEC proliferation in the LN.[62] In this study, they demonstrated that colon cancer cell-derived EV regulates lymphangiogenesis via macrophages and the production of VEGF-C. Ablation of macrophages by clodrosome or EV from IRF-2 knockdown cancer cells prevented the remodeling of lymphatic network and sentinel lymph node metastasis.[62] Although IRF-2 response element was predicted at promoter region of VEGFC, the regulation of VEGF-C by IRF-2 was not further studied.

Podoplanin (PDPN) is a transmembrane glycoprotein that is essential for the development of lymphatic vascular system and its expression has been used as a marker of lymphatic vessels. Increased expression of PDPN in tumors has been observed and contributed to malignancy. PDPN mRNA and protein are incorporated in both exosomes and MVs.[63] Knockdown of PDPN in human HN5 squamous carcinoma cells reduced EV production and inhibited EMT program and tumorigenesis. EVs containing PDPN promote lymphatic vessel formation, which can be neutralized by a specific monoclonal antibody.[63] Interestingly, proteins implicated in the regulation of intracellular vesicle trafficking were enriched in EV of PDPN-expressing cells, suggesting a regulatory role of PDPN on EV biogenesis or secretion. However, it remains to be evaluated whether EV-PDPN interacts with a cell-surface receptor or how it is internalized. Furthermore, the downstream signaling involved in EV-PDPN induced lymphangiogenesis remains to be investigated.

Increased expression of a 7-transmembrane G-protein chemokine receptor CXCR4 in breast cancer cells mediates its migration toward lymph node where CXCL12, the ligand for CXCR4, is highly expressed.[64] It was found that EV-CXCR4 from lymph node metastatic hepatocellular carcinoma cells (Hca-F) promotes its parental cell migration and invasion ability.[65] Horizontal transfer of EV-CXCR4 from Hca-F also promotes proliferation and tube formation in LECs. CXCL12 of LECs bound to CXCR4 of cancer cells and enhanced the secretion of MMP9, MMP2, and VEGF-C, creating a positive feedback loop that may facilitate lymphatic metastasis.

A systematic analysis of the association between 33 cytokines and EVs in different cell lines and body fluids demonstrated that a cytokine could be released in a soluble form or an EV-associated form, depending on the stimulation and biology systems.[66] It was also found that cytokines can be surface-bound or EV-encapsulated. It was recently found that VEGF-C is predominantly associated with EV from pancreatic cancer cells.[23] By transmission electron microscope and proteinase K treatment, topology determination showed that VEGF-C is associated with EVs surface. Proteolytic activation of VEGF-C determines its function and activity[67] and different modes of VEGF-C activation have been proposed.[68] Interestingly, EV-associated VEGF-C is the functional form, and VEGF-C processing might occur within pancreatic cancer cells.[23] While it remains to be determined whether surface bound EV-VEGF-C is more stable than free form, deposition via EV should be more efficient in the close vicinity of target cells (LECs) than release into the ECM. As ECM contains many proteoglycans and heparin sulfates that may trap peptide growth factors such as VEGF-C, being carried by EVs provides a better way to transport VEGF-C to a remote site where its target cells reside.

The uptake of EV by endothelial cells

The surface components of EV may determine the uptake mechanism and efficiency of the recipient cells. EVs derived from lymph node metastatic oral squamous cell carcinoma (OSCC) cells enhanced LECs migration, tube formation, and were uptaken by LECs more effectively.[69] Laminin-332 was further identified due to its upregulated expression on plasma EVs from OSCC patients with lymphatic metastasis. Laminin is a major glycoprotein component in the basement membrane, which binds to integrins and mediates cell adhesion, migration, or differentiation. Knockdown of integrins in LECs showed $\beta 1$, $\beta 4$, $\alpha 3$, and $\alpha 6$ reduced uptake of EV from OSCC cells, while only integrins $\alpha 3$ is important in mediating laminin-332/gamma2-enhanced EVs uptake of LECs. Interestingly, laminin-332 is involved in lymph node draining of EV since reduced draining to lymph node of laminin-332-deficient EVs was observed compared to the control EVs.

CD47, an integrin associated transmembrane protein, was involved in EV uptake in human umbilical vein endothelial cells (HUVEC). $CD47^{+}$EV from breast cancer cells exhibited greater uptake by HUVEC compared to $CD47^{-}$ EVs.[70] $CD47^{+}$EV alters the signaling pathways, including angiogenesis, lymphangiogenesis, EMT in the recipient endothelial cells. However, CD47 alters EV uptake in a cell-type dependent manner as CD47 blocking antibody inhibits EV uptake by macrophage but not by HUVEC. Therefore, integrin networks may play an important role in mediating EV uptakes while the underlying mechanism in LEC and HUVEC may be different.

Perspective

As more compelling studies indicate that lymph node metastasis is not merely prognosis factor but also

contributes to the seeding, selection, and progression of metastasis,[7,11] a more comprehensive understanding of lymphatic metastasis is required to provide therapeutic targets. Higher incidence of sentinel lymph node metastasis is often accompanied by increased tumor lymphangiogenesis, by which facilitates the spreading of tumor cells. EVs are now recognized as important mediators in cancer progression and studies about EVs in lymphangiogenesis have been discovered in recent years. Current evidence indicates that tumor-secreted EVs act on LECs to promote proliferation or tube formation via RNAs or protein cargos; however, EV cargo is far more complicated than we have already known because distinct metabolites (lactic acid and glutamic acid) are also contained within EVs.[71] Since metabolic reprogramming of cancer cells adapt themselves to the dynamic changes in the microenvironment, it is possible that EV-metabolites also act on LECs.

Remodeling of lymph nodes occurs prior to the dissemination of cancer cells, which includes lymph node lymphangiogenesis, chemokine and cytokine production, and alteration of immune cell composition.[11] Tumor derived EV-mediated conditioning of lymph nodes and preparing the pre-metastatic niche for further metastasis.[72] The feedback effect of LECs derived EV on tumor cells or in the microenvironment is also important to accomplish the process. It has been demonstrated that inflammatory LECs-secreted EV increases the directional movement of CX3CR1-expressing dendritic cells and prostate carcinoma cells.[73] Therefore, the influence of LEC-EV on tumor cells homing to lymph nodes or in immune modulation deserved further studies.

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Both authors contributed to the composing, writing, and editing of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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