Roles of signaling and transcriptional networks in pathological lymphangiogenesis

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Abstract

Lymphangiogenesis, the generation of new lymphatic vessels, plays important roles in cancer metastasis. Outstanding progress during the past decade have dramatically increased the novel knowledge and insights of the mechanisms underlying the generation of new lymphatic vessels, the roles of transcription factors and lymphangiogenic growth factors during physiological development and pathological processes such as cancer and inflammation. Furthermore, an understanding of the molecular consequences during tumor lymphangiogenesis has provided chances to develop better diagnostic and therapeutic approaches that aim to limit the progression of cancer. In this article, we will explain the current knowledge of how lymphatic function is altered in various pathological conditions including cancer progression.

1. Introduction

The maintenance of fluid homeostasis of vertebrates is regulated by the blood and lymphatic vessels which are distributed throughout the body. In addition to their physiological roles, it is widely known that tumors require microvessel formation in the vicinity in order to incorporate oxygen and nutrition to grow and/or metastasize [1]. Cancer cells migrate to distant tissues via either blood or lymphatic vessels [2]. Lymphatic vessel was reported to be the main metastatic route for oral squamous cell carcinoma [3]. In this review, we will discuss the major findings that have led to the understanding of critical roles for the lymphatic vascular system during cancer metastasis with a specific focus on the transcription factors and signaling cascades involved, and development of novel strategies to therapeutically target the lymphatic vessels in cancer.

Footnotes for Definition of technical terms

In this review, to avoid misunderstanding technical term definition, we define metastasis as follows. Most tumors that grow in size to some extent recruit both blood and lymphatic vessels. The tumors need these blood vessels or lymphatic vessels to metastasize to distant organs. Hematogenous metastasis is a process in which cancer cells migrate from primary tumors via blood vessels and settle down to distant organs. Lymph node metastasis is a process in which cancer cells migrate from primary tumors to lymph nodes via lymphatic vessels. Please note that cancer cells which pass through

lymph nodes further metastasize to distant organs via blood vessels. Metastasis is a migrating process of cancer cells to distant organs. Therefore, when we simply mention “metastasis” in this review, it includes both hematogenous metastasis and lymph node metastasis.

In addition, we define types of lymphatic vessels as follows. Lymphatic vessel is frequently and widely used as vessels through which lymph fluids flow. When ‘lymphatics’ is used, it exactly means lymphatic vessels. Lymphatic capillaries are lymphatic microvessels in peripheral tissues which includes the smallest lymphatic vessels and initial lymphatics (very tips incorporating lymph fluids). ‘Lymphatic vascular’ means lymphatic vessel’s (possessive) as in lymphatic vascular system.

2. Lymphatic vessels in physiological conditions

2-1. Lymphatic Development

More than 100 years ago, Dr. Sabin postulated that lymphatic vascular system arises by sprouting from blood vessels [4]. This “centrifugal” theory has been confirmed during the last 15 years [5]. During embryogenesis, the lymphatic vessels start to develop after the blood vascular system is established. Around embryonic day (E) 9.0 in mice, shortly after the separation of veins and arteries, a distinct group of BECs of the anterior cardinal vein starts to express LYVE-1, one of the lymphatic endothelial markers. Around E9.75, a population of LYVE-1-positive cells of the cardinal vein starts to express Prox1 transcription factor (Fig. 1) [6, 7]. In E10.5 mouse embryos, when the Prox1-positive cells become committed to differentiating into lymphatic ECs, they sprout and bud off the cardinal vein, and then migrate toward the surrounding tissue that express vascular endothelial growth factor (VEGF)-C, where they develop primary lymphatic vasculature. Expansion and extension of the lymphatic vessels by sprouting and proliferation leads to the development of the primitive lymphatic sacs (Fig. 1).

The maturation step of the lymphatic vasculature starts approximately at E14.5 and continues until after birth (Fig. 1). During these processes, the primitive lymphatic plexus develops into a hierarchical structure consisting of lymphatic capillaries and collecting lymphatic vessels through the structural remodeling of collecting vessels including the formation of lymphatic valves and the smooth muscle coverage (Fig.

1) The separation of lymphatic vessels from the blood vessels depends on multiple transcription factors and signaling pathways, whose roles have been reviewed elsewhere in more details [5, 8].

2-2. Functions of lymphatic vessels

The lymphatic vasculature is an essential organ of the vertebrate vascular system and plays a number of important roles in homeostasis and disease [5, 8]. The mature lymphatic system in mammals consists of the lymphatic vessels and the lymphoid organs including the lymph nodes, tonsils, thymus, Peyer's patches and spleen. The lymphatic vasculature travels over most of the body, with the exception of cartilage, epidermis, cornea, and retina. While it had long been thought that lymphatic vessels are absent in the central nervous system and bone marrow, recent reports have identified lymphatic vasculature in central nervous system [9, 10]. Lymphatic vasculature functions to return interstitial fluid and protein to the blood circulation, to traffic immune cells, and to absorb dietary fatty acids. For example, almost all of body water from peripheral tissues is collected by veins but excessive water that cannot be collected by veins are collected by lymphatic vessels. The reason why lymphatic vessels absorb fat and fatty acid has not been well described yet. However, the need for collecting aqueous solution and hydrophobic fatty emulsion by blood vessels and by lymphatic vessels respectively is a reasonable/possible explanation. Trafficking immune cells to lymph nodes is a critical role of lymphatic vessels since lymphocytes, committed effector cells and macrophages in lymph nodes attack and eliminate pathogens to maintain pathogen-free conditions in the body.

2-3. Structures of lymphatic vessels

The blood and lymphatic vessels are lined by endothelial cells (ECs). The lymphatic vasculature is formed by lymphatic endothelial cells (LECs), which have highly similar properties to blood vascular endothelial cells (BECs). While LECs represent distinct cell populations from BECs, LECs are differentiated from BECs during embryogenesis and are capable of de-differentiate to BECs in the presence of BMP-9 signals [11-13]. The lymphatic vascular network starts with blind-ended lymphatic capillaries in the peripheral tissues (Fig. 2). Lymphatic capillaries consist of a single-cell layer of overlapping LECs which are interconnected by discontinuous

1 button-like junctions without intercellular tight junctions or adherens junctions [14].
2 Lymphatic capillaries have minimal basement membranes, or are not covered by
3 smooth muscle cells (Fig. 2). They are tethered by anchoring filaments to collagen
4 fibers of the extracellular matrix. Under high interstitial pressure, pulling of anchoring
5 filaments induces the opening of EC junctions, followed by influx of tissue fluid into
6 the lymphatic vessel. The lymphatic capillaries first descend into pre-collecting
7 lymphatics, which eventually converge into larger secondary collecting lymphatic
8 vessels (Fig. 2). Collecting lymphatic vessels possess endothelial cells which exhibit
9 continuous zipper-like junctions, and are covered by the smooth muscle cells that
10 provide contractile and relaxing activity to assist pushing lymph flow forward [14]. In
11 order to make a unidirectional flow of the lymph from the peripheral tissues towards the
12 bloodstream, lymphatic collecting vessels contain bi-leaflet lymphatic valves. The
13 driving forces of lymph flow are generated by contractions of the lymphatic endothelial
14 sheets and by contractions of skeletal muscles from the outside. The lymph of the body
15 is drained into the thoracic duct and the right lymphatic duct, which convey the lymph
16 back into the blood circulation at the joints connecting with the left and right subclavian
17 veins, respectively.

19 **3. Lymphatic vessels in pathological conditions**

20 **3-1. Introduction to pathological lymphangiogenesis**

21 Lymphatic vessels have been implicated in the pathogenesis of a number of
22 human diseases. Impairment of lymphatic vascular functions often gives rise to
23 lymphedema, whereas the aberrant growth of lymphatic vessels shown as higher density
24 of lymphatic vessels is involved in the progression of many types of cancers such as
25 melanoma [15, 16], breast cancer [17], colorectal cancer [18-20] and lung cancer [21].
26 Since tumor cells utilize lymphatic vessels to migrate to local lymph nodes and, after
27 egressing the lymph nodes, migrate (metastasize) to secondary tumor sites via blood
28 vessels, the presence of lymph node metastases typically indicates strong correlation
29 with poor prognosis [22, 23]. Therefore, an understanding of the molecular mechanisms
30 underlying the generation of the lymphatic vessels will aid in developing of novel
31 therapeutic strategies to interfere with the cancer metastasis to lymph nodes.

3-2. Tumor lymphangiogenesis

3-2-1. General overview of research history of tumor lymphangiogenesis

Cancer tissues consist of various components including cancer cells, tumor vessels, cancer-associated fibroblasts (CAFs) and inflammatory cells. In addition, the formation of lymphatic vessels is observed in many types of cancer tissues. During cancer progression, angiogenesis plays important roles in the proliferation and hematogenous metastasis of cancer cells. In response to hypoxia, cancer cells secrete pro-angiogenic factors such as VEGF-A, which induces the proliferation and migration of BECs in tumor stroma. Consequently, cancer cells are supplied with oxygen and nutrients through newly-formed blood vessels, and proliferate further. After cancer cells acquire the ability to invade to surrounding tissues, newly-formed blood vessels provide metastatic pathways to distant organs. Therefore, angiogenesis has become a critical therapeutic target for metastatic tumors. In 2004, bevacizumab [24], an anti-VEGF neutralizing antibody, has been placed on the market as a drug for curing metastatic colon cancer. Thus, tumor vascular biology has made great progress and ideal research development has been accomplished in ranging from the discovery of VEGF-A [25-28] to the development of anti-angiogenic therapy-based drugs [29]. On the other hand, the research on tumor lymphangiogenesis had been delayed although they are critical prognosis markers equivalent to angiogenesis. However, at the beginning of the twenty-first century, the discovery of several markers for LECs opened up the dramatic research progress on lymphangiogenesis [6, 30-33]. In 2001, a study using a mouse model of breast carcinoma revealed that lymphangiogenesis observed in tumors promotes metastasis of cancer cells to sentinel lymph nodes [34] (Fig. 3).

After the identification of several lymphatic vessel markers, a growing number of researchers began to study the role of tumor lymphangiogenesis in cancer metastasis. In human head and neck cancer, intratumoral lymphatic vessel density was shown to correlate with lymphatic metastasis, and in melanoma, tumor lymphangiogenesis was proved to be a better predictor of cancer metastasis and survival rate than tumor size [35, 36]. Correlation between lymphatic metastasis and the secretion of VEGF-C/D which are lymphangiogenic factors, is observed in various cancers such as breast cancer and prostate cancer where cancer cells are prone to metastasize [37]. Xenograft model of cancer cell lines in mice was used as experimental models of tumor lymphangiogenesis in early stages of basic research [38-42]. These studies have evoked more clinical investigations, leading to the identification of tumor lymphangiogenesis in many types

of solid malignant tumors, and to the recognition as a novel prognosis marker for cancer patients.

We will describe multi-step changes in lymphatic vessels during cancer progression in the following section: 1. Lymphangiogenesis in the periphery of tumors; 2. Lymphangiogenesis inside tumors (may not be critical); 3. Cancer cell entry/invasion into lymphatic vessels; 4. Lymphangiogenesis in lymph nodes; 5. Lymphatic remodeling in lymph node metastasis.

3-2-2. Roles and characteristics of tumor lymphatic vessels during cancer progression

While embryonic lymphatic vessel formation occurs by the differentiation of LECs from BECs [43], tumor lymphangiogenesis occurs by elongation of pre-existing lymphatic vessels in the same manner as tumor angiogenesis (Fig. 3). Lymphangiogenic factors such as VEGF-C/D which are secreted by cancer and inflammatory cells promote the proliferation and migration of LECs in tumor-surrounding lymphatic vessels [44, 45]. While blood vessels have been clearly demonstrated to contribute to tumor growth, the roles of lymphatic vessels in the growth and progression of tumors remain to be elucidated.

In many types of cancer, tumor lymphatic vessels are observed as the ones surrounding the periphery of cancer tissues. High interstitial pressure in tumors may hinder lymphatic vessels from intussuscepting into tumors. These facts can be explained by the following reason. Blood capillaries are covered by pericytes but lymphatic capillaries lack pericytes. Only collecting lymphatics in the lymphatic vasculature are covered by pericytes/ smooth muscle cells, and they have discontinuous basal layers without tight junction, which result in loose cell-cell adhesion and dilated caliber [46-48]. This property may also facilitate cancer cell entry into lymphatic capillaries.

Since lymphangiogenic factors such as VEGF-A, -C and -D have been shown to be secreted by some tumors, they are critical factors to determine the susceptibility of lymphatic vessels to intussuscept into tumors. However, despite of these properties, in some tumors, lymphatic vessels are intratumorally distributed [13, 83] (see Figure 4 which shows the presence of peripheral lymphatic vessels in an experimental tumor model). Since the intratumoral lymphatic vessels occasionally appear to be flattened by high interstitial fluid pressure, it is not clear whether they have functional tube-like

structures. Lymphatic vessels surrounding cancer tissues may easily incorporate invading cancer cells, partly due to their loose structure. Cancer cells are not thought to be actively collected by lymphatic capillaries. However, migratory cancer cells that have undergone epithelial-to-mesenchymal transition (EMT) are liable to be collected passively by them through high interstitial pressures and then the cancer cells migrate towards lymph nodes by interacting with lymphatic vessels. The interaction has been shown to be mediated by the molecular interaction between lymphatic endothelial-derived SDF-1 or CCL21 and CXCR4 or CCR7 on some cancer cells, respectively [49-51].

Pre-existing lymphatics including lymphatic capillaries and collecting lymphatics become enlarged by tumor-derived lymphangiogenic factors [34, 35, 44, 52]. In this manner, the environment is readily arranged where cancer cells invading lymphatic vessels are prone to migrate into sentinel lymph nodes. Taken together, lymphatic vessels play a greater role for tumor metastasis during late stages of cancer progression than for tumor growth in early-to-intermediate stages.

3-2-3. Tumor lymphangiogenesis and remodeling of lymphatic vessels distant from primary tumors

Tissue fluid incorporated by peripheral lymphatics flows into lymph nodes through collecting lymphatic vessels and reflux into veins through the thoracic duct. Cancer cells incorporated by lymphatic capillaries metastasize into distant organs all the way through blood flow (Fig. 3). Accumulated lines of evidence suggest that during pre-metastatic stage, lymphangiogenic factors from primary tumors may alter the characteristics of lymphoid tissues to facilitate metastases of cancer cells.

VEGF-A, -C and -D secreted by primary tumors are hypothesized to become collected to their draining lymphatics and then to stimulate pre-existing collecting lymphatics which connect to lymph nodes. This hypothesis was postulated by the observation that lymphatics connecting between tumors and their sentinel lymph nodes are enlarged and that both lymph flow and its promoting pulse increase [2, 53-56].

During lymph node metastases, cancer cells are drained to lymphatic capillaries, and migrate to lymph nodes via its collecting lymphatics. The caliber size of collecting lymphatics has reported to be enlarged in metastatic tumors of human patients and an

experimental mouse model of metastatic tumors. In a mouse model of VEGF-D-expressing tumors, VEGF-D regulates production of prostaglandins by collecting lymphatic endothelium and then blocks the contraction of their smooth muscle cells [55]. In pathological condition, observation of lymphatic vessel growth in lymph nodes even prior to tumor metastases has led to the acknowledgement of ‘pre-metastatic lymphatic niche’ as a key phenomenon during lymphatic metastasis [57].

Taken together, various growth factors secreted by cancer tissues induce the growth and remodeling of pre-existing lymphoid tissues to facilitate metastases of cancer cells. We will dictate such lymphangiogenesis-regulating factors in the following section.

4. Transcription factors and signaling cascades involved in lymphatic vessel formation

4-1. Transcription factors involved in physiological and pathological lymphangiogenesis

Identification of LEC specific markers and functional studies of regulators by generating genetically engineered animals and *in vitro* cell-based studies have identified Prox1 as the key transcription factor critically required for the specification of LEC fate [58]. We will describe the following critical signaling pathways in tumor lymphangiogenesis

4-1-1. Prox1

Prox1 is a homeobox transcription factor and is related to and named after *Drosophila prospero* [6]. At E9.75 during mouse embryogenesis, Prox1 starts to be expressed in a group of BEC of the cardinal vein, from which they sprout to develop primary lymph sacs [6, 7]. Multiple studies have suggested that Prox1 is necessary and sufficient for the specification of the LEC phenotype in venous BECs. In *Prox1*-deficient mice, sprouting of LEC progenitors from the veins appears unaffected at E10.5, but their migration is arrested at around E11.5-E12.0, resulting in a complete absence of the lymphatic vessels, suggesting that Prox1 is required to specify LEC

phenotypes in a subset of venous ECs. Furthermore, it has been reported that Prox1, a transcription factor, induces the expression of LEC markers, and suppresses that of BEC markers [11, 59, 60]. These *in vitro* and *in vivo* findings suggest that Prox1 regulates the differentiation program of embryonic BECs to LECs via functioning as a binary transcriptional switch, turning the LEC program on and the BEC program off.

4-1-2. Sox18

Since Prox1 is recognized as a master transcription factor of lymphatic development, molecular mechanisms how Prox1 expression is induced during lymphatic development have been extensively studied. Francois and colleagues reported that Sox18, a member of Sox (SRY-related HMG-box) family of transcription factors, directly binds to Prox1 promoter and induces its expression during the differentiation of LECs from venous BECs [61]. Subsequent study revealed that Prox1 expression is induced by the cooperation of Sox18 with COUP-TFII nuclear receptor, which is expressed in venous BECs [62]. However, Sox18 expression in LECs decreases after E14.5 in murine embryogenesis, and becomes undetectable in adulthood. These results suggest that Sox18 plays critical roles in LEC fate specification, but is not required to maintain LEC characteristics.

During tumor growth, Sox18 is re-expressed in newly-formed BECs, and has been implicated in the proliferation and migration of ECs and vascular integrity [63-65]. In addition, Sox18 is also upregulated in tumor lymphangiogenesis [66]. Genetic deletion of Sox18 in mice suppressed tumor lymphatic vessel formation and metastasis in melanomas. Although these analyses were not conducted in EC-specific or LEC-specific manner, Sox18 can be a therapeutic target for tumor lymphangiogenesis and metastasis.

4-1-3. Ets family members

Prox1 is expressed not only in LECs but also in various types of cells including pancreas, liver and lens [6], suggesting that other transcriptional co-factors regulate the expression of various LEC markers in cooperation with Prox1. Yoshimatsu et al. reported that Ets-2 transcription factor is expressed in embryonic BECs and LECs, and induces expression of VEGFR3 cooperatively with Prox1 [67]. Consistent with its

effects on VEGFR3 expression, Ets-2 promotes the migration of LECs toward VEGF-C. Endogenous Ets-2 associates with Prox1 in LECs and that both Ets-2 and Prox1 bind to the promoter of VEGFR3 gene in the vicinity in intact chromatin. Furthermore, when adenoviral delivery of Ets-2 and a dominant negative form of Ets-1 (TM-Ets-1) was conducted to murine diaphragms on which inflammatory lymphangiogenesis is induced by thioglycollate, Ets-2 significantly enhanced the lymphangiogenesis but TM-Ets-1 suppressed it. These findings suggest that Ets-2 functions as a transcriptional co-factor which enhances Prox1-induced lymphangiogenesis.

4-2. Signaling cascades involved in physiological and pathological lymphangiogenesis

Various *in vitro* and *in vivo* studies have identified various growth factors that regulate the growth and migration of LECs, which leads to the generation of lymphatic vessels [68]. We will describe the following critical signaling pathways in tumor lymphangiogenesis which are relatively well-established.

4-2-1. VEGF family members (figure 6)

VEGF-C and VEGF-D bind and signal through their cognate receptor VEGFR3 [69], and promote the proliferation and migration of LECs *in vitro* [70-72]. Furthermore, adenoviral or transgenic expression of VEGF-C enhances lymphangiogenesis *in vivo* [73-75]. Studies using knockout mice showed that allelic deletion of VEGF-C results in the pathogenesis of severe lymphedema, and homozygous VEGF-C deletion leads to the complete failure to form lymphatic vasculature. In *Vegfc*-null mouse embryos, although the specification of LEC occurs, the embryos lack a lymphatic vasculature because the committed LECs fail to sprout from the cardinal vein [76]. In contrast to VEGF-C, genetic ablation of VEGF-D displays no apparent phenotypes on the formation of lymphatic vasculature [77]. Since VEGFR3 is expressed in all BECs during the early stages of embryogenesis, loss of VEGFR3 signals in this stage leads to embryonic lethality due to vascular failure before the development of lymphatic vascular systems starts [78]. Expression of VEGFR3 becomes restricted to LECs later in development (~E12.5) [79], when faulty VEGFR3 signaling inhibits the development of a proper lymphatic vasculature. Mutations in the tyrosine kinase domain of VEGFR3 cause a defective signaling activity and result in

lymphatic hypoplasia and lymphedema in human (Milroy Disease) [80] and in mice (Chy mutant mice) [81].

4-2-2. PDGF family members (figure 7)

Platelet-derived growth factor BB (PDGF-BB), one of the PDGF family members, is originally identified as factors secreted from activated platelets and is expressed in endothelial cells. PDGF Receptor β (PDGFR β), one of the specific receptors for PDGF-BB, is expressed in vascular pericytes and PDGF-BB/PDGFR β signal between endothelial cells and pericytes has been implicated in vascular remodeling. Multiple groups including ours have reported that PDGFR β is also expressed in LECs and that PDGF-BB promotes the proliferation and migration of LECs [82, 83]. Moreover, thioglycollate-induced inflammatory lymphangiogenesis and xenografted tumor-driven lymphangiogenesis in mice are blocked by a small-molecule inhibitor for PDGFR β or PDGFR β Fc chimeric receptor (Fig. 5). These results suggest that the activity of endogenous PDGF signals is detected at a considerable level to modulate the formation of lymphatic vessels during cancer progression and inflammation, indicating the importance of PDGF-BB/ PDGFR β signal in lymphangiogenesis in adult.

4-2-3. TGF- β superfamily members (figure 8)

While many of pro-lymphangiogenic factors have been identified, anti-lymphangiogenic factors are poorly understood. We have identified the members of transforming growth factor (TGF)- β superfamily including TGF- β and bone morphogenetic protein (BMP)-9 as factors that negatively regulate lymphangiogenesis. TGF- β superfamily consists of more than 30 cytokines which share basic molecular structures. TGF- β s and BMPs respectively regulate the expression of their specific target genes through their specific receptors and intracellular signal transducing molecules. We reported that both TGF- β and BMP-9 negatively regulate the proliferation of LECs via similar mechanisms [13, 84]. We showed that cell proliferation and tube-like structure formation of human dermal LECs (HDLECs) are suppressed by TGF- β but promoted by its small-molecule inhibitor of TGF- β receptor

kinases such as LY364947 and SB431542. In a mouse model of thioglycollate-induced aseptic peritonitis, inflammatory lymphangiogenesis is promoted by LY364947. In a xenograft model of human pancreatic carcinoma BxPC3 cells, tumor lymphangiogenesis is also promoted by the inhibitor. These data suggest that TGF- β negatively regulates lymphangiogenesis [84].

Activin receptor-like kinase (ALK)-1, a receptor for BMP-9, is preferentially expressed in endothelial cells and has been identified as a responsible gene for one of the human hereditary vascular diseases; hereditary hemorrhagic telangiectasia. We found that deficiency in mice for both BMP-9 and ALK-1 develop lymphatic vessel hypertrophy during perinatal development, compared to the control mice. Furthermore, BMP-9 inhibited the proliferation of HDLECs and suppressed both inflammatory and tumor lymphangiogenesis in mice, suggesting that BMP-9/ALK-1 signals negatively regulate lymphangiogenesis [13]. The underlying mechanism for the anti-proliferative effect of TGF- β and BMP-9 on lymphatic endothelial cells has been clarified as decreased expression of Prox1. Since Prox1 has been found to regulate several cyclin families [13], a novel mechanism of lymphatic vessel formation via Prox1 expression began to be clarified.

4. Therapeutic approaches

Recent findings have revealed that tumor lymphangiogenesis plays important roles in lymph node metastases, and makes negative impacts on patients' prognosis. The next action to be taken for us is to inhibit lymphangiogenesis and block lymph node metastasis. The development of therapeutic strategies to target lymph node metastasis has been actively in progress.

Since the central signals for tumor lymphangiogenesis had been shown to be VEGF-C, D/VEGFR3, a number of trials has been conducted by shutting down these signals in order to block lymphangiogenesis and then to diminish lymph node metastasis of tumor cells. Monoclonal antibodies against VEGF-C, D and soluble receptors for VEGF families have been developed [85-91] and small molecule inhibitors [92-96] to block tyrosine kinases of VEGFR3 have been applied to clinical therapies. Monoclonal antibodies against neuropilin-2, a co-receptor for VEGFR3, have been shown to block lymphangiogenesis [97]. In some tumors, cancer cells themselves

express VEGFR3 and lung adenocarcinoma cells such as A549 or H928 migrate and infiltrate in response to VEGF-C [98]. This suggests that, when infiltration and metastasis of cancer cells are analyzed on VEGF-C/VEGFR3 signal, we should consider not only lymphatic vessels but also a wide variety of components of tumor tissues and their response to the signal.

Other than VEGF-C/VEGFR3 signals, a target for blocking lymphangiogenesis is ANG1, 2/TIE2 signal [99, 100]. A peptibody neutralizing ANG1, 2 and a small molecule inhibitor for TIE2 have been developed [99]. Regorafenib, a small molecule inhibitor targets receptor tyrosine kinases such as KIT, PDGFR and RET, which suppress the proliferation of cancer cells, in addition to targeting receptor tyrosine kinases such as VEGFR1, R2, R3 and TIE2, which regulate angiogenesis [101]. This multi-kinase inhibitor has been already approved as an anti-cancer drug.

To take plausible strategies in order to block tumor lymphangiogenesis and lymphatic metastasis, we should consider the plasticity of components of tumor tissues which readily acquire resistance to primary treatment of drugs. We could remind the lessons from treatment of bevacizumab, an anti-VEGF-A neutralizing antibody. Although the antibodies have been effective to some extent to lessen some tumors, other tumors have acquired resistance by utilizing other angiogenic signals. Thus we should expect this biological response of tumor tissues and target multiple lymphangiogenic signals other than VEGF-C/VEGFR3 signals for anti-lymphangiogenesis and anti-lymphatic metastasis therapies. As we discuss earlier, good candidates of such signals can be ANG-1,2/TIE2, SDF-1/CXCR12 and CCL21/CCR7. It has still been argued whether cancer cells metastasize via blood vessels or lymphatic vessels depends on their affinities to respective vessels. In the future, it is greatly expected to reveal biological properties of the interaction between multiple types of cells in the tumor microenvironment when cancer cells metastasize and to identify novel therapeutic targets implicated in tumor lymph node metastases.

5. Conclusion

Multiple lines of evidence have suggested that tumor lymphangiogenesis and lymph node lymphangiogenesis represent crucial prognostic indicators for the risk of future metastasis and overall survival in an increasing incidence of tumors. The

previous reports described above have vastly expanded our understanding of how the networks of multiple transcription factors and signaling cascades regulate the multiple steps during the formation of tumor lymphatic vasculature. A better understanding of the molecular mechanisms how lymphangiogenesis is controlled by the transcription factor networks will contribute to the development of novel therapeutic strategies. Growing lines of evidence have suggested that tumor-associated lymphatic vessels play pivotal roles in tumor metastasis to sentinel lymph nodes. Furthermore, defects in the formation of lymphatic vessels cause the pathogenesis of lymphedema. Therefore, it is of crucial importance to devise and develop strategies to control lymphangiogenesis in order to prevent the metastasis of tumors and to cure lymphedema.

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Figure legends

Graphic abstract. Roles of positive and negative regulators of lymphangiogenesis

during cancer metastasis. Cancer cells and infiltrating immune cells in primary tumor produce various factors that regulate lymphangiogenesis positively (e.g. VEGF-C/D) and negatively (TGF- β and BMP-9). Generation and remodeling of new lymphatic vessels promotes the tumor cells to metastasize to lymph nodes and eventually to distant organs. Understanding the signaling and transcriptional networks regulating these processes will aid in developing novel therapeutic strategies for cancer.

Fig. 1. Structure of lymphatic vessels. Lymphatic capillaries are lymphatic microvessels in peripheral tissues which include the smallest lymphatic vessels and initial lymphatics (very tips incorporating lymph fluids). While both lymphatic capillaries and collecting lymphatic vessels are lined by lymphatic endothelial cells (LECs), they differ in multiple points. LECs of lymphatic capillaries lack tight junctions without coverage by smooth muscle cells. They partly overlap, and form valve-like openings, which allow access for lymph into the vessel lumen in collaboration with the anchoring filaments connecting LECs of lymphatic capillaries to the surrounding extracellular matrix. Under high interstitial pressure, pulling of anchoring filaments induces the opening of EC junctions, followed by influx of tissue fluid into the lymphatic vessel. The lymphatic capillaries first descend into pre-collecting lymphatics, which eventually converge into larger secondary collecting lymphatic vessels. The lymph drains from the lymphatic capillaries to collecting lymphatic vessels, and is incorporated to veins in the jugular region. The collecting lymphatic vessels have tight junctions and are covered by smooth muscle cells, which have intrinsic contractile activity to promote lymph flow. They also contain lymphatic valves that prevent the backflow of the lymph.

Fig. 2. Embryonic formation of lymphatic vessels. (A) Around embryonic day (E) 9.0 during mouse embryogenesis, a subset of venous endothelial cells (green) express Prox1 transcription factor and become committed to differentiate into the lymphatic endothelial cells (LECs). (B) Differentiating LECs express vascular endothelial growth factor receptor 3 (VEGFR3) and migrate towards neighboring mesenchymal cells expressing VEGF-C, a ligand for VEGFR3, and form primary lymph sacs. (C, D) The primary lymphatic vascular plexus (green) becomes separated and apart from the blood vessels (red) (C), and undergoes remodeling and maturation to construct the hierarchy consisting

of lymphatic capillaries and collecting lymphatic vessels (D). Adapted from Karpanen and Alitalo, 2008.

Fig. 3. Formation and remodelling of lymphatic vessels during progression and metastasis of cancer. Various growth factors secreted from cancer and immune cells in tumor promote pre-existing lymphatic vessels to induce generation of new vessels and enlargement of collecting vessels. These lymphangiogenic growth factors also induce generation of new lymphatic vessels in the sentinel lymph nodes (SLNs), enlargement of tumor-draining collecting lymphatics that are proximal and distal to SLNs. These alteration of lymphatic vessels have been implicated in metastasis of tumor cells to distant organs via lymph node metastasis. Adapted from Stacker et al., 2014.

Fig. 4. Lymphatic vessels inside and in the periphery of tumors.

Most tumors induce the formation of lymphatic vessels in the periphery of them. Some tumors recruit lymphatic vessels inside (intratumoral lymphatic vessels) but others do not. BxPC3, human pancreatic adenocarcinoma cells, were xenografted to immuno-deficient mice. The upper region above the dotted line are BxPC3-derived tumor foci, and the lower one is derived from mice (periphery of tumors). Lymphatic vessels and blood vessels are stained with anti-LYVE-1 (red) and anti-PECAM-1 (green) antibodies, respectively. Nuclei are counterstained with TOTO-3. Lymphatic vessels are formed inside the tumors less than in the periphery or hardly found inside in some tumors.

Fig. 5. Inhibition of the inflammatory and tumor lymphangiogenesis by targeting PDGF-BB/PDGFR β signals. (A, B) Repeated intraperitoneal administration of inflammation-inducing thioglycollate led to the formation of lymphangiogenic plaques on the murine diaphragm. Diaphragms from mice were subject to whole-mount immunostaining with anti-LYVE-1 antibody. In order to target the PDGF-BB/PDGFR β signals, imatinib (A) or adenoviruses encoding PDGFR β /Fc (B) were also intraperitoneally administered. PBS (A) or adenoviruses encoding control-Fc were used as negative controls. (C) BxPC3 cells that were infected with lentiviruses encoding control-Fc or PDGFR β /Fc were subcutaneously inoculated in immuno-deficient mice. After 7 weeks, tumors were excised and were subject to immunostaining for LYVE-1.

Figure 6. VEGFR signal transduction in lymphatic endothelial cells.

1 VEGF-C/D is known as one of the most crucial prolymphangiogenic growth factors.
2 VEGF-C/D signals are mediated via VEGFR2/3 in lymphatic endothelial cells.
3 VEGFRs are autophosphorylated upon ligand binding and induce intracellular signaling
4 cascades such as Ras/Raf/MEK/ERK pathway, PI3 dependent Akt pathways, and
5 JNK1/2 pathways. There are several membrane proteins which have been shown to
6 interact with VEGFRs. Neuropilin-2 (Nrp2) is a co-receptor which binds to VEGFR2/3
7 and promote VEGFR3 signaling. Ephrin B2 regulates VEGFR internalization and
8 promote VEGF signaling. Integrin induces ligand independent phosphorylation of
9 VEGFR intracellular domain, and activates the JNK1/2 pathway.

10
11 **Figure 7. PDGFR signal transduction in lymphatic endothelial cells.**

12 PDGF-BB/PDGFR β signaling has been reported to be involved in lymphangiogenesis.
13 Ligand bound PDGFRs are dimerized and autophosphorylated, and induce several
14 intracellular signaling pathways such as Ras/Raf/MEK/ERK pathway and PI3
15 dependent Akt pathway. The role of each signaling pathway in lymphangiogenesis
16 remain to be elucidated. PDGFR α may be involved in this signaling by
17 heterodimerizing with PDGFR β but the involvement in lymphangiogenesis is not well
18 described yet.

19
20 **Figure 8. TGF- β superfamily signal transduction in lymphatic endothelial cells.**

21 In endothelial cells, TGF- β and BMP-9/10 are transmitted through TGF- β type I
22 receptor, TGF β RI /ALK5 and BMP Type I receptor, ALK1, respectively. TGF- β induces
23 phosphorylation of Smad2/3 via TGF β RI, whereas BMP9/10 binds to ALK1 and
24 phosphorylate Smad1/5/8. Both of activated Smads form heteromeric complex with
25 Smad4, and these complexes regulate gene expression by interaction with various
26 co-factors. There also exist several noncanonical (Smad-independent) pathways such as
27 Ras/Raf/MEK/ERK pathway and JNK pathway in the downstream of TGF- β /BMP
28 signaling, and these pathways also interact with Smad-dependent pathways by affecting
29 phosphorylation of Smad2/3.

30 TGF- β and BMP9 negatively regulate lymphangiogenesis, downregulating expression
31 of several lymphatic markers such as Prox1, LYVE-1, and VEGFR3.

A. E9-E9.5

B. E10-E11

C. E11.5-E14.5

D. E14.5-postnatal

Yoshimatsu, Fig. 4

Yoshimatsu, Fig. 6

Yoshimatsu, Fig. 7