

Molecular mechanisms of lymphangiogenesis in development and cancer

IMKE ALBRECHT and GERHARD CHRISTOFORI*

Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Basel, Switzerland

ABSTRACT The lymphatic system, also named the second vascular system, plays a critical role in tissue homeostasis and immunosurveillance. The past two decades of intensive research have led to the identification and detailed understanding of many molecular players and mechanisms regulating the formation of the lymphatic vasculature during embryonic development. Furthermore, clinical and experimental data clearly demonstrate that the formation of new lymphatic vessels by sprouting lymphangiogenesis from pre-existing lymphatic vessels, or by the *de novo* formation of lymphatic capillaries also occurs in various pathological conditions, such as cancer and organ transplant rejection, while lymphangiogenesis is non-functional in primary edema. In cancer, lymphatic vessels are one major gateway for invasive tumor cells to leave the primary tumor site and to establish distant organ metastasis. Therefore, the specific targeting of the lymphatic vasculature at the tumor site could be a promising approach to prevent metastasis formation.

KEY WORDS: *angiogenesis, cancer, development, lymphangiogenesis, metastasis, signaling*

Introduction

The lymphatic system is composed of a network of lymphatic vessels and lymphoid organs and fulfils three principal roles (Figure 1A). Firstly, it ensures the maintenance of the blood and tissue volume. For the supply of tissue and organs with nutrients and oxygen, fluid from the blood vasculature system constantly discharges into the surrounding tissue. Only 90% of this interstitial fluid is transported back via the venous system and, thus, the lymphatic vessel network is responsible for the drainage of most of the excess interstitial fluid back to the circulation (up to 2 liters daily). In doing so, it also removes catabolic products from tissue and organs. Secondly, the lymphatic system is also a pivotal component of the immune system. Lymphoid organs are the places where immune cells are generated, where they can monitor for the presence of pathogens, and where an immune response is initiated. Finally, the lymphatic system is responsible for the transport of dietary fat from the intestine to the liver.

However, in pathological conditions, the functional role of the lymphatic system is often misused. In cancer patients, the lymphatic vessels provide conduits for metastasizing cancer cells to leave the primary tumor site and to establish secondary tumors in regional lymph nodes and in distant organs. For long, the process of lymphatic metastasis has been considered a passive process based on the random existence of lymphatic vessels nearby the growing

tumor known (Sleeman *et al.*, 2009; Tammela and Alitalo, 2010). Today, it is well established that tumors and the lymphatic vasculature interact with each other in promoting metastasis formation. Therefore, understanding the molecular mechanisms underlying the development and the function of the lymphatic system may allow the design of novel therapeutic approaches to interfere with metastasis formation.

Structural composition of the lymphatic system

All vertebrates have a lymphatic system, yet with differing complexities (Butler *et al.*, 2009). In mammals, lymphatic vessels are found in all vascularized organs and tissues, with the exception of the brain and retina where drainage of interstitial fluid occurs perivascular or via a venous network (Fig. 1A).

The lymphatic vasculature is formed by lymphatic endothelial cells (LEC), which are highly related to blood endothelial cells (BEC). Although BEC and LEC represent two distinct cell populations (Podgrabinska *et al.*, 2002; Petrova *et al.*, 2002; Hirakawa *et al.*, 2003; Nelson *et al.*, 2007; Wick *et al.*, 2007), they can under certain circumstances gain the characteristics of the other cell type. Until recently, the identification of lymphatic vessels was hampered by

Abbreviations used in this paper: BEC, blood endothelial cell; LEC, lymphatic endothelial cell

*Address correspondence to: Gerhard Christofori. Institute of Biochemistry and Genetics, Department of Biomedicine, University of Basel, Mattenstrasse 28, CH-4058 Basel, Switzerland. Fax: +41-61-267-3566. e-mail: gerhard.christofori@unibas.ch

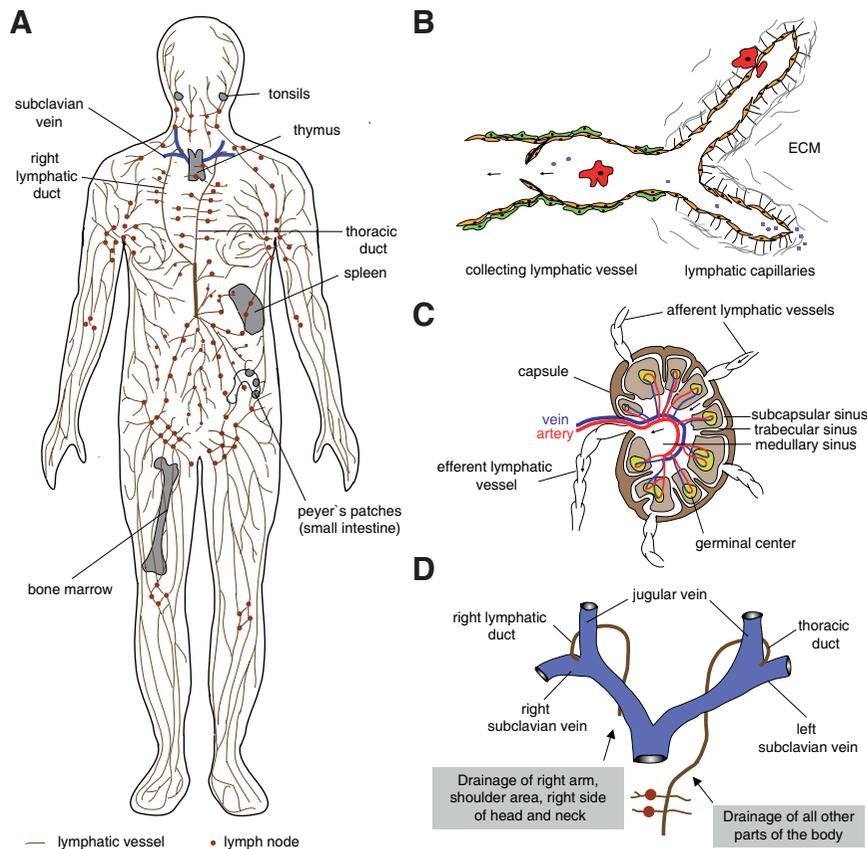


Fig. 1. The lymphatic system. (A) Schematic illustration of the human lymphatic vascular system. (B) Structure of lymphatic vessels. Blind-ending lymphatic capillaries are the main entry site for interstitial fluid, macromolecules and cells into the lymphatic system. Capillaries consist of single endothelial cell layers and are anchored to the surrounding ECM by filaments. Capillaries descend into collecting lymphatic vessels that have a basement membrane, are covered by smooth muscle cells. Valves in the collecting vessels prevent fluid backflow. (C) Schematic representation of a lymph node. The lymph enters via afferent lymphatic vessels the lymph node and is drained through the subcapsular, trabecular and medullary sinus to the efferent lymphatic vessel as indicated by arrows. During passage through the lymph node, the lymph fluid is surveyed by immune cells for antigens. (D) Connection of the lymphatic system with the blood vasculature at the subclavian veins.

the lack of specific LEC markers. With the discovery of proteins specifically expressed by LEC research on lymphangiogenesis and lymphatic vessel homeostasis and function has been impressively progressed. Specific markers for LEC include Lyve-1 (a lymphatic vessel endothelial hyaluronan receptor (Banerji *et al.*, 1999), podoplanin (a type-1 transmembrane sialomucin-like glycoprotein; (Breiteneder-Geleff *et al.*, 1999), the transcription factor Prox1 (the orthologue of the *Drosophila* homeobox transcription factor prospero (Wigle and Oliver, 1999; Wigle *et al.*, 2002), and vascular endothelial growth factor receptor-3 (VEGFR3) (Kaipainen *et al.*, 1995). However, the identification of lymphatic vessels should not exclusively be based on only one of these markers, since they are also expressed by other cell types and their expression may vary among different LEC populations.

The vascular network of the lymphatic system starts in the periphery with blind-ending lymphatic capillaries of a diameter between 30–80 μm (Fig. 1B). They are composed of a single layer of oak-leaf shaped LECs. The cells are loosely connected with each other by discontinuous “button-like structures” made of VE-cadherin

and other junctional proteins (Baluk *et al.*, 2007). Lymphatic capillaries have no basement membrane and are not covered by smooth muscle cells. They are tethered by anchoring filaments composed mainly of emillin-1 and fibrillin to collagen fibers of the extracellular matrix (Danussi *et al.*, 2008). Under low interstitial pressure (IFP) the vessels are normally collapsed. An increase in the amount of extracellular fluid leads to tissue swelling and, via tension on the anchoring filaments, to the formation of gaps in the lymphatic endothelial cell lining where fluid and cells can now enter. The lymphatic capillaries descend into collecting lymphatic vessels via so-called pre-collector vessels, which have only a sparse coverage with smooth muscle cells. In contrast, collecting lymphatic vessels structurally resemble veins with endothelial cells continuously connected to each other in a “zipper-like” structure, with a basement membrane and with a layer of smooth muscle cells (Baluk *et al.*, 2007). In order to ensure a unidirectional flow of the lymph from the periphery towards the blood circulation, collecting vessels contain bi-leaflet valves that consist of a matrix core anchored to the vessel wall and covered on both sides with LEC. In mammals the driving forces for the generation of lymph flow are not provided by a central pump but are generated both intrinsically by contractions of the lymph vessel coat as well as extrinsically by skeletal muscle contractions and respiratory movements. The lymph of the body is drained into two main lymphatic vessels, the right lymphatic duct and the thoracic duct. Both transport the lymph back into the circulation via connections with the right and left subclavian veins, respectively (Fig. 1D).

Besides the lymph conducting system, the lymphatic system holds the lymphoid tissue, which is involved in immune surveillance and the generation of immune responses. Depending on the role, we distinguish between primary, secondary and tertiary lymphoid organs. Production and selection of lymphocytes from immature progenitor cells take place in the primary lymphoid organs, the thymus and bone marrow. The secondary lymphoid organs, including lymph nodes, spleen and mucosa-associated lymphoid tissue (MALT), such as tonsils and Peyer's patches, provide an environment where lymphocytes can encounter foreign antigens and initiate a specific immune response (Fig. 1C). Tertiary lymphoid tissues are ectopic accumulations of immune cells formed randomly in the adult during chronic inflammation. Organogenesis of the lymphoid tissue is beyond the scope of this review and we refer the interested reader to the relevant literature.

Development of the lymphatic vasculature

Establishment of lymphatic endothelial cell identity

The lymphatic vessel network forms after the blood vascular system has been established. Shortly after the separation of arteries and veins, around embryonic day (E) 9.0 in mice (gestation week 6 in humans), a distinct population of VEGFR3+ cells of the anterior

cardinal vein starts to express Lyve-1 (Fig. 2A). The signal driving Lyve-1 expression, possibly the first step in acquiring LEC identity, has not been identified yet. Also, Lyve-1's functional contribution to lymphatic vasculature development remains obscure: Lyve-1 has been found to be non-functional in HA binding in LEC (Nightingale *et al.*, 2009), and its ablation in Lyve-1-knockout mice has no discernable effect on the development of the lymphatic vasculature (Gale *et al.*, 2007; Luong *et al.*, 2009).

In contrast to Lyve-1, the transcription factor Prox1 is essential for the development of the lymphatic vasculature (Wigle and Oliver, 1999; Wigle *et al.*, 2002). Prox1 is expressed in the VEGFR3+/Lyve-1+ cells of the cardinal vein before these cells sprout and bud off to form the primary lymphatic sacs (Fig. 2). Mice lacking Prox1 fail to develop primary lymphatic sacs and consequently a lymphatic vasculature and die before birth. The failure is due to the inability of the blood-endothelium-derived VEGFR3+/Lyve-1+ cells to upregulate LEC-specific genes and to acquire an LEC identity. Indeed, forced expression of Prox1 in blood endothelial cells *in vitro* and *in vivo* is sufficient to repress the expression of BEC-specific genes and to gain expression of LEC-specific markers (Hong *et al.*, 2002, 2004; Petrova *et al.*, 2002).

The transcriptional activity of Prox1 is partly co-regulated by COUP-TFII (chicken ovalbumin upstream promoter-transcription factor II), a transcription factor required for maintenance of venous endothelial cell identity. COUP-TFII directly interacts with Prox1 thereby inducing the expression of genes, such as VEGFR3, Cyclin E1, and FGF3 (Yamazaki *et al.*, 2009; Lee *et al.*, 2009). Specific inactivation of COUP-TFII during early development of the lymphatic vasculature demonstrates its requirement for the establishment of LEC identity by ensuring the venous character of the cardinal vein. Interestingly, loss of COUP-TFII at later developmental stages correlates with the loss of LEC identity without altering Prox1 expression, suggesting that COUP-TFII and Prox1 together are required to maintain LEC specification (Lin *et al.*, 2010).

The regulation of Prox1 expression is not completely understood. Treatment of cultured endothelial cells with IL3 and IL7 is sufficient to induce Prox1 expression (Groger *et al.*, 2004; Al-Rawi *et al.*, 2005). Recent results from mouse genetic experiments suggest that Prox1 gene expression is directly induced by the transcription factor Sox18 (Francois *et al.*, 2008). During mouse embryogenesis Sox18 is found to be expressed prior to Prox1 in VEGFR3+/Lyve-1+ endothelial cell population of the cardinal vein committed to develop into LEC. Conversely, genetic ablation of Sox18 function in knockout mice or in the "ragged" mouse mutant results in a loss of Prox1 expression in these cells. As a result, similar to Prox1 knockout mice, Sox18-deficient mice develop edema and die before birth. However, the signal driving Sox18 expression in VEGFR3+/Lyve-1+ cells is not known and the "master switch" of lymphatic development still remains elusive.

Together, these results seem to settle a longstanding controversy about the origin of LEC in mammals. In 1908, Huntington and McClure have proposed that the first arising lymphatic vessel is formed by mesenchymal cells (centripetal model) (Huntington and McClure, 1908), while Sabin has claimed in 1902 that the primary lymphatic sacs originate from venous cells, which then later, by budding and sprouting into the surrounding tissue, give rise to the lymphatic vasculature (centrifugal model) (Sabin, 1902). The complete failure in the formation of a lymphatic vasculature after preventing the establishment of an LEC identity in venous-derived

BEC, clearly demonstrates that the mammalian lymphatic system has a venous origin. Nevertheless, in the developing embryo there seems to be mesenchymal cells expressing LEC markers. Whether and to which extent these cells contribute to the develop-

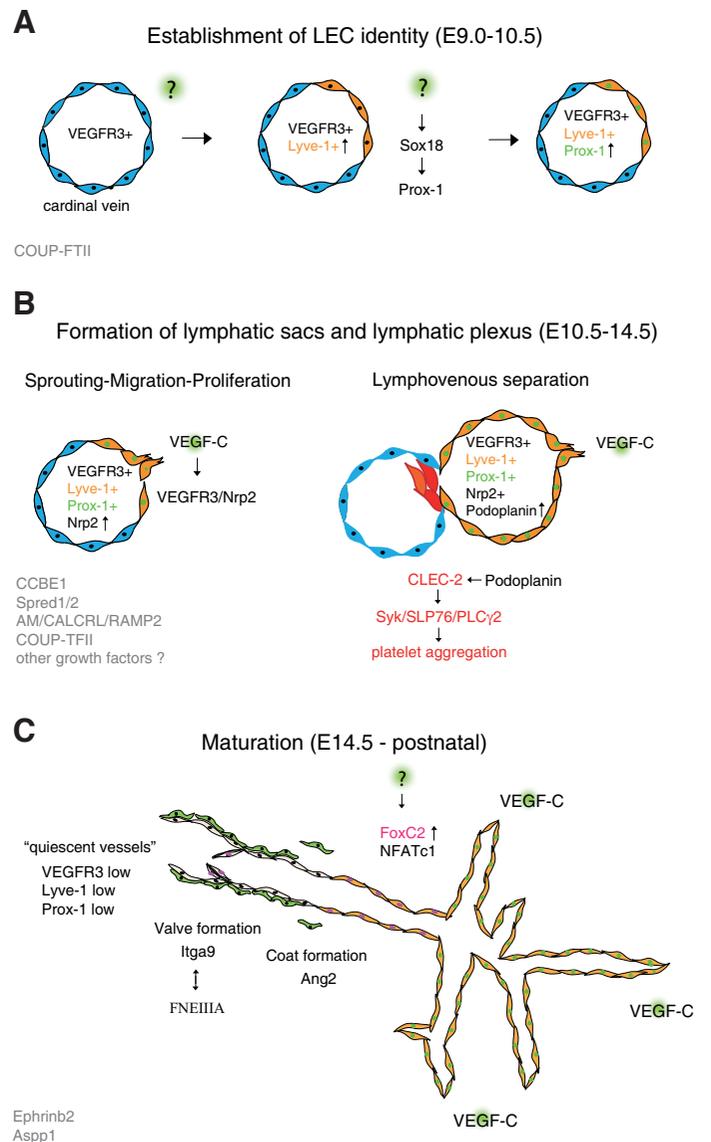


Fig. 2. Development of the murine lymphatic vascular network. (A) At embryonic day (E) 9.0, after arterial-venous separation, cells of the cardinal vein start to lose blood endothelial characteristics and acquire a lymphatic endothelial cell (LEC) identity. This process is controlled by the sequential expression of Lyve-1, Sox18 and Prox1. **(B)** At E10.5, LEC then bud off the cardinal vein, migrate into the surrounding tissue and form primary lymphatic sacs. This process is dependent on VEGF-C/VEGFR3/Nrp2 signaling. Subsequently, the primary lymphatic sacs separate from the cardinal vein and by further growth and spreading into the tissue, gives rise to a primitive lymphatic plexus. **(C)** At E14.5, remodeling of the primitive lymphatic vasculature begins and lasts until after birth. During this period a hierarchical network consisting of collecting lymphatic vessels and lymphatic capillaries are formed. This maturation process involves changes in protein expression leading to a quiescent, non-growing vessel, the formation of lymphatic valves and the acquisition of a smooth muscle coat. In the figure, the molecules involved in the different steps of lymphatic vessel formation are displayed. Their functional contribution is described in the text.

ping lymphatic vasculature remains to be investigated (Buttler *et al.*, 2006, 2008). In contrast to mammals, in lower vertebrates the origin of LEC seems more diverse. In the zebra fish *D. rerio*, lineage tracing experiments suggest a venous origin of LEC (Yaniv *et al.*, 2006; Kuchler *et al.*, 2006), while in *X. laevis* tadpoles and in chicken the mesenchyme contributes to the formation of lymphatic vasculature (Ny *et al.*, 2005; Wilting *et al.*, 2006).

Formation of the primary lymphatic sacs and plexus

Lymphatic endothelial cell proliferation and migration

Around E10.5, when the cells have been committed to their LEC character, they bud off the cardinal vein, migrate in a polarized manner into the surrounding tissue, where they proliferate and form the primary lymphatic sacs (6 sacs in humans and 8 sacs in mice). Expansion of the lymphatic sacs by sprouting and proliferation leads to the formation of the primitive lymphatic vasculature (Fig. 2B). Similar to the blood vasculature, sprouting lymphatic vessels contain specialized tip cells at their leading edge, which sense and respond to chemoattractants. One major attractant is VEGF-C, which is expressed by mesenchymal cells adjacent to the growing lymphatic vessels.

VEGF-C, VEGF-D and their cognate receptor VEGFR3 are commonly regarded as the lymphangiogenic branch of the vascular endothelial growth factor signaling axis (Lohela *et al.*, 2009). VEGF-C and D promote migration and proliferation of LEC *in vitro* and adenoviral or transgenic expression of these VEGFR3 ligands induced lymphangiogenesis *in vivo* (Jeltsch *et al.*, 1997; Enholm *et al.*, 2001; Byzova *et al.*, 2002). During development, allelic loss of VEGF-C is sufficient to cause severe lymphedema, and homozygous VEGF-C deletion results in the complete loss of the lymphatic vasculature. Apparently, despite correct LEC specification, in VEGF-C-deficient mice the committed cells fail to sprout from the cardinal vein (Karkkainen *et al.*, 2004). In contrast to VEGF-C, genetic ablation of VEGF-D has no discernable effect on the development of the lymphatic vasculature (Baldwin *et al.*, 2005).

In their unprocessed form VEGF-C and VEGF-D bind exclusively VEGFR3 (Lohela *et al.*, 2009). In the early stages of development all endothelial cells express VEGFR3. Loss of VEGFR3 signaling in this period results in embryonic lethality due to vascular failure before the lymphatic vascular development has started (Dumont *et al.*, 1998). At E12.5 VEGFR3 expression becomes restricted to LEC (Kaipainen *et al.*, 1995), when defective VEGFR3 signaling interferes with the development of a proper lymphatic vasculature. Mutations in the tyrosine kinase domain of VEGFR3 results in a loss of signaling activity and leads to lymphatic hypoplasia and lymphedema in patients (Milroy Disease) and in mice (Chy mutant mice) (Irrthum *et al.*, 2000; Karkkainen *et al.*, 2001).

An integral part of the VEGF-VEGFR signaling complexes are neuropilins (Nrp), which were first identified as axon guidance molecules in the central nervous system. Nrp do not possess an own enzymatic signaling activity, yet they function as co-receptors of VEGF receptors by modulating the ligand binding to the receptor (Karpanen *et al.*, 2006a). Nrp1 is expressed by arterial BEC, while Nrp2 is expressed by venous BEC and by LEC. Genetic deletion of Nrp2 or its neutralization by specific anti-Nrp2 antibody reduces the number of small lymphatic vessels and capillaries (Yuan *et al.*, 2002). Blocking of Nrp2 function affects outgrowth of lymphatic sprouts by inhibiting tip cell migration (Xu *et al.*, 2010). Nrp2 gene expression in LEC is at least in part regulated by COUP-TFII, and

silencing of COUP-TFII during embryogenesis reduces Nrp2 expression and blocks lymphatic sprout formation (Lin *et al.*, 2010).

VEGF-C/VEGFR3 signaling is also controlled by the action of Spred (Sprouty-related) proteins, negative regulators of growth factor-induced MAP kinase signaling. Spred 1 and 2 suppress LEC proliferation and differentiation by blocking VEGF-C/VEGFR3 signaling. Spred1/2 double-knockout mice exhibit subcutaneous hemorrhages, edema and blood-filled dilated lymphatic vessel and die at E12.5-15.5 (Taniguchi *et al.*, 2007). Another negative regulator of the VEGF-C/VEGFR3 signaling is TGF β . TGF β signaling blocks proliferation, migration and expression of lymphatic markers in LEC stimulated with VEGF-C. Conversely, loss of TGF β signaling enhances lymphangiogenesis (Clavin *et al.*, 2008; Oka *et al.*, 2008). Whether TGF- β also modulates VEGF-C/VEGFR3 signaling in the early stages of lymphatic vasculogenesis has to be investigated. In contrast, blockage of TGF- β signaling at late stages of development severely affects the maturation and remodeling of the lymphatic vasculature indicating that TGF- β might also regulate other signaling pathways (Niessen *et al.*, 2010).

Besides VEGFR3-mediated signal transduction, other signaling pathways have also been implicated in lymphatic vessel development. Blockade of adrenomedullin (AM) signaling achieved by genetic ablation of either the AM ligand, the AM receptor (CALCRL), the intracellular receptor-activity modifying protein (RAMP2), or the AM processing enzyme (PAM) leads to decreased LEC proliferation, to the occurrence of smaller lymphatic sacs and to the development of interstitial lymphedema (Fritz-Six *et al.*, 2008; Czyzyk *et al.*, 2005). Conversely, treatment with AM induces lymphangiogenesis and can resolve lymphedema (Jin *et al.*, 2008).

A complete loss of the lymphatic vasculature has been recently reported to occur in Zebrafish upon genetic ablation of CCBE1 (collagen and calcium binding EGF-domain-1 protein). CCBE1 is a secreted protein that is not expressed by LEC. However, similar to VEGF-C, it is essential for LEC in order to sprout from the veins and to form the primary lymphatic sacs (Hogan *et al.*, 2009). In humans, mutations in CCBE1 are found to cause a form of congenital lymphedema (Hennekam syndrome) (Alders *et al.*, 2009). Whether CCBE1 only functions as a guidance molecule for growing lymphatics or whether it also promotes LEC proliferation awaits further analysis.

Finally, a number of growth factors display lymphangiogenic properties *in vitro* and *in vivo*, including VEGF-A (Nagy *et al.*, 2002), IGF1/2 (Bjorndahl *et al.*, 2005a), PDGF-BB (Cao *et al.*, 2004), HGF (Kajiyama *et al.*, 2005), GH (Banziger-Tobler *et al.*, 2008), and FGF2 (Kubo *et al.*, 2002; Chang *et al.*, 2004; Shin *et al.*, 2006). However, the extent and mode of action by which these growth factors contribute to the formation of the lymphatic system warrants further investigations.

Separation of blood and lymphatic vasculature

Except for the two connections between the veins in the neck region and the right lymphatic and thoracic duct, the lymphatic system is completely separated from the blood vasculature. The molecular mechanisms underlying this separation seem to involve the function of podoplanin, the tyrosine kinase Syk, phospholipase-C γ 2 (PLC γ 2), and the signaling adaptor SLP-76: knockout of either of these genes in mice results in blood filled lymphatic vessels (Abtahian *et al.*, 2003; Uhrin *et al.*, 2010; Ichise *et al.*, 2009). The defect in lympho-venous separation in these mice is likely based

on a defect in platelet aggregation, which is required for constriction of the opening between the cardinal vein and the lymphatic sacs. Platelet aggregation at the separation zone is induced by LEC via binding of podoplanin to CLEC-2 (C-type lectin-like protein) expressed on the surface of platelets. Binding of podoplanin to CLEC-2 triggers an intracellular signaling cascade, which is dependent on Syk, SLP-76 and PLC γ 2 (Fig. 2B).

Maturation of the lymphatic system

Maturation of the lymphatic system begins at around E14.5 and lasts until birth. During this time period, the primitive lymphatic plexus develops into a hierarchical network of collecting lymphatic vessels and lymphatic capillaries. The structural remodeling of collecting vessels involves the formation of intraluminal valves and the attainment of smooth muscle coverage. This maturation steps are in parts accompanied by reduced expression of LEC markers implicated in actively growing vessels, including Lyve-1, Prox1 and VEGFR3 (Fig. 2C).

One major regulator for the establishment of the collecting vessel phenotype is the forkhead transcription factor FoxC2. Mutations in the FoxC2 gene were identified as the cause for the human hereditary lymphedema-distichiasis syndrome of puberty onset limb edema (Fang *et al.*, 2000). Inactivation of FoxC2 in mice phenocopies the human disease with impaired development of lymphatic vessel valves and an increased coverage of capillaries with smooth muscle cells (Petrova *et al.*, 2004). During development, FoxC2 expression is upregulated between E14.5 and E15.5 by a thus far unknown mechanism in maturing lymphatic vessels. The transcription factor NFATc1, whose expression is controlled by VEGF-C/VEGFR3 signaling, cooperates with FoxC2 to trigger the genetic program involved in the maturation process (Norrmen *et al.*, 2009).

With the accumulation of basement membrane proteins at E16.5 recruitment of NG2-positive mural cells begins to finally generate the smooth muscle cell coverage observed in major lymphatic vessels (Norrmen *et al.*, 2009). Similar to the remodeling of the blood vasculature, angiopoietins (Ang) are implicated in lymphatic vessel maturation by binding to their bona fide tyrosine kinase receptor Tie2 (Augustin *et al.*, 2009). Besides defects in the vascular remodeling process, Ang2 knockout mice exhibit a lymphatic phenotype with edema formation, abnormal patterning of lymphatic vessels, and a block in lymphatic vessel maturation. Furthermore, lymphatic capillaries in Ang2-deficient mice are covered with periendothelial cells, suggesting that Ang2 may be necessary to prevent premature coverage of lymphatic vessels with smooth muscle cells (Gale *et al.*, 2002; Shimoda *et al.*, 2007; Dellinger *et al.*, 2008). During the maturation process, FoxC2 and NFATc1 repress Ang2 expression, thus initiating the recruitment of pericytes and smooth muscle cells to the maturing vessels (Norrmen *et al.*, 2009). Ang1, although reported to promote lymphangiogenesis in a mouse cornea assay, is dispensable for lymphatic development (Morisada *et al.*, 2005). On the contrary, loss of Tie1 leads to edema and abnormal patterning of the lymphatic sac, suggesting a role of this thus far poorly understood receptor in lymphatic vessel formation (Sato *et al.*, 1995; Puri *et al.*, 1995; D'Amico *et al.*, 2010). Defects in lymphatic vessel maturation were also reported in mice with inactive EphrinB2 and in Aspp1 (apoptosis-stimulating protein of p53) knockout mice (Makinen *et al.*, 2005; Hirashima *et al.*, 2008).

The formation of lymphatic valves is dependent on the presence

of α_9 -integrin. Expression of α_9 -integrin is increased in Prox1+/FoxC2+ LEC during the maturation process and later becomes restricted to LEC located on valves. Loss of α_9 -integrin results in abnormal valve formation similar to that seen in fibronectin-E11A knockout mice (Bazigou *et al.*, 2009; Muro *et al.*, 2003). Apparently, the interaction between α_9 -integrin and fibronectin-E11A induces fibronectin fibril assembly, which is essential for the proper assembly of the extracellular matrix and lymphatic valves.

In the adult, the lymphatic vasculature is rather quiescent, and survival of LEC is no longer dependent on VEGFR3 signaling (Karpanen *et al.*, 2006b). Under normal physiological conditions the *de novo* formation of lymphatic vasculature is restricted to the endometrium during pregnancy. However, in several pathological situations, including wound healing, tissue repair, inflammation, organ transplant rejection and cancer, new lymphatic vasculature is generated.

Lymphangiogenesis in cancer

In a number of cancer types, including breast cancer, melanoma, prostate cancer, gastric cancer and colon cancer, the occurrence of metastasis in the tumor draining lymph node (the "sentinel" lymph node) is commonly regarded the first step in metastatic cancer cell dissemination. This notion is based on the observation that cancer cells access the lymphatic vasculature at the tumor site from where they spread via the lymphatic system first to draining regional lymph nodes and further on to the blood stream and to distant organs (Fig. 3D). Therefore, the examination of sentinel lymph nodes for metastasis is part of the regular clinical routine and is critical to stage disease progression, to determine patient prognosis and to select the appropriate treatment strategies. Yet, parameters associated with lymphatic vessels growth and with tumor cell invasion into the lymphatic vasculature also hold predictive weight. In cancer patients, an increase in these "lymphatic parameters", including lymphatic vessel density (LVD), lymphovascular invasion (LVI), and lymphangiogenic growth factor levels, generally correlates with tumor recurrence and increased regional and distant organ metastasis (Tammela and Alitalo, 2010; Sleeman *et al.*, 2009).

Animal models have been instrumental in demonstrating that active lymphangiogenesis at the tumor site is sufficient to promote lymphatic metastasis. For example, the forced expression of the lymphangiogenic factors VEGF-C and VEGF-D in tumors of xenograft-transplanted or of transgenic mice has lead to activated lymphangiogenesis, the formation of tumoral lymphatic vessels and increased lymph node and distant organ metastasis (Stacker *et al.*, 2001; Kopfstein *et al.*, 2007; Mandriota *et al.*, 2001; Skobe *et al.*, 2001; Karpanen *et al.*, 2001; Yanai *et al.*, 2001; He *et al.*, 2002; Padera *et al.*, 2002). Lymphangiogenesis and subsequent lymphatic metastasis has also been seen in a mouse model of skin cancer upon transgenic expression of VEGF-A and in a murine fibrosarcoma model by the forced expression of PDGF-BB (Cao *et al.*, 2004; Hirakawa *et al.*, 2005; Bjorndahl *et al.*, 2005b). Notably, while PDGF-BB-mediated lymphangiogenesis seems independent of VEGFR3 signaling, VEGF-A exhibits its lymphangiogenic action mainly by attracting macrophages which are known to express VEGF-C and D and thus promote lymphangiogenesis. Moreover, VEGF-A may also induce VEGF-C expression in LEC.

Besides overexpression of lymphangiogenic factors, tissue re-

modeling can also contribute to lymphangiogenesis. For example, the deletion of the neuronal cell adhesion molecule (NCAM) in a mouse model of pancreatic insulinoma (Rip1 Tag2 mouse model) causes loss of β_1 -integrin-mediated adhesion of tumor cells and results in upregulated expression of VEGF-C and VEGF-D and thus promotes lymphangiogenesis (Crnic *et al.*, 2004; Kren *et al.*, 2007). However, an increase in proliferating LEC and in lymphatic vessel density at the tumor site is not always detected in the presence of lymph node metastasis. Rather than inducing lymphangiogenesis, tumors can also co-opt existing lymphatic vessels, especially when they are located in a tissue with high lymphatic vessel density (Sleeman *et al.*, 2009).

Phenotype of tumor-derived lymphatic vessels

Tumor lymphatics can be found intratumoral as well as peritumoral. Similar to tumor blood vessels, tumor-associated lymphatic vessels are structurally different from their quiescent counterparts in healthy tissue. In particular, intratumoral lymphatic vessels are usually disorganized and are in most cases non-functional as they are collapsed due to the high intratumoral pressure to which they are exposed. They may not contribute to lymphogenic metastasis (Padera *et al.*, 2002). However in some cancer types, such as gastric, ovarian and renal cancer, intratumoral lymphatic density positively correlates with lymph node metastasis (Lee *et al.*, 2010; Li *et al.*, 2009; Horiguchi *et al.*, 2008).

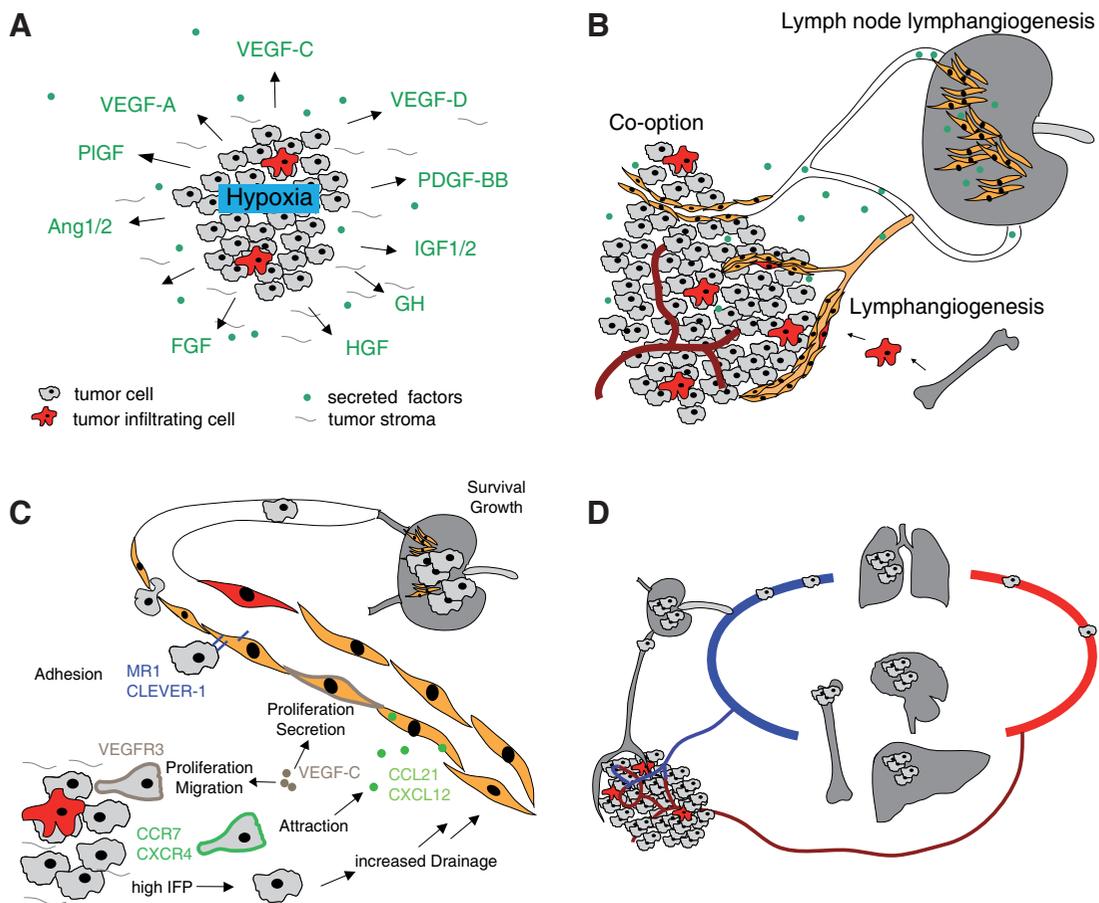


Fig 3. Tumor lymphangiogenesis and its contribution to metastatic spreading. (A) Induced by hypoxia and other signals, tumor cells, stromal cells and tumor-infiltrating cells secrete angiogenic and lymphangiogenic factors, such as VEGF-C, VEGF-D, VEGF-A, PDGF-BB, Angiopoietin-1 and 2 and PIGF, which all can contribute to tumor lymphangiogenesis. **(B)** Secreted lymphangiogenic factors act on existing lymphatic vessels at the tumor site inducing vessel sprouting, LEC proliferation and subsequent formation of intra and peritumoral lymphatic vessels (lymphangiogenesis). Bone-marrow-derived LEC precursor cells and myeloid lineage cells directly integrate into newly formed tumor lymphatics. Finally, lymphangiogenic factors draining from the tumor to neighboring lymph nodes, induce lymphangiogenesis in the lymph nodes prior to the onset of metastasis. Besides inducing the formation of new lymphatic vessels, tumors can co-opt existing lymphatics at the tumor site. **(C)** The lymphatic vasculature contributes to metastasis formation in several ways. The entry of the tumor cells into lymphatic vessels is promoted by increased fluid drainage due to high intratumoral interstitial fluid pressure (IFP). LECs can secrete chemokines (CCL21, CXCL12), which actively recruit tumor cells expressing the cognate chemokine receptors (CCR7, CXCR4) to lymphatic vessels. Secreted growth factors and chemokines can also increase tumor cell migration. Adhesion of tumor cells to the lymphatic vessels is in part mediated by MR1 and CLEVER-1. Lymph node lymphangiogenesis as well as changes in the lymph nodes' immune cell composition promotes survival of disseminated tumor cells and outgrowth of metastases in lymph nodes. **(D)** Lymphogenic vs. hematogenic tumor cell dissemination. Tumor cells gain access to tumoral lymphatic vessels (grey) from where they are transported to the blood circulation (lymphogenic dissemination). Establishment of sentinel lymph node metastasis is the first sign of metastatic spreading. Invasive cancer cells can also reach the blood circulation (blue, red) directly at the primary tumor site and disseminate via the blood circulation to distant organs where they induce the formation of secondary tumors (hematogenic dissemination).

Genome-wide transcriptional profiling analysis reveals that tumor-derived LEC are different from LEC isolated from normal tissue (Clasper *et al.*, 2008; Wu *et al.*, 2010). Tumor-derived lymphatic vessels resemble activated, growing vessels and, thus, tumor-derived LEC express many factors implicated in lymphangiogenesis. For example, VEGFR3 and Nrp2, known to be functional during embryonic lymphangiogenesis, are specifically expressed by tumor-associated lymphatic vessels (Caunt *et al.*, 2008). Lymphatics at the tumor site are also exposed to a variety of inflammatory molecules and, due to increased intratumoral IFP, exhibit increased lymph flow. Together these pathophysiological changes in the tumor microenvironment alter LEC gene expression and cell-cell and cell-matrix interactions (Miteva *et al.*, 2010; Clasper *et al.*, 2008).

Mechanisms of tumor lymphangiogenesis

Tumor lymphangiogenesis is mainly based on sprouting lymphangiogenesis and thereby mainly observed in tumors that are growing in lymphatic-rich tissue. In response to lymphangiogenic factors, lymphatic vessels start to form sprouts and, driven by LEC proliferation, new lymphatic vessels are formed in the periphery or within the tumor. The hypoxic conditions and the growth stimulatory environment in a growing tumor instigate tumor cells, cells of the tumor stroma, and tumor-infiltrating inflammatory cells to express a variety of growth factors, including VEGFs, FGFs, PDGFs, Angiopoietins, HGF, GH and IGFs, and thus inducing the formation of new blood vessels in the tumor (referred to as the angiogenic switch). Furthermore, the presence of the classical lymphangiogenic factors VEGF-C and VEGF-D at the tumor site induces the formation of tumor lymphatic vessels. To which degree the other angiogenic factors, which in many cases are also lymphangiogenic, contribute to tumor lymphangiogenesis remains to be determined (Fig. 3A).

In addition to sprouting lymphangiogenesis, precursor LEC and bone marrow-derived cells can also directly contribute to the formation of lymphatic vessels during cancer and other, mainly inflammatory pathological conditions. For example, after transplantation of GFP+ hematopoietic stem cells (HSC) into tumor-bearing mice, 1-3% of the cells of tumor lymphatics express GFP. This result indicates that HSC can give rise to LEC, which integrate into tumor-associated lymphatic vessels (Jiang *et al.*, 2008). Moreover, in a mouse model of fibrosarcoma, the presence of bone-marrow-derived cells in newly formed tumor lymphatics has been detected (Religa *et al.*, 2005). Besides LEC precursor cells, which thus far have not been well characterized, also differentiated cells of bone marrow origin are able to become part of the lymphatic endothelium. For example, in human renal transplants, in a cornea inflammation model, and in two mouse models of cancer (the Rip1Tag2 transgenic model of pancreatic β cell carcinogenesis and the TRAMP-C syngeneic transplantation model of prostate cancer) approximately 3% of all cells found in newly formed lymphatic vasculature are derived from the myeloid-monocyte lineage (Kerjaschki *et al.*, 2006; Maruyama *et al.*, 2005; Zumsteg *et al.*, 2009). Apparently, the integrated, bone marrow-derived cells lose expression of their macrophage markers and gain the expression of all LEC markers. Notably, this transdifferentiation process can be recapitulated *in vitro* and seems to depend on FGF signaling (Zumsteg *et al.*, 2009). Whether these integrated macrophages provide special functions to ongoing pathological lymphangiogenesis remains to be determined. However, there are also reports failing to detect

any contribution of bone-marrow-derived cells to tumor lymphatic vessels (He *et al.*, 2004), raising the possibility that such contribution may depend on specific, possibly inflammatory conditions in the tumor microenvironment.

Contribution of tumor-associated lymphatics to metastatic spreading

Tumor associated lymphatic vessels can support metastatic spreading in several ways. Firstly, the lymphatic vasculature provides both the conduits (lymphatic vessels) as well as the means of transport (lymph flow) allowing the invasive cancer cell to leave the primary tumor site and to travel to distant organ sites. In general, tumor blood vessels are immature and leaky which results in a high IFP in tumors. As a consequence, drainage of the tumor is increased and high IFP and high fluid flow facilitate the entry of tumor cells into lymphatic vessels. In particular, lymphatic capillaries are easily accessible with their discontinuous cell-cell contacts and the lack of a basement membrane and of smooth muscle cell coverage.

Secondly, lymphatic vessels also express a variety of molecules involved in attraction, adhesion and homing of peripheral-tissue-residing immune cells, such as macrophages and dendritic cells, to lymphoid organs. Tumor cells can hijack these molecular mechanisms in order to promote their escape from the primary tumor site (Fig. 3C). Secondary lymphoid cytokine (SLC, CCL21) is secreted by LEC and is involved in homing of CCR7+ dendritic cells to lymph nodes. In cancer it serves as chemoattractant for tumor cells thus promoting metastasis formation. For example, CCR7-expressing melanoma cells migrate towards CCL21-expressing LEC and metastasize at a higher frequency to the draining lymph node (Shields *et al.*, 2007a; Wiley *et al.*, 2001). Notably, CCR7-expressing tumor cells that also secrete CCL21 can migrate in an autocrine chemotactic manner towards lymphatics. Thereby the CCL21 gradient is generated by the interstitial fluid flow around the tumor cells (Shields *et al.*, 2007b). In patients, CCR7 expression correlates with lymph node metastasis in a variety of cancers, including gastric cancer, breast cancer, pancreatic cancer, colorectal cancer, NSCLC and squamous cell carcinoma (Mashino *et al.*, 2002; Cabioglu *et al.*, 2005; Nakata *et al.*, 2008; Schimanski *et al.*, 2005; Takanami, 2003; Ding *et al.*, 2003). Interestingly, VEGF-C as well as increased lymph flow upregulates CCL21 expression in LEC (Issa *et al.*, 2009; Miteva *et al.*, 2010).

The chemokine stromal-cell derived factor 1 (SDF-1, CXCL12) and its receptors (CXCR4 and CXCR7) also play an important role in metastasis. It has been shown that CXCR4-expressing cancer cells preferentially home to organs with high CXCL12 expression, such as lung, liver and bone marrow via the blood and lymphatic systems. Similarly to the CCL21/CCR7 signaling axis, CXCL12 and CXCR4 can facilitate the entry of cancer cells into the lymphatics by tumor-associated LEC expressing CXCL12. The expression of CXCL12 by LEC can be induced by hypoxia, which also leads to upregulated expression of CXCR4 on cancer cells (Irigoyen *et al.*, 2007; Hirakawa *et al.*, 2009).

Besides chemoattraction, enhanced adhesion to the lymphatic vasculature also seems to improve the entry of cancer cells into the lymphatic vasculature. Two molecules, macrophage mannose receptor 1 (MR1) and CLEVER-1 (stabilin1) have been implicated in this process (Fig. 3C). MR1 is expressed on lymphatic vessels where it is involved in leukocyte trafficking. Loss of MR compromises

the ability of tumor cells to metastasize to regional lymph nodes. This decrease in metastasis is due to altered adhesion of cancer cells to lymphatic vessels, as no change in tumor cell chemoattraction and motility and lymphatic vessel density has been found (Marttila-Ichihara *et al.*, 2008). In patients, the expression of MR in intratumoral lymphatics of breast cancer correlates with increased lymph node metastasis (Irljala *et al.*, 2003). Also, CLEVER-1 is involved in the regulation of leukocyte trafficking in the lymphatic system. It is expressed in tumor-associated lymphatic vessels and may also play a role in cancer cell adhesion (Irljala *et al.*, 2003).

Finally, tumors may actively contribute to increased lymph flow by inducing lymphatic vessel hyperplasia in draining lymph nodes, and such premetastatic changes in lymphatic vessels as well as in lymph nodes could support metastasis by ensuring tumor cell survival and promoting tumor cell growth in the lymphatic system (Fig. 3B) (Harrell *et al.*, 2007; Ruddell *et al.*, 2008a; Hoshida *et al.*, 2006). Proliferation of LEC in the tumor draining lymph node has been first reported in chemically induced skin tumors expressing VEGF-C or VEGF-A (Hirakawa *et al.*, 2005, 2007). Lymph node lymphangiogenesis, as this process is called, begins even before the arrival of metastasizing tumor cells and is independent of the lymphangiogenesis induced at the tumor site (Harrell *et al.*, 2007; Ruddell *et al.*, 2008b). Lymph node lymphangiogenesis can also be promoted by VEGF-A secreted by B-cells accumulating in tumor-draining lymph nodes (Harrell *et al.*, 2007; Shrestha *et al.*, 2010). Lymph node lymphangiogenesis increases lymph flow to the draining lymph node and in doing so, facilitates entry of metastasizing cancer cells into lymphatics. Moreover, the modified microenvironment in the tumor draining lymph node might provide favorable conditions for the survival and growth of arriving tumor cells.

Tumor-induced changes in the sentinel lymph node also include changes in the immune cell composition of the lymph node. Comparison of the immune repertoire of sentinel lymph nodes with non-sentinel lymph nodes in melanoma and breast cancer patients revealed significant changes in the density and maturation state of dendritic cells and T-cells, in the cytokine composition, and the ability of the immune cells to generate a specific immune response (Cochran *et al.*, 2006). Altogether these changes contribute to the generation of an immunosuppressive environment in the sentinel lymph node, again favoring tumor cell survival and growth.

These results clearly show that lymphatic metastasis is an active process promoted by the interaction of tumor-associated lymphatic vessels with tumor cells. Nevertheless, VEGF-C at the tumor site does not only bind to VEGFR3 on LEC to activate lymphangiogenesis, but also stimulates VEGFR3 on cancer cells themselves, thus promoting tumor cell proliferation and tumor cell motility (Su *et al.*, 2006; Issa *et al.*, 2009).

Towards anti-lymphangiogenic therapy

The involvement of tumor-associated lymphatic vessels in metastasis makes the lymphatic vasculature an attractive target for interfering with metastasis formation. Due to its major contribution to the formation of a new tumor lymphatic vasculature, the VEGF-C/VEGF-D/VEGFR3 signaling axis has received major attention in the search for anti-lymphangiogenic therapy. Furthermore, survival of quiescent lymphatic vessels in healthy tissue does not depend on VEGFR3 function, indicating that growing lymphatic vessels at the tumor site can be selectively targeted (Karpanen *et al.*,

2006b). Silencing of the VEGF-C/VEGF-D/VEGFR3 signaling axis is achieved either through ligand sequestration by soluble receptor constructs (trap constructs), small molecular weight inhibitors and monoclonal antibodies against VEGFR3, or through repression of ligand expression by RNAi-technology (Sleeman *et al.*, 2009). In most of these experimental approaches, blockade of the VEGFR3 signaling axis has efficiently repressed tumor lymphangiogenesis and with it metastasis to regional lymph nodes and distant organs. As discussed above, the contribution of the VEGF-A/VEGFR2 signaling axis to tumor lymphangiogenesis is less well defined. Blockade of VEGFR2 signaling activities in the tumor context substantially represses tumor angiogenesis, yet inhibits lymphatic metastasis only in combination with anti-VEGFR3 treatment (Burton *et al.*, 2008; Roberts *et al.*, 2006; Shibata *et al.*, 2008). From animal experiments, targeting the VEGFR3 axis seems to be the most promising approach. However the failure in blocking tumor lymphangiogenesis by VEGFR3 inhibition in some mouse models indicates that tumor lymphangiogenesis may also rely on other signaling pathways and can become independent of VEGFR3 signaling (Schomber *et al.*, 2009; Cao *et al.*, 2004).

Tumor lymphangiogenesis can also be targeted indirectly by interfering with the infiltration and function of tumor-associated macrophages, which are a major source of tumoral VEGF-C. Treatment with an anti-PlGF antibody diminishes the recruitment of VEGFR1+ macrophages into tumors resulting in reduced tumoral VEGF-C levels, lymphatic vessel density and lymph node metastasis (Fischer *et al.*, 2007). Moreover, in several human malignancies, the expression of cyclooxygenase-2 (Cox-2) correlates with tumor lymphangiogenesis and lymph node metastasis. In a mouse model of gastric cancer, Cox-2 inhibition reduces VEGF-C expression in the tumor-infiltrating macrophages and thus represses tumor lymphangiogenesis and lymph node metastasis (Iwata *et al.*, 2007). Finally, inhibition of Nrp2 ligand binding with an anti-Nrp2 antibody effectively reduced tumor lymphangiogenesis and lymph node and lung metastasis in glioma and breast cancer, and repression of PDGF-BB signaling blocked lymphangiogenesis (Caunt *et al.*, 2008; Cao *et al.*, 2004).

In conclusion, first data into the therapeutic potential of interfering with tumor lymphangiogenesis reveal that by targeting growing lymphatic vessels at the tumor site, lymphatic metastasis can be decreased or prevented. Whether or not this holds true in cancer patients awaits the results of relevant clinical trials. Furthermore, active lymphangiogenesis may not always be the basis for the lymphogenic spreading of metastatic cancer cells; treatment regimen targeting lymphatic function rather than lymphatic growth may be warranted. To this end, future investigations are required to identify suitable therapeutic target molecules that are specific for tumor-associated lymphatics. Besides, blocking lymphatic drainage may lead to accumulation of tissue fluid and cause lymphedema, an unwanted adverse side-effect. Another open question relates to the timing when anti-lymphangiogenic therapy may be applied. Should it be preventive to avoid pre-metastatic lymph node lymphangiogenesis or should it be interventional to interfere with ongoing lymphangiogenesis and metastasis? Clinical studies comparing the survival rates of patients undergoing regional lymph node dissection with patients without lymph node dissection has generated controversial results, indicating that further efforts are needed to completely understand the contribution of the lymphatic vasculature to metastatic spreading.

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