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REVIEW

Developmental and pathological lymphangiogenesis: from models to human disease

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Abstract The lymphatic vascular system, the body's second vascular system present in vertebrates, has emerged in recent years as a crucial player in normal and pathological processes. It participates in the maintenance of normal tissue fluid balance, the immune functions of cellular and antigen trafficking and absorption of fatty acids and lipid-soluble vitamins in the gut. Recent scientific discoveries have highlighted the role of lymphatic system in a number of pathologic conditions, including lymphedema, inflammatory diseases, and tumor metastasis. Development of genetically modified animal models, identification of lymphatic endothelial specific markers and regulators coupled with technological advances such as high-resolution imaging and genome-wide approaches have been instrumental in understanding the major steps controling growth and remodeling of lymphatic vessels. This review highlights the recent insights and developments in the field of lymphatic vascular biology.

Keywords Lymphangiogenesis · Lymphedema · Tumor metastasis · Inflammation

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Introduction

The complexity of the circulatory networks of animals tends to increase as species increase in size. Apart from the cardiovascular system, vertebrates also possess a lymphatic system that consists of the lymphatic vessels and the lymphoid organs such as lymph nodes, mucosal-associated lymphoid tissue (MALT) (tonsils, Peyer's patches, and lymphoid tissues associated to the bronchial and nasal systems), spleen, and thymus. Blood and lymphatic vessels comprise two interdependent vascular networks in most tissues; however, their organization and function are distinct. The cardiovascular system forms a continuous loop around which the heart pumps blood, whereas the lymphatic system comprises a one-way, open-ended transit network without a central driving force. Blood vessels deliver blood cells, nutrients, hormones, and oxygen to tissues, whereas lymphatic vasculature removes macromolecules, microbes, and other substances from interstitial space.

The lymphatic system comprises a network of blind-ended lymphatic capillaries (also called initial lymphatic vessels) that collect the excess extravasated tissue fluid that has originated as capillary infiltration from the blood serum (Fig. 1). After being collected by the lymphatic capillaries, the lymph is transported through a system of lymphatic vessels of progressively larger size, to pre-collector lymphatic vessels (Sacchi et al. 1997) then larger collecting lymphatic vessels, which converge into lymphatic trunks, and the lymph is finally returned to the venous circulation via a connection with subclavian veins (Casley-Smith 1980; Moore 1985). Thus the main function of the lymphatic system is to maintain normal tissue fluid balance by restoring interstitial fluid to the cardiovascular system. In addition, in the digestive tract, lacteal lymphatic vessels inside the intestinal villi absorb and transport fat-soluble vitamins

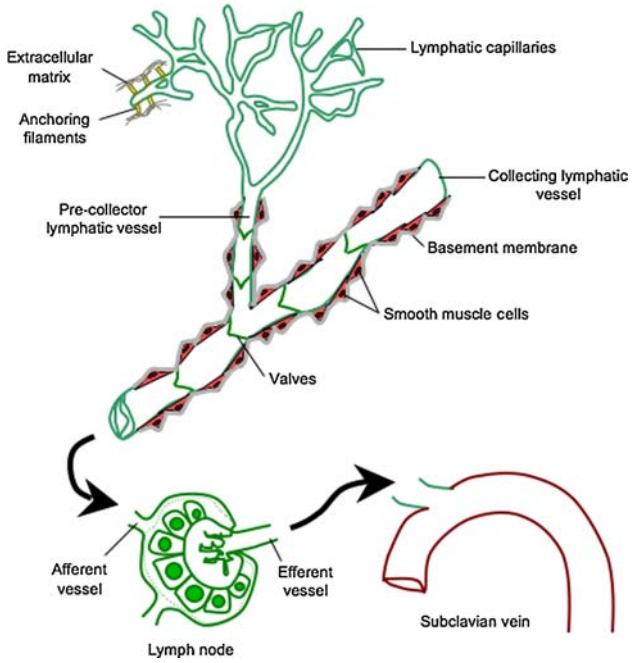

Fig. 1 Organization of lymphatic vascular system. The lymph is collected by a network of blind-ended lymphatic capillaries and is transported by pre-collector lymphatic vessels and collecting lymphatic vessels, which are emptied into veins in the jugular region. Lymphatic capillaries consist of thin-walled lymphatic endothelial cells with overlapping junctions which look like valves. Anchoring filaments, containing fibrillin, connect lymphatic capillaries to the extracellular matrix and prevent vessel collapse during increased interstitial pressure. The pre-collector and collecting lymphatic vessels have a basement membrane, are surrounded by smooth muscle cells (*red*) and contain intraluminal valves that prevent lymph blackflow

(A, D, E, and K) and dietary fat, released by enterocytes in the form of lipid particles called chylomicrons. Furthermore, the lymphatic system is an important part of immune surveillance by carrying antigens and antigen presenting cells from the interstitium to be displayed for B and T cells in the lymph nodes. In addition to these physiological tasks the lymphatic system plays a major role in a number of pathologic conditions, including lymphedema, inflammatory diseases, and tumor metastasis.

Present in the skin and in most internal organs, with the exception of avascular structures such as epidermis, hair, nails, cartilage, and cornea, and some vascularised organs such as the brain, bone marrow, and retina, the lymphatic vasculature is composed of vessels with distinct morphological features (Fig. 1). Similar to blood capillaries, lymphatic capillaries consist of a single layer of thin-walled, non-fenestrated lymphatic endothelial cells (LECs), but they differ in that they are not ensheathed by pericytes or smooth muscle cells (SMCs), and have an absent or poorly developed basement membrane. In addition, they lack tight junctions and adherens junctions, which allow easy access for fluid, macromolecules, and cells into the vessel lumen (Leak 1976). Endothelial cells of lymphatic capillaries are oak leaf shaped and are interconnected by specialized discontinuous button-like junctions, whereas collecting lymphatic vessels downstream have continuous zipper-like junctions found also in blood vessels (Baluk et al. 2007). Overlapping endothelial cell–cell contacts (also called primary valves) in initial lymphatic vessels prevent fluid escaping back into the interstitial space (Schmid-Schönbein 2003; Trzewik et al. 2001). Lymphatic capillary endothelial cells are closely linked to the surrounding extracellular matrix by elastic fibers known as anchoring filaments (Gerli et al. 1991; Leak and Burke 1968), which prevent vessel collapse in conditions of high interstitial pressure, indeed in this condition the junctions of the initial lymphatics open and the anchoring filaments stretch, allowing fluid to move into the vessel. Anchoring filaments are made of fibrillin (Gerli et al. 2000; Solito et al. 1997), a large glycoprotein that contains an Arg–Gly–Asp (RGD) motif capable of binding $\alpha v\beta 3$ integrins, which are transmembrane glycoproteins that cluster at focal adhesion plaques. Pre-collector lymphatic vessels are characterized by the alternation of areas with the same structural simplicity as initial lymphatic capillaries and areas with a well-developed muscular coat, but the ultrastructural features of these different portions do not differ, and anchoring filaments are present in both (Scavelli et al. 2004). The pre-collecting lymphatic vessels merge into collecting lymphatic vessels, and the two types of vessels have a basement membrane, and are surrounded by SMCs with intrinsic contractile activity to promote lymph flow. The latter is also due to the contraction of surrounding skeletal muscles, as well as arterial pulsations. Like veins, the larger lymphatic vessels contain one-way intraluminal valves that aid in lymph propulsion by preventing backflow (Leak and Burke 1966; von der Weid and Zawieja 2004). Compared to blood vessels, the lymphatic vasculature is a low flow and low pressure system.

Development of lymphatic vasculature

Embryonic development concepts

The first description of the lymphatic system dates back to the seventeenth century, when Gasparo Aselli identified lymphatic vessels as ‘‘milky veins’’ in the mesentery of a ‘‘well-fed’’ dog (Asellius 1627). However, the embryonic origin of lymphatic vessels remained unclear for a long time until F. Sabin (1902, 1909) and F. Lewis (1905) postulated, at the beginning of the twentieth century, that LECs are derived from the venous endothelium. This “centrifugal” theory proposes that endothelial cells bud off from the veins during early embryonic development and form primitive lymph sacs in the jugular region (reviewed by Oliver

2004). From these sacs, budding endothelial cells centrifugally sprout towards the periphery, forming capillaries that surround tissues and organs. An alternative, "centripetal" theory, suggests that LECs are derived from mesenchymal progenitor cells, which, nowadays, are called lymphangioblasts (Huntington and Mc Clure 1910). Several recent studies in genetically engineered mouse models (Srinivasan et al. 2007; Wigle et al. 2002; Wigle and Oliver 1999) and in vivo imaging of developing lymphatic vasculature in zebrafish (Yaniv et al. 2006) provide strong support for the venous origin of lymphatic vessels. Lineage tracing experiments demonstrate that LECs originating in embryonic veins are the main source for the developing lymphatic vasculature; they have also identified some of the molecular determinants that control the step-wise process of lymphatic competence, commitment, differentiation and maturation (reviewed in Adams and Alitalo 2007; Karpanen and Alitalo 2008; Oliver 2004).

An intermediate position favouring a dual origin from embryonic veins and mesenchymal lymphangioblasts is also proposed. Grafting experiments in avian embryos suggest that while the deep parts of the lymph sacs are derived from adjacent veins, the superficial parts of the jugular lymph sacs and the dermal lymphatics arise from local lymphangioblasts (Wilting et al. 2006). In *Xenopus* tadpoles, PROX1-positive mesodermal precursor cells, lymphangioblasts, which share a common origin with vascular progenitor cells, contribute to lymphatic vessel formation (Ny et al. 2005). In murine embryos, scattered mesenchymal cells, which coexpress leukocyte (CD45) and lymphatic endothelial markers (LYVE-1, PROX1), were detected in the regions of new lymphatic vessel growth; and it was suggested that the triple positive cells (LYVE-1^+, $PROX1^+$, $F4/80^+$) with characteristics of LECs and macrophages may integrate into lymphatic vessels (Buttler et al. 2006, 2008a). The functional role of such cells remains unclear, as formation of lymph sacs is not affected in *Runx1*$^{-/-}$ mice, which have defective hematopoiesis, and lineage tracing studies failed to demonstrate contribution of hematopoietic cells to lymphatic endothelium up to the embryonic day E16.5 (Buttler et al. 2008b; Srinivasan et al. 2007). A possibility remains that hematopoietic cells contribute to developmental lymphangiogenesis in a non-cell autonomous manner, as described previously for angiogenesis (Takakura et al. 2000).

In adults, similar to blood vessels, the lymphatic vasculature remains in quiescent state with the exception of situations of tissue and organ regeneration, wound healing, tumor growth, and in inflammation. New lymphatic vessels primarily grow by sprouting from existing ones; in addition, the hematopoietic cell-derived circulating endothelial progenitors and transdifferentiating macrophages are putative sources of LECs during pathological lymphangiogenesis, such as during chronic renal transplant rejection or corneal lymphangiogenesis (Maruyama et al. 2005; Religa et al. 2005; Schledzewski et al. 2006). However, bone marrow-derived progenitor cells did not incorporate significantly into the endothelium of newly formed lymphatic vessels in mouse tumor xenografts (He et al. 2004), and more generally, a growing body of evidence suggest that circulating bone marrow-derived progenitor cells may promote growth of vessels mainly by acting as supporting cells (Grunewald et al. 2006; Purhonen et al. 2008).

Mechanisms of lymphatic vascular development

Lymphatic endothelial cell fate commitment

During embryogenesis, the development of lymphatic vessels starts after the establishment of a functional blood vasculature. LYVE-1, lymphatic endothelial hyaluronan receptor-1, is the first indicator of lymphatic endothelial commitment, and is expressed in mice from embryonic day E9 in a polarized manner in the LECs differentiating in venous endothelium (Oliver 2004). It has been proposed that LYVE-1 expression in the embryonic cardinal veins signifies competence of the venous endothelium to receive an inductive signal that results in lymphatic endothelial cell fate specification (Wigle et al. 2002). In adults, LYVE-1 is downregulated in the collecting lymphatic vessels but remains high in lymphatic capillaries (Makinen et al. 2005). In addition to lymphatic endothelium, LYVE-1 is also expressed on blood vessels of the yolk sac and intra-embryonic arterial and venous endothelium at early embryonic stages, on endothelial cells of the lung and endocardium throughout embryogenesis, as well as in liver sinusoids (Gordon et al. 2008). LYVE-1 is thought to participate in hyaluronan transport and leukocyte migration across the lymphatic vessel wall (Jackson 2004). *Lyve1*$^{-/-}$ mice do not display lymphatic vascular defects, suggesting the existence of compensatory mechanisms (Gale et al. 2007).

The transcription factor prospero-related homeobox 1, PROX1, controls the initial steps of lymphangiogenesis and is the most specific lineage marker for lymphatic endothelium (reviewed in Oliver and Detmar 2002). Targeted inactivation of *Prox1* in mice leads to a complete absence of lymphatic vessels, while blood vasculature development proceeds normally (Wigle et al. 2002; Wigle and Oliver 1999). Around E9.5 of mouse development, polarized expression of PROX1 is observed on one side of the anterior cardinal veins and soon thereafter these cells start budding and migrating in a polarized manner, eventually forming lymph sacs. PROX1 acts as a master regulator of lymphatic endothelial differentiation, as in the absence of PROX1 endothelial cells continue to express blood vascular endothelial cell (BECs) markers, while the ectopic expression of PROX1 in human BECs induces expression

of lymphatic-specific genes and suppresses many blood vascular-specific transcripts (Hong et al. 2002; Petrova et al. 2002; Wigle et al. 2002). At present, the signals that induce PROX1 expression in a restricted subpopulation of endothelial cells are not well understood. Interleukin (IL)-3 and IL-7 have been shown to induce PROX1 expression in human cultured BECs, but whether they provide signals for lymphatic differentiation in vivo has not yet been addressed (Al-Rawi et al. 2005; Groger et al. 2004). In addition, direct PROX1 target genes that mediate its function during the process of lymphangiogenesis are not known. In cultured endothelial cells, PROX1 has been shown to induce the expression of important genes regulating lymphatic development, such as vascular endothelial growth factor receptor-3 (VEGFR-3) (Petrova et al. 2002; Saharinen and Petrova 2004; Wigle et al. 2002) and integrin-α9 (Mishima et al. 2007) thereby promoting the migration of LECs towards the VEGFR-3 ligand, VEGF-C, and regulating the endothelial sheet formation (Mishima et al. 2007). PROX1 also regulates the expression of fibroblast growth factor receptor-3 (FGFR-3) in vitro (Shin et al. 2006), however, the role of FGFR-3 in lymphatic vascular development in vivo remains to be investigated.

Lymphatic sprouting

Endothelial receptor tyrosine kinase VEGFR-3 and its ligand VEGF-C are required for the survival, maintenance, and migration of lymphatic vessels during embryonic development (reviewed by Karpanen and Alitalo 2008; Tammela et al. 2005a) and a 2 week postnatal period, after which lymphatic vessels become independent of VEGFR-3 (Karpanen et al. 2006b). During early development, VEGFR-3 is expressed in all endothelial cells and is important for blood vascular remodeling (Dumont et al. 1998; Hamada et al. 2000), indeed *Vegfr3*-deficient mice die at E9.5 owing to cardiovascular failure. At the onset of lymphangiogenesis, VEGFR-3 expression becomes restricted to newly forming LECs (Dumont et al. 1998). However, VEGFR-3 is upregulated in the blood microvasculature of tumors (Valtola et al. 1999) and wounds (Paavonen et al. 2000). Moreover, VEGFR-3 is highly expressed in angiogenic sprouts and blocking of VEGFR-3 signaling results in decreased sprouting, vascular density, vessel branching and endothelial cell proliferation (Tammela et al. 2008). The two known ligands of VEGFR-3, VEGF-C, and VEGF-D, can induce lymphangiogenesis in vivo and stimulate proliferation, survival, and migration of LECs in vitro (Jeltsch et al. 1997; Mäkinen et al. 2001; Veikkola et al. 2001). VEGF-C is expressed by mesenchymal cells surrounding the cardinal veins, and is essential for the initial sprouting and directed migration as well as for the subsequent survival of LECs. Consequently, in the absence of *Vegfc*, $PROX1^+$ LECs arise normally in the embryonic veins, but are unable to sprout and migrate from their original location, and *Vegfc* deficient mice do not develop a lymphatic vasculature (Karkkainen et al. 2004). VEGF-D is dispensable for lymphatic vascular development, and $Vegfd^{-/-}$ embryos display only mild hypoplasia of the pulmonary lymphatic vasculature, although exogenous VEGF-D protein rescues the impaired vessel sprouting in $Vegfc^{-/-}$ embryos (Baldwin et al. 2005; Karkkainen et al. 2004).

After proteolytic cleavage, fully processed, mature forms of VEGF-C and VEGF-D serve as also ligands for the major regulator of angiogenesis, VEGFR-2 (Achen et al. 1998; Joukov et al. 1997). It has recently been suggested that cooperative signaling between VEGFR-2 and VEGFR-3 is required for LECs migration and proliferation, whereas VEGFR-3 is redundant with VEGFR-2 for the organization of LECs into functional capillaries (Goldman et al. 2007). However, when activated by overexpression of VEGF or the related VEGFR-2-specific ligand VEGF-E, VEGFR-2 signals only promote lymphatic vessel enlargement and are not involved in vessel sprouting to generate new lymphatic vessels in vivo (Wirzenius et al. 2007). Moreover, a mutant form of VEGF-C unable to stimulate VEGFR-2 is sufficient for stimulating lymphangiogenesis (Joukov et al. 1998; Veikkola et al. 2001).

The importance of VEGF-C/VEGFR-3 pathway for lymphatic vascular development has been recently highlighted by studies in zebrafish, which resolved a long standing controversy about the presence of lymphatic vessels in teleost fishes (Küchler et al. 2006; Vogel and Claviez 1981; Yaniv et al. 2006). Zebrafish lymphatic vessels express high levels of *Prox1*, *Nrp2 (neuropilin-2)*, *Vegfr3*, and *Angpt2* (*angiopoietin-2*) and, knock-down of *Prox1* and *Vegfc* or inhibition of VEGFR-3 signaling by expression of VEGF-C trap VEGFR-3-Ig fusion protein prevents the formation of lymphatic vasculature (Küchler et al. 2006; Yaniv et al. 2006). Use of zebrafish or *Xenopus* embryos as small vertebrate models offers unparalled opportunities for identification of novel regulators of lymphatic vascular development and detailed analysis using in vivo imaging approaches (Ny et al. 2005; Yaniv et al. 2006).

In addition to VEGF-C and VEGF-D, extracellular matrix proteins, collagen, and fibronectin, enhance tyrosine phosphorylation of VEGFR-3 through activation of integrin β1, which interacts directly with VEGFR-3 (Wang et al. 2001). As mice deficient in both *Vegfc* and *Vegfd*, unlike *Vegfr3* null mice, have normal blood vascular development (Haiko et al. 2008), it is likely that other VEGFR-3 ligands, such as integrins, contribute to VEGFR-3 signaling in blood vascular endothelium. Additional VEGF-C signal transduction pathways were also described, such as interaction of VEGF-C with neuropilin-2 (NP2) and integrin α9. Indeed, NP2 is cointernalized along with VEGFR-3 in the

endocytic vesicles of lymphatic endothelial cells upon stimulation with VEGF-C or VEGF-D, suggesting that NP2 modulates VEGFR-3 signaling (Karpanen et al. 2006a). The formation of small lymphatic vessels and capillaries is abnormal in *Nrp2* knockout mice, these vessels are absent or severely reduced until postnatal stages, whereas larger lymphatic vessels develop normally (Yuan et al. 2002). It may be significant that integrin $\alpha 9\beta 1$ binds VEGF-C and VEGF-D, and endothelial adhesion to and migration on the lymphangiogenic vascular endothelial growth factors (VEGF-C and -D) are $\alpha 9\beta 1$-dependent (Vlahakis et al. 2005). Mice deficient in the integrin $\alpha 9$ chain die of chylothorax during the early postnatal period, suggesting an underlying function for integrin $\alpha 9\beta 1$ in the normal development of the lymphatic system, including the thoracic duct (Huang et al. 2000).

Several other growth factors with lymphangiogenic function have been proposed, including fibroblast growth factor-2 (FGF-2), platelet-derived growth factors (PDGFs), insulin-like growth factors (IGFs), hepatocyte growth factor (HGF) and growth hormone (GH) (Banziger-Tobler et al. 2008; Björndahl et al. 2005; Cao et al. 2004, 2006; Chang et al. 2004; Kajiya et al. 2005; Kubo et al. 2002; Saito et al. 2006; Shin et al. 2006). While all of them are capable of stimulating the proliferation of lymphatic endothelial cells in vitro or sometimes upon overexpression in vivo, their definitive role in developmental or pathological lymphangiogenesis via loss-of-function analysis remains to be established. Indirect lymphangiogenic effect in vivo, for example through an increased recruitment of VEGF-C/D expressing inflammatory cells, is a plausible alternative for FGF-2 and HGF action (Cao et al. 2006; Chang et al. 2004; Kubo et al. 2002).

Separation of blood and lymphatic vasculature

The primary lymphatic plexus, which begins to form in mice around E10.5–E11, is composed of capillary-like vessels and is initially connected to the blood vasculature. In adults, apart the lymphatico-venous communications in the renal, hepatic, and adrenal veins, and in the lymph nodes, the main connections of blood and lymphatic vasculature locate at the junction where thoracic and right lymphatic ducts empty their contents into the subclavian veins. This suggests that mechanisms exist for keeping the blood and lymphatic vascular compartments separate. Tyrosine kinase Syk and adaptor protein Slp76 are important for this separation (Abtahian et al. 2003; Sebzda et al. 2006), as deficiency in *Syk* or *Slp76* results in arterio-venous shunting and mixing of blood and lymphatic endothelial cells, which ultimately leads to haemorrhaging and perinatal death. The model proposed is that the exit of progenitors from veins is defective, and this leads to the formation of lymphovenous connections. Syk and Slp76 are expressed almost exclusively in hematopoietic cells, suggesting a role for this cell type in regulating the separation of the blood and lymphatic vascular networks.

Fasting-induced adipose factor (Fiaf) (or angiopoietin-like protein 4 (Angptl4)) is another factor involved in the regulation of lymphatic and blood vessel separation in the intestine. Mice deficient in Fiaf developed normally until birth, but displayed blood-filled intestinal lymphatic vessels post-natally, and decreased *Prox1* expression was observed on the LYVE-1^+ vessels in *Fiaf*$^{-/-}$ mice (Bäckhed et al. 2007). However, the underlying mechanisms, including the identification of the Fiaf receptor, remain to be investigated. This study suggests that active and organ-specific mechanisms are required after birth to preserve the separation of the blood and lymphatic vascular systems.

Maturation of the lymphatic vasculature

The primary lymphatic plexus undergoes a further dramatic change to form a hierarchically organized network of lymph vessels, composed of capillaries devoid of basement membrane and pericytes, and collecting lymphatic vessels containing intraluminal valves, basement membrane, and covered with SMCs. This complex process of reorganization of lymph vessels is referred as "lymphatic vascular maturation", during which new capillaries arise by sprouting from the pre-existing vasculature, SMCs on collecting vessels are recruited and luminal valves are formed. Angiopoietin-2, EphrinB2, FOXC2, and Podoplanin have been shown to play important roles at late steps of lymphatic vascular development.

Angiopoietins and Tie receptors are involved in the remodeling and stabilization of lymphatic vessels. Three members of the Angiopoietin family (Ang1, Ang2, and Ang3/4) are ligands for the Tie1 and/or Tie2 receptor tyrosine kinases, which are expressed both in blood vascular and lymphatic endothelia (Iljin et al. 2002; Morisada et al. 2005; Tammela et al. 2005b). Ang1 can activate both Tie1 and Tie2 that appear as preformed heteromeric complexes between the two receptor molecules (Saharinen et al. 2005). Overexpression of *Ang1* in adult mice stimulates lymphatic endothelial cell proliferation and promotes vessel enlargement and generation of new sprouts (Morisada et al. 2005; Tammela et al. 2005b). Ang2 is required for postnatal blood vascular remodeling and proper development of the lymphatic vasculature. *Angpt2*-deficient mice display a disorganization and hyperplasia of the vessels caused by impaired recruitment of SMCs onto collecting lymphatic vessels and an irregularly patterned hypoplastic lymphatic capillary network. Interestingly, replacement of Ang2 by Ang1 was sufficient to rescue the lymphatic phenotype (Gale et al. 2002).

During vascular development, EphrinB2 plays important roles in the remodeling of the arterial-venous capillary

plexus and in the postnatal maturation of the lymphatic vasculature. EphrinB2 is expressed in collecting lymphatic vessels, whereas its receptor EphB4 is detected both in collecting lymphatic vessels and in lymphatic capillaries. Mice lacking the PDZ interaction domain of EphrinB2, develop an apparently normal blood vasculature, but fail to establish a hierarchically organized lymphatic vessel network consisting of lymphatic capillaries and collecting vessels. These mutants display defects in the formation of luminal valves in the collecting vessels, ectopic SMC coverage in lymphatic capillaries, and persistent expression of LYVE-1 in all lymphatic vessels (Makinen et al. 2005).

FOXC2 is a forkhead transcription factor, which is involved in the specification of the lymphatic capillary versus collecting lymphatic vessel phenotype. This factor is highly expressed in the developing lymphatic vasculature and in the luminal valves in adult lymphatic vessels (Dagenais et al. 2004; Petrova et al. 2004). Initial development of the lymphatic vasculature proceeds normally in the absence of FOXC2; however, later the patterning of the lymphatic vasculature becomes abnormal. Collecting lymphatic vessels in *Foxc2*$^{-/-}$ mice fail to develop valves while the lymphatic capillaries acquire an excessive coverage by SMCs and components of the basal lamina, and begin to express some blood vascular endothelial cell markers such as Endoglin and PDGF-B (Petrova et al. 2004). Mice heterozygous for both *Foxc2* and *Vegfr3* display a phenotype very similar to *Foxc2*$^{-/-}$ mice, suggesting that FOXC2 and VEGFR-3 act through a common genetic pathway to establish some of the distinct properties of the lymphatic vasculature. The human disease lymphedema-distichiasis (LD), an autosomal dominant disease, is caused by heterozygous-loss-of-function mutations of *Foxc2* (Fang et al. 2000). Unlike in congenital lymphedema, the lymphatic vasculature in LD is normal or hyperplastic, but there is lymph backflow, presumably due to abnormal lymphatic valves, defective patterning and the presence of ectopic SMCs (Brice et al. 2002; Petrova et al. 2004). Interestingly, a recent study showed that *Foxc2* mutations are associated with primary venous valve failure in both the superficial and deep veins in the lower limb. Thus, this gene appears to be important for the normal development and maintenance of both venous and lymphatic valves (Mellor et al. 2007).

Podoplanin, a small transmembrane mucin-like protein, is highly expressed in lymphatic endothelial cells. In mice, its expression starts around E11 and remains high both in collecting lymphatic vessels and in lymphatic capillaries in the adult (Breiteneder-Geleff et al. 1999; Schacht et al. 2003). *Pdpn*-deficient mice die at birth due to abnormal lung development and have defects in lymphatic but not blood vessel formation and patterning, which result in impaired lymphatic transport, dilation of lymphatic vessels, and congenital lymphedema (Schacht et al. 2003). Podoplanin is upregulated in the invasive front of many human carcinomas and promotes collective cell migration by filopodia formation via the downregulation of the activities of small Rho family GTPases (Wicki et al. 2006). In contrast to the role in tumor cells, siRNA mediated knockdown of *Podoplanin* in lung LECs prevented activation of RhoA during capillary morphogenesis and impaired localization of phosphorylated ezrin/radixin/moesin proteins to plasma membrane (Navarro et al. 2008).

Other regulators of lymphatic vascular development

Spred-1 and Spred-2, two negative regulators for growth factor- and cytokine-induced ERK activation, were reported as mediators of the separation of lymphatic vessels from the parental vein, and *Spred-1/Spred-2* double-knockout phenotype resembles that of *Syk*- and *Slp-76*-deficient mice. Spred proteins were shown to regulate VEGF-C signaling by suppressing VEGFR-3-mediated ERK and Akt activation (Taniguchi et al. 2007). However, the phenotype of *Spred-1/Spred-2* knockout mice differs from the one observed in *Vegfc* knockout or *Vegfr3* mutant Chy mice (Karkkainen et al. 2004), therefore alternative explanations should be also considered.

Adrenomedullin (AM), a vasodilatator and diuretic peptide, is necessary for murine lymphatic vascular development (Fritz-Six et al. 2008; Jin et al. 2008). Genetic loss of AM or components of its receptor complex, calcitonin receptor-like receptor and receptor activity-modifying protein 2 (RAMP2), leads to non-hemorrhagic edema and embryonic lethality at midgestation (Fritz-Six et al. 2008). The initial development of lymphatic vasculature was not affected; however, loss of AM signaling resulted in severely hypoplastic jugular lymph sacs while the development of retroperitoneal and skin lymphatic vessels was normal. Administration of AM was also shown to improve secondary lymphedema in mouse tail lymphedema model, and to promote lymphangiogenesis (Jin et al. 2008). Interestingly, in contrast to Fritz-Six et al., another study of *RAMP2* deficient mice concluded that AM has blood vascular functions, such as regulation of vascular stability and permeability, and that embryos lacking AM signaling die due to leaky and unstable blood vessels (Ichikawa-Shindo et al. 2008), therefore further work may be necessary to reconcile these results and to unravel details of AM signaling in the vasculature during embryonic development.

Aspp1 (apoptosis stimulating protein of p53), an endothelial-specific gene functioning in mouse embryogenesis, was recently described as an important player in the lymphatic vessel assembly. *Aspp1*$^{-/-}$ mice showed a disorganized lymphatic plexus and impaired lymphatic drainage function in embryonic skin, whereas the lymphatic drainage defect was resolved in adults. Moreover, collecting lymphatic

vessels of *Aspp1*$^{-/-}$ adult mice possess luminal valves. These results indicate that Aspp1 is important for the initial assembly of lymphatic endothelial cells but dispensable for lymphatic remodeling (Hirashima et al. 2008). The lymphatic vascular function of Aspp1 is independent of p53, and may be linked instead to C-terminal Src kinase (Csk), given the fact the Drosophila homologue of Aspp1 acts as a positive regulator of dCsk in the control of epithelial integrity (Langton et al. 2007).

Recently, Sphingosine-1-phosphate (S1P), a potent bioactive lipid that is implicated in a variety of biologic processes such as inflammatory responses and angiogenesis, was reported as a lymphangiogenic mediator (Yoon et al. 2008). S1P induces the migration and capillary-like tube formation of lymphatic endothelial cells in vitro and lymphangiogenesis in vivo. Furthermore, the use of pertussis toxin, intracellular Ca^{2+} chelator, and phospholipase C (PLC) inhibitor efficiently blocks S1P-induced in vitro lymphangiogenesis and intracellular Ca^{2+} mobilization of HLECs, suggesting that S1P promotes lymphangiogenesis by stimulating S1P1/Gi/phospholipase C/Ca^{2+} signaling pathways.

TGF-β signaling negatively regulates lymphangiogenesis in inflammatory tissues as well as in certain tumor tissues. In addition to inhibiting the proliferation and migration of human dermal lymphatic microvascular endothelial cells, TGF-β decreases the expression of LEC-related genes, including *Prox1* and *Lyve1*, in these cells. Moreover, TGFβR-I inhibitor potentiates lymphangiogenesis in the presence of VEGF-C in vivo (Oka et al. 2008).

Finally, Emilin1, an elastic microfibril-associated protein, is expressed in lymphatic endothelial cells, and plays a role in the regulation of lymphatic vessel patterning and proliferation (Danussi et al. 2008). *Emilin1*$^{-/-}$ mice have hyperplastic and disorganised lymphatic vessels with reduced number of anchoring filaments, and they also show impaired lymphatic drainage function. These data highlight the importance of interactions between LECs and surrounding ECM (Danussi et al. 2008).

Pathology of lymphatic vessels

The lymphatic vascular system is a dynamic structure that responds to a changing environment and evolves during the life of individual. In the adult, many pathological conditions affect the lymphatic vessels and they respond by undergoing neo-lymphangiogenesis. Such events include inflammation and immune responses, tumorigenesis and after trauma, infections, surgery and radiation. Many of the known regulators of developmental lymphangiogenesis have also been implicated in neo-lymphangiogenesis and pathological conditions.

Lymphedema

Congenital or acquired dysfunction of the lymphatic system can result in the formation of lymphedema, due to the stagnation of lymph and accumulation of tissue fluid because of an impairment of the capacity to remove fluid from the interstitium (Fig. 2a). This may be caused by abnormal lymphatic vessel development or damaged lymphatic vessels. Lymphedema is a progressive disease characterized by swelling of the affected limb, accompanied by fibrosis, fatty degeneration of the connective tissue and susceptibility to infections (reviewed by Rockson 2001). The treatment of lymphedema is currently based on manual lymphatic drainage by physiotherapy, compression garments, and occasionally surgery. Lymphedema is usually divided into two main categories. Primary lymphedema is a condition with no identifiable antecedent cause that can be present at birth (congenital), at puberty (praecox) or, more rarely, at adulthood (tarda). Secondary, acquired lymphedema develops when the lymphatic vessels are damaged by infection, for example as a result of parasitic infection (filiariasis), radiation therapy, or when lymph nodes are surgically removed.

Milroy's disease or primary congenital lymphedema (Online Mendelian Inheritance in Man, OMIM, number 153100), becomes apparent at birth, affects primarily the legs and feet and is characterized by an absence or reduced number of lymphatic vessels (Brice et al. 2005). This disease has been associated with missense mutations encoding inactive tyrosine kinase VEGFR-3 proteins (Butler et al. 2007; Irrthum et al. 2000; Karkkainen et al. 2000). Mutations in *Vegfr3* were identified in mutant mouse strain Chy, which has defective lymphatic vessels (Karkkainen et al. 2001). This model has been used to test VEGF-C gene therapy, which promoted the formation of functional lymphatic vessels in these mice. In the case of lymphedema-distichiasis (LD, OMIM 153400), mutations in the forkhead transcription factor FOXC2 have been identified (Fang et al. 2000; Finegold et al. 2001). This disorder is characterized by distichiasis (a double row of eyelashes) at birth and bilateral lower limb lymphedema at puberty. LD is associated with abnormal lymph vessel patterning, agenesis of lymphatic valves, and abnormal recruitment of SMCs (Petrova et al. 2004). Generated *Foxc2*$^{\pm}$ and *Foxc2*$^{-/-}$ mice constitute useful animal models for this disorder (Kriederman et al. 2003; Petrova et al. 2004). A recent study reported renal disease and diabetes mellitus in combination with LD caused by a *Foxc2* mutation, reflecting the developmental role of FOXC2 in multiple tissues (Yildirim-Toruner et al. 2004). Finally, mutations in the transcription factor SOX18 were recently identified in recessive and dominant forms of hypotrichosis–lymphedema–telangiectasia (Irrthum et al. 2003).

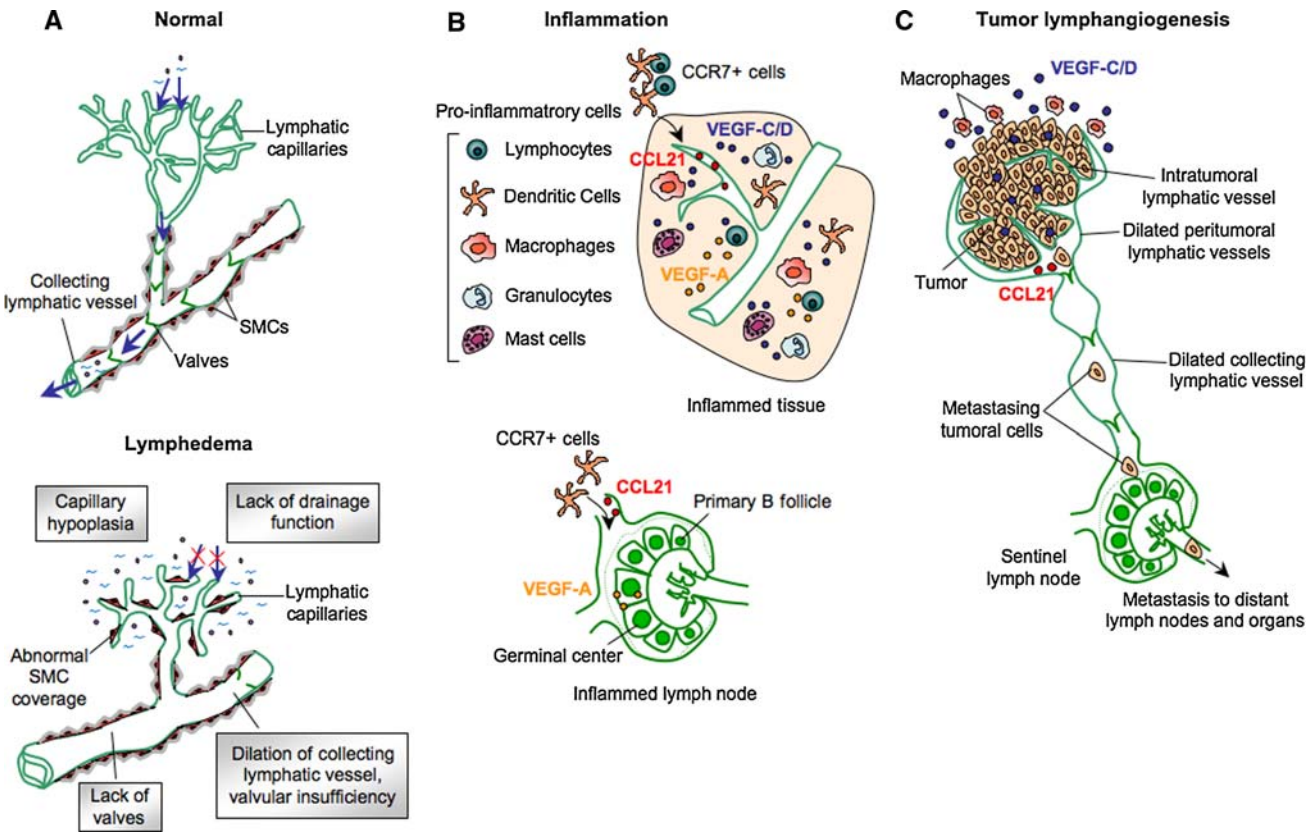

Fig. 2 Lymphatic vasculature in disease. **a** Malformation of lymphatic capillaries, damage to collecting lymphatic vessels and lack or insufficient function of lymphatic valves can lead to lymphedema. Abnormal coverage by smooth muscle cells (SMC), observed in some lymphedema patients, may also compromise the function of lymphatic capillaries and leads to insufficient lymph drainage. **b** Inflammatory cells produce proinflammatory cytokines and lymphangiogenic factors that stimulate lymphatic vessel growth. Lymphatic endotheial cells produce CCL21, which further attracts $CCR7^+$ lymphocytes and dendritic cells. VEGF-A is prominently expressed by follicular B cells and is a potential mediator of the increase in lymphatic vessels and DC migration in inflamed lymph nodes. **c** Tumor and tumor-associated macrophages secrete factors, such as VEGF-C and VEGF-D, which can induce lymphatic vessel growth in the periphery of and/or inside the tumor as well as in the draining sentinel lymph nodes. The lymphatic endothelial cells may also actively attract some tumor cells through the secretion of chemokines, such as CCL21. Tumor-associated and lymph node lymphangiogenesis can lead to enhanced metastasis to sentinel lymph nodes and beyond to distant organs

Edema of the arm after axillary lymph node dissection is probably the most common cause of lymphedema in industrialized countries. The reported incidence of edema after mastectomy, approximately 6–30% of operated patients, is increased by radiotherapy, but its etiology and pathophysiology are still not fully understood and appear to be multifactorial (reviewed by Rockson 2001). A recent study, using a newly established axillary lymphadenectomy mouse model, showed that collecting lymphatic vessels can be regenerated and fused to lymph node transplants after lymph node removal. Treatment with adenovirally delivered VEGF-C or VEGF-D induces robust growth of the lymphatic capillaries, which can mature and become functional even without the presence of lymph nodes. Furthermore, VEGF-C therapy greatly improves the success of lymph node survival; without VEGF-C the transplantation leads to atrophy of lymph node structure. Combination of VEGF-C therapy and lymph node transplantation thus provides a working model for treating individuals with a history of cancer (Tammela et al. 2007).

Worldwide, the main cause of lymphedema is filariasis, in over 80 tropical and subtropical countries, with about 120 million people affected (Wynd et al. 2007). This disease is caused by infection with the mosquito-borne parasites *Wuchereria bancrofti* and *Brugia malayi*, which live and reproduce in the lymphatic system. This leads to a massive damage of lymphatic vessels with a complete and permanent disruption of lymphatic transport, resulting in chronic lymphedema of the legs and genitals (reviewed by Melrose 2002). Current drugs (diethylcarbamazine or ivermectin, usually given with albendazole) effectively killed the microfilariae (larval offspring of the parasite), but their effect on the macrofilariae (adult worms) was incomplete. Doxycycline was suggested as a novel strategy against

bancroftian filariasis. This drug depletes *Wolbachia*, the intracellular bacterial symbiont of filarial parasites, and kills most adult worms (Hoerauf et al. 2003; Stolk et al. 2005).

Inflammation

Inflammation and immune system dysfunction are intimately associated with increased lymphangiogenesis. VEGF-C is upregulated in response to proinflammatory cytokines, presumably through NF-κB-mediated promoter activation, suggesting a role for the regulation of lymphatic vessel growth during inflammation (Ristimaki et al. 1998). Interestingly, NF-κB is also constitutively active in at least some lymphatic vessels (Saban et al. 2004). Lymphatic vessels actively regulate inflammatory responses by transporting leukocytes from the site of inflammation to secondary lymphoid organs (Fig. 2b). Upon exposure to an inflammatory stimulus and recognition of pathogen-associated molecular patterns, dendritic cells (DC) capture antigens in peripheral tissues and migrate through afferent lymphatic vessels into lymph nodes. After their maturation, DC express higher levels of the specific lymphoid chemokine receptor CCR7 (Sallusto et al. 1998) that promotes their migration into lymph node, attracted by the ligand of CCR7, the chemokine CCL21 (or SLC, Secondary Lymphoid tissue Chemokine) produced by the lymphatic vascular endothelium of afferent vessels (Ohl et al. 2004; Saeki et al. 1999; Sallusto et al. 1998). Antigenic peptide-loaded DC are transported by lymph to the subcapsular sinus and enter the paracortex zone where they aggregate preferentially around high endothelial venules (HEV), on the entry route of T cells into the paracortex, and thus may foster the presentation of major histocompatibility complex (MHC)-peptide complexes to passing T cells and optimize initial T cell activation (Bajenoff et al. 2003). In addition, native antigen-loaded DC could encounter B cells that enter the lymph node by HEV and activate them (Qi et al. 2006). Other cell types are also transported by afferent vessels, such as T and B lymphocytes and macrophages (Mackay et al. 1988). Indeed DC, T, and B cells leave peripheral tissues and migrate towards lymph nodes owing to their expression of chemokine receptor CCR7 (Bromley et al. 2005; Debes et al. 2005). Additional mechanisms that could also control this afflux of lymphocytes include interactions of lymphocytes with adhesion molecules expressed by lymphatic endothelium as CLEVER-1 (Common Lymphatic Endothelial and Vascular Receptor-1) (Salmi et al. 2004) and Mannose receptor 1 (Irjala et al. 2001). Finally, naive T and B cells, which have failed to find their specific antigen, or activated T cells, will go through medullary cords before reaching efferent vessels, and then return to bloodstream. Exit of lymphocytes depends on the expression of a G protein coupled receptor, Sphingosine-1-phosphate 1 (S1P1) by lymphatic endothelial cells and its ligand S1P expressed by lymphocytes (Mandala et al. 2002; Matloubian et al. 2004). Immunisation leads to enhanced lymph node lymphangiogenesis, an event that in turn leads to improved DC mobilisation. Of particular note, lymphangiogenesis occurred not only at the infection site and the draining lymph node, but also in uninvolved peripheral tissue that drains to the same lymph node. VEGF-A, ligand of VEGFR-2, which is highly expressed by follicular B cells, is a possible mediator of the increase lymphangiogenesis and DC migration (Angeli et al. 2006).

Macrophages are suggested to have a dual role in inflammation-induced lymphangiogenesis (Kerjaschki 2005). Macrophages recruited into the inflammation site can transform from naive monocytes into VEGF-C/D-producing cells, which can stimulate the growth of existing LECs (Schoppmann et al. 2002). They might also contribute to lymphangiogenesis by trans-differentiating to lymphatic endothelial cells, which incorporate into the lymphatic endothelium (Maruyama et al. 2005). Furthermore, human kidney transplant rejection is frequently accompanied by lymphangiogenesis, and these lymph vessels produced CCL21, which further attracted $CCR7^+$ lymphocytes and DC, and might actively promote the inflammatory process (Kerjaschki et al. 2004). In addition, other inflammatory diseases seem to be associated with lymphatic activation and dysfunction. For example, lymphatic hyperplasia is observed in UVB-irradiation-induced skin inflammation, in a mouse model of chronic skin inflammation resembling psoriasis, and in human psoriatic skin lesions (Kajiya et al. 2006; Kunstfeld et al. 2004). In chronic airway inflammation induced by *Mycoplasma pulmonis*, a massive lymphangiogenesis is induced by VEGF-C/-D-producing inflammatory cells, and its blocking resulted in bronchial lymphedema, thus demonstrating the importance of lymphangiogenesis for compensation of vascular leakage during the inflammation (Baluk et al. 2005). Finally, the role of VEGF-C/D–VEGFR-3 signaling in inflammatory lymphangiogenesis is also suggested by overexpression of VEGF-C in the joint synovium of rheumatoid arthritis patients (Paavonen et al. 2002). These studies underline the close relationship between inflammation, the immune response and the adaptability of a mature lymphatic network.

Tumor lymphangiogenesis

Tumor metastasis to regional lymph nodes often represents the first step of tumor dissemination and serves as a major prognostic indicator for the progression of human cancers. A variety of mechanisms may contribute to the dissemination of primary malignant cancer cells: local tissue invasion, systemic metastasis via tumor-associated blood vessels to distant organs, and lymphatic metastasis via

tumor-associated lymphatic vessels to the draining (sentinel) lymph node, distal lymph nodes, and from there to distal organs. The latter pathway constitutes the most common pathway of initial metastasis for many types of solid human tumors (reviewed by Stacker et al. 2002b). The extent of lymph node metastasis is a major determinant for the staging and the prognosis of most human malignancies and often guides therapeutic decisions. Despite this, the molecular mechanisms of lymphatic metastasis are not completely understood.

Dissemination of tumor cells from the primary sites to the lymphatic system is accomplished either by invasion into pre-existing lymphatic vessels in the surrounding tissues or by invasion into intratumoral lymphatic networks (Achen et al. 2005; Alitalo et al. 2004; Cao et al. 2004; Cao 2005; Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001a). In some cases of human cancers the presence of lymphatic vessels inside the tumor was reported to correlate positively with lymph node metastasis and poor prognosis (Beasley et al. 2002; Kyzas et al. 2005). However, the issue whether intratumoral lymphatic vessels are important for tumor spread is controversial; indeed, intratumoral lymphatic vessels might be poorly functional and not required for lymphatic metastasis (Padera et al. 2002; Wong et al. 2005). In contrast to intratumoral vessels, peritumoral lymphatic vessels certainly are functional and have a drainage function (He et al. 2002, 2004; Padera et al. 2002). In addition, tumor cells produce lymphangiogenic factors that could dilate the pre-existing lymphatic vessels surrounding the tumor tissue. Some of these factors are able to facilitate the transmigration of tumor cells through the lymphatic endothelium (Alitalo et al. 2004).

Tumor cells and tumor-associated macrophages can express lymphangiogenic factors VEGF-C and VEGF-D, thus they could play a role in peritumoral lymphangiogenesis and subsequent dissemination in human cancer (Schoppmann et al. 2002) (Fig. 2c). Studies in animal tumor models have provided direct experimental evidence that increased levels of VEGF-C or VEGF-D promote active tumor lymphangiogenesis and lymphatic tumor spread to regional lymph nodes (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001b). These effects are suppressed by specific inhibition of the VEGFR-3 pathway—using either blocking VEGFR-3 antibody, VEGF-C/D trap (VEGFR-3 extracellular domain fused with immunoglobulin Fc portion) or VEGF-C targeting siRNA—that prevents cancer metastasis to lymph nodes and beyond (e.g. (He et al. 2002; Karpanen et al. 2001; Lin et al. 2005; Roberts et al. 2006), underlining the direct link between VEGF-C or VEGF-D expression and metastasis. Moreover, a large number of clinicopathological studies of human cancers have shown a direct correlation between expression of VEGF-C or VEGF-D by tumor cells and lymphatic invasion, lymph node and distant metastasis, and poor patient survival, but not necessarily with the density of tumor-associated lymphatic vessels (Achen et al. 2005; Pepper et al. 2003; Stacker et al. 2002a; Tobler and Detmar 2006). Combination of siRNA therapy targeting both VEGF-C and VEGF-A inhibits both lymph node and lung metastasis in a mouse immunocompetent mammary cancer model, rendering this combined therapy more beneficial than either alone (Shibata et al. 2008).

VEGF-C stimulates the growth of new tumor-associated lymphatic vessels, and in this process nearby LECs send filopodia toward VEGF-C producing tumor cells, and then form tumor-directed vessel sprouts, where the vessel lumen opens up and allows facilitated access of tumor cells to the lumen (He et al. 2005). The lymphatic endothelium may also actively participate in metastasis formation by secreting chemokines such as CCL21, whose receptor is expressed on some malignant cells (Shields et al. 2006; Zlotnik 2004). Moreover, intraluminal VEGF-C also promotes the dilation of collecting lymphatic vessels through the process of endothelial proliferation in the vessel wall, and further enhances the delivery of tumor cells to sentinel lymph nodes, probably by increasing the lymph flow rate (He et al. 2005; Hoshida et al. 2006).

Overexpression of VEGF-C or VEGF-A under the control of keratinocyte-specific promoter can induce active proliferation of tumor-associated lymphatic vessels in a multi-step skin carcinogenesis model, leading to enhanced tumor metastasis to the sentinel and distant lymph nodes (Hirakawa et al. 2005, 2007). This effect is preceded by lymphangiogenesis in sentinel lymph nodes, which suggests that primary tumors can prepare their future metastatic site in advance of their arrival, partly by producing lymphangiogenic factors. Induction of lymph node lymphangiogenesis and increased lymph flow through tumor-draining lymph nodes, prior to tumor cell arrival, was suggested to be B cell-dependent (Harrell et al. 2007). In addition, not only can cancer cells ‘prepare the ground’ for metastasis, but also stromal cells might serve as important mediators for tumor-induced lymphangiogenesis. A recent study, using a conditional transgenic mouse model of breast cancer, showed that hyaluronan-rich tumor microenvironment plays an important role in promoting tumor lymphangiogenesis, thus demonstrating the importance of the tumor stromal cells and extracellular matrix in educating malignant cells to secrete specific lymphangiogenic factors (Koyama et al. 2008).

Concluding remarks

In recent years, novel molecules regulating the development, differentiation, morphogenesis and functional expression

of lymphatic endothelial cells have been successively identified, resulting in marked advances in the field of lymphangiogenesis. Progress in technical tools such as sophisticated animal models, better imaging techniques that allow work at cellular or subcellular resolution and in live animals during dynamic studies and high throughput genomic or proteomic screens, have made vascular morphogenesis much more accessible to research. However, many questions concerning some fundamental processes of physiological and pathological lymphangiogenesis are yet remain unresolved.

Early steps of lymphatic endothelial cell commitment are not yet completely understood, and the mechanisms of lymphatic vascular remodeling and maturation are only beginning to be elucidated. It will be important to clarify the importance of mesenchymal lymphangioblasts as a secondary source of LECs, other than the venous endothelium. Furthermore, signals that induce PROX1 expression in a restricted subpopulation of endothelial cells and direct PROX1 target genes that act as its effectors in lymphatic endothelium remain to be identified. Lymphangiogenesis bears a close resemblance to angiogenesis, yet it appears that lymphatic vasculature uses distinct molecular mechanisms. It will be important to understand the basis for such differences and identify signaling pathways that control different stages of lymphatic vascular development. Further studies are needed to pinpoint the mechanisms that keep the emerging lymphatic vessels separate from blood vessels. This process appears to be complex and it is likely that includes other factors in addition to Syk/Slp76. Furthermore, the lymphatic vascular tree is composed of a system of lymphatic vessels of different size and morphology; it would be interesting to study the phenotypic and genotypic differences of the LECs that comprise these vessels, and to characterize endothelial, molecular, and phenotypic variability through the lymphatic vasculature system.

Answers to these questions will help to provide a comprehensive picture of lymphatic vascular development, and will contribute to our understanding of the mechanisms involved in lymphatic vascular dysfunction in lymphedema, inflammation, and cancer. In the case of lymphedema-distichiasis, it is necessary to extend the study of the role of FOXC2, and to identify direct FOXC2 target genes to enhance our understanding of the morphogenesis and differentiation of intraluminal valves.

The involvement of lymphatic vessels in inflammation should be explored in several contexts, and the impact of inflammation on the phenotype and function of lymphatic vessels needs to be investigated. In this way, a clear vision of physiological and ectopic lymph node neogenesis should provide information about the link between lymphatic vessels and lymph nodes. Finally, it is of great importance to have a better understanding of the importance of tumor-associated lymphangiogenesis in the spread of cancer to distant organs. The elucidation of the molecular mechanisms of lymphatic metastasis represents another challenge that will be instrumental in our understanding of how to control the spread of cancers in patients. It has become evident over the last decade that VEGFR-3 and its ligands VEGF-C and VEGF-D are critical targets for new drug development. The question now concerns the potential efficacy of targeting such lymphangiogenic growth factors in the management of cancer. In addition, the involvement of tumor microenvironment in neo-lymphangiogenesis has to be addressed, and with this the identification of stromal and immune determinants. That also implies studying the interactions between stromal cells and LECs, which could promote further development of lymphatic vessels. Immune cells have been shown to participate in tumor progression, and tumor immunomodulation could also form a part along with lymphangiogenic growth factor signaling, of processes that promote metastasis through the lymphatic system. The challenge for future studies is to identify new molecular and cellular players and increase our understanding of the basic biology of lymphatic development, which will contribute to designing novel therapeutic strategies for treatment of different lymphatic disorders.

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