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Review

Molecular mechanisms of lymphatic vascular development

T. Mäkinen[a], C. Norrmén[b] and T. V. Petrova[b]*

[a]Cancer Research UK London Research Institute, 44 Lincoln's Inn Fields, London WC2A 3PX (UK)
[b]Molecular and Cancer Biology Program, Biomedicum Helsinki, P.O.B. 63 (Haartmaninkatu 8), 00014 University of Helsinki (Finland), Fax: +358 9 1912 5610, e-mail: Tatiana.Petrova@helsinki.fi

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Abstract. Lymphatic vasculature has recently emerged as a prominent area in biomedical research because of its essential role in the maintenance of normal fluid homeostasis and the involvement in pathogenesis of several human diseases, such as solid tumor metastasis, inflammation and lymphedema. Identification of lymphatic endothelial specific markers and regulators, such as VEGFR-3, VEGF-C/D, PROX1, podoplanin, LYVE-1, ephrinB2 and FOXC2, and the development of mouse models have laid a foundation for our understanding of the major steps controlling growth and remodeling of lymphatic vessels. In this review we summarize recent advances in the field and discuss how this knowledge as well as use of model organisms, such as zebrafish and *Xenopus*, should allow further in depth analysis of the lymphatic vascular system.

Keywords. Lymphangiogenesis, vascular remodeling, PROX1, VEGFR-3, FOXC2, Ephs/ephrins.

The lymphatic vasculature

The adult lymphatic system is composed of blind-ended capillaries, collecting vessels and lymphoid organs, such as lymph nodes, tonsils and Peyer's patches. Lymph capillaries are characterized by the presence of loose intercellular junctions, little or absent basement membrane and the absence of surrounding smooth muscle cells or pericytes (Fig. 1a, b). Thin fibrillar structures, called anchoring filaments, connect the abluminal surface of lymphatic endothelial cells to the extracellular matrix, providing a way to sense the expansion of the interstitium during edema and preventing the collapse of lymphatic capillaries under high pressure conditions. Collecting lymph vessels, which transport lymph to the lymph nodes, are surrounded by a basement membrane and smooth muscle cells, which form a thinner and more disorganized layer than in blood vessels of a similar caliber. A distinguishing feature of collecting lymphatic vessels is intraluminal valves, which prevent lymph back flow (Fig. 1a, c). Lymphatic vessels are present in nearly all tissues, with the exceptions of the central nervous system, bone marrow, cartilage, cornea and epidermis. Virchow-Robin spaces, formed by prolongation of meninges, surround blood vessels as they enter the brain, and collect the interstitial fluid, thus playing a lymphatic vessel-like role.

One of the main functions of lymphatic vasculature is the maintenance of fluid homeostasis. Unlike the cardiovascular system, the lymphatic vasculature has no central pump and is not closed: interstitial proteins and water, extravasated from blood capillaries, are absorbed by lymphatic capillaries and transported to the blood circulation by the peristalsis of collecting lymphatic vessels and contractions of surrounding skeletal muscles. Another important function of the

* Corresponding author.

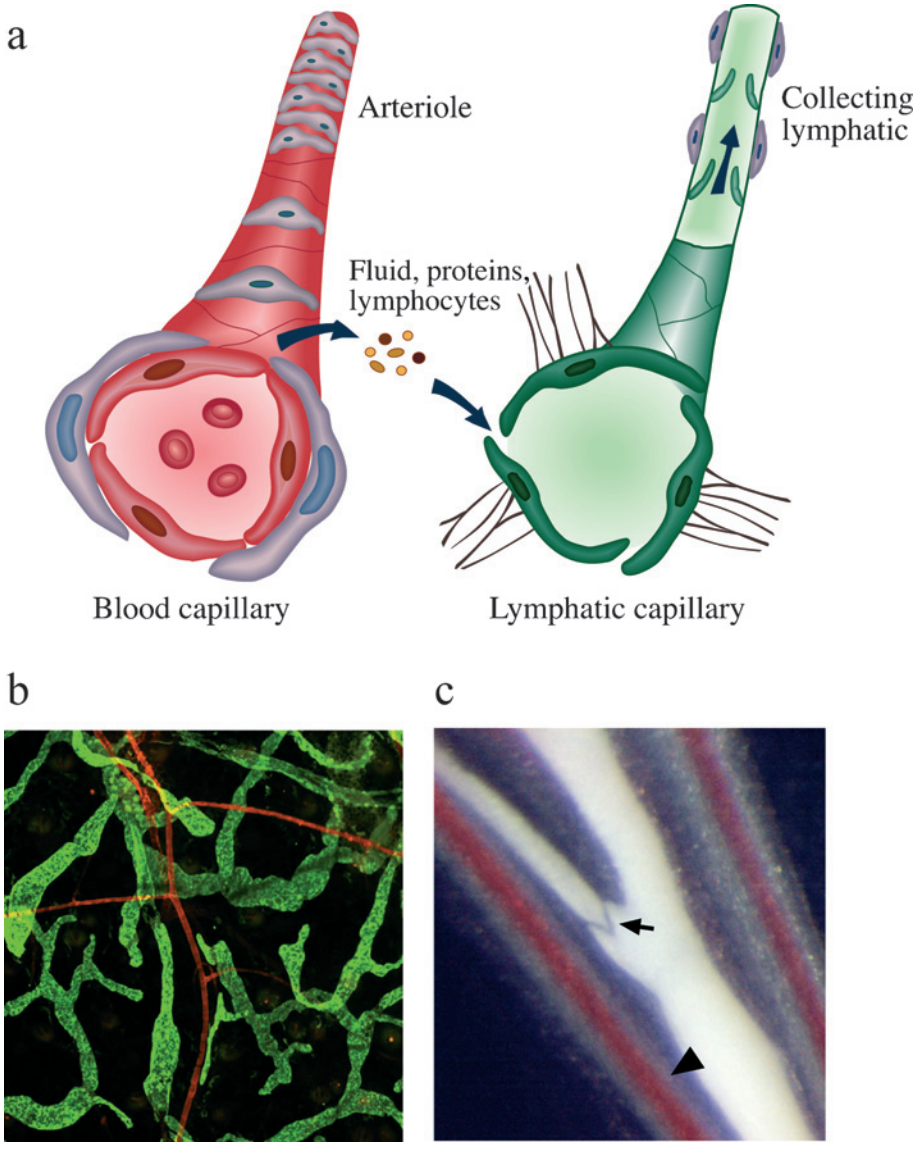

Figure 1. Function and features of lymphatic vasculature. (*a*) Mechanism of fluid and particle uptake by lymphatic capillary. (*b*) Blind-ended lymphatic capillaries in adult mouse ear. Whole mount staining for lymphatic endothelial specific marker LYVE-1 (green) and smooth muscle actin (red), which identifies smooth muscle cell-covered blood vessels. (*c*) Chyle-filled collecting lymphatic vessel in mouse mesentery. Arrow, lymphatic valve; arrowhead, blood vessel.

lymphatic system is immune surveillance: lymphocytes and antigen-presenting dendritic cells are transported by lymphatic vessels from the interstitium to the lymph nodes, where specific immune responses may be initiated. Lymphatic vessels also play a major role in the absorption of dietary fat, which is secreted by enterocytes in the form of lipid particles or chylomicrons. This task is carried out by lacteals, lymphatic capillaries located inside the villi of the small intestine. Chylomicrons may constitute up to 5–15 % of lymph volume after ingestion of a fat containing meal, and up to 90 % of dietary fat is absorbed from the gut in this manner.

In addition to their role in homeostasis, lymphatic vessels are involved in several human diseases, such as tumor metastasis, lymphedema and inflammation. In many solid tumors, peritumoral lymphatic vessels provide a route for the escape of tumor cells to the regional lymph nodes, whereas blocked or absent lymphatic vessels cause lymphedema. Secondary lymphedema, following lymph node removal, is an important cause of chronic disability in cancer patients. The role of lymphatic vasculature in human diseases has been recently reviewed [1–3], and we refer the reader to these sources for more detailed information. In the present review we will focus on the molecular mechanisms involved in the formation, growth and remodeling of the lymph vasculature during embryonic and early postnatal development.

Models for the development of lymphatic vasculature

Most of the available mouse molecular genetics data support the theory of centrifugal sprouting for the development of lymphatic vessels, which was first formulated by Florence Sabin, who based her conclusions on morphological observations in pig fetuses [4]. According to this theory, lymphatic endothelial cells arise at specific locations of embryonic veins, from which they migrate to form primary lymph sacs (Fig. 2a). Lymphatic vessels further sprout from these to form a primary lymphatic plexus, which is remodeled to produce a hierarchically organized network of lymphatic capillaries and collecting lymph vessels. An alternative view, proposed by Huntington and McClure, suggested that lymphatic endothelial cells arise independently from blood vessels from the mesenchymal precursor cells [5]. This hypothesis is mainly supported by data in birds, in which in addition to blood vessel-derived lymphatic endothelial cells, the allantoic mesoderm and paraxial/somitic mesoderm of the avian wing were shown to contribute to the lymphatic endothelium [6]. At present, it is not known whether similar resident mesenchymal precursors contribute to the lymphatic vasculature during normal development in mammals. However, circulating lymphatic endothelial progenitor cells participate in lymphangiogenesis in several pathological situations, such as chronic renal transplant rejection [7]. At the molecular level, a number of regulators of the crucial stages of lymphatic vascular development, including the transcription factors Prox1 and Foxc2, tyrosine kinases and their ligands, such as VEGFR-3/VEGF-C, Tie/Ang2 and ephrinB2, have been identified in recent years (Fig. 2b), and their role is discussed here in more detail.

Lymphatic endothelial cell fate commitment and early sprouting

Prox1

The atypical homeobox transcription factor Prox1 is a mammalian homolog of Drosophila Prospero. Prox1 is an important regulator of cell differentiation and organogenesis in several tissues, such as liver, pancreas, lens, retina and lymphatic vessels [8–12]. In mice, Prox1 expressing endothelial cells appear at embryonic day (E) 10 in the jugular vein, from which they migrate to form the first lymphatic sprouts [8]. Targeted inactivation of *Prox1* completely arrests the lymphatic vascular development without affecting blood vasculature. In the absence of Prox1, endothelial cells bud from the cardinal vein but are unable to migrate further and fail to establish a lymphatic endothelial cell-specific gene expression profile [13]. Accordingly, overexpression of PROX1 in blood vascular endothelial cells suppresses many blood vascular-specific genes and up-regulates lymphatic endothelial cell-specific transcripts [14, 15]. Prox1 is also necessary for the maintenance of the lymphatic vasculature during later stages of development and in adulthood, as *Prox1*$^{+/-}$ mice develop chylous ascites, and show disorganized and abnormally patterned lymphatic vessels [16]. Notably, impaired lymphatic vascular function in *Prox1* heterozygotes and in mice with conditional deletion of *Prox1* in endothelial cells causes adult onset obesity, indicating an important link between lymph drainage and adipogenesis. At present the signals leading to the expression of Prox1 in cardinal vein and its target genes in lymphatic endothelial cells, are largely unknown. Treatment of cultured endothelial cells with interleukin-3 and -7 was shown to induce the expression of PROX1 and podoplanin; however, the relevance for the *in vivo* regulation of Prox1 expression remains to be elucidated [17, 18]. The ability of Prox1 to repress the transcriptional activity of nuclear receptors LRH-1 and Ff1b in mammals and zebrafish raises the intriguing possibility that the regulation of nuclear receptor signaling may underlie the master regulatory function of Prox1 in lymphatic endothelial cells [19–21].

VEGF-C/D – VEGFR-3

Receptor tyrosine kinase VEGFR-3 (Flt4) and its ligand VEGF-C are essential for lymphatic endothelial cell proliferation, survival and migration. Before the onset of lymphatic vascular differentiation, VEGFR-3 is highly expressed in blood vascular endothelial cells, but its expression becomes gradually restricted to lymphatic endothelial cells after midgestation [22–24]. VEGF-C is produced by vascular smooth muscle cells and in the mesenchymal cells in the regions adjacent to the sites of initial sprouting of lymphatic endothelial cells from veins [23, 25]. Homozygous deletion of *Vegfc* leads to a complete absence of lymphatic vasculature and embryonic death at around E15, whereas *Vegfc*$^{+/-}$ mice survive into adulthood but display severe lymphatic hypoplasia [25]. In *Vegfc*$^{-/-}$ mice, Prox1^{+} lymphatic endothelial cells arise normally in the cardinal vein, but do not sprout from their initial location, demonstrating that VEGF-C is an essential chemotactic and survival factor during embryonic lymphangiogenesis. In contrast, mice with targeted inactivation of the other VEGFR-3 ligand, V*egfd*, develop normal lymphatic vasculature [26]. Overexpression of either VEGF-C or VEGF-D in the skin of transgenic mice induces lymphangiogenesis of

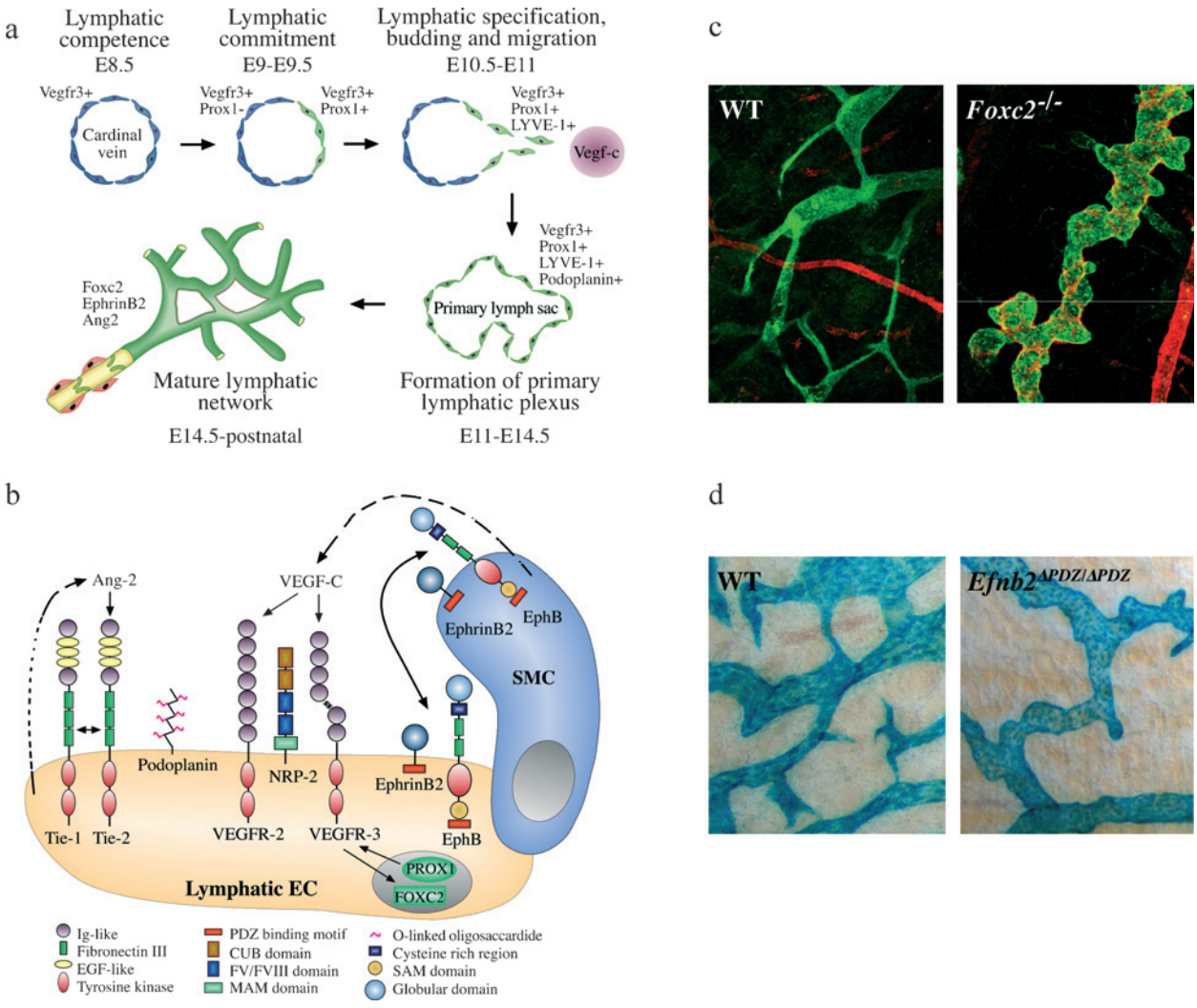

Figure 2. Major steps and regulators of lymphatic vascular development in vertebrates. (*a*) Current model of lymphatic vascular development in mice. (*b*) Regulators of lymphatic vascular development. Mesenchymal or smooth muscle cells (SMC) are a major source of VEGF-C; Ang2 is mainly produced by endothelial cells. A genetic interaction exists between VEGFR-3 and FOXC2 pathways [64], while PROX1 controls the expression of *VEGFR3* in lymphatic endothelial cells [13, 15]. (*c*) Abnormal patterning and association of smooth muscle cells with lymphatic vessels in *Foxc2*$^{-/-}$ mice [64]. Staining for VEGFR-3 (green) and smooth muscle actin (red). (*d*) Defective lymphatic vessel remodeling in the skin of *Efnb2*$^{\Delta PDZ/\Delta PDZ}$ mice, lacking the PDZ binding motif of ephrinB2 [50]. WT, wild type. Mice are crossed with *Vegfr3*$^{Lacz/+}$ mice to visualize lymphatic vessels by X-gal staining.

cutaneous lymphatic vessels without affecting blood vessel growth [27, 28].

In agreement with its early blood vascular expression, homozygous *Vegfr3* deletion leads to defective blood vessel remodeling and embryonic death at E9.5, while *Vegfr3*$^{+/-}$ mice have normal lymphatic vasculature [24]. The phenotype of *Chy* mice, which carry a heterozygous missense point mutation that impairs the kinase activity of VEGFR-3, is very similar to *Vegfc*$^{+/-}$ mice. *Chy* mice develop hypoplasia of cutaneous lymphatic capillaries and lymphedema of hind limbs, which makes them a useful model for studies of hereditary lymphedema and its therapy [29]. In humans, similar heterozygous missense point mutations in *VEGFR3* were found in several families with Milroy disease (OMIM 153100), a rare autosomal dominant lymphedema, characterized by hypoplastic lymphatic capillaries in the skin [30]. Furthermore, transgenic overexpression of VEGFR-3-Ig fusion protein, which traps soluble VEGFR-3 ligands, prevents growth of skin lymphatic vessels [31]. Interestingly, in contrast to cutaneous lymphatic vessels, growth of internal lymphatic vessels in K14-VEGFR-3-Ig mice is affected only transiently, and they re-grow post-natally, suggesting a tissue-specific role for VEGFR-3 in the regulation of lymph vessel stability and survival [31]. Recent studies of post-natal lymphangiogenesis have shown that lymphatic capillaries require VEGFR-3 to be activated by the soluble ligands for up to two weeks after birth, after which

they become insensitive to VEGFR-3 inhibition, demonstrating the existence of a postnatal lymphatic vascular maturation mechanism [32].

VEGFR-3 downstream signaling has been studied in several *in vitro* systems [33–35]. Full-length VEGF-C and VEGF-D have the highest binding affinity for VEGFR-3; however, proteolytically processed forms of the VEGF-C or VEGF-D homodimers interact with and activate another member of the vascular endothelial growth factor receptor family, VEGFR-2, primarily involved in the induction of angiogenic response in blood vessels [36, 37]. 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 [38]. Recent studies have revealed additional VEGF-C signal transduction pathways, such as interaction of VEGF-C with Neuropilin-2 and integrin α9, and VEGFR-3 with HHV-8 envelope protein gB6 [39–41]. Ligand induced formation of homo- and heterodimeric VEGFR-2 and VEGFR-3 receptor complexes leads to tyrosine phosphorylation of their intracellular domains, the recruitment of intracellular signal transduction proteins adaptor molecules Shc, Grb-2 and CRKI/II, and the activation of PI3 and MAP kinases ERK1/2 and JNK, and the member of the FAK non-receptor tyrosine kinase family RAFTK [34, 35, 42]. VEGFR-3 and VEGFR-2 also form heterodimers, in which VEGFR-2 phosphorylates only a subset of the VEGFR-3 tyrosine residues phosphorylated upon its homodimerization [43]. It is likely that VEGF-C activated endothelial cell sprouting is mediated *via* both VEGFR-2 homodimers and VEGFR-3/VEGFR-2 heterodimers [27, 34]; however, at present the differences in intracellular signaling originating from VEGFR-3 and VEGFR-2 homodimers and VEGFR-3/VEGFR-2 heterodimers is not generally understood. One example of such differential signaling is the induction of angiopoietin-2 mRNA expression in response to stimulation by VEGF-C- but not the VEGFR-3-specific mutant VEGF-C156S [44].

LYVE-1

LYVE-1 (lymphatic vessel hyaluronan receptor-1), a homolog of hyaluronan receptor CD44, is one of the most widely used markers of lymphatic endothelial cells both in normal and tumor tissues [45]. In addition to lymphatic endothelium, LYVE-1 is also present in endothelial cells of liver sinusoids, lung blood vessels, high endothelial venules and in activated tissue macrophages [46–49]. LYVE-1 is the earliest known marker of lymphatic endothelial commitment, and in mice it is expressed in a polarized manner starting from E9. Interestingly, in adults, the expression of LYVE-1 in collecting lymphatic vessels is down-regulated [50]. TNF-α suppresses the expression of LYVE-1 in cultured lymphatic endothelial cells; however, the mechanism of LYVE-1 down-regulation *in vivo* has not been elucidated [51]. The physiological role of LYVE-1 remains unclear; LYVE-1-deficient mice have a normal lymphatic vasculature and secondary lymphoid tissue, with normal trafficking of cutaneous dendritic cells to draining lymph nodes and skin inflammation responses [52].

Separation of blood and lymphatic vasculature

Although there are many similarities in the mechanisms regulating the growth and development of blood and lymphatic vasculature, these two types of vessels are normally connected only at few and very precise locations, such as at the junctions of subclavian and jugular veins. This in turn suggests the existence of mechanisms for keeping apart blood and lymphatic vascular compartments. One such mechanism may use tyrosine kinase Syk and its substrate adaptor molecule Slp76, which are highly expressed in lymphocytes and were initially studied in the context of hematopoietic development. Deficiency in Syk or Slp76 results in arterio-venous shunting and mixing of blood and lymphatic endothelial cells, manifested as blood-filled lymphatic vessels with mosaic expression of LYVE-1 [53, 54]. Expression of Slp76 in a subset of hematopoietic cells rescues this phenotype, while the deficient cells confer the cell mixing phenotype in chimeric embryos, suggesting the contribution of hematopoietic cells to lymphatic vascular development [54]. The current model proposes that the main defect is due to an incomplete exit of circulating lymphatic endothelial precursor cells from blood vessels; however, the mechanism, which may be linked to defective integrin signaling, remains to be investigated. Signals other than Slp76-Syk-mediated signals must exist to prevent fusion of emerging lymphatic capillaries with pre-existing blood vessels, but no molecular candidates have been identified so far.

Vessel guidance, remodeling and maturation

Remodeling of the blood vasculature is an important step in the formation of a fully functional cardiovascular system, which transforms the uniformly sized primary capillary plexus into a hierarchical vascular tree, composed of arteries and veins. A morphologically similar remodeling process takes place in the lymphatic vasculature, leading to the formation of a

network of lymphatic capillaries and collecting lymphatic vessels. In the past few years several regulators of this process have been identified. Interestingly, in all these cases the genes or their functions appear to be specific to lymphatic vessels, suggesting that in spite of the morphological similarities between blood vessel and lymphatic vessel remodeling, the underlying molecular mechanisms may differ.

Forkhead transcription factors Foxc2 and Foxc1

The forkhead family of transcription factors in mammals is composed of more than 40 members, characterized by the presence of a winged-helix or forkhead DNA binding domain. Forkhead proteins, which in most cases act as transcriptional activators, play an important role in many developmental processes, including the establishment of right-left asymmetry and morphogenesis of various tissues (reviewed in [55]). The specific role for the forkhead transcription factors in the regulation of lymphatic vascular function was first demonstrated in human hereditary disease lymphedema-distichiasis (LD) (OMIM 153400), an autosomal dominant condition characterized by late onset leg lymphedema and metaplasia of meibomian glands. Meibomian glands are specialized sebaceous glands at the rim of the eyelids, which secrete sebum to prevent evaporation of tear film. In LD patients they develop as pylosebaceous units, resulting in a double row of eyelashes (distichiasis), which is fully penetrant and often the diagnostic feature of this disease. Lymphedema usually develops at or after puberty; however, neonatal cases have also been described. In addition to lymphedema, venous insufficiency, due to incompetent venous valves, is a frequent feature of LD [128]. In most LD cases, insertions, deletions or nonsense mutations in the coding region of FOXC2, which lead to a frameshift, premature peptide termination, and loss of transcriptional activity, have been identified [56–58]. Missense mutations, which affect DNA binding of FOXC2, as well as two cases of LD without mutations in the coding region, have also been described. Although the mutations can be found throughout the gene, there is a hotspot in the region 893–930 bp, characterized by the presence of a GCCGCCGC element on either side of a 14-bp sequence, which is likely responsible for misalignment [59]. A number of *de novo* FOXC2 mutations have recently been identified, suggesting that LD may be a much more common cause of adult-onset lymphedema than originally thought [59].

Foxc2 deficiency in mice leads to perinatal lethality due to aortic arch malformations [60]. In addition mice have mild skeletal defects, cleft palate and abnormal kidney and lung development [60–63]. *Foxc2* is highly expressed in the developing lymphatic vessels as well as in lymphatic valves in adults [64, 65]. Initial development of lymphatic vasculature proceeds normally in the absence of Foxc2; however, the specification of the lymphatic capillaries *versus* collecting lymphatic vessels at later stages of embryogenesis is severely affected [64]. Collecting lymphatic vessels in $Foxc2^{-/-}$ mice fail to develop valves while the lymphatic capillaries acquire ectopic coverage by basal lamina components and smooth muscle cells and begin to express some blood vascular endothelial cell markers such as endoglin and PDGF-B [64] (Fig. 2c). Mice heterozygous for both *Foxc2* and *Vegfr3* display a phenotype very similar to $Foxc2^{-/-}$ mice, suggesting a genetic interaction between these two pathways.

Foxc1 encodes a transcription factor with the DNA binding domain almost identical to that of Foxc2, and the two transcription factors are co-expressed in most target tissues with the exception of lymphatic endothelial cells [65, 66]. Recent studies showed that Foxc2 and Foxc1 cooperate in the regulation of early lymphatic vascular development. Indeed, compound $Foxc1^{+/-}$; $Foxc2^{-/-}$ embryos show reduced lymphatic vascular density in the areas of initial sprouting of lymphatic endothelial cells from the primary lymph sacs, which is likely a result of reduced expression of VEGF-C in the surrounding mesenchymal cells [67].

Ephs and ephrins

Eph receptor tyrosine kinases and their membrane-bound ligands; ephrins, regulate a variety of developmental processes, including axon guidance, proliferation of neural stem cells and angiogenesis. During vascular development, ephrinB2 and its receptors play important roles in the remodeling of the arterial-venous capillary plexus and in the postnatal maturation of lymphatic vasculature [50, 68, 69]. EphrinB ligands have intrinsic signaling capacities; their cytoplasmic domains can be phosphorylated on tyrosine residues and they have a C-terminal motif for binding of PDZ domain containing proteins. While deletion of the entire cytoplasmic domain of ephrinB2 leads to failure in cardiovascular development [70], mouse mutants deficient either in the PDZ binding motif or lacking the conserved tyrosine residues in the intracellular domain, develop apparently normal blood vasculature [50]. However, the ΔPDZ mutants fail to establish a hierarchically organized lymphatic vessel network consisting of lymphatic capillaries and collecting vessels (Fig. 2d). In addition, they lack luminal valves in the collecting vessels and acquire ectopic smooth muscle cell coverage in lymphatic capillaries. These results suggest an important role for ephrinB2 signaling in the postnatal remodeling of lymphatic vessels [50].

The cell contact mediated receptor-ligand interactions between (lymphatic) endothelial cells and smooth muscle cells or cells in the surrounding tissue play a critical role in activating Eph/ephrin signaling. However, individual endothelial cells and smooth muscle cells often co-express several ligands and receptors. Recent studies suggest that in addition to the *trans*-configuration, where receptor and ligand are expressed in neighboring cells, Eph-ephrin proteins may also interact in *cis* and mediate signaling in the absence of cell-cell contact. Co-expression of EphA/ephrinA proteins in the same cell can mask binding sites required for the *trans*-interaction, and attenuate *trans*-activated signaling [71, 72]. On the other hand, in neurons, EphAs and ephrinAs were shown to segregate into distinct membrane domains and mediate opposing effects on growth cone guidance [73]. Finally, removal of ephrinB2 from smooth muscle cells was shown to lead to cell contact-independent defects in cell spreading and polarized migration [74]. Bi-directional signaling, cell contact-independent activation and overlap between expression patterns and cognate receptor ligand interactions cause a high degree of complexity in the regulation of Eph-ephrin signaling. Cell and tissue context and the involvement of other signaling components play an important role in determining the appropriate signaling response, which may sometimes have opposite effects; the ephrinB2-EphB4 interaction provides the repulsive signals required to set arterial-venous boundaries, while in other contexts the ephrinB-EphB interaction can stimulate endothelial cell sprouting [69, 75].

Endothelial cells in lymphatic capillaries co-express at least ephrinB2 and EphB4 [50], and the interaction of ephrinB2 in *cis* with EphB4, or in *trans* with other EphB receptors expressed in the surrounding tissue, may regulate endothelial cell sprouting during the remodeling process. Furthermore, EphB-ephrinB signaling appears to regulate interactions between lymphatic endothelial and smooth muscle cells. Deletion of ephrinB2 specifically in pericytes and vascular smooth muscle cells, or deletion of the PDZ binding domain in ephrinB2 leads to ectopic recruitment of smooth muscle cells into lymphatic capillaries [50, 74]. On the other hand, expression of ephrinB2 in endothelial cells of collecting vessels may play an important role in the acquisition and maintenance of smooth muscle cell coverage.

Other neuron guidance molecules in lymphatic development

Several guidance molecules, which were initially discovered in the nervous system regulating the path finding of axonal growth cones, have been more recently implicated in blood vessel guidance. These molecules include netrins, slits, and semaphorins, which bind and activate their respective receptors, Unc5b, Robo and Plexin/Neuropilins, in endothelial cells (reviewed in [76]). Neuropilin-2 (Nrp2) is also expressed in a subset of lymphatic vessels and is required for the normal development of lymphatic capillaries [77]. In addition to binding class III semaphorins, Nrp2 binds the lymphangiogenic growth factors VEGF-C and VEGF-D and forms a complex with VEGFR-3 [41]. Therefore, Nrp2 may exert its functions in lymphatic development by acting as a co-receptor for VEGFR-3. Alternatively Nrp2 may function as co-receptor for Plexin signaling, although no lymphatic endothelial Plexin has been reported so far. Furthermore, the expression and potential function of other blood vessel guidance molecules in the lymphatic endothelium remain to be explored.

Podoplanin

Podoplanin is a small transmembrane mucin-like protein, which is highly expressed in lymphatic endothelial cells [78, 79]. It is also expressed in podocytes, keratinocytes, cells of choroid plexus, alveolar lung cells, as well as at the invasive front in some human carcinomas. Targeted inactivation of podoplanin results in abnormal lung development and perinatal lethality. Podoplanin-knockout mice develop paw lymphedema and display an abnormal lymphatic function and patterning [80]. Podoplanin overexpression induces haptotactic cell migration and filopodia formation in immortalized endothelial cells, while its loss affects lymphatic endothelial cell adhesion. In tumor cells, podoplanin interacts with ezrin and moesin and promotes filopodia formation and cell migration by modulating activities of small Rho family GTPases [81, 82].

Angiopoietins and Tie1/Tie2

The Tie2 endothelial specific receptor tyrosine kinase interacts with three members of the angiopoietin family; Ang1, Ang2, and Ang3/4. Ang1 can activate both Tie1 and Tie2, which form heteromeric complexes [83, 84]. In contrast, Ang2 is proposed to act as a context-dependent agonist or antagonist of Tie2 signaling because it counteracts blood vascular tube stabilization, but was shown to be required for proper patterning of the lymphatic vasculature [85, 86]. In mouse models lacking Tie1, Tie2 and Ang1, initial blood vascular development proceeds normally, but embryonic remodeling and maturation of the vessels are defective [87–91]. *Tie2*$^{-/-}$ mice die at E9.5–12.5 due to a failure in remodeling the primary capillary plexus and cardiac defects, such as poor association between

endothelial cells and the underlying matrix, and lack of myocardial trabecular projections. *Angpt1*$^{-/-}$ mice die by E12.5 and show a similar vascular phenotype, suggesting that Ang1-induced activation of Tie2 is required for endothelial-mesenchymal cell–cell and cell–matrix interactions. Further supporting this view, a constitutively active mutant form of Tie2 was found in human hereditary venous malformations, characterized by dilated vessels with poor smooth muscle cell coverage [92]. Homozygous deletion of *Tie1* leads to embryonic or neonatal lethality, hemorrhages, edema, increased vessel number, and abnormal vascular endothelial cell development [88, 93, 94]. In contrast, *Angpt2*$^{-/-}$ mice develop normally, but demonstrate defective postnatal blood and lymphatic vessel remodeling, such as persistence of hyaloid vasculature and defects of retinal vascularization and abnormally patterned and leaky lymphatic vessels [85, 95]. The lymphatic but not the blood vascular phenotype can be rescued by Ang1, demonstrating that Ang2 acts as Tie2 agonist in lymphatic but not blood vascular endothelial cells [85]. *In vivo*, overexpression of Ang1 stimulated lymphatic endothelial cell proliferation and promoted vessel enlargement and generation of new sprouts [96, 97]. Ang1 up-regulated the expression of VEGFR-3, and a soluble form of VEGFR-3 inhibited the observed lymphatic sprouting, suggesting a cross-talk between VEGF and angiopoietin signaling during lymphatic development [96].

Several cell signaling cascades and downstream targets of Tie2 signaling have been identified in endothelial cells *in vitro*. Ang1 is only weakly mitogenic for cultured endothelial cells, while signaling through PI3 kinase and Akt is essential for Ang1-induced survival, sprouting, migration and capillary tube formation. Activated Tie2 recruits adaptor proteins Grb2, Grb7, Grb14 and ShcA and protein tyrosine phosphatase SHP2 [98–100]. Tie2 also associates with adaptor protein Dok-R, which leads to the recruitment of Nck and p21-activated kinase (PAK) [101, 102]. The Dok-R-PAK- and ShcA-mediated pathways are involved in the Ang1-induced migration [100, 102]. Ang1 may also regulate the MAPK signaling cascade by modulating phosphorylation of ERK1/2 and p38 MAPKs by PI3-K [103, 104].

In addition to the genes described above, defects in lymphatic vascular development have been described in several other mouse mutants. In many cases the underlying mechanisms remain to be elucidated; genes and the corresponding phenotypes are presented in Table 1.

New tools for lymphatic vascular biology research

In vitro models: cultured lymphatic endothelial cells and embryoid bodies

Important differences exist both in the function and the *in vivo* responses of blood vascular endothelial and lymphatic endothelial cells, therefore *in vitro* cell culture models, which allow studying these two cell lineages, can provide important insights into the signaling mechanisms operating in these two vascular compartments. Cultured dermal microvascular endothelial cells represent a mixed population of blood vascular and lymphatic endothelial cells, which can be separated using antibodies to lymphatic endothelial cell specific surface markers, such as VEGFR-3, podoplanin and LYVE-1 or by negative selection with antibodies against CD34 [34, 105–107]. Blood vascular endothelial cells (BECs) and lymphatic endothelial cells (LECs) represent relatively stable, but highly related cell lineages, which differ in about 2 % of expressed genes, as determined by the genome-wide comparison of their expression profiles [15, 34, 105, 107]. Differential expression of several groups of genes reflects the distinct *in vivo* functions of BECs and LECs. For example, BECs express significantly higher levels of pro-inflammatory cytokines, chemokines and chemokine receptors, which is consistent with their ability to regulate the homing of lymphocytes. Cultured BECs also have higher levels of β-catenin and display increased numbers of actin stress fibers, reflecting the fact that *in vivo* BECs are subject to higher blood flow and pressure, and they therefore need stronger intercellular adhesions than LECs, which are exposed to a sluggish lymph flow, and which must allow access of fluid from the extracellular milieu. LECs and BECs also show differential responses to shear stress, and organize in phenotypically distinct structures when cultured in fibrin gel in the presence of interstitial flow [108]. Although many typical features of lymphatic endothelium are preserved in cultured cells, such as high expression of PROX1, LYVE-1, podoplanin, VEGFR-3 and mannose receptor 1, responsiveness to VEGF-C and VEGF-D, and the preferential LEC-LEC homotypic interactions, recent studies show that the transfer from *in vivo* to *in vitro* conditions significantly affects the gene expression profile of lymphatic endothelial cells [109, 129], indicating further room for improvement of the *in vitro* model. Together with the availability of lymphatic endothelial cells from skin and other tissues [34, 105, 107, 110] this should allow in depth analyses of their molecular and functional characteristics, as well as dissection and comparison of signaling mechanisms operating in lymphatic and blood vascular endothelial cells.

Table 1. Developmental lymphatic vascular phenotypes in mouse knockout and transgenic models.

Knockout models	**Phenotype**
Angpt2 (angiopoietin-2)	Lymphatic vascular hypoplasia, transient chylous ascites, eye hyaloid vasculature fails to regress ($^{-/-}$) [85], abnormal inflammatory response ($^{-/-}$) [117].
Efnb2$^{\Delta PDZ/\Delta PDZ}$ (ephrinB2)	Mutation in the PDZ binding intracellular domain. Retrograde lymph flow, chylothorax, ectopic mural cells, absent valves ($^{-/-}$) [50], lung development defects (R. Klein, personal communication), postnatal lethality.
Elk3 (Net)	Lymphangiectasis, chylothorax, postnatal lethality ($^{-/-}$) [118], impaired wound and tumor angiogenesis ($^{+/-}$) [119].
Foxc2	Abnormal lymphatic patterning, presence of mural cells, absent valves ($^{-/-}$) [64]; lymphatic vessel and lymph node hyperplasia ($^{+/-}$) [120]. Aortic arch malformations, heart septal defects, abnormal kidney and urethra development ($^{-/-}$) [60–62].
Foxc1/Foxc2	Decreased lymphatic endothelial cells sprouting from the vein (Foxc1$^{+/-}$; Foxc2$^{-/-}$); arterio-venous malformations and loss of expression of arterial markers [67].
Itga9 (integrin α9)	Lymphedema, chylothorax ($^{-/-}$) [121].
Xlkd1 (LYVE-1)	No discernible lymphatic vascular phenotype [122].
Nrp2 (Neuropilin-2)	Transient lymphatic capillary hypoplasia [77], defects in neural fasciculation and guidance [123], postnatal lethality ($^{-/-}$).
Pi3kr1 (PI3-kinase p85α)	Chylous ascites; liver necrosis, enlarged skeletal muscle fibers, brown fat depositions, calcification of heart tissue ($^{-/-}$) [124].
Pdpn (Podoplanin, gp36)	Lymphangiectasis, abnormal lymph transport, lymphedema, respiratory failure due to abnormal lung development ($^{-/-}$) [80].
Prox1	Absent lymphatic vessels, lethality at E14.5 ($^{-/-}$), chylous ascites and obesity, lethality on most backgrounds ($^{+/-}$) [8, 16]. Obesity and lymphatic vascular defects are recapitulated upon endothelial specific deletion of Prox1 [16]. Abnormal eye, liver and pancreas development ($^{-/-}$) [9–12].
Lcp2 (Slp76) Syk	Failure of separation of blood and lymphatic vasculature, chylous ascites (Slp76 or Syk$^{-/-}$) [53, 54]; Failure of T cell development and fetal hemorrhage (Slp76); block of B cell development and fetal hemorrhage (Syk).
Sox18^{rag}	Spontaneous missense mutations. Edema and chylous ascites ($^{-/-}$), lack of vibrissae and coat hairs, generalized edema and cyanosis due to cardiovascular defects [125].
Vegfc	No lymphatic vessels ($^{-/-}$), hypoplasia and chylous ascites ($^{+/-}$) [25].
Vegfd	Viable, no discernible lymphatic vascular phenotype ($^{-/-}$) [26].
Vegfr3 (Flt4)	Failure of remodeling of primitive blood vascular plexus ($^{-/-}$), embryonic death at E10.5 [24].
Vegfr3^{Chy}	Hypoplasia, chylous ascites ($^{+/-}$); failure of remodeling of primitive blood vascular plexus ($^{-/-}$), embryonic death at E10.5 [29].
Vezf1	Lymphatic hyperplasia ($^{+/-}$), blood vascular remodeling defects and loss of vascular integrity ($^{-/-}$), death between E12.5 and birth ($^{-/-}$) [126].
Transgenic models	**Phenotype**
K14-VEGF-C	Keratinocyte specific overexpression of human VEGF-C. Hyperplasia of cutaneous lymphatic vasculature [28].
K14-VEGF-C156S	Keratinocyte specific overexpression of human VEGF-C mutant, which binds only VEGFR-3. Hyperplasia of cutaneous lymphatic vasculature [27].
K14-VEGF-D	Keratinocyte specific overexpression of human VEGF-D. Hyperplasia of cutaneous lymphatic vasculature [27].
K14-VEGFR3-Ig	Keratinocyte specific overexpression of a trap for soluble VEGFR-3 ligands. Hypoplasia of cutaneous lymph vessels, limb edema, transient hypoplasia of internal lymphatic vessels [31].
Trisomy16 (Ts16)	Nuchal edema, abnormal size and structure of jugular lymph sacs from E14; multiple cardiac or craniofacial development defects, lethality at E16–20 [127].

Recently, a promising approach for studying lymphatic endothelial development was introduced using directed differentiation of mouse embryonic stem (ES) cells in so-called embryoid bodies, where ES cell aggregates form upon culturing in non-adhering conditions. Culture of embryoid bodies in the presence of VEGF and VEGF-C leads to the development of blood vascular capillaries, which further give rise to lymphatic endothelial cells in a process that apparently recapitulates early lymphatic vessel sprouting during mouse embryonic development [111, 112]. Such an *in vitro* system, used in conjunction with ES cells from mutant mice, represent a powerful tool to manipulate and understand the processes of early lymphatic differentiation.

Novel *in vivo* systems: fish and frogs

The vast amount of information obtained as a result of genome-wide screens creates one of the major challenges in the post-genomic era, namely assigning the biological function to the proteins and identifying the most important genes implicated in developmental or pathological processes. Small animal models, such as zebrafish (*Danio rerio*) and frogs (*Xenopus laevis* and *Xenopus tropicalis*), represent a formidable opportunity to answer these questions. Their embryos are transparent, allowing direct observation during organogenesis, and they can be obtained in large numbers for embryological manipulation and microinjection of RNA, DNA, or protein, as well as for chemical compound screening. Moreover, since the embryos can live by diffusion for several days, they are particularly suitable for studies that perturb cardiovascular development. Zebrafish, frog and human genomes show a significant degree of conservation, although zebrafish and *X. laevis* genomes have undergone duplication and therefore may contain several paralogs for a single gene in mammals. Short generation time and nearly completed sequence of genome make zebrafish the organism of choice for the large-scale genetic manipulations. On the other hand, *Xenopus* may be more suitable for studies of some aspects of higher vertebrate cardiovascular development. One of the strong advantages in studying zebrafish embryos and frog tadpoles is the possibility to do reverse genetics by repressing the expression of specific genes using morpholino oligonucleotide analogs, which prevent the production of gene product by sterical blocking of translation or correct splicing into a mature mRNA.

Recently, the lymphatic vasculature has been characterized both in zebrafish and frog tadpoles [110, 113, 114]. The discovery of lymphatic vessels in zebrafish has resolved a long-standing controversy in developmental vascular biology, as it has been widely believed, based on the earlier morphological studies, that teleost fishes do not have a lymphatic system [115]. Similar to their mammalian counterparts, lymphatic vessels in zebrafish and frog do not have a basal membrane and associated mural cells, and they are connected to the surrounding extracellular matrix by anchoring filaments. Zebrafish lymphatic endothelial cells furthermore express high levels of *Prox1*, *Nrp2, Vegfr3* and *Ang2* [113, 114]. Importantly, mechanisms of the early development of the lymphatic vasculature in frog and zebrafish show a high degree of conservation with higher vertebrates. Morpholino mediated knock down of Prox1 and VEGF-C prevented the formation of lymphatic vasculature in both of these organisms, as did the inhibition of VEGFR-3 signaling by expression of VEGFR-3-Ig fusion protein [113, 114, 116]. Two-photon live time-lapse imaging of transgenic zebrafish, expressing nuclear green fluorescent protein in endothelial cells, demonstrated that LECs forming the thoracic duct arise from venous endothelial cells in the parachordal vessel, a derivative of the posterior cardinal vein [114], which further confirms evolutionary conservation of the mechanisms for early lymphatic vascular development. Using a lineage tracing approach it will be possible to determine in the future whether peripheral lymphatic vessels in zebrafish arise *via* proliferation of these initial LECs, or through mesenchymal lymphangioblasts, as proposed in avian and *Xenopus* models [116, 117].

Mouse, because of its close relation to humans and the vast genetic and molecular knowledge of its genome, undeniably is a valuable model for studying lymphatic vessels. However, genetic manipulation in mice is time consuming and expensive, and allows only analysis on a gene-per-gene basis, which creates a bottleneck in the analysis of potentially interesting lymphatic endothelial genes. Zebrafish and *Xenopus* systems have already greatly contributed to answering many important questions of vertebrate development, and the establishment of small vertebrate models for studies of lymphatic vasculature opens a highly promising new area of research, especially suitable for the discovery and high throughput analysis of novel mediators of lymphangiogenesis, and advanced molecular imaging studies of developing lymph vessels.

Concluding remarks

Significant progress has been made in our understanding of how the lymphatic vasculature is formed and remodeled, mostly through the discovery of lymphatic endothelial markers, growth factors and gene targeting studies in mice. However, much work remains to be done. The mechanisms for the establishment of lymphatic endothelial cell identity are at present not fully understood. How is the stability of lymphatic capillaries controlled in the absence of mural cells? What mechanisms prevent the fusion of sprouting lymphatic and blood vascular capillaries and why do the former remain blind-ended? What are the mechanisms regulating the development of lymphatic valves? What is the role of interstitial fluid flow and mechano-sensory transduction in the regulation of lymphatic vessel function and morphogenesis? Answers to these questions will help to provide a comprehensive picture of lymphatic vascular development and will be instrumental in designing therapeutic

approaches for treatment of lymphatic vascular dysfunction in cancer, lymphedema and inflammation.

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