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Ying Yang, Guillermo Oliver

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Review Series

The two vascular systems of our body are the blood and lymphatic vasculature. Our understanding of the cellular and molecular processes controlling the development of the lymphatic vasculature has progressed significantly in the last decade. In mammals, this is a stepwise process that starts in the embryonic veins, where lymphatic EC (LEC) progenitors are initially specified. The differentiation and maturation of these progenitors continues as they bud from the veins to produce scattered primitive lymph sacs, from which most of the lymphatic vasculature is derived. Here, we summarize our current understanding of the key steps leading to the formation of a functional lymphatic vasculature.

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Ying Yang and Guillermo Oliver

Department of Genetics, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

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Origin and specification of lymphatic ECs

Origin of lymphatic ECs in the mammalian embryo. The lymphatic vasculature is essential for maintaining interstitial fluid homeostasis. Deficiency in development and/or function of the lymphatic vasculature causes various lymphedema syndromes in humans, and active lymphangiogenesis plays a significant role in chronic inflammation and tumor metastasis. Research of the lymphatic vasculature dates back to the 17th century, but the anatomic features of the developing lymphatic vasculature were most extensively characterized during the 20th century by using pig and mouse embryos (1, 2). Florence Sabin first suggested that the lymphatic vessels arise from preexisting blood vessels, specifically the cardinal veins (CVs) (1). More than 100 years later, experiments using detailed lineage tracing in mammals and live imaging in zebrafish demonstrated that lymphatic EC (LEC) progenitors originate in the veins from venous ECs (VECs) (3, 4). Recent work in mouse embryos shows that the intersomitic veins (ISVs) and the superficial venous plexus are additional sources of LEC progenitors (5, 6). These findings support previous observations that LEC progenitors are of venous origin and that they leave the veins via specific migratory paths that extend radially from the dorsal half of the CV (7, 8). Additionally, they help to explain the rapid appearance of large numbers of migrating LECs within the mesenchymal tissue (Figure 1).

LEC fate specification. Development of the mammalian lymphatic vasculature is a stepwise process in which LEC progenitors are first specified in the embryonic veins and then bud from the veins to form the primitive lymph sacs from which most of the lymphatic vasculature will eventually be derived (9). During the past few years, several key regulatory molecules and specific markers of the lymphatic endothelium have been identified (Table 1). The expression of the transcription factor *Prox1* in a subpopulation of VECs (referred to as LEC progenitors) in the embryonic CVs at approximately E9.5 (3, 5, 7) is the initial step in the formation of the lymphatic vasculature. The transcription factors *Sox18* and *COUP-TFII* are required for activation of *Prox1* expression in VECs (Figure 1 and refs. 10, 11), and loss of either *Sox18* or *COUP-TFII* results in the absence of LEC progenitors (Tables 2, 3, and 4 and refs. 10–12).

COUP-TFII mediates venous cell fate differentiation and is required for the specification of LEC progenitors. Its activity in the veins is regulated epigenetically by the chromatin-remodeling enzyme BRG1, a member of the SWI/SNF protein family (13). In the CV, COUP-TFII represses *Notch* activity, is a direct in vivo activator of *Prox1* during early LEC specification, and is also required to maintain PROX1 expression in LECs (11). Functional inactivation of COUP-TFII in LEC progenitors reduces the number of LECs (11). Several studies have shown that Notch signaling inhibits LEC fate and LEC sprouting in vitro (14, 15). Recently, it was shown to also play a role in LEC specification during embryonic development in vivo (16). Downregulation of Notch activity leads to an increased number of LEC progenitors, indicating that Notch signaling is a negative regulator of LEC specification (16). This new finding highlights the relationship between the COUP-TFII and Notch pathways in arterial-venous-lymphatic cell fate decisions (Figure 1).

Mutations in *SOX18* have been identified as the cause of hypotrichosis-lymphedema-telangiectasia in humans (17), and MAPK/ERK signaling is a newly discovered activator of *Sox18* in the embryonic veins (18). *RAF1* mutations that activate MAPK/ERK signaling are associated with Noonan syndrome, a disease that includes lymphangiectasia (19, 20).

Gain- and loss-of-function studies revealed that *Prox1* plays an important role in both the induction (21, 22) and the maintenance (23) of LEC fate. An early knockout mouse study revealed that loss of *Prox1* led to absence of the lymphatic vasculature, as LEC fate was never acquired (7). PROX1 expression levels are also crucial for normal lymphatic vasculature formation, as *Prox1*-heterozygous embryos have significantly fewer LEC progenitors in their embryonic veins and all develop edema, a typical sign of lymphatic vascular dysfunction (11, 24). *Prox1* haploinsufficiency causes perinatal death and, in most mouse strains, pups exhibit chylothorax and chylous ascites (Tables 2–4). On an NMR imaging background, 10%–20% of *Prox1* heterozygous mice live to adulthood; however, their lymphatic vessels are leaky, and they develop adult-onset obesity (24). Interestingly, not all PROX1$^+$ LEC progenitors bud from the veins; a small group of those cells remain in place, where they contribute to the formation of the lymphovenous valves at the main connection between the jugular lymph sacs and the adjacent veins at approximately E11.5 (ref. 25 and Figure 2).

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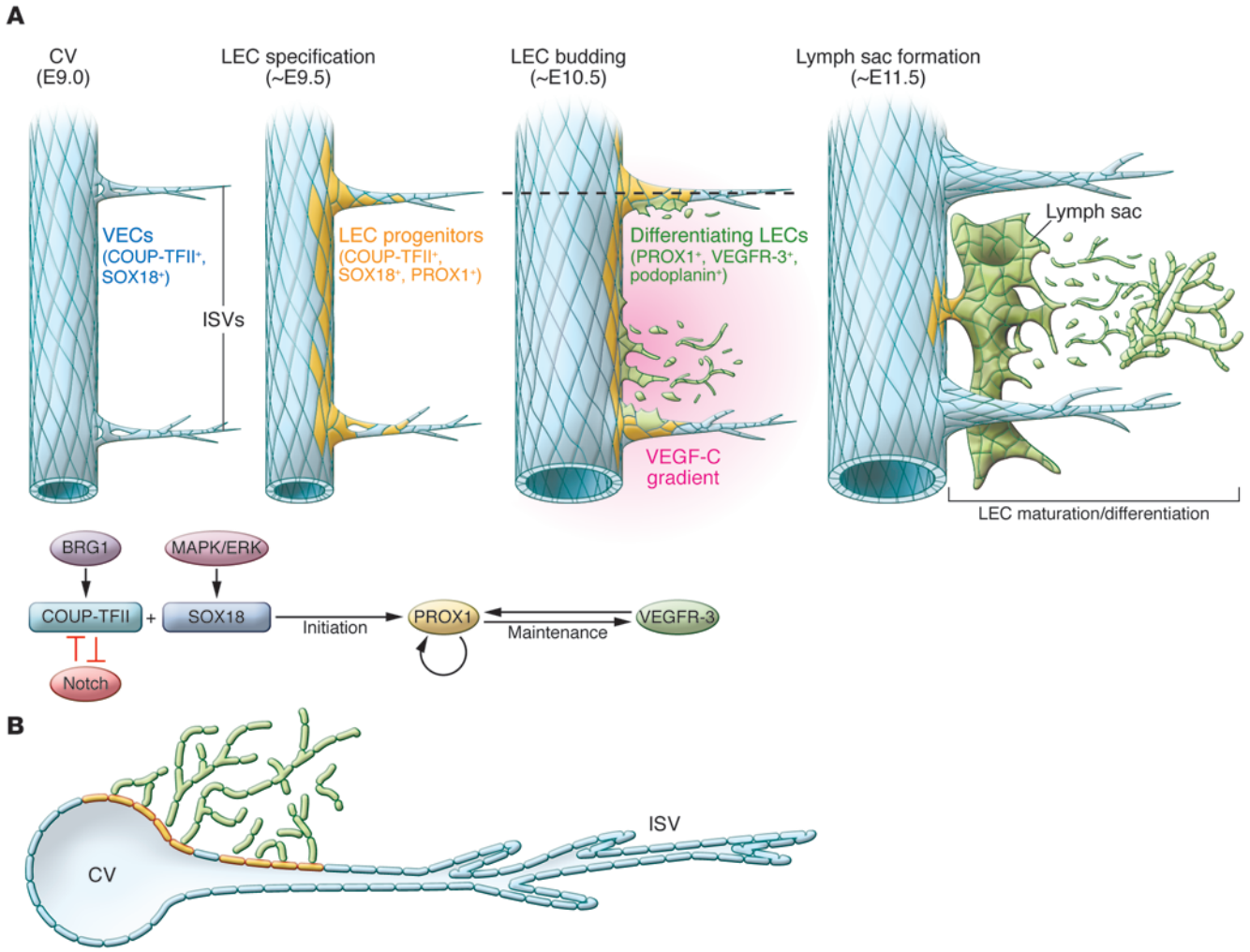

Figure 1

Schematic representation of the development of the mammalian lymphatic vasculature. (**A**) Sagittal view of the key temporal events that take place along the CV from E9.0 to E11.5 in the mouse embryo. The CV is the main source of LECs. Initially, among others, blood ECs in the CV and ISVs express the transcription factors COUP-TFII and SOX18. A few hours later (E9.5), the activity of SOX18 and COUP-TFII induces PROX1 expression in a subpopulation of venous ECs. The initiation of PROX1 expression indicates that LEC specification has started, and venous PROX1-expressing ECs are considered LEC progenitors. At around E10.5, most of those progenitors start to bud from the CV and ISVs. This process requires the graded expression of VEGF-C in the surrounding mesenchyme. Mediated by PROX1, budding LECs maintain the expression of VEGFR-3 and begin expressing PDPN once outside of the CV. The combined expression of these genes indicates that lymphatic differentiation is progressing. As LECs bud off in an interconnected manner, they assemble together, and at approximately E11.5 they start to form different lymph sacs. Following LEC proliferation and sprouting, the majority of the lymphatic network arises from these sacs. (**B**) Transverse representation of the LEC budding process. At around E10.5, PROX1$^+$/PDPN$^+$/VEGFR-3$^+$-differentiating LECs bud from the CV and ISVs. The budding LECs migrate as an interconnected group of cells dorsally and longitudinally into the surrounding mesenchyme in the anterior region of the embryo.

Formation of lymph sacs

Budding of LECs from the veins. Once PROX1-expressing LEC progenitors are specified in the embryonic veins at approximately E9.5, they start to bud into the surrounding mesenchyme (Figure 1). Electron microscopy of E10.5 mouse embryos revealed that LEC progenitors bud as groups of cells interconnected by adhesion junctions expressing high levels of VE-cadherin. These junctions ensure that venous endothelial integrity is not compromised during budding (5). These results are consistent with a previous report showing that PROX1$^+$ cells actively budding from the CVs at E12.5 are joined by zipper-like junctions expressing VE-cadherin at their cell borders (26). When the cell-cell junction was disrupted, lymphatic vasculature development was disturbed and edema developed (27, 28).

Although *Prox1*-deficient mice are devoid of a lymphatic vasculature, initial analysis suggested that ECs were able to bud off from the CV in *Prox1*-null embryos (7), indicating that PROX1 is vital for the specification of LEC fate but not for LEC budding from the veins. However, a more recent evaluation using confocal microscopy of semi-thick sections of *Prox1*-null embryos revealed that PROX1 activity is also required for the budding of LECs from the CVs (5). This analysis showed that cells with an activated *Prox1*

Table 1
Marker expression during mammalian lymphatic vasculature development

Cell type	Embryonic stage	Tissue site	Marker expression
LEC progenitor	E9.5–E12.5	Embryonic veins	PROX1^{med}/PDPN^{-}/LYVE1^{+}/SOX18^{+}/VEGFR-3^{med}/NRP2^{med}
Migrating specified LECs	E10.5–E12.5	Mesenchymal tissue outside the veins	PROX1^{hi}/PDPN^{+}/LYVE1$^{partially+}$/VEGFR-3^{hi}/NRP2^{hi}
Lymphovenous valves LECs	E12.5–adult	Lymphovenous valves	PROX1^{hi}/PDPN^{-}/LYVE1 (patchy)/VEGFR-3^{-}/FOXC2^{hi}/GATA2^{+}
LECs in collecting lymphatics	E15.5–adult	Collecting lymphatics	PROX1^{+}/PDPN^{+}/LYVE1^{lo}/Reelin (extracellular)/VEGFR-3^{lo}
LECs in capillaries	E15.5–adult	Lymphatic capillaries	PROX1^{+}/PDPN^{+}/LYVE1^{+}/Reelin (intracellular)/VEGFR-3^{+}
LECs in lymphatic valves	E16–adult	Collecting lymphatics	PROX1^{hi}/FOXC2^{hi}/LYVE1^{lo}/GATA2^{+}/Laminin-α5^{+}/Integrin-α9^{+}/VEGFR-3^{hi}/PDPN^{+}

promoter are located in the ISVs rather than in the mesenchyme surrounding the CVs of *Prox1*-null embryos, demonstrating that LEC progenitors had failed to leave the CVs and ISVs (5).

Budding of LEC progenitors is not only associated with cell and nuclear shape change (6), but also with changes in LEC marker expression. The expression of podoplanin (PDPN) is an important molecular distinction between LEC progenitors inside the veins and those that budded. PDPN is expressed by budding PROX1-expressing LECs only when they fully exit the endothelium of the embryonic veins (5, 29), allowing a molecular distinction of venous PROX1-expressing LEC progenitors from differentiating PROX1/PDPN-expressing LECs that contribute to lymph sac formation outside the embryonic veins (Figure 1). The mechanism by which PDPN expression is activated in LECs remains to be elucidated. The expression pattern of PDPN suggests that it is either repressed by the venous environment or induced by factors outside the veins.

VEGF-C/VEGFR-3 signaling is required for budding of LECs from the CVs (30, 31), as PROX1-expressing LEC progenitors fail to detach from the CV in *Vegfc*-deficient mice (ref. 31 and Tables 2–4) and *Vegfc*$^{+/-}$ embryos and adult mice develop cutaneous lymphatic hypoplasia and lymphedema (31). Conversely, overexpression of VEGF-C in the skin of transgenic mice induces selective hyperplasia of the lymphatic vasculature (32). As the primary receptor of VEGF-C, VEGFR-3 expression is restricted to LECs after E10.5 (33). Mutation of *Vegfr3* in mouse embryos demonstrated that the tyrosine kinase activity of *Vegfr3*, but not the ligand-binding domain, is essential for budding and separation of LEC progenitors from the veins (34). Heterozygous mutations in both mouse and human VEGFR-3 tyrosine kinase domains leads to lymphatic vascular defects; *Chy* mice display lymphedema and chylous ascites, while humans develop primary lymphedema (Milroy's disease) (35–38). In addition to VEGFR-3, VEGF-C also binds the coreceptor neuropilin-2 (NRP2) (39). NRP2 colocalizes and interacts directly with VEGFR-3 in lymphatic vessels (39, 41). The interaction between NRP2 and VEGFR-3 mediates proper lymphatic vessel sprouting (40). Blocking VEGF-C binding to NRP2 inhibits LEC tip cell sprouting in vivo (40). Although loss of NRP2 function does not affect the budding of LECs from the veins, the number of small lymphatic vessels and capillaries is severely reduced in NRP2 knockout mice (41).

The secreted protein CCBE1 was identified as a regulator of LEC budding in a zebrafish genetic screen (42). In that study, zebrafish embryos with mutated *Ccbe1* alleles lacked a lymphatic vasculature and displayed severe edema (42). In early mouse embryos, *Ccbe1* is expressed in cardiac progenitors and in mesenchyme near the nascent lymphatics (43, 44). When *Ccbe1* is functionally inactivated in mice, the specified LEC progenitors remain in the veins and the lymphatic vasculature does not develop (Tables 2–4 and refs. 6, 44). Unlike *Vegfc*$^{-/-}$ embryos, *Ccbe1*$^{-/-}$ embryos display dysmorphic sprouts and projections from the ISVs at E10.5 and E11.5, respectively (6). CCBE1 induces lymphangiogenesis by enhancing VEGF-C function independently of the tyrosine phosphorylation activity of VEGFR-3 (44). Moreover, human mutations in *CCBE1* cause a type of lymphatic dysplasia known as Hennekam syndrome (45–47). Because loss of either *Vegfc* or *Ccbe1* arrests the budding of LEC progenitors, both molecules appear to be required for this process.

Lymph sac formation. Immunostaining of semi-thick sections of E10.5–E12.5 mouse embryos showed that after LEC progenitors bud from the veins, they merge along the embryo's anterior-posterior axis to form unique intermediate structures called lymph sacs (5). At mid-gestation, the lymph sacs are the main source of LECs required for the formation of the entire lymphatic vasculature. Like the lymphatic vessels, the lymph sacs have a luminal structure comprising a single EC layer. Unlike the lymphatic vessels, mammalian lymph sacs have an irregular, sac-like shape when viewed in sagittal section (Figure 1). Because mouse embryos are relatively opaque, deep-tissue live imaging of the early steps in lymph sac formation remains technically challenging. The current model suggests that LEC progenitors bud from the veins to form lymphatic plexuses that develop into lymph sacs (1, 2). Recent analysis of this process by ultramicroscopy and by 3D reconstructed confocal images of semi-thick sections suggested that lymph sacs form by a stepwise process (5, 6). The initial LEC progenitors were shown to bud and migrate as an interconnected stream of cells that eventually assembled into a capillary-like structure along the anterior and posterior axes of the embryo. This structure condensed and organized to form the lymph sacs after further sprouting and migration (Figure 1 and refs. 5, 6).

Several factors that influence lymph sac formation in mammals have recently been identified (Table 1). For example, loss of any of the components of adrenomedullin signaling (adrenomedullin and its receptors *Calcr1* and *Ramp2*) results in hypoplastic lymph sacs and subcutaneous edema (48). Mouse embryos with macrophage-specific *PU.1* deficiency exhibit hypoplastic jugular lymph sacs at E14.5 (49). *TIE1*-deficient (angiopoietin receptor–deficient) embryos displayed enlarged lymph sacs and nuchal edema (50, 51). Lymph sacs in mice lacking the transcription factor *Nfatc1* showed decreased luminal area, indicating that NFATC1 also regulates lymph sac formation (52). Lymph sacs appeared hypoplastic in *Gata2*-knockout embryos (53). Interestingly, mutations in *GATA2*

Table 2
Loss-of-function phenotypes of genes associated with lymphatic vasculature development and disease

Gene	Loss-of-function phenotype in animal models	Related human vascular disease	Reference(s)
Defective LEC progenitor specification			
Coup-TFII	Severe subcutaneous edema; embryos lack LECs and lymphatic vasculature due to failure in LEC progenitor specification		11, 12
Notch1	Loss of Notch1 results in an increased number of PROX1$^+$ LEC progenitors in the veins and outside the CV with significant lymphatic overgrowth, incomplete separation of veins, and formation of lymphatics		16
Prox1	Severe subcutaneous edema; embryonic lethality at E14.5; embryos lack LECs and lymphatic vasculature due to failure in LEC progenitor specification; in most genetic backgrounds, *Prox1* haploinsufficiency results in defects in LEC fate maintenance, perinatal death with chylothorax, and chylous ascites; in the NMRI strain, surviving *Prox1* heterozygous mice exhibit adult onset obesity, leaky lymphatics, and lack of lymphovenous valves		7, 8, 23–25
Sox18	Severe subcutaneous edema; embryonic lethal at E14.5; embryos lack LECs and lymphatic vasculature due to failure in LEC progenitor specification in certain genetic backgrounds	Hypotrichosis-lymphedema-telangiectasia syndrome	10, 17
Defective budding of LEC progenitors and lymph sac formation			
AM	*AM*-null embryos die at mid-gestation with interstitial edema and abnormal jugular lymphatics due to defective LEC proliferation		48
Calcrl	Severe interstitial edema, embryonic lethal, hypoplastic lymph sacs		48
Ccbe1	Severe subcutaneous edema, budding of LEC progenitors arrested in the CV, lack of lymphatic vasculature	Hennekam syndrome	42, 44–47
Gata2	Hypoplastic lymph sacs and abnormal separation of venous and lymphatic vessels	Emberger syndrome	53, 54
Nfatc1	Enlarged lymph sacs		52
PU.1	Hypoplastic lymph sacs, hyperplastic lymphatic vessels		49
Ramp2	Severe interstitial edema, embryonic lethal, hypoplastic lymph sacs		48
Tie1	Reduction in TIE1 levels results in abnormal lymphatic patterning and dilated and disorganized lymphatics; homozygous null embryos are lethal at E14.5 and exhibit nuchal edema, hemorrhages, enlarged lymph sacs, dilated lymphatic vessels and impaired lymphatic drainage		50
Vegfc	Embryonic lethal at E14.5; severe subcutaneous edema, budding of LEC progenitors arrested in the CV, lack of lymphatic vasculature		31
Vegfr3	Primary receptor for VEGF-C, blood vasculature malfunction in loss of function embryos, required for survival and migration of LECs	Milroy disease	33, 35, 36, 38, 116

are associated with Emberger syndrome, myelodysplastic syndrome, acute myeloid leukemia, and MonoMAC syndrome with primary lymphedema (54, 55). GATA2 is also expressed in lymphatic valves, suggesting that it may play a role in lymphatic valve formation (55).

Separation of the venous and lymphatic vasculatures

Histologic evidence has shown that the jugular lymph sacs and jugular veins remain connected by small apertures after the lymph sacs form at approximately E12.5 (2, 56). Each lymph sac maintains its connection to the adjacent vein, and lymphovenous valves at the junctions of the subclavian veins and jugular lymph sacs prevent the reflux of blood into the lymphatic vessels (ref. 57 and Figure 2). Recent work molecularly characterized the formation of the lymphovenous valves, showing that they form through the intercalation of lymph sac–derived PROX1$^+$ LECs with a previously unidentified population of PROX1$^+$ ECs in the adjacent veins. These venous ECs correspond to a small subpopulation of PROX1-expressing ECs that do not become LEC progenitors and do not bud from the veins or acquire LEC characteristics, but instead remain within the veins (25).

Several mouse mutant models have shown defects in the separation of the lymphatic and blood vasculatures, resulting in blood-filled lymphatic vessels (Tables 2–4 and ref. 58). For example, mice deficient in *Syk*, *SLP76*, *Runx1*, or PDPN all develop blood-filled lymphatics at specific embryonic time points (3, 59–61). Interestingly, *Syk*, *SLP76*, and *Runx1* are essential for hematopoiesis (62–66), which suggested that hematopoietic cells play a role in keeping the two vascular systems separated. The nature of the cell lineages involved and the mechanisms by which they control blood and lymphatic vessel separation remained unclear until several reports proposed that platelets are required for separation of the forming lymph sacs from the CVs at their connecting points (56, 67–69). In addition to being an early marker of LEC specification/differentiation, PDPN is important in initiating platelet aggregation (70, 71). During the embryonic separation of lymphatics from blood vessels, LEC-expressing PDPN activates the platelet receptor CLEC2, initiating downstream SYK-SLP76 signaling. Mice lacking the megakaryocyte transcription factors *Meis1* and *Clec2* displayed blood-filled lymphatic vessels, further supporting the essential role of platelets in separating the blood and lymphatic vasculature dur-

Table 3
Loss-of-function phenotypes of genes associated with lymphatic vasculature development and disease

Gene	Loss-of-function phenotype in animal models	Related human vascular disease	Reference(s)
Defective lymphatic vessel maturation and valve formation			
Akt1	Reduced diameter and EC number in lymphatic capillaries; loss of valves in the smaller collecting lymphatic vessels in the superficial dermal layer of the ear skin		111
Bmp9	Mutant pups exhibit hyperplastic mesenteric collecting vessels with abnormally high LYVE1 expression, reduction in the number and in the maturation of mesenteric lymphatic valves		108
Celsr1	Disorganized cell-cell junction, defects in cell reorientation during lymphatic valve formation, lack of lymphatic valves		112
Cnb1	Defects in the demarcation of the valve territory		79
Cx37	Mutant mice exhibit lymphedema and chylothorax and have defective valve formation in collecting lymphatics; regulates jugular lymph sacs size		89
Cx43	Loss of lymphatic vessels in the diaphragm, absence of lymphatic valves in mesenteric collecting lymphatic vessels		89
Cx47	Expressed in lymphatic valves, currently uncharacterized developmental function	Primary lymphedema	89, 91, 92
Emilin1	Defective lymphatic valve structure and lymph flow		102
Ephrin B2	Hyperplastic collecting lymphatic vessels, lack of valves, abnormally high level of expression of LYVE1 in the lymphatic vessels		107
Fn1	Defects in the formation and extension of the valve leaflets		101
Foxc2	Embryonic lethal perinatally; unusual mural cell recruitment on the collecting lymphatic vessels with abnormal high level of LYVE1 expression, lack of lymphatic valves, and lymph backflow	Lymphedema-distichiasis syndrome	79, 81–84
Integrin-α9	Reduced number of valves leads to failure in the formation of the matrix core of the valves (leaky, lymph backflow)	Congenital chylothorax	101, 103
Nrp1	A mutation in the SEMA3A binding site of NRP1 leads to smaller lymphatic valves and ectopic SMC coverage on the valve region		106
Plxna1	Smaller lymphatic valves		106
Reelin	Reduced SMC coverage on the collecting lymphatic vessels with abnormally high level of LYVE1 expression, dilated and leaky collecting lymphatic vessels, and reduction in the rate of lymph flow		80
Sema3a	Smaller lymphatic valves; ectopic SMC coverage on the valve region; aberrant lymph drainage		106

ing lymphangiogenesis (67–69). It has also been shown in cultured LECs that overexpression of PDPN promotes EC migration, adhesion, and tube formation (61). A more recent proposal suggested that PDPN/CLEC2 binding could activate platelets in the gaps between the lymph sacs and the CVs, enabling blood/lymphatic vessel separation by inhibiting LEC migration, proliferation, and tube formation in the developing embryo (72). Endothelial-specific deletion of the Rho GTPase *Rac1* also resulted in blood-filled lymphatics, indicating that RAC1 helps to ensure proper lymphatic-blood vessel separation by regulating LEC budding and migration (73). The cell adhesion molecule coxsackie and adenovirus receptor (CXADR) also plays an essential role in lymphatic vasculature development. CXADR is localized in cell-cell junctions in LECs, and its deletion in E12.5 mouse embryos results in embryonic death. These embryos exhibit dilated dermal lymphatic vessels, subcutaneous edema, defective cell-cell junctions in LECs, and blood-filled lymphatics (28). In addition to the blood-filled lymphatic phenotype, animals deficient in fasting-induced adipose factor (FIAF) develop gut-specific dilated intestinal lymphatic vessels and blood-filled small intestinal villi due to the defective separation of the intestinal lymphatic vasculature from the blood vasculature (74).

Formation of lymphatic vessels and valves

As the lymph sacs form, LECs continue to proliferate and migrate into the mesenchymal tissue. During this process, the primitive lymphatic plexus further differentiates to form the two distinct mammalian lymphatic vessel types: collecting lymphatic vessels (larger) and lymphatic capillaries (smaller) (75). Although precollectors have been considered a third type of lymphatic vessels, their definition is ambiguous. In general, precollectors have features of both collecting vessels and capillaries, as they lack SMC coverage but contain valves, a feature typical of collecting lymphatics. Lymphatic capillaries are thin-walled, blind-ended vessels within the tissue spaces that absorb interstitial fluid and transport it to the larger, collecting lymphatic vessels. Collecting lymphatics are surrounded by SMCs that facilitate the transport of lymph against hydrostatic pressure (76). Intraluminal valves like those in the great veins are present to prevent the backflow of lymph and divide the collecting lymphatic vessels into functional pumping units termed lymphangions (77, 78). By contracting, collecting lymphatic vessels force the lymph into the venous circulation at the sites of the lymphovenous valves.

FOXC2-calcineurin/NFATC1 signaling. Collecting lymphatics and their valves develop almost simultaneously (detected at approximately E14.5–E15.5 and E16.0, respectively) (79). As collecting lymphatic vessels mature, lymphatic capillary markers such as PROX1, VEGFR-3, and LYVE1 are downregulated in most LECs, ECM is deposited around the vessels, and SMCs begin to cover the vessels (79, 80). However, in lymphatic valve–forming cells, expression of PROX1 and FOXC2 remains elevated (79). Several

Table 4
Loss-of-function phenotypes of genes associated with lymphatic vasculature development and disease

Gene	Loss-of-function phenotype in animal models	Related human vascular disease	Reference(s)
Defective blood and lymphatic vessel separation			
Clec2	Inactivation of this PDPN receptor results in defects similar to those reported for *Pdpn* and *SLP76*; mutant embryos show blood-filled intestinal and mesenteric lymphatic vessels		68, 69
Fiaf	Dilated and blood-filled lymphatic vessels in the intestine		74
Plcg2	Blood-filled lymphatic vessels		117
Pdpn	Embryonic lethal at birth; lymphedema and dilated and blood-filled lymphatic vessels		56, 61
Rac1	Conditional deletion results in embryonic lethality before birth, edema, and blood-filled lymphatic vessels		73
SLP76	Severe subcutaneous edema, peritoneal hemorrhage, and chylous ascites		59, 60, 68
Syk	Severe subcutaneous edema and blood-filled lymphatic vessels.		59, 69
Defective lymphatic vessel growth			
Afadin	Modulates RhoA; severe subcutaneous edema with severe disruption of VE-cadherin–mediated cell-cell junctions in lymphatic vessels of the skin		27
Ang2	Subcutaneous edema, chylous ascites, lymphatic vessel hypoplasia, and mispatterned lymphatic vessels in the mesentery		118
Aspp1	Null embryos exhibit subcutaneous edema, defective lymphatic drainage, and mispatterned collecting lymphatic vessels		113
Cxadr	Conditional deletion at E12.5 results in subcutaneous edema, hemorrhage, and embryonic death with dilated subcutaneous lymphatic vessels that appear structurally abnormal, exhibiting gaps and holes in LEC cell-cell junctions; blood-filled lymphatics show defects in the separation of the blood and lymphatic vasculatures		28
Cx26	Conditional deletion in the ectoderm results in embryonic death before birth, severe subcutaneous edema, and reduced dermal lymphatic capillary network		90
Integrin-β1	Edema and hemorrhages; embryonic lethality; reduced LEC numbers and LEC proliferation; smaller lymph sacs; complete lack of dermal and mesenteric lymphatic vasculature at E15.5		99
Nrp2	Absence or severe reduction of small lymphatic vessels and capillaries		41
Ptpn14	Lymphedema; lymphatic hyperplasia; interacts with VEGFR-3	Lymphedema-choanal atresia syndrome	119
Rasa1	Hyperplasia, dilation, and leakage of lymphatic vessels and chylothorax	Capillary and arteriovenous malformation	120
Tbx1	Regulates VEGFR3; conditional deletion in ECs results in embryonic edema and postnatal lethality between 2 and 4 days after birth; mice exhibit chylous ascites and lack of mesenteric lymphatic vessels	DiGeorge syndrome	114, 115
TGFBRI or TGFBRII	Severe edema; blood-filled lymphatic vessels; reduced lymphatic branching; aberrant lymphatic vessel network		110
Vezf1	Lymphatic hypervascularization, edema, and hemorrhaging the jugular region of heterozygous embryos		121

crucial signaling pathways are associated with the formation of collecting lymphatic vessels (Tables 2–4). The best-characterized pathway is FOXC2/calcineurin/NFATC1 signaling, which is indispensable for both the maturation of collecting lymphatics and the formation and maintenance of lymphatic valves. The transcription factor *Foxc2* was previously shown to be necessary for correct lymphatic patterning and mural cell recruitment during the maturation of collecting lymphatics (81). More recently, in *Foxc2*-knockout mice the primitive lymphatic plexus was shown to maintain high expression of capillary markers (e.g., PROX1, VEGFR-3, and LYVE1) without differentiating into functional collecting lymphatics and valves, as indicated by the backflow of lymph (81, 82). Intriguingly, point mutations in human *FOXC2* are associated with lymphedema-distichiasis syndrome (83, 84), in which lymphatic and venous valves are defective (85). Genome-wide ChIP-on-ChIP analysis showed that FOXC2 cooperates with the cardiac valve development transcription factor calcineurin/NFATC1, which is present in LECs during the maturation of collecting lymphatic vessels (82, 86–88). Moreover, inducible deletion of the calcineurin regulatory subunit *Cnb1* at any mouse embryonic stage leads to defects in the formation of the lymphatic valve territory and the lymphatic valves themselves (79). Several studies have shown that gap junction proteins of the connexin family (CX26, CX37, and CX43) are important for lymphangiogenesis during development (refs. 89, 90, and Tables 2–4). For example, *Cx37* knockout mice lack lymphatic valve–forming cells and have no lymphatic valves (79, 89), and mutations in *CX47* are associated with primary lymphedema in humans (91, 92). Strikingly, in vitro flow analyses revealed that calcineurin/NFATC1 activation is markedly reduced when *Cx37* is depleted, and that PROX1, FOXC2, and oscillatory shear stress regulate the expression of *Cx37* (79). Taken together, these results indicate that this pathway is crucial for the maturation of collecting lymphatic vessels and the formation and maintenance of lymphatic valves.

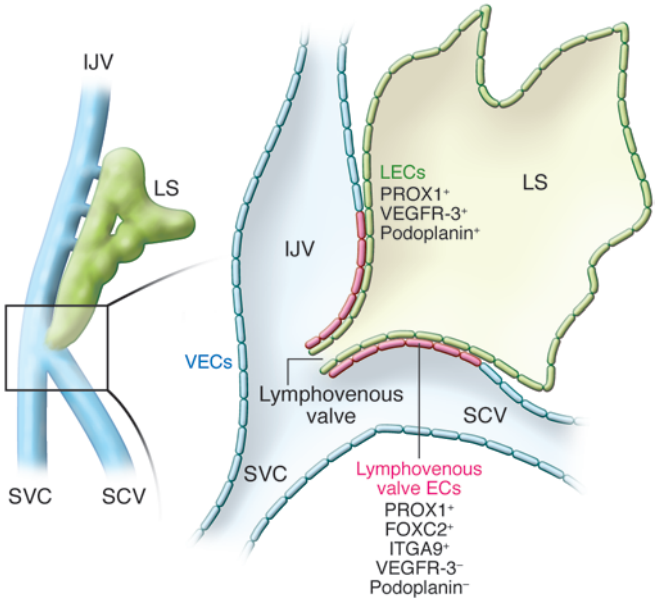

Figure 2
Diagrammatic representation of the lymphovenous valves. Although most PROX1-expressing LEC progenitors bud off from the veins, a small subpopulation remains and forms the lymphovenous valves at the junction of the jugular and subclavian veins (SCV). This unique population of ECs is negative for PDPN and VEGFR-3. Each of the valve's two leaflets has two layers of PROX1+ ECs: an inner PROX1+/PDPN+ layer continuous with the lymph sac and an outer PROX1+/PDPN− layer continuous with the veins. Left: The region of an E13.5 embryo in which the jugular and subclavian veins join to form the lymphovenous valves. Right: A frontal view of the boxed region shown at left. EJV, external jugular vein; IJV, internal jugular vein; LS, lymph sac; LV, lymphovenous valve; SVC, superior vena cava.

Reelin signaling. The reelin pathway is also important for the formation of collecting lymphatic vessels. Immunofluorescence studies detected reelin expression in LECs of collecting lymphatics and lymphatic capillaries. Interestingly, the reelin signal was intracellular in lymphatic capillaries and extracellular in collecting lymphatic vessels, especially near the valve regions, indicating that LECs of the collecting lymphatics secrete reelin into the ECM near the valves (80). Moreover, this LEC secretion of reelin protein is strongly associated with the migration and adherence of SMCs to collecting lymphatics. In reelin-mutant mice, the dermal collecting lymphatics are dilated and retain abnormally high expression of LYVE1, and SMC recruitment to collecting lymphatic vessels is reduced (80). The function of these vessels is also impaired in reelin knockout mice, whose collecting lymphatics are leaky and show a reduced rate of lymphatic flow in real-time imaging of indocyanine green dye uptake (Tables 2–4). Therefore, reelin serves an important and unique function in the formation of collecting lymphatics as an LEC-specific matrix molecule.

ECM components in lymphatic valve formation. As ECM deposition is a characteristic feature of collecting lymphatic vessels maturation, it is not surprising that ECM protein receptors are involved in development of the lymphatic vasculature. For example, many reports have described the function of integrins during lymphangiogenesis. Integrin signaling can promote the migration, proliferation, and survival of LECs (93–99). The integrin family contains eight α and 18 β subunits, which can form 24 integrin transmembrane heterodimers to mediate cell-cell and cell-ECM interactions (100). Specifically, integrin-α9 has fibronectin as its ligand, and its interaction with the ECM protein EMILIN1 plays a crucial role during lymphatic valve morphogenesis (101, 102). Integrin-α9 is highly expressed in mature and developing lymphatic valves, which are reported to be reduced in number and be morphologically abnormal in integrin-α9–deficient mice. The matrix core of the valves fails to form in these mice, leading to leakage and backflow of lymph (101). The finding of congenital chylothorax in human fetuses with mutations in the *ITGA9* gene (103) is consistent with these reports.

Axonal guidance genes. It is well established that during blood vessel growth the endothelial tip cells and the axon growth cones are guided by common signaling cues. Many of these proteins also play significant roles in lymphangiogenesis (104). The axonal guidance genes semaphorin 3A (*Sema3a*) and its receptors *Nrp1* and plexin A1 (*Plxna1*) are expressed in collecting lymphatic vessels and lymphatic valves (105, 106). *Sema3A* is required for lymphatic valve formation. The valve area is significantly smaller in *Sema3a*$^{-/-}$ and *Plxna1*$^{-/-}$ mice (106). Inhibition of SEMA3A/NRP1 binding leads to irregularly shaped collecting lymphatic vessels, abnormally small lymphatic valves, and defects in postnatal lymph flow (105, 106). In addition to these morphological and functional problems, ectopic SMCs cover collecting lymphatic vessels and integrin-α9, and the integrin-α9 ligand FN1 is aberrantly expressed on lymphatic valve leaflets in the absence of the SEMA3A signal (105). These valvular defects result in impaired lymphatic flow (105). In summary, both integrin-α9 and SEMA3A-NRP1 signaling are required in later stages of valve development but do not affect early valve specification. Another axon guidance molecule, ephrin B2, a member of the Eph receptor tyrosine kinase family, has also been identified as an essential regulator of lymphatic development (107). That study demonstrated that the C-terminal PDZ interaction site of ephrin B2 is required to mediate the function of ephrin B2 in the formation of collecting lymphatic vessels and lymphatic valves, and hyperplastic collecting lymphatics and absence of valves were observed in ephrin B2 PDZ domain knockout mice (107). Similar to the *Foxc2* and reelin deficiency, loss of ephrin B2 PDZ domain affected the specification of collecting lymphatic identity, as shown by an abnormally high level of expression of LYVE1 in the hyperplastic lymphatics (107).

Other important factors in lymphatic vessel and valve formation. BMP9, a ligand of the TGF-β family type 1 receptor ALK1, was recently identified as a participant in the maturation of lymphatic capillaries, collecting lymphatic vessels, and lymphatic valves (ref. 108 and Tables 2–4). *Bmp9*-knockout mice exhibit abnormal patterning of both lymphatic capillaries and collecting lymphatic vessels, and the enlarged collecting lymphatic vessels retain LYVE1 expression, indicating that their maturation is affected. In primary cultured human LECs, BMP9 regulates LYVE1 expression through its receptor ALK1 (108). Moreover, pups lacking *Bmp9* have a significantly reduced total number of valves, and therefore decreased lymph flow. Mechanistically, *Bmp9* induces expression of FOXC2, CX37, ephrin B2, and NRP1 in an ALK1-dependent manner (108), consistent with the previous finding that ALK1 signaling regulates postnatal lymphatic vasculature patterning (109). Therefore, BMP9 is essential for development of the lymphatic capillaries and collecting vessels as well as for the

formation of lymphatic valves. In addition to ALK1, conditional deletion of the TGF-β receptors *Tgfbr1* and *Tgfbr2* in LECs leads to a severe reduction in lymphangiogenic sprouting (110).

AKT-mediated signaling plays an important role in development of the lymphatic vasculature (111). *Akt1*$^{-/-}$ mice have smaller lymphatic capillaries, and their small collecting lymphatic vessels lack valves; however, valves are present in the large collecting lymphatic vessels. The collecting lymphatic vessels in *Akt1*$^{-/-}$ mice are enlarged in diameter and show abnormal SMC coverage and defective lymph flow (111).

It has recently been revealed that the planar cell polarity proteins CELSR1 and VANGL2 also participate in lymphatic valve formation (112). During valve leaflet morphogenesis, LECs undergo elongation, reorientation, and collective migration into the vessel lumen. *Celsr1*- or *Vangl2*-null mice lack valves because LECs fail to rearrange and adopt perpendicular orientation at valve initiation sites (112).

Finally, apoptosis-stimulating protein of p53 (ASPP1) knockout mice show subcutaneous edema and mispatterned lymphatic collecting vessels (113). Functional analysis has revealed impaired lymphatic drainage in *Aspp1*$^{-/-}$ embryos. Although it has been reported that ASPP1 enhances apoptotic activity of p53, the function of ASPP1 in lymphatic vasculature development is independent of p53 (113).

Conclusions

Although our knowledge of developmental lymphangiogenesis has drastically improved, many interesting questions remain to be analyzed in the years to come. For example, what determines the number of LEC progenitors that will bud from the veins? What determines the location of the forming lymph sacs? Many key regulators of the maturation of collecting lymphatics and the formation of lymphatic valves have been identified; however, additional factors and signaling pathways are likely to participate in this process. In particular, it will be interesting to identify those factors essential for the formation of more specialized lymphatics, such those of mesenteric lymphatics or other specific organs. For example, *Tbx1*-deficient mice have hyperplastic lymphatic vessels in the heart, diaphragm, and skin, and they lack the entire gastrointestinal lymphatic vasculature (114). Interestingly, *Tbx1* is associated with DiGeorge syndrome; however, lymphatic defects so far have been reported just once in patients with this syndrome (115). It is likely that a better understanding of the morphogenetic process leading to the formation of the mature lymphatic network will facilitate the identification of additional functional roles of the lymphatic vasculature that, when defective, could lead to different pathological conditions. Also, it will be interesting to better understand the functional importance of EC fate plasticity — does this reprogramming potential occur only under certain circumstances, such that blood and LECs can switch fates?

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Address correspondence to: Guillermo Oliver, Department of Genetics, St. Jude Children's Research Hospital, 262 Danny Thomas Place, Memphis, Tennessee 38105, USA. Phone: 901.595.2697; Fax: 901.595.6035; E-mail: guillermo.oliver@stjude.org.

Ying Yang's present address is: University of Missouri, Bond Life Sciences Center, Columbia, Missouri, USA.

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