The Transcriptional Control of Lymphatic Vascular Development

More than 100 years ago, Florence Sabin suggested that lymphatic vessels develop by sprouting from preexisting blood vessels, but it is only over the past decade that the molecular mechanisms underpinning lymphatic vascular development have begun to be elucidated. Genetic manipulations in mice have identified a transcriptional hub comprised of Prox1, CoupTFII, and Sox18 that is essential for lymphatic endothelial cell fate specification. Recent work has identified a number of additional transcription factors that regulate later stages of lymphatic vessel differentiation and maturation. This review highlights recent advances in our understanding of the transcriptional control of lymphatic vascular development and reflects on efforts to better understand the activities of transcriptional networks during this discrete developmental process. Finally, we highlight the transcription factors associated with human lymphatic vascular disorders, demonstrating the importance of understanding how the activity of these key molecules is regulated, with a view toward the development of innovative therapeutic avenues.

Lymphatic Vessels in Health and Disease

The lymphatic vasculature is an essential component of the vertebrate vascular system and plays a number of critical roles in homeostasis and disease. Lymphatic vessels function to return interstitial fluid and protein to the bloodstream, to absorb dietary fatty acids, and to traffic cells of the immune system (36). Lymphatic vessels are involved in the pathogenesis of a number of human diseases. Reduced lymphatic vascular function can lead to lymphedema, whereas the aberrant growth and development of lymphatic vessels has been implicated in obesity, hypertension, and inflammatory diseases (62, 72). During cancer progression, tumors have been demonstrated to secrete vascular endothelial growth factor C (VEGFC) or D (VEGFD), which promote the growth of lymphatic vessels (46, 71). Tumor cells can subsequently utilize peri- and intratumoral lymphatic vessels to metastasize to local lymph nodes and secondary tumor sites, and the presence of lymph node metastases is typically correlated with poor prognosis (1, 3, 21, 73).

Initial Events During Lymphatic Vascular Development

Lymphangiogenesis is initiated in the mammalian embryo when a subset of endothelial cells delaminates from the walls of the embryonic veins to give rise to the lymph sacs and early lymphatic vascular plexus (FIGURE 1, A AND B) (63, 76, 77). In the mouse embryo, the initial event preceding morphogenesis is the specification of a population of lymphatic endothelial cell (LEC) precursors that are located within the dorsolateral walls of the cardinal veins (~9.5 days post coitum, dpc). These precursors are marked by the expression of key transcriptional regulators that act to program LEC identity (18, 70, 74b, 75). Once specified, LEC precursors delaminate from the veins and migrate into the mesenchyme in dorso-anterior and dorsolateral streams between 9.5 and 11.5 dpc to form the embryonic lymph sacs (FIGURE 1B). Current thought suggests that the LEC precursor pool that originates in the embryonic veins goes on to generate the mature lymphatic vasculature via remodeling and expansion of the lymph sacs and early lymphatic plexus (63, 69, 74, 76). Studies using alternative vertebrate models have shown that the mechanism of dorsal sprouting from embryonic veins is a highly conserved process in lymphangiogenesis across different species, although some species appear to develop early lymph sacs only in the anterior of the embryo (7, 23, 24, 51, 81).

The sprouting of LEC precursors from the embryonic veins depends on Vegfc, which acts through its receptor Vegfr3 (33, 35). This signaling pathway is indispensable for lymphangiogenesis and is tightly regulated by the activity of co-receptors (45, 74a, 76a).
78), secreted inhibitors (2), and alternate ligand combinations (27, 60). The roles of these key molecules have been reviewed elsewhere (72). In this review, we will discuss the transcription factors inherent to endothelial cells that play critical roles in embryonic lymphangiogenesis. We will explore the roles of these factors during LEC fate specification, differentiation, the formation of distinct lymphatic vascular structures, and the maintenance of LEC identity.

**Specification of Lymphatic Endothelial Cell Precursors in the Cardinal Veins**

Specification of a population of LEC precursors within the dorsolateral walls of the embryonic veins is a crucial process during vertebrate development. The transcription factors indispensable for LEC fate specification are Sox18, CoupTFII, and Prox1. Recent data in cultured endothelial cells and zebrafish has also suggested a role for the Notch signaling pathway in regulating Prox1 and lymphatic vascular development.

**Prospero-Related Homebox Domain**

Prospero-related homebox domain 1 (Prox1) is a homeobox domain 1 transcription factor that plays key roles during the development of diverse organ systems. The use of Prox1 to regulate developmental decisions is conserved throughout evolution. In the *Drosophila* nervous system, the Prox1 homolog (*prospero*) regulates neural cell fate specification, whereas in mammals Prox1 drives the differentiation of cell types in the retina, lens, and hemopoietic compartment (18a, 28, 74b).

In the first study to identify a central transcriptional regulator of LEC identity, Wigle and Oliver (76) described the embryonic mouse phenotype caused by the targeted inactivation of *Prox1*. *Prox1* expression labeled a polarized subpopulation of endothelial cells in the dorsolateral walls of the anterior cardinal veins, as well as endothelial cells sprouting from this region in a dorsolateral direction (FIGURE 1C, TOP, AND FIGURE 2). Expression analyses revealed that these *Prox1*-positive cells were LECs. At later stages of development, *Prox1* expression was reduced in the veins and became restricted to the lymphatic vasculature.

**FIGURE 1.** The anatomy of lymph sac development in the mouse embryo

*A*: whole-mount immunofluorescence for Pecam (green; left) reveals the organization of the vasculature in an 11.5-dpc whole-mount mouse embryo. The red plane indicates the orientation of the section shown in *B*. *B*: schematic adapted from Van der Putte’s representation of the anatomy of the developing lymphatic vascular system in the mouse embryo (74). A dorsal view of the left side of an 11.5-dpc embryo is depicted indicating major arteries, veins, and location of the jugular anterior lymph sac (jals); the region most commonly examined in studies of embryonic lymphangiogenesis and the focus of *C*. *C*: top: cross section double immuno and in situ hybridization fluorescence staining for *Prox1* (green), Sox18 (red, in situ hybridization), and DAPI (blue) in the mouse embryo. At 10.5 dpc, LEC precursors are distributed on the dorsolateral side of the cardinal vein. LEC precursors that have delaminated from the cardinal vein can be seen migrating within the mesenchyme. Bottom: cross section triple immunofluorescence staining for *Prox1* (red), Podoplanin (green), and Pecam (blue). At 14.5 dpc, LECs have assembled into jugular (jals) lymph sacs. Right: a schematic diagram is shown for each stage. Jals, jugulo-axillary lymph sac; acv, anterior cardinal vein; dao, dorsal aorta; ao, aorta; ccv, common cardinal vein; puv, primitive ulnar vein; pcv, posterior cardinal vein; D, dorsal; L, lateral.
Prox1-deficient animals were devoid of lymphatic vessels and, as a result, exhibited striking edema (76). Interestingly, endothelial cells were still observed to sprout from the dorsolateral walls of the cardinal veins in Prox1-null animals, but they retained the expression of blood vascular endothelial cell (BEC) markers and failed to express markers of arteries.

**Figure 2. Overview of the transcriptional network that controls lymphatic vascular development in the embryo**

**A:** During arterio-venous specification, CoupTflI and Sox18 regulate the venous phenotype (blue cells). The Notch-Dll4 pathway promotes arterial development, but is repressed by CoupTflI (82). Hey1/2 represses Coup TflI in arterial cells. The molecular cues responsible for polarization of the vein have not yet been identified.

**B:** At 9.5 dpc, a subset of endothelial cells located in the dorsolateral side of the cardinal vein express Prox1, which is directly activated by CoupTflI and Sox18. This cell subpopulation constitutes the pool of LEC precursors (yellow). C: From 10.5 dpc until 11.5 dpc, LEC precursors delaminate from the wall of the vein and migrate within the mesenchyme. Cellular events underlying the assembly of lymph sacs remain poorly described. Once LEC precursors have detached from the vessel wall, additional lymphatic markers (e.g., Podoplanin) are acquired (green). During this stage, Prox1 expression is maintained by a transient cooperative mechanism involving Prox1 and CoupTflI. Prox1 continues to play a role in maintaining LEC identity from this point onward (32).

**D:** At 14.5 dpc, the lymphatic plexus has developed, and the maturation of the vessel network is starting. FoxC2 and NfatC1 play key roles controlling valve morphogenesis and maturation of collecting vessels. The remodeling of the lymphatic vascular tree is at least partly controlled by Tbx1 regulation of Vegfr3. The expression levels of key transcription factors during the initial steps of lymphangiogenesis (9–14.5 dpc) is depicted at left through A–D. Cre, CoupTflI responsive element; Sre, SoxF responsive element; Tre, Tbox1 responsive element. Note that a number of targets of these transcription factors that have been identified only in vitro and have not been validated in vivo have been omitted from this figure (see Refs. 20, 40, 50, 53, 67).
differentiated LECs (75). These studies revealed that Proxl is essential for the specification of LEC fate but not initial sprouting from the veins. Mouse mutants deficient in VegfC retain Proxl-positive cells in the walls of the cardinal veins but fail to undergo dorsolateral sprouting (35), illustrating that Proxl and VegfC play distinct roles in specification and sprouting, respectively.

Several lines of evidence have revealed that Proxl is not only necessary but sufficient to drive LEC fate specification. Adenoviral-mediated expression of Proxl in BECs results in elevated expression of lymphatic markers (e.g., Podoplanin, Vegf3, Nrpx2, Integrin-α) and decreased expression of BEC markers (e.g., Icam1 and Integrin-α) (26, 58). Moreover, ectopic expression of Proxl under control of the Tiel promoter also induced the expression of LEC markers in the blood vasculature in vivo (38). Importantly, Proxl homologs have been shown to be necessary for the formation of the lymphatic vasculature in all vertebrate models subjected to detailed analysis thus far, suggesting a conserved role in lymphatic development throughout evolution (51, 81, 12).

**SRY-Related HMG-box 18 and SoxF Group Transcription Factors**

SRY-related HMG-box 18 (Sox18) is a member of the F-group of Sox transcription factors, a subfamily that also contains the closely related Sox7 and Sox17 proteins (5). SoxF transcription factors are known to play a number of iterative roles during vascular development and often function redundantly in these processes (13, 17, 48). Sox18, for example, is required for arteriovenous specification but acts redundantly with Sox7 in this process (8, 22, 55).

It was recently shown that the Proxl promoter region contains two conserved SoxF consensus binding sites (18). These sites were bound by Sox18 and were essential for transactivation of the Proxl promoter both in vitro and in vivo during LEC fate induction (FIGURE 2). In addition to the Sox18-dependent induction of Proxl expression in these contexts, ectopic Sox18 activity was sufficient to force the expression of Proxl in vitro, in both BECs and a model of LEC differentiation from ES cells (18).

The physiological relevance of the Sox18- Proxl transcriptional relationship was confirmed in *Ragged Opposum* (Ra0p) mutant mice, which carry a natural mutation rendering Sox18 dominant negative in function (56, 68). These mice failed to initiate venous Proxl expression, resulting in arrested lymphatic vascular development (18). Interestingly, Sox18 knockout mice (a targeted deletion, not the Ra0p allele) displayed a complete loss of venous Proxl expression in a pure C57Bl/6 background but did not exhibit this phenotype in a mixed genetic background. Although Sox7 and Sox17 are not normally expressed in LEC precursors during the course of lymphatic differentiation, genetic linkage analyses identified these two transcription factors as the strain-specific modifiers responsible for differences in the lymphatic vascular phenotype of Sox18 mutant mice. It was found that, as a result of Sox18 loss of function in a mixed genetic background, Sox7 and Sox17 expression was upregulated in LEC precursors with the redundant biochemical properties of these transcription factors leading to the rescue of Proxl transcription (29).

Importantly, mutations in SOX18 in humans have been previously characterized as causative for hypotrichosis-lymphedema-telangiectasia (HLT) (30) (Table 1). These mutations typically render SOX18 dominant negative in function and indicate a conserved and crucial role for SOX18 and possibly SOX7/17 in human lymphangiogenesis. In support of a role for additional SOXF group members in human lymphangiogenesis, mutations in SOX17 have been linked with human primary lymphedema (16).

Intriguingly, during LEC fate induction, Sox18 expression in the vasculature is not restricted to venous endothelial cells. Sox18 is also expressed in arterial endothelial cells, which do not go on to express Proxl. This observation suggests that arterial-specific repressor(s) interfere with the ability of Sox18 to drive Proxl expression in arterial endothelial cells or that a venous-restricted cofactor cooperates with Sox18 to induce Proxl expression specifically in the veins. In contrast to Proxl, Sox18 expression in the lymphatic vasculature was not detected during later stages of embryonic lymphangiogenesis that correspond to the expansion and maturation of the lymphatic plexus (~14.5 dpc). These data suggest that Sox18 may play no further role in ongoing LEC differentiation or the maintenance of LEC identity. The mechanism by which Sox18 is repressed in the lymphatic vasculature remains to be elucidated.

**Chicken Ovalbumin Upstream Transcription Factor II**

Chicken ovalbumin upstream transcription factor II (CoupTFII) is an orphan nuclear receptor that modulates the activity of transcriptional binding partners. CoupTFII is expressed in the embryonic veins from 8.5 dpc and in LECs throughout embryogenesis and adulthood (43). Endothelial knockout of CoupTFII resulted in a loss of venous cell identity; embryonic veins instead adopted arterial characteristics (82), and in this context the specification of lymphatic endothelial precursor cells was arrested (70).

A series of in vitro assays suggested a specific role for CoupTFII in LECs, with yeast two-hybrid and immuno-precipitation assays demonstrating...
that CoupTFII was capable of directly binding to Prox1 in vitro (42, 70, 80). Furthermore, a conserved consensus Coup-TF responsive element in the Prox1 promoter was capable of driving CoupTFII-mediated Prox1 expression in vitro (70) (FIGURE 2). Supporting these data, ectopic expression of Prox1 and CoupTFII was demonstrated to synergistically induce the expression of the Prox1 target gene \textit{Fgfr3} in cultured BECs, and siRNA-mediated depletion of Prox1 and CoupTFII together in LECs synergistically reduced the expression of lymphatic markers (42). Together, these data suggest that CoupTFII and Prox1 work cooperatively to control LEC identity in vitro.

Recent work has revealed an important role for CoupTFII during lymphangiogenesis in vivo. Prox1 and CoupTFII are co-expressed by developing LECs at 11.5 dpc during the dorsal sprouting of LECs from the cardinal veins (43, 70). Srinivasan and colleagues (70) simultaneously deleted CoupTFII and the Notch pathway effector Rbpj (Suh) (which drives arterial development) in the vasculature. In this context, venous fate was partially rescued in double mutant endothelial cells, overcoming epistasis posed by loss of venous identity, and Prox1 expression remained significantly reduced. These results suggested that CoupTFII is required for the initiation of Prox1 expression and the specification of LEC identity during development. CoupTFII was also shown to play a role in the early maintenance of Prox1 expression [described below (70)].

The induction of Prox1 expression in response to CoupTFII and Sox18 signifies the specification of LEC fate. Lymphatic precursors that express Prox1 are considered specified (52). Interestingly, CoupTFII expression is not polarized to the dorsolateral aspects of the cardinal veins, and yet Prox1 expression is only induced in the dorsolateral cells (18, 76). This suggests that a factor other than CoupTFII must provide additional positional cues that polarize downstream gene expression in the embryonic cardinal veins.

### The Notch Pathway

Recent work has suggested that the Notch pathway is likely to play a role in lymphatic vascular development. Notch receptors and their ligands mediate crucial and diverse roles in blood vascular development and angiogenesis; Notch signaling controls arterio-venous cell fate specification, angiogenic sprouting, and vascular smooth muscle development (61).

Kang et al. (34) demonstrated that ectopic expression of the Notch intracellular domain (NICD; the main effector of Notch pathway function) in cultured LECs simultaneously repressed Prox1 and CoupTFII transcription. In addition, treatment of LECs with soluble, recombinant forms of Notch ligands Dll4 and Jag1 led to consistent downregulation of Prox1 and CoupTFII mRNA...
levels. This repression could also be induced by overexpression of Notch target transcription factors Hey1 and Hey2, and the siRNA-mediated depletion of Hey1 and Hey2 rescued the repression of Prox1 expression on ectopic NICD expression. Taken together, these findings indicated that classical Notch signaling represses Prox1 and CoupTFII expression and the induction of LEC fate in vitro. Consistent with this model, a reciprocal relationship was identified between the levels of Notch receptor expression and Podoplanin expression in human dermal lymphatic vessels (34).

To date, this in vitro molecular pathway remains to be confirmed in vivo. In fact, recent data from zebrafish and mouse models are conflicting and suggest that Notch signaling either plays a positive regulatory role in lymphangiogenesis or is not required at all for the initial steps of lymphatic vascular development. In zebrafish, depletion of Notch receptors or the Notch ligand Dll4 resulted in robust inhibition of embryonic lymphatic development (19). In Dll4 morpholino injected animals, cells still sprouted dorsally from the vein but failed to contribute to the lymphatic vasculature; instead an increase in the number of venous endothelial sprouts contributing to the blood vasculature was observed. This suggested that Notch signaling might regulate LEC fate specification or sprouting morphogenesis from the veins (19). Complicating the interpretation of these data, zebrafish Dll4 was not found to be expressed in veins or lymphatics, so it is likely to act non-autonomously to regulate lymphangiogenesis in this setting.

In the mouse embryo, endothelial deletion of the transcriptional regulator Rbpj (Suh), which is required for active Notch signaling, did not interfere with the early stages (10.0 dpc) of lymphatic development at all (70). Although an analysis of later stages of lymphatic development remains to be reported, this study indicates that the very early steps of lymphatic vascular development occur independently of Rbpj (suh) and canonical Notch signaling.

Taken together, these findings suggest that Notch signaling is capable of regulating lymphangiogenesis in some capacity and likely plays a role in some contexts in vivo. However, further analyses are required to define the role of Notch signaling during embryonic lymphangiogenesis. The expression of Notch pathway components in both blood and lymphatic vessels, and the context-dependent and often transient nature of Notch pathway functions, makes the experimental dissection and interpretation of their roles in discrete blood and lymphatic vascular processes challenging.

**Lymphatic Vessel Differentiation**

The lymphatic vasculature is made up of a number of distinct vessel types and contains several critical functional components. Collecting lymphatic vessels contain well defined valves, have a basement membrane, and are surrounded by smooth muscle cells. Lymphatic capillaries lack smooth muscle cells and valves, and their basement membrane is anchored to the extracellular matrix by thin anchoring filaments. The mechanisms by which these different cellular identities and substructures are defined require changes in the transcriptional output of LECs that are driven by key transcription factors.

**Forkhead Box Protein c2**

FoxC2 is a forkhead transcription factor that plays reiterative roles in vascular development (41). Initially, FoxC2 plays an essential role in regulating arterio-venous differentiation in cooperation with FoxC1. Animals with loss of function of both FoxC1 and FoxC2 exhibit arterio-venous shunts and express venous markers in the arteries (65).

In the developing mouse embryo, the transcriptional network controlling LEC specification does not require FoxC2 function. FoxC2 expression is observed in developing LECs from as late as 14.5 dpc, and FoxC2-deficient animals retain normal polarized venous and early LEC expression of Prox1 (57). The expression of Foxc2 is high at 14.5 dpc, but as the LECs mature into collecting lymphatics it is downregulated (along with Prox1) and remains high specifically in lymphatic valves (50). FoxC2 mutants display severe morphological defects in developing lymphatic vessels and fail to form lymphatic valves (50, 57). Interestingly, FoxC2 mutant lymphatic capillaries also ectopically recruit pericytes (57). In humans, mutations in FOXC2 underlie the pathogenesis of lymphedema distichiasis, in which patients display defects in lymphatic vascular morphogenesis that include an absence of normal valve structures and aberrant pericyte recruitment (15, 47) (Table 1). Together, these findings define a conserved role for FoxC2 in lymphatic valve formation and pericyte recruitment to lymphatic vessels in mice and humans (57).

Recent data have shown that as well as lymphatic collecting vessel valve formation, FoxC2 regulates venous valve formation (49). FoxC2 functions closely with another transcription factor, NfatC1, to modulate lymphatic vessel maturation (50) (see below). Intriguingly, NfatC1 also controls cardiac valve formation (11, 59), suggesting that different endothelial valvular morphogenesis
programs may be controlled by common transcriptional regulators.

**Nuclear Factor of Activated T-Cells, Cytoplasmic 1**

Nuclear factor of activated T-cells, cytoplasmic 1 (NfatC1) is a calcium-sensitive transcription factor activated by the Calcineurin pathway that interacts directly with a number of developmental transcription factors (for review, see Ref. 10). NfatC1 is expressed by the Prox1-positive LEC precursors of the cardinal veins and the LECs of the developing lymph sacs. In two independent studies, NfatC1 was shown to be required for normal lymphatic vascular patterning (40, 50). Kulkarni et al. (40) showed that, although NfatC1-null mice were capable of forming lymph sacs, their patterning was dysmorphic, displaying reduced luminal area and a decrease in the number of Prox1-positive cells. NfatC1 activation and nuclear localization is dependent on Calcineurin, which dephosphorylates NfatC1. On inhibition of Calcineurin phosphatase activity by Cyclosporine (CsA) treatment, lymph sacs developed in a disorganized manner and showed a reduction in Fgfr3 and Podoplanin staining, although the expression of Prox1 and Vegfr3 was unaltered (40, 50). These analyses indicated a role for NfatC1 that is independent or downstream of Prox1, although the precise role of this transcription factor and its targets remains to be described.

The loss of Foxc2 function and the inhibition of the NfatC1 pathway by CsA treatment in vivo led to abnormal lymphatic vessel patterning, lymphatic vessel backflow, and maturation defects. In a detailed study, Norrmen et al. (50) showed that the loss of one Foxc2 allele enhanced the effects of Nfat pathway inhibition by CsA treatment. Using a genome-wide ChIP analysis, a large set of FoxC2 binding sites were identified and mapped; data showed consistent enrichment of Nfat binding sites co-located with FoxC2 binding sites (50). These data, together with characterization of the NfatC1 and FoxC2 phenotypes, illustrate cooperation between FoxC2 and NfatC1 signaling in the transcriptional control of lymphatic vessel differentiation and maturation.

**T-Box 1**

The most well characterized signaling pathway controlling patterning of the lymphatic vasculature is the VegfC/Vegfr3 pathway; however, the cell-autonomous transcriptional regulators that modulate the ability of LECs to respond to VegfC remain to be characterized.

Tbx1 is expressed in lymphatic vessels, and endothelial deleted Tie2-Cre/Tbx1lox/lox knockout mice exhibit a marked reduction of Vegfr3/Lyve1-positive lymphatic vessels in the developing mesentery (9). Chen and colleagues demonstrated that Tbx1 is required for normal Vegfr3 expression in HUVECs and in human microvascular LECs by siRNA knockdown and went on to identify an intronic consensus T-box binding element (TBE) in the Vegfr3 gene. Using ChIP, this TBE was shown to be bound by Tbx1 and to activate downstream gene expression in vitro. Furthermore, by the construction of a transgenic mouse model in which this specific Vegfr3 enhancer fragment was fused to a LacZ reporter gene, Tbx1 was demonstrated to directly activate Vegfr-3 in vivo. Importantly, Tbx1 does not induce Prox1 expression, suggesting that Tbx1 is required for differentiation independent of specification of LECs (9). This specific regulation of Vegfr3 levels in LECs provides an alternative pathway to regulate the response of LECs to exogenous VEGF-C, thereby controlling the ongoing expansion of the lymphatic vasculature.

**Maintenance of Lymphatic Endothelial Cell Identity**

After the early stages of LEC specification and sprouting, Sox18 expression is not detectable in mature LECs (from 14.5 dpc), yet Prox1 expression is maintained in adult lymphatic vessels at reduced levels (37, 50). These studies suggested that distinct mechanisms regulate the induction of LEC fate specification and the maintenance of LEC identity. Recent work has begun to delineate the transcriptional hierarchies that underpin the maintenance of LEC identity during later stages of development, and in adult tissues.

Almost a decade ago, in vitro analyses suggested plasticity between mature LEC and BEC cell phenotypes (58). Recently, these findings have been validated in vivo in a series of experiments demonstrating an ongoing role for Prox1 in the maintenance of LEC identity. Johnson et al. (32) used an inducible conditional knockout model to inactivate Prox1 during embryogenesis, during postnatal stages, and during adulthood. At all stages examined, loss of Prox1 led to increased expression of BEC markers in LECs, with lymphatic vessels taking on blood vascular characteristics (32). These data revealed a constant role for Prox1 in maintaining LEC identity and a remarkable plasticity in the mature LEC phenotype, suggesting that committed, functional LECs may be re-programmed under certain conditions.

In addition to temporally controlled Prox1 inactivation, the use of tamoxifen-inducible Prox1-CreErT2 animals to delete CoupTFII at 10.5, 11.5, and 12.5 dpc led to dramatic defects in the early maintenance of LEC identity assayed at 15.5 dpc (70). In a separate study, Lin et al. (43) performed conditional knockout experiments for CoupTFII...
during later developmental stages (13.5 dpc) and found this to result in a loss of LEC fate and impaired lymphatic vessel sprouting via a Neuropilin 2-dependent mechanism. These studies together demonstrated that, in addition to regulating the specification of LEC fate, the binding partners Prox1 and CoupTFII play essential roles in the early maintenance of LEC identity in the 10.5- to 13.5-dpc mouse embryo. Intriguingly, the loss of CoupTFII in adult vessels did not affect lymphatic identity, but neo-lymphangiogenesis was impaired, suggesting that CoupTFII and Prox1 have distinct roles in mature lymphatic vessels (43).

Interestingly, two recent studies revealed that the microRNAs, mir-181 and mir-31, regulate Prox1 mRNA and protein levels in endothelial cells (37, 54). This work established that Prox1 expression is also controlled at the posttranscriptional level, complementing previous studies showing that Prox1 activity is modulated at the posttranslational level by sumoylation (66, 53). Ectopic expression of mir-181 or mir-31 in cultured LECs reduced Prox1 levels, resulting in the reversion of the LEC phenotype toward a BEC identity. High levels of mir-181 are expressed by embryonic BECs compared with embryonic LECs, suggesting that mir-181 might contribute to the mechanism of Prox1 silencing in the embryonic blood vasculature. These data suggest that posttranscriptional and posttranslational modifications are likely to provide an important layer of regulation to fine tune the activity of transcription factors responsible for the specification of LEC fate and maintenance of LEC identity.

Future Perspectives

The key experiments outlined above represent work that has vastly expanded our knowledge of how transcription factors regulate the development of lymphatic vessels. Looking forward, it is clear that significant questions remain to be answered. It is surprising that the mechanism behind the earliest step in the process of lymphatic development—the molecular polarization of the cardinal vein—remains enigmatic. How is it that Sox18 and Prox1 expression is activated in the dorsolateral CV, or actively repressed in the ventrolateral CV? Moreover, not a single target of Prox1 has been identified with a demonstrated functional role in lymphatic development in vivo. How do these transcription factors define cell fate? Beyond the Sox18-CoupTFII-Prox1 driven pathways, transcription factors such as Foxc2, NfatC1, and Tbx1 regulate the ongoing differentiation and maturation of lymphatic vessels, but how are these different transcriptional inputs integrated by a developing or latent LEC? We currently think of LEC identity as being overlayed onto a venous endothelial cell identity; LECs can even revert back to a BEC phenotype in mutant contexts. But does LEC-BEC reversion reflect a physiologically relevant mechanism that is utilized by endothelial cells in normal or disease conditions? Another gap in our current knowledge is the lack of molecular markers that discriminate between subclasses of LECs or lymphatic vessel subtypes.

These questions, and others, will continue to drive investigation of transcription factors that regulate this unique process, whereby one vascular system derives from another during development. In the present era of whole genome studies, deciphering the pathways that act downstream of known transcription factors is becoming increasingly more achievable. A comprehensive genome-wide analysis of the targets of key transcriptional regulators (e.g., Sox18, Prox1, CoupTFII, Foxc2, NfatC1) may provide a more comprehensive model of lymphangiogenic transcriptional pathways. From a medical perspective, deciphering the molecular hub that controls lymphangiogenesis would identify novel therapeutic targets and lead to potential therapeutic avenues complementing existing (anti-VEGF) strategies.

We thank Dr. Dagmar Wilhelm for providing data.

M. Francois was supported by an NHMRC project grant (626959) and also by a CDA fellowship (1011242). B. M. Hogan was supported in part by an NHMRC CJ Martin training fellowship (433628) and in part by an ARC Future Fellowship (FT100100165). N. L. Harvey was supported by a Medvet Fellowship and an NHMRC project grant (626959).

No conflicts of interest, financial or otherwise, are declared by the author(s).

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