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Molecular and cellular mechanisms of lymphatic vascular maturation

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Abstract

Lymphatic vasculature is necessary for maintaining fluid homeostasis in vertebrates. During embryogenesis lymphatic endothelial cells originate from the veins as a homogeneous population. These cells undergo a series of changes at the morphological and molecular levels to become mature lymphatic vasculature that consists of lymphatic capillaries, collecting lymphatic vessels and valves. In this article we summarize our current knowledge about these steps and highlight some black boxes that require further clarification.

Introduction

Lymphatic vasculature is required to return interstitial fluid and digested lipids (collectively known as lymph) back to blood circulation. In healthy humans this amounts to approximately 1–2 liters of lymph per day (Tammela and Alitalo 2010). In addition to fluid homeostasis, lymphatic vessels play a profound role in maintaining health. Lymphatic vessels regulate immune surveillance, reduce inflammation, modulate blood pressure and mediate reverse cholesterol transport (Randolph et al. 2005; Machnik et al. 2009; Dieterich et al. 2013; Lim et al. 2013; Martel et al. 2013; Wiig et al. 2013). Abnormal lymphatic vascular function leads to lymphedema, atherosclerosis, tumor metastasis, fibrosis, obesity, inflammatory bowel disease and sepsis (Harvey et al. 2005; Jang et al. 2013; Jurisic et al. 2013; Martel et al. 2013; Song et al. 2013). Despite having such important functions, lymphatic vasculature historically did not get the attention it deserved from the research community, partially due to the difficulty in identifying and visualizing lymphatic vessels. In the past decade, significant progress has been made in our understanding of lymphatic vasculature due to the advent of gene targeting technology and the identification of lymphatic vessel-specific markers. A few excellent review articles have described our current understanding about the development of lymphatic vasculature, the molecular players that drive lymphatic development and the role of lymphatic vasculature in health and disease (Cueni and Detmar 2008; Oliver and Srinivasan 2008; Jurisic and Detmar 2009;

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Oliver and Srinivasan 2010; Tammela and Alitalo 2010; Wang and Oliver 2010; Francois et al. 2011; Norrmen et al. 2011; Koltowska et al. 2013). Here we provide a short treatise on the stepwise patterning and maturation of lymphatic vasculature, highlighting some unexplored or understudied aspects of the process. We hope this will invigorate interest in the developmental biology of the lymphatic vasculature and help refine the existing models.

1. Specification of Lymphatic endothelial cell progenitors on the embryonic veins

Lymphatic endothelial cells (LECs) originate from the embryonic veins (Srinivasan et al. 2007). In mouse embryos around embryonic day ten (E10) a sub-population of venous endothelial cells turn on expression of the homeobox transcription factor PROX1 (Wigle and Oliver 1999; Srinivasan et al. 2007). These are the LEC progenitor cells. PROX1 is necessary for maintaining the identity of these progenitors, as indicated by the observation that the number of LEC progenitors is significantly reduced in *Prox1*^{+/-} embryos (Srinivasan and Oliver 2011). However, we currently do not know what besides PROX1 expression molecularly distinguishes progenitor cells from other venous endothelial cells nor the mechanisms that maintain LEC progenitor cell identity.

So far two transcription factors have been shown to be critical for the activation of PROX1 expression in the LEC progenitors, COUP-TFII and SOX18. PROX1⁺ LEC progenitors are absent in both *Sox18*^{-/-} and in *Tie2-Cre;Coup-TFII*^{fl/fl} (endothelial cell-specific knock-out) embryos (Srinivasan et al. 2007; Francois et al. 2008; Srinivasan et al. 2010). COUP-TFII binds to a highly conserved site in the regulatory elements of *Prox1* (Srinivasan et al. 2010). Therefore, COUP-TFII is hypothesized to be a direct activator of *Prox1* expression in the LEC progenitors. However, since COUP-TFII is expressed in all venous endothelial cells (You et al. 2005), it is unclear how COUP-TFII activates *Prox1* expression only in a subset of venous endothelial cells. In contrast to COUP-TFII, SOX18 is highly enriched in a subset of venous endothelial cells that become the LEC progenitors (Francois et al. 2008). SOX18 also directly binds to *Prox1* regulatory elements. Therefore, it is likely that COUP-TFII and SOX18 cooperate in activating *Prox1* expression to specify the LEC progenitors (Oliver and Srinivasan 2010; Srinivasan et al. 2010).

COUP-TFII and SOX18 probably interact with additional transcriptional coregulators and epigenetic modifiers to mediate LEC progenitor specification. For example, HMG box transcription factors like SOX18 are known to bend DNA and provide access to other transcription factors. Such transcription factors might include TBX1 or GATA2, which have been implicated in lymphatic development (Chen et al. 2010; Kazenwadel et al. 2012; Lim et al. 2012). Additionally, *Coup-TFII* and *Sox18* are themselves regulated by complex signaling and transcriptional mechanisms (Davis et al. 2013; Deng et al. 2013). Therefore, much remains to be clarified about the factors that influence *Prox1* transcription and LEC progenitor specification.

2. Separation of lymphatic and blood vessels

After specification, the PROX1⁺ progenitor cells migrate out from the veins between E10 and E13.5 (Wigle and Oliver 1999). This process does not occur in *Vegfc*^{-/-}, *Ccbe1*^{-/-} and *Prox1*^{-/-} embryos (Karkkainen et al. 2004; Bos et al. 2011; Yang et al. 2012; Hagerling et al. 2013). VEGFC is a chemo-attractant expressed in the mesenchyme that triggers the migration of LECs (Karkkainen et al. 2004). CCBE1 regulates the processing of VEGFC into a potent form that can associate with its cognate receptor VEGFR3 (Jeltsch et al. 2014; Le Guen et al. 2014). PROX1 likely activates the expression of VEGFR3 in LECs prior to migration (Wigle et al. 2002). During migration, LECs undergo significant changes in cell shape, upregulate their rate of proliferation, and initiate expression of LEC markers such as podoplanin and NRP2 (Hagerling et al. 2013). Expression of PROX1 and VEGFR3 are also enhanced. No LEC progenitors are seen on the veins after E13.5 (Srinivasan and Oliver 2011).

As the LECs migrate out from the veins, it is important to exclude blood cells from entering the newly forming lymphatic vasculature. Pioneering work from Mark Kahn's lab showed that the hematopoietic signaling proteins PLCγ2, SYK and SLP-76 regulate this separation process during embryonic development (Abtahian et al. 2003). Two important findings built upon this work to elucidate the molecular mechanisms underlying blood-lymphatic separation. In 2008, Fu et al reported that mice with endothelial cell-specific deletion of T-synthase (also known as *C1galt1*), a critical glycosyltransferase for biosynthesis of O-glycans, exhibited defective separation of blood and lymphatic vessels during embryonic development (Fu et al. 2008). This study showed that podoplanin is a T-synthase substrate and that podoplanin stability is impaired upon loss of O-glycosylation (Fu et al. 2008). Importantly, this study also revealed that *podoplanin*^{-/-} embryos shared a blood-filled lymphatic vessel phenotype with endothelial T-synthase mutants (Fu et al. 2008). In 2010, a separate study confirmed that podoplanin is required for blood and lymphatic separation during development (Uhrin et al. 2010). Podoplanin is known to interact with C-type lectin-like receptor 2 (CLEC-2) on platelets and activate platelet aggregation via PLCγ2, Syk and SLP-76 (Suzuki-Inoue et al. 2007). These crucial findings set the stage for further dissection of the mechanisms involved in blood-lymphatic separation. Subsequent research demonstrated that platelets and CLEC-2 are necessary for lymphatic vascular integrity (Bertozzi et al. 2010).

Despite such a strong molecular evidence for the role of platelets and podoplanin-CLEC2-PLCγ2/ Syk /SLP-76 pathway in blood-lymphatic separation, we do not yet completely understand the cellular and morphological mechanisms involved. LEC migration from the vein is the most logical step during which blood-lymphatic separation is initiated. Therefore, we will discuss the two available models describing LEC migration from the vein and evaluate their relevance to blood-lymphatic separation.

The budding model

Two groups have shown that LECs migrate out from the anterior cardinal vein, intersomitic veins, and peripheral venous plexus as interconnected clusters of cells (Yang et al. 2012; Hagerling et al. 2013). The integrity of the venous vessel wall is maintained during this

process to prevent vascular leakage. This “budding” model (Figure 1A, B) raises some questions about how blood-lymphatic separation mediated by CLEC2-podoplanin could be achieved. LEC progenitors on the vein do not express podoplanin; these cells turn on the expression of podoplanin only after they have completely exited the vein (Yang et al. 2012; Hagerling et al. 2013). So how do platelets that are within the blood vessels interact with podoplanin that is expressed on LECs outside the vein? We can predict three non-exclusive possibilities: (1) podoplanin could be turned on in venous LEC progenitors and interact with platelets immediately before the cells migrate away from veins, (2) there could be micropores within the venous vessel wall through which LECs and platelets interact, and (3) LECs and platelets may exclusively interact at lymphovenous valves, which start forming around E11.5 and are the only sites where blood and lymphatic vessels connect directly (Srinivasan and Oliver 2011).

The ballooning model

Francois et al proposed an alternative “ballooning” LEC migration model in which PROX1⁺ cells first assemble on the walls of the vein as “pre-lymphatic clusters” (Francois et al. 2012). These clusters undergo delamination much like the inflation of a balloon to form the lymph sac (Figure 1C, D). The connection between veins and lymph sacs are later resolved. A few LECs are also seen migrating directly to the periphery of the embryo. According to this model, blood cells enter the balloon-like lymph sacs, thus providing opportunities for LEC-platelet interactions. These blood cells are later pushed back into the veins as the connection between the veins and lymph sacs are resolved. Using a similar model, Uhrin et al proposed that LEC-platelet interaction might resolve these structures either through platelet aggregates or through the recruitment of mural cells to these sites (Uhrin et al. 2010).

Further investigation is needed to elaborate, verify and refine these two models due to the major conceptual differences. Also, these studies have focused on the migration of LECs from the anterior portion of the mouse embryo, primarily around the anterior cardinal vein. Although the anterior cardinal vein makes a major contribution to the total LEC population, LECs also arise from other venous sources, such as the posterior cardinal vein, iliac vein, and azygous vein (van der Putte 1975). It is possible that the LEC migration could be quite distinct from these different sources.

3. Patterning of the lymphatic vessels

LECs that migrate out of the vein interact to form the lymph sacs (Srinivasan et al. 2007). A total of eight lymph sacs are present in mice (Oliver 2004). Jugular, subclavian and posterior lymph sacs are present as pairs on the left and right sides of the body. Jugular and subclavian lymph sacs are present in the anterior portion of the body at the junction of the jugular and subclavian veins. Posterior lymph sacs, which are also known as the iliac lymph sacs, form at the junction of the iliac and posterior cardinal veins. The cisterna chyli and retroperitoneal lymph sac form as individual structures at the junction of inferior vena cava and portal veins. Most of our current understanding about lymph sacs comes from the analysis of jugular lymph sacs, as these are the largest of the lymph sacs and are morphologically easy to identify. Jugular lymph sacs start forming around E11.0, gradually

increasing in size until E13.5. However, the mechanisms controlling the formation of lymph sacs are not completely understood.

LECs begin to sprout from the jugular lymph sacs at E13.5, giving rise to the primitive lymphatic plexus of the limbs, lungs, heart and skin in the anterior portion of the body (Srinivasan et al. 2007). It is possible that at least a sub-population of LECs from the veins could also directly migrate to the tissues (Francois et al. 2012). Nevertheless, lymph sacs are considered to be the primary source of most of the lymphatic vessels. Consequently, jugular lymph sacs are largely reduced in size by E17.5. Rapid embryonic growth likely contributes to this change in jugular lymph sac architecture. Other lymph sacs would also be expected to undergo size reduction while giving rise to the lymphatic vessels in their vicinities of the growing embryo. The only clearly visible sac-like structure in adult mammals is the cisterna chyli, which collects lymph from the intestine and other abdominal tissues and transfers it to the thoracic duct.

The developmental necessity for the formation of lymph sacs is currently unclear, since it is conceivable that LECs could migrate directly from the veins to tissues to form lymphatic vessels. Lymph is returned back to blood circulation only at the junction of the jugular lymph sacs with the jugular and subclavian veins. Lymphovenous valves form at this site, regulating the unidirectional flow of lymph while preventing blood from entering into the lymphatic vessels (Srinivasan and Oliver 2011). So, it is tempting to speculate that the formation of lymph sacs minimizes the number of sites at which lymphatic and blood vessels interact, thus minimizing the chances of blood entering into the lymphatic vessels.

Sprouting of LECs from the lymph sacs is a stereotypic process that results in a honeycomb-like patterned lymphatic plexus in the skin (Figure 2A). Unlike retinal blood vessels, which have a similar pattern (Figure 2B) dictated by the intricate crosstalk between Vegf, ephrin-B2 and Notch signaling pathways (Adams and Alitalo 2007), the mechanisms controlling the patterning of the lymphatic plexus are poorly understood. However, *Vegfc*, *Prox1*, *Foxc2*, *Nrp2* and *Aspp1* are known to be necessary for its proper formation (Yuan et al. 2002; Karkkainen et al. 2004; Petrova et al. 2004; Harvey et al. 2005; Hirashima et al. 2008). Mutation in these genes results in hypoplasia of the lymphatic plexus. A reduction in LEC proliferation was reported in *Nrp2*^{-/-} embryos (Yuan et al. 2002). In *Aspp1*^{-/-} embryos, LECs remained as isolated ball-like structures (lymphatic islands) that never fully fused to form the lymphatic plexus (Hirashima et al. 2008). Future research will likely link these and other molecules in a mechanistic manner.

At E15.5 the lymphatic plexus lacks any obvious structural heterogeneity and undergoes maturation in a stepwise manner (Yao et al. 2012). Fully developed lymphatic vasculature consists of lymphatic capillaries and lymphatic collecting vessels (Tammela and Alitalo 2010).

Lymphatic capillaries

Lymph is collected from the interstitial space through the lymphatic capillaries. LECs that are loosely connected to each other through discontinuous button-like junctions are the only cellular components of lymphatic capillaries (Baluk et al. 2007). Lymphatic capillaries are

devoid of basement membrane or perivascular mural cells such as pericytes and smooth muscle cells (Figure 1E). This allows efficient interaction between LECs and the interstitial fluid. LECs in the capillaries interact with the extracellular matrix through anchoring filaments (Figure 1E). When the interstitial fluid pressure increases, anchoring filaments pull the LECs to create intercellular openings that allow the entry of lymph into the capillaries (Tammela and Alitalo 2010).

LECs in the primitive lymphatic plexus are connected to each other through zipper-like tight junctions (Yao et al. 2012). The mechanisms that control the reorganization of these cell junctions in the mature lymphatic capillaries are not understood. We also have very little information regarding the formation and function of anchoring filaments. A secreted glycoprotein called Emilin1 is the only molecule that has so far been shown to be important for the formation of anchoring filaments (Danussi et al. 2008).

The lymphatic capillaries in *Foxc2*^{-/-} embryos occasionally contain blood and are abnormally covered with mural cells (Petrova et al. 2004). Platelet-derived growth factor subunit B (PDGFB) is normally expressed in blood endothelial cells to recruit mural cells (Gaengel et al. 2009). Angiopoietin-2 (ANG2) is normally expressed in the collecting lymphatic vessel LECs for the same purpose (Gale et al. 2002). However, in *Foxc2*^{-/-} embryos PDGFB and ANG2 are abnormally expressed in the lymphatic capillaries, presumably resulting in the recruitment of mural cells (Petrova et al. 2004; Norrmen et al. 2009). Interestingly, the lymphatic capillaries of *Ang2*^{-/-} mice are also abnormally covered with mural cells (Dellinger et al. 2008). These apparently conflicting sets of data indicate that mechanical forces such as lymph flow could dictate the maturation of lymphatic capillaries. Dysfunctional lymphatic vessels might recruit mural cells to prevent vascular rupture or to pump out the lymph. In support of this proposal, mice in which *Prox1* was deleted after the formation of lymphatic vessels develop blood-filled lymphatic vessels and mural cell coverage on capillaries (Johnson et al. 2008). Endothelial cell-specific deletion of T-synthase also results in the blood-filled lymphatic capillaries and abnormal mural cell coverage (Fu et al. 2008).

Lymphatic collecting vessels

Lymph from the capillaries drains into lymphatic collecting vessels. Collecting vessels have LECs with continuous zipper-like junctions and are covered with pericytes and smooth muscle cells that “pump” lymph (Baluk et al. 2007; Muthuchamy and Zawieja 2008). Collecting vessels also have valves that regulate the unidirectional flow of lymph (Norrmen et al. 2009).

Mouse mesenteric lymphatic vessels have so far been the primary model used to characterize the maturation of collecting lymphatic vessels. Using this model, Tatiana Petrova's lab elegantly characterized the steps involved in the maturation of collecting lymphatic vessels (Norrmen et al. 2009). At E15.5 mesenteric lymphatic vessels have a highly branched mesh-like appearance. In the subsequent days (E16.5-P10) these vessels minimize the number of branches and the vessel diameter. They also acquire mural cell coverage and interluminal valves. During these steps, expression of LEC markers like PROX1 and VEGFR3 are downregulated in the collecting vessels. ANG2 and FOXC2 are

known to be necessary for the proper maturation of lymphatic vessels. In *Ang2*^{-/-} mice the mesenteric lymphatic plexus remains highly branched, and the mural cells do not properly cover these vessels (Gale et al. 2002). In *Foxc2*^{-/-} embryos, LECs in the mesenteric lymphatic plexus express high levels of PROX1, LYVE1 and VEGFR3 (Norrmen et al. 2009). It is not clear if mural cells are recruited to the mesenteric plexus of *Foxc2*^{-/-} embryos or not.

A subpopulation of cells within the collecting vessels upregulate the expression of PROX1 and FOXC2 (Norrmen et al. 2009). Upregulation of additional genes such as Integrin- α 9, Connexin 43, Connexin 37 and Gata2 leads to the maturation of lymphatic valves (Bazigou et al. 2009; Kanady et al. 2011; Kazenwadel et al. 2012). The labs of Tatiana Petrova, Taija Makinen and Alex Simon have done pioneering work on the development of lymphatic valves and have identified numerous genes that are involved in their development. These findings are reviewed elsewhere (Bazigou and Makinen 2013).

Collecting vessels either directly drain into the jugular lymph sacs or into the thoracic duct, which is the longest collecting lymphatic vessel that drains into the jugular lymph sac. Starting around E13, the thoracic duct develops from the azygous vein as distinct clusters of cells eventually fusing with each other to form a single collecting vessel that lies parallel to the dorsal aorta (van der Putte 1975). This developmental distinction indicates that the mechanisms controlling thoracic duct formation could be different from that of other collecting vessels. In support of this hypothesis, *Connexin 43*^{-/-} embryos have tortuous, immature thoracic duct although their mesenteric vessels are morphologically normal except for the lack of valves (Kanady et al. 2011).

Since lymphatic plexus formation, collecting vessel maturation and valve formation are interlinked processes we need to be cautious while interpreting the phenotypes of mutant mice. It is possible that a valve deficiency phenotype in a mutant mouse line could be secondary to defective plexus formation or collecting vessel maturation. It is also possible that the same gene could play distinct roles at different time points. For example, *Foxc2* is modestly expressed in all endothelial cell types, although its expression is enhanced in venous and lymphatic valves (Petrova et al. 2004; Seo et al. 2006; De Val et al. 2008). FOXC2 is also important for the development of vascular smooth muscle cells (Lagha et al. 2009). Consistently, *Foxc2*^{-/-} embryos have a complex phenotype with defects in lymphatic plexus formation, lymphatic capillaries, lymphatic collecting vessels and valves (Petrova et al. 2004; Norrmen et al. 2009). Therefore, cell type- and time specific- deletion of *Foxc2* might allow us to better dissect the role of FOXC2 in lymphatic development.

4. Maintenance of proper blood-lymphatic separation

Lymphatic vessels return lymph to the blood circulation. But, as described earlier, blood and lymphatic vessels interact with each other only at the junction of the jugular and subclavian veins through two pairs of lymphovenous valves (Srinivasan and Oliver 2011). Lymph is unidirectionally drained into the venous circulation through these valves, while blood is prevented from entering into the lymphatic vessels (Srinivasan and Oliver 2011). Except for dendritic cells and other antigen presenting cells, very few blood cells if any are observed

within lymphatic vessels. The same podoplanin-CLEC2 signaling pathway that regulates the establishment of blood-lymphatic separation during development actively maintains this separation in mature adults (Abtahian et al. 2003; Fu et al. 2008; Hess et al. 2014). Treating adult animals with CLEC2-targeting antibodies results in blood-filled lymphatic vessels (Hess et al. 2014). Although valves are considered to be passive regulators of fluid flow, this recent finding showed that platelet aggregates at lymphovenous valves prevent the entry of blood into the lymphatic vessels (Figure 1E). In mouse models with defective lymphovenous valves, the platelets aggregate on the upstream lymphatic collecting valves to prevent the aberrant entry of blood further into the lymphatic system (Hess et al. 2014). This important finding raises numerous questions. What is the average size of these platelet aggregates? How do these platelet aggregates prevent blood regurgitation while allowing lymph flow? What is the rate of platelet aggregate turn over? Since platelet aggregates on cardiac and venous valves result in inflammation and valve damage, how are lymphovenous valves not damaged? And, can the platelet aggregates on lymphovenous valves dislodge into the blood circulatory system and result in thromboembolism?

In addition to thrombus formation, platelets might regulate blood-lymphatic separation by other mechanisms. Since podoplanin-CLEC2 interactions trigger platelet aggregation with a long lag phase, secretion of platelet granules might contribute to blood-lymphatic separation. Platelet derived BMP-9 was proposed to seal blood-lymphatic connections by constricting of endothelial tubes (Suzuki-Inoue et al. 2007; Osada et al. 2012). Likewise, interaction between podoplanin and CLEC2 maintains vascular integrity in the high endothelial cell venules of lymph nodes by triggering the secretion of sphingosine-1-phosphate (S1P) from platelets, which stabilizes endothelial cell adherens junctions (Herzog et al. 2013). S1P signaling might likewise stabilize blood endothelial cell junctions at blood-lymphatic interfaces to promote separation of the systems.

Clearly our understanding of platelet function in lymphatic vascular integrity is incomplete. Future research will hopefully provide novel insights about this intriguing phenomenon.

5. Looking forward

We currently know more about angiogenesis (growth of blood vessels), than about lymphangiogenesis primarily due to the availability of simple and established models for studying angiogenesis, such as the retinal angiogenesis and three-dimensional matrix/endothelial cell culture assays. Preliminary results from such assays help generate hypotheses that are genetically testable using mouse and zebrafish models. Intravital imaging is a popular tool for imaging angiogenesis in mouse embryos (Lawson and Weinstein 2002; Udan et al. 2013). Efforts are ongoing to build such a repertoire of models and technologies to study lymphangiogenesis. A lymphatic ring assay was recently reported for ex vivo analysis (Bruyere et al. 2008). For in vivo studies, *Xenopus* may become a useful model to screen for genes and small molecules that regulate lymphatic vascular development in a high-throughput manner (Ny et al. 2005). Recently generated mouse models can likewise providing an opportunity to perform live imaging of developing lymphatic vessels (Choi et al. 2011; Truman et al. 2012; Hagerling et al. 2013). And, zebrafish are becoming increasingly important models for studying lymphatic vascular development and function

(Kuchler et al. 2006; Yaniv et al. 2006). But, we have to be aware of the limitations and differences between mice and zebrafish. For example, lymph sacs and valves are absent in fish (Koltowska et al. 2013). Moreover, conflicting reports debate the dispensability of *Prox1*, *Coup-TFII* and *Sox18* for lymphatic development in zebrafish (Yaniv et al. 2006; Aranguren et al. 2011; Cermenati et al. 2013; van Impel et al. 2014). This raises concerns about the validity of zebrafish as a useful model for making functionally significant interpretations about mammalian lymphatic vessels. Is it possible that the zebrafish and mammalian lymphatic vessels are functionally similar but morphologically and developmentally unique (like the gills and lungs)? It is worth pointing out that podoplanin and Clec2 are absent in zebrafish.

In conclusion, in the last 20 years we have made significant progress in understanding lymphatic vascular development and maturation. But, as highlighted throughout this article, important questions remain. Undoubtedly, new information will eventually allow us to achieve our goal of developing therapies for diseases related to abnormal lymphatic vascular development and function.

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Highlights

1. Lymphatic endothelial cells (LECs) originate from embryonic veins.
2. LECs become lymphatic capillaries, lymphatic collecting vessels and valves.
3. Perivascular cells surround collecting lymphatic vessels and propel lymph.
4. Lymph is returned back to blood via two-pairs of lymphovenous valves.
5. Platelets regulate blood and lymphatic vascular separation.

Figure 1. Development of lymphatic vasculature

A, B) Budding model: LECs originate from the veins as interconnected clusters of cells to maintain vascular integrity. These LECs interact to form the lymph sacs. Platelets likely initiate the separation of blood and lymphatic vasculatures at the lymphovenous valve rudiments.

C, D) Ballooning model: LECs form pre lymphatic clusters on the vein which balloon out and inflate to form the lymph sacs. Platelets interact directly with the ballooning LECs and cause the separation between blood and lymphatic vasculatures. A few LECs directly sprout out from the veins.

E) LECs sprout from the lymph sacs to form the primitive lymphatic plexus. Subsequent maturation results in lymphatic capillaries and lymphatic collecting vessels. Lymphatic capillaries collect lymph from the interstitium and drain it into the collecting lymphatic vessels. Collecting lymphatic vessels have perivascular cell coverage to propel lymph and valves to prevent regurgitation. Thoracic duct is the longest collecting lymphatic vessel in the mammalian body that returns lymph to the blood circulation via the lymphovenous valves. The proper separation between blood and lymphatic vasculatures is maintained by platelet aggregates at the lymphovenous valves.

CV, cardinal vein; LS, lymph sac; IJV, internal jugular vein; SCV, subclavian vein; TD, thoracic duct; LC, lymphatic capillary; VEC, venous endothelial cell; LEC, lymphatic endothelial cell; LECP, lymphatic endothelial cell progenitor; PLC, pre lymphatic cluster;

LVV, lymphovenous valve; SMC, smooth muscle cell; LV, lymphatic valve; AF, anchoring filament.

Figure 2. Lymphatic and blood vessels have similar pattern

A) Immunohistochemistry on the peripheral skin of E15.5 embryos using LEC-specific Prox1 (red) and Vegfr3 (green) antibodies.

B) Blood vessels in the retina of a 10-days old mouse pup immunostained using PECAM1.