Lymphangiogenesis: Origin, Specification, and Cell Fate Determination

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Annu. Rev. Cell Dev. Biol. 2016. 32:677–91

First published online as a Review in Advance on June 1, 2016

The *Annual Review of Cell and Developmental Biology* is online at cellbio.annualreviews.org

This article's doi: 10.1146/annurev-cellbio-111315-124944

Keywords

lymphatic vessels, lymphatic endothelial cell, cardinal vein, endothelial cell, Prox1, mouse embryo

Abstract

The two vascular systems of our body are the blood and the lymphatic vasculature. Our understanding of the genes and molecular mechanisms controlling the development of the lymphatic vasculature network has significantly improved. The availability of novel animal models and better imaging tools led to the identification of lymphatics in tissues and organs previously thought to be devoid of them. Similarly, the classical textbook list of established functional roles of the lymphatic system has been expanded by the addition of novel findings. In this review we provide a historical perspective of some of the important landmarks that opened the doors to researchers working in this field. We also summarize some of the current views about embryonic lymphangiogenesis, particularly about the source(s), commitment, and differentiation of lymphatic endothelial cells.

Contents

INTRODUCTION

The lymphatic vascular system is a one-way network of thin-walled capillaries and larger vessels coated by a continuous layer of endothelial cells (ECs). Among its main functions, it maintains fluid homeostasis; drains the protein-rich lymph, small molecules, resorbed fat, and cells (collectively termed lymph) from the tissue extracellular spaces; and returns these substrates to the venous system via the thoracic duct or the right lymphatic duct (Proulx et al. 2013, Tammela & Alitalo 2010, Wigle & Oliver 1999). Other functional roles of the lymphatics include reverse cholesterol transport, absorption of dietary fat, transport of antigen-presenting cells to lymph nodes, management of immune cell trafficking and inflammation, regulation of blood pressure, and metabolic and adipose tissue regulation (Dieterich et al. 2014, Hirakawa et al. 2003, Kerjaschki 2014, Lim et al. 2013, Machnik et al. 2009, Randolph et al. 2005, Wiig et al. 2013). Failure of lymphatic vasculature function leads to a diverse type of phenotypes such as lymphedema, atherosclerosis, 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). A complete absence of lymphatic vessels is incompatible with life (Tammela & Alitalo 2010, Wigle & Oliver 1999).

In the past decade, our understanding of the molecular mechanisms regulating the formation of the lymphatic vasculature network has considerably improved, thanks mainly to the increase in the number of investigators interested in lymphatic research and to the use of new technologies. Both of these factors helped with the identification of genes whose activity proved to be necessary during different aspects of developmental and postnatal lymphangiogenesis in health and disease. Here, first we summarize some of the remarkable pioneering early reports describing the lymphatic system. Next we summarize our current knowledge about the origin(s) of lymphatic endothelial cells (LECs). Finally, we review the stepwise process leading to LEC cell fate commitment and specification.

HISTORICAL CONTEXT

Historically, some of the initial reports about what we know today are some of the components of the lymphatic system can be tracked back to ancient civilizations. Hippocrates (460–377 BC) coined the terms white blood and chyle, and Herophilus (335–280 BC) first noticed the existence of mesenteric lymph nodes and "milky veins" (lacteals) (Chikly 1997). Through the dissection of cadavers, Erasistratus of Chios (310–250 BC) later observed that digested food was fragmented into droplets termed chyle (Chikly 1997). The rediscovery of the mesenteric vessels was mostly

due to the physician Gasparo Aselli (1581–1626), who noted the lacteal vessels of the lymphatic system while dissecting a well-fed dog in 1622. He observed thin, white vessels distributed within the mesentery and above the upper intestinal wall. When the vessels were cut, a thin, milky-white liquid "gushed out" (Ambrose 2007). Aselli later confirmed his observations in cats, sheep, calves, cows, horses, and goats (Chikly 1997). In 1629, Jacques Mentel (1599–1670) observed that mesenteric lacteals end in the thoracic duct before entering into the bloodstream (Chikly 1997). In 1647, Jean Pecquet (1622–1674) discovered the cisterna chyli in a canine. After removal of the heart, Pecquet noticed white fluid (chyle) emerging from the superior vena cava, and after tracing its source, he found a chyle reservoir that he termed "receptaculum chyli," which corresponds to what we know today as cisterna chyli. Later, in 1651 he observed that the lacteals connect with the cisterna chyli and with the thoracic duct at its abdominal level and concluded that the chyle enters the venous system at the junction of the left subclavian and jugular veins. He also described the presence of valves within lymphatics (Ambrose 2007). Almost simultaneously and without knowing Pecquet's results, Olaus Rudbeck (1630–1702) also noticed the thoracic duct tracking back the "white liquid" emanating after heart removal from a calf. By performing a retrograde dissection of such a duct, he observed a slight enlargement that he termed "saccus lacteus" or "vesicula chyli" (cisterna chyli) (Chikly 1997). Rudbeck continued to perform vivisections in different mammals and concluded that lacteals, vesicula chyli, and the thoracic duct end in the jugular subclavian veins of the neck and that the lymphatic network is an integrated system of closed vessels inside the whole body similar to arteries and veins (Chikly 1997).

WHAT ABOUT ARTERIES AND VEINS?

The vertebrate blood vascular system is one of the first organs to form during embryogenesis. In the mouse, the first blood vessels form by the aggregation of angioblasts, the precursors of blood endothelial cells (BECs), at approximately embryonic day (E)8.0 (Drake & Fleming 2000). The first embryonic vessels to acquire a pattern are the arteries, which differentiate in a stepwise manner; veins emerge slightly later, progressively forming and following the turning of the embryo (the 9–11-somite stage) (Chong et al. 2011, Kim et al. 2008). The dorsal aorta (DA) is the first vessel to form during murine vascular morphogenesis, and its formation is concomitant with heart formation (Calnek et al. 1978). The DA emerges before the cardinal vein (CV) by de novo differentiation and aggregation of angioblasts (Coffin & Poole 1988, Drake & Fleming 2000, Kim et al. 2008, Lindskog et al. 2014).

Approximately a century ago and using cultured chick embryos, Florence Sabin proposed that the primitive CV begins as a growth from the wall of the aorta as "longitudinal anastomosis between direct branches of the aorta" (Sabin 1917). Years later, studies using electron microscopy in chick embryos and immunostaining in quail embryos refuted this aortic origin. Those studies proposed that the CV forms in situ by the de novo differentiation of angioblasts segregated from the mesoderm (Coffin & Poole 1988, Hirakow & Hiruma 1981). However, a recent characterization of ECs in mouse and zebrafish embryos argued that the CV is established by selective migration of venous-fated angioblasts from the primitive DA (Herbert et al. 2009, Lindskog et al. 2014). The developing murine DA transiently contains a heterogeneous population of ECs expressing either the venous molecular markers Coup-TFII and ephrin (Eph)B4 or the arterial molecular markers EphB2, Notch, and connexin 40. Approximately 15% of those ECs are venous fated at the 4–5-somite stage, and subsequently the DA starts to exhibit uniform expression of arterial markers (Lindskog et al. 2014). Thus, according to those results the DA forms by vasculogenesis, whereas CV formation apparently involves a mechanism requiring selective sprouting of venous-fated ECs from the DA. Furthermore, recent results in zebrafish proposed that the CV is a heterogeneous

structure that contains a pool of specialized angioblasts within the floor, with the potential to result in an arterial, a venous, or a lymphatic fate (Nicenboim et al. 2015).

ABOUT THE ORIGIN: HOW MANY SOURCES OF LYMPHATIC ENDOTHELIAL CELLS ARE NEEDED TO FORM FUNCTIONAL LYMPHATICS?

Until the late nineteenth century it was thought that "lymphatics arose by the dilatation of tissue spaces caused by the fluid that exuded from the blood vessels, and that this dilation of the spaces began in the periphery" (Sabin 1913). Therefore, the idea that lymphatic vessels developed from the periphery to the center was widely accepted. This idea started to change thanks to studies done by Louis Ranvier from 1895 to 1897. By injecting lymphatics in a variety of embryos, he published a long series of articles on the development of the lymphatic system. These studies established that lymphatic vessels develop from the center to the periphery and proposed the hypothesis that lymphatics originate from veins (Sabin 1913). With this hypothesis in mind, a few years after Ranvier's studies, Sabin (1902) performed a series of elegant systematic experiments in pig embryos and concluded that lymphatics originate from veins. By injecting ink into these embryos, she showed that the first lymphatics bud from the anterior CV to form two jugular lymph sacs near the junction of the subclavian and anterior CV. Later, another pair of sacs originate from the mesonephric vein and veins in the dorsomedial edge of the Wolffian bodies. The retroperitoneal lymph sac forms near the primitive inferior vena cava and mesonephric veins, and the posterior lymph sacs appear at the junctions of the primitive iliac veins and the posterior CVs (Sabin 1902). "At the same time there is a growth of ducts along the dorsal line following the aorta to make a thoracic duct from which the lymphatics grow to the various organs" (Sabin 1902). Sabin's results led her to conclude that there are eight lymph sacs: three paired and two unpaired. The paired lymph sacs are the jugular, subclavian, and posterior lymph sacs, and the unpaired lymph sacs are the cisterna chyli and the retroperitoneal (mesenterial) lymph sac (Wilting et al. 2006). The jugular sacs drain the anterior half of the body, the iliac sacs drain the posterior half of the body, the retroperitoneal sac drains the diaphragm and the abdominal viscera, and the cisterna chyli and the thoracic duct connect the jugular and renal lymphatics (Sabin 1913).

Together, the results from Ranvier (1897), Sabin (1902, 1913), and Lewis (1905, 1909) indicated that the lymphatic endothelium originated from a few sprouts derived from the venous system (van der Putte & van Limborgh 1980). Although this was the most popular model at that time, Huntington & McClure (1910), in contrast, proposed a centripetal model. Their observations were based upon the study of wax reconstructions of work performed in cat embryos and led them to conclude that lymphatics originate from the fusion of perivascular mesenchyme–derived cells that only secondarily communicate with the veins (van der Putte & van Limborgh 1980). For more than a century, work performed in different animal models favored one or the other hypothesis or led to more conciliatory ideas indicating that both mechanisms were probably involved in lymphatic vessel formation.

Many years later, a systematic anatomical characterization of mice and human embryos (van der Putte 1975, van der Putte & van Limborgh 1980) provided further support to Sabin's views. These investigators concluded that, at least in mammals, all the main lymphatics are derived from the endothelial lining of the venous wall. Once the initial sacs form, they rapidly enlarge; fuse with each other; and produce sprouts that move into peripheral regions, usually along veins (van der Putte & van Limborgh 1980). Four decades later, additional support for this view was provided using detailed Cre/loxP-based lineage-tracing approaches in mice that led to the conclusion that most, if not all, embryonic mammalian LECs are venous derived (Srinivasan et al. 2007). It was proposed

that in the mouse embryo, the lymphatic vascular network begins to form in the endothelium of the CV and intersomitic vessels at approximately E9.5 (Hagerling et al. 2013, Srinivasan et al. 2007, Yang et al. 2012). Years later, in vivo imaging in transgenic zebrafish embryos (Yaniv et al. 2006) provided further support to the venous origin of LECs. Yaniv et al. (2006) showed that in zebrafish, the initial thoracic duct sprouts emerge adjacent to the DA and then grow and spread across the trunk to join with other segments and form a continuous thoracic duct (Yaniv et al. 2006).

However, the debate was not over, as quail-chick chimera homotopical grafting experiments argued that in birds, the lymphatic vasculature originated from two sources: The deep parts of the jugular lymph sacs were derived from the jugular segments of the CVs, whereas the superficial parts developed by integration of lymphangioblasts from the nearby somites (Schneider et al. 1999, Wilting et al. 2006). A dual origin was also suggested for *Xenopus* tadpoles (Ny et al. 2005). The same group that reported on the dual lymphatic origin in birds also proposed alternative sources for at least some murine LECs. This group suggested that scattered mesenchymal cells that upregulate lymphatic endothelial characteristics and downregulate leukocyte features integrate into the venous-derived growing lymphatics (Buttler et al. 2006).

Debate about the origin of the lymphatic vasculature continues. Some recent evidence agrees with the proposal that most embryonic lymphatics are indeed venous derived (Srinivasan et al. 2007), but other evidence suggests that additional sources contribute to LECs in specific organs or tissues. For example, a detailed characterization of the vascularization of the intestine using chick-quail chimeras and transgenic mice revealed a dual origin of gut lymphatics: Prior arterial growth is a necessary step to form local lymphatics and a subsequent connection of lymphatics with the vascular network (Mahadevan et al. 2014). Similarly, other recent results using lineage-tracing analyses in mice also supported a regional hybrid mechanism of lymphatic vascular development in which lymphatic vessels in different regions of the skin (cervical versus lumbar) develop via different mechanisms (Martinez-Corral et al. 2015). Martinez-Corral et al. (2015) showed that the embryonic dermal vasculature contains isolated cells expressing LEC markers but that these cells are physically separated from the developing lymphatic network. Further examination by fluorescence-activated cell sorting suggested that approximately one-third of the skin lymphatic vasculature is not derived from Tie2 lineage-labeled cells. On the basis of those results, these authors proposed a centrifugal-centripetal dual origin of the mammalian dermal vessels: Lymphatic vessels of the cervical and thoracic skin develop via sprouting from venous-derived lymph sacs, whereas some dermal vessels of the lumbar area form by assembly of non–venous lineage cells into clusters and vessels through a process that the authors defined as lymphvasculogenesis (Martinez-Corral et al. 2015) (**Figure 1**). In this same work, using *Vav-Cre* mice for lineage tracing, the authors also excluded cells from the definitive hematopoietic lineage as a source of LECs (Martinez-Corral et al. 2015). This same group also argued that part of the mesenteric lymphatic vasculature originates from cKit lineage of hemogenic endothelial origin (Stanczuk et al. 2015). A heterogeneous cellular origin of LECs was also recently proposed for cardiac lymphatic vessels in mice, in which approximately one-fifth of the cardiac lymphatic network was proposed to be formed independently of venous sprouting (Klotz et al. 2015).

In summary, all these data seem to indicate that, a couple of centuries after lymphatic research began, the debate about lymphatic origin continues, and although it is evident that most LECs are venous derived, a small percentage of organ- and/or tissue-specific LECs may originate from some other source(s) that has not yet been fully identified. It will now be important to determine whether removal of that small percentage of LECs originating from sources other than the veins has any functional impact on embryonic or postnatal lymphangiogenesis.

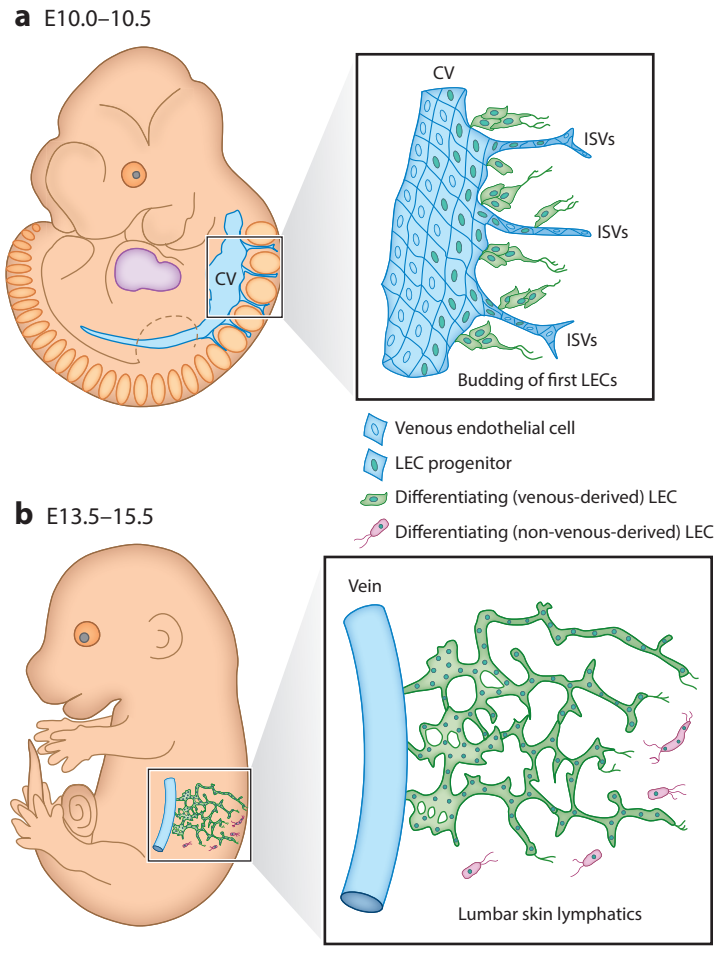

Figure 1

Schematic representation of the development of the mouse lymphatic vasculature. (*a*) Venous sprouting of Prox1-positive lymphatic endothelial progenitor cells emerging from the cardinal veins (CVs) and intersomitic veins (ISVs) of the mouse embryo at approximately E9.5 generates the different lymph sacs. (*b*) In the mouse embryonic skin, most lymphatic vessels originate from venous sprouting (*green* plexus). However, new reports argue that there is also a small contribution from nonvenous progenitors to the formation of lymphatic vessels of the thoracic and lumbar regions (*magenta*). LEC denotes lymphatic endothelial cell.

COMMITMENT AND DIFFERENTIATION OF MAMMALIAN LECS

As discussed above, most embryonic mammalian LECs have a venous origin; therefore, a very important question that remains partially unanswered is how venous BECs become eventually committed toward a LEC precursor cell fate. Lymphatic vascular development starts later than development of the blood vascular system. In the mouse, the first LEC precursors are detected in the jugular region of the CV at approximately E9.5 (Srinivasan et al. 2007). These first committed LEC precursors can be identified by the specific expression of the transcription factor Prospero Homeobox 1 (Prox1) (Wigle & Oliver 1999). We proposed that Prox1 expression in those LEC precursors in the CV is the earliest indication that venous BECs are committed toward a LEC

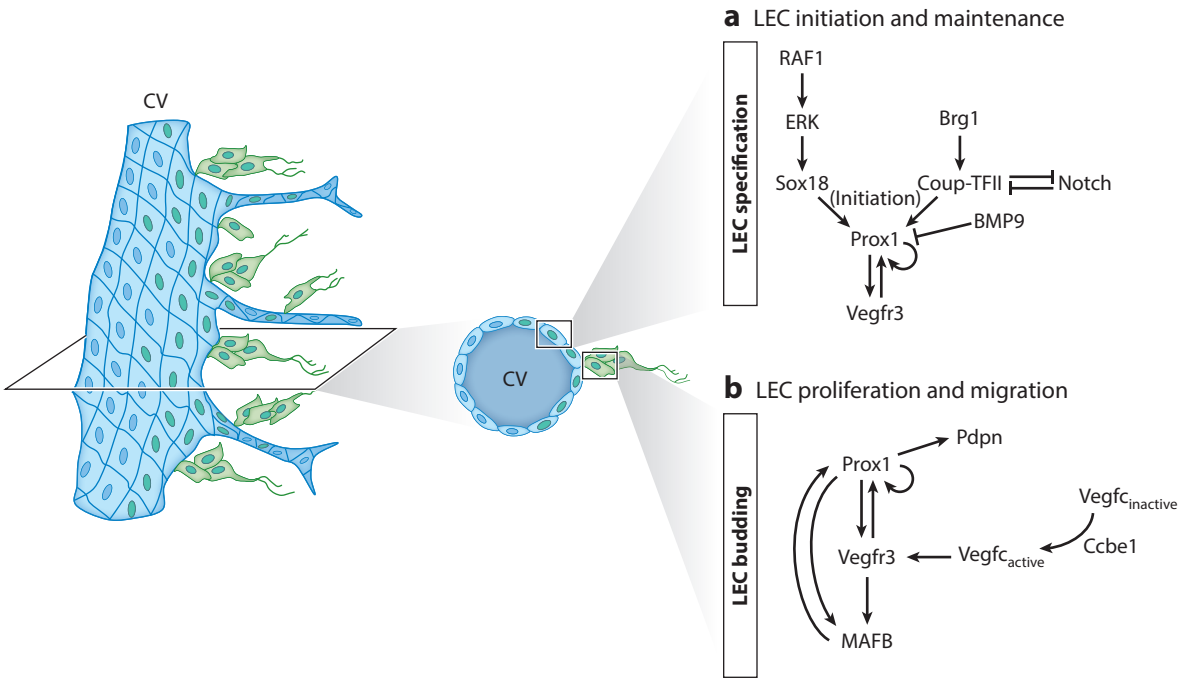

Figure 2

Schematic model of molecular players and signaling pathways involved in mammalian lymphatic endothelial cell (LEC) specification and budding. The cardinal vein (CV) is the main source of LECs. (*a*) Early during development, the mouse CV expresses Coup-TFII and Sox18. These two transcription factors induce the expression of Prox1 in a subpopulation of venous endothelial cells that will become specified as LEC progenitors. Brg1, a member of the SWI/SNF protein family, can epigenetically regulate Coup-TFII activity by direct binding. Additionally, Notch signaling is repressed in the specified LEC progenitors by Coup-TFII activity. Prox1 maintains Vegfr3 expression in LECs, and in turn, Vegfr3 regulates Prox1 by establishing a feedback loop required to maintain LEC identity. BMP9 downregulates the expression of Prox1, which is necessary to maintain LEC identity. (*b*) At approximately E10.5, most of the LEC progenitors start to bud off from the CV and intersomitic veins, guided by a Vegfc gradient in the surrounding mesenchyme and in a Vegfr3-dependent manner. Podoplanin (Pdpn) expression in budding LECs is an indication that lymphatic differentiation has begun. As LECs bud off, they assemble together and start to form the different lymph sacs (at ~E11.5), intermediate structures from which, after proliferation and sprouting, most of the lymphatic network will be derived. Ccbe1 modulates Vegfc activity by converting inactive, full-length Vegfc into an active form. MAFB is a transcription factor specifically activated by Vegfr3 and induces Prox1 expression in differentiated LECs.

fate (Oliver 2004, Oliver & Alitalo 2005, Oliver & Srinivasan 2010). As lymphatic development progresses, the number of Prox1-expressing LEC precursors in the CV increases, and at approximately E10.5, they begin to bud off from the CV to eventually give rise to the different lymph sacs (Wigle & Oliver 1999, Wigle et al. 2002).

Even when both blood and lymphatic vessels are lined by ECs and they share the expression of common markers, they are also distinguishable by the differential expression of specific molecular markers and morphological features. In the last decade, our knowledge about the genes and mechanisms regulating the formation of the lymphatic vascular network has considerably improved. In **Figure 2**, we illustrate the identified regulatory network required to convert venous endothelium into lymphatic endothelium. Additionally, below we briefly summarize the earliest and best-characterized players involved in LEC fate commitment and differentiation.

Prox1 was the first gene to be identified whose activity was necessary for lymphatic vascular development, and it is considered to be the master regulator of the program specifying LEC fate (Hong & Detmar 2003, Oliver 2004, Wigle & Oliver 1999). The polarized expression of Prox1 in the CV is one of the earliest signs that the program leading to the commitment and specification of LEC fate has initiated. In the mouse, Prox1 expression begins at approximately E9.5 in a few scattered BECs in the anterior CV (Wigle & Oliver 1999). At this point, these first Prox1-positive cells are considered to be LEC progenitors. At E10.5, the number of venous LECs has increased, and they begin to bud off from the CV and the intersomitic vessels into the surrounding mesenchyme in a dorso-anterior manner (Wigle & Oliver 1999, Wigle et al. 2002, Yang et al. 2012). By E12.5, the number of Prox1-positive cells adjacent to the CV has clearly increased (Wigle et al. 2002). In vitro studies have also shown that Prox1 overexpression is sufficient to induce a lymphatic fate in blood vascular endothelium by suppressing the expression of a large number of BEC-specific transcripts and by acting as a switch in the program specifying LEC fate (Hong et al. 2002, Petrova et al. 2002). Besides promoting a LEC phenotype, Prox1 activity is also required to maintain LEC fate identity. Conditional downregulation of Prox1 during the embryonic, postnatal, or adult stages is sufficient to reprogram LECs into BECs (Johnson et al. 2008). Therefore, the LEC phenotype depends on constant Prox1 activity for its maintenance, and LEC fate is arguably plastic and reprogrammable (Oliver & Srinivasan 2010).

The transcription factor SRY (Sex-Determining Region Y)-Box 18 (Sox18) is a member of the F-group of Sox genes needed to turn on Prox1 expression in the CV (François et al. 2008). Sox18 binds to two conserved SoxF consensus-binding sites in the Prox1 promoter region (François et al. 2008). These sites are essential for transactivation of the Prox1 promoter both in vitro and in vivo during LEC fate induction. Additionally, Sox18 overexpression in BECs induces Prox1 expression and other lymphatic endothelial markers (François et al. 2008). Similar to the *Prox1*-null phenotype, *Sox18*-null embryos show subcutaneous edema and fetal lethality at approximately E14.5 (François et al. 2008). Additionally, mutations in *SOX18* in humans have been characterized as causing hypotrichosis-lymphedema-telangiectasia (Irrthum et al. 2003). Despite the role of Sox18 in the early induction of Prox1 expression in venous ECs, arterial ECs express Sox18 but do not express Prox1, a result indicating that Sox18 activity, although necessary, is not sufficient for Prox1 expression. Additionally, Sox18 expression is not observed during later stages of embryonic lymphangiogenesis (François et al. 2011).

Chicken ovalbumin upstream promoter transcription factor (Coup-TFII) (also known as Nr2f2), a member of the orphan nuclear receptor superfamily, plays a major role in the acquisition of venous fate by ECs (Chen et al. 2012, You et al. 2005). Coup-TFII is expressed in the venous endothelium at E8.5 (You et al. 2005) and in LECs throughout embryogenesis and adulthood (Lin et al. 2010). Conditional ablation of Coup-TFII at an early embryonic stage resulted in failed formation of lymphatic vessels (Lin et al. 2010). A series of in vitro assays demonstrated that Coup-TFII physically interacts with Prox1, acting as a coregulator of Prox1 to maintain LEC fate (Lee et al. 2009, Srinivasan et al. 2010, Yamazaki et al. 2009). Moreover, Coup-TFII in LECs is also required to maintain the expression of VEGFR3 (Yamazaki et al. 2009). In vivo data revealed that Coup-TFII has a time-dependent role in the regulation of lymphatic vascular development. Initially, Coup-TFII activity is required to maintain Prox1 expression during early stages of LEC specification and differentiation. However, such activity is no longer required after E13.5, as Prox1 expression in specified LECs becomes independent of Coup-TFII expression (Srinivasan et al. 2010).

The vascular endothelial cell growth factor receptor 3 (VEGFR3) is required for angiogenesis and lymphangiogenesis. During embryogenesis, Vegfr3 is expressed in all BECs until E10.5 (Dumont et al. 1998), but after approximately this stage its expression begins to become more

restricted to LECs and is downregulated in BECs (Dumont et al. 1998, Kaipainen et al. 1995, Wigle et al. 2002). Different in vitro and in vivo data demonstrated that Vegfr3 is a target of Prox1 in LECs (Mishima et al. 2007, Pan et al. 2009, Petrova et al. 2002, Srinivasan et al. 2014, Yamazaki et al. 2009). Interestingly, Vegfr3 also regulates Prox1, establishing a feedback loop required to maintain the identity and the number of LEC progenitors (Srinivasan et al. 2014). Similar results were recently reported in zebrafish (Koltowska et al. 2015).

Vegfc is the best-characterized Vegfr3 ligand. In mouse embryos Vegfc expression is detected in mesenchymal cells at E10.5, particularly near the regions where Vegfr3-expressing LECs undergo budding from embryonic veins (Karkkainen et al. 2004, Kukk et al. 1996). In the absence of Vegfc, BECs still commit to the lymphatic lineage fate, as Prox1 expression remains; however, Prox1$^+$ LEC progenitors fail to bud off from the CV (Karkkainen et al. 2004). Therefore, Vegfc is not needed for cell commitment to the LEC lineage within the CV but is required for LEC budding and the subsequent formation of the lymph sacs (Karkkainen et al. 2004, Srinivasan et al. 2014).

Collagen- and calcium-binding EGF domain 1 (CCBE1) is also essential for budding and/or migration of LECs from the anterior CVs (Facucho-Oliveira et al. 2011). By E9.5, Ccbe1 is expressed in the proximity of the anterior CV (Facucho-Oliveira et al. 2011) and acts at the same stage of development as Vegfc by regulating the processing and activation of Vegfc (Hogan et al. 2009, Koltowska et al. 2015). In the same way as Vegfc, targeted inactivation of CCBE1 resulted in decreases in the number of Prox1-positive and lymphatic vessel endothelial HA receptor-1 (Lyve-1)-positive LECs at the level of the CV and lymph sacs; such decreases led to a defective lymphatic vasculature, severe edema, and prenatal death (Bos et al. 2011).

Lyve-1 is widely used as a LEC marker and provides the first indicator of lymphatic endothelial competence (Banerji et al. 1999, Karpanen & Alitalo 2008, Oliver & Srinivasan 2008, Tammela & Alitalo 2010). Lyve-1 is expressed early in development of the venous lymphatic endothelium (Banerji et al. 1999, Gordon et al. 2008, Prevo et al. 2001). It is also expressed on a subpopulation of macrophages (Cursiefen et al. 2004, Schledzewski et al. 2006, Wigle et al. 2002); in blood vessels of the embryonic mouse yolk sac (Gordon et al. 2008); and in the sinusoidal endothelium of the liver, spleen, and lymph nodes (Banerji et al. 1999, Mouta Carreira et al. 2001, Prevo et al. 2001). During mouse development it is uniformly expressed at E9.5–10.5 in the ECs of the CV and intersomitic vessels, prior to the induction of Prox1 expression (Gordon et al. 2008, Wigle et al. 2002). Lyve-1 gene-targeted mice develop normally and exhibit a functional network of lymphatic vessels (Gale et al. 2007). These results indicate that Lyve-1 is nonessential for normal lymphatic development or function; however, the early expression of Lyve-1 together with that of Prox1 is probably one of the first indications that lymphangiogenesis has started. As development proceeds, this subpopulation of Lyve-1- and Prox1-positive LEC progenitors start to express additional lymphatic specification markers and begin to bud from the veins.

Podoplanin (Pdpn), also known as gp38, aggrus, and T1α, is a mucin-type O-glycoprotein expressed in LECs and the fibroblastic reticular cells in lymph nodes (Pan & Xia 2015). In LECs, Pdpn expression in the mouse begins to be detected at approximately E10.5 in the migrating front of the Prox1-positive LECs (Schacht et al. 2003, Yang et al. 2012), only after these cells are out of the CV (Yang et al. 2012) in a process that requires transcriptional activation of Pdpn by Prox1 (Pan & Xia 2015). In vitro data have identified four putative binding elements for Prox1 in the 5′ upstream regulatory region of the Pdpn gene. Prox1 directly binds these binding elements in LECs, indicating that Pdpn functions downstream of Prox1 during lymphatic vasculature development (Pan et al. 2014). The fact that Pdpn expression starts only after LECs leave the CV is probably one of the first molecular indications that the LEC specification and differentiation process has started. Actually, Pdpn is required to initiate and maintain separation of blood and lymphatic vessels via a process that involves Ppdn on the LEC surface and CLEC2, a receptor that activates platelets in

response to LECs, on the platelet surface, resulting in platelet aggregation (Bertozzi et al. 2010, Carramolino et al. 2010, Pan & Xia 2015, Suzuki-Inoue et al. 2010). Pdpn$^{-/-}$ pups die soon after birth with severe lymphedema, a consequence of highly abnormal lymphatic vascular patterning and function (Schacht et al. 2003). Although it was initially thought that aggregated platelets seal off the CV, allowing LECs to bud off (Bertozzi et al. 2010, Uhrin et al. 2010), Hess et al. (2014) reported that such an essential role for platelets in preventing blood from entering the lymphatic system occurs only at the lympho-venous junction. Loss of CLEC2 resulted in backfilling of the lymphatic network with blood from the thoracic duct in both neonatal and mature mice (Hess et al. 2014).

NOVEL FINDINGS

It has been assumed that lymphatic vessels exist in all vascularized organs and tissues except the brain, spinal cord, bone marrow, cartilage, and retina. However, recent findings have challenged some of these assumptions. It has generally been accepted that, because of its lack of lymphatics, the brain is an immune-privileged organ. This belief has been maintained for centuries, despite old papers reporting the presence of these vessels in the head. In 1787 Mascagni described lymphatics in the human dura mater (the most external of the meningeal layers covering the brain) (Bucchieri et al. 2015). Later in 1869, Schwalbe reported the "appearance of colored markers in the cervical lymph nodes after injection into the subarachnoid spaces and contended that the lymphatic system constituted a major drainage pathway for the cerebrospinal fluid (CSF)" (quoted from Foldi 1996). In the 1960s, a Hungarian group described the existence of lymphatics in the central nervous system and noted that lymph drainage played an important part in fluid circulation of the brain (Mezey & Palkovits 2015). Miura et al. (1998) described the structural organization of the lymphatics in the monkey spinal dura mater and lymphatic drainage of the CSF, thus showing the existence of the epidural lymphatics. It was not until 2015 that, by taking advantage of better animal models and imaging resources, two groups confirmed the presence of a lymphatic vascular network in the brain's meninges (Aspelund et al. 2015, Louveau et al. 2015). These lymphatic vessels have anatomical and molecular features characteristic of initial lymphatic vessels, as they express Prox1, Lyve-1, Vegfr3, Pdpn, CCL21, VE-cadherin, Pecam1, and integrin α9 (Aspelund et al. 2015, Louveau et al. 2015). Dural lymphatic vessels absorb CSF from the adjacent subarachnoid space and brain interstitial fluid and then transport it to deep cervical lymph nodes. This function may be important for the clearance of macromolecules from the brain (Aspelund et al. 2015). Dural lymphatic vessels were absent in K14-VEGFR3-Ig transgenic mice that have impaired VEGFC/D-VEGFR3 signaling (Aspelund et al. 2015, Makinen et al. 2001), but injection of recombinant Vegfc into the cisterna magna resulted in an increase in the diameter of the meningeal lymphatic vessels (Louveau et al. 2015). These data indicate that Vegfr3-Vegfc signaling is important for the proper function of dura mater lymphatic vessels and that K14-VEGFR3-Ig transgenic mice represent a model for studying the functional effects of the absence of lymphatic drainage from the brain. Although the dura mater is not a component of the central nervous system (CNS) (Bucchieri et al. 2015), the fact that it covers the brain and the spinal cord argues that a better characterization of dural lymphatics could open up new directions for research on CNS diseases.

Another recent unexpected finding has to do with Schlemm's canal (SC) in the eye. SC is a circumferential channel formed by an endothelium-lined vessel that encircles the cornea. SC is essential for drainage in the eye. Its functions consist of collecting aqueous humor from the anterior chamber and delivering it to episcleral blood vessels via aqueous veins. Blockage of aqueous outflow into SC increases intraocular pressure and results in glaucoma. Glaucoma is a group of eye diseases that produce damage to the optic nerve, which can then lead to irreversible blindness (Aspelund

et al. 2014, Yucel et al. 2009). Until recently, it was not known whether SC was a component of the blood or the lymphatic vascular system; however, new data now argue that SC is a component of the lymphatic vascular network, although with a very unique molecular profile (Aspelund et al. 2014, Karpinich & Caron 2014, Park et al. 2014, Truong et al. 2014). Detailed characterization of SC showed high Prox1 expression on the SC endothelium during the initial stages of SC development (Aspelund et al. 2014, Kizhatil et al. 2014, Truong et al. 2014). SC also expresses Vegfr3, CCL21, integrin α9, CD31, collagen IV, VE-cadherin, and angiopoietin/Tie2 but is negative for (or is very low in expression of) Lyve-1 and Pdpn (Aspelund et al. 2014, Park et al. 2014). Additionally, Vegfc and its receptor VEGFR3 are essential for SC development, and a single intraocular injection of recombinant VEGFC induces SC enlargement that is associated with a trend of decreased intraocular pressure (Aspelund et al. 2014). Moreover, lineage-tracing studies indicated that, similar to normal lymphatic vessels, SC has a blood vascular origin (developing postnatally from trans-scleral veins) and does not originate from the nearby preexisting lymphatic vasculature (Aspelund et al. 2014, Kizhatil et al. 2014). Instead, the ECs of SC acquire lymphatic identity through upregulation of Prox1 as development progresses (Karpinich & Caron 2014, Park et al. 2014). These findings of lymph-like qualities of SC in the eye, and the role of these qualities in aqueous humor drainage, open a novel therapeutic target for glaucoma treatment.

CONCLUSIONS AND FUTURE DIRECTIONS

Although our knowledge of developmental lymphangiogenesis has considerably improved over time, many questions remain unanswered. In particular, the rediscovery of the dural lymphatics and the lymphatic vessels in SC opens new directions in the understanding of novel lymphatic functions in tissues that before were considered alymphatic. Additionally, and thanks to the development of new techniques and technologies, we will be able to better unravel the complexity of specific molecular networks operating during different stages of lymphatic vascular development and in different vascular beds, resulting in data that could be used for the modulation of lymphatic function in particular tissues or organs.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

G.O. is supported by National Institutes of Health grant R01-HL073402 and the Leducq Foundation.

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