

# Getting out and about: the emergence and morphogenesis of the vertebrate lymphatic vasculature

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## Summary

The lymphatic vascular system develops from the pre-existing blood vasculature of the vertebrate embryo. New insights into lymphatic vascular development have recently been achieved with the use of alternative model systems, new molecular tools, novel imaging technologies and growing interest in the role of lymphatic vessels in human disorders. The signals and cellular mechanisms that facilitate the emergence of lymphatic endothelial cells from veins, guide migration through the embryonic environment, mediate interactions with neighbouring tissues and control vessel maturation are beginning to emerge. Here, we review the most recent advances in lymphatic vascular development, with a major focus on mouse and zebrafish model systems.

**Key words:** Lymphatic vasculature, Morphogenesis, Vertebrate embryo

## Introduction

Organ development is a complex process involving reciprocal signals between cells and their surrounding tissues to drive specification, differentiation, growth and morphogenesis. During embryonic development, the blood vasculature develops from embryonic mesoderm via a process known as vasculogenesis: the migration and cohesion of a population of endothelial progenitor cells (Eilken and Adams, 2010; Geudens and Gerhardt, 2011). The embryonic blood vasculature further elaborates via angiogenesis, vessel remodelling and functional specialisation (Eilken and Adams, 2010). Remarkably, it also gives rise to a second vascular system, the lymphatic vasculature (Sabin, 1902; Oliver and Srinivasan, 2010). Lymphatic endothelial cell (LEC) progenitors are specified from venous blood endothelial cells (BECs) in defined locations, and subsequently migrate away from the embryonic veins to form lymphatic vessels (Sabin, 1902). The resulting lymphatic vasculature is a hierarchical network comprising initial, or absorptive, vessels and larger collecting vessels, specialised for lymph transport, that act in a coordinated manner to return lymph to the blood stream. Lymphatic vessels are also crucial for fatty acid absorption and immune cell trafficking (see Box 1). A number of disease states are associated with lymphatic vascular dysfunction (reviewed by Alitalo, 2011), and the drive to understand the roles of lymphatic vessels in human disease, together with recent technical advances, have seen a resurgence of research into lymphatic vascular development. Our understanding of the cellular

and molecular mechanisms underlying formation and morphogenesis of the developing lymphatic vasculature has progressed significantly in recent years. This Review will summarise our current understanding of lymphatic vascular development and discuss recent findings pertaining to the molecular and cellular events driving this process in mouse and zebrafish, the two species on which most research has focussed.

## Overview of lymphatic vascular development

### Mouse

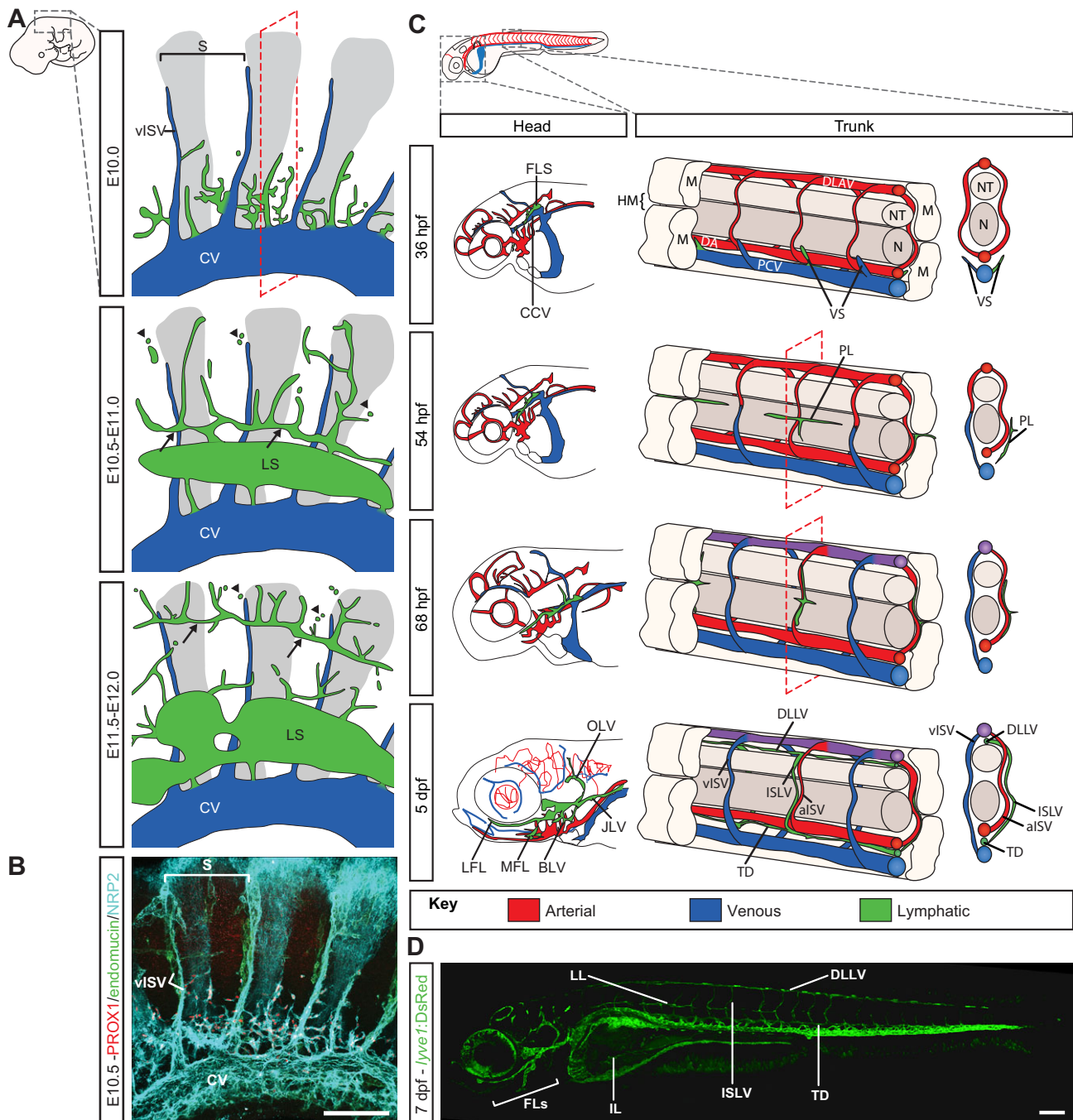
The earliest evidence that lymphangiogenesis has been initiated in the mouse embryo is the onset of expression of the transcription factor PROX1 in a subpopulation of venous BECs located in the dorsolateral walls of the paired anterior cardinal veins (CVs) at approximately embryonic day (E)9.5 (Wigle and Oliver, 1999; François et al., 2008). PROX1-positive LEC progenitors exit the walls of the veins at multiple sites throughout the embryo and migrate away (commencing ~E10.0–E11.5) in a dorsolateral direction to form lymph sacs and superficial lymphatic vessels (Fig. 1A) (Wigle and Oliver, 1999; Oliver, 2004; François et al., 2012; Yang et al., 2012; Hägerling et al., 2013). This event was originally proposed in the early nineteenth century by Florence Sabin (Sabin, 1902) and recently validated by lineage-tracing experiments in mice (Srinivasan et al., 2007). By E14.5, the subcutaneous lymphatic vascular network has been generated via a process assumed to involve angiogenic outgrowth and remodelling from the lymph sacs and superficial vessels. PROX1-positive cells are more abundant in the anterior part of the CVs than in the posterior, corresponding with elevated numbers of LEC

### Box 1. Lymphatic vessel form and function

The cellular architecture of lymphatic vessels underlies the primary function of the network. Lymphatic capillaries, or initial lymphatic vessels, absorb the interstitial fluid and protein (lymph) that is exuded from blood capillaries (Delamere and Cuneo, 1903). The ECs that make up these vessels contain specialised 'button-like' junctions (Baluk et al., 2007) and adhere to the ECM via anchoring filaments, both of which enable these vessels to sense interstitial pressure and open to absorb lymph (Leak and Burke, 1968). The deeper, collecting lymphatic vessels contain valves that maintain the unidirectional flow of lymph and are lined with vascular smooth muscle cells that rhythmically contract to drive lymph flow (Kampmeier, 1928; Smith, 1949). Draining lymph is returned to the venous vasculature via connections between the major lymphatic trunks and the subclavian veins, thereby maintaining fluid homeostasis. Lymphatic vessels are punctuated by lymph nodes that house immune cells, including antigen-presenting cells, T and B lymphocytes, and macrophages. The flow of lymph through lymph nodes enables the constant surveillance of lymph, is important for the effective mounting of immune responses and facilitates immune cell trafficking. Specialised lymphatic vessels in the intestine (lacteals) are essential for the absorption of fatty acids.

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**Fig. 1. Cellular events in early lymphatic morphogenesis in mouse and zebrafish.** **(A)** Stepwise model of lymphatic development in the jugular region of the mouse embryo. At E10.0, LECs (green) bud dorsolaterally from the cardinal (CV; blue) and intersomitic (viSV; blue) veins to form a mesh-like plexus. Migrating lymphatic endothelial cells (LECs; green) often track along the junction between anterior (grey) and posterior somite (S) halves. Further sprouting and migration generates the lymph sacs (LS) by E10.5-E11.0. 'Stalk-like' connections are temporarily maintained between the LS and CV. Dorsal to the LS, the peripheral longitudinal vessel (PLL, arrows), lymphatic plexus and superficial LECs (arrowheads) are indicated. Red plane indicates orientation of the transverse cross-section depicted in Fig. 2. **(B)** Lateral confocal projection of an E10.5 mouse embryo stained with antibodies to PROX1 (red), endomucin (green) and NRP2 (cyan). Scale bar: 150  $\mu$ m. Image by Michaela Scherer (Centre for Cancer Biology, SA Pathology, Adelaide, Australia). **(C)** Stepwise model of morphological events in head (left panels) and trunk (right panels) regions during lymphatic vascular development in zebrafish. Facial lymphatics (FLs) consist of the lateral facial lymphatics (LFL), medial facial lymphatics (MFL) and branchial lymphatic vessels (BLV), and sprout dorsally forming the otolith lymphatic vessel (OLV) and jugular lymphatic vessel (JLV). Trunk lymphatics consist of the dorsal longitudinal lymphatic vessel (DLLV), thoracic duct (TD) and intersomitic lymphatic vessels (ISLV). For further details refer to the main text. Red plane indicates the orientation of the transverse cross-section depicted in Fig. 4. Dorsal aorta (DA), dorsal longitudinal anastomosing vessel (DLAV), horizontal myoseptum (HM; black bracket), myotome (M), neural tube (NT), notochord (N), common cardinal vein (CCV) and facial lymphatic sprout (FLS) are indicated. **(D)** Lateral confocal projection of a 7 dpf *Tg(-5.2lyve1:DsRed)<sup>nz101</sup>* zebrafish demonstrating robust labelling of the lymphatic vascular network. DsRed is pseudocoloured green. Scale bar: 150  $\mu$ m. Image from the Hogan laboratory. aISV, arterial intersomitic vessel; IL, intestinal lymphatic vessels; LL, lateral lymphatic vessels; PCV, posterior cardinal vein; PL, parachordal lymphangioblast; VS, venous sprout.

progenitors migrating away from the anterior region (van der Putte, 1975; Wigle and Oliver, 1999; Karkkainen et al., 2004; François et al., 2012). Ultimately, the mouse embryo develops its lymphatic vasculature progressively from anterior to posterior (van der Putte, 1975; Wigle and Oliver, 1999; Yang et al., 2012; Hägerling et al., 2013).

Three recent studies employing high resolution whole-mount confocal microscopy techniques have described the migratory paths taken by LECs during lymphatic vascular development in the mouse embryo and have also uncovered previously unappreciated morphogenetic events during the exit of LEC progenitors from the veins (François et al., 2012; Yang et al., 2012; Hägerling et al., 2013). The first study by François and colleagues suggested that within the wall of the CV, PROX1-positive LEC progenitor cells assemble in defined territories along the anteroposterior axis of the veins, termed pre-lymphatic clusters (PLCs). Live imaging of this process revealed that PROX1-positive progenitor cells dynamically aggregate into PLCs and further increase their *Prox1* levels (François et al., 2012). The same study suggested that these cells then exit the veins in streams and clusters of a few cells, but also via a ballooning mechanism from the PLCs, whereby cells collectively migrate from the PLCs to form small sacs that later fuse to generate lymph sacs. Open connection points between the lymph sacs and CVs could be seen in this study, providing an explanation as to why blood cells are found within early developing lymph sacs (François et al., 2012). An additional observation, revealed by the employment of whole-mount immunostaining techniques, was that isolated single cells and small clusters of cells were apparent ahead of the sprouting lymphatic vascular plexus (Fig. 1A, arrowheads), suggesting that some cells may break away and migrate ahead of those sprouting from the vein. The signals responsible for directing the assembly of PLCs remain to be described and further work is needed to understand the relative contributions of PLC ballooning and cell sprouting during exit from the CVs.

In a second study, high resolution confocal and electron microscopy illustrated that LEC progenitors leave the CV (beginning at E10.0) as an interconnected group of cells via a sprouting mechanism, ensuring that integrity of the veins is maintained (Yang et al., 2012). Yang et al. proposed that venous integrity is achieved during LEC progenitor exit by the presence of 'zipper-like' junctions containing VE-cadherin (vascular endothelial cadherin; also known as cadherin 5 – Mouse Genome Informatics), which connect LEC progenitors within the CV to those actively budding away (Yang et al., 2012). Based on images of fixed samples, these cells appear to migrate in streams in a dorsolateral manner (Fig. 1B). In addition to their location in the CVs, LEC progenitors were found within the venous intersomitic vessels (vISVs), and sprouting LEC progenitor populations were shown to merge together at ~E10.5 to form a rudimentary capillary-like plexus along the anteroposterior axis of the embryo (François et al., 2012; Yang et al., 2012). Yang et al. proposed that further morphogenesis of this plexus leads to the formation of lymph sac-like structures, which gradually become more defined by E12.5 (Yang et al., 2012). Whereas LYVE1 and PROX1 are expressed by LEC progenitors within the veins, podoplanin expression is solely detected in LEC progenitors that have completely delaminated from the CV, suggesting that exit from the venous wall is tightly associated with the induction of the LEC differentiation programme (François et al., 2012; Yang et al., 2012). It was also proposed that, although the majority of PROX1-expressing LEC progenitors eventually exit the veins and

contribute to the developing lymphatic vasculature, a subpopulation persists within the veins and form the lymphovenous valves; these develop via the intercalation of lymph sac-derived LECs with a subpopulation of PROX1-positive venous BECs at the junction of the jugular and subclavian veins (Srinivasan and Oliver, 2011).

The third and most recent study, by Hägerling and colleagues (Hägerling et al., 2013), used ultramicroscopy to examine lymphatic development in carefully staged whole-mount mouse embryos. This approach revealed the process in single-cell resolution and built on existing models. In a carefully quantified analysis consistent with previous findings (François et al., 2012; Yang et al., 2012), it was shown that individual initial LECs take on both distinct spindle morphologies and distinct protein expression profiles as they emerge from the CV. Refining and suggesting significant changes to existing models, it was proposed that two main populations of initial LECs emerge from the veins before coalescing bilaterally to contribute to a dorsal peripheral longitudinal lymphatic vessel (PLLV), as well as a ventral primordial thoracic duct (pTD; the structure referred to as jugular lymph sacs in previous studies) close to the CV. The PLLV was proposed to give rise ultimately to superficial LECs and might in part arise from the superficial lateral venous plexus (sVP), a previously unappreciated source of embryonic LECs.

Taken together, these studies build on a number of previous analyses (Wigle and Oliver, 1999; Karkkainen et al., 2004) and significantly extend models that had lacked descriptive cellular detail. All consistently propose a sprouting mechanism involving progressive migration of individual and groups of LEC progenitors away from the CV to form lymph sacs (or the pTD) and superficial lymphatic vessels. However, there are differences in the models proposed, suggesting the need for further detailed analyses. These variations are likely to be due to the different methodological approaches used to examine developing embryos and the inherent problems in drawing conclusions about dynamic cell behaviours from fixed embryonic samples. A model summarising these events is outlined in Fig. 1A.

## Zebrafish

Although lymphatics in fish were described centuries ago (Hewson and Hunter, 1769), the zebrafish lymphatic vascular system was first described as recently as 2006 (Küchler et al., 2006; Yaniv et al., 2006). Use of zebrafish has tremendously aided our understanding of the cellular events and genetic pathways important for lymphatic vascular development. In particular, new insights have come from live imaging of cellular processes, rapid gene knockdown, and gene discovery using forward genetic screens. In zebrafish, the most studied lymphatic vessels are those of the trunk lymphatic network, which consists of the thoracic duct (TD), intersomitic lymphatic vessels (ISLVs) and dorsal longitudinal lymphatic vessels (DLLVs) (Fig. 1C,D) (Küchler et al., 2006; Yaniv et al., 2006; Hogan et al., 2009a). The process of embryonic lymphangiogenesis starts at ~32 hours post-fertilisation (hpf). At this stage, the dorsal aorta (DA), posterior cardinal vein (PCV), arterial intersomitic vessels (aISVs) and dorsal longitudinal anastomosing vessel (DLAV) have already formed by the processes of vasculogenesis and primary (arterial) angiogenic sprouting (Fig. 1C) (Isogai et al., 2003). Lymphangiogenesis is linked to secondary (venous) angiogenic sprouting, when BECs start sprouting dorsally from the PCV. Approximately half of the venous sprouts undergo anastomoses with the intersomitic arteries to produce venous intersomitic vessels (vISVs); on average, every second venous sprout (Bussmann et al., 2010) becomes a lymphatic



precursor migrating to the horizontal myoseptum (HM) by 48 hpf to form a parachordal lymphangioblast (PL). PLs remain at the HM until ~60 hpf and, subsequently, they migrate ventrally to give rise to the TD and the ventral part of the ISLV or dorsally to form the DLLV and the dorsal half of the ISLV (Yaniv et al., 2006; Hogan et al., 2009a). It is tempting to draw parallels with the streaming of lymphatic precursors from the CV in mice (Karkkainen et al., 2004; Srinivasan and Oliver, 2011; François et al., 2012; Yang et al., 2012; Hägerling et al., 2013) but there are anatomical differences between these models, notably the absence of lymph sac intermediates in zebrafish and dramatic differences in overall LEC numbers (Küchler et al., 2006; Yaniv et al., 2006; Hogan et al., 2009a). It should also be noted that the most commonly studied regions in mouse (the jugular region) and zebrafish (trunk) embryos are not anatomically homologous.

The recent generation of new, robust transgenic lines that label the venous and lymphatic vascular systems in zebrafish has uncovered previously uncharacterised lymphatic vascular beds and modes of embryonic lymphangiogenesis (Flores et al., 2010; Okuda et al., 2012). These lymphatic vessels include the facial lymphatics (FLs) [also observed previously (Yaniv et al., 2006; Bussmann et al., 2010)] (Fig. 1C, left panels), the lateral lymphatics (LL) and the intestinal lymphatics (IL), which connect to the TD (Okuda et al., 2012) (Fig. 1D). Interestingly, the formation of the FLs occurs in a different manner to that of the trunk lymphatic vascular network. The FLs consist of three primary vessels: the lateral facial lymphatic (LFL), medial facial lymphatic (MFL) and otolith lymphatic vessel (OLV) (Fig. 1C, left panels). The cellular mechanisms by which the FLs develop resemble those by which the jugular lymphatic vessels develop in mouse: the intermediate facial lymphatic sprout (FLS), somewhat reminiscent of a lymph sac intermediate, forms first from the common cardinal vein (CCV) and then appears to remodel into lymphatic vessels (Yaniv et al., 2006; Okuda et al., 2012). Subsequently, cells continue to sprout out from the CCV, but from different, more anterior, CCV origins than those that formed the FLS; these will join sprouts emanating from the FLS to form individual facial lymphatic vessels using precursor cells from multiple venous origins. Upon maturation of the FLs, the initial connection to the CCV is lost and the FL system forms a connection to the TD via the jugular lymphatic vessel (JLV) (Okuda et al., 2012) (Fig. 1C, left panel).

### Evolutionary comparisons

Anatomists have identified a surprising level of morphological divergence in different vertebrate lymphatic vascular systems (see Box 2) and have suggested that intrinsic differences between the fluid homeostasis needs of terrestrial compared with aquatic vertebrates are likely to be responsible for such divergence (Kampmeier, 1969). Indeed, zebrafish probably lack both lymph nodes and lymphatic valves, two defining features of the mature mammalian lymphatic vasculature (Kampmeier, 1969; Steffensen and Lomholt, 1992; Dahl Ejby Jensen et al., 2009). Frogs and reptiles have additional structures, the contracting lymph hearts, important for lymph propulsion, highlighting the anatomical divergence of lymphatic systems throughout evolution. However, despite the anatomical and functional divergence, it is clear that the early, larger lymphatic vessels in mouse and zebrafish develop through very similar cellular processes of dorsal sprouting from embryonic veins, migration and remodelling (Küchler et al., 2006; Yaniv et al., 2006). Furthermore, zebrafish genetics can be applied to understand the formation of lymphatic vessels with relevance to mammalian development (Alders et al., 2009; Hogan et al., 2009a;

### Box 2. Evolutionary divergence in lymphatic vascular systems

Lymphatic systems are found exclusively in vertebrates, yet different organisms have adopted a surprising level of morphological divergence during evolution to provide adequate functions within different environments (reviewed by Kampmeier, 1969; Isogai et al., 2009). As early as the 18th century, advances in understanding the mammalian lymphatic vasculature were made by Olaus Rudbeck and Thomas Bartholin (reviewed by Lord, 1968). In 1769, Hewson and Hunter described the lymphatic system in fish, turtle and birds (Hewson and Hunter, 1769).

Some major points of evolutionary divergence are highlighted below:

**Fish.** In teleosts, lymph flow is thought to occur as a result of the contraction of skeletal muscles (Kampmeier, 1969). The lymphatic system in teleosts lacks lymphatic valves and lymph nodes (Hewson and Hunter, 1769).

**Amphibians.** Frogs have an additional level of complexity to their lymphatic vasculature: lymphatic hearts, which develop from an intermediate lymph sac (Ny et al., 2005; Peyrot et al., 2010). These help pump lymph through the body and probably evolved because of the change from an aquatic to a terrestrial environment (Knower, 1908).

**Reptiles.** Reptilian lymphatic networks resemble those of amphibians, but possess only posterior lymph hearts (Hoyer, 1934).

**Birds.** Birds have primitive lymphatic valves to facilitate lymph flow but have no lymph hearts (Clark, 1915). Interestingly, work in chick has suggested that LECs arise from venous and somitic origins, adding further complexity to our understanding of lymphatic development (Wilting et al., 2006).

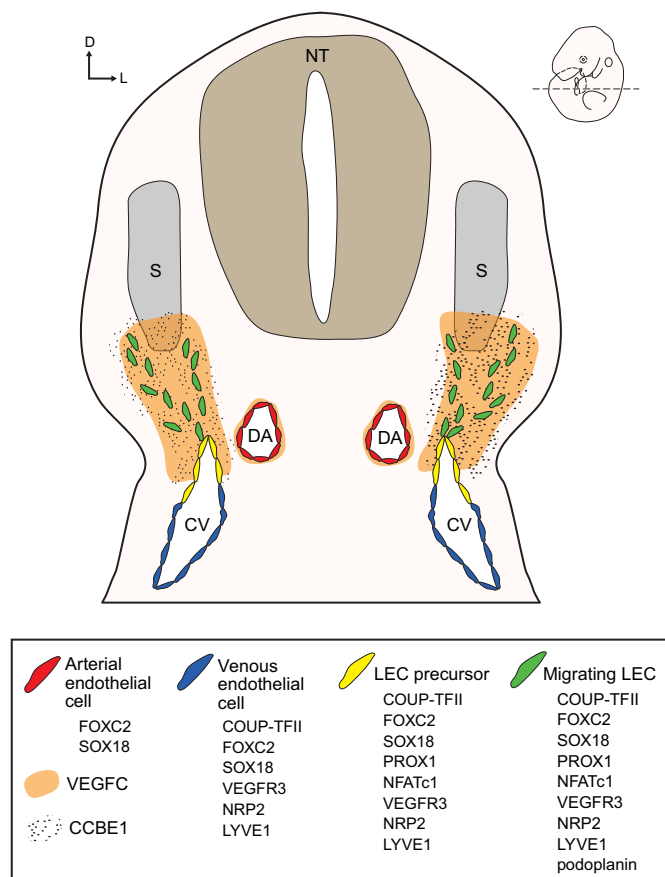
**Mammals.** All mammals have a complex lymphatic vasculature with highly developed lymphatic valves, nodes and hierarchical vessel subtypes (Sabin, 1902; Delamere and Cuneo, 1903).

Bussmann et al., 2010; Lim et al., 2011; Cha et al., 2012). Future studies exploring the developmental mechanisms underpinning the evolution of lymphatic systems are likely to greatly inform our understanding of embryonic lymphangiogenesis.

### Acquiring LEC identity and the induction of sprouting

#### Molecular mechanisms of LEC specification

The first molecular indicator of LEC competence in the mouse is the expression of the SOXF family transcription factor SOX18 in the dorsolateral wall of the CV (François et al., 2008) (Fig. 2). Inactivation of SOX18, by gene knockout or the presence of a point mutation creating a dominant-negative protein, disrupts the development of lymphatic vessels (François et al., 2008; Hosking et al., 2009). SOX18 binds to *cis*-acting regulatory regions of the *Prox1* gene and activates its transcription (François et al., 2008). PROX1 has long been considered the master regulator of LEC fate and serves as the most reliable marker of LEC identity (Wigle and Oliver, 1999; Rodriguez-Niedenführ et al., 2001). PROX1 is crucial for lymphatic development; *Prox1* knockout mice fail to form a lymphatic vasculature and prospective LEC precursors retain BEC marker gene expression, failing to exit the veins (Wigle and Oliver, 1999; Wigle et al., 2002; Yang et al., 2012). PROX1 is also sufficient to specify LEC fate; both *in vitro* when *Prox1* is introduced into BECs (Hong et al., 2002) and *in vivo* when it is ectopically expressed in BECs from the *Tie1* promoter (Kim et al., 2010). Sox18 function remains to be explored in zebrafish lymphatic development, but Prox1a (PROX1 homologues are duplicated in zebrafish) is likely to play a role in lymphatic



**Fig. 2. Regulators of early lymphatic sprouting in mouse.** By E10.5, *Prox1* is apparent in a polarised subpopulation of venous ECs located in the dorsolateral walls of the paired cardinal veins (CVs). *Prox1*-positive lymphatic endothelial cell (LEC) precursors (yellow) delaminate and progressively migrate away from the CV coursing a dorsolateral trajectory defined by VEGFC (orange) and CCBE1 (black speckle) expression. LEC precursors that have detached from the CV begin to express lymphatic markers, including podoplanin (green). FOXC2 and SOX18 are also expressed in arterial endothelial cells (red). Red plane in Fig. 1A indicates the orientation of this transverse cross-section. D, dorsal; DA, dorsal aorta; L, lateral; NT, neural tube, S, somite.

development in zebrafish, because its expression correlates with a role in venous endothelium during the initiation of dorsal sprouting and knockdown of *Prox1a* can lead to absence of a TD (Yaniv et al., 2006; Del Giacco et al., 2010; Tao et al., 2011; Cha et al., 2012).

The process of LEC fate specification in the mouse also involves the activity of COUP-TFII (NR2F2 – Mouse Genome Informatics), an orphan nuclear receptor transcription factor. COUP-TFII is expressed throughout the veins and is a direct binding partner of PROX1 (Lee et al., 2009; Yamazaki et al., 2009). COUP-TFII works together with PROX1 to initiate the expression of known target genes and also has a distinct role in the initiation of PROX1 expression in the veins (Srinivasan and Oliver, 2011). The level of cooperative control of early LEC fate induction is intriguing and it remains to be understood whether COUP-TFII and SOX18 can also cooperate during this process. Importantly, the key transcription factors have distinct and combinatorial functions throughout embryonic and post-embryonic lymphangiogenesis. SOX18 is only expressed during the initial stages of induction of

PROX1 expression and probably plays no role in the maintenance of LEC identity (François et al., 2008). PROX1 is necessary both for LEC specification and for ongoing maintenance of LEC identity in adults (Johnson et al., 2008), but COUP-TFII, although essential for specification, is not needed for maintenance of identity beyond early embryonic stages (Johnson et al., 2008; Lin et al., 2010). In zebrafish and *Xenopus*, it has been shown that COUP-TFII is essential for the formation of the lymphatic vasculature (Aranguren et al., 2011). Transcriptional interactions important for lymphatic vascular development have been recently reviewed in detail elsewhere (Oliver and Srinivasan, 2010).

The signals responsible for the early polarisation of gene expression during LEC specification in the veins remain enigmatic. Studies have revealed a role for Notch signalling upstream of PROX1 during mammalian lymphangiogenesis *in vitro* and in neolymphangiogenesis in the adult but the role of this pathway in the context of embryonic induction of LEC fate remains unclear (Kang et al., 2010; Niessen et al., 2011; Zheng et al., 2011). In zebrafish, in the absence of Delta-like ligand 4 (*Dll4*), the cells that leave the vein during secondary sprouting all acquire BEC identity and give rise solely to vISVs (Geudens et al., 2010). However, the mouse knockout of *Rbpj* (*Suh*), which encodes the primary mediator of canonical Notch signalling, in lymphatic precursors (*Prox1:CreERT2*) showed no defects in LEC specification (Srinivasan et al., 2010). Further work is needed to determine the extent of Notch function during the induction of LEC fate *in vivo*.

### Molecular mechanisms underlying LEC sprouting

Following specification, LEC precursors delaminate from the vein and start migrating in streams and clusters of cells to give rise to the earliest lymphatic structures. Following exit from the veins, LECs acquire additional lymphatic specific markers and progressively downregulate BEC markers (Wigle et al., 2002; Hägerling et al., 2013). The exit of LEC progenitor cells from the veins is dependent upon the ligand VEGFC (vascular endothelial growth factor C). VEGFR3 (FLT4 – Mouse Genome Informatics), the receptor for VEGFC, is expressed by all endothelial cells (ECs), but at particularly high levels in PROX1-positive lymphatic precursors and angiogenic blood vessels (Kukuk et al., 1996; Tammela et al., 2008). *Vegfr3*-null mice die during embryogenesis because of a crucial role for VEGFR3 in cardiovascular development (Dumont et al., 1998). Heterozygous kinase-inactivating mutations in both mouse *Vegfr3* and human *VEGFR3* result in dramatic lymphatic vascular defects; *Chy* mice present with lymphoedema and chylous ascites (extravasation of milky chyle in the peritoneal cavity due to lymphatic defects), modelling the lymphoedema observed in human patients that carry inactivating *VEGFR3* mutations (Irrthum et al., 2000; Karkkainen et al., 2000; Karkkainen et al., 2001) (see also Table 1). These phenotypes may be due in part to a dominant-negative effect of mutant VEGFR3 receptors (Dumont et al., 1998; Karkkainen et al., 2001). VEGFC is expressed in the jugular mesenchyme where lymph sacs first form (Fig. 2) and in *Vegfc*-null mice, PROX1-positive cells fail to sprout from the CVs (Karkkainen et al., 2004). Studies in zebrafish have shown that *Vegfc* and *Vegfr3* functions are highly conserved and regulate dorsal sprouting from the CV in the zebrafish embryo (Küchler et al., 2006; Yaniv et al., 2006; Hogan et al., 2009b).

Other perturbations of the VEGFC/VEGFR3 pathway also effect lymphatic sprouting. Jeltsch et al. showed that VEGFC overexpression in mouse induces excessive lymphangiogenesis (Jeltsch et al., 1997), whereas expression of a soluble VEGFR3

Table 1. Lymphoedema and developmental genes

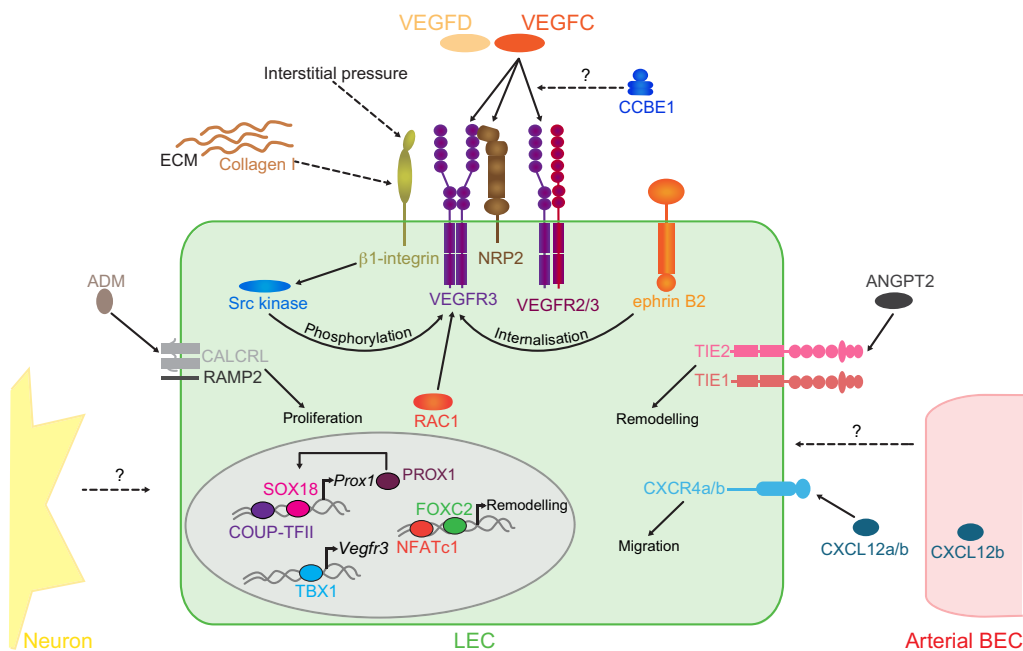
Gene name	Syndrome	Syndrome characteristics	Molecular/developmental function	References
<i>FLT4</i> (encoding VEGFR3)	Milroy disease	Congenital lymphoedema, hypoplastic cutaneous lymphatic vessels and functional insufficiencies in interstitial fluid absorption and transport	Receptor tyrosine kinase for VEGFC. Required for survival, maintenance and migration of LECs.	(Dumont et al., 1998; Ferrell et al., 1998; Irrthum et al., 2000; Karkkainen et al., 2000; Veikkola et al., 2001; Karkkainen et al., 2004; Küchler et al., 2006; Yaniv et al., 2006; Hogan et al., 2009b; Mellor et al., 2010)
<i>FOXC2</i>	Lymphoedema-distichiasis	Late-onset lymphoedema, a double row of eyelashes and varicose veins. Abnormal patterning and retrograde lymph flow due to lymphatic valve incompetence.	Transcription factor regulating lymphatic maturation and lymphatic valve morphogenesis	(Fang et al., 2000; Finegold et al., 2001; Brice et al., 2002; Petrova et al., 2004; Norrmén et al., 2009; Sabine et al., 2012)
<i>SOX18</i>	Hypotrichosis-lymphoedema-telangiectasia syndrome	Alopecia, lymphoedema of the lower extremities and dilation of small blood vessels	Transcription factor required for LEC specification	(Irrthum et al., 2003; François et al., 2008)
<i>CCBE1</i>	Hennekam syndrome	Lymphoedema, facial abnormalities, growth retardation and mental retardation	Extracellular matrix protein required for sprouting of lymphatic precursor cells	(Alders et al., 2009; Hogan et al., 2009a; Connell et al., 2010; Bos et al., 2011)
<i>GJC2</i> (encoding Cx47)	Primary lymphoedema	Lymphoedema	Gap junction protein expressed in lymphatic valves	(Ferrell et al., 2010; Kanady et al., 2011; Ostergaard et al., 2011a)
<i>GATA2</i>	Emberger syndrome	Lymphoedema associated with predisposition to myelodysplasia/acute myeloid leukaemia	Transcription factor important for lymphatic vascular development	(Ostergaard et al., 2011b; Kazenwadel et al., 2012; Lim et al., 2012)
<i>KIF11</i> (encoding EG5)	Microcephaly-lymphoedema-chorioretinal dysplasia (MLCRD) syndrome	Lymphoedema, craniofacial features and eye abnormalities	Kinesin motor protein with currently uncharacterised developmental function	(Hazan et al., 2012; Ostergaard et al., 2012)
<i>PTPN14</i>	Lymphoedema-choanal atresia syndrome	Lymphoedema, choanal atresia (blockage of the nasal passage) and pericardial effusion	Protein tyrosine phosphatase that might interact with VEGFR3 to promote lymphangiogenesis	(Au et al., 2010)
<i>ITGA9</i>	Congenital chylothorax	Pleural effusion	Cell matrix adhesion receptor required for fibronectin matrix assembly during lymphatic valve morphogenesis	(Huang et al., 2000; Ma et al., 2008; Bazigou et al., 2009)

ligand trap blocks lymphangiogenesis (Mäkinen et al., 2001). Many other factors that influence lymphangiogenesis in mice are known modulators of VEGFC/VEGFR3 signalling. Neuropilins are VEGFR co-receptors that bind to VEGFs (Soker et al., 1998; Mäkinen et al., 1999; Wise et al., 1999). Neuropilin 2 (*Nrp2*) is present at high levels on early migrating LECs in the mouse embryo (Fig. 1B) and *Nrp2* mutant mice exhibit disrupted, hypoplastic lymphatic vessels (Yuan et al., 2002). *Nrp2* loss of function has an earlier and more profound impact during zebrafish and *Xenopus* lymphatic development where an interaction with an additional regulator of early lymphangiogenesis, Synectin (also known as Gipc1), was recently described to promote the sprouting of LEC precursors from the veins (Hermans et al., 2010). Ephrin B2 is capable of modulating signalling through VEGFR3 by controlling the internalisation of VEGFR3 receptor complexes (Wang et al., 2010); ephrin B2 loss of function in the mouse is associated with developmental lymphatic vascular defects (Mäkinen et al., 2005; Wang et al., 2010). Another receptor,  $\beta$ 1-integrin, acts via regulation of c-Src (CSK – Mouse Genome Informatics) to modulate the intracellular phosphorylation of the VEGFR3 kinase domain. Collagen I can also influence this

signalling event, possibly acting through  $\beta$ 1-integrin (Galvagni et al., 2010; Planas-Paz et al., 2012; Tammela et al., 2011). In addition, the Rho GTPase RAC1 regulated VEGFR3 levels during LEC budding and migration from the CVs (D'Amico et al., 2009). Finally, the transcription factor T-box 1 (TBX1) activates *Vegfr3* transcription and regulates lymphatic vessel morphogenesis (Chen et al., 2010). Taken together, this series of findings highlight the importance of precise modulation of the activity of the VEGFC/VEGFR3 pathway in lymphatic vascular growth and development (Fig. 3).

Less well-understood pathways that influence the early stages of LEC migration and lymph sac formation include the adrenomedullin and CCBE1 pathways. Mice carrying targeted mutations of *Adm* (adrenomedullin), *Ramp2* or *Calcr1* (receptors for adrenomedullin) exhibit hypoplastic jugular lymph sacs and pronounced subcutaneous oedema (Fritz-Six et al., 2008). The mechanism by which adrenomedullin signalling regulates lymphatic vascular development remains to be established, though it has been proposed that adrenomedullin promotes LEC proliferation (Fritz-Six et al., 2008). Likewise, little is known about the molecular mechanism by which the recently identified collagen





**Fig. 3. Signalling pathways and their regulators driving lymphangiogenesis.** Schematic of factors controlling lymphatic vascular development. PROX1 and its regulators COUP-TFII and SOX18 drive lymphatic endothelial cell (LEC) specification in mice. The VEGFC/VEGFR3 signalling pathway is tightly regulated on a number of levels and is central in LEC migration and the formation of initial lymphatic structures. Additional influential pathways and molecules include adrenomedullin, chemokine (CXCL12a/b) signalling, CCBE1, TIE-ANGPT, VEGFD and cellular interactions of LECs with neurons and arteries. BEC, blood endothelial cell.

and calcium binding EGF-domain containing protein 1 (CCBE1) acts. CCBE1 is essential for embryonic lymphangiogenesis in both zebrafish and mice (Hogan et al., 2009a; Bos et al., 2011) and is mutated in the human primary lymphoedema syndrome, Hennekam syndrome (Alders et al., 2009; Connell et al., 2010) (see also Table 1). CCBE1 is a secreted protein and loss-of-function phenotypes closely resemble those of *Vegfc* and *Vegfr3* in zebrafish and VEGFC in mice (Hogan et al., 2009a; Bos et al., 2011). Perhaps suggestive of a role in that pathway, *Ccbe1/Vegfc* double heterozygous knockout mice have recently been shown to display synergistic phenotypic interactions (Hägerling et al., 2013) but more work is required to understand the mechanistic relevance of this genetic interaction.

Importantly, many of the key developmental regulators described above are required for the development of the lymphatic vasculature in humans. Mutations that impair lymphatic vascular development or function lead to primary (inherited) forms of lymphoedema. Table 1 provides a summary of the genes currently established to underlie primary lymphoedema in the human population.

### Induction and guidance of lymphangiogenesis by cell extrinsic factors

Recent work has revealed that the interactions of migrating LECs with their surrounding environment play key roles during lymphatic vascular development. Several tissues are known to be involved in this process.

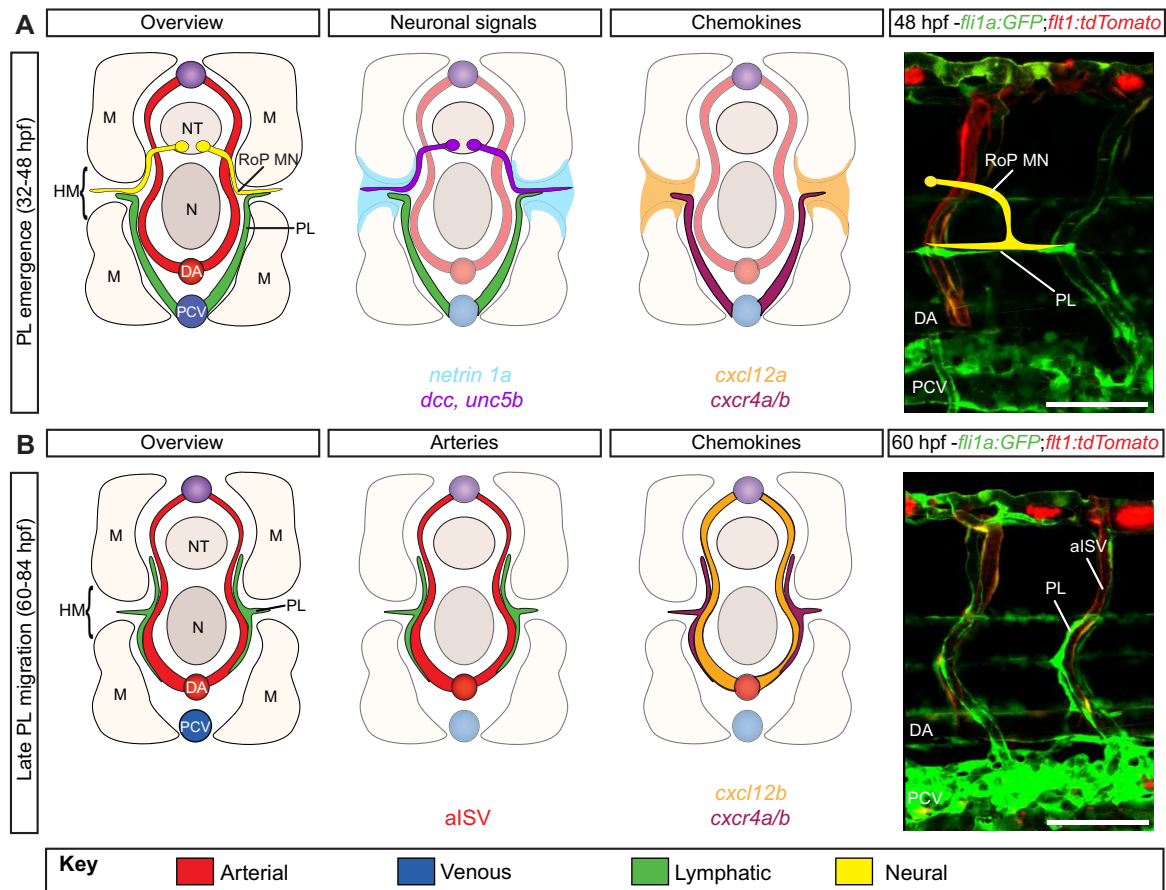
#### Neurons

Migrating LECs have recently been shown to be guided by tracts of adjacent neurons during lymphatic development (Navankasattusas et al., 2008; Lim et al., 2011) (Fig. 4A). In zebrafish, motoneurons line the pathway that is taken by migrating PLs into the HM and the genetic depletion of motoneurons using *Olig2* and *Islet1* knockdown, or physical ablation of these cells, disrupts PL migration and subsequent TD formation (Lim et al., 2011). Hence, the appropriate guidance and migration of motoneurons into the HM is a prerequisite for PL migration. *netrin*

*1a* mediates this neuronal migration, acting from the adjacent muscle pioneer cells and signalling via its receptor *Dcc* (Deleted in colorectal cancer) expressed on the motoneurons (Lim et al., 2011). Upon depletion of either *netrin 1a* or *dcc*, the LEC precursors sprout from the vein and extend dorsally but fail to turn laterally and to extend anteroposteriorly to rest at the HM. Likewise, knockdown of the *Netrin 1a* receptor *Unc5b* (Wilson et al., 2006; Navankasattusas et al., 2008) also phenocopies depletion of *netrin 1a* and *dcc*, further supporting this guidance mechanism. The signalling pathways mediating the interaction between PLs and motoneurons remain to be uncovered.

#### Blood vasculature

Evidence for tissue guidance of lymphatic precursor migration in zebrafish came from the observation that ISLVs always form alongside arterial intersomitic vessels (aISVs) (Bussmann et al., 2010) (Fig. 4B). It had been previously observed that initial sprouting and migration of lymphatic precursors to the HM (32–48 hpf) occurs independently of arteries in *plcg1<sup>p10</sup>* mutants, which lack arteries (Lawson et al., 2003). However, in *kdr<sup>th5088</sup>* mutant embryos, in which some aISVs fail to form along the dorsal aspect of the embryo, there is defective migration of PLs away from HM at later stages in development, specifically in regions of the embryo lacking aISVs (Bussmann et al., 2010). Live imaging and comprehensive quantification in this study identified a strong, almost exclusive, association between migrating PLs and aISVs, once PLs leave the HM from 60 hpf onwards. Although the mechanism responsible for this process was initially unclear, a role for chemokine signalling has recently emerged (Cha et al., 2012) (Fig. 4A,B). The chemokine receptors *cxc4a* and *cxc4b* are expressed in migrating lymphatic precursors. Their ligands *cxc12a* and *cxc12b* are expressed in cells that line the changing pathway taken by migrating PLs through the embryo: *cxc12a* in muscles of the HM, where it is required for migration of PLs into the HM region, and *cxc12b* in arterial BECs, guiding PLs as they subsequently migrate out of the HM alongside arteries. This study suggests that reiterative chemokine cues from multiple, adjacent tissues regulate the progressive migration of PLs through the



**Fig. 4. Tissue cross-talk driving lymphatic vascular development in zebrafish.** (A) Between 32 and 48 hpf, lymphatic precursor cells migrate from the posterior cardinal vein (PCV), past the dorsal aorta (DA) and towards the horizontal myoseptum (HM; black bracket), making up the parachordal lymphangioblast (PL) population. The rostral primary motoneurons (RoP MN; yellow) express *deleted in colorectal cancer* (*dcc*) and *unc5b* (purple), encoding receptors for the ligand Netrin 1a (blue), which is expressed in muscle pioneer cells. Formation of RoP MN at the HM is necessary for PL migration into the HM. Interaction of chemokine receptor Cxcr4a/b (dark red) and its cognate ligand Cxcl12a (orange) promotes the migration of PLs into the HM. The right-hand panel shows a lateral confocal projection of a 48 hpf *Tg(fli1a:EGFP;-0.8flt1:tdTomato)* zebrafish showing the PLs at the HM with schematic representation of a guiding RoP MN (yellow). (B) Between 60 and 84 hpf, dorsoventral migration of PLs depends on signals arising from arterial intersomitic vessels (alSV; red). Cxcr4a/b (dark red), activated by Cxcl12b (orange), which is expressed in arteries, promotes this migration event. The right-hand panel shows a confocal projection of a 60 hpf *Tg(fli1a:EGFP;-0.8flt1:tdTomato)* zebrafish showing close interaction of a PL (green) with an alSV (red) during migration from the HM. Red plane in Fig. 1B (left panel) indicates the orientation of this transverse cross-section. M, myotome; NT, neural tube; N, notochord. Scale bars: 70  $\mu$ m. Images from the Hogan laboratory.

zebrafish embryonic environment and provides a mechanistic explanation for the location of lymphatic vessels alongside arteries (Cha et al., 2012). Supporting the likely conservation of chemokine signalling in lymphangiogenesis, CXCR4 is expressed in mammalian LECs and Cxcl12a promotes LEC migration in culture (Zhuo et al., 2012). However, it is interesting to note that phenotypes in knockdown and mutant zebrafish were not fully penetrant, perhaps suggesting that redundancy exists within the chemokine pathways or with other, yet to be described, guidance factors.

#### Haematopoietic cells

Macrophages share an intimate spatial localisation with lymphatic vessels in the mouse embryo and have been shown to influence lymphatic vessel patterning during development (Böhmer et al., 2010; Gordon et al., 2010). *PU.1* (*Sfp1* – Mouse Genome Informatics) and *Csf1r* mutant mice, both largely devoid of macrophages, exhibit hyperplastic dermal lymphatic vessels and hypoplastic jugular lymph sacs (Gordon et al., 2010). Myeloid

cells, particularly those that express the cytoplasmic tyrosine kinase SYK, have been demonstrated to potentiate lymphangiogenesis by producing pro-lymphangiogenic stimuli including VEGFC, VEGFD (FIGF – Mouse Genome Informatics), MMP2 and MMP9 (Böhmer et al., 2010; Gordon et al., 2010). *Syk* mutant mice exhibit elevated numbers of macrophages in embryonic skin and dramatic hyperplasia of the dermal lymphatic vasculature (Böhmer et al., 2010). It is tempting to speculate that macrophages might deposit guidance cues or matrix-remodelling factors as they migrate through the tissues, paving the way for the developing lymphatic system. However, further studies that dissect the relative contribution of macrophage-derived factors will be needed to harmonise insights from the studies described above and to understand the precise roles of macrophages in lymphatic vascular development.

Platelets have been shown by a number of groups to play a role in mediating separation of the lymphatic vasculature from blood vessels (Bertozzi et al., 2010; Carramolino et al., 2010; Uhrin et al., 2010). CLEC-2 (CLEC1B – Mouse Genome Informatics), a C-type



lectin receptor on platelets that binds to podoplanin on LECs, is important for platelet aggregation at the junctions between developing lymph sacs and the CVs (Bertozi et al., 2010; Suzuki-Inoue et al., 2010; Osada et al., 2012). Mice deficient in megakaryocytes, platelets or platelet aggregation, or with mutations disrupting podoplanin, O-glycosylation (a key post-translational modification of podoplanin), CLEC-2, or SLP-76 (LCP2– Mouse Genome Informatics) signalling in platelets, all exhibit blood-filled lymphatic vessels (Fu et al., 2008; Uhrin et al., 2010; Carromolino et al., 2010; Bertozi et al., 2010; Suzuki-Inoue et al., 2010; Debrincat et al., 2012; Osada et al., 2012), reflecting the aberrant maintenance of blood-lymphatic vascular connections. Whether separation of the two vascular compartments is mediated solely by platelets acting as a physical barrier to ‘plug’ openings between veins and lymph sacs/lymphatic vessels, or whether platelet-LEC interaction results in downstream signalling events important for blood-lymphatic vascular separation remains to be established. Together, these data provide a cellular and molecular mechanism to explain earlier observations that signalling via the Syk/SLP-76/PLC $\gamma$  axis in haematopoietic cells was important for separation of the blood and lymphatic vascular networks (Abtahian et al., 2003; Sebzda et al., 2006; Ichise et al., 2009).

### Mechanical effects

An additional, cell-extrinsic signal involved in the regulation of lymphatic development in the embryo is the mechanical force exerted by elevated tissue fluid pressure (Planas-Paz et al., 2012). Planas-Paz and colleagues have shown that tissue fluid pressure increases in the dorsal interstitium of the mouse embryo at E11.0, both spatially and temporally, correlating with the induction of outgrowth of lymphatic vessels from the lymph sacs. In a series of technically challenging ‘gain-’ and ‘loss-of-fluid’ studies, it was found that increasing or decreasing interstitial pressure reciprocally regulates lymphatic vessel outgrowth from lymph sacs (Planas-Paz et al., 2012). At the molecular level,  $\beta$ 1-integrin activation correlated with higher interstitial pressure, and in the absence of  $\beta$ 1-integrin, VEGFR3 tyrosine phosphorylation was decreased, consistent with the known c-Src-mediated cross-talk between  $\beta$ 1-integrin and VEGFR3 (Fig. 3) (Galvagni et al., 2010). These studies concluded that high interstitial pressure mechanically induces  $\beta$ 1-integrin-mediated phosphorylation of the VEGFR3 kinase domain to modulate the activation of VEGFR3 and induce embryonic lymphangiogenesis (Planas-Paz et al., 2012).

Taken together, the series of studies outlined above highlight the fact that genesis of the lymphatic vasculature is a highly orchestrated process involving a number of sequential, tissue-specific cues. Together, these signals trigger LEC migration and navigate LECs through their changing embryonic environment.

## Forming functional lymphatic vessels

### Vessel remodelling and maturation

By E14.5 in the mouse, the lymphatic vasculature has extended throughout the embryo to form a primitive lymphatic plexus. Commencing at E15.5 and continuing post-natally, this primary network undergoes remodelling to form a mature lymphatic network composed of superficial lymphatic capillaries, or initial lymphatic vessels, pre-collectors and collecting lymphatic vessels (Norrmén et al., 2009) (Box 1). Initial lymphatic vessels constitute the absorptive component of the lymphatic vasculature and are blind-ended vessels characterised by a single layer of overlapping ‘oak-leaf’ shaped ECs. These ECs adhere to one another via discontinuous ‘button-like’ junctions and are anchored to the

extracellular matrix (ECM) via specialised anchoring filaments (Leak and Burke, 1968). Initial lymphatic vessels have little basement membrane and lack a supporting pericyte layer. By contrast, collecting vessels, into which the initial lymphatics drain, exhibit continuous ‘zipper-like’ inter-endothelial junctions, a substantial basement membrane layer, pericyte coverage and the presence of intraluminal valves, all of which facilitate lymph transport (Baluk et al., 2007).

Work from a number of laboratories has shed light on the molecular pathways that are important for maturation of the primary lymphatic plexus into a hierarchical lymphatic vascular network. Analysis of mice deficient in forkhead box protein C2 (*Foxc2*) revealed that although the initial stages of lymphatic vascular development proceeded normally, initial lymphatic vessels acquired ectopic basement membrane and pericyte coverage (Petrova et al., 2004). In addition, lymphatic collectors retained markers characteristic of initial lymphatic vessels and failed to develop valves. These data demonstrate that FOXC2 has an important role in the separation between the initial and collecting vessel phenotypes (Petrova et al., 2004). Moreover, cooperation between FOXC2 and nuclear factor of activated T-cells c1 (NFATc1) is required for collecting vessel identity; NFATc1 has been demonstrated to bind to FOXC2-binding enhancers in LECs (Norrmén et al., 2009). *Nfatc1*<sup>−/−</sup> mice and embryos treated with a pharmacological inhibitor of NFAT signalling phenocopy the lymphatic vascular remodelling defects observed in *Foxc2*<sup>−/−</sup> mice.

Ephrins and their cognate Eph tyrosine kinase receptors are established regulators of axonal guidance in the nervous system (reviewed by Flanagan and Vanderhaeghen, 1998) and blood vascular development (reviewed by Kuijper et al., 2007), and also play key roles in lymphatic vessel maturation (Mäkinen et al., 2005). Mice lacking the C-terminal PDZ domain of ephrin B2 exhibit hyperplastic collecting lymphatic vessels, an absence of luminal valves and failure of the primary lymphatic plexus to remodel into a hierarchical network (Mäkinen et al., 2005). Whereas Eph receptor B4 (EphB4) is present in both initial and collecting lymphatics, ephrin B2 is present selectively in collecting lymphatic vessels, suggesting a mechanism whereby ephrinB2-EphB4 interactions contribute to establishing the distinction between initial and collecting lymphatic vessel identity (Mäkinen et al., 2005).

The angiopoietin/Tie pathway, which is well established as a key regulator of blood vascular remodelling and maturation (reviewed by Augustin et al., 2009), is also important for lymphatic vascular remodelling. Mice hypomorphic for *Tie1* exhibit dilated and disorganised lymphatic vessels with impaired lymphatic drainage capacity (D’Amico et al., 2010; Qu et al., 2010) and mice deficient in the gene encoding the TIE2 (TEK – Mouse Genome Informatics) ligand, *Angpt2*, display abnormal lymphatic vessel architecture, aberrant remodelling of the initial superficial capillary plexus and a failure of luminal valve formation (Gale et al., 2002; Dellinger et al., 2008).

### Lymphatic valve formation

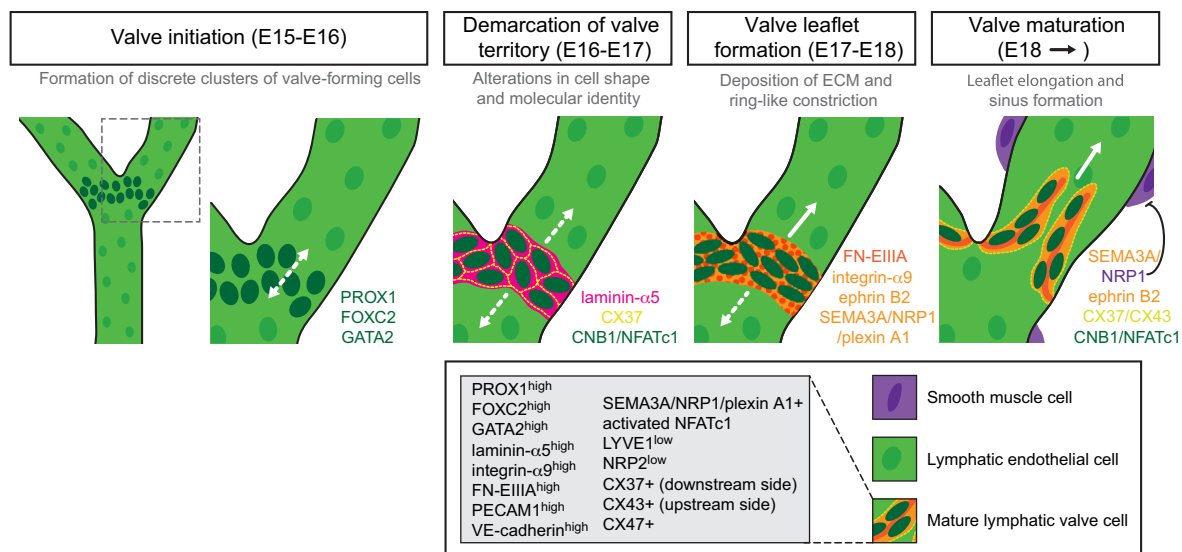
Intraluminal valves are a defining feature of collecting vessels and are imperative for unidirectional lymph transport in mammals; failure of these specialised structures to form or function results in lymphoedema (see Table 1). Mature lymphatic valves are characteristically bicuspid; they comprise two valve leaflets, each consisting of a central connective tissue core, lined by a layer of ECs (Lauweryns and Boussauw, 1973; Navas et al., 1991; Bazigou et al., 2009). Despite the established clinical significance of

lymphatic valves, the molecular mechanisms regulating their morphogenesis (recently reviewed by Bazigou and Makinen, 2012) are only now beginning to emerge. Most of what is currently known comes from extensive analysis of the developing embryonic mesenteric lymphatic vessels in both wild-type and genetically modified mice using high-resolution imaging techniques (summarised below and in Fig. 5).

The initiation of lymphatic valve development in the mouse embryo can be first recognised at ~E15.5, when PROX1, FOXC2 and GATA2 become visible at high levels within discrete clusters of cells in the initial lymphatic plexus (Bazigou et al., 2009; Norrmén et al., 2009; Kazenwadel et al., 2012; Sabine et al., 2012). GATA2, a zinc finger transcription factor, regulates expression of both *Prox1* and *Foxc2* *in vitro* in cultured LECs, and might therefore represent an upstream regulator of lymphatic valve development (Kazenwadel et al., 2012). Although the exact 'trigger' of lymphatic valve morphogenesis remains elusive, recent elegant work by Sabine and colleagues demonstrated that exposure of LECs to disturbed flow regulates the expression levels of key molecules, including FOXC2, that are important for lymphatic valve development (Sabine et al., 2012). That lymphatic valves are often located at sites of vessel bifurcation (Bazigou et al., 2009; Sabine et al., 2012) supports the hypothesis that fluid flow dynamics and shear stress are important factors in determining where valves ultimately form. Accordingly, the initiation of lymphatic valve formation in the mouse embryo correlates with the onset of lymph flow (Sabine et al., 2012). Recent work revealed that ECs located directly adjacent to venous and lymphatic valves display distinct morphological characteristics; ECs immediately upstream of the valve are elongated, whereas rounded ECs

predominate downstream of the valve (Bazigou et al., 2011). To date, the molecular players responsible for transducing the mechanosensory signals that initiate valve development remain elusive.

Elevated levels of PROX1 and FOXC2 in prospective valve-forming cells, in combination with oscillatory shear stress, activate two crucial downstream regulators of valve morphogenesis: connexin 37 (CX37; GJA4 – Mouse Genome Informatics) and calcineurin/NFAT signalling. CX37 is a gap junction protein crucial for the formation of ring-like constrictions of ECs in early developing valves, and calcineurin (CNB1; PPP3R1 – Mouse Genome Informatics) signalling regulates nuclear translocation of NFATc1 to control the demarcation of lymphatic valve territory (Norrmén et al., 2009; Kanady et al., 2011; Sabine et al., 2012). Once specified, valve-forming cells undergo a transition from a squamous to a cuboidal-shaped morphology, re-orientate perpendicular to the longitudinal axis of the vessel and protrude into the vessel lumen (Sabine et al., 2012; Bazigou, 2009). Concomitant with cell rearrangement, ECM components, including laminin- $\alpha$ 5, collagen IV and the EIIIA splice isoform of fibronectin (FN-EIIIA) are deposited and expression of the cell-matrix adhesion receptor, integrin- $\alpha$ 9, is elevated in valve-forming cells (Bazigou et al., 2009; Norrmén et al., 2009). Both integrin- $\alpha$ 9 and its ligand, FN-EIIIA, are crucial for the development of valve leaflets; mice in which either gene has been inactivated exhibit abnormal valve leaflets (Bazigou et al., 2009). The axonal guidance molecule SEMA3A, expressed in lymphatic vessels, has recently been suggested to regulate valve leaflet formation through communication with valve-forming cells expressing the semaphorin receptors NRP1 and plexin A1 (Bouvrée et al., 2012;



**Fig. 5. Model of mouse embryonic lymphatic valve morphogenesis.** Clusters of endothelial cells (ECs) expressing high levels of PROX1, FOXC2 and GATA2 become apparent at sites of vessel bifurcation and regions of disturbed flow (white dashed arrows). Valve territory is established upon the expression of connexin 37 (CX37) and activation of calcineurin (CNB1)/NFATc1 signalling, reorientation of cell clusters and the deposition of laminin- $\alpha$ 5. Valve leaflets form a circumferential ring-like structure dependent on interaction of integrin- $\alpha$ 9 with its ligand, fibronectin (FN-EIIIA), and on SEMA3A/NRP1/plexin A1 signalling. Subsequent cell invagination, sinus formation and leaflet elongation generate a mature lymphatic valve, consisting of two bi-layered leaflets encompassing a thick extracellular matrix (ECM) core (FN-EIIIA; dark orange). Following valve maturation, flow becomes mostly unidirectional (white solid arrows). Repulsive signalling between SEMA3A (expressed by valve LECs) and its receptor, NRP1 (expressed by smooth muscle cells; purple), probably maintains valve regions devoid of smooth muscle cell coverage. Continuous CNB1/NFATc1 and ephrin B2 signalling are important for maintenance of lymphatic valve leaflets. Lymphatic valve-forming cells undergo distinct morphological changes during maturation from round, to cuboidal, to a more elongated shape, and are defined by a molecularly distinct profile (grey shaded box). Dashed box in left-hand panel indicates area depicted at higher magnification in the other schematics.

Juriscic et al., 2012). In addition, SEMA3A has been proposed to maintain valve regions as 'smooth muscle cell-free' zones by repelling smooth muscle cells that express the NRP1 receptor away from valves (Bouvrée et al., 2012; Juriscic et al., 2012). Valve-forming cells exhibit a distinct molecular and morphological profile compared with neighbouring non-valve LECs; they have elevated levels of the junctional markers PECAM1 and VE-cadherin, reduced expression of LYVE1 and NRP2, and are enriched in specific connexins [CX37, CX43 (GJA1) and CX47 (GJC2)] (Kanady et al., 2011; Bouvrée et al., 2012; Sabine et al., 2012). CX47 is present on mature lymphatic valves, whereas CX37 and CX43 are differentially localised on the valve leaflets: CX37 on the downstream side of the valve and CX43 on the upstream side (Kanady et al., 2011; Sabine et al., 2012). Interestingly, graded expression of PROX1 and FOXC2 has been described on either side of the developing valves during the initial stages of valve morphogenesis, suggesting a role in regulating the subsequent orientation of lymphatic valve formation (Sabine et al., 2012).

The transmembrane ligand ephrin B2 is also important for lymphatic valve formation and maintenance (Mäkinen et al., 2005), although the exact mechanism by which ephrin B2 functions and the receptor(s) that mediate this activity currently remain unknown. Intriguingly, it was recently shown that ephrin B2 regulates the internalisation of VEGFR3 (Fig. 3) (Wang et al., 2010), which is also present at elevated levels in developing and mature lymphatic valves (Petrova et al., 2004; Normén et al., 2009). More work is required to uncover the exact roles played by ephrin B2 and VEGFR3 in lymphatic valve morphogenesis.

Although beyond the scope of this review, it is important to point out that the morphogenetic process of valve development occurs similarly in the venous vasculature; genes important for lymphatic valve morphogenesis also play key roles in venous valve formation (Bazigou et al., 2011; Munger et al., 2013). Furthermore, Srinivasan and Oliver have recently demonstrated that *Prox1* dosage is crucial for the formation of embryonic lymphovenous valves, which separate the developing lymphatic vasculature from the jugular and subclavian veins (Srinivasan and Oliver, 2011). FOXC2 and PROX1 are also present at high levels in these valves (Srinivasan and Oliver, 2011). These studies highlight common pathways important for venous, lymphovenous and lymphatic valve development and suggest that shared molecular mechanisms driving valve morphogenesis are likely to be further illuminated in the future.

## Conclusions and future perspectives

In recent years, we have seen a rapid expansion of knowledge in our understanding of the mechanisms by which the lymphatic vasculature emerges and differentiates in the vertebrate embryo. From the molecular control of LEC fate specification and maintenance, to selective modulation of the VEGFC/VEGFR3 signalling pathway, flow induction of lymph sac outgrowth and valve formation, and the identification of tissue-specific guidance cues, a picture has begun to emerge of a non-linear process in which morphogenesis and molecular regulation are tightly linked and co-dependent. As often seems to be the case in modern developmental biology, the ability to observe phenotypic changes with increased resolution and in new developmental contexts leads to new insights, directions and paradigms. Thus, molecular events that programme LEC identity occur concomitantly with morphological changes, LEC precursors are constantly communicating with their neighbours along their pathway through the embryo and physical forces are capable of inducing molecular

changes that drive lymphangiogenesis and differentiation. How tightly are molecular and morphological processes linked? How influential and widespread are mechanical forces in regulating lymphatic development and the molecular processes underpinning it? Is our current view of distinct transcriptional programming and growth factor responsiveness too inflexible and does a level of cross-collaboration exist? Recent discoveries of new molecular regulators of lymphangiogenesis appear to signal a level of complexity that remains to be fully dissected, but how many new pathways and processes remain undefined? Although much has been uncovered in recent years, it seems that a new set of questions have begun to emerge. Answering some of these questions will not only inform our understanding of a fascinating biological process, but will be applicable to the generation of novel diagnostic tools and new therapeutics for the treatment of human lymphatic vascular pathologies, including lymphoedema, inflammatory diseases and metastasis.

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## Competing interests statement

The authors declare no competing financial interests.

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