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REVIEW

When form meets function: the cells and signals that shape the lymphatic vasculature during development

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ABSTRACT

The lymphatic vasculature is an integral component of the cardiovascular system. It is essential to maintain tissue fluid homeostasis, direct immune cell trafficking and absorb dietary lipids from the digestive tract. Major advances in our understanding of the genetic and cellular events important for constructing the lymphatic vasculature during development have recently been made. These include the identification of novel sources of lymphatic endothelial progenitor cells, the recognition of lymphatic endothelial cell specialisation and heterogeneity, and discovery of novel genes and signalling pathways underpinning developmental lymphangiogenesis. Here, we review these advances and discuss how they inform our understanding of lymphatic network formation, function and dysfunction.

KEY WORDS: Lymphatic, Lymphangiogenesis, Vascular development, Valve development, Endothelial cell heterogeneity, Vascular malformations, Lymphoedema

Introduction

Lymphatic vessels are structurally and functionally distinct from blood vessels, in accordance with the unique functions that the blood and lymphatic vascular networks perform (Oliver et al., 2020; Petrova and Koh, 2020; Potente and Makinen, 2017). Whereas blood vessels transport oxygen-, nutrient- and cell-rich blood throughout the body, lymphatic vessels absorb interstitial fluid that is extruded from the bloodstream at the level of capillary beds and return it, together with macromolecules, to the bloodstream. In addition to this function, which is crucial for tissue fluid homeostasis, lymphatic vessels play key roles in directing immune cell traffic, mediating lipid absorption from the digestive tract and facilitating reverse cholesterol transport from tissues to the bloodstream (Oliver et al., 2020; Petrova and Koh, 2020). Abnormalities in the embryonic development or function of lymphatic vessels underlie a number of human disorders, including vascular malformations, non-immune foetal hydrops and primary lymphoedema. Recent discoveries have also implicated lymphatic vessels in pathological conditions such as obesity, glaucoma, tumour metastasis, tumour immunity, cardiovascular disease and neurological disease (Oliver et al., 2020; Petrova and Koh, 2020). Understanding how the lymphatic system arises, and how it is maintained, is therefore key for understanding the aetiology of lymphatic vascular disorders and diseases.

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Recent years have seen major discoveries in the field of developmental lymphangiogenesis. These include the identification of distinct, tissue-specific sources of lymphatic endothelial progenitor cells, the recognition of cell heterogeneity across different aspects of the lymphatic vasculature and between different organs, and the delineation of novel mechanical signals including fluid flow and tension that play important roles in the control of lymphatic vessel morphogenesis. Here, we review these recent discoveries and discuss their implications for understanding the development of the lymphatic vasculature and the aetiology of lymphatic vascular diseases. Where applicable, we also speculate very briefly on how these discoveries could be leveraged to develop novel therapeutics able to effectively treat diseases involving the lymphatic vasculature.

Lymphatic vessel structure and heterogeneity

Interstitial fluid, macromolecules and immune cells first enter the lymphatic vasculature via initial lymphatics (also known as lymphatic capillaries, although they are blind ended and distinct from blood vascular capillaries). The lymphatic endothelial cells (LECs) comprising initial lymphatics are interconnected via relatively loose, button-like junctions (Baluk et al., 2007), and are surrounded by low levels of extracellular matrix (ECM) proteins and anchored into their surrounding environment by elastin-rich anchoring filaments (Leak and Burke, 1968; Pullinger and Florey, 1935) (Fig. 1). Each of these features facilitates the entry of fluid, macromolecules and cells into the initial lymphatics. Upon entry, this fluid (lymph) is conveyed via pre-collecting vessels to collecting lymphatics, which are specialised for lymph transport. The LECs of collecting lymphatics are held together by tighter, zipper-like junctions (Baluk et al., 2007), and are surrounded by higher levels of ECM and ensheathed by specialised lymphatic muscle cells that contract to aid lymph propulsion (Muthuchamy et al., 2003; Pullinger and Florey, 1935) (Fig. 1). In addition to lymphatic muscle cell contraction, which is regulated by the autonomic nervous system (Bachmann et al., 2019; Choe et al., 2015), the return of lymph to the bloodstream is facilitated by skeletal muscle contraction and arterial pulsation (Gashev, 2002). Moreover, unidirectional lymph flow is facilitated by valves that are composed of a specialised population of LECs, which sandwich an important structural matrix core (Bazigou and Makinen, 2013). Lymphovenous valves, situated at the sites where the right lymphatic and thoracic ducts meet with the jugular and subclavian veins, are also important for efficient function of the lymphatic vasculature. These specialised valves allow lymph to return to the bloodstream while preventing blood from entering the lymphatics (Srinivasan and Oliver, 2011).

Although morphological differences in the pattern and density of lymphatic vessels in different tissues have long been recognised, more recent studies have provided molecular insight to the genes differentially expressed in LECs, both in different parts of the lymphatic vasculature and in distinct organs (Petrova and

Development (2021) 148, dev167098. doi:10.1242/dev.167098

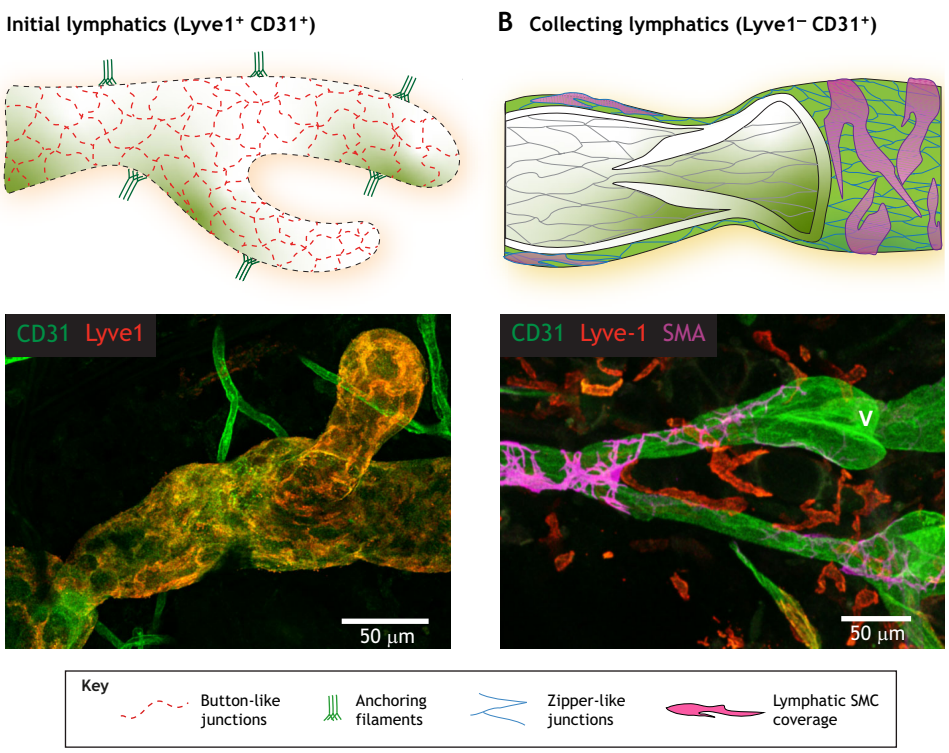

Fig. 1. Hierarchical structure of lymphatic vessels. (A) Top: Initial lymphatics are comprised of a single layer of Lyve1-positive lymphatic endothelial cells (LECs) with button-like intercellular junctions. Anchoring filaments attach the initial lymphatics to the surrounding tissue and, in response to interstitial fluid accumulation, contribute to junctional opening and lymph influx. Bottom: Immunofluorescence image of dermal lymphatic initial vessel from mouse ear. (B) Top: Collecting lymphatics are comprised of Lyve1-negative LECs connected through tighter, zipper-like junctions and are covered with lymphatic smooth muscle cells (SMC), which contract to assist lymph flow. The unidirectional flow of lymph in collecting lymphatics is ensured by the presence of specialised lymphatic valves. Bottom: Immunofluorescence image of dermal lymphatic collecting vessel from mouse ear. V, valves. Scale bars: 50 µm. Immunofluorescence images generated by Anna Oszmiana.

Koh, 2018; Potente and Makinen, 2017). As has been revealed in landmark studies annotating endothelial cell heterogeneity in the blood vasculature (Kalucka et al., 2020; Nolan et al., 2013), LECs isolated from different organs exhibit tissue-specific molecular signatures. For example, LECs isolated from initial lymphatics in the skin play crucial roles in immune surveillance and coordinating adaptive immunity, and express molecules including Lyve1 and Ccl21 that regulate the entry of immune cells into this part of the lymphatic vasculature (Johnson et al., 2017; Russo et al., 2016). By contrast, LECs comprising the lacteals within intestinal villi, which are specialised for lipid absorption, are enriched in molecules such as liprin β1 (also known as Ppfibp1) that are important for lymphatic vessel integrity (Norrmen et al., 2010). Lacteal integrity is also regulated by a subset of fibroblasts within intestinal villi that produce vascular endothelial cell growth factor c (Vegfc) in a Yap1-dependent manner (Hong et al., 2020). Intriguingly, in contrast to lymphatic vessels in other tissues examined to date, lacteals appear to be in a constant regenerative state; this state is regulated by the Notch ligand Delta-like 4 (Dll4) and vascular endothelial growth factor receptor 2/3 (VEGFR2/VEGFR3) signalling (Bernier-Latmani et al., 2015; Nurmi et al., 2015). Gene expression profiling of collecting lymphatic vessels isolated from the mouse mesentery revealed a number of genes, including *Reln* (which encodes the ECM glycoprotein reelin), that are enriched in collecting lymphatic vessels compared with arteries and veins, and are important for controlling collecting vessel maturation (Lutter et al., 2012). In addition, recent single cell RNA-sequencing studies profiling LECs within lymph nodes identified six distinct populations of LECs in this specialised environment, revealing substantially greater heterogeneity between LECs in a single tissue than had previously been appreciated (Fujimoto et al., 2020; Takeda et al., 2019; Xiang et al., 2020). This is perhaps not surprising given the highly orchestrated architecture of lymph nodes and the degree to which precisely controlled immune cell traffic impacts the generation of specific immune responses. Further studies employing single cell RNA-sequencing promise to provide much deeper insight to LEC heterogeneity between tissues and within distinct aspects of the lymphatic vasculature, enhancing our understanding of the genes important for mediating the cellular events that underpin lymphatic vessel development, maturation and organotypic functions. Single cell profiling studies also promise to yield valuable insight into the changes in LEC identity that occur during disease.

In addition to identifying structural and organotypic lymphatic vessel heterogeneity, recent studies have identified unique hybrid vessels exhibiting both blood vessel and lymphatic vessel characteristics. Although these vessels exhibit some markers and/or functions of lymphatic vessels, they are not true lymphatic vessels. These specialised vessels include: Schlemm's canal of the eye, which is important for draining aqueous humour from the intraocular chamber into the venous circulation (Aspelund et al., 2014; Kizhatil et al., 2014; Park et al., 2014; Thomson et al., 2014); ascending vasa recta of the kidney, which mediate fluid absorption in the renal medulla (Kenig-Kozlovsky et al., 2018); and remodelled spiral arteries of the placenta, which undergo extensive transitions in size and shape to deliver maternal blood-derived factors that fuel the metabolic demands of the developing foetus (Pawlak et al., 2019). In each of these cases, blood vessels acquire the expression of characteristic lymphatic markers, including Prox1 and VEGFR3, although a full complement of lymphatic markers (including podoplanin, Lyve1 and Ccl21) is not achieved. Also common to each of these examples of hybrid vessels is endothelial cell expression of Tie2 (Tek) and regulation of vessel development and/or function by angiopoietin 1/angiopoietin 2/Tie2 signalling (Goldman-Wohl et al., 2000; Kenig-Kozlovsky et al., 2018; Kizhatil et al., 2014; Park et al., 2014; Thomson et al., 2014). Although a prominent role in fluid absorption is a key feature of both the Schlemm's canal and ascending vasa recta, it is not a feature of remodelled spiral arteries, suggesting that *Prox1* expression has distinct effects in a venous compared to an arterial

Development (2021) 148, dev167098. doi:10.1242/dev.167098

setting, likely as a result of the distinct transcriptional machinery in each of these vascular contexts. In addition to being expressed in the Schlemm's canal, ascending vasa recta and remodelled spiral arteries, *Prox1* is expressed prominently in the endothelial cells comprising venous valves (Bazigou et al., 2011), lymphovenous valves (Srinivasan and Oliver, 2011) and cardiac valves (Rodriguez-Niedenfuhr et al., 2001). It will be fascinating to unravel the transcriptional components regulating Prox1 activity, together with the suite of genes regulated by Prox1 in each of these vascular contexts, to understand how unique programs of endothelial cell identity are driven in each scenario.

In addition to endothelial cells that comprise the lymphatic vessel wall, recent work has identified a unique population of cells surrounding the blood vessels in the meninges of zebrafish and mice that exhibit characteristic features of LECs, including the expression of *Prox1*, *Lyve1* and *Vegfr3*. These cells are distinct from both macrophages and pericytes, and have been variously termed mural LECs (muLECs) (Bower et al., 2017a), fluorescent granular perithelial cells (FGPs) (Venero Galanternik et al., 2017), brain lymphatic endothelial cells (bLECs) (van Lessen et al., 2017) and leptomeningeal lymphatic endothelial cells (LLECs) (Shibata-Germanos et al., 2020); for simplicity, we refer to them here collectively as muLECs. A key feature of these cells is their high expression levels of scavenger receptors including *Mrc1* and *Stab1*, rendering them highly endocytotic (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017). This function has been proposed to fulfil various roles including the internalisation and degradation of waste products carried in the blood vasculature that enter the interstitial space. Like cells of the lymphatic endothelium, muLECs are venous-derived and their development and patterning is dependent on Vegfc/Vegfd/Ccbe1/Vegfr3 signalling (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017). This population initially forms as a lymphatic vascular plexus that later disassembles into individual cells that are intimately associated with the blood vasculature (Bower et al., 2017a). Intriguingly, muLECs promote development of the meningeal blood vasculature in a model of regeneration in zebrafish, suggesting that they provide important patterning and/or vessel maintenance cues (Bower et al., 2017a). Many fascinating questions remain to be answered to fully resolve the function and distinction of these unique muLECs. How is it that cells with features of lymphatic endothelium reside individually in a perivascular location rather than forming lumenised vessels? Are these cells restricted to the brain and do they perform brain-specific functions? How are they distinct from the LECs that comprise lymphatic vessels in different tissues? Answers to these questions will further define the roles of LECs during development, homeostasis and disease. In addition, the identification of muLECs and their cellular origin points towards a growing complexity in LEC identity and suggests that the gene regulatory networks underpinning lymphangiogenesis can be rewired to give rise to distinct cell types.

Initiation of lymphatic vascular development in the embryo

When do lymphatic vessels first arise in the vertebrate embryo and how are they built? The first evidence that specification of LEC fate has been initiated in the vertebrate embryo is the appearance of *Prox1* expression in the embryonic cardinal and intersomitic veins (Wigle and Oliver, 1999). Intriguingly, in the mouse embryo, Prox1-positive cells are not localised uniformly throughout the cardinal veins, but are polarised to the dorso-lateral aspect of the veins in the anterior part of the embryo and are more uniformly distributed throughout the veins in the posterior part of the embryo (Francois et al., 2012; Wigle and Oliver, 1999). Prox1 is both necessary and sufficient to programme LEC identity from a venous endothelial state (Hong et al., 2002; Petrova et al., 2002; Wigle et al., 2002; Wigle and Oliver, 1999). Initiation of *Prox1* expression in murine veins is dependent on the transcription factors Sox18 and Nr2f2 (CouptfII) (Francois et al., 2008; Srinivasan et al., 2010). The pattern and number of Prox1-positive progenitor cells in the cardinal veins is regulated by retinoic acid signalling (Bowles et al., 2014), myeloid-derived Wnt signals (Muley et al., 2017), Notch signalling (Murtomaki et al., 2013), BMP signalling (Dunworth et al., 2014) and RAS/RAF-mediated ERK activity (Deng et al., 2013). Prox1-positive progenitor cells then bud off and migrate away from the veins to form an initial lymphatic plexus. This event is dependent on both the key pro-lymphangiogenic factor Vegfc (Karkkainen et al., 2004) and two proteins crucial for Vegfc binding and proteolytic cleavage: Ccbe1 (Bos et al., 2011; Hagerling et al., 2013; Hogan et al., 2009; Le Guen et al., 2014) and Adamts3 (Bui et al., 2016; Janssen et al., 2016; Jeltsch et al., 2014). A Prox1-Vegfr3 auto-regulatory loop is important for controlling the number of LECs specified, as well as their exit from the veins and the maintenance of their identity (Srinivasan et al., 2014). Prox1 itself plays an important role in maintaining Prox1 levels in specified murine LECs, and additional transcriptional components including Nr2f2 (Srinivasan et al., 2010), Mafb (Dieterich et al., 2015), Gata2 (Frye et al., 2018; Kazenwadel et al., 2012; 2015) and Hhex (Gauvrit et al., 2018) are important for regulating Prox1 levels post-LEC specification. In addition, a recent study investigating metabolism in endothelial cells revealed that fatty acid oxidation is significantly higher in LECs than in blood vascular endothelial cells and demonstrated that Prox1-mediated elevation of fatty acid oxidation regulates LEC gene expression epigenetically by producing acetyl CoA, which promotes histone acetylation at key lymphatic target genes (Wong et al., 2017).

In the zebrafish embryo, Prox1-positive progenitor cells are first observed in the cardinal vein at 32 hpf (Koltowska et al., 2015). Prox1-positive cells then divide in a Vegfc-regulated manner, with one daughter cell maintaining high levels of Prox1 and exiting the dorsal side of the vein, while the other daughter cell reduces its Prox1 expression and remains within the vein (Koltowska et al., 2015). As is the case in the mouse embryo, the exit of lymphatic progenitor cells from the veins is dependent on Vegfc, Vegfr3, Ccbe1 (Hogan et al., 2009), Adamts3 and the closely related protease Adamts14 (Wang et al., 2020), and is regulated by Bmp signalling (Dunworth et al., 2014). Intriguingly, the origin of Prox1-positive cells exiting the dorsal wall of the cardinal vein to form lymphatic vessels in the zebrafish embryo was recently mapped to a population of haemogenic endothelial cells residing within the ventral floor of the cardinal vein that also exhibit the capacity to generate arterial and venous endothelial cells (Nicenboim et al., 2015). In this setting, the specification of LEC identity was dependent on Wnt5b signalling originating from the neighbouring endoderm (Nicenboim et al., 2015). In line with this, the addition of WNT5B to human embryonic stem cell cultures subjected to endothelial cell lineage specification conditions enhances LEC differentiation, suggesting that WNT5B has the capacity to induce LEC fate in mammals (Nicenboim et al., 2015). However, whether *Prox1* expression in the cardinal veins of mice is dependent on *Wnt5b* remains to be addressed. Although there is no doubt that the induction of *Prox1* expression in venous endothelial cells is crucial to programme LEC identity, a complete picture of the signals and transcriptional components responsible for initiating *Prox1* expression in a restricted pool of progenitor cells remains to be clarified.

Development (2021) 148, dev167098. doi:10.1242/dev.167098

Mapping the origins of lymphatic endothelial progenitor cells during organogenesis

Lineage tracing studies performed in mouse and zebrafish have demonstrated that the majority of LECs in the lymphatic vasculature originate from venous Prox1-positive progenitors (Srinivasan et al., 2007; Yaniv et al., 2006). However, other work in mice, birds and frogs, though not based on lineage tracing, has suggested that non-venous-derived sources of LECs also contribute to the lymphatic vasculature during development (Buttler et al., 2006; Ny et al., 2005; Wilting et al., 2006). More recently, studies employing genetic lineage tracing and high-resolution imaging techniques in both mice and zebrafish have confirmed the contribution of non-venous progenitors to the developing lymphatic vasculature. Moreover, these studies have demonstrated that distinct sources of progenitors are employed in a tissue-specific manner (Fig. 2).

The dermal lymphatic vasculature in the dorsal lumbar region of the mouse embryo was the first lymphatic vessel bed in which a proportion of LECs was established to have a non-venous origin (Martinez-Corral et al., 2015), although the source of these LECs remains to be defined. More recently, isolated Prox1-positive cells have been observed to bud from the blood capillary plexus in the dorsal midline of the mouse embryo, generating small clusters of LECs that integrate with venous-derived LECs to form an interconnected dermal network (Pichol-Thievend et al., 2018). How *Prox1* expression is initiated and restricted to a few cells within the capillary bed is not yet established, but the exit of Prox1-positive cells from the capillary bed was shown to be dependent on *Ccbe1* (Pichol-Thievend et al., 2018), as is the case with Prox1-positive cells exiting the cardinal veins. The mesenteric lymphatic vasculature in mice originates from both venous and haemogenic endothelial cell origins and is particularly dependent on Vegfr3/phosphoinositide-3 kinase (PI3K) signalling (Stanczuk et al., 2015). Lineage tracing employing *cKit-Cre*^{ERT2} mice to inducibly label haemogenic endothelial cells at embryonic day (E)10-E11, revealed that haemogenic endothelium-derived LECs contribute to the mesenteric lymphatic vasculature and assemble via the progressive amalgamation of clusters of cells in a process termed lymphvasculogenesis (Stanczuk et al., 2015). It will be fascinating in future studies to determine the specific population of haemogenic endothelial cells within the embryo or extra-embryonic environment that give rise to LECs of the mesentery. A third tissue in which an additional, non-venous progenitor cell source contributes to the developing lymphatic vasculature is the heart. Here, a population of LECs postulated to derive from yolk sac haemogenic endothelium was reported (Klotz et al., 2015), and second heart field-derived progenitor cells were demonstrated to contribute to the formation of cardiac lymphatics on the ventral surface of the embryonic heart (Lioux et al., 2020; Maruyama et al., 2019).

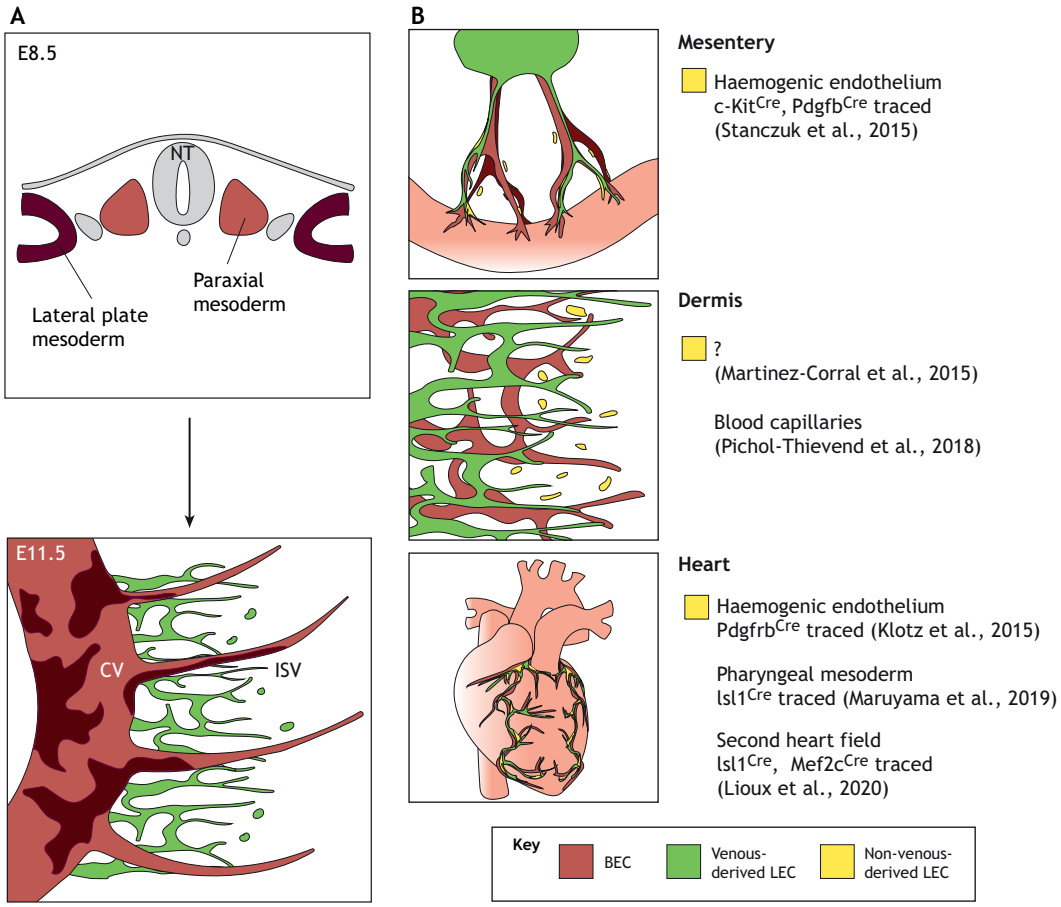

Fig. 2. Developmental origins of lymphatic endothelial cells. (A) Most Prox1-positive lymphatic endothelial cells (LECs) in the mouse embryo descend (at ~E8.5) from paraxial and lateral plate mesoderm-derived venous endothelial cells and contribute to the lymphatic endothelium of multiple organs and tissues (top). By E11.5 in the mouse, Prox1-positive LEC progenitors migrate from the cardinal vein (CV) and intersomitic veins (ISVs) to form a lymphatic plexus and lymph sacs (bottom). (B) In addition to venous-derived LECs (green), the contribution of non-venous progenitors (yellow) to the developing lymphatic vasculature has been confirmed in organs including the mesentery, dermis and heart; note that blood endothelial cells (BEC) are depicted in red.

Development (2021) 148, dev167098. doi:10.1242/dev.167098

The cardiac lymphatic vasculature in zebrafish was also recently demonstrated to originate from dual sources that exhibit distinct mechanisms of growth and differential dependence on Cxcl12/Cxcr4 or Vegfc/Vegfr3 signalling for their development (Gancz et al., 2019). Moreover, trunk lymphatics in zebrafish are primarily venous-derived (Kuchler et al., 2006; Yaniv et al., 2006), whereas facial lymphatics are generated from both venous and non-venous sources, with a population of LECs derived from lymphangioblasts originating from a region near the ventral aorta (Eng et al., 2019). Facial and trunk lymphatics exhibit a differential dependence on Vegfr signalling; whereas the development of trunk lymphatics is directed by Vegfc-initiated Vegfr3 signalling, facial lymphatics are dependent on signal transduction mediated via Vegfd binding to Kdr/Vegfr2 (Bower et al., 2017b; Vogrin et al., 2019). In future studies, it will be informative to map the sources of LECs employed during pathology-stimulated lymphangiogenesis to investigate whether mechanisms that underpin developmental lymphangiogenesis are recapitulated, or unique, in settings of disease.

A common feature of LEC progenitors derived from non-venous sources in the mesentery, skin and heart appears to be their mode of assembly (Gancz et al., 2019; Pichol-Thievend et al., 2018; Stanczuk et al., 2015). These cells first appear as small clusters of cells that proliferate and subsequently join up with venous-derived LECs to generate an integrated network. Similar isolated cell clusters have been observed in the developing meningeal lymphatics (Antila et al., 2017) and kidney (Jafree et al., 2019) during mouse development, although lineage tracing has not yet definitively determined the origin of LEC clusters in these tissues. An intriguing, recent lineage-tracing study in mouse employed a *Pax3-Cre* driver line to reveal that most Prox1-positive LECs in the mouse embryo are derived from paraxial mesoderm, identifying for the first time a marker of prospective LECs before the initiation of *Prox1* expression in endothelial cells of the cardinal veins (Stone and Stainier, 2019). In the future, it will be fascinating to determine the mechanisms by which these cells are distributed throughout the embryo and to understand how they are primed and programmed to turn on *Prox1* to specify LEC fate. Whether unique functions are ascribed to LECs derived from venous versus non-venous sources remains to be investigated, as does assessing the impact of removing selected pools of progenitor cells on the genesis of the lymphatic vasculature. These studies will benefit from the recent advent of novel, more selective, genetic lineage-tracing methods, including dual recombinase targeting approaches (He et al., 2018; Liu et al., 2020), together with the use of appropriate controls (Alvarez-Aznar et al., 2020; Brash et al., 2020), enabling the specific targeting of discrete populations of cells while minimising confounding off-target effects. These advances should overcome current limitations with genetic lineage tracing, which include variability in Cre-mediated recombination efficiency, even within a single litter (Heffner et al., 2012), parent-of-origin-dependent Cre specificity (Heffner et al., 2012), Cre-mediated toxicity in the absence of tamoxifen-mediated Cre recombination (Brash et al., 2020; Naiche and Papaioannou, 2007), and 'leaky' reporter gene expression in the absence of tamoxifen administration (Alvarez-Aznar et al., 2020).

Expansion and maturation of the lymphatic vasculature

Key events underpinning expansion and maturation of the lymphatic vascular network during development include cell migration, navigation, vessel anastomosis, lumen formation, valve development, lymphatic smooth muscle recruitment and distinction between initial and collecting vessel identity. LECs that exit the veins to form an initial vascular plexus remodel to form lymph sacs, which reportedly fuse along the anterior-posterior axis to generate the thoracic duct (Francois et al., 2012; Hagerling et al., 2013; Yang et al., 2012). Continued sprouting and migration of LECs from the lymph sacs, from more superficial veins and from non-venous sources elaborates an interconnected network of vessels. Signals that direct the guidance of lymphatic vessels during development remain largely enigmatic, although the alignment of large collecting lymphatic vessels with arteries and veins suggests that blood vessel-derived cues are important for lymphatic guidance. In line with its role in axon guidance, repulsive signalling via Sema3F/G binding to plexin D1 on LECs is important for patterning the dermal lymphatic vasculature in mice, regulating both the pattern of lymphatic vessel branching and the alignment of lymphatics in the skin with arteries (Liu et al., 2016; Uchida et al., 2015). Vegfc, a crucial cue for lymphatic vessel sprouting and migration, was initially thought to be sufficient to promote directional LEC migration (Karkkainen et al., 2004). However, recent work in zebrafish has identified neuronal structures and a population of fibroblasts as key cellular sources of Vegfc, Adamts3/14 and Ccbe1, revealing that migration routes followed by sprouting cells are precisely coordinated by all three of these factors (Wang et al., 2020). In zebrafish, Cxcl12a and Cxcl12b produced by the horizontal myoseptum and arterial intersegmental vessels are important for guiding Cxcr4-positive lymphatic progenitors that exit the cardinal vein to form the parachordal lymphangioblast and promote further sprouting and alignment with the intersegmental arteries in the trunk (Bussmann et al., 2010; Cha et al., 2012). In mice, arteries and their surrounding smooth muscle also express significant levels of Vegfc (Antila et al., 2017; Karkkainen et al., 2004), suggesting that Vegfc might contribute to arterial-lymphatic alignment during development. A recent study in zebrafish identified the extracellular secretion of type II collagen by notochord sheath cells as an important factor for the patterning and migration of LECs following their exit from the cardinal vein, further demonstrating the importance of ECM proteins in LEC guidance and migration (Chaudhury et al., 2020).

An important signalling axis regulating LEC proliferation and expansion of the lymphatic vasculature is that involving the peptide hormone adrenomedullin (AM) and its receptor complex, which is comprised of calcitonin receptor like receptor (Calcrl) and receptor activity-modifying protein Ramp2. Deletion of the AM gene (*Adm*), *Calcrl* or *Ramp2* in mice results in profound lymphatic vascular defects and embryonic lethality (Fritz-Six et al., 2008; Ichikawa-Shindo et al., 2008). A recently described decoy receptor for AM, CXCR7 (Ackr3), also regulates lymphatic vascular development; *Cxcr7* deletion in mice results in hyperproliferation of LECs as a result of a gain-of-function in AM-mediated signalling (Klein et al., 2014). Signalling via the fibroblast growth factor (FGF) pathway is also important for driving expansion of the lymphatic vasculature during development. In this example, Fgf2 binding to Fgf receptor 1/3 in LECs promotes LEC proliferation and migration via elevation of c-Myc regulated expression of the key glycolytic enzyme hexokinase 2 (HK2), driving glycolysis to fuel LEC metabolism (Yu et al., 2017).

Yap and Taz, key transcriptional effectors of the Hippo pathway, have also recently been shown to play important roles during growth and maturation of the lymphatic vasculature (Cha et al., 2020; Cho et al., 2019; Grimm et al., 2019). Although Yap and Taz have been demonstrated to be dispensable for lymphatic endothelial progenitor cell specification in zebrafish (Grimm et al., 2019), work in both zebrafish and mice has shown that Yap and Taz are required for the sprouting and migration of Prox1-positive LECs from the cardinal veins (Cho et al., 2019; Grimm et al., 2019) and for lymphatic vessel

Development (2021) 148, dev167098. doi:10.1242/dev.167098

valve morphogenesis (Cha et al., 2020; Cho et al., 2019). In zebrafish, nuclear localisation of Yap1 is promoted by Vegfc, and Yap1 is essential for Vegfc-mediated proliferation (Grimm et al., 2019). It will be informative in future work to determine the transcriptional targets of Hippo pathway activity that function in this context, together with the upstream mechanisms regulating Hippo pathway activity during developmental lymphangiogenesis.

The recruitment of lymphatic smooth muscle cells to collecting lymphatics is important for lymphatic vessel maturation and function, and is a key factor distinguishing initial lymphatics from collecting lymphatics. As is the case in the blood vasculature, platelet-derived growth factor B (PDGFB) expression in LECs of the collecting vessels and tethering of PDFGB in the ECM surrounding collecting vessels are important for muscle cell recruitment (Wang et al., 2017). The ECM glycoprotein reelin also plays a role in lymphatic smooth muscle cell recruitment to collecting vessels; *Reln*-deficient mice exhibit reduced numbers of smooth muscle cells associated with collecting lymphatic vessels and less efficient lymphatic transport than their wild-type counterparts (Lutter et al., 2012). Angiopoietin 2-deficient mice also exhibit abnormally patterned collecting lymphatic vessels that fail to recruit lymphatic muscle cells and are defective in function (Dellinger et al., 2008; Gale et al., 2002). Intriguingly, lymphatic smooth muscle cells are not recruited in the close vicinity of lymphatic vessel valves, which is presumably important for valve formation and/or function. Signalling via semaphorin 3a and its receptors neuropilin 1 and plexin A1 is important for preventing the recruitment of muscle cells to valve regions; in the absence of any of these genes or abrogation of this signalling pathway, aberrant association of smooth muscle cells with valves is observed (Bouvree et al., 2012; Jurisic et al., 2012). Lack of proper recruitment of smooth muscle cells clearly impairs lymphatic vessel function but does not give rise to a change in LEC identity (Wang et al., 2017). Although the heterogeneity among endothelial cells comprising lymphatic vessels is beginning to be appreciated, the possibility that heterogeneity exists in the muscle cells surrounding collecting lymphatics has not yet been explored and will be fascinating to evaluate.

Mechanical signals important for lymphatic vascular development

Recent studies have discovered key mechanical stimuli, including flow and cell stretch, that are transduced by LECs and have a major impact in shaping the developing lymphatic vasculature. Such mechanical signals have been established to regulate a number of cellular events, including proliferation, sprouting, control of lumen diameter and valve development.

Lymphatic vessel valves, which begin to develop at approximately E16 in the mouse dermis and mesentery, usually form at vessel branch points, leading to the hypothesis that lymphatic valve morphogenesis is regulated by oscillatory shear stress (OSS) (Sabine et al., 2012) (Fig. 3). Indeed, the passage of fluid through lymphatic vessels exerts shear stress, although this is much lower than that experienced by blood vessels (Dixon et al., 2006). In the context of heart development, high resolution imaging of blood flow patterns in zebrafish has provided compelling evidence that spatiotemporal patterns and directionality of flow forces drive valve morphogenesis (Boselli et al., 2017; Vermot et al., 2009). In LECs, this was initially tested *in vitro* by exposing human LECs to shear, revealing that oscillatory flow elevates levels of FOXC2 and GATA2 and leads to acquisition of features displayed by valve endothelial cells *in vivo* (Kazenwadel et al., 2015; Sabine et al., 2012). More recently, the importance of fluid shear forces in lymphatic valve formation was confirmed *in vivo* using *Clec2* (*Clec1b*)-deficient mice, in which lymphatics develop normally but lymphatic flow is opposed by the influx of blood to the lymphatic vasculature. These mice form 80% fewer valves in neonatal mesenteric lymphatic vessels than do control animals and they fail to remodel the primary mesenteric lymphatic plexus into a hierarchical network (Sweet et al., 2015). In addition to oscillatory

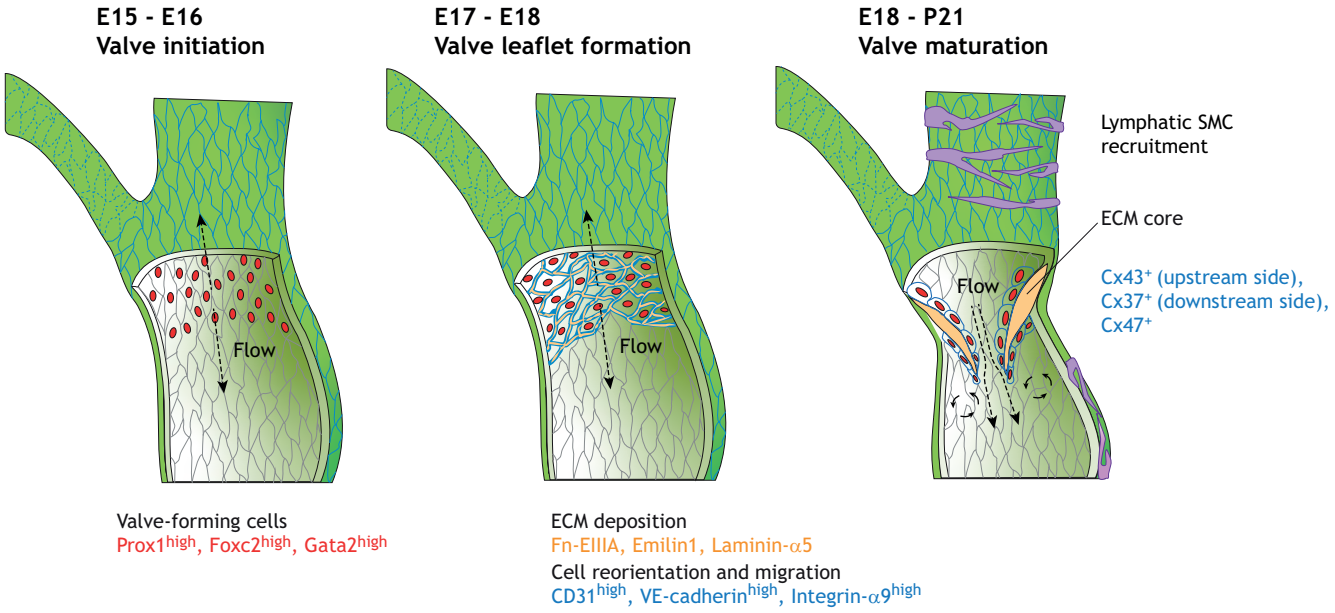

Fig. 3. Stages of lymphatic vessel valve morphogenesis. Valve formation begins (at ∼E15-E16) as clusters of lymphatic endothelial cells near sites of vessel bifurcation upregulate their expression of Prox1, Foxc2 and Gata2 (red). At ∼E17-E18, cells within valve-forming territories then align perpendicular to the vessel axis, remodel their junctions (blue) and begin to deposit extracellular matrix components (ECM; orange). Valve cells then migrate into the vessel lumen to form V-shaped bi-layered leaflets intercalated by an ECM core (at ∼E19-E21). Subsequent elongation of leaflets results in lymph flow becoming unidirectional. Valve development occurs in parallel to the recruitment of lymphatic smooth muscle cells (SMC; purple) to the surface of collecting lymphatic vessels.

Development (2021) 148, dev167098. doi:10.1242/dev.167098

shear activating key genetic pathways directing valve formation, laminar flow has been shown to regulate cell alignment and cell polarity during lymphatic vascular development (Betterman et al., 2020; Norden et al., 2020; Wang et al., 2016).

How LECs sense different shear forces during valve development is not fully understood (Fig. 4). Early studies of mechanosensory responses to flow focused on blood vascular endothelial cells, which are exposed to far greater shear forces than LECs. These studies revealed that a mechanosensory complex comprised of Pecam1 (which senses shear stress and initiates Src phosphorylation), VE-cadherin (which functions as an adaptor) and Vegfr2 [which activates PI(3)K signalling] mediates flow responses in blood vascular endothelial cells (Conway et al., 2013; Tzima et al., 2005). More recently, Vegfr3 was also shown to

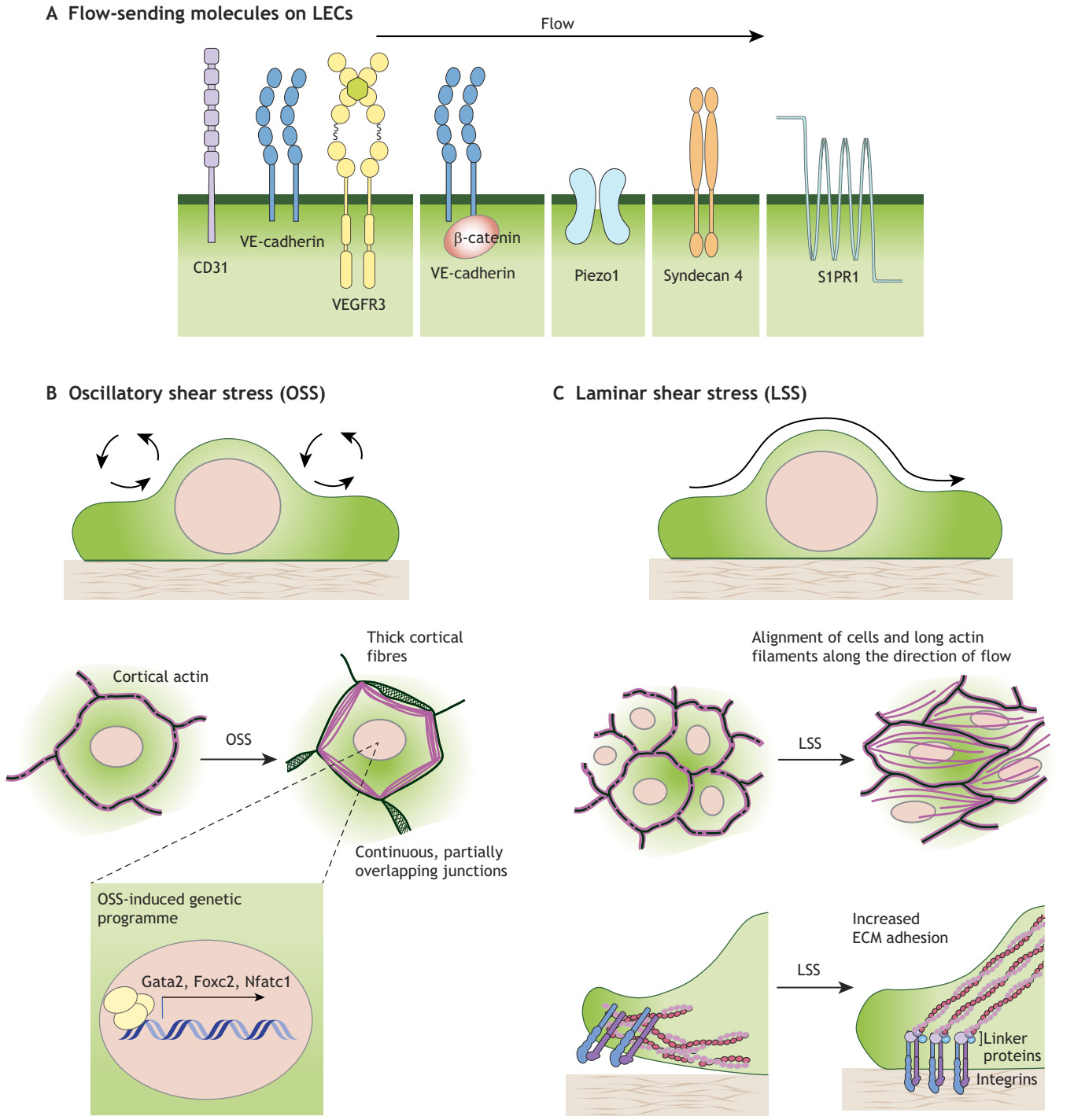

Fig. 4. Mechano-transduction in lymphatic endothelial cells. (A) Sensing of shear stress in lymphatic endothelial cells (LECs) is controlled by multiple molecules present at the cell surface. These allow LECs to react to flow in various ways. (B) Exposure to oscillatory shear stress (OSS) induces LECs to adopt a cuboidal shape, elevates the levels of transcription factors including Foxc2, Nfatc1 and Gata2, and results in the accumulation of thick cortical actin fibres. (C) In contrast, laminar shear stress (LSS) triggers elongation of LECs, elevates levels of transcription factors including Klf2, promotes the assembly of long actin filaments aligned with the direction of flow, and increases adhesion to the extracellular matrix.

Development (2021) 148, dev167098. doi:10.1242/dev.167098

participate in shear stress sensing by interacting with this complex (Coon et al., 2015), and the relative levels of Vegfr3 were proposed to control sensitivity to flow, accounting for the fact that LECs respond to lower shear forces than their blood vascular endothelial cell counterparts (Baeyens et al., 2015). However, our understanding of the receptors and signalling pathways important for mechanosensation in the lymphatic vasculature has increased substantially in recent years. In line with a flow-sensing role in blood vascular endothelial cells, Pecam1 has been shown to be important for lymphatic valve maturation by regulating LEC alignment in response to flow (Wang et al., 2016). In addition, imaging of the mesenteric lymphatic vasculature in *Pecam1* null mice at E18.5 revealed abnormally branched vessels and immature valves with randomly oriented Prox1-high valve-forming cells. The same study also identified the transmembrane heparin sulphate proteoglycan syndecan 4 as an important regulator of lymphatic vascular remodelling in response to flow. This built on earlier work showing that syndecan 4 is required specifically for sensing flow direction, but not for other flow responses, in human umbilical vein endothelial cells (Baeyens et al., 2014). Similar to *Pecam*-null mice, mesenteric lymphatic vessels of *Sdc4*-null animals fail to fully remodel into a hierarchical network and display less mature valves than their control counterparts. However, despite that fact that *Pecam1*- and *Sdc4*-null mice exhibit similar phenotypes, Pecam1 and syndecan 4 appear to regulate flow-mediated signalling via distinct mechanisms. Indeed, $Sdc4^{-/-}$;$Pecam1^{-/-}$ double null embryos exhibit a more severe lymphatic phenotype than embryos deficient in either gene alone, with reduced survival, blood-filled lymphatic structures and/or obvious oedema. In primary human LECs transfected with siRNA against *SDC4* and subjected to laminar flow, reducing the levels of the planar cell polarity protein Vangl2 restores the ability of LECs to align under flow, indicating that syndecan 4 acts by regulating Vangl2 expression (Wang et al., 2016).

A recent study examined the involvement of VE-cadherin in lymphatic valve formation and maintenance, revealing that deletion of *Cdh5* in the embryonic lymphatic vasculature interrupts lymphovenous and lymphatic vessel valve development (Yang et al., 2019), whereas postnatal *Cdh5* deletion results in valve regression (Yang et al., 2019). Defects in mechanotransduction were proposed to underlie this phenotype; LECs within developing VE-cadherin-deficient vessels appear rounder and randomly oriented. In addition, the major transcription factors controlling valve development, Gata2 and Foxc2, that are normally upregulated within valve-forming regions in response to OSS, are uniformly distributed throughout vessels. Consistently, *CDH5*-knockdown in human dermal LECs results in the failure to upregulate GATA2 and FOXC2 in response to OSS *in vitro*. It was further shown that both expression of a constitutively active β-catenin or direct pharmacologic activation of AKT *in vivo* partially rescues valve development in VE-cadherin-deficient mice (Yang et al., 2019). These two parallel pathways – β-catenin and AKT signalling – were therefore deemed responsible for the transmission of mechanically initiated VE-cadherin signalling to the nucleus.

Two recent studies independently uncovered a role for the ion channel Piezo1 in mechanosensing during lymphatic valve formation (Choi et al., 2019; Nonomura et al., 2018). The findings of these studies were broadly complementary in establishing that Piezo1 is required at multiple stages during the formation and maintenance of valves. However, there were important differences between the two studies. For example, in cultured human LECs, Piezo1 appears to be important for driving the elevation of signature lymphatic valve genes, such as *FOXC2*, *GATA2*, *CX37* (*GJA4*) and *LAMA5*, in response to OSS (Choi et al., 2019). In contrast, the levels of Foxc2 or Nfatc1 in lymphatic vessel valve-forming territories in mice appear to be the same between control and *Tie2-Cre;*$Piezo1^{cKO}$ mice, with clusters of Foxc2-high or Nfatc1-high nuclei overlapping with Prox1-high nuclei in mesenteric lymphatic vessels from both groups (Nonomura et al., 2018). Subsequent events important for re-orientation of valve forming cells and elongation into leaflets are, however, arrested in *Tie2-Cre;*$Piezo1^{cKO}$ mice. These differences are likely to arise, at least in part, from the different experimental systems employed, and more studies are clearly needed to fully understand these discrepancies.

Intriguingly, although zebrafish were believed to be devoid of lymphatic vessel valves, a recent study documented valves located selectively in the facial lymphatic vasculature of zebrafish larvae (Shin et al., 2019). These valves display a similar ultrastructure to those found in mammals, together with a dependence on key genes including *Gata2a* and *Itga9* that are important for mammalian valve development (Shin et al., 2019). Future work investigating valve development in zebrafish will no doubt provide further insight into the cellular and genetic events important for lymphatic vessel valve morphogenesis. Together, these recent studies shed some light on how fluid flow-induced signalling is transduced in LECs. However, precisely how these signals are coordinated spatiotemporally to ensure appropriate cellular responses and, in turn, drive valve morphogenesis remains to be determined.

Cytoskeletal remodelling during lymphatic vessel morphogenesis

Mechanical force sensing in cells is closely linked to changes in cytoskeletal organisation. The cytoskeleton has at least three important functions during lymphatic vessel morphogenesis; enabling cell adhesion to the ECM, regulating cell shape and migration, and driving the remodelling of cell-cell junctions.

Interactions between cells and the ECM are crucially important for the growth and remodelling of tissues. The role of the ECM in facilitating valve formation has been extensively studied in the context of heart development (Camenisch et al., 2002; Fondard et al., 2005). In the lymphatic vasculature, ultrastructural studies demonstrated a physical association between the ECM and valve endothelial cells decades ago (Lauweryns and Boussauw, 1973; Navas et al., 1991). Later studies using mice deficient in ECM components and their receptors confirmed the important role of the ECM in providing structural integrity during lymphatic valve formation (Bazigou et al., 2009; Danussi et al., 2013). Cells sense their surrounding matrix through transmembrane receptors such as integrins that trigger cytoskeletal remodelling; reciprocally, forces applied by the cytoskeleton can induce strengthening of integrin-mediated adhesions (Carisey et al., 2013; Friedland et al., 2009). Integrin α9 and its ligand fibronectin EIIIA are highly expressed within valve-forming regions and contribute to the assembly of an ECM core during lymphatic valve development. Accordingly, reduced numbers of lymphatic valves and abnormal leaflet elongation are observed in the lymphatic vasculature of both integrin α9- and fibronectin EIIIA-deficient mouse embryos (Bazigou et al., 2009). The binding of integrin α9β1 to the ECM protein Emilin1 was also suggested to have an important role in valve maintenance postnatally (Danussi et al., 2013), as indicated by the reduced number of valves and the immature valve phenotype observed in Emilin1-deficient pups compared with wild-type controls or fibronectin EIIIA-deficient pups (Danussi et al., 2013).

Development (2021) 148, dev167098. doi:10.1242/dev.167098

β1 Integrin is also important for the response of LECs to mechanical signals. In a series of elegant 'loss-of-fluid' and 'gain-of-fluid' experiments, it was demonstrated that β1 integrin mediates mechano-induction of Vegfr3 signalling and is required for Vegfr3 tyrosine phosphorylation, LEC proliferation and lymphatic vessel expansion in response to cell stretch (Planas-Paz et al., 2012). Later work revealed that mechanosensitive Vegfr3 signalling during embryonic development is controlled by integrin-linked kinase (ILK) and that mechanical stimulation abolishes ILK binding to β1 integrin, enabling the interaction between Vegfr3 and β1 integrin (Urner et al., 2019). ECM stiffness is also known to control a broad range of cellular processes, such as cell growth, shape, migration and differentiation (Mammoto et al., 2009). In LECs, levels of the transcription factor GATA2, which controls valve development, are regulated not only by exposure to oscillatory shear, but also by ECM stiffness; GATA2 levels are elevated in response to decreased substrate stiffness (Frye et al., 2018). Intriguingly, only a small subset of stiffness-regulated genes overlaps with the OSS-regulated genes known to control valve morphogenesis (Frye et al., 2018). Thus, different mechanical stimuli trigger unique responses in LECs.

Although it is not fully understood how genetic programmes interact with cytoskeletal changes during lymphatic vessel remodelling, there is considerable evidence that GTPase signalling, specifically that mediated by RhoA, Rac1 and Cdc42, is important for this process. The roles of Foxc1 and Foxc2 in regulating cytoskeletal dynamics during the maturation of lymphatic valves were carefully dissected in studies employing a range of endothelial- and lymphatic-specific inducible Cre mouse strains (Norden et al., 2020; Sabine et al., 2015). Sabine and colleagues proposed a model whereby Foxc2 controls contractility of the actomyosin cytoskeleton and remodelling of cell-cell junctions to promote LEC quiescence in response to OSS during lymphatic vascular maturation. Later studies showed that Foxc1, which is regulated by laminar flow, also contributes to valve maturation (Fatima et al., 2016; Norden et al., 2020). Consistently, knockdown of FOXC1 or FOXC2 in cultured human LECs leads to altered organization of the actin cytoskeleton and the formation of abnormal cell-cell junctions, and these differences were potentiated in response to flow. Together, these studies established that FOXC1 and FOXC2 act to suppress RhoA/ROCK activation in the context of mechanical stress. Indeed, ROCK inhibition abolishes hypercontractility of the actin cytoskeleton in FOXC1- and FOXC2-deficient cells *in vitro* and partially rescues lymphatic valve loss in *Foxc2*-deficient lymphatic vessels *in vivo* (Norden et al., 2020).

RhoA signalling has been also proposed to control lymphatic vessel barrier integrity. In a recent study, deletion of *S1pr1* (which encodes sphigosine-1-phosphate receptor 1) in the lymphatic vasculature was shown to enhance RhoA activity and, in turn, reduce expression of the tight junction protein claudin 5 in developing lymphatic vessels (Geng et al., 2020). S1pr1 function appears to have an impact on maturation of the lymphatic vasculature, as indicated by the lack of mural cell recruitment and fewer lymphatic valves in *S1pr1*-deficient dermal lymphatic vessels, but it remains unclear whether S1pr1 directly regulates valve development. Intriguingly, the same study demonstrated a role for S1pr1 in regulating the magnitude of signalling via the Vegfc/Vegfr3 signalling pathway in response to flow. Given the role of Vegfr3 in sensing shear stress (Baeyens et al., 2015), it could be postulated that S1pr1 might interact with Vegfr3 in the context of a mechanosensory complex. Rac1-/RhoA-mediated contractility of the actin cytoskeleton and junctional stability has also recently been shown to be regulated by ephrin B2/EphB4 signalling (Frye et al., 2020). In this case, deletion of either *Efnb2* or *Ephb4* in mice results in disruption of LEC junctions, and inhibition of ephrin B2 signalling in cultured human LECs results in increased actin remodelling and reduced junctional integrity, effects that can be rescued using the Rho kinase inhibitor Y-27632 (Frye et al., 2020). Together, these data suggest that targeting either the ephrin B2/EphB4 or Rac1/RhoA signalling axes could provide a pathway toward therapeutic targeting of LEC barrier integrity and lymphatic vessel leakage in pathological settings.

Another recently identified regulator of GTPase signalling in endothelial cells is Rasip1 (Liu et al., 2018; Xu et al., 2011). In blood vascular endothelial cells, depletion of Rasip1 blocks lumen formation *in vivo* and *in vitro*, alters organisation of the cytoskeleton and reduces integrin-dependent adhesion to the ECM as a result of increasing RhoA/ROCK/myosin II activity, thereby blocking Cdc42 and Rac1 signalling (Xu et al., 2011). In cultured LECs, knockdown of Rasip1 results in punctuated F-actin organization and is linked to reduced Cdc42 activity (Liu et al., 2018). Similar changes in the appearance of actin filaments are noticeable in the mesenteric lymphatic vessels of *Rasip1* conditional null mice, together with disorganised junctions and reduced levels of junctional proteins. Both Rasip1 and Cdc42 are important for valve formation and remodelling of collecting lymphatic vessels, as indicated by dilated collecting vessels and significant loss of valves in *Rasip1*- and *Cdc42*-deficient mice (Liu et al., 2018). Another recent study also reported loss of valves in *Cdc42*-deficient mesenteric lymphatic vessels, concurrent with impaired lymphatic muscle cell recruitment and lymphatic vessel remodelling (Jin et al., 2020).

Despite these exciting recent insights to the control of LEC biology by mechanical forces, it remains unclear how different mechanical stimuli are integrated on a single cell level and how these signals interact with genetic programs to drive lymphatic vessel morphogenesis *in vivo*. Studies in optically clear zebrafish have revealed important insights into cellular sensing and force responsiveness in live tissues. For example, a recent and elegant study coupled high-resolution live imaging with transcriptional profiling analyses to demonstrate that blood flow and flow-responsive genes regulate synthesis of the ECM component Fibronectin 1b, which is important for coordinating cell movements during heart valve formation (Steed et al., 2016). Another innovative approach based on an *in vivo* tension sensor was used to quantify changes in VE-cadherin tension occurring during arterial maturation in zebrafish (Lagendijk et al., 2017). The development of similar cutting-edge approaches employing advanced imaging techniques that are applicable to the mouse embryo should enable us to address the gaps in our knowledge of the impact of mechanical signals on lymphatic vessel morphogenesis and valve development.

Conclusions and future directions

The last decade has seen incredibly exciting progress in the field of lymphangiogenesis research. The recognition of LEC heterogeneity, both within distinct compartments of the lymphatic vasculature and in distinct organs, has provided important insight into our understanding of the mechanisms by which lymphatic vessels work to control fluid homeostasis, lipid absorption and immune cell trafficking. Each of these functions of the lymphatic vasculature is crucial for tissue homeostasis and, when disrupted, has implications for human disease. The aetiology of lymphatic diseases including lymphatic vascular anomalies, non-immune foetal hydrops and primary lymphoedema is developmental in nature and, accordingly, deepening our understanding of the fundamental biology underlying lymphangiogenesis will provide new insights into our understanding of the causes of lymphatic disease.

Next generation sequencing and rapid genome editing technologies have vastly accelerated the rate at which we are able to define the genetic and developmental basis of human disease and generate animal models of disease, providing new opportunities to establish innovative pre-clinical model systems to screen established therapeutic agents and develop new ones. Recognition of LEC heterogeneity has been fuelled by single cell technologies, facilitating the identification of new cell types important for lymphatic vascular function. The employment of these techniques to profile lymphatic vessels in different tissues throughout development, and in settings of disease, stands to increase our knowledge of the cellular identities and heterogeneities that underpin vessel function and dysfunction. Moreover, although major advances in the field have had a particular focus on the behaviour of individual cells or discrete cell populations, it would be informative to take advantage of the plethora of studies modelling collective epithelial cell behaviour during organogenesis to start filling the gap in our understanding of how LEC collectives are coordinated to govern vessel formation.

Progress in the development of tissue clearing and high-resolution real time imaging technologies has also provided substantial insight to our understanding of developmental lymphangiogenesis, particularly in the zebrafish embryo. Application of these techniques to the mouse embryo will continue to yield important information, particularly for events such as lymphatic vessel valve development and branching morphogenesis. Finally, coupling our rapidly developing understanding of the transcriptional regulation of LEC identity with stem cell programming and bioengineering approaches will provide the ability to generate or reprogramme LECs for therapeutic applications *ex vivo*. Overall, advancing our understanding of developmental lymphangiogenesis will provide new opportunities to dissect 'what goes wrong' in lymphatic disease, together with novel opportunities to develop therapeutics applicable to the growing number of pathologies involving the lymphatic vasculature.

Competing interests

The authors declare no competing or financial interests.

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