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Organ-specific mechanisms of vascular development in the mesentery

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Abstract

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Understanding how the vascular systems are formed has significant clinical importance. General mechanisms underlying vascular development have been extensively studied during the past decades. However, the mechanisms regulating the development and function of the blood and lymphatic vessels in specific organs are poorly understood.

The aim of this thesis was to investigate lymphatic vascular development in the mesentery, which is a fold of peritoneum that attaches the intestine to the abdominal wall, and contains arteries, veins, lymphatic vessels, nerves and lymph nodes. We found that mesenteric lymphatic vessels were formed through lymphvasculogenesis - coalescence of isolated lymphatic endothelial cell (LEC) clusters, rather than by lymphangiogenesis - sprouting from the veins or pre-existing lymphatic vessels. The lymphvasculogenic process was selectively sensitive to inhibition of the vascular endothelial growth factor receptor 3 (VEGFR3)/ phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway. Using genetic lineage tracing, we uncovered that part of the mesenteric lymphatic vasculature was derived from *cKit* lineage cells likely originating from the blood-forming hemogenic endothelium of major arteries (**Paper I**). This is in contrast to the previously accepted dogma that all mammalian lymphatic vessels are of venous endothelial origin. By characterizing a mouse mutant lacking the non-venous-derived LEC progenitors we found that an alternative venous source of LECs could however compensate to build a functional mesenteric lymphatic vasculature (**Paper IV**). We further described in the developing mesentery that a transient loss of venous integrity, characterized by the formation of inter-endothelial cell gaps, was accompanied by extravasation of red blood cells, which were cleared by the developing lymphatic vessels. By studying mice with defective platelet function, we revealed a previously unappreciated role of platelets in maintaining the integrity of the remodeling embryonic blood vasculature and thus preventing excessive blood-filling of lymphatic vessels (**Paper III**). We also studied the mechanism of vessel maturation into functional lymphatic vessels, which involves smooth muscle cell recruitment. Analysis of mice with LEC-specific deletion of *Pdgfb*, encoding the platelet derived growth factor B (PDGFB), showed that LEC-autonomous PDGFB was required for the recruitment of smooth muscles cells that in turn control lymphatic vessel size and function (**Paper II**).

Keywords: Lymphatic vasculature, mesentery, hemogenic endothelium, lymphvasculogenesis, endothelial integrity, platelet, blood-filled lymphatic vessel, morphogenesis and maturation, compensation

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There is pleasure in recognizing old things from a new viewpoint.

Richard Feynman

To my family

致我的家人

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Stanczuk, L., Martinez-Corral, I., Ulvmar, M.H., **Zhang, Y.**, Laviña, B., Fruttiger, M., Adams, R.H., Saur, D., Betsholtz, C., Ortega, S., Alitalo, K., Graupera, M., and Mäkinen, T. (2015) *cKit* lineage hemogenic endothelium-derived cells contribute to mesenteric lymphatic vessels. *Cell Reports* 10*:* 1708-1721

II Wang, Y., Jin, Y[*]., Mäe M.A[*]., **Zhang, Y.**, Ortsäter, H., Betsholtz, C., Mäkinen, T[‡]., and Jakobsson, L[‡]. (2017) Smooth muscle cell recruitment to lymphatic vessels requires PDGFB and impacts vessel size but not identity. *Development* 144, 3590-3601 (*These authors contributed equally to this work. [‡]Authors for correspondence)

III **Zhang, Y.**, Daubel, N., Stritt, S., and Mäkinen, T. (2018) Transient loss of venous integrity during developmental vascular remodeling leads to red blood cell extravasation and clearance by lymphatic vessels. *Development* 145: pii: dev156745

IV **Zhang, Y.**, Stritt, S., Martinez-Corral, I., Laviña, B., Betsholtz, C., and Mäkinen, T. (2018) Alternative lymphatic endothelial progenitor cells compensate for the loss of non-venous derived progenitors to form mesenteric lymphatic vessels. *Manuscript*

Contents

Abbreviations

AGM	Aorta-gonad-mesonephros
ANG	Angiopoietin
BBB	Blood-brain barrier
BEC	Blood endothelial cell
bFGF	Basic fibroblast growth factor
BM	Basement membrane
BMP	Bone morphogenetic protein
CCBE1	Calcium-binding EGF-like domain 1
CCM1	Cerebral cavernous malformation 1
CCV	Common cardinal vein
CDK5	Cyclin-dependent kinase 5
CNS	Central nervous system
COUP-TFII	COUP transcription factor 2
CV	Cardinal vein
CX	Connexin
DC	Dendritic cell
Dll4	Delta-like 4
EC	Endothelial cell
ECM	Extracellular matrix
EHT	Endothelial-to-hematopoietic transition
eNOS	Endothelial nitric oxide
EPH	Ephrin receptor
ERK	Extracellular signal reguated kinase
ETS	E26 transforming-specific
E-number	Embryonic day
GPVI	Glycoprotein VI
HDAC3	Histone-modifying enzyme histone deacetylase 3
HE	Hemogenic endothelium
HEC	Hemogenic endothelial cell
HEY	Hairy- and enhancer of split-related with YRPW motif
HSC	Hematopoietic stem cell

HSPC	Hematopoietic stem and progenitor cell
IAHC	Intra-aortic hematopoietic cluster
IHH	Indian hedgehog
ITAM	Immunoreceptor tyrosine-based activation motif
KLF	Krüppel-like factor
LEC	Lymphatic endothelial cell
LN	Lymph node
NICD	Notch intracellular domain
NPAS4L	Neuronal PAS domain-containing protein 4-like protein
NRP	Neuropilin
PAR3	Partitioning defective 3
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDPN	Podoplanin
PI3K	Phosphoinositide 3-kinase
PROX1	Prospero hemeobox 1
PLCγ2	Phospholipase Cγ2
P-number	Postnatal day
RASIP1	RAS-interacting protein 1
RBC	Red blood cell
ROS	Reactive oxygen species
SCF	Stem cell factor
SEMA	Semaphorin
SIM	Structure illumination microscopy
SLP76	Src homology 2 domain-containing leukocyte protein of 76 kDa
SMC	Smooth muscle cell
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
TJ	Tight junction
VE-cad	VE-cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	Von Willebrand factor
4-OHT	4-hydroxytamoxifen

Introduction

Structure and function of the vascular system

The vascular system is composed of the blood vasculature and the lymphatic vasculature. The blood vasculature delivers nutrients, metabolites, oxygen, carbon dioxide, hormones, and blood cells to almost all the tissues in the body, and it supplies paracrine factors to the adjacent perivascular tissues (Potente and Mäkinen, 2017). The lymphatic system is indispensable in maintenance of tissue fluid homeostasis, immune surveillance, and dietary fat absorption (Tammela and Alitalo, 2010).

The blood vasculature is a closed system consisting of arteries, arterioles, veins, venules and the interconnecting capillaries. Histologically, most of the blood vessels have three distinct layers: *tunica intima*, *tunica media*, and *tunica adventitia* (Figure 1). The *tunica intima*, facing the lumen, consists of the single endothelial cell (EC) layer, adjacent basement membrane (BM), fibro-elastic connective tissues, and the internal elastic lamina. Smaller vessels in the *tunica intima* are covered by pericytes. The *tunica media*, the middle layer, comprises mainly smooth muscle cells (SMCs), collagen, and the external elastic lamina. The *tunica adventitia* contains fibro-elastic connective tissues, collagen, mast cells, macrophages, dendritic cells, autonomic nerves, lymphatic vessels and small vessels named *vaso vasorum*. (Majesky et al., 2011; Pugsley and Tabrizchi, 2000).

There are two types of morphologically and functionally different arteries – elastic and muscular arteries. The elastic arteries like the aorta contain a great amount of elastic fibers and laminae interspersed with SMCs. Thus, these arteries are adapted to the high conductance of blood, which in turn can reduce the pulsatile flow and pressure to ensure normal blood flow from the heart to the downstream organs. In contrast, the muscular arteries such as mesenteric arteries have discontinuous elastic fibers and a large number of SMCs, which facilitates the efficient blood transportation (Leloup et al., 2015). The blood passes the arteries into the network of arterioles that are the smallest arterial vessels and covered by the SMCs and pericytes. The main function of the arterioles is to reduce the blood flow and protect the fragile blood capillaries (Figure 1).

The blood capillaries cover a large surface area and thus promote exchange of nutrients, solutes, and water between blood and the adjacent organs and tissues. They are surrounded by BM and pericytes rather than SMCs. There are three different types of blood capillaries: continuous, fenestrated, and discontinuous (Figure 1). The continuous capillaries are the most common capillaries and present in many organs such as heart and lung. These capillaries are much less permeable due to continuous endothelium and BM (Aird, 2007a; Pugsley and Tabrizchi, 2000). The fenestrated capillaries are mainly found in organs involved in filtration or secretion such as endocrine glands and the glomeruli of the kidney. BM of the fenestrated capillaries is continuous, whereas the endothelium contains small filtration pores (~70 nm in diameter). These pores allow solutes and water to pass but restrict protein passage (Aird, 2007; Pavelka and Roth, 2010; Pugsley and Tabrizchi, 2000). The discontinuous capillaries are predominant in the specialized organs such as the liver and bone marrow. Neither endothelium nor BM is continuous in the discontinuous capillaries. Moreover, there are bigger pores (100 – 200 nm in diameter) between the ECs allowing macromolecules and blood cells to pass through (Figure 1).

The venous vasculature is composed of venules and veins (Figure 1). Like in the arterioles and arteries, there is a gradual increase of collagen and elastic fibers, and appearance of SMCs though to a lesser extent in the postcapillary venules and veins (Carroll, 2006). Of note, the postcapillary venules are the main sites of leukocyte transmigration during inflammation (Muller, 2011). Moreover, luminal valves can be found in medium and large veins, and they are critical in maintaining the unidirectional flow of blood (Figure 1).

The lymphatic system is blind-ended and unidirectional (Aspelund et al., 2016a). It includes lymphatic vessels, lymph nodes, and associated lymphoid organs. Lymphatic vessels contain three vessel compartments: lymphatic capillaries, pre-collecting, and collecting lymphatic vessels (Aspelund et al., 2016a) (Figure 1). Lymphatic capillaries are blind-ended structures and lack a continuous BM and mural cells. There is a single layer of oak leaf-shaped lymphatic endothelial cells (LECs) showing discontinuous button-like intercellular junctions, which facilitate uptake of interstitial fluid and entrance of immune cells into the lymphatic vessel lumen. Lymphatic capillaries connect to the extracellular matrix (ECM) through anchoring filaments to control valve-like opening of the vessels and maintain their structure under conditions of increased interstitial pressure (Alitalo, 2011; Schulte-Merker et al., 2011) (Figure 1). In addition to this paracellular route, LECs have recently been demonstrated to transport lymph via vesicle formation and transcytosis (Triacca et al., 2017). The pre-collecting vessels share characteristics with both lymphatic capillaries and collecting lymphatic vessels: oak leaf-shaped LECs, coverage of mural cells though sparse, and valves. The collecting lymphatic vessels are composed of a series of lymphangions, the functional and

structural unit separated by intraluminal valves (Figure 1). LECs in collecting lymphatic vessels form continuous zipper-like junctions, and they are covered by a continuous BM and mural cells. Valves lack SMCs and have two leaflets that either open or close in response to upstream and downstream pressure differences (Davis et al., 2011) (Figure 1). The aforementioned characteristics of the collecting lymphatic vessels ensure unidirectional flow of lymph and prevent leakage during lymph transportation (Aspelund et al., 2016a; Schulte-Merker et al., 2011). The afferent lymphatic vessels transport lymph, blood-derived naive lymphocytes with antigens and antigen-presenting dendritic cells (DCs) from the peripheral tissues to the lymph nodes (LNs). After leaving the LNs, lymph returns to the venous circulation via the lymphvenous junctions of the subclavian and internal jugular veins (Aspelund et al., 2016a).

ECs in different organs show distinct morphologies and functions, as aforementioned, to complement the organotypic functions. Here I focus on ECs in a few representative vascular beds.

Blood-brain barrier (BBB), composed of ECs, pericytes, and astrocytes, is the specialized microvasculature of the central nervous system (CNS). The microvasculature in the BBB is continuous. The ECs are connected to each other via tight junctions (TJs) that are intercellular adhesion complexes limiting the paracellular passage of molecules and ions (Daneman and Prat, 2015; Zihni et al., 2016). Moreover, transcytosis in these ECs is at an extremely low level, which minimizes the transcellular transportation. Collectively, the CNS ECs form a physical barrier to control cellular transportation (Aird, 2007). To maintain the CNS function, these ECs have developed a couple of unique transport properties including solute carrier and ATP-binding cassette transporters (Abbott et al., 2010). Additionally, they express much lower level of leukocyte adhesion molecules compare to the ECs in other organs and/or tissues, which helps to minimize leukocyte adhesion and maintain the CNS immune privilege (Aird, 2007; Ben-Zvi et al., 2014; Daneman and Prat, 2015; Nguyen et al., 2014). Taken together, the specific properties of CNS ECs ensure the formation of highly selective BBB that is essential for CNS homeostasis.

In the bone marrow, the blood vessels provide vascular niches to regulate the homeostasis of the hematopoietic stem and progenitor cells (HSPCs). It has been found that the more permeable sinusoid endothelium promotes HSPCs activation and proliferation, whereas the arterial vessels promote HSPC quiescence by maintaining low level or reactive oxygen species (ROS) (Itkin et al., 2016). Though the presence of the arterial vascular niche is still controversial, a recent study demonstrated that the stem cell factor (SCF) is specifically produced by the arterial endothelial cells in the bone marrow (Acar et al.,

2015; Xu et al., 2018). It suggests that the functional heterogeneity of the ECs even exists in the same organ.

Indeed, ECs in the heart show different structural and molecular characteristics, which leads to specific functions. For instance, endocardial ECs are larger and with many microvilli, which largely increase the surface area in the endocardium. The endocardial ECs highly express von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS). eNOS serves to modulate heart contraction, relaxation and rate, which are the major functions of endocardium (Aird, 2007b). The myocardial capillaries are continuous endothelium and in close contact with the cardiomyocytes (~ 1μm). The organization does not only ensure the efficient exchange of oxygen and nutrients, but reciprocal modulation between ECs and muscle cells (Aird, 2007b). ECs in the heart also contribute to the cardiac metabolism by transporting blood-borne fatty acid to the adjacent tissues via transporters and binding proteins that are exclusively expressed in heart ECs (Coppiello et al., 2015). It has been reported that paracrine vascular endothelial growth factor B (VEGFB) and 3-hydroxy-isobutyrate, an amino acid metabolite, regulate fatty acid transport through the cardiac ECs respectively (Hagberg et al., 2010; Jang et al., 2016). However, Alitalo and coworkers demonstrated that VEGFB plays no role in fatty acid transportation in heart (Kivela et al. 2014).

LECs also show the organ-specific characteristics. Lacteals, the specialized lymphatic capillaries in the villi of intestine, can uptake the dietary fats and transport microbial antigens and antigen-presenting DCs (Kim et al., 2007b). Notably, the lymphatics in lacteal share the typical structures of lymphatic capillaries and collecting lymphatic vessels by showing a mix of continuous and discontinuous junctions, which are maintained by Notch ligand delta-like 4 (Dll4) signaling (Bernier-Latmani et al., 2015). Recent advances have also uncovered the lymphatic or lymphatic-like vessels in the CNS system such as meningeal lymphatics and the Schlemm's canal in the eye (Aspelund et al., 2015; Louveau et al., 2015; Kizhatil et al., 2014; Park et al., 2014; Aspelund et al., 2014). The meningeal lymphatics, expressing the lymphatic capillary markers, are involved in draining cerebrospinal fluid, immune cells, and small molecules from the CNS to the deep cervical LNs (Aspelund et al., 2015; Louveau et al., 2015), and the Schlemm's canal, showing a mixture of both blood and lymphatic vascular phenotypes, is essential for aqueous humor drainage from the eye (Kizhatil et al., 2014; Park et al., 2014; Aspelund et al., 2014).

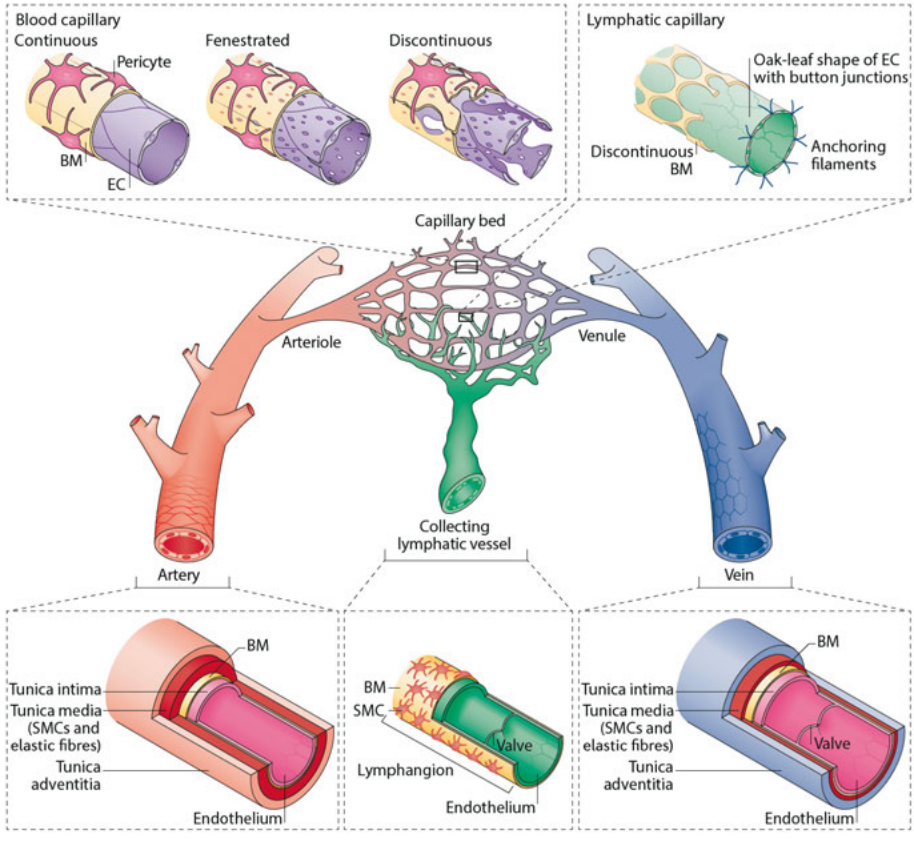

Figure 1. Structure of the blood vasculature and of the lymphatic vasculature. The blood vasculature is a closed system including arteries, arterioles, capillaries, venules, and veins. Both arteries and veins have three histologically different layers: *tunica intima*, *tunica media*, and *tunica adventitia*. They are covered by continuous BM and SMCs. There are three types of capillaries: continuous, fenestrated, and discontinuous. The capillaries are covered by BM and pericytes. BM in continuous and fenestrated capillaries is continuous, whereas in the discontinuous capillaries is discontinuous. There are pores in the endothelium of both fenestrated and discontinuous capillaries, and the sizes of pores are around 70 nm and 100-200 nm in diameter respectively. The lymphatic vasculature is comprised of lymphatic capillaries, pre-collecting lymphatics, and collecting lymphatics. In the lymphatic capillaries, LECs are of oak-leaf shape and show discontinous button-like junctions. BM is discontinuous in the lymphatic capillaries, and there is no SMC coverage. The anchoring filaments maintain the structure and control the loose valve-like openings of capillary lymphatic vessel. Collecting lymphatic vessels are surrounded by BM and covered by SMCs, and contain bileaflet intraluminal valves. LECs in collecting lymphatic vessels contain zipper-like junctions. The lymphangions are the functional and structural units separated by the valves. **Reprinted with permission (Potente and Mäkinen, 2017)**.

More recently, a new population of isolated perivascular cells surrounding the meningeal blood vessels has been identified in zebrafish by three different groups (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017). There is no consensus on the name of these cells so far, and they are named mural LECs (Bower et al., 2017a), brain LECs (van Lessen et al., 2017), and zebrafish fluorescent granular perithelial cells (Venero Galanternik et al., 2017), respectively. These cells do not form lumenized vessels (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017), but, like lymphatic vessels, they depend on VEGFC/vascular endothelial growth factor receptor 3 (VEGFR3) signaling for development (Bower et al., 2017a; van Lessen et al., 2017). They not only express lymphatic markers like LYVE1 and prospero homeobox 1 (PROX1), but also a perivascular macrophage marker – mannose receptor. They uptake and store macromolecules such as lipids and low-density lipoproteins from the meningeal blood vessels and regulate meningeal vascularization but not maintenance (Bower et al., 2017a; van Lessen et al., 2017; Venero Galanternik et al., 2017).

In brief, ECs adapt unique structural and molecular phenotypes in different vascular beds to meet the distinct functions across different organs and tissues. It is of great interest to delineate the molecular mechanisms regulating the EC heterogeneity.

Development of the blood and lymphatic vasculatures

The blood vasculature is formed to meet the increased demands of oxygen and nutrients during development. Two different mechanisms – vasculogenesis and angiogenesis, contribute to new blood vessel formation. In vasculogenesis, blood ECs (BECs) are differentiated from the mesoderm-derived endothelial progenitors (angioblasts) and assembled to form the vascular lumen and then vascular network. In angiogenesis, new blood vessels grow from the preexisting vessels via expansion or remodeling (Herbert and Stainier, 2011). Shortly after the blood flow starts, a subpopulation of venous ECs become specified to LECs, which in turn form the whole lymphatic vasculature through proliferation and sprouting, and this process is named lymphangiogenesis (Yang and Oliver, 2014). Notably, latest advances have revealed non-venous origins of LECs and organotypic mechanisms of lymphatic vessel formation, which will be discussed later.

Vasculogenesis

There are two waves of vasculogenesis in mice: extraembryonic and intraembryonic (Figure 2a). The extraembryonic vasculogenesis starts in the yolk sac

(Chong et al., 2011). The extra-embryonic mesoderm derived hematopoietic precursors and angioblasts assemble to form blood islands around E7, in which the hematopoietic precursors are surrounded by the angioblasts. The blood islands give rise to the primitive vascular network through coalescence. Additionally, dispersed angioblasts in the yolk sac are also shown to contribute to the vasculature formation (Drake and Fleming, 2000). Moreover, allantois is an alternative site of extraembryonic vasculogenesis (Drake and Fleming, 2000). Intraembryonic ECs differentiate from the angioblasts without concomitant differentiation of hematopoietic cells with the exception of a small region in the aorta (Risau and Flamme, 2003). Through coalescence or migration, the angioblasts form the first functional blood vasculatures including endocardium, the dorsal aortea and the cardinal veins during the intraembryonic vasculogenesis (Drake and Fleming, 2000).

Molecular signals mediating vasculogenesis are mainly derived from the endoderm though there is evidence showing that endoderm is dispensable for angioblast differentiation in xenopus and chick (Marcelo et al., 2013; Vokes and Krieg, 2002). Indian hedgehog (IHH) derived from the visceral endoderm has been shown to be sufficient for both endothelial and hematopoietic specification via its downstream effector bone morphogenetic proteins (BMPs) (Astorga and Carlsson, 2007; Dyer et al., 2001). Genetic inactivation of *Ihh* leads to defective vasculogenesis in the yolk sac (Astorga and Carlsson, 2007). VEGF signaling is essential for endothelial lineage commitment. VEGFA is expressed in the endoderm, and VEGFR2, the receptor, is expressed in the mesodermal progenitors (Breier et al. 1995; Motoike et al. 2000). Loss of a single copy of *Vegfa* leads to compromised vasculature development and in turn lethality of the mouse embryos (Carmeliet et al., 1996; Ferrara et al., 1996). Similarly, *Vegfr2*-deficient embryos have no organized blood vessels in the yolk sac due to EC differentiation defects (Shalaby et al., 1995). Interestingly, Herzog and colleagues recently revealed that VEGFA is not required for angioblast specification or migration in zebrafish. Instead, they found that two hormones – Apelin and Elabela, which bind to and activate the Apelin receptor in the angioblasts, mediate migration of angioblasts to the midline in zebrafish (Helker et al., 2015). VEGFA also binds to semaphorin (SEMA) receptors neuropilin1 and 2 (NRP1 and NRP2), which are coreceptors for VEGFR2 (Soker et al., 1998). *Nrp1* and *2* double-knockout mice show avascular yolk sac, whereas the phenotypes in the single knockout mutants are less severe (Takashima et al., 2002). One of the key regulators of *Vegfr2* expression is Cloche (Liao et al., 1997). *Cloche* mutant zebrafish show defects in both endothelial and hematopoietic differentiation (Liao et al., 1997). Cloche has recently been identified as the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein neuronal PAS domain-containing protein 4-like protein (NPAS4L) (Reischauer et al., 2016). However, NPAS4, which is the highest homologue of Npas4l in mice and can rescue the loss-of-function mutants of

Npas4l in zebrafish, is not essential for development, although it is transiently expressed during EC specification (Reischauer et al., 2016). Additionally, a group of transcription factors such as Gata proteins, members of the Krüppel-like factors (KLFs), and the E26 transformation-specific (ETS) proteins, are required for endothelial differentiation (Ferguson et al., 2005).

Arterial or venous fate is established in response to intrinsic and extrinsic signals. Expression of hairy- and enhancer of split-related with YRPW motif (HEY) basic helix-loop-helix transcription factors HEY1 and HEY2, which are induced by Notch signaling, promotes arterial specification; whereas the nuclear receptor COUP transcription factor 2 (COUP-TFII) suppresses the Notch signaling and induces venous differentiation (Herbert and Stainier, 2011; You et al., 2005). Kohli et al recently demonstrated that arterial and venous progenitors are located in the distinct regions of the lateral plate mesoderm, which is mediated by VEGFA and Hedgehog concentrations (Kohli et al., 2013). In addition, Herzog and colleagues have showed that the angioblasts giving rise to common cardinal veins (CCVs) are distinct from the ones generating the lateral dorsal aortae (Helker et al., 2013). Surprisingly, they uncovered that the formation of CCVs is through lumen ensheathment and that EC proliferation within the growing CCVs depends on VEGFC derived from the red blood cells in the circulation (Helker et al., 2013). Furthermore, they showed that mesenchymal SEMA3D controls the migration of the EC sheets during CCV outgrowth through plexin signaling and the autocrine SEMA3D regulates actin network organization and junction formation to stabilize the EC sheet (Hamm et al., 2016).

Angiogenesis

Angiogenesis is a multistep process involving sprouting, lumen formation, anastomosis and remodeling and maturation into functional vascular system, and each step is tightly regulated by distinct molecular mechanisms (Figure 2b).

VEGFA is one of the most important proangiogenic factors (Herbert and Stainier, 2011). Upon binding to VEGFA, VEGFR2 dimerization and transphosphorylation creates docking sites for intracellular signaling molecules (Koch and Claesson-Welsh, 2012). The VEGFR2 phosphorylation leads to activation of various downstream pathways including mitogen-activated protein kinases, phosphoinositide 3-kinases (PI3Ks), AKT, phospholipase C_{γ} and small GTPases (Napione et al., 2012; Simons et al., 2016). This signaling network modulates different aspects of angiogenesis including EC proliferation, filopodial extension, BM degradation and chemotaxis (Simons et al., 2016). VEGFA also binds to VEGFR1 with high affinity, but induces weak kinase

activity. Thus, VEGFR1 functions as a decoy receptor antagonizing proangiogenic signaling (Hiratsuka et al., 2005). Moreover, soluble VEGFR1s, which sequester free VEGFA, can direct proper sprouting as a spatial cue (Chappell et al., 2009). Loss of *Vegfr1* leads to aberrant angiogenesis and embryonic lethality in mice (Fong et al., 1999; Fong et al., 1995; Hiratsuka et al., 2005).

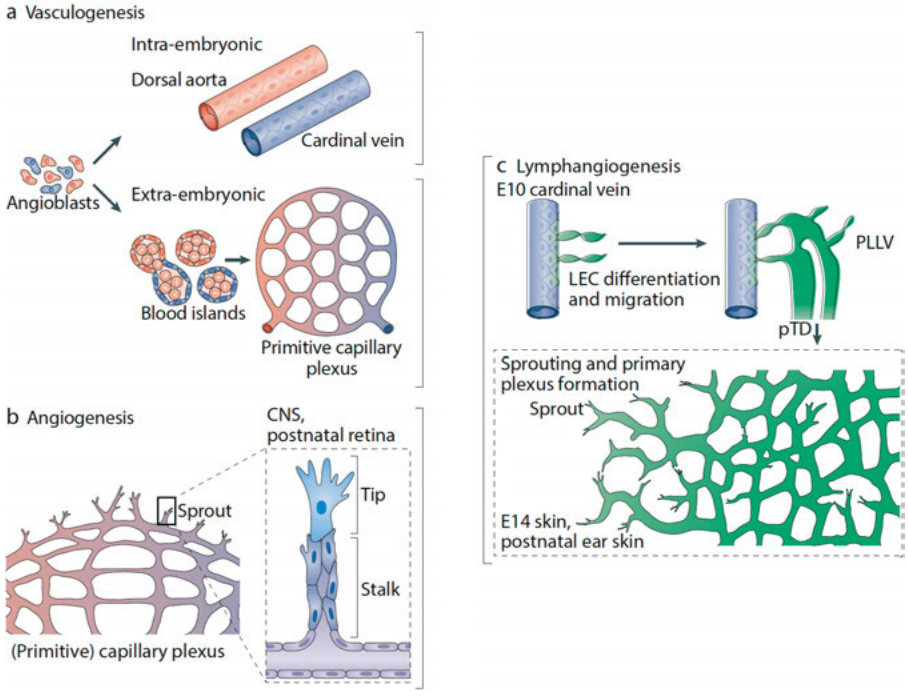

Figure 2. Development of the blood and lymphatic vasculatures. **a.** Through two waves of vasculogenesis (intra- and extra- embryonic), the angioblasts differentiate into either arterial or venous ECs, and the newly formed BECs give rise to major blood vessels including cardinal vein and dorsal aorta in the embryo proper and primitive extra-embryonic vascular network respectively. **b.** Angiogenesis generates new vessels from the preexisting blood vessels via sprouting, branching, lumen formation, anastomosis, and remodeling. Specified BECs named tip and stalk cells are involved in sprouting angiogenesis. **c.** Subpopulation of ECs in the cardinal vein acquire LEC identity at around E9.5 in mice. These LECs bud off and assemble to form the lymph sacs at around E10.5 and E11.5 respectively. The lymphatic network arises from the lymph sac through LEC proliferation and sprouting. **Reprinted with permission (Potente and Mäkinen, 2017)**.

In their seminal work, Betsholtz group characterized tip and stalk ECs during angiogenesis of retina, and showed that tip cells extend many filopodia extensions to sense the adjacent microenvironment and migrate towards the specific cues and that stalk cells are much less motile and more proliferative (Gerhardt

et al., 2003). Notch signaling is required for specification of tip and stalk cells (Phng and Gerhardt, 2009; Roca and Adams, 2007). Tip cells express higher level of the transmembrane Notch ligand Dll4 that activates Notch signaling via NOTCH1 in the adjacent stalk cells, which leads to inhibition of tip cell fate acquisition by stalk cells and maintain the sprouting EC hierarchy (Hellström et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). Higher expression of Dll4 in tip cells is mediated by VEGFA- VEGFR2 signaling through MEF2 transcription factors (Sacilotto et al., 2016). Blocking the Notch signaling increases expression of tip cell-associated genes, which leads to abnormal tip cell formation and sprouting, increased EC proliferation, and vascular mispatterning (Hellström et al., 2007; Leslie et al., 2007; Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). In zebrafish, constitutively active Notch expressing ECs are excluded from the tip cell positions of the sprouting vessels (Siekmann and Lawson, 2007). Dll4-Notch signaling suppresses the tip cells fate by modulating VEGFR signaling. Notch signaling inhibits VEGFR2 function and blocks VEGFR3 expression in the stalk cells (Lobov et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007), yet Notch activation upregulates expression of *Vegfr1* to block tip cell specification (Funahashi et al., 2010). Using genetic mosaic sprouting assay, Jakobsson and colleagues showed that ECs compete for the tip cell positions through dynamic changes of expression of *Vegfr1* and *Vegfr2* (Jakobsson et al., 2010). However, two latest studies in both fish and mice respectively showed that Dll4 is dispensable for maintenance of the tip cell position (Hasan et al., 2017; Pitulescu et al., 2017). Unlike tip cells, stalk cells show lower level of Dll4 expression but highly express Jagged 1, a Notch ligand without induction of productive Notching signaling. Jagged 1 functions as a negative regulator of Dll4-Notch signaling via antagonizing Dll4 binding, which leads to suppression of Notch signaling in tip cells (Benedito et al., 2009). Moreover, posttranslational modification functions as another layer of regulation of Notch signaling in the ECs specification during angiogenesis (Guarani et al., 2011; Moretti and Brou, 2013). For instance, increased acetylation of Notch intracellular domain (NICD), which is released upon activation and translocates into nucleus to induce gene expression, leads to enhanced stability of NICD and consequently increased Notch signaling (Guarani et al., 2011).

In addition, axonal guidance signals control EC directional migration and vascular patterning as a consequence of neurovascular congruency (Andreone et al., 2015). Slit proteins particularly Slit2 bind to roundabout 1 and 2 receptors to elicit angiogenesis by promoting EC motility and polarity, and this is achieved by interacting with VEGFA through the scaffolding proteins Nck1 and 2 and Rac1 (Dubrac et al., 2016; Rama et al., 2015). SEMA4D binds to PlexinB to activate RhoA GTPase signaling, which in turn induces actin cytoskeleton reorganization and consequently EC migration and tube formation

(Sakurai et al., 2012). SEMA3E-PlexinD1 signaling causes filopodia retraction in the tip cells and disassembly of focal adhesions, thus inhibiting angiogenesis (Sakurai et al., 2010). Furthermore, Ephrin receptor (EPH)/ephrin signaling such as EPHA2-EphrinA1 and EPHB4-EphrinB2, is known to regulate angiogenesis (Barquilla and Pasquale, 2015). EphrinB2 induces VEGF receptor endocytosis and thus enhances the angiogenic signaling (Nakayama et al., 2013; Sawamiphak et al., 2010; Wang et al., 2010b).

Lumen formation involves EC apical-basal polarity establishment, redistribution of junctional proteins, and cellular shape change (Lammert and Axnick, 2011). VE-cadherin (VE-cad) is required to establish the apical-basal polarity by localizing CD34-sialomucins to the EC contact sites, which defines the apical polarity and initiates EC separation (Strilić et al., 2009). CD34-sialomucins also recruits F-actin and Moesin to the apical cell surface to promote lumen formation (Strilić et al., 2009; Wang et al., 2010a). RAS-interacting protein1 (RASIP1) has been shown to regulate endothelial polarity through modulating GTPase and CDC42/Rac signaling (Xu et al., 2011). *Rasip1*-deficient embryos lack lumenized blood vessels due to abnormal intercellular junction distribution and failure to adhere to the extracellular matrix (Xu et al., 2011). In addition, cerebral cavernous malformation 1 (CCM1) coordinates with VE-cad to regulate junctional localization of the polarity proteins and as a consequence control EC polarity establishment (Lampugnani et al., 2010). The integrin-extracellular signaling also plays a critical role. Loss of β1 integrin results in decreased expression of partitioning defective 3 (PAR3), a key regulator of cell polarity in both epithelium and endothelium, and thus disrupted EC polarity and defective lumen formation (Zovein et al., 2010a). Junctional localization of PAR3 is essential for EC polarity establishment, and it is regulated by VE-cad and RASIP1 (Strilić et al., 2009; Wang et al., 2010a; Xu et al., 2011).

Tip cells contact and fuse with other tip cells or the ECs of the functional vessels to add newly formed blood vessels to the pre-existing vascular network during anastomosis. VE-cad is expressed in the tip of the filopodia from the sprouting ECs, and is required for contact formation (Almagro et al., 2010). Macrophages have been showed to facilitate the process of anastomosis, though they are dispensable for angiogenesis (Fantin et al., 2010; Geudens and Gerhardt, 2011). Newly formed connections proceed to vessel pruning, regression and remodeling to become functional and mature. ECs can sense the blood flow via distinct mechanosensors such as VE-cad, PECAM1, and PIEZO1 (Baratchi et al., 2017). The mechanotransduction of blood flow plays a critical role in the aforementioned processes. Shear stress promotes EC survival and vessel dilation through activation of KLF2 and related downstream signaling (Dekker et al., 2005). Blood flow difference leads to loss of EC symmetry. ECs experiencing low flow retract, then migrate and incorporate into

the high flow regions (Franco et al., 2015; Lenard et al., 2015). By fine-tuning expression of vasoactive genes, Dll4/Notch regulates blood flow and vessel regression, which is preceded by blood flow loss (Lobov et al., 2011). Two recent studies elegantly showed that the EC migration contributes to artery formation via CXCL12/CXCR4 signaling pathway (Pitulescu et al., 2017; Xu et al., 2014). Moreover, endoglin, the TGF-β co-receptor, has been shown to be required for flow-induced EC migration and vessel size determination (Jin et al., 2017; Sugden et al., 2017). Notably, endoglin and activin receptor-like kinase 1 mutations in humans causes hereditary hemorrhagic telangiectasia, in which patients show arteriovenous malformations (Pardali et al., 2010). In addition, oxygenation and nutrient delivery can downregulate VEGF signaling and thus induce EC apoptosis and vessel regression (Korn and Augustin, 2015).

Stabilization of the newly formed vessels requires BM deposition, mural cell (pericytes and SMCs) recruitment, and junction formation. BM matrix formation is mediated by pericyte recruitment and EC-pericyte interaction (Stratman et al., 2009; Stratman et al., 2010). Pericytes cover the immature and small blood vessels such as capillaries, whereas SMCs are associated with major vessels including arteries and veins (Gaengel et al., 2009). EC-derived platelet-derived growth factor B (PDGFB) stimulates pericyte migration and proliferation via platelet-derived growth factor receptor β (PDGFRβ) in the pericytes (Hellström et al.; Lindahl et al.). Of note, requirement of the PDGFB/PDGFRβ signaling is context-dependent. Loss of either *Pdgfb* or *Pdgfrβ* causes significant reduction of pericyte recruitment in various vascular beds but liver (Hellström et al. 1999; Lindahl et al. 1997). Sphingosine-1-phosphate receptor (S1PR) is responsible for redistribution of N-cadherin to the abluminal side to enhance the EC-pericyte interaction and thus blood vessel stabilization (Paik et al., 2004). Additionally, heparin-binding epidermal growth factor signaling, stromal-derived factor 1-a /CXCR4, sonic hedgehog signaling, NOTCH3, and EPH-Ephrin are controlling pericyte recruitment to the ECs (Armulik et al., 2011). Failure of pericyte recruitment leads to various vascular dysfunctions, for example, leaky BBB (Armulik et al., 2010).

Lymphangiogenesis

Lymphangiogenesis includes LEC specification, LEC budding and migration, and lymph sac formation, from which most of lymphatics are arising through LEC proliferation and sprouting (Figure 2c).

In mice, lymphatic vessel development starts at E9.5-E10 when a subset of ECs starts expressing the lymphatic markers in the anterior cardinal vein (CV), the intersomitic veins, and the superficial venous plexus, (Aspelund et al., 2016b). The homeobox transcription factor PROX1 is the master regulator in

LEC fate induction and maintenance (Yang and Oliver, 2014). *Prox1*-deficient mice are devoid of lymphatic vessels due to failure of LEC budding and sprouting (Wigle and Oliver, 1999; Yang et al., 2012). *In vitro* studies have demonstrated that overexpression of *Prox1* can reprogram BECs into LEC phenotype by suppression of BEC-specific and induction of LEC-specific genes (Hong et al., 2002; Petrova et al., 2002). A recent study has revealed that PROX1 regulates key lymphatic genes epigenetically by modulating fatty acid β-oxidation (Wong et al., 2016). PROX1 also regulates the number of LEC progenitors in a dose-dependent manner via PROX1/VEGFR3 positive feedback loop (Srinivasan et al., 2014). In addition, the *Prox1* dosage is important for lymphovenous valve formation (Srinivasan and Oliver, 2011). Moreover, PROX1 is required for maintenance of LEC identity. Conditional deletion of *Prox1* at embryonic, postnatal, or adult stages leads to reversal of LEC fate towards BEC fate (Johnson et al., 2008). PROX1 expression in the venous ECs is regulated by SOX 18, which binds to the *Prox1* promoter region (Francois et al., 2008). *Sox18*$^{-/-}$ embryos, on a C57BL/6 genetic background, show no *Prox1* expression in BECs in cardinal vein at E10.5 and consequently lack lymphatic vessels (Francois et al., 2008). Conversely, overexpression of *Sox18* in BECs induces expression of *Prox1* and other LEC markers (Francois et al., 2008). Recently, it has been shown that SOX18 is required for normal expression of *Mafba*, a newly identified key regulator of lymphangiogenesis (Koltowska et al., 2015b). SOX18 activity is positively controlled by the extracellular signal regulated kinase (ERK) signaling, which is inhibited by the PI3K/AKT signaling pathway in endothelial cells (Deng et al., 2013; Ren et al., 2010). Of note, contrary to PROX1, SOX18 is not expressed in the LECs at later stages of embryonic development, indicating SOX18 is required for inducing but not maintaining *Prox1* expression (Francois et al., 2011). Furthermore, COUP-TFII promotes and maintains expression of PROX1 in the LECs during specification and differentiation, but not in the mature LECs (Srinivasan et al., 2010). It has also been found that COUP-TFII interacts with PROX1 as a co-regulator mediating lymphatic specific gene expression such as VEGFR3 and LYVE1 to maintain LEC identity (Lee et al., 2009; Srinivasan et al., 2010; Yamazaki et al., 2009). In addition, COUP-TFII promotes LEC fate acquisition via inhibition of Notch signaling pathway (Srinivasan et al., 2010), which functions as a negative regulator of LEC specification (Murtomäki et al., 2013).

During development, LYVE1 is expressed in LEC progenitors before PROX1 expression (Gordon et al., 2008; Wigle et al., 2002). However, LYVE1 gene-targeted mice do not show abnormal lymphatic development and function, suggesting it is not essential for either lymphatic development or function (Gale et al., 2007). NOTCH1 is expressed in the endothelial cells located in the CV in the period of LEC differentiation from the CV endothelium (Murtomäki et al., 2013). Conditional knock out *Notch1* in LEC progenitors leads

to increased number of PROX1+ venous endothelial cells, enlarged lymph sac, mild edema and blood-filled lymphatics in the embryonic skin (Murtomäki et al., 2013). In contrast, constitutive activation of NOTCH1 in PROX1+ endothelial cells causes loss of PROX1+ endothelial cells, abnormal thoracic duct, severe edema and blood-filled lymphatics (Murtomäki et al., 2013). Choi and colleagues demonstrated that laminar flow induces LEC sprouting by suppression of NOTCH1 activity (Choi et al., 2017). However, other studies in zebrafish and neonatal mice have revealed that Notch signaling enhances lymphangiogenesis (Geudens et al., 2010; Niessen et al., 2011). Species and/or developmental stages and context-dependent Notch signaling may contribute to the discrepancy, and further studies are required.

After exiting the veins, LECs migrate as streams of cells and assemble into the first lymphatic vessels called lymph sacs at E10.5-11.5 (Hägerling et al., 2013; Yang et al., 2012). Lymph sac is composed of a single layer of LECs and serves as the major source of LECs during mid-gestation (Yang and Oliver, 2014). The migrating LECs are interconnected via intercellular junctions (Yang et al., 2012). During this process, the expression of LEC markers changes. For instance, expression of Podoplanin (PDPN), a type-I transmembrane sialomucin-type *O*-glycoprotein, starts in the LEC progenitors only after they exit embryonic veins (Francois et al., 2012; Pan and Xia, 2015; Yang et al., 2012).

The key regulator of LEC sprouting is the VEGFC/CCBE1/VEGFR3/NRP2 pathway. VEGFC is expressed in smooth muscle cells and mesenchymal cells that are located adjacent to the region where the lymphatic vessels develop (Karkkainen et al., 2004). Inactivation of *Vegfc* in mice does not affect LEC fate acquisition, but arrests PROX1+ LEC migration from the veins and lymph sac formation (Karkkainen et al., 2004). In contrast, skin-specific overexpression of *Vegfc* in mice induces LEC proliferation, which in turn leads to hyperplasia of superficial lymphatic network in skin (Jeltsch, 1997; Veikkola et al., 2001). Using different strategies, two groups independently identified transcription factor Mafba as a downstream effector of VEGFC signaling pathway (Dieterich et al., 2015; Koltowska et al., 2015b). In zebrafish, VEGFC is required for LEC progenitor division and PROX1 expression (Koltowska et al., 2015a). VEGFD is the other known activating ligand for VEGFR3. Overexpression of *Vegfd* in the dermis results in lymphatic hyperplasia without affecting blood vessel in mice (Veikkola et al., 2001). It can also stimulate tumor lymphangiogenesis by activating VEGFR3 (Stacker et al., 2001). However, deletion of *Vegfd* has no effect on lymphatic vascular development in mice (Baldwin et al., 2005). Surprisingly, taking advantage of TALEN-mediated mutagenesis, Hogan and colleagues created a new strain of *Vegfd* mutant zebrafish and observed serious defects in facial lymphangiogenesis but normal trunk lymphatic vessel formation (Bower et al., 2017b). They further revealed

that VEGFC and VEGFD regulate lymphatic vessel development cooperatively, though VEGFC alone is efficient for trunk lymphatic vessel formation, suggesting the context-specific role of VEGFD in lymphangiogenesis (Bower et al., 2017b). Expression analysis of collagen and calcium-binding EGF-like domain 1 (CCBE1) revealed that it is expressed in the proximity of the PROX1+ LEC progenitors within the anterior cardinal vein at E9.5 in mice (Facucho-Oliveira et al., 2011). CCBE1 induces lymphangiogenesis by regulating the proteolytic processing of VEGFC through interaction with the A disintegrin and metalloprotease with thrombospondin motifs 3 (ADAMTS3) to increase the amount of bioactive VEGFC (Bos et al., 2011; Bui et al., 2016; Jeltsch et al., 2014; Roukens et al., 2015). *Ccbe1* deficiency in mice results in abnormal sprouting, followed by downregulation of *Prox1* and loss of all lymphatic vessels (Hägerling et al., 2013). More recently, lethality and massive edema due to lack of lymphatic vessel development have been observed in *Adamts3*$^{-/-}$ embryos at E15 (Janssen et al., 2016).

After LEC fate acquisition, VEGFR3 upregulation in LECs is accompanied with downregulation in BECs. Later, its expression is largely restricted to LECs except for the fenestrated blood vessels in endocrine organs and angiogenic blood vessels (Kaipainen et al., 1995; Partanen et al., 2000). *In vivo* and *in vitro* studies have demonstrated that VEGFR3 is a direct target of PROX1 (Mishima et al., 2007; Pan et al., 2009; Petrova et al., 2002; Srinivasan et al., 2014; Yamazaki et al., 2009), while VEGFR3 is required for PROX1 expression and maintenance of LEC identity and the number of LEC progenitors (Srinivasan et al., 2014). Activation of VEGFR3 by VEGFC is important for LEC growth, migration, and survival (Mäkinen et al., 2001b). VEGFC stimulation of LECs can cause heterodimer formation between VEGFR2 and VEGFR3, thus may induce distinct combinatorial signaling of these two receptors (Dixelius et al., 2003). A further study has doonstrated that the VEGFR2/VEGFR3 heterodimer is necessary for AKT activation, whereas the VEGFR3 homodimer is required for ERK activation in LECs upon VEGFC activation (Deng et al., 2015). Missense mutation in the tyrosine kinase domain in *Chy* mice results in hypoplasia of cutaneous lymphatic capillaries, lymphedema and chylous ascites due to the defects of lymphatic vasculature (Karkkainen et al., 2000; Karkkainen et al., 2001). Transgenic overexpression of VEGFR3-Ig fusion protein (VEGF-C/D trap) in mouse skin inhibits dermal lymphatic vascular formation, and leads to a regression of the fetal lymphatic vasculature, suggesting the essential role of VEGFR3 in maintenance of lymphatic vasculature (Mäkinen et al., 2001a). Interestingly, although activation of VEGFR3 by its soluble ligands is required to maintain the lymphatic capillaries in the first two weeks after birth, its activation becomes dispensable after two weeks, suggesting a stage-dependent molecular mechanism controlling lymphatic vessel maturation (Karpanen et al., 2006b).

VEGFR2, a receptor tyrosine kinase, is expressed in blood vessels, collecting lymphatic vessels, and lymphatic capillaries in the process of lymphangiogenesis (Nagy et al., 2002; Wirzenius et al., 2007). It is activated by VEGFA and VEGFC, and the viral VEGFE. *In vitro* studies demonstrated that VEGFR2 signaling can promote LEC proliferation, migration, and tube formation, indicating its critical role in lymphangiogenesis (Dellinger and Brekken, 2011; Hong et al., 2004; Veikkola et al., 2003). Ectopic expression of $Vegfe_{NZ7}$, a virus-encoded ligand that specifically binds to VEGFR2, in skin leads to hyperplastic lymphatic vessels by enhancing LEC proliferation in postnatal mice (Wirzenius et al., 2007). Nevertheless, selective activation of VEGFR2 does not affect lymphatic vessel sprouting (Wirzenius et al., 2007).

NRP2 functions as a co-receptor for VEGFR3 in LECs. Upon VEGFC and VEGFD stimulation, it becomes internalized with VEGFR3 (Karpanen et al., 2006a). It is involved in regulating LEC sprouting rather than LEC specification (Xu et al., 2010). $Nrp2^{-/-}$ mice show abnormal development of small lymphatic vessels and capillaries (Yuan et al., 2002). The genetic interaction between NRP2 and VEGFR3 is critical for proper lymphatic vessel sprouting (Xu et al., 2010). Double heterozygous $Nrp2^{+/-};Vegfr3^{+/-}$ mice display reduced lymphatic vessel sprouting and branching in adult (Xu et al., 2010). It has been shown that COUP-TFII regulates lymphatic development by stimulating expression of NRP2 and modulating the VEGFR3/NRP2 signaling pathway (Lin et al., 2010). Recently, NRP2 has been demonstrated to mediate lymphatic vascular patterning by binding to SEMA3G through a receptor complex with PlexinD1, though no abnormal artery-lymph alignment was observed in the back skin of *Nrp2-null* mouse embryos (Liu et al., 2016).

There are several factors regulating lymph sac formation. For example, polycystin signaling has been shown to play a crucial role in LEC progenitor migration after budding off from the veins (Coxam et al., 2014; Outeda et al., 2014). In *Pkd1*/*Pkd2*-deficient mouse embryos, LEC progenitors can bud off and migrate from the veins, but sprouting is decreased and progenitor migration is not directed compared to the *wild type* ones. In addition, components of the adrenomedullin signaling pathway such as adrenomedullin and its receptors CALCR1 and RAMP2 are important in controlling LEC proliferation and morphological integrity of the jugular lymphatic vessels, but not in early stages of LEC differentiation, budding and migration (Fritz-Six et al., 2008). Lack of the adrenomedullin signaling results in smaller lymph sacs and subcutaneous edema in the embryos (Fritz-Six et al., 2008).

After lymph sacs are formed, they maintain connections to the veins (Uhrin et al., 2010). LECs from the lymph sacs intercalate with the PROX1+ venous endothelial cells to form lymphovenous valves at the junctions between the lymph sac and the vein (Hägerling et al., 2013; Srinivasan and Oliver, 2011).

Lymphovenous valves prevent blood backflow into the lymphatic system (Hess et al., 2014). Recently, Geng et al. characterized lymphovenous valve morphogenesis during mouse embryogenesis and described the role of several previously known regulators of lymphatic (valve) development such as PROX1, FOXC2, CONNEXIN37 (CX37), and GATA2 in this process (Geng et al., 2016). GATA2 has been further shown to control the development and maintenance of both lymphovenous and lymphatic vessel valves via regulating expression of *Prox1* and *Foxc2* (Kazenwadel et al., 2015). Interestingly, mutations in *Gata2* leads to primary lymphedema in human (Kazenwadel et al., 2012; Ostergaard et al., 2011).

Cyclin-dependent kinase 5 (CDK5), a proline-directed serine/threonine kinase, has been shown to play an important role in lymphatic development through regulating FOXC2 activity by phosphorylation (Liebl et al., 2015). Endothelial cell-specific knockout of *Cdk5* caused defective lymphatic vessel development including lymphovenous and lymphatic valve formation (Liebl et al., 2015). More recently, it was reported that LEC-specific inactivation of *Ephb4*, leads to failed formation of lymphovenous valves and subcutaneous edema (Martin-Almedina et al., 2016). Interestingly, inactivating *Ephb4* mutations in humans lead to hydrops fetalis, which is characterized by excessive accumulation of fluid (Martin-Almedina et al., 2016).

Platelets have also been proposed to play an essential role in 'lymphovenous separation', due to a blood-filled lymphatic vessel phenotype exhibited by several mouse mutants with defective platelet function (Welsh et al., 2016). The functions of platelets in this process will be discussed later.

LECs continue to proliferate and migrate from the lymph sacs into mesenchymal tissue (Tammela and Alitalo, 2010). The resulting primitive lymphatic plexus gives rise to two different types of lymphatic vessels: collecting and capillary lymphatic vessels, in a step-wise manner (Schulte-Merker et al., 2011). The lymphatic capillaries lack basement membrane or mural cell coverage, while the collecting lymphatic vessel maturation includes valve formation and mural cell recruitment accompanied by the downregulation of LEC markers such as PROX1, LYVE1, and VEGFR3 in non-valve cells (Bazigou and Mäkinen, 2013; Norrmén et al., 2009).

A balanced Angiopoietin (ANG)/TIE signaling is required for lymphatic vasculature maturation, valve formation, and maintenance of vessel integrity (Tammela and Alitalo, 2010; Zheng et al., 2014). ANG2 is highly expressed in the LECs, whereas the expression of the receptors TIE2 and its homolog TIE1 is low (Norrmén et al., 2009). *Tie1* mutant embryos show mispatterned lymph sac and edema from E12.5 (D'Amico et al., 2010). Knockout of *Ang2* in mice does not compromise the embryonic vasculature development (Gale

et al., 2002). Blocking the ANG2/TIE2 signaling using the ANG2 antibody impairs lymphatic vessel sprouting, LEC proliferation, valve formation, and SMC recruitment (Zheng et al., 2014). Moreover, ANG2 is also found to play a role in junctional transformation in the capillary lymphatics and maintenance of LEC junctions in collecting lymphatic vessels (Zheng et al., 2014). Interestingly, replacing the *Ang2* gene with cDNA of *Ang1* can rescue the defective lymphatic phenotypes in *Ang2*$^{-/-}$ mice (Gale et al., 2002). Overexpression of *Ang1*, *2*, *3*, or *4* increases the number of sprouts from lymphatic vessels in adult mice (Kim et al., 2007a). During development, overexpression of *Ang2* promotes LEC proliferation and increases vessel caliber but not vessel density (Zheng et al., 2014).

Mice with a mutation in the C-terminal PDZ domain of EphrinB2 show hyperplastic collecting lymphatic vessels that are devoid of valves, and failed remodeling of primary lymphatic capillary plexus into a mature vessel network, suggesting the EphrinB2-depdendent reverse signaling is required for lymphatic remodeling and maturation (Mäkinen et al., 2005). In addition, combining specific antibody targeting and *EphrinB2* mutant mice, Yan and colleagues demonstrated that EPHB4 forward signaling is indispensable for lymphatic valve formation (Zhang et al., 2015). Inducible deletion of *Cnb1*, the calcineurin regulatory subunit, results in abnormal formation of lymphatic valve region and valves themselves (Sabine et al., 2012). Furthermore, the gap junction proteins of the connexin family (CX26, CX37, CX43, and CX47) are required for lymphatic development (Kanady et al., 2011; Kanady et al., 2015; Munger et al., 2016). For instance, there are no lymphatic valve-forming cells in *Cx37* knockout mice (Kanady et al., 2011; Sabine et al., 2012). Lymphatic-specific loss of *Cx43* in mice leads to chylothorax due to reduction of valve numbers, delayed lymphatic development, and aberrant leaflet morphology (Munger et al., 2017).

Mechanotransduction induced by lymph flow also contributes to lymphatic valve formation through upregulating the expression of key transcription factors including PROX1, FOXC2, and GATA2 in the valve-forming cells (Kazenwadel et al., 2015; Sabine et al., 2012; Sweet et al., 2015). Moreover, epigenetic modification is involved in regulating lymphvenous valve and lymphatic valve formation. Loss of histone-modifying enzyme histone deacetylase 3 (HDAC3) leads to aberrant development of both lymphvenous and lymphatic valves (Janardhan et al., 2017). Upon oscillatory shear stress, HDAC3 interacts with TAL1, GATA2, and ETS1/2 and is recruited to an intragenic enhancer of *Gata2*. Sequentially, histone acetyltransferase Ep300 is recruited to HDAC3 to form an enhanceosome complex that enhances *Gata2* expression (Janardhan et al., 2017).

SMC recruitment and coverage to the collecting lymphatics is concomitant with the vessel remodeling and maturation. As discussed above, SMC recruitment is preceded by matrix protein deposition in the BM and coincides with LYVE1 downregulation in the LECs (Lutter et al., 2012). Reelin, an extracellular matrix glycoprotein, is produced by the LECs. In response to SMC contact, it is secreted efficiently from the LECs in the collecting lymphatic vessels and thus mediates SMC recruitment (Lutter et al., 2012). *Reelin*$^{-/-}$ mice show decreased SMC coverage and sustained LYVE1 expression in the collecting vessels in mouse ear skin (Lutter et al., 2012). Of note, the mesenteric collecting vessel maturation seems not to adapt the same mechanism, highlighting the organ-specific lymphatic development. Indeed, SMC recruitment in the mesenteric collecting vessels is negatively regulated by flow and fluid shear stress. Lack of lymph flow in the *Clec1b*-deficient neonates results in abnormal SMC coverage (Sweet et al., 2015). SEMA3A is highly expressed in LECs, and its receptor NRP1 is expressed in both perivascular cells and valve LECs (Jurisic et al., 2012). Disruption of the SEMA3A/NRP1 signaling leads to defective lymphatic vessel function, abnormal lymphatic vessel morphology, and aberrant SMC coverage in the valve areas (Bouvrée et al., 2012; Jurisic et al., 2012). Nevertheless, the signal molecules regulating the initial SMC-LEC contact remain elusive. PDGFB, which is also expressed in the LECs, has been proposed to be involved in SMC recruitment to the collecting lymphatic vessels (Tammela et al., 2007). FOXC2, the forkhead transcription factor, is highly expressed in the lymphatic vessels during development and in the lymphatic valves in adulthood. It cooperates with VEGFR3 to regulate the lymphatic vascular patterning (Aspelund et al., 2016b). The collecting lymphatic vessels maintain high level expression of VEGFR3, PROX1 and LYVE1, and are highly branched without intraluminal valve formation in *Foxc2*-deficient mice (Norrmén et al., 2009). *Foxc2*-deficiency leads to aberrant SMC recruitment and coverage in the lymphatic capillaries, which has been proposed to be mediated by PDGFB upregulation due to loss of FOXC2 suppression (Meinecke et al., 2012; Petrova et al., 2004; Tammela et al., 2007). However, FOXC2 is also expressed in the SMC-covered collecting vessels (Dagenais et al., 2004). Moreover, the ectopic recruitment of SMCs is observed in ANG2 antibody treated embryos, but PDGFB was barely detected in lymphatic capillaries (Zheng et al., 2014). Altogether, differential SMC recruitment to the collecting vessels and capillaries cannot be explained by FOXC2-mediated suppression of PDGFB signaling. Additionally, loss of *Ang2* leads to disorganized lymphatic vessel network and sparse coverage of SMCs postnatally, and thus chylous ascites, lymphedema and hypoplastic lymphatic vasculature abnormal phenotypes (Gale et al., 2002; Shimoda et al., 2007). Dellinger et al. further analyzed the *Ang2*$^{-/-}$ mice and revealed that these animals fail to establish the collecting lymphatic vessel phenotype and show abnormal recruitment of SMCs to the capillary lymphatics (Dellinger et al., 2008).

Hemogenic endothelium (HE)

In addition to giving rise to lymphatics, BEC can generate HE during hematopoiesis. There are two waves of hematopoiesis: primitive and definitive. HE is a subset of the specified ECs that can generate multiple-lineage hematopoietic stem/progenitor cells during definitive hematopoiesis (Lacaud and Kouskoff, 2017). The hemogenic ECs (HECs) expresses both endothelial and hematopoietic cell markers such as VE-cad, PECAM1, cKIT, RUNX1 and GATA2 (Gritz and Hirschi, 2016). Emergence of these specialized ECs is spatially and temporally restricted, and the corresponding progenies show distinct potentialities. In mice, for instance, HECs are found in the yolk sac at E8.25 and are able to give rise to the erythromyeloid progenitors, which in turn give rise to definitive erythrocytes and other myeloid lineages (McGrath et al., 2015). In the later stage, from E9 to E9.5, HECs appear in both yolk sac and para-aortic splanchnopleura tissues and produce both B and T progenitor cells (Yoshimoto et al., 2011; Yoshimoto et al., 2012). The endocardial cells in the heart also possess hemogenic activity and are involved in transient hematopoiesis (Nakano et al., 2013). HECs become specified at different sites such as the dorsal aorta of the aorta-gonad-mesonephros (AGM) region around E9.5, and produce the hematopoietic stem cells (HSCs) from E10 to E11.5 (Chen et al., 2009; Gordon-Keylock et al., 2013; Li et al., 2012; Yokomizo et al., 2011; Zovein et al., 2010b). All the aforementioned hematopoietic stem/progenitor cells are produced through endothelial-to-hematopoietic transition (EHT), in which the HECs bud off from the vessel wall and form the clusters of round cells expressing both endothelial and hematopoietic markers (Klaus and Robin, 2017). Of note, not all the HECs are arterial. For example, the ECs in veins in yolk sac can also contribute to the formation of hematopoietic progenitors (Frame et al., 2015). Interestingly, similar cell clusters are also observed in the omphalomesenteric arteries, suggesting the presence of HECs as well, although they do not develop into HSCs (Rybtsov et al., 2014). Taken together, HE is heterogenous considering distinct locations, developmental stages and progenies.

Most of the studies of EHT have been focused on the AGM region so far. During EHT, the intra-aortic hematopoietic clusters (IAHCs) are formed and attached to the endothelium of the dorsal aorta, and these clusters have been showed to contain the HSC precursors that will mature into the HSCs one day later (Boisset et al., 2014). Unlike other vertebrates in which IAHCs are only found in the ventral side of the dorsal aorta, mice show the clusters in both ventral and dorsal sides (Taoudi and Medvinsky, 2007). Though cells of the ventral and dorsal IAHCs share the similar transcriptome and colony-forming hematopoietic activity, only the ones in the ventral side can generate HSCs (Baron et al., 2018; Taoudi and Medvinsky, 2007).

Both intrinsic and extrinsic factors regulate the EHT. Among them, RUNX1, a transcription factor, is the pivotal regulator of EHT and HSC formation. It is expressed in the hematopoietic cells and HECs (North et al., 1999; Yokomizo et al., 2001). Deletion of *Runx1* results in failure of HSC emergence in mice, but does not affect HE specification (Cai et al., 2000; Chen et al., 2009; Lancrin et al., 2009). Another study further elucidated the function of RUNX1 in this process and found that it plays a crucial role in the cellular transition from VE-cad^{+}CD41^{+}CD45^{-} to VE-cad^{+}CD41^{+}CD45^{+} (Liakhovitskaia et al., 2014). Additionally, ectopic expression of *Runx1* in the non-hemogenic ECs can induce blood cell formation, though specifically in the certain period during development (Yzaguirre et al., 2018). RUNX1's function in EHT is through suppression of the endothelial program and promotion of the hematopoietic program (Gritz and Hirschi, 2016). This can be achieved by its own and/or its downstream targets such as GFI1 and GFI1B (Lancrin et al., 2012; Thambyrajah et al., 2016). RUNX1 can inhibit expression of arterial genes like SOX17 in the HE forming cells to ensure the hematopoietic fate change (Lizama et al., 2015). In the *Gfi1* and *Gfi1B* double knockout mice, there are neither IAHCs nor HSCs formed in the AGM region (Thambyrajah et al., 2016). Additionally, RUNX1 has been shown to activate expression of genes mediating cellular adhesion and migration in the HE to facilitate HSC budding off (Lie-A-Ling et al., 2014). The activity of RUNX1 during EHT requires the +23 enhancer that is bound by GATA2, ETS transcription factors, RUNX1, and stem cell leukemia factors, which in turn are indispensable for EHT and definitive hematopoiesis (Nottingham et al., 2007).

In addition to RUNX1 and related transcriptional network that it is involved in, Notch signaling, Wnt/β-catenin signaling, HOX and SOX genes, epigenetic regulation, and G-protein coupled receptors also contribute to EHT and HSC generation (Gritz and Hirschi, 2016). Moreover, blood flow plays an important role in hematopoietic development by regulating RUNX1 expression and nitric oxide production (Adamo et al., 2009; North et al., 2009).

Origins of endothelial cells

Arteries, veins, and lymphatics not only possess distinct anatomies, structures, and functions, but different molecular profiles. All these differences suggest their unique developmental paths.

BEC origins

As discussed above, mesoderm-derived precursors – angioblasts generate blood ECs in response to signals from the adjacent visceral endoderm. Historically, the first blood ECs were assumed to be homogenous before the onset

of blood flow, which will determine the vascular phenotype in the later developmental stage (Risau, 1997). However, a pioneering work from Anderson lab demonstrated that the arterial and venous fates were genetically determined prior to the blood flow (Wang et al., 1998). They showed that EphrinB2 and its receptor EPHB4 specifically mark the arteries and veins respectively (Wang et al., 1998). Adams and colleagues further showed that complex Ephrin ligand-receptor interactions are involved in arterial-venous separation (Adams et al., 1999). Studies in both mice and zebrafish revealed that a subset of specified arterial and venous ECs progenitors coalesce to form primitive vascular plexus and then separate from each other through migration to from dorsal aortae and CVs respectively, and that the predetermined angioblasts can only give rise to either arterial or venous ECs (Herbert et al., 2009; Lindskog et al., 2014; Red-Horse et al., 2010; Zhong et al., 2001). Additionally, Kohli et al used fate mapping and lineage tracing in zebrafish to uncover two distinct endothelial populations contributing to formation of dorsal aorta and CV differentially (Kohli et al., 2013).

Even though the arterial and venous fate is predetermined during early development, ECs show great plasticity of the phenotypes to adapt to changes of the environmental stimuli and/or of the physiological needs in different tissues and organs. In zebrafish, some arterial ECs can become venous EC during vascular remodeling by downregulating Notch signaling (Quillien et al., 2014). Venous ECs can also generate arterial ECs. Xu and colleagues found that vein-derived endothelial tip cells migrate against the sprouting vascular front and contribute to the artery formation (Xu et al., 2014). Two more recent studies showed Notch signaling is involved in regulating this process in both mice and zebrafish (Hasan et al., 2017; Pitulescu et al., 2017). During heart development, coronary arteries were thought to from via assembly of endothelial tubes from the proepicardium-derived progenitors. Taking advantage of clonal analysis, Red-horse et al. have showed that angiogenic sprouting from the sinus venosus leads to coronary artery formation (Red-Horse et al., 2010). The sprouting venous ECs dedifferentiate into arterial and capillary ECs in the process of migration (Chen et al., 2014; Red-Horse et al., 2010). Single-cell RNA sequencing analysis revealed that the venous to arterial transition is through the pre-artery state in a gradual and simultaneous way (Su et al., 2018). Additional studies have uncovered the endocardial and proepicardial cells as alternative origins of the coronary vessels (Katz et al., 2012; Tian et al., 2014; Wu et al., 2012; Zhang et al., 2016a). Interestingly, loss of sinus venosus-derived coronary vessels in *Apelin* or *Elabela* mutant mice can induce expansion of the endocardium-derived vasculature to compensate and normalize the coronary vasculature and thus heart function (Sharma et al., 2017). This compensatory mechanism adds another layer of complexity of the phenotype switch. It is of great interest to understand physiological functions for the ves-

sels from distinct origins. In addition to contribute to coronary arteries formation, endocardium of the sinus venosus is a source of the liver vasculature (Zhang et al., 2016b). Moreover, ECs can give rise to hematopoietic cells through the intermediate hemogenic endothelium, as discussed above.

In the adult, it has been proposed that bone marrow-derived endothelial progenitor cells in the circulation can participate in neovascularization through transdifferentiating into endothelial cells (Yoder 2018). However, other studies have not been able to confirm these findings (Wagers et al., 2002; Ziegelhoeffer et al., 2004). Endothelial colony-forming cells, which are isolated from the umbilical cord blood or peripheral blood, have been shown to be capable to form new blood vessels (Banno and Yoder 2017). Additionally, it has been recently reported that the resident endothelial progenitor/stem cells can be activated and proliferate to form new blood vessels in response to injury (Wakabayashi et al., 2018; McDonald et al., 2018).

LEC origins

Discovery and characterization of the lymphatic vessel dated back to the 17th century (Chikly, 1997). However, the mechanisms of lymphatic vascular development were not studied until the 20th century (Yang and Oliver, 2014). Sabin carried out ink injection studies in pigs and proposed a centrifugal model, in which lymphatic vasculature originated from embryonic veins and formed by sprouting (Sabin, 1902). An alternative centripetal model was proposed by Huntington and McClure, which suggested that lymphatic vasculature had a mesenchymal cell origin and was formed via fusion between the newly formed lymphatic network and the venous system in cats (Huntington and McClure, 1910). In 1930s, Van Der Jagt described the development of the anterior lymph-sacs showing both mesenchymal and venous origins in sea turtles (Van der Jagt, 1932). Finally, in late 2000's, Oliver and colleagues demonstrated by lineage tracing that murine lymphatic vasculature is derived from the cardinal vein (Srinivasan et al., 2007), which supported Sabin's model and firmly established the dogma on the sole venous origin of the mammalian lymphatic vasculature. Further studies in mouse embryo have demonstrated that the intersomitic veins and the superficial venous plexus could also give rise to lymphatic vessels (Hägerling et al., 2013; Yang et al., 2012). Moreover, lymphatic vasculature in *Xenopus laevis* and zebrafish has been shown to share the venous origin (Ny et al., 2005; Yaniv et al., 2006). However, the latest studies from our lab and others have shown non-venous origins of the lymphatics, which challenge the previously accepted dogma that all lymphatics share the venous origin. It will be discussed later.

The role of platelets in angiogenesis and lymphangiogenesis

Platelets are anucleate cells in the circulation, and they play a vital role in maintaining vascular homeostasis and thrombosis. In addition, their roles in angiogenesis are emerging. Upon platelet activation, various kinds of bioactive molecules are released in microparticles and/or α-granules. These molecules include growth factors such as VEGF, basic fibroblast growth factor (bFGF), PDGF, cytokines, and phospholipids like sphingosine-1-phosphate (S1P) that can induce angiogenesis (Walsh et al., 2015) and vascular stability through S1PR1 by counteracting VEGF function and mediating endothelial cell adhesion (Gaengel et al., 2012). For example, an *in vitro* study showed that platelets could promote endothelial cell tube formation (Synetos et al., 1998). Brill et al. found that both platelets and their releasate could promote endothelial cell migration and thus angiogenesis *in vitro* and *in vivo* (Brill et al., 2004). Furthermore, the same group showed that microparticles from platelets also participates in the induction of angiogenesis through activities of VEGF, heparanase, and PDGF in different *in vitro* and *in vivo* models (Brill et al., 2005). VEGF and bFGF from platelets synergize to induce capillary sprouting, and release phospholipids that contribute to endothelial cell migration and proliferation (Rhee et al., 2004). Depletion of platelet or anti-platelet therapy inhibits neovascularization in the model of "retinopathy of prematurity" (Rhee et al., 2004). More interestingly, Wagner and colleagues reported that platelets do not only stimulate angiogenesis but stabilize and prevent hemorrhage from the sprouting blood vessels through platelet adhesion and secretion of distinct growth factors (Kisucka et al., 2006). A recent study revealed that failure of platelet aggregation on the vessel wall results in hemorrhage due to compromised vascular integrity during development (Lowe et al., 2015). It has been further proposed that secreted molecules from the activated platelets mediate mural cell recruitment to the endothelial cells to facilitate their maturation (Lowe et al., 2015). These findings highlight the involvement of platelets in angiogenesis. However, it is generally thought that platelets are not required for maintenance of blood vessel integrity during embryogenesis.

In contrast, platelets are essential for normal lymphatic vascular development during embryogenesis. This conclusion is based on characterisation of several mouse mutants with dysfunctional platelets that show blood-filled lymphatic vessels. It has been proposed that platelets are activated and aggregate at the lymphovenous junctions through interaction between CLEC1B and PDPN, which are expressed in platelets and LECs respectively (Hess et al., 2014; Welsh et al., 2016). The formation of platelet aggregation at the level of lymphovenous valves has been further proposed to maintain lymphovenous homeostasis by preventing blood from entering the lymphatic system (Hess et

al., 2014; Welsh et al., 2016). The evidence supporting the above conclusions is discussed below.

Loss of megakaryocytes/platelets in *Meis1* mutant embryos results in blood-filled lymphatic vessel phenotype (Carramolino et al., 2010). In addition, targeted ablation of megakaryocytes/platelets using *PF4-Cre* line combined with *R26R-LacZbpa-DTA* line in mouse embryos could recapitulate the same phenotype as in the *Meis1*-deficient embryos (Carramolino et al., 2010). This study provided the direct evidence of the role of platelets in lymphatic vessel development. Interestingly, however, *Nf-e2*$^{-/-}$ mice that lack platelets do not show a similar blood-filled lymphatic vessel phenotype (Shivdasani et al., 1995). It was argued that the platelet-like particles in *Nf-e2* mutant mice could be sufficient to fulfill the related function (Bertozzi et al., 2010; Uhrin et al., 2010). In *Kindlin3*-deficient mice, in which there is a failure of platelet aggregation, blood-filled lymphatic vessel phenotype is also present (Uhrin et al., 2010).

PDPN is widely expressed in different types of cells including LECs, osteoblasts, kidney podocytes, fibroblastic reticular cells, and some tumor cells (Pan and Xia, 2015). PDPN is not only an early marker of LECs, but is crucial for inducing platelet aggregation on LECs (Kaneko et al., 2004; Kato et al., 2003b). *Pdpn*-deficient mice have lymphatic vessel defects including decreased lymphatic transport, congenital lymphedema and dilation of lymphatic vessels (Schacht et al., 2003), and exhibit blood-filled lymphatic vessel phenotype (Uhrin et al., 2010). Employing the lymphatic-specific *Pdpn* knockout mice, Detmar and coworkers revealed that PDPN expression in the LECs is indispensable to prevent blood-filled lymphatic phenotype postnatally (Bianchi et al., 2017). Loss of T-synthase, which is essential for the biosynthesis of core1-derived O-glycans present in PDPN, also leads to blood-filled lymphatic vessels (Fu et al., 2008).

CLEC1B is a glycosylated type II transmembrane protein that is expressed in the subpopulation of myeloid cells, platelets, and megakaryocytes (Colonna et al., 2000; Senis et al., 2007; Suzuki-Inoue et al., 2006). CLEC1B induces strong platelet activation via Src and Syk tyrosine kinases, and different adaptor and effector proteins such as Src homology 2 domain-containing leukocyte protein of 76 kDa (SLP76) to activate phospholipase Cγ2 (PLCγ2) (Watson et al., 2010). Blood-filled lymphatic vessels can be observed in mice with homozygous deletion of *Clec1b* (Bertozzi et al., 2010; Finney et al., 2012; Osada et al., 2012). Also, *Plcγ2*-null mice show non-separation phenotype between blood and lymphatic vasculature (Ichise et al., 2009). So far PDPN is the only known endogenous ligand for CLEC1B (Suzuki-Inoue et al., 2007). The interaction between CLEC1B and PDPN leads to reciprocal activation of respective downstream signaling pathways (Watson et al., 2014). It has been

found that CLEC1B-PDPN interaction-induced activation of platelets, which results in release of BMP9 that inhibits LEC migration, proliferation, and tube formation, is required for blood-lymphatic vessel separation (Osada et al., 2012).

Spleen tyrosine kinase (Syk) signaling pathway is crucial for adaptive immune responses, but it has also been shown to play a critical role in platelet function and lymphatic vascular development (Mócsai et al., 2010). Syk mediates glycoprotein VI (GPVI) and CLEC1B signaling in a immunoreceptor tyrosine-based activation motif (ITAM)-dependent way, and outside-in signaling by integrin αIIbβ3 non-ITAM dependently in platelets (Hughes et al., 2015; Mócsai et al., 2010). *Syk*-deficient mouse embryos show subcutaneous hemorrhage and edema and blood filled lymphatic vessels (Abtahian et al., 2003; Cheng et al., 1996; Finney et al., 2012; Sebzda et al., 2006). Kahn and colleagues first proposed that the defective circulating endothelial cell progenitors from the mutant animals contribute to the abnormal blood-lymphatic connection (Sebzda et al., 2006). Later, Kiefer et al. ruled out the possibility of contribution of Syk-expressing circulating endothelial cells by fate mapping, and discovered that Syk+ myeloid cells instead regulate lymphangiogenesis by secretion of VEGFC. Deregulation of Syk+ myeloid cells in *Syk*-deficient embryos leads to lymphatic hyperplasia, which causes abnormal connections between veins and lymphatic vessels (Bohmer et al., 2010). Recently, loss of ITAM-dependent signaling has been shown to be mainly responsible for the blood-lymphatic connections (Hughes et al., 2015). Interestingly, mice deficient of glycoprotein VI (GPVI), which shares the similar signaling pathway (Syk, Slp76, and PLCγ2) with CLEC1B, do not show the same phenotype (Kato et al., 2003a; Lockyer et al., 2006). This suggests a specific function of PDPN-CLEC1B signaling pathway in ‘lymphovenous separation’.

Aims of the thesis

In addition to the common functions, the blood and lymphatic vasculatures in different organs possess organotypic functions, and the corresponding endothelial cells show unique characteristics of cellular morphology and gene expression. Though the general mechanisms regulating vascular development have been well studied, organ-specific mechanisms of vessel formation remain elusive. In this thesis, we aimed to understand vascular development in a specific organ – the mesentery. The specific aims are listed below:

I To investigate the origin(s) of the lymphatic vasculature in the mesentery.

II To study the mechanisms of mesenteric lymphatic vessel formation and remodeling, with the focus on smooth muscle cell recruitment.

III To study the regulation of blood vessel integrity during developmental remodeling of the mesenteric vasculature.

Present investigations

Paper I

cKit lineage hemogenic endothelium-derived cells contribute to mesenteric lymphatic vessels (Stanczuk et al., 2015)

All mammalian lymphatic vessels were previously thought to arise from the veins. However, the dogma was mainly based on the studies of development of the paired jugular lymph sacs at the junction of the internal jugular and subclavian veins. Considering that certain hereditary lymphatic-associated diseases only affect certain tissues or organs, we hypothesized that the mechanisms of lymphatic vascular development may show regional differences. We focused our investigation on the lymphatic vessel development in the mesentery.

We first found that the vascular endothelial growth factor receptor 3 (VEGFR3)/phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) signaling pathway was selectively required for the lymphatic vessel formation in the mesentery rather than for other organs such as the skin and diaphragm during development. Surprisingly, we observed the presence of isolated LEC clusters, which emerged from E13, alongside the blood vessels, and their coalescence to lymphatic vessels by E14.5. This is contrast to the dogma in which the mesenteric lymphatic vessels form via sprouting from the mesenteric lymph sac. To characterise these LEC progenitors, we performed immunofluorescence staining using various endothelial cell (blood and lymphatic) and hematopoietic cell markers, and found that these cells showed neither blood endothelial nor hematopoietic markers.

Considering the fact that GFP perdurance can be used as a lineage readout, we employed *Cldn5-GFP* transgenic mice, in which vasculatures including lymph sacs are labelled by strong GFP fluorescence, to investigate the origin of the LECs. We did not detect GFP expression in $PROX1^{+}$ cells that were in small clusters composed of one to two cells. This suggested that these progenitors were not derived by sprouting from the local blood vessels or from the mesenteric lymph sac.

Additionally, we performed genetic lineage tracing to study the cellular origin of the mesenteric LECs. In general, we combined inducible and constitutive *Cre* lines with the *R26mTmG* reporter, in which Cre recombinase expressing cells and their progenies replace membrane Tomato with membrane GFP expression. Accordingly, we could trace the progenies by GFP labelling. We first used endothelial specific *Pdgfb-CreER^{T2};R26mTmG* mice. Upon 4-hydroxytamoxifen (4-OHT) induction at E8-E9, we could see efficient GFP labelling in the mesenteric LEC clusters, but no or poor labelling in the mesenteric blood vessels. Through immunofluorescence staining and flow cytometry analysis, we found that both major arteries and cKit^{+} (hemogenic) endothelial cells in the yolk sac were targeted in the E9.5 embryos upon 4-OHT treatment at E8-E8.5. This suggested that the mesenteric LEC progenitors are derived from arterial or hemogenic endothelium. To validate whether hemogenic endothelium gives rise to the mesenteric LECs, we employed *cKit-CreER^{T2};R26mTmG* mouse line. 4-OHT treatment at E10 or E11 led to GFP labelling in the mesenteric LEC clusters at E13.5, suggesting that mesenteric LECs originate from cKit^{-}lineage cells, which include hemogenic endothelium. Moreover, we could not see any tracing in these LECs using *Vav-Cre;R26mTmG*, a hematopoietic-specific *Cre* line. Taken together, our results suggest that *cKit* lineage hemogenic endothelium-derived progenitors provide a source of mesenteric LECs.

In summary, we demonstrated that mesenteric lymphatic vessels are formed through lymphvasculogenesis rather than lymphangiogenesis, and the lymphvasculogenic process selectively requires VEGFR3/PI3K signalling. We also showed that *cKit* lineage hemogenic endothelium-derived progenitors contribute to the mesenteric lymphatic vessel formation, which is against the previously accepted dogma that all mammalian lymphatic vessels are of venous origin.

Paper II

Smooth muscle cell recruitment to lymphatic vessels requires PDGFB and impacts vessel size but not identity (Wang et al., 2017)

It is well known that the collecting lymphatic vessels are covered by smooth muscle cells (SMCs). Nevertheless, the roles of SMCs in lymphatic morphogenesis, maturation and function are not fully understood. To this end, we investigated mechanisms regulating SMC recruitment and their roles in lymphatic development and function.

We first studied the expression of the platelet-derived growth factor B (PDGFB) and the its receptor – platelet-derived growth factor receptor β (PDGFRβ) using two different mouse strains: *Pdgfb-CreER^{T2}-IRES-egfp;R26-mTmG*, in which PDGFB^{+} cells will show GFP upon tamoxifen induction, and *Pdgfrβ-GFP*. We found that PDGFB is exclusively expressed in the LECs of the collecting lymphatic vessels, and that PDGFRβ in the SMCs surrounding the collecting lymphatics in the P21 mouse ear skin, suggesting the role of PDGFB signalling in SMC recruitment to the collecting lymphatics. We then specifically deleted PDGFB in LECs or ECs using *Prox1-CreER^{T2}; Pdgfb$^{flox/flox}$; R26R-eYFP* (*Pdgfb^{iLECKO}*) and *Cdh(PAC)-CreER^{T2}; Pdgfb$^{flox/flox}$; R26R-eYFP* (*Pdgfb^{iECKO}*) mice respectively. We observed loss of SMC coverage and dilation in the collecting lymphatic vessels but no effect on either capillary lymphatics or blood vessels, suggesting that SMC recruitment requires LEC-derived PDGFB and that lymphatic hierarchy establishment via lymphatic remodeling is SMC-independent. Moreover, we only saw larger LEC cells in the collecting lymphatics of the *Pdgfb^{iLECKO}* mice.

We also analyzed SMC recruitment to the large collecting vessels in the *Pdgfb^{iLECKO}* mice during development. We saw significant reduction in SMC coverage in both mesenteric and popliteal collecting lymphatics, whereas vessel dilation was observed only in the mesenteric collecting lymphatic vessels. Postnatally, LEC-derived PDGFB is necessary for SMC expansion and maintenance in the hind limb rather than in the mesentery. These data suggest the organ-specific crosstalk between collecting lymphatic LECs and SMCs. Furthermore, we examined the biological consequences upon loss of SMC coverage in the collecting lymphatics by tracer injection and high-frequency non-invasive imaging. We noted there were significant decrease of contraction sites and a lack of contraction in the collecting lymphatics. However, the clearance function was not affected.

To understand whether PDGFB is sufficient for SMC recruitment, we expressed *Pdgfb* ectopically in lymphatic capillaries using *Prox1-CreER^{T2};R26hPDGFB$^{+/+}$* mice. We did not detect any SMC coverage on the capillaries. Instead we noticed a modest but significant increase of SMC coverage on the collecting vessels, suggesting that PDGFB alone is not sufficient for SMC recruitment. We reasoned that extracellular matrix (ECM) retention of PDGFB, which was shown to be critical in mural cell recruitment to the blood vessels, could play a role. To test this possibility, we employed *Pdgfb$^{ret/ret}$* mice lacking heparin sulphate-binding domain of PDGFB, and saw lower SMC coverage and dilation of collecting lymphatic vessels in ear skin. It thus confirmed the important role of ECM binding of PDGFB in SMC recruitment. We also observed prominent differences in ECM deposition be-

tween collecting and capillary vessels, which explains the discrepancy between collecting and capillary vessels regarding SMC recruitment and further supports the function of ECM.

In conclusion, our work elucidates how both LEC-autonomous PDGFB and its interaction with ECM mediate SMC recruitment to the collecting lymphatic vessels rather than the capillary lymphatic vessels.

Paper III

Transient loss of venous integrity during developmental vascular remodeling leads to red blood cell extravasation and clearance by lymphatic vessels (Zhang et al., 2018)

Maintenance of vascular integrity in homeostasis has been extensively studied. In contrast, little is known about how vascular integrity is maintained during development. Here, we investigated this process in the developing mesentery.

We observed red blood cells (RBCs), both nucleated and enucleated, were in the extravascular space and associated with LEC clusters in the *wild type* E14 mesenteries. We next assessed the frequency of extravascular RBCs along each blood vessel segment in mesenteries at different stages - E13, E13.5, E14, and E16. There were rare extravascular RBCs at E13-E13.5. But the frequency increased to 7.38±7.33% at E14, and more than 80% of the extravascular RBCs were interacting with membrane protrusions and/or cell bodies of the LECs in the LEC clusters. RBCs were found in the lumen of the developing lymphatic vessels at E15. However, RBCs were barely seen after E16, when the lymph flow initiates (Sweet et al., 2015). By genetic mosaic labelling of LECs, we investigated LEC-RBC interactions at high resolution. The direct contacts between LEC protrusions and RBCs were confirmed by structured illumination microscopy (SIM) imaging. Together, these data suggest that RBC extravasation is transient and that the developing mesenteric lymphatic vessels possess the clearance function.

We reasoned RBC extravasation could be caused by the loss of vascular integrity, which has been shown in certain leaky tumours (Hashizumi et al., 2000). We used *Cldn5-GFP* reporter mice to visualize the blood vasculatures in the developing mesenteries at E13-E13.5. Extensive vascular remodeling in both arteries and veins was observed. Furthermore, we saw fragmented basement membrane (BM) and sparse mural cell coverage in the veins. In contrast, arteries were invested by even BM and abundant mural cells. Though the arteries showed continuous intercellular junctions between adjacent ECs, yet the

veins showed disrupted junctions in the E14 mesenteries. Additionally, we found the intercellular gap structures in the veins. These gaps were 6-10 μm in diameter allowing both nucleated and enucleated RBCs to extravasate. The observations above suggest that, during vascular remodeling, there is a transient loss of venous integrity that correlates to RBC extravasation in the E14 mesentery.

Platelets are crucial in maintaining vascular integrity in primary homeostasis and vascular injuries. We therefore hypothesized that platelets may participate in vessel integrity maintenance during mesenteric vascular remodeling. By IF staining, we detected that platelets adhered to the venous endothelium and were present in the intercellular gaps but not found in the extravascular space. These platelets were spreading and extending protrusions, a reminiscence of platelet activation. Moreover, we did not detect platelet adherence to the blood vessels in E15 mesentery. We used *Clec1b*$^{-/-}$ mice, in which platelets show activation defects, to further validate the role of platelets. In the *Clec1b* deficient E14 mesenteries and skins, there were excessive number of extravascular RBCs and platelets, which were found inside the lumenized lymphatic vessels, entrapped in the LEC clusters, or located in the interstitial space, suggesting that platelets play a critical role in maintaining the venous integrity and that failure of platelet activation leads to the blood-filled lymphatic phenotype in mesentery of the *Clec1b*$^{-/-}$ mice.

Taken together, we described the loss of venous integrity during mesenteric vascular remodeling and concomitant RBC extravasation and clearance function of the developing mesenteric lymphatic vessels, revealed the essential role of platelets in maintaining the vascular integrity during development, and provided novel insights of the cause of the blood-filled lymphatic phenotype in the *Clec1b* mutant mice.

Paper IV

Alternative lymphatic endothelial progenitor cells compensate for the loss of non-venous-derived progenitors to form mesenteric lymphatic vessels (Manuscript)

Our previous work has demonstrated that both venous and non-venous origin progenitors contribute to the formation of mesenteric lymphatic vasculature. The interactions between these two distinct pools of progenitors to form the functional lymphatic vasculature remain to be investigated.

We observed delayed emergence of the mesenteric LEC progenitors at E13.5 and delayed vessel formation at E14 in *Cldn5-GFP* reporter mice in our previous study (Stanczuk et al., 2015). To understand the mesenteric lymphatic vessel development in these mice, we performed the time-course analysis. In the *wild type* embryos, the mesenteric LEC progenitors assembled into LEC clusters from E13 to E14; these clusters coalesced into a mesh-like lymphatic network at E15; the primitive vessel network was formed at E17; the functional lymphatic vessels were formed at E18. In *Cldn5-GFP* embryos, we observed LEC clusters were depleted at E15; thin and fragmented lymphatic vessels re-emerged at E16; primitive lymphatic plexus was formed at E17; most of the vessel segments, running from the mesentery root to the intestine, showed either absence of lymphatic vessels or truncated lymphatic vessels in the E18 mesenteries, though mature collecting lymphatic vessels were formed along a few blood vessel segments. Notably, the dermal lymphatic development including the thoracic and lumbar lymphatics, which are formed via venous sprouting and lymphvasculogenesis respectively, was not affected in *Cldn5-GFP* embryos. These data suggest an alternative origin of LECs that can compensate to build the mesenteric lymphatic vessels in response to loss of the non-venous origin progenitors.

We saw a significant increase of venous sprouting in the E15-E16 *Cldn5-GFP* mesenteries, which was coincident with the depletion and re-emergence of the lymphatic vessels. We hypothesized that mesenteric venous endothelial cells could compensate to form new lymphatic vessels via sprouting lymphangiogenesis. In fact, we could detect PROX1 expression in the venous sprouts in *Cldn5-GFP* mesenteries. Furthermore, lineage tracing analysis using *Pdgfb-CreER*^{2}*; R26-tdTom; Cldn5-GFP* mice, in which the mesenteric blood vasculatures rather than the lymphatic vessels are labelled by Tomato upon optimized dosage of 4-hydroxytamoxifen administration at E10, demonstrated increased number of Tomato^{+} LECs in the *Cldn5-GFP* mesenteries compared to the *wild type* mesenteries. These data indicate that the venous-derived LECs can compensate for the mesenteric lymphatic vessel regrowth in the *Cldn5-GFP* embryos.

We aimed to examine whether the mesenteric lymphatic vessels in the *Cldn5-GFP* mice were functional. We first assessed the Mendelian ratio of the *Cldn5-GFP* animals. *Cldn5-GFP* embryos were observed in expected Mendelian ratio (50%), yet the ratios of the P1 neonates (35%) and P21 weaning-age mice (37%) were lower than expected. The decreased viability suggests defective mesenteric lymphatic vessels, which leads to compromised dietary fats absorption and in turn increased perinatal lethality. We next analysed the mesenteric lymphatic vessels in the *Cldn5-GFP* P1 neonates, and found chyle leakage and dilated lymphatic vessels. Of note, there was no difference in size between the *Cldn5-GFP* animals and the *wild type* littermates. The detailed

analysis showed truncated lymphatic vessels along the vessel segments and lower smooth muscle cell coverage in different parts of the mesentery, which could lead to the aforementioned phenotypes in the *Cldn5-GFP* animals. Nevertheless, the *Cldn5-GFP* mice and the *wild type* mice showed comparable mesenteric lymphatic vessels at later stages. Therefore, the vein-derived lymphatic vessels in the *Cldn5-GFP* mice are capable to fulfill the physiological function.

In brief, we uncovered a compensatory mechanism that maintains the robustness of mesenteric lymphatic vascular development in the mesentery in the *Cldn5-GFP* mice.

Outlook

Organotypic functions of the vasculatures are well known, and endothelial cell heterogeneity has been revealed. However, organ-specific development of the vasculatures remains elusive. We aimed to answer this question by using the developing mesentery as our model system. The work in this thesis, as summarized above, has made contributions to our understanding of the organotypic development of the vasculatures. Additionally, it has opened a number of new avenues to explore.

We found that the *cKit*$^{+}$ hemogenic endothelium-derived progenitors contribute to mesenteric lymphatic vessels through lymphvasculogenesis (Stanczuk et al., 2015). This is contrast to the previously accepted dogma that all mammalian lymphatic vessels arise from the veins via lymphangiogenesis (Yang and Oliver, 2014). Recent advances have provided additional evidence for the non-venous origin of the lymphatic vessels. Using lineage tracing, my colleagues revealed that lumbar lymphatic vessels are derived from non-*Tie2*-lineage and thus non-venous progenitors, and these vessels are also formed via lymphvasculogenesis (Martinez-Corral et al., 2015). Similarly, non-*Tie2*-lineage progenitors were shown to contribute to cardiac lymphatic vessel formation (Klotz et al., 2015). Yaniv and co-workers demonstrated that LEC progenitors arise from specialized angioblasts located in the posterior cardinal vein of zebrafish, which depends on Wnt5b signaling (Nicenboim et al., 2015). Notably, two different pools of LEC progenitors were found to contribute to the mesenteric lymphatic vessels in chick embryos (Mahadevan et al., 2014). To better understand the mesenteric lymphatic development, further studies are required to address the following questions: where do the mesenteric lymphatic endothelial cell (LEC) progenitors come from? How do they reach the mesentery? What are the molecular mechanisms mediating their differentiation into the mesenteric LECs? How are the mesenteric lymphatic vessels formed? Because cKit is expressed in both (hemogenic) endothelial and hematopoietic cells (Solaimani Kartalaei et al., 2015), a hemogenic endothelium-specific mouse model will be needed to verify the precise origin of the mesenteric LEC progenitors. Alternatively, single-cell RNA sequencing (scRNA-seq) can be employed to reconstruct the developmental trajectory of the mesenteric LECs and provide insight into the origin (Briggs et al., 2018; Farrell et al., 2018; Wagner et al., 2018). Furthermore, my colleagues are

working on transcriptomic profiling of the mesenteric progenitors to identify key molecules involved in mesenteric lymphatic vessel development.

Recent studies have revealed a number of novel lymphatic vascular beds and lymphatic-like vessels such as meningeal lymphatics (Absinta et al., 2017; Aspelund et al., 2015; Louveau et al., 2015), and Schlemm's canal (Kizhatil et al., 2014; Park et al., 2014; Aspelund et al., 2014), which possess distinct functions. Interestingly, isolated LEC clusters were observed to connect to each other through long filopodia during meningeal lymphatic development, which is similar to the lymphvasculogenesis in embryonic mesentery and skin (Antila et al., 2017; Martinez-Corral et al., 2015; Stanczuk et al., 2015). Thus, it is of great interest to investigate how the LEC heterogeneity in different vascular beds arises: the origin(s) of the organotypic LECs, and the intrinsic and extrinsic mechanisms regulating organ-specific lymphatic vessel development.

In addition to the novel origin of the mesenteric LEC progenitors, we discovered an unexpected compensatory mechanism for regrowth of the mesenteric lymphatic vessels after depletion of the original hemogenic endothelium-derived progenitors in *Cldn5-GFP* mice. This finding highlights endothelial cell plasticity in the mesentery. Interestingly, two sources of progenitors, in response to distinct growth cues, contribute to the cardiac coronary vasculature formation during development (Sharma et al., 2017). Similar to our observations in the mesentery, stunted development of the sinus venosus-derived coronary vasculature causes a compensatory expansion of the endocardium-derived progenitors to form the functional cardiac vasculature in the adult (Sharma et al., 2017). However, it is still unclear whether the venous sprouting plays a role in normal mesenteric lymphatic development. We also would like to know the signal 'sensor' detecting the depletion of the mesenteric LEC progenitors and the signal 'trigger' initiating the venous sprouting. Due to the importance of VEGFC in LEC progenitor migration from the veins (Vaahtomeri et al., 2017), it is possible that increased VEGFC availability and/or signaling, which can be caused by LEC depletion in the *Cldn5-GFP* mice, acts as the 'sensor' and the 'trigger'. This requires further investigation to test.

In the process of vascular morphogenesis, we observed intercellular gap formation in the mesenteric veins that leads to transient loss of venous integrity and concomitant RBC extravasation, and uncovered previously unappreciated role of platelets in maintaining vessel integrity during development (Zhang et al., 2018). It was previously thought that loss of vessel integrity only happens under pathological conditions or upon injury (Dejana et al., 2009; Hashizume et al., 2000). Our finding is likely owing to the organotypic microenvironment. Indeed, there is ECM remodeling and redistribution of intercellular adhesions

in the mesenchyme, which leads to an asymmetric outgrowth in the mesentery during gut looping (Davis et al.; Kurpios et al., 2008). Notably, we also detected RBCs in the extravascular space in the embryonic skin, but failed to detect similar gap structure in the dermal veins (Zhang et al., 2018). Platelets have been shown to participate in vascular remodeling through secreted chemokines (Koenen, R.R. and Weber, C. 2010). An interesting possibility is that platelet activation at the intercellular gaps may promote venous remodeling in addition to their role in maintaining vessel integrity. Indeed, activated platelet can produce S1P that is critical in stabilizing blood vasculature and promoting blood flow (Gaengel et al., 2012; Jung et al., 2012). S1P signaling has also been reported to mediate directional migration of LECs under fluid shear stress (Surya et al., 2016). Consequently, further studies are needed regarding the role of platelets in this process. Additionally, RBCs also store and generate S1P (Gazit et al., 2016). Do the extravasated RBCs contribute to mesenteric lymphatic formation through providing chemical cues? Our results argue against this possibility by showing random localization of the extravasated RBCs and random protrusions from the LECs that are in the vicinity of these RBCs. Additionally, it has been recently shown that S1P derived from RBCs or platelets alone is dispensable for vascular development and maintenance of vessel integrity (Gazit et al., 2016).

Additionally, we demonstrated that failure to maintain the venous integrity contributes to the blood-filled lymphatics in the mesentery of *Clec1b*-deficient mice. Thus, it is important to consider defective blood vessel integrity as a cause of the blood-filled lymphatic anomality.

Lastly, we investigated maturation of the mesenteric lymphatic vessels focusing on SMC recruitment. Our study revealed that LEC-autonomous PDGFB is necessary for SMC recruitment that in turn regulates vessel size and function in the mesentery (Wang et al., 2017). It will be of interest to dissect how reciprocal interactions between LECs and SMCs control vessel size and function.

Collectively, our research on the organ-specific vascular development in the mesentery not only deepens understanding of the endothelial cell heterogeneity and plasticity, but provides insights into the cause of organotypic vasculature-related diseases.

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