REVIEW

Lymphangiogenesis guidance by paracrine and pericellular factors

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Lymphatic vessels are important for tissue fluid homeostasis, lipid absorption, and immune cell trafficking and are involved in the pathogenesis of several human diseases. The mechanisms by which the lymphatic vasculature network is formed, remodeled, and adapted to physiological and pathological challenges are controlled by an intricate balance of growth factor and biomechanical cues. These transduce signals for the readjustment of gene expression and lymphatic endothelial migration, proliferation, and differentiation. In this review, we describe several of these cues and how they are integrated for the generation of functional lymphatic vessel networks.

Some of the most dense lymphatic networks are located under various epithelia that form the interface between the body and the outside environment; for example, in the skin and in the gut. In these locations, the immune cell trafficking functions of the lymphatics are of special importance; for instance, for the launching of adaptive immune responses against pathogens. The lymphatic system is also essential for the transport of interstitial fluid and associated solutes, metabolites, and macromolecules, which have extravasated from blood vessels. Blind-ended lymphatic capillaries form the portal of entry for interstitial fluid, antigen-presenting cells, and lymphocytes (Aebischer et al. 2014; Aspelund et al. 2016; Betterman and Harvey 2016). From the capillary network, the interstitial fluid—now called lymph—flows via precollector and collector vessels and through a series of lymph nodes back into the systemic circulation via the thoracic duct, leading to entry of substances transported in lymph into the bloodstream (Schulte-Merker et al. 2011; Koltowska et al. 2013). The lymphatic network is a low-pressure system, where lymph is propelled forward by the squeezing action of smooth muscle cells (SMCs) that surround the lymphangions between valves of the collecting vessels and by vasomotion and breathing that promote suction in the downstream collector vessels (Bazigou and Makinen 2013).

With the exception of the Schlemm's canal in the eyes, meningeal lymphatic vessels, and the majority of the (lacteal) lymphatic vessels in the intestine, most lymphatic networks are generated during embryonic development (Kim et al. 2007; Aspelund et al. 2014, 2015; Kizhatil et al. 2014; Nurmi et al. 2015). However, they also undergo dynamic changes in adults. Lymphatic vessels can grow in length and caliber (lymphangiogenesis) in various pathological conditions, such as inflammation, wound healing, tumorigenesis, and in association with tissue transplantation. A common feature in many of these conditions is tissue edema and inflammation, which increase the demand for fluid drainage and immune cell trafficking. When the lymphatic network undergoes remodeling, the enlarged vessels with their increased tissue drainage capacity may benefit the resolution of inflammation by enabling enhanced removal of accumulated tissue fluid, immune cells, tissue debris, chemokines, growth factors, etc. (Aebischer et al. 2014; Betterman and Harvey 2016). Increased lymphatic function can sometimes also lead to adverse effects. For example, lymphangiogenesis can increase the severity of transplant rejection (Dashkevich et al. 2016). In cancer, it can facilitate the spread of tumor cells to the lymph nodes and from there to the systemic circulation, with subsequent metastatic colonization of distant organs (Alitalo 2011; Stacker et al. 2014). As these examples indicate, development of molecular tools to control lymphangiogenesis would be beneficial for the treatment of several diseases.

The stepwise process of lymphangiogenesis has similarities to the better-studied blood vascular angiogenesis and the growth of the gas-transporting tracheal system in *Drosophila melanogaster* (Ochoa-Espinosa and Affolter 2012). Lymphangiogenic growth starts upon exposure of lymphatic endothelial cells (LECs) to growth factors or biomechanical stimuli, which in many cases leads to activation of vascular endothelial growth factor (VEGF)

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receptor 3 (VEGFR3) (Fig. 1). Available data support the view that, in lymphangiogenesis, as in angiogenesis, the growing lymphatic vessels are guided by migrating tip cells, which display filopodia and cellular protrusions that sample the pericellular environment in search of guidance cues (Figs. 1, 2; Gerhardt et al. 2003; Zheng et al. 2011). The tip cell guides the forming branch, and endothelial proliferation behind the tip cell allows the elongation of the branch (Gerhardt et al. 2003; Baluk et al. 2005). The growth of new branches ceases upon decreased growth factor exposure, or, in some cases, growth is stalled by an increase of inhibitory signals, such as IFN-γ, TGF-β, endostatin, neostatin-7, or thrombospondin, which act directly on LECs or via control over growth factor production by other cell types (Fig. 1; Brideau et al. 2007; Clavin et al. 2008; Kojima et al. 2008; Oka et al. 2008; Avraham et al. 2010; Cursiefen et al. 2011; Kataru et al. 2011; Ou et al. 2011; Zampell et al. 2012). After some pruning of the newly formed branches, some of them are stabilized to form capillaries or collector vessels. The maturation of collectors involves the development of valves and SMC investment (Bazigou and Makinen 2013; Martinez-Corral and Makinen 2013). The intercellular cadherin junctions of the capillaries undergo a switch from a zipper-like structure to button-like connections (Yao et al. 2012), and this is accompanied by the formation of anchoring filaments that connect the LECs to the pericellular matrix (Leak and Burke 1968). Interestingly, during embryonic growth, the LEC junctions are zippers and change to buttons slowly around birth but revert back to zippers upon stimulation by growth factor or inflammatory processes (Yao et al. 2012).

In this review, we first outline the main principles of the formation of lymphatic vessel networks during development and their expansion in pathological conditions such as inflammation and tumorigenesis. We then describe the mechanisms of lymphangiogenesis; i.e., how VEGF-C activates its cognate receptor, VEGFR3, in LECs, leading to sprouting lymphangiogenesis. We next discuss the modulation of VEGFR3 activity by its coreceptors. We also describe how mechanical cues, such as tissue fluid pressure and tissue structures such as arteries and extracellular matrix (ECM), contribute to lymphangiogenesis guidance. Finally, we describe some of the well-established mouse models for lymphangiogenesis (Fig. 2). Throughout the review, we focus on the guidance mechanisms of lymphangiogenesis in comparison with angiogenesis in mammals and zebrafish.

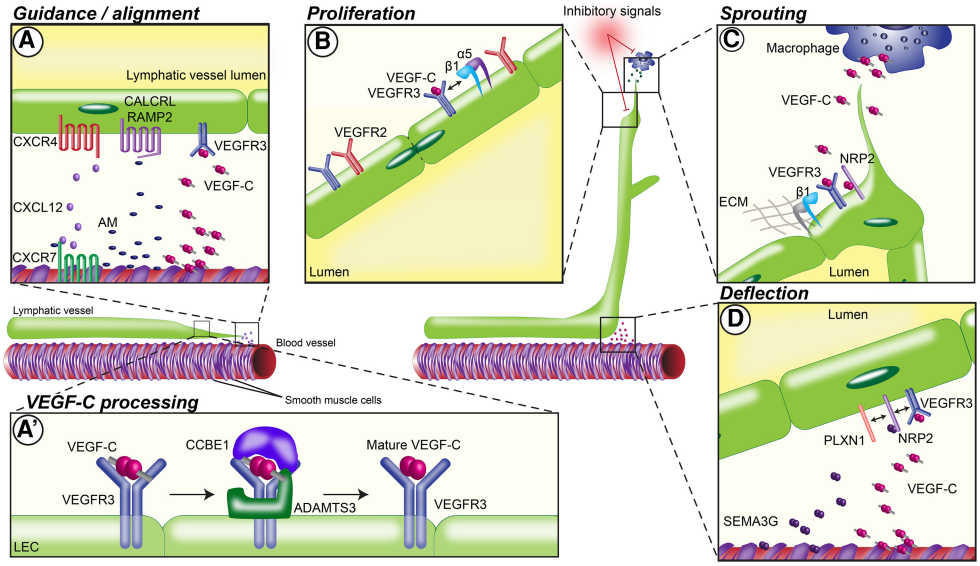

Figure 1. Pericellular cues that guide lymphatic vessel growth. (*A*,*A*′) Arterial endothelial cells and SMCs secrete lymphangiogenic guidance cues that contribute to the alignment of large lymphatic collectors with arteries. VEGF-C binds to pericellular matrix and LEC surface proteins, such as VEGFR3, neuropilin 2 (NRP2), and syndecan-4, and is processed upon its interaction with extracellular matrix (ECM) adapter, collagen- and calcium-binding EGF domain-containing protein 1 (CCBE1), and the ADAMTS3 protease as shown in *A*′. In zebrafish and mice, CXCL12 produced by blood vascular endothelial cells guides lymphatic growth via binding to its receptor, CXCR4, on LECs. Adrenomedullin (AM) binds to the RAMP2 and CALCRL receptors in mice. The chemokine sink CXCR7 regulates these interactions by sequestering both CXCL12 and adrenomedullin. (*B*) Upon growth factor-induced activation, both VEGFR3 and VEGFR2 can stimulate LEC proliferation, and VEGFR3 interaction with β1 integrins, such as α5β1, enhances the lymphangiogenic signals. (*C*) The sprouting and branching of lymphatic vessels is dependent on VEGF-C signaling via the VEGFR3–NRP2 receptor complex. Integrin α5β1 ligands fibronectin and collagen in the ECM increase VEGFR3 phosphorylation in the absence of a VEGFR3 ligand; they also potentiate VEGF-C-induced VEGFR3 activation and LEC migration. Macrophages provide a major source of VEGF-C in lymphangiogenesis associated with inflammation. The growth-promoting factors are counteracted by inhibitory signals, such as TGF-β and INF-γ, which act directly on LECs or affect VEGF-C production by, e.g., macrophages (see the overview figure). (*D*) The deflection of lymphatic vessel sprouts away from arteries has been suggested to be driven by arterial expression of semaphorin 3G (SEMA3G), which induces LEC repulsion via a plexin 1 (PLXN1)–NRP2–VEGFR3 receptor complex.

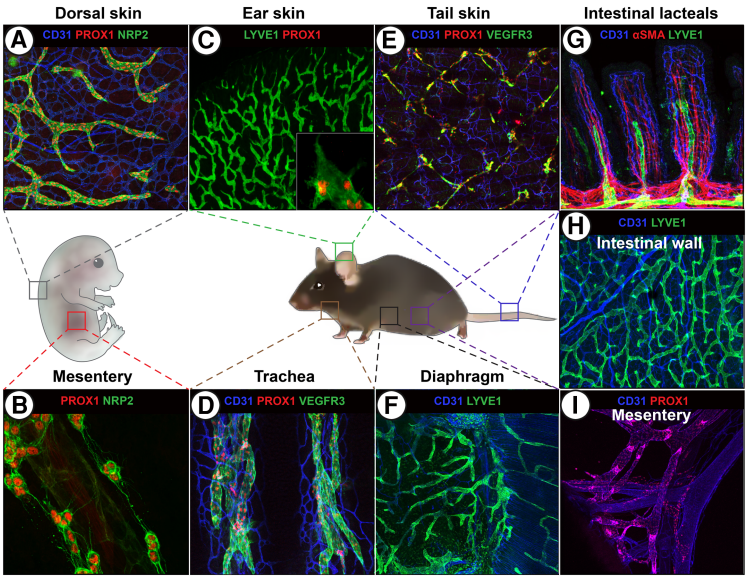

Figure 2. Examples of ongoing lymphangiogenesis in mouse embryos and postnatal mice. (*A*) Embryonic skin dermis at embryonic day 14 (E14) stained for CD31 (blue), PROX1 (red), and NRP2 (green). (*B*) LEC clusters in the process of assembling to form mesenteric lymphatic vessels at E14, here stained for PROX1 (red) and NRP2 (green). (*C*) LYVE1 (green) staining of developing lymphatic vessels in the ventral part of the ear at postnatal day 16 (P16). The *inset* shows one of the growing lymphatic vessel tips, with the LEC nuclei indicated using PROX1 (red). (*D*,*E*) CD31 (blue), PROX1 (red), and VEGFR3 (green) whole-mount staining of the trachea (*D*) and tail dermis (*E*) at P5. (*F*) CD31 (blue) and LYVE1 (green) staining of the pleural side of a P5 diaphragm. (*G*,*H*) CD31 (blue), α-smooth muscle actin (αSMA; red), and LYVE1 (green) staining of (lacteal) lymphatic vessels in intestinal villi (*G*) and the intestinal wall (*H*) in adult mice. (*I*) CD31-stained (blue) and PROX1-stained (red) mesenteric lymphatic vessels at P7.

Lymphangiogenesis in development

Most of the lymphatic vessels in mice and zebrafish arise from LECs sprouting from embryonic veins (Sabin 1902; Wigle and Oliver 1999; Isogai et al. 2003; Yaniv et al. 2006; Srinivasan et al. 2007). In zebrafish, phagocytic perivascular cell populations resembling LECs have been found recently in the brain that do not form vessels but are required for the formation of the cerebral blood vessels (Bower et al. 2017a; van Lessen et al. 2017; Venero Galanternik et al. 2017). An interesting case of lymphatic vessel specialization in fish is their involvement in fin erection and thus locomotion in tunas (Pavlov et al. 2017). In mouse embryos, the first committed LEC progenitors appear in the cardinal vein at embryonic day 9.5 (E9.5). These cells express SOX18 (a SRY-related HMG-box transcription factor) and differ from the majority of other venous cells by expression of homeobox transcription factor PROX1 and LYVE1 (Wigle and Oliver 1999; Schacht et al. 2003; Francois et al. 2008; Hagerling et al. 2013). SOX18 induces expression of the downstream *Prox1*, which is essential for LEC specification and the subsequent formation of lymphatic vessel networks (Wigle and Oliver 1999; Francois et al. 2008; Johnson et al. 2008). PROX1 drives lymphatic identity and its maintenance by directly inducing expression of LEC-specific genes and suppressing blood endothelial cell (BEC)-specific genes in collaboration with its binding partners, such as the nuclear receptor COUP-TFII (Petrova et al. 2002; Wigle et al. 2002; Lin et al. 2010; Srinivasan et al. 2010).

Based on live imaging of zebrafish embryos, the initial LEC specification takes place on the ventral side of the cardinal vein, where Wnt5b, secreted by the neighboring endoderm, induces the specification of LEC lineage cells (Nicenboim et al. 2015). These cells subsequently migrate to the dorsal side of the cardinal vein. It has been suggested that on the dorsal side, the committed LECs arise via asymmetric fate determination following cell division; the daughter cells expressing increased levels of Prox1 then become destined to the lymphatic lineage (Koltowska et al. 2015). Also in mouse embryos, PROX1-positive LEC progenitors are spatially restricted to the dorsal side of the cardinal vein at E10.5 (Wigle and Oliver 1999). WNT activation can increase *Prox1* expression via transcription factor 4 (TCF4)-binding sites upstream of the *Prox1* gene in lymphatic endothelial, neuronal, and tumor cells (Petrova et al. 2008; Karalay et al. 2011; Cha et al. 2016). However, β-catenin deletion in mouse embryos from E9.5 onward did not interfere with LEC differentiation. Rather, β-catenin was necessary for lymphatic vasculature morphogenesis and valve formation, possibly via shear stress sensing and regulation of FOXC2 expression (Cha et al. 2016).

After delamination, the PROX1-positive LECs migrate dorsally as strings of loosely connected spindle-shaped cells and, at E11.5, form the first lumenized lymphatic structures ("lymph sacs"), the peripheral longitudinal lymphatic vessel (PLLV), and the primordial thoracic duct (pTD) (Yang et al. 2012; Hagerling et al. 2013). It has been suggested that instead of LEC proliferation, abundant LEC delamination from the cardinal vein and possibly from the superficial blood vessel plexus comprises the major source of migrating LECs (Hagerling et al. 2013). Formation of additional lymph sacs takes place in other anatomical locations at later developmental time points. For example, the PROX1-positive LECs in the superior mesenteric vein are specified at E12.5 and form the mesenteric lymph sac by E14 (Kim et al. 2007; Stanczuk et al. 2015). Lymph sacs will subsequently give rise to most of the primitive lymphatic vessel plexuses in embryos via vigorous LEC sprouting and proliferation (Hagerling et al. 2013). Thereafter, additional pruning and further sprouting sculpt the final lymphatic vessel network, consisting of lymphatic capillaries, precollectors, collectors, and lymph nodes organized in a hierarchical manner. The lymphatic network patterns in various tissues differ

greatly from each other, reflecting the physical constraints and functional demands imposed by the host tissue (Ulvmar and Makinen 2016).

Although most of the lymphatic vascular plexuses originate from *Prox1*-positive venous endothelia (Srinivasan et al. 2007), recent reports have indicated a contribution by nonvenous sources in diverse tissues (for review, see Ulvmar and Makinen 2016). Mesenteric lymphatic vessels are formed from isolated clusters of LECs (Fig. 2B), whose origin was traced to progenitors derived from blood-forming hemogenic endothelium (Stanczuk et al. 2015). In the lumbar dermis and heart, some of the LECs were negative for *Tie2-Cre* lineage tracing, suggesting that these cells had a nonendothelial origin (Klotz et al. 2015; Martinez-Corral et al. 2015). According to Klotz et al. (2015), these LECs are similar to cells in a VAV1-positive hematopoietic cell lineage in the heart. The precise contribution of alternative LEC sources and their importance for the formation of different lymphatic vascular beds are still unclear. It is not understood why certain lymphatic networks are composed of LECs derived from various sources. It is possible that LECs from diverse backgrounds could have differential functions within the mature network or ensure efficient and rapid (re)vascularization. These findings also call for studies on the contribution of lymphvasculogenesis in pathological conditions.

Lymphangiogenesis in pathological conditions

In adults, lymphangiogenesis is reactivated in inflammation, wound healing, and tumorigenesis. Although lymphangiogenesis in adults operates with the same principles as in embryos, it is less well coordinated by the appropriate signals in pathological processes, and thus lymphatic vessels often become malformed and poorly functional. Lymphatic vessel density increases locally at sites of inflammation in tissues and in their downstream lymph nodes, which receive lymphangiogenic signals from the inflamed site (for review, see Aebischer et al. 2014; Kim et al. 2014). Substantial lymphatic vasculature is required for the resolution of inflammation and efficient tissue clearance. Increased lymphatic vessel density in transgenic animals overexpressing VEGF-C improves the resolution of tissue edema in models of cutaneous contact hypersensitivity, UV irradiation, or lipopolysaccharide-mediated inflammation (Kataru et al. 2009; Huggenberger et al. 2011). On the other hand, inhibition of lymphangiogenesis prolongs the resolution of tissue edema in acute inflammation of the mouse ear or peritoneum and in TNFα-induced arthritis (Guo et al. 2009; Kataru et al. 2009; Kim et al. 2009). However, blocking lymphangiogenic signals can alleviate rejection of transplanted cardiac, corneal, and pancreatic tissue allografts by preventing antigen presentation in the draining lymph nodes (Chen et al. 2004; Zhang et al. 2009; Dietrich et al. 2010; Nykanen et al. 2010; Dashkevich et al. 2016).

Inflammation-associated lymphangiogenesis is induced by inflammatory cytokines such as TNFα and IL-1 (which stimulate target cells), and leukocytes (e.g., macrophages) can produce substantial amounts of VEGF-C (Enholm et al. 1997; Matsui et al. 2003; Kataru et al. 2009; Kim et al. 2009). Macrophages and other bone marrow-derived cells have also been reported to intercalate in between the LECs at a very low rate during the formation of lymphatic vessels and, in some cases, have been detected to express the LEC markers LYVE1 and PROX1 (Maruyama et al. 2005; Religa et al. 2005; Jiang et al. 2008; Zumsteg et al. 2009; Lee et al. 2010; Hall et al. 2012; Hirai et al. 2013). However, there is no lineage tracing evidence that these cells would directly contribute to expansion of lymphatic vessels in inflammation.

The stability of inflammation-induced lymphatic neovessels varies between tissues. In the cornea, lymphatic capillaries induced by a surgical suture started to regress immediately upon suture removal, but, 6 mo later, short fragments still persisted (Cursiefen et al. 2006). It was suggested that they can act as seeds of accelerated lymphangiogenesis in recurring inflammation (Kelley et al. 2013). Furthermore, in the trachea, entire lymphatic vessel networks generated during inflammation persisted for long time periods after the resolution of inflammation (Baluk et al. 2005), whereas in lymph nodes treated with a single injection of lipopolysaccharide, the lymphatic capillary area peaked 3 d later and returned to normal in 2 wk (Kataru et al. 2011). The reasons for such variation in lymphatic vessel regression are not known (for review, see Kim et al. 2014).

Increased expression of lymphangiogenic factors occurs also in a variety of tumors that promote lymphangiogenesis in the peritumoral area and enlarge the downstream collecting lymphatic vessels as well as the subcapsular sinus network of the draining lymph nodes (for review, see Alitalo 2011; Karaman and Detmar 2014; Stacker et al. 2014). Lymphatic vessels can also grow intratumorally (Beasley et al. 2002; Dadras et al. 2003). Intratumoral vessels either have penetrated the tumor or represent pre-existing lymphatics trapped by the growing tumor (Stacker et al. 2014). VEGF-C produced by tumor cells and by inflammatory cells in the tumor stroma promotes lymphangiogenesis (Salven et al. 1998; Achen et al. 2001; Karpanen et al. 2001; Schoppmann et al. 2002), which facilitates the dissemination of tumor cells into the lymphatic vessels and lymph nodes (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001).

It has been suggested that lymphatic and lymph node colonization facilitates tumor cell entry into the systemic circulation (Karpanen and Alitalo 2001). Indeed, tumor-induced lymphangiogenesis is associated with increased lymph node metastasis and worse disease-free/overall survival of patients, and surgical removal of cancer cell-harboring lymph nodes can improve patient survival (Moertel et al. 1995; Dadras et al. 2003, 2005; Nakamura et al. 2005; Renyi-Vamos et al. 2005; Saad et al. 2006; Takanami 2006; Tobler and Detmar 2006; Adachi et al. 2007; Kaneko et al. 2007; Matsumoto et al. 2007; Doekhie et al. 2008; Mumprecht and Detmar 2013). However, metastases can also occur via an exclusive hematogenous route. Reconstruction of phylogenetic trees of primary tumors and associated metastases from colon cancer

patients showed that 35% of liver and lymph node metastases had the same subclonal origin in the primary tumor, reflecting either the metastatic route or the capability of a metastatic tumor clone to efficiently spread to several independent locations (Naxerova et al. 2017). In mouse models, lymphangiogenic factors produced by tumor cells facilitate lymph node metastases, and blocking of lymphangiogenesis in various tumor models attenuates tumor dissemination (Karpanen et al. 2001; Mandriota et al. 2001; Skobe et al. 2001; Stacker et al. 2001; He et al. 2002, 2005; Krishnan et al. 2003; Lin et al. 2005; Roberts et al. 2006). However, increased marginal lymphatic vessels and a high "immunoscore" (i.e., density of tumor-infiltrating cytotoxic $CD8^+$ and memory $CD45RO^+$ T cells) in human colorectal carcinoma are associated with protection against the generation of distant metastases (Galon et al. 2006; Kirilovsky et al. 2016; Mlecnik et al. 2016). In melanoma, VEGF-C levels correlated with tumor infiltration of naïve T cells and enhanced response to immunotherapy (Fankhauser et al. 2017). Thus, lymphatic vessels likely have a dual role during tumor progression, allowing metastatic escape but also regulating the immune recognition and critical checkpoints in anti-tumor responses.

VEGF-C and other lymphangiogenic growth factors

VEGF-C is so far the only specific growth factor that is essential for embryonic lymphangiogenesis (Karkkainen et al. 2004). In the absence of VEGF-C, PROX1-positive endothelial cells are specified in the cardinal vein of developing mouse embryos but fail to delaminate, which leads to failure of primary lymph sac and lymphatic network formation and prenatal death (Karkkainen et al. 2004; Hagerling et al. 2013). Heterozygous embryos survive but develop hypoplastic lymphatic vessels (Karkkainen et al. 2004). Conditional deletion of *Vegfc* at a later developmental time point leads to absence of, e.g., lacteals in the intestine and hypoplastic Schlemm's canal in the eyes (Aspelund et al. 2014; Nurmi et al. 2015). Furthermore, VEGF-C/D sequestration by epidermally expressed soluble VEGFR3-Ig protein (VEGF-C/D trap) inhibits cutaneous and meningeal lymphatic vessel development (Makinen et al. 2001; Haiko et al. 2008; Aspelund et al. 2015).

VEGF-C is also indispensable for lymphangiogenesis in adult tissues, as shown in models of acute inflammation in which an inflammatory response was induced in the trachea by *Mycoplasma pulmonis*, in the peritoneum by lipopolysaccharide, or in the ear dermis by lipopolysaccharide or lipoteichoic acid and muramyl dipeptide (Baluk et al. 2005; Kataru et al. 2009; Kim et al. 2009). In these models, lymphangiogenesis was prevented by the VEGF-C/D trap. Interestingly, the maintenance of lymphatic capillaries seems to be dependent on constant VEGF-C signaling in some tissues. Deletion of *Vegfc* in adult mice caused slow degeneration of intestinal lacteals, whereas the maintenance of dermal lymphatic vessels was independent of a constant supply of VEGF-C (Aspelund et al. 2014; Nurmi et al. 2015). Furthermore, in contrast to dermal LECs, it was reported that the lacteal LECs proliferate at a slow rate, and some of the lacteal tips display a tip cell phenotype even in adult mice (Bernier-Latmani et al. 2015).

Although VEGF-C and perhaps Wnt5a (see above; Nicenboim et al. 2015) are the only indispensable growth factors for lymphangiogenesis, several other growth factors can also induce lymphatic growth. For instance, the VEGF-C-related VEGF-D induces lymphangiogenesis when overexpressed (Stacker et al. 2001), and its deletion results in mild lymphatic vessel hypoplasia in the lungs and slightly decreased lymphatic vessel caliber in the dermis (Baldwin et al. 2005; Paquet-Fifield et al. 2013). In zebrafish, VEGF-D is indispensable for facial lymphangiogenesis (Astin et al. 2014; Bower et al. 2017b). Ectopic expression of FGF2 can also induce lymphangiogenesis, and *Fgfr1* and *Fgfr3* double-mutant mice show reduced growth of dermal lymphatic vessels at E15.5 (Kubo et al. 2002; Cao et al. 2004b; Chang et al. 2004; Yu et al. 2017). Ectopic expression of several other growth factors, such as VEGF (Nagy et al. 2002; Cao et al. 2004b; Cursiefen et al. 2004; Kunstfeld et al. 2004), angiopoietin 1 (Gale et al. 2002; Morisada et al. 2005; Tammela et al. 2005), angiopoietin 2 (Gale et al. 2002), PDGF-BB (Cao et al. 2004a), EGF (Marino et al. 2013), IGF-1 (Bjorndahl et al. 2005), and HGF (Kajiya et al. 2005; Cao et al. 2006; Gibot et al. 2016), can induce lymphangiogenesis in mouse tissues. At least FGF2-, angiopoietin 1-, and HGF-induced lymphangiogenesis is inhibited by the VEGF-C/D trap (Kubo et al. 2002; Chang et al. 2004; Tammela et al. 2005; Cao et al. 2006). Thus, in addition to direct effects on LECs, these growth factors may act by recruiting leukocytes, which can produce VEGF-C/D (for review, see Zumsteg and Christofori 2012). Another possibility is that they induce VEGF-C expression in blood vascular endothelium or associated SMCs, which leads to lymphangiogenesis via angiocrine mechanisms (Kubo et al. 2002; Cao et al. 2006).

Sources of VEGF-C

When overexpressed, VEGF-C provides directional cues for LEC migration and lymphatic vessel extension. For example, LECs delaminating from the jugular vein migrate toward the paracrine VEGF-C source (Karkkainen et al. 2004). VEGF-C induces directed LEC migration in vitro (Joukov et al. 1996), and beads soaked in recombinant VEGF-C were capable of recruiting LECs in *Vegfc*-deleted embryos (Karkkainen et al. 2004). Lymphatic vessels also grow toward VEGF-C-expressing tumors and encircle the tumor foci, occasionally penetrating into the tumor stroma (Stacker et al. 2014). A point source of VEGF-C may form a LEC-guiding gradient, or the tip LEC may follow a source of VEGF-C that advances ahead of the growing lymphatic capillary tip. Furthermore, extracellular processing of VEGF-C by the ADAMTS3 metallprotease and associated collagen- and calcium-binding EGF domain-containing protein 1 (CCBE1) may shape active VEGF-C gradients (see below). Such mechanisms are known from other model systems. For example, angiogenesis toward the midline in the hindbrain is regulated by a

VEGF gradient and neuropilin binding (Ruhrberg et al. 2002), and tracheal vessel growth in *Drosophila* is directed by a gradient formed by a point source of FGF (Sutherland et al. 1996). In the developing retina, the angiogenic vessel front (the leading edge) follows the advancing border of hypoxic tissue, which shows high VEGF expression by the underlying astrocytes (Stone et al. 1995; Gerhardt et al. 2003). So far, it has been difficult to explore such mechanisms in the case of VEGF-C because of the lack of specific reagents for the localization of the activated form of VEGF-C.

Macrophages are often detected in the vicinity of forming lymphatic vessels in embryos and in inflamed and tumor tissues in adults (Kelley et al. 2013; Lee et al. 2014; Ochsenbein et al. 2016). However, claims that macrophages incorporate to lymphatic vessels and transdifferentiate into proliferating LECs have not been supported by hematopoietic lineage tracing using *Vav-Cre* or *Csf1r-iCre* mice (Maruyama et al. 2005; Religa et al. 2005; Kerjaschki et al. 2006; Bertozzi et al. 2010; Gordon et al. 2010; Martinez-Corral et al. 2015). However, macrophages are essential for lymphangiogenesis associated with inflammation. They are known to produce angiogenic cytokines, including VEGF-C and VEGF-D (Fig. 1C; Schoppmann et al. 2002). Clodronate liposomes, which have been used to deplete macrophages systemically, inhibited lymphangiogenesis induced by lipopolysaccharide in the ear, skin, and diaphragm; by corneal suture in the eye; and by a high-salt diet in the skin (Maruyama et al. 2005; Kataru et al. 2009; Kim et al. 2009; Machnik et al. 2009).

Although macrophages stimulate LEC proliferation in vitro (Gordon et al. 2010), the role of macrophages in developmental lymphangiogenesis is less obvious. *op/op* mice, which lack macrophage colony-stimulating factor (M-CSF and CSF-1)-dependent macrophages, show delayed development of dermal and tracheal lymphatic vessels (Kubota et al. 2009). Lymphangiogenesis induced by ischemia and tumors was also attenuated in the *op/op* mice (Kubota et al. 2009). Furthermore, defective CCL2–CCR2 chemokine signaling led to decreased association of macrophages with the lymphatic vessels and decreased density of the dermal lymphatic network, possibly by reducing the concentration of VEGF-C/D that interacts with its receptor on the LECs (Lee et al. 2014). Interestingly, depletion of the PU.1 transcription factor or M-CSF-dependent macrophages in the corresponding gene targeted mice led to LEC hyperproliferation and lymphatic vessel dilation. Although it was suggested that *PU.1*- and *Csf1r*-dependent cells inhibit lymphangiogenesis, VEGF-C/D expression was increased in *PU.1* embryos, suggesting alternative overcompensating mechanisms (Gordon et al. 2010).

Immunohistochemistry for VEGF-C shows staining in the endothelium and arterial SMCs in adult mice, reflecting its binding to the vascular endothelial cell surfaces and production by SMCs and possibly BECs (Skobe et al. 1999; Partanen et al. 2000; Tammela et al. 2008). β-Galactosidase staining of tissues from heterozygous $Vegfc^{wt/lacZ}$ mice confirmed *Vegfc* expression in SMCs in E10.5 embryos and intestinal arteries in adults (Karkkainen et al. 2004; Nurmi et al. 2015). Furthermore, the circular smooth muscle fibers of the intestinal wall and elongated SMCs extending into the gut villi were positive for β-galactosidase (Nurmi et al. 2015). VEGF-C production by SMCs could explain why the intestinal lacteals run parallel to the intestinal SMC fibers and why lymphatic vessels accompany major arteries, although the CXCL12/CXCR4 chemokine signaling system is also involved (see below; Fig. 1A; Cha et al. 2012). However, the proof of SMC-produced VEGF-C function would require targeted deletion of VEGF-C in these cells. Overall, cell- and tissue-specific patterns of VEGF-C expression and physical constraints for the growth of lymphatic vessels may explain the tissue-specific patterns of many lymphatic networks.

VEGF-C activation

Unlike other members of the VEGF family, VEGF-C and VEGF-D are produced as precursor proteins, which require processing of their C-terminal and N-terminal propeptides to achieve full activity toward their cognate receptors, VEGFR3 and VEGFR2 (Joukov et al. 1997). Concomitant with its secretion, the VEGF-C precursor undergoes C-terminal cleavage by proprotein convertases (Siegfried et al. 2003). The resulting VEGF-C form is poorly active, but subsequent N-terminal processing greatly potentiates its receptor binding. Recent studies have revealed that the processing of the N terminus is a complex mechanism, requiring the scaffold protein CCBE1 (Jeltsch et al. 2014; Le Guen et al. 2014; Jha et al. 2017). Both CCBE1 and the ADAMTS3 metalloproteinase are essential for cleavage of VEGF-C into its active form in vivo and in vitro (Jeltsch et al. 2014; Janssen et al. 2016). Importantly, *CCBE1*-inactivating mutations in the collagen domain, calcium-binding EGF domain, or cysteine-rich domain upstream of the EGF domain have been found in patients with Hennekam syndrome, which involves severe lymphedema (Alders et al. 2009, 2013; Connell et al. 2010). Furthermore, homozygous *Ccbe1* mutations prevent the formation of all primitive lymphatic structures in mice and zebrafish (Hogan et al. 2009; Bos et al. 2011). In *Ccbe1* mutant mice, LECs are specified and able to delaminate from the cardinal vein in small amounts but fail to migrate and form PLLV and pTD (Hagerling et al. 2013). The fact that LECs still delaminate in *Ccbe1* mutants, but not in *Vegfc* mutants, suggests that the unprocessed VEGF-C also has some activity toward VEGFR3. Alternatively, low levels of VEGF-C cleavage may occur in the absence of CCBE1. In line with the developmental phenotype, conditional deletion of *Ccbe1* in adult mice abolishes lymphangiogenesis induced by VEGF-C overexpression (Bui et al. 2016). Moreover, CCBE1 overexpression in adult mice synergizes with VEGF-C expression for improved lymphangiogenesis (Jeltsch et al. 2014). The ADAMTS3 protease that activates VEGF-C was previously considered to be of major importance for the processing of interstitial procollagens to collagen (for review, see Fernandes et al. 2001). It was therefore surprising that the *Adamts3*-deleted mice had no connective tissue phenotype but instead lacked

lymphatic vessels and had severe tissue swelling as embryos, resulting in prenatal death (Janssen et al. 2016).

Mechanistically, CCBE1 could present a scaffold that directly binds ADAMTS3, and complex formation may be required for the binding of pro-VEGF-C in vitro (Jeltsch et al. 2014; Bui et al. 2016). The N terminus of CCBE1 interacts also with VEGFR3 and the pericellular matrix components vitronectin and collagens I, IV, and V (Bos et al. 2011; Jeltsch et al. 2014). Immature VEGF-C also binds VEGFR3 and cell surface/ECM heparan sulfates in vitro (Yin et al. 2011; Jeltsch et al. 2014; Johns et al. 2016). Most VEGF-C activation may thus occur on the endothelial cell surface or in the pericellular matrix. It is also interesting that the C-terminal propeptide contains a repetitive arrangement of cysteine residues, which is otherwise exclusively known from the salivary proteins of silk-weaving mosquito larvae of the genus *Chironomus*, which can form fibrous structures (Joukov et al. 1996; Jha et al. 2017). Whether latent VEGF-C is present in fibrillar silk-like matrix structures in mammalian tissues is not known yet, but it is clear that spatiotemporal regulation of VEGF-C activity may be achieved by either regulated VEGF-C production or three-dimensional (3D) integration of the components of its activation machinery. Furthermore, differences in ECM composition or VEGFR3 protein levels likely create hot spots for VEGF-C activation and localized/guided lymphangiogenesis.

The VEGF-C–VEGFR3 signaling complex in lymphangiogenesis

Several lines of evidence support the key role of VEGFR3 in lymphangiogenesis (Fig. 1). Processed "mature" VEGF-C binds to and activates its primary receptor, VEGFR3; the main angiogenic receptor VEGFR2; and, to some extent, their heterodimers (Joukov et al. 1996; Dixelius et al. 2003). In humans and mice, heterozygous *VEGFR3* and *VEGFC* mutations lead to lymphedema (lymphedema type 1A and 1D, *Chy* mice) (Irrthum et al. 2000; Karkkainen et al. 2000, 2001; Gordon et al. 2013; Brouillard et al. 2014). Furthermore, combined *Vegfc* and *Vegfr3* heterozygosity leads to embryonic lethality, and expression of the VEGF-C/D trap in the developing epidermis prevents lymphatic vessel development in the skin (Makinen et al. 2001; Haiko et al. 2008). VEGFR3 is also expressed in developing blood vessels and in fenestrated endothelia and the tip cells of angiogenic vessel sprouts in adults (Valtola et al. 1999; Partanen et al. 2000; Siekmann and Lawson 2007; Tammela et al. 2008). Accordingly, a homozygous deletion of *Vegfr3* leads to failure of cardiovascular development before the first lymphatic vessels develop from embryonic veins at E9.5 (Dumont et al. 1998). Intriguingly, the role of VEGFR3 in embryonic angiogenesis seems to be ligand-independent, as deletion of both of its identified ligands, VEGF-C and VEGF-D, led to the absence of lymphatic but not blood vasculature in E13.5 embryos (Haiko et al. 2008). Moreover, mutation of the VEGFR3 ligand-binding domain or kinase domain prevented lymphangiogenesis but not angiogenesis (Zhang et al. 2010). In the absence of ligand-induced VEGFR3 kinase activity, other kinases (such as the SRC kinases) activated by integrin signaling can phosphorylate the cytoplasmic tail of VEGFR3, providing docking sites for VEGFR3 downstream signaling components (Galvagni et al. 2010). However, experiments so far have shown that lymphangiogenesis is strictly dependent on an intact VEGF-C–VEGFR3 signaling pathway.

While *Vegfr3* has been shown to be necessary for LEC proliferation, sprouting, and migration (Karkkainen et al. 2001), the role of VEGFR2 in LECs seems more context-dependent. The VEGFR2-specific ligand VEGF-E induced LEC proliferation but not sprouting, suggesting that VEGFR2 has a secondary role in the modulation of lymphatic vessel caliber (Wirzenius et al. 2007; Zarkada et al. 2015). However, *Vegfr2* deletion had no effect on developmental lymphangiogenesis, whereas *Vegfr3* expression was essential for postnatal lymphangiogenesis and even the maintenance of some lymphatic vessel segments in adult skin (Zarkada et al. 2015).

VEGF-C binding to VEGFR3 induces endocytosis of the ligand–receptor complex, which may be necessary for full activation of VEGFR3-derived signals. Ephrin B2, localized to cellular filopodia in active LECs, was necessary for VEGFR3 endocytosis after ligand binding and increased filopodia number and length in response to VEGF-C exposure (Wang et al. 2010). In line with this, deletion of the intracellular C-terminal PDZ domain of ephrin B2 led to defective expansion and pruning of the primary lymphatic capillary plexus, resulting in blunted lymphatic capillary sprouts (Makinen et al. 2005). Ephrin B2 was also needed for the internalization of VEGFR2 (Sawamiphak et al. 2010). Accordingly, antibody-mediated inhibition of ephrin B2 led to attenuation of tumor-associated lymphangiogenesis and angiogenesis (Abengozar et al. 2012). After its internalization, growth factor-activated VEGFR3 triggers several intracellular signal transduction pathways (for review, see Coso et al. 2014; Secker and Harvey 2015). The ubiquitin-binding adaptor proteins epsin 1 and epsin 2 bind to VEGFR3 and mediate its internalization and degradation, resulting in termination of VEGFR3 signaling. Interestingly, mice with LEC-specific deficiency of epsin 1 and epsin 2 had dilated lymphatic capillaries, abnormally high VEGFR3 abundance in collecting lymphatics, immature lymphatic valves, and defective lymph drainage (Liu et al. 2014).

VEGFR3 activity is modulated by its coreceptor, neuropilin 2 (NRP2), initially identified as an axon guidance receptor, which is also expressed in lymphatic vessels and veins (Yuan et al. 2002). VEGF-C binds NRP2 directly, promoting its interaction with VEGFR3 (Karkkainen et al. 2001; Favier et al. 2006). *Nrp2* deletion or antibodies blocking the NRP2–VEGF-C interaction attenuated LEC migration and sprouting but not proliferation (Fig. 1B,C; Caunt et al. 2008; Xu et al. 2010). Accordingly, lymphatic vessels in the dermis of *Nrp2* mutant embryos are larger and less branched (Uchida et al. 2015), although this phenotype is partially compensated in adults (Yuan et al. 2002). A similar phenotype with increased cell

proliferation but decreased branching was observed upon mutation of *Tgfβr1* or *Tgfβr2* (James et al. 2013). Interestingly, TGFβ up-regulates NRP2 and VEGFR3 levels, simultaneously inhibiting LEC proliferation in vitro, which suggests that TGFβ-driven lymphatic vessel sprouting/branching is NRP2-dependent (James et al. 2013). It is curious that NRP2 signaling does not promote VEGFR3-dependent LEC proliferation; perhaps lymphatic vessel branching/sprouting requires a higher VEGFR3 activity threshold than LEC proliferation. On the other hand, NRP2 could be necessary for only some VEGFR3 downstream signaling pathways, similarly to NRP1, which is specifically required for full activation of the p38MAPK signaling pathway downstream from VEGFR2 (Kawamura et al. 2008).

According to Johns et al. (2016), the cell surface heparan sulfate proteoglycan syndecan-4 interacts with VEGFR3 and potentiates its activity. It was also suggested that the heparan sulfate side chains of syndecan-4 bind immature VEGF-C via the charged heparan sulfate side chains and that these could provide a tissue reservoir or sink of VEGF-C (Johns et al. 2016; Jha et al. 2017). In line with this, lymphatic endothelial-specific deletion of heparan sulfates leads to attenuation of tumor-induced lymphangiogenesis, possibly because of decreased VEGFR3 signaling (Johns et al. 2016). Strikingly, however, syndecan-4 deletion led to excessive expansion of lymphatic vasculature during embryonic development (Wang et al. 2016), suggesting that syndecan-4 is not needed for VEGFR3 signaling, at least in the developmental setting. Furthermore, a chimeric VEGF-C containing the VEGF homology domain of VEGF-C in fusion with the high-affinity heparan sulfate-binding domain of VEGF induced a unique lymphatic vessel growth pattern along blood vessels (Tammela et al. 2007). The syndecan-4 heparan sulfates could act as a reservoir or sink of VEGF-C in a context-dependent manner, and the CCBE1–ADAMTS3 complex could provide the required switch to activate the syndecan-4-bound latent VEGF-C (Jeltsch et al. 2014; Jha et al. 2017).

The integrin β1 subunit has been shown to interact with VEGFR3 in response to LEC adhesion to fibronectin or VEGFR3 stimulation with VEGF-C (Fig. 1B,C). Formation of the integrin β1–VEGFR3 complex increases VEGFR3 phosphorylation and LEC migration in vitro (Wang et al. 2001; Zhang et al. 2005). LECs are in contact with the ECM during lymphangiogenesis and with the basement membrane in mature quiescent lymphatic vessels; thus, the function of the VEGFR3–integrin β1 interaction differs in these two settings. By binding to the ECM, integrins are able to translate changes in extracellular tension to cellular responses via outside-in signaling. Interestingly, interstitial pressure/edema also leads to VEGFR3 activation and lymphangiogenesis in an integrin β1-dependent manner (see below; Planas-Paz et al. 2012). Recently, other cell surface transmembrane proteins, such as CLP24 and CLEC14a, have been shown to interact with VEGFR3, but their exact roles in lymphangiogenesis are not yet known (Saharinen et al. 2010; Lee et al. 2017).

Lymphatic vessel sprouting

Several principles of blood vessel sprouting apply also to lymphangiogenesis, although differences are evident. Blood vascular endothelial tip cells are considered to sample the microenvironment with long thin filopodia that guide the establishment of the leading BEC lamellopodia and the direction of vessel growth, whereas BEC proliferation (and thus sprout elongation) occurs most intensely in the vessel stalk (Gerhardt et al. 2003). In tracheal lymphatic vessels, most of the LEC proliferation in response to *M. pulmonis*-induced inflammation was found to occur ~60 µm behind the lymphatic capillary tip cell (Baluk et al. 2005), suggesting similarity between blood and lymphatic vessel growth.

In growing blood vessel sprouts, the endothelial tip cells have high VEGFR2 activity (Jakobsson et al. 2010; Costa et al. 2016). VEGFR2 activation induces expression of the membrane-bound delta-like ligand 4 (DLL4), especially in the tip cells, and subsequent NOTCH activation in the sprouts (Hellstrom et al. 2007; Lobov et al. 2007; Ubezio et al. 2016; Hasan et al. 2017; Pitulescu et al. 2017). DLL4 in turn suppresses further sprouting, as evidenced by hyperbranching of blood vasculature upon *Dll4* deletion or attenuation of NOTCH signaling (Sainson et al. 2005; Hellstrom et al. 2007; Lobov et al. 2007; Suchting et al. 2007). Similarly, VEGF-C induces DLL4 expression in LECs (Zheng et al. 2011). Suppression of NOTCH signaling by a soluble DLL4, an inhibitor of NOTCH signaling, led to hypersprouting of lymphatic vessels in adult mouse skin (Zheng et al. 2011). Lymphangiogenesis triggered by Notch inhibition was suppressed by a VEGFR2-blocking antibody as well as soluble VEGF and VEGF-C/VEGF-D ligand traps (Zheng et al. 2011). In the embryonic dermis, deletion of *Notch1* caused hyperbranching of the lymphatic vessels and increased LEC proliferation (Murtomaki et al. 2013; Fatima et al. 2014). However, *Dll4* deletion in adult mice caused shortening of lacteal vessels, which are typically unbranched and may constantly renew in homeostatic conditions (Bernier-Latmani et al. 2015; Nurmi et al. 2015). *Dll4* deletion had no effect on mature dermal lymphatic vessels (Bernier-Latmani et al. 2015). Antibody-mediated NOTCH inhibition in postnatal mice during the development of the dermal lymphatic network caused inhibition of both lymphatic vessel growth and sprouting (Niessen et al. 2011). In this context, inhibition of NOTCH signaling was associated with down-regulation of ephrin B2 (Niessen et al. 2011), which is necessary for VEGFR3 internalization and signaling (Wang et al. 2010). These experiments indicate that NOTCH signaling has context-dependent effects in lymphangiogenesis.

Interstitial fluid pressure, edema, and flow regulate lymphangiogenesis

One of the key functions of lymphatic vessels is to remove interstitial fluid and return it to the blood circulation. An obvious question is whether accumulation of interstitial fluid (and thus increased pressure) stimulates

lymphangiogenesis. Indeed, expansion of the lymphatic network during development, inflammation, and hypertension correlates with interstitial fluid accumulation (Machnik et al. 2009; Planas-Paz et al. 2012; Kim et al. 2014). In mouse embryos, interstitial fluid pressure peaks at about E12.0, and this coincides with the proliferation of LECs and formation of lymph sacs and the associated superficial lymphatic plexus (Planas-Paz et al. 2012). Interstitial pressure was shown to stretch LECs and induce their proliferation in vivo, and, in vitro, stretch synergized with VEGF-C in stimulating LEC proliferation (Planas-Paz et al. 2012). The shear stress associated with lymph flow has been shown to potentiate VEGF-C-induced LEC sprouting in two-dimensional and 3D cell cultures (Helm et al. 2005; Kim et al. 2016; Choi et al. 2017b). Recently, flow was shown to suppress NOTCH signaling via the calcium influx mediated by the ORAI1 calcium channel. Accordingly, *Orai1* calcium channel-deficient embryos displayed hypobranching of dermal lymphatic vessels (Choi et al. 2017b). Furthermore, VEGF expression by tumor cells drives peritumoral interstitial convection, which could potentially stimulate lymphangiogenesis associated with tumorigenesis (Dafni et al. 2002). Interstitial pressure and fluid flow also modulate the regeneration of lymphatic capillaries. Lymphangiogenesis associated with skin wound healing in mouse tails was attenuated upon decreased interstitial flow through the regenerating region, and the capacity of ectopic VEGF-C to induce lymphangiogenesis was blunted in conditions of decreased flow (Rutkowski et al. 2006; Goldman et al. 2007). It was suggested that the need for flow is related to the channeling of growth factor and protease cues (Boardman and Swartz 2003).

It seems that lymphatic vessel specification and identity are determined in part by the level of fluid shear stress. In *Slp-76* (*Lcp2*) mutant mice, in which the access of blood into some lymphatic vessels leads to lymphatic vessel exposure to high shear stress, PROX1 is down-regulated and LECs start to display features of blood vascular endothelia (Abtahian et al. 2003; Chen et al. 2012a). In vitro, even low shear stress induces LEC alignment with the direction of flow in a pattern similar to the in vivo situation in lymphatic vessels (Ng et al. 2004; Sabine et al. 2012). Upon oscillatory flow, which mimics turbulent flow in the valve-forming areas, cultured LECs adopt a cuboidal shape similar to that of valve-forming cells (Sabine et al. 2012). Interestingly, only the PROX1-high LECs respond to oscillatory flow, whereas all LECs respond to shear stress caused by laminar flow (Sabine et al. 2012). It is not known whether flow contributes to lymphangiogenesis in already lumenized sprouts by modulating LEC proliferation in the stalks of the sprouts.

A variety of fluid pressure/flow sensors has been implicated in lymphangiogenic responses. It has been suggested that interstitial fluid accumulation and increased pressure impacts the reorganization of stretched ECM, thereby affecting integrin β1 and subsequent VEGFR3 activation in embryos (Fig. 1B,C; Planas-Paz et al. 2012). Interestingly, VEGFR3 may also provide a mechanosensory function when complexed with VE-cadherin (Coon et al. 2015), raising the possibility of interstitial pressure sensing at the level of LEC–LEC junctions, which mediate tension between the cells. The endothelial transmembrane protein PECAM1 (CD31) functions as a mechanosensor in BEC–BEC junctions of the blood vascular endothelium (Osawa et al. 2002; Tzima et al. 2005). Interestingly, *Pecam1*-deleted mouse embryos have increased branching of mesenteric lymphatics, suggesting that PECAM1 could provide a similar function also in the lymphatic vessels (Wang et al. 2016). Furthermore, loss of syndecan-4 or β-catenin function leads to defective lymphatic vasculature patterning in the embryonic mesentery and dermis, respectively (Cha et al. 2016; Wang et al. 2016). These mutant phenotypes may be caused by defective flow sensing, which leads to increased proliferation of LECs or lack of pruning of the lymphatic sprouts, resembling the defective blood vessel pruning in decreased flow conditions (for review, see Korn and Augustin 2015). Laminar flow has also been shown to induce ORAI1-dependent calcium signaling, which stimulated LEC proliferation and sprouting during development (Choi et al. 2017a,b). However, the actual sensor that activates ORAI1 has not yet been identified. Mutations of mechanosensitive calcium-permeable channel *PIEZO1* have been linked to hereditary lymphedema (Fotiou et al. 2015; Lukacs et al. 2015). Although the exact role of *PIEZO1* in lymphatic function is still unclear, studies on blood vasculature have suggested a role for PIEZO1 in transducing shear stress to polarized BEC orientation (Li et al. 2014; Ranade et al. 2014). In addition, deletion of *Pdk1* or *Pdk2*, which have been implicated in mechanosensitive calcium signaling, led to failure of thoracic duct development in zebrafish embryos and attenuated branching of cutaneous lymphatic vessels in mouse embryos (Coxam et al. 2014; Outeda et al. 2014). Although most of the in vivo investigations so far have focused on developmental lymphangiogenesis, it would be interesting to know whether similar mechanisms regulate regenerative lymphangiogenesis.

Lymphatic vessel guidance by arteries and nerves

As lymphatic vessels drain the tissue fluid extravasated from blood vessels, the codevelopment of these two vascular systems is critical. Indeed, large lymphatic collectors align with major blood vessels in mice and humans, indicating that the growth of the two vascular systems is interconnected (Fig. 1A; Sabin 1902). Lymphatic and blood vessels display close association already in the chorioallantonic membrane of chicken embryos (Oh et al. 1997). Furthermore, lymphatic vessel development is dependent on prior arteriogenesis in the mouse mesentery (Mahadevan et al. 2014). In zebrafish, a recently identified population of cells resembling LECs in the brain was shown to migrate along the mesencephalic vein during development; the cells remain positioned in close proximity to meningeal blood vessels in adult fish without forming a lumenized structure (Bower et al. 2017a; van Lessen et al. 2017; Venero Galanternik et al. 2017). Interestingly, migration of these LEC-resembling cells was shown to be

vegfr3-, *vegfc*-, *vegfd*-, and *ccbe1*-dependent (Bower et al. 2017a; van Lessen et al. 2017). Whether this reflects Vegfc production by the BECs or the associated SMCs (see above; Fig. 1A) requires further investigation. Similarly, patterning of the first lymphatic vessels in zebrafish is dependent on LEC migration along intersegmental arteries whose mispatterning alters lymphatic vessel patterning, indicating that the blood vasculature provides guidance for the developing lymphatic vessels (Bussmann et al. 2010). The arterial chemokine Cxcl12 and its receptor, Cxcr4, in LECs provide such a guidance function in zebrafish (Cha et al. 2012; Zhuo et al. 2012). Consistent with this, a specific inhibitor of the CXCL12–CXCR4 interaction blocks suture-induced corneal lymphangiogenesis in mice (Du and Liu 2016).

Blood vessels can regulate lymphatic vessel growth and maintenance also via the peptide hormone adrenomedullin, which is essential for proper lymphatic vessel development. Accordingly, deletion of adrenomedullin or either of its two receptors, *Calcrl* or *Ramp2*, leads to an edematous embryonic phenotype (Fritz-Six et al. 2008; Ichikawa-Shindo et al. 2008), whereas overexpression of adrenomedullin by tumor cells results in increased lymphangiogenesis (Karpinich et al. 2013). Adrenomedullin levels are regulated by the atypical chemokine receptor CXCR7, which acts as a sink of adrenomedullin. Because of this, *Cxcr7* deletion leads to increased adrenomedullin levels and hypersprouting of lymphatic vessels (Klein et al. 2014). Interestingly, CXCR7 is expressed predominantly in BECs, whereas CALCRL and RAMP2 are more prominent in LECs, and CXCR7 acts also as a sink for CXCL12 (Boldajipour et al. 2008; Fritz-Six et al. 2008). Thus, dynamic modulation of CXCL12, adrenomedullin, and CXCR7 levels in the two vascular systems could potentially regulate their alignment and separation from each other at later developmental stages (Boldajipour et al. 2008).

Although the major collecting lymphatic vessels accompany arteries, lymphatic capillaries display a distribution pattern that is distinct from blood vessels. The separation of these two networks is an active process that uses, for example, semaphorin 3G, a repulsive cue in axon pathfinding (Uchida et al. 2015; Liu et al. 2016). In vitro, semaphorin 3G induces LEC contraction and repulsion, which is dependent on semaphorin 3G receptors plexin and NRP2 (Uchida et al. 2015; Liu et al. 2016). Developing arteries in the embryonic dermis express semaphorin 3G, suggesting that the altered lymphatic pattern in semaphorin 3G-deleted mice results from failure of lymphatic vessel separation from the arteries (Fig. 1D; Uchida et al. 2015; Liu et al. 2016). The nonarterial semaphorins 3C and 3F may also inhibit lymphangiogenesis during development and tumorigenesis (Doci et al. 2015; Mumblat et al. 2015).

Although nerves have not been directly implicated in lymphatic vessel growth in mammals, it is well established that cutaneous neurons guide developing arteries in embryonic skin (Mukouyama et al. 2002). They could thus indirectly affect lymphatic guidance. In zebrafish, however, LECs migrate along motoneurons, which in turn are directed by netrin 1, secreted by the underlying muscle pioneers in the horizontal myoseptum. Both netrin 1 down-regulation and laser-mediated motoneuron ablation prevented proper LEC migration and the parachordal sprouting of LECs (Lim et al. 2011). Vegfc from the preformed dorsal aorta guides the axon growth of secondary motoneurons in zebrafish (Kwon et al. 2013). These examples indicate the existence of cross-talk between the developing neuronal and lymphatic vascular networks.

Basement membranes in lymphangiogenesis

Quiescent lymphatic vessels are invested with a basement membrane, which is very thin and porous around lymphatic capillaries, being thicker and continuous around the collector vessels (Sauter et al. 1998; Pflicke and Sixt 2009; Lutter et al. 2012). These differences reflect functional specialization of these two vessel types. The porous basement membrane allows leukocyte entry via LEC–LEC junctions into the lymphatic capillary lumen (Pflicke and Sixt 2009), whereas the basement membrane around collectors is critical for endothelial cell–SMC interactions, as in blood vessels (Lutter et al. 2012). The lymphatic vessel basement membrane is composed of LEC-expressed laminin α4/5, β1/2, and γ1 chains; collagens IV and XVII; reelin; and nidogen 1 that cross-links the laminin and collagen layers (Vainionpaa et al. 2007; Pflicke and Sixt 2009; Lutter et al. 2012). The functional significance of the lymphatic vessel basement membrane and its constituents are less well known than in the case of blood vessel basement membranes. Embryoid bodies that have a mutation in the laminin γ1 gene lack a structured basement membrane and have dilated blood vessels and altered vessel branching (Jakobsson et al. 2008). Deletion of the laminin α4 gene in vivo led to blood vessel hypersprouting in mouse retinas in an integrin β1-dependent manner (Stenzel et al. 2011). As in blood vessels, basement membranes seem to stabilize lymphatic vessels. Reelin was shown to be essential for lymphatic collector maturation via stabilization of interactions between LECs and SMCs (Lutter et al. 2012). Furthermore, basement membrane matrix (Matrigel) inhibited sprouting lymphangiogenesis in explants of the thoracic duct in vitro (Detry et al. 2012).

During angiogenesis, matrix metalloproteases digest basement membranes and the interstitial matrix, thus revealing new integrin-binding epitopes and releasing growth factors that facilitate sprout formation (Arroyo and Iruela-Arispe 2010). Thin and porous lymphatic capillary basement membranes should allow an interaction between LECs and the components of the interstitial matrix. Thus, the extension of LEC sprouts may be much less protease-dependent (for review, see Paupert et al. 2011). Indeed, LECs in general express fewer proteases than BECs (Petrova et al. 2002). Nevertheless, increased expression of matrix metalloproteinase 2 (MMP2) and MMP9 is associated with wound healing and FGF2-induced lymphangiogenesis (Chang et al. 2004; Rutkowski et al. 2006). MMP2 was also up-regulated in lymphangiogenesis in

response to corneal injury, and *Mmp2* deletion led to tortuous lymphatic capillaries in the cornea (Detry et al. 2012). In zebrafish, *mmp2* deletion reduced the length of the thoracic duct, possibly via attenuated processing of collagen (Detry et al. 2012). In contrast, spontaneous lymphangiogenesis was observed in corneas of MT-MMP1-deficient mice (Wong et al. 2016). These examples suggest that metalloproteases may control lymphangiogenesis, e.g., via modulation of basement membrane components and by exposing hidden matrix components.

Integrins and the interstitial matrix in lymphatic sprouting

During lymphangiogenesis, lymphatic vessel tip cells interact with fibrillar and provisional matrix components, such as collagen I and fibronectin, in the remodeling tissue. Several of these components are known to modulate lymphangiogenic responses. The ECM components that directly interact with integrins in LECs lead to activation of signal transduction pathways involved in the regulation of migration and proliferation (for review, see Chen et al. 2012b). For example, injection of a collagen I gel stimulated lymphangiogenesis associated with wound healing in mice (Clavin et al. 2008), and the EDA epitope-containing fibronectin form, which is abundant in regenerating tissues, stimulated LEC proliferation in vitro (Ou et al. 2010).

Several integrins expressed in LECs are essential for lymphangiogenesis. The integrin α9 gene (*ITGA9*) is an important Prox1-regulated LEC signature gene (Petrova et al. 2002; Mishima et al. 2007). Mice deleted of the α9 subunit (*Itga9*) of integrin α9β1 failed to survive beyond postnatal day 12 due to development of chylothorax, which has also been reported in patients with missense mutations of the *ITGA9* (Huang et al. 2000; Liao et al. 2002; Ma et al. 2008). α9β1 integrin is necessary for the formation of lymphatic valves; it acts via binding to the fibronectin EDA domain, emilin1, and polydom (Bazigou et al. 2009; Danussi et al. 2013; Karpanen et al. 2017; Morooka et al. 2017). Polydom deletion recapitulates the chylothorax phenotype of *Itga9* mutant mice and also leads to severe defects in lymphatic vessel sprouting, which has not been reported for *Itga9* mutants (Morooka et al. 2017). Furthermore, it was suggested that defective lymphatic vessel sprouting in Polydom-deficient mice depends on attenuated angiopoietin 2 signaling (Morooka et al. 2017).

Expression of the major fibronectin and collagen receptors is upregulated in LECs in lymphangiogenic conditions. The fibronectin receptor integrin α5β1 is induced in lymphangiogenic sprouts of inflamed tracheal mucous membranes, and small molecules that block α5β1 inhibited lymphangiogenesis but not angiogenesis associated with tracheal or corneal inflammation (Dietrich et al. 2007; Okazaki et al. 2009). The fibronectin and VCAM receptor α4β1 is up-regulated in lymphangiogenesis, and its genetic deletion or antibody-mediated inhibition attenuated tumor lymphangiogenesis (Garmy-Susini et al. 2010). Furthermore, collagen receptors integrin α1β1 and α2β1 were induced upon VEGF exposure in vitro, and antibodies against α1β1 and α2β1 attenuated wound healing-associated lymphatic vessel density in mice treated with VEGF-expressing implants (Hong et al. 2004). Inhibition of α1β1 also attenuated suture-induced inflammatory lymphangiogenesis (Grimaldo et al. 2011). Lymphangiogenic integrins contain the β1 chain, which interacts with VEGFR3 and stimulates its activity (Fig. 1B,C); thus, the above results could be mediated at least in part by regulation of VEGFR3 activation.

Outlook

Recent findings in the field of lymphangiogenesis and lymphatic biology include the identification of meningeal lymphatic vessels (Aspelund et al. 2015; Louveau et al. 2015) and finding of nonvenous endothelial cell contribution to lymphatic vessel growth in various tissues (Klotz et al. 2015; Martinez-Corral et al. 2015; Stanczuk et al. 2015). These will undoubtedly provide additional insights for our understanding of the normal and pathological functions of lymphatic vasculature. Improvements in genetic reporters and lineage tracing tools and the ongoing deep and single-cell RNA sequencing should allow the identification of distinct molecular signatures of various types of lymphatic vessels in different organs as well as identification of novel lymphatic vessel-specific markers, which can be used to develop highly specific lineage tracing and Cre-deleter mouse strains. Together with advanced whole-mount imaging techniques, these tools should enable studies of lymphangiogenesis in the context of hierarchical lymphatic networks, which may reveal unexpected heterogeneity among seemingly similar LECs and allow studies on lymphatic vessel interactions with other anatomical structures. The possibility of postnatal manipulation of lymphatic vessel development in vivo allows studies of molecules that are essential for vascular growth and indispensable for embryonic development. These studies should provide additional insights into the general mechanisms of vascular growth and disease.

The importance of lymphatic vessels has been shown in the pathogenesis of several diseases, and modulation of lymphangiogenesis provides opportunities for therapeutic interventions. In preclinical models, inhibition of lymphangiogenesis decreases tumor dissemination, whereas stimulation of lymphangiogenesis results in enhanced resolution of inflammation. The studies done so far have targeted VEGFR3 ligand availability or signaling activity directly. However, detailed knowledge of other paracrine and pericellular mechanisms of lymphangiogenesis should provide additional possibilities to treat diseases whose pathogenesis involves lymphatic vessels.

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