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Review

Lymphangiogenesis: Molecular Mechanisms and Future Promise

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The growth of lymphatic vessels (lymphangiogenesis) is actively involved in a number of pathological processes including tissue inflammation and tumor dissemination but is insufficient in patients suffering from lymphedema, a debilitating condition characterized by chronic tissue edema and impaired immunity. The recent explosion of knowledge on the molecular mechanisms governing lymphangiogenesis provides new possibilities to treat these diseases.

Introduction

Angiogenesis, the growth of blood vessels, has received considerable attention over the past few decades, and the first antiangiogenic pharmaceuticals have recently entered the clinics. However, the molecular mechanisms regulating lymphangiogenesis, the growth of lymphatic vessels, are far less explored. Yet understanding the functions and regulatory pathways of this vascular system will undoubtedly lead to novel and significant paradigms in both biology and clinical medicine.

Lymphatic vessels regulate tissue fluid homeostasis, immune cell trafficking, and absorption of dietary fats. The absence of lymphatic vessels is incompatible with life, and individuals with dysfunctional lymphatic vessels often suffer from chronic edema and impaired immune responses. Our knowledge of the molecular mechanisms controlling lymphangiogenesis has improved considerably over the past few years, mainly thanks to progress in the identification of regulatory molecules and markers specific to the lymphatic endothelium. The genetic programs that determine lymphatic endothelial cell (LEC) differentiation and growth, and make them distinct from blood vessels, involve a number of newly described signal transduction pathways.

Importantly, lymphangiogenesis occurs in adult tissues during inflammation, wound healing, and tumor metastasis. Consequently, administration of lymphatic growth factors or their antagonists provides the possibility of targeting lymphatic vessels in human disease. In this Review we discuss the recent advances that have greatly improved our understanding of the biology behind lymphangiogenesis, including the management of inflammation, obesity, hypertension, tumor metastasis, and lymphatic vessel dysfunction.

Functions of the Lymphatic Vascular System

Large multicellular organisms such as humans need a circulatory system to distribute oxygen, nutrients, hormones, and even cells to tissues, as well as to collect carbon dioxide and other metabolic waste products. The blood contains a variety of colloid proteins that help to maintain its water content higher than in the surrounding tissues. Blood pressure causes plasma constituents to filtrate continuously from the arterial side of the capillary bed into the interstitial space. Approximately 90% of the extravasated water is reabsorbed at the venous side of the capillary bed, where the colloid osmotic pressure of the blood exceeds the blood pressure. The main function of the lymphatic vasculature is to return the remaining 10% back to the blood vascular system (Földi and Strössenreuther, 2004).

Fluid, macromolecules, and certain leukocytes enter blind-ended lymphatic capillaries, which connect with larger collecting lymphatic vessels (Figure 1). Lymph from the left side of the body, abdomen, and both lower limbs ends up in the thoracic duct, the largest lymphatic vessel that runs alongside the aorta, and finally connects with the left subclavian vein. Lymph from the right upper arm, thorax, and head is returned to the right subclavian vein via the right lymphatic trunk (Jeltsch et al., 2003). In healthy adult individuals, the lymphatic system returns approximately 1–2 liters of interstitial fluid with 20–30 g of protein per liter to the venous circulation every day (Levick, 1999). The collecting lymphatic vessels connect with chains of lymph nodes that perform a sieving function (Figure 1C). Thus the lymphatic vascular system also plays an important role in immune responses by serving as a conduit for extravasated leukocytes and activated antigen-presenting cells. In the small intestine, lacteal lymphatic vessels inside the intestinal villi absorb dietary lipids released by intestinal epithelial cells in the form of chylomicrons.

Lymphatic vessels are typically found in all vascularized tissues, with the notable exception of bone marrow and the central nervous system. However, some connections between the cerebrospinal fluid and lymphatic vascular systems exist, and lymphatic vessels are capable of partly compensating for the drainage of intracranial fluid when clearance of the cerebrospinal fluid is compromised (Johnston et al., 2004). Besides the fat-absorbing small intestine, tissues that frequently become in contact with foreign antigens, such as the skin and mucous membranes, are particularly rich in lymphatic vessels. The lymphatic vascular system is found at least in vertebrates such as teleost fish, amphibians, reptiles, and mammals (Jeltsch et al., 2003; Kuchler et al., 2006; Ny et al., 2005; Yaniv et al., 2006),

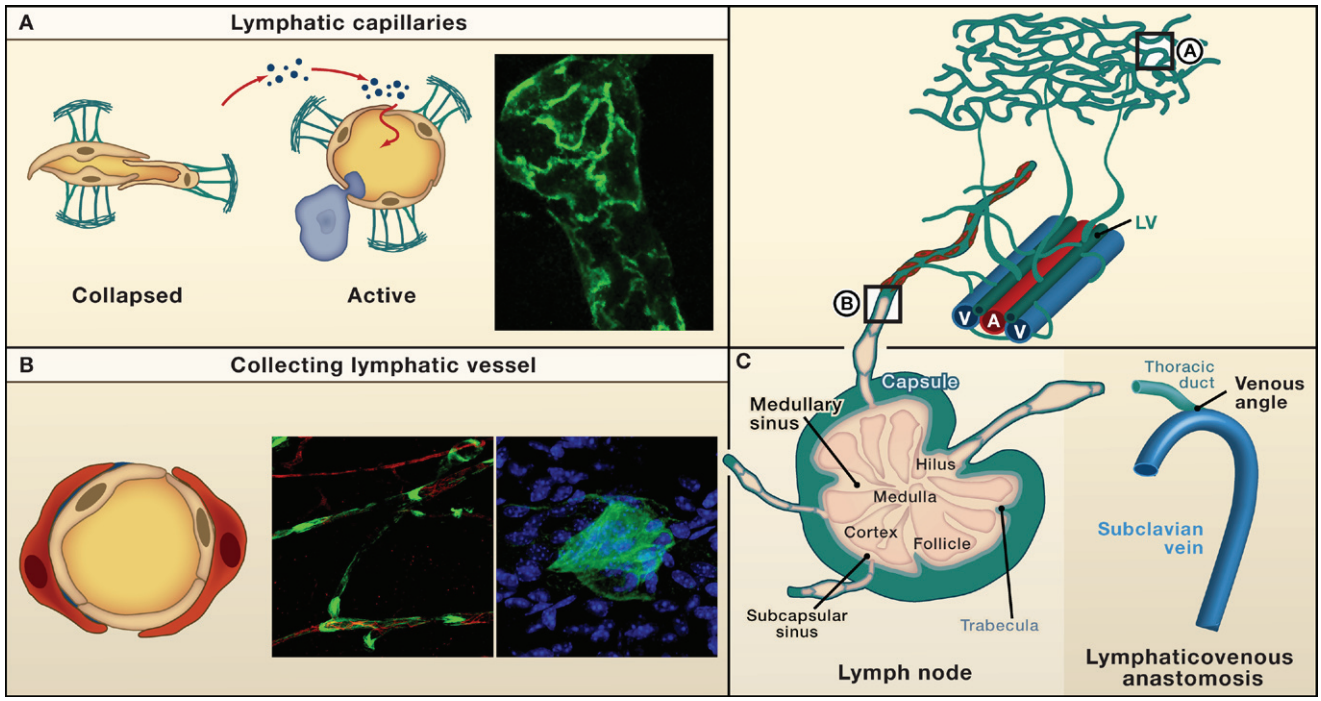

Figure 1. Anatomy of the Lymphatic Vascular System

(A) Interstitial fluid, macromolecules, and cells enter lymphatic capillaries through interendothelial gaps, which are sealed by overlapping oak-leaf-shaped lymphatic endothelial cells (LECs) when the vessel is filled. Lymphatic vessel hyaluronan receptor-1 (LYVE-1) immunostaining (green) shows a blind end of a lymphatic capillary in a whole-mount preparation of the mouse ear skin.

(B) From the capillaries lymph moves to precollectors and on to collecting vessels, directed by minute changes in interstitial fluid pressure and the negative pressure within the lymphatic vascular system. Collecting lymphatic vessels are specialized for the transport of lymph; they do not absorb fluid from surrounding tissues. These vessels are surrounded by a basement membrane and a smooth muscle cell layer and contain bileaflet intralumenal valves to prevent lymph backflow. Confocal images are of whole-mount preparations of the mouse ear following intradermal injection of *Lycopersicon esculentum* lectin (green), which marks lymphatic valves, and immunostaining with antibodies to smooth muscle α-actin (red). Nuclear staining in blue.

(C) Afferent collecting vessels connect with lymph nodes, which process foreign antigens that are found soluble in the lymph as well as presented by antigen-presenting cells. Lymph leaves the lymph node via an efferent vessel and finally reaches the venous system via the thoracic duct or the right lymphatic duct that connect with the subclavian veins at the venous angles. Images adapted by permission from Macmillan Publishers Ltd: *Nature* (Tammela et al., 2007), copyright 2007. LV, lymphatic vessel.

whose complex cardiovascular system and relatively large body size require the presence of a secondary vascular system for the maintenance of fluid balance.

The lymphatic capillaries form the absorptive part of the lymphatic vascular tree, being responsible for the uptake of interstitial fluid, macromolecules, and cells that collectively are called lymph, once inside the lymphatic vessels. The lymphatic capillaries are blind-ended and thin-walled vessels of approximately 30–80 μm in diameter and composed of a single layer of LECs, which are not ensheathed by pericytes or smooth muscle cells, have little or no basement membrane, and display distinct gene expression patterns (Alitalo et al., 2005; Maby-El Hajjami and Petrova, 2008; Wick et al., 2007) (Figure 1). In contrast to continuous interendothelial junctions in blood vessels, lymphatic capillaries have discontinuous or "button-like" junctions (Baluk et al., 2007; Tammela et al., 2007). The interjunctional gaps act as sites of leukocyte entry into the vessels. These structural features render the walls of the lymphatic capillaries highly permeable, which enables optimal uptake of lymph components. Furthermore, the lymphatic capillaries are connected to the surrounding extracellular matrix by anchoring filaments, which attach to collagen fibers so that they become taut in conditions of tissue swelling, resulting in opening of the lymphatic vessel lumen, decreased intralumenal pressure, and increased uptake of tissue fluid (Figure 1A). The anchoring filaments are mainly composed of emilin-1 and fibrillin, which may attach to the LECs via adhesion molecules such as $\alpha v\beta 3$ integrin (Danussi et al., 2008; Maby-El Hajjami and Petrova, 2008).

Lymph moves from the lymphatic capillary bed into precollector vessels, which are sparsely covered by smooth muscle cells (Maby-El Hajjami and Petrova, 2008). The precollector vessels fuse with collecting lymphatic vessels, which are characterized by the presence of a smooth muscle cell layer, a basement membrane, continuous "zipper-like" interendothelial junctions, and bileaflet valves (Alitalo et al., 2005; Baluk et al., 2007). The intrinsic contractility of smooth muscle cells as well as the contraction of surrounding skeletal muscles and arterial pulsations are necessary for lymph propulsion, whereas valves prevent lymph backflow (Bazigou et al., 2009) (Figure 1B). In this respect, the collecting lymphatic vessels resemble small veins.

The LECs are terminally differentiated cells distinct from blood vascular endothelial cells (Saharinen et al., 2004; Wick et al., 2007). This has enabled the discovery of lymphatic

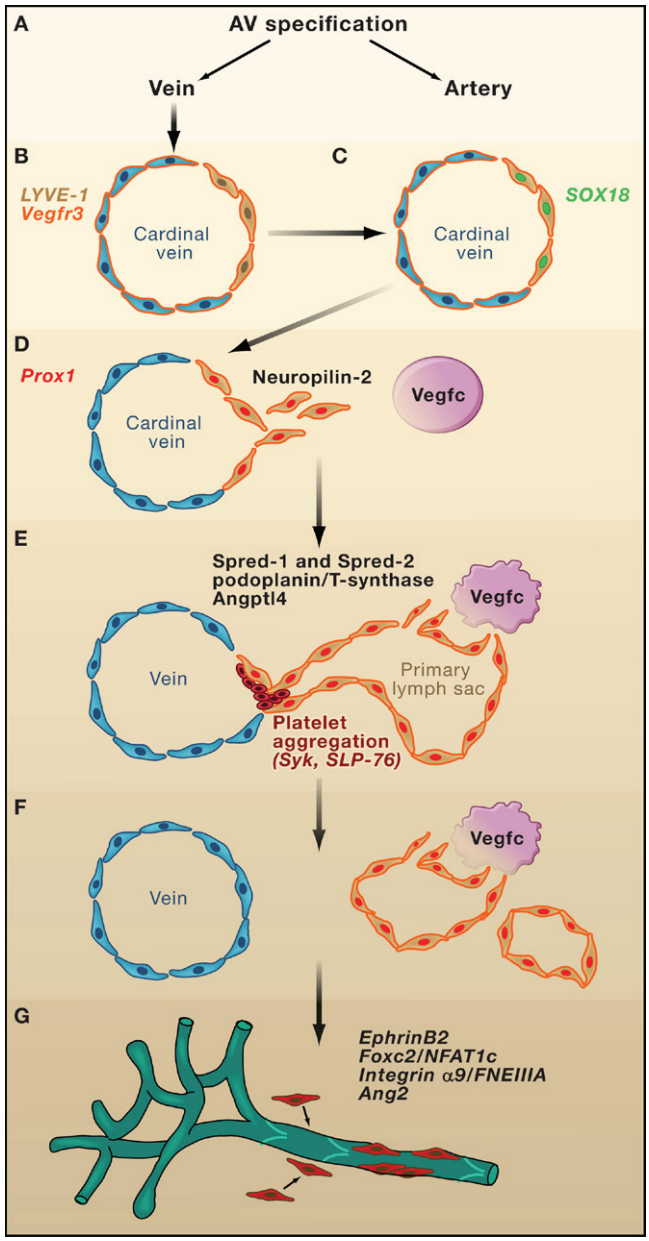

Figure 2. Development of the Mammalian Lymphatic Vasculature

(A) After differentiation from angioblasts, endothelial cells undergo arterial-venous (AV) specification.

(B) Embryonic veins express high levels of VEGFR-3, whereas a subpopulation of endothelial cells in the large central veins upregulate LYVE-1 (lymphatic vessel hyaluronan receptor-1).

(C) The transcription factor SOX18 is induced in the LYVE-1-positive lymphatic endothelial cell (LEC) precursors.

(D) SOX18 induces Prox1 expression, the first marker for LEC determination. At about this time, VEGFR-3 expression is downregulated in the blood vessels, but it remains high in the LEC precursors, which also begin to express neuropilin-2, rendering them more responsive to VEGF-C signals arising from the lateral mesenchyme. These signals are required for sprouting of the LECs, which form lymph sacs lateral to the central veins.

(E) The LECs begin to express podoplanin, which via the CLEC-2 receptor activates the Syk tyrosine kinase in platelets. This leads to platelet aggregation, which blocks lymphatico-venous connections and helps to separate the blood and lymphatic vascular systems.

(F) Further centrifugal growth of the lymphatic vessel network ensues, driven by VEGF-C/VEGFR-3 and possibly Ccbe1 signals.

(G) The final steps of lymphatic vascular development involve differentiation to lymphatic capillaries and collecting lymphatic vessels. The latter form intraluminal valves, recruit smooth muscle cells, develop continuous interendothelial junctions, and produce a basement membrane.

vascular-specific molecules that are used for identification of lymphatic vessels in tissues, as well as for finding targets for the specific induction or inhibition of lymphatic vessel growth in pathological conditions (reviewed in Saharinen et al., 2004). These include the prospero-related homeodomain transcription factor Prox1, the membrane glycoprotein podoplanin, vascular endothelial growth factor receptor-3 (VEGFR-3), and lymphatic vessel hyaluronan receptor-1 (LYVE-1).

Development of the Lymphatic Vascular Tree

Commitment to Lymphatic Endothelium

The lymphatic vessels arise after the cardiovascular system is established and functional (Figure 2). Lymphatic vessel development starts at about embryonic weeks 6–7 in humans and at embryonic day (E) 9.5–10.5 in mice, when distinct subpopulations of endothelial cells in the lateral parts of the anterior cardinal veins commit to the lymphatic lineage and then sprout laterally to form primordial lymphatic vascular structures, the lymph sacs (Alitalo et al., 2005; Oliver, 2004; Wigle and Oliver, 1999). The peripheral lymphatic vasculature is then generated by centrifugal sprouting of lymphatic vessels from the lymph sacs, followed by merging of the separate lymphatic capillary networks and remodeling and maturation of the primitive lymphatic capillary plexus (Figure 2).

Genetic experiments in mice have validated that mammalian lymphatic vessels originate from embryonic veins (Karkkainen et al., 2004; Oliver, 2004; Srinivasan et al., 2007; Wigle and Oliver, 1999), as postulated already in 1902 by the American anatomist Florence Sabin. Further validation of this view has been obtained from lineage-tracing experiments in mice (Srinivasan et al., 2007), and dynamic imaging in developing zebrafish embryos has elegantly demonstrated that this process is conserved in evolution (Kuchler et al., 2006; Yaniv et al., 2006). These pioneering studies have demonstrated the versatility and speed of the zebrafish as a model organism in the discovery of new molecular mechanisms regulating the development of the lymphatic vessels, although it should be noted that the differentiation of collecting lymphatic vessels has not been reported in fish (Hogan et al., 2009) (Figure S1 available online).

LYVE-1, one of the most specific and widely used lymphatic endothelial markers, is expressed in a subset of endothelial cells in the large central veins and currently provides the first indicator of lymphatic endothelial competence (Jurisic and Detmar, 2009; Maby-El Hajjami and Petrova, 2008) (Figure 2B). In adults its expression in collecting lymphatic vessels decreases and remains high only in lymphatic capillaries (Makinen et al., 2005). However, gene targeting in mice indicates that LYVE-1 is dispensable for normal lymphatic development or function (Gale et al., 2007).

Prox1 is specific for lymphatic vessels in the vascular system. In mice, the first Prox1-positive endothelial cells are detected as a restricted subpopulation on one side of the anterior cardinal

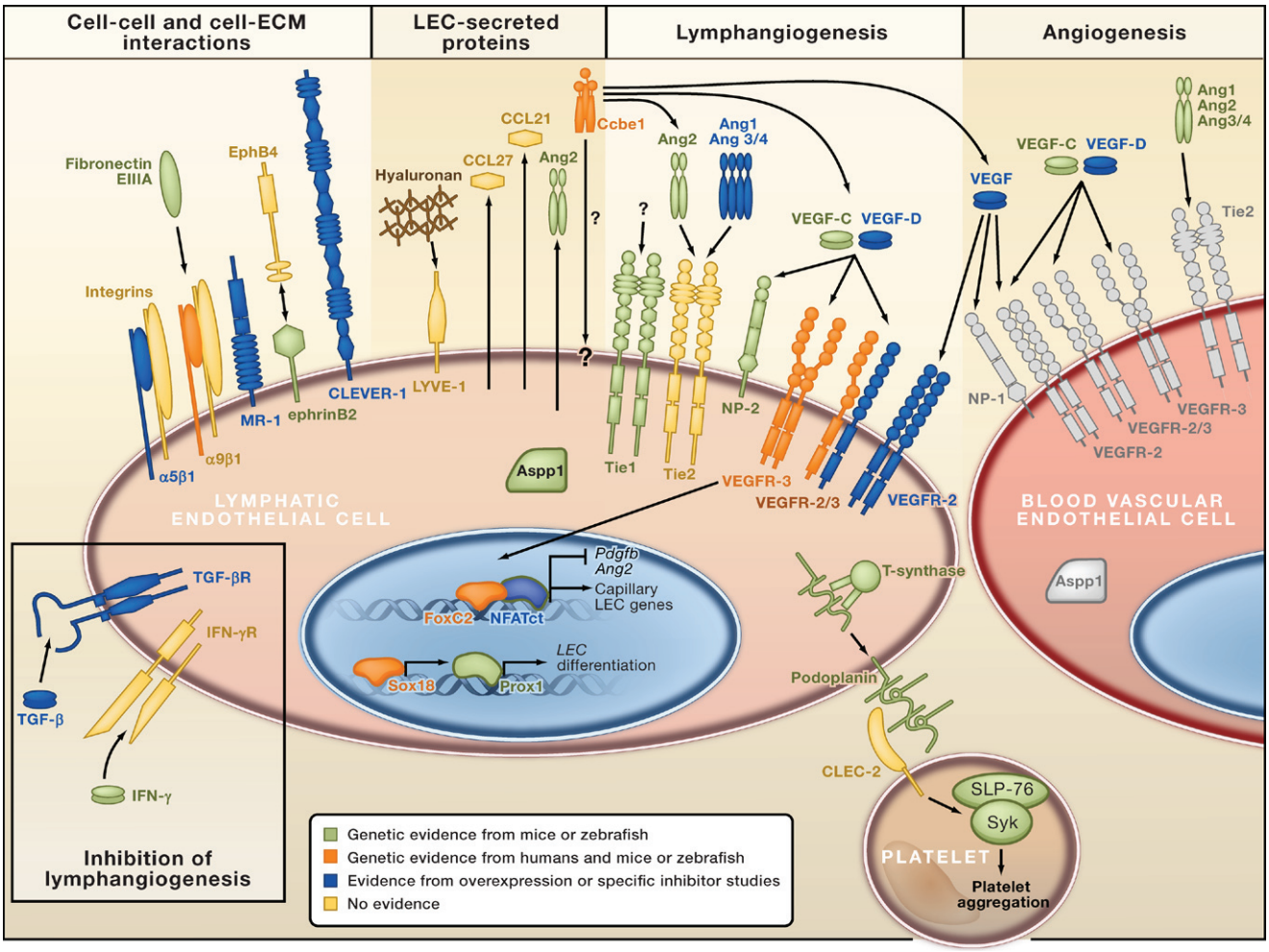

Figure 3. Key Molecular Pathways Regulating Lymphangiogenesis

The transcription factor Prox1 drives expression of lymphatic endothelial-specific genes, whereas FOXC2 and NFAT1c regulate differentiation of the lymphatic collecting vessel phenotype. Podoplanin activates the CLEC-2 receptor in platelets, leading to the activation of the tyrosine kinase Syk and platelet aggregation. This mechanism is important for the separation of blood and lymphatic vascular systems (also see Figure 2). TGF-β and IFN-γ act as endogenous inhibitors of lymphangiogenesis. Note VEGFR and Tie expression in both lymphatic and blood vascular endothelial cells.

vein at E9.5, and soon thereafter these cells start budding from the vein and migrating in a polarized manner, eventually forming lymph sacs (Oliver, 2004; Wigle and Oliver, 1999) (Figures 2D and 2E). *Prox1* knockout embryos lack lymph sacs and lymphatic vessels. The *Prox1*-deficient endothelial cells initially bud and sprout from the cardinal vein, although in an unpolarized manner, but their migration is soon arrested. These cells fail to express lymphatic endothelial markers and instead retain their blood vascular endothelial phenotype (Oliver, 2004; Wigle and Oliver, 1999). Overexpression of Prox1 in human blood vascular endothelial cells (BECs) suppresses the expression of several genes specific for the blood vascular endothelium and upregulates LEC-specific gene expression (Alitalo et al., 2005; Jurisic and Detmar, 2009; Oliver, 2004), which further suggests a function for Prox1 as a master switch that determines LEC fate.

The signals leading to polarized expression of PROX1 in differentiating LECs are currently poorly understood. In a recent study, Francois et al. demonstrated that the homeobox transcription factor SOX18 is expressed in cardinal vein endothelial cells prior to *Prox1*, and that the *Prox1* promoter contains SOX18-binding sites, indicating that SOX18 is required for initiation of the LEC differentiation program upstream of *Prox1* (Francois et al., 2008) (Figure 2C). Gene targeting of the *SOX18* locus or homozygous expression of a dominant-negative mutant SOX18 abolished Prox1 expression and led to edema and embryonic lethality (Francois et al., 2008). Interestingly, loss of even one allele or heterozygous dominant-negative mutation of SOX18 leads to defects in the patterning of the cutaneous lymphatic vessels (Francois et al., 2008). However SOX7 and SOX17 can be upregulated in the absence of SOX18, which may modify the mutant phenotype (Hosking et al., 2009). At present, the signals leading to the induction of SOX18 in cells committing to the LEC lineage remain unknown.

Studies in nonmammalian species have led to an alternative view of a dual origin from embryonic veins and mesenchymal lymphangioblasts. Grafting experiments in avian embryos

suggest that although the deep parts of the lymph sacs are derived from adjacent veins, the superficial parts of the jugular lymph sacs and the dermal lymphatics arise from local lymphangioblasts (Wilting et al., 2006). In *Xenopus laevis* tadpoles, Prox1-positive mesodermal precursor cells, lymphangioblasts, which share a common origin with vascular progenitor cells, contribute to lymphatic vessel formation (Ny et al., 2005). It is not yet known how these cells relate to the parachordal lymphatic precursors described in zebrafish (Figure S1). Also in murine embryos, scattered mesenchymal cells, which coexpress leukocyte (CD45) and lymphatic endothelial markers (LYVE-1, Prox1), were detected in the regions of new lymphatic vessel growth, and it is suggested that these cells with characteristics of LECs and macrophages integrate into lymphatic vessels (Buttler et al., 2006, 2008). However, the formation of lymph sacs is not affected in *Runx1* gene-targeted mice, which have defective hematopoiesis, and lineage-tracing studies fail to confirm contribution of hematopoietic cells to lymphatic endothelium (Buttler et al., 2008; Srinivasan et al., 2007). It is conceivable that hematopoietic cells contribute to developmental lymphangiogenesis by providing paracrine factors, as has been described previously for angiogenesis (Tammela et al., 2005a). At least macrophages appear to be important sources of lymphangiogenic factors, as postnatal lymphatic vessel development is delayed in *op/op* mice, which harbor an inactivating mutation in the *csf1* gene that encodes monocyte colony-stimulating factor (M-CSF), leading to loss of macrophages (Kubota et al., 2009).

Expansion of the Lymphatic Vascular Tree by Migration and Proliferation

The receptor tyrosine kinase VEGFR-3 was one of the first lymphatic endothelial markers to be discovered (Alitalo et al., 2005; Karpanen and Alitalo, 2008). VEGFR-3 is activated by VEGF-C and VEGF-D, both members of the VEGF family of growth factors (Achen et al., 2005; Karpanen and Alitalo, 2008). VEGFR-3 can form heterodimers with VEGFR-2 upon binding of the mature, proteolytically processed forms of VEGF-C and VEGF-D, which may lead to unique combinatorial signals by the intracellular domains of the two receptors (Olsson et al., 2006) (Figure 3). VEGFR-3 is present in all endothelia during early stages of development, and *Vegfr3* gene-targeted mice die at around E10.5 due to defective development of the cardiovascular system (Alitalo et al., 2005; Karpanen and Alitalo, 2008). Endothelial cells committed to the lymphatic lineage express high levels of VEGFR-3, and as the lymphatic vascular system begins to develop, VEGFR-3 expression becomes restricted exclusively to LECs with the exception of the fenestrated blood vessels found in endocrine organs such as the thyroid, the adrenal glands, and pancreas (Tammela et al., 2005a).

Whereas VEGF, PlGF, and VEGF-B isoforms are formed through alternative splicing, the different forms of VEGF-C and VEGF-D are the result of proteolytic processing. Both growth factors are produced as precursor proteins, which are activated by intracellular proprotein convertases (Achen et al., 2005; Alitalo et al., 2005; Karpanen and Alitalo, 2008). The secreted, disulphide-linked VEGF-C subunits only bind VEGFR-3, but the factor is further proteolyzed in the extracellular environment by plasmin and other proteases to generate non-disulfide-linked homodimeric proteins with high affinity for both VEGFR-2 and VEGFR-3 (Alitalo et al., 2005; Karpanen and Alitalo, 2008). VEGF-C and VEGF-D both induce proliferation, migration, and survival of endothelial cells (Tammela et al., 2005a).

During development, VEGF-C is expressed predominantly in regions where lymphatic vessels develop (Karkkainen et al., 2004; Karpanen and Alitalo, 2008) (Figure 2C). In mice, *Xenopus* tadpoles, and zebrafish where *Vegfc* has been inactivated, LECs initially differentiate in the embryonic veins but fail to migrate and form the primary lymph sacs (Karkkainen et al., 2004; Kuchler et al., 2006; Yaniv et al., 2006). Homozygous deletion of *Vegfc* leads to the complete absence of a lymphatic vascular system in mouse embryos, and *Vegfc* heterozygous mice display severe lymphatic hypoplasia, indicating an analogous haploinsufficient requirement of VEGF-C for lymphangiogenesis as has been described for VEGF in angiogenesis (Karkkainen et al., 2004). In contrast, deletion of *Vegfd* does not affect development of the lymphatic vasculature in mice (Baldwin et al., 2005; Karkkainen et al., 2004). Interestingly, compound deletion of both *Vegfc* and *Vegfd* fails to recapitulate the early embryonic lethality observed in *Vegfr3* null mice (Haiko et al., 2008), suggesting the existence of as yet unidentified factors capable of activating VEGFR-3.

Transgenic overexpression of a soluble VEGFR-3—immunoglobulin G Fc-domain fusion protein ("VEGF-C/D Trap")—from E14.5 onward in mouse embryos results in severe hypoplasia of the lymphatic vessels (Makinen et al., 2001), indicating that VEGF-C/VEGFR-3 signaling plays a key role in further development of the lymphatic vascular tree following formation of the lymph sacs. Also an endogenous soluble form of VEGFR-2 appears to block VEGF-C signaling in the cornea, which is devoid of lymphatic vessels (Albuquerque et al., 2009). Interestingly, the VEGF-C/D Trap or VEGFR-3 blocking monoclonal antibodies induce regression of the already developed lymphatic vessels during the first 2 postnatal weeks, but lymphatic vessel regrowth is observed at 4 weeks of age despite sustained VEGFR-3 pathway inhibition (Karpanen et al., 2006b). Prolonged inhibition does not affect adult lymphatic vessels (Lin et al., 2005), indicating that VEGF-C/VEGFR-3 signaling is not required for the maintenance of the lymphatic vasculature in adulthood.

The axon guidance receptor neuropilin-2 (NP-2) is expressed in veins and lymphatic vessels, and *Np2* mutant mice have lymphatic capillary hypoplasia (Yuan et al., 2002) (Figure 3D). Interestingly, both VEGF-C and VEGF-D bind to NP-2, which is internalized with VEGFR-3 upon ligand stimulation (Karpanen et al., 2006a). NP-2 does not have enzymatic signaling activity, but NP-2 and VEGFR-3 may collaborate to increase the affinity of LECs toward VEGF-C/D to enable maximal sensing of growth factor gradients (Figure 3). Interestingly, NP-2 blocking antibodies arrest initial lymphatic sprout elongation stimulated by VEGF-C but do not affect further growth of lymphatic vessels (Xu et al., 2010).

Overexpression of VEGF-C or VEGF-D or their VEGFR-3-specific forms in adult tissues stimulates lymphangiogenesis (Karpanen and Alitalo, 2008). Importantly, damaged collecting lymphatic vessels can also be regenerated via lymphatic capillaries undergoing an intrinsic maturation program in response

to VEGF-C or VEGF-D stimulation (Ikomi et al., 2008; Tammela et al., 2007). VEGFR-2 is expressed at low levels in the LECs, and adenoviral or transgenic overexpression of VEGF in the skin induces mainly lymphatic vessel enlargement but very little sprouting (Alitalo et al., 2005; Karpanen and Alitalo, 2008; Wirzenius et al., 2007).

Although fibroblast growth factor 2 (FGF-2), insulin-like growth factor 1 (IGF-1), IGF-2, hepatocyte growth factor (HGF), endothelin-1 (ET-1), and PDGF-B have been reported to induce lymphangiogenesis in various contexts, most of these effects may be secondary to the induction of VEGF-C and VEGF-D in a variety of cell types, such as inflammatory cells and fibroblasts (Alitalo et al., 2005; Karpanen and Alitalo, 2008). Because of other early-onset phenotypes and lethality resulting from global gene targeting of these growth factors, it is not known whether they directly contribute to the development of the lymphatic vascular system.

A recent study using forward genetic screening in the zebrafish identified *ccbe1* as a regulator of lymphatic vessel formation (Hogan et al., 2009). *Ccbe1* encodes a secreted collagen and calcium-binding EGF-domain-1 protein, whose receptor is as yet unidentified. Lymphangioblasts in *ccbe1* gene-targeted fish fail to sprout from the primitive veins and form the parachordal lymphatic precursor, a lumenless single chord of precursor cells acting as a source of LECs; this leads to a complete lack of lymphatic vessels, similar to when *vegfc* is targeted (Hogan et al., 2009) (Figures 2C and 2D). The expression of *ccbe1* overlaps with *vegfc* in the somitic mesoderm (Hogan et al., 2009), suggesting a connection between the two signaling pathways, but further studies, such as the identification of the Ccbe1 receptor, are required to understand its function (Figure 3). Also knockdown of the lysophosphatidic acid (LPA) receptor *lpa1* in zebrafish prevents formation of the thoracic duct, although the mechanism remains unclear, as it is not known whether *lpa1* is expressed in the lymphatic endothelium (Lee et al., 2008).

In adults, lymphangiogenesis occurs physiologically during development of the corpus luteum and wound healing (Alitalo et al., 2005; Otsuki et al., 1986). Lymphatic vessel growth is also associated with a number of pathological conditions, including tumor metastasis, inflammation, and transplant rejection (Achen et al., 2005; Alitalo et al., 2005; Cueni and Detmar, 2008). Adult lymphangiogenesis occurs primarily by sprouting from pre-existing vessels (Alitalo et al., 2005; He et al., 2004, 2005), although bone-marrow-derived cells, such as macrophages, may transdifferentiate into lymphatic endothelium at least in human kidney transplants (Kerjaschki et al., 2004, 2006), in a mouse model of corneal injury (Maruyama et al., 2005), and in murine tumor models (Zumsteg et al., 2009).

Association of Lymphatic Vessels with the Extracellular Matrix

LEC anchorage to the extracellular matrix is dramatically different when compared to BECs, as evidenced by the vestigial basement membrane surrounding LECs. The molecular mechanisms regulating LEC association with the extracellular matrix are largely unknown. The elastic microfibril-associated protein Emilin1 is a component of the anchoring filaments in lymphatic vessels (Danussi et al., 2008). *Emilin1*-deficient mice have hyperplastic and disorganized lymphatic vessels with a reduced number of anchoring filaments and dysfunctional junctions, and they also show impaired lymphatic drainage function (Danussi et al., 2008).

Interestingly, targeted inactivation of integrin α9, which forms heterodimers with integrin β1, causes fatal chylothorax in mice (Huang et al., 2000). Recent exciting work by Bazigou et al. (2009) reveals that endothelial cell-specific deletion of *Itga9* (encoding integrin α9) in mouse embryos results in the formation of dysplastic lymphatic valve leaflets, characterized by disorganized fibronectin matrix, short cusps, and retrograde lymphatic flow. Similar morphological and functional defects are observed in mice lacking the EIIIA domain of fibronectin, which is a ligand for integrin α9 (Bazigou et al., 2009) (Figure 3), indicating that LEC interactions with the extracellular matrix play a key role in lymphatic valve formation.

Disconnection from the Blood Vessels

During the course of development, connections between the lymphatics and veins are lost, except at the sites where lymph mixes with the blood in the subclavian veins (Figure 2E). Mice with homozygous mutations in either the tyrosine kinase *Syk* or its adaptor protein SLP-76 (*Lcp2*) develop arterio-venous shunts and abnormal lymphatico-venous connections (Abtahian et al., 2003). *Syk* and *Slp76* are expressed almost exclusively in hematopoietic cells, suggesting that these cells contribute to the separation of the two vascular systems (Abtahian et al., 2003; Sebzda et al., 2006). Interestingly, genetic ablation of *Syk* causes accumulation of leukocytes that is associated with lymphatic hyperproliferation and lymphatic vessel dilation, ultimately resulting in the formation of blood-lymphatic shunts (F. Kiefer, personal communication). A phenotype that closely resembles the one observed in *Syk* or *Slp76* gene-targeted embryos (Abtahian et al., 2003) occurs in mice deleted of Spred-1 and Spred-2, which suppress the phosphorylation of the MAP kinase ERK following VEGF-C/VEGFR-3 signaling. This results in embryonic lethality by E15.5 with marked subcutaneous hemorrhage, edema, and dilated lymphatic vessels filled with blood (Taniguchi et al., 2007).

Podoplanin (also known as T1α, E11 antigen, gp38, and PA2.26) is a small O- and N-glycosylated transmembrane protein expressed at E11.5 in the cardinal vein and later in Prox1-positive LECs (Breiteneder-Geleff et al., 1999; Schacht et al., 2003). Among other phenotypes, podoplanin gene-targeted mice have dilated and dysfunctional lymphatic vessels, as well as lymphedema (Schacht et al., 2003). Importantly, the separation of the lymphatic vessels from the blood vessels critically involves platelet activation by podoplanin. Platelet aggregates build up in wild-type embryos at the separation zone of podoplanin-positive lymph sacs and cardinal veins but not in *podoplanin* knockout embryos or in embryos where platelet aggregation is inhibited pharmacologically (Uhrin et al., 2010). Podoplanin activates C-type lectin receptor 2 (CLEC-2) in platelets and promotes platelet aggregation with a long lag phase (Suzuki-Inoue et al., 2007). Interestingly, CLEC-2 activation leads to activation of Syk via SLP76 in the platelets (Suzuki-Inoue et al., 2006), indicating a mechanism for the initial steps of blood and lymphatic vessel separation during development (Figures 2E and 3).

Also mice lacking T-synthase, a glycosyltransferase encoded by the gene *C1galt1* that is essential for the biosynthesis of core 1-derived O-glycans, in hematopoietic and endothelial cells display embryonic or neonatal lethality associated with disorganized and blood-filled lymphatic vessels, mosaic expression of LEC markers, and incomplete separation of blood and lymphatic vessels (Fu et al., 2008). Podoplanin expression is dramatically reduced in the lymphatic vessels of *C1galt1* mutant mice, suggesting that O-glycosylation is required for normal podoplanin function (Fu et al., 2008).

Notably, fasting-induced adipose factor (Fiaf, or angiopoietin-like protein 4, Angptl4) appears to regulate the separation of lymphatic and blood vessels specifically in the intestine. *Angptl4* gene-targeted mice develop normally until birth but have blood-filled intestinal lymphatic vessels and decreased Prox1 expression postnatally (Backhed et al., 2007). Interestingly, Angptl4 is an inhibitor of lipoprotein lipase (Sukonina et al., 2006), which points to a connection between the intestinal phenotype and processing of dietary fats.

Remodeling and Maturation of Primitive Lymphatic Vessels

Remodeling of the blood vasculature into arteries, capillaries, and veins is required for the development of a functional blood vessel network. Similarly, remodeling of the lymphatic vasculature includes sprouting of lymphatic capillaries from the primary lymphatic plexus, whereas deeper lymphatic vessels recruit smooth muscle cells and develop lymphatic valves, acquiring a collecting vessel phenotype (Karpanen and Alitalo, 2008; Maby-El Hajjami and Petrova, 2008) (Figure 2G).

The ephrins and their Eph tyrosine kinase receptors have been implicated in repulsive axon guidance in the nervous system and in controlling blood vessel remodeling (Adams, 2002). Mice having a mutant C-terminal PDZ domain of ephrinB2 have normal blood vasculature, but they have hyperplasia of the collecting lymphatic vessels, lack lumenal valve formation, and fail to remodel the primary lymphatic capillary plexus (Makinen et al., 2005).

The angiopoietin (Ang) growth factors bind to the receptor tyrosine kinase Tie2 that is expressed almost exclusively in endothelial cells and regulate interactions between endothelial cells and mural cells (Augustin et al., 2009). Tie2 and its close homolog Tie1 are expressed at low levels in LECs, which express abundant Ang2 levels in both culture conditions and in vivo (Alitalo et al., 2005; Norrmén et al., 2009; Wick et al., 2007) (Figure 3). Interestingly, Tie1 gene-targeted embryos have dysplastic lymph sacs and edema starting from E12.5, indicating that Tie1 may modulate Ang signals in LECs (D'Amico et al., 2009). *Ang2* knockout mice show incomplete regression of hyaloid blood vessels and defective lymphatic vessel maturation, including impaired smooth muscle cell recruitment to the collecting vessels and hypoplastic lymphatic capillaries, suggesting that Ang2 is needed for lymphatic vessel stabilization (Gale et al., 2002). Furthermore, transgenic overexpression of Ang2 in LECs produced a similar collecting vessel phenotype (M. Lohela, G. D'Amico, K.A., et al., unpublished data), indicating a requirement for a delicate balance in Ang/Tie signaling during lymphatic vessel maturation. Notably, replacement of the Ang2 gene with a cDNA encoding Ang1 is sufficient to rescue the lymphatic phenotype but not the blood vascular phenotype (Gale et al., 2002). Overexpression of Ang1, Ang2, and Ang3/Ang4 in adult tissues promotes lymphangiogenic sprouting in several in vivo model systems, with Ang1 being the most potent lymphangiogenic factor (Kim et al., 2007; Morisada et al., 2005; Tammela et al., 2005b).

The forkhead transcription factor FoxC2 is highly expressed in the developing lymphatic vessels as well as in lymphatic valves in adults (Dagenais et al., 2004; Petrova et al., 2004). Although the early development of lymphatic vessels proceeds normally in the absence of *Foxc2*, the collecting lymphatic vessels lack valves, and the lymphatic capillaries acquire an ectopic coverage by basement membrane components and smooth muscle cells (Petrova et al., 2004). This indicates that FoxC2 controls the genetic program responsible for the specification of the lymphatic capillary versus collecting lymphatic vessel phenotype (Figure 3). In a recent study, Norrmén et al. find that, downstream of VEGFR-3, the nuclear factor of activated T cells (NFATc)-1 and FoxC2 cooperatively control the expression of the set of genes required for the differentiation of lymphatic capillaries and valves (Norrmén et al., 2009). Upon further maturation, the collecting lymphatic vessels downregulate FoxC2, which leads to reduced expression levels of Prox1, VEGFR-3, and LYVE-1 (Norrmén et al., 2009). Interestingly, *FoxC2* null mice and mice treated with the NFATc-1 inhibitor cyclosporin A display increased expression of Ang2 in the lymphatic endothelium, pointing to an intriguing link with the phenotype observed in *Ang2* gene-targeted mice (Gale et al., 2002; Norrmén et al., 2009). These observations suggest that Ang2 stimulates smooth muscle cell recruitment to the collecting vessels downstream of NFATc-1 and FoxC2 (Figure 3).

Mice deficient in the vasodilator and diuretic peptide adrenomedullin (AM) or components of its receptor complex (calcitonin receptor-like receptor and receptor activity-modifying protein 2 [RAMP2]) develop edema and embryonic lethality at midgestation (Fritz-Six et al., 2008). Loss of AM signaling results in severely hypoplastic jugular lymph sacs, whereas peripheral lymphatic vessel development is not affected (Fritz-Six et al., 2008). Systemic administration of AM stimulates both lymphangiogenesis and angiogenesis at a site of injury to mouse lymphatic vessels (Jin et al., 2008). However, another study of RAMP2 gene-targeted mice concludes that the embryonic edema observed in these mice is due to hyperpermeable blood vessels (Ichikawa-Shindo et al., 2008).

Apoptosis stimulating protein of p53 (Aspp1) is expressed specifically in endothelial cells during development. *Aspp1* gene-targeted mice have a disorganized and poorly functional cutaneous lymphatic vasculature during embryonic development, but some of the functional capacity of the lymphatic vessels is restored in adult mice (Hirashima et al., 2008). However, *p53* null mice have normal lymphatic vessels, indicating that Aspp1 acts via other mechanisms in LECs (Hirashima et al., 2008). A summary of the molecular pathways contributing to development of the lymphatic vessels is presented in Table S1.

Development of the Lymph Nodes

The first lymph nodes begin to develop as protrusions of connective tissue into the lymph sacs at around E12.5. Lymph node induction is initiated by lymphoid tissue inducer (LTi) cells of hematopoietic origin, which express interleukin-7-receptor-α (IL-7Rα), CD45, and CD4 but lack CD3 (Drayton et al., 2006; Mebius, 2003). These cells differ-

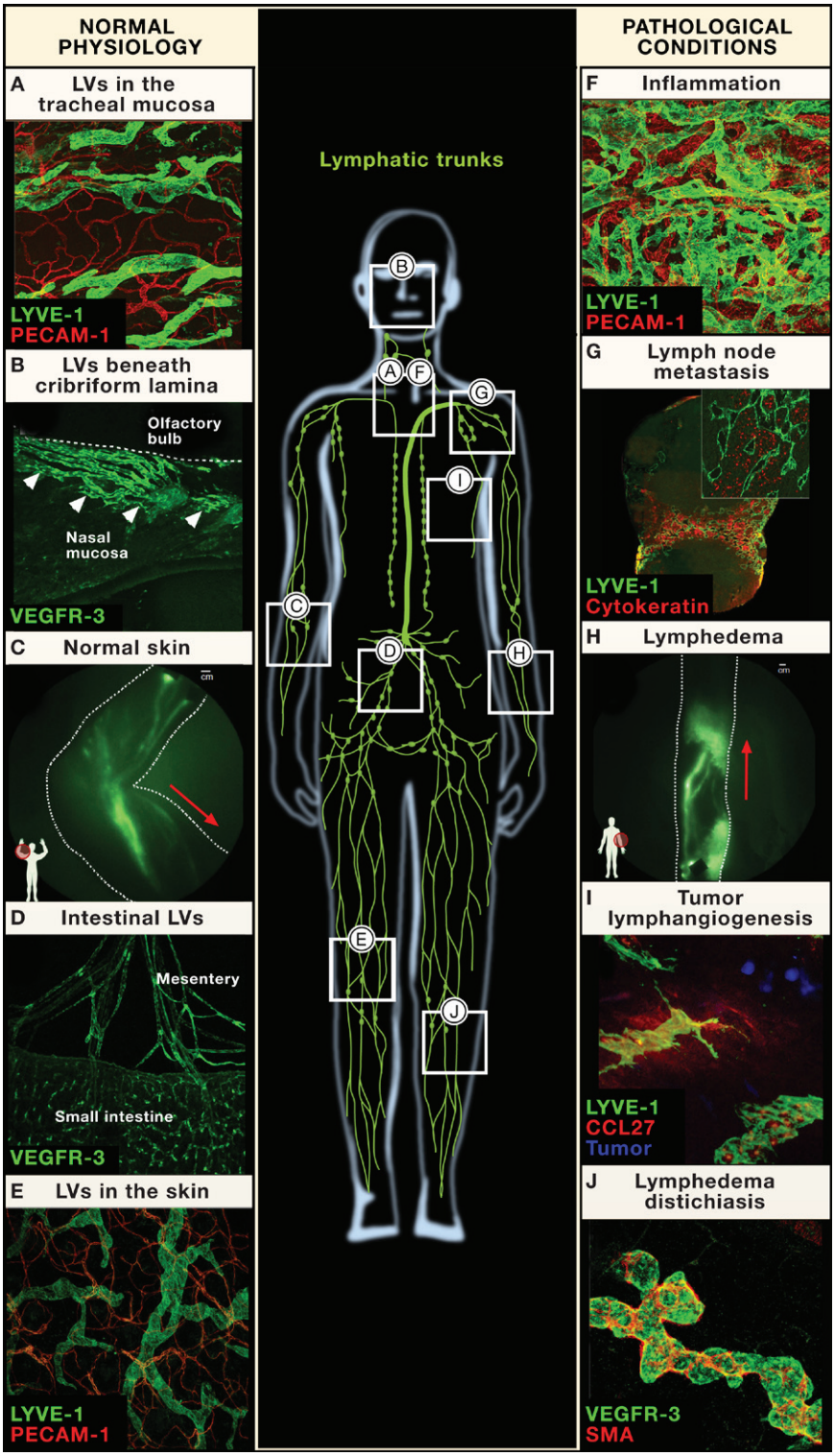

Figure 4. Lymphatic Vessels in Physiological and Pathological Conditions

(A) Lymphatic vessels (LVs, green) and blood vessels (red) in the tracheal mucosa of a mouse. The indentation lacking lymphatic vessels overlies the tracheal cartilage ring.

(B) Sagittal cross-section of a *Vegfr3/LacZ* mouse cranium showing VEGFR-3-positive lymphatic vessels (arrowheads) located anteroventrally from the cribriform lamina and the olfactory bulb (dotted line). These lymphatic vessels have connections with the cerebrospinal fluid system (Johnston et al., 2004).

(C) Live near-infrared fluorescence lymphangiography of the arm (dotted lines) in a healthy human subject. The red arrow indicates direction of lymph flow toward the axilla.

Lymphatic vessels (green) and blood vessels (red) in the mouse small intestine (D) and the ear skin (E).

(F) Hyperplasia of lymphatic vessels (green) and blood vessels (red) in the mouse tracheal mucosa following infection with *Mycoplasma pulmonis* (modified from Baluk et al., 2005).

(G) A mouse lymph node infiltrated by metastatic cells (red) originating from a human tumor xenograft. Note the location of the tumor cells near LYVE-1 (lymphatic vessel hyaluronan receptor-1) positive lymphatic vessels (green).

(H) Live near-infrared lymphangiography of the arm in a patient suffering from lymphedema. Note lymph stagnation and leakage. The red arrow indicates direction of the axilla.

(I) Lymphatic capillary (green) sprouting (arrowheads) and release of the CCL27 chemokine (red) near green fluorescent protein-positive LNM35 tumor cells (blue) in the mouse ear.

(J) Abnormal coverage of a lymphatic capillary (green) with smooth muscle cells (red) in a *FOXC2* gene-targeted mouse, a model of human lymphedema-distichiasis.

Image (A) courtesy of C. Norrmén. Panels (C) and (H) were kindly provided by E. Sevick-Muraca (Rasmussen et al., 2009; Sevick-Muraca et al., 2008). Panel (G) was kindly provided by Y.J. Koh and G.Y. Koh.

entiate from $CD45^-/CD4^-/CD3^-$ precursor cells in response to tumor necrosis factor(TNF)-related activation-induced cytokine (TRANCE) (Drayton et al., 2006; Vondenhoff et al., 2009a). Notably, signaling via IL-7Rα induces the LTi cells to produce lymphotoxin-α1β2 (LTα1β2), a member of the TNF family, and to associate with mesenchymal organizer cells expressing vascular cell adhesion molecule-1 (VCAM-1), the LTβ-receptor (LTβR), TRANCE, and IL-7 (Drayton et al., 2006; Mebius, 2003). Both LTα1β2 and LTβR are absolutely required for lymph node development, which highlights the importance of this signaling pathway in lymphoid organogenesis (Cupedo and Mebius, 2005).

Interestingly, LTα1β2 also induces VEGF-C in the organizer cells, suggesting a possible mechanism for the induction of developmental lymph node lymphangiogenesis (Vondenhoff et al., 2009a). Furthermore, at least in superficial analysis, the lymphatic vessels in mice lacking LTßR are not affected, indicating that the extranodal lymphatic vessel development is not driven via the LTßR pathway (Vondenhoff et al., 2009a). Importantly, the lack of

lymph sacs does not prevent the early steps of lymph node development at least in mice lacking Prox1 (Vondenhoff et al., 2009b).

The lymphoid chemokine CXCL13 made by the LTi cells activates its receptor CXCR5 in an autocrine loop, leading to expression of $\alpha 4\beta 1$ integrin (Drayton et al., 2006; Mebius, 2003). Stromal cell VCAM-1 activates the integrin, resulting in increased expression of adhesion molecules and secreted chemokines, such as CCL19, CCL21, CXCL12, and CXCL13 (Drayton et al., 2006; Mebius, 2003). This leads to the amplification of both the LTi and the stromal cell populations, and presumably also to the differentiation of resident blood vessels into high-endothelial venules (Drayton et al., 2006; Mebius, 2003). The emergence of these vessels allows T and B cells, attracted by the same chemokine signals, to enter the lymph node from the bloodstream (Cupedo and Mebius, 2005). The chemokines are also required for the organization of lymph nodes into B cell follicles that are surrounded by T cell zones (Cupedo and Mebius, 2005; Drayton et al., 2006).

Continuous influx of antigen-presenting cells through the afferent lymphatic vessels is required for the maintenance of organized lymph nodes (Mebius et al., 1991). The lymph nodes are highly plastic organs, but complete lymph nodes do not form after embryogenesis. However, so-called tertiary lymphoid organs consisting of clonally expanding B cell follicles and T cells are commonly found at sites of chronic inflammation. The organization of these structures seems to involve many of the chemokines involved in lymph node development (Drayton et al., 2006; Mebius, 2003). The involvement of lymphangiogenic factors and the mechanisms of recruitment of lymphatic vessels to such structures are beginning to be understood (Furtado et al., 2007).

Lymphatic Vessels and Disease

The lymphatic vessels contribute to a wide range of human pathologies. A summary of their functions in physiological homeostasis and pathological conditions is illustrated in Figure 4.

Inflammation

The lymphatic vessels serve as the principal conduit for soluble antigens and antigen-presenting cells from peripheral tissues to the lymph nodes and other secondary lymphoid organs. They also help to clear other types of leukocytes from sites of resolving inflammation. The LECs in afferent lymphatic vessels attract activated dendritic, T, and B cells expressing the chemokine receptor CCR7 by producing its ligand CCL21 (also known as secondary lymphoid chemokine, SLC) (Forster et al., 2008). Conversely, the CCR10-positive T cells are guided into the afferent vessels by a subpopulation of LECs expressing low levels of podoplanin and CCL21 but high levels of the CCR10 ligand CCL27 and Duffy blood group antigen receptor for chemokines (DARC) (Wick et al., 2008). Leukocyte trafficking in the lymphatic vessels appears to also be regulated by cell adhesion molecules such as CLEVER-1 (common lymphatic endothelial and vascular receptor-1) and Mannose receptor 1 (Salmi and Jalkanen, 2005). An interesting mechanism was recently revealed whereby inflamed lymphatic endothelium is able to attenuate dendritic cell maturation via CD11b interaction with the ICAM-1 receptor in LECs (Podgrabinska et al., 2009).

Sphingosine-1-phosphate (S1P) is a bioactive lipid, synthesized by sphingosine kinases, that is involved in paracrine signaling of BECs with pericytes and inflammatory cells. Lymphocytes express the G protein-coupled S1P receptor S1P1 (also known as EDG-1) and exit from the lymph nodes to the lymph following a gradient of S1P, likely produced by LECs (Ledgerwood et al., 2008; Schwab and Cyster, 2007). S1P1 is expressed in LECs, and S1P stimulates lymphangiogenesis in vitro as well as in a mouse model (Yoon et al., 2008; Pham et al., 2009). Interestingly, LEC-specific deletion of sphingosine kinase 1 in a sphingosine kinase 2 gene-targeted background inhibits the formation of the button-like junctions in lymphatic capillaries in mice (Pham et al., 2009), suggesting that S1P regulates lymphatic vessel maturation either directly or by lymphocyte-mediated mechanisms.

Antigen-presenting cells such as dendritic cells first reach the subcapsular sinus and then move into the paracortex where they aggregate around high-endothelial venules (Alvarez et al., 2008). This localization allows newly arrived dendritic cells to present recently acquired peripheral antigens to both B and T cells that constantly enter the lymph node across the high-endothelial venules from the blood (Mempel et al., 2004; Qi et al., 2006). However, antigens do not necessarily require antigen-presenting cells to reach the lymph nodes. Elegant studies employing intravital microscopy have elucidated that small soluble antigens reach the subcapsular sinus of mouse popliteal lymph nodes within minutes following subcutaneous injection into the footpad (Roozendaal et al., 2008). Interestingly, extracellular matrix conduits that extend from the subcapsular sinus into the B cell follicles enable rapid delivery of the small antigens to cognate B cells and follicular dendritic cells (Roozendaal et al., 2008). Larger soluble antigens can also reach the lymph nodes with the lymph, but they are captured by resident dendritic cells and macrophages in the cortical region to initiate early T cell priming events already within the first four hours following antigenic challenge (Alvarez et al., 2008). However, it appears that the second wave of antigen-bearing dendritic cells from the skin is required for inducing maximal lymphocyte responses (Alvarez et al., 2008).

Mast cells in the inflamed peripheral tissues were recently demonstrated to signal to the draining lymph nodes by releasing stable submicrometer heparin-based particles that contain TNF-α and other proteins (Kunder et al., 2009). The complexes are taken up by the lymphatic vessels and transported to the draining lymph nodes, enabling rapid delivery of proinflammatory signaling molecules in concentrated and stable form (Kunder et al., 2009). It is conceivable that similar cytokine packages are also released by other cell types into the lymph.

Lymphangiogenesis typically occurs at sites of tissue inflammation, for example in immunization and in bacterial infection (Alitalo et al., 2005; Baluk et al., 2005). Accordingly, VEGF-C is induced in response to proinflammatory cytokines such as TNF-α in several cell types, presumably via the activation of the NF-κB pathway (Alitalo et al., 2005; Baluk et al., 2009). Lymphangiogenesis at sites of inflammatory insults is induced by macrophages and granulocytes, which produce ample amounts of VEGF-C and VEGF-D (Alitalo et al., 2005; Baluk et al., 2005). Blocking these factors with the soluble VEGFR-

3-Ig ligand trap suppresses lymphangiogenesis and reactive lymphadenitis and exacerbates pulmonary edema in a mouse model of *Mycoplasma pulmonis* infection (Baluk et al., 2005). Interestingly, lymphangiogenesis is greatly attenuated in T or B cell-deficient mice in the *M. pulmonis* model, possibly due to reduced VEGF-D and FGF-2 expression in the inflamed airways (Aurora et al., 2005). Blocking integrin $\alpha5\beta1$ signaling with small-molecule inhibitors also suppresses lymphangiogenesis in this model presumably by directly inhibiting LEC proliferation and migration (Okazaki et al., 2009).

The lymphatic vascular system and the molecular pathways regulating inflammatory responses are intimately associated, and LECs at least in some tissues constitutively express NF-κB (Saban et al., 2004). Activation of the NF-κB pathway in LECs upregulates Prox1 and VEGFR-3, which renders the lymphatic vessels more sensitive to VEGF-C and VEGF-D produced by leukocytes (Flister et al., 2009). NF-κB is activated for example downstream of Toll-like receptor 4 binding to lipopolysaccharide in the LECs, which induces activation and production of leukocyte chemoattractants such as CCL2, CCL5, and CX3CL1 to promote leukocyte homing to the lymphatic vessels and eventually to the draining lymph node (Kang et al., 2009). Lymphangiogenesis in inflammatory settings facilitates the resolution of tissue edema and enhances immune responses by promoting macrophage and dendritic cell mobilization (Baluk et al., 2005; Kataru et al., 2009). Notably, lymphangiogenesis is not observed in atherosclerotic lesions in human coronary arteries despite prolonged inflammation and upregulation of VEGF-C in the atherosclerotic lesions (Nakano et al., 2005). This lack of leukocyte exit routes suggests one reason for the accumulation of the so-called foam cells in the atherosclerotic intima.

Lymphangiogenesis (sinusoidal hyperplasia) is also observed in lymph nodes that drain inflamed tissues (Angeli et al., 2006). According to Angeli et al., lymphangiogenesis is stimulated by VEGF produced by the follicular B cells in the lymph nodes (Angeli et al., 2006). Interestingly, T cells appear to antagonize B cell-driven lymphangiogenesis in the lymph node via interferon-γ signals, suggesting a mechanism for regression of inflammation-induced lymphatic vessels during resorption of immune responses (R. Kataru and G.-Y. Koh, personal communication). Also, transforming growth factor β (TGF-β) signaling inhibits the proliferation and migration of cultured human LECs as well as lymphangiogenesis in inflammatory settings and tumors (Clavin et al., 2008; Oka et al., 2008). Conversely, lymphangiogenesis induced by VEGF-C is potentiated in the presence of a TGF-β receptor inhibitor in vivo (Oka et al., 2008). These findings are in line with the generally anti-inflammatory role of TGF-β (Shull et al., 1992).

Lymphangiogenesis is also associated with chronic inflammation, such as psoriasis or rheumatoid arthritis (Alitalo et al., 2005; Kajiya and Detmar, 2006; Kunstfeld et al., 2004). Elegant studies of human kidney transplants show that transplant rejection is frequently associated with lymphangiogenesis, and that CCL21 produced by host-derived lymphatic vessels in the transplant attracts CCR7-expressing dendritic cells (Kerjaschki et al., 2004), which elicit primary alloantigen recognition events in the lymph nodes that drain the graft. Interestingly, blocking VEGFR-3 reduces CCL21 in the allograft LECs and suppresses adaptive immune responses toward heart transplants in a mouse model (A. Nykänen, H. Sandelin, K. Lemström, K.A., et al., unpublished data). These studies indicate that blocking lymphangiogenesis and associated dendritic cell migration provide an effective anti-inflammatory mode of therapy for the prevention of auto- and alloimmunization.

Tumor Metastasis

Regional lymph node metastasis represents the first step of tumor dissemination for a variety of common human cancers, such as carcinomas of the breast, colon, and prostate as well as melanoma (Achen et al., 2005; Karpanen and Alitalo, 2008). Currently it is not clear if tumor cells are trapped by lymph nodes, resulting in a lag phase in tumor dissemination, if lymph node metastases just serve as evidence of tumor cell transit through the lymph node, or whether the lymph nodes promote spreading of the tumor cells by amplifying the tumor and by serving as a launch pad for further systemic metastasis (Joyce and Pollard, 2009; Sleeman and Thiele, 2009). The extent of lymph node metastasis is a major determinant for the staging and the prognosis of most human malignancies, which often guides therapeutic decisions. Despite this, the molecular mechanisms of lymphatic metastasis are incompletely understood.

It is known that tumor cells enter the lymphatic vasculature by invading pre-existing lymphatic vessels in the tumor periphery or by eliciting lymphangiogenesis via growth factor production (Achen et al., 2005; Alitalo et al., 2005; Tobler and Detmar, 2006). The density of lymphatic vessels correlates with the incidence of lymph node metastasis and poor prognosis in some human cancers (Achen et al., 2005; Kyzas et al., 2005). However, mechanistic studies have elucidated that intratumoral lymphatic vessels may be poorly functional due to high intratumoral pressure and not required for lymphatic metastasis (Padera et al., 2002; Wong et al., 2005). Conversely, lymphatic vessels in the tumor periphery are functional and can drain colloids from the tumor (Achen et al., 2005; Alitalo et al., 2005; He et al., 2005; Padera et al., 2002; Tobler and Detmar, 2006). Furthermore, lymphangiogenic growth factors produced by tumor cells and tumor-associated macrophages stimulate growth and dilation of the peritumoral lymphatic vessels, as well as facilitate tumor cell entry through the lymphatic endothelium (Alitalo et al., 2005; Joyce and Pollard, 2009). Recent elegant work by Giampieri et al. shows that blocking TGF-β signaling prevents breast cancer cells from moving singly in vivo but does not inhibit collective movement in clusters. Interestingly the cells restricted to collective invasion are capable of lymphatic invasion but not blood-borne metastasis (Giampieri et al., 2009).

According to a study utilizing anti-colony stimulating factor-1 (CSF-1) antibodies and small-molecule CSFR-1 receptor inhibitors, macrophages are involved in tumor lymphangiogenesis and metastasis (Kubota et al., 2009). Also other cells within the tumor stroma likely contribute to lymphangiogenesis induced by tumors. For instance, tumor-associated fibroblasts producing high levels of hyaluronan promoted tumor lymphangiogenesis, presumably by stimulating malignant cells to secrete specific lymphangiogenic factors (Koyama et

al., 2008). On the other hand, LECs produce cytokines that can guide the tumor cells toward the lymphatic vessels utilizing similar mechanisms as when attracting leukocytes. For example, CCL21 produced by the LECs appears to attract some tumor cells that express its receptor CCR7 (Issa et al., 2009; Shields et al., 2007) (Figure 4I).

VEGF is essential for tumor angiogenesis, and monoclonal antibodies targeting this factor have been approved for clinical use in the treatment of several human tumors (Kowanetz and Ferrara, 2006). Overexpression of VEGF has also been shown to promote tumor lymphangiogenesis and lymph node metastasis in experimental models, but it is difficult to assess how direct this activity is toward the lymphatic endothelium, as VEGF effectively recruits VEGFR-1-expressing macrophages that can produce VEGF-C and VEGF-D (Hirakawa et al., 2005). Furthermore, blocking VEGFR-2, the key angiogenesis pathway, inhibits tumor growth and angiogenesis but not lymph node metastasis in a prostate tumor model (Burton et al., 2008). Nevertheless, a combination of small-interfering RNA (siRNA) silencing both VEGF-C and VEGF-A is more effective in suppressing both lymph node and lung metastases in a mouse-immunocompetent mammary cancer model than knockdown of either factor alone (Shibata et al., 2008).

Forced expression of VEGF-C or VEGF-D in xenografts and in transgenic tumors results in tumor lymphangiogenesis and increased tumor dissemination to regional lymph nodes (Achen et al., 2005; Alitalo et al., 2005; Tobler and Detmar, 2006). This applies even to tumors that normally do not metastasize to the lymph nodes (Achen et al., 2005; Alitalo et al., 2005; Tobler and Detmar, 2006). Conversely, inhibiting the VEGFR-3 pathway either by the VEGF-C/D Trap or VEGFR-3 blocking antibodies suppresses approximately 60%–70% of lymph node metastasis in a variety of experimental tumor models (Achen et al., 2005; Alitalo et al., 2005; Burton et al., 2008; He et al., 2005; Lin et al., 2005; Roberts et al., 2006; Tobler and Detmar, 2006). Also NP-2 blocking antibodies have recently been shown to reduce tumor lymphangiogenesis and lymph node metastasis at least partly by suppressing LEC migration (Caunt et al., 2008).

Studies of human cancers have shown a direct correlation between VEGF-C and VEGF-D expression and lymphatic invasion, lymph node and distant organ metastasis, and poor prognosis of survival, but not with the density of tumor-associated lymphatic vessels (reviewed in Achen et al., 2005; Alitalo et al., 2005; Tobler and Detmar, 2006). This observation suggests that VEGF-C and VEGF-D qualitatively modulate the lymphatic vasculature to promote tumor metastasis. Indeed, studies in mouse models utilizing high-resolution imaging demonstrate that the lymphangiogenic sprouting stimulated by VEGF-C results in the formation of intercellular gaps (Tammela et al., 2007), which facilitate tumor cell entry into the lymphatic vessels (Alitalo et al., 2005; He et al., 2005). VEGF-C also promotes circumferential enlargement of the collecting vessels, leading to increased lymph flow and transport of tumor cells, as well as accommodation of larger tumor cell clusters (Alitalo et al., 2005; He et al., 2005; Hoshida et al., 2006; Ruddell et al., 2008; T.T. et al., unpublished data). Importantly, the collecting lymphatic vessels can also act as host tissue for secondary tumor formation at least in experimental models (T.T. et al., unpublished data).

Furthermore, lymphangiogenic growth factors produced by the primary tumor appear to act at a distance by inducing lymphangiogenesis (sinusoidal hyperplasia) in the sentinel lymph node even before the arrival of the first metastatic cells (Harrell et al., 2007; Hirakawa et al., 2005, 2007; Kozlowski and Hrabowska, 1975). It has been suggested that in this way, the tumor cells prepare the "soil" in the lymph node beforehand to render it more hospitable for secondary tumor formation (Tobler and Detmar, 2006). An elegant study utilizing noninvasive imaging suggests that lymph node lymphangiogenesis and increased lymph flow through the tumor-draining lymph nodes are B cell dependent, as in inflammatory conditions (Harrell et al., 2007).

Lymphatic Insufficiency

Impairment of lymphatic transport capacity due to abnormal vessel development or obstruction or obliteration of the lymphatic vessels causes stagnation of proteins and associated water in the interstitium, resulting in lymphedema, usually a progressive and lifelong condition for which curative treatments are not available at present. The protein-rich interstitial fluid initiates a persistent inflammatory response, leading to fibrosis, impaired immune responses, and accumulation of subcutaneous fat. Lymphedema is classified into primary (congenital) lymphedema and secondary (acquired) lymphedema, based on the mechanism of pathogenesis (Warren et al., 2007).

Although primary lymphedema is a rare condition, identification of the underlying genetic causes has provided valuable insight into the molecular mechanisms regulating the development and function of the lymphatic vasculature. Heterozygous tyrosine kinase-inactivating missense point mutations of the *VEGFR3* gene have been identified as a major cause of Milroy disease (OMIM #153100), a form of lymphedema due to hypoplasia of lymphatic capillaries and typically present at birth (Alitalo et al., 2005; Connell et al., 2009; Karpanen and Alitalo, 2008; Tammela et al., 2007). Mutations in the transcription factor *FOXC2* have been linked to lymphedema-distichiasis (LD, OMIM #153400), characterized by late-onset lymphedema, a double row of eyelashes, and varicose veins (Maby-El Hajjami and Petrova, 2008; Mellor et al., 2007). Analysis of *Foxc2* mutant mice reveals that LD is due to ectopic smooth muscle cell and basement membrane coverage of the lymphatic capillaries and loss of valves in the collecting vessels (Figure 4J); similar defects are also observed in samples obtained from LD patients (Petrova et al., 2004). Heterozygous missense mutations in *ITGA9* encoding for α9 integrin underlie congenital chylothorax in human fetuses (Ma et al., 2008), recapitulating the results obtained by gene targeting in mice (Bazigou et al., 2009; Huang et al., 2000).

Dominant-negative mutations of the homeobox transcription factor *SOX18* have been linked with hypotrichosis-lymphedema-telangiectasia syndrome (HLTS, OMIM #607823) (Irrthum et al., 2003). A similar phenotype involving reduced hair, blood vessel ruptures, and lymphatic vessel malformations is observed in so-called *Ragged* spontaneous mutant mice, which harbor a single-base frameshift mutation in *SOX18* that abolishes transcriptional transactivation (Francois et al., 2008; Pennisi et al., 2000). Finally, mutations in the NF-κB regulatory protein *NEMO* associate with a rare and complex syndrome

involving lymphedema (anhidrotic ectodermal dysplasia with immunodeficiency, osteopetrosis, and lymphedema, OL-EDA-ID, OMIM #300301) (Döffinger et al., 2001; Saban et al., 2004). Interestingly, mutations in human collagen and calcium-binding protein-1 (*CCBE1)* were recently found to underlie lymphatic vessel dysplasia in primary lymphedema patients (Alders et al., 2009), suggesting a conserved function for this gene in the regulation of lymphatic vessel development from zebrafish to mammals (Hogan et al., 2009).

Over 99% of lymphedema cases worldwide are secondary to acquired damage to the lymphatic vessels (Radhakrishnan and Rockson, 2008; Warren et al., 2007). Filariasis (elephantiasis) is an infection of the lymphatics by the parasitic worms *Wuchereria bancrofti*, *Brugia malayi*, or *Brugia timori*, which leads to obstruction and scarring of lymphatic vessels and chronic lymphedema of the lower limbs or genital organs. The onset of filariasis is triggered by a filarial-specific inflammatory reaction that stimulates the production of VEGF, VEGF-C, and VEGF-D, which leads to persistent hyperplasia of the lymphatic vessels (Pfarr et al., 2009). Interestingly, individuals with genetically attenuated immune responses to the filaria appear to develop markedly milder forms of the disease (Pfarr et al., 2009). Filariasis is the principal cause of lymphedema worldwide, affecting approximately 100 million people (Hoerauf, 2006; Pfarr et al., 2009).

Breast cancer surgery is the leading cause for secondary lymphedema in industrialized countries (Alitalo et al., 2005; Radhakrishnan and Rockson, 2008). The metastatic tumor cells that frequently spread to the lymph nodes necessitate radical surgery and radiotherapy, which destroy the lymphatic vessel network and lead to impairment of afferent lymphatic flow (Alitalo et al., 2005; Radhakrishnan and Rockson, 2008; Warren et al., 2007). For example, approximately 20%–30% of patients that have undergone radical axillary lymph node dissection develop lymphedema of the upper limb later on (Clark et al., 2005; Warren et al., 2007). The predisposition to develop post-lymphadenectomy lymphedema appears to depend on genetic factors, but susceptibility genes have thus far not been identified (Radhakrishnan and Rockson, 2008; Warren et al., 2007). Damage to the lymphatics may also result from bacterial infections of the skin (such as erysipelas) or the lymphatic vessels (lymphangitis), which are typically caused by streptococcal or staphylococcal infections, respectively (Radhakrishnan and Rockson, 2008; Warren et al., 2007).

Unlike primary lymphedema, secondary lymphedema is typically due to damage to the collecting lymphatic vessels. Spontaneous recanalization of collecting vessels may occur in minimal lesions, but formation of new lymphatic vessels is typically not observed in lymphedema patients, although pre-existing vessels dilate to accommodate the increased fluid (Ikomi et al., 2006; Stanton et al., 2009; Tabibiazar et al., 2006). Hypoxia is a ubiquitous stimulus for the initiation of angiogenesis, but it is not known if, analogously, intrinsic edema-induced mechanisms for engaging lymphangiogenic gene expression programs exist.

The treatment of lymphedema is currently based on physiotherapy, compression garments, liposuction, and occasionally surgery (Radhakrishnan and Rockson, 2008; Warren et al., 2007), but means to reconstitute the collecting lymphatic vessels and cure the condition are rarely successful (Baumeister et al., 1981; Becker et al., 2006). VEGF-C gene transfer via adenoviruses, adeno-associated viruses, or naked plasmids, as well as the application of recombinant VEGF-C protein stimulate the formation of new lymphatic capillaries and alleviate edema in preclinical animal models of lymphedema (Alitalo et al., 2005), pointing to possibilities to restore lymphatic vessels in lymphedema patients.

As a therapeutic advance, we recently showed that lymphatic capillaries induced by adenoviral VEGF-C or VEGF-D expression mature into collecting vessels in a mouse model of axillary lymph node dissection (Tammela et al., 2007). Recombinant VEGF-C has also been shown to promote collecting vessel formation after removal of popliteal lymph nodes (Ikomi et al., 2008). There are few known molecular players that regulate lymphatic vessel maturation in adult tissues. Whether the lymphatic vessels mature or not appears to be tissue specific, as lymphatic capillaries induced by VEGF-C do not acquire all hallmarks of collecting vessels in mouse ear (T.T. et al., unpublished data). Therefore it is likely that lymph flow in the nascent vessels contributes to the remodeling (Ng et al., 2004). Notably, VEGF-C is also a chemoattractant for monocytes/macrophages, and these cells may also play a role in lymphatic vessel maturation (Saaristo et al., 2006; Skobe et al., 2001). Prolonged VEGF-C stimulation may also directly promote LEC differentiation.

In order to comprehensively restore the anatomy of the axilla following surgery, combined VEGF-C therapy with lymph node transplantation was applied in mice (Tammela et al., 2007). The lymph nodes transduced with adenoviral VEGF-C survive, form connections with the pre-existing lymphatic vessel network, and can trap metastatic tumor cells, whereas the majority of control-treated nodes regress, indicating that VEGF-C therapy can improve the success rate of lymph node transplantation (Becker et al., 2006; Tammela et al., 2007). Importantly, the combination of VEGF-C gene transfer and lymph node transplantation also appears successful in a porcine model, indicating the feasibility of such an approach for humans (M. Lähteenvuo, T. Tervala, A. Saaristo, K.A., et al., unpublished data).

Interestingly, artificial lymph nodes composed of collagen scaffolds have been shown to attract lymphocytes, which elicit immune responses in mouse models, suggesting an approach for the replacement of damaged lymph nodes or for the augmentation of regional immune responses (Okamoto et al., 2007). This approach and VEGF-C/D therapy should be an attractive combination for the future.

Obesity and Fat Metabolism

The lymphatic vessels are essential for the adsorption of hydrophobic nutrients, such as triglycerides, cholesterol, and other lipid molecules from the small intestine. The enterocytes lining the intestinal villi package these molecules into water-soluble chylomicrons 75 to 1200 nm in diameter and secrete them to the ablumenal side. The chylomicrons are taken up by a centrally located lymphatic vessel in the villus, called the lacteal due to the milky appearance of the lipid-rich intestinal lymph. The lacteal vessels connect with larger mesenterial collecting vessels. In fact, the first mention of lymphatic vessels in the literature dates from 1627 when the Italian anatomist Gasparo Aselli observed “milky veins” (lacteis venis) in the mesentery of a dog that had just been fed.

Prox1 is required for the maintenance of LECs. *Prox1* heterozygous mice that survive until adulthood, as well as mice with an endothelial-specific deletion of *Prox1*, develop late-onset chylous ascites and obesity, indicating a link between impaired lymph drainage and tissue adiposity (Harvey et al., 2005). Interestingly, human and mouse lymphedema are also characterized by the accumulation of fat in peripheral tissues such as the subcutaneous space (Alitalo et al., 2005; Radhakrishnan and Rockson, 2008; Warren et al., 2007). These observations suggest that the cutaneous lymphatic vessels may be required for lipid metabolism or mobilization of tissue fat reserves much like in the small intestine. Alternatively, lymph may contain adipogenic factors, which need to be cleared by the lymphatic vessels in order to maintain tissue architecture (Harvey, 2008).

Hypertension

As mentioned above, the lymphatic vessels are specialized for the uptake of fluid and macromolecules from the interstitium, which is essential for the maintenance of tissue fluid homeostasis. Recent exciting work by Machnik et al. suggests that the lymphatic vessels proliferate in response to a high-salt diet, possibly via upregulation of VEGF-C in monocytic cells infiltrating the interstitium of the skin (Machnik et al., 2009). Blocking lymphangiogenesis with VEGFR-3-Ig appears to increase the blood pressure of the salt-loaded, but not control mice, suggesting that the lymphatic vessel hyperplasia is required in the regulation of salt storage to the interstitium. On the other hand, VEGF-C can also lower the blood pressure directly via a VEGFR-2/eNOS-mediated mechanism (Machnik et al., 2009; T.T. and G. Zarkada, unpublished data). At least tonicity-responsive enhancer binding protein (TonEBP) and lens epithelium-derived growth factor (LEDGF/p75) stimulate VEGF-C transcription in conditions of cellular stress (Cohen et al., 2009; Machnik et al., 2009). Further work should clarify if these observations can lead to additional modalities of blood pressure control.

Conclusions

Because of the specific functions of the lymphatic vessels in the maintenance of tissue fluid balance, immunosurveillance, and the uptake of dietary fats, they differ greatly from the blood vessels in their structure, their function, and the molecular mechanisms that regulate their development and growth. Considerable progress has been made in the elucidation of these mechanisms during the past years, mainly because of the use of molecular genetic analyses in mouse, zebrafish, and *Xenopus* as well as comparison of LECs and BECs in cell biological and transcriptional studies. The lymphatic vascular system actively facilitates tumor metastasis, as well as the elicitation of auto- and alloimmune responses. Inhibiting lymphangiogenesis in such settings appears to be a promising therapeutic avenue. Conversely, stimulation of lymphatic vessel growth with factors such as VEGF-C or VEGF-D has become a realistic treatment modality for patients suffering from lymphatic insufficiency or tissue edema. Such progress should provide clinicians with therapeutic targeting of the lymphatic system in the treatment of an expanding spectrum of human pathologies.

Supplemental Information

Supplemental Information includes one figure and one table and can be found with this article online at 10.1016/j.cell.2010.01.045.

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