

# Lymphangiogenesis in development and human disease

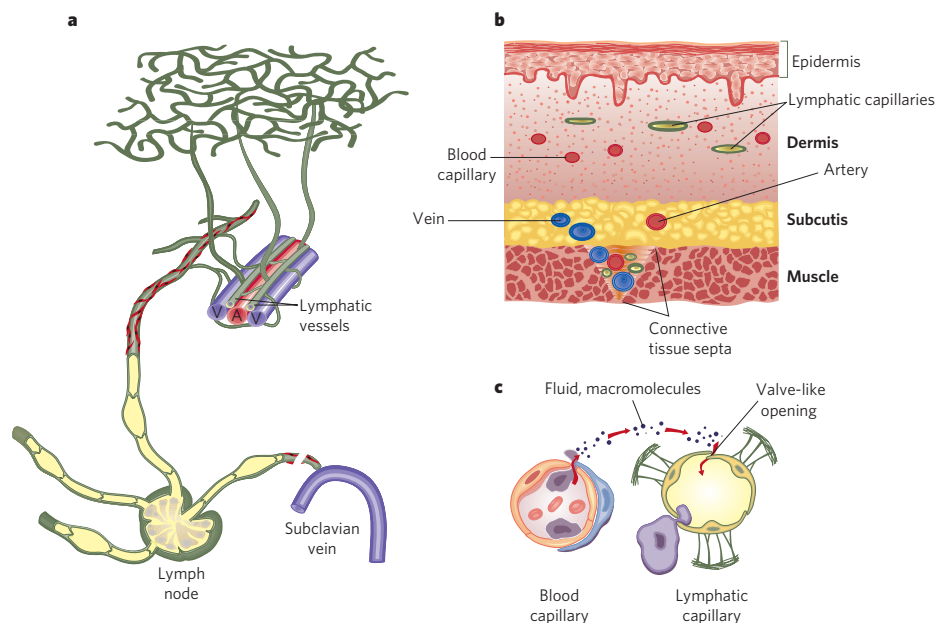
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**The lymphatic vasculature forms a vessel network that drains interstitial fluid from tissues and returns it to the blood. Lymphatic vessels are also an essential part of the body's immune defence. They have an important role in the pathogenesis of several diseases, such as cancer, lymphoedema and various inflammatory conditions. Recent biological and technological developments in lymphatic vascular biology will lead to a better understanding and treatment of these diseases.**

Oxygen, nutrients and hormones are delivered to tissues by blood vessels, and capillaries are involved in the molecular exchange of these compounds with the surrounding tissues. Blood pressure causes plasma to leak continuously from the capillaries into the interstitial space. The main function of the lymphatic vasculature is to return this protein-rich fluid back to the circulating blood. Fluid, macromolecules and cells, such as extravasated leukocytes and activated antigen-presenting cells, enter the blind-ended lymphatic capillaries. From here, lymph is transported towards collecting lymphatic vessels and is returned to the blood circulation through the lymphatico-venous junctions in the jugular area (Fig. 1a, b). On its way, lymph is filtered through the lymph nodes, where foreign particles taken up by antigen-presenting cells are used to initiate specific immune responses. In the small intestine, lacteal lymphatic vessels inside the intestinal villi

absorb the dietary fat released by enterocytes in the form of lipid particles called chylomicrons. Lymphatic capillaries are present in the skin and in most internal organs, with the exception of the central nervous system, bone marrow and avascular tissues such as cartilage, cornea and epidermis. The lymphatic vascular system is a characteristic feature of higher vertebrates, whose complex cardiovascular system and large body size require the presence of a secondary vascular system for the maintenance of fluid balance (Box 1).

The lymphatic capillaries are thin-walled, relatively large vessels, composed of a single layer of endothelial cells. Lymphatic capillaries are not ensheathed by pericytes or smooth muscle cells, and have little or no basement membrane (Fig. 1c). Collecting lymphatic vessels have a smooth muscle cell layer, basement membrane and valves. The contraction of smooth muscle cells, and surrounding skeletal muscles, as



**Figure 1 | Organization of lymphatic vasculature. a**, Interstitial fluid, collected by the initial lymphatic capillary plexus, is transported by pre-collector lymphatic vessels to larger collecting lymphatic vessels and returned to the circulation through the thoracic duct. Collecting lymphatic vessels have smooth muscle cell coverage (red) and luminal valves to propel and maintain unidirectional lymph flow. Deep lymphatic vessels run along arteries and veins. **b**, Schematic cross-section of skin, showing the relative positions of blood and lymphatic vessels. **c**, Mechanism of interstitial tissue fluid uptake by a lymphatic capillary. Plasma components, extravasated white blood cells and particulate matter, such as bacteria, enter the lymphatic vessels through loose valve-like openings. Lymphatic vessels are linked to the extracellular matrix by anchoring filaments. The latter are very thin (4–10 nm) fibrillin-containing filaments, which are inserted into the endothelial cell plasma membrane. Anchoring filaments prevent vessel collapse in conditions of high interstitial pressure.

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**Box 1 | Evolutionary aspects of lymphatic vascular development**

Molluscs and arthropods have an open circulatory system, which combines both blood and lymphatic functions. Blood (or haemolymph) freely diffuses to the tissues to distribute oxygen, nutrients and to collect metabolic wastes. Vertebrates and some invertebrates have closed circulatory systems, where blood is always contained within vessels. With increasing animal size and associated complexity of the cardiovascular system, an additional mechanism is necessary to clear the tissue of the proteins, microbes and other substances that blood vessels are unable to absorb. Lymphatic vessels are present in amphibia and reptiles. An interesting feature of the lymphatic system in several of these animals is the presence of enlarged, rhythmically contracting portions of lymphatic vessels called lymph hearts. Lymph hearts contain valves and are necessary to propel lymph and to prevent lymph backflow. Recently, the *Xenopus* tadpole has become the system of choice for the developmental genetic analysis of lymphangiogenesis. For example, prox-1-GFP transgenic tadpoles can be used to visualize lymphatic vessels *in vivo*, and morpholino oligonucleotides can be used to downregulate lymphangiogenic genes (P. Carmeliet, personal communication; ref. 96). In birds and amphibians, Prox1-expressing lymphangioblasts also develop independently in the mesenchyme<sup>96,97</sup>. However, it is not known whether similar precursors exist in mammals. At least in mouse xenograft experiments, very few, if any, bone-marrow-derived cells are incorporated into growing lymphatic vessels<sup>98</sup>. Lymph nodes are present only in mammals and some aquatic birds; their number is significantly lower in birds.

well as arterial pulsations, contribute to lymph propulsion, and valves prevent backflow.

Lymphatic vessels were first described in the beginning of the seventeenth century; however, the first growth factors and molecular markers specific for these vessels were discovered only ten years ago. In retrospect, this may seem surprising, given the well-known importance of the lymphatic system in maintaining the fluid balance in the body, and its involvement in the pathogenesis of many diseases, including cancer. Recent developments in lymphatic vascular biology research include the discovery of lymphangiogenic factors, identification of lymphatic vascular markers, isolation of lymphatic endothelial cells and the development of animal models to study lymphangiogenesis. The molecular mechanisms of lymphatic growth and development have been recently reviewed<sup>1-3</sup>. In this review, we summarize the recent progress in this fast-growing field of vascular biology with particular emphasis on the understanding and management of lymphatic dysfunction, inflammation and tumour metastasis.

**Mechanisms of lymphangiogenesis**

Studies over the past ten years have revealed a signal-transduction system for lymphatic endothelial cell growth, migration and survival. This system is formed by vascular endothelial growth factors (VEGF) C and D and their receptor VEGFR-3 (Fig. 2a, b)<sup>4-8</sup>. VEGF-C and VEGF-D also bind to neuropilin-2 (Nrp2), a semaphorin receptor in the nervous system that is also expressed in lymphatic capillaries<sup>9</sup>. Consistent with these findings, Nrp2-deficient mice have lymphatic hypoplasia<sup>10</sup>. Proteolytically processed VEGF-C and VEGF-D also activate VEGFR-2 and can induce blood-vessel growth<sup>5,11-14</sup>. Conversely, VEGF-A, which binds to VEGFR-2, can induce lymphatic hyperplasia but cannot substitute for VEGF-C in lymphatic development<sup>15,16</sup>. By contrast, in the chick chorioallantoic membrane and in a mouse insulinoma tumour model, VEGF-A stimulates only angiogenesis<sup>17,18</sup>. At least some of the effects of VEGF-A on lymphatic vessels may be secondary to the induction of vascular hyperpermeability and to the recruitment of the inflammatory cells that produce VEGF-C and VEGF-D<sup>19,20</sup>.

The recent identification of co-receptors and novel signalling complexes for lymphangiogenic signalling suggests a greater complexity than previously thought. *In vitro* studies show that upon binding to matrix fibronectin,  $\beta$ 1 integrin interacts with VEGFR-3 and induces weak activation of its tyrosine kinase<sup>21</sup>. Integrin  $\alpha$ 9 binds VEGF-C and inactivation of *Itga9* causes chylothorax in mice<sup>22,23</sup>. Furthermore,

Kaposi sarcoma herpesvirus envelope glycoprotein gB interacts with VEGFR-3 and  $\alpha$ 3 $\beta$ 1 integrin and can activate both, resulting in increased endothelial cell growth and migration<sup>24</sup> (Box 2; Fig. 2b). In addition to the two VEGF family members, fibroblast growth factor 2, platelet-derived growth factor B and hepatocyte growth factor stimulate lymphatic vessel growth<sup>25-27</sup>.

**Mechanisms of embryonic and postnatal lymphangiogenesis**

In humans, lymph sacs appear in 6-7-week-old embryos, and in the mouse, lymph-vessel development begins around embryonic day 10 (E10). So far, experimental data from mice support the hypothesis proposed by Florence Sabin about 100 years ago that lymphatic endothelial cells arise by sprouting from embryonic veins in the jugular and perimesonephric areas. From here they migrate to form primary lymph sacs and the primary lymphatic plexus, which is composed of capillary-like vessels<sup>28</sup> (Fig. 3; Table 1). The homeobox transcription factor Prox1 and VEGF-C are essential for these initial developmental events. These and other factors involved are addressed below.

**Prox1**

In mice, Prox1-expressing endothelial cells are first observed at E10 in the jugular vein, from which they migrate to form the first lymphatic sprouts<sup>29</sup>. *Prox1* deletion leads to a complete absence of the lymphatic vasculature; endothelial cells bud from the cardinal vein but fail to express lymphatic endothelial markers and do not migrate further (Fig. 3)<sup>30</sup>. Accordingly, PROX1 overexpression in human blood vascular endothelial cells suppresses many blood vascular-specific genes and upregulates lymphatic endothelial-cell-specific transcripts<sup>31,32</sup>. At present, the signals leading to the polarized expression of Prox1 and its target genes in lymphatic endothelial cells are not known. *Prox1*<sup>+/-</sup> mice die perinatally in most genetic backgrounds except in the outbred NMRI background, in which they develop chylous ascites and adult-onset obesity<sup>33</sup>. Notably, endothelium-specific deletion of *Prox1* at least partly recapitulates the obese phenotype, indicating a link between abnormal lymphatic vessel development, impaired lymph drainage and tissue adiposity<sup>33</sup>.

**VEGF-C/D and VEGFR-3**

Homozygous deletion of *Vegfc* leads to the complete absence of the lymphatic vasculature in mouse embryos, whereas *Vegfc*<sup>+/-</sup> mice display severe lymphatic hypoplasia<sup>16</sup>. In *Vegfc*-null mice, lymphatic endothelial cells initially differentiate in the cardinal veins but fail to migrate and to form primary lymph sacs. This demonstrates that VEGF-C is an essential chemotactic and survival factor during embryonic lymphangiogenesis<sup>16</sup>. By contrast, deletion of *Vegfd* does not affect development of the lymphatic vasculature, although exogenous VEGF-D protein rescues the impaired vessel sprouting in *Vegfc*<sup>-/-</sup> embryos<sup>16,34</sup>. *Vegfr3* deletion leads to defects in blood-vessel remodeling and embryonic death at mid-gestation, indicating an early blood vascular function<sup>35</sup>.

**Box 2 | Origin of Kaposi sarcoma spindle cells**

Kaposi sarcoma is a neoplasm characterized by vascular nodules in the skin, mucous membranes and internal organs. It is endemic in sub-Saharan regions in Africa and is frequently encountered in AIDS patients. The nodules are composed of clusters of spindle-shaped tumour cells and characterized by a prominent vasculature. The spindle cells express both blood and lymphatic endothelial cell markers, suggesting their endothelial origin. Development of Kaposi sarcoma is associated with infection by the human herpesvirus-8 (HHV-8). The transcriptional profile of Kaposi sarcoma cells is closely related to normal lymphatic endothelial cells<sup>99,100</sup>. Furthermore, *in vitro* infection of blood vascular endothelial cells with HHV-8 resulted in the expression of several lymphatic endothelial cell-specific genes, although HHV-8-infected lymphatic endothelial cells also showed some infidelity of phenotypic gene expression<sup>99,100</sup>. Growth signals may also be provided directly by HHV-8 through interactions between one of its capsid proteins and VEGFRs or integrins on endothelial cells<sup>24</sup>.

Heterozygous missense point mutations, which lead to tyrosine-kinase inactivation, have been found in *VEGFR3* in patients with Milroy disease (OMIM 153100), a rare autosomal dominant lymphoedema characterized by hypoplasia of cutaneous lymphatic vessels<sup>36</sup>. Chy mice, derived from an ethylnitrosourea mutagenesis screen, have a similar mutation and develop lymphoedema. They are a useful model for studies of hereditary lymphoedema and its therapy<sup>9</sup>.

### LYVE-1

LYVE-1 (lymphatic vessel hyaluronan receptor-1) is one of the most widely used markers for lymphatic endothelial cells<sup>37</sup>. LYVE-1 is the first marker of lymphatic endothelial commitment, and in mice it is expressed in a polarized manner in venous endothelium starting from E9 (Fig. 3). In adults, LYVE-1 expression is downregulated in the collecting lymphatic vessels but remains high in lymphatic capillaries<sup>38</sup>. The role of LYVE-1 in the regulation of lymphatic vascular function is not known, but mice lacking this receptor have normal lymphatic vessels (G. Thurston, personal communication).

### Syk and SLP76

A connection to the thoracic duct at the junction of the left subclavian and the internal jugular veins is the main point of entry of lymph to the bloodstream. Additional lymphatico-venous communications occur in the renal, hepatic and adrenal veins, in the lymph nodes and in other

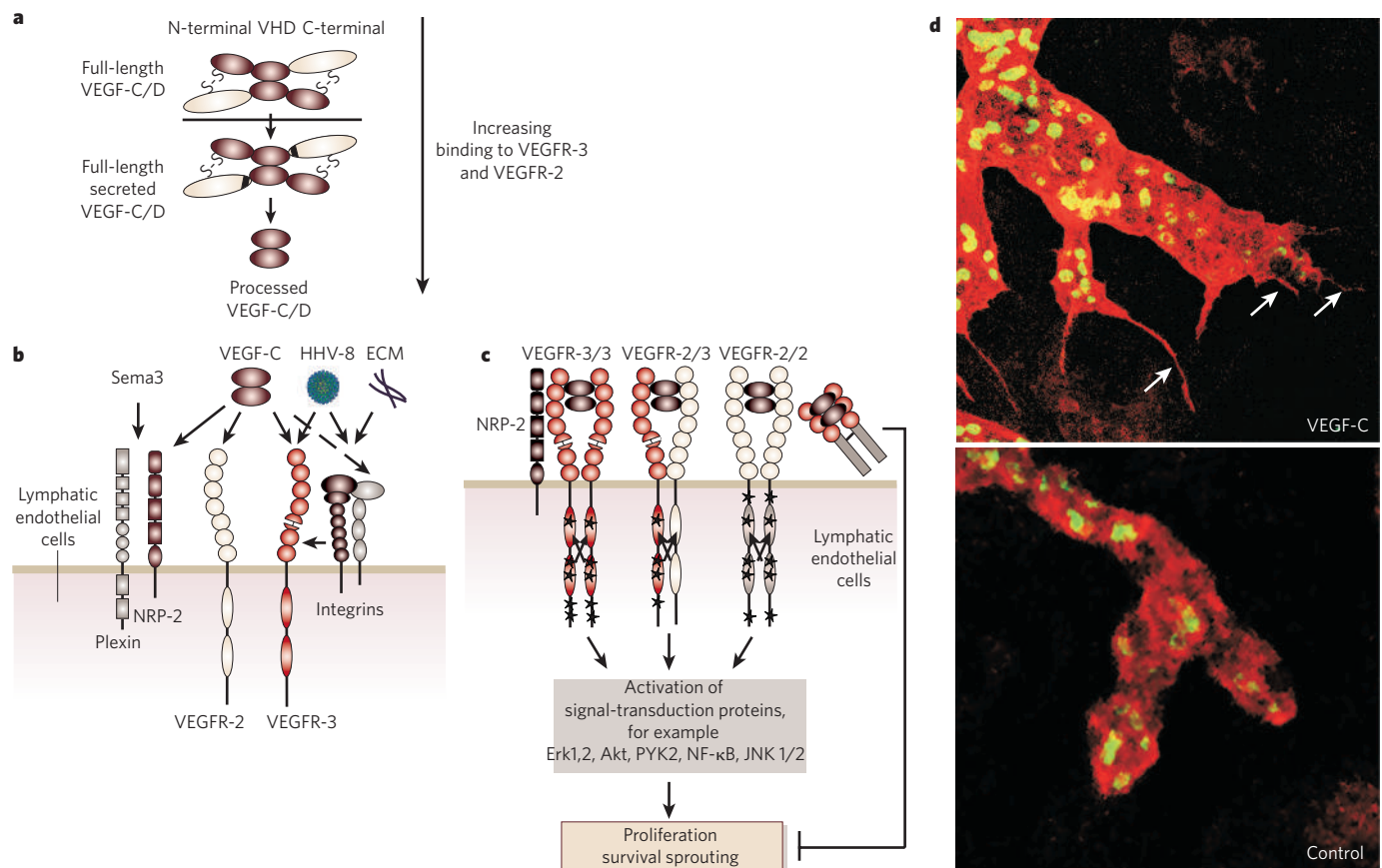
peripheral locations<sup>39,40</sup>. Lymphatico-venous anastomoses are frequently observed in lymphoedema, chylous ascites and chylothorax, where they are an adaptive response to lymphatic hypertension.

The tyrosine kinase Syk and adaptor protein SLP76 are involved in controlling the separation of the lymphatic and blood vascular systems. Mice with mutations in these molecules have arterio-venous shunts and abnormal lymphatico-venous communications. Syk and SLP76 are expressed almost exclusively in haematopoietic cells, suggesting that these cells contribute to the separation of the two vascular systems<sup>41</sup>.

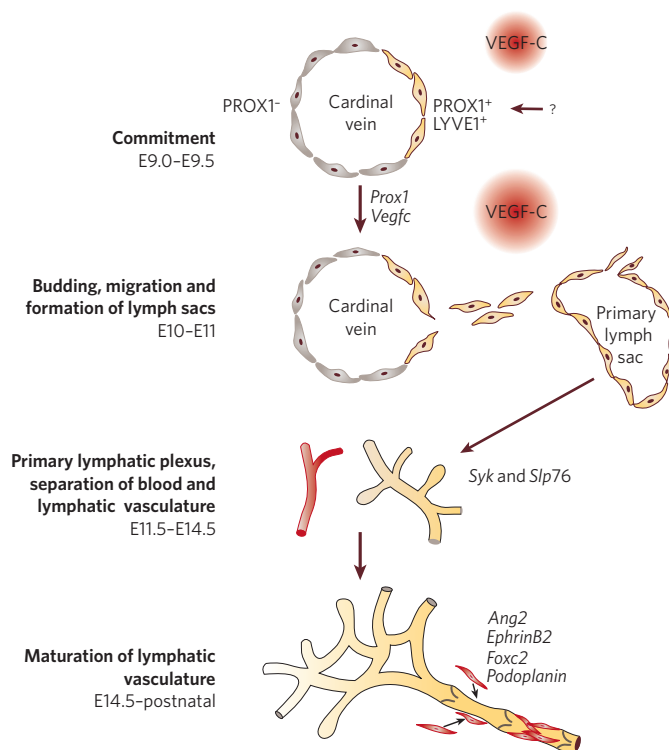
Further development of the lymphatic vessels involves remodelling during late embryogenesis and postnatally to form a superficial capillary plexus and collecting lymphatic vessels. Genetic ablation experiments point to the involvement of several genes in this process; these are highlighted below (also see Table 1).

### Angiopoietins and Tie receptors

Little is known of the functions of the angiopoietins (Ang) in the lymphatic vasculature. The Ang receptors Tie1 and Tie2 are expressed by lymphatic endothelial cells<sup>42</sup>, and Ang2 is considered to be an endogenous inhibitor of Tie2 in endothelial cells, although in some conditions it can act like the agonistic Ang1. *Angpt2*-gene-targeted mice display lymphatic hypoplasia, but replacement of *Angpt2* with *Angpt1* was sufficient to rescue the lymphatic vascular phenotype<sup>43</sup>. Furthermore,



**Figure 2 | VEGF-C/D-VEGFR-3 pathway in the regulation of the lymphatic vessel growth.** **a**, Stepwise proteolytic processing of the VEGF-C or VEGF-D homodimers results in gradually increased binding to VEGFR-3 and VEGFR-2. VEGF-C and VEGF-D are activated by intracellular secretory proprotein convertases. The secreted subunits of these factors are disulphide bonded by means of their propeptides, but they are further proteolysed in the extracellular environment to generate non-disulphide-linked homodimeric proteins (reviewed in ref. 39). VHD, VEGF homology domain. **b**, Extracellular matrix proteins (ECM), such as collagen and fibronectin, enhance tyrosine phosphorylation of VEGFR-3 through activation of integrin  $\beta 1$ . Additional, less explored VEGF-C signal-transduction pathways include interaction of VEGF-C with NRP-2 and integrin  $\alpha 9$ , and VEGFR-3 with integrin  $\beta 1$  and HHV-8 envelope protein  $gB^{9,21,22,24}$ . NRP-2 also serves as a plexin co-receptor for type III semaphorins (Sema 3). The importance of these interactions for the *in vivo* signalling through VEGFR-3 still needs to be demonstrated. **c**, Formation of homo- and heterodimeric VEGFR-2 and VEGFR-3 receptor complexes leads to tyrosine phosphorylation (stars), the recruitment of intracellular signal-transduction proteins, and enhanced endothelial cell migration, proliferation and survival<sup>94,95</sup>. **d**, Adenoviral delivery of VEGF-C induces lymphangiogenesis in mouse skin. Lymphatic vessels are visualized by staining for VEGFR-3 (red) and PROX1 (green). Arrow points at sprouts of growing lymphatic vessels.



**Figure 3 | Model for the development of mouse lymphatic vasculature** (see explanations in the main text). For more detailed discussion of developmental lymphangiogenesis see ref. 2.

Ang1 induces lymphatic vessel growth in adult tissues<sup>44,45</sup>. It is unclear how the angiopoietins convey lymphangiogenic signals.

### EphrinB2

Postnatal remodelling 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<sup>38</sup>. Mice expressing a mutated form of the transmembrane growth factor ephrinB2, which lacks the carboxy-terminal site for binding PDZ-domain-containing proteins, have a normal blood vasculature but display a disturbed postnatal remodelling of the lymphatic vasculature. This leads to hyperplasia of the collecting lymphatic vessels, lack of luminal valve formation and a failure to remodel the primary lymphatic capillary plexus<sup>38</sup>. The ephrins and their Eph receptors have been implicated in repulsive axon guidance in the nervous system and in the control of blood-vessel remodelling (ref. 46; see also the review by Coultas, Chawengsaksophak and Rossant in this issue, p. 937). The new data suggest that there are interesting differences in the remodelling processes between blood and lymphatic vascular systems.

### Foxc2

The forkhead transcription factor Foxc2 is involved in the specification of the lymphatic capillary versus collecting lymphatic vessel phenotype. Foxc2 is highly expressed in the developing lymphatic vessels as well as in lymphatic valves in adults<sup>47,48</sup>. The early development of lymphatic vessels proceeds normally in the absence of Foxc2, but later the patterning of lymphatic vasculature becomes abnormal. Moreover, collecting lymphatic vessels in *Foxc2*<sup>-/-</sup> mice lack valves, whereas the lymphatic capillaries acquire ectopic coverage by basal lamina components and smooth muscle cells<sup>47</sup>.

Ectopic smooth muscle cells surrounding abnormal lymph vessels are also found in humans suffering from lymphoedema-distichiasis (LD, OMIM 153400), an autosomal dominant disease caused by heterozygous loss-of-function mutations of *FOXC2* (ref. 49). LD is char-

acterized by late-onset lymphoedema and a double row of eyelashes. Unlike in Milroy disease, the lymphatic vasculature in LD is normal or hyperplastic, but there is lymph backflow, presumably due to abnormal lymphatic valves, defective vessel patterning and the presence of ectopic smooth muscle cells<sup>47,50</sup>. Many LD patients also suffer from incompetent venous valves (P. Mortimer, personal communication), suggesting that *FOXC2* is also important for their development.

### Podoplanin

Podoplanin is transmembrane mucin-type glycoprotein that is highly expressed in podocytes, keratinocytes, cells of choroid plexus, alveolar lung cells and lymphatic endothelial cells. Podoplanin deficiency leads to abnormal lung development and perinatal lethality. Podoplanin knockout mice displayed paw lymphedema and abnormal lymphatic function and patterning, perhaps due to impaired migration of lymphatic endothelial cells<sup>51</sup>.

### Molecular blueprint of lymphatic endothelial cells

The discovery of cell-surface markers, such as VEGFR-3, podoplanin, LYVE-1 and CD34, that distinguish blood vascular from lymphatic endothelial cells has allowed the isolation of pure populations of these two cell types from human skin<sup>52-55</sup>. Growth of cultured lymphatic endothelial cells is dependent on VEGF-C, which in mixed cultures is supplied by the blood vascular endothelial cells. Interestingly, both cell types show preferentially homotypic interactions, even *in vitro*<sup>52</sup>. Approximately 2% of transcribed genes are differentially expressed between lymphatic and blood vascular endothelial cells, and this difference may reflect their distinct *in vivo* functions<sup>32,54,55</sup>. Detailed discussion of the expression-profiling studies has been provided in recent reviews<sup>1,3</sup>. Although the transcripts expressed by lymphatic and blood vascular endothelial cells are significantly modified soon after their isolation from tissues (P. Saharinen and N. Wick, personal communication), several genes potentially important in the regulation of lymphatic vascular function have been identified. Further analysis of their functions by gene knockout and knockdown should provide a comprehensive view of lymphatic vascular biology in the coming few years.

### Lymphatic vascular insufficiency and its treatment

Impairment of the lymphatic-transport capacity because of abnormal vessel development or damaged lymphatic vessels causes stagnation of proteins and associated water in the interstitium, and leads to lymphoedema, usually a progressive and lifelong condition for which no curative treatment exists. The protein-rich interstitial fluid initiates an inflammatory reaction, leading to fibrosis, impaired immune responses and fatty degeneration of the connective tissue. Although primary, congenital lymphoedema is commonly the result of inherited genetic damage, secondary lymphoedema is caused by filariasis (elephantiasis) or by traumas due to radiation therapy, surgery or infection. Filariasis is a parasitic infection of lymphatic vessels by *Wuchereria bancrofti* or *Brugia malayi* worms, transmitted by mosquito bites. This leads to damage of lymphatic vessels and chronic lymphoedema of legs or genitals. Filariasis is the main cause of lymphoedema in tropical countries, with some 100 million people affected worldwide, whereas breast-cancer surgery is a leading cause for secondary lymphoedema in industrialized countries<sup>56</sup>.

Chylous ascites and chylothorax are caused by accumulation of high-fat-containing fluid or chyle in the abdomen or thorax as a result of trauma, obstruction or abnormal development of lymphatic vessels<sup>40</sup>. This leads to lymphatic hypertension, lymph extravasation and loss of proteins, lipids and leukocytes as well as abdominal inflammation and intestinal adhesions. Chylous ascites or chylothorax may accompany other types of lymphatic vascular dysfunction, especially in mouse models (see Table 1), whereas peripheral lymphoedema is often inconspicuous in these animals, probably due to their small size and the low hydrostatic pressure in the limbs.

Recently, promising lymphoedema treatment results have been

**Table 1 | Genes that mediate lymphatic vasculature formation and patterning**

Gene	Function	Lymphatic phenotype	Other defects	Lethality
<i>Angpt2</i> , gene targeted <sup>43</sup>	Growth factor, ligand of Tie-2	Hypoplasia, chylous ascites ( <sup>-/-</sup> )	Eye hyaloid vasculature fails to regress <sup>43</sup> , abnormal inflammatory response (H. Augustin, personal communication)	<sup>-/-</sup> : perinatal or normal
<i>Efnb2</i> , PDZ-binding mutant <sup>38</sup>	Ligand of EphB receptors	Retrograde lymph flow, chylothorax, ectopic mural cells, absent valves ( $\Delta V/\Delta V$ )	Lung development defects (R. Klein, personal communication)	<sup>-/-</sup> : perinatal <sup>+/-</sup> : normal
<i>Foxc2</i> , gene targeted	Transcription factor	Abnormal lymphatic patterning, presence of mural cells, absent valves ( <sup>-/-</sup> ) <sup>47</sup> , lymphatic vessel and lymph node hyperplasia ( <sup>+/-</sup> ) <sup>89</sup>	Aortic arch malformations, heart septal defects, abnormal kidney and urethra development	<sup>-/-</sup> : E12.5-perinatal <sup>+/-</sup> : normal
<i>Itga9</i> , gene targeted <sup>23</sup>	Adhesion receptor	Lymphoedema, chylothorax ( <sup>-/-</sup> )	Not reported	<sup>-/-</sup> : perinatal <sup>+/-</sup> : normal
<i>Elk3</i> (Net), gene targeted <sup>90</sup>	Transcription factor Mutant form lacks DNA-binding domain	Lymphangiectasis, chylothorax ( <sup>-/-</sup> )	Impaired wound and tumour angiogenesis <sup>+/-</sup>	<sup>-/-</sup> : perinatal <sup>+/-</sup> : normal
<i>Nrp2</i> , gene targeted <sup>10</sup>	Receptor for VEGF165, VEGF145, PIGF, VEGF-C and class 3 semaphorins	Transient hypoplasia of lymphatic capillaries ( <sup>-/-</sup> )	Defects in neural fasciculation and guidance	<sup>-/-</sup> : perinatal <sup>+/-</sup> : normal
Podoplanin ( <i>Gp38</i> ), gene targeted <sup>51</sup>	Membrane glycoprotein	Lymphangiectasis, abnormal lymph transport, lymphoedema ( <sup>-/-</sup> )	Respiratory failure due to abnormal lung development	<sup>-/-</sup> : perinatal <sup>+/-</sup> : normal
<i>Pik3r1</i> , gene targeted <sup>91</sup>	Regulatory subunits of class I <sub>A</sub> PI(3)K	Chylous ascites ( <sup>-/-</sup> )	Liver necrosis, enlarged skeletal muscle fibres, brown fat depositions, calcification of heart tissue	<sup>-/-</sup> : perinatal (129Sv × C57Bl6) or 30% survival (outbred) <sup>+/-</sup> : normal
<i>Prox1</i> , gene targeted or endothelial specific deletion <sup>29,33</sup>	Transcription factor	No lymphatic vessels ( <sup>-/-</sup> ) Chylous ascites, adult onset obesity ( <sup>+/-</sup> )	Abnormal eye, liver and pancreas development	<sup>+/-</sup> : perinatal in most backgrounds <sup>-/-</sup> : E14.5
<i>Lcp2</i> (SLP-76) and <i>Syk</i> , gene targeted <sup>41</sup>	Tyrosine kinase ( <i>Syk</i> ); adaptor protein (SLP-76)	Failure of separation of blood and lymphatic vasculature, chylous ascites ( <sup>-/-</sup> )	Failure of T-cell development and fetal haemorrhage (Slp76) Block of B-cell development and fetal haemorrhage ( <i>Syk</i> )	<i>Lcp2</i> <sup>-/-</sup> : perinatal <i>Lcp2</i> <sup>+/-</sup> : normal <i>Syk</i> <sup>-/-</sup> : perinatal <i>Syk</i> <sup>+/-</sup> : normal
<i>Sox18</i> (ragged) <sup>92</sup>	Transcription factor Spontaneous missense mutations	Edema and chylous ascites ( <sup>-/-</sup> )	Lack of vibrissae and coat hairs, generalized oedema and cyanosis due to cardiovascular defects	<sup>-/-</sup> : perinatal <sup>+/-</sup> : normal
Trisomy 16 (Ts16) <sup>93</sup>	Many	Nuchal oedema, abnormal size and structure of jugular lymph sacs from E14	Multiple cardiac or craniofacial development defects	E16-E20
<i>Vegfc</i> , gene targeted <sup>16</sup>	Growth factor, ligand of VEGFR-3	No lymphatic vessels ( <sup>-/-</sup> ) hypoplasia, chylous ascites ( <sup>+/-</sup> )	Not reported	<sup>-/-</sup> : E17-E19 <sup>+/-</sup> : perinatal or normal
<i>Vegfr3</i> ( <i>Chy</i> , ethylnitrosourea-induced mutation) <sup>9</sup>	Receptor tyrosine kinase, kinase-inactivating mutation I1053F	Hypoplasia, chylous ascites ( <sup>+/-</sup> )	Failure of remodelling of primitive blood vascular plexus ( <sup>-/-</sup> )	<sup>-/-</sup> : E10 <sup>+/-</sup> : perinatal or normal

achieved in preclinical models using viral gene-transfer vectors that encode lymphangiogenic growth factors (reviewed in ref. 57). For example, *VEGF-C* gene-transduction induces growth of functional lymphatic vessels<sup>58</sup>, whereas the mature form of *VEGF-D* is a very powerful inducer of angiogenesis<sup>14</sup>. Lymphatic vascular growth without concomitant blood vascular side effects was selectively induced with the VEGFR-3-specific ligand VEGF-C156S (ref. 58). VEGF-C gene therapy was effective even in *Chy* mice that suffer from lymphoedema caused by a heterozygous inactivating mutation of VEGFR-3 (ref. 9). *ANG1* gene transfer to mouse skin promoted lymphangiogenesis, simultaneously inhibiting vascular hyperpermeability. This factor could also be used for the treatment of tissue oedema<sup>45,59</sup>. The pathophysiology of vascular permeability has been recently reviewed elsewhere<sup>60</sup>.

### Tumour metastasis to lymph nodes and its inhibition

Metastatic tumour spread through the blood or lymphatic vessels occurs in most forms of human cancer, with regional lymph-node metastasis often being the most important prognostic factor for carcinoma patients<sup>61</sup>. From the sentinel lymph node, which is the first regional lymph node to which tumour cells metastasize, further dissemination may occur to other nodes and distant organs. At present, it is not clear whether lymphatic metastasis selects cells with increased

potential for subsequent organ metastasis or simply indicates that the tumour has become metastatic in general.

Growth-factor stimulation of lymphatic vessels enhances lymphatic metastasis. Several studies have found positive correlations between VEGF-C or VEGF-D expression and vascular invasion, lymphatic vessel and lymph node involvement, distant metastasis and, in some instances, poor clinical outcomes<sup>62</sup>. VEGF-C expression in tumour cells may be induced by growth factors or proinflammatory cytokines, and some may be derived from inflammatory cells in tumours. High levels of VEGF-C or VEGF-D also enhance lymphatic metastasis in various experimental models<sup>63-67</sup>. Furthermore, in some tumours, proteolytically processed VEGF-C or VEGF-D may be generated, which targets VEGFR-2 or VEGFR-3 that is often upregulated in tumour blood vessels<sup>68</sup>. A direct link between VEGF-C or VEGF-D expression and metastasis was established with the use of a soluble VEGFR-3-immunoglobulin fusion protein (VEGF-C/D trap) or blocking anti-VEGF-D antibodies<sup>63,66,67</sup>. In some models lymphatic, but not lung, metastases were blocked with the VEGF-C/D trap, whereas in others the treatment inhibited both types of metastases<sup>63,69</sup>. Although these experiments provide support for the contribution of VEGF-C, VEGF-D, and their receptor, VEGFR-3, in lymphatic spread in malignancy, the mechanisms of these effects have only recently been addressed.

Proliferating intratumoural lymphatic vessels are present in certain

human cancers, such as melanomas, head and neck carcinomas and xenograft tumour models overexpressing lymphangiogenic factors<sup>70,71</sup>. However, they may not be a prominent feature, and may in fact not be required for enhanced metastasis in most solid tumours. At least in animal models, intratumoural lymphatic vessels may not be completely functional, because these vessels collapse under high intratumoural pressure<sup>72</sup>, and at least in one study they were not conducive of lymphatic metastasis<sup>73</sup>. We favour the view that local lymphatic vessels at the tumour margin are more important for spreading tumour cells, through the process of vessel sprouting under the influence of interstitial fluid hypertension and tumour-secreted VEGF-C<sup>74,75</sup>. In this process endothelial cells send long filopodia towards the VEGF-C-producing tumour cells and then form tumour-directed vessel sprouts, where the vessel lumen opens up and allows facilitated access of tumour cells to the lumen (Fig. 4). Tumour lymphatic vessels carry specific markers, such as CD34, and their heterogeneity can be used for their targeting (H. Augustin, personal communication; ref. 76).

Some evidence indicates that the lymphatic endothelium actively participates in metastasis formation by secreting chemokines, such as CCL21 (SLC, 6CKine and Exodus), whose receptor (CCR7) is expressed on some tumour cells<sup>77</sup>. Furthermore, the collecting lymphatic vessels draining fluid from the tumour area are stimulated by intraluminal VEGF-C to dilate through the process of endothelial proliferation in the vessel wall<sup>75</sup>. Clumps of metastatic tumour cells could then undergo an easier transit in lymph, flowing in the dilated hyperplastic vessels. The VEGF-C/D trap inhibited the sprouting and vessel dilation and seemed to restore the integrity of the vessel wall<sup>75</sup>. Similarly, blocking monoclonal antibodies that target VEGF-C, VEGF-D or their receptor(s) and small molecules that inhibit the tyrosine kinase catalytic domain of these receptors could be used for the inhibition of tumor metastasis. Further work should soon tell if these same molecules inhibit further systemic metastasis or angiogenesis in some tumours. In this case such compounds would undoubtedly proceed to clinical trials. However, it should be noted that also VEGF can stimulate lymphatic metastasis<sup>78</sup>.

Lymphatic vessels proliferate during inflammation<sup>79</sup>. Pro-inflammatory cytokines induce VEGF-C messenger RNA transcription, presumably through NF- $\kappa$ B-mediated promoter activation, suggesting that they regulate lymphatic vessel growth during inflammation<sup>80</sup>. Interestingly, constitutive NF- $\kappa$ B activity is detected in the lymphatic endothelium *in vivo*, but its role in the lymphatic endothelial cells remains enigmatic<sup>81</sup>.

### Lymphangiogenesis in inflammation

Inflammatory infiltrates in human kidney transplants undergoing rejection contain proliferating host lymphatics<sup>82</sup>. Infection of mouse airway epithelial cells with the respiratory pathogen *Mycoplasma pulmonis* resulted in robust lymphangiogenesis driven by VEGF-C- and VEGF-D-expressing immune cells that could be inhibited by using a VEGF-C/D trap<sup>20</sup>. Importantly, VEGFR-3 inhibition resulted in severe exacerbation of mucosal oedema and reactive lymphadenitis decreased. This is consistent with the importance of the lymphatic vascular system as an exit route for immune cells and fluid<sup>20</sup>. In a rabbit cornea model of inflammatory angiogenesis and lymphangiogenesis, either a VEGF inhibitor or selective depletion of the VEGF-C and VEGF-D producing macrophages blocked lymphangiogenesis, demonstrating that inflammatory cells recruited by VEGF can mediate the formation of lymphatic vessels<sup>19</sup>. Moreover, dendritic cells expressing both VEGFR-3 and VEGF-C could be detected in a mouse model of corneal inflammation, suggesting that immune cells may both respond to lymphangiogenic signals and induce lymphangiogenesis (ref. 83). Indeed, blockade of VEGFR-3 signalling suppressed trafficking of corneal dendritic cells to draining lymph nodes and inhibited induction of delayed-type hypersensitivity and rejection of corneal transplants<sup>84</sup>.

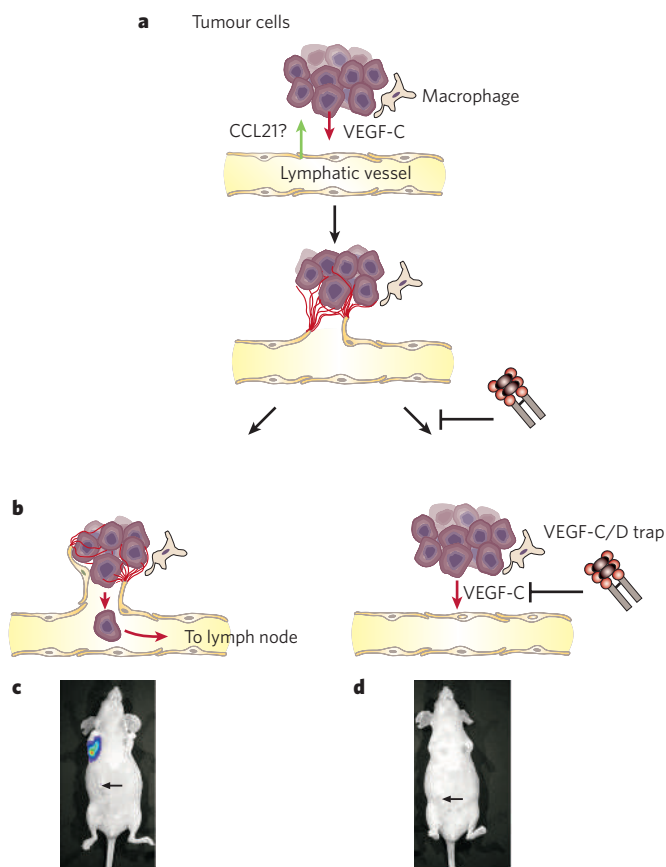
Lymphatic vessels participate in the regulation of inflammatory response through their role in transport of lymphocytes to the lymph nodes. Migration of dendritic cells is mediated by the chemokine receptor CCR7, whereas lymphatic vessels express the ligand CCL21<sup>85</sup>. Furthermore, mannose receptor 1 and common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) control lymphocyte traffic in lymphatic vessels<sup>86,87</sup>. Human lymphatic endothelial cells also express the D6 chemokine receptor, which is involved in the post-inflammatory clearance of beta-chemokines<sup>88</sup>.

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### Future directions

Recent progress in the area of lymphatic vascular biology has provided various genetic mouse models and new molecular tools for isolation and growth regulation of lymphatic vessels. Coupled with high-throughput genomic, proteomic and functional screens, these methods will undoubtedly reveal additional possibilities for therapeutic intervention in diseases where the lymphatic vascular system has a significant pathophysiological function. Below we have briefly outlined the questions that we believe should be addressed in the next few years.

The early steps of lymphatic endothelial cell commitment are not yet understood, and the mechanisms of lymphatic vascular remodeling, patterning and maturation are only beginning to be elucidated. Studies of blood vascular development have shown that Notch,



**Figure 4 | Role of VEGF-C/D in lymphatic metastasis in cancer.** **a**, Tumour cells and tumour-associated macrophages secrete lymphangiogenic growth factor VEGF-C or VEGF-D, which induces sprouting of nearby lymphatic vessels, facilitating the access of tumour cells into the vessel lumen. The lymphatic endothelial cells may also actively attract some tumour cells through the secretion of chemokines, such as CCL21. **b**, Aggregates of tumour cells are transported to the regional lymph node, from which they can spread to distant organs through either blood or lymphatic vessels. Blockage of VEGFR-3 signalling inhibits metastasis in most mouse tumour xenograft models by stabilizing lymphatic vessels. **c**, Nude mice were implanted with luciferase-tagged tumour cells, which metastasize to the ipsilateral axillary lymph node in control adenovirus-treated mice. **d**, By contrast, metastasis was abolished in mice treated with adenovirus encoding a VEGF-C/D trap<sup>75</sup>. Note that the primary tumours have been excised (arrows).

Eph/ephrin, Shh and TGF- $\beta$  pathways have an important role in the specification of arterial versus venous cell fates, whereas neural-guidance molecules such as netrins, semaphorins, plexins and members of Slit/Robo family are essential for vessel remodelling and navigation (see also p. 937). Furthermore, interaction of endothelial cells and pericytes, mediated in part through PDGF-B/PDGFR $\beta$ , is necessary during blood-vessel maturation. It will be important to determine which signalling pathways control different stages of lymphatic vascular development and to what extent they are similar to the ones operating in the blood vessels.

Lymphangiogenesis research has so far provided imminent therapeutic applications for human diseases such as lymphoedema and other tissue oedemas that will enter clinical development in the near future. A crucial question concerns the possibility of inhibiting lymphatic metastasis in cancer patients. The importance of lymph-node metastasis in the spread of cancer to distant organs needs to be better understood before the new knowledge can be applied to patients. In this context, the possible roles of VEGF-C, VEGF-D and VEGFR-3 upregulation in tumour angiogenesis need to be explored for additional therapeutic applications.

Understanding the mechanisms of lymphatic metastasis, including the identification of stromal and tumour determinants that are important for the spread of tumour cells through lymphatic vessels, represents another challenge for tumour vascular biology researchers. Furthermore, characterization of lymphatic endothelial cells from different vascular beds including various tumour types will provide important novel targets for therapy, along with new information about normal and diseased lymphatic vascular function. Finally, the involvement of lymphatic vessels in inflammation should be explored in several contexts. ■

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