LYMPHATIC VASCULATURE DEVELOPMENT

Guillermo Oliver

Although the process of blood vasculature formation has been well documented, little is known about lymphatic vasculature development, despite its importance in normal and pathological conditions. The lack of specific lymphatic markers has hampered progress in this field. However, the recent identification of genes that participate in the formation of the lymphatic vasculature denotes the beginning of a new era in which better diagnoses and therapeutic treatment(s) of lymphatic disorders could become a reachable goal.

LYMPHANGIOGENESIS
The growth of lymphatic vessels.

LYMPHOEDEMA
Occlusion of lymphatic drainage followed by the abnormal accumulation of interstitial fluid and swelling in the affected body part.

LYMPHATIC METASTASIS
Invasion of detaching primary tumour cells through the lymphatic system to lymph nodes. After re-entering of the lymphatic vessels into the blood vasculature, tumour cells will also spread to distant organs.

Department of Genetics, St Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, Tennessee 38105, USA.
e-mail: guillermo.oliver@stjude.org
doi:10.1038/nri1258

Lymphatic vessels were first described in the seventeenth century by G. Aselli[1]. However, until a few years ago, the lymphatic system was not considered an interesting topic among most scientists. Despite the importance of the lymphatic system and the belief that metastatic tumour cells are disseminated through the lymphatic vasculature, modern anatomy textbooks generally include only a minimal description of the development of this system. Interest in basic lymphatic research has radically increased in the past few years, in part, thanks to the identification of lymphatic-specific markers and growth factors, the debate about tumour LYMPHANGIOGENESIS, and the efforts of patients suffering from LYMPHOEDEMA and their families, who have attracted public and governmental attention by creating the Lymphatic Research Foundation (see further information for website).

Although few life-threatening diseases (BOX 1) result from malfunction of the lymphatic system, failure of lymph transport promotes lymphoedema — a disfiguring, disabling and occasionally life-threatening disorder for which present treatment is limited and ineffective[2]. Malignant tumours can directly activate lymphangiogenesis and LYMPHATIC METASTASIS[3–8]; so, understanding the basic biology that is involved in lymphatic development is vital. Metastasis of most cancers mainly occurs through the lymphatic system, and the extent of lymph-node involvement is a useful prognostic indicator. The lymphatic system is also essential for the immune response to infectious agents, for example, during exposure to an inflammatory agent, afferent lymphatic vessels are the route through which, after antigen uptake, dendritic cells migrate to the lymph nodes and lymphoid organs. Recent studies indicate that spindle cells and cells lining the irregular spaces in Kaposi's sarcoma have a lymphatic endothelial origin[9,10]. An increased understanding of normal lymphatic development should allow us to address pathological lymphatic conditions that lead to inflammation, autoimmunity and cancer, and to improve the clinical treatment of primary and secondary forms of lymphoedema. Here, I discuss a working model for normal lymphatic vasculature development that focuses mainly on the early aspects, and that summarizes findings from different laboratories through the characterization of newly identified lymphatic markers and available mouse mutant lines. In this model, blood venous endothelial cells are the ground condition from which a lymphatic endothelial cell phenotype will be progressively acquired by the stepwise expression of different gene products.

Historical considerations

During embryonic development, blood vessels originate from mesodermally derived endothelial cell precursors (vasculogenesis) (FIG. 1). These vessels grow and remodel into the mature network by endothelial sprouting and splitting (angiogenesis). The lymphatic vasculature appears after the blood vasculature forms, which was the first indication that lymphatics might have a blood vasculature origin.

Box 1 | **Roles of the lymphatic system in health and disease**

In health
- Drains lymph fluid from the extracellular spaces
- Absorbs lipids from the intestinal tract
- Maintains the fluid homeostasis
- Transports white blood cells and antigen-presenting cells to lymphoid organs

Disease
- Congenital and acquired defects of the lymphatic system
- Lymphoedema: imbalance between lymph formation and adsorption into lymphatic vessels
- Lymphangitis: inflammation of lymphatic vessels
- Lymphangioma: benign neoplasm of lymphatic vessels
- Lymphangiosarcoma: malignant neoplasm of lymphatic vessels
- Role in metastasis: migration of tumour cells

PERICYTES
Smooth-muscle-like cells that cover the outer surface of the endothelial cells of blood vessels.

WOLFFIAN BODIES
A series of tubules that constitute the mesonephros.

The lymphatic system (FIG. 2) is composed of a vascular network of blind-ended, thin-walled capillaries and larger vessels that drain protein-rich interstitial fluid (lymph) from the extracellular spaces within organs into larger collecting ducts. In contrast to blood capillaries, lymphatic capillaries lack fenestrations, a continuous basal membrane and PERICYTES; instead, these vessels are lined with a continuous, single layer of overlapping endothelial cells that form loose intercellular junctions. These characteristics make the lymphatic capillaries highly permeable to large macromolecules, pathogens and migrating cells.

Lymph returns to the venous circulation through the thoracic duct and the larger lymphatic collecting vessels, which contain a muscular layer and an adventitial layer. The lymphatic system is crucial for maintaining the colloid osmotic volume (or pressure) and for absorbing lipids from the intestinal tract. The lymphatic system also includes lymphoid organs such as the lymph nodes, tonsils, Peyer's patches, spleen and thymus, all of which have key roles in the immune response to infectious agents.

The lymphatic system develops in parallel, but secondary to the development of the blood vascular system, and lymphatic vessels are not normally present in avascular structures such as the epidermis, hair, nails, cartilage and cornea, nor are they present in some vascularized organs such as the retina and probably the brain. From an evolutionary point of view, specialized lymph hearts first appeared in amphibians and reptiles[11]; later, lymph nodes appeared in birds, and the final refinement of the lymphatic vasculature occurred in mammals[2].

In 1902, Florence Sabin[12,13] proposed the most widely accepted model of lymphatic vasculature development. Sabin injected dye into pig embryos (FIG. 3), and her results culminated in the proposal that endothelial cells bud from veins to form primary lymphatic sacs. From these sacs, budding cells then centrifugally sprout towards the periphery, forming capillaries that surround tissues and organs. In this model, the two jugular lymph sacs develop near the junction of the subclavian and anterior cardinal veins (FIG. 3d). From these sacs, lymphatic capillaries spread to the head, neck, arms and thorax. Later, the remaining lymph sacs originate from the mesonephric vein and veins in the dorsomedial edge of the WOLFFIAN BODIES. The retroperitoneal lymph sac forms near the primitive inferior vena cava and mesonephric veins, and lymphatics spread to the abdominal viscera and diaphragm. The thoracic duct, which carries most lymph back to the blood vascular system, originates in the cisterna chyli — a dilated sac that lies anterior to the uppermost lumbar vertebrae — which forms near the wolffian bodies. The posterior lymph sacs occur near the junctions of the primitive iliac veins and the posterior cardinal veins; from these sacs, lymphatics spread to the abdominal wall, pelvic region and legs (FIG. 3d) (for a detailed anatomical description of the lymphatic system in mice see REF. 14).

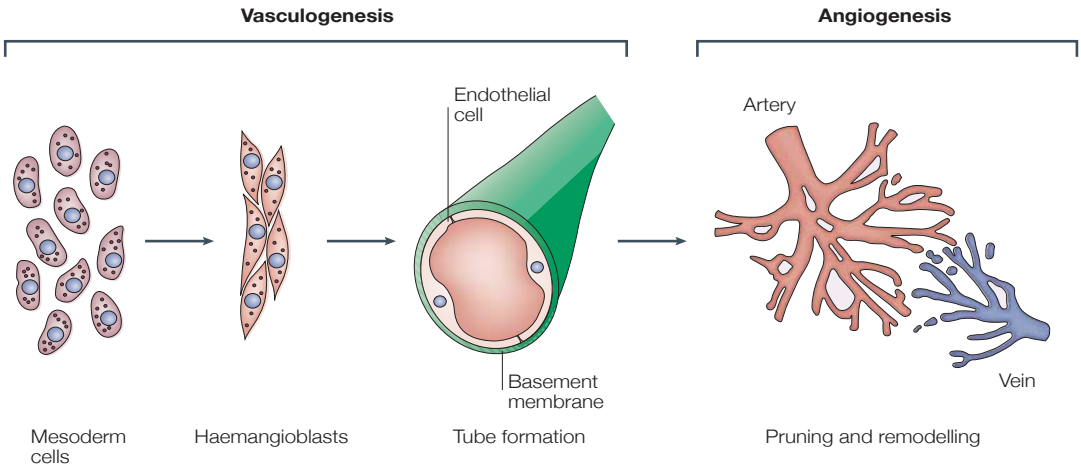

Figure 1 | **Blood vascular development.** Mesodermally derived haemangioblasts give rise to angioblasts, the precursors of blood and vascular endothelial cells that will form the lining of the blood vessels (vasculogenesis). The remodelling of the primary capillary network and the formation of new blood vessels from pre-existing vessels by endothelial sprouting and splitting (angiogenesis) leads to the formation of mature arteries and veins. Figure reproduced, with permission, from REF. 68 © Sinauer Associates, Inc. (2003).

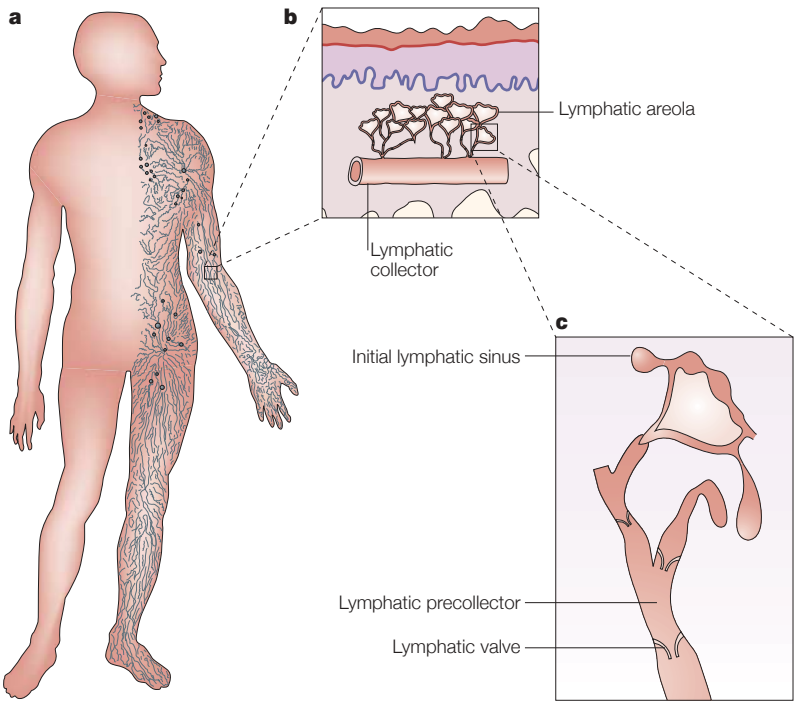

Figure 2 | **Human lymphatic system.** Diagram of the network of superficial lymphatic capillaries present in human skin (**a**). Differences in capillary density occur among the body regions. A representation of the capillaries and vessels of the dermis is shown in **b** and **c**. Figure reproduced, with permission, from REF. 69 © Society of Nuclear Medicine (2003).

In 1910, Huntington and McClure[15] proposed an alternative model. In this less popular model, primary lymph sacs arise in the mesenchyme independent of the veins and then establish venous connections. This proposal has been supported by a recent study in chickens[16], which suggested that lymphatic vessels forming in early wing buds are derived not only by sprouting from the lymph sacs, but also from embryonic mesenchyme.

Until a few years ago, the differentiation between the blood and lymphatic vasculature within a tissue was defined by morphological criteria at the histological level (for example, lymph vessels have thin walls and lack pericytes). Although several blood vascular markers were available, most of them are also expressed by the lymphatic vessels. Recently, investigators have identified a few lymphatic-vasculature-specific markers; in some cases, the specificity of expression of the gene product is acquired only as embryonic development progresses (for example, vascular endothelial growth-factor receptor 3, VEGFR3); so, differentiation of the lymphatic vasculature is probably a stepwise process[7,8,17]. The overlap in molecular markers supports the close structural and developmental relationship between the blood and lymphatic systems.

According to these arguments, we can speculate that during evolution, the expression of a limited number of additional genes by pre-existing blood vascular endothelial cells might have been sufficient to confer the subsequent lymphatic vascular phenotype.

A model for lymphatic vasculature development

In vertebrates, cell types are determined before morphological differentiation. Activation of certain genes before determination could be an early indication that the biological properties of the cell types are changing.

On the basis of our results, as well as those of others, we recently proposed a working model for the development of the lymphatic vasculature that considers the classic embryological concepts of lens-cell-type determination (for review see REF. 18). My colleagues and I proposed[7,8,17] that the development of the mammalian lymphatic vasculature takes place in four stages (FIG. 4): lymphatic endothelial cell (LEC) competence, LEC bias, LEC specification, and lymphatic vessel differentiation and maturation.

The identification of gene products that regulate these stages and the generation of animal models in which loss- or gain-of-function approaches affect the activity of those gene products should allow us to carry out embryological manipulations that will further corroborate or dismiss this working model in mammals.

Lymphatic endothelial cell competence. Competence is the capacity of cells to respond to an initial inducing signal; this stage seems to be autonomous and controlled by a developmental timer[18]. To understand the mechanisms that are involved in lymphatic vasculature development, we must first determine the location and timing of its initiation programme. Where are the LEC progenitors located? What signalling mechanisms initiate the lymphangiogenic pathway? Are lymphangioblast cells present in the developing mammalian embryo?

According to Sabin's model, one of the first events that should take place during embryonic lymphatic development is that venous endothelial cells become competent to respond to an initial lymphatic-inducing signal. The initiation of the programme that leads to lymphatic vasculature development lags behind the formation of the blood vasculature network. Therefore, we can conclude that the presence of the blood vasculature system alone is not sufficient to initiate lymphatic vasculature development; some additional gene product(s) must be expressed for venous endothelial cells to become progressively competent to respond to a local lymphatic-inducing signal.

Although the molecular factor(s) that regulates this initial stage of lymphatic competence remains unknown, the expression of the lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1) (TABLE 1) by a few of the endothelial cells that line the anterior cardinal vein of mice at embryonic day (E) 9.0–9.5 could be considered the first morphological indication that venous endothelial cells are already competent to respond to a lymphatic-inducing signal (FIG. 4). Lyve1 was recently cloned as a homologue of CD44 (REFS 19,20), and although it is not expressed by most blood vessel endothelia, it is expressed by embryonic and adult lymphatic vessels, liver and spleen sinusoids and macrophages[7,21].

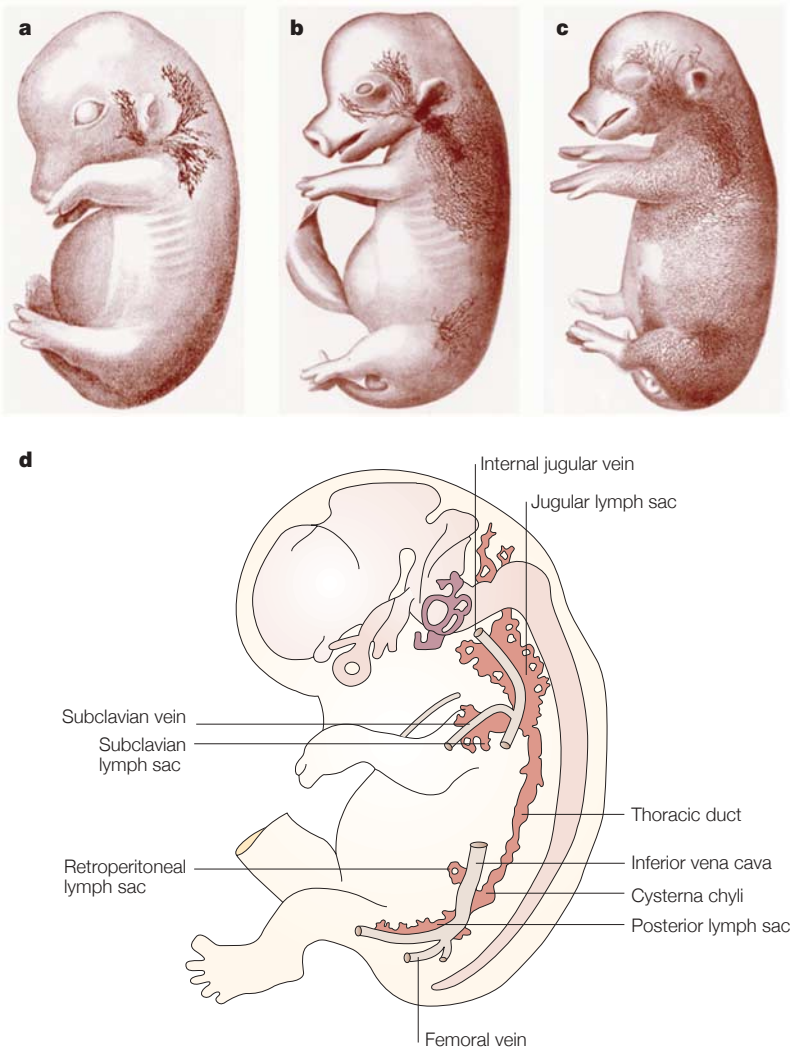

Figure 3 | **Sequential growth of lymphatic vessels in the mammalian embryo.** Growth of lymphatic vessels in pig embryos visualized by the ink injection method of Sabin[12,13]. According to her proposal, the peripheral lymphatic system originates from the primary lymph sacs, then spreads by endothelial sprouting into the surrounding tissues and organs, where local capillaries are formed. **a** | Embryo, 3.0 cm in length, showing the lymphatic ducts in the side of the neck growing in three directions; the back of the head behind the ear, the scapular region and the region of the jaw. **b** | Embryo, 4.3 cm in length, showing the ducts of the primary plexus, which have grown to the median line in the back and anastomosed with those on the other side. Many of the lymphatics in the lower part of the body have already reached the skin. **c** | Embryo, 5.5 cm in length, in which the ducts have now covered most of the body (reproduced from REFS 12,13,84). **d** | Diagram of a 9-week-old human embryo; localization of the primary lymph sacs and the developing thoracic duct are highlighted. Image modified, with permission, from REF. 70 © Elsevier (1999).

Hyaluronan — a mediator of cell migration during embryogenesis and tumour metastasis[22] — could also be involved in lymph-node homing by CD44$^+$ leukocytes and tumour cells[22,23]. Lyve1 might also participate in transporting hyaluronan across the lymphatic vessel wall and in regulating leukocyte migration throughout the lymphatics[23].

The lack of any obvious lymphatic alteration in mice with a targeted deletion of *Lyve1* (N.W. Gale, G. Thurston and G.D. Yancopolous, personal communication) indicates that venous endothelial cells do not require this gene activity to acquire lymphatic competence. Instead, another unidentified molecule must initiate lymphatic competence in venous endothelial cells (FIG. 4). This initial stage is considered to be independent of tissue interactions, and expression of the competence factor should be sufficient to make embryonic veins capable of producing LECs after exposure to a lymphatic-inducing signal. Is competence restricted to venous endothelial cells, or would expression of this unidentified competence factor by other blood vascular endothelial cells sustain the development of the lymphatic vasculature, if placed in the correct embryonic environment? At present, we do not know the answers to these questions; however, if this working model is correct, then functional inactivation of the competence factor in mice should prematurely arrest the programme that leads to lymphatic development.

Lymphatic endothelial cell bias. Following the initial stage of lymphatic competence, cells acquire the ability to give rise to a particular cell type or structure (lymphatic bias); this stage depends on the inducing tissue[18]. Around E9.5, a few hours after Lyve1 expression is initiated in the anterior cardinal vein, expression of the transcription factor prospero-related homeobox 1 (Prox1) is also observed in that tissue[24]. Unlike Lyve1 expression, which is uniformly expressed by all endothelial cells of the anterior cardinal vein at this stage, expression of Prox1 is restricted to a subpopulation of endothelial cells on one side of the vein[24] (FIG. 4). In addition to this initial polarized localization of the LEC progenitors in the veins, their subsequent budding is also polarized[24] (FIGS 5a and 5b); a finding that indicates the presence of some type of guidance mechanism.

The *Prox1*$^{-/-}$ mouse embryo is the only model of early lymphatic developmental arrest that has been reported[24]. *Prox1*$^{-/-}$ embryos have no lymphatic vasculature. Localization of Prox1 expression in the venous endothelial cells that give rise to the entire lymphatic system provides strong support for Sabin's original model of a venous origin of the lymphatic vasculature.

Although Prox1 is expressed by various cell types including embryonic and adult LECs, it is not expressed by blood vascular endothelial cells[7,24]. The initial restricted expression of Prox1 by a subpopulation of venous endothelial cells is probably an indication that the expressing cells are already committed to the lymphatic pathway and, therefore, could be considered as LEC precursors. The absence of lymphatic vasculature in *Prox1*$^{-/-}$ embryos is not necessarily caused by an arrest in LEC budding, but instead by a failure in lymphatic cell-type specification[7]. Endothelial cells that bud from the *Prox1*$^{-/-}$ anterior cardinal vein on E11.0–11.5 do not express LEC markers; instead, they express blood vascular markers[7]. Therefore, we proposed that first, Prox1 activity is required and sufficient to confer a LEC phenotype (LEC bias) to cells that would otherwise remain blood vascular endothelial cells, and second, blood vascular endothelial cells is the default ground

state, and after expression of Prox1 these cells adopt a lymphatic cell-type phenotype[7,8,17]. This proposal was recently corroborated by experiments in which over-expression of Prox1 was sufficient to reprogramme cultured blood vascular endothelial cells into LECs; Prox1 induced LEC-specific gene expression while repressing the expression of some blood vascular endothelial cell markers[25,26]. In addition, Prox1 is expressed by LECs in adult and tumour tissues[7]. Therefore, Prox1 is a reliable, specific marker of embryonic and adult LECs in normal and pathological conditions.

In summary, Prox1 seems to be required and sufficient to bias a subpopulation of competent venous endothelial cells (lymphatic progenitors) into the lymphatic programme. However, we do not yet know whether the restricted expression of Prox1 on one side of the anterior cardinal vein results from a localized inductive signalling mechanism or from heterogeneity in the venous endothelial cells.

According to these results, venous endothelial cells could be considered the source of LEC progenitors, which, after Prox1 expression, become biased (committed) to the lymphatic specification pathway. However, we do not know whether these venous LEC progenitors are lymphangioblast cells. Once the expression of Prox1 is initiated by the venous endothelial cells, the expression of some receptor–ligand signalling system might be required to guide the subsequent polarized budding of LECs towards the receptor–ligand source, thereby ensuring the precise location of the primary lymph sacs (FIG. 3d). The source and identity of the Prox1-inducing factor in the veins remains unknown.

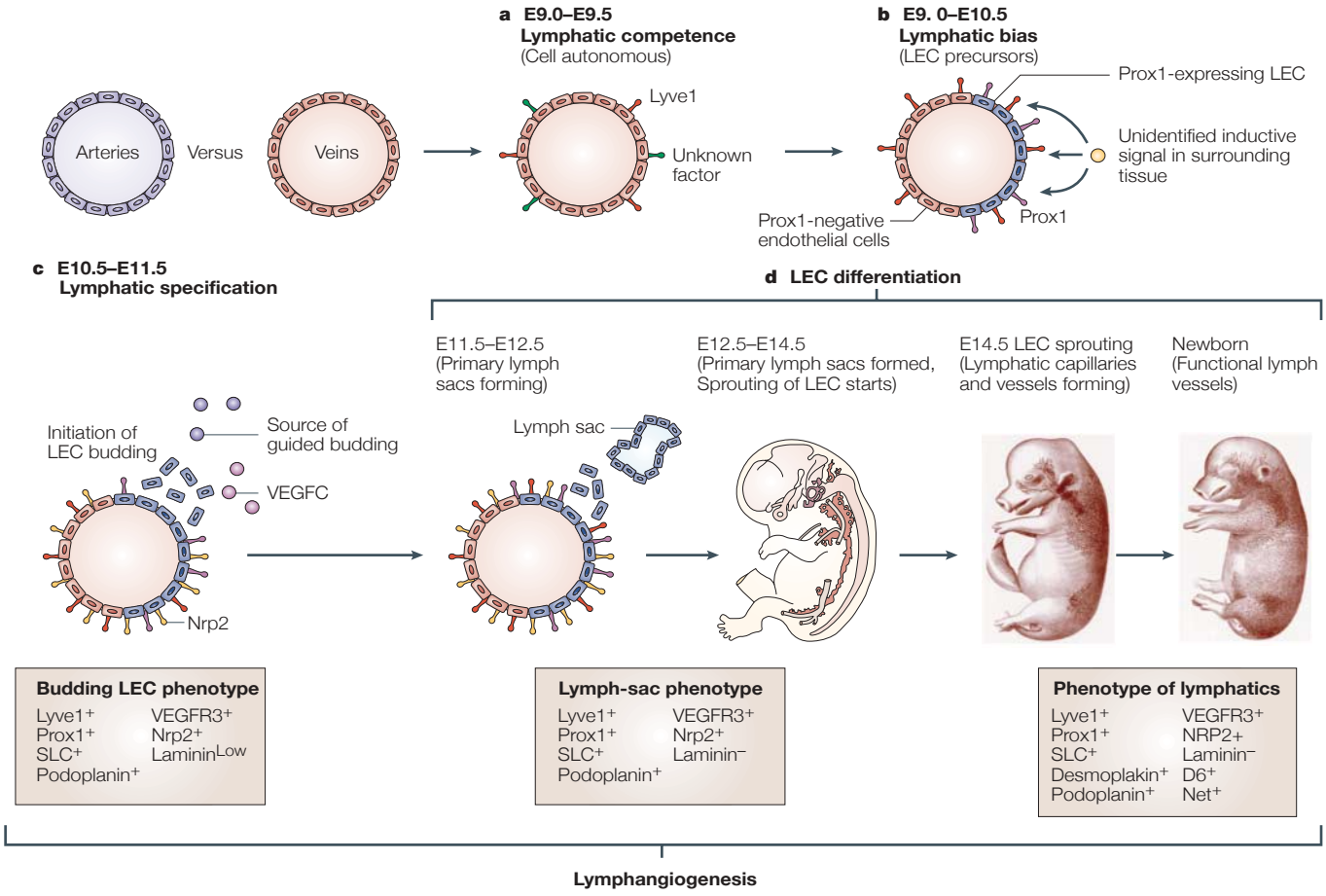

Figure 4 | **Model for differentiation of the lymphatic vasculature.** The proposed four-step model for lymphatic vasculature formation is summarized in this scheme. **a** | Lymphatic endothelial cell (LEC) competence is the autonomous ability of venous endothelial cells to respond to a specific, inductive signal; this stage is characterized, for example, by the appearance or disappearance of a receptor or by different expression levels of certain transcription factors. Competence is not a passive state, but an actively acquired condition that occurs only in venous endothelial cells; arterial endothelial cells should not respond, even if exposed to the inducer. **b** | LEC bias, or an intermediate state of bias towards LEC determination, is eventually lost in other venous endothelial cells that are not exposed to the inductive signals. Expression of prospero-related homeobox 1 (Prox1) might be crucial for this step. **c** | LEC specification occurs when biased venous endothelial cells differentiate independently from the regional context; for example, the anterior cardinal vein should give rise to LECs, even if transplanted to another part of the embryo. **d** | Finally, lymphatic vessel differentiation and maturation occurs in a stepwise manner leading to synthesis of all of the main lymphatic vessel components. BEC, blood vascular endothelial cell; D6, β-chemokine receptor D6; E, embryonic day; Lyve1, lymphatic vessel endothelial hyaluronan receptor 1; Nrp2, neuropilin 2; SLC, secondary lymphoid chemokine; VEGFC, vascular endothelial growth factor C; VEGFR3, vascular endothelial growth-factor receptor 3.

Specification of lymphatic endothelial cells. Specification is the stage at which cells will differentiate into the desired phenotype, even if isolated and cultured alone[18]. Lymphatic bias and specification are closely linked. Specification of LECs is the first step towards final lymphatic vascular differentiation. During this stage, the expression of more lymphatic markers begins whereas the expression of some blood vascular markers (for example, CD34 and laminin) progressively decreases[7]. A clear morphological indication of

Table 1 | **Genes expressed and/or required during lymphatic vasculature formation**

Gene	Expression	Animal model	References
Angiopoietin 2 (*Ang2*)	Smooth muscle cells of large arteries, large veins and venules. Expression is induced by the endothelium of smaller vessels at sites of vascular remodelling.	*Ang2* $^{-/-}$ mice have defects in postnatal angiogenesis and lymphatic patterning, chylous ascites and peripheral oedema. They die within two weeks of birth. In *Ang2* $^{-/-}$ embryos, larger lymphatic channels have structural defects, and the smaller lymphatic vessels show abnormal patterning.	49,71,72
β-chemokine receptor D6 (*D6*)	Expressed by a subset of mature lymphatic vessels of the skin, intestine and lymph nodes.	Not available.	73
Desmoplakin (*Dsp*)	Desmosome-associated transmembrane glycoprotein, expressed by mature lymphatic vessels, epithelial cells and cardiac muscle, but not by blood vessels. It attaches intermediate filaments to the plasma membrane of epithelial cells.	Not available.	74–76
FoxC2	Paraxial, presomitic mesoderm and developing somites during mouse embryogenesis. Expression later becomes restricted to condensing mesenchyme of the vertebrae, head, limbs and kidney.	Most *FoxC2*$^{-/-}$ mice die prenatally and have cardiovascular defects, such as interrupted aortic arch, but some survive to birth, with many craniofacial and vertebral column defects. Adult heterozygote mice have generalized lymphatic-vessel and lymph-node hyperplasia and uniformly displayed distichiasis. In humans, *FOXC2* mutations cause lymphoedema–distichiasis, but could also be involved in other lymphoedema syndromes.	60,61,77
Lyve1	Embryonic and adult LECs (detected as early as E9.0 in the anterior cardinal vein and budding LECs), macrophages, liver and spleen sinusoids.	No obvious lymphatic-vasculature alteration	19,20
Net	A member of the Ets-domain transcription factor family and the ternary complex factor family; Net represses immediate early genes *in vitro* through serum response elements. Expressed by the embryonic and adult vasculature: LECs and muscle layer in the thoracic duct, intestine and skin lymphatic vessels in midgestation.	*Net*$^{-/-}$ mice die soon after birth due to accumulation of chyle in the thoracic cage (chylothorax). They have dilated lymphatic vessels as early as E16.5; indicating that Net is involved in lymphatic-vasculature formation.	78,79
Neuropilin 2 (*Nrp2*)	Embryonic and adult LECs (detected as early as E10.0 in the anterior cardinal vein and budding LECs). Floor plate and ventral spinal cord, dorsal root and sympathetic ganglia.	*Nrp2*$^{-/-}$ mice show absence or severe reduction of small lymphatic vessels and capillaries during development.	29
Podoplanin (*T1α*)	Embryonic and adult LECs (detected as early as E11.0 in budding LECs), kidney podocytes, osteoblasts, lung alveolar cells, epithelial cells of the choroid plexus.	Podoplanin$^{-/-}$ mice have lymphatic defects associated with diminished lymphatic transport, congenital lymphoedema and dilation of lymphatic vessels.	34,34,80
Prox1	Embryonic and adult LECs (detected as early as E9.5 in the anterior cardinal vein), hepatocytes, heart, CNS, lens, pancreas and retina.	Non-surviving *Prox1*$^{+/-}$ mice have chylous ascites and oedema. *Prox1*$^{-/-}$ are embryonic lethal at E14.5, and completely lack lymphatic vasculature	7,24,81–83
Secondary lymphoid chemokine (*SLC*)	Embryonic and adult LECs (detection starts at ~E10.5 in budding LECs), T-cell zones in lymph nodes and lymphoid tissues.	Not available.	7,84,85
VEGFR3	BECs and LECs early in development, downregulated by BECs, but remain high in LECs later during embryogenesis (~E10.5) and in the adult, tumour blood vessels.	*VEGFR3*$^{-/-}$ embryos are lethal at E9.5 and have defective blood-vessel development, leading to cardiovasculature defects. *Chy* (lymphoedema) mutant mice have inactivating mutations in *VEGFR3*. Mutations in *VEGFR3 are* seen in primary human lymphoedema (Milroy disease).	7,30,31,37, 47,53,86
VEGFC	Mesenchymal cells around embryonic veins, weakly in the spinal cord, lymph nodes, heart, lung, kidney, placenta, skeletal muscle and ovary.	Transgenic mice, generated by overexpression in the skin under the human keratin-14 promoter, develop hyperplastic lymphatic vessels. Adenovirus injection into mouse ear skin promotes lymphangiogenesis. Virus-mediated gene therapy through the generation of functional lymphatics in lymphoedema (*Chy*) mice.	39,41,42, 44,45

BEC, blood vascular endothelial cell; CNS, central nervous system; E, embryonic day; *FoxC2*, forkhead box C2; LEC, lymphatic endothelial cell; *Lyve1*, lymphatic vessel endothelial hyaluronan receptor 1; *Prox1*, prospero-related homeobox 1; *VEGFC*, vascular endothelial growth factor C; *VEGFR3*, vascular endothelial growth-factor receptor 3.

lymphatic specification is the budding of immature LECs from mouse veins on E10.5 (FIGS 5b, 6). The initiation of LEC budding is independent of Prox1 function[7,8] and is the first morphological indication that lymphangiogenesis has begun. At E10.5, both Prox1 and Lyve1 are expressed by venous endothelial cells and budding LECs (FIGS 4, 5, 6).

In mice, LECs that bud from the anterior cardinal vein can be observed as late as E12.5, when the primary lymph sacs become apparent. A typical characteristic of the lymphatic capillaries is their lack of a continuous basement membrane. From around E10.5–11.0, expression of extracellular matrix components laminin or collagen IV starts to decrease in the budding LECs[7].

Neuropilin 2 (Nrp2) (TABLE 1) mediates axonal guidance during neuronal development[27]. Nrp proteins also bind various vascular endothelial growth factors (VEGFs) and have a role in VEGF–VEGFR signalling[28]. At around E10.0, uniform Nrp2 expression is detected in the anterior cardinal vein, in the budding LECs and in the surrounding mesoderm[29] (N. Harvey and G.O., unpublished observations). *Nrp2*$^{-/-}$ mice show an absence or severe reduction in the number of small lymphatic vessels and capillaries — a phenotype that is associated with reduced DNA synthesis in the LECs[29].

VEGFR3 (also known as Fms-like tyrosine kinase 4, Flt4) (TABLE 1) was one of the first lymphatic markers associated with lymphatic differentiation[30]. VEGFR3 binds the secreted glycoproteins VEGFC and VEGFD. Early during mouse development, VEGFR3 is widely expressed by both the blood and lymphatic endothelia, including the anterior cardinal vein[30–32] (FIG. 4). This expression is subsequently downregulated in blood vasculature and becomes mostly restricted to the lymphatic endothelium during late development (FIG. 6c) and adulthood[7,30]. The pattern of expression of VEGFR3 and its ligand VEGFC (see later) in developing mice has provided additional support for Sabin's model.

The number and distribution of budding LECs increases markedly between E10.5 and E12.5. From around E11.0, expression of the cell-surface mucin-type glycoprotein T1α/podoplanin[33–35] also starts to be detected in the budding LECs (FIG. 4). In addition, podoplanin-deficient mice have lymphatic defects associated with diminished lymphatic transport, congenital lymphoedema and dilation of lymphatic vessels[35].

At E12.5, the number of budding Prox1^{+}Lyve1^{+} Nrp2^{+}podoplanin^{+}VEGFR3^{+} LECs adjacent to the anterior cardinal vein has clearly increased[7]; expression of secondary lymphoid chemokine (SLC) is now also evident in these cells[7], but expression of blood vascular markers, such as CD34 and laminin, is not seen[7,8,17] (FIG. 4); levels of VEGFR3 expression by blood vascular endothelial cells are now reduced[7,30,36]. So, the maintenance of high levels of VEGFR3 and the expression of podoplanin and SLC by the budding Prox1^{+}Lyve1^{+}Nrp2^{+} immature lymphatic endothelial cells indicates that, at E12.5, the budding venous endothelial cells are now specified into the lymphatic programme. Blood vascular markers are mostly undetectable in those budding cells[7,8,17] and primary lymph sacs appear along the embryo at around this stage (FIGS 3d, 5c).

The expression of Prox1 might maintain VEGFR3 expression by LECs during lymphangiogenesis. Unfortunately, functional inactivation of VEGFR3 causes cardiovascular failure and defects in the remodelling of the primary vascular networks; therefore, these embryos die before the lymphatic vessels emerge[31]. Nonsense mutations in *VEGFR3* have been identified in patients with hereditary lymphoedema[37]. Together, these findings show that VEGFR3 regulates the development of the blood vascular and the lymphatic vascular systems[38].

Further support for the importance of the VEGFR3-signalling pathway during lymphatic development is provided by studies of its ligand VEGFC (TABLE 1), which was the first lymphangiogenic growth factor to be identified[39]. Although no functional inactivation of this ligand has been reported, much evidence supports an important role for this growth factor during lymphatic development. VEGFC was identified as a potent inducer of lymphatic sprouting[40].

VEGFC is mainly expressed in mesenchyme of areas where embryonic lymphatics are developing[39]. Recombinant VEGFC promotes lymphangiogenesis in the chick chorioallantoic membrane[41]. Overexpression of VEGFC in the skin of transgenic mice results in hyperplasia of cutaneous lymphatic vessels[42], and overexpression of VEGFC through the use of a recombinant adenovirus promotes lymphangiogenesis in adult mouse skin[43]. *VEGFC* gene transfer to the skin of mice with lymphoedema induces regeneration of the cutaneous lymphatic vessel network[44]. In addition, VEGFC stimulates blood vascular angiogenesis in the mouse cornea *in vivo* — a result that is probably due to an interaction with VEGFR2, which is expressed by blood vessels[45]. VEGFC also stimulates the migration and proliferation of endothelial cells[46] and promotes tumour lymphangiogenesis[5]. VEGFD, another VEGFR3 ligand, also stimulates lymphangiogenesis in the skin of transgenic mice[47]. Both VEGFC and VEGFD stimulate angiogenesis and lymphangiogenesis, depending on whether they are bound to VEGFR2 (mainly an angiogenic receptor) or VEGFR3 (mainly a lymphangiogenic receptor)[48].

Differentiation and maturation of lymphatic vessels and lymphangiogenesis. The final stage — differentiation — results from an induction event promoted by a nearby structure[18]. At around E14.5, the lymphatic vasculature has spread throughout the developing embryo (FIG. 5d) by budding and sprouting from the primary lymph sacs, and the immature LECs are in the process of terminal differentiation. As differentiation and lymphangiogenesis progress, additional lymphatic markers are expressed by the forming lymph vessels and capillaries.

As proposed earlier, it is probable that the differentiation of the lymphatic vasculature is a stepwise process that occurs over several days[17]. Therefore, the detection, as well as the level of expression, of some of the markers

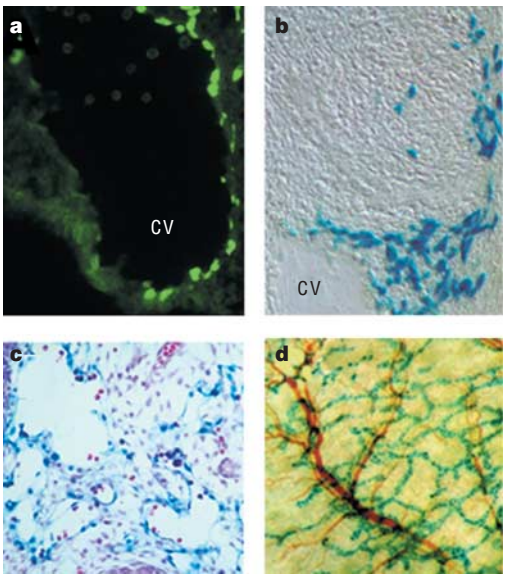

Figure 5 | **Prox1 is a specific marker for lymphatic endothelial cells. a** | At embryonic day (E) 9.5, prospero-related homeobox 1 (Prox1) expression (green fluorescence) is polarized — that is, it is detected only on endothelial cells that are located on one side of the anterior cardinal vein. **b** | At E10.5, polarized budding of lymphatic endothelial veins from the anterior cardinal vein is evident (blue X-gal-stained lymphatic endothelial cells (LECs) in *Prox1*$^{+/-}$ embryos). **c** | At around E12.5, Prox1 expression (blue) is observed in the developing primary lymph sacs. **d** | At E16.5, an extensive network of Prox1-expressing lymphatic capillaries and vessels (blue) is intermingled with the blood vasculature. Images reprinted, with permission, from REF. 24 © Elsevier (1999). CV, anterior cardinal vein.

on the differentiating lymphatics depends on the developmental stage of the embryo, the type of tissue being analysed or both. Only near the time of birth do lymphatic vessels express the complete profile of markers that are found in adult lymphatics. As previously mentioned, expression of podoplanin and SLC starts to be detected at around the time that LECs begin to bud from the anterior cardinal vein. Expression of additional markers, such as desmoplakin and β-chemokine receptor D6, later in embryogenesis (FIG. 4 and TABLE 1) is probably an indicator of final stages of lymphatic differentiation/maturation.

Although expression of angiopoietin 2 (Ang2) by embryonic lymphatic vessels has not yet been reported, recent data have indicated that its function is required for proper remodelling and maturation of the lymphatic vasculature[49]. Ang2 activates the endothelial cell-specific Tie2 receptor on some cells and blocks it on others. Ang2 might regulate vascular remodelling by participating in vessel sprouting and vessel regression[49]. Members of the VEGF and Ang2 families are partners during development of the blood vasculature[50,51]. Functional inactivation of Ang2 indicated that this molecule is required for postnatal blood vascular remodelling and proper development of the lymphatic vasculature[49]. According to the authors, normal lymphatic development requires local expression of Ang2 to act on Tie2 receptors in the lymphatics[49]. In addition, it was suggested that VEGFC and VEGFD require Ang2 to form functional lymphatics through remodelling and maturation of the lymphatic vessels. Interestingly, replacement of Ang2 by Ang1 was sufficient to rescue the lymphatic phenotype[49]. Therefore, the growth factor–receptor signalling pathways (Ang1/Ang2 through Tie2; VEGFs through VEGFRs and Nrp co-receptors) could regulate the growth of blood and lymphatic vessels.

It can be speculated that defects in later stages of lymphatic development or in lymphatic patterning could also have an earlier blood vasculature origin, if the activity of a gene product has a non crucial, but important, function in blood vascular endothelial cells. For example, precursor venous endothelial cells that lack the functional activity of such a gene could presumably still become specified into an LEC phenotype; however, alterations in the characteristics of mutant venous endothelial cells might give rise to lymphatic vessels with disrupted structure, patterning or both.

Blood and lymphatic vasculature separation

As lymphatic vessels and capillaries spread along the embryo, they must remain separated from pre-existing blood vessels. The adaptor protein SLP76 (SRC homology 2-domain-containing leukocyte protein of 76 kDa) and the tyrosine kinase SYK — two molecules that are mainly expressed by haematopoietic cells — are involved in the separation of the blood and lymphatic vasculature[52]. Mice that have mutations in these molecules have abnormal blood–lymphatic connections, embryonic haemorrhage and arteriovenous shunting. Expression of these gene products by haematopoietic-cell- or bone-marrow-derived endothelial progenitor cells mediates the separation of these two systems. This idea was supported by experiments in which *Slp76*-mutant bone marrow was transplanted into lethally irradiated wild-type mice. The intestines of the transplanted mice failed to regenerate lymphatic vessels and blood vessels; instead, they developed blood-filled mesenteric lymph vessels[52]. How SLP76- and SYK-signalling pathways mediate the separation of these two vascular systems remains unknown. In adults and during vascular regeneration, SYK signalling might repress the junction between the lymphatic and blood vasculature. At the same time, other unknown mechanisms must maintain the proximity between the developing lymphatic vessels and the pre-existing blood vessels.

Normal and pathological lymphangiogenesis

In addition to its important role during embryonic development, lymphangiogenesis is an essential feature of tissue repair and inflammation in most organs. Angiogenesis supplies oxygen and nutrients to the regenerating tissue, and transient lymphangiogenesis probably occurs concurrently[53]. After blood capillaries appear, lymphatic capillaries sprout from pre-existing lymphatics, but do not anastomose with blood capillaries[14,53]. Do lymphatic vessels also grow *de novo* from lymphangioblast cells, or do they grow *de novo* through

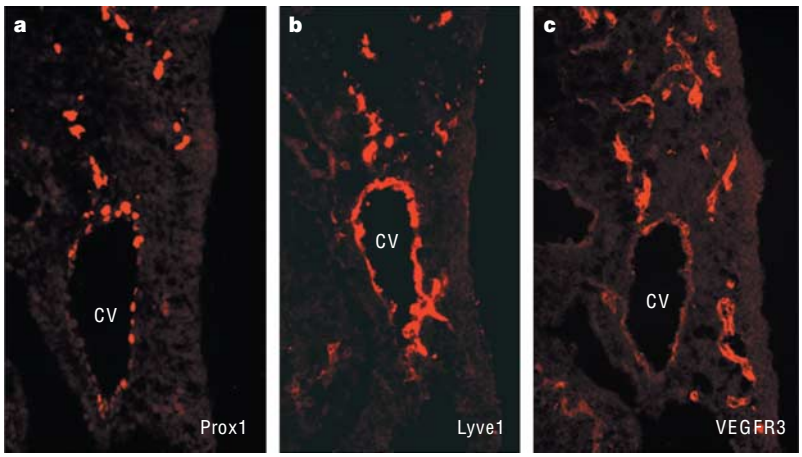

Figure 6 | **Specification of lymphatic endothelial cells. a** | At around embryonic day (E) 10.0–10.5, lymphatic endothelial cells (LECs) begin to bud from the anterior cardinal vein in a polarized manner. Prospero-related homeobox 1 (Prox1) (red fluorescence) is expressed by a subpopulation of venous endothelial cells and by the budding LECs. **b** | In an adjacent section, lymphatic vessel endothelial hyaluronan receptor 1 (Lyve1; red fluorescence) is uniformly expressed by venous endothelial cells and budding LECs. **c** | At this stage, the expression of vascular endothelial growth-factor receptor 3 (VEGFR3; red fluorescence) is downregulated by the venous endothelial cells of the cardinal vein, but is maintained by the budding LECs. Images reprinted, with permission, from REF. 7 © Oxford University Press (2002).

the budding of LECs from newly formed blood vessels? In the future, use of the newly available lymphatic-specific markers should allow us to address some of these important questions.

Disorders of the lymphatic system. Congenital or acquired dysfunction of the lymphatic system can result in the formation of lymphoedema[2] — a disorder that results in thickening of the skin and accumulation of adipose tissue. Primary lymphoedema has a genetic origin; however, secondary lymphoedema arises as a consequence of surgery, infection (for example, filiariasis, which is caused by parasitic infection) or radiation therapy (particularly after the treatment of breast cancer)[2]. Finally, lymphoedema can be present at birth. For example, Milroy disease — a form of primary (inherited) lymphoedema — is characterized by the absence or reduced number of lymphatic vessels[54,55].

Recently, specific genetic defects were identified in hereditary diseases associated with lymphatic hypoplasia and dysfunction (that is, primary lymphoedemas). Mutations in *VEGFR3* cause congenital hereditary lymphoedema[37,56,57]. Mutations in this gene are also responsible for the mutant mouse strain *Chy*, which has defective lymphatic vessels[58]. This model has been used to test targeted gene therapy; adenoviral expression of VEGFC promoted the formation of functional lymphatic vessels in these mice[58]. Recombinant VEGFC was also used to promote therapeutic lymphangiogenesis in a rabbit model of acquired lymphoedema[44] and plasmid-DNA-mediated gene transfer was also successfully used in a similar model of secondary lymphoedema[59]. Similarly, mutations in the forkhead transcription factor *FOXC2* have been identified in patients with lymphoedema–ditichiasis syndrome[60]. This disorder is characterized by distichiasis (a double row of eyelashes) at birth and bilateral lower limb lymphoedema at puberty[61]. Recently generated *FoxC2* heterozygous mice[61] could be a useful animal model for this disorder.

Finally, mutations in the transcription factor *SOX18* were recently identified in recessive and dominant forms of hypotrichosis–lymphoedema–telangiectasia[62].

Role of the lymphatic system and lymphangiogenesis in metastasis. During cancer progression, metastatic spreading of malignant cells occurs through the lymphatic and blood vessels; however, the manner by which tumour cells enter the lymphatic system is not clear. Using lymphatic-specific molecular markers, many groups have shown that tumour cells activate peritumoral and intratumoral lymphangiogenesis[3,5–7,63,64]. It remains controversial whether metastasizing cancer cells reach the lymph nodes through intratumoral lymphatics. Some recent findings indicated that the presence of functional lymphatics around the tumour is sufficient for lymphatic metastasis and that those tumours do not contain intratumoral lymphatics[65].

The induction of tumour lymphangiogenesis by VEGFC promotes breast cancer metastasis[5]. VEGFD induces the formation of intratumoral lymphatic vessels in a mouse tumour model, and VEGFD expression by tumour cells facilitates the spreading of the tumour to lymph nodes[6]. VEGFR3 expression is upregulated by the endothelium of blood vessels in breast cancer, and VEGFC secreted by the intraductal carcinoma cells acts mainly as an angiogenic growth factor[66]. Increased expression of VEGFC by primary tumours correlates with the dissemination of tumour cells to regional lymph nodes, and VEGFC-induced lymphangiogenesis mediates tumour-cell dissemination[63]. Levels of expression of VEGFC by some types of primary tumour seem to correlate with the degree of lymph-node metastasis[3].

Concluding remarks

In conclusion, it can be speculated that during evolution, the addition of a single gene product into the pre-existing venous endothelial cells was sufficient to promote the subsequent determination of the lymphatic vascular phenotype. Recent data have indicated that the arterial or venous fate of endothelial cells could be determined even before circulation[67]. Recent work carried out in zebrafish has indicated that Notch signalling is required to promote arterial cell fate by repressing venous cell fate[67]. It could be argued that later during evolution, expression of Prox1 by venous endothelial cells was sufficient to initiate the programme leading to lymphatic endothelial cell-type specification. This possibility is supported by the fact that, so far, only the lack of Prox1 represses the whole programme of lymphatic differentiation; the lack of other lymphatic markers seems to affect only the later aspects of lymphangiogenesis.

Identifying additional components in the molecular pathway the leads to lymphatic vasculature differentiation is important for the advancement of this field. Lymphatic-specific markers will also allow us to

determine precisely the mechanisms that are involved in lymphatic vessel formation in adults during tissue regeneration, wound healing and tumour formation. These studies will help to determine the functional role of peritumoral and intratumoral lymphatics during tumour progression, and could eventually lead to the generation of anti-lymphangiogenic therapy. In addition, the generation of other animal models of lymphatic disorders will increase our understanding of the molecular mechanisms that underlie the normal and pathological formation of the lymphatic vessels and facilitate the development of gene therapy for patients suffering from lymphatic disorders.

Although great progress has been made during the past few years towards understanding the biology of the lymphatic system, important questions remain unanswered; are new lymphatic vessels and capillaries formed only by the proliferation of endothelial cells from pre-existing lymphatics, or can they also be formed from endothelial precursors, lymphangioblast cells or by endothelial budding from veins? Are lymphangioblast cells present in both mammalian embryos and adults? What signalling mechanisms guide the budding of LECs in embryos? What molecules and mechanisms are involved in the separation of the lymphatic and blood vessels, and which ones maintain their close proximity?

Information gathered during this new molecular era, combined with previously gained knowledge suggests that gene therapy to promote or inhibit lymphatic growth should become available in the near future.

1. Aselli, G. De Lacteibus sive Lacteis Venis, Quarto Vasorum Mesarai corum Genere novo invento Mediolani, Milano (1627).
2. Witte, M. H. *et al.* Lymphangiogenesis and lymphangiodysplasia: from molecular to clinical lymphology. *Microsc. Res. Tech*. **55**, 122–145 (2001).
3. Karpanen, T. *et al.* Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res.* **61**, 1786–1790 (2001).
4. Mandriota, S. J. *et al.* Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J*. **20**, 672–682 (2001).
5. Skobe, M. *et al.* Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Med*. **7**, 192–198 (2001).
This paper presents important results about the role of tumour lymphangiogenesis.
6. Stacker, S. A. *et al.* VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nature Med*. **7**, 186–191 (2001).
7. Wigle, J. T. *et al.* An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J*. **21**, 1505–1513 (2002).
The first report that budding venous endothelial cells are a default ground state and after expression of prospero-related homeobox 1 (Prox1) they acquire a lymphatic phenotype.
8. Oliver, G. & Detmar, M. The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. *Genes Dev.* **16**, 773–783 (2002).
9. Jussila, L. *et al.* Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res*. **58**, 1599–1604 (1998).
10. Skobe, M. *et al.* Vascular endothelial growth factor-C (VEGF-C) and its receptors KDR and flt-4 are expressed in AIDS-associated Kaposi's sarcoma. *J. Invest. Dermatol*. **113**, 1047–1053 (1999).
11. Kampmeier, O. F. *Evolution and Comparative Morphology of the Lymphatic System*. (Springfield, Illinois, Charles C. Thomas, 1969).
12. Sabin, F. R. On the origin of the lymphatic system from the veins, and the development of the lymph hearts and thoracic duct in the pig. *Am. J. Anat*. **1**, 367–389 (1902).
A remarkable, almost accurate, description of the mechanisms involved in lymphatic vasculature development carried out 100 years ago.
13. Sabin, F. R. On the development of the superficial lymphatics in the skin of the pig. *Am. J. Anat*. **3**, 183–195 (1904).
14. van der Putte S. C. The early development of the lymphatic system in mouse embryos. *Acta Morphol. Neerl. Scand.* **13**, 245–286 (1975).
15. Huntington, G. S. & McClure, C. F. W. The anatomy and development of the jugular lymph sac in the domestic cat (*Felis domestica*). *Am. J. Anat.* **10**, 177–311 (1910).
16. Schneider, M., Othman-Hassan, K., Christ, B. & Wilting, J. Lymphangioblasts in the avian wing bud. *Dev. Dyn*. **216**, 311–319 (1999).
17. Oliver, G. & Harvey, N. A stepwise model of the development of lymphatic vasculature. *Ann. NY Acad. Sci*. **979**, 159–165 (2002).
18. Grainger, R. M. Embryonic lens induction: shedding light on vertebrate tissue determination. *Trends Genet*. **8**, 349–355 (1992).
19. Banerji, S. *et al.* LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J. Cell Biol*. **144**, 789–801 (1999).
20. Prevo, R. *et al.* Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J. Biol. Chem*. **276**, 19420–19430 (2001).
21. Mouta-Carreira, C. *et al.* LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and downregulation in human liver cancer and cirrhosis. *Cancer Res*. **61**, 8079–8084 (2001).
22. Knudson, C. B. & Knudson, W. Hyaluronan-binding proteins in development, tissue homeostasis, and disease. *FASEB J*. **7**, 1233–1241 (1993).
23. Jackson, D. G., Prevo, R., Clasper, S. & Banerji, S. LYVE-1, the lymphatic system and tumor lymphangiogenesis. *Trends Immunol.* **22**, 317–321 (2001).
24. Wigle, J. T. & Oliver, G. Prox1 function is required for the development of the murine lymphatic system. *Cell* **98**, 769–778 (1999).
The first report of a gene product, targeted inactivation of which leads to embryos without any lymphatic vasculature.
25. Hong, Y. K. *et al.* Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev. Dyn.* **225**, 351–357 (2002).
26. Petrova, T. V. *et al.* Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J*. **21**, 4593–4599 (2002).
27. Chen, H. *et al.* Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* **19**, 547–559 (1997).
28. Soker, S. *et al.* Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor. *Cell* **92**, 735–745 (1998).
29. Yuan, L. *et al.* Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **129**, 4797–4806 (2002).
30. Kaipainen, A. *et al.* Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc. Natl Acad. Sci*. USA **92**, 3566–3570 (1995).
The first identification of a gene product with restricted expression in the lymphatic system.
31. Dumont, D. J. *et al.* Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* **282**, 946–949 (1998).
32. Karkkainen, M. J. & Alitalo, K. Lymphatic endothelial regulation, lymphoedema, and lymph node metastasis. *Semin. Cell Dev. Biol.* **13**, 9–18 (2002).
33. Wetterwald, A. *et al.* Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. *Bone* **18**, 25–32 (1996).
34. Breiteneder-Geleff, S. *et al.* Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am. J. Pathol.* **154**, 385–394 (1999).
35. Schacht, V. *et al.* T1α/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J*. **22**, 3546–3556 (2003).
36. Partanen, T. A. *et al.* VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J.* **14**, 2087–2096 (2000).
37. Karkkainen, M. J. *et al.* Missense mutations interfere with VEGFR-3 signalling in primary lymphoedema. *Nature Genet.* **25**, 153–159 (2000).
Identification that mutations in vascular endothelial growth-factor receptor 3 (*VEGFR3*) are responsible for certain forms of hereditary lymphoedema.
38. Jussila, L. & Alitalo, K. Vascular growth factors and lymphangiogenesis. *Physiol. Rev.* **82**, 673–700 (2002).
39. Kukk, E. *et al.* VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* **122**, 3829–3837 (1996).
40. Saaristo, A. *et al.* Lymphangiogenic gene therapy with minimal blood vascular side effects. *J. Exp. Med.* **196**, 719–730 (2002).
41. Oh, S. J. *et al.* VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev. Biol.* **188**, 96–109 (1997).
42. Jeltsch, M. *et al.* Hyperplasia of lymphatic vessels in VEGF-C transgenic mice. *Science* **276**, 1423–1425 (1997).
43. Enholm, B. *et al.* Adenoviral expression of vascular endothelial growth factor-C induces lymphangiogenesis in the skin. *Circ Res.* **88**, 623–629 (2001).
44. Szuba, A. *et al.* Therapeutic lymphangiogenesis with human recombinant VEGF-C. *FASEB J*. **16**, 1985–1987 (2002).
45. Cao, Y. *et al.* Vascular endothelial growth factor C induces angiogenesis *in vivo*. *Proc. Natl Acad. Sci.* USA **95**, 14389–14394 (1998).
46. Makinen, T. *et al.* Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J*. **20**, 4762–4773 (2001).
47. Veikkola, T *et al.* Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J*. **20**, 1223–1231 (2001).
48. Alitalo, K. & Carmeliet, P. Molecular mechanisms of lymphangiogenesis in health and disease. *Cancer Cell*. **1**, 219–227 (2002).
49. Gale, N. W. *et al.* Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Dev. Cell*. **3**, 411–423 (2002).
50. Sato, T. N. *et al.* Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376**, 70–74 (1995).
51. Suri, C. *et al.* Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171–1180 (1996).
52. Abtahian, F. *et al.* Regulation of blood and lymphatic vascular separation by signaling proteins SLP-76 and Syk. *Science* **299**, 247–251 (2003).
Intriguing results providing indication of genes and mechanisms that are involved in the separation of the blood and lymphatic vasculature.
53. Paavonen, K., Puolakkainen, P., Jussila, L., Jahkola, T. & Alitalo, K. Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am. J. Pathol*. **156**,1499–1504 (2000).

54. Milroy, W. F. An undescribed variety of hereditary oedema. *NY Med. J.* **56**, 503 (1892).
55. Meige, H. Dystophie oedematoeuse hereditaire. *Presse Med*. **6**, 341–343 (1898).
56. Irrthum, A. *et al.* Congenital hereditary lymphedema caused by a mutation that inactivates VEGFR3 tyrosine kinase. *Am. J. Hum. Genet.* **67**, 295–301 (2000).
57. Ferrell, R. E. *et al.* Hereditary lymphedema: evidence for linkage and genetic heterogeneity. *Hum. Mol. Genet*. **7**, 2073–2078 (1998).
58. Karkkainen, M. J. *et al.* A model for gene therapy of human hereditary lymphedema. *Proc. Natl Acad. Sci*. USA **98**, 12677–12682 (2001).
An important demonstration that gene therapy could eventually be achieved in patients with lymphoedema.
59. Yoon, Y. S. *et al.* VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. *J. Clin. Invest.* **111**, 717–725 (2003).
60. Fang, J. *et al.* Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema–distichiasis syndrome. *Am. J. Hum. Genet*. **67**, 1382–1388 (2000).
The authors identified forkhead box C2 (*FOXC2*) as a gene responsible for the hereditary lymphoedema –distichiasis syndrome.
61. Kriederman, B. M. *et al.* FOXC2 haploinsufficient mice are a model for human autosomal dominant lymphedema–distichiasis syndrome. *Hum. Mol. Genet*. **12**, 1179–1185 (2003).
62. Irrthum, A. *et al.* Mutations in the transcription factor gene SOX18 underlie recessive and dominant forms of hypotrichosis–lymphedema–telangiectasia. *Am. J. Hum. Genet*. **72**, 1470–1478 (2003).
63. Mandriota, S. J. *et al.* Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J*. **20**, 672–682 (2001).
64. Beasley, N. J. P. *et al.* Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res*. **62**, 1315–1320 (2002).
65. Padera, T. P. *et al.* Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* **296**, 1883–1886 (2002).
References 64 and 65 provide antagonistic points of view about the role of intratumoral lymphatics in tumour progression.
66. Valtola, R. *et al.* VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am. J. Pathol.* **154**, 1381–1390 (1999).
67. Lawson, N. D. & Weinstein, B. M. Arteries and veins: making a difference with zebrafish. *Nature Rev. Genet*. **3**, 674–682 (2002).
68. Gilbert, S. F. *Developmental Biology*. 7th edn (Sinauer Associates Inc., Massachusetts, 2003).
69. Szuba, A., Shin, W. S., Strauss, H. W. & Rockson S. The third circulation: radionuclide lymphoscintigraphy in the evaluation of lymphedema. *J. Nucl. Med*. **44**, 43–57 (2003).
70. Carlson, B. M. *Human Embryology and Developmental Biology*. 2nd edn (Mosby Inc., USA, 1999).
71. Stratmann, A., Risau, W. & Plate KH. Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am. J. Pathol.* **153**,1459–1466 (1998).
72. Zagzag, D. *et al. In situ* expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis. *Exp. Neurol*. **159**, 391–400 (1999).
73. Nibbs, R. J. *et al.* The β-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am. J. Pathol*. **158**, 867–877 (2001).
74. Virata, M. L., Wagner, R. M., Parry, D. A. & Green, K. J. Molecular structure of the human desmoplakin I and II amino terminus. *Proc. Natl Acad. Sci.* USA **89**, 544–548 (1992).
75. Schmelz, M., Moll, R., Kuhn, C. & Franke, W. W. Complexus adhaerentes, a new group of desmoplakin-containing junctions in endothelial cells: II. Different types of lymphatic vessels. *Differentiation* **57**, 97–117 (1994).
76. Ebata, N. *et al.* Desmoplakin as a specific marker of lymphatic vessels. *Microvasc. Res*. **61**, 40–48 (2001).
77. Winnier, G. E., Hargett, L. & Hogan, B. L. The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo. *Genes Dev*. **11**, 926–940 (1997).
78. Ayadi, A., Suelves, M., Dolle, P. & Wasylyk, B. Net, an Ets ternary complex transcription factor, is expressed in sites of vasculogenesis, angiogenesis, and chondrogenesis during mouse development. *Mech Dev*. **102**, 205–208 (2001).
79. Ayadi, A. *et al.* Net-targeted mutant mice develop a vascular phenotype and upregulate egr-1. *EMBO J*. **20**, 5139–5152 (2001).
80. Breiteneder-Geleff, S. *et al.* Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is downregulated in puromycin nephrosis. *Am. J. Pathol*. **151**, 1141–1152 (1997).
81. Wigle, J. T., Chowdhury, K., Gruss, P. & Oliver, G. Prox1 function is crucial for mouse lens-fibre elongation. *Nature Genet*. **21**, 318–322 (1999).
82. Sosa-Pineda, B., Wigle, J. T. & Oliver, G. Hepatocyte migration during liver development requires Prox1. *Nature Genet*. **25**, 254–255 (2000).
83. Dyer, M. A., Livesey, F. J., Cepko, C. L. & Oliver, G. Prox1 function controls progenitor cell proliferation and horizontal cell genesis in the mammalian retina. *Nature Genet*. **34**, 53–58 (2003).
84. Gunn, M. D. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl Acad. Sci.* USA **95**, 258–263 (1998).
85. Gunn, M. D. *et al.* Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J. Exp. Med.* **189**, 451–460 (1999).
86. Witmer, A. N. *et al.* VEGFR-3 in adult angiogenesis. *J. Pathol*. **195**, 490–497 (2001).
87. Yoffey, J. M. & Courtice, F. C. *Lymphatics, Lymph and the Lymphomyeloid Complex*. (Academic Press, London, 1970).

Acknowledgements

I thank J. Wigle and N. Harvey, whose outstanding work in my laboratory has been instrumental in the progress of many of the ideas presented here, J. Morgan and S. Rockson for help with the figures, N. Harvey for critical reading of the manuscript and A. McArthur for scientific editing. This work was supported by the National Institutes of Health, Cancer Center Support from the National Cancer Institute and the American Lebanese Syrian Associated Charities (ALSAC).

Competing interests statement

The author declares that he has no competing financial interests.

Online links

DATABASES

The following terms in this article are linked online to:

LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/

Ang1 | Ang2 | β-chemokine receptor D6 | desmoplakin | *FOXC2* | Lyve1 | Nrp2 | podoplanin | Prox1 | SLC | *SOX18* | Tie2 | VEGFC | VEGFD | VEGFR2 | VEGFR3

FURTHER INFORMATION

Lymphatic Research Foundation:

http://www.lymphatic research.org

Access to this interactive links box is free online.