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Lymphangiogenesis: *in vitro* and *in vivo* models

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ABSTRACT **Lymphangiogenesis, the formation of new lymphatic vessels from preexisting ones, is an important biological process associated with diverse pathologies, such as metastatic dissemination and graft rejection. In addition, lymphatic hypoplasia characterizes lymphedema, usually a progressive and lifelong condition for which no curative treatment exists. Much progress has been made in recent years in identifying molecules specifically expressed on lymphatic vessels and in the setting up of *in vitro* and *in vivo* models of lymphangiogenesis. These new tools rapidly provided an abundance of information on the mechanisms underlying lymphatic development and the progression of diseases associated with lymphatic dysfunction. In this review, we describe the common *in vitro* and *in vivo* models of lymphangiogenesis that have proven suitable for investigating lymphatic biology and the interactions occurring between lymphatic vessels and other cells, such as immune cells and cancer cells. Their rationales and limitations are discussed and illustrated by the most informative findings obtained with them.—Bruyère, F., Noël, A. Lymphangiogenesis: *in vitro* and *in vivo* models. *FASEB J.* 24, 000–000 (2010). www.fasebj.org**

Key Words: lymphatic endothelial cell · lymphedema · metastatic dissemination · graft rejection

The lymphatic vascular system plays a key role in tissue–fluid homeostasis, as a tissue-drainage system, and contributes to the immunosurveillance by providing a route for migrating cells. The terminal vessels of this network collect extravasated fluids, macromolecules, lymphocytes, and antigen-presenting cells from the tissues and return them to the blood circulation *via* larger collecting lymphatic vessels and the thoracic duct (1). Lymphatics are also essential for the absorption of long-chain dietary triglycerides and lypophilic compounds released in the intestine in the form of chylomicrons. Impairment of the lymphatic-transport capacity because of abnormal vessel development or damaged vessels causes stagnation of water and proteins in the interstitium and leads to lymphedema. Lymphangiogenesis, the formation of new lymphatic vessels, is associated with several pathological conditions, such as chronic inflammation (Crohn's disease, psoriasis) (2–4), renal or corneal graft rejection (5, 6), malignancies, and metastatic dissemination (1, 7).

The structure of the lymphatic system, the lymphatic endothelial cell (LEC) markers, and the molecular mechanisms underlying lymphatic system development and function in pathologies have been largely reviewed (1, 8, 9) and are beyond the scope of this review. In contrast to angiogenesis research, lymphatic vessel research was long hampered by the lack of valuable markers and experimental models. Lymphatic system identification in small animals, transgenic mouse generation, tool development and validation, and recent *in vitro* models of 2- and 3-dimensional (2-D and 3-D) endothelial cell culture pave the way for a new area in the field of lymphangiogenesis. Obviously, no single model is able to elucidate the entire process of lymphangiogenesis associated with various pathological situations. Although *in vivo* assays are obviously more relevant than *in vitro* assays, they are time consuming and expensive, and the part played by the inflammatory response in them renders interpretation of the results difficult. Therefore, *in vitro* models, under more controlled and defined conditions, are complementary to *in vivo* experiments.

The present review aims to help researchers in their experimental exploration of the lymphatic system. We describe the common *in vitro* and *in vivo* models of lymphangiogenesis that have proven valuable for studying this complex biological process. The different experimental systems are presented and discussed in order of increasing complexity, starting from primary monolayer cell cultures and progressing to *in vivo* studies. We critically examine their advantages and limitations and illustrate them with the pertinent information derived from them.

LEC CULTURES

Isolation methods

LECs are isolated from collecting vessels, such as the lymphatic thoracic duct, or lymphatic capillaries, primarily in dermal tissue. Gnepp *et al.* (10) isolated LECs from canine and human lymphatic thoracic duct by enzymatic digestion. Later on, this method was applied

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to bovine, porcine, and rat species, and has generated cells with morphological features consistent with those of intact lymphatic endothelia (11–16). However, because that enzymatic method yielded barely enough cells for molecular analyses (17), especially from rodents, LECs were immortalized by using *tsA58*-transgenic rats harboring a simian virus 40 (SV40)-mutant temperature-sensitive large T-antigen gene (18). To isolate LECs from lymphatic capillaries, adult or newborn human foreskin is the most widely used source.

Lacking known specific markers, LECs and blood endothelial cells (BECs) were initially separated by excluding BECs through the isolation of a nonvascularized area of skin (19). That approach led to the identification of several lymphatic cell surface markers (20). LEC selection is now obtained by immunopurification, either with fluorescence-activated cell sorting (FACS) or magnetic beads (21–25). CD31, the hyaluronan-binding protein, lymphatic vessel endothelial receptor-1 (Lyve-1), and podoplanin are antigens widely used for LEC isolation.

To overcome the limited growth potential of primary cells, cell populations can be transformed with the human telomerase reverse transcriptase (hTERT; human dermal LECs) and achieve an extended life span (26). These LECs were molecularly characterized by gene arrays under physiological conditions, treatment with vascular endothelial growth factor-C (VEGF-C), or after transfection with the transcription factor Prospero-related homeobox 1 gene (*prox-1*) (22, 23, 27, 28). Dermal LEC isolation has the advantage of providing many more cells than enzymatic digestion of the thoracic duct. However, the immunoselection methods often give rise to biased results in gene- and protein-expression profiles, depending on the antibodies used. Moreover, both methods (using the lymphatic thoracic duct or the dermis) isolate cells that need to be cultured in supplemented medium, and close attention must be paid to the growth factors added. Indeed, LECs can be contaminated by BECs expressing lymphatic markers, as was reported for interleukin-3-treated BECs, which express two lymphatic antigens (Prox-1 and podoplanin) (29, 30). Inversely, BEC cultures are often contaminated with LECs or differentiate into LECs (31).

Recently, different LECs were isolated from the microlymphatic vessels in various tissues. A rat mesenteric LEC line (RMLEC) was established and characterized according to its morphology, phenotypic stability, lymphatic marker presence, cell-adhesion molecule expression (32), molecular profile, and proliferative response (33). Yamaguchi *et al.* (34) reported the isolation and long-term culture of organ-specific LECs issued from transgenic mice expressing the SV40 tsA58 large-T antigen. Little information is available about the different morphological and functional properties of LECs issued from microlymphatic (initial) and macrolymphatic (collecting) vessels. Kawai *et al.* (35) reported heterogeneous immunohistochemical, genomic, and biological properties of initial and collecting LECs based on their comparison of human newborn dermal LECs and LECs derived from afferent collecting lymph vessels of the sentinel lymph nodes of breast cancer patients. However, further studies are needed to establish and clarify that suspected heterogeneity. A LEC-selection procedure avoiding the use of antibodies is based on LEC differentiation from embryonic stem cells (36), which can be achieved by adding growth factors (VEGF-C and VEGF-A) or by coculturing cells with OP9 mouse stromal cells (37–39).

LECs for cultures were also isolated from lymphangiomas, which are lesions induced by incomplete Freund's adjuvant injection into the peritoneal cavity of mice (40) or rats (41). Lymphatic vessels from this lesion are mechanically disrupted, and LEC is enzymatically isolated before seeding onto a 2-D substrate (42, 43) or being embedded in a fibrin gel for 3-D cultures (44). Lymphangioma-derived cells were used to generate two monoclonal antibodies enabling LECs and BECs to be distinguished (45). Once again, cell immortalization was achieved by isolating cells from SV40 *tsA58*-transgenic mice and inducing their large-T–antigen expression with interferon-γ (46). This system has the advantage of being applicable to knockout mice and thus to allow the comparison of LECs from animals deficient or not for one or another gene.

All these mammal LEC-isolation models have their own advantages and disadvantages. When isolated from nontransformed animals, only a limited number of cells are available, and immortalization can change their features. Moreover, the LEC cultures can suffer from dedifferentiation phenomena. Two elegant studies compared the gene-expression profiles of LECs and BECs directly isolated from tissues, with or without expansion in cell culture (31, 47). Transcriptional analyses of *ex vivo* and *in vitro* 2-D LEC and BEC cultures indicated that cell culture introduces substantial changes in gene expression. LECs do not always retain all lymphatic properties *in vitro*, for instance, the Lyve-1 expression or CCL21 chemokine production (48). Interestingly, in such culture systems, the inflammatory cytokines such as tumor necrosis factor (TNF-α) regulate the production of Lyve-1, chemokines, and adhesion molecules, including VCAM, ICAM, and E-selectin (48–50).

2-D LEC cultures

In most assays, LECs are seeded as monolayers on culture plates or onto the surface of matrix-coated plates. Such 2-D cultures are suitable to evaluate the effects of putative lymphangiogenic stimulators or inhibitors on specific LEC properties. While none of the 2-D cultures can undergo all steps of lymphatic vessel formation, all culture systems contribute to analyzing each step individually, using various assays of cell activities (*e.g.*, gene expression profiling), cell proliferation, apoptosis, adhesion, migration (wound scratch assay, boyden chamber assay), and morphogenesis (tu-

bulogenesis) (**Fig. 1**). These assays are reviewed in the context of angiogenesis research (51) and illustrated in a recent study on the lymphangiogenic effect of somatotropin (52) or Galectin-8 (53). LEC monolayer cultures have also proven suitable to explore the molecular mechanisms of transmigration across LECs (49). They have the advantages of achieving defined experimental conditions, using a relatively uniform endothelial cell population, and being easily quantifiable. With these models, studies on an individual gene or protein are applicable by up-regulating them with recombinant molecules or down-regulating them through a small interfering RNA approach, for instance.

3-D LEC cultures

LECs differentiated from human embryonic stem cells can be cultured easily as 3-D structures called embryoid bodies in 3-D matrix. These embryonic spheroids grown in a gel can then be grafted with fibroblasts into mice and should adhere to the mouse circulation under appropriate conditions (54, 55). These spheroids offer the opportunity to study the formation of the initial lymphatic system during embryonic stages, but their pertinence to the biology of mature lymphatic vessels remains to be proven. To mimic the *in vivo* context in which LECs are subjected to interstitial flow from the extracellular matrix, Ng *et al.* (56) improved capillary morphogenesis by introducing an artificial flow. Moreover, in the presence of matrix-binding VEGF, flow induced local VEGF gradients, which further improved capillarogenesis (57).

A novel model mimicking the formation of entire lymphatic capillaries was recently reported (58, 59). Small fragments of mouse lymphatic thoracic duct were embedded in a collagen gel and produced outgrowths of lymphatic vessels with a lumen. This model is suitable for screening lymphangiogenic factors but also for phenotyping transgenic mice. For example, this lymphatic-ring assay helped identify matrix metalloprotease-2 (MMP-2) as a key regulator of LEC sprouting (58). Such 3-D cultures of LECs from rat lymphatic ducts were described previously, but they had been contaminated by other vascular cell types (60). Indeed, we confirmed the cell population heterogeneity obtained with rat lymphatic thoracic duct (unpublished observations). In sharp contrast, when mouse lymphatics were used, sprouting cells were exclusively LECs (58). The advantages of the lymphatic-ring assay are that LECs are not preselected by immunoisolation and/or passaging; inflammatory complications are avoided, offering the possibility to investigate direct effects on LECs; and it is an accessible method to examine LEC biology in transgenic knockout or knock-in mice.

While 2-D cultures address separately the different steps of the vessel formation (see above), 3-D cultures bridge the gap between *in vitro* and *in vivo* assays and are available to follow the sprouting process and LEC morphogenesis (Fig. 1). The 3-D cultures in complex matrices of embryonic bodies (37) or LECs with interstitial flow (61) allow tube formation and are suitable for examining the formation of a complex plexus, similar to that observed during embryogenesis. The lymphatic-ring assay (58) appears as a potent tool for the study of pathological lymphangiogenesis, defined as the abnormal formation of new lymphatic vessels from preexisting ones.

Organotypic models

Ex vivo studies on excised vessels can also be undertaken to analyze functional features of larger lymphatic vessels. For example, the Rho–Rho kinase pathway was shown to be involved in lymphangiogenic myogenic tone (62). The pump activity was investigated on iliac afferent lymph vessels transferred into an organ chamber, where the duct was mounted on pipettes to ensure its perfusion. In this system, vessel permeability was evaluated through the endothelial barrier (63). Similarly, the lymphatic flow pathway of fluorescent substances was visualized in isolated rat lymph nodes (64) or the lymphatic thoracic duct (65, 66). Lymph nodes were also reconstructed *in vitro* by using a bioreactor that allows self-assembly of human lymphatic tissues, with reestablishment of immune-cell traffic (67). Such scaffold-engineering models also exist for studying angiogenesis; seeding endothelial cells in matrix gels that have open channels spanning the gels forms tubes. Over time, these endothelial tubes developed functional behaviors typical of microvessels *in vivo*, *e.g.*, functioning as a barrier and serving as a leukocyte-adhesion site (68, 69). Similarly, such systems are well engineered to study the physiological functions of larger lymphatic vessels, especially lymph pumping, which is highly specific to vessels with a muscle-cell layer (70).

IN VIVO ASSESSMENT OF LYMPHATIC VESSELS

Several models of lymphangiogenesis are available (**Fig. 2**). Most were designed to investigate this process in a particular physiological or pathological condition. Here, we consider the models according to their relevance in various situations: physiological processes, embryogenesis, and pathological lymphangiogenesis associated with genetic disorders, edema, or cancer. Particular attention has been paid to the tools used to visualize the lymphatic vessels.

Visualization of the lymphatic tree under physiological conditions

Lymphatic vessels are present in almost every vertebrate organ other than avascular tissues, such as the epidermis, cartilage, and cornea, and some vascularized tissues, such as the brain, bone marrow, and retina (71). Immunohistochemical labeling of specific LEC antigens is still the most widely used method to identify

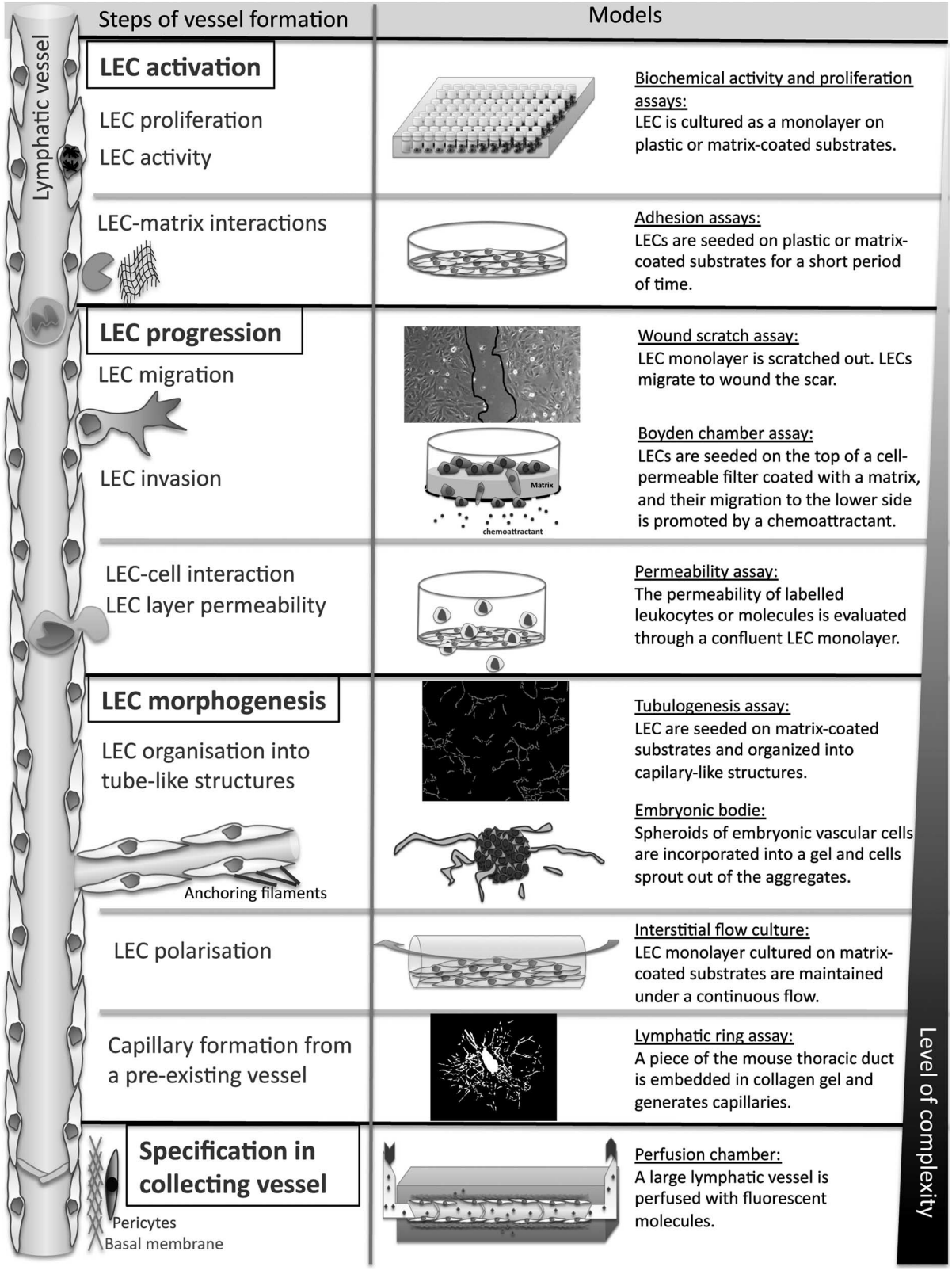

Figure 1. The different *in vitro* assays to unravel lymphangiogenesis. The level of complexity increases from 2-D cultures of LECs to lymphatic organotypic cultures. The 2-D cultures of LECs isolated from the different sources indicated in the text are powerful tools to study individually the different steps of lymphangiogenesis, *i.e.*, LEC proliferation, migration, invasion and morphogenesis (tubulogenesis). Cell morphogenesis can be improved under a continuous flow. The 3-D cultures, including the embryonic body and the lymphatic ring assay, are more appropriate models to dissect the mechanisms underlying the whole process of vasculogenesis or lymphangiogenesis. The perfusion of lymphatic organs or vessels are suitable for investigating vessel functionality.

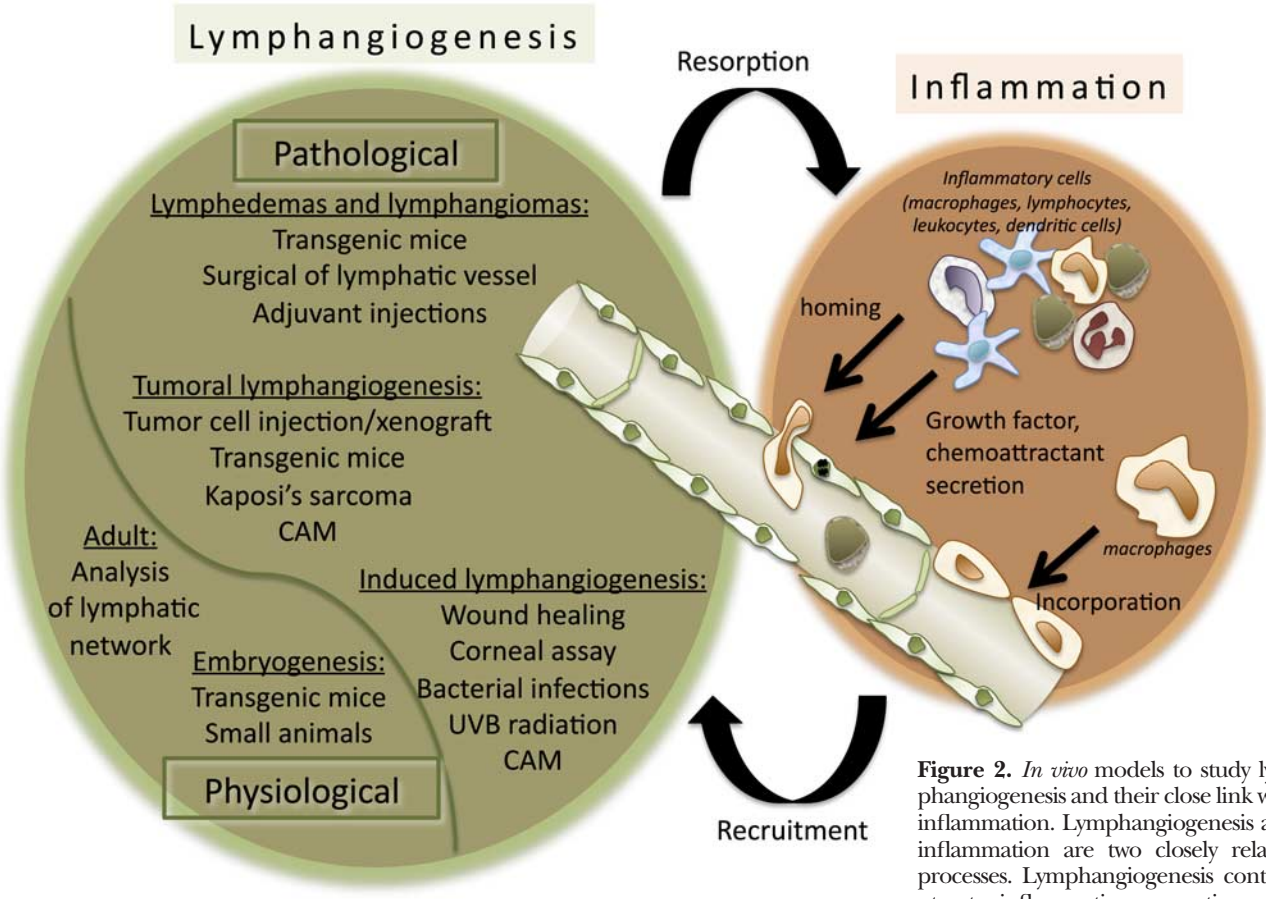

Figure 2. *In vivo* models to study lymphangiogenesis and their close link with inflammation. Lymphangiogenesis and inflammation are two closely related processes. Lymphangiogenesis contributes to inflammation resorption, and *vice versa*, inflammatory cells influence lymphangiogenesis through the secretion of stimulatory factors such as, at least, VEGF-C. Moreover, macrophages were reported to incorporate into the lymphatic vessel wall. The inflammatory cell recruitment *in vivo* has to be considered carefully in interpreting the results. The lymphatic network formed under physiological conditions (in adults or during embryogenesis) is independent of inflammation and can be assessed on tissue sections of, for instance, normal skin, gut, intestine, trachea and ears, using transgenic mice or small animals (*Xenopus* and zebrafish). In contrast, lymphangiogenesis induced in various pathological conditions is influenced by the presence of the inflammatory reaction. In these systems, one must pay attention to the putative indirect effects of inflammatory cells on lymphangiogenesis.

lymphatics. Among specific lymphatic markers recently reviewed (20), Lyve-1, Prox-1, podoplanin, and VEGF-receptor-3 (VEGFR-3) are the most frequent molecules examined. Under physiological conditions, lymphatic vessel densities and morphologies are routinely assessed after immunolabeling of tissue sections of normal skin, gut, intestine, trachea, and ears (58, 72–75).

As a complementary approach, lymphatic vessel functionality is assessed by exploiting the capacity of lymphatics to absorb fluids and molecules according to hydrostatic and oncotic pressure gradients. After subcutaneous injection, a macromolecule transits into the lymphatic network before reaching the blood circulation. The lymphatic vessels and lymph nodes drain Evans blue stain, a high-molecular-weight protein, within 15 min (76). Evans blue staining can be used to visualize lymph nodes and the thoracic duct in healthy mice (58), as well as in pathological lymphangiogenesis (74, 77). Similarly, fluorescence microlymphangiography (FITC–dextran) (78), can be used *in vivo* and is suitable to visualize the lymphatic tail network, for instance (79, 80). Lymphatic ferritin incorporation allows subsequent histochemical staining with Prussian blue (potassium ferrocyanide and HCl) detection of Fe^{III} (81). Other more complex and expensive approaches include microlymphographic procedures, such as radiolymphangiography scanning (82) or magnetic resonance lymphangiography (83). The injection of two different fluorescent quantum dots (nanometer-size particles) into two different animal loci (84, 85) allows the simultaneous analysis of the flows of two different lymphatic vessels. A new approach uses hyaluronic acid-conjugated quantum dots to visualize lymphangiogenesis in real time *in vivo* (86). Finally, the function of intestinal lymphatic vessels that transport absorbed fat to the liver can be exploited to observe lipid absorption using fluorochrome-coupled molecules (87, 88).

Embryonic lymphangiogenesis

The origin of lymphatic vessels is still controversial and the object of debate. The initial theory of lymphatic vessel origin, advanced by Florence Sabin and based on ink-injection experiments in pigs (89), supports the concept of centrifugal sprouting from the primary

lymph sacs that arise from embryonic veins (90). Investigation of pre- or postnatal lymphangiogenesis has primarily been based on the use of transgenic mice. For example, the homeobox gene *prox-1* is the main factor known to make part of the jugular vein commit to lymphatic development. Notably, Prox-1–deficient mice do not have a lymphatic network (91). Similar approaches demonstrated the crucial role of VEGF-C, among other factors, in the sprouting of the first lymphatic vessels from embryonic veins (92). Moreover, the injection of recombinant adenovirus-encoded soluble VEGFR-3–Ig into newborn mice on d 1–7 led to the almost complete absence of lymphatic vessels (71). Other lymphatic-gene mouse models are available, such as mice deficient for Net (93), podoplanin (94), neuropillin-2 (NRP-2) (95), angiopoietin-2 (Ang2) (96), integrin-α_9 (97), p85 subunit of PI3K (98), intracellular signaling protein Slp76, trisomy-16 (99), or vascular endothelial zinc finger-1 (Vezf1) (100) (reviewed in ref. 101). Another theory for the lymphatic vessel formation refers to a mesodermal origin of LECs and is sometimes combined with the vessel-sprouting origin (102). The avian chorioallantoic membrane (CAM) was used to support this concept of progenitor cell recruitment and to demonstrate its dependence to VEGF-C (103, 104).

New models in small animals were recently validated for the study of lymphangiogenesis. The *Xenopus laevis* tadpole was first described as having a lymphatic system only in 2005 (105), and the adult frog is known to have a lymphatic network with beating lymphatic hearts (106). Studies on the lymphatic network of this amphibian demonstrated that this system is dependent on the lymphangiogenic factors Prox-1 and VEGF-C, and showed the critical role of VEGFR-3 in embryonic lymphatic development and functions (105, 107). Kalin *et al.* (108) used this model for a chemical library screening approach, identifying 32 active compounds interfering with blood and/or lymphatic development. A year later, two independent groups reported the presence of a lymphatic system in zebrafish (109, 110). Zebrafish lymphatic vessels have characteristics similar to those of other species: conserved anatomy, morphological features (*i.e.*, anchoring filaments), conserved gene expression (Prox-1, VEGF-C, NRP-2, Ang2), and functional features (111). The role of VEGF-D in this vasculature was examined and compared to its role in blood-vessel angiogenesis (112). These small-animal models are valuable to dissect the molecular mechanisms underlying lymphatic vessel development.

Lymphedemas and lymphangiomas

Lymphedema is defined as tissue swelling, usually the skin, caused by the failure of proper lymphatic drainage that can be due to lymphatic hypoplasia or malformation, impaired lymphatic transport function, or obstruction of lymph flow (113, 114). Primary lymphedemas result from genetic defects, whereas secondary (acquired) lymphedemas are mainly caused by surgical removal or irradiation of lymph nodes, or by chronic infection (filariasis). Once again, the generation of transgenic animals targeting specific genes represents a potent tool to study hereditary lymphedemas.

A model of human Milroy's disease, *chy*-mutant mice are characterized by chylous ascites accumulation in their abdomens and express a heterozygous inactivating point mutation in *vegfr-3* (115). Crossing those *chy* mutants with *K14–vegf-*C_{156S} mice, which overexpress the VegfR-3–specific ligand Vegf-C_{156S}, led to the restoration of lymphatic function in the double-transgenic offspring (115). In such experiments, the lymphatic network and its functions were visualized either by fluorescence microscopy of the ears after intradermal injection of FITC–dextran or after Evans blue injection into deeper lymphatic vessels. Transcapillary fluid-balance parameters can also be assessed (116).

Transgenic mouse models with features of human lymphedema also generate transgenic overexpression of the soluble form of the Vegf-C or Vegf-D receptor (sVegfR-3) following the keratin 14 gene promoter that achieved regression of the lymphatic vessels in several organs, *i.e.*, the skin, and lymphedema (73). Forkhead box C2 (*foxc2*)-haploinsufficient mice have defective lymphatic recruitment of pericytes, the mechanism underlying the pathogenesis of the lymphedema-distichiasis syndrome (74, 117). Intriguingly, lymphatic vessels could be regenerated in these lymphatic system-defective mice by using adenovirus- or adenovirus-associated virus (AAV)-mediated transduction of *vegf-C* or its *vegfR-3*–specific point-mutant form (*vegf-*C_{156S}) (118, 119). Mice deficient in podoplanin (94), Net (93), integrin-α_9 (97), Vezf1 (100), Chy-3 (120), or with trisomy-16 (99) or heterozygous *vegf-C* (121), also suffer from edema (reviewed in refs. 101, 113).

Secondary lymphedemas can be experimentally induced in several ways. Some models ligate or cauterize deeper lymphatic trunks (79, 122, 123), where lymphatic vessel formation is then initiated along preestablished routes of fluid flow. Fluid channels form within the extracellular matrix, and then LECs migrate along these channels and organize to form a functional lymphatic network (124). In this system, hepatocyte growth factor (HGF) expression, induced by intramuscular plasmid injection, reduces lymphedema by promoting lymphangiogenesis (125). The lymphatic vessels are detected by several methods: fluorescence microlymphangiography with FITC-conjugated dextran (124, 126) or tetramethylrhodamine-conjugated dextran (127, 128), lymphoscintigraphy using ^{99m}Tc–sulfur colloid (123), or gadolinium-enhanced indirect magnetic resonance lymphangiography (83). In this model, *in vivo* bioluminescence imaging could visualize the traffic of immune cells from a transgenic mouse expressing the firefly *luc* under the control of the β-actin promoter (123). Similarly, experimental lymphedema was induced in rabbit ears (129–131) and reversed by supplying VEGF-C to the ear (132, 133). Lymphedema

could also be induced in rodents (rat or rabbit) by surgical removal of a groin lymph node, followed or not by regional irradiation (134–137). Lymphatic vessels were detected by scintigraphy, magnetic resonance imaging, protein refractometry or plain X-rays.

Lymphangiomas are benign malformations of the lymphatic system (138). They can be induced in mice (40) or rats (41) by injecting Freund's adjuvant. This induction, initially performed on a mouse's ear (139, 140), was first devised to isolate LECs, as mentioned above. It was also applied to challenge transgenic mice and evaluate the effect of a gene deficiency on the lymphangiogenic response (58, 88). However, it must be kept in mind that lymphangioma is accompanied by inflammation, which can bias the evaluation of lymphatic vessels.

Induced lymphangiogenesis

The wound-healing process is a complex cascade of events that relies on several mechanisms, including inflammation, angiogenesis, and lymphangiogenesis, which is enhanced by the injection of VEGF-C or VEGF-A (141, 142). Lymphatic vessels appear in the wound concurrently with blood vessels but regress earlier (143). Cicatrization was induced either on superficial areas, such as skin (141, 142), or on localized deeper loci requiring interruption of the popliteal or axillary prenodal lymphatic collector vessel (144). The removal of a circumferential band of the skin from the midpoint of the mouse's tail is a wound-healing model in which lymphangiogenesis can be readily studied (126, 145).

Under physiological conditions, the cornea is devoid of lymphatics (146, 147). Corneal lymphangiogenesis was observed during wound healing after thermal cauterization (148), suturing (149), or alkali injuries (150). Lymphatic vessels were recruited from the corneal limbus. Once again, this lymphangiogenic process appeared to be dependent on the VEGF-C–VEGFR-3 pathway and could be fully inhibited by a blocking antibody raised against the receptor without affecting angiogenesis (150, 151). It is worth noting that age-related changes in corneal lymphangiogenesis have been reported: Young mice had better lymphatic responses than older mice (152). The pertinence of this model is obvious in the context of inflammation and graft-rejection studies (148, 153, 154). Furthermore, the lack of vasculature within cornea was also exploited by grafting pellets releasing putative lymphangiogenic factors. Various growth factors [*e.g.*, fibroblast growth factor-2 (FGF-2), platelet-derived growth factor (PDGF), HGF, Ang1] were implanted individually in a corneal micropocket and were shown to enhance lymphangiogenesis (155–158).

Infection-induced lymphangiogenesis was assessed after infecting mice with *Mycoplasma pulmonis*, which led to inflammatory cell influx, angiogenesis, mucosal edema, epithelial changes, and fibrosis. In this model, lymphangiogenesis persisted in the airway mucosa, even after the resolution of inflammation and blood-vessel remodeling. This lymphangiogenesis appeared to be driven by VEGF-C and VEGF-D released from inflammatory cells (72). Similar effects were observed in a model of allergic airway inflammation induced by ovalbumin sensitization and challenge. In this system, the EphrinB2 implication was highlighted in lymphatic and blood vessel development (159).

Oxazolone-induced delayed-type hypersensitivity reaction in mouse ears triggers lymphatic vessel proliferation and enlargement, which might contribute to stronger inflammatory responses (4). These inflammatory tissues closely resemble human psoriasis, and VEGF-A increased lymphatic recruitment to this lesion and the draining lymph nodes (4, 160).

Ultraviolet-B irradiation of the skin resulted in prominent lymphatic vessel enlargement (161). These enlarged lymphatics were functionally impaired and more permeable and could be restored by the activation of the VEGFR-3 pathway through VEGF-C (162). Intriguingly, an inhibitor of the nitric oxide receptor (soluble guanylate cyclase) prevented irradiation-induced lymphatic vessel enlargement, skin inflammation, and edema (163).

Artificial lymph-node-like tissues could be generated by implanting biocompatible scaffolds with embedded stromal cells into the renal subcapsular spaces of mice (164). Like natural lymph nodes, they contained T, B, and dendritic cells and lymph vessels. Moreover, germinal centers developed in these artificial lymph nodes following immunization, as in intact lymphoid tissue organoids (165). This model is suitable to examine lymphangiogenesis inside lymph nodes, a process associated with pathological conditions, such as cancer dissemination (166, 167).

It is worth noting that all models cited here are associated with inflammation, and particular attention must be paid to the indirect effects that inflammatory factors could exert on lymphangiogenesis. For example, VEGF-A stimulates lymphangiogenesis in the corneal neovascularization assay through the recruitment and activation of macrophages, which are known to produce VEGF-C in this system too (149, 168).

Cancer lymphangiogenesis

Lymphatic metastases are well documented, but the understanding of lymphatic tumor dissemination is currently controversial. It is not clear yet whether tumor cells induce an active lymphangiogenesis, promote vessel hyperplasia, or passively enter the lymphatic circulation to drive tumor invasion into lymphatics (for reviews, see refs. 7, 169, 170). Several cancer models have aimed to address this important issue (113). Lymphangiogenesis is studied intensely in breast cancer or melanoma because lymphatic metastases often occur in patients with these malignancies. Carcinoma progression and lymphangiogenesis were assessed in an orthotopic mammary-tumor model using either MCF-7 or MDA-MB-435 cells overexpressing

VEGF-C (171–174) or 293EBNA cells overexpressing VEGF-D (175). VEGF-C expression induced more lymphatic vessel invasion inside the tumor and increased the number of metastases that were thereby inhibited by sVEGFR-3 or a kinase inhibitor. Lymphatic vessel functionality was revealed by Evans blue drainage.

Xenotransplantations of different types of cancer-cell lines, *e.g.*, fibrosarcoma cells (176) or colorectal cancer cells (177, 178), into orthotopic sites corroborated the importance of VEGF-C-mediated lymphangiogenesis in facilitating tumor spread. Indeed, subcutaneous injections of lung cancer (179), melanoma (176), or other tumor cells (77) into immunocompetent rats confirmed the involvement of the VEGF-C–VEGFR-3 pathway in tumor-induced lymphangiogenesis and metastatic spread. Moreover, vector-based expression of small-interfering RNA targeting VEGF-C and/or VEGF-A limited tumor lymphangiogenesis and reduced lymph node and lung metastases in a syngeneic model of mammary cancers (180). In addition, a complex dialogue between lymphatics and tumor cells was pinpointed. Chemotaxis for tumor cells can be induced by LECs through laminin-421 (181). Inversely, tumor cells can attract LECs through, at least, angiopoietin-1 (182). Tumor cell transplantation is also applicable onto the avian chorioallantoic membrane (CAM) for studying lymphangiogenesis (183, 184).

The use of syngeneic rat or mouse models has the great advantages of not using immunodeficient mice and taking into account the entire inflammatory response. Tumor lymphangiogenesis and lymph-node metastases were studied in the chemically induced skin carcinogenesis model of *K14-vegf-C* transgenic mice. Notably, lymphangiogenesis was induced in the sentinel lymph node by Vegf-C release and it promoted cancer metastasis beyond the sentinel lymph nodes (166, 167). These data support the emerging concept of the premetastatic niche (185, 186). New findings obtained with these tumor models include the capacity of PDGF-BB, among other factors, to induce the formation of intratumor lymphatic vessels; that ability was highlighted by several experiments including subcutaneous coinjection of human MCF7 cells and tumor-associated fibroblasts (187). A murine dorsal chamber model was also used to examine the lymphatic network of the skin. In this model, Vegf-C–overexpressing cells recruited functional lymphatic vessels within the chamber, as assessed by Evans blue drainage (188).

Transgenic (*Ripvegf-C*) mice, which express Vegf-C specifically in pancreatic β cells, were generated. They develop a lymphatic network around those β cells. These mice were crossed with a second transgenic (*Rip1Tag2*) strain that is known to develop nonmetastatic pancreatic β-cell tumors (189). These double-transgenic mice exhibited Vegf-C–induced lymphangiogenesis around the β-cell tumors and developed metastases to pancreatic and regional lymph nodes (190). Furthermore, the loss of neural adhesion molecule up-regulated the expression of lymphangiogenic factors and increased lymphangiogenesis, features that make this model a valuable tool resembling endogenous tumor lymphangiogenesis and lymph-node metastasis in immunocompetent mice (191). This model highlighted the contribution of integrin-β_1 to lymphangiogenesis (192).

Kaposi's sarcoma is a skin cancer associated with human herpesvirus-8 (HHV8), but its precise origin remains controversial. Kaposi's sarcoma expresses lymphatic markers, like podoplanin, VEGFR-3, and Prox-1, and the plasma levels of lymphangiogenic molecules are elevated in patients with such lesions (193, 194). Blood vascular endothelial cells infected with Kaposi's sarcoma-associated HHV8 seem to undergo lymphatic reprogramming (195). Symptoms include edema of the lower extremities that is treated with lymphedema therapy (196). Thus, this tumor may help to unravel some molecular mechanisms of a new lymphatic-associated pathology.

CONCLUSIONS

The lymphatic vessel system is an emerging field of research. It has long been overshadowed by research on angiogenesis because of the lack of markers to identify and distinguish lymphatic and blood endothelial cells. These tools are now available but the lymphatic circulation remains difficult to study because of the absence of appropriate culture models. These difficulties were overcome partially during the past few years by the development and validation of various 2-D and 3-D culture systems, and novel *in vivo* models in rodents and small animals. These new experimental models contributed to the rapid expansion of our knowledge on the mechanisms underlying lymphatic development and the diseases associated with lymphatic dysfunction. More information is still needed concerning the discrimination of the direct effects on lymphangiogenesis and inflammation-induced lymphangiogenesis, as both events are very closely related (Fig. 2). It is demonstrated that inflammatory cells can stimulate LECs by growth factor secretion and by a transdifferentiation of macrophages (168). The understanding of the molecular pathways that regulate lymphatic network formation, mostly in pathological conditions, and the discrimination between those that act directly on lymphatic endothelial cells and those modulating the inflammatory process are mandatory to pave the way to the development of new therapies for cancers, lymphedemas, and inflammation-related diseases (*e.g.*, psoriasis, graft rejection). FJ

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