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Understanding Lymphangiogenesis in Knockout Models, the Cornea, and Ocular Diseases for the Development of Therapeutic Interventions

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Abstract

A major focus of cancer research for several decades has been understanding the ability of tumors to induce new blood vessel formation, a process known as angiogenesis. Unfortunately, only limited success has been achieved in the clinical application of angiogenesis inhibitors. We now know that lymphangiogenesis, the growth of lymphatic vessels, likely also plays a major role in tumor progression. Thus, therapeutic strategies targeting lymphangiogenesis or both lymphangiogenesis and angiogenesis may represent promising approaches for treating cancer and other diseases. Importantly, research progress toward understanding lymphangiogenesis is significantly behind that related to angiogenesis. A PubMed search of ‘angiogenesis’ returns nearly 80,000 articles, whereas a search of ‘lymphangiogenesis’ returns approximately 2,635 articles. This stark contrast can be explained by the lack of molecular markers for identifying the invisible lymphatic vasculature that persisted until less than two decades ago combined with the intensity of research interest in angiogenesis during the past half-century. Still, significant strides have been made in developing strategies to modulate lymphangiogenesis, largely using ocular disease models. Here, we review the current knowledge of lymphangiogenesis in the context of knockout models, ocular diseases, the biology of activators and inhibitors, and the potential for therapeutic interventions targeting this process.

Keywords

VEGF-C; VEGFR-3; LYVE-1; prox-1; podoplanin; dry eye disease; corneal transplant; herpetic stromal keratitis; glaucoma; ocular tumor

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CONFLICT OF INTEREST

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1. INTRODUCTION

The lymphatic vasculature is responsible for collecting excess fluid and macromolecules from capillary beds, returning these elements to the blood circulation, and capturing and delivering antigens to lymph nodes to induce immunological responses.[1] Thus, lymphangiogenesis, the generation of new lymphatic vessels from pre-existing lymphatics, serves important functions during embryonic development and wound healing, but disruption of the fine balance between pro-lymphangiogenic and anti-lymphangiogenic factors can cause certain pathologies. For example, excess lymphangiogenesis can result in tumor metastasis[2; 3] and deficient lymphangiogenesis can lead to lymphedema. In the eye specifically, transmission of immunogenic stimuli from a corneal graft through both lymphatic and blood vessels can lead to graft rejection, and the presence of lymphatic vessels in the host prior to corneal transplantation has been shown to be a key predictor of poor outcome, thus demonstrating the importance of lymphatics in transplantation.[4; 5; 6; 7; 8] Because the identification and visualization of normally invisible lymphatic vessels was originally difficult, the contribution of these vessels in graft rejection was largely overlooked. However, the discovery of molecular markers for lymphatic vessels has helped advance lymphangiogenesis research in the past 20 years, including the recently discovery of a classical lymphatic drainage system in the central nervous system. [9]

Normally, the cornea lacks lymphatic vessels and can tolerate foreign antigens without mounting a systemic immune response, a concept termed “immune privilege.”[10; 11] This makes corneal transplant acceptance possible without human leukocyte antigen (HLA) matching.[12] The eye has an anterior chamber-associated immune deviation (ACAID) through which inflammatory and immune cells are naturally suppressed when foreign antigens are introduced into the anterior chamber, preventing a systemic immune response.[12] When a corneal graft is introduced, it forms the anterior surface of the anterior chamber, and through ACAID, rejection can be avoided.[12] However, in the presence of corneal neovascularization, the integrity of the ACAID is lost, and the risk associated with graft rejection increases significantly because immune privilege is no longer applicable.[13]

The normal adult cornea is both avascular and alymphatic, and it is, therefore, an ideal model for easily assessing both forms of vessel formation.[10; 11; 14] Lymphangiogenesis in the cornea occurs when lymphatic vessels either grow from pre-existing vessels in the limbus of the eye or form *de novo*.[15] Under hypoxic and inflammatory conditions, various members of the vascular endothelial growth factor (VEGF) family are released by inflammatory cells to stimulate both angiogenesis and lymphangiogenesis.[16; 17; 18] Elucidation of the mechanisms by which lymphangiogenesis occurs in the cornea can lead to the development of therapeutics targeted at reducing corneal neovascularization and may be extrapolated to the prevention of tumor cell metastasis in cancer patients.[19; 20; 21]

In this review, we first provide an overview of lymphatic markers and knockout models currently used to study lymphatic development and emphasize why the cornea serves as a great model for studying lymphangiogenesis. We also provide updated descriptions of the normal ocular surface anatomy, factors involved in regulating lymphangiogenesis, and

ocular diseases associated with lymphangiogenesis. Finally, we discuss strategies for modulating lymphangiogenesis in the context of disease.

2. CORNEAL LYMPHANGIOGENESIS AND ANGIOGENESIS

The cornea is normally avascular and serves as an ideal model in which to study both angiogenesis and lymphangiogenesis.[1; 4; 11; 15] However, in various ocular pathologies, [22] corneal angiogenesis and lymphangiogenesis are induced, and corneal transparency is lost. The presence of newly formed blood and lymphatic vessels induces leakage of proteins, lipids, and calcium within the cornea, which results in a reduction in visual acuity and increases the risk of graft rejection after corneal transplantation.[4; 5; 6; 7; 8] Prior to the discovery of lymphatic markers, corneal neovascularization was believed to only involve blood vessels, since the lymphatic vessels are biomicroscopically undetectable.[23] However, we now know that pathologic lymphangiogenesis is usually present with angiogenesis and occurs in the setting of an inflammatory insult directly to the cornea, overriding the angiogenic and lymphangiogenic privilege of the cornea.[24] Members of the VEGF family (VEGF-A, -B, -C, and –D) are the primary mediators of both angiogenesis and lymphangiogenesis. Lymphangiogenesis is primarily mediated by VEGF-C and VEGF-D binding to vascular endothelial growth factor receptor 3 (VEGFR-3) on lymphatic endothelial cells (LECs).[25; 26] Bone marrow-derived cells, such as macrophages, produce both VEGF-C and VEGF-D.[11; 27; 28] If inflammation occurs after corneal transplantation, macrophages will enter via the blood vasculature in response to cytokines and other mediators of inflammation, whereas antigens are transported by antigen-presenting cells to regional lymph nodes via the lymphatic vasculature.[28]

3. LYMPHATIC MARKERS

A major breakthrough in the study of lymphangiogenesis occurred with the introduction of lymphatic-specific markers that made lymphatic vessel visualization more accessible and facilitated significant scientific advancements. The ideal characteristic of a lymphatic-specific marker is its exclusive expression on LECs.[29; 30] However, this is rarely the case because many of the lymphatic-specific markers currently used are also expressed on certain nonendothelial cells. Thus, the most important feature is that these markers are not expressed on blood vessels and can be used to distinguish lymphatic vessels from blood vessels. A summary of lymphatic-specific markers is provided in Table 1.

3.1. LYVE-1

Banerji et al. discovered lymphatic vessel endothelial hyaluronan (HA) receptor 1 (LYVE-1), a member of the Link superfamily of HA-binding proteins, by searching the expression sequence tag database for cDNAs homologous to CD44, the only other major member of the Link superfamily of HA receptors.[31] Although both CD44 and LYVE-1 bind to HA, CD44 is not expressed on lymphatic vessels and is instead primarily located on blood vascular endothelial cells. In contrast, LYVE-1 is highly expressed on lymphatic vessels and serves as a lymphatic- specific marker.[31] LYVE-1 binds to and internalizes HA[31; 32] and is speculated to assist HA in its involvement with cell migration in the lymphatic system[33] and to transport HA to the liver or regional lymph nodes for degradation.[34] Platanova et al.

reported that LYVE-1 and FGF2 have a functional relationship in which LYVE-1 and FGF2 interaction inhibits FGF2-induced lymphangiogenesis and also prevents TNF-β–dependent down-regulation of LYVE-1.[35]

LYVE-1 mRNA has been detected in a variety of organs where its cellular expression pattern was analyzed to determine its status as a lymphatic-specific marker. An abundant amount of LYVE-1 mRNA was detected in the human spleen sinusoidal endothelium, lymph nodes, heart, lung, and fetal liver.[31; 36] LYVE-1 mRNA was also detected in smaller amounts in the human appendix, muscles, placental syncytiotrophoblasts, bone marrow, and adult liver sinusoidal endothelium.[31; 36] In the liver, LYVE-1 is expressed in both lymphatic and blood endothelial cells and cannot be used to differentiate lymphatic vessels from blood vessels.[37] Instead, prospero homeobox 1 (Prox-1) is present on liver lymphatic vessels, but not on liver blood vessels, and is a better lymphatic marker for the liver.[37] When different diseased states of the liver were examined, it was found that blood vessels in cirrhotic livers express less LYVE-1 than blood vessels in healthy livers, whereas blood vessels of hepatocellular carcinomas express no LYVE-1.[37] In both of these states, LYVE-1 expression in the liver lymphatic vessels remained the same.[37] Aside from its expression in the liver, LYVE-1 is confined to LECs in adult humans and mice and can overall be used as a lymphatic-specific marker.[31] LYVE-1 expression is most visible in the draining lymphatic vessels of the gastrointestinal system, the lacteals that drain intestinal villi, and the subdermal lymphatic vessels of the skin.[31] On the lymphatic vessel, LYVE-1 has a bipolar distribution and is present on both the luminal and abluminal surfaces of the lymphatic endothelium.[31] LYVE-1 and FGF2 have a bifunctional relationship in which FGF2 regulates LYVE-1 expression by reversing the TNF-β–dependent downregulation of LYVE-1 and increasing the overall expression of LYVE-1.[35] In contrast, LYVE-1 acts on FGF2 and inhibits FGF2-dependent lymphangiogenesis.[35] LYVE-1 expression in corneal lymphatics is variable, with an increase in the number of LYVE-1 absent gaps along corneal lymphatic vessels up until 8 weeks of age, when LYVE-1 expression begins to increase.[38] Nakao et al. reported that the LYVE-1 absent regions potentially serve as microvalves that facilitate unidirectional lymphatic flow and as immunological hot spots for stromal macrophage re-entry into the lymphatic vessels.[38]

LYVE-1 expression in corneal lymphatics is variable, with an increase in the number of LYVE-1 absent gaps in the lymphatic vessels from birth until week 8.

Through the detection of LYVE-1 with anti-LYVE-1 antibodies, the presence of lymphangiogenesis in the cornea,[15] inflammatory diseases,[39] and the skin via VEGF-C induction[40] has been established. The major advantages associated with using LYVE-1 as a lymphatic marker are its specificity and its use as a method to visualize lymphatic vessels *in vivo* through anti-LYVE-1 antibodies conjugated with fluorophores.[41]

3.2. VEGFR-3

VEGFR-3 interaction with VEGF-C and VEGF-D induces lymphatic vessel sprouting from the venous system, an early step in lymphangiogenesis.[42] The complete absence of VEGFR-3 is embryonic lethal (for more information regarding VEGFR-3 knockout, refer to section 4.4.).[42] VEGFR-3 is expressed on all blood vessels embryonically, but becomes

predominantly expressed in LECs later in development.[43; 44] Although VEGFR-3 is frequently used in research as a marker for lymphatic vessels, alone it is not sufficient because VEGFR-3 can be expressed on blood vessels.[45] Specifically, studies have identified VEGFR-3 on the tip cells of the retina blood vessels,[46, 47] in chronic inflammatory wounds,[48; 49; 50; 51] in tumors during neovascularization,[48; 49; 50; 51] and on fenestrated capillaries of normal tissues.[46] VEGFR-3 loses its lymphatic specificity in tumors because its expression is upregulated on blood vessels.[50] Previous studies using VEGFR-3 as a lymphatic marker have identified the presence of lymphatic vessels in cutaneous lymphangiomatosis,[52] hemangiomas,[52] and regions surrounding lymphomas[53] and breast carcinomas.[53; 54] Furthermore, the presence of VEGFR-3 has revealed the lymphatic endothelium origin of AIDS-associated Karposi's sarcoma.[53] These studies also used antibodies against vascular-specific markers (i.e., CD31, PAL-E, and von Willebrand factor) to ensure that the detected VEGFR-3 was indeed on lymphatic vessels and not on blood vessels.[52; 53]

3.3. Prox-1

The transcription factor Prox-1 is a lymphatic-specific marker and required for LEC commitment from blood endothelial cells (BECs).[55] Prox-1 expression is restricted to lymphatic vessels and has no effect on the development or function of blood vessels.[55] Thus, the embryonic lethality of Prox-1 knockout murine models is attributed to defects in the lymphatic system (for more information regarding Prox-1 knockout, refer to section 4.1.). Prox-1 is predominantly restricted to LECs of normal tissues and tumors.[56] In addition to LECs, Prox-1 is also expressed in hepatocytes,[37] some photoreceptors in the liver,[37] cardiomyocytes,[57; 58] and pancreatic epithelial cells.[57; 58] Prox-1 is co-expressed with CD31/PECAM-1, a transmembrane protein present on both blood and lymphatic vessels, and staining for both Prox-1 and CD31 is the most reliable way to identify LECs given that Prox-1 can be present on non-endothelial cells.[59; 60] One of the major advantages of using Prox-1 as a lymphatic marker is that it is absent on blood vessels, and unlike LYVE-1, Prox-1 can be used to identify lymphatic vessels in the liver.[37] Wilting et al. compared Prox-1 to VEGFR-3 and concluded that it is a much better lymphatic marker due to its lymphatic specificity in all tissue types.[59] Overall, Prox-1 is a reliable lymphatic marker in normal and pathological human tissue.[59]

3.4. Podoplanin

Podoplanin was first described by Wetterwald et al., who identified the protein on osteocytes and osteoblasts and called it E11 antigen.[61] Soon after, Breiteneder-Geleff et al. discovered the same protein on rat podocytes and named it podoplanin.[62] Alternate names for podoplanin include OTS-8,[63] M2A antigen,[64] T1α,[65] Aggrus,[66] and glycoprotein 36.[67] In all organs examined, podoplanin is expressed on LECs and not BECs, but it is also expressed on many nonendothelial cell types.[68] The adult lung is a major site for T1α/podoplanin expression,[65] and other sites include osteocytes,[61; 63] the choroid plexus,[61] podocytes,[62] skin endothelium,[69] and central nervous system ependymal cells.[68]

Before Schacht et al. discovered that mouse monoclonal D2-40 antibody can recognize human podoplanin, it was very difficult to study human podoplanin expression due to a lack

of protein detection methods.[68] An obstacle in detecting lymphatic vessel through immunohistochemistry is the absence of a single exclusive lymphatic marker; thus, current guidelines recommend the use of at least two lymphatic-specific markers for identifying lymphatic vessels in the eye.[70] Cursiefen et al. used both podoplanin and LYVE-1 to confirm that lymphangiogenesis occurs in the cornea.[15] Currently, podoplanin is used to identify many different types of tumors: lymphangiomas,[69; 71] Karposi's sarcomas,[69; 71; 72] angiosarcomas,[69; 71; 73] and squamous cell carcinomas.[74] Additionally, podoplanin has also been found to be a potential diagnostic marker for epitheliod mesothelioma[73; 75; 76] and gonadal and extragonadal germ cell tumors.[64; 68; 77]

4. KNOCKOUT AND MUTANT MODELS FOR STUDYING LYMPHANGIOGENESIS

Many factors have been implicated in the process of lymphangiogenesis with varying degrees of importance. Knockout models allow researchers to gain insight into the specific functions these factors have in the development of the lymphatic system. Factors can be involved in the initial development of the lymphatic system or in downstream maturation events (Fig. 1). A clear understanding of the process of lymphatic system development and the unique roles each factor plays serves as an important clinical tool for developing treatments for diseases involving the lymphatic system. A summary of factors involved in lymphangiogenesis and their corresponding knockout models is provided in Table 2.

4.1. Prox-1 knockout models

Prox1, prospero-related homeobox transcription factor gene, is expressed in the developing central nervous system, lens, pancreas, heart, and liver of mice.[58] Wigle and Oliver found that *Prox1* is present in a subset of BECs that eventually gives rise to the lymphatic system.[55] Through two primary types of murine *Prox1* knockout models, *Prox1's* critical regulatory role in the development of the lymphatic system and the commitment of BECs into LECs was discovered.[55]

The *Prox1* heterozygous knockout murine model, $Prox1^{+/-}$, is viable at birth, but lethal 2–3 days later.[55] Hemizygous $Prox1^{+/-}$ mice develop dysfunctional lymphatic vessels and a chylous ascites phenotype, which presents with chyle in the intestines, at the time of death.[55; 78] This is in contrast to the nullizygous murine knockout model, $Prox1^{-/-}$, which is lethal at E14.5 and has no lymphatic vasculature present.[55; 78] At E14.5, both $Prox1^{+/-}$ and $Prox1^{-/-}$ mice present with severe edema, suggesting the haploinsufficiency of *Prox1* in lymphatic development.[55]

A more detailed analysis of the embryonic development of $Prox1^{+/-}$ and $Prox1^{-/-}$ mice reveals *Prox1*'s specific effects on the development of the lymphatic system. Early lymphatic commitment and development involves two main steps: budding and sprouting. In budding, endothelial venous cells bud out to form lymphatic sacs. In sprouting, endothelial cells from the lymphatic sacs sprout to give rise to lymphatic vessels. At E10.5, $Prox1^{+/-}$ *and* $Prox1^{-/-}$ mice are developmentally similar and both exhibit normal budding.[55] However, by E11.5, $Prox1^{-/-}$ mice have suppressed levels of endothelial budding with

random migration due to defective polarization, and by E12, complete arrest of budding is observed.[55; 81] These findings reveal that *Prox1* is not required for the initial endothelial cell budding from the cardinal vein but is required for the maintenance of further endothelial cell budding and sprouting.[78] At E13.5, *Prox1*$^{+/-}$ mice show normal endothelial sprouting, whereas *Prox1*$^{-/-}$ mice exhibit no sprouting and an absence of lymphatic vessels.[81] During the process of budding and sprouting, *Prox1* plays a role in the lymphatic commitment of budding endothelial cells by upregulating the lymphatic-specific markers VEGF-C, LYVE-1, and secondary lymphoid tissue chemokine (SLC) to help BECs commit to a lymphatic fate.[81] Budding endothelial cells in *Prox1*$^{+/-}$ mice are similar to those in wild-type mice in that they begin to express these lymphatic-specific markers and suppress expression of their original blood vascular markers.[81] *Prox1*$^{-/-}$ mice on the other hand do not express LYVE-1 or SLC and only express low levels of VEGFR-3, while also expressing high levels of the blood vascular markers CD34 and laminin, overall adopting a blood vascular phenotype.[81]

After the discovery that Prox-1 is necessary for lymphatic development from the embryonic venous blood, Hong et al. and Petrova et al. performed experiments using human dermal microvascular endothelial cells (HDMECs) and found that Prox-1 expression can reprogram BECs into LECs.[82; 83] The introduction of Prox-1 to HDMECs successfully induced the expression of lymphatic-specific markers VEGFR-3 and podoplanin and suppressed the expression of some of the previously elevated blood vascular markers.[82; 83]

4.2. Sox18 knockout models

SRY-related HMG-box 18 (Sox18) is a transcription factor that is expressed on lymphatic vasculature precursor cells and is necessary for inducing Prox-1 expression.[84] Mutations in *Sox18* cause the autosomal recessive and dominant forms of hypotrichosis-lymphoedema-telangiectasia syndrome in humans.[85]

Heterozygous *Sox18*$^{+/-}$ mice survive to adulthood but have very dense and branched lymphatic vessels.[84] Homozygous *Sox18*$^{-/-}$ mice die at 14 days post coitum (d.p.c.) and have no Prox-1 venous expression and subsequent lymphatic development.[84] Thus, Sox18 is required only during initial lymphatic development, and its absence leads to a complete halt in further development.[84]

4.3. VEGF-C and VEGF-D knockout models

Vascular endothelial growth factors C and D (VEGF-C and VEGF-D) are the only known high affinity ligands for VEGFR-3 (flt-4), a receptor unique to adult lymphatic vessels.[44] These ligands play a critical role in regulating lymphangiogenesis and are necessary for LEC migration and survival.[42] Schoppmann et al. found that tumor-associated macrophages express high levels of VEGF-C and VEGF-D, which are necessary for the induction of peritumoral lymphangiogenesis and contribute to cancer metastasis.[86] The development of VEGF-C and VEGF-D murine knockout models revealed the involvement of VEGF-C in LEC sprouting from embryonic veins during early lymphatic development and the comparatively trivial role that VEGF-D plays in lymphangiogenesis.[42; 87]

Hemizygous *Vegfc*$^{+/-}$ murine models presented with lymphatic vessel defects, emphasizing the necessity for two functional *Vegfc* alleles for normal lymphatic development.[42] In

contrast to nullizygous *Vegfc*$^{-/-}$ mice, a fraction of *Vegfc* haploinsufficient mice survive to adulthood, but many die perinatally.[42] When examined at E13, *Vegfc*$^{+/-}$ mice have normal or only slightly reduced lymph sac formation, a process primarily guided by *Prox1* and as a whole unaffected by VEGF-C levels.[42] At the time of birth, *Vegfc*$^{+/-}$ mice present with lymphatic hypoplasia in all of the studied organs, including the skin.[42] During the first few postnatal weeks, lymphatic capillaries slowly grow into most of these organs; however, this effort is not sufficient to completely revert the mice to a wild-type phenotype.[42] The lymphatic defects persist in the skin, and the cutaneous lymphatic vessel hypoplasia does not improve with time, contrary to what is seen in the other organs.[42] When the mice reach adulthood, lymphedema and abdominal chylous ascites are present as a result of the lymphatic hypoplasia.[42] The lymphatic phenotype seen in *Vegfc*$^{+/-}$ mice can be rescued by VEGF-D overexpression as seen in Haiko et al's K14-hVEGF-D; *Vegfc*$^{+/-}$ compound mice, indicating some overlap in the functions of VEGF-C and VEGF-D.[87] Küchler et al. investigated the role of VEGF-C in zebrafish and found that lymphatic vessel development in zebrafish was also sensitive to levels of VEGF-C and VEGFR-3 signaling.[88]

Nullizygous *Vegfc*$^{-/-}$ mice all die embryonically, with most dying between E15.5 and E17.5.[42] Defects presented as lymphedema are seen as early as E12.[42] Without VEGF-C, lymph sacs and lymphatic vessels are completely absent, because Prox-1-positive venous endothelial cells cannot proliferate, survive, or sprout into lymphatic vessels and thus undergo apoptosis as evidenced histologically by macrophage infiltration.[42] Similar results were seen in *Xenopus laevis* tadpole VEGF-C knockout models.[89] Typically, VEGFR-3 is initially expressed on all blood and lymphatic vessels, but through the course of normal development, blood vessel VEGFR-3 is downregulated and the receptor can only be found on lymphatic vessels. In *Vegfc*$^{-/-}$ mice, the VEGFR-3 on blood vessels is never downregulated, but it is in *Vegfc*$^{+/-}$ mice, suggesting that VEGF-C participates in the downregulation of VEGFR-3.[42, 26] Overall, the primary effect of complete VEGF-C knockout is the absence of lymphatic vessels, which has been proven to be embryonic lethal.

To examine the specific role of VEGF-D in lymphangiogenesis, Haiko et al. developed *Vegfd*$^{-/-}$ mice and *Vegfc*$^{-/-}$;*Vegfd*$^{-/-}$ double knockout mice.[87] *Vegfd*$^{-/-}$ mice show no lymphatic abnormalities, indicating that VEGF-D plays a secondary role to VEGF-C in regulating lymphangiogenesis.[87] The *Vegfc*$^{-/-}$;*Vegfd*$^{-/-}$ double knockout mice are phenotypically similar to the *Vegfc*$^{-/-}$ mice, with both models presenting no lymphatic vessels at the time of embryonic death.[87]

Through these various knockout models, the specific and necessary roles of VEGF-C in LEC proliferation, migration, and sprouting from the lymph sacs have been determined.[42] Although VEFG-C is not required for the Prox-1 dominated BEC commitment to LECs, it is needed for the continued development of lymphatic vessels.[42]

4.4. VEGFR-3/flt-4 knockout models

VEGFR-3 (or flt-4) is initially present on all blood and lymphatic vessels during development. Eventually, VEGFR-3 is upregulated in Prox-1–expressing cells that sprout from the venous system to form the lymphatic system and is downregulated in blood vessels. By the time development is complete, VEGFR-3 is almost exclusively present in lymphatic

vessels.[43; 46] Upon VEGF-C and VEGF-D binding to VEGFR-3, downstream signaling leads to the proliferation, migration, and survival of cultured human LECs.[90] In humans, VEGFR-3's role in inducing lymphangiogenesis has been established, and a missense mutation in VEGFR-3, the cause of primary human lymphedema or Milroy's disease, leads to lymphatic hypoplasia.[91; 92; 93] Because VEGF-C and VEGF-D are the only known ligands of VEGFR-3, knockout models can be used to reveal potential functions of VEGFR-3 that are independent of VEGF-C and VEGF-D. VEGFR-3 knockout, conditional knockout, and missense mutation models are clinically important tools for investigating the role of VEGFR-3 in lymphangiogenesis and Milroy's disease.

Chy mice have a missense mutation in one *Vegfr3* allele and serve as a murine model for Milroy's disease.[93; 94] Both Chy mice and $Vegfc^{+/-}$ mice present with abdominal chylous ascites and a deficiency in subcutaneous lymphatic vessels.[42; 94; 95] $Vegfr3^{+/neo}$ mice, a conditional heterozygous knockout model, are phenotypically similar to $Vegfc^{+/-}$ and Chy mice, with all three presenting with abdominal chylous ascites.[42] The haploinsufficient nature of *Vegfr3* can be seen when comparing $Vegfr3^{+/neo}$ and $Vegfr3^{neo/neo}$ conditional knockout models. $Vegfr3^{neo/neo}$ mice, similar to $Vegfc^{-/-}$ mice, are embryonic lethal, but $Vegfr3^{+/neo}$ mice can survive to adulthood.[42] When examined at E17.5, $Vegfr3^{neo/neo}$ mice lack lymphatic vessels in their skin, whereas $Vegfr3^{+/neo}$ mice retain some lymphatic vessel remnants.[42] The lymphatic phenotypes present in VEGFR-3 knockout mice are similar to those seen in VEGF-C knockout mice; however, $Vegfr3^{-/-}$ mice embryos were found to have cardiovascular failure, a phenotype not present in $Vegfc^{-/-}$ mice.[42] This finding indicates that VEGFR-3 has a ligand other than VEGF-C and VEGF-D that plays a role in angiogenesis.

4.5. Podoplanin knockout models

Podoplanin is a transmembrane glycoprotein initially discovered for its ability to control human kidney podocyte shape.[96] Podoplanin and VEGF-C both play integral roles in lymphatic vasculature development and are both regulated by the homeobox gene *Prox1*.[82] After further investigation, it was found that podoplanin is primarily expressed on the lymphatic epithelium and is also one of the most highly expressed lymphatic-specific markers.[69; 83] Schacht et al. produced hemizygous and nullizygous knockout models of murine podoplanin (found on alveolar type 1 cells of the lung) to examine the exact role that podoplanin plays in lymphangiogenesis.[69; 97; 98]

Unlike the previously discussed genes involved in lymphangiogenesis, *podoplanin* is not essential for life, and $T1\alpha/podoplanin^{+/-}$ mice are healthy, fertile, and differ minimally from wild-type mice. Upon examining the intestinal and cutaneous lymphatic networks in $T1\alpha/podoplanin^{+/-}$ mice, Schacht et al. saw that the lymphatic vessels are dense, well-organized, and have no deficiencies in lymphatic transportation.[98] The only differences present in $T1\alpha/podoplanin^{+/-}$ mice compared to their wild-type counterparts were the presence of some dilated lymphatic vessels and a few regions with incomplete network formation, which were both not significantly detrimental.[98]

Nullizygous $T1\alpha/podoplanin^{-/-}$ mice die at birth due to respiratory failure from the loss of T1α in alveolar type 1 cells.[99] Podoplanin plays a role in the later stages of

lymphangiogenesis, and its absence does not prevent the lymphatic system from developing. Although a lymphatic system is detected at the time of death, it contains severe organizational and functional defects attributed to podoplanin's involvement in lymphatic pattern formation.[98] These defects include a diminished lymphatic transport capability, congenital lymphedema, lymphangectasia in abdominal and cutaneous lymphatic vessels, undetectable small lymphatic capillaries, and absent intestinal lymphatic lacteals for dietary lipid absorption.[98] Many of these defects, including the lymphangiectasia, may potentially be caused by the lack of anastomosing lymphatic vessels typically present between superficial and subcutaneous lymphatic networks.[98] In another study, Bertozzi et al. found that complete absence of podoplanin leads to an incomplete separation between blood and lymphatic vessels.[100] This nonseparation phenotype is similar to that seen in *slp-76*$^{-/-}$ and *Clec-2*$^{-/-}$ mice (discussed later).[100; 101] Podoplanin, C-type lectin-like receptor 2 (CLEC-2), SLP-76, and Syk are all components of a signaling pathway involved in the separation between blood vascular and lymphatic systems, and the complete absence of podoplanin leads to the presence of chyle and blood in mice mesenteric and intestinal lymphatic vessels.[100]

After observing the effects of podoplanin through knockout models, Schacht et al. confirmed their conclusions by observing the effects of podoplanin overexpression.[98] When two different cell lines (human microvascular endothelial cells and murine hemangioendothelioma-derived EOMA cells) were overexpressed T1α/podoplanin, their lymphatic cells showed very long and thin cell extensions, increased migration and adhesion, and an increased propensity for tubule formation.[98] Even though podoplanin is not involved in early lymphatic development, it plays a crucial and necessary role in the later stages of lymphatic patterning and networking.

4.6. Ang2 knockout models

Angiopoietin 2 (Ang2) is a member of the vascular growth factor family. Ang2 has contradictory roles in angiogenesis and lymphangiogenesis depending on a number of factors. In angiogenesis, Ang2 promotes vessel regression in the absence of VEGF, but promotes vessel growth in the presence of VEGF.[102; 103; 104] Ang2 carries out its effects through binding to Tie2, a tyrosine kinase receptor specific to BECs, and through unknown mechanisms, Ang2 can either activate or repress Tie2 signaling.[105; 106; 107] Although its name implies a primary effect on vascularization, Ang2 knockout models reveal that Ang2 is integral in the fundamental processes of lymphatic maturation and remodeling.[108; 109]

Ang2's involvement in lymphatic vasculature maturation and remodeling makes it an indispensible factor in normal lymphatic functioning as seen in *Ang2*$^{-/-}$ mice. Most *Ang2*$^{-/-}$ mice die within 2 weeks of birth, and the few that survive to adulthood exhibit severe lymphatic dysfunctions including chylous ascites, chylothorax, and lymphedema.[108; 110] In wild-type mice, lymphatic remodeling occurs between P0–P5 and involves sprouting of the secondary lymphatic plexus from the primary plexus and the transformation of the remaining primary plexus into collecting lymphatic vessels with valves.[109; 111] Dellinger et al. compared wild-type and *Ang2*$^{-/-}$ mice at three different postnatal time points and found that *Ang2*$^{-/-}$ mice have hypoplastic primary and secondary lymphatic networks and do not

adopt the collecting lymphatic phenotype, which is characterized by elongated LECs and downregulated LYVE-1 levels.[108] Overall, *Ang2*$^{-/-}$ mice display a disorganized lymphatic system with sparse lymphatic vessels that do not surround and follow major blood vessels.

4.7. EphrinB2 knockout models

EphrinB2, a transmembrane ligand that binds to the receptor tyrosine kinase EphB4, participates in a unique bidirectional signaling pathway. The forward signaling is EphB4-dependent and involves ephrinB2 binding to EphB4, inducing EphB4 autophosphorylation and activating downstream signaling pathways.[112; 113] The reverse signaling is ephrinB2-dependent and involves the ephrinB2's C-terminal PDZ-binding motif signaling back to its ephrinB2 host cell.[114; 115] Thus, when a cell containing ephrinB2 encounters an EphB4-containing cell, the signal has the potential of propagating in both the forward and reverse directions. EphrinB2 is highly expressed in the LECs of collecting lymphatic vessel valves and plays an important role in regulating lymphatic valve formation and morphology along with lymphatic capillary sprouting.[111; 116; 117; 118; 119] Complete and correct valve formation ensures the unidirectional flow of lymph and is imperative for a proper functioning lymphatic system, especially at the lymphatic venous junctions.

Bazigou et al. discovered that complete deletion of *efnb2* in mature lymphatic vessels leads to a decreased number and deformed morphology of the lymphatic luminal valves, suggesting that a continuous presence of ephrinB2 is necessary for valve maintenance.[120] The amount of ephrinB2 expression is also correlated with the amount and integrity of valves formed. In a mouse model with a heterozygous deletion of *efnb2*, fewer lymphatic valves are present in the cornea in comparison to the number in wild-type mice.[121]

Because ephrinB2 carries out both forward and reverse signaling, many researchers have aimed to determine the type of ephrinB2 signaling and the specific region of ephrinB2 responsible for lymphatic valve formation. Makinen et al. created *ephrinB2*$^{\Delta V/\Delta V}$ mice by removing the valine in the C-terminal PDZ-binding motif of ephrinB2 and found that this motif executes reverse signaling and is involved in lymphatic valve formation.[111] The *ephrinB2*$^{\Delta V/\Delta V}$ mice die within 3 weeks after birth and present with chylothorax, absent luminal valves, lymphatic leakage and backflow, and incomplete separation between blood vessels and lymphatic vessels.[111] Zhang et al. extended the *ephrinB2*$^{\Delta V/\Delta V}$ model by also replacing the six critical intracellular tyrosine residues with phenylalanine to prevent tyrosine phosphorylation in EphB4-dependent forward signaling.[122] The impaired forward signaling in these *ephrinB2*$^{6YF\Delta V/6YF\Delta V}$ mice was confirmed through observation of a decrease in the number of phosphorylated EphB4 receptors.[122] These *ephrinB2*$^{6YF\Delta V/6YF\Delta V}$ mice die perinatally, lack lymphatic valve development, and have an overall phenotype similar to that of *ephrinB2*$^{\Delta V/\Delta V}$ mice.[122] Interestingly, the administration of an EphB4 agonistic antibody can save *ephrinB2*$^{6YF\Delta V/+}$ mice from lymphatic valve defects and suggests that forward signaling is also required for proper valve formation.[122] Having already removed the critical signaling regions in *ephrinB2*$^{6YF\Delta V/6YF\Delta V}$ mice, Zhang et al. replaced the entire intracellular region of ephrinB2 with β-gal to create *ephrinB2*$^{lacZ/lacZ}$ mice.[122] Although *ephrinB2*$^{lacZ/lacZ}$ mice are perinatally lethal, they show normal lymphatic development with an abundance of lymphatic valves at E18.[122] This suggests that the PDZ-

binding motif and subsequent reverse ephrinB2 signaling is not required for lymphatic valve formation.[122] To confirm this, Zhang et al. mated their previous two mutant models to form *ephrinB2*$^{lacZ/6YF\Delta V}$ mice, which survive to adulthood and exhibit a normal lymphatic phenotype, implying that *ephrinB2*^{lacZ} can compensate for the *ephrinB2*$^{6YF\Delta V}$ allele.[122]

4.8. FOXC2 knockout models

Forkhead Box C2 (FOXC2, also named mesenchymal fork head-1) is a transcription factor that is expressed in both developing embryos and adults.[123; 124; 125; 126] FOXC2's role in lymphangiogenesis is of particular importance because of the phenotypic similarities seen between Foxc2 heterozygous mutant mice and humans with lymphatic-distichiasis syndrome (LD), a rare autosomal dominant genetic disorder caused by mutations in *FOXC2* and characterized by the presence of primary lymphedema and distichiasis.[127; 128; 129] *Foxc2* is required for lymphatic maturation, remodeling, and valve formation, and the consequences of its absence can be seen in hemizygous and nullizygous knockout models.

Heterozygous *Foxc2*$^{+/-}$ mice survive to adulthood and appear grossly normal.[127] However, upon further examination, common lymphatic defects include lymphatic hyperplasia and dilation, lymphatic reflux from the cisterna chili into dilated lymphatic channels in the hepatic hilum, and increases in the number and size of lymph nodes.[127] All of the hemizygous mice present with distichiasis, a condition in which an extra row of eyelashes is present.[127] Overall, *Foxc2*$^{+/-}$ mice are a good model for the lymphatic and ocular phenotypes seen in LD.[127]

The absence of both *Foxc2* alleles is embryonic lethal, with a majority of nullizygous *Foxc2*$^{-/-}$ mice dying by E12.5.[130; 131] Severe cardiovascular and skeletal defects are present in all embryos, indicating that *Foxc2* has an indispensible role in cardiovascular remodeling and skeletal formation.[130; 131] Lymphatic development arrests at the primary plexus stage in *Foxc2*$^{-/-}$ mice, suggesting that Foxc2 is not required for initial lymphatic development; however, maturation of the primary plexus into collecting vessels and formation of luminal valves does not occur.[125; 132] Typically, lymphatic vessels develop a collecting vessel phenotype through downregulation of Prox-1, LYVE-1, VEGFR-3, and SLC expression. However, these markers are not downregulated in *Foxc2*$^{-/-}$ mice, and their lymphatic vessels remain in an immature capillary-like state characterized by high expression of VEGFR-3.[132]

4.9. Nrp2 knockout models

Neuropilin-2 (Nrp2) was initially discovered as a transmembrane nervous system receptor that binds to class 3 semaphorins to guide the direction of axon development.[133; 134; 135] Later, it was found that Nrp2 also binds to VEGF-C and plays a role in lymphangiogenesis.[94] Nrp2 is initially expressed in the developing nervous, vascular, and lymphatic systems, but by E13, it is downregulated in the nervous and vascular systems and only highly expressed in the lymphatic system.[136] Although the exact effects of Nrp2 on lymphangiogenesis are not known, the clinical significance of Nrp2 is highlighted by Caunt et al.'s finding that blocking the interaction between Nrp2 and VEGF-C decreases tumor lymphangiogenesis and metastasis.[137] Through various mutant murine models, research

groups have found that Nrp2 exerts its main lymphangiogenic effects by promoting lymphatic sprouting from pre-existing lymphatic vessels.[136; 138]

Three different murine *nrp2* knockout models have been created, $nrp2^{+/-}$, $nrp2^{-/-}$, and $nrp2^{+/};vegfr3^{+/-}$, to examine the effects of Nrp2 on lymphangiogenesis.[136; 138] Of these models, $nrp2^{+/-}$ mice appear to be similar to wild-type mice and lack any detectable lymphatic defects.[136] The $nrp2^{-/-}$ and $nrp2^{+/-};vegfr3^{+/-}$ double heterozygous mice show significant overlap and similarities in lymphatic defects.[138] Although both of these mice are viable, they reproduce at a reduced Mendelian ratio, indicating embryonic death.[136; 138] Because the superficial dermal lymphatic network is formed through sprouting from deeper major lymphatic vessels, the dermis is an ideal region to observe the effects of Nrp2.[136] The skin lymphatic vessels in both $nrp2^{-/-}$ and $nrp2^{+/-};vegfr3^{+/-}$ mice are characterized by a reduced area of lymphatic coverage with enlarged and poorly branched lymphatic vessels.[138] Furthermore, $nrp2^{-/-}$ mice have skin lymphatic vessels located in atypical regions of the dermis.[136] Nrp2 guides the process of lymphatic sprouting from pre-existing lymphatic vessels by modulating tip cell stability in sprouting.[136; 138] In $nrp2^{-/-}$ and $nrp2^{+/-};vegfr3^{+/-}$ mice, the number of tip cells is reduced with a majority of the tip cells failing to extend their filopodia in sprouting.[136; 138] With impaired lymphangiogenesis, the lymph sac becomes enlarged in these mice and severe edema is detected by E15.5.[136; 138]

Studies so far indicate that Nrp2 is not required for the initial lymphatic sprouting from the venous system but rather for the further growth of the lymphatic network from pre-existing lymphatic vessels.[136] Nrp2 modulates this sprouting by directly inhibiting tip cell retraction and stalling to increase its overall stability and thus indirectly promoting tip cell extension in response to VEGF-C.[136] Because both VEGFR-3 and Nrp2 are co-expressed on lymphatic vessels and both bind to VEGF-C, it is speculated that Nrp2 is a VEGFR-3 co-receptor that mediates VEGF-C–induced lymphangiogenesis without activating downstream signaling pathways directly.[94; 136; 139; 140]

4.10. SLP-76 and Syk knockout models

Adaptor protein SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) and spleen tyrosine kinase (Syk) are intracellular signaling proteins on hematopoietic cells that are involved in the separation of blood vessels from lymphatic vessels.[141; 142] Both $Slp\text{-}76^{-/-}$ and $Syk^{-/-}$ knockout mice display a similar phenotype in which blood vessels and lymphatic vessels are not completely separated in the developing mice. This nonseparation phenotype is also seen in podoplanin and CLEC-2 knockout mice.[100; 101] Specifically, $Slp\text{-}76^{-/-}$ and $Syk^{-/-}$ mice lack arterio-venous-lymphatic shunts and have a direct vascular and lymphatic connection that leads to blood-filled lymphatic vessels.[142] SLP-76 and Syk in hematopoietic cells prevent LECs from connecting to pre-existing blood vessels and are essential in forming the separation between the closed blood vascular system and the open lymphatic system.[142; 143]

4.11. CLEC-2 knockout models

CLEC-2 is a receptor present on platelet cells that binds to its endogenous ligand, podoplanin, present on LECs.[144] CLEC-2 is the mediator between podoplanin and the

SLP-76 and Syk pathway and an important component in inhibiting developing lymphatic vessels from fusing with blood vessels.[145; 146] *Clec-2*$^{+/-}$ mice are healthy, fertile, and do not present with lymphatic defects.[100] *Clec-2*$^{-/-}$ mice, on the other hand, are embryonic lethal or die within the first few weeks after birth due to respiratory failure.[100; 101] The nonseparation phenotype of *Clec-2*$^{-/-}$ mice can be explained by CLEC-2's interactions with podoplanin, SLP-76, and Syk. During the course of lymphatic development at the venous lymphatic junction, podoplanin present on LECs binds to the CLEC-2 on platelet cells to activate the platelet cells.[101] Once activated, the platelets release granules with contents that inhibit lymphangiogenesis *in vivo*.[101] The platelet releasates involved in platelet-mediated inhibition of lymphangiogenesis include bone morphogenetic protein-9 (BMP-9), transforming growth factor-β (TGF-β), platelet factor 4, angiostatin, and endostatin.[101] Of these releasates, BMP-9 is thought to be the most important and potent because it is the only one that inhibits tube formation by LECs.[101]

4.12. Elk3 (Net) knockout model

Elk3 (or Net) is a member of the Ets-domain transcription factor and ternary complex factor families.[147] The exact mechanism of Elk3's involvement in lymphangiogenesis is unknown, but it co-localizes with VEGFR-3 in the thoracic duct and the intestinal and cutaneous lymphatic vessels.[147] *Elk3*$^{-/-}$ mice die shortly after birth and present with chylothorax and lymphangiectasia.[147]

5. LYMPHATIC STRUCTURE IN THE LIMBUS

The limbus is the border between the opaque sclera and transparent cornea and contains blood vessels that arise primarily from the anterior ciliary arteries and lymphatic vessels that connect to the conjunctival lymphatic network.[151] Despite the small size of the limbus, it has several vital ocular functions, which include maintaining nourishment for the cornea and containing the pathways for outflow from the aqueous humor.[151] The lymphatic and blood vasculature within the limbus are not evenly distributed and may be heterogeneic in patients.[152; 153] Understanding the molecular mechanisms driving lymphatic heterogeneity and the lymphangiogenic or angiogenic response during ocular pathologies and transplant rejection may provide insight into the etiology of some of these diseases and strategies for certain procedures and treatments.[153] Ecoiffier et al. found that lymphatic vessels were nasally dominated in the limbus and in the cornea under inflammatory conditions.[152] Because the lymphatic vessels are more prone to develop on the nasal side, treatments using subconjunctival injection of anti-lymphangiogenic reagents or surgical procedures such as corneal and limbal transplantation may be more effectively approached via the nasal side.[152] Age may also factor into the distribution of limbal lymphatic vessels.[154] Hos et al. found that older mice have fewer resting limbal lymphatic vessel sprouts than young mice, and that younger mice show greater lymphatic corneal neovascularization in response to an inflammatory stimulus.[154]

The limbus is believed to serve a crucial role in preventing corneal neovascularization and maintaining corneal avascularity.[155] Significant increases in inflammation and corneal neovascularization have been demonstrated in experimental limbal damage or pathological

limbal stem cell deficiency, suggesting that the limbus serves as a physical barrier to angiogenesis and lymphangiogenesis.[156; 157; 158] Collin et al. found that corneal lymphangiogenesis arises from limbal lymphatics in the vascularized rabbit cornea.[159] Lymphatic vessel formation from the limbus is primarily mediated by VEGFR-3 binding to its ligands VEGF-C and VEGF-D.[2; 3; 160; 161; 162; 163; 164; 165; 166] The presence of other mechanisms for corneal lymphangiogenesis has not been established. Our research group has questioned the role of the limbus as merely a physical barrier and found that removal of half of the limbus results in corneal neovascularization from the opposite side.[167] Further research is needed to understand the molecular pathways by which the limbus maintains corneal avascularity.

6. DISEASES ASSOCIATED WITH CORNEAL LYMPHANGIOGENESIS

6.1. Dry eye disease

Dry eye disease (DED) is an immune-inflammation mediated disorder that affects the ocular surface resulting in abnormal tear composition.[168] More than 10 million Americans 50 years and older have this disease.[169] DED severely affects patients' vision-related quality of life and may lead to psychological and physically debilitating symptoms.[168] Cyclosporine is the only treatment for DED currently approved by the U.S. Food and Drug Administration (FDA), but its use is primarily palliative.[170] Inflammation of the ocular surface is maintained by the entry of primarily $CD4^+$ T cells.[171] In DED, lymphangiogenesis in the cornea occurs without hemangiogenesis, and the lymphatics grow from the limbal areas to the central cornea.[172] Because DED occurs without hemangiogenesis, inflammation leads to selective upregulation of VEGF-C, VEGF-D, and VEGFR-3.[172] Proinflammatory cytokines, such as interleukin (IL)-17 and IL-1β, are present at increased levels in corneas of patients with DED and are essential for regulating the gene expression of VEGFR-3 and VEGF ligands.[173; 174; 175]

Goyal et al. administered systemic anti-VEGF-C treatment in a murine model before inducing DED and found significant reductions in lymphatic area and caliber compared to the untreated group.[176] VEGF-A binding to VEGFR-2 can also induce lymphangiogenesis, but these lymphatic vessels have a poorly functional phenotype, exhibiting dilated and leaky vessels.[177] When VEGF-C is interrupted with anti-VEGF-C treatment, the residual lymphatics are likely less effective at carrying antigen-presenting cells.[176] Mice that are treated with anti-VEGF-C also show decreased expression of interferon gamma (IFNγ) and IL-17, which are potent inflammatory attractants.[176] By disrupting the VEGF-C/VEGFR-3 axis, the afferent arm of the immune cycle is interrupted. The anti-VEGF-C treatment also suppresses epithelial disease associated with DED, likely by reducing the expression of proinflammatory cytokines.[176] Lee et al. also found therapeutic efficacy of epigallocatechin gallate (EGCG), a principal extract of green tea, in murine dry eyes by suppressing expression of inflammatory cytokines (IL-1β and CCL2), VEGF-A and VEGF-D, and CD11b+ cells.[178] Thrombospondin-1 (TSP-1) has recently been identified as a key endogenous inhibitor for corneal lymphangiogenesis related to DED and may have potential as a therapeutic target for DED.[179] TSP-1 promotes the cleavage of TGF-β into the active form, which has anti-inflammatory roles and induces regulatory T cells.[179] TSP-1 inhibits

lymphangiogenesis by binding to CD36 present on macrophages and downregulating their production of VEGF-C.[180] Without TSP-1's anti-lymphangiogenic effects, TSP-1–deficient mice have spontaneous isolated lymphatic outgrowths.[180] TSP-1 null mice also have a lacrimal gland and corneal inflammation and are used as mouse models of Sjögren syndrome.[181] Cho et al. recently found that surgical insults such as corneal suture or incision aggravate preoperative DED in a murine model.[182] Thus, dry eye may be part of a pre-lymphangiogenic milieu that is amplified upon corneal injury.[182] Surgeons may need to use more aggressive anti-inflammatory treatment in patients with pre-existing DED.

6.2. Corneal transplant rejection

Corneal transplantation is one of the most prevalent and successful ocular procedures. Because the cornea is avascular and immunologically privileged, corneal transplantation does not require HLA matching or the use of high-dose immunosuppressants.[183] However, complications can arise if the recipient has severely inflamed and prevascularized beds prior to transplantation, providing a mechanism by which antigen-loaded dendritic cells from the graft gain immediate access to the lymph nodes where allosensitization can occur.[5; 184] The rejection rates in high-risk patients can exceed 70%.[185; 186; 187] Effector cells reject the graft by entering through the vascularized cornea, and thus, the requirements of both hemangiogenesis and lymphangiogenesis for graft rejection are met. Recent research has focused on modulating the afferent arm of the immune system to prevent graft rejection. The risk of human corneal graft rejection is correlated with lymphangiogenesis, and the presence of lymphangiogenesis is a signal for poor prognosis of the allograft.[188] Using a suture-induced corneal neovascularization assay, selective lymphangiogenesis inhibitors have been evaluated.[8] Grafts placed into a hemvascularized recipient bed show similar survival rates as grafts placed into a completely avascular recipient bed.[8] However, if the recipient had lymphatic vessels within the bed, graft survival is significantly lower, suggesting that lymphatic vessels are more important for graft rejection than blood vessels.[8] Patients that carry a certain combination of single nucleotide polymorphisms in IL-17F, VEGF-A, and tumor necrosis factor (TNF)-α of the ACGTCT haplotype may be at increased risk of developing corneal transplant rejection, which may require the surgeon to adjust the therapeutic approach according to a patient's genetics.[189] The immune response against allogeneic corneal tissue is also intensified in the presence of allergic conjunctivitis, which has been shown to have corneal lymphangiogenesis involvement.[190; 191]

Corticosteroid therapy remains the mainstay for preventing corneal transplant rejection, and the initiation time point for treatment is crucial for graft survival.[192] Specifically, presurgical corticosteroid treatment improves graft survival in high-risk patients than extended treatment.[192] The anti-lymphangiogenic and anti-angiogenic potency of corticosteroids depends on the specific corticosteroid used. Corticosteroids with stronger anti-inflammatory activity, such as dexamethasone, exert stronger *in* vivo anti-lymphangiogenic and anti-angiogenic effects in comparison to prednisone and fluoromethalone, corticosteroids with weaker anti-inflammatory properties.[193] The strength of corticosteroid used and its adverse drug reactions should both be considered when administering steroids for promoting graft survival. Cho et al. reported that combining steroid treatment with VEGFR-1 morpholino, a synthetically produced molecule that binds mRNA to inhibit translation or alternative

splicing, decreases both angiogenesis and lymphangiogenesis and improves corneal graft survival in a high-risk corneal transplantation murine model.[194] In addition, a combination of Flt23k nanoparticles that deliver plasmids expressing anti-VEGF intrareceptor Flt23k and steroid treatment significantly reduces lymphangiogenesis and improves graft survival in a mouse model of corneal transplantation.[195]

Initial approaches to inhibiting lymphangiogenesis begin by targeting members of the lymphangiogenesis signaling pathway. Studies show that inhibiting VEGF-A in the postoperative corneal transplant reduces both angiogenesis and lymphangiogenesis, improving the overall graft survival.[5; 6; 7;8] Additionally, targeting VEGFR-3 has also been shown to potentiate anti-lymphangiogenic effects beneficial in prolonging corneal graft survival.[4]

By administering a soluble form of VEGFR-2 (sVEGFR-2) that specifically traps VEGF-C, Albuquerque et al. observed a significant and selective reduction in lymphangiogenesis without affecting hemangiogenesis.[196] Thus, treatment with sVEGFR-2 has the therapeutic potential to improve corneal allograft survival. Emami-Naeini et al. used a soluble form of VEGFR-3 (sVEGFR-3), which consisted of the ligand-binding domain of VEGFR-3 fused to the F_c domain of immunoglobulin, and evaluated its therapeutic effects on corneal transplant survival.[197] Treatment of mice after corneal allogeneic transplantation and intrastromal suture placement with sVEGFR-3 inhibited lymphangiogenesis, improved graft survival, reduced the frequency of allosensitized T cells, and decreased the frequency of IFN-γ–secreting T cells compared to a control group.[197] Zhang et al. showed that the combined blockade of VEGFR-3 and very late antigen-1, a mediator of corneal inflammatory lymphangiogenesis *in vivo*, using neutralizing antibodies against the two receptors increases high-risk transplant survival.[198]

Maruyama et al. administered podoplanin-neutralizing antibody *in vivo* to understand the role of podoplanin in corneal transplant rejection.[199] They found that neutralization of podoplanin reduced lymphatic vessel growth and the presence of macrophages, suggesting the potential role of podoplanin as a therapeutic target.[199] Bucher et al. used photodynamic therapy to regress mature lymphatic vessels prior to corneal transplantation in a murine model.[200] Photodynamic therapy followed by corneal instrastromal photosensitizer verteporfin injection resulted in selective regression of lymphatic vessels.[200] This may prove to be an effective strategy to reduce the risk of graft rejection in high-risk patients prior to surgery.[200]

Hua et al. showed the therapeutic efficacy of resolvin D1 analogue (RvD1a), a potent lipid mediator of anti-inflammatory effects, in a murine model.[201] Mice treated with RvD1a had decreased T-cell infiltration into the corneal graft and a reduced frequency of IFN-γ–secreting T cells in draining lymph nodes.[201] RvD1a inhibits the maturation of dendritic cells as well as alloimmune sensitization after corneal transplantation.[201] Recently, Dohlman et al. compared the efficacies of VEGF trap, anti-VEGF-C, and sVEGFR-3 *in vivo*.[202] They found that the VEGF trap aflibercept, which primarily neutralizes VEGF-A, is significantly more effective than current therapies at reducing the rate of corneal transplant rejection,

suggesting that the efferent arm of the immune system via hemangiogenesis plays a more important role than lymphangiogenesis in corneal transplant rejection.

Tang et al. reported that knockdown of neuropilin-2, a co-receptor for VEGF-C, selectively inhibits lymphangiogenesis *in vivo* prior to corneal transplantation.[203] By employing an artificial microRNA to knockdown neuropilin-2 in a mouse model of high-risk corneal transplantation, they determined that inhibiting neuropilin-2 may selectively inhibit lymphangiogenesis to improve graft survival rate.[203]

Hos et al. reported that blockade of IRS-1, a pro-lymphangiogenic factor, results in *in* vivo inhibition of lymphangiogenesis.[204] GS-101 antisense oligonucleotide against IRS-1, under the trade name Aganirsen, is currently in phase III clinical trials for the treatment of corneal graft rejection. Cursiefen et al. reported that GS-101 eye drops administered at a daily dose of 86 μg/day effectively inhibit active corneal angiogenesis and lead to its regression.[205; 206]

6.3. Herpetic stromal keratitis

The most severe form of corneal herpes simplex virus-1 (HSV-1) infection, herpetic stromal keratitis (HSK), is the leading cause of blindness in developing countries.[207] HSV-1 is a very infectious human pathogen with a seroconversion rate between 50–90%.[208] In HSK, lymphangiogenesis is induced in the alymphatic cornea and immune privilege is lost. During the initial stage of HSK development, inflammatory lymphangiogenesis is induced exclusively through VEGF-A and VEGFR-2 signaling pathways, and the lymphatic vessels persist even after the infection has subsided.[209] This differs from inflammatory lymphangiogenesis that occurs during bacterial infections or wound healing, which involves recruitment of macrophages and VEGF-C and VEGF-D activity.[209] The reported upregulation of VEGF-A is attributed to VEGF-A production by infected corneal epithelial cells and is an atypical host response to a viral infection, making it rather a unique characteristic of HSV-1.[209] The lymphatic vessels produced from VEGF-A and VEGFR-2 interaction are more dilated and leaky than the typical VEGF-C–induced lymphatic vessels.[210; 211; 212] Although the integrity of the VEGF-A–induced lymphatic vessels is inferior, they are still capable of transporting antigens to draining lymph nodes and impeding immune privilege.[209] Infiltrating neutrophils[213; 214] and CD4+ T cells[215; 216] in later stages of HSK development are responsible for most of the damage seen in HSK.

Current standard treatments for HSK, including topical antivirals to antagonize viral replication and corticosteroids to limit the immune response, do not address the presence of corneal lymphatic vessels. Corneal lymphangiogenesis in HSK leads to stromal opacity, and corneal transplantation may be required to restore vision.[209] However, transplant rejection rates are higher in HSK patients.[217] Because VEGF-A–dependent lymphangiogenesis occurs in HSK, VEGF-A serves as a promising therapeutic target, and drugs targeting VEGF-A such as bevacizumab,[218; 219; 220; 221] ranibizumab,[193] and VEGF trap[5; 222] have shown positive results as potential anti-lymphangiogenic treatments for HSK. Although the exact role of insulin receptor substrate-1 in lymphangiogenesis remains unknown, anti-sense oligonucleotides against IRS-1 (insulin receptor substrate-1) inhibit lymphangiogenesis in both mouse models[204] and human HSK patients[205; 206] and are potential therapeutic targets for the suppression of lymphangiogenesis.

GS-101 antisense oligonucleotide blockade of IRS-1 has also been shown to inhibit lymphangiogenesis and angiogenesis as a treatment for corneal keratitis patients and is currently in clinical trials as the first topical anti-angiogenic agent for the cornea.[206]

6.4. Glaucoma

Glaucoma, a neurodegenerative disease that leads to optic nerve neuropathy, is caused by impaired aqueous humor drainage, which subsequently leads to an increased intraocular pressure (IOP). The two passageways for draining the aqueous humor are the conventional or trabecular outflow pathway[223] and the unconventional or uveoscleral outflow pathway involving the ciliary body[224; 225]. The presence or absence of “true” lymphatic vessels in the human eye is still a controversial topic. Heindl et al. pointed out that there is no sufficient evidence that ciliary body lymphatic vessels are true lymphatic vessels without extraocular extension[226] and that the presence of intraocular lymphangiogenesis in ciliary body melanomas with extraocular extensions is associated with a poorer prognosis[227]. In addition, immunohistochemical staining for LYVE-1 on postmortem human adult eyes reveals the absence of lymphatic vessels.[228] New guidelines for the identification of lymphatic vessels in the eye, including using at least two different immunohistochemical stains, aim to clarify the identification of lymphatic vessels.[70]

Some studies show that compared to the rest of the eye, the ciliary body has the most lymphatic vessels.[229] Current glaucoma treatments target the trabecular and uveoscleral outflow pathways to lower the IOP, but if these medications do not work, surgical treatment with the risk of vision loss may be required. Latanoprost, a prostaglandin F2 analog, is a commonly prescribed topical antiglaucoma drug that acts on the uveoscleral outflow pathway.[230; 231] Recently, Tam et al. reported that latanoprost increases lymphatic drainage via the uveoscleral pathway by 400%, suggesting that the efficacy of the treatment lies in its effects on the lymphatic system.[230] However, Hos et al. found that tafluprost, a prostaglandin F2 analog, has no effect on murine corneal angiogenesis or lymphangiogenesis and can be used to treat inflammation without affecting the vascular and lymphatic profile of the cornea.[232] With this knowledge, a better understanding of the lymphatics of the eye and their role in glaucoma can lead to the development of better therapeutic interventions for glaucoma and other ocular pathologies involving an increased IOP.

6.5. Ocular tumors

Many types of cancers can occur in the eye, with lymphangiogenesis involvement depending on the cell type and location. Heindl et al. reported that the transformation of a conjunctival intraepithelial neoplasia into an invasive squamous cell carcinoma (SCC) of the conjunctiva involves conjunctival lymphangiogenesis.[233] Furthermore, the risk of SCC recurrence is associated with the amount of lymphangiogenesis present.[233] In another study, Heindl et al. reported that intraocular lymphangiogenesis increases tumor size and metastasis, and is thus correlated with mortality in patients with malignant melanoma and extraocular extensions.[227] Recently, Hos et al. described the role of tumor-associated lymphangiogenesis in ocular malignancies and its potential role in future therapeutic development.[20]

7. MODULATION OF LYMPHANGIOGENESIS

Many endogenous and pharmaceutical factors have been found to modulate lymphangiogenesis, either by activating or inhibiting it. Because fewer anti-lymphangiogenic factors have been identified in the literature compared to pro-lymphangiogenic factors, we focus here on inhibitors of lymphangiogenesis. Many molecules inhibit lymphangiogenesis by modulating members of the VEGF and VEGFR family (Fig. 2). Table 3 and Fig. 3 summarize both endogenous and pharmaceutical activators and inhibitors of lymphangiogenesis, and a more detailed explanation of pro-lymphangiogenic factors can be found in Zheng et al.[234].

7.1 Pharmacological modulation

7.1.1 Anti-VEGF-A—Although VEGF-A is primarily associated with angiogenesis in solid tumors, several studies have demonstrated that VEGF-A also regulates lymphangiogenesis.[5; 236; 237] VEGF-A has been shown to induce lymphatic vessel formation in both mouse[5] and rat[238] models of corneal injury. Inhibition of VEGF-A in these studies inhibited the growth of lymphatic vessels, suggesting that VEGF-A activates both angiogenesis and lymphangiogenesis through multiple mechanisms.[239] The current belief is that VEGF-A directs lymphangiogenesis via indirect and direct mechanisms. Through its indirect mechanism, VEGF-A recruits inflammatory cells that supply lymphangiogenic factors, VEGF-C and VEGF-D.[238] The direct mechanism involves VEGF-A directly binding to VEGFR-2 on pre-existing lymphatic vessels.[29; 240; 241] Whitehurst et al. showed that anti-VEGF-A neutralizing antibody 2C3 suppresses tumor lymphangiogenesis and metastasis in an orthotopic breast tumor model, suggesting that VEGF-A neutralizing therapeutics may prove to be clinically useful for preventing metastasis in breast cancer patients.[242] Bevacizumab is a recombinant, humanized, monoclonal antibody that targets VEGF-A and is approved by the FDA for various metastatic carcinomas. Bock et al. found that bevacizumab inhibits not only corneal angiogenesis but also lymphangiogenesis.[221]

Serum eye drops are prescribed for ocular surface disorders associated with reduced trophic factors.[243] Because these eye drops contain growth factors, they often induce lymphangiogenesis and angiogenesis.[244] Thus, serum eye drops have antagonistic effects relative to bevacizumab both *in vitro* and *in vivo*, suggesting that the opposing effects of serum eye drops and bevacizumab must be taken into account when considering combinatory therapies.[245] Li et al. have shown that by blocking insulin-like growth factor-I receptor and administering bevacizumab in a human gastric cancer cell line, both angiogenesis and lymphangiogenesis are greatly reduced; thus, this combination may be a potential therapeutic approach for gastrointestinal cancers.[246] The combination of fotemustine and bevacizumab has been shown to be clinically efficacious in untreated metastatic melanoma patients during a phase II clinical trial in Italy.[247]

7.1.2 Anti-VEGFR-2—Because anti-VEGFR-2 antibodies have been found to be effective at inhibiting angiogenesis *in vitro*[248] and *in vivo*,[249] their effects on lymphangiogenesis have also been evaluated. Kodera et al. found that anti-VEGFR-2 treatment results in only partial reduction in LEC growth and function.[250] Using an orthotopic spontaneous breast cancer

metastasis model, Roberts et al. observed that anti-VEGFR-2 antibodies suppress tumor lymphangiogenesis, and the combination of anti-VEGFR-2 and anti-VEGFR-3 antibodies is more potent at decreasing lung and lymph node metastases than either antibody alone.[251] Other research groups also have found that anti-VEGFR-2 antibodies inhibit the growth of lymphatic vessels, but their efficacy is limited compared to other pharmacological modulators of lymphangiogenesis.[252; 253]

7.1.3. Anti-VEGFR-3—VEGFR-3 interaction with VEGF-C and VEGF-D is essential in normal lymphatic development. Using a mouse monoclonal anti-VEGFR-3 antibody that antagonizes binding of VEGFR-3 to VEGF-C, Pytowski et al. demonstrated that the neutralizing antibody can block normal and VEGF-C-enhanced lymphangiogenesis in wild-type and tumor murine models without affecting the integrity of previously formed lymphatic vessels.[254] Direct targeting of VEGFR-3 has been shown to almost completely inhibit ingrowth of lymphatic vessels without affecting angiogenesis.[255] The use of VEGFR-3 neutralizing antibodies also has been implicated in reducing corneal lymphangiogenesis,[256] lymph node metastasis and lymphatic vessel density in primary tumors,[256] and lymphangiogenesis in gastric cancer.[257] The major disadvantage of anti-VEGFR-3 therapy as an anti-tumor treatment is the timing of tumor detection, because anti-VEGFR-3 therapy does not affect previously formed lymphatic vessels and will only prevent future lymphangiogenic events.[254] Still, the anti-lymphangiogenic effects of anti-VEGFR-3 therapy show clinical potential for a variety of diseases.

7.1.4 VEGF Trap—VEGF trap is a soluble chimera of the binding domains of VEGFR-1 and VEGFR-2 coupled to a human F_c fragment.[258] VEGF trap has been shown to have similar pharmacokinetics as monoclonal anti-VEGF antibodies but has a higher affinity for VEGF.[258] By suppressing lymphangiogenesis and angiogenesis, VEGF trap suppresses the inflammatory response in murine corneal suture models[238] and improves graft survival in mice after normal-risk corneal transplation.[5] Fukasawa and Kore reported that VEGF trap also suppresses pancreatic ductal adenocarcinoma lymph node enlargement, growth, and metastasis.[259] Two side effects of systemic administration of VEGF trap are hypertension and proteinuria, and thus, caution should be used when considering VEGF trap treatment in patients with a history of hypertension or impaired renal function.[260]

Intravitreal aflibercept, also known as VEGF trap-eye, is a form of VEGF trap that is purified and formulated for intraocular injection.[261] Heier et al. reported that aflibercept injections elicit similar results as ranibizumab for the treatment of age-related macular degeneration (AMD), but to achieve the same results, ranibizumab injections must be administered twice as frequently as aflibercept injections.[261] However, a recent study comparing aflibercept and ranibizumab treatment patterns for AMD showed that the number of injections patients receive is comparable in the first year of treatment with aflibercept or ranibizumab; this may render the previously reported aflibercept-associated benefits of a decreased treatment and compliance burden and injection-related risks inapplicable.[262] In another study, aflibercept was found to have greater efficacy for improving visual acuity in patients with diabetic macular edema in comparison to bevacizumab and ranibizumab, while also allowing for a greater interval of time between injections.[263] The FDA has approved

aflibercept under the trade name Eylea for the treatment of wet AMD and diabetic retinopathy in patients with diabetic macular edema.

Topical application of ranibizumab has been shown to directly inhibit corneal lymphangiogenesis and angiogenesis *in vitro* and *in vivo*, and thus, ranibizumab is a potential therapeutic agent for conditions with limbal stem cell deficiency, such as chemical burns.[264] The FDA approved pegaptanib, an antibody against the human isoform of VEGF-A, for the treatment of AMD. Unlike the other therapies discussed above, pegaptanib has anti-angiogenic effects, but no effects on lymphangiogenesis.[265]

7.1.5. sVEGFR-3—sVEGFR-3 is composed of the soluble extracellular ligand-binding domain of VEGFR-3. sVEGFR-3 inhibits lymphangiogenesis and causes regression of previously formed lymphatic vessels by binding to and trapping VEGF-C.[266] The anti-lymphangiogenic effects of sVEGFR-3 make it a potential therapeutic agent for the treatment of various cancers. In a murine orthopic urinary bladder cancer model, sVEGFR-3 gene therapy was found to suppress lymphangiogenesis and metastasis.[267] Additionally, sVEGFR-3 gene therapy in an endometrial cancer model reduces lymph node and lung metastasis, two important prognostic factors in endometrial cancer.[197] When administered in conjunction with chemotherapy, sVEGFR-1, -2, and -3 gene therapy reduces lymphangiogenesis and tumor growth in ovarian cancer models and is more efficacious than treatment with sVEGFR-1, -2, or -3 alone.[268; 269] sVEGFR-3 also has been shown to increase corneal allograft survival through the suppression of lymphangiogenesis, T cell allosensitization, and corneal allograft rejection.[197; 270]

7.1.6. Other—Additional molecules reported to exhibit anti-lymphangiogenic properties include modified forms of endogenous molecules, synthetic drugs, and herbal components. Many endogenous molecules have anti-lymphangiogenic properties, but obstacles such as a short half-life and insufficient therapeutic concentrations prevent these molecules from suppressing lymphangiogenesis without modification. Recombinant forms of canstatin[271] and lamstatin[272], fragments found at the C-terminal of type IV collagen's α2 and α6 chains, respectively, reduce LEC migration and exhibit anti-lymphangiogenic properties. When administered in melanoma mice models, the 16K N-terminal region of human prolactin has been reported to decrease the number of lymphatic vessels in both the primary tumor and sentinel lymph nodes through the inhibition of LEC proliferation, migration, and tube formation.[273] Small peptides derived from somatotropin-containing domains have also been shown to exhibit anti-angiogenic properties.[274]

Another approach to inhibiting lymphangiogenesis is to pharmacologically target endogenously produced pro-lymphangiogenic molecules. Members of the matrix metalloproteinase (MMP) family, MMP-2 and MMP-9, exhibit pro-lymphangiogenic properties[275] and are inhibited by the synthetic MMP inhibitor MMI270.[276] Cyclooxygenase-2 (COX-2) expression is implicated in many different cancers,[277; 278; 279] and COX-2 inhibitors celecoxib,[280; 281] under the trade name Celebrex, and rofecoxib,[46] under the trade name Vioxx, have been reported to inhibit lymphangiogenesis. Another pharmaceutical method for inhibiting lymphangiogenesis involves the use of siRNA gene

silencing to downregulate the expression of VEGF-C, a potent stimulator of lymphangiogenesis.[282]

Curcurmin, a natural component of the plant turmeric, has also been reported to show anti-lymphangiogenic potential.[283]

7.2. Endogenous modulation

Endogenous anti-lymphangiogenic molecules play important regulatory roles in preventing excess lymphatic vessel development. During tumor development and metastasis, the balance between anti-lymphangiogenic and pro-lymphangiogenic factors is offset, with lymphangiogenesis activators overpowering inhibitors. The current literature related to endogenous anti-lymphangiogenic molecules is limited, and thus, these factors represent an avenue for further research.

sVEGFR-2 is a monomer composed of the soluble extracellular domain of membrane-bound VEGFR-2 and created through alternative splicing. sVEGFR-2 functions as an anti-lymphangiogenic factor by antagonizing VEGF-C.[196] sVEGFR-2 is produced by the corneal epithelium and plays an integral role in maintaining the alymphatic nature of the cornea.[196] Administration of exogenous sVEGFR-2 can inhibit cornea suture-induced and corneal transplant-induced lymphangiogenesis to increase corneal allograft survival.[196] Furthermore, a reduction in sVEGFR-2 levels leads to lymphatic invasion of the cornea and skin and is implicated in the progression of neuroblastoma.[196; 284] The primary advantage of administering sVEGFR-2 to inhibit lymphangiogenesis is its specificity in targeting the lymphatic system and its lack of influence on the vascular system.[196]

Sempahorins were initially discovered for their ability to guide axon growth cones upon binding to Nrp2 during neuronal development. Members of class 3 sempahorins are believed to inhibit lymphangiogenesis through their interactions with Nrp2.[136] Moreover, class 3 sempahorins exhibit anti-tumorigenic activity in certain cancer cells by inducing apoptosis.[285; 286; 287; 288]

Vasohibin-1 is an anti-lymphangiogenic protein that is endogenously produced by BECs and has been reported to inhibit tumor lymphangiogenesis, lymph node metastasis, and corneal lymphangiogenesis.[289]

Endostatin is the C-terminal fragment of type XVIII collagen that exhibits both anti-angiogenic and anti-lymphangiogenic properties. [290] Specifically, endostatin inhibits VEGF-stimulated LEC proliferation and migration.[291]

TSP-1, an ECM protein, endogenously inhibits lymphangiogenesis in the cornea and other tissues by binding to CD36 on macrophages and downregulating their production of VEGF-C.[180]

TRAIL is a type II transmembrane protein member of the TNF family expressed in epithelial and endothelial layers of the cornea.[292] Corneal expression of TRAIL has been shown to inhibit lymphangiogenesis.[293; 294]

CONCLUSION

Lymphangiogenesis has now been implicated in many diseases for which treatments with angiogenic inhibitors have failed, and several research groups are on the brink of uncovering pathophysiological mechanisms involving lymphangiogenesis. Recent and ongoing research continues to show us that lymphangiogenesis may be as important as angiogenesis in regulating physiological processes and in the development of certain pathologies. Thus, a thorough understanding of lymphangiogenesis in the contexts of knockout models, the cornea, and ocular diseases has the potential to support the development of therapeutics for various diseases in which lymphangiogenesis is upregulated or downregulated, such as cancer, lymphedema, and some ocular diseases.

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Abbreviations

HLA	human leukocyte antigen
ACAID	anterior chamber-associated immune deviation
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
LEC	lymphatic endothelial cell
LYVE-1	lymphatic vessel endothelial hyaluronan receptor 1
TNF	tumor necrosis factor
HA	hyaluronan
Prox-1	prospero homeobox 1
BEC	blood endothelial cell
SLC	secondary lymphoid tissue chemokine
HDMEC	human dermal microvascular endothelial cell
Sox18	SRY-related HMG-box 18
CLEC-2	C-type lectin-like receptor 2
Ang	angiopoietin
Foxc2	forkhead box c2
LD	lymphatic-distichiasis syndrome
Nrp2	neuropilin 2
Syk	spleen tyrosine kinase

BMP	bone morphogenetic protein
DED	dry eye disease
IL	interleukin
EGCG	epigallocatechin gallate
TSP-1	thrombospondin-1
IFNγ	interferonγ
RvD1a	resolving D1 analogue
HSK	herpetic stromal keratitis
HSV-1	herpes simplex virus-1
IOP	intraocular pressure
SCC	squamous cell carcinoma
AMD	age-related macular degeneration
IRS-1	insulin receptor substrate-1

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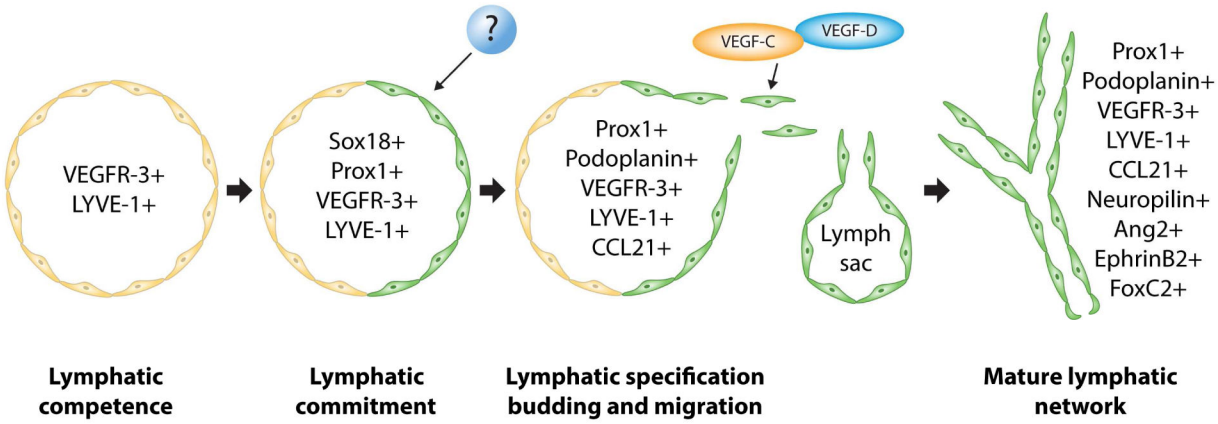

Fig. 1.

Diagram of proteins essential to specific steps in the development of the lymphatic system. (Adapted from Cueni and Detmar[45], with permission from Nature Publishing group).

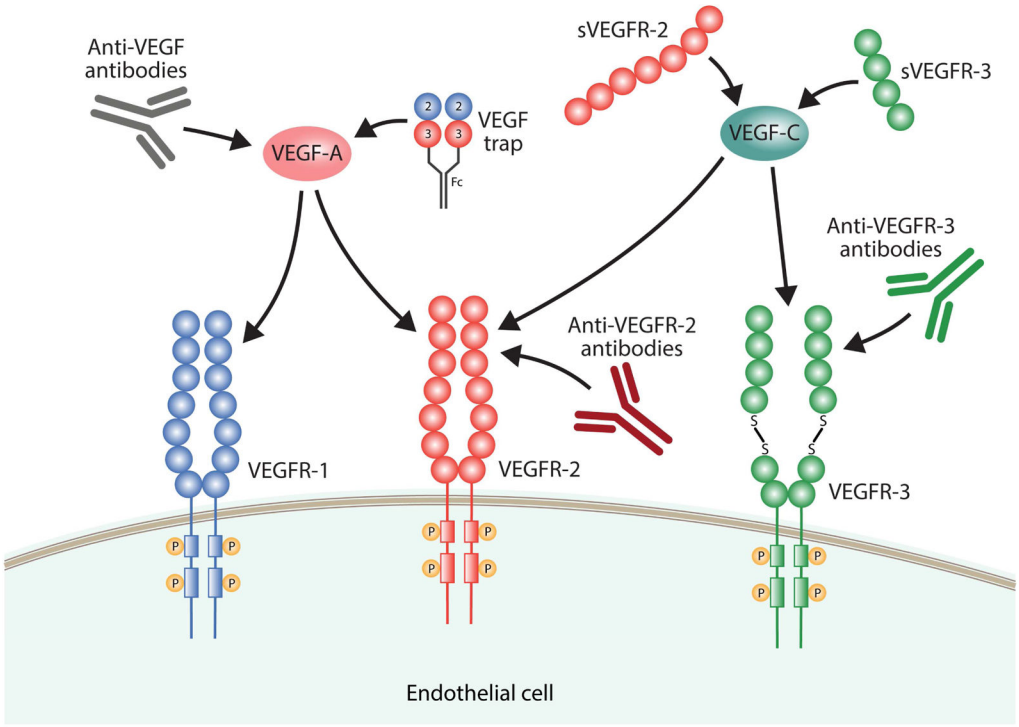

Fig. 2.

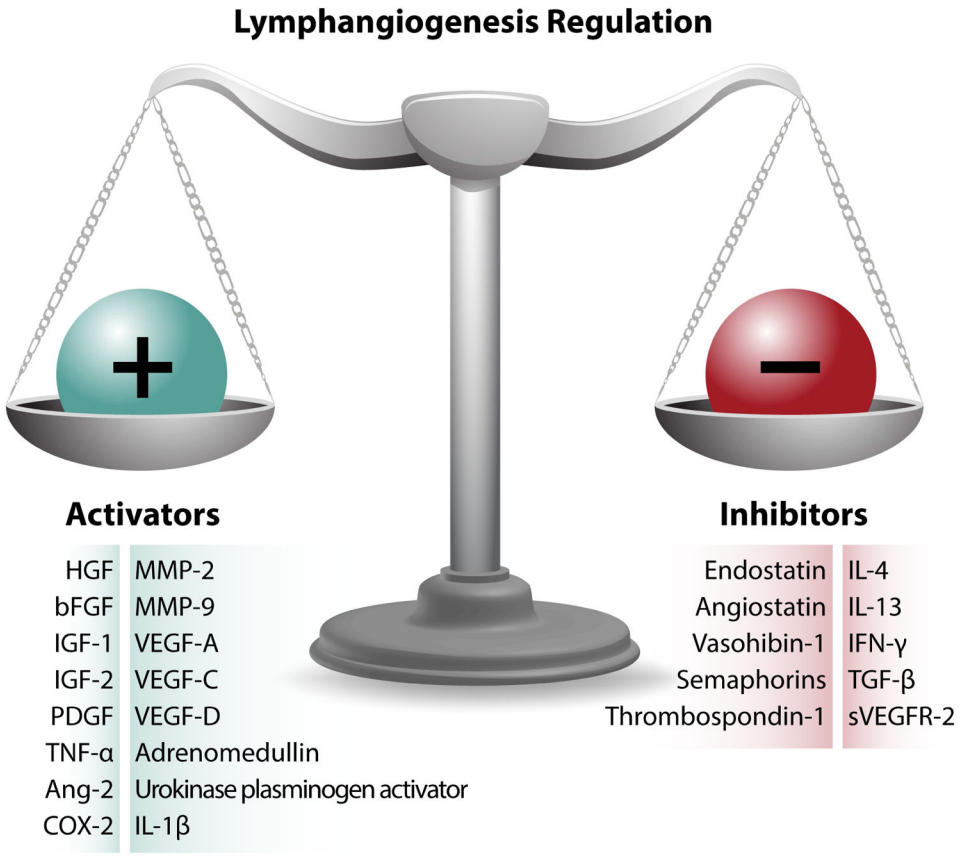

Fig. 3.

Lymphangiogenesis is highly regulated via a balance between various activators and inhibitors. Lymphangiogenesis occurs when either the levels of activators are increased or inhibitors are decreased, whereas lymphedema may develop if either the activators are downregulated or the inhibitors are upregulated. (Adapted from Bruce R. Zetter[235], with permission from Nature Publishing group).

Table 1

Lymphatic markers and their presence on lymphatic and/or blood vessels

Lymphatic Marker	Present on Lymphatic Vessels	Present on Blood Vessels	Reference(s)
LYVE-1	++	+[a]	31; 37
VEGFR-3	+	+[b]	43; 44
Prox-1	++	–	78
Podoplanin	++	–	61; 69
Desmoplakin	+	–	79
CCL21	+	–	80

[a]LYVE-1 is present on blood vessels in the liver.

[b]VEGFR-3 is present on blood vessels in many organs

Table 2

Murine knockout models and their lymphatic phenotypes reveal gene functions in lymphangiogenesis

Murine Model	Lethality	Lymphatic Phenotype	Gene Function	Reference(s)
Prox-1				
Prox1$^{+/-}$	P2–P3	Abdominal chylous ascites. Detectable but dysfunctional lymphatic vessels.	Prox-1 is required for the maintenance of lymphatic cell budding and sprouting and is critical in BEC commitment to LECs.	55; 78; 8
Prox1$^{-/-}$	E14.5	Arrested sprouting with no lymphatic vessels present. Venous ECs lack lymphatic markers LYVE-1 and SLC but contain blood markers CD34 and laminin.		
SRY-related HMG-box 18 (Sox18)				
Sox18$^{+/-}$	Normal	Fine, dense, and more branched lymphatic vessels.	Sox18 is a transcription factor that is responsible for inducing the expression of Prox-1 on lymphatic vasculature precursor cells.	84; 85
Sox18$^{-/-}$	14.5 d.p.c.	No lymphatic vessels present. No venous Prox-1 expression.		
Vascular endothelial growth factors C and D (VEGF-C and VEGF-D)				
Vegfc$^{+/-}$	Normal or perinatal	Abdominal chylous ascites. Cutaneous lymphatic hypoplasia with lymphedema in adults.	VEGF-C controls the process of Prox-1–positive LEC sprouting and migration from embryonic veins. VEGF-C is required for LEC proliferation and survival. VEGFD plays a similar but not as impactful role as VEGF-C.	42; 87
Vegfc$^{-/-}$	E17–E19	No detected lymphatic vessels. Severe embryonic lymphedema.		
Vegfd$^{-/-}$	Normal	Normal lymphatic vasculature.		
Vegfc$^{-/-}$ x *Vegfd*$^{-/-}$	E16.5	Same phenotype as *Vegfc*$^{-/-}$.		
Vascular endothelial growth factor receptor 3 (VEGFR-3)				
Vegfr3$^{+/neo}$	Normal	Leaky and dysfunctional lymphatic vessels. Transient abdominal chylous ascites after birth.	VEGFR-3 is regulated by VEGF-C and VEGF-D and is necessary for LEC proliferation and survival. VEGFR-3 is also necessary for angiogenesis.	42; 148
Vegfr3$^{neo/neo}$	Perinatal	No detectable lymphatic vessels. Cardiovascular failure.		
Vegfr3$^{lz/lz}$	E10.5	No detectable lymphatic vessels.		
Podoplanin				
T1α/podoplanin$^{+/-}$	Normal	Similar to wild-type mice. Some regions of dilated lymphatic vessels with incomplete lymphatic network formation.	Podoplanin plays a critical role in lymphatic patterning and network formation. Specifically, podoplanin promotes LEC migration, adhesion, and tubulogenesis.	98; 100
T1α/podoplanin$^{-/-}$	Perinatal	Congenital lymph edema, impaired lymphatic transport, lymphangiectasia, undetectable small lymphatic capillaries, absent abdominal lacteals, and lack of anatamosing lymphatic vessels between superficial and subcutaneous lymphatic networks. Incomplete separation between blood vascular and lymphatic vascular systems.		
Angiopoietin 2 (Ang2)				
Ang2$^{+/-}$	Normal	Collecting lymphatics with valves are present. Needs additional investigation.	Ang2 is required for the remodeling and maturation of the primary lymphatic plexus. This includes the emergence of	108; 110

Murine Model	Lethality	Lymphatic Phenotype	Gene Function	Reference(s)
Ang2$^{-/-}$	Perinatal or normal	Abdominal chylous ascites and chylothorax. Lymphedema present. Lack of collecting lymphatic vessels with valves. Primary and secondary lymphatic plexus hypoplasia. Impaired lymphatic maturation and remodeling.	a secondary lymphatic plexus along with the development of collecting lymphatic vessels with valves.	
EphrinB2				
EphrinB2$^{\Delta V/\Delta V}$	Perinatal	No lymphatic valves. Chylothorax.	EphrinB2 is essential in regulating valve formation and morphology in developing lymphatics. Data regarding whether ephrinB2 forward or reverse signaling is responsible for valve formation are conflicting.	111; 114; 122
EphrinB2$^{6YF\Delta V/6YF\Delta V}$	Perinatal	No lymphatic valves. Chylothorax.		
EphrinB2$^{lacZ/lacZ}$	Perinatal	Normal lymphatic valves detected at E18.		
EphrinB2$^{6YF\Delta V/lacZ}$	Normal	Normal lymphatic development.		
Forkhead box c2 (Foxc2)				
Foxc2$^{+/-}$	Normal	Lymphatic hyperplasia and reflux. Distichiasis.	Foxc2 is necessary for the lymphatic vessel maturation and valve formation.	125; 127; 130; 131; 132
Foxc2$^{-/-}$	E12.5	Cardiovascular and skeletal defects. Impaired lymphatic maturation, remodeling, and valve formation.		
Neuropilin-2 (NRP2)				
Nrp2$^{+/-}$	Normal	Appears unaffected.	Nrp2 is necessary for new lymphatic vessels sprouting from pre-existing lymphatic vessels. Nrp2 inhibits tip cell retraction and thus promotes VEGF-C induced tip cell extension from the lymphatic sprout.	94; 136; 138
Nrp2$^{+/-}$*vegfr3*$^{+/-}$	Reduced Mendelian ratio	Lymphatic hypoplasia in the skin with lymphatic vessel enlargement and increased branching. Reduced number of tip cells with defective filopodia extension from existing tip cells. Enlarged lymph sac and severe edema present.		
Nrp2$^{-/-}$	Reduced Mendelian ratio			
SLP-76 and Syk				
Slp-76$^{-/-}$ or *Syk*$^{-/-}$	Perinatal	Nonseparation phenotype. Chylous ascites. Lymphatic vessels are filled with blood.	Slp-76 and Syk are hematopoietic intracellular signaling proteins required for the separation of the closed blood vascularsystem from the open lymphatic system.	142; 143
C-type lectin-like receptor 2 (CLEC-2)				
Clec-2$^{+/-}$	Normal	Normal	CLEC-2 serves as the intermediate between podoplanin and SLP-76/Syk. LEC podoplanin binds to CLEC-2 to activate platelet cells and induce release of granules that inhibit lymphangiogenesis.	100; 101; 145; 146
Clec-2$^{-/-}$	Perinatal	Nonseparation phenotype.		
Elk3 (Net)				
Elk3$^{-/-}$	Perinatal	Chylothorax and lymphangiectasis.	N/A	147
Integrin α9				
α9$^{-/-}$	Perinatal	Chylothorax and lymphangiectasis.	Integrin-α9 binds to VEGF-C and VEGF-D and also interacts with VEGFR-3.	149; 150

Table 3

Endogenous and pharmaceutical compounds that modulate lymphangiogenesis.

	Mechanism of Action	Reference(s)
Lymphangiogenesis Activators		
VEGF-A	Directly binds to VEGFR-2 on pre-existing lymphatic cells. Indirectly recruits inflammatory cells that produce VEGF-C and VEGF-D.	221; 238; 242
VEGF-C and VEGF-D	Directly binds to VEGFR-3 on lymphatic cells. Indirectly stimulates lymphangiogenesis.	295
PDGF[a]	A direct lymphangiogenic factor that binds to tyrosine kinase receptors on LECs and induces a signaling pathway similar to VEGF-C binding to VEGFR-3.	296
bFGF[b]	bFGF or FGF-2, binds to VEGFR-3 and indirectly stimulates lymphangiogenesis. LYVE-1 can bind to bFGF and inhibit bFGF-dependent lymphangiogenesis.	35; 256
IGF-1 and IGF- 2[c]	IGF induces phosphorylation of intracellular signaling factors and extracellular kinases in LECs	297
HGF[d]	Mechanism is unknown, but it is believed that HGF can bind to VEGFR-3 and induce lymphangiogenesis.	298
Ang	Mechanism is unknown, but it is believed that Ang-2 and Ang- 2 induces lymphangiogenesis through binding to its Tie2 receptor.	299; 300; 301; 302
IRS-1	Mechanism is unknown, but it is believed that IRS-1 promotes macrophage infiltration, which plays a role in lymphangiogenesis. Inhibition of IRS-1 by GS-101 (Aganirsen) suppresses lymphangiogenesis.	204; 205; 206
Lymphangiogenesis Inhibitors		
sVEGFR-2	Binds to VEGF-C and prevents it from binding to VEGFR-3 and inducing lymphangiogenesis.	196
Class 3 Semaphorins	Binds to Nrp2 on lymphatic cells. Believed to inhibit VEGF-C phosphorylation of VEGFR-3.	136; 303
Endostatin	Competes with VEGF-C for binding to VEGFR-3.	291
Vasohibin-1	Believed to inhibit the pro-lymphangiogenic effects of VEGF- A.	289; 304
Anti-VEGFR-2	A neutralizing antibody that directly binds to VEGFR-2 and prevents it from interacting with VEGF-C.	248; 249
Anti-VEGFR-3	A neutralizing antibody that binds VEGFR-3 and prevents VEGF-C binding to VEGFR-3.	254
VEGF trap	A soluble decoy receptor that binds and traps VEGF, preventing it from binding to VEGFR-1 and VEGFR-2 and inducing lymphangiogenesis.	259; 5; 238; 261
sVEGFR-3	A soluble receptor that binds to and traps VEGF-C, preventing it from binding VEGFR-3 and inducing lymphangiogenesis.	266
Canstatin	Inhibits Ang-1–induced lymphangiogenesis.	271
Lamstatin	Believed to stimulate apoptosis.	272
16K N terminal human prolactin	Induces LEC apoptosis.	273
Small peptide derived from somatotrophin-contained domain	Induces LEC apoptosis.	274
MMPI270	Inhibits the actions of MMP2 and MMP9, two pro- lymphangiogenic members of the MMP family.	276
COX-2 inhibitors	Inhibits the action of COX2 enzymes implicated in lymphangiogenesis.	280; 281; 46
siRNA	Downregulates VEGF-C expression by gene silencing.	282
Curcurmin	Believed to inhibit tubule formation through Akt and MMP2 pathways.	283

Note. – PDGF = platelet-derived growth factor; bFGF = basic fibroblast growth factor; IGF = insulin-like growth factor; HGF = hepatocyte growth factor.