Commentary

When It Comes to Blocking Lymphatics, It Is All a Question of Time

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The lymphatic system comprises a group of interconnected, thin-walled vessels that drain lymph from the extracellular spaces into larger collecting tubes that eventually join the blood circulatory system. In addition to regulating extracellular fluids, lymphatics are also responsible for immune homeostasis and absorption of lipids from the intestinal tract and, more recently, have been implicated in obesity.

In pathological settings, lymphatics have received attention because of several hereditary disorders that lead to lymphedema and because of their association with metastatic events. The fact that metastatic seeding generally is detected in lymph nodes has supported the notion that metastatic spreading occurs, at least in part, via the lymphatic system. Consequently, understanding the mechanisms that regulate lymphatic expansion has paved the way to translational research aiming at suppression of lymphangiogenesis. Targeting vascular endothelial growth factor (VEGF)-C has been a popular option for therapeutic exploration because growth and differentiation of lymphatic vessels appears to be exquisitely dependent on signaling via this pathway. Nonetheless, this advantage is a double-edged sword because the requirement of VEGF-C signaling to normal lymphatics might be equally important. Thus, concerns related to side effects have always clouded the excitement for this approach. A study published in this issue of *The American Journal of Pathology*¹ puts some of these concerns to rest and brings new insights into the regulation of lymphatic growth.

Early Regulation of Lymphatic Growth—The VEGFR3-VEGF-C Signaling Axis

The development of the lymphatic system occurs mostly in tandem with the blood vascular system but subsequent to the initial formation of the primitive vascular plexus. The

first lymphatic vessels originate from assembly and differentiation of a small group of endothelial cells that depart from the cardinal vein at approximately E10.5 in the mouse.^{2–4} The molecular underpinnings that regulate the departure of venular endothelial cells and their differentiation into lymphatics are only now being unraveled. The process can be first noted by the presence of Lyve-1, a marker that identifies hyaluronan receptor 1, and the expression of prox-1, a transcription factor responsible for lymphatic commitment.^{4,5} Prox1 (prospero-related homeobox-1) is not exclusive to the lymphatic system, but it is certainly specific to this endothelial cell type, because it is not detected in any endothelium of blood vascular origin. Loss-of-function studies in mice have provided strong evidence that prox1 is essential for the initial development of lymphatics. In fact, inactivation of prox1 in mice results in absence of lymphatic vessels and lethality at mid-gestation.^{6,7} Expression of prox1 is required for commitment to the lymphatic lineage and for the subsequent steps related to expansion and assembly of lymphatic endothelial cells into cords.⁷ In fact, overexpression of prox 1 in endothelial cells from venular origin is sufficient to induce a lymphatic fate and reprogram their venular characteristics.^{8,9}

In addition to prox-1, the VEGF signaling pathway is also essential for lymphatic growth. VEGFR3 (also known as Fms-like tyrosine kinase 4, Flt4) can be detected very early during the process of lymphatic differentiation.¹⁰ This receptor has been known to interact with both VEGF-C and VEGF-D.^{11–13} Initially, VEGFR3 is expressed throughout the vascular endothelium (lymphatic and blood-related), but as development proceeds expression becomes more restricted and eventually exclusive to lymphatic vessels. This dual developmental expression of VEGFR3 has made loss-of-function studies not as informative as expected, because inactivation of the gene

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results in generalized cardiovascular failure with subsequent lethality before the development of lymphatic vessels.¹⁴ Nonetheless, the fact that some types of hereditary lymphedema have been linked to VEGFR3 provides sufficient evidence to implicate this molecule in the regulation of lymphangiogenesis and lymphatic homeostasis.

Ligands to VEGFR3 are VEGF-C and VEGF-D. Both proteins are secreted as inactive precursors and require proteolytic processing for activation.^{12,15} Although VEGF-C can bind with high affinity to both VEGFR2 and VEGFR3, VEGF-D is specific for VEGFR3.^{16,17} During development, however, VEGF-C is far more predominant than VEGF-D. Genetic ablation of VEGF-C has provided critical evidence for the absolute requirement of this signaling system in the development of lymphatic vessels.¹⁸ Homozygous mutants for the targeted allele showed no lymphatics and lethality at mid-gestation. Together, the information gathered thus far supports the notion that although prox-1 is essential for commitment of lymphatic endothelial cells, VEGF-C is subsequently needed for further budding and proliferation of prox-1-expressing cells from the cardinal veins.

These genetic studies also demonstrate the importance of gene dosage. Although VEGF-C heterozygous mice showed normal development of lymphatics in most organs, these mice display progressive accumulation of chyle in the peritoneal cavity, hypoplasia of cutaneous lymphatic vessels, and lymphedema. Together the findings indicate that haploinsufficiency is not compatible with normal lymphatic function.¹⁸ The phenotypes can be rescued by recombinant VEGF-C and to an extent by VEGF-D but not VEGF-A.¹⁸ The poor rescue by VEGF-D is interesting and begs the question as to why: if VEGF-D can activate VEGFR3 to the same extent as VEGF-C, why is the rescue not identical? This paradox leads to the speculation that either VEGF-C activates other receptors in lymphatic vessels in addition to VEGFR3 or that the activation of VEGFR3 by VEGF-C and VEGF-D results in distinct signaling cascades. Clearly these findings have revealed important nuances mediated by VEGF-C and VEGF-D that were not previously considered and should be the focus of future investigations. It should also be stressed that in addition to the VEGF system, other molecules have been shown to affect and modulate lymphatic growth and function, including angiopoietins/Tie, foxc2, podoplanin, ephrin B2, and neuropilin-2.³ However, the comments here are mostly focused on the VEGF-VEGFR3 signaling axis.

Adult Lymphatics and Lymphatic Homeostasis

Morphogenesis and expansion of the lymphatic vasculature is completed by E14.5 in the mouse. However, similar to the blood vascular system, full differentiation of lymphatics proceeds thereafter. In addition to a constant state of budding, remodeling, regression, and regrowth, the transcriptional profiles of lymphatic endothelial cells are similar to adult lymphatics only at birth.^{19,20} Thus, progressive cellular differentiation of the lymphatic endo-

thelium continues long after morphogenesis of lymphatic network has been concluded. Furthermore, it has been shown that acquired lymphedema is often associated with impaired VEGF signaling, indicating that the VEGF axis is indeed used in the adult and is required for lymphatic homeostasis. More recently it has been shown that inflammatory states associated with transplantation and rejection lead to expansion of the lymphatic system by incorporation of cells from the bone marrow.²¹ Interestingly, a subpopulation of bone marrow-derived CD11⁺ cells has also been shown to express high levels of prox-1 and podoplanin, indicating their commitment to the lymphatic lineage. Although the actual incorporation of these cells into lymphatic vessels remains to be proven, it is highly possible that CD11⁺/prox⁺/podoplanin⁺ cells are indeed lymphatic endothelial progenitors. Together, these data would indicate that the lymphatic network is constantly renewed and remodeled in response to physiological and pathological conditions. Consequently, interference with the key molecular factors that trigger their growth has been a source of potential concern and of argument against the development of therapies that target VEGF-C, VEGF-D, and/or VEGFR3.

Therapeutic Explorations for Manipulation of Lymphatics in Vivo

There are two main pathologies that have fueled the development of therapies to regulate lymphatic growth: lymphedema and cancer. Lymphedema is clinically associated with chronic swelling, fibrosis, susceptibility to infections, and impaired wound healing.²² The condition can be hereditary (primary lymphedema) or acquired (secondary lymphedema). The latter is more frequent and develops as a sequelae to radiation therapy, surgery, or infection. The hereditary form can affect one or more of the following genes: *VEGFR3*,^{18,23} *FOXC2*,²⁴ *SOX18*,²⁵ or *REELIN*.²⁶

In terms of cancer progression, it is accepted that tumor metastasis to either lymph nodes or other organs takes place through either lymphatics or blood vessels.²⁷ Because lymph nodes are often the first site where carcinomas expand, lymphatics have received attention as potential conduits of metastatic cells. Supporting the notion that lymphangiogenesis is important for metastatic expansion, preclinical studies have shown that overexpression of VEGF-C results in a higher rate of regional lymph node metastases.²⁸ More importantly, blockade of VEGF-C, VEGF-D, or VEGFR3 can result in reduction of metastatic events.^{29–32} In sum, exploration of therapeutic intervention using animal models has shown the benefit to both increased lymphatic growth in the case of lymphedema and suppression of metastatic spread in the case of tumors.

A central concern of both vascular and lymphatic intervention is the possibility of severe side effects to normal vessels. This has become an important point in lymphatic biology because several animal models (transgenic and knockouts) have reiterated the notion stated previously that, although fully developed, the lymphatic

system is in a constant state of remodeling. Thus, will there be long-term consequences to VEGFR3 blockade to normal tissue homeostasis? Karpanen and colleagues¹ address this question in this issue of *The American Journal of Pathology*. Their study reports the outcome of preclinical trials in mice exposed to either recombinant adenovirus encoding a soluble VEGFR3 protein (Ad-VEGFR3-Ig), recombinant VEGFR3-Ig protein, or blocking antibodies against VEGFR3. As anticipated, blockade of VEGFR3 in young mice leads to the regression of lymphatic capillaries and medium-sized lymphatics. Surprisingly, within the time frame used in these experiments, the treatment did not alter larger collecting lymphatics or blood vessels. The results suggest that larger lymphatics might be phenotypically different from smaller lymphatics and are likely under different regulatory controls. The unexpected outcome, however, was that lymphatics grew back at 4 weeks even in the presence of sustained pharmacological inhibition of VEGFR3. Interpretation of this result presents two possibilities: endogenous compensation of the pharmacological blockade by up-regulation of VEGFR3 or its ligands or alternative mechanism for induction of lymphatic growth independent of VEGFR3 activation. Although not entirely discarded by the investigators, up-regulation of the VEGFR3 axis is unlikely because the pharmacological blockade was at multifold excess. The second possibility was preferred by the authors, and if correct, it opens a new dimension to our understanding as to how lymphatics grow. This possibility implies distinct modes of regulation for lymphangiogenesis in the embryo and in the adult—a point that gains further credence by the outcome of experiments exploring gain- and loss-of-function of VEGF-C and VEGF-D. VEGF-C regulates lymphatic growth in the embryo; however, as development proceeds, lymphatic endothelial cells acquire sensitivity to VEGF-D while decreasing their response to VEGF-C. Thus lymphatic expansion in the neonate is more dependent on VEGF-D than VEGF-C (Figure 1). Interestingly, both VEGF-C and VEGF-D signal via the same receptor, VEGFR3. Consequently the molecular underpinnings that explain this temporal switch in sensitivity are yet to be understood.

The outcome of the studies by Karpanen and colleagues¹ still beg the question: what is (are) the mechanism(s) that regulate VEGFR3-independent growth of lymphatics in the adult? Clearly this point will be the subject of much research in the near future. An interesting alternative is the potential contribution of bone-marrow-derived progenitors. Although the investigators searched for circulating VEGFR3-positive cells and detected no increase, a recent publication suggests that lymphatic progenitors are indeed VEGFR3-/podoplanin⁺/prox⁺.²¹ Thus, endothelial progenitors remain a viable alternative to explain lymphatic expansion.

Getting back to therapeutics, the central finding of Karpanen and colleagues¹ argues that VEGFR3-targeted therapy is innocuous for normal lymphatics but toxic for tumor lymphatics as demonstrated by multiple studies.^{29–32} However, would VEGFR3 therapy in tumors be long lasting? Or would tumor lymphatics regrow in a VEGFR3-independent manner similarly to normal lymph-

Figure 1. Response of lymphatic endothelial cells to VEGFR3 signaling is developmentally regulated. **A:** Growth and morphogenesis of lymphatic endothelial cells requires activation of VEGFR3 via VEGF-C, leading to the expansion of lymphatic endothelium by E10.5 to E11.5 and their progressive organization into a network of lymphatic vessels during mid and late gestation. After birth, the sensitivity of VEGF-C decreases, in contrast to the stronger lymphangiogenic potential of VEGF-D. **B:** Blockade of VEGFR3 has been shown to affect tumor lymphatics and metastasis. Interestingly, although pharmacological inhibition of VEGFR3 led to the initial suppression of lymphatic after birth, these vessels regenerate at 4 weeks, even with a constant blockade of VEGFR3.

phatics after 2 weeks? These are key challenges that will likely direct future experimental exploration. As for now, the publication by Karpanen and colleagues¹ has redefined our understanding of lymphatic growth and propelled investigations of therapeutic intervention.

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