

## Ordered Chaos: Harnessing Developmental Pathways in Tumor-Induced Lymphangiogenesis

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### Abstract

Over the past decade, research investigating the development of the lymphatic vascular system has become a key focus within the fields of developmental biology and cancer biology. Critical discoveries relating to the specification of lymphatic endothelial cells and lymphatic morphogenesis during embryonic development have helped to identify novel molecular drivers of pathological lymphangiogenesis; that is, the sprouting of a new lymphatic vessel from, or the enlargement of, pre-existing lymphatic vessels. These pathways may also constitute potentially useful therapeutic targets, which may be exploited to restrict tumor spread via the lymphatic system. Here, we discuss the current knowledge of both developmental and tumor-induced lymphangiogenesis, and draw parallels between the two processes to describe the molecular pathways that are re-capitulated during the growth of tumor lymphatics, and which promote metastasis.

**Keywords:** Lymphangiogenesis; Transcription factor; Metastasis; Growth factors; Developmental genetics; Solid cancer

### Introduction

The lymphatic vasculature forms a complex network that parallels the blood vascular system to drain fluid from, and thus regulate the homeostasis of, the interstitial tissues. The lymphatic vascular tree is a key feature of vertebrate physiology and plays a central role in lipid and hormone transport as well as immune cell trafficking. For over a century, the cellular origin of lymphatic endothelial cells (LECs) has been debated [1,2]. More recently, molecular advances in developmental genetics and imaging have shed light onto the processes that instruct lymphangiogenesis; both in development and as part of several key human diseases. In adult vertebrates, lymphangiogenesis plays an important role in cancer metastasis, a process that determines patient mortality [3-5]. Discoveries of the molecular pathways that underpin lymphangiogenesis are therefore pivotal to developing novel therapeutic avenues to restrict cancer spread [6]. In this review we highlight parallels between the fundamental molecular processes underlying lymphatic vascular development, and the same processes that become dysregulated during tumor-induced lymphangiogenesis.

### Molecular Pathways Driving Embryonic Lymphangiogenesis

In both fish and mammals, the lymphatic vasculature has been shown to arise from venous endothelial cell precursors [7,8]. Major sources of LEC-precursors that contribute to establishing the primitive lymphatic plexus (9.5 day post-coitum (dpc)-14.5 dpc in the mouse) have a venous origin and were identified by lineage tracing

experiments and advanced imaging methods [9-12]. Vascular beds that establish a pool of LEC-precursors include the cardinal and inter-somitic veins and the superficial venous plexus (Figure 1A).

In order to shift from a venous endothelial cell identity to a LEC fate, a subset of the venous endothelial cells begins to express a finely-tuned combination of transcription factors and growth factors/receptors that subsequently induce the development and morphogenesis of the lymphatic vascular plexus [13].

### Transcriptional control of LEC specification

Gain- and loss-of-function experiments using transgenic mouse models have demonstrated that only a handful of transcription factors have been shown to guide lymphangiogenesis. Following the onset of arterio-venous specification (around 7.75-9 dpc in the mouse), a sub-population of the endothelium that expresses SOX18 (Sry-related HMG box containing-18) and COUP-TFII (chicken ovalbumin upstream promoter or NR2F2) becomes restricted to the dorso-lateral part of the anterior cardinal vein to form pre-lymphatic clusters, which in turn form a reservoir of LEC-precursors (up to 14.5 dpc) [9]. At this time point, the primary lymphatic structure has been established and is composed of lymphatic sacs, also described as pre-thoracic ducts [10] that later form the deeper collecting lymphatic vessels. In contrast, the superficial lymphatic vascular network is still expanding and completes the colonization of organs such as the skin or heart at significantly later embryonic stages (e.g. 17-18 dpc).

A critical transcription factor is SOX18, which -along with the endothelium-specific SOX7 and SOX17 -belongs to the SOX-F group. One function of SOX18 is to directly transactivate a downstream transcription factor, *Prox1* [14], which act as the gatekeeper of LEC identity [7,15]. Recent work has shown that gain-of-function of the

RAF1/MEK/ERK pathway is able to constitutively modulate SOX18 activity and trigger excessive lymphangiogenesis [16].

Similarly, COUP-TFII, which is essential for maintenance of venous identity, has also been shown to directly induce *Prox1* transcription [17,18]. Later during development, COUP-TFII additionally causes direct transactivation of neuropilin-2 (NRP2), a co-receptor of VEGF-R3 that is essential in driving LEC migration within mesenchymal tissues [19].

Once LEC cell fate has been induced by SOX18 and COUP-TFII, the expression of *Prox1* is also required for maintenance of LEC identity and for acquiring additional lymphatic-specific markers [20]. Conditional deletion of the *Prox1* gene in adult LECs led to a reversal from a LEC identity into a blood endothelial cell phenotype [21]; whereas SOX18 signaling ceases after 14.5 dpc and is not essential to the maintenance of a LEC phenotype.

### External cues that influence lymphangiogenesis

Despite the lymphatic potential of the venous endothelium, not all venous cells commit to a LEC fate. Rather, some segments of the vein must remain intact and spared from undergoing lymphangiogenesis in order to preserve the functional integrity of the blood circulatory system. To this end, negative regulation of lymphangiogenesis has been shown to be mediated by at least three independent pathways: *Cyp26b1* (a retinoic acid (RA) degradation enzyme), *Notch* and TGF-B/BMP signaling. Supporting this negative regulatory role, loss of *Cyp26b1* function *in vivo* was shown to lead to an increased local concentration of RA, which in turn stimulated LEC proliferation, aberrant lymphatic vessel formation and enlarged lymph sacs [22,23].

More recently, Notch signaling was also established as an essential factor in confining lymphatic differentiation to the dorso-lateral side of the anterior cardinal veins [24-26]. Targeted gene disruption of *Notch1* in LEC precursors gave rise to a localized expansion of lymphatic specific markers in non-lymphangiogenic segments of the veins. Another growth factor, TGF-B was recently reported to perform a dual role during dermal lymphangiogenesis; whilst able to promote lymphatic vessels sprouting and branching complexity, TGF-B also inhibited LEC proliferation [27]. Finally, bone morphogenic proteins BMP2 and BMP9 were also reported as negative modulators of lymphatic vessel growth and differentiation. *In vitro*, BMP9-induced ALK1 was found to directly inhibit PROX1 activity; while loss-of-function of either *Bmp9* or *Alk1* lead to dysmorphic lymphatic vessels [28]. Further, BMP2 was reported to inhibit *Prox1* via the induction of micro RNAs (miR-31 and miR181a) [29] to negatively regulate LEC identity.

### Growth factors and lymphatic vascular remodelling

Embryonic animal models have shown that once the venous endothelium became committed to a LEC fate, the acquisition of lymphatic-specific markers such as podoplanin, LYVE1, VEGF-R3 and NRP2 enabled LECs to become responsive to growth factor stimulation (predominantly VEGF-C), in order to assemble a vascular plexus within the mesenchyme (from 10.5 dpc until 14.5 dpc) [30]. Later, this plexus was remodeled (14.5 dpc until 18.5 dpc) and finally matured to establish a functional lymphatic network. The major signaling axis controlling LEC migration and remodeling was controlled by the VEGF-C/Collagen and Calcium-Binding Epidermal growth factor domains 1 (CCBE1)/VEGF-R3 pathway -a signaling axis that promoted LEC migration, proliferation and survival, and was thus

indispensable for both embryonic and adult lymphangiogenesis [31]. *Vegf-c* loss-of-function experiments revealed that, despite LEC specification still occurring in the absence of this key growth factor, the embryos lacked a complete lymphatic vasculature, due to defective lymphangiogenic sprouting. Further, a positive feedback loop between VEGF-R3 and PROX1 has been proposed to maintain the identity and the number of LEC progenitor in the cardinal vein [32].

Studying the maturation process of VEGF-C protein, Jeltsch et al. and Leguen et al. demonstrated a regulatory pathway of VEGF-C activation that varies depending on the activity of CCBE1 [33,34]. It was shown that CCBE1 could promote cleavage from a minimally-active form of VEGF-C to a mature and active form, via the A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS-3) protease [33]. This discovery not only suggested that CCBE1/ADAMTS-3-inhibition may represent an exciting novel therapeutic avenue to limit tumor lymphangiogenesis; but also further re-enforced the concept that tumor location (e.g. next to a source of CCBE1) may be a key determinant of the ability of normal lymphatics to sprout -even in the presence of established pro-lymphangiogenic cues -during both embryonic and pathological lymphangiogenesis [35].

### Bridging the Gap between Embryonic and Tumor-Induced Lymphangiogenesis

#### Re-activation of embryonic pathways during tumor-induced lymphangiogenesis

As tumor lymphatics mimic normal initial lymphatic capillaries in their role as the entry point for fluid absorption and cellular escape into the lymphatic system, it is reasonable to draw parallels between major embryonic pathways that instruct lymphatic formation and morphogenesis and the pathways that become dysregulated and/or reactivated under tumor conditions (Figure 1B).

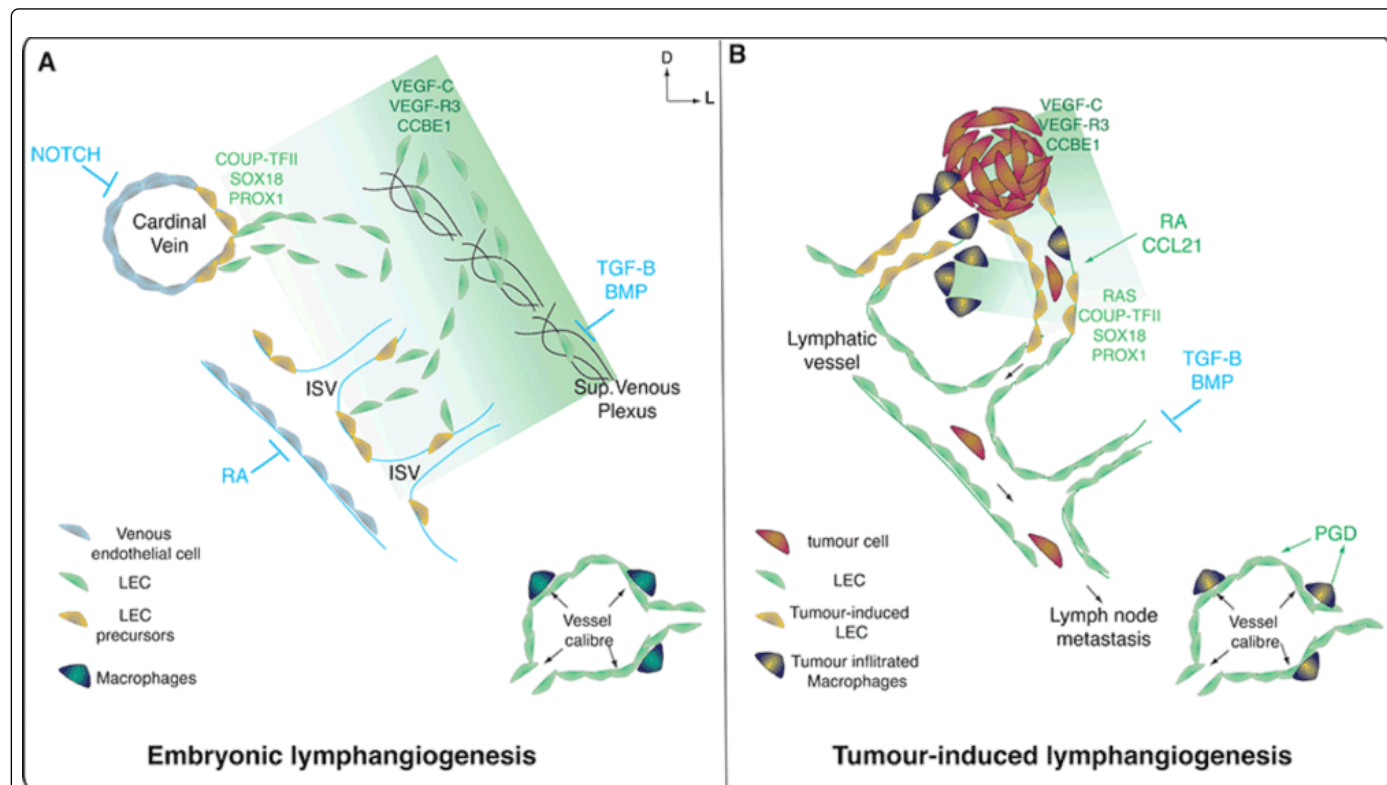
A key mechanism found to contribute to tumor lymphangiogenesis was outgrowth from pre-existing initial lymphatic vessels in and around the primary tumor mass in response to multiple stimuli, including VEGF-C, VEGF-D, PDGF-B, FGF2 and angiopoietin [35]. Similar to their functions during embryonic development, these growth factors were seen to trigger peri-tumoral lymphangiogenesis and increase metastasis in mice [36]. Each of these signaling molecules was reported to play key functions during embryonic angiogenesis and lymphangiogenesis, in both mouse and fish model systems [31].

Within the tumor microenvironment, multiple cellular sources of VEGF-C have been identified. Moussai et al. and Schoppmann et al. showed that tumor-infiltrating macrophages secrete this growth factor [37,38], while in cancer cells such as MCF7 human breast cancer cell line, SIX1 was also shown to promote VEGF-C gene expression, which stimulated cancer metastasis [39]. Further, LECs were demonstrated to act in an autocrine fashion in response to VEGF-C signaling, by up-regulating chemokines such as CCL21, which enhanced tumor chemoinvasion [40].

Other developmental lymphangiogenic pathways 're-awakened' in cancer-related lymphangiogenesis include NRP2, a receptor for class III semaphorins and VEGF-C co-receptor [19] that can stimulate both VEGFR-2 and VEGFR-3 signaling [41]. Yuan et al. demonstrated that genetic disruption of *Nrp2* results in reduced LEC proliferation and tissue lymphatic density during development [41]. In contrast to the

situation found in developing lymphatics, *Nrp2* is not expressed in quiescent lymphatics; however, Caunt et al. demonstrated not only that *Nrp2* became expressed during active lymphangiogenesis in and

around a primary tumor -but that blockade of NRP2 signaling restricted tumoral lymphangiogenesis [42].



**Figure 1:** “Ordered chaos”: Key developmental pathways activated during embryonic development face re-activation under tumor conditions to drive aberrant lymphangiogenesis and metastatic spread. (A) Schematic representation of embryonic lymphangiogenesis and the major genetic pathways that instruct lymphatic endothelial cell fate (SOX18, COUP-TFII, PROX1), maintain venous identity (Notch, RA) and govern morphogenesis of the lymphatic vascular plexus (VEGF-C, VEGF-R3, CCBE1, TGF-B, BMP9 and BMP2) from 9 dpc-14.5 dpc in mice. During development venous endothelial cells provide LEC precursor cells and macrophages control lymphatic vessel calibre. (B) Under tumor-induced conditions, embryonic pathways are re-activated or dysregulated to cause expansion of the pre-existing lymphatic vasculature in and around tumor tissues. Neo-lymphatics form an ‘on-ramp’ for tumor cells and fluid draining to the lymph node basin. Reactivation of the developmental program is influenced by the inflammatory response, components like tumor-infiltrated macrophages produce VEGF-C and change their molecular profile to acquire a LEC-like molecular signature. These factors contribute to produce a pseudo-functional lymphatic vasculature.

LEC: Lymphatic Endothelial Cells; VEGF: Vascular Endothelial Growth Factor; TGF: Transforming Growth Factor; BMP: Bone Morphogenetic Protein; COUP-TFII: Chicken-Ovalbumin Upstream Transcription Factor II; PROX1: Prospero-Related Homeobox-1; SOX18: SRY-Related HMG Box containing Domain 18; CCBE1: Collagen and Calcium Binding EGF Domain; RA: Retinoic Acid; CCL21: Chemokine (C-C motif) Ligand 21; VEGF-R3: VEGF-Receptor3. ISV: Inter-Somitic Vessels; PGD: Prostaglandin-D

Fewer functional tumor lymphatics were generated in this tumor model, thereby contributing to reduced metastasis to lymph nodes and distant organs [42]. Similarly critical for lymphatic development, the ligand for endothelial Tie2 receptor tyrosine kinase angiopoietin 2 (*Ang2*; also known as *Angpt2* or *Agpt2*) was shown to be specifically required for developmental lymphatic patterning [43]. It was subsequently demonstrated that angiopoietin expression induced in animal models additionally promoted peri-tumoral lymphangiogenesis, and that an ANG2-inhibitory antibody reduced

tumor lymphangiogenesis and metastasis to both regional lymph nodes and the lungs [44,45]. Cao et al. used a mouse corneal model to investigate a developmental role for FGF2 in lymphangiogenesis [46]. They found that FGF2 activity was mediated by FGFR-1 expressed on LECs; however, that this lymphangiogenic pathway required synergistic VEGFR-3 activation by VEGF-C - particularly for tip cell initiation of lymphatic sprouting [46]. In the analogous process of lymphangiogenesis induced in a tumor model, the authors found that VEGFR-3-mediated lymphangiogenesis incorporated synergistic VEGF-C/FGF2-driven tumoral lymphatic formation, which was associated with tumor metastasis. Neutralising antibody against the common VEGFR-3 pathway was able to restrict lymphatic ingrowth and tumor spread [46].

Finally, numerous other genes have been linked with abnormal developmental remodeling or maturation, and altered resulting phenotypes lymphatics, without yet being implicated in tumor lymphangiogenesis. Examples include genes such as angiopoietin-like



4 (*Angptl4*) [47] apoptosis stimulating protein of p53 (*Aspp1*; *Ppp1r13b*) [48] and T-synthase (*C1galt1*) [49]. Other abnormalities such as dysfunctional pericyte recruitment, impaired valve formation [50], atypical patterning or hypoplastic/hyperplasia have been linked to chylothorax and lymphoedematous phenotypes, some of which have been linked to analogous human syndromes (such as *lymphoedema distichiasis* [51-53]). Collectively, these pathways and are now considered potential targets for molecular therapeutics to restrict tumor-induced lymphangiogenesis [36]; while restricting the negative regulators of these pathways may facilitate therapeutic lymphangiogenesis in patients suffering from secondary lymphedema following lymph node surgery [54].

### The cellular origin of LECs in tumor lymphatics

The utilisation of fate mapping experiments during embryonic development has established, at least in the early steps of lymphangiogenesis that LEC precursors arise from multiple venous vascular beds [55]. Wilting et al. showed in an a vein limb bud grafting experiment that homotopically grafted distal wing buds of chick into quail embryos formed lymphatics composed of both chick and quail endothelial cells; suggesting that the lymphatics of the wing bud do not exclusively develop from sprouts from nearby lymph sacs, but also involve recruitment of local so-called 'lymphangioblasts' [55].

In the tumor micro-environment several additional factors such as inflammation may alter the manifestation of these developmental processes. Tumor-induced neo- lymphangiogenesis is thought to originate predominantly from outgrowths from pre-existing lymphatic vessels. However, it still remains to be established whether cell-autonomous contributions to neo-lymphatic formation in the tumor setting are complemented by trans-differentiation from other cell types [56].

A potential non-endothelial LEC-progenitor proposed in the literature are macrophages or circulating endothelial progenitor [57,58]. The role of tumor-associated macrophages (TAMs) in promoting angiogenesis and acquiring an angiogenic phenotype has been well established [59]; but it was not until recently that TAM were suggested as a potential source of LECs. This hypothesis derived from observations that TAMs -which expressed integrin family member cluster of differentiation molecule 11b (*Cd11b*) -acquire lymphatic specific markers including *Prox1*, *Lyve1*, *Podoplanin* and *Vegf-r3*; meanwhile undergoing an observable down-regulation of myeloid markers within the same cells [60]. These TAMs were able to integrate into growing lymphatic vessels in an experimental mouse tumor model [60]. This partial, transient reprogramming of the myeloid identity into a LEC profile suggested that TAMs may contribute to the lymphatic vasculature [61]. Conversely, studies in developing mouse embryos suggested that macrophages solely contributed to modulation of lymphatic vessel caliber by regulating LEC proliferation, but that they did not trans-differentiate to integrate into forming vessels or act as a cellular reservoir for LEC-precursors [62]. More detailed fate mapping experiments in the tumor setting and advanced *in vivo* live imaging will enable researchers to answer definitely whether or not TAM trans-differentiate into LECs, or if they merely act as intermediate cell clusters to bridge newly-formed intra- and peritumoral lymphatics.

A key feature of lymphangiogenesis during embryonic development is the trans- differentiation of venous endothelial cells into LECs [14]. Despite advances our in understanding of the genetic pathways that govern LEC differentiation, studies have yet to explore the hypothesis

that a subset of neo-lymphatics could also arise from pre-existing veins or other blood vascular structures in the adult. Several studies support this concept: COUP-TFII and SOX18 were each shown to be individually required for tumor-induced lymphangiogenesis [63,64]. Further, VEGF-R3 expression has been shown to become reactivated in a subset of the blood vessels [65-67]. Future studies based on fate mapping experiments are required to identify the cellular origin of LEC progenitors in a tumor setting.

### Remodeling of Pre-Existing Adult Lymphatic Vessels in Solid Tumors

#### Peripheral and central tumoral lymphatic sprouting/remodelling

Whilst originally considered a passive conduit, the lymphatic system has been more recently acknowledged as an active, dynamic participant in cancer metastasis [68]. Further, the ways in which the individual vessel subtypes within the hierarchical lymphatic network respond to, and interact with, external cues have also become recognized as critical to cancer spread [35]. Neo-lymphatics formed within the primary tumor have been the focus of much animal and human research, and have been shown to be associated with enhanced rates of metastasis [69]. The location within the tumor (whether central or peripheral) in which the lymphatics form, has also aroused interest; both in terms of what role vessels in each location might play in metastasis and what the different lymphangiogenic mechanisms favoring neo- lymphatics in each location might be [35].

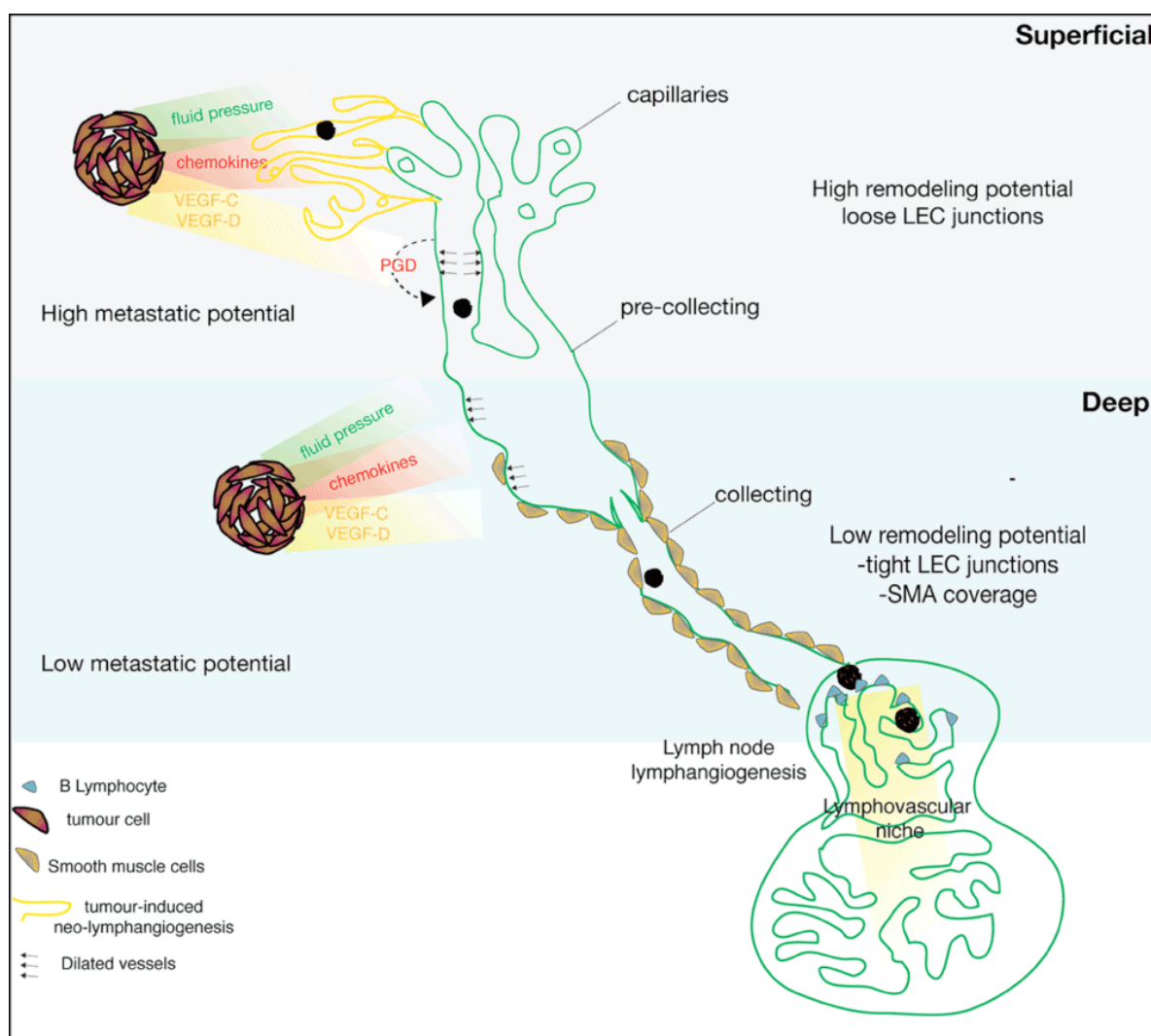
In normal physiology, interstitial fluid bathing the extravascular (interstitial) tissues cycles via the lymphatics back into the blood vascular circulation. Absorbed by thin-walled 'initial' or capillary lymphatics, lymph is transported via progressively enlarging vessels that also adopt a more developed mural structure, consisting of muscular and adventitial layers surrounding LEC-lined lumens. They exhibit both valves and a contraction system that aid lymph movement against gravity [69-71]. For the most part, tumor-induced lymphatics have been shown to resemble a disorganized version of initial lymphatics [72] both mimicking the absorptive function as an entry point to the lymphatic network and sharing structural similarities with their parent vessel of origin [35]. Tumor lymphatics derived from nearby normal initial lymphatics sprout then undergo directional in-growth toward tumor-derived lymphangiogenic cues reminiscent of those seen during development. Further, normal lymphatics surrounding the tumor undergo remodeling in the form of dilatation and increased caliber in response to VEGF-D [72,73] and VEGF-C [74], potentially augmenting the flow into the collecting lymphatic vessels and onward to the draining lymph nodes (Figure 2); a feature associated with enhanced metastasis [68,75].

#### Remodeling of collecting lymphatics

The small caliber initial lymphatics and larger collecting vessels that make up the lymphatic vasculature differ in their morphology, structure and anatomical locations; and perform distinct specialized functions that contribute toward their respective normal physiological roles [76,77]. Most research investigating mechanisms behind lymphogenous metastasis of cancer cells has focused on elucidating the influences of lymphangiogenic growth factors on the initial lymphatic vessel subtypes within or around a primary tumor, whilst the collecting lymphatics that drain tumor tissues towards regional

lymph nodes, have remained largely ignored [78]. Whereas ambient smaller lymphatics largely respond to lymphangiogenic factors by proliferation and/or sprouting to generate tumor-associated lymphangiogenesis (Figure 2), the larger collecting lymphatics respond to the same lymphangiogenic stimuli in a unique and quite distinct manner [35,75,79,80]. Far from passive conduits of metastatic cells, more recent findings suggest that collecting vessels undergo a significant remodeling of their own, which contributes critically to the process of tumor spread [68,75] (Figure 2). Observations of murine models of VEGF-C-over-expressing metastatic tumors demonstrated that drainage from the primary tumor via the collecting lymphatics performs an active role in enhancing tumor dissemination, through increasing fluid flow by dilating-an increase in collecting lymphatic

diameter attributed to LEC proliferation [75,79,80]. Similarly, it was shown that VEGF-D secreted by a flank xenograft tumor model also induced dilatation of the collecting lymphatics draining from the primary tumor to the axilla that was critical for cancer spread to the sentinel lymph node [68]. In contrast to the VEGF-C model and to the effect that VEGF-D had on initial lymphatics [72]; however, the mechanism by which circumferential dilation occurs is not by endothelial proliferation but through specific prostaglandin-mediated responses to VEGF-D [68]. Importantly, treatment with non-steroidal anti-inflammatory drugs not only reversed the VEGF-D-driven morphological remodeling of collection lymphatics, but also reduced the rate of tumor metastasis to draining lymph nodes and distant organs [68].S



**Figure 2: The remodeling of lymphatic vessels influences tumor metastasis.** The proximity of the primary tumor to small, plastic and hyperdense initial or capillary vessels is a key parameter that modulates metastatic events. In small vessels the lymphatic endothelium is only anchored to the matrix by filaments and zipper-like cell-cell junctions that favor the entry of tumor cells. Conversely larger lymphatic vessels surrounded by a layer of smooth-muscle cells and adventitia are less prone to sprouting, however they remodel via alternative prostaglandin mediated mechanisms. The combination of various components from the tumor micro-environment, such as fluid pressure, chemokines and growth factor signaling stimulate neo-lymphangiogenesis and remotely prepare a lymphovascular niche in the tumor draining lymph nodes.

## The influence of tissue forces on lymphatic remodelling

In addition to growth factors, pro-lymphangiogenic cues may also include mechano-biological stimuli such as hydrostatic pressure and tissue flow, which then become translated into lymphangiogenic molecular pathways [71,81]. Whilst the importance of these forces in generating tumor lymphatics is less well understood, emerging work indicates that mechano-induction may also be an important additional determinant of the density and nature of lymphatics generated within different regions of the tumor [71,81]. Both mechano-induction and lymphangiogenic signaling pathways are process 'borrowed' from embryogenesis. During development, mechanosensory complexes formed by Integrin  $\beta 1$  and VEGFR-3 can translate increased interstitial/ECM stiffness into lymphangiogenic signals [70,71]. Increased interstitial fluid pressure leads to activation of Integrin  $\beta 1$ , which in turn induces VEGFR-3 tyrosine phosphorylation [82]. This VEGFR-3 activation is thought to be mediated by Src family kinases (SFKs) in a VEGF-C-independent pathway [83], thus resulting in VEGF-C-independent LEC proliferation [82]. Further, fibrillin anchoring-filaments were shown to be capable of binding transmembrane integrin glycoproteins to activate intracellular signaling pathways [77,84]. These signals translate ECM stiffening into cytoskeletal alterations that increase cell permeability in order to facilitate improved fluid uptake and flow [77,84]. In a tumor, higher fluid pressure within the centre or increased lymph flow toward the periphery may also determine the nature of lymphatics generated in different regions. High intra-tumoral pressure due to leaky nascent lymphatics and blood vessels may both induce intra-tumoral vessels to grow and lead to their collapse (due to poorly developed supporting structures) and dysfunction, contributing to further fluid accumulation [85]. This accumulation of non-absorbed fluid in turn produces a pressure gradient favoring flow towards the lower pressure tumor periphery, where the vessels remain functional due to lower ambient pressure and VEGF-C/D-mediated dilatation [73] (Figure 2). Whilst a specific role for hydrostatic pressure in driving tumor lymphangiogenesis remains to be defined in cancer, fluid flow gradients have been shown to drive tumor lymphangiogenesis [86].

Regardless of the underlying stimulus, however, tumors that recreate developmental lymphangiogenesis can enhance metastasis. The respective contributions of newly- formed vessels and the pre-formed mature lymphatics to the metastatic process has been debated [69]. The role/functionality of the nascent lymphatics within the substance of the tumor, and the degree of vessel dilation/collapse compared with those induced in peripheral areas of the tumor (or immediately adjacent tissues), remains controversial [87,88]. Intratumoral lymphatics were shown to be present in a murine model, yet predominantly collapsed -in contrast to the apparently dilated peri-tumoral lymphatics [87]. Analysis of human tumors showed that peri-tumoral lymphatics were the most important for metastasis; while intra-tumoral lymphatics exhibit proliferative markers [88], these vessels were collapsed and unable to transport tumor cells, despite a higher vessels density [42,75,89]. Whether peri-tumoral lymphatics represented pre-existing vessels compressed by an expanding tumor, or 'neo-lymphatics' generated through lymphangiogenesis remains unclear [89,90]. Authors postulate that there is both a greater concentration of stromal and inflammatory cells secreting VEGF-family members, and a greater density of pre-existing vessels to provide a 'source' from which tumor neo-lymphatics can sprout, in the periperal tumor microenvironment [91-93]. Studies of human melanomas found that peri-tumoral vessel density and caliber were significantly increased in metastatic lesions and were associated with

regional metastasis, poor disease-free and overall patient survival [91,94,95]. A more recent study matching melanoma samples for all other prognostic indicators found that a high ratio of peri-tumoral-to-intra-tumoral lymphatic vessel density was associated with a higher rate of metastasis to the draining lymph node basin [35]. Human breast cancer specimens also exhibited collapsed intra-tumoral vessels (poorly-staining with proliferation markers) and increased densities of peri- tumoral lymphatics, that contained tumor emboli [88,96].

## Lymphangiogenesis in the sentinel lymph node

Comparatively little is known about the development of lymph nodes or other lymphoid tissue. During development, these structures are situated along collecting lymphatics within a lymph node 'anlagen' or precursor tissue, at the site of future lymph nodes. Lymph nodes originate from connective tissues protruding into primitive lymph sacs, and integrate lymphatics and blood vessels with haemopoietic cells and stromal supportive cells [97,98]. This process incorporates mesenchymal cells differentiation into aggregates of CD45+CD4+CD3+ lymphoid 'tissue inducer cells' and stromal organiser cells [98]. These distinct cell types interact within the anlagen to stimulate adhesion molecule expression on stromal organiser cells, and release chemokines such as CCL19, CCL21 and CXCL13; signals which, in turn, attract additional lymphoid 'tissue-inducer cells' and other haemopoietic cells [98,99]. Importantly, mouse models showed that lymph node development is genetically independent from lymphatic vascular embryogenesis [100].

## A lymph node and distant organ metastatic niche

Lymph nodes represent a key 'staging-post' in tumor dissemination from the primary cancer toward distant organs. The pro-metastatic effects of tumor-derived lymphangiogenic growth factors are not limited only to the primary tumor microenvironment and may also induce lymphangiogenesis and modulate existing lymphatics and blood vessels within draining lymph nodes 'downstream' of the tumor; often prior to the arrival of metastatic cells [101,102]. Harrell et al. observed that lymphangiogenesis and enhanced lymph flow preceded melanoma metastasis, and was associated with increased levels of B-lymphocytes within the nodes [103]. These observations are consistent with the 'seed' and 'soil' theory of metastasis articulated by Paget in 1889. He suggested that primary cancer cells can spread preferentially to specific distant areas. Thus, increase lymphatic vessel density in a sentinel lymph node prior to any detectable metastasis could promote enhanced tumor transport to the lymph nodes and could serve an accurate predictor of lymphogenous spread [104] (Figure 2). The enhancement of the lymphatic network within these draining nodes is referred as the 'lymphovascular niche', which is akin to the 'vascular niche' seen in the formation and maintenance of hemopoietic stem cells within lymph nodes and bone marrow [104,105]. The lymphovascular niche may be able to tailor the microenvironment and, therefore, immune responses against cancer cells transiting toward the lymph node. Additionally, this niche may influence the conditions that preempt then support the survival and growth of metastatic deposits [106].

## Future Approaches to Manipulating Cancer Lymphatics

Most of the novel anti-lymphangiogenic strategies to restrict tumor metastasis revolve around the VEGF-C/VEGF-R3 signaling axis, with



the view to complementing anti-angiogenic therapies. Data from pre-clinical models suggest a clear benefit in targeting tumor-induced lymphangiogenesis to reduce solid tumor metastasis [107]. The next step is now to assess the clinical outcome of targeting the lymphatic vasculature in human cancer. Some anti-angiogenic molecules that target pathways involving the VEGF-family of growth factors also restrict lymphangiogenesis via the inhibition of the VEGFR-3 pathway; however, specific benefit of anti-lymphangiogenic therapies in this setting needs to be assessed more thoroughly, aside from evaluating tumor burden in the draining lymph nodes. In rare lymphatic vascular disorders, it is possible to manipulate LEC proliferation; as demonstrated by using Sildenafil (Viagra) treatment to restrict the growth of pediatric orbital lymphangioma [108]. So far, only two active phase I clinical trials targeting the VEGF-C/VEGF-R3 pathway have been initiated. The first combined Bevacizumab (avastin, anti-VEGF-A) with VGX-100 (anti-VEGF-C blocking antibody, NCT01514123) and another targeted VEGF-R3 (NCT01288989) in advanced solid adult malignancy, however, the outcomes are still pending.

Most of the novel anti-lymphangiogenic strategies to restrict tumor metastasis revolve around the VEGF-C/VEGF-R3 signaling axis, with the view to complementing anti-angiogenic therapies. Data from pre-clinical models suggest a clear benefit in targeting tumor-induced lymphangiogenesis to reduce solid tumor metastasis [107]. The next step is now to assess the clinical outcome of targeting the lymphatic vasculature in human cancer. Some anti-angiogenic molecules that target pathways involving the VEGF-family of growth factors also restrict lymphangiogenesis via the inhibition of the VEGFR-3 pathway; however, specific benefit of anti-lymphangiogenic therapies in this setting needs to be assessed more thoroughly, aside from evaluating tumor burden in the draining lymph nodes. In rare lymphatic vascular disorders, it is possible to manipulate LEC proliferation; as demonstrated by using Sildenafil (Viagra) treatment to restrict the growth of pediatric orbital lymphangioma [108]. So far, only two active phase I clinical trials targeting the VEGF-C/VEGF-R3 pathway have been initiated. The first combined Bevacizumab (avastin, anti-VEGF-A) with VGX-100 (anti-VEGF-C blocking antibody, NCT01514123) and another targeted VEGF-R3 (NCT01288989) in advanced solid adult malignancy, however, the outcomes are still pending. Despite major advances in our understanding of the molecular pathways involved in VEGF biology, the development of anti-VEGF drugs in human cancer has already started to show some limitations. Long-term clinical benefit of VEGF-based therapies has remained marginal due to resistance mechanisms mediated by a combination of both VEGF-axis-dependent alterations and non-VEGF-dependent pathways [109]. In some cases, anti-angio/lymphangiogenic approaches even led to the opposite effect and enhanced tumor metastasis [110]. To complement anti-VEGF-A strategies, other monoclonal antibody inhibitors have been developed against targets such as angiopoietin signaling antagonists [111,112]. In order to avoid developing resistance phenomena similar to limitations as 'Avastin-escape' seen in anti-VEGF-A therapies, it will be essential to further explore the potential of VEGF-C- and VEGF-D-independent strategies. Additionally, immuno-modulatory regulating effects of the VEGF protein growth factor family is an aspect of their biology that remains relatively neglected in designing anti-angiogenic therapeutic strategies; for example VEGF-A was shown to promote priming of natural killer cells in mouse tumor models [113,114], while VEGF-C was reported to enhance immunity in a B16 melanoma model [115]. Further, manipulating the remodeling of the lymphatic

network may also affect immune cell trafficking and therefore influence the inflammatory micro-environment of the tumor. These findings have led to the reassessment of the approaches used to target the VEGF family of proteins in the tumor setting.

Lastly, the heterogeneity of the both the blood and lymphatic tumor vasculature may also contribute to limiting the success of the anti-VEGF therapies, as only a subset of the remodeling vasculature is affected by the blockade of VEGF signaling [116]. Further, the broadening of the therapeutic focus from simply targeting newly-sprouting tumor lymphatics and nearby initial lymphatics under the influence of the tumor microenvironment, to also encompass the collecting lymphatics may provide further avenues for therapeutic intervention. A more detailed understanding of the biological characteristics of the different lymphatic subtypes and their role in metastasis is thus, fundamental. An example of detailed mechanistic understanding leading to novel therapeutic approaches is the elucidation of the role of PGE2 in metastasis resulting from VEGF-D-mediated down-regulation of the enzyme prostaglandin dehydrogenase by Karnezis et al.; and the inhibition of metastasis by using non-steroidal anti-inflammatory drugs [68]. Several subsequent clinical studies have corroborated an anti-metastatic benefit to breast cancer patients treated with Aspirin [117,118].

Overall, therefore, it is critical to further explore the developmental pathways that govern lymphangiogenesis in order to facilitate the identification, characterisation and optimisation of molecular targets in tumor lymphangiogenesis, and to provide new directions in treatments to restrict the spread of cancer.

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