

OPINION

## Emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis

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**Abstract** | Malignant tumours can spread to lymph nodes through lymphatic vessels. Recent studies show that tumours produce a range of growth factors that directly or indirectly stimulate lymphatic vessel growth (lymphangiogenesis) and lymphatic metastasis. These findings indicate that tumour lymphangiogenesis, similar to haemangiogenesis, is a complex process that is regulated by multiple growth factors. Understanding the underlying mechanisms by which tumours induce lymphangiogenesis might provide important information for the therapeutic intervention of metastatic spread.

Metastasis of malignant tumours to regional lymph nodes is one of the early signs of cancer spread in patients, and it occurs at least as frequently as haematogenous metastasis. In certain types of cancer, such as breast cancer, lymphatic metastasis is one of the predominant routes of cancer spread<sup>1–6</sup>. From the lymphatic system, cancer cells can be transported to the circulation and can spread to distal organs and tissues through blood vessels<sup>7–9</sup>. Dissemination of tumour cells from the primary sites to the lymphatic system occurs either by invasion into pre-existing lymphatic vessels in surrounding tissues or by invasion into intratumoural lymphatic networks<sup>10,11</sup>.

The physiological function of lymphatic vascular networks in the body is to collect extravasated fluid, macromolecules and leukocytes at regional lymph nodes for

immune surveillance, and then transport them to the blood vessels for circulation<sup>11–13</sup>. Lymphatic microvessels consist of a thin endothelium, which is usually sparsely coated with pericytes and vascular smooth muscle cells (VSMCs)<sup>14,15</sup>. Although accumulating evidence shows that intratumoural lymphatic networks are vital for lymphatic metastasis, little is known about possible structural and functional differences between healthy lymphatic vessels and those present in tumours. Tumour blood vessels usually consist of disorganized, leaky and tortuous vasculatures, indicating that tumour lymphatic capillaries might have similar characteristics. Recent studies demonstrate that peritumoural and intratumoural lymphatic networks also consist of disorganized microvessels that might lack drainage function<sup>16,17</sup>. The structural irregularity and leaky features of tumoural lymphatic vessels might make them more susceptible for invasion by malignant cells<sup>18</sup>. In addition, lymphatic endothelial cells (LECs) of tumour-associated lymphatic networks have been reported to interact with tumour cells and to facilitate their transmigration through the endothelium<sup>12,19,20</sup>.

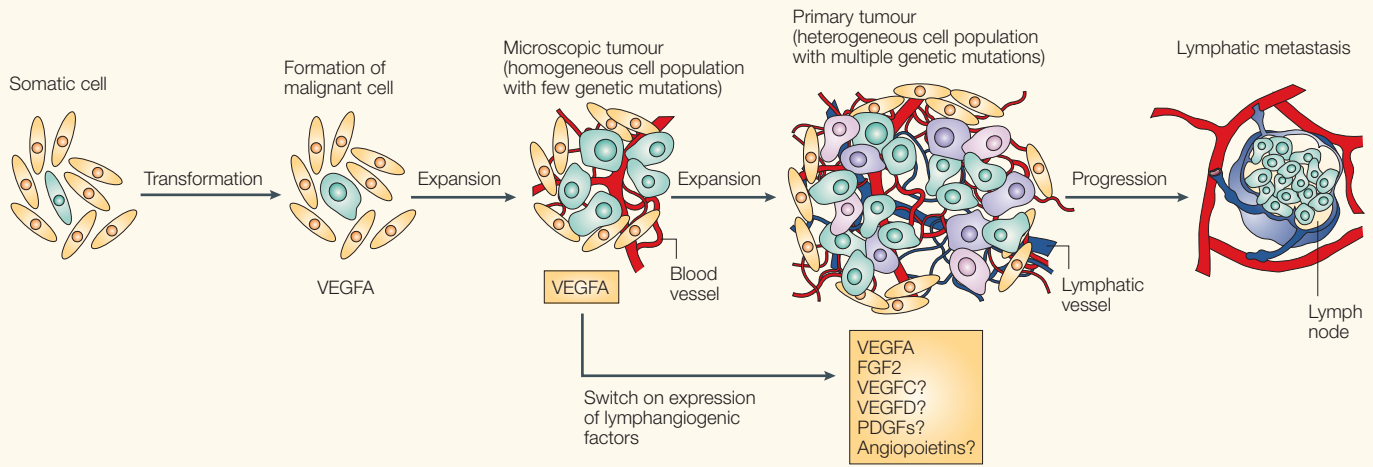
Similar to the blood vasculature, lymphatic vessels in most adult tissues and organs are quiescent under physiological conditions. Based on what we have learnt from haemangiogenesis, it seems plausible that a lymphangiogenic phenotype must

be switched on to initiate lymphatic vessel growth. The ‘lymphangiogenic switch’ in tumours might represent a mirror image of the haemangiogenic switch, with overproduction of lymphangiogenic factors and downregulation of lymphangiogenesis inhibitors. A range of lymphangiogenic factors that are produced by tumour cells, stromal cells or inflammatory cells have recently been identified<sup>21–27</sup>. These findings indicate that lymphangiogenesis is a complex process that is controlled by multiple factors produced by various cell types, and that the functional outcome might be dependent on the combined effect of these factors. Therefore, combinations of different antagonists targeting multiple lymphangiogenic factors should be considered for therapeutic suppression of lymphangiogenesis and lymphatic metastasis. Although many endogenous inhibitors of haemangiogenesis, including angiostatin, endostatin and tumstatin, have been shown to suppress tumour angiogenesis<sup>28–30</sup>, it is still unclear whether these molecules can also block lymphangiogenesis. The fact that lymphangiogenesis can occur independently from haemangiogenesis suggests the development of specific inhibitors that target lymphatic vessels is warranted for prevention of lymphatic metastasis.

### A lymphangiogenic switch?

The formation of malignant cells that lack the ability to switch on angiogenesis rarely leads to clinically detectable cancers<sup>31,32</sup>. Spreading of tumour cells to the lymphatic system might also require stimulation of lymphangiogenesis by malignant cells<sup>10,20,33</sup>. Experimental evidence to support the concept of the ‘lymphangiogenic switch’ is currently lacking. But, based on haemangiogenesis, it seems likely that the acquisition of new lymph vessels to supply the tumour is triggered at some point during the development of the tumour.

Accumulation of sequential genetic alterations in tumour cells might turn on the expression or release of several haemangiogenic and lymphangiogenic



**Figure 1 | The haemangiogenic switch and potential lymphangiogenic switch.** Mutations of vital oncogenes and tumour-suppressor genes cause the transformation of a normal somatic cell into a tumour cell. At early stages of malignancy, a tumour at microscopic size contains a relatively homogenous cell population with a limited number of mutations in their genome. The tumour cells might only produce vascular endothelial growth factor A (VEGFA) as an angiogenic factor. However, during tumour progression, genomic instability of tumour cells often leads to the accumulation of genetic alterations that switch on the expression of multiple angiogenic and potentially lymphangiogenic factors and therefore promote cancer metastasis. The switching on of lymphangiogenesis during tumour progression is still a hypothesis. FGF2, fibroblast growth factor 2; PDGF, platelet-derived growth factor.

factors<sup>34–36</sup>. For example, during the multistep development of a fibrosarcoma in a mouse genetic model, secretion of fibroblast growth factor 2 (FGF2) occurs during the transition from hyperplasia to malignant fibrosarcoma, indicating that an accumulation of genetic mutations is required to switch on angiogenesis and assumedly also lymphangiogenesis<sup>19,37</sup>. Wild-type p53 — tumour suppressor protein — inhibits FGF2 expression at both the transcriptional and post-transcriptional levels, and the frequent occurrence of mutations in the TP53 gene (more than 50% of all human tumours) might increase the expression of FGF2 and promote lymphangiogenesis<sup>23,25,38–40</sup>.

In addition to FGF2, loss of p53 function owing to genetic alterations also abrogates wild-type p53-mediated repression of the expression of different isoforms of vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs). Also affected by the disruption of p53 expression are the different isoforms of platelet-derived growth factors (PDGFs) and PDGF receptors (PDGFRs) — two receptor–ligand systems that transduce active signals for lymphangiogenesis<sup>41–43</sup>.

The von Hippel–Lindau (VHL) tumour suppressor protein represses expression of PDGF-BB. The VHL protein also negatively controls VEGFA expression through targeting of hypoxia-inducible factor (HIF)- $\alpha$  for proteolytic degradation<sup>44,45</sup>. Because both PDGF-BB and VEGFA induce lymphangiogenesis, loss of VHL function might result in increased lymphangiogenic activity by these

factors. Germline alterations of the VHL gene in humans result in multiple tumour types with increased metastatic capacities<sup>46</sup>. Oncogenes such as RAS and MYC lead to increased expression of lymphangiogenic factors, including members of the VEGF, FGF and PDGF families<sup>47,48</sup>. The heterogeneity of tumour-cell populations in a relatively large tumour might further select for the expression of other lymphangiogenic factors such as VEGFC, VEGFD and angiopoietins<sup>41,49</sup> (FIG. 1). These factors might function as joint switches for tumour lymphangiogenesis.

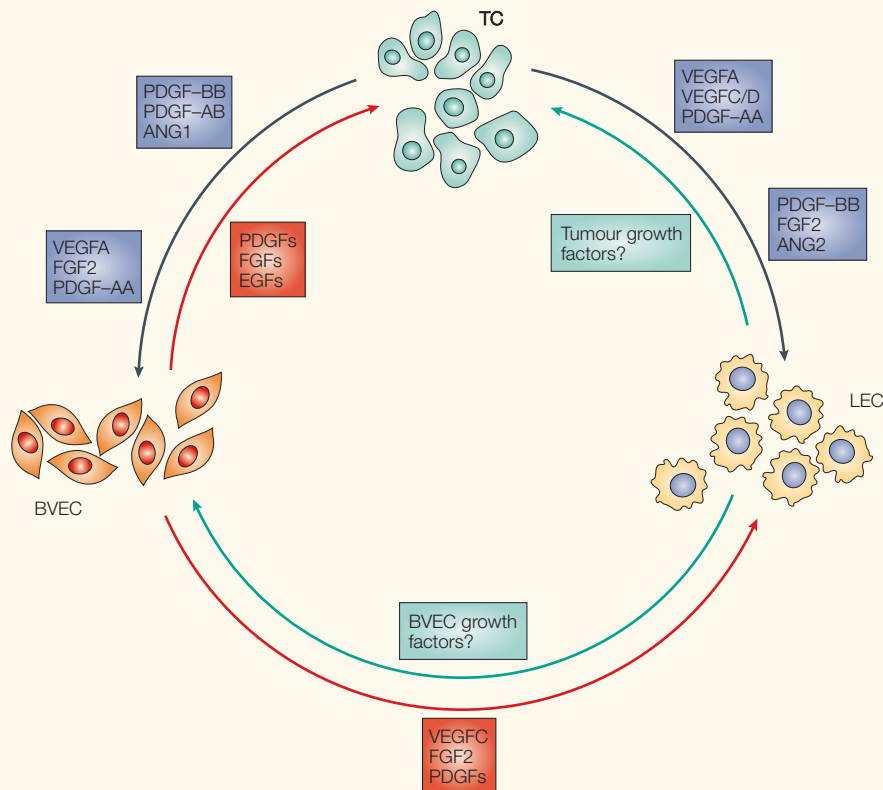
**Why tumour lymphangiogenesis?**

Outgrowth of blood vessels into a solid tumour supplies oxygen and nutrients for tumour cells and removes metabolic waste products. But it is uncertain how lymphatic vessels contribute to tumour growth. As not all solid tumours induce intratumoural lymphangiogenesis, the lymphatic vasculature itself is apparently not a prerequisite for tumour growth. Why then do certain types of solid tumour induce lymphatic vessel growth? What is the role of lymphatic vessels in tumour growth and invasion? Lymphatic vessels might contribute to tumour growth and invasion by several mechanisms.

**Crosstalk between tumour cells, LEC and blood vessel endothelial cells.** Although the exact roles of LECs in tumours need to be defined, it seems plausible that the infiltration of lymphatic vessels into the tumour tissue might also establish a paracrine signalling pathway for tumour-cell growth

through the release of specific, but as-yet-uncharacterized, growth factors (FIG. 2). These LEC-derived growth factors might also function as haemangiogenic factors, and might further enhance tumour-induced haemangiogenesis. Blood vessel endothelial cells (BVECs) produce lymphangiogenic factors such as VEGFC, FGF2 and PDGFs to facilitate tumour-induced lymphangiogenesis<sup>34,50</sup>. In addition, BVEC-derived paracrine growth factors such as PDGFs, epidermal growth factors and FGFs also stimulate tumour-cell growth<sup>34</sup>. The bilateral interplays of these three compartments are all beneficial for tumour growth. BVECs and LECs produce matrix metalloproteinases (MMPs) and urokinase plasminogen activator (uPA). These proteases directly (or indirectly) potentiate the invasiveness of tumours in the surrounding tissues and their spread into remote tissues<sup>50</sup>.

**Lowering of interstitial fluid pressure in the tumour.** Many solid tumours have increased interstitial fluid pressure (IFP) owing to their disorganized and leaky vasculatures<sup>51,52</sup>. In some tumours, high IFP forms a barrier to transcapillary transport and restricts blood flow within the tumour tissue<sup>51,52</sup>. Although microvessel density is usually high, these microvessels can remain occluded owing to the high IFP. Therefore, most tumour tissues are exposed to a hypoxic/ischaemic environment, which limits tumour growth. The growth of lymphatic vessels within a tumour would, in principle, increase the drainage of interstitial fluids,



**Figure 2 | Crosstalk between tumour cells, blood vessel endothelial cells and lymphatic endothelial cells.** Tumour cells (TCs) produce multiple haemangiogenic and lymphatic angiogenic factors that stimulate the growth of blood vessel endothelial cells (BVECs) and lymphatic endothelial cells (LECs). The BVEC compartment also produces growth factors to promote tumour-cell growth. In addition, tumour-infiltrated BVECs also produce lymphangiogenic factors to stimulate the growth of LECs. LECs might also produce growth factors that stimulate the growth of tumour cells and BVECs, but this remains to be shown. It is therefore possible that all three compartments benefit from these bilateral interactions. ANG, angiopoietin; EGF, epidermal growth factor; FGF2, fibroblast growth factor 2; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

facilitating tumour growth by lowering IFP and increasing blood perfusion within the tumour tissue. But this issue remains controversial and needs to be studied further. A recent investigation shows that stimulation of lymphangiogenesis with VEGFC did not lower IFP in tumours, indicating that lymphatic vessels within tumours are not functional<sup>17,19</sup>. It is unclear if other lymphangiogenic factors can induce functional vessels in tumours.

#### Structural basis for tumour-cell invasion.

Lymphatic vessels consist of a single, thin LEC layer, and have larger diameters than blood vessels<sup>14,21,53,54</sup>. The endothelium of microlymphatic vessels lacks tight junctions and is present on a discontinuous layer of basement membrane. In addition, small lymphatic vessels are not coated with pericytes or VSMCs, and lymphatic endothelial cells are attached to the extracellular matrix by elastase fibres that keep these vessels open. These structural features of

lymphatics make them more accessible for tumour-cell invasion and enable access to regional lymph nodes. Invasion of tumour cells into lymphatic vessels might therefore be easier than invasion into blood vessels. The interaction of LECs with tumour cells has been shown to facilitate tumour-cell invasion into the lymphatics. Mihaela Skobe and colleagues have shown that lymphatic capillaries activated by factors produced from tumours, such as VEGFC, promote tumour-cell invasion by increasing tumour-cell transendothelial migration<sup>19</sup>. This effect could in part be due to the expression of CC-type chemokine ligand 1 (CCL1) on LECs and its receptor CC-type chemokine receptor 8 (CCR8) on tumour cells<sup>19</sup>.

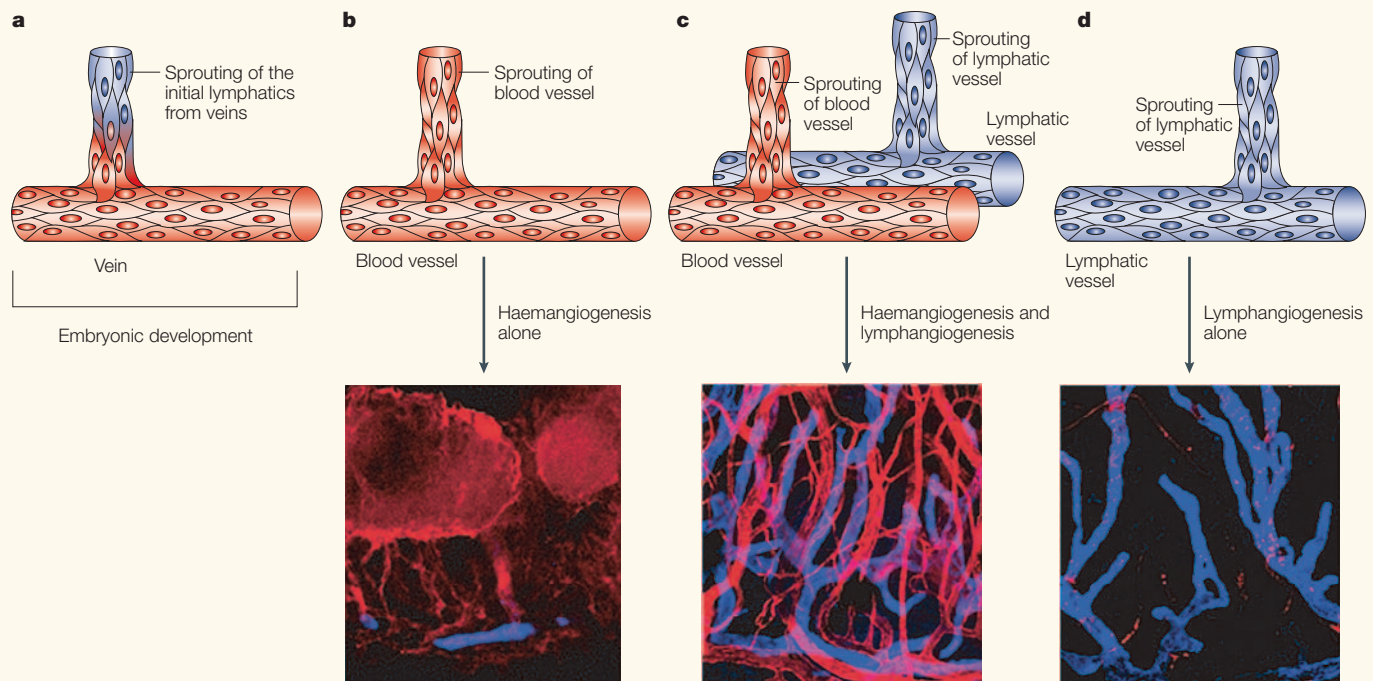
#### Lymphatic metastasis

A clinically detectable metastatic lesion in lymph nodes represents a consequence of complex multistep processes, including the dissemination of tumour cells from

the primary site to the lymphatic vessels, the transport of tumour cells through the lymphatic system to the lymph nodes, the settlement of tumour cells in lymph nodes, and the growth of the metastatic lesion to a detectable mass. As discussed above, dissemination of tumour cells to the lymphatic system requires physical contacts between tumour cells and lymphatic vessels<sup>17,19,55–57</sup>. It is unclear whether this contact is mediated by specific adhesive events or by high IFP that drives cells into the lymphatic vessels. The size of peritumoural lymphatic vessels has been indicated as the most important factor that contributes to lymph-node metastasis in human malignant melanoma<sup>57</sup>. However, other studies show that intratumoural lymphatic vessels are vital for lymphatic metastasis<sup>17,20,55,57–59</sup>. The retrospective analyses of human cancer samples have shown a positive correlation between tumoural lymphatics and lymphatic metastases<sup>55,60</sup>.

Tumour, stromal and inflammatory cells that are present in the tumour microenvironment produce multiple lymphangiogenic factors that might stimulate intratumoural lymphangiogenesis<sup>21,23–25,54,58,61,62</sup>. Factors including VEGFA, VEGFC, VEGFD and PDGFBB have been reported to induce lymphatic metastasis<sup>10,11,20,21,33,63</sup>. Transport of tumour cells through the lymphatic system to the regional lymph nodes might indicate that tumour lymphatic systems have drainage functions, despite tumour lymphatic vessels reportedly lacking this capacity<sup>17</sup>. Several studies show that intratumoural lymphatic vessels are often occluded with tumour cells at the time when lymph-node metastases are detectable<sup>17,20,58</sup>. It is therefore possible that tumour lymphatic vessels have normal drainage functions at early stages, but that these functions are blocked at later stages of tumour progression. In addition, the high IFP in tumours might force tumour cells to enter the lymphatic system. Further work is warranted to confirm this possibility.

Although sprouting of new lymphatic microvessels is a key process of intratumoural lymphangiogenesis, it is unclear if recruitment of precursor LECs also participate in the formation of intratumoural lymphatic vessels. *In vitro* studies show that bone-marrow-derived stem cells differentiate into LEC-like precursor cells<sup>64</sup>, although *in vivo* transplantation experiments indicate that bone-marrow-derived cells do not significantly contribute to tumour lymphangiogenesis<sup>65</sup>.



**Figure 3 | Relationship between haemangiogenesis and lymphangiogenesis.** **a** | During the development of early embryos, the first lymphatic vessel sprouts from a vein. *PROX1* is required for differentiation of blood vessel endothelial cells into lymphatic endothelial cells, and vascular endothelial growth factor receptor 3 (VEGFR3) signalling is essential for sprouting. **b** | Haemangiogenesis without lymphangiogenesis. In the mouse cornea model of angiogenesis, VEGFA at day 5 after implantation only stimulates the growth of blood vessels (CD31, red), but not lymphatic vessels (LYVE-1, blue) as shown in the fluorescent image. **c** | Concomitant haemangiogenesis and lymphangiogenesis. At day 12 after implantation, fibroblast growth factor 2 (FGF2) induces both blood (CD31, red) and lymphatic vessel (LYVE-1, blue) growth in the mouse cornea, as shown in the fluorescent image. **d** | Lymphangiogenesis without haemangiogenesis. At day of 12 of implantation, FGF2 stimulates only lymphatic vessel (LYVE1, blue), but not blood vessel (CD31, red) growth on the opposite circumferential sphere of the growth-factor-implanted surface of the corneal globe as shown in the fluorescent image.

### Haem- and lymphangiogenesis

Lymphangiogenesis and haemangiogenesis are tightly linked. During the development of early embryos, the first lymphatic vessel is known to sprout from a vein. *VEGFR3* is required for differentiation of BVECs expressing the homeobox gene *PROX1* into LECs<sup>66,67</sup> (FIG. 3a). The formation of new blood vessels involves the sprouting of new capillaries from pre-existing vessels (angiogenesis), the recruitment of stem cells that differentiate into endothelial precursor cells (vasculogenesis), and the division of large ‘mother’ vessels into smaller ‘daughter’ vessels (intussusception)<sup>34,68–70</sup>. In the adult, lymphangiogenesis occurs mainly through the sprouting of new lymphatic vessels from the pre-existing lymphatic system<sup>11,15,19,54,61</sup>. Blood vessels can grow alone, without lymphatics<sup>71</sup>; for example, VEGFA only induces neovascularization, and not lymphangiogenesis, in the cornea within 3–5 days after growth-factor implantation (FIG. 3b). However, under most situations, it seems likely that lymphatic vessels grow concomitantly with blood vessels (FIG. 3c). Interestingly, results from our own laboratory and the published results of another group show that haemangiogenic/lymphangiogenic

factors such as FGF2, when present at particular sites of the corneal tissue, only induce lymphangiogenesis without blood vessels<sup>23</sup> (Y.C., unpublished observations; FIG. 3d). This observation has revealed two interesting points. First, lymphangiogenesis occurs independently from haemangiogenesis and in the absence of pre-existing blood vessels (the cornea lacks pre-existing vessels). Second, haemangiogenic or lymphangiogenic factors might differentially induce haemangiogenesis or lymphangiogenesis depending on where they are expressed in a tissue.

Separation of lymphangiogenesis from haemangiogenesis might provide important clues to understand the mechanism that underlies the formation of new lymphatic vessels, as well as the occurrence of cancer metastases only in the lymphatic system. Such findings would aid the development of therapeutic agents to specifically target lymphangiogenesis. Currently, it is unknown why potent haemangiogenic factors such as FGF2 only induce lymphangiogenesis under certain conditions. Although this question remains to be answered, recent data indicate that angiogenic-factor-induced inflammatory cells contribute to lymphangiogenesis<sup>61,72</sup>.

An interesting observation is that irradiation of bone marrow significantly blocks lymphangiogenesis<sup>73</sup>. These findings indicate that FGF2, and probably other angiogenic factors, can indirectly stimulate lymphangiogenesis by attracting bone-marrow-derived inflammatory cells, which might then produce cytokines that specifically induce lymphangiogenesis. For example, VEGFA functions as a chemotactic factor for inflammatory macrophages through the VEGFR1 receptor<sup>61,73,74</sup>.

Chang *et al.* show that FGF2 can differentially induce haemangiogenesis and lymphangiogenesis, or lymphangiogenesis alone, depending on the doses of FGF2 that are applied to the cornea<sup>23</sup>. At a low dose, FGF2 only stimulates lymphangiogenesis but not blood vessel growth, indicating that LECs are more sensitive than BVECs to FGF2 stimulation. Therefore, it is possible that FGF2 produced by tumours at low levels might preferentially switch on lymphangiogenesis and in turn facilitate lymphatic metastasis. Mechanistically, it is not clear why LECs are more sensitive than BVECs to FGF2. Similar to VEGFA, FGF2 also induces accumulation of inflammatory cells that might also contribute to lymphangiogenesis<sup>23</sup>.

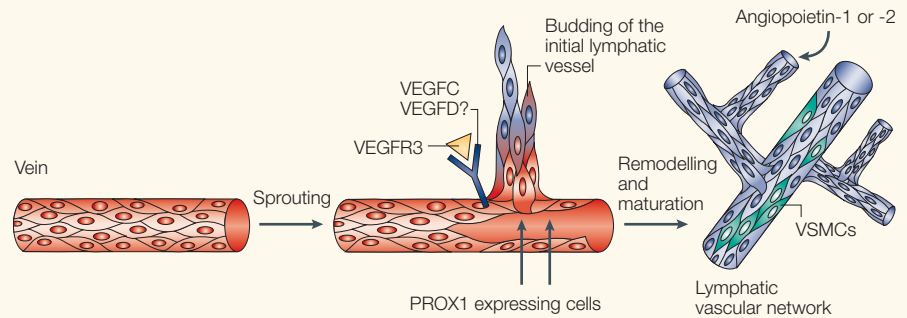
### Lymphangiogenic factors

Among known lymphangiogenic factors, the VEGFC/VEGFD–VEGFR3 pathway is the best-characterized signalling system. It has a vital role in the budding of initial lymphatics from PROX1-expressing vein endothelium. Elimination of either *Vegfc* or *Prox1* genes in mice results in failure to form the initial lymphatic vasculature in embryos<sup>66,67</sup>.

Recently, several lymphangiogenic factors have been reported. In addition to members of the VEGF family, these factors include members of the FGF, PDGF and angiopoietin families, and they seem to have interdependent or collaborative roles with each other or with VEGFs in the establishment of functional lymphatics. For example, VEGFC is required for the formation and growth of initial lymphatic vessels, whereas angiopoietins and their receptors are essential for later stages of remodelling and maturation<sup>24</sup> (FIG. 4). Without angiopoietin-2, VEGFC is unable to establish functional lymphatic vessels in the adult. These studies highlight that the regulation of lymphatic vessel formation and growth is complex and requires collaborative action among these different factors.

### PDGFs promote lymphangiogenesis and metastasis.

The PDGF family includes three well-characterized members — the homodimers PDGF-AA and PDGF-BB, and the heterodimer PDGF-AB — and two new homodimer members PDGF-CC and PDGF-DD<sup>75,76</sup>. Their biological functions are mediated by activation of the tyrosine-kinase receptors, PDGFR $\alpha$ , PDGFR $\beta$  and PDGF $\alpha\beta$ , which are encoded by two genes<sup>75,76</sup>. Among all PDGFs, PDGF-BB is the only ligand that can activate all three forms of PDGFR. The complexity of various ligand–receptor interactions indicates that members of the PDGF family have important and non-overlapping physiological functions. Inactivation of genes that code for PDGFs or PDGFRs have been found to result in early embryonic lethality owing to defective development of certain tissues and organs<sup>77,78</sup>. Intriguingly, PDGFB and PDGFR $\beta$ -knockout mice exhibit a haemorrhagic and oedematous phenotype<sup>77–79</sup>. Although this phenotype could, in part, be attributed to defective development and recruitment of pericytes and VSMCs, it is also possible that defective development of a functional lymphatic vasculature in these knockout mice might contribute to tissue oedema. This possibility remains to be further investigated.



**Figure 4 | Collaborative signalling systems.** While the vascular endothelial growth factor C (VEGFC)–VEGFD–VEGFR3 (VEGF receptor 3) system is required for sprouting of the initial lymphatic vessels from the venous system, angiopoietin-2–TIE2 is essential for development, remodelling and maturation of functional lymphatic networks. VSMC, vascular smooth muscle cell. TIE2; Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains.

In the mouse cornea, PDGF-AA, PDGF-AB and PDGF-BB can all stimulate lymphatic vessel growth, PDGF-BB being the most potent<sup>21</sup>. These findings indicate that both PDGFR $\alpha$  and PDGFR $\beta$  are involved in lymphatic vessel growth. PDGFR $\alpha$  and PDGFR $\beta$  are expressed on isolated LECs and the newly formed lymphatic vessels<sup>21</sup>. In isolated human, rat and mouse LECs, PDGFs stimulate cell migration, as indicated by an increase in the levels of phosphorylated SRC, extracellular signal-regulated kinase (ERK) and AKT. This stimulation can be inhibited by the PDGFR inhibitor imatinib (Gleevec), but not by VEGFC, VEGFD or VEGFR3 antagonists. These results indicate that PDGFs function as direct lymphangiogenic factors. However, it is also probable that PDGFs indirectly stimulate lymphangiogenesis through other growth factors and cytokines.

PDGFs, including PDGF-AA and PDGF-BB, are often highly expressed in tumour tissues that have an increased incidence of lymphatic metastasis<sup>80,81</sup>. The lymphangiogenic nature of the PDGFs indicates that they might promote tumour lymphangiogenesis and lymphatic metastasis. PDGF-BB-overexpressing tumour tissue implanted in the mouse cornea induces extensive, but disorganized, neovascularization compared with wild-type tumours. PDGF-BB also induces sprouting of new lymphatic vessels, which are leaky and disorganized and seem to accumulate at the borders of the tumour tissue and occasionally penetrate into deeper regions of the tumour. Furthermore, in a mouse subcutaneous fibrosarcoma model, PDGF-BB-expressing tumour-bearing mice develop metastatic lesions in the axillary lymph nodes after removal of primary tumours<sup>21</sup>. Therefore, peritumoural lymphatic vessels might aid the formation of metastases. Taken together with other published data<sup>82–84</sup>, these results indicate

that PDGFs promote malignant progression through three mechanisms; direct effects on tumour cells; stimulation of tumour haemangiogenesis and haematogenous metastasis; and stimulation of tumour lymphangiogenesis and lymphatic metastasis (FIG. 5). Therefore, suppressing the functions of PDGFs is an important approach for cancer therapy and prevention of lymphatic metastasis.

**FGF2 and lymphangiogenesis.** FGF2 is an angiogenic molecule that has potent stimulatory activity on endothelial cells *in vitro* and on angiogenesis *in vivo*<sup>21,85</sup>. It is the prototype of perhaps the largest family of growth factors, with 23 current members<sup>86</sup>. Unlike most other growth factors, FGF1 and FGF2 lack a classical signal peptide for secretion<sup>87–89</sup>, so how these two factors are secreted by cells remains enigmatic, although alternative secretory pathways have been suggested<sup>37,90</sup>. It seems that export of FGF2 from malignant cells is highly regulated and is responsible for the angiogenic switch during the development of certain types of tumour. Studies of clinical samples taken from patients with various different cancers show remarkably increased levels of FGF2 in the body fluids compared with healthy controls<sup>91</sup>. A positive correlation between the expression of FGF2 and cancer spread, including lymphatic metastasis, has been reported<sup>91</sup>. This finding indicates that FGF2 might function to promote lymphatic metastasis.

*In vitro*, FGF2 stimulates proliferation, migration and tube formation of isolated LECs, indicating a direct role in lymphatic vessel growth<sup>23,92</sup>. However, when FGF2-expressing tumours are implanted into mouse corneal tissue this induces a massive infiltration of leukocytes, including macrophages, granulocytes, dendritic cells and other mononuclear cells, all of which

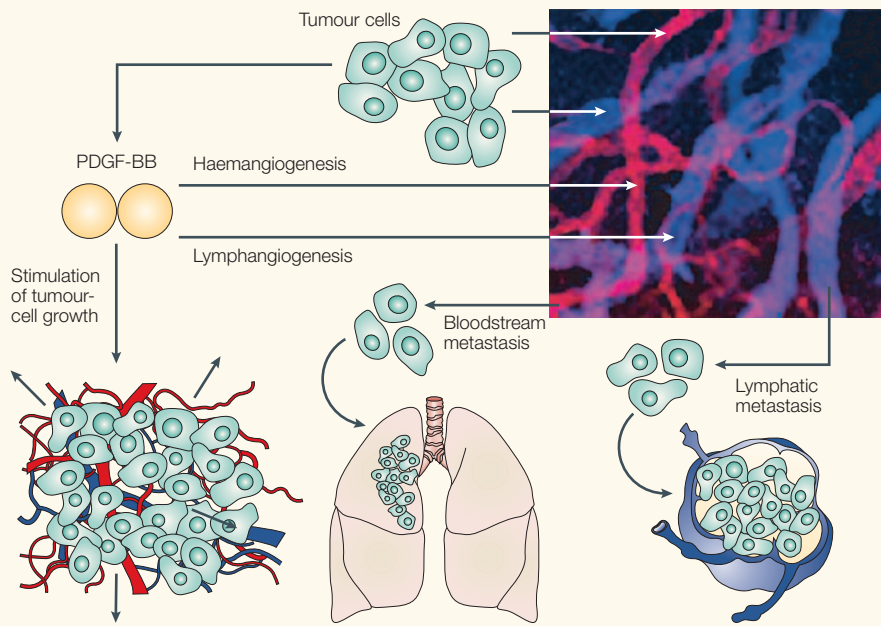


Figure 5 | **Multiple roles of platelet-derived growth factor-BB.** Platelet-derived growth factor-BB (PDGF-BB) that is produced from tumour cells stimulates further tumour-cell growth<sup>83,84</sup>, haemangiogenesis<sup>82,102</sup> and lymphangiogenesis<sup>21</sup>. Peri- and intratumoural lymphatic vessels and blood vessels can also facilitate cancer metastasis.

are rich sources of VEGFC and VEGFD<sup>93,94</sup>. This increases the possibility that FGF2 functions through other cytokines to indirectly stimulate lymphangiogenesis<sup>23</sup>. Furthermore, two independent studies show that FGF2-induced lymphangiogenesis can be completely blocked with VEGFR3 antagonists<sup>23,25</sup>. These findings indicate not only that FGF2-induced lymphangiogenesis is mediated by the VEGFR3 pathway, but also that suppression of VEGFR3 signalling might block a common lymphangiogenic pathway that is triggered by several factors.

**Angiopoietins and lymphangiogenesis.**

Angiopoietin-1 and angiopoietin-2 have been shown to regulate vascular stability through the TIE2 (Tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) receptor tyrosine kinase that is expressed on blood vessel endothelial cells<sup>95</sup>. Whereas angiopoietin-1 stabilizes the nascent vasculature by recruiting pericytes and VSMCs, angiopoietin-2 destabilizes blood vessels by interfering with angiopoietin-1-TIE2-induced endothelial-pericyte and VSMC interactions<sup>95</sup>. Both angiopoietin-1 and -2 have coordinate roles with other known angiogenic factors. For example, in the presence of high levels of VEGF, upregulation of angiopoietin-2 in tumours can cause endothelial cell growth by detaching pericytes and VSMCs from blood vessels, leading to the sprouting of new blood

vessels. In the absence of VEGFA, overproduction of angiopoietin-2 results in regression of the vasculature, as seen in the corpus luteum during the menstruation cycle<sup>95</sup>.

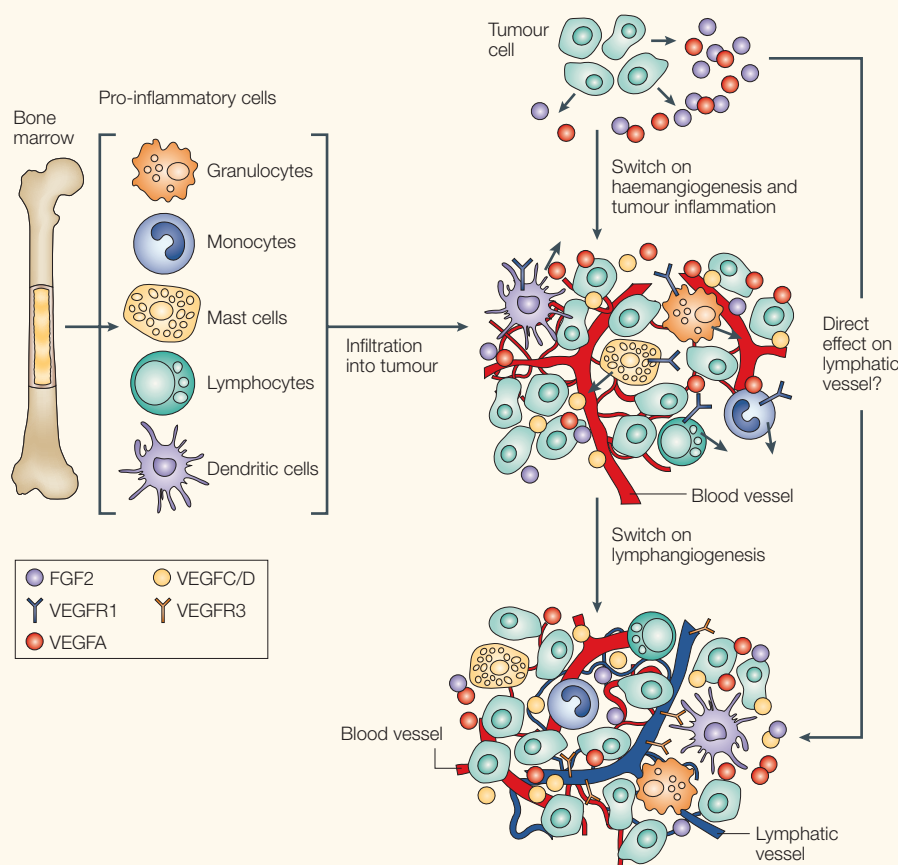
Deletion of angiopoietin-2 in mice produces an expected result, with defective blood vessel development in certain organs — for example, the regression of blood vessels in the retina<sup>24</sup>. However, a surprising finding is that these mice have disorganized and hypoplastic dermal and intestinal lymphatic capillaries<sup>24</sup>. Microlymphatic vessels in angiopoietin-2-null mice also show abnormal patterning. The aberrant development of lymphatics in these tissues leads to subcutaneous oedema and chylous ascites (a milky fluid that is composed of lymph fluid and emulsified fats, which have been absorbed by the lymphatic vessels in the small intestine) at an early stage during postnatal development. The latter phenotype resembles that seen in mice expressing a VEGFR3-inactive mutant<sup>96</sup>. This finding implies that both lymphatic vessel growth and maturation are essential for establishment of functional lymphatic networks. In addition to malformation of microlymphatic vessels, large lymphatics in the mesentery seemed to be similarly leaky and to have similar ragged vascular structures, although they were positioned normally. The underlying mechanism of the abnormal architecture of the large lymphatic vessels is due, in part, to the poor association of VSMCs.

One of the most remarkable findings in the study of angiopoietin-2-knockout mice is that the lymphatic defects, but not the pathological haemangiogenic phenotype, can be rescued by angiopoietin-1 (REF. 24). In support of this, angiopoietin-1 has recently been reported to promote lymphangiogenesis<sup>97</sup>. This indicates that these two factors have overlapping or redundant functions on lymphatic vessels, but distinct roles in blood vessel development. The angiopoietin system provides (to the best of the author's knowledge) the first example of two growth factors that bind the same TIE2 receptor and that function either as agonists or antagonists, depending on the tissue in which they are expressed. The roles of angiopoietins in tumour lymphangiogenesis and lymphatic metastasis need to be further elucidated.

**Inflammation and lymphangiogenesis.**

Whereas the role of inflammation in angiogenesis has been well studied, little is known about how acute and chronic inflammation contributes to lymphatic vessel growth. Various physiological and pathological conditions, including wound healing and tumour development, are associated with inflammation. Activated leukocytes in inflammatory sites produce a broad spectrum of growth factors, including members in the FGF, VEGF and PDGF families, as well as pro-inflammatory cytokines and chemokines that might stimulate proliferation, migration and survival of isolated LECs. Macrophages have been shown to be a rich source of lymphangiogenic factors such as VEGFC and VEGFD<sup>93,94</sup>. In a mouse corneal wound model, VEGFA attracts macrophages to the injury site through activation of the VEGFR1 receptor, and the activated inflammatory cells produce VEGFC and VEGFD to induce lymphangiogenesis<sup>61</sup>. FGF2 uses the same mechanism to promote lymphangiogenesis<sup>23,25</sup> (FIG. 6). Suppression of inflammatory-cell numbers by bone-marrow irradiation inhibits lymphangiogenesis in the cornea. Moreover, independent evidence indicates that COX2 inhibitors and non-steroid, anti-inflammatory drugs have potent anti-lymphangiogenesis activity<sup>19</sup>.

Inflammatory responses in tumour tissues have been associated with malignant progression and metastasis<sup>98</sup>. Infiltration of inflammatory cells into tumours could promote lymphatic metastasis. Accumulation of inflammatory cells in tumour tissues might lead to the growth of intratumoural lymphatic vessels, which in turn might promote lymphatic metastasis. The inhibition of inflammatory pathways might therefore suppress lymphatic metastasis.



**Figure 6 | The role of inflammation in lymphangiogenesis.** Tumours produce vascular endothelial growth factor A (VEGFA) and/or fibroblast growth factor 2 (FGF2) that can directly switch on haemangiogenesis for tumour growth. Both VEGFA and FGF2 can also induce the infiltration of bone marrow-derived inflammatory cells into tumours. The tumour-cell-activated inflammatory cells produce cytokines and lymphangiogenic factors that stimulate intratumoural lymphatic vessel growth and possibly metastasis. VEGFR, vascular endothelial growth factor receptor.

### Patterns of metastasis

Different switches of haemangiogenesis and lymphangiogenesis in tumours might be manifested as different patterns of haematogenous and lymphatic metastases in relation to primary tumours (TABLE 1). Although invasion of cancer cells into the pre-existing vasculature of the surrounding tissues might contribute to metastasis, metastases are, in most cases, probably mediated through intratumoural lymphatics and blood vessels.

The existence of both peritumoural and intratumoural lymphatics have been positively correlated with lymphatic metastasis<sup>11,17,20,21,33,55,57,63</sup>. Both types of lymphatic vessel are probably involved in lymphatic metastasis. Whereas haemangiogenesis is a prerequisite for tumour growth, infiltration of blood vessels into tumours does not always result in haematogenous metastasis (TABLE 1, type III/IV). In some cases, tumours metastasize through the blood to remote tissues and organs (TABLE 1, type I/II). In a number of cases haematogenous metastases

occur while primary tumours remain undetected (TABLE 1, type V/VII), indicating that invasion of primary tumour cells into pre-existing blood vessels might be crucial for metastasis. A primary tumour can be spread to other tissues and organs through blood and lymphatic systems (TABLE 1, type I). In type I tumours, the presence of peritumoural and intratumoural lymphatic vessels is usually detectable<sup>55,57,60</sup>. Lymphatic vessels might be more vulnerable to tumour-cell invasion, as lymphatic metastases are often detectable in the absence of haematogenous metastasis (TABLE 1, type IV and VI).

In some common cancer types, metastases in regional lymph nodes are detectable before the primary tumours (TABLE 1, type VI/VII)<sup>5</sup>. Such tumour types are particularly common in patients with breast cancer<sup>99</sup>. Regular examination of subaxillary lymph nodes has therefore become a recommended routine for early detection of breast cancers<sup>100</sup>. Such cases might result when lymphatic vessels grow in the absence of haemangiogenesis. Primary

tumours would then remain microscopic in size and dormant owing to the lack of blood vessels. The development of different antagonists that target blood and lymphatic vessels should be considered to inhibit or prevent lymphatic and haematogenous metastases. In some patients with rare cancers, both haematogenous and lymphatic metastases are detected when the primary tumours are not easily identifiable (TABLE 1, type VII).

### Lymphangiogenesis inhibitors

Lymphangiogenesis inhibitors can be classified as direct and indirect inhibitors. Direct lymphangiogenesis inhibitors refer to those molecules or compounds that function directly on LECs by blocking a common pathway of lymphatic vessel growth that is stimulated by various lymphangiogenic stimuli. For example, LECs express MMPs, including MMP2, MMP9, and membrane type 1-MMP (MT1-MMP)<sup>101</sup>. MMI270, a general inhibitor of these MMPs, significantly suppresses the invasion of LECs in a matrigel assay, and lymphatic tube formation is stimulated by various lymphangiogenic factors<sup>101</sup>. *In vivo*, MMI270 significantly reduces lymph-node metastasis in a mouse model of Lewis lung carcinoma<sup>101</sup>. Judah Folkman and colleagues have shown that celecoxib (Celebrex; Pfizer) and rofecoxib (Vioxx; Merck), two cyclooxygenase-2 inhibitors, are powerful inhibitors of lymphangiogenesis and that they might function by blocking a common pathway for cell proliferation<sup>19</sup>. In addition to exogenous inhibitors, certain types of tumour, such as fibrosarcoma, produce systemic lymphangiogenesis inhibitors. However, the identity of these inhibitors remains unknown.

Indirect lymphangiogenesis inhibitors are molecules that neutralize the functions of lymphangiogenic factors. According to their mode of action, these inhibitors target different levels of growth-factor-receptor-activated signalling pathways, ranging from neutralization of growth factors with soluble receptors and antibodies to inhibition of receptors with anti-receptor antibodies, to inhibition of intracellular tyrosine-kinase receptors and their downstream signalling components with small chemical compounds. Current development of therapeutic anti-lymphangiogenic agents is almost exclusively focused on the VEGFC/VEGFD-VEGFR3 signalling pathway<sup>11</sup>. The discovery of VEGFA and non-VEGF-related factors as novel lymphangiogenic factors indicates that neutralizing or blocking agents for these other factors might also be useful for the inhibition of lymphangiogenesis

Table 1 | **Patterns of haematogenic and lymphatic metastasis**

Type	Primary tumour	Haematogenous metastasis	Lymphatic metastasis	Tumour blood vessels	Tumour lymphatic vessels	Examples of clinical tumours*
I	+	+	+	+	+	Breast, lung, colon, liver
II	+	+	-	+	-	Liver, prostate, kidney, most sarcomas
III	+	-	-	+	-	Brain, <i>in situ</i> carcinomas
IV	+	-	+	+	+	Breast, cervical, endometrial carcinoma
V	-	+	-	-	-	Lung, prostate, sarcomas
VI	-	-	+	-	+	Breast, head and neck, ovarian
VII	-	+	+	-	+	Prostate, lymphoma, teratoma

\*The various tumours listed for each pattern of haematogenic and lymphogenic spread are illustrative examples, and many other tumours also show these patterns of metastasis.

and lymphatic metastasis. Therefore, combinations of these antagonists would in principle be more effective in blocking lymphangiogenesis.

**Future directions**

The recent discovery of specific lymphatic vessel markers, the isolation of LECs from various tissues, the establishment of *in vivo* lymphangiogenesis animal models, the identification of new lymphangiogenic molecules, and the understanding of the roles of lymphatic vessels in developing healthy and malignant tissues have distinguished lymphangiogenesis as an independent research field. The VEGFC/VEGFD-VEGFR3 signalling pathway was previously thought to be the only system for lymphangiogenesis. Identification of several non-VEGF-related molecules as lymphangiogenic factors has demonstrated that lymphangiogenesis is a complex process that is regulated by several factors. The collaboration between the VEGFC/VEGFD-VEGFR3 and the angiopoietin-TIE systems to produce new lymphatics during embryogenesis is one such example. As more lymphangiogenic factors are discovered, the molecular interplay among these factors will probably become more complex at the different levels. Warranting further investigation are the lymphangiogenic factors or cytokines that only induce lymphatic vessel growth, but not haemangiogenesis, and the

lymphangiogenesis inhibitors produced by specific tumours.

To develop anti-lymphangiogenesis therapeutic agents, it is important that these inhibitors block a common signalling pathway of lymphangiogenesis that is triggered by various growth factors. Otherwise, inefficient suppression of lymphangiogenesis and potential drug resistance could be encountered. So far, we lack information on structural differences of lymphatic vessels in healthy and malignant tissues. Do lymphatics in the malignant tissues express specific markers that can be used for targeting? A recent study by Ekki Ruoslahti and colleagues seems to have found an answer to this question by demonstrating that an angiogenic targeting peptide specifically recognized tumour lymphatic vessels<sup>19</sup>. It remains to be seen if this type of targeting peptide can guide a lymphangiogenesis inhibitor specifically to the tumour lymphatic system. More in-depth molecular mechanistic studies of lymphangiogenesis will help us to understand the roles of lymphatic vessels in pathological conditions and to design therapeutic agents to target lymphatic vessels.

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## Competing interests statement

The authors declare no competing financial interests.

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