Oncogene (2012) **31,** 4499 – 4508

www.nature.com/onc

REVIEW

Interaction of tumor cells and lymphatic vessels in cancer progression

A Alitalo and M Detmar

Metastatic spread of cancer through the lymphatic system affects hundreds of thousands of patients yearly. Growth of new lymphatic vessels, lymphangiogenesis, is activated in cancer and inflammation, but is largely inactive in normal physiology, and therefore offers therapeutic potential. Key mediators of lymphangiogenesis have been identified in developmental studies. During embryonic development, lymphatic endothelial cells derive from the blood vascular endothelium and differentiate under the guidance of lymphatic-specific regulators, such as the prospero homeobox 1 transcription factor. Vascular endothelial growth factor-C (VEGF-C) and VEGF receptor 3 signaling are essential for the further development of lymphatic vessels and therefore they provide a promising target for inhibition of tumor lymphangiogenesis. Lymphangiogenesis is important for the progression of solid tumors as shown for melanoma and breast cancer. Tumor cells may use chemokine gradients as guidance cues and enter lymphatic vessels through intercellular openings between endothelial cell junctions or, possibly, by inducing larger discontinuities in the endothelial cell layer. Tumor-draining sentinel lymph nodes show enhanced lymphangiogenesis even before cancer metastasis and they may function as a permissive 'lymphovascular niche' for the survival of metastatic cells. Although our current knowledge indicates that the development of anti-lymphangiogenic therapies may be beneficial for the treatment of cancer patients, several open questions remain with regard to the frequency, mechanisms and biological importance of lymphatic metastases.

Oncogene (2012) **31,** 4499 – 4508; doi:10.1038/onc.2011.602; published online 19 December 2011

Keywords: lymphangiogenesis; metastasis; lymph node; VEGF-C; VEGF; therapy

INTRODUCTION

The role of the lymphatic vascular system in promoting cancer metastasis has received increased research effort and clinical attention in the past 15 years. In 2007, an estimated 12 million people were diagnosed with cancer which is the second most common cause of death after only heart disease.[1] In 2011, more than 300 000 patients in the United States and more than 400 000 patients in Europe will likely be diagnosed with breast cancer or melanoma, for which spread through the lymphatic system has been studied most.[2,3]

The growth of new lymphatic vessels, called lymphangiogenesis, is largely absent in adults, but can be induced in pathological processes, such as inflammation, wound healing and cancer. Significant progress in our understanding of lymphatic metastasis has been achieved through the use of lymphatic-specific markers in clinical studies of primary tumors and lymph node material, and through proof-of-principle preclinical studies employing lymphangiogenic growth factors and their inhibitors.

This review highlights the development of the lymphatic system and discusses the major molecules involved, as a potential source of anti-lymphangiogenic drug targets, as well as research on lymphatic metastasis and key elements of anti-lymphangiogenic therapy. Research into the lymphatic system is currently undergoing another revolution with new emerging concepts on the role of the tumor microenvironment and with the real-time imaging of lymphatic metastasis.

FUNCTIONAL ANATOMY OF THE LYMPHATIC SYSTEM

The lymphatic vessels, first described by the Milanese surgeon GaspareAselli in 1622, consist of one-way endothelium-lined conduits from the peripheral tissues to the blood circulation. Excess tissue fluid extravasated from the blood circulation is drained by lymphatic vessels and returned to the blood circulation. In addition to fluid and solutes, the lymphatic vessels also transport cells–under physiological conditions immune cells and in pathological conditions also infectious agents or cancer cells–to lymphoid tissues. The lymphatic vasculature begins as blind-ended capillaries in the peripheral tissues. Under conditions of high interstitial tissue pressure, the lymphatic capillaries are kept open by forces applied through anchoring filaments that link the vessels to the extracellular matrix. The capillaries drain to pre-collecting vessels and thereafter to collecting lymphatic vessels. These are coated by a periendothelial smooth muscle cell layer and contain valves to prevent backflow. The collecting vessels connect as afferent vessels to sentinel lymph nodes, which are the first organs to receive cells and fluid that have entered lymphatic vessels in peripheral tissues. The efferent vessels of the sentinel lymph node further transfer cells and fluid to distal lymph nodes. The main lymphatic vessel trunks connect to the blood vasculature by draining into the subclavian veins.

LYMPHATIC VESSEL DEVELOPMENT

During the past 15 years, the lymphatic endothelium has been found to express specific markers, enabling its reliable identifica-

Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland. Correspondence: Professor M Detmar, Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, ETH Zurich, Wolfgang Pauli-Str. 10, HCI H303, 8093 Zurich, Switzerland.
E-mail: michael.detmar@pharma.ethz.ch
Received 1 October 2011; revised 21 November 2011; accepted 22 November 2011; published online 19 December 2011

tion and distinction from blood vascular endothelium in clinical samples and mouse disease models. In several species, including human, mouse and zebrafish, developmental lymphangiogenesis, that is, the growth of lymphatic vessels within the embryo, is regulated by a plethora of molecules. These molecules are also likely to play an important role in tumor lymphangiogenesis and are investigated as potential targets for blocking tumor lymphangiogenesis and metastasis.

Development of the lymphatic system has been studied extensively in mouse embryos in which it follows a general mammalian scheme. During embryogenesis, the blood circulatory system is first to evolve, followed by specification of lymphatic endothelial progenitor cells from blood vascular endothelial cells and budding of these cells from the cardinal veins. This occurs on the dorsolateral sides of the cardinal veins, particularly in the neck area. The primary effectors altering transcription factor profiles and thus the fate of the endothelial cells in this region are still unknown. In the cells committing to lymphatic endothelial fate, a transcription factor cascade, including the paired box factor Sox18, leads to the expression of the master regulator of lymphatic cell specification, the prospero homeobox 1 (Prox-1). Prox-1 expression is critical as in Prox-1-deficient knockout mice; the specification and budding of lymphatic endothelial cells is blocked, leading to absence of lymphatic vessels and edema (so-called lymphedema).[4] During early steps of lymphatic endothelial cell specification, Ets-family transcription factors are also abundantly expressed in blood vascular endothelial cells and, when co-expressed with Prox-1, enhance the transcriptional activator activities of Prox-1.[5] Prox-1, with or without Ets and the later expressed transcription factor COUP-TFII, upregulates the expression of further lymphangiogenic signaling molecules such as vascular endothelial growth factor receptor 3 (VEGFR-3) and integrin α9. Lymphatic endothelial progenitor cells expressing Prox-1 migrate from the cardinal veins into the adjacent mesenchyme, where they form the primary lymphatic plexus.[4] This budding and migration of lymphatic endothelial progenitor cells is mediated by VEGF-C signals and is critically modulated by collagen- and calcium-binding EGF domain 1 protein.[6,7] COUP-TFII and Prox-1 are essential for the maintenance of the identity of the lymphatic endothelial cells following their differentiation.[8] The primary lymphatic networks then enlarge by sprouting lymphangiogenesis induced by the VEGFR-3 ligand VEGF-C. By upregulation of VEGFR-3 expression independently of Prox-1, T-box transcription factor 1 supports the growth and maintenance of the gastrointestinal lymphatic vessel network.[9]

Lymphatic vessel subtypes are specialized and patterned by a remodeling program. Upon the expression of the transcription factors Foxc2 and NFATc1, the collecting vessels acquire basement membranes, smooth muscle cell coverage and valves.[10] The sialoglycoprotein podoplanin, used as a marker to distinguish the lymphatic endothelium in both experimental mouse models and in clinical samples, is expressed at high levels in lymphatic capillaries and at lower levels in precollector vessels.[11] Podoplanin is essential for the correct maturation of the vessels, and podoplanin-deficient mouse embryos show a blood–lymphatic vascular mixing phenotype with blood-filled lymphatic vessels. The functions of podoplanin on lymphatic endothelial cells include binding to CLEC-2 receptors expressed on platelets in the circulating blood. CLEC-2–podoplanin interaction leads to SLP-76 adaptor–SYK tyrosine kinase signaling in platelets and formation of platelet aggregates at points of contact between blood and lymphatic vasculature. These aggregates seal off the embryonic connections between the blood and lymphatic vessels.[12–15]

The *in vivo* function of the hyaluronan receptor LYVE-1, expressed by the lymphatic endothelial cells, is currently unknown.[16] Augmented by retinoic acid-induced signaling, LYVE-1 is expressed early on in the cardinal vein lymphatic progenitor cells.[17,18] Since its discovery, LYVE-1 has been used extensively in clinical and experimental models as a lymphatic vessel marker despite its low expression levels in mature collecting vessels.[19] LYVE-1 expression has also been reported in the pulmonary blood microvasculature, liver blood sinusoids and in a so-called M2 subpopulation of macrophages active in tissue remodeling.[20–24]

THE ROLE OF VEGFS IN LYMPHANGIOGENESIS

In addition to the above-mentioned lymphatic vessel-specific effectors, members of the VEGF family play major roles in

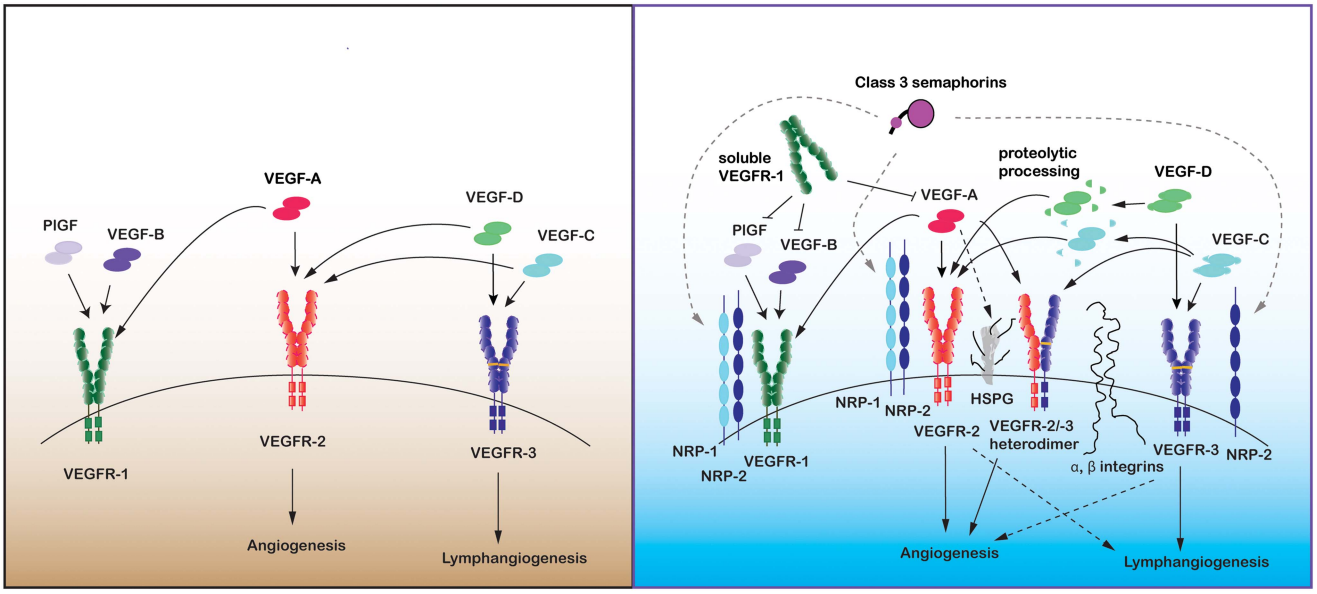

Figure 1. Once viewed as a simple signaling system, the VEGF receptors are now known to involve several co-receptors, receptor and ligand modifications, competing interactions and receptor heterodimerization. Although VEGR-2 signaling strongly promotes angiogenesis and VEGFR-3 signaling lymphangiogenesis, both receptors may be involved in both processes. PlGF, placental growth factor; HSPG, heparin sulfate proteoglycan.

lymphangiogenesis. VEGFs were originally characterized as factors that promote either angiogenesis or lymphangiogenesis without major overlaps between the two processes (Figure 1). During subsequent studies, however, this view has become more complex as various forms of VEGFs and several co-receptors have been discovered.

During early embryonic development, the main lymphangiogenic receptor VEGFR-3 is widely expressed in the blood vessels and is essential for the development of the blood circulatory system. Once developmental angiogenesis is brought to completion, VEGFR-3 expression is restricted to the lymphatic endothelium.[25] However, upon reactivation of angiogenesis, VEGFR-3 expression may be upregulated, most prominently in angiogenic vessel sprouts.[26,27] VEGFR-3 may function differentially on blood and lymphatic endothelial cells. VEGF ligand-induced activation of VEGFR-3 is indispensable for the development of the lymphatic vascular system, but not for blood vascular development. Mutation of the VEGFR-3 kinase domain, deletion of the ligands VEGF-C and VEGF-D or of their binding domain in VEGFR-3 all lead to the development of hypoplastic lymphatic vessels, but the blood vascular system is less affected and functional.[28–30] However, VEGFR-3 expression is required for blood vascular development as VEGFR-3-deficient mice die owing to cardiovascular defects.[31] VEGFR-3 may function to limit excessive angiogenic signaling through VEGFR-2 on blood vascular endothelial cells.[32] This effect of VEGFR-3 seems to be independent of the intrinsic kinase activity of the receptor. Upon cell attachment to the extracellular matrix component collagen I and activation of integrin β1 expressed on the cell membrane, VEGFR-3 can be phosphorylated by Src kinase independently of VEGF ligand binding or activity of the VEGFR-3 kinase domain.[32,33] Recent studies have also shown that both VEGFR-2 and VEGFR-3 are expressed by the blood vascular endothelial cells, where VEGF-C may induce VEGFR-2/VEGFR-3 heterodimerization and downstream signaling in part explaining the redundancy of VEGFR-3 ligands in blood vascular development.[34]

VEGF-C and VEGF-D may also function in monocyte and macrophage recruitment as VEGFR-3 expression has been found on some of these cells.[35–38] Macrophages may orchestrate different aspects of lymphangiogenesis in development and in inflammation.[39–41]

VEGFR-2 is a potent mediator of angiogenic signaling. The first target of an anti-angiogenic therapy was its ligand VEGF-A. However, VEGF-A was also shown to promote lymphangiogenesis in tumor, contact-induced hypersensitivity and wound healing models and after adeno-viral delivery to mouse ear skin.[42–46] The expression levels of VEGFR-2 on lymphatic endothelial cells and thus the response to VEGF-A seem to vary depending on the tissue microenvironment.[42,43,45,47–49] Recently, studies indicate that VEGF-A mainly induces enlargement of lymphatic vessels, but induces only little lymphatic vessel sprouting unless Notch receptor signals are inhibited.[47,50] VEGF-A may also induce inflammation, including leukocyte recruitment that contributes to lymphangiogenesis.[51,52]

Further complexity is added to the interactions of VEGF-C and VEGF-D with putative receptors by processing proteases and co-receptor binding (Figure 1). Proteolytic cleavage of the VEGF-C and VEGF-D at their N and C termini modulates the affinities of the factors to VEGFR-2 and VEGFR-3, as well as to neuropilin (NRP) co-receptors.[35,53–55] NRP-2 binds class III semaphorins, VEGFR-3 and VEGFs. The importance of NRP-2 during the initiation of new lymphatic vessel sprouts is evident from the hypoplastic lymphatic vessels observed in NRP-2 gene targeted mice.[56] Among other structures, NRP-2 is expressed on veins and upregulated in tumor-associated lymphatic vessels, where it binds VEGF-C and VEGF-A, in addition to partially processed VEGF-D.[57–59] Anti-lymphangiogenic therapy targeting NRP-2 reduced metastasis in a mammary tumor model.[57] A complex of NRP-2 and VEGFR-3 may include integrin β1 and possibly simultaneously interact with semaphorin 3 components for correct lymphangiogenic signaling.[33,60]

A continuously growing number of additional factors are studied as potential modulators of lymphangiogenesis, including integrin α9 expressed specifically by lymphatic valve endothelial cells, hepatocyte growth factor,[61] the Tie-2–angiopoietin signaling system,[27] fibroblast growth factor 2[62] and platelet-derived growth factor BB.[63] The selection of the most promising anti-lymphangiogenic targets is based on previous experiences of, for example, blocking tumor angiogenesis. But how targetable is the lymphatic system in cancer and what would be the utility of blocking lymphangiogenesis?

LYMPHATIC SYSTEM AND CANCER METASTASIS

The discovery of the lymphatic growth factors, VEGF-C and VEGF-D, and lymphatic vessel markers, VEGFR-3, LYVE-1, podoplanin and Prox-1, has enabled detailed studies on the role of the lymphatic system in human cancer. According to the classical view of metastasis, malignant cells reaching the sentinel lymph node may disseminate further to distal lymph nodes, reach systemic circulation and subsequently form organ metastases. Thus, together with excision of the primary tumor, lymph node dissection or, more recently, sentinel lymph node excision is commonly carried out in, for example, breast cancer and melanoma therapy. The excised primary tumor and lymph node material are used for the analysis of tumor blood and lymphatic vasculature and the occurrence of intralymphatic tumor cells serves as a negative prognostic parameter. To further develop prognostic markers, long-term studies are required to correlate patient survival and incidence of metastasis with the expression of lymphangiogenic molecules. Metastatic cells may also adopt a dormant phenotype on their way to clinically apparent metastasis, thus ceasing to proliferate for an undefined time. Such quiescent cells are resistant to chemotherapy targeting proliferating cell populations. Therefore, potential anti-lymphangiogenic therapies should target the lymphatic dissemination and survival of malignant cells. Several questions need to be clarified to develop and use such therapeutics successfully (Figure 2), namely:

(1) What is the significance of lymphatic metastasis for tumor progression?
(2) How is tumor lymphangiogenesis induced?
(3) Do intra- or peritumoral lymphatic vessels transport tumor cells?
(4) How do tumor cells gain access to lymphatic vessels?
(5) How do tumor cells reach lymph nodes?
(6) Do lymph nodes act as barriers for metastasis?

What is the significance of lymphatic metastasis for tumor progression?

It has been estimated that 80% of metastasis of solid cancers, such as breast cancer and melanoma, disseminate through the lymphatic system, while 20% of metastases may occur through the blood vasculature or by direct seeding.[64] In fact, lymph node metastasis is the first sign of tumor progression in the majority of epithelial malignancies. Malignant cells disseminating through lymphatic vessels may also produce locoregional metastasis (so-called in transit or satellite lesions) by proliferation *in situ* inside the vessel.[65,66] Metastatic cells reaching lymph nodes may survive and proliferate there or they may enter a dormant stage of variable duration. Current knowledge on the prognostic significance of individual metastatic cells, small metastatic cell clusters or micrometastases (0.2–2 mm in diameter) in lymph nodes varies depending on tumor type.[64] In the case of melanoma, treatment follow-up studies indicate that metastases under 0.1 mm in diameter might not have an impact on prognosis.[67] However, larger size metastatic melanoma lesions in lymph nodes correlate

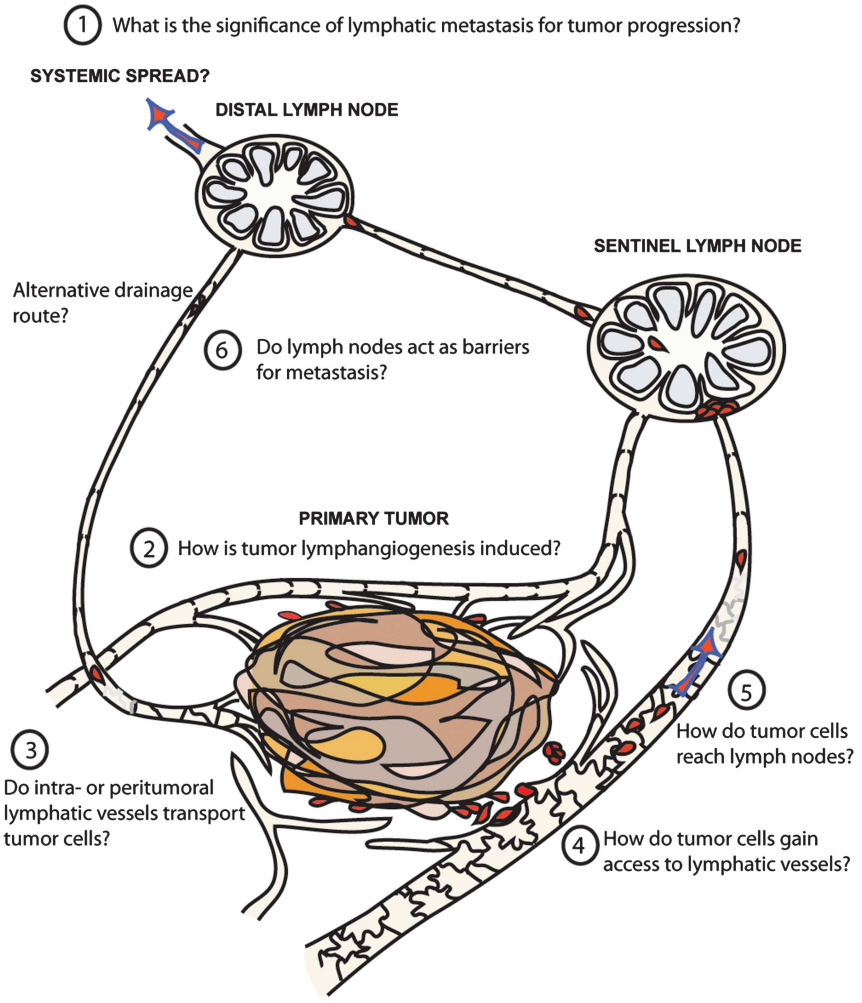

Figure 2. Possible paths of metastatic tumor cells in the lymphatic system. Questions marked 1–6 in the figure are discussed in detail in the text. According to the classical view, metastatic cells enter tumor draining lymphatic vessels and are passively drained to sentinel lymph nodes, from where further dissemination may occur to distal lymph nodes, the blood circulation and distant organs. Tumors may activate lymphangiogenesis in the tumor periphery or inside the tumor mass. It seems possible that tumor cells induce holes in endothelial cell layers or pass between endothelial cells to enter through the vessel wall. Alternative drainage pathways bypassing the sentinel lymph nodes and draining directly to a distal lymph node may exist. It is currently unclear where tumor cells engaged in the metastatic process enter the blood circulation. Possibilities include direct entry in the primary tumor, entry into the high endothelial blood venules in the lymph nodes or lymphatic drainage to subclavian veins.

with shorter progression-free survival (reviewed in Leong *et al.*[64]). Thus, lymphatic metastasis is common, at least in selected cancer types, and is associated with increased lethality.

How is tumor lymphangiogenesis induced?

If tumor cells utilize pre-existing vessels for metastasis, anti-lymphangiogenic therapies might not be sufficient for the prevention of tumor metastasis. Increased density of peritumoral lymphatic vessels and the existence of intratumoral lymphatic vessels indicate activation of lymphangiogenesis within the tumor. It has long been debated whether tumor cells play an active role in tumor lymphangiogenesis. Recently, the role of the tumor microenvironment also has been emphasized by description of tumor-associated macrophages that may function as a second source of lymphangiogenic factors.[68,69] In the majority of clinical studies, a significant correlation has been observed between lymphatic vessel density and lymph node and organ metastasis (see meta-analyses in refs 70–72). This has led to the concept that the denser the lymphatic vasculature is within or close to the tumor, the more potential entry sites the tumor cells have to vessels that could be used as highways for metastatic spread.

High expression levels of the lymphangiogenic factor VEGF-C in patient samples correlate with lymph node metastasis in a number of tumor types (see meta-analysis in refs 72, 73). Using mouse models, overexpression of VEGF-C or VEGF-D has been shown to increase lymphatic vessel density, vessel diameter and lymph node and organ metastasis of many cancer types.[74–78] The effects of VEGF-C have been described in detail in a lung cancer model (*LNM35*). VEGF-C caused dilation of peritumoral lymphatic vessels and sprouting of new lymphatic vessels to closely surround the malignant cells.[74] VEGF-C-induced dilation of collecting vessels around tumors may allow the entry of clusters of metastatic tumor cells.[78,79] Even tumors that rarely metastasize through the lymphatic vessels, for example, the fibrosarcoma T241 and the prostate cancer cell line LAPC9, have been observed to do so when manipulated to overexpress VEGF-C.[20,65,75] Although VEGF-D overexpression in mouse models resulted in increased lymphangiogenesis and metastasis,[72,80] correlation between VEGF-D expression levels and lymph node metastasis in patients has been found only in less than half of the studies.[73] VEGF-D may play a limited role in tumor lymphangiogenesis, as low expression levels have been found in most patient-derived cell lines and

tumors.[26] Inhibition of the VEGF-C and VEGF-D receptor VEGFR-3, either by blocking antibodies or by a soluble receptor acting as a ligand trap, has been shown to inhibit lymphangiogenesis, and to a moderate degree angiogenesis, and is also found to restrict lymph node metastasis without effects on mature vessels in surrounding tissues.[26,74,75,81]

In addition to VEGF-C and VEGF-D, overexpression of VEGF-A may also lead to the activation of lymphangiogenesis. We have observed intratumoral lymphatic vessels and enlargement of peritumoral lymphatic vessels in VEGF-C- or VEGF-A-overexpressing squamous cell carcinomas.[43,82]

Do intra- or peritumoral lymphatic vessels transport tumor cells?

Controversy has prevailed on the presence and significance of intratumoral lymphatic vessels. In patient material, intratumoral lymphatic vessels have been observed in at least head and neck squamous cell carcinomas and melanoma.[83,84] Variable results on intratumoral vessels may be due to different staining methods, tumor types or due to inconsistency in their definition, such as whether lymphatic vessel within stromal structures inside the tumor mass is regarded as intratumoral.[85] When vessels in intratumoral stromal structures were considered intratumoral, they were observed in 80% of breast cancer cases,[86] whereas when vessels in stromal structures were not included, intratumoral vessels were observed in only 10% of ductal breast carcinomas.[87] Invasively growing tumors could co-opt pre-existing lymphatic vessels, whereas expansive growth could recruit lymphangiogenesis.[86] In clinical samples, intravasated tumor cells, called lymphovascular invasion, have been observed in both peritumoral and intratumoral lymphatic vessels.[88]

Intratumoral lymphatic vessels and increased metastasis have been observed in VEGF-C-overexpressing tumors implanted to mice.[76,78,86] Use of different prostate cancer models has shown a VEGF-C dependency of intratumoral lymphatic vessels.[75,89] Furthermore, in a VEGF-C-expressing prostate cancer model (PC3), ablation of intratumoral vessels by a VEGF-C and VEGF-D binding decoy receptor did not decrease metastasis, suggesting that peritumoral lymphatic vessels were sufficient for tumor metastasis in this model.[89]

Some imaging studies have shown that intratumorally injected tracers may be deposited in tumors instead of being cleared by lymphatic drainage.[20,90] A high interstitial pressure within tumors may cause intratumoral vessels to collapse to a non-functional state.[91] Thus, the lymphatic vessels observed inside a tumor, are not necessarily functional with regard to fluid drainage or transport of tumor cells, at least in some experimental models. In support of this interpretation, although intratumoral lymphatic vessels were found more frequently in patients with lymph node metastasis than nodal metastasis-negative patients, the extent of peritumoral lymphangiogenesis was concluded to be the most important prognostic factor for the metastasis of melanoma.[84] Yet, intratumoral lymphatic vessels may serve as an indication of an aggressive, poorly differentiated tumor type that is more likely to metastasize.[83]

How do tumor cells gain access to lymphatic vessels?

It is poorly known how tumor cells enter the lymphatic vessels (Figure 2). Suggestions include envelopment of tumor cells by the lymphangiogenic sprouts and transmigration through the endothelium, similar to leukocyte transmigration, in channels that were recently shown to lie between button-like intercellular junctions of lymphatic capillaries.[19,74,92] One recent study has indicated that breast cancer cells can actively invade lymphangiogenic vessels inside the lymph nodes by inducing the formation of holes in the endothelial cell layer. This mechanism may also be active *in vivo* as the knockdown of a key enzyme, 15-lipoxygenase-1, in xenografted cells blocked metastasis. Furthermore, in breast cancer patients, the expression level of 15-lipoxygenase-1 in sentinel lymph node metastasis correlated with metastasis-free survival.[88] Whether other malignant cell types also induce holes in the lymphatic endothelium remains to be determined. Tumor cell aggregates were observed in transit through breaches in the lymphatic vessel wall, and also within the vessel.[88] In contrast, in an experimental tumor model in mice, fluorescently labeled breast cancer cells have been found to enter lymphatic vessels initially as single cells and later to occur as cell aggregates within the vessel.[93] However, a consistent cohesive migration pattern does not seem to impede metastasis through lymphatic vessels.[94] Transforming growth factor β signaling may be essential in the selection of cohesive or single-cell migration pattern by metastatic cells.[95]

To reach the lymphatic vessel, the migrating cells need to sense direction in the extracellular environment. The coming together of the cells and the vessel may occur owing to their migration or growth toward each other. The lymphatic vessel endothelium may express CXCL12 (stromal-derived factor 1) and CCL21 chemokines, which when bound to CXCR4 or CCR7 receptors on malignant cells lead to chemoattraction.[96,97] This chemoattraction mechanism is used in leukocyte homing. Thus, cancer cells, when expressing receptors for lymphatic-derived factors, may use such chemokine gradients to sense direction in the tumor microenvironment. High CXCL12 expression has been shown in proximity of the malignant cells in lymphatic vessels, in lymph nodes and distant organs. Simultaneously, the malignant cells induced lymphangiogenesis in their vicinity by secretion of VEGF-A and VEGF-C.[97] Thus, a crosstalk seems to exist between the lymphatic vessel expressing the chemoattractant CXCL12 and malignant cells expressing its receptor CXCR4 in addition to lymphangiogenic factors. Importantly, antagonists or neutralizing antibodies to these chemokines or their receptors have been shown to reduce metastasis of breast cancer and melanoma cells.[97–99] For a more detailed review on the chemoattraction of tumor cells and the immunomodulatory effects of lymphatic factors and lymphatic endothelium, the reader is referred to a recent review by Christiansen and Detmar.[100]

How do tumor cells reach lymph nodes?

Increased interstitial pressure within tumors has been proposed to be the driving force for movement of the cells and fluid within the lymphatic drainage pathway.[93] This suggests passive translocation of the tumor cells toward sentinel lymph nodes once inside the vessel lumen. Therefore, the flow patterns in tumor draining lymphatic vessels are relevant for the transport of tumor cells. In a melanoma mouse model, fluid drainage was increased in the legs bearing tumors in the footpad compared with contralateral legs.[101,102] Abnormal multidirectional flow has been observed in lymphatic vessels draining VEGF-C-overexpressing melanomas implanted in dorsal back skin of mice, suggesting insufficient function of lymphatic valves in that model.[65,90] However, valve function in tumor draining lymphatic vessels may not be insufficient in all tumors, as we have observed functional lymphatic valves by *in vivo* imaging of lymphatic vessels in footpad melanoma and subcutaneous breast cancer models (Proulx S *et al*., unpublished data). Upon progression, the arrival of metastatic cells into the lymph node may block the lymphatic sinuses, decreasing fluid flow from the tumor.[93,102]

Abnormal flow patterns may permit transient stagnation of flow and the observed growth of tumor cells *in situ* in the vessel.[65,79] These tumor cell aggregates may grow to the critical size limit of passive oxygen diffusion and induce growth of blood vessels within the metastatic foci. Such regional metastasis bears the risk of recurrent cancer after excision of the primary tumor with or without lymph nodes. It was recently shown that destroying tumor draining lymphatic vessels by photodynamic activation of a

phototoxic compound taken up by draining lymphatic vessels efficiently removed tumor cell aggregates also from within the vessels.[79]

Do lymph nodes act as barriers for metastasis?

Changes in the receiving sentinel lymph nodes may precede arrival of metastatic cells. Tumor-secreted factors arrive in the sentinel lymph node through drainage of fluid and solutes from the periphery. These factors promote enlargement of the lymphatic networks inside the node, known as sinusoidal hyperplasia,[43,82] and possibly also affect blood vasculature in some models.[43,103] These changes have been suggested to 'prepare the soil' for successful metastasis at a later stage. Lymph node metastases themselves are very rarely harmful, but what happens to tumor cells within lymph nodes? The lymph node could be rapidly transited by the tumor cells, leaving some cells behind, or it could be used as an in-transit amplifier, a selective launch pad for further metastasis or as a site for tumor dormancy.

The primary tumor or its lymph node metastasis may influence not only regional but also distant sites of metastasis. For example, the chemokine CXCL12 was found highly expressed by the lymphatic endothelium in the lungs of mice bearing cutaneous melanomas, but not in non-tumor-bearing mice.[97] We and others have shown that VEGF-C-overexpressing tumors send metastasis more frequently not only to the sentinel lymph nodes, but also to distal lymph nodes and distant organs, and that at least in some models, this can be inhibited by blocking VEGFR-3.[43,75] VEGF-C-induced lymphangiogenesis in sentinel lymph nodes may provide tumor cells numerous enlarged entry sites to efferent lymphatic conduits and may improve tumor cell survival inside the lymph node. Tumor draining lymph nodes may also act as a conductive 'lymphovascular niche' for 'cancer stem cell' survival.[104]

Another view of lymph node metastasis is that it indicates a tumor type capable of dissemination through lymphatic vessels and survival in lymphatic tissue. Concurrent metastasis of primary tumor to other sites could occur independently of lymphatic spread. As lymph node metastasis rarely causes major morbidity, leaving lymph node metastasis *in situ* would be an option if the cells would not survive elsewhere in the body.[105] Such an approach seems risk prone as the triggers of further dissemination of metastatic cells are not well established. Sentinel lymph node excision is routinely performed and seems to provide local control of metastasis in breast cancer patients without clinical metastasis.[106] In these patients, excision of all regional lymph nodes from the axilla compared with excision of sentinel lymph node alone did not result in additional benefit.[107,108] Therefore, it was concluded that removal of the sentinel lymph node might be sufficient and more extensive removal of axillary nodes was not recommended in patients without sentinel lymph node metastasis.

At present, the stepwise progression of malignant cells from tumor to sentinel lymph node and from thereon to distal lymph nodes and organs has not been shown conclusively, as clinicians and researchers have not been able to observe what happens after the tumor cells arrive in the sentinel lymph node. Although we know that lymph node metastasis is a prognostic factor for organ metastasis in many, but not all, cancers,[109,110] the precise route the metastatic cells exit from lymph nodes needs to be investigated. This has been called 'the black box' phenomenon, where we can only observe the end result (distant organ metastasis), but not the process itself.[94] In the near future, inducible labeling of cells as they transit the lymph node holds great promise to enlighten the malicious path used by metastatic cells.

DEVELOPMENT OF ANTI-LYMPHANGIOGENIC THERAPIES FOR CANCER

At present, solid cancers are mainly treated using stage-dependent combinations of surgery and radiation and chemotherapy (Figure 3). Anti-angiogenic therapy using the VEGF-A blocking antibody bevacizumab can be used for selected cancers in combination with chemotherapy. Over 15 years of research into the factors inducing lymphatic vessel growth in development, cancer and inflammatory diseases have now led to the first clinical trial testing anti-lymphangiogenic therapy.[111]

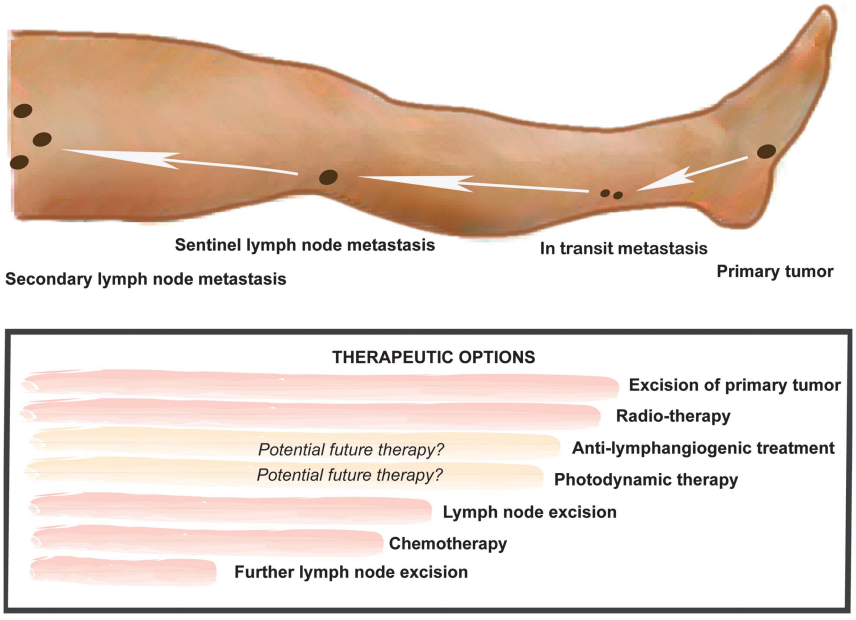

Figure 3. Anti-lymphangiogenic therapies may provide non-invasive options to inhibit tumor metastasis in addition to established cancer therapies. Anti-lymphangiogenic treatment may reduce further lymphatic metastasis by inhibiting the growth of new lymphatic vessels at the primary tumor site, in lymph nodes and at metastatic sites. Photodynamic therapy may eliminate tumor draining lymphatic vessels and, possibly, intralymphatic tumor cells.

Who will benefit from anti-lymphangiogenic cancer therapy?

In light of our current knowledge, we believe that to prevent lymphatic dissemination of tumor cells, anti-lymphangiogenic therapy should ideally be administered before lymph node metastasis (Figure 3). Whether metastatic cells disseminate through a blood or lymph vascular route from the metastatic lymph node is of interest considering treatment of patients with already established metastasis in lymph nodes. In addition, lymphatic metastasis may continue from the remaining foci of malignant cells after excision of primary tumor and possibly affected lymph nodes.

Can a suitable target molecule be identified so as to block lymphangiogenesis efficiently?

Furthest advances in this regard have been made in targeting VEGFR-3 signaling. VEGFR-3 was the earliest molecule identified as essentially required for lymphangiogenesis. This target has provided promise of efficacy, although not complete blockage of metastasis, in several pre-clinical models.[69,74,75,81,112] Thus, there is a need to identify additional mediators of pathological lymphangiogenesis.

What are the risks of adverse side effects?

As an advantage of using biomolecular targeting, off-target effects are less likely than with most small molecular inhibitors. For example, VEGFR-3 signaling has been targeted by receptor blocking antibodies, a ligand trap, ligand blocking antibodies and by blocking translation of mRNA. The advantageous diffusion efficiency and oral uptake of small molecular kinase inhibitors are counterweighed by their 'off-targets', for example, blockage of many tyrosine kinases. Regardless of the type of inhibitory molecule, on-target side effects are possible. Such would include cells other than lymphatic endothelium expressing the target. VEGFR-3 may provide a reasonably specific target, although blood monocytes, tissue macrophages and possibly cells in the bone marrow may be also affected.[37,38,76] More research needs to be carried out to investigate the role of these cells in tumor progression and the effects of inhibition of VEGFR-3 signaling on these cells. Recently, NRP-2 blocking antibodies have proven efficacy in blocking lymph node metastasis in a pre-clinical model, but one concern in their use is the wide NRP-2 expression outside the lymphatic system.[57]

Lymphatic vessels are largely quiescent in adults, and thus lymphangiogenesis could provide a safe target. However, lymphangiogenesis appears to play a role in promoting wound healing.[112] In the case of invasive surgery to remove the primary tumor or metastases, concurrent inhibition of lymphangiogenesis at wound sites might delay wound closure in susceptible patients.[37] Thus, targeting tumor-associated lymphatic vessels specifically may provide advantages. This necessitates the identification of tumor lymphatic vessel-specific markers. Lymphatic vessels may provide more resistance to regression than blood vessels, as shown after adenoviral delivery of VEGF-A,[42] and in an inflammation model where spontaneous regression of blood vessels, but not lymphatic vessels, was observed after resolution of tracheal *Mycoplasma pulmonis* infection.[48] Lymphatic endothelial cells may also sustain commonly used doses of radiation therapy.[114] Therefore, anti-lymphangiogenic and radiotherapy might be combined to inhibit lymphatic metastasis of potentially remaining tumor cells postoperatively in cases not complicated by postoperative lymphedema (Figure 3).

Selection of anti-lymphangiogenic therapy, dosing, duration and follow-up

Some controversy remains with regard to the correlation between lymphatic vessel density and organ metastasis. It remains to be tested if patients for anti-lymphangiogenic therapy should first be tested for activated lymphangiogenesis. Screening tests for biomarkers of activated lymphangiogenesis, for example, levels of lymphangiogenic factors in blood samples, have limited promise as many of the factors involved seem to act in a paracrine manner and low levels are found in the systemic circulation. On the basis of our recent results, imaging techniques may prove a solution for targeting anti-lymphangiogenic therapy to the right patients at the right time. We have shown that activated lymphangiogenesis can be visualized using labeled antibodies recognizing the lymphatic endothelial marker LYVE-1 in positron emission tomography.[115] Also, using this non-invasive method, lymph node excision can be avoided in cases where there is no indication of activated lymphatic expansion at the lymph node. This would limit surgical trauma and risk of post-surgical lymphedema due to ablation of lymphatic drainage pathways. In an anti-lymphangiogenic treatment trial, dose selection has to be extrapolated from animal studies. However, highly specific targeting, such as therapies based on the epitope recognition sites of antibodies, would reduce concerns of off-target effects and provide a broader therapeutic window. Eventually, it has to be considered whether the initiated therapy should be discontinued or whether life-long treatment would be required in fear of dormant cancer cells somewhere in the body. Molecular imaging techniques may provide a non-invasive method for screening reactivation of lymphangiogenesis after successful remission.

OUTLOOK

Our increased understanding of the role of the lymphatic vascular system in cancer metastasis allowed us to advance in anti-lymphangiogenic cancer therapy. Studies on developmental lymphangiogenesis have led to the identification of strong regulators of lymphangiogenesis that have been studied further in mouse models of cancer. Identification of the vessels conducting metastasis from the primary tumor and beyond lymph nodes seems within reach, enabling a better understanding of the metastatic process. Inducible gene expression and cell tracing systems provide a technical opportunity to overcome the challenge of imaging tumor cells undergoing the metastasis process. Analysis of tumor-induced changes and intralymphatic invasion of tumor cells within lymph nodes offer prognostic potential. It seems that malignant cells may interact with lymphatic vessels to induce a conductive microenvironment for their survival in lymph nodes and at sites of distant metastasis. A further understanding of such a 'lymphovascular niche' should be obtained to target dormant chemoresistant cancer cells that maintain a risk of recurrent disease. Successful anti-lymphangiogenic therapy should be administered to selected cancer patients in risk of lymphatic metastasis, at a therapeutic dosage, duration and formulation and, most likely, as a combination therapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Work in the authors' lab is supported by the Swiss National Science Foundation (grant numbers 3100A0108207and 31003A-130627); Commission of the European Communities (grant number LSHCCT2005518178); Advanced European Research Council (grant LYVICAM); and Oncosuisse and Krebsliga Zurich (to MD).

REFERENCES

1 Garcia M, Jemal A, Ward EM, Center MM, Hao Y, Sieger RL *et al. Global Cancer Facts & Figures 2007*. American Cancer Society: Atlanta, GA, 2007.

2 Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* 2011; **61**: 212 - 236.
3 Ferlay J, Parkin DM, Steliarova-Foucher E. Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* 2010; **46**: 765 - 781.
4 Wigle JT, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* 1999; **98**: 769 - 778.
5 Yoshimatsu Y, Yamazaki T, Mihira H, Itoh T, Suehiro J, Yuki K *et al.* Ets family members induce lymphangiogenesis through physical and functional interaction with Prox1. *J Cell Sci* 2011; **124**: 2753 - 2762.
6 Bos FL, Caunt M, Peterson-Maduro J, Planas-Paz L, Kowalski J, Karpanen T *et al.* CCBE1 is essential for mammalian lymphatic vascular development and enhances the lymphangiogenic effect of vascular endothelial growth factor-C *in vivo*. *Circ Res* 2011; **109**: 486 - 491.
7 Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV *et al.* Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 2004; **5**: 74 - 80.
8 Kang J, Yoo J, Lee S, Tang W, Aguilar B, Ramu S *et al.* An exquisite cross-control mechanism among endothelial cell fate regulators directs the plasticity and heterogeneity of lymphatic endothelial cells. *Blood* 2010; **116**: 140 - 150.
9 Chen L, Mupo A, Huynh T, Cioffi S, Woods M, Jin C *et al.* Tbx1 regulates Vegfr3 and is required for lymphatic vessel development. *J Cell Biol* 2010; **189**: 417 - 424.
10 Norrmen C, Ivanov KI, Cheng J, Zangger N, Delorenzi M, Jaquet M *et al.* FOXC2 controls formation and maturation of lymphatic collecting vessels through cooperation with NFATc1. *J Cell Biol* 2009; **185**: 439 - 457.
11 Wick N, Haluza D, Gurnhofer E, Raab I, Kasimir MT, Prinz M *et al.* Lymphatic precollectors contain a novel, specialized subpopulation of podoplanin low, CCL27-expressing lymphatic endothelial cells. *Am J Pathol* 2008; **173**: 1202 - 1209.
12 Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N *et al.* T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO J* 2003; **22**: 3546 - 3556.
13 Bertozzi CC, Hess PR, Kahn ML. Platelets: covert regulators of lymphatic development [Review]. *Arterioscler Thromb Vasc Biol* 2010; **30**: 2368 - 2371.
14 Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M *et al.* Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. *Blood* 2010; **116**: 661 - 670.
15 Cueni LN, Chen L, Zhang H, Marino D, Huggenberger R, Alitalo A *et al.* Podoplanin-Fc reduces lymphatic vessel formation *in vitro* and *in vivo* and causes disseminated intravascular coagulation when transgenically expressed in the skin. *Blood* 2010; **116**: 4376 - 4384.
16 Jackson DG. Immunological functions of hyaluronan and its receptors in the lymphatics [Review]. *Immunol Rev* 2009; **230**: 216 - 231.
17 Oliver G, Srinivasan RS. Lymphatic vasculature development: current concepts [Review]. *Ann NY Acad Sci* 2008; **1131**: 75 - 81.
18 Marino D, Dabouras V, Brandli AW, Detmar M. A role for all-*trans*-retinoic acid in the early steps of lymphatic vasculature development. *J Vasc Res* 2011; **48**: 236 - 251.
19 Pflicke H, Sixt M. Preformed portals facilitate dendritic cell entry into afferent lymphatic vessels. *J Exp Med* 2009; **206**: 2925 - 2935.
20 Padera TP, Kadambi A, di Tomaso E, Carreira CM, Brown EB, Boucher Y *et al.* Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 2002; **296**: 1883 - 1886.
21 Schledzewski K, Falkowski M, Moldenhauer G, Metharom P, Kzhyshkowska J, Ganss R *et al.* Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue *in vivo* and in bone marrow cultures *in vitro*: implications for the assessment of lymphangiogenesis. *J Pathol* 2006; **209**: 67 - 77.
22 Cho CH, Koh YJ, Han J, Sung HK, Jong Lee H, Morisada T *et al.* Angiogenic role of LYVE-1-positive macrophages in adipose tissue. *Circ Res* 2007; **100**: e47 - e57.
23 Shaul ME, Bennett G, Strissel KJ, Greenberg AS, Obin MS. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet-induced obesity in mice. *Diabetes* 2010; **59**: 1171 - 1181.
24 Mouta Carreira C, Nasser SM, di Tomaso E, Padera TP, Boucher Y, Tomarev SI *et al.* LYVE-1 is not restricted to the lymph vessels: expression in normal liver blood sinusoids and down-regulation in human liver cancer and cirrhosis. *Cancer Res* 2001; **61**: 8079 - 8084.
25 Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D *et al.* Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 1995; **92**: 3566 - 3570.
26 Laakkonen P, Waltari M, Holopainen T, Takahashi T, Pytowski B, Steiner P *et al.* Vascular endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. *Cancer Res* 2007; **67**: 593 - 599.
27 Tammela T, Zarkada G, Wallgard E, Murtomaki A, Suchting S, Wirzenius M *et al.* Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature* 2008; **454**: 656 - 660.
28 Haiko P, Makinen T, Keskitalo S, Taipale J, Karkkainen MJ, Baldwin ME *et al.* Deletion of vascular endothelial growth factor C (VEGF-C) and VEGF-D is not equivalent to VEGF receptor 3 deletion in mouse embryos. *Mol Cell Biol* 2008; **28**: 4843 - 4850.
29 Zhang L, Zhou F, Han W, Shen B, Luo J, Shibuya M *et al.* VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis. *Cell Res* 2010; **20**: 1319 - 1331.
30 Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K *et al.* A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci USA* 2001; **98**: 12677 - 12682.
31 Dumont DJ, Jussila L, Taipale J, Lymboussaki A, Mustonen T, Pajusola K *et al.* Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science* 1998; **282**: 946 - 949.
32 Tammela T, Zarkada G, Nurmi H, Jakobsson L, Heinolainen K, Tvorogov D *et al.* VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nat Cell Biol* 2011; **13**: 1202 - 1213.
33 Galvagni F, Pennacchini S, Salameh A, Rocchigiani M, Neri F, Orlandini M *et al.* Endothelial cell adhesion to the extracellular matrix induces c-Src-dependent VEGFR-3 phosphorylation without the activation of the receptor intrinsic kinase activity. *Circ Res* 2010; **106**: 1839 - 1848.
34 Nilsson I, Bahram F, Li X, Gualandi L, Koch S, Jarvius M *et al.* VEGF receptor 2/-3 heterodimers detected *in situ* by proximity ligation on angiogenic sprouts. *EMBO J* 2010; **29**: 1377 - 1388.
35 Harris NC, Paavonen K, Davydova N, Roufail S, Sato T, Zhang YF *et al.* Proteolytic processing of vascular endothelial growth factor-D is essential for its capacity to promote the growth and spread of cancer. *FASEB J* 2011; **25**: 2615 - 2625.
36 Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K *et al.* Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 2001; **159**: 893 - 903.
37 Saaristo A, Tammela T, Farkkila A, Karkkainen M, Suominen E, Yla-Herttuala S *et al.* Vascular endothelial growth factor-C accelerates diabetic wound healing. *Am J Pathol* 2006; **169**: 1080 - 1087.
38 Schoppmann SF, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C *et al.* Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol* 2002; **161**: 947 - 956.
39 Gordon EJ, Rao S, Pollard JW, Nutt SL, Lang RA, Harvey NL. Macrophages define dermal lymphatic vessel calibre during development by regulating lymphatic endothelial cell proliferation. *Development* 2010; **137**: 3899 - 3910.
40 Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S *et al.* Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* 2010; **116**: 829 - 840.
41 Kim KE, Koh YJ, Jeon BH, Jang C, Han J, Kataru RP *et al.* Role of CD11b+ macrophages in intraperitoneal lipopolysaccharide-induced aberrant lymphangiogenesis and lymphatic function in the diaphragm. *Am J Pathol* 2009; **175**: 1733 - 1745.
42 Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ *et al.* Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med* 2002; **196**: 1497 - 1506.
43 Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M. VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* 2005; **201**: 1089 - 1099.
44 Halin C, Tobler NE, Vigl B, Brown LF, Detmar M. VEGF-A produced by chronically inflamed tissue induces lymphangiogenesis in draining lymph nodes. *Blood* 2007; **110**: 3158 - 3167.
45 Hong YK, Lange-Asschenfeldt B, Velasco P, Hirakawa S, Kunstfeld R, Brown LF *et al.* VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. *FASEB J* 2004; **18**: 1111 - 1113.
46 Kunstfeld R, Hirakawa S, Hong YK, Schacht V, Lange-Asschenfeldt B, Velasco P *et al.* Induction of cutaneous delayed-type hypersensitivity reactions in VEGF-A transgenic mice results in chronic skin inflammation associated with persistent lymphatic hyperplasia. *Blood* 2004; **104**: 1048 - 1057.
47 Wirzenius M, Tammela T, Uutela M, He Y, Odorisio T, Zambruno G *et al.* Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *J Exp Med* 2007; **204**: 1431 - 1440.
48 Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ *et al.* Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* 2005; **115**: 247 - 257.

49 Veikkola T, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV *et al.* Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001; **20**: 1223 - 1231.
50 Zheng W, Tammela T, Yamamoto M, Anisimov A, Holopainen T, Kaijalainen S *et al.* Notch restricts lymphatic vessel sprouting induced by vascular endothelial growth factor. *Blood* 2011; **118**: 1154 - 1162.
51 Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C *et al.* VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 2004; **113**: 1040 - 1050.
52 Murakami M, Zheng Y, Hirashima M, Suda T, Morita Y, Ooehara J *et al.* VEGFR1 tyrosine kinase signaling promotes lymphangiogenesis as well as angiogenesis indirectly via macrophage recruitment. *Arterioscler Thromb Vasc Biol* 2008; **28**: 658 - 664.
53 Leppanen VM, Jeltsch M, Anisimov A, Tvorogov D, Aho K, Kalkkinen N *et al.* Structural determinants of vascular endothelial growth factor-D receptor binding and specificity. *Blood* 2011; **117**: 1507 - 1515.
54 Anisimov A, Alitalo A, Korpisalo P, Soronen J, Kaijalainen S, Leppanen VM *et al.* Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. *Circ Res* 2009; **104**: 1302 - 1312.
55 Rissanen TT, Markkanen JE, Gruchala M, Heikura T, Puranen A, Kettunen MI *et al.* VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circ Res* 2003; **92**: 1098 - 1106.
56 Xu Y, Yuan L, Mak J, Pardanaud L, Caunt M, Kasman I *et al.* Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. *J Cell Biol* 2010; **188**: 115 - 130.
57 Caunt M, Mak J, Liang WC, Stawicki S, Pan Q, Tong RK *et al.* Blocking neuropilin-2 function inhibits tumor cell metastasis. *Cancer Cell* 2008; **13**: 331 - 342.
58 Gluzman-Poltorak Z, Cohen T, Herzog Y, Neufeld G. Neuropilin-2 is a receptor for the vascular endothelial growth factor (VEGF) forms VEGF-145 and VEGF-165 [corrected]. *J Biol Chem* 2000; **275**: 18040 - 18045.
59 Yuan L, Moyon D, Pardanaud L, Breant C, Karkkainen MJ, Alitalo K *et al.* Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* 2002; **129**: 4797 - 4806.
60 Neufeld G, Kessler O. The semaphorins: versatile regulators of tumour progression and tumour angiogenesis [Review]. *Nat Rev Cancer* 2008; **8**: 632 - 645.
61 Kajiya K, Hirakawa S, Ma B, Drinnenberg I, Detmar M. Hepatocyte growth factor promotes lymphatic vessel formation and function. *EMBO J* 2005; **24**: 2885 - 2895.
62 Chang LK, Garcia-Cardena G, Farnebo F, Fannon M, Chen EJ, Butterfield C *et al.* Dose-dependent response of FGF-2 for lymphangiogenesis. *Proc Natl Acad Sci USA* 2004; **101**: 11658 - 11663.
63 Cao R, Bjorndahl MA, Religa P, Clasper S, Garvin S, Galter D *et al.* PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell* 2004; **6**: 333 - 345.
64 Leong SP, Nakakura EK, Pollock R, Choti MA, Morton DL, Henner WD *et al.* Unique patterns of metastases in common and rare types of malignancy [Review]. *J Surg Oncol* 2011; **103**: 607 - 614.
65 Hoshida T, Isaka N, Hagendoorn J, di Tomaso E, Chen YL, Pytowski B *et al.* Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. *Cancer Res* 2006; **66**: 8065 - 8075.
66 Catalano O, Caraco C, Mozzillo N, Siani A. Locoregional spread of cutaneous melanoma: sonography findings [Review]. *Am J Roentgenol* 2010; **194**: 735 - 745.
67 van Akkooi AC, de Wilt JH, Verhoef C, Schmitz PI, van Geel AN, Eggermont AM *et al.* Clinical relevance of melanoma micrometastases (<0.1 mm) in sentinel nodes: are these nodes to be considered negative? *Ann Oncol* 2006; **17**: 1578 - 1585.
68 Moussai D, Mitsui H, Pettersen JS, Pierson KC, Shah KR, Suarez-Farinas M *et al.* The human cutaneous squamous cell carcinoma microenvironment is characterized by increased lymphatic density and enhanced expression of macrophage-derived VEGF-C. *J Invest Dermatol* 2011; **131**: 229 - 236.
69 Yang H, Kim C, Kim MJ, Schwendener RA, Alitalo K, Heston W *et al.* Soluble vascular endothelial growth factor receptor-3 suppresses lymphangiogenesis and lymphatic metastasis in bladder cancer. *Mol Cancer* 2011; **10**: 36.
70 Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. *Pathophysiology* 2010; **17**: 229 - 251.
71 Rinderknecht M, Detmar M. Tumor lymphangiogenesis and melanoma metastasis [Review]. *J Cell Physiol* 2008; **216**: 347 - 354.
72 Thiele W, Sleeman JP. Tumor-induced lymphangiogenesis: a target for cancer therapy? [Review]. *J Biotechnol* 2006; **124**: 224 - 241.
73 Rinderknecht M, Detmar M. Molecular mechanisms of lymph node metastasisi. In: Stacker SA, Achen MG (eds). *Lymphangiogenesis in Cancer Metastasis*. Springer Science+Business Media BV, 2009.
74 He Y, Rajantie I, Pajusola K, Jeltsch M, Holopainen T, Yla-Herttuala S *et al.* Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. *Cancer Res* 2005; **65**: 4739 - 4746.
75 Burton JB, Priceman SJ, Sung JL, Brakenhielm E, An DS, Pytowski B *et al.* Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. *Cancer Res* 2008; **68**: 7828 - 7837.
76 Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P *et al.* Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001; **7**: 192 - 198.
77 Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R *et al.* Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 2001; **20**: 672 - 682.
78 Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M *et al.* Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001; **61**: 1786 - 1790.
79 Tammela T, Saaristo A, Holopainen T, Yla-Herttuala S, Andersson LC, Virolainen S *et al.* Photodynamic ablation of lymphatic vessels and intralymphatic cancer cells prevents metastasis. *Sci Transl Med* 2011; **3**: 69ra11.
80 Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R *et al.* VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* 2001; **7**: 186 - 191.
81 Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y *et al.* Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res* 2006; **66**: 2650 - 2657.
82 Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M. VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* 2007; **109**: 1010 - 1017.
83 Kyzas PA, Geleff S, Batistatou A, Agnantis NJ, Stefanou D. Evidence for lymphangiogenesis and its prognostic implications in head and neck squamous cell carcinoma. *J Pathol* 2005; **206**: 170 - 177.
84 Dadras SS, Lange-Asschenfeldt B, Velasco P, Nguyen L, Vora A, Muzikansky A *et al.* Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. *Mod Pathol* 2005; **18**: 1232 - 1242.
85 Van den Eynden GG, Van der Auwera I, Colpaert CG, Dirix LY, Van Marck EA, Vermeulen PB. Letter to the editor: lymphangiogenesis in primary breast cancer. *Cancer Lett* 2007; **256**: 279 - 281.
86 Van der Auwera I, Van den Eynden GG, Colpaert CG, Van Laere SJ, van Dam P, Van Marck EA *et al.* Tumor lymphangiogenesis in inflammatory breast carcinoma: a histomorphometric study. *Clin Cancer Res* 2005; **11**: 7637 - 7642.
87 van der Schaft DW, Pauwels P, Hulsmans S, Zimmermann M, van de Poll-Franse LV, Griffioen AW. Absence of lymphangiogenesis in ductal breast cancer at the primary tumor site. *Cancer Lett* 2007; **254**: 128 - 136.
88 Kerjaschki D, Bago-Horvath Z, Rudas M, Sexl V, Schneckenleithner C, Wolbank S *et al.* Lipoxygenase mediates invasion of intrametastatic lymphatic vessels and propagates lymph node metastasis of human mammary carcinoma xenografts in mouse. *J Clin Invest* 2011; **121**: 2000 - 2012.
89 Wong SY, Haack H, Crowley D, Barry M, Bronson RT, Hynes RO. Tumor-secreted vascular endothelial growth factor-C is necessary for prostate cancer lymphangiogenesis, but lymphangiogenesis is unnecessary for lymph node metastasis. *Cancer Res* 2005; **65**: 9789 - 9798.
90 Isaka N, Padera TP, Hagendoorn J, Fukumura D, Jain RK. Peritumor lymphatics induced by vascular endothelial growth factor-C exhibit abnormal function. *Cancer Res* 2004; **64**: 4400 - 4404.
91 Fukumura D, Duda DG, Munn LL, Jain RK. Tumor microvasculature and microenvironment: novel insights through intravital imaging in pre-clinical models [Review]. *Microcirculation* 2010; **17**: 206 - 225.
92 Azzali G. Tumor cell transendothelial passage in the absorbing lymphatic vessel of transgenic adenocarcinoma mouse prostate. *Am J Pathol* 2007; **170**: 334 - 346.
93 Dadiani M, Kalchenko V, Yosepovich A, Margalit R, Hassid Y, Degani H *et al.* Real-time imaging of lymphogenic metastasis in orthotopic human breast cancer. *Cancer Res* 2006; **66**: 8037 - 8041.
94 Sahai E. Illuminating the metastatic process [Review]. *Nat Rev Cancer* 2007; **7**: 737 - 749.
95 Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGFbeta signalling switches breast cancer cells from cohesive to single cell motility. *Nat Cell Biol* 2009; **11**: 1287 - 1296.
96 Kabashima K, Shiraishi N, Sugita K, Mori T, Onoue A, Kobayashi M *et al.* CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol* 2007; **171**: 1249 - 1257.
97 Kim M, Koh YJ, Kim KE, Koh BI, Nam DH, Alitalo K *et al.* CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. *Cancer Res* 2010; **70**: 10411 - 10421.
98 Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; **410**: 50 - 56.

99 Wiley HE, Gonzalez EB, Maki W, Wu MT, Hwang ST. Expression of CC chemokine receptor-7 and regional lymph node metastasis of B16 murine melanoma. *J Natl Cancer Inst* 2001; **93**: 1638 - 1643.
100 Christiansen A, Detmar M. Lymphangiogenesis and Cancer. *Genes Cancer* (e-pub ahead of print 3 October 2011).
101 Ruddell A, Harrell MI, Minoshima S, Maravilla KR, Iritani BM, White SW *et al.* Dynamic contrast-enhanced magnetic resonance imaging of tumor-induced lymph flow. *Neoplasia* 2008; **10**: 706 - 713.
102 Proulx ST, Luciani P, Derzsi S, Rinderknecht M, Mumprecht V, Leroux JC *et al.* Quantitative imaging of lymphatic function with liposomal indocyanine green. *Cancer Res* 2010; **70**: 7053 - 7062.
103 Qian CN, Berghuis B, Tsarfaty G, Bruch M, Kort EJ, Ditlev J *et al.* Preparing the 'soil': the primary tumor induces vasculature reorganization in the sentinel lymph node before the arrival of metastatic cancer cells. *Cancer Res* 2006; **66**: 10365 - 10376.
104 Hirakawa S. From tumor lymphangiogenesis to lymphvascular niche [Review]. *Cancer Sci* 2009; **100**: 983 - 989.
105 Cady B. Regional lymph node metastases; a singular manifestation of the process of clinical metastases in cancer: contemporary animal research and clinical reports suggest unifying concepts [Review]. *Ann Surg Oncol* 2007; **14**: 1790 - 1800.
106 Viehl CT, Langer I, Guller U, Zanetti-Dallenbach R, Moch H, Wight E *et al.* Prognostic impact and therapeutic implications of sentinel lymph node micro-metastases in early-stage breast cancer patients [Review]. *J Surg Oncol* 2011; **103**: 531 - 533.
107 Krag DN, Anderson SJ, Julian TB, Brown AM, Harlow SP, Costantino JP *et al.* Sentinel-lymph-node resection compared with conventional axillary-lymph-node dissection in clinically node-negative patients with breast cancer: overall survival findings from the NSABP B-32 randomised phase 3 trial. *Lancet Oncol* 2010; **11**: 927 - 933.
108 Louis-Sylvestre C, Clough K, Asselain B, Vilcoq JR, Salmon RJ, Campana F *et al.* Axillary treatment in conservative management of operable breast cancer: dissection or radiotherapy? Results of a randomized study with 15 years of follow-up. *J Clin Oncol* 2004; **22**: 97 - 101.
109 Moehrle M, Schippert W, Rassner G, Garbe C, Breuninger H. Micrometastasis of a sentinel lymph node in cutaneous melanoma is a significant prognostic factor for disease-free survival, distant-metastasis-free survival, and overall survival. *Dermatol Surg* 2004; **30**: 1319 - 1328.
110 Leong SP, Zuber M, Ferris RL, Kitagawa Y, Cabanas R, Levenback C *et al.* Impact of nodal status and tumor burden in sentinel lymph nodes on the clinical outcomes of cancer patients [Review]. *J Surg Oncol* 2011; **103**: 518 - 530.
111 Clinicaltrials. *Trial Identifier NCT01288989*. US National Institutes of Health: Bethesda, MD.
112 Lin J, Lalani AS, Harding TC, Gonzalez M, Wu WW, Luan B *et al.* Inhibition of lymphogenous metastasis using adeno-associated virus-mediated gene transfer of a soluble VEGFR-3 decoy receptor. *Cancer Res* 2005; **65**: 6901 - 6909.
113 Maruyama K, Asai J, Ii M, Thorne T, Losordo DW, D'Amore PA. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. *Am J Pathol* 2007; **170**: 1178 - 1191.
114 Sung HK, Morisada T, Cho CH, Oike Y, Lee J, Sung EK *et al.* Intestinal and peri-tumoral lymphatic endothelial cells are resistant to radiation-induced apoptosis. *Biochem Biophys Res Commun* 2006; **345**: 545 - 551.
115 Mumprecht V, Honer M, Vigl B, Proulx ST, Trachsel E, Kaspar M *et al. In vivo* imaging of inflammation- and tumor-induced lymph node lymphangiogenesis by immuno-positron emission tomography. *Cancer Res* 2010; **70**: 8842 - 8851.