# Focus on lymphangiogenesis in tumor metastasis

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## Introduction

The major cause of cancer mortality is the metastatic spread of tumor cells that can occur via multiple routes, including the lymphatic vasculature. Recent research has indicated that growth of lymphatic vessels (lymphangiogenesis) in the vicinity of solid tumors correlates with lymphatic metastasis, and has identified protein growth factors and receptors, principally vascular endothelial growth factor C (VEGF-C), VEGF-D, and VEGF receptor-3 (VEGFR-3), as drivers of lymphangiogenesis. Studies in animal models and clinicopathological data indicate that these signaling molecules also contribute to tumor lymphangiogenesis and metastatic spread. This signaling system is therefore a promising target for inhibitors of lymphangiogenesis designed to restrict metastasis. Although most primary tumors are surgically removed from cancer patients, an antimetastatic approach would be useful for restricting metastatic spread from inoperable primary tumors or from remnants of primary tumors that were not completely removed by surgery. Importantly, this approach could also be beneficial in restricting further spread from existing cancer metastases.

## Lymphatic vasculature in health and disease

The lymphatic vasculature is essential for homeostasis of tissue fluid, immune function, and absorption of dietary fat. It maintains appropriate fluid volume in healthy tissues by transporting extravasated fluid and macromolecules from tissues to the bloodstream (Witte et al., 2001). Abnormalities of the lymphatic vasculature that restrict fluid drainage from tissues can be a cause of lymphedema—a condition that initially presents as a swelling of the limbs and often becomes debilitating and chronic (Witte et al., 2001; Alitalo and Carmeliet, 2002; Baldwin et al., 2002). The role of the lymphatic vasculature in immunity involves entry of antigen-presenting cells and lymphocytes into blind-ended lymphatic capillaries in the periphery, followed by migration of these cells through lymphatic vessels to lymph nodes, where they elicit immune responses. The entry of lymphocytes into lymphatic capillaries is made easier by the structural features of these vessels, which consist of a single thin layer of endothelial cells lacking tight junctions. Furthermore, the basement membrane of these vessels is discontinuous, and they are not ensheathed by pericytes or smooth muscle cells. The endothelial cells of lymphatic capillaries are attached to the extracellular matrix by elastic fibers (Leak and Burke, 1966), keeping the vessels open during changes in interstitial pressure (Saharinen et al., 2004). In contrast, larger collecting lymphatics have perivascular smooth muscle cells with contractile function to propel lymph fluid within the vessels.

The lymphatic vessels play a major role in cancer biology, as the spread of tumor cells to lymph nodes implicates the lymphatics as an important route of metastasis and is often an early event in metastatic disease. The presence of tumor cells in local lymph nodes is significant for the staging of cancer, hence the relevance of sentinel lymph node biopsy for planning of therapeutic strategies (Tuttle, 2004) and of sentinel lymphadenectomy as a surgical approach (Fidler, 1997). However, in some cases, the initial spread of cancer is not to lymph nodes, indicating that this process may be complex and that the mechanisms determining the first sites to develop metastases require further study. It was long supposed that lymphatic metastasis was a passive process whereby detached tumor cells reached lymph nodes via drainage through preexisting local lymphatic vessels. More recently, it has become apparent that lymphangiogenesis can contribute actively to metastasis.

## Detection of lymphatics

Analysis of lymphatic vessels in cancer was hampered by a lack of markers that discriminated between lymphatics and blood vessels. This changed recently with discovery of improved markers such as the transcription factor Prox-1, which is essential for lymphatic development (Wigle et al., 2002; Alitalo and Carmeliet, 2002), is a determinant of lymphatic endothelial cell (LEC) phenotype (Figure 1) (for review see Oliver, 2004), and is a useful marker for LECs in human tissues (Wilting et al., 2002). Podoplanin is a glomerular podocyte membrane mucoprotein expressed by LECs (Breiteneder-Geleff et al., 1999) that is required for lymphatic development and promotes endothelial cell adhesion, migration, and tube formation (Schacht et al., 2003). Although expressed in some nonendothelial cell types, podoplanin is not expressed in blood vessels and is a useful marker of small lymphatics. The lymphatic vessel hyaluronan receptor 1 (LYVE-1) is a homolog of CD44 expressed by embryonic and adult lymphatics (Prevo et al., 2001). Although expressed by liver and spleen sinusoids and macrophages, it has been a useful marker of lymphatics in mouse and man. VEGFR-3 (also known as Flt4) is a cell surface receptor tyrosine kinase that signals for lymphangiogenesis and is predominantly expressed on lymphatic endothelium in adult tissues but also detected on some fenestrated blood vessels (Partanen et al., 2000). Its utility as a marker for lymphatics in cancer is limited because of upregulation on some tumor blood vessels (Valtola et al., 1999).

Antibodies to podoplanin, LYVE-1, and VEGFR-3 have facilitated separation of LECs from blood vascular endothelial cells using magnetic beads or flow cytometry (for review, see Pepper and Skobe, 2003). Cultures of pure LECs allow in vitro analysis of this cell type for signaling that controls cell proliferation, differentiation, and cell adhesion (Mäkinen et al., 2001). Further, the phenotype of these cells can be compared to blood vascular endothelium by DNA array (Petrova et al., 2002; Podgrabinska et al., 2002; Hirakawa et al., 2003; Pepper and Skobe, 2003) or proteomic technologies which will surely identify more markers specific for blood vessels or lymphatics and indicate novel approaches for targeting therapeutics to these vessels in tumors.

## Signaling for lymphangiogenesis

The best characterized lymphangiogenic growth factors are VEGF-C and VEGF-D, secreted glycoproteins that activate VEGFR-3 (Joukov et al., 1996; Achen et al., 1998), a receptor

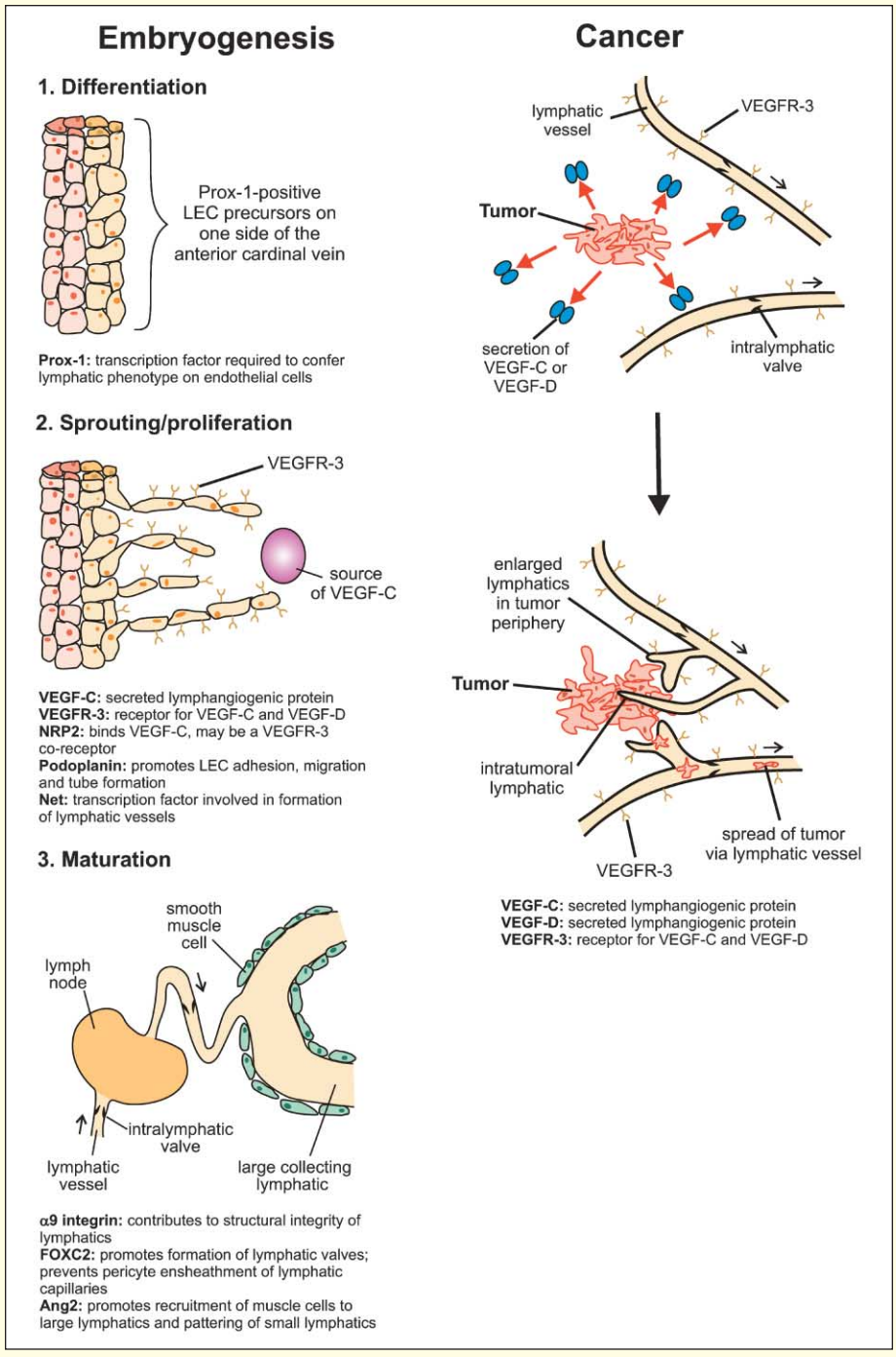


**Figure 1.** Development of lymphatic vessels in embryogenesis and cancer

Some of the proteins that are important in these events are shown underneath each section. Arrows denote the direction of lymph flow in the lymphatic vessels. A more extensive review of these processes with full lists of supporting references is presented elsewhere (Baldwin et al., 2002; Oliver, 2004). A recent report has called into question the involvement of LEC precursors in tumor lymphangiogenesis (He et al., 2004b). The role of FOXC2 has recently been delineated (Petrova et al., 2004).

expressed on lymphatic endothelium in adults (Lymboussaki et al., 1998). VEGFR-3 activation induces proliferation of LECs in vitro (Mäkinen et al., 2001) and lymphangiogenesis in vivo (Veikkola et al., 2001). Conversely, some human hereditary lymphedemas are associated with missense VEGFR-3 mutations which inactivate the tyrosine kinase domain and impair signaling (Karkkainen et al., 2000). Likewise, expression of a soluble form of VEGFR-3 that sequesters VEGF-C and VEGF-D in transgenic mice inhibits lymphangiogenesis and induces lymphedema (Mäkinen et al., 2001). Inactivation of the *Vegfr-3* gene in mice demonstrated that it also plays a role in formation of blood vessels, before emergence of the lymphatics (Dumont et al., 1998). Inactivation of the gene for neuropilin-2, a cell surface receptor that binds VEGF-C and may be a VEGFR-3 coreceptor (Karkkainen et al., 2001), causes abnormal lymphatic vessel development (Yuan et al., 2002), further emphasizing the importance of VEGF-C/VEGFR-3 signaling for lymphangiogenesis (Figure 1).

Human VEGF-C and VEGF-D also activate VEGFR-2 (known as Flk1 in mouse; KDR in man) (Joukov et al., 1996; Achen et al., 1998), a receptor expressed on the endothelium of blood vessels that is thought to signal for angiogenesis, although it can be expressed on lymphatics (Saaristo et al., 2002). VEGF-C and VEGF-D are initially secreted in full-length forms that appear to activate VEGFR-3 but not VEGFR-2; however, upon proteolytic processing, they are converted to mature forms that bind both VEGFR-2 and VEGFR-3 with high affinity (Joukov et al., 1997; Stacker et al., 1999). Recent in vitro studies have demonstrated that the serine protease plasmin (McColl et al., 2003) and members of the proprotein convertase family (Siegfried et al., 2003) are able to proteolytically process VEGF-C and VEGF-D. The receptor binding affinities explain why full-length VEGF-C and VEGF-D are predominantly lymphangiogenic (Jeltsch et al., 1997; Enholm et al., 2001; Rissanen et al., 2003), whereas the mature forms induce growth of both blood vessels and lymphatics (Cao et al., 1998; Byzova et al., 2002; Rissanen et al., 2003).

The function of VEGF-C and VEGF-D has been explored in numerous animal models. Ablation of the mouse *Vegf-c* gene showed that it is essential for emergence of the first lymphatic vessels from the cardinal vein during embryogenesis (Karkkainen et al., 2004), whereas the *Vegf-d* gene does not play a crucial role in embryonic lymphatic vessel development (Baldwin et al., 2005). Full-length VEGF-C and VEGF-D, and a VEGFR-3-specific mutant of VEGF-C (VEGF-C156S) (Joukov et al., 1998), induced predominantly lymphangiogenesis in rabbit hindlimb muscle (Rissanen et al., 2003), whereas mature VEGF-D induced both angiogenesis and lymphangiogenesis, suggesting that different forms of these proteins may find application for therapeutic angiogenesis or lymphangiogenesis (Alitalo and

**Table 1.** Inhibitors of the VEGF-C/VEGF-D/VEGFR-3 lymphangiogenic signaling pathway

| Inhibitor | Class of compound | Target[1] | Clinical trial status[2] | Reference |
|---|---|---|---|---|
| Soluble VEGFR-3 | Ig fusion protein | VEGF-C, VEGF-D, and any unknown VEGFR-3 ligands | Not in clinical trial | Karpanen et al., 2001; He et al., 2002; Krishnan et al., 2003 |
| VEGF-D antibody | Monoclonal antibody | VEGF-D | Not in clinical trial | Achen et al., 2000; Stacker et al., 2001 |
| VEGFR-3 antibody | Monoclonal antibody | VEGFR-3 EC[3] | Not in clinical trial | Kubo et al., 2000; Persaud et al., 2004 |
| BAY 43-9006 (Sorafenib) | Bi-aryl urea | VEGFR-3 TK[4], VEGFR-2 TK, Raf-1 S/TK[4], PDGFR-β TK, c-Kit TK, Flt3 TK, FGFR-1 TK, BRAF S/TK | Phase III for renal cell cancer; Phase II for CML and prostate, ovarian, pancreatic, head and neck, breast, thyroid, and lung cancers; Phase I for glioma | Wilhelm et al., 2004 |
| CEP-7055 | *N,N*-dimethyl glycine ester | VEGFR-3 TK, VEGFR-2 TK, VEGFR-1 TK, Flt3 TK, Mlk1 S/TK, Mlk3 S/TK | Phase I for various malignancies | Ruggeri et al., 2003 |
| MAE87, MAE106, MAZ51 | Indolinone | VEGFR-3 TK, VEGFR-2 TK | Not in clinical trial | Kirkin et al., 2001 |
| PTK787/ZK 222584 (Vatalanib) | Chloroanilino-pyridylmethyl phthalazine succinate | VEGFR-3 TK, VEGFR-2 TK, VEGFR-1 TK, c-Kit TK, c-Fms TK, PDGFR-β TK | Phase III for colorectal cancer; Phase II for mesothelioma and myelodysplastic syndromes; Phase I and II for CML, AML, and AMM | Lin et al., 2002 |

[1]Some of the inhibitors also target kinases that are not involved in lymphangiogenic signaling. For the small molecule inhibitors, targets or groups of targets that these compounds were initially designed or screened to inhibit are underlined. VEGF-A, FGF-2, and PDGF-BB have been reported to induce lymphangiogenesis in various models, but these targets are not included here, as their mechanisms of action in lymphangiogenesis have yet to be fully delineated.
[2]These trials are either active, or approved but not yet active, as cited on the Cancer Trials page of the website of the National Cancer Institute (http://www.cancer.gov/clinicaltrials), with the exception of the CEP-7055 trials that are referred to elsewhere (Ruggeri et al., 2003).
[3]EC denotes extracellular domain.
[4]TK denotes protein tyrosine kinase, and S/TK denotes protein serine/threonine kinase.

Carmeliet, 2002). Indeed, administration of VEGF-C promotes lymphangiogenesis in animal models of lymphedema that may ameliorate the condition (for example, see Szuba et al., 2002), and *VEGF-C* gene therapy restores lymph flow across incision wounds (Saaristo et al., 2004), suggesting approaches for reducing tissue edema in skin and muscle flaps generated during reconstructive surgery. Further, adenoviral delivery of mature VEGF-D induced transmural angiogenesis and increased myocardial perfusion in porcine heart (Rutanen et al., 2004).

Little is known about intracellular lymphangiogenic signaling. VEGFR-3 induces the phosphoinositide 3-kinase pathway leading to Akt activation, and the mitogen-activated protein kinase pathway, including activation of p42/p44 kinases, probably by protein kinase C (Mäkinen et al., 2001). However, signaling that controls specific events in lymphangiogenesis, e.g., tube formation, remains uncharacterized.

Other growth factors have been reported as lymphangiogenic, namely VEGF-A (Nagy et al., 2002), FGF-2 (Kubo et al., 2002), and PDGF-BB (Cao et al., 2004). The lymphangiogenic effect of FGF-2 appears to be indirect, i.e., via VEGF-C or VEGF-D. VEGF-A may induce lymphangiogenesis directly by signaling via VEGFR-2 on LECs; however, an indirect mechanism dependent on VEGF-C or VEGF-D could be responsible. In one recent report, PDGF-BB was observed to induce motility of isolated LECs and lymphangiogenesis in mouse cornea in vivo (Cao et al., 2004). Interestingly, the lymphangiogenesis induced by PDGF-BB could not be restricted by inhibitors blocking interaction of VEGF-C or VEGF-D with VEGFR-3, suggesting that, in this model, PDGF-BB exerts its effect via an independent pathway that may involve PDGF receptors on lymphatic vessels (Cao et al., 2004). Nevertheless, it is still possible that activation of PDGF receptors could lead to activation of VEGFR-3 in LECs by intracellular mechanisms. The importance of PDGF signaling for lymphangiogenesis during embryogenesis is unknown. Finally, the secreted protein angiopoietin-2 (Ang-2), a ligand for the Tie2 receptor tyrosine kinase, is critical for establishing the lymphatic vasculature, as mutant mice deficient for Ang-2 have defects in lymphatic organization and smooth muscle association, although the mice do possess peripheral lymphatics (Gale et al., 2002). Comparison with Vegf-c-deficient mice suggests that VEGF-C/VEGFR-3 signaling is a key primary proliferation pathway for lymphatic vessels, whereas Ang-2 is important in later remodeling stages (Figure 1) (Gale et al., 2002; Karkkainen et al., 2004).

## Role of lymphangiogenesis in cancer

The existence of functional intratumoral lymphatics in human cancer was doubted, because lymphatic vessels might not penetrate into expanding primary tumors due to the physical pressure inside tumors (Padera et al., 2002). Furthermore, it was envisaged that lymphangiogenesis was not required to explain lymphatic metastasis, as tumor cells might spread via preexisting lymphatic vessels (Pepper, 2001). However, intratumoral lymphatics and lymphangiogenesis were recently detected in head and neck cancer, thyroid carcinoma, and melanoma, and may contribute to lymph node metastasis (Beasley et al., 2002; Hall et al., 2003; Straume et al., 2003). Moreover, peritumoral lymphatics located immediately adjacent to tumors or in the peritumoral stroma, which can be dilated or enlarged, are known to be associated with human tumors (Pepper, 2001; Dadras et al., 2004; Bono et al., 2004).

### *Experimental models of tumor lymphangiogenesis*

Studies in animal models have indicated the important role of VEGF-C/VEGF-D/VEGFR-3 lymphangiogenic signaling in tumor biology (Figure 1). For example, VEGF-C induced intratumoral lymphangiogenesis when expressed in human breast cancer cells orthotopically transplanted onto nude mice that

resulted in enhanced metastasis to regional lymph nodes and to the lung (Skobe et al., 2001b). In similar breast cancer models, VEGF-C promoted growth of tumor-associated lymphatics, commonly infiltrated with tumor cells (Karpanen et al., 2001), and metastatic spread to local lymph nodes (Mattila et al., 2002). In a transgenic mouse model system, animals overexpressing VEGF-C in β cells of the pancreas exhibited extensive lymphangiogenesis around the endocrine islets of Langerhans, in contrast to wild-type controls (Mandriota et al., 2001). When tumors were induced in these islets, tumor cells entered surrounding lymphatics and established metastases in draining lymph nodes, in contrast to tumors of mice lacking the *VEGF-C* transgene, which did not metastasize.

VEGF-D promoted formation of intratumoral lymphatics and metastasis to lymph nodes in a mouse tumor xenograft model (Stacker et al., 2001). It also enhanced tumor angiogenesis and solid tumor growth that may reflect proteolytic processing to mature VEGF-D capable of activating VEGFR-2 and signaling for angiogenesis. The enhanced metastasis induced by VEGF-D in this model could be blocked by a neutralizing antibody targeting the central domain of VEGF-D (Stacker et al., 2001), the first direct evidence for the prevention of lymphatic metastasis in a tumor model (Jain and Padera, 2002). VEGF-C also induced tumor angiogenesis, and lymphangiogenesis, when human melanoma cells were transplanted onto nude mice (Skobe et al., 2001a). Use of blocking VEGFR-2 antibodies suggested that VEGF-C signaling for tumor angiogenesis is via VEGFR-2 (Kadambi et al., 2001). Nevertheless, a role for VEGFR-3 in tumor blood vessels is possible, as it can be upregulated in blood vessels in human cancer (Valtola et al., 1999), and a blocking antibody to VEGFR-3 suppressed tumor growth in a mouse model by inhibiting angiogenesis and inducing microhemorrhage in the tumors (Kubo et al., 2000).

Despite the presence of intratumoral lymphatics in some of the models above, other VEGF-C models suggested that these lymphatics are not functional, and that lymphatics in the tumor margin alone are sufficient for lymphatic metastasis (Padera et al., 2002). Irrespective of the importance of lymphatic vessel location for metastasis, lymph node metastasis can certainly be suppressed by inhibitors of lymphangiogenic signaling such as a soluble form of VEGFR-3 (Karpanen et al., 2001; He et al., 2002; Krishnan et al., 2003) or a monoclonal antibody to VEGF-D (Stacker et al., 2001). The findings that VEGF-C and VEGF-D can promote tumor angiogenesis suggest that targeting these proteins in cancer may bring the double benefit of restricting growth of blood vessels and lymphatics.

A recent study suggested that PDGF-BB may promote lymphangiogenesis in cancer. Expression of PDGF-BB in murine fibrosarcoma cells induced tumor lymphangiogenesis in syngeneic mice, causing formation of intratumoral lymphatics and enhanced metastasis in lymph nodes (Cao et al., 2004). PDGF-BB also promoted tumor angiogenesis and solid tumor growth in this model.

### *Clinicopathological findings*

Expression of VEGF-C or VEGF-D correlates with lymphatic metastasis in a range of human cancers, whereas involvement of PDGF-BB is poorly characterized. This topic has been reviewed extensively (Stacker et al., 2002a, 2002b; He et al., 2004a), and so is discussed here only briefly. Many studies indicate that VEGF-C levels in primary tumors correlate with lymph node metastasis in thyroid, prostate, gastric, colorectal, lung, and esophageal carcinomas (for review, see Stacker et al., 2002a). Moreover, patients with gastric cancers expressing high levels of VEGF-C were reported to have poorer prognoses than those with tumors expressing low levels of VEGF-C (Yonemura et al., 1999). Similarly, VEGF-C has been reported as a potential prognostic factor in cervical and ovarian carcinomas (Ueda et al., 2002; Nishida et al., 2004). Analysis of VEGF-D suggests that it also promotes metastasis in human cancer. VEGF-D has been reported as an independent prognostic marker for disease-free and overall survival in colorectal carcinoma that correlates with lymphatic involvement (White et al., 2002). Expression of VEGF-D and VEGFR-3 in endometrial carcinoma may predict myometrial invasion and lymph node metastasis, and may prospectively identify patients at increased risk for poor outcome (Yokoyama et al., 2003a). Furthermore, VEGF-D was found to be an independent predictor of poor outcome in epithelial ovarian carcinoma (Yokoyama et al., 2003b) and to be elevated in primary prostate tumors with sentinel lymph node involvement compared to those lacking lymph node involvement (Stearns et al., 2004). In a minority of studies, expression of VEGF-C or VEGF-D did not correlate with lymphatic involvement or cancer progression (for review, see Stacker et al., 2002b); for example, VEGF-D mRNA was found to be downregulated in breast cancer tissue and inversely correlated with lymph node metastasis (Koyama et al., 2003). However, in another study, VEGF-D protein was reported to be upregulated in breast cancer and to correlate with lymph node metastasis and poor patient prognosis (Nakamura et al., 2003).

## Tumor lymphangiogenesis as a therapeutic target

The findings from experimental models of lymphatic metastasis and clinicopathological analyses of human cancer identify the VEGF-C/VEGF-D/VEGFR-3 lymphangiogenic signaling pathway as a target for anticancer therapeutics designed to limit metastatic spread. The observations that VEGF-C and VEGF-D can also promote tumor angiogenesis make targeting these molecules more attractive, particularly in light of the clinical success with bevacizumab (also known as Avastin), an inhibitory monoclonal antibody to the angiogenic protein VEGF-A, for enhancing survival in patients with metastatic colorectal cancer (Hurwitz et al., 2004).

Numerous strategies could be used to inhibit this lymphangiogenic signaling pathway (Table 1), including blocking the proteolytic activation of VEGF-C and VEGF-D or blocking the binding of VEGF-C or VEGF-D to VEGFR-2 and VEGFR-3 with neutralizing antibodies against the ligands or the receptors. Neutralizing antibodies to VEGF-D (Achen et al., 2000; Stacker et al., 2001), VEGFR-2 (Bocci et al., 2004) and VEGFR-3 (Kubo et al., 2000; Persaud et al., 2004) have been reported. Alternatively, this could be achieved with soluble versions of the receptors (Karpanen et al., 2001; He et al., 2002; Krishnan et al., 2003). A third strategy includes blocking the tyrosine kinase activity of VEGFR-2 and VEGFR-3 using orally active small molecule inhibitors that enter the cell; small molecules inhibiting both VEGFR-2 and VEGFR-3 include BAY 43-9006 (Wilhelm et al., 2004), CEP-7055 (Ruggeri et al., 2003), and PTK787/ZK222584 (Lin et al., 2002), although these compounds are not specific, as they inhibit multiple other kinases (Table 1). Irrespective of the approach, it will be important to identify appropriate cohorts of cancer patients to allow effects on metastatic spread to be monitored.

## Concluding remarks

Important questions remain regarding the role of lymphatic vessels and lymphangiogenesis in metastasis. Debate continues as

to the relative contribution of intratumoral and peritumoral lymphatics to this process. It is not known if enlargement of preexisting lymphatics in the tumor periphery is sufficient, or if formation of new lymphatics is required to promote lymphatic metastasis. Recently, it has been speculated that, in addition to stimulating growth of tumor lymphatics, VEGF-C and VEGF-D might contribute to metastasis by altering the permeability or cell adhesive properties of lymphatic endothelium; in vitro analyses of LECs may shed light on this. The importance of other potential lymphangiogenic growth factors, in particular members of the angiopoietin and PDGF families, for metastatic spread of human cancer is unknown. Despite these uncertainties, there is abundant evidence from experimental and clinicopathological studies to justify targeting the VEGF-C/VEGF-D/VEGFR-3 lymphangiogenic signaling pathway in the clinic. This will be a priority for the future.

**Acknowledgments**

M.G.A. and S.A.S. are supported by Senior Research Fellowships from the National Health and Medical Research Council of Australia (NHMRC) and the Pfizer Foundation, respectively, and by a Program Grant from the NHMRC. B.K.M. is a recipient of a Melbourne Research Scholarship from the University of Melbourne. We thank Tony Burgess for helpful comments and acknowledge researchers whose work could not be cited here due to space restrictions. M.G.A. and S.A.S. declare that they have ownership of a company with interests related to the topic discussed in this article.

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