

# PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis

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## Summary

**Cancer metastases are commonly found in the lymphatic system. Like tumor blood angiogenesis, stimulation of tumor lymphangiogenesis may require the interplay of several tumor-derived growth factors. Here we report that members of the PDGF family act as lymphangiogenic factors. In vitro, PDGF-BB stimulated MAP kinase activity and cell motility of isolated lymphatic endothelial cells. In vivo, PDGF-BB potently induced growth of lymphatic vessels. Expression of PDGF-BB in murine fibrosarcoma cells induced tumor lymphangiogenesis, leading to enhanced metastasis in lymph nodes. These data demonstrate that PDGF-BB is an important growth factor contributing to lymphatic metastasis. Thus, blockage of PDGF-induced lymphangiogenesis may provide a novel approach for prevention and treatment of lymphatic metastasis.**

## Introduction

The major cause of cancer mortality is metastasis, which relies on de novo formation of blood and lymphatic vessels (Fidler, 2003). While blood vessels support tumor growth by providing nutrients and oxygen, and by removing waste products, the function of tumor lymphatic vessels remains poorly understood (Dyer et al., 2003). One might expect that sprouting of lymphatic vessels in the tumor would facilitate tumor immunity by providing lymph node conduits for antigen-presenting cells. In reality, however, these conduits facilitate the spread of tumor cells to regional lymph nodes. Indeed, the lymphatics are the dominant route for tumor cell spread in some of the most common cancer types, such as breast and colon/rectal cancers (Fidler, 2003). Thus, understanding the molecular mechanisms that control lymphangiogenesis and its functions will be an important step in the development of therapeutic agents for prevention and treatment of cancer metastasis.

Tumors stimulate blood and lymphatic vessel growth by producing angiogenic factors. Although it is known that induction of tumor angiogenesis is a complex process that involves the interplay of a dozen or more tumor-derived growth factors (Carmeliet and Jain, 2000; Folkman, 1995; Hanahan and Folkman, 1996), it is poorly understood how tumors induce lymphangiogenesis, and what its role is in tumor spread (Beasley et al., 2002; Williams et al., 2003). Many of the recent research efforts in studying lymphangiogenesis are focused on two members of the VEGF family, VEGF-C and VEGF-D, which interact with VEGFR-3, a lymphatic endothelial receptor (Alitalo and Carmeliet, 2002). Indeed, both VEGF-C and VEGF-D have been shown to enhance lymphatic metastasis when expressed at high levels in tumors (Makinen et al., 2001; Skobe et al., 2001; Stacker et al., 2001).

In addition to members of the VEGF family, tumors with high lymphatic metastatic ability also express other growth factors at high levels (Relf et al., 1997). For example, high levels of

## SIGNIFICANCE

Lymphatic metastases are common in solid malignant tumors. However, it remains poorly understood how cancer cells invade the lymphatic system. Our study provides novel mechanistic insights into tumor lymphatic vessel growth stimulated by tumor cell-derived lymphangiogenic factors. Here we report that members of the PDGF family, in particular PDGF-BB, which is a growth factor frequently overexpressed in cancers, are novel potent lymphangiogenic factors. Expression of PDGF-BB in transplanted primary murine sarcomas leads to regional lymph node metastases in mice. These findings demonstrate that PDGF-BB plays a critical role in stimulating tumor lymphangiogenesis and lymphatic metastasis. Thus, development of antagonists targeting the PDGF family will be an important approach in blocking tumor lymphatic vessel growth and metastasis.

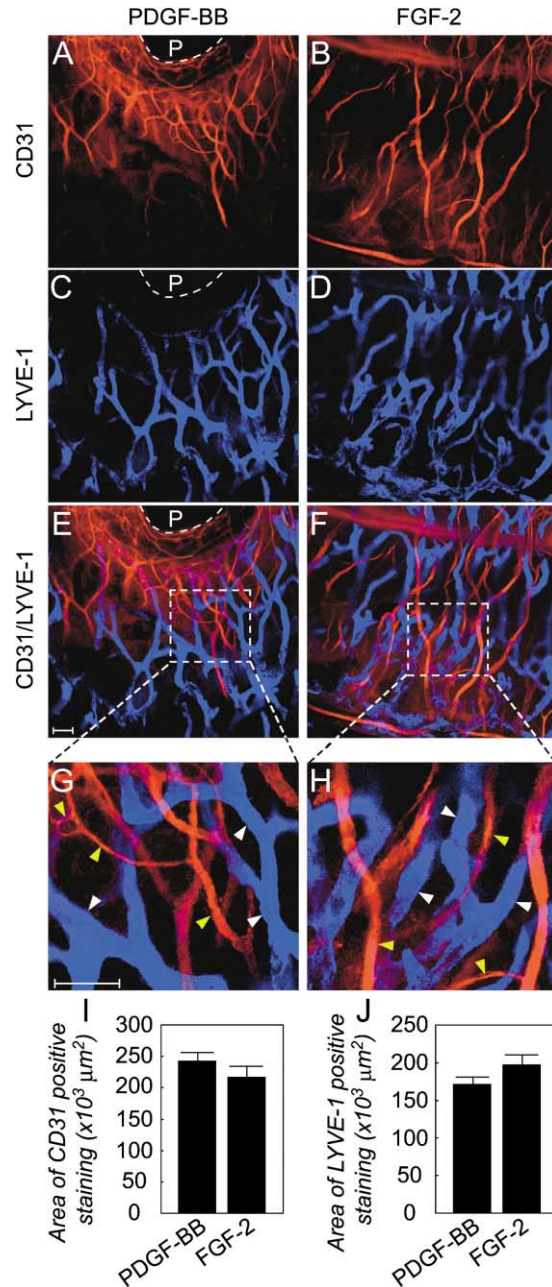
PDGF-BB expression have been found in breast cancer tissues (Coltrera et al., 1995). These distribution studies have raised the possibility that members of the PDGF family may contribute to tumor lymphangiogenesis and lymphatic metastasis. The PDGF family includes at least four structurally related members, PDGF-AA, -BB, -CC, and -DD (Kazlauskas, 2000), that can form both homodimers and heterodimers (Heldin and Westermark, 1999). Members of the PDGF family bind to tyrosine kinase receptors encoded by two genes, PDGFR- $\alpha$  and - $\beta$ . PDGF-BB is the only ligand that can activate both the PDGFR- $\alpha$  and - $\beta$  (Hammacher et al., 1989). Both genetic studies and our own recent work have demonstrated that members of the PDGF family are important angiogenic factors (Cao et al., 2003; Lindahl et al., 1997). Here we have established a murine lymphangiogenesis model to assess whether PDGF-BB plays a direct role in promoting lymphangiogenesis and metastasis. Our data demonstrate that PDGF-BB induces tumor lymphangiogenesis and metastatic spread to lymph nodes, and show that growth factors other than VEGFs can play key roles in these processes.

## Results

### PDGF-BB stimulates lymphatic vessel growth in vivo

To study the lymphangiogenic properties of PDGF-BB, we used a mouse corneal lymphangiogenesis model in combination with confocal microscopy to reveal specific markers that are exclusively expressed on lymphatic endothelial cells. Similar to FGF-2, PDGF-BB induced a robust angiogenic response in the mouse cornea (Supplemental Figures S1C–S1F and S1H–S1K). Within 2 weeks after growth factor implantation, PDGF-BB-induced corneal neovascularization was almost as potent as FGF-2-induced angiogenesis in this model (Supplemental Figures S1B, S1D–S1G, and S1I–S1K). Thus, PDGF-BB is a potent angiogenic factor in vivo. Under physiological conditions, the corneal tissue lacks both blood vessels and lymphatic vessels (Cursiefen et al., 2002). The only lymphatic vessels detected, using the lymphatic marker LYVE-1 (Banerji et al., 1999; Prevo et al., 2001), were the preexisting lymphatics in the limbus (Supplemental Figure S3A, LYVE-1). Implantation of the slow-release polymer without growth factors did not result in the growth of new lymphatic vessels (Supplemental Figure S3A, PBS). However, the inclusion of PDGF-BB potently stimulated growth of new lymphatic vessels sprouting from the existing limbal lymphatics (Figures 1C, 1E, and 1G). These newly formed LYVE-1 positive lymphatic vessels were of larger diameter than newly formed blood vessels (Figures 1E and 1G), displayed a different distribution, and generally lacked expression of PECAM-1 (CD31). Similar to PDGF-BB, FGF-2 was also able to induce lymphangiogenesis in the corneal model (Figures 1D, 1F, and 1H). Quantitative analysis showed that PDGF-BB and FGF-2 are almost equally potent in inducing blood and lymphatic angiogenesis (Figures 1I and 1J). These data demonstrate that PDGF-BB is a potent lymphangiogenic factor in vivo.

To further confirm their identity as lymphatic vessels, LYVE-1-positive structures were costained with antibodies against podoplanin and VEGFR-3, two other specific markers expressed on lymphatic vessels (Breiteneder-Geleff et al., 1999; Kaipainen et al., 1995). Consistent with previous findings, both VEGFR-3 and podoplanin are codistributed on the newly formed lymphatic vessels (Kubo et al., 2002; Schacht et al., 2003). As expected,



**Figure 1.** Stimulation of lymphangiogenesis by PDGF-BB

**A–H:** Confocal analysis of mouse corneal blood vessels and lymphatic vessels induced by PDGF-BB and FGF-2 at day 25 by immunofluorescent double labeling for CD31 (red) and LYVE-1 (blue) (**A–H**). Scale bar = 100  $\mu\text{m}$ . P = pellet. White arrowheads point to lymphatic vessels and yellow arrowheads point to blood vessels.

**I and J:** Quantification of CD31- (**I**) and LYVE-1- (**J**) positive signals from 5–7 optical fields of different sections.

completely overlapping immunostaining patterns were detected between LYVE-1 and podoplanin (Supplemental Figures S2A–S2C) or VEGFR-3 (Supplemental Figures S2D–S2F). To demonstrate that LYVE-1 did not stain blood vessels, corneal sections were double-stained with antibodies against LYVE-1 and CD34 (a specific marker expressed on blood vessels). Similar to CD31/LYVE-1 double staining, LYVE-1 positive vessels lacked CD34

signals (Supplemental Figures S2G–S2L). These data confirm the LYVE-1 positive structures in neovascularized cornea as lymphatics and further validate the specificity of LYVE-1 antibodies for lymphatic rather than blood vessel endothelium. Based on these findings, we chose LYVE-1 as a lymphatic specific marker for the rest of our studies.

### Stimulation of lymphangiogenesis by PDGF-AA and PDGF-AB

To determine whether other members of the PDGF family could also induce lymphangiogenesis and to compare their lymphangiogenic activity, the same amounts of PDGF-AA, -AB, and -BB were implanted in mouse corneas. Similar to PDGF-BB, PDGF-AA and -AB were also able to induce corneal lymphangiogenesis. However, the number of lymphatic vessels induced by PDGF-AA was significantly less than that induced by PDGF-AB and -BB (Figures 2D–2I and 2K). PDGF-AB and -BB induced similar numbers of lymphatic vessels in this system (Figure 2K). Since PDGF-AB and -BB, but not PDGF-AA, bind to PDGFR- $\beta$  (Heldin and Westermark, 1990), these findings suggest that the PDGFR- $\beta$  plays a critical role in mediating PDGF-induced lymphangiogenesis. Similar to lymphangiogenesis, all three factors were also able to induce hemangiogenesis (Figures 2A–2C and 2G–2I). However, unlike lymphangiogenesis, the levels of hemangiogenesis were approximately equal in each case (Figure 2J). Due to its potent lymphangiogenic activity, the remainder of our study was focused on PDGF-BB.

### Remodeling of nascent lymphatic vessels

Like blood angiogenesis, the formation of lymphatic vessels stimulated by PDGF-BB was readily detectable at day 5 after implantation, and a maximal response was detectable at day 14 after implantation (Supplemental Figures S3B, S3C, and S3G). During this period, the primitive newly formed lymphatic vessels underwent remodeling. By day 14, vascular tree-like structures were found in the PDGF-BB-induced lymphatic vessels, and some lymphatic vessels penetrated into the implanted PDGF-BB pellets. In addition, PDGF-BB-induced corneal blood vessels were remodeled from the primitive vascular plexuses into well-organized vasculatures (Supplemental Figures S3B and S3C). Similar vascular remodeling was also found in FGF-2-induced lymphatic and blood vessels (Supplemental Figures S3D and S3E). Quantification analysis showed that PDGF-BB and FGF-2 induced approximately equal numbers of lymphatic vessels (Supplemental Figures S3G).

### Blockage of VEGF-C/-D/VEGFR-3 does not inhibit PDGF-BB-induced lymphangiogenesis

One possible explanation for PDGF-BB-stimulated lymphangiogenesis is that the growth factor induced secondary activation of the well-characterized VEGF-C/-D/VEGFR-3 system. To exclude this possibility, we investigated the effects of an anti-mouse VEGFR-3 antibody and a soluble VEGFR-3-Fc, both of which are antagonists of the VEGF-C/-D/VEGFR-3 system. Firstly, we validated the ability of both reagents to block VEGF-C-induced endothelial cell activity in vitro by using the VEGFR-3-overexpressing porcine aortic endothelial (VEGFR-3/PAE) cell line. In the presence of VEGF-C, these cells underwent dramatic morphological changes from regular cell shapes into elongated spindle-like structures (Figures 3A, 3B, and 3E). This VEGF-C-induced cell morphological change was almost completely

blocked by the anti-VEGFR-3 antibody and the sVEGFR-3-Fc (Figures 3C–3E). To further assess the blocking activity of these antagonists, we performed a modified Boyden chamber assay. Both antagonists effectively blocked the migration of VEGFR-3/PAE cells, the sVEGFR-3-Fc being more potent than the anti-VEGFR-3 antibody (Figure 3F).

Having shown that these antagonists efficiently inhibited the VEGF-C/-D/VEGFR-3 system in vitro, we further investigated their antagonistic effect in vivo. As expected, in the mouse corneal assay, the anti-VEGFR-3 antibody selectively blocked VEGF-C-induced lymphangiogenesis but had no significant effects on blood angiogenesis (Figures 3I, 3N, and 3O). The sVEGFR-3-Fc blocked both VEGF-C-induced blood and lymphatic angiogenesis (Figures 3J, 3N, and 3O). By contrast, however, neither the anti-VEGFR-3 antibody nor the sVEGFR-3-Fc had any effect on PDGF-BB-induced blood or lymphatic angiogenesis (Figures 3K–3O). Hence these results demonstrate that PDGF-BB-induced lymphangiogenesis is not mediated via the VEGF-C/-D/VEGFR-3 pathway.

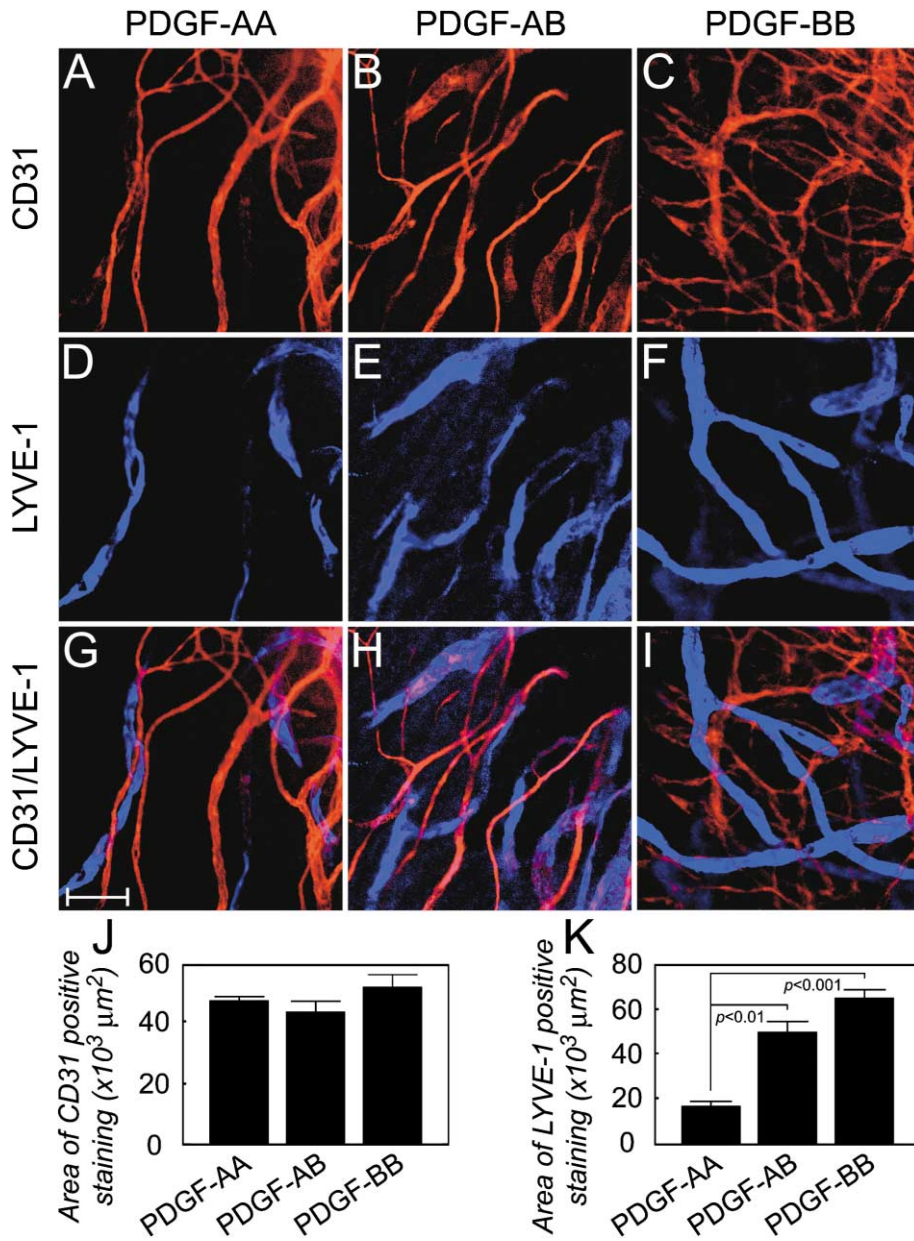
### PDGF-BB is a chemoattractant for primary lymphatic endothelial cells

The fact that the VEGF-C/-D/VEGFR-3 neutralizing agents had no inhibitory effect on PDGF-BB-induced lymphangiogenesis suggests that PDGF-BB acts directly on lymphatic endothelial cells (LECs). To investigate this possibility, human, mouse, and rat primary LECs were isolated. These LECs expressed lymphatic markers such as podoplanin and LYVE-1 (Figures 4A–4C). PDGF-BB and -AA stimulated migration of mouse and human LECs in a concentration-dependent manner (Figures 4D and 4E). Similarly, VEGF-C, a known chemoattractant for LECs, also induced cell motility in a concentration-dependent fashion. As expected, STI571, a potent PDGFR inhibitor, attenuated both PDGF-BB- and -AA-induced cell migration (Figures 4D and 4E). Interestingly, this PDGFR inhibitor also partially inhibited VEGF-C-induced cell migration, suggesting that its inhibitory effect was not only limited to the PDGFR-signaling pathway. Similar to mouse and human LECs, PDGF-BB significantly stimulated the migration of rat LECs (Figures 4F and 4G), but in common with PDGF-AA and VEGF-C, it did not regulate the expression levels of Prox-1 in mouse LECs (Figure 4H). The anti-VEGF-C/-D/VEGFR-3 antagonists could not block PDGF-BB-induced cell migration of human LECs, whereas both neutralizing agents blocked VEGF-C-induced migration significantly (Figure 3G).

In light of reports that the Tie2 receptor ligand angiopoietin-2 (ang-2) can induce lymphangiogenesis (Veikkola and Alitalo, 2002), we considered the possibility that PDGF-BB-induced lymphangiogenesis might operate via upregulation of Ang-2. However, Ang-2 expression levels were not affected by PDGF-BB stimulation in cells that are known to express PDGF receptors (Figure 4I) (Furuhashi et al., 2004). These data further support the notion that PDGF-BB has a direct effect on LECs.

### Activation of intracellular signaling pathways of isolated primary LECs

Stimulation of LEC motility by PDGF-BB suggested that this factor could activate intracellular signaling components. Indeed, increased levels of phosphorylated *Src* (P-*Src*), *Erk* 1/2 (MAP kinases, P-*Erk*1/2), and *Akt* (protein kinase B) were detected in rat LECs upon PDGF-BB stimulation. The activation of these intracellular signaling components was both concentration- and



**Figure 2.** Stimulation of lymphangiogenesis by members of the PDGF family

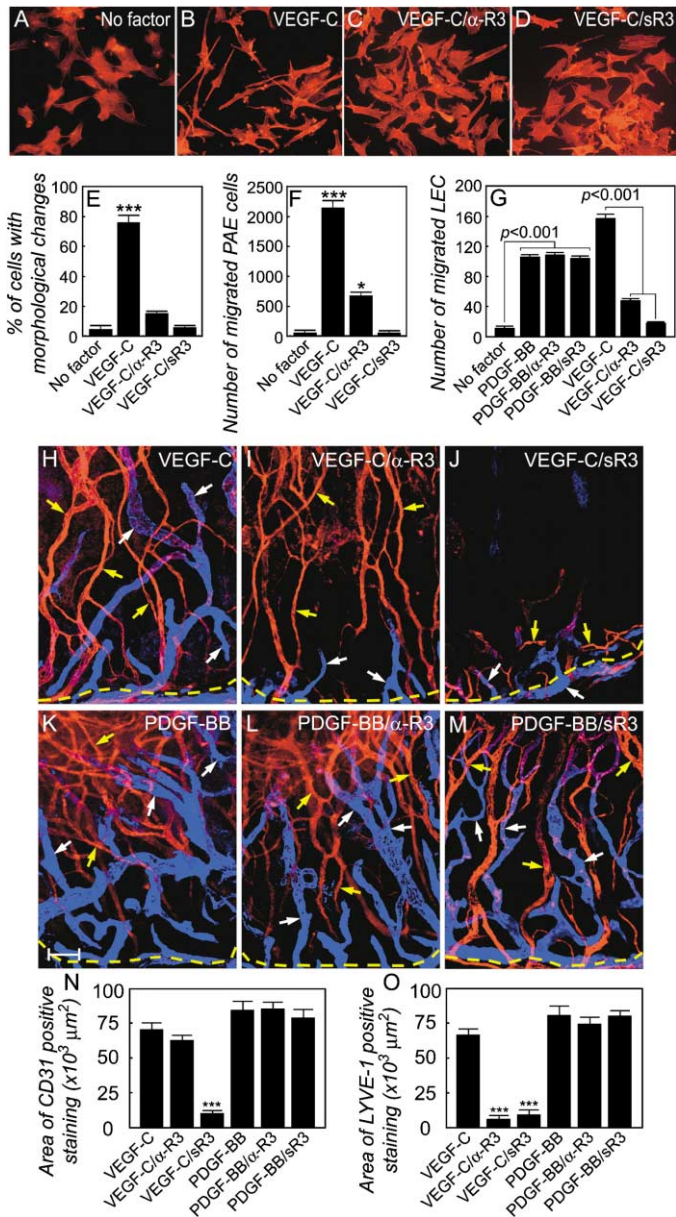
**A–I:** Implantation of the same amount of PDGF-AA, -AB, or -BB in mouse corneas induced both blood vessel (CD31, red) and lymphatic vessel (LYVE-1, blue) growth. Scale bar = 100  $\mu\text{m}$ .

**J and K:** Quantification of CD31- (**J**) and LYVE-1- (**K**) positive signals from 5–7 optical fields of different sections.

time-dependent (Figures 5A and 5B). Similarly, PDGF-AA and VEGF-C also induced increased levels of P-Src and P-Erk as compared with those of controls (Figures 5C and 5D). As expected, the protein kinase inhibitor STI571 inhibited the phosphorylation levels of these intracellular components, in response to PDGF-BB and -AA, in a concentration-dependent manner (Figures 5E and 5F). Interestingly, this PDGFR inhibitor also significantly suppressed VEGF-C-induced phosphorylation of Src and Erk1/2 (Figure 5G), suggesting that this compound has a broad inhibitory effect on several tyrosine kinases. However, it should be emphasized that STI571 had little effect on VEGF-C-tumor growth in vivo. Although the underlying mechanism of the differential effects by which STI571 acts in vitro and in vivo is not known, it is possible that VEGF-C could utilize other signaling pathways to promote blood and lymphatic angiogenesis.

#### Detection of PDGFRs on newly formed lymphatic vessels

PDGF-BB exerts its biological functions via activation of two tyrosine kinase receptors, PDGFR- $\alpha$  and - $\beta$  (Heldin and Westermark, 1999). To study the expression of these receptors on corneal lymphatics, we used a combination of in situ RNA hybridization and antibody staining to colocalize mRNAs coding for PDGFR- $\alpha$  and - $\beta$  with LYVE-1-positive vessels. Lymphatic vessels were detected using an anti-LYVE-1-specific antibody (Figures 6C and 6D, brown color), and PDGF receptor signals were determined using probes specific for PDGFR- $\alpha$  and - $\beta$  (Cao et al., 2003). Both PDGFR- $\alpha$  and - $\beta$  positive signals were detected on PDGF-BB-induced lymphatic vessels (Figures 6C and 6D, arrows) and on blood vessel endothelial cells (Cao et al., 2003). PDGFR- $\alpha$  and - $\beta$  were also detected at the protein level in preimplanted corneal lymphatics as assessed by double



**Figure 3.** Inhibition of PDGF-BB- and VEGF-C-induced corneal blood and lymphatic angiogenesis by VEGF-C/D/VEGFR-3 neutralizing reagents

Blockage of VEGF-C-induced VEGFR3/PAE morphological changes (B–E) and chemotaxis (F) by a rat anti-mouse VEGFR-3 antibody or a sVEGFR-3-Fc. Nonstimulated VEGFR3/PAE cells were used as a negative control (A). Blockage of PDGF-BB and VEGF-C-induced migration of human LEC by a rat anti-mouse VEGFR-3 antibody or a sVEGFR-3-Fc (G). Nonstimulated human LEC served as a negative control. Quantification of the percentage of morphological changes counted from 10 different optical fields (20×) (E). Quantification of the numbers of migrated cells counted from 6 wells of each sample (F and G). \**p* < 0.05 and \*\*\**p* < 0.001. VEGF-C alone- (H), VEGF-C/anti-VEGFR-3 antibody- (I), VEGF-C/sVEGFR-3-Fc- (J), PDGF-BB alone- (K), PDGF-BB/anti-VEGFR-3 antibody- (L), or PDGF-BB/sVEGFR-3-Fc-implanted corneal sections (M) were double stained with an anti-CD31 antibody (red) and an anti-LYVE-1 antibody (blue) (H–M). Quantification of CD31- (N) and LYVE-1- (O) positive signals (*n* = 10 different optical fields). \*\*\**p* < 0.001. Scale bar = 100 μm.

staining for PDGFR $\alpha/\beta$  and LYVE-1 (Figures 6E–6J, white arrows point to double positive signals in I and J). Further, to determine whether LECs in tissues other than cornea also expressed PDGFRs, we carried out RT-PCR using cDNA prepared from primary mouse dermal LEC. Again, both PDGFR- $\alpha$  and - $\beta$  were detected in these cells (Figures 6K and 6L). These findings were further supported by detection of expression of both types of PDGF receptors on the mouse LEC using Affymetrix gene array analysis (S.C. and D.G.J., unpublished data).

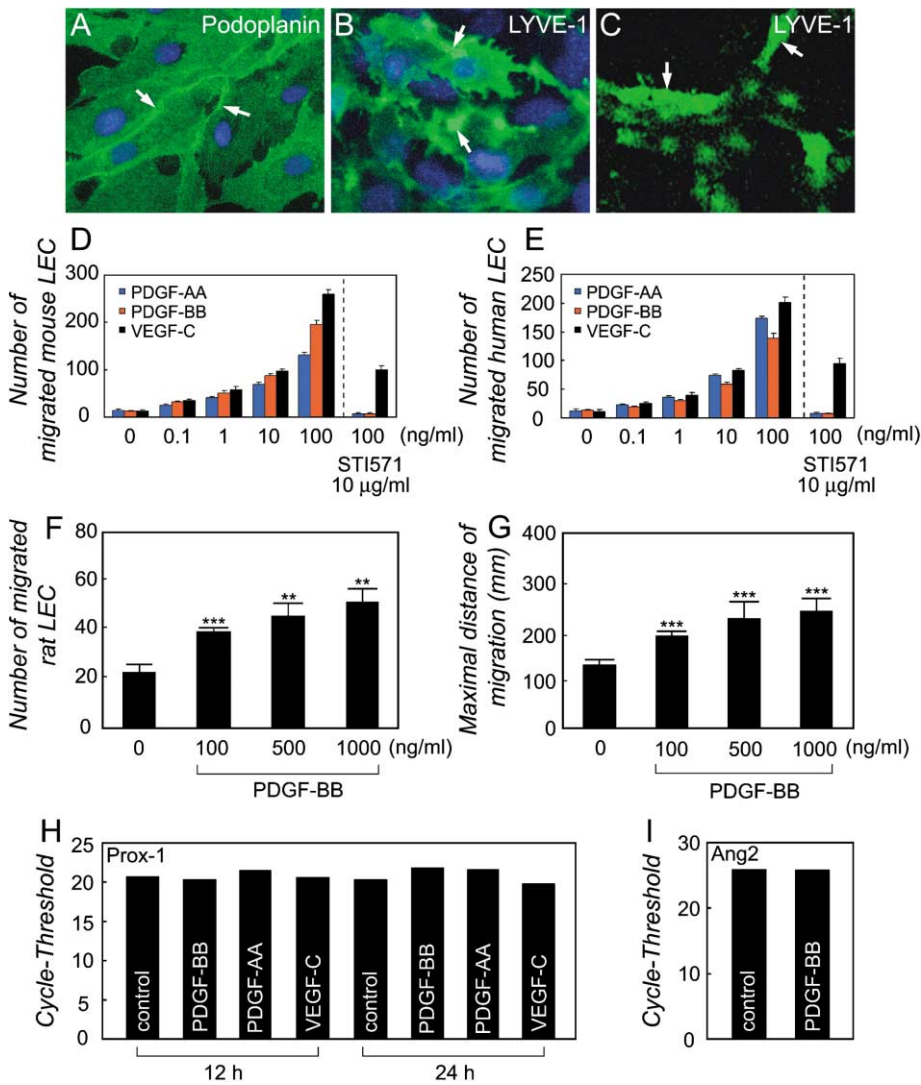
### PDGF-BB promotes primary tumor growth

To determine the role of PDGF-BB in promoting tumor growth, we transfected a murine fibrosarcoma cell line (T241) with cDNA coding for human PDGF-BB. Approximately 100 ng/ml PDGF-BB was secreted into the conditioned medium as quantified by a sensitive immunodetection assay. Despite a high level of expression, PDGF-BB did not appear to affect the growth rate of tumor cells in vitro (Figure 7A), suggesting that these cells lacked expression of the PDGFRs. However, implantation of PDGF-BB-expressing tumor cells into C57Bl/6 syngeneic mice resulted in accelerated tumor growth as measured by both tumor volume and weight (Figures 7B and 7C). Similarly, expression of VEGF-C, a known lymphangiogenic and blood angiogenic factor, significantly stimulated tumor growth in vivo without affecting the growth rate of tumor cells in vitro. The stimulatory effect of PDGF-BB on primary T241-tumor growth was statistically significant as compared with control tumors (*p* < 0.001) (Figures 7B and 7C). Confocal microscopy analysis using CD31 as a blood vessel marker revealed a significantly higher density of microvessels present in the PDGF-BB-expressing tumors as compared with controls (Figures 7D, 7E, 7G, and 7H). These tumor vessels appeared as disorganized and tortuous vascular plexuses with dilated microvessels (Figure 7G). A high density of malformed tumor vessels was also found in the VEGF-C-expressing tumors (Figure 7F, CD31). In contrast, relatively well-organized tumor blood vessels were found in wt and vector-transfected tumor sections (Figures 7D and 7E). Thus, it appears that both PDGF-BB and VEGF-C are able to stimulate blood vessel growth in primary tumors.

### PDGF-BB induces tumor lymphangiogenesis

Like blood-borne metastasis, lymphatic metastasis may rely on the outgrowth of host lymphatic vessels into the tumor tissue. To determine if PDGF-BB could induce tumor lymphangiogenesis, serial sections of subcutaneous tumors from the margin toward the center were stained with an anti-LYVE-1 antibody. Vector-, PDGF-BB-, and VEGF-C-transfected tumors expressed GFP that defined the margins of tumors (Figures 7E–7G). Interestingly, a high density of lymphatic vessels was found in the PDGF-BB-expressing tumors (Figure 7G). These lymphatic vessels were distributed throughout the entire tumor tissue. Similarly, tumor lymphatic vessels were also detected in the entire area of the VEGF-C-expressing tumors (Figure 7F). In contrast, host lymphatic vessels were located only at the margins of wt and vector-transfected tumors, and no tumor lymphatics were found (Figure 7D and 7E). Quantitative analysis showed that the numbers of lymphatic vessels induced by PDGF-BB- and VEGF-C-expressing tumors were significantly greater than those of controls (Figure 7I).

To further delineate the role of PDGF-BB in induction of tumor lymphangiogenesis, we established the mouse corneal



**Figure 4.** In vitro effects of PDGF-BB, -AA, and VEGF-C on isolated primary LECs

**A–C:** Isolated human (**A**), mouse (**B**), and rat (**C**) LECs were stained for cell surface podoplanin (**A**) and LYVE-1 (**B** and **C**) expression. A FITC-conjugated secondary antibody was used for signal detection.

**D and E:** The chemotactic effects of various concentrations of PDGF-AA (blue bar), -BB (red bar), and VEGF-C (black bar) on mouse (**D**) and human (**E**) LECs were analyzed using a modified Boyden chamber assay. Migrating cells in the presence or absence of STI571 were counted from 12 wells of each sample.

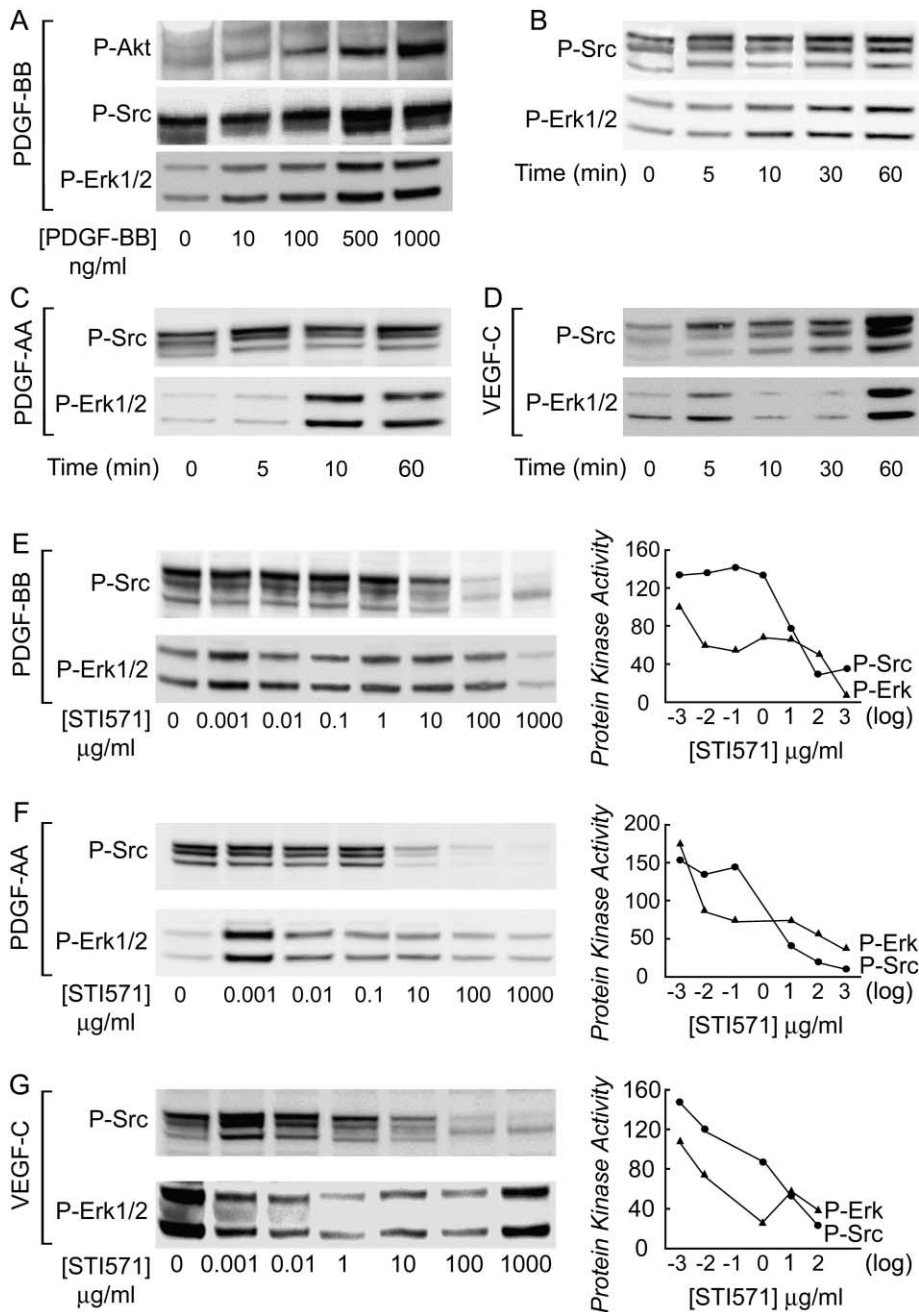
**F and G:** The number (**F**) and maximal distance (**G**) of migrating rat LECs in the absence and presence of various concentrations of PDGF-BB. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

**H and I:** Real-time PCR was used to quantify the expression levels of prox-1 mRNA in PDGF-BB-, -AA, or VEGF-C-treated and nontreated mouse LECs at 12 and 24 hr posttreatment (**H**), and Ang-2 mRNA in PDGF-BB-treated and nontreated mouse melanoma cells (**I**).

tumor model. Because of the corneal avascularity and lack of lymphatics, tumor growth in the cornea excludes a primary involvement of any preexisting blood and lymphatic vessels. Implantation of tumor tissues into the corneal micropockets resulted in the growth of tumors expanding from the micropockets to the limbus (Supplemental Figures S4A and S4F). Corneal tumor neovascularization became directly visible by gross examination 2 weeks after implantation. These newly formed tumor vessels, sprouting from the limbal vessels, infiltrated the entire area of the tumor tissue. PDGF-BB-expressing tumors were more potent stimulators of angiogenesis in the cornea than wt tumors, and some of the PDGF-BB-induced vessels appeared as disorganized vascular plexuses (Supplemental Figure S4F). Indeed, immunohistochemical analysis revealed that PDGF-BB-expressing tumors contained a high vascular density with potentially tortuous and leaky features at the leading edges (Supplemental Figure S4G, arrows). In contrast, wt tumors lacked such a disorganized vascularity (Supplemental Figure S4B).

Like blood neovascularization, lymphatic vessels sprouting from the existing limbal lymphatics infiltrated the central region of PDGF-BB-expressing tumors as defined by LYVE-1 and GFP

positive signals (Supplemental Figures S4H–S4J). Notably, lymphatic vessels outside the borders of tumors were relatively well-organized with branches (dashes in B–E and G–J). In contrast, tumor lymphatic vessels appeared as disorganized vascular plexuses generally lacking defined lymphatic vessel structures (Supplemental Figure S4H). Similar to tumor blood vessels, these premature tumor lymphatic vessels seemed to be leaky and resulted from fusion of lymphatic capillaries into large lumens. Triple staining of tumor cells (GFP-positive) and blood and lymphatic vessels revealed that these premature tumor lymphatic vessels were distributed throughout the PDGF-BB-expressing tumors, with nonoverlapping patterns from blood vessels (Supplemental Figure S4J). In contrast to PDGF-BB-expressing tumors, the distribution of wt lymphatic vessels was only limited to the border of the implanted tumor, and no tumor lymphatics were detected (Supplemental Figures S4C and S4E). Most of these lymphatic vessels remained outside the tumor tissue and appeared as well-structured vessels. Quantification analysis showed that tumor-produced PDGF-BB significantly increased the number of blood and lymphatic vessels in this mouse tumor model (Supplemental Figure S4K and S4L). These



**Figure 5.** Activation of intracellular signaling pathways of primary LECs by PDGF-BB, PDGF-AA, and VEGF-C

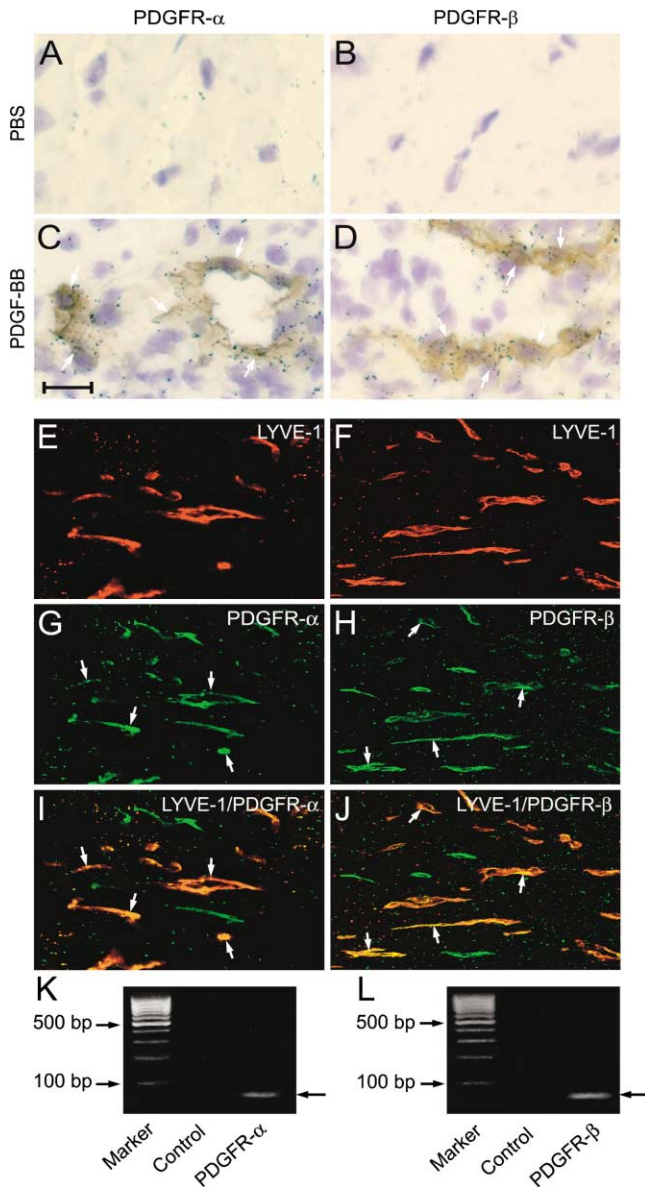
Rat LEC were starved in serum-free medium for 12 hr and incubated with various concentrations of PDGF-BB for 10 min (A), with 100 ng/ml of PDGF-BB at different time points (B), with 100 ng/ml of PDGF-AA at different time points (C), or with 500 ng/ml VEGF-C at different time points (D) as indicated. Rat LECs were incubated for 10 min with 100 ng/ml of PDGF-BB- (E) and -AA (F), or 500 ng/ml of VEGF-C (G), in the absence and presence of various concentrations of STI571 as indicated. Equal amounts of cell lysates were analyzed by SDS-PAGE/Western blotting and probed with specific antibodies against active forms of Src, Erk1/2 or Akt. The intensity of each band was quantified.

data demonstrate that PDGF-BB induces tumor growth of lymphatic vessels that consist of relatively malformed, premature, and probably leaky structures. Like blood vessels, such premature lymphatic vessels may contribute to lymphatic metastasis.

#### PDGF-BB promotes lymphatic metastasis

Stimulation of tumor lymphangiogenesis by PDGF-BB raised the possibility that this factor might promote lymphatic metastasis. We established a mouse lymphatic metastatic model in which subcutaneous T241 tumors were grown in the middle dorsum. After removal of primary tumors, autopsy analysis showed that a majority of the PDGF-BB tumor-bearing animals developed metastatic lesions of axillary lymph nodes ( $n = 8$ ,

Figures 8A–8D). H&E staining revealed the presence of invasive tumor cells in lymph nodes of PDGF-BB- and VEGF-C-tumor bearing mice. These lymphatic tumor lesions were approximately 20-fold larger than the lymph nodes of healthy animals or animals with wt or vector-transfected tumors as measured by weight and volume (Figures 8E and 8F). Remarkably, it appeared that PDGF-BB-induced lymphatic metastatic lesions were larger than VEGF-C-induced lymphatic metastases ( $p < 0.05$ , Figure 8E). In contrast, neither wt nor vector-transfected tumors resulted in visible metastases in regional lymph nodes (Figures 8A and 8D–8F). Histological examination confirmed that GFP-positive PDGF-BB or VEGF-C transfected tumor cells were present in axillary lymph nodes, whereas lymph nodes of control



**Figure 6.** Localization of PDGFRs on newly formed lymphatics

PBS-implanted corneas (A and B) or PDGF-BB-induced (C and D) lymphatic vessels were stained for LYVE-1 (brown color). The same sections were hybridized with oligonucleotide probes for mouse PDGFR- $\alpha$  and  $\beta$  (black dots). A–D are bright-field images. Arrowheads indicate LYVE-1 positive signals. Scale bar = 10  $\mu$ m. PDGF-BB-implanted corneal sections (day 14) were double stained for LYVE-1/PDGFR- $\alpha$  (E, G, and I) or LYVE-1/PDGFR- $\beta$  (F, H, and J). Arrows in I and J point to double positive signals. RT-PCR was performed to amplify PDGFR- $\alpha$  (K) and - $\beta$  (L) using cDNA prepared from mouse LECs. Arrows in K and L point to positive signals.

mice lacked detectable GFP-positive tumor cells (Figures 8A–8C). None of the PDGF-BB, VEGF-C, or control tumor-bearing mice had visible or microscopic pulmonary metastases as detected by histological examination (data not shown). In summary, these results demonstrate that PDGF-BB is a potent lymphangiogenic factor that promotes lymphatic metastasis in mice.

### Blockage of PDGF receptor activation inhibited PDGF-BB-induced lymphangiogenesis and tumor growth

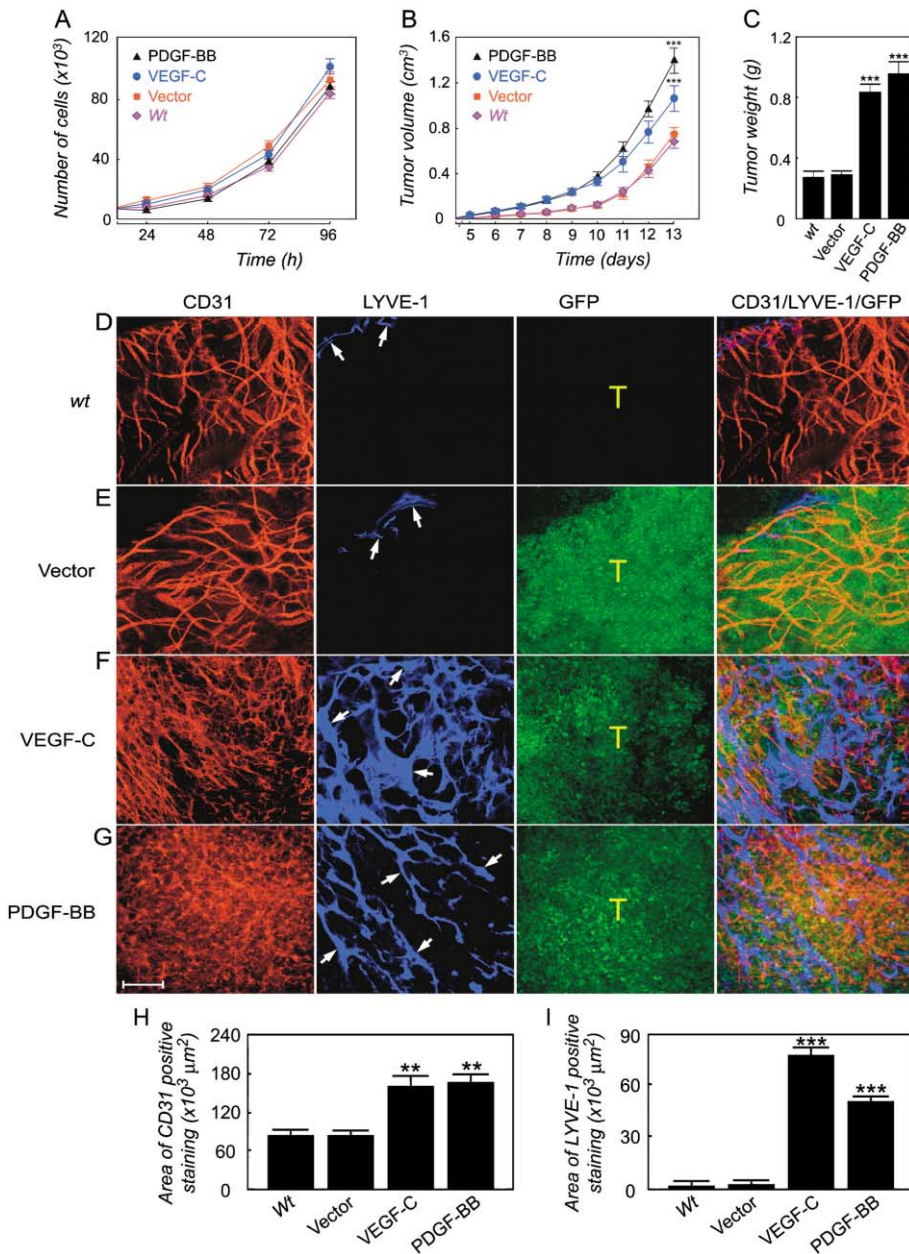
To determine if inhibitors of the PDGF receptors could affect PDGF-BB-induced tumoral blood and lymphatic vessel growth, STI571 (50 mg/kg body weight/day) was used to treat tumor-bearing mice. This dose was previously reported to sufficiently block PDGFR activation (Hwang et al., 2003; Uehara et al., 2003). Administration of STI571 significantly reduced tumor burden as measured by weight and volume after treatment (Supplemental Figures S5A and S5B). Interestingly, the number of tumor lymphatic vessels was lower in the STI571-treated tumors (Supplemental Figure S5C, S5D, and S5H) than in the PBS-treated tumors. Similarly, the number of blood vessels in the STI571-treated tumors was also significantly reduced compared to controls (Supplemental Figure S5G). In contrast, STI571 did not significantly block VEGF-C-induced tumor growth, blood angiogenesis, or lymphangiogenesis (Supplemental Figures S5A and S5E–S5H). These data suggest that activation of PDGF receptors is critical for PDGF-BB-induced lymphangiogenesis.

### Discussion

Spontaneous lymphatic metastases are the consequence of a complex metastatic process that includes: (1) dissemination of malignant cells from a primary tumor to the lymphatics; (2) transport of tumor cells via the lymphatics to local lymph nodes; (3) settlement of tumor cells in the lymph nodes; and (4) growth of metastatic tumors in the lymph nodes. Each of these steps is critical in facilitating clinical detection of lymphatic metastases in cancer patients. The question of whether tumor lymphatics are functional remains controversial (Padera et al., 2002; Skobe et al., 2001; Stacker et al., 2001). To enter the lymphatic system, tumor cells must remain in physical contact with lymphatic vessels. Although the question of how tumor cells enter the lymphatic vessels remains to be solved, tumor cells may gain access into the lymphatic system by inducing intratumoral lymphangiogenesis or by co-opting preexisting lymphatics in the surrounding tissue (Beasley et al., 2002; Williams et al., 2003).

A great deal of the recent work in lymphangiogenesis research has been focused on two members of the VEGF family, VEGF-C and -D, which interact with VEGFR-3. Both VEGF-C and -D have been found to act on isolated lymphatic endothelial cells in vitro and induce lymphangiogenesis in vivo (Jussila and Alitalo, 2002). Moreover, overexpression of VEGF-C or -D promotes lymphatic metastasis (Makinen et al., 2001; Skobe et al., 2001; Stacker et al., 2001). However, it seems unlikely that these are the sole factors regulating such processes. For example, the parallel process of hemangiogenesis involves not only VEGFs but also many other growth factors such as angiopoietins, FGFs, PDGF, etc. The instability of the tumor cell genome that may switch on many growth factors during malignant progression further suggests that multiple factors may be involved in controlling lymphangiogenesis in tumors (Folkman, 2002). Among cancer-produced growth factors, expression of members of the PDGF family has constantly been reported at high levels, particularly in breast cancers (Ariad et al., 1991). PDGF-AA and -BB do not require proteolytic processing for conversion into active forms (Heldin and Westermark, 1999). Previous work on elucidating the role of PDGFs in tumor growth has mainly focused on their direct impact on tumor cells (Uehara et al., 2003). Recent work performed in our lab, alongside other labs,





**Figure 7.** Stimulation of tumor growth, angiogenesis, and lymphangiogenesis

**A:** Growth rates of vector-, PDGF-BB-, VEGF-C-transduced or wt tumor cells in vitro. T241 tumor cells were seeded at a density of  $1 \times 10^4$  cells/well in 24-well plates, and triplicates of each cell line were counted at indicated time points ( $\pm$  SEM).

**B:** Tumor volumes were measured at indicated time points and the data are presented as mean determinants ( $\pm$  SEM).

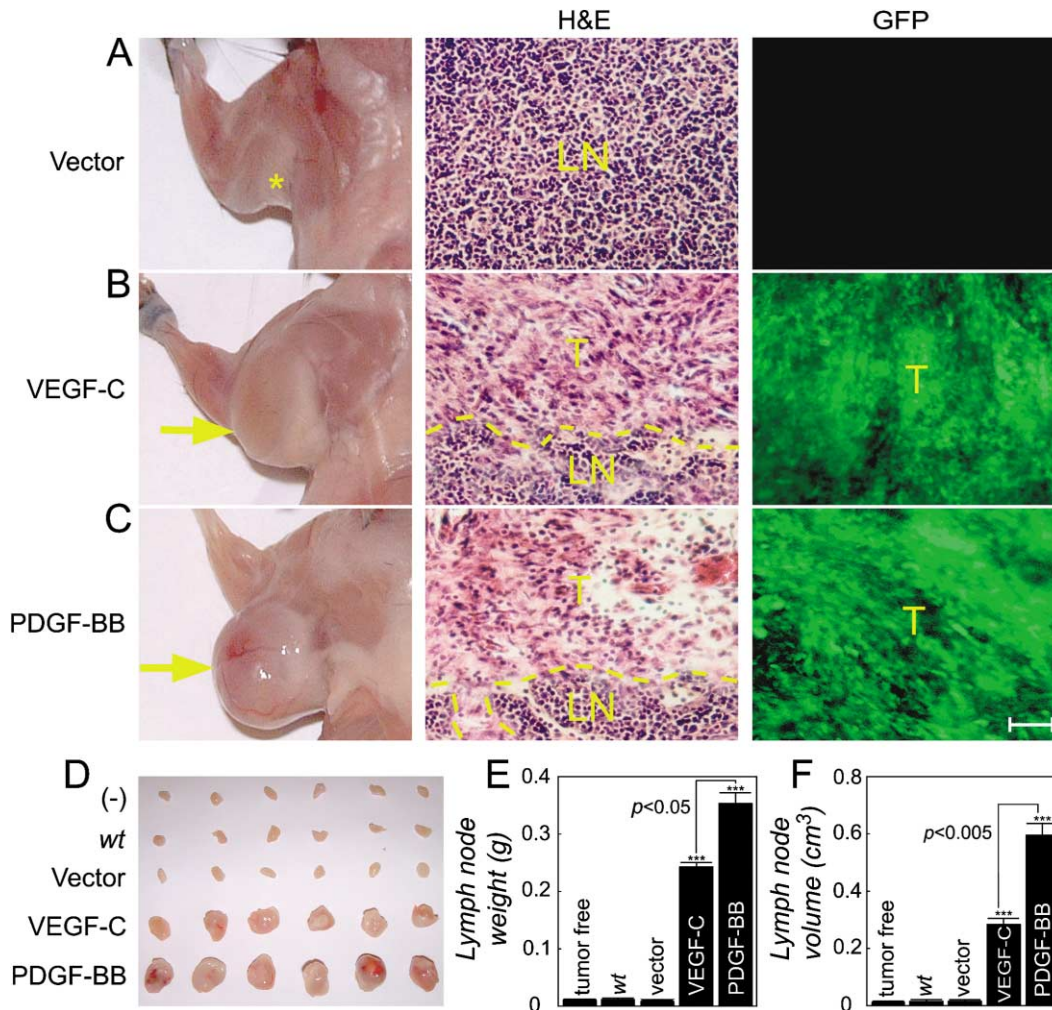
**C:** Tumors were weighed at day 13 after implantation and the data are presented as mean determinants ( $\pm$  SEM). \*\*\* $p < 0.001$ .

**D-G:** Tumor blood vessels and lymphatic vessels were double labeled for CD31 (red) and LYVE-1 (blue). Tumor cells in **E-G** are GFP-positive (green). Arrows in LYVE-1 panel point to newly formed lymphatic vessels. T = tumor. Scale bar = 100  $\mu$ m. Quantification of CD31 (**H**) and LYVE-1 (**I**) positive signals ( $n = 7$  different optical fields). \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

has demonstrated that PDGF-BB and other members of this family are important angiogenic factors (Cao et al., 2002, 2003). In this paper we show that members of the PDGF family display direct potent lymphangiogenic activity. This is based on the following evidence: (1) both mRNA and proteins of the PDGF receptors are localized on newly formed lymphatic vessels; (2) both PDGFR- $\alpha$  and - $\beta$  are detected in isolated primary lymphatic endothelial cells; (3) PDGF-BB stimulates motility of primary LEC in vitro; (4) in isolated LEC, PDGF-BB activates intracellular signaling pathways, such as the MAP kinases *Erk1/2* and *Akt*, in a way similar to that observed for VEGF-activated signaling in endothelial cells (Eriksson et al., 2003); and (5) VEGF-C/-D-VEGFR-3 neutralizing reagents do not block PDGF-BB-induced lymphangiogenesis in vitro and in vivo. We conclude that PDGF-BB is a direct lymphangiogenic factor. Our work demonstrates

that PDGF-BB is as potent as VEGF-C in inducing tumor lymphangiogenesis and in promoting lymphatic metastasis. Thus, lymphangiogenesis and lymphatic metastasis are regulated by multiple factors. Further, our work suggests that PDGF-BB may be a survival factor for newly formed lymphatics since PDGF-BB activates the *Akt* kinase, which promotes antiapoptotic signaling.

Deletion of PDGF-B or PDGFR- $\beta$  genes leads to homozygous lethality at birth. Among the cardiovascular abnormalities, microvascular hemorrhage and development of edema seem to be the major cause of lethality. Although lack of pericytes/vascular smooth muscle cells (PC/VSMC) in the developing vasculatures is the most obvious reason for this hemorrhagic phenotype and blood vessel leakage (Leveen et al., 1994; Soriano, 1994), tissue edema could also be, in part, due to inappropriate



**Figure 8.** Promotion of lymphatic metastasis

Implanted subcutaneous T241 tumors were removed and axillary lymph node metastases were visible approximately 3 weeks after removal of primary tumors. Arrows point to grossly visible lymphatic metastases, and asterisks mark the tumor-free axillary lymph nodes (A–C). Resected lymph nodes and lymphatic metastases were photographed (D), weighed (E), and the volumes were measured (F). Histological examination of axillary lymph nodes with H&E staining. LN = lymph node tissues. T = tumor tissues. Dashed lines mark the borders between tumor tissues and lymph node tissues in B and C. \*\*\* $p < 0.001$ . Scale bar = 25  $\mu$ m.

development of the lymphatic system. In the present study, we have shown that PDGF-BB is a potent lymphangiogenic factor in the adult animals. Our studies should be validated in the future by the use of genetic models including PDGFs and their receptor knockin and knockout mice. It is possible that this factor also plays a critical role in the establishment of the lymphatic vasculature during embryonic development. The tissue edema observed in the PDGF-B/PDGFR- $\beta$  knockout mice could also be due to a default drainage function of the lymphatic system. In physiological conditions, only initial lymphatics lack mural cells. It still remains unknown, however, if the lymphatic vessels in the PDGF-B/PDGFR- $\beta$  knockout mice lack PC/VSMC, which are required to maintain the physiological function of the lymphatic system. These interesting issues remain to be further studied using genetic PDGF-B or PDGFR- $\beta$  knockout models.

The homeobox gene Prox-1 is an essential early transcription factor for lymphatic development. Deletion of the Prox-1

gene in mice arrests lymphatic budding from veins, resulting in embryos lacking lymphatic vasculature (Wigle and Oliver, 1999). Thus, it is important to study whether Prox-1 regulates expression levels of PDGF-BB. Two recent independent studies using Affymetrix gene array analysis show that overexpression of Prox-1 cDNA in blood vascular and lymphatic endothelial cells does not seem to induce PDGF-BB expression (Hong et al., 2002; Petrova et al., 2002). Prox-1 induces differentiation of blood vascular endothelial cells toward the lymphatic lineage. The fact that PDGF-BB does not induce Prox-1 expression suggests that PDGF-BB-induced lymphatic endothelial cell differentiation and assembly of lymphatic vessels are mediated via a pathway separated from that activated by Prox-1. These findings further suggest that although PDGF/PDGFR and VEGF-C/VEGFR-3 have overlapping developmental functions on lymphatic system, the molecular mechanisms by which PDGF/PDGFR- and VEGF-C/-D/VEGFR-3 induce lymphangiogenesis may be regulated by different sets of genes. It is possible that

there is a tissue-specific overlapping role between the VEGF-C/VEGFR-3 and the PDGF/PDGFR signaling pathways in regulation of lymphangiogenesis. This issue should be addressed using tissue-specific VEGF-C/VEGFR-3 or PDGF/PDGFR knockin and knockout mice. The differential roles of VEGF-C/VEGFR-3 and PDGF/PDGFR signaling in the regulation of lymphangiogenesis should also be further studied.

The Prox-1 gene is involved in regulating early events of lymphatic development (Hong et al., 2002; Wigle and Oliver, 1999). Perhaps a Prox-1 independent control gene exists that regulates lymphatic vessel growth, which could be driven by the PDGF system at later events. It is also possible that the expression of Prox-1 in newly sprouting vessels is maintained after PDGF-BB treatment, even though the expression level is not increased. This basal expression level may already be sufficient for maintaining lymphatic differentiation. Further, proliferation of LEC may not necessarily lead to elevation of Prox-1 expression. These important issues need to be further explored using genetic models.

The mouse corneal tumor model is particularly useful to distinguish newly formed lymphatic vessels induced by tumors and the preexisting host vessels, as the cornea lacks both blood vessels and lymphatic vessels. Our data clearly demonstrate that all tumors, including controls and transfectants, were able to induce new lymphatics in this model, whereas only PDGF-BB tumors contained tumor lymphatic vessels. The infiltration of lymphatic vessels deeper into the tumor tissue may facilitate lymphatic metastasis by several mechanisms. First, interactions between lymphatic endothelial cells and tumor cells are bilaterally beneficial. While tumor cells produce PDGF-BB and VEGF-C that stimulate lymphatic endothelial cell growth and migration, lymphatic endothelial cells are able to produce tumor growth-promoting factors. This could potentially stimulate tumor cell growth. Second, the structure of tumor lymphatic vessels appeared to be disorganized, tortuous, and leaky as demonstrated in our confocal analysis of LYVE-1 positive vessels. These leaky tumoral lymphatics could provide a vulnerable structural basis for tumor cell invasion into the lymphatic system. Indeed, the high interstitial pressure in the tumor environment would further force tumor cells into the leaky lymphatics. Finally, lymphatic endothelial cells may become activated by tumor cells, and they may assist tumor cell transport into the lymphatics.

In summary, our results show that PDGF-BB is an important lymphangiogenic factor that contributes to lymphatic metastasis. PDGF-BB is a potent pleiotropic factor that promotes tumor growth and metastasis through the following three mechanisms: (1) direct stimulation of tumor cell growth, (2) stimulation of angiogenesis, and (3) stimulation of lymphangiogenesis and metastasis. Thus, development of antagonists for PDGF-BB and other members of the PDGF family may be an important approach for control of tumor growth and metastasis.

#### Experimental procedures

##### Animals

Female and male 6- to 7-week-old C57Bl/6 mice were acclimated and caged in groups of six or less. Animals were anaesthetized by an injection of a mixture of dromicum and hypnorm (1:1) before all procedures and sacrificed by a lethal dose of CO<sub>2</sub> followed by cervical dislocation. All animal studies were reviewed and approved by the animal care and use committee of the North Stockholm Animal Board.

##### Mouse corneal neovascularization assay

The mouse corneal angiogenesis assay was performed as previously described (Cao et al., 2003) (see Supplemental Experimental Procedures at <http://www.cancer.org/cgi/content/full/6/4/333/DC1/> for a detailed description).

##### Mouse corneal tumor model

Implantation of tumor tissues in the mouse cornea was carried out according to previously described procedures (Muthukkaruppan and Auerbach, 1979) (see Supplemental Experimental Procedures for a detailed description).

##### VEGFR3/PAE cell shape changes and motility assay

Analysis of VEGFR-3/PAE cell shape changes and motility was carried out as previously published (Cao et al., 1998) (see Supplemental Experimental Procedures for a detailed description).

##### Lymphatic endothelial cell motility assay

Murine LECs were isolated from skin by immunobead selection using an antibody targeting mouse LYVE-1. Human LECs were isolated as previously described (Nisato et al., 2004). Expression of podoplanin and LYVE-1 signals was detected by staining nonpermeabilized cells with a rabbit anti-human podoplanin antibody or a rabbit anti-mouse LYVE-1 antibody. The motility responses of human and murine LECs to PDGF-AA, -BB, or VEGF-C were assayed using a modified Boyden chamber technique previously described (Cao et al., 1998). The neutralizing effect of an anti-mouse VEGFR-3 antibody or an sVEGFR-3-Fc on PDGF-BB-induced cell migration of human LECs was analyzed (see Supplemental Experimental Procedures for a detailed description).

Rat LECs were isolated from the thoracic duct as previously described (Mizuno et al., 2003). LEC migration assay was performed according to previously published procedures (Cao et al., 1998). Briefly, confluent LEC cultures grown in 35 mm<sup>2</sup> petri dishes were scraped with a razor blade, resulting in a cell-free zone of 4 mm<sup>2</sup> per dish. The remaining cells were washed three times and incubated with serum-free endothelial cell basal medium-2 in the absence or presence of PDGF-BB (100, 500, or 1000 ng/ml). After 72 hr incubation, cells were fixed with methanol and stained with Giemsa. Two areas of maximal cellular migration/zone were determined. The number of cell nuclei and the maximal distance of migration from the starting line were measured (n = 8).

##### Real-time PCR and reverse transcriptase PCR (RT-PCR)

Two sets of nucleotide primers specific for mouse Ang2 or Prox1 and FAM-labeled probes and primers were used for real-time PCR analysis. As an internal loading control, 18S ribosomal RNA reagents were used. The following amplification cycle program (95°C for 10 min, followed by 50 cycles of 95°C for 15 s → 60°C for 1 min) was used to amplify Prox-1 signals from cDNA prepared from mouse LECs stimulated with or without PDGF-BB, -AA, or VEGF-C, and to amplify Ang-2 signals from cDNA prepared from murine melanoma B16 cells. Relative standard curves were prepared by serial dilutions of the positive control. Recalculation was performed as described in the manufacturer's manual for the system.

RT-PCR was performed to amplify PDGFR- $\alpha$  and - $\beta$  using cDNA prepared from mouse LECs. Primer sequences for PDGFR- $\alpha$ : forward; 5'-AACGGAACCTTCAGCGTGG-3' and reverse; 5'-GGTACGGTTGCTTGGAGCC-3'; and for PDGFR- $\beta$ : forward; 5'-GGACGCTGCGGGTGG-3' and reverse; 5'-AACTCGTGGTCTTGAACGTC-3'. The amplification program consisted of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for a total of 35 cycles.

##### Western blot analysis

Western blot analyses of lysates from primary rat LECs were performed according to previously published methods (Eriksson et al., 2003) (see Supplemental Experimental Procedures for a detailed description).

##### In situ hybridization

Detection of PDGFR- $\alpha$  and - $\beta$  in corneal tissues by in situ hybridization was performed according to recent published procedures using two independent oligoprobes complementary to PDGFR- $\alpha$  (nucleotides 423–470 and 3083–3130) and PDGFR- $\beta$  (946–996 and 2610–2657) (Cao et al., 2003). Briefly, slides were exposed on phosphorimager screen overnight to confirm the in situ hybridization. For immunohistochemistry, the same sections were

stained with a rabbit anti-mouse LYVE-1 antibody, and positive signals were developed with a streptavidin peroxidase kit (Vectastain ABC kit, Vector laboratories, Burlingame, CA, USA) combined with a Sigma-Fast-DAB peroxidase substrate (Sigma Aldrich, Steinheim, Germany). To visualize the in situ hybridization signal, slides were dehydrated, dipped into photo emulsion, and exposed for 6 weeks in the dark. Finally, sections were developed, counterstained with cresyl violet, and analyzed under light and dark field of a Zeiss Axiophot microscope.

#### Immunohistochemistry

Frozen corneal sections of 6  $\mu\text{m}$  thickness were double-stained for LYVE-1 and each of the PDGFRs according to standard immunohistochemical procedures. A mixture of a rabbit anti-mouse LYVE-1 antibody (Banerji et al., 1999) and a mouse monoclonal anti-human PDGFR- $\alpha$  or - $\beta$  antibody (BD Biosciences Pharmingen, San Diego, CA) was used. A mixture of a goat anti-rabbit Cy5 (Chemicon International, Temecula, CA) and a rat anti-mouse IgG-FITC antibody (Vector Laboratories, Inc., Burlingame, CA) was used for signal detection. Positive signals were photographed under a fluorescence microscope (20 $\times$ ). The lymph node tissues were fixed in 3% PFA, dehydrated, and embedded in paraffin. Thin sections (6  $\mu\text{m}$ ) were counterstained with hematoxylin/eosin. The sections were examined and photographed under a light microscope at 20 $\times$  magnification.

#### Retroviral vector design and tumor cell transduction

Cloning and transfection of human PDGF-BB and VEGF-C cDNA into murine T241-tumor cells were carried out according to previously published procedures (Eriksson et al., 2002) (see Supplemental Experimental Procedures for a detailed description).

#### Tumor cell proliferation assay

Nontransduced, vector-, hPDGF-BB-, and hVEGF-C-transduced T241 fibrosarcoma cells were seeded at a density of  $1 \times 10^4$  cells/well in 24-well plates in DME medium (10% FCS), and incubated at 37°C. Cells were trypsinized, resuspended in Isoton II solution (Beckman Coulter, Sweden), and counted in a Coulter Counter at various time points. Triplicates were used for each sample and all experiments were performed three times.

#### Tumor growth assay

Approximately  $1 \times 10^6$  tumor cells of wt, vector-, hPDGF-BB, or hVEGF-C-transduced tumor cells were implanted subcutaneously on the back of 6- to 7-week-old female C57Bl/6 mouse, and tumor volumes were measured as previously reported (Cao et al., 1999). In an additional tumor study, STI571 (Novartis Pharma AG, Basel, Switzerland) (50 mg/kg body weight/day) was injected i.p. into tumor-bearing mice. Tumor-bearing mice treated with PBS served as controls.

#### Lymphatic metastasis assay

Subcutaneous primary tumors implanted in the dorsal midline were grown to sizes close to, but smaller than, 1.5 cm<sup>3</sup>. Primary tumors were surgically removed in all groups. About 3 weeks after removal of primary tumors, visible metastases were present in axillary lymph nodes of hPDGF-BB and hVEGF-C tumor-bearing mice. Mice of all groups were sacrificed at the same time point and axillary lymph nodes were removed for histological examination.

#### Whole-mount staining and confocal analysis

Growth factor- and tumor-implanted mouse eyes, and primary tumors were double stained for CD31 and LYVE-1 using a whole-mount staining protocol (see Supplemental Experimental Procedures for a detailed description).

#### Statistical analyses

Statistical analysis of the in vitro and in vivo results was made by a standard two-tailed Student's *t* test using Microsoft Excel 5, and by one-way analysis of variance (ANOVA) followed by Tukey-Kramer and Bonferroni/Dunn post-hoc tests (Newman-Keuls) using Stat-View. *p* values below 0.05 (\*) and <0.001 (\*\*\*) were deemed as significant and highly significant, respectively.

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