Expression of platelet-derived growth factor receptor β is maintained by Prox1 in lymphatic endothelial cells and is required for tumor lymphangiogenesis

Hideki Miyazaki,[1,2,5] Yasuhiro Yoshimatsu,[1,5] Yuichi Akatsu,[1] Koichi Mishima,[1] Masashi Fukayama,[2] Tetsuro Watabe[1,3,4] and Kohei Miyazono[1]

[1]Departments of Molecular Pathology; [2]Human Pathology, Graduate School of Medicine, The University of Tokyo, Tokyo; [3]Laboratory of Oncology, School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo; [4]PRESTO, Japan Science Technology Agency, Kawaguchi, Japan

correspondence:

Tetsuro Watabe, Laboratory of Oncology, School of Life Sciences, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji-city, 192-0392 Tokyo, Japan.
Tel: +81-426-76-5246; Fax: +81-426-76-5246;
E-mail: t-watabe@umin.ac.jp

[5]These authors contributed equally to this work.

The lymphatic system plays important roles not only in the physiological processes, such as maintenance of tissue fluid homeostasis, but also in pathological processes including the lymph node metastasis of tumor cells. Therefore, understanding of the molecular mechanisms underlying lymphatic vessel formation is crucial. Previous studies have shown that proliferation and migration of lymphatic endothelial cells (LECs) are activated by multiple types of signals mediated by tyrosine kinase receptors such as vascular endothelial growth factor receptor (VEGFR) 3. Although signals mediated by platelet-derived growth factor receptor β (PDGFRβ) have been implicated in lymphangiogenesis, the mechanisms explaining how PDGFRβ expression is maintained in LECs remain to be fully elucidated. In the present study, we show that PDGFRβ expression in LECs is maintained by Prox1 transcription factor. Knockdown of Prox1 expression in human dermal LECs decreased the expression of PDGFRβ, leading to the lowered migration of human dermal LECs towards PDGF-BB. Furthermore, we found that PDGF signals play important roles in inflammatory lymphangiogenesis in a chronic aseptic peritonitis model. Intraperitoneal administration of imatinib, a potent inhibitor of PDGFRβ, and transduction of PDGFRβ/Fc chimeric protein by an adenoviral system both reduced inflammatory lymphangiogenesis induced by thioglycollate in mice. We also found that the expression of PDGFRβ/Fc reduced tumor lymphangiogenesis in a BxPC3 human pancreatic cancer xenograft model. These findings suggest that PDGFRβ is one of the key mediators of lymphatic vessel formation acting downstream of Prox1.

Cancer mortality is mainly caused by metastasis. Lymphatic metastases are commonly found in many types of solid tumors. Multiple lines of evidence have suggested that the newly formed lymphatic vessels provide a major pathway for tumor metastasis, and regional lymph node metastasis is correlated with cancer progression.[1] Therefore, understanding of the molecular mechanisms underlying lymphangiogenesis is clinically critical for the development of novel therapeutic strategies to cure cancer.

Previous studies have revealed various signaling components that play important roles in the formation of lymphatic vessels. Vascular endothelial growth factor receptor 3 (VEGFR3) serves as a lymphatic-specific receptor for VEGFs, VEGF-C, and VEGF-D, and plays central roles in the embryonic and postnatal formation of lymphatic vessels.[2,3] Vascular endothelial growth factor-C induces the phosphorylation of VEGFR3, leading to the activation of intracellular signals, which results in the proliferation and migration of lymphatic endothelial cells (LECs).[4,5] In addition, neolymphangiogenesis is also induced by multiple types of receptor tyrosine kinases, VEGFR2, insulin-like growth factor receptor, hepatocyte growth factor receptor (also known as c-Met), fibroblast growth factor receptor 3 (FGFR3), and Tie2, which serve as receptors for VEGF-A, insulin-like growth factors, hepatocyte growth factor, FGF-2, and angiopoietin-1, respectively.[6–10]

In addition to signaling molecules, certain transcription factors also play essential roles in the formation and maintenance of lymphatic vessels. During embryogenesis, Prox1, a homeobox-containing transcription factor, is expressed in a subset of blood vascular endothelial cells (BECs) of the cardinal vein.[11,12] The Prox1-expressing BECs differentiate into LECs and sprout to form primary lymph sacs.[13] In *Prox1*-null mice, the migration of embryonic LECs is perturbed, resulting in the absence of the lymphatic vasculature. Prox1 has been shown to upregulate the expression of LEC markers in BECs.[14,15] Notably, Prox1 induces the expression of VEGFR3 and FGFR3 in BECs. Furthermore, decrease in Prox1 expression in *in vitro* cultured LECs results in the decreased expression of VEGFR3.[15] In accordance with these *in vitro* data, conditional deletion of the *Prox1* gene in postnatal LECs results in the loss of LECs. These findings suggest that Prox1 regulates the program of differentiation of embryonic BECs to LECs and maintenance of LECs by regulating the profiles of expression of specific markers of BECs and LECs.

Many types of tumors express members of platelet-derived growth factor (PDGF). The PDGF family regulates a diverse array of cellular processes including cell proliferation and migration.[16] Platelet-derived growth factor exists in the form of a homodimer or heterodimer of PDGF-A and -B chains (PDGF-AA, PDGF-BB, and PDGF-AB), and two forms of homodimers, PDGF-CC and PDGF-DD. Their biological activities are mediated by three forms of receptor tyrosine kinases encoded by two gene products, PDGF receptor (PDGFR) α and PDGFRβ. Signaling pathways mediated by PDGF family members play important roles in many biological processes. Platelet-derived growth factor-B and PDGFRβ knockout mice show hemorrhagic and tissue edema phenotypes in early embryos due to defective development and recruitment of mural cells (pericytes and smooth muscle cells) to blood vessels.[16] In addition to angiogenesis, Cao and colleagues reported that PDGF-B/PDGFRβ signals play important roles in lymphangiogenesis.[17] Platelet-derived growth factors directly induce cell migration of LECs isolated from different species. Isolated LECs express both PDGFRα and PDGFRβ. Administration of PDGF-BB increased *in vivo* growth of lymphatic vessels in a mouse corneal model and in a murine fibrosarcoma xenograft model, leading to increased lymphatic metastasis. Furthermore, they showed that inhibition of PDGFRβ signals using STI571 (imatinib) inhibited tumor lymphangiogenesis. Although these results suggest that the PDGFRβ expressed in LECs plays important roles in lymphangiogenesis, the mechanisms through which the PDGFR expression is maintained in LECs have not been elucidated.

In the present study, we show that Prox1 maintains the expression of PDGFRβ in LECs. We also showed that blockade of PDGFRβ signals using PDGFRβ/Fc chimeric receptor, which is a more specific inhibitor of PDGFRβ signals than imtinib, suppressed lymphangiogenesis in mouse models of chronic aseptic peritonitis and human pancreatic cancer xenograft. These findings suggest that Prox1-induced PDGFRβ signals play important roles in various types of lymphangiogenesis.

Materials and Methods

Cell culture. Human umbilical vein endothelial cells, human dermal lymphatic endothelial cells (HDLECs), and pulmonary arterial smooth muscle cells were obtained from Lonza (Basel, Switzerland), and cultured as described.[18] PDGF-BB and Imatinib mesylate were obtained from R&D (Minneapolis, MN, USA) and Novartis Pharma (Basel, Switzerland), respectively.

RNA interference and oligonucleotides. Small interfering RNAs were introduced into cells as described previously.[18] The target sequence for human Prox1 siRNA was 5′-AATTCTCCGAACGTGTCACGT-3′. Control siRNAs were obtained from Qiagen.

RNA isolation and RT-PCR. Total RNA was prepared with RNeasy Reagent (Qiagen Hilden, Germany) according to the manufacturer's instructions and reverse-transcribed by random priming and a Superscript first strand synthesis kit (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR analysis was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences are available online as indicated in Table S1.

Capillary-like tube formation assay. Matrigel Matrix (500 μL; BD Biosciences, San Jose, CA, USA) was added to each well of a 6-well plate and allowed to polymerize. A total of

7×10^5 HDLECs were seeded per well and incubated for 24 h, and were subjected to quantitative RT-PCR analyses.

Migration assay. Chemotaxis was determined as described previously.[15] Briefly, a total of 5×10^4 cells were seeded onto the upper chamber of a Cell Culture Insert (8-μm pore size; BD Biosciences), containing 100 ng/mL PDGF-BB (chemoattractant) in the lower chamber. Migrated cells at the bottom of the membrane were fixed and stained with crystal violet 0.2% /methanol 20% for counting. Assays were carried out in triplicate at least three times.

Model of chronic aseptic peritonitis. The model of chronic aseptic peritonitis was described previously.[19,20] Briefly, we i.p. administered 2 mL of 3% thioglycollate medium (BBL thioglycollate medium; BD Biosciences) into BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) every 2 days for 2 weeks to induce peritonitis. Imatinib was also given i.p. every day during the same period. The mice were then killed, and their diaphragms were excised and prepared for immunostaining as described below. Results were statistically examined using the two-sided Student's *t*-test. Differences were considered significant at $P < 0.05$. All experimental protocols were carried out in accordance with the policies of the Animal Ethics Committee of the University of Tokyo (Tokyo, Japan).

Lentivirus production and infection. A lentiviral expression system was used to establish the BxPC3 cells expressing PDGFRβ/Fc chimeric receptor as previously described.[21] Briefly, PDGFRβ/Fc cDNA was subcloned into the pENTR201 vector, and subsequently transferred into the pCS-EF-RfA lentiviral expression vector (Invitrogen). For lentiviral infection, 1×10^5 BxPC3 cells were infected with lentivirus vectors in suspension and plated in 6-well culture plates.

BALB/c nude mouse model of human pancreatic cancer. BALB/c nude male mice aged 5–6 weeks were obtained from Clea Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan). A total of 5×10^6 BxPC3 tumor cells in 100 μL PBS ($n = 4$ mice per group) were injected s.c. into the left flank of each mouse and allowed to grow for 7 weeks, when the major axis of tumors was approximately 10 mm long. Determination of the pericyte coverage of blood vessels was carried out by counting the numbers of CD31-positive vessels with and without associated NG2-positive cells for each tumor ($n = 3$).

Immunocytochemistry, immunohistochemistry and immunoblot analysis. Monoclonal antibodies to mouse PECAM1 (Mec13.3), α-tubulin, and total Erk were obtained from BD Pharmingen (San Diego, CA, USA), Sigma (St. Louis, MO, USA), and Upstate Biotechnology (Merck-Millipore, Darmstadt, Germany), respectively. Manufacturers of polyclonal antibodies were as follows: mouse LYVE-1, Abcam (Cambridge, United Kingdom); phospho-Erk, Cell Signaling Technology (Danvers, MA, USA); human PDGFRβ, Santa Cruz Biotechnology (Dallas, TX, USA); mouse and human Prox1, R&D Systems; human podoplanin, eBioscience (San Diego, CA, USA); mouse NG2, Chemicon (Merck-Millipore). Western blotting, immunocytochemistry, and immunohistochemistry were carried out as previously described.[22] Briefly, for immunoblotting, after transfer, PDVF membranes blocked with 5% skim milk or 5% BSA were subject to immunoblot with antibodies. For immunocytochemistry, cells fixed with methanol for a few minutes were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) including 10% FBS and were subjected to treatment with antibodies. For immunohistochemistry, frozen tissues were sectioned in a cryostat, fixed with 4% paraformaldehyde, and then blocked with Blocking One and treated with

antibodies. Nuclear counterstaining was carried out using TOTO-3 or DAPI (Invitrogen).

Results

Platelet-derived growth factor-BB activates intracellular signals in HDLECs. We first attempted to examine whether PDGF-BB activates intracellular signals in HDLECs. Platelet-derived growth factor-BB triggers the intracellular signaling pathways including activation of MAPK, which leads to the phosphorylation of Erk1/2. As shown in Figure S1, treatment of HDLECs with PDGF-BB resulted in phosphorylation of Erk in a dose-dependent manner. Phosphorylation of Erk was observed at 2 min after PDGF-BB treatment and lasted for 30 min (Fig. 1). These results suggest that HDLECs are capable of transducing PDGF signals.

Human dermal LECs express PDGFRβ. Platelet-derived growth factor-BB exerts its biological functions through activation of tyrosine kinase receptor complexes consisting of PDGFRα and β. In order to examine whether HDLECs express these PDGFRs, we attempted to detect their expression by semiquantitative RT-PCR analyses. As shown in Figure 2(a), transcripts for PDGFRβ as well as other LEC markers including Prox1, VEGFR3, and podoplanin were detected in HDLECs. Cao and colleagues described the expression of PDGFRα in mouse LECs,[17] however, we were not able to detect its transcripts, although its expression was detected in pulmonary arterial smooth muscle cells.

Expression of PDGFRβ in HDLECs was also examined at the protein level by immunostaining with an anti-PDGFRβ antibody. As shown in Figures 2(b) and S2, PDGFRβ protein was detected on the surface of HDLECs expressing Prox1 and podoplanin, both of which are markers for LEC. These findings suggest that PDGF signals are transduced by way of PDGFRβ in *in vitro* cultured HDLECs.

Expression of PDGFRβ and Prox1 increases during tube formation of HDLECs. During postnatal lymphangiogenesis, new lymphatic vessels are formed from existing vessels by multiple steps of activation of LECs, including proliferation, migration towards lymphangiogenic stimuli, and re-organization to form the necessary 3-D vessel structure. One of the most widely used *in vitro* assays to model the reorganization stage of lymphangiogenesis is the tube formation assay, in which the ability of LECs, plated with the appropriate ECM support, to form capillary-like structures is measured. When HDLECs were plated on Matrigel, they form capillary-like structures. To our interest, the expression of PDGFRβ increased when HDLECs were plated on Matrigel (Fig. 3a). We attempted to study the molecular mechanisms underpinning how PDGFRβ expression is regulated in HDLECs. Multiple lines of evidence have sug-
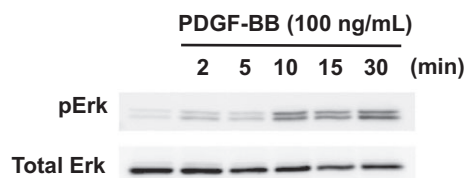

Fig. 2. Expression of platelet-derived growth factor receptors (PDGFR) in human dermal lymphatic endothelial cells (HDLECs). (a) RNA samples from HUVECs, HDLECs, and pulmonary arterial smooth muscle cells (PASMCs) were analyzed by RT-PCR for expression of various endothelial markers, PDGFRs, and the housekeeping genes (β-actin and GAPDH) to normalize the amount of total cDNA in each sample. As controls, a PCR reaction for each set of primers was run against H_2O. (b) Immunofluorescence staining was carried out on HDLECs using antibodies for PDGFRβ (green), Prox1 (blue), and podoplanin (red). Signals generated by anti-PDGFRβ antibody was not detected in the absence of primary antibody. VEGFR, vascular endothelial growth factor receptor. Scale bar: 50 μm.

gested that Prox1, a homeobox-containing transcription factor, is expressed in LECs and is required for the expression of LEC markers including VEGFR3.[15] When HDLECs were cultured on Matrigel, the Prox1 expression was also increased (Fig. 3b). In order to examine whether the ECM in Matrigel regulates Prox1 expression in HDLECs, we 2-D cultured HDLECs on fibronectin, collagen I, or different concentrations of Matrigel, followed by quantitative RT-PCR analysis for Prox1 expression. As shown in Figure S3(a), Prox1 expression was not altered when HDLECs were cultured on fibronectin or collagen I, but was increased when cultured on Matrigel in a dose-dependent manner. Furthermore, this effect of Matrigel on Prox1 expression was not dependent on the growth factors in Matrigel, as the induction of Prox1 expression was observed when Growth Factor Reduced Matrigel was used (Fig. S3b). These results suggest that Prox1 expression in the HDLECs is induced by 3-D culture on Matrigel, which results in the enhancement of PDGFRβ expression.

Fig. 1. Activation of intracellular signals in human dermal lymphatic endothelial cells by platelet-derived growth factor (PDGF)-BB. Cells were starved in serum-free medium for 6 h and incubated with 100 ng/mL PDGF-BB at different time points. Equal amounts of cell lysates were analyzed by Western blotting using specific antibodies against phosphorylated (top panel) and total (bottom panel) Erk1/2.
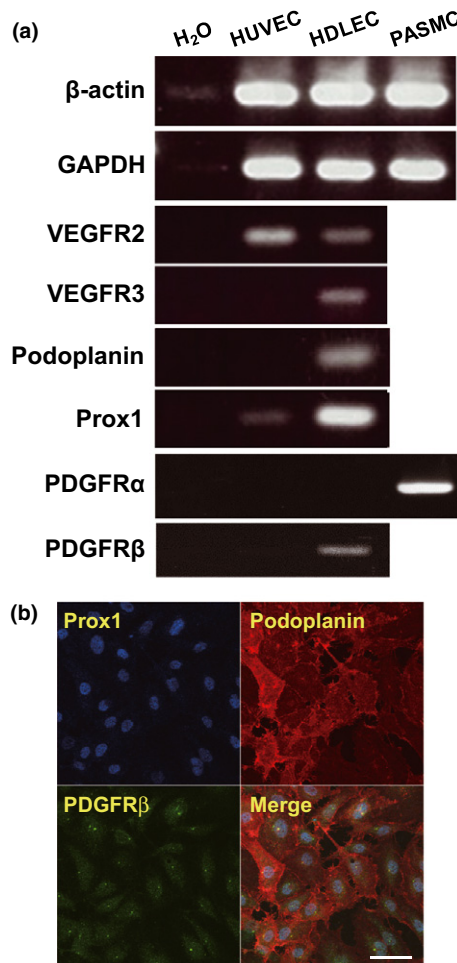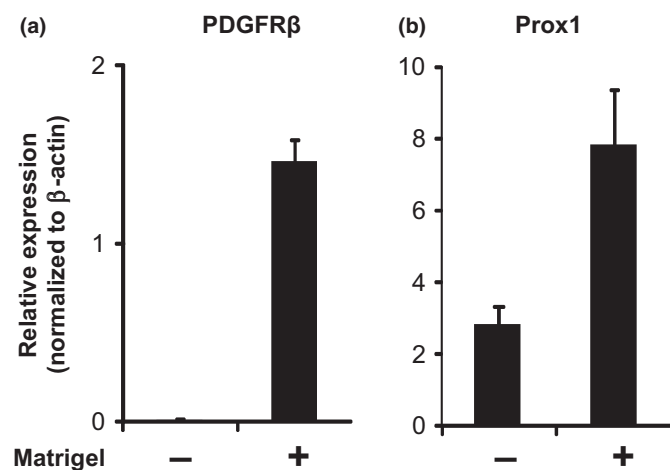
Fig. 3. Expression of platelet-derived growth factor receptor (PDGFR) β and Prox1 during tube formation of human dermal lymphatic endothelial cells. Cells were cultured in without (−) or with Matrigel (+), which led to tube formation, and were subjected to quantitative RT-PCR analyses for the expression of PDGFRβ (a) and Prox1 (b). Each value represents the mean of triplicate determinations; bars, SD.

Prox1 is required for maintenance of PDFGRβ expression in HDLECs and their migration towards PDGF-BB. Next, in order to examine whether Prox1 is involved in the PDGFRβ expression in HDLECs, we studied the effects of knockdown of Prox1 expression in HDLECs on PDGFRβ expression and their characteristics. When the levels of endogenous Prox1 expression were decreased by siRNA (Fig. 4a), the expression levels of PDGFRβ as well as those of VEGFR3 and podoplanin were decreased (Fig. 4b, unpublished data), which was confirmed at a protein level (Fig. 4c). These results suggest that Prox1 maintains the expression of PDGFRβ in HDLECs.

Mature LECs have been reported to migrate towards VEGF-C and PDGF-BB.[2,17] In order to study the effects of Prox1 knockdown on the behavior of HDLECs, we examined their chemotaxis towards PDGF-BB. Human dermal LECs migrated towards PDGF-BB; Prox1 knockdown decreased the migration of HDLECs towards PDGF-BB (Fig. 4d). Furthermore, HDLECs failed to form capillary-like structures in 3-D culture on Matrigel when endogenous Prox1 expression was knocked down (Fig. S4). These results suggest that Prox1 maintains the properties of LECs by sustaining the expression of LEC marker proteins.

Inhibition of endogenous PDGF signals by imatinib suppresses inflammatory lymphangiogenesis. Although Cao and colleagues reported that inhibition of endogenous PDGF signals by imatinib suppressed tumor lymphangiogenesis,[17] the roles of PDGF signals in inflammatory lymphangiogenesis have not yet been elucidated. To examine whether endogenous PDGFRβ signals are required for lymphangiogenesis *in vivo*, we used a mouse model of chronic inflammatory lymphangiogenesis.[19,20,23] In this model, thioglycollate medium was given i.p. three times a week as an inflammation-inducing agent to evoke chronic aseptic peritonitis in immunocompetent BALB/c mice. To investigate the function of PDGFRβ, imatinib, a potent inhibitor of PDGFRβ, was given daily for 2 weeks. By day 16, inflammatory plaques consisting mainly of macrophages had formed on the peritoneal surface of the diaphragm. Diaphragms from killed mice were subjected to immunostaining for LYVE-1, a marker for LECs. Although LYVE-1 is also expressed in macrophages, we found that most LYVE-1-positive cells in the diaphragms were LECs as they also expressed
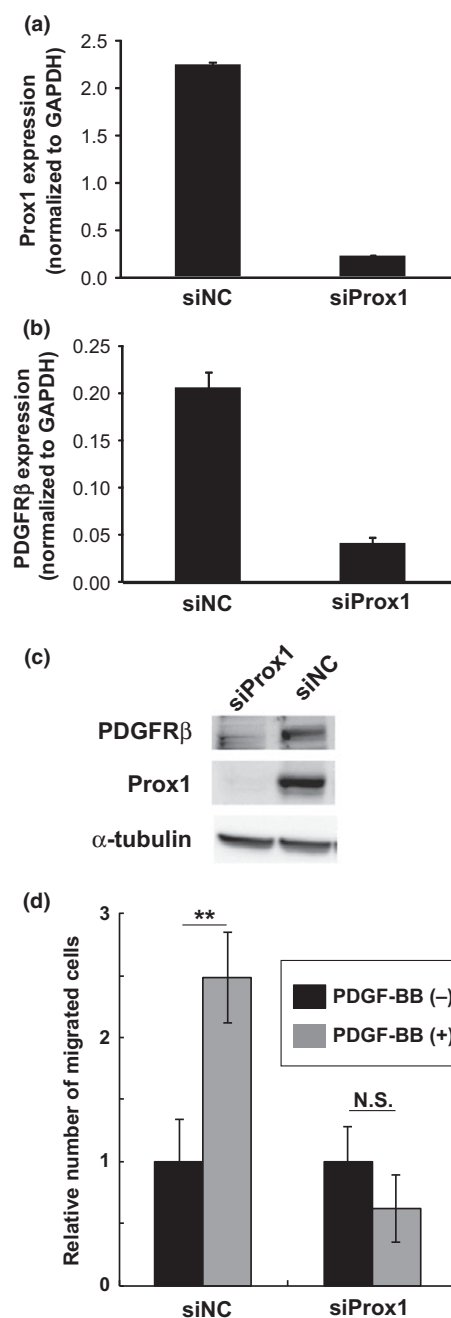

Fig. 4. Roles of endogenous Prox1 expression in human dermal lymphatic endothelial cells (HDLECs) in the maintenance of platelet-derived growth factor receptor (PDGFR) β expression and their migration to PDGF-BB. (a, b) HDLECs were transfected with negative control siRNA (siNC) or specific siRNA for Prox1 (siProx1), and were subjected to quantitative RT-PCR analyses for the expression of Prox1 (a) and PDGFRβ (b) and Western blot analysis (c) for PDGFRb (top) and Prox1 (middle) and α-tubulin (bottom). (d) Effects of Prox1 knockdown on the chemotaxis of HDLECs towards PDGF-BB. Cell migration was measured by Boyden chamber. HDLECs were transfected with scrambled siRNAs (siNC) or Prox1 siRNAs (siProx1) and plated on the upper chambers, with PDGF-BB (100 ng/mL) placed in the lower chambers. Results were expressed as the ratio of number of migrated cells normalized to control (no PDGF-BB). Each value represents the mean of triplicate determinations. Error bars represent SD. **$P < 0.01$. N.S., not significant (evaluated by Student's t-test).

Prox1 (Fig. S5). Compared to control diaphragms from mice injected with PBS (carrier alone), those from imatinib-treated mice showed decreased LYVE-1-positive areas on the
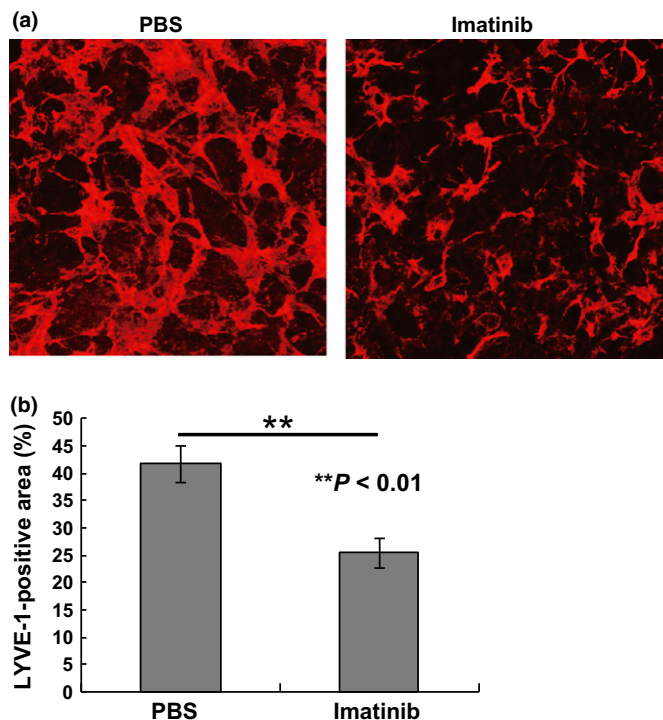
(a)

PBS   **Imatinib**

(b)

Fig. 5. Effects of imatinib on inflammatory lymphangiogenesis in the diaphragm. (a) Repeated i.p. injection of inflammation-inducing thioglycollate led to formation of lymphangiogenic plaques on the peritoneal side of the murine diaphragm. Imatinib or PBS (negative control) was also injected i.p. every day, and the diaphragms from mice killed after 16 days were subjected to whole-mount immunostaining with anti-LYVE-1 antibody. (b) Densities of LYVE-1-positive diaphragmatic lymphatic vessels were measured in each defined area, and values are presented as the ratio of LYVE-1-positive area to total area of the field. Error bars represent SE. **$P < 0.01$ (evaluated by Student's t-test).

diaphragm (Fig. 5a), as confirmed quantitatively (Fig. 5b). These findings imply that inhibition of tyrosine kinase activity of PDGFRβ suppresses inflammatory lymphangiogenesis *in vivo*.

Inhibition of inflammatory lymphangiogenesis by PDGFRβ/Fc decoy receptor. Imatinib is a selective inhibitor for PDGFRβ tyrosine kinase, but it also has multiple targets such as c-Kit and v-Abl at low doses and VEGFR3, -R2 and, -R1 at high doses.[24] Therefore, the inhibitory effects of imatinib on inflammatory lymphangiogenesis may have been caused in a PDGFRβ-independent fashion. In order to target PDGF signals more specifically, we generated adenoviruses encoding PDGFRβ/Fc, which consists of the extracellular domain of PDGFRβ and Fc moiety of human IgG (Fig. 6a). Addition of the conditioned medium containing PDGFRβ/Fc decreased the PDGF-BB-induced Erk phosphorylation in HDLECs (unpublished data). Adenoviruses containing PDGFRβ/Fc or control-Fc were injected into mice twice a week during the 16-day inflammatory assay period. Compared to control diaphragms from mice injected with control-Fc, those from PDGFRβ/Fc-injected mice showed decreased LYVE-1-positive areas on the diaphragm (Fig. 6b,c). Taken together with the assay using imatinib, these findings suggest that endogenous PDGF signals contribute to inflammatory lymphangiogenesis in the diaphragm.

Endogenous PDGF signals are involved in tumor lymphangiogenesis in a human pancreatic cancer xenograft model. We next tried to extend our finding that PDGFRβ/Fc inhibits lymphan-

(a) **PDGFRβ**
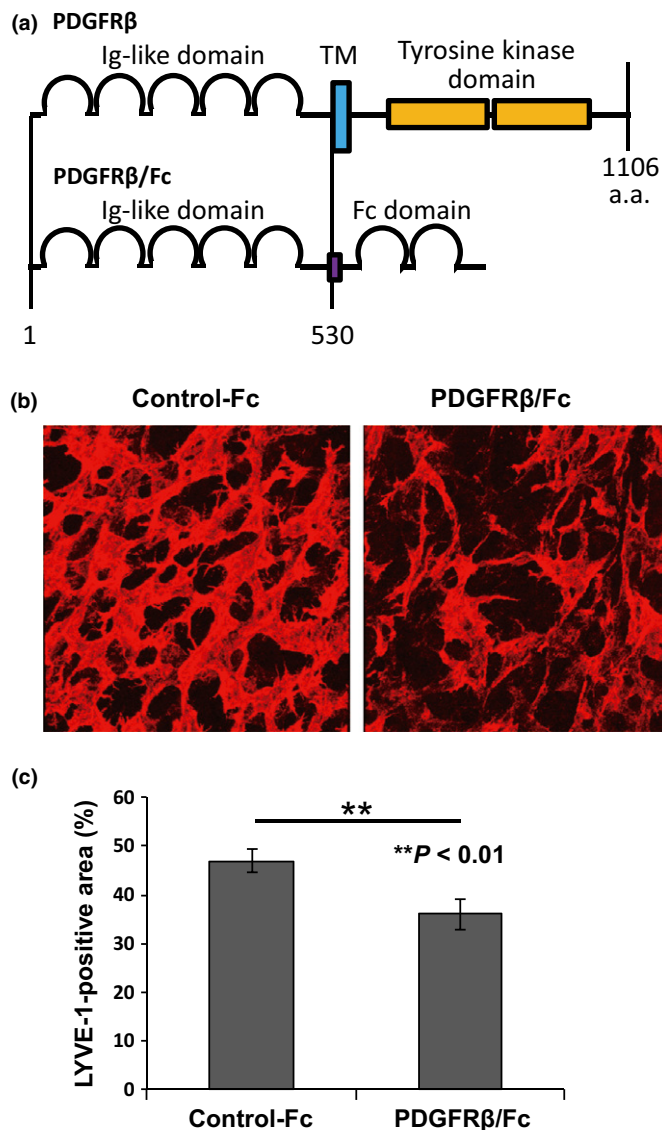

(b)

Control-Fc   **PDGFRβ/Fc**

(c)

Fig. 6. Effects of platelet-derived growth factor receptor (PDGFR) β/Fc decoy receptor on inflammatory lymphangiogenesis in the diaphragm. (a) Structure of PDGFRβ (top) and PDGFRβ/Fc chimeric receptor (bottom). a.a., amino acids; TM, transmembrane domain. (b) Repeated i.p. injection of inflammation-inducing thioglycollate led to formation of lymphangiogenic plaques on the peritoneal side of the murine diaphragm. Adenoviruses encoding control-Fc or PDGFRβ/Fc were also i.p. injected repeatedly, and the diaphragms from mice killed after 16 days were subjected to whole-mount immunostaining with anti-LYVE-1 antibody. (c) Densities of LYVE-1-positive diaphragmatic lymphatic vessels were measured in each defined area, and values are presented as the ratio of LYVE-1-positive area to total area of the field. Error bars represent SE. **$P < 0.01$ (evaluated by Student's t-test).

giogenesis in inflammation to another model of lymphangiogenesis. As our previous studies showed that human pancreatic adenocarcinoma BxPC3 cells formed xenograft tumors in immunodeficient mice that were relatively rich in lymphatic vessels as well as blood vessels,[25] we used this model to examine the effect of PDGF signals on tumor lymphangiogenesis.

We established BxPC3 cells that express and secrete control-Fc or PDGFRβ/Fc. When conditioned medium prepared from BxPC3-PDGFRβ/Fc cells was added to NIH/3T3 cells before they were treated with PDGF-BB, phosphorylation of

Erk was decreased compared with addition of medium from BxPC3-control Fc cells (unpublished data). These cells did not show any difference in cell proliferation (unpublished data), suggesting that inhibition of PDGF signals does not influence the proliferation of BxPC3 cells.

Both types of BxPC3 cells were s.c. grafted to immunocompromised BALB/c nude mice to obtain tumors. Tumor blood and lymphatic vasculatures were examined by staining for PECAM1 and LYVE-1, respectively. As shown in Figure 7, the formation of lymphatic vessels in BxPC3-driven tumors was inhibited by PDGFRβ/Fc more significantly than by control-Fc. In contrast, PDGFRβ/Fc did not affect the formation of blood vessels. However, in agreement with previous reports,[16] we found that the coverage of blood vessels with pericytes was disturbed by PDGFRβ/Fc (Fig. S6). Taken together, these findings suggest that PDGF signals contribute to tumor lymphangiogenesis to a considerable extent, and function specifically to lymphatic vessels in this tumor model.

Discussion

Recent studies have revealed that lymphangiogenesis is regulated by signaling cascades mediated by various tyrosine kinase receptors.[1,4] The present study showed that expression of PDGFRβ tyrosine kinase in LECs plays important roles in various types of lymphangiogenesis in inflammation and tumor.

Cao and colleagues previously reported that rat LECs express both PDGFRα and PDGFRβ.[17] The difference between their finding and the present finding regarding the expression of PDGFRs (Fig. 2a) may be derived from the heterogeneity of LECs, which is observed among various types of endothelial cells.[26] Although phosphorylation of PDGFRβ by PDGF-BB in HDLECs was not shown in the present study, phosphorylation of Erk by PDGF-BB (Fig. 1) is likely to be mediated by PDGFRβ as PDGFRα is not expressed in HDLECs. As the physiological ligand for PDGFRβ homodimers is PDGF-BB,[16] the present study focused on the importance of PDGF-BB during lymphangiogenesis. The PDGF-B chain is expressed in multiple types of tumors.[16] The autocrine loops of PDGF-BB/PDGFRβ signals have been implicated in the growth and metastasis of cancer cells, but the roles of PDGF-BB in the lymphatic vessel formation in various types of tumors need to be clarified in the future.

We, for the first time, found that PDGFRβ expression in LECs is maintained by Prox1 transcription factor (Fig. 4). We previously reported that Prox1 is required for the expression of VEGFR3 and other LEC markers in HDLECs.[15] Furthermore, we found that Prox1 collaborates with Ets2 transcription factor to enhance VEGFR3 expression.[23] As Prox1 plays important roles in the expression of FGFR3 tyrosine kinase in LECs,[9] Prox1 appears to be a master regulator that maintains the expression of various pleiotropic tyrosine kinase receptors. Consistent with this notion, the decrease in Prox1 expression in HDLECs suppresses the proliferation of HDLECs.[18]

Prox1 has been implicated in the embryonic differentiation of venous BECs into LECs through the regulation of multiple signaling cascades that play important roles in lymphangiogenesis.[13,27] Although such differentiation is not observed in adults, suppression of Prox1 expression in cultured LECs and postnatal deletion of the *Prox1* gene in LECs result in the dedifferentiation of LECs to BECs.[18,28] The deletion of PDGFRβ results in embryonic lethality due to cardiovascular defects.[16] The roles of PDGFRβ signals in embryonic lym-
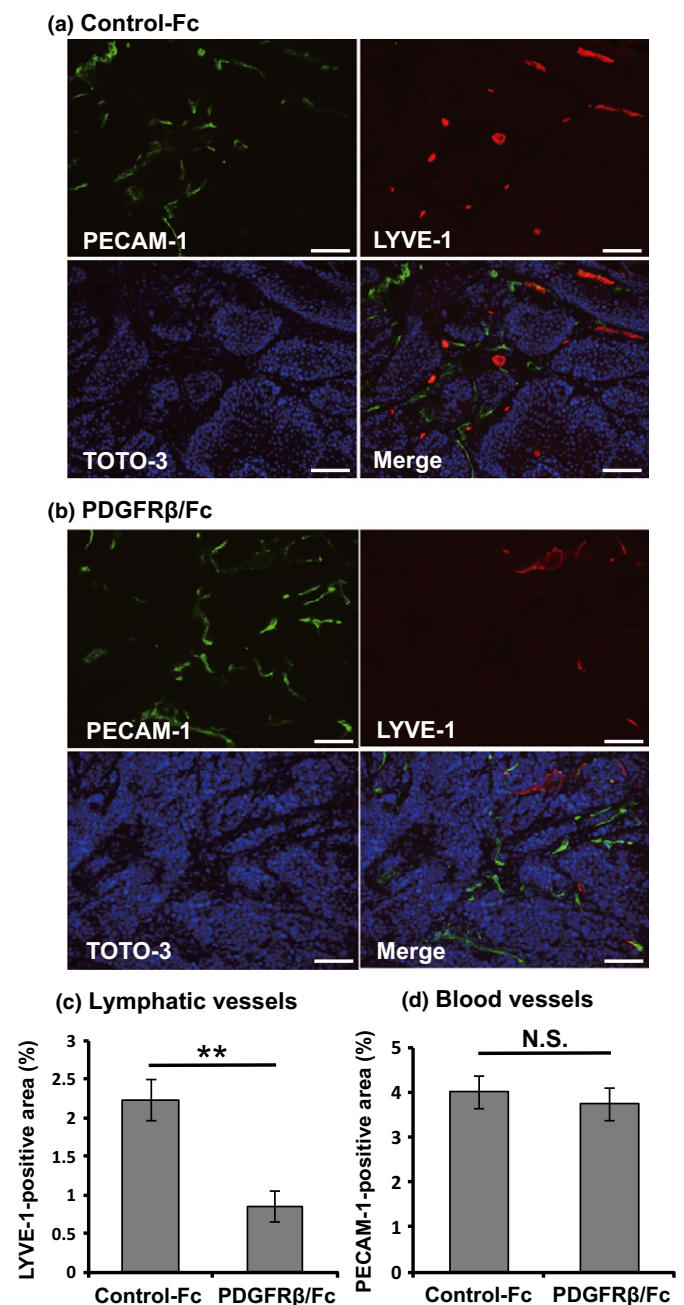

Fig. 7. Effects of platelet-derived growth factor receptor (PDGFR) β/Fc decoy receptor on tumor lymphangiogenesis in a mouse xenograft model of human pancreatic cancer. BxPC3 human pancreatic adenocarcinoma cells that were infected with lentiviruses encoding control-Fc or PDGFRβ/Fc were s.c. inoculated in BALB/c nude mice. After 7 weeks, tumors were excised and examined for vascular density. (a, b) Immunostaining for PECAM-1 (green) and LYVE-1 (red) of sections obtained from tumors of BxPC3 cells expressing control-Fc (a) and PDGFRβ/Fc (b). All samples were counterstained with TOTO-3 (blue) to visualize nuclei. Scale bar: 100 μm. (c, d) Quantitative analyses for the density of lymphatic vessels (percent LYVE-1-positive area) (c) and that of blood vessels (percent PECAM-1-positive area) (d). Each value represents the mean of four sections; error bars represent SE. **P < 0.01. N.S., not significant.

phangiogenesis and the PDGFRβ expression pattern in embryonic LECs need to be studied in the future.

During tumorigenesis and inflammation, multiple types of infiltrating inflammatory cells secrete lymphangiogenic factors including VEGF-A and VEGF-C, leading to the formation of

lymphatic vessels. Expression of PDGF is spatio-temporally regulated *in vivo*.[16] As PDGF-B is mainly expressed in endothelial cells and inflammatory cells, PDGF-BB is expected to be abundantly present in tumor and inflamed tissues. These reports, taken together with the present findings, suggest that manipulation of PDGF-BB/PDGFRβ signals is useful as a therapeutic strategy.

We succeeded in targeting PDGF-BB/PDGFRβ signals using imatinib and PDGFRβ/Fc decoy receptor and showed that endogenous PDGF-BB/PDGFRβ signals are required for lymphangiogenesis *in vivo* (Figs 5–7). As PDGFRβ/Fc theoretically interferes only with PDGF-BB/PDGFRβ signals, the specificity of this assay is supposed to be superior to imatinib. In tumor, PDGFRβ/Fc expression decreased the formation of lymphatic vessels but not blood vessels (Fig. 7). This result is consistent with the present finding that PDGFRβ is not expressed in BECs (Fig. 2).

The lymphatic vasculature plays important roles in the pathogenesis and progression of various conditions and diseases such as inflammation and cancer metastasis. The findings of the present study suggest the possibility that manipulation of PDGF-BB/PDGFRβ signals may be useful as a therapeutic strategy to inhibit tumor lymphangiogenesis and prevent metastasis.

Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Activation of intracellular signals in human dermal lymphatic endothelial cells by different doses of platelet-derived growth factor (PDGF)-BB.

Fig. S2. Confirmation of signals generated by anti-platelet-derived growth factor receptor (PDGFR) β antibody.

Fig. S3. Expression of Prox1 in human dermal lymphatic endothelial cells cultured on various types of ECM.

Fig. S4. Roles of endogenous Prox1 expression in human dermal lymphatic endothelial cells in the formation of cord-like structures.

Fig. S5. Expression of Prox1 in LYVE-1-positive cells in the diaphragm.

Fig. S6. Effects of platelet-derived growth factor receptor (PDGFR) β∕Fc decoy receptor on pericyte coverage of blood vessels in a mouse xenograft model of human pancreatic cancer.

Table S1. List of PCR primers used in this study.