and TβR-I inhibitor, respectively (Fig. 1F), suggesting the presence of autocrine loop of TGF-β1 signaling in HDLECs.

TGF-β induces expression of LEC markers in HDLECs

We next investigated whether TGF-β signaling regulates the expression of LEC markers in HDLECs. We recently reported that VEGFR3 signaling induces the expression of LYVE-1, but not other LEC markers, in embryonic stem cell (ESC)-derived endothelial cells.[30] In contrast, the homeobox transcription factor Prox1 induces expression of most LEC markers in ESC-derived endothelial cells.[28]

We examined the expression of Prox1 protein in HDLECs by immunocytochemistry and immunoblotting (Fig. 2A and B). Prox1 was observed in the nuclei of most of the untreated HDLECs, although expression levels of Prox1 in them varied with some cells displaying very weak or no significant staining by Prox1 antibody (Fig. 2A). When the cells were treated with TGF-β1 for 24 h, expression of Prox1 was strongly suppressed, and the number of cells with weak or no staining of Prox1 had increased. In contrast, TβR-I inhibitor LY364947 induced expression of Prox1 in almost all HDLECs after 24 h. Immunoblot analysis using anti-Prox1 antibody confirmed the results of immunocytochemistry (Fig. 2B).

Regulation of Prox1 expression as well as that of LYVE-1 by TGF-β signaling was further examined at the mRNA level. Total RNA was isolated from HDLECs treated with or without TGF-β1 or LY364947 for 24 h, and levels of expression of Prox1 and LYVE-1 mRNAs were determined by quantitative RT-PCR (Fig. 2C and D). TGF-β1 suppressed the expression of Prox1 and LYVE-1, while LY364947 strongly induced their expression. Similar results were obtained by another TβR-I inhibitor SB431542 (Supplementary Fig. S3A and B).

The induction of Prox1 and LYVE-1 by TβR-I inhibitor was abolished by suppression of protein synthesis with cycloheximide treatment of HDLECs, while that of PAI-1 was not suppressed (Fig. 2C-E). These findings suggest that TGF-β signaling indirectly regulates the expression of Prox1 and LYVE-1 in lymphatic endothelial cells. In contrast, Prox1 was not induced by TβR-I inhibitor in HUVECs, although LYVE-1 was upregulated (Supplementary Fig. S3C and D).

Induction of cord formation and migration of HDLECs by TGF-β signaling

We examined the formation of cord-like structures by HDLECs in three-dimensional type I collagen gels. Quantification of the total length of cord-like structures after 3 days of cultivation confirmed significant decrease and increase in cord formation of HDLECs by treatment with TGF-β1 and TβR-I inhibitor, respectively (Fig. 3A).

Next, migration of HDLECs was examined by Boyden chamber assay. HDLECs were treated or not with TGF-β1 or TβR-I inhibitor LY364947, and their migration towards VEGF-C (50 ng/ml) was determined after 6-h incubation. In the absence of VEGF-C, migration of HDLECs was not strongly induced, and there was no significant difference in migration between untreated cells and those treated with TGF-β1 or TβR-I inhibitor (data not shown). VEGF-C induced the migration of HDLECs, which was strongly suppressed by TGF-β1, whereas TβR-I inhibitor weakly enhanced it (Fig. 3B and C).

Induction of early lymph vessel development by TβR-I inhibitor in ES cells

To determine whether TGF-β signaling regulates lymphatic vessel development, mouse R1 ES cells were aggregated to form embryoid bodies, and cultured in three-dimensional collagen in the presence of VEGF-A and -C. Although VEGF-A and -C induced formation of

lymphatic vessel structures as previously reported,[32,33] addition of TβR-I inhibitor LY364947 further induced the production of a network of LYVE-1-positive lymphatic vessel-like structures, whereas addition of TGF-β1 reduced the production of these structures (Fig. 4A and B). Immunostaining of Prox1 in PECAM1-positive areas also increased with TβR-I inhibitor and decreased with TGF-β1 (Fig. 4C), indicating that the effects of TGF-β signaling on lymphatics are not limited to HDLECs.

Inhibition of endogenous TGF-β signaling accelerates lymphangiogenesis in a mouse model of chronic peritonitis

We next examined whether inhibition of TGF-β signaling regulates lymphangiogenesis in vivo. Since chronic inflammation is reported to induce lymphangiogenesis, possibly through production of VEGF-C by F4/80-positive macrophages.[37] we induced chronic peritonitis in mice as a model of lymphangiogenesis. Formation of inflammatory plaques containing lymphatic vessels and macrophages was induced on the peritoneal side of the diaphragm by intraperitoneal injection of thioglycollate in BALB/c mice. Mice were injected intraperitoneally with 2 ml of 5% thioglycollate and TβR-I inhibitor LY364947 (1 mg/kg) three times a week for two weeks. Lymphangiogenesis in the plaques of the diaphragms was then examined by immunostaining using LYVE-1 antibody. Formation of lymphatic plaques was observed after two weeks in the mice treated with thioglycollate, and TβR-I inhibitor significantly increased the LYVE-1-positive areas in the plaques (Fig. 5A and B).

Since macrophages have been suggested to be the major sources of lymphangiogenic growth factors, including VEGF-C, we obtained peritoneal macrophages from thioglycollate-treated mice, and determined the production of VEGF-C by quantitative RT-PCR. As shown in Fig. 5C, production of VEGF-C was not induced by TβR-I inhibitor,

suggesting that the observed effect of TβR-I inhibitor on lymphangiogenesis may be primarily induced by its direct action on LECs.

Induction of lymphangiogenesis by TβR-I inhibitor in models of cancer

Since TβR-I inhibitor induced growth, migration and cord formation of LECs in vitro and lymphangiogenesis in a mouse model of chronic peritonitis in vivo, we examined whether it induces lymphangiogenesis in tumor xenograft models using BxPC3 and MIA PaCa-2 pancreatic adenocarcinoma cells. Pancreatic adenocarcinoma cells were inoculated subcutaneously into BALB/c nude mice. The mice were then injected with 1 mg/kg of TβR-I inhibitor LY364947 three times a week for three weeks.

When the BxPC3 cells were mixed with or without 1 μg/ml of VEGF-C, and inoculated into nude mice, we found that the number of LECs stained by LYVE-1 antibody was slightly increased in the presence of VEGF-C. Interestingly, TβR-I inhibitor significantly increased the LYVE-1-positive areas in tumor tissues in the presence of VEGF-C (Fig. 6A and B). Z-stack analysis of tumor tissues by a confocal microscope revealed that the LYVE-1-positive cells tended to form tube-like structures in the tumor tissues (data not shown). In order to confirm that the LYVE-1-positive cells were indeed LECs, tumor tissues treated with VEGF-C and TβR-I inhibitor were stained with Prox1 and podoplanin antibodies. The LYVE-1-postive cells were co-stained by Prox1 and podoplanin antibodies (Fig. 6C), whereas most PECAM1-positive cells were not stained by LYVE-1 or Prox1 antibodies (Fig. 6D). The LYVE-1-positive cells in the BxPC3 xenografts were thus LECs.

Similar experiments were conducted using BxPC3 cells overexpressing VEGF-C. BxPC3 cells were infected with a lentivirus containing VEGF-C, and increase in the production of VEGF-C mRNA by the VEGF-C-lentivirus-infected cells was confirmed by

quantitative RT-PCR (Fig. 6E). Similar to the addition of VEGF-C protein, TβR-I inhibitor significantly increased the LYVE-1-positive areas in BxPC3 tumor tissues expressing VEGF-C (Fig. 6F).

The effects of TβR-I inhibitor on lymphangiogenesis were also examined using MIA PaCa-2 cells with or without 1 μg/ml of VEGF-C protein. As shown in Fig. 7A and B, TβR-I inhibitor significantly increased the LYVE-1-positive areas in tumor tissues in the presence of VEGF-C. In addition, we have tested the effect of overexpression of TGF-β1 ligand in tumor cells using a lentivirus expression system in the MIA PaCa-2 model, which resulted in reduction of lymphangiogenesis (Supplementary Fig. S4).

Discussion

TGF-β transduces signals in HDLECs and regulates the expression of LEC markers in vitro

TGF-β is a potent growth inhibitor on vascular endothelial cells, and also inhibits their migration and cord formation in vitro. In the presence of TβR-II, TGF-β binds to TβR-I (ALK-5) and an endothelial-specific type I receptor ALK-1 in vascular endothelial cells, and activates Smad2/3 and Smad1/5, respectively. ALK-5 has been reported to be responsible for inhibition of growth and migration of endothelial cells.[38] We have found that phosphorylation of Smad2 is induced by TGF-β and suppressed by TβR-I inhibitor in HDLECs. Migration of HDLECs towards VEGF-C, and formation of cord-like structures by these cells, were thus negatively regulated by TGF-β treatment.

TGF-β also plays pivotal roles in regulating the differentiation of blood endothelial cells. Inhibition of endogenous TGF-β signaling by the TβR-I kinase inhibitor SB431542 results in the proliferation and formation of sheet-like structures of mouse ESC-derived

endothelial cells.[39] In the present study, we found that TβR-I inhibitor upregulated the expression of some LEC-related genes, including Prox1 and LYVE-1. We also found that TβR-I inhibitor induced early lymph vessel development in mouse ES cells. Thus, the effects of TβR-I inhibitor on LECs are not limited to HDLECs.

LYVE-1 is a hyaluronan receptor specifically expressed in LECs. However, LYVE-1-deficient mice do not exhibit abnormalities in lymphatic vessels, and the function of LYVE-1 in LECs is unknown.[40] In contrast, Prox1 functions as a key transcriptional factor in the differentiation of LECs. Prox1 upregulates the expression of various LEC-specific genes, and induces a shift in the transcriptional program from blood endothelial cells to that of LECs. During embryonic development, Prox1 is expressed in a subset of vein endothelial cells, which begin to express LEC-markers and form primary lymphatic sacs.[24,25] Elucidation of the signals that induce expression of Prox1 in blood endothelial cells is thus important to understand the mechanisms of lymphangiogenesis. VEGFR3 signaling does not induce the expression of Prox1,[30] while interluekin-3 signaling has been reported to induce expression of Prox1 in dermal blood endothelial cells.[41] Infection by Kaposi's sarcoma-associated herpes virus also induces Prox1 expression in human dermal microvascular endothelial cells.[42] However, signaling pathways that regulate the expression of Prox1 in LECs have not been fully determined. The finding that TβR-I inhibitor induces the expression of Prox1 in HDLECs suggests that TGF-β signaling is a novel pathway that regulates Prox1 expression.

Induction of lymphangiogenesis by TβR-I inhibitor in vivo

We have shown that TβR-I inhibitor induces lymphangiogenesis in a chronic peritonitis model and in pancreatic carcinoma xenograft models. We used a low dose of TβR-I inhibitor for in vivo treatment, which has been shown to decrease the coverage of endothelium by

pericytes and promote efficient accumulation of macromolecules to tumors through leakiness of tumor blood vessels. The low-dose TβR-I inhibitor acted on blood cells and vascular cells, and suppressed the phosphorylation of Smad2 in these cells but not in tumor cells.[8] It will thus be of interest to examine whether the low-dose TβR-I inhibitor also acts on LECs and regulates lymphangiogenesis.

In the chronic peritonitis model we examined, lymphangiogenesis was induced in the diaphragm of immunocompetent mice through induction of chronic inflammation by repeated injection of thioglycollate. Accumulation of inflammatory cells, e.g. macrophages, and LECs could be observed in the plaques.[43] Some LECs were positive for Ki67, suggesting that these LECs were actively proliferating. Under these conditions, TβR-I inhibitor was able to induce lymphangiogenesis without addition of exogenous growth factors. Macrophages may produce VEGF-C[37] and other cytokines in sites of chronic inflammation; however, TβR-I inhibitor did not enhance the secretion of VEGF-C from inflammatory macrophages in the present study, suggesting that it may primarily induce lymphangiogenesis through direct action on LECs.

TβR-I inhibitor induced lymphangiogenesis in both xenografts of BxPC3 and those of MIA PaCa-2 cells in the presence of VEGF-C, suggesting that TβR-I inhibitor may induce proliferation of LECs once lymphatic vessels have been formed in the tumors by VEGF-C. An important question is whether TβR-I inhibitor induces lymphatic metastasis of tumors. The present findings suggest that TβR-I inhibitor may induce lymphangiogenesis in tumors which express VEGF-C or -D. However, Laakkonen et al. reported that only certain types of cancers secrete VEGF-C.[21] We have also found in an orthotopic transplantation model of diffuse-type gastric carcinoma OCUM-2MLN that treatment with low-dose TβR-I inhibitor did not affect the extent of lymph node metastasis 16 days later.[8,43] Moreover, Ge et al. reported that

metastasis of certain breast tumors was prevented by TβR-I inhibitor through activation of immune function.[44]

Dendritic cells, cytotoxic T cells, and natural killer cells, which could be involved in anti-tumor immune responses, are known to be functionally inhibited by TGF-β signaling.[43] Dendritic cells are reported to migrate from sites of inflammation to regional lymph nodes through lymphatic vessels for presenting antigens to initiate further immune responses.[46,47] Therefore, it is possible that the use of TβR-I inhibitor may enhance anti-tumor immune responses via providing more routes for dendritic cells to migrate from tumors to regional lymph nodes, and recovering functions of various immune cells including dendritic cells inhibited by TGF-β ligands. These issues, however, remain for further investigation.

In conclusion, we have shown that inhibition of endogenous TGF-β signaling results in induction of lymphangiogenesis. Although TβR-I inhibitor induces lymphangiogenesis in the presence of VEGF-C and possibly via other lymphangiogenic cytokines, it remains to be determined whether it can induce the spread of tumors through lymphatic vessels or suppress it through activation of immune function.

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Authorship

Masako Oka performed the research and wrote the manuscript. Caname Iwata collected data, contributed vital new reagents or analytical tools, and analyzed and interpreted the data. Hiroshi I. Suzuki performed the research and collected the data. Kunihiko Kiyono collected the data, and contributed vital new reagents or analytical tools. Yasuyuki Morishita collected the data. Tetsuro Watabe contributed vital new reagents or analytical tools. Akiyoshi Komuro, collected the data. Mitsunobu R. Kano designed and performed the research, analyzed and interpreted the data, performed statistical analysis, wrote the manuscript. Kohei Miyazono designed the research, and wrote the manuscript.

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Figure legends

Figure 1. Transduction of TGF-β signals in HDLECs. (A) Immunoblotting of phospho-Smad2 after TGF-β or TβR-I inhibitor treatment. HDLECs were treated with TGF-β1 or with two kinds of TβR-I inhibitors (LY364947 or SB431542, shown as Inhib1 or Inhib2, respectively) in the presence and absence of TGF-β1 for 1 h, and subjected to immunoblot analysis using phospho-Smad2 antibody (upper panel) and Smad2/3 antibody (lower panel). Ctrl, control. (B and C) Real-time PCR of Smad7 and PAI-1. HDLECs were treated as in (A), and expression of Smad7 and PAI-1 mRNAs was determined at 1 h and 24 h after stimulation, respectively. ***$p<0.001$. (D) Regulation of growth of HDLECs by TGF-β and TβR-I inhibitor. HDLECs were seeded at a density of 2×10^3 cells/well in 96-well plates, and cells were treated or not with TGF-β1 (1 ng/ml) or LY364947 (3 μM). Photographs of the cells were taken at day 2. Cell numbers were determined by WST assay in triplicate at day 2. Error bars represent standard deviations. *$p<0.05$, and **$p<0.01$. (E and F) Autocrine TGF-β signaling in HDLECs. Expression of TGF-β1 mRNA in HDLECs treated with TGF-β1 (1 ng/ml) or TβR-I inhibitors (3 μM) as in (A) was determined by real-time PCR (E). Production of TGF-β1 protein by HDLECs treated as in (A), but with TGF-β3 (1 ng/ml) as the stimulant, was examined in conditioned medium using an ELISA kit (F). LY364947 was used as TβR-I inhibitor (Inhib). HUVECs were used as a control. Error bars represent standard deviations. *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Figure 2. TGF-β signaling regulates the expression of LEC-related genes in HDLECs. (A) Expression levels of Prox1 determined by immunostaining in HDLECs. HDLECs were untreated (left) or treated with TGF-β1 (1 ng/ml) (middle) or TβR-I inhibitor LY364947 (3

μM) (right) for 24 h and subjected to immunocytochemical examination. Bars 50 μm. (B) Expression levels of Prox1 in HDLECs treated as described in (A) were determined by immunoblotting. α-tubulin levels were monitored as a loading control for whole-cell extracts. (C-E) Expression levels of Prox1, LYVE-1, and PAI-1 mRNAs were analyzed by real-time PCR at 24 h after TGF-β1 or TβR-I inhibitor treatment. In the right three columns, cells were treated with 1 μM cycloheximide (CHX) for 24 h before they were treated with TGF-β1 or TβR-I inhibitor for 24 h. Values were normalized to amounts of GAPDH mRNA. Error bars represent standard deviations. *$p<0.05$, **$p<0.01$, and ***$p<0.001$

Figure 3. Formation of cord-like structures and migration of HDLECs are increased by inhibition of TGF-β signaling. (A) Total lengths of cord-like structures of HDLECs in three-dimensional culture were quantified. Cells were mixed with type I collagen gel at a density of 1 $\times 10^4$ cells/well and seeded onto culture-slide wells. HDLECs were treated with TGF-β1 (1 ng/ml) or TβR-I inhibitor (3 μM). Formation of cord-like structures was observed by video microscopy, and total lengths of cord-like structures of cells were quantified 3 days after cultivation. Error bars represent standard deviations. *$p<0.05$ and **$p<0.01$. Effects of TGF-β signaling on migration of HDLECs were determined by Boyden chamber assay. Cells were seeded at 4 $\times 10^4$ cells/well in the upper chambers coated with type I collagen. (B) Medium containing VEGF-C (50 ng/ml) was placed in the lower chamber, while that in the upper chamber did not contain VEGF-C but did contain TGF-β1 (1 ng/ml) or TβR-I inhibitor (3 μM). Migration of cells was determined after 6 h. Bars 100 μm. (C) Migration of HDLECs was quantified. Cells that had migrated to the lower chambers were counted after 6 h in triplicate. Error bars represent standard deviations. *$p<0.05$ and **$p<0.01$.

Figure 4. Enhancement of early lymph vessel development in ES cells cultured in three-dimensional collagen by inhibition of TGF-β signaling. Mouse R1 ES cells were cultured in collagen gel with VEGF-A (30 ng/ml) for first 4 days and subsequently with VEGF-C (30 ng/ml) and VEGF-A (30 ng/ml) for 14 days to form embryoid bodies exhibiting early lymph vessel formation. The embryoid bodies were also treated with TGF-β1 (1 ng/ml) or TβR-I inhibitor (3 μM) for the last 7 days. (A, B) The embryoid bodies were stained by PECAM1 (red) and LYVE-1 (green) (A). Bars 50 μm. Quantification of LYVE-1-stained areas was performed in 5 low-magnification microscopic fields on 3 embryoid bodies (B). Error bars represent standard deviations. ***$p<0.001$. (C) Immunostaining for PECAM1 (red) and Prox1 (green) confirmed the effect of modulation of TGF-β signaling on expression of Prox1 in PECAM1-positive structures.

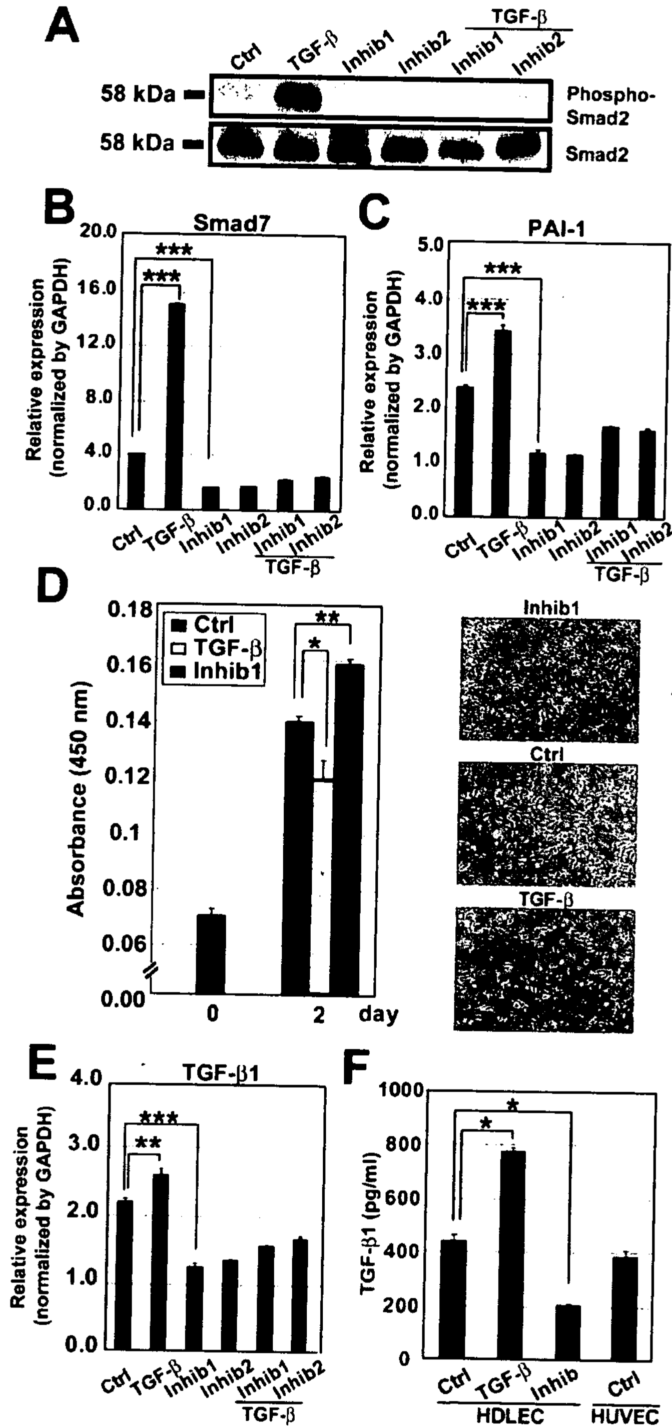
Figure 5. Enhancement of lymphangiogenesis by TβR-I inhibitor in a mouse model of chronic peritonitis. Mice were treated with 5% thioglycollate (2 ml) and TβR-I inhibitor (LY364947, 1 mg/kg) three times a week for two weeks. Their diaphragms were then examined for lymphangiogenesis in plaques. (A) LYVE-1 immunostaining (shown in red) of diaphragms treated without (control, left panels) or with TβR-I inhibitor (right panels). Sections were also stained for Mac1 (green) (lower panels). Bars 50 μm. (B) Quantification of LYVE-1-positive area in plaques of the diaphragms treated without (control) or with TβR-I inhibitor (n=3 for each group). Error bars represent standard errors. ***$p<0.001$. (C) Expression of VEGF-C in inflammatory macrophages in the presence and absence of TβR-I inhibitor. Inflammatory macrophages were harvested from ascites fluid of mice four days after induction of peritonitis by intraperitoneal injection of thioglycollate, seeded at 1×10^6, and treated with or without TβR-I inhibitor for 24 h. Error bars represent standard deviations.

*p<0.05.

Figure 6. Lymphangiogenesis is increased by TβR-I inhibitor in pancreatic adenocarcinoma BxPC3 xenograft models. (A-D) Effects of TβR-I inhibitor on lymphangiogenesis were examined in a xenograft model using a human pancreatic cancer cell line, BxPC3. BxPC3 cells mixed with or without VEGF-C (1 μg/ml) were subcutaneously inoculated in BALB/c nude mice. After tumors had formed, the mice were injected intraperitoneally with TβR-I inhibitor (LY364947, 1 mg/kg) three times a week for three weeks. They were sacrificed at the end of the experiment and excised tumors were examined histologically. (A) Immunostaining of BxPC3 xenograft sections by LYVE-1 antibody (shown in green). Bars 50 μm. (B) LYVE-1-positive areas in the BxPC3 xenograft sections were determined in the presence and absence of VEGF-C and TβR-I inhibitor (n=3 for each group). Error bars represent standard errors. n.s., not significant, and *p<0.05. (C) Immunostaining of BxPC3 xenograft sections for Prox1 (left panel, red), podoplanin (right panel, red), and LYVE-1 (green). Bars 20 μm. (D) Immunostaining of BxPC3 xenograft sections for PECAM1 (red), LYVE-1 (left panel, green), Prox1 (right panel, green), and TOTO-3 (blue). Bars 100 μm. (E and F) Effects of TβR-I inhibitor on lymphangiogenesis were examined in a xenograft model using BxPC3 cells overexpressing VEGF-C by infection of the VEGF-C-lentivirus. BxPC3 cells infected with a lentivirus containing GFP were used as a control. (E) Upregulation of VEGF-C mRNA in BxPC3 cells after infection of the VEGF-C-lentivirus was determined by real-time PCR. (F) LYVE-1-positive areas in the GFP- and VEGF-C-expressing BxPC3 xenograft sections treated with or without TβR-I inhibitor were determined (n=3 for each group). Error bars represent standard errors. n.s., not significant, and ***p<0.001.

Figure 7. Induction of lymphangiogenesis by TβR-I inhibitor in MIA PaCa-2 xenograft models. (A and B) Effects of TβR-I inhibitor on lymphangiogenesis were examined in a xenograft model using a TGF-β-non-responsive human pancreatic cancer cell line, MIA PaCa-2. MIA PaCa-2 cells mixed with or without VEGF-C (1 μg/ml) were subcutaneously inoculated in BALB/c nude mice, and treated with TβR-I inhibitor (1 mg/kg) as described in Figure 7. Bars 50 μm. (A) Immunostaining of MIA PaCa-2 xenograft sections by LYVE-1 (shown in green). (B) LYVE-1-positive areas in the MIA PaCa-2 xenograft sections in the presence or absence of VEGF-C and TβR-I inhibitor were determined (n=3 for each group). Error bars represent standard errors. n.s., not significant, and $^*p<0.05$.

Figure 1



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