Discussion

HSEC are characterized by their unique morphological feature and functions [24]. As the blood flows from the gut into the liver, one important function of the liver is to eliminate various foreign substances absorbed in the gut. HSEC play a key role as a filter by their strong endocytic activity [6]. Lyve1 and Stab2 are such scavenger receptors, implicated in the uptake of hyaluronan as well as other scavenger receptor-ligands [25–27]. We used Lyve1 and Stab2 to analyze EC differentiation, because they are functional receptors involved in the scavenger function of HSEC, and their expression is the earliest sign of HSEC differentiation [7,11].

The features of EC developed with Tbr1ki2 were similar to those of fetal immature HSEC, because (i) they expressed Stab2, (ii) they also expressed CD34, (iii) they exhibited a higher endocytic activity than control EC, and (iv) expression of FcgR proteins was undetectable by flow cytometric analysis (Nonaka et al. unpublished observation), though the transcript was detected in EC developed with Tbr1ki2 on culture day 6 (Fig. 3), and (v) fenestrae was not observed (Nonaka et al. unpublished observation). Further maturation of HSEC seems to require an additional factor. Yoshida et al. previously demonstrated that EC with fenestrae could be induced from rat E13.5 HSEC by the inhibition of TGFβ/activin signaling [16]. Since their culture system contains hepatoblasts, stellate cells, and others besides HSEC, paracrine factors from hepatic cells may promote HSEC maturation cooperatively with the TGFβRI inhibition.

The negative impact of TGFβ signaling on Lyve1 expression was also observed in human dermal LEC and EC induced from ESC in 3-dimensional culture [15]. We show in this paper that the TGFβRI inhibitor also upregulated HSEC markers, but not LEC markers such as Pdpn and Ccl21, while the previous study showed that the inhibition of TGFβ/activin signaling upregulated another LEC marker, Prox1 [15]. Since LEC and HSEC share some common characteristic features, it is possible they use the same signaling cascade for regulating their differentiation. An intriguing question is how the same signaling cascade, TGFβ/activin signaling, elicits different biological outputs, LEC/HSEC differentiation.

Since differentiation of ESC often mirrors a normal developmental process [28], to establish a tissue-specific EC differentiation system from ESC will also contribute to a better understanding of the development of EC heterogeneity. Interestingly, a recent study demonstrated that transplantation of healthy HSEC might be useful for cell therapy of relevant disorders [29]. Because a large number of homogenous cells can be generated from ESC, our successful induction of Stab2$^+$ EC from ESC raises an intriguing possibility for their use in future clinical applications.

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Ras signaling directs endothelial specification of VEGFR2$^+$ vascular progenitor cells

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Vascular endothelial growth factor receptor 2 (VEGFR2) transmits signals of crucial importance to vasculogenesis, including proliferation, migration, and differentiation of vascular progenitor cells. Embryonic stem cell–derived VEGFR2$^+$ mesodermal cells differentiate into mural lineage in the presence of platelet derived growth factor (PDGF)–BB or serum but into endothelial lineage in response to VEGF-A. We found that inhibition of H-Ras function by a farnesyltransferase inhibitor or a knockdown technique results in selective suppression of VEGF-A–induced endothelial specification. Experiments with ex vivo whole-embryo culture as well as analysis of H-*ras*$^{-/-}$ mice also supported this conclusion. Furthermore, expression of a constitutively active H-Ras[G12V] in VEGFR2$^+$ progenitor cells resulted in endothelial differentiation through the extracellular signal-related kinase (Erk) pathway. Both VEGF-A and PDGF-BB activated Ras in VEGFR2$^+$ progenitor cells 5 min after treatment. However, VEGF-A, but not PDGF-BB, activated Ras 6–9 h after treatment, preceding the induction of endothelial markers. VEGF-A thus activates temporally distinct Ras–Erk signaling to direct endothelial specification of VEGFR2$^+$ vascular progenitor cells.

Introduction

Blood vessel formation is a fundamental process in organogenesis during embryonic development (Coultas et al., 2005; Ferguson et al., 2005). Vascular progenitor cells are thought to first appear in the posterior primitive streak as vascular endothelial growth factor receptor 2–positive (VEGFR2$^+$) mesodermal cells. These cells are specified for the hematopoietic and/or vascular lineage (hemangioblasts or angioblasts) and then migrate into extraembryonic sites, including the yolk sac and allantois as well as intraembryonic sites, in VEGF-A–dependent fashion (Huber et al., 2004; Hiratsuka et al., 2005). These precursor cells differentiate and assemble to form primary capillary plexuses or directly aggregate into the dorsal aorta or cardinal vein, followed by a process of remodeling through sprouting/nonsprouting angiogenesis and fusion of vessels. Finally, maturation of the nascent vasculature is accomplished by recruitment and adhesion of mural cells to endothelial cells.

VEGFR2 (also known as Flk1 and KDR), one of the receptors for the VEGF family of growth factors, plays essential roles during vascular development. VEGFR2-deficient mice die in utero between 8.5 and 9.5 d postcoitum because of lack of endothelial cells and hematopoietic cells (Shalaby et al., 1995). Subsequent analysis suggested that the role of VEGFR2 signaling in vascular development in vivo includes proliferation, migration, and differentiation of progenitor cells (Shalaby et al., 1997). Because VEGFR2$^+$ mesodermal cells can give rise to multiple lineages other than endothelial and hematopoietic cells, including vascular mural cells, skeletal muscle cells, and cardiomyocytes (Motoike et al., 2003; Ema et al., 2006), differentiation of VEGFR2$^+$ cells should be appropriately specified. However, the signal transduction pathways leading to endothelial specification downstream of VEGFR2 are poorly understood, although those for cell proliferation and migration have been well explored in mature endothelial cells (Shibuya and Claesson-Welsh, 2006).

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Abbreviations used in this paper: AcLDL, acetylated low-density lipoprotein; αSMA$^+$, α–smooth muscle actin positive; E, embryonic day; ESC, embryonic stem cells; Erk, extracellular signal-related kinase; HMEC, human microvascular endothelial cell; miRNA, microRNA; PECAM1$^+$, platelet-endothelial cell adhesion molecule-1 positive; VEGFR2$^+$, vascular endothelial growth factor receptor 2 positive.

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Use of differentiating embryonic stem cells (ESCs) is advantageous for the study of signaling for lineage specification because migration of progenitor cells to the correct microenvironment is unnecessary. Using mouse ESC-derived VEGFR2$^+$ cells, an in vitro system for analysis of ligand-dependent endothelial specification has recently been established (Hirashima et al., 1999; Yamashita et al., 2000). In this system, ESC-derived VEGFR2$^+$ cells differentiate into endothelial cells in response to VEGF-A, whereas they differentiate into α–smooth muscle actin–positive (αSMA$^+$) mural cells resembling vascular smooth muscle cells in the presence of PDGF-BB or serum (Yamashita et al., 2000; Ema et al., 2003; Watabe et al., 2003). VEGFR2 appears to transmit a specific signal for induction of endothelial differentiation of VEGFR2$^+$ progenitor cells because signaling from either VEGFR1 or 3 fails to induce it (Yamashita et al., 2000; Suzuki et al., 2005).

In the present study, we investigated the signaling pathway downstream of VEGFR2 for specification of endothelial lineage. Using pharmacological inhibitors, a gene silencing approach, and a gain-of-function approach, we concluded that Ras signaling is involved in endothelial specification induced by VEGF-A. Although PDGF-BB fails to induce endothelial differentiation, it also activates Ras in VEGFR2$^+$ progenitor cells. We found that VEGF-A activates the Ras pathway at periods distinct from PDGF-BB, thus directing endothelial differentiation from VEGFR2$^+$ vascular progenitor cells. These findings also provide mechanistic insights into signaling for cell specification through widely shared effector molecules.

Results

A farnesyltransferase inhibitor, FTI-277, inhibits VEGF-A–induced endothelial specification of ESC-derived VEGFR2$^+$ cells

To determine the signaling components required for VEGF-A–induced endothelial differentiation from vascular progenitor cells, we used in vitro vascular differentiation systems (Yamashita et al., 2000). VEGFR2$^+$ cells derived from CCE mouse ESCs were cultured in medium containing serum with or without VEGF-A. In the absence of VEGF-A, most cells differentiated into αSMA$^+$ mural cells, whereas in the presence of VEGF-A, platelet-endothelial cell adhesion molecule-1–positive (PECAM1$^+$) endothelial cells emerged (Fig. 1 A; Yamashita et al., 2000).

We first examined the effects of various inhibitors targeting signal molecules. Among those tested, we found that FTI-277 (Lerner et al., 1995), a farnesyltransferase inhibitor, had a selective inhibitory effect on endothelial differentiation. When FTI-277 was added, VEGF-A–induced appearance of PECAM1$^+$ cells was suppressed, whereas that of αSMA$^+$ cells was not markedly altered (Fig. 1 A). To determine whether the reduction in number of PECAM1$^+$ cells by FTI-277 was caused by inhibition of differentiation, we next performed quantitative analyses using a limiting dilution assay (Fig. 1 B). When VEGFR2$^+$ cells were seeded at low density (90–120 cells/cm^2), they formed single-cell–derived colonies in 4 d. We counted the number of colonies after immunostaining for PECAM1 and αSMA, which reflects the fate of differentiation. In the absence of FTI-277, stimulation with VEGF-A increased PECAM1$^+$ colonies and decreased αSMA$^+$ colonies, indicating that VEGF-A directs endothelial differentiation at the expense of mural differentiation. In the presence of FTI-277, the number of PECAM1$^+$ colonies was decreased and that of αSMA$^+$ colonies was increased, whereas the total number of colonies was not markedly changed. These findings indicate that FTI-277 specifically inhibits endothelial differentiation of ESC-derived VEGFR2$^+$ cells. Similar results were obtained using MGZ5 ESCs (unpublished data).

To determine when FTI-277–sensitive signal is transmitted, we added FTI-277 at different time points after VEGF-A stimulation (Fig. 1 C). When FTI-277 was added 3 h after stimulation, the appearance of PECAM1$^+$ cells was suppressed but when FTI-277 was added 6 h after stimulation, it was not. We concluded that the FTI-277–sensitive signal for endothelial specification is transmitted later than 3 h after VEGF-A stimulation.

We also performed ex vivo whole-embryo culture assay to investigate the effects of FTI-277 on vascular development in mouse embryo. Embryonic day (E)–6.75 concepti were picked out from the uteri of pregnant mice and cultured for 3 d, during which PECAM1$^+$ blood vessels were formed in the yolk sac. In the presence of FTI-277, however, PECAM1$^+$ vessels were diminished, although overall development of the yolk sac was not affected (Fig. 1 D). We then examined the expression of vascular markers by quantitative RT-PCR. FTI-277 treatment resulted in decrease in the level of expression of PECAM1 and VE-cadherin compared with control, whereas expression of αSMA was unchanged (Fig. 1 E). These findings suggest that FTI-277 suppresses vascular development.

Loss of H-Ras abrogates endothelial differentiation of VEGFR2$^+$ cells

Because the principal targets of FTI-277 include H-Ras, it appeared possible that Ras signaling could be involved in VEGF-A–induced endothelial differentiation of vascular progenitor cells. To examine the effect of H-Ras inactivation on vascular development, we investigated the vascular phenotype of H-*ras* knockout mice. Heterozygous H-*ras*$^{+/-}$ mice produced homozygous H-*ras*$^{-/-}$ offspring in Mendelian ratio (+/+, 17; +/−, 36; and −/−, 17), as described previously (Ise et al., 2000; Esteban et al., 2001). We therefore focused on vascular phenotypes during early development, and found vascular aberration in the periphery of the brain of 73% (8/11) of H-*ras*$^{-/-}$ embryos studied at E9.5, although they contained similar numbers of somites, as did wild-type and heterozygous littermates (Fig. 2 A). H-*ras*$^{+/-}$ embryos exhibited no clear difference from wild-type embryos. We further double stained the cephalic region for PECAM1 and VEGFR2, the earliest marker of differentiation of endothelial cells (Fig. 2 B). In H-*ras*$^{+/-}$ embryos, complex vascular networks were stained for both PECAM1 and VEGFR2, whereas in H-*ras*$^{-/-}$ embryos, vascular structures positive for either PECAM1 or VEGFR2 were strikingly reduced. Furthermore, we found that vascular structures were rare in cross sections of the head region of H-*ras*$^{-/-}$ embryos (Fig. 2 C). This vascular aberration was transient,

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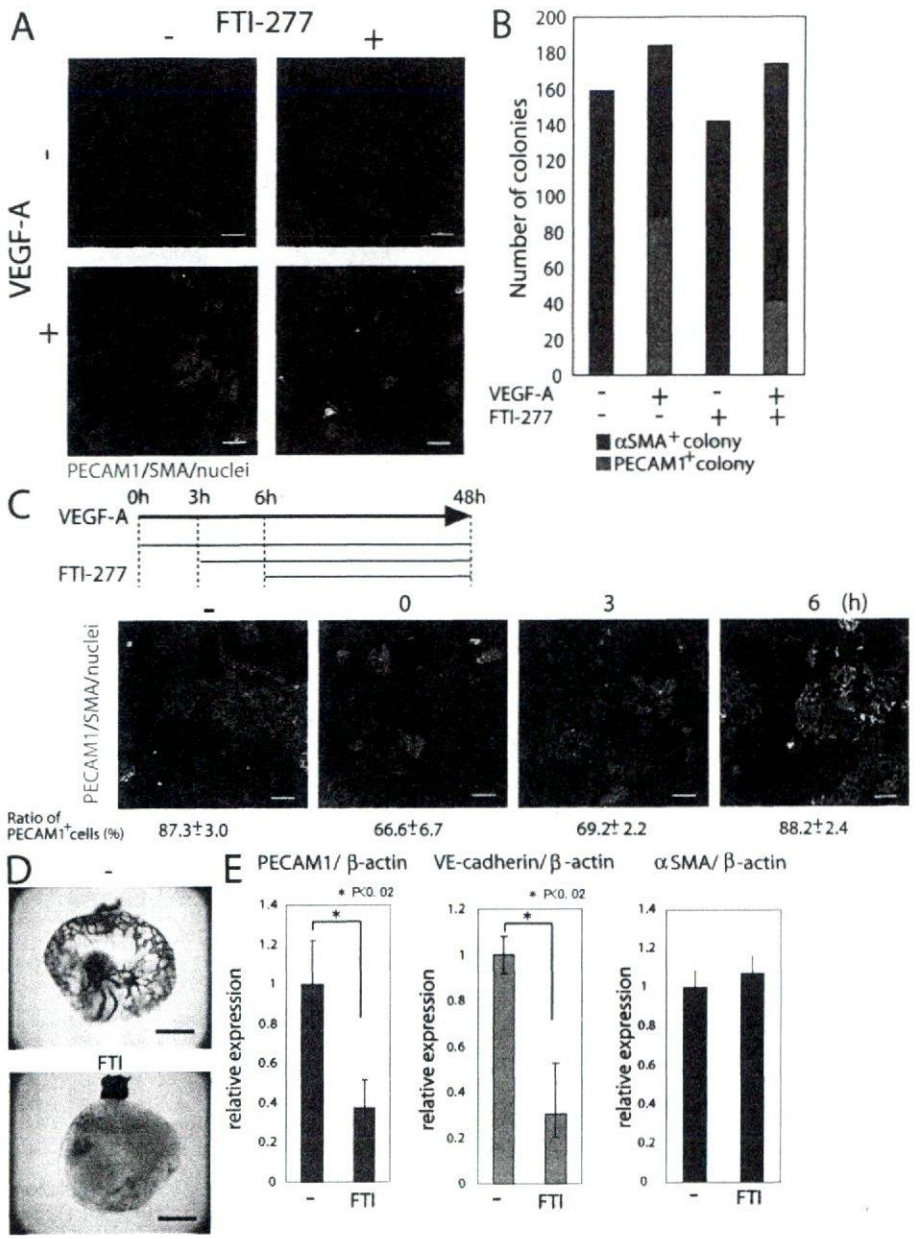

Figure 1. **Inhibitory effect of FTI-277 on endothelial differentiation.** (A) ESC-derived VEGFR2$^+$ cells were cultured for 2 d with 1 μM FTI-277 and/or 30 ng/ml VEGF-A, followed by immunostaining for PECAM1 (green), αSMA (red), and nuclei (blue). Bars, 100 μm. (B) Quantification of colony formation. FTI-277 was used at 3 μM. Representative results for three independent experiments are shown. (C) Time course of changes in FTI-277 sensitivity of VEGF-A-dependent endothelial differentiation. 1 μM FTI-277 was added at 0, 3, and 6 h after stimulation with VEGF-A, and cultured until 48 h. Cells were immunostained for PECAM1 (green), αSMA (red), and nuclei (blue). Bars, 100 μm. Quantification of appearance of PECAM1$^+$ cells is indicated below the panels (% of PECAM1$^+$ cells; means ± SD from three independent fields). (D) Ex vivo culture of mouse embryo E6.75 concepti were picked out and cultured with or without 10 μM FTI-277 for 3 d. Vasculature in yolk sacs of concepti were immunostained for PECAM1 (blue). Bars, 1 mm. (E) Quantitative RT-PCR analysis of PECAM1, VE-cadherin, and αSMA of ex vivo–cultured whole concepti. Each value is normalized to expression of β-actin. Values are the means ± SD of triplicate measurements. *, $P < 0.02$ (Student's *t* test).

and no obvious abnormality was observed in E10.5 H-*ras*$^{-/-}$ embryos (unpublished data).

To determine whether H-Ras regulates endothelial differentiation from vascular progenitor cells in vitro, we established stable ESC lines in which expression of H-Ras can be knocked down by microRNA (miRNA) under the control of tetracycline (Tc) because siRNA duplex was only minimally incorporated into ESC-derived VEGFR2$^+$ cells. A premiRNA sequence targeting H-Ras was knocked into the *ROSA26* locus in MGZ-RTcH cells (Tc-miR-H-Ras; Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). In MGZRTcH cells, expression of transgene at the *ROSA26* locus was induced by removal of Tc (Masui et al., 2005). In Tc-miR-H-Ras cells, expression of endogenous H-Ras was knocked down in the absence of Tc (Fig. S1 B). Limiting dilution assay was then performed for Tc-miR-H-Ras–derived VEGFR2$^+$ cells in the presence or absence of Tc. In the absence of Tc (Ras–knocked down condition), VEGF-A–induced PECAM1$^+$ colonies decreased in number, whereas αSMA$^+$ colonies increased compared with those in the presence of Tc (Fig. 2 D). In Tc-miR-NTC cells expressing negative control miRNA, PECAM1$^+$ colonies did not decrease in number (unpublished data). These findings suggest that H-Ras plays a role in endothelial specification of VEGFR2$^+$ progenitor cells.

Constitutively active G12V mutant of H-Ras induces PECAM1$^+$ cells from VEGFR2$^+$ progenitor cells

We next established ESC lines carrying a Tc-regulatable active form of H-Ras (Tc-H-Ras[G12V]) or no transgene (Tc-empty). In Tc-H-Ras[G12V] cells, Ras is expressed at high levels in the absence of Tc but is not expressed in the presence of Tc (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). We then examined differentiation of VEGFR2$^+$

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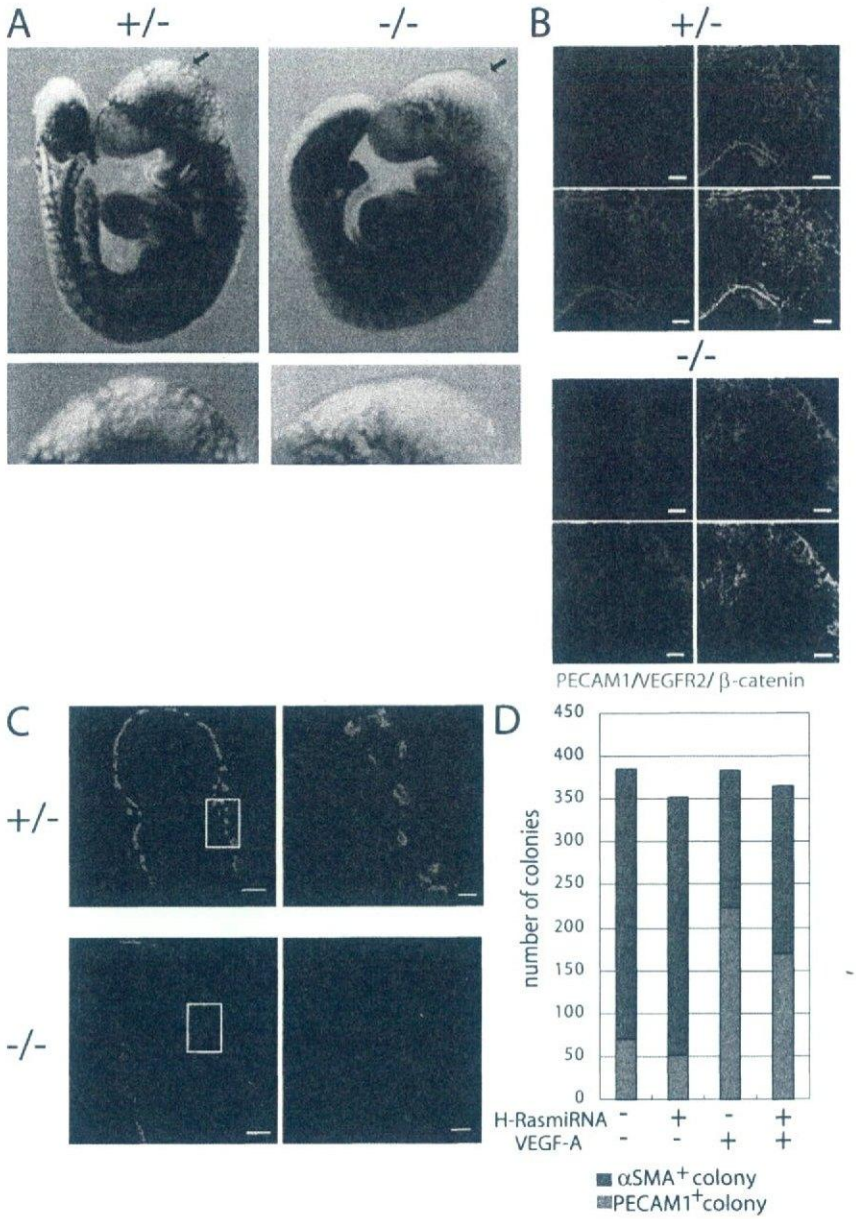

Figure 2. **Loss of H-Ras impairs vascular development.** (A) Whole-mount PECAM1 staining of E9.5 H-*ras*$^{+/}$ and H-*ras* $^{/}$ mice. Magnifications of the areas marked with arrows in the top are shown in the bottom. (B) Immunostaining for PECAM1 (green), VEGFR2 (red), and β-catenin (blue) of cephalic region of E9.5 H-*ras*$^{+/}$ and H-*ras* $^{/}$ mice. Bars, 100 μm. (C) Immunostaining for PECAM1 (green) of cross sections of cephalic region of E9.5 H-*ras*$^{+/}$ and H-*ras* $^{/}$ mice. Magnifications of the boxed areas in the left are shown in the right. Bars: (left) 100 μm; (right) 20 μm. (D) Quantification of colony formation of Tc-miR-H-Ras cells, in which H-Ras has been knocked down by miRNA in the absence of Tc. Representative results of three independent experiments are shown.

cells derived from these cell lines. When H-Ras[G12V] was not expressed in the presence of Tc, appearance of PECAM1$^+$ cells was VEGF-A–dependent (Fig. 3 A, H-Ras[G12V](-)). Upon expression of H-Ras[G12V] by removal of Tc, PECAM1$^+$ cells appeared even in the absence of VEGF-A (Fig. 3 A, H-Ras[G12V](+)). Among Tc-empty cells, PECAM1$^+$ cells were not induced by removal of Tc (unpublished data). We further confirmed that the appearance of PECAM1$^+$ cells induced by H-Ras[G12V] was inhibited by FTI-277 (Fig. S3 A).

These PECAM1$^+$ cells were also positive for other endothelial markers, including VE-cadherin (Fig. 3 B), CD34, and endoglin (not depicted), and they incorporated acetylated low-density lipoprotein (AcLDL; Fig. 3 C) and expressed mRNA for endothelial nitric oxide synthase and claudin-5 (not depicted). We next examined the ability of VEGFR2$^+$ cells to form vascular structures in three-dimensional culture upon expression of H-Ras[G12V]. Aggregated VEGFR2$^+$ cells derived from Tc-H-Ras[G12V] cells were cultured in type I collagen gel for 7 d. When active Ras was inducibly expressed, cells formed tube-like structures even in the absence of VEGF-A (Fig. 3 D). Furthermore, we performed in vivo vascular formation assay. Tc-empty and Tc-H-Ras[G12V] cells were labeled with retrovirus carrying YFP. These cells were differentiated in vitro and subcutaneously injected, together with Matrigel, into the abdominal region of mice. After 10 d, Matrigel was harvested, frozen sectioned, and immunostained for PECAM1 and αSMA. When Tc-H-Ras[G12V] cells were injected, PECAM1$^+$ blood vessels surrounded by αSMA$^+$ cells were observed. These PECAM1$^+$ cells were also positive for YFP, indicating that they originated from Tc-H-Ras[G12V] cells (Fig. 3 E). In contrast, PECAM1$^+$ vessels were not observed when Tc-H-Ras[G12V] cells were injected, but H-Ras[G12V] expression was suppressed by treatment with Tc or when Tc-empty cells were injected. These findings suggest that active Ras induces differentiation of cells with characteristics of endothelial cells from VEGFR2$^+$ progenitor cells.

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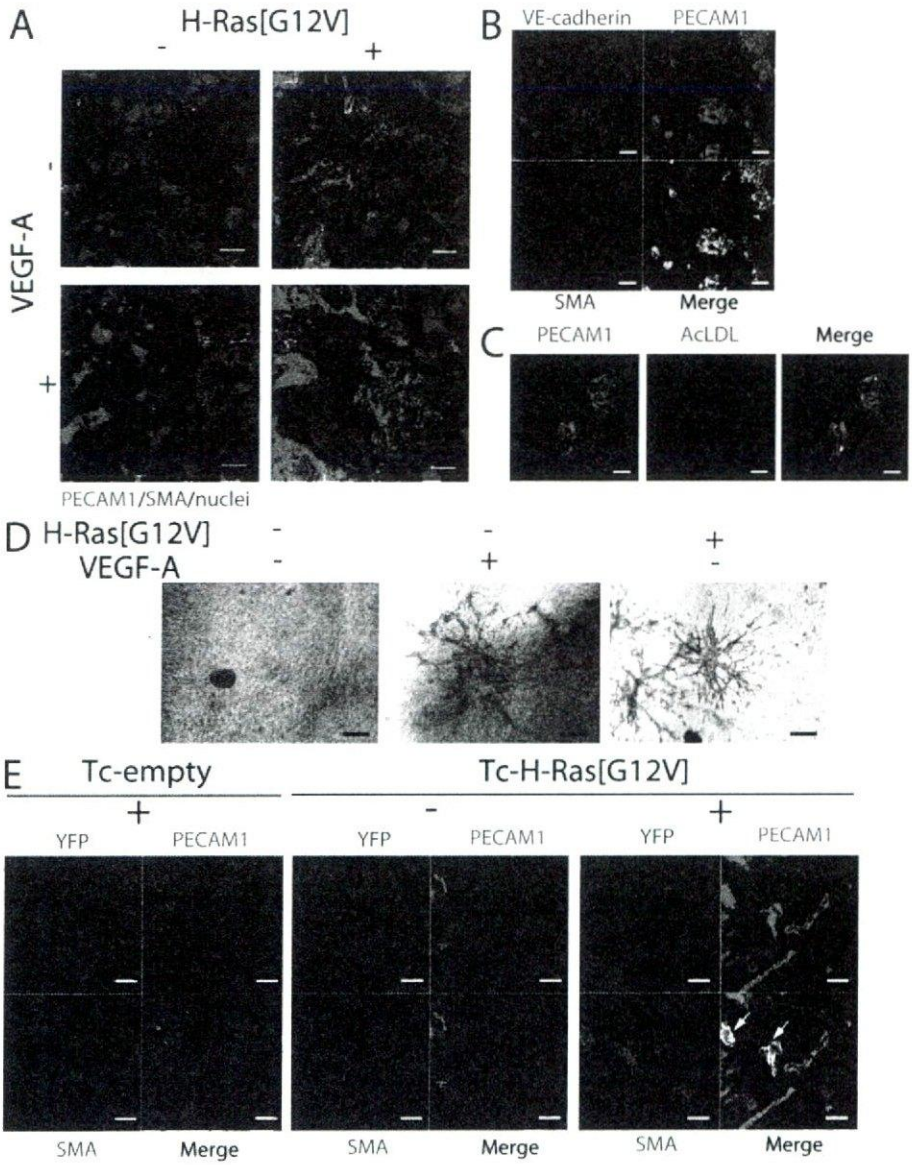

Figure 3. **Induction of PECAM1$^+$ cells by H-Ras[G12V].** (A) Differentiation of VEGFR2$^+$ Tc-H-Ras[G12V] cells in which constitutively active form of Ras is induced. Tc-H-Ras[G12V] ESCs were cultured on type IV collagen-coated dishes in the absence of LIF for 4 d during which expression of H-Ras[G12V] was suppressed by addition of Tc. ESC-derived VEGFR2$^+$ cells were then sorted and further cultured for 2 d with or without 1 μg/ml Tc and/or 30 ng/ml VEGF-A, followed by immunostaining for PECAM1 (green), αSMA (red), and nuclei (blue). Bars, 100 μm. (B) Immunostaining of H-Ras[G12V]–induced PECAM1$^+$ cells for VE-cadherin. PECAM1, green; αSMA, blue; and VE-cadherin, red. Bars, 100 μm. (C) PECAM1 staining (green) and AcLDL uptake (red) of PECAM1$^+$ cells induced by H-Ras[G12V]. Bars, 50 μm. (D) Three-dimensional culture of Tc-H-Ras[G12V] cells. ESC-derived VEGFR2$^+$ cells were cultured for 12 h on Petri dishes with 1 μg/ml Tc and/or 30 ng/ml VEGF-A. Aggregates formed were suspended in type I collagen gel and cultured for 7 d in medium containing 1 μg/ml Tc and/or 30 ng/ml VEGF-A, followed by microscopic observation. Bars, 200 μm. (E) In vivo vascular formation assay. Tc-empty and Tc-H-Ras[G12V] cells were labeled with retrovirus encoding YFP. After in vitro differentiation, Tc-H-Ras[G12V] or Tc-empty cells were mixed with Matrigel and subcutaneously injected into 129svJ mice. In vivo suppression of transgene was maintained by adding 1 μg/ml Tc in Matrigel and supplementing drinking water with 2 mg/ml doxycycline. After 10 d, Matrigels containing ES-derived cells were picked out and frozen sectioned, followed by immunostaining for PECAM1 (green) and αSMA (red). Fluorescence of YFP is also shown (blue). +, transgene-induced condition (-Tc); -, transgene-suppressed condition (+Tc). Arrows indicate PECAM1$^+$ vessels covered with αSMA$^+$ cells. Bars, 20 μm.

Signaling for endothelial specification is mediated through the Ras-Erk pathway

To investigate whether Ras signaling is involved in cell fate determination, we next performed a limiting dilution assay (Fig. 4 A). When H-Ras[G12V] was expressed, the total number of colonies increased. PECAM1$^+$ colonies dramatically increased in number. Notably, αSMA$^+$ colonies decreased in number. These findings suggest that expression of active Ras leads to endothelial differentiation at the expense of mural differentiation. To confirm the causal relationship between Ras expression and endothelial differentiation, cells were immunocytochemically examined for Ras expression (Fig. 4 B). Cells that successfully expressed Ras at high levels were positive for PECAM1, whereas those that failed to express Ras were positive for αSMA. These findings suggest that expression of constitutively active Ras directs endothelial specification of VEGFR2$^+$ cells.

Ras signaling is known to induce the expression of VEGF-A (Rak et al., 1995; Grugel et al., 1995; Arbiser et al., 1997). It thus appeared possible that VEGF-A induced by signaling from H-Ras[G12V] directed differentiation of VEGFR2$^+$ progenitor cells to PECAM1$^+$ cells in the present experimental system. To exclude this possibility, we examined Ras-induced endothelial differentiation in the presence of SU5614 (Spiekermann et al., 2002), an inhibitor of VEGFR2 kinase, as well as VEGFR1 (Flt1)-Fc chimera protein, which competes with VEGFR2 for binding with VEGF-A. Ras-induced endothelial differentiation was not inhibited under these conditions (Fig. S3 B and not depicted). Furthermore, Ras-induced endothelial cells formed tube-like structure in the presence of SU5614 (Fig. S3 C). These findings suggest that differentiation depends primarily on intracellular signal transduction from Ras protein.

We next established ESC lines in which H-Ras effector mutants H-Ras[G12V, T35S] or H-Ras[G12V, Y40C] can be inducibly expressed to examine the signaling pathway mediating Ras-induced endothelial specification. H-Ras[G12V, T35S] and H-Ras[G12V, Y40C] preferentially activate the Raf–MEK–Erk and PI3K–Akt pathways, respectively (Joneson et al., 1996; Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1).

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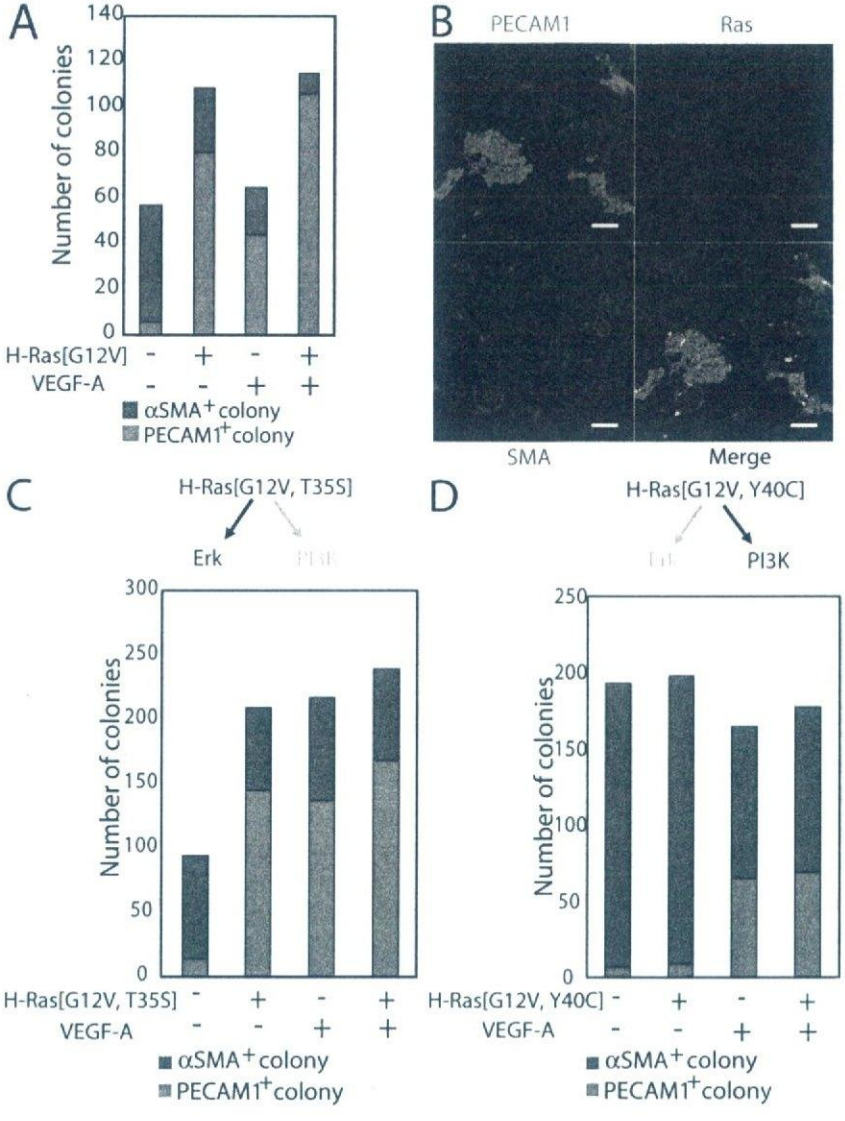

Figure 4. **Induction of endothelial differentiation by Ras–Erk signaling.** (A) Quantification of colony formation of Tc-H-Ras[G12V] cells. Representative results of three independent experiments are shown. (B) Cells overexpressing Ras were positive for PECAM1. ESC-derived VEGFR2$^+$ cells were cultured for 2 d in the absence of Tc and VEGF-A, followed by immunostaining for PECAM1 (green), αSMA (red), and Ras (blue). Bars, 100 μm. (C and D) Quantification of colony formation of Tc-H-Ras[G12V, T35S] (C) and Tc-H-Ras[G12V, Y40C] (D) cells. Representative results of three independent experiments are shown.

We performed a limiting dilution assay using these cells. When H-Ras[G12V, T35S] was expressed, PECAM1$^+$ colonies increased in number, whereas αSMA$^+$ colonies decreased (Fig. 4 C). In contrast, when H-Ras[G12V, Y40C] was expressed, numbers of PECAM1$^+$ colonies and αSMA$^+$ colonies were unchanged (Fig. 4 D). Additionally, among H-Ras[G12V, T35S] cells, those expressing Ras at high levels all differentiated into PECAM1$^+$ cells, whereas among H-Ras[G12V, Y40C] cells, those expressing Ras differentiated into either PECAM1$^+$ cells or αSMA$^+$ cells (unpublished data). These findings suggest that the Ras–PI3K pathway does not affect determination of cell fate. We concluded that the Ras–Erk pathway transmits signals required to specify endothelial differentiation of VEGFR2$^+$ progenitor cells.

The window of time within which Ras is specifically activated by VEGF-A

Ras proteins are known to be activated by various extracellular stimuli including cytokines and growth factors. In the present experimental system, ESC-derived VEGFR2$^+$ cells differentiate into endothelial cells upon stimulation with VEGF-A but not upon stimulation with PDGF-BB. Utilization of Ras by VEGFR2 appears to be different from that by PDGF receptors. In this respect, it is notable that FTI-277 was still effective in inhibiting endothelial differentiation when added 3 h after VEGF-A stimulation (Fig. 1 C). The specificity of Ras signaling induced by VEGFR2 can be attributed to the timing of Ras activation. We therefore investigated the window of time within which Ras protein is specifically activated by VEGF-A, focusing on the period more than 3 h after stimulation with VEGF-A. We first examined levels of phosphorylation of Erk, a downstream effector of Ras, 3–12 h after stimulation with VEGF-A (Fig. 5 A). Erk phosphorylation peaked at 6 and 9 h after stimulation, suggesting that Ras may be activated with a similar time course. We next examined activation of Ras in cells stimulated with VEGF-A or PDGF-BB for 6 h (Fig. 5 B). Activated Ras was detected by pulldown assay using the Raf-Ras binding domain. We found that Ras activation in response to VEGF-A or PDGF-BB was markedly different at 6 h after stimulation. VEGF-A caused intense activation of Ras and Erk, whereas PDGF-BB failed to activate both Ras and Erk. At 5 min after stimulation with VEGF-A, when VEGF-A efficiently activates Erk (Takahashi et al., 1999; Yashima et al., 2001), the levels of activation of Ras and

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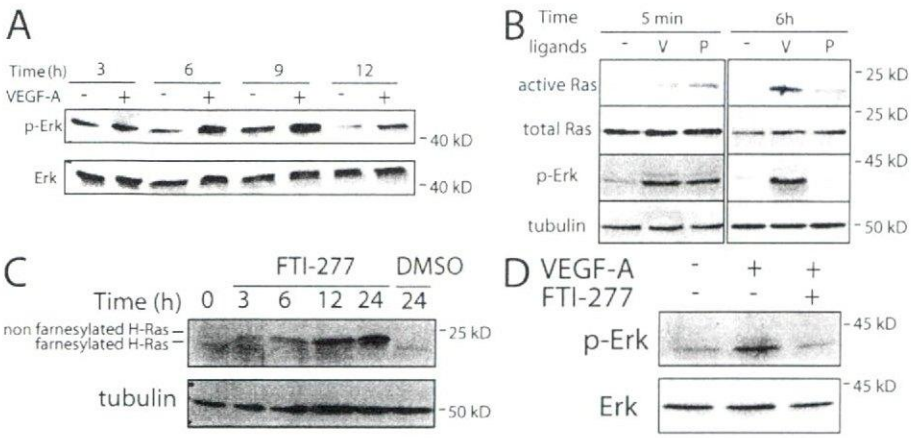

Figure 5. **Window of time within which Ras is specifically activated by VEGF-A.** (A) Time course of phosphorylation of Erk 3–12 h after stimulation with VEGF-A. VEGFR2$^+$ cells were stimulated with 30 ng/ml VEGF-A and lysed at the indicated time. Protein lysates were subjected to immunoblot analysis using anti–p-44/42 antibody and anti-Erk antibody. (B) Ras activation at 5 min and 6 h after stimulation with VEGF-A or PDGF-BB. VEGFR2$^+$ cells were stimulated with 30 ng/ml VEGF-A or 15 ng/ml PDGF-BB. At 5 min and 6 h after stimulation, cells were lysed and 1 mg of lysates was subjected to pulldown assay using a Ras activation assay kit (top). Residual lysates were subjected to immunoblot analysis (bottom three panels) using anti-Ras antibody, anti-p44/42 antibody, and anti-tubulin antibody (loading control). −, no ligand control cells; V, VEGF-A–stimulated cells; P, PDGF-BB–stimulated cells. (C) Time course of change in ratios of farnesylated and nonfarnesylated H-Ras after addition of FTI-277. 1 μM FTI-277 and 30 ng/ml VEGF-A were added to ESC-derived VEGFR2$^+$ cells. At 0, 3, 6, 12, and 24 h after addition, cells were lysed and lysates were subjected to immunoblot analysis using anti–H-Ras antibody. The top bands correspond to nonfarnesylated H-Ras and the bottom bands to farnesylated H-Ras. DMSO was used as vehicle control. Tubulin expression is shown as loading control. (D) Inhibitory effect of FTI-277 on phosphorylation of Erk. At 6 h after the addition of 1 μM FTI-277 and 30 ng/ml VEGF-A, cells were lysed and lysates were subjected to immunoblot analysis using anti-p44/42 and anti-Erk antibodies.

Erk were not notably different from those induced by PDGF-BB (Fig. 5 B). Activation of Ras and Erk by VEGF-A was also observed at 9 h but not at 3 h (unpublished data).

We next compared phosphorylation of Erk 3–12 h after stimulation with VEGF-A, PDGF-BB, FGF-2, and PlGF (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). Two ligands, PDGF-BB and PlGF, which lack ability to induce endothelial differentiation of VEGFR2$^+$ progenitor cells (Yamashita et al., 2000), failed to activate Erk during the period. FGF-2 that modestly supports endothelial differentiation of VEGFR2$^+$ progenitor cells (Kano et al., 2005), however, resulted in strong and sustained activation of Erk from 3 to 9 h after stimulation. These findings indicate that activation of Erk at late time points is specific for ligands that induce endothelial differentiation of VEGFR2$^+$ vascular progenitor cells. We also examined time course of phosphorylation of Erk after stimulation of human microvascular endothelial cells (HMECs) with VEGF-A (Fig. S5 B, available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). Erk was intensively activated 5–15 min after stimulation but not at later time points. These findings indicate that activation of Erk at later time points is not a common feature of VEGFR2 signaling.

We further examined the farnesylation status of H-Ras after treatment with FTI-277 (Fig. 5 C). Nonfarnesylated Ras began to increase 3 h after treatment and was constant after 6 h. Consistent with this finding, FTI-277 inhibited phosphorylation of Erk 6 h after VEGF-A-stimulation (Fig. 5 D). We thus confirmed that activation of Ras around 6 h after VEGF-A stimulation is sensitive to FTI-277. These findings suggest that activation of the Ras–Erk pathway 6–9 h after stimulation with VEGF-A directs endothelial specification of VEGFR2$^+$ progenitor cells.

VEGF-A-induced Ras activation precedes the expression of endothelial markers

We next examined the expression of vascular markers over time during in vitro differentiation of ESC-derived VEGFR2$^+$ cells by VEGF-A (Fig. 6 A). The expression of the endothelial markers PECAM1 and VE-cadherin began to increase from 12 h after stimulation with VEGF-A. Interestingly, the level of expression of VEGFR2 in VEGF-A–stimulated cells was similar to that in unstimulated cells up to 6 h after stimulation. During the period beyond 12 h after stimulation, VEGFR2 expression increased in VEGF-A–stimulated cells, whereas it decreased in nonstimulated cells. These findings suggest that endothelial specification occurs between 6 and 12 h after stimulation with VEGF-A, which is preceded by VEGF-A–induced Ras activation. Consistent with these findings, the level of expression of αSMA, a mural cell marker, began to increase later than 24 h. Genes up-regulated at 48 h after VEGF-A stimulation were analyzed by oligonucleotide microarray (Affymetrix) and listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). We observed induction of PECAM1 and VE-cadherin, as well as VEGFR2, e-NOS, Tie1, and other genes expressed in endothelial cells by treatment with VEGF-A.

We further determined expression of vascular markers after induction of H-Ras[G12V] (Fig. 6 B). mRNA for H-Ras[G12V] was detected at 3–6 h, followed by induction of PECAM1 and VE-cadherin later than 12 h. Earlier induction of these endothelial markers may be caused by the earlier onset of Ras signaling through expression of the constitutively active form. Up-regulation of VEGFR2 was, however, delayed. The reason for this delay remains to be elucidated.

We conclude that VEGF-A stimulation of VEGFR2$^+$ vascular progenitor cells specifically induces Ras–Erk activation around 6–9 h after stimulation, which in turn specifies endothelial differentiation.

Discussion

The development of multicellular organisms requires the orchestrated growth, migration, and differentiation of numerous cells. Various extracellular factors, as well as intracellular signaling molecules, are involved in the robust regulation of the behaviors

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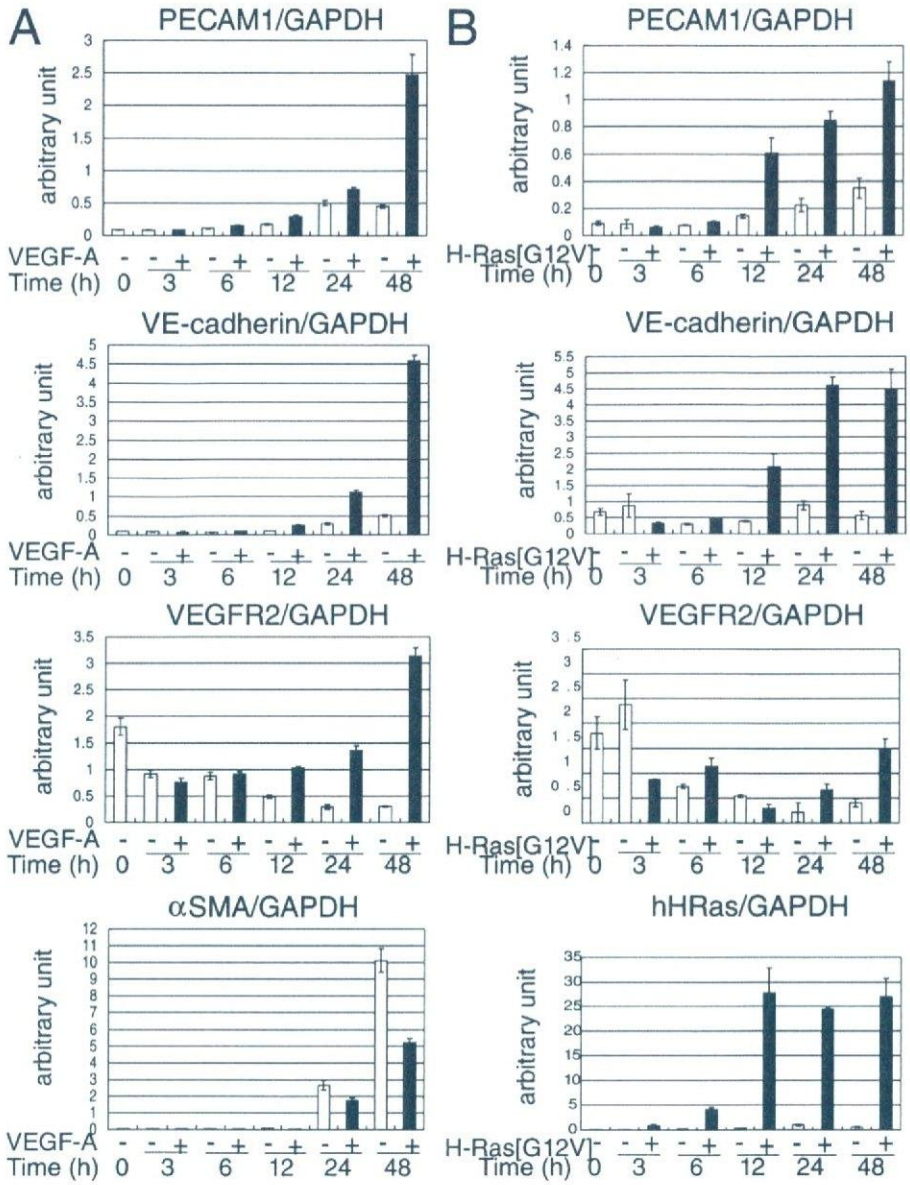

Figure 6. **Expression of vascular markers over time after in vitro differentiation of VEGFR2$^+$ cells.** Quantitative RT-PCR analysis of vascular markers of cells stimulated with 30 ng/ml VEGF-A (A) or H-Ras[G12V] (B) in the presence of serum. Each value is normalized to the expression of GAPDH. Error bars represent SD.

of cells during development. VEGFR2 signaling plays a central role in de novo blood vessel formation (vasculogenesis). In extraembryonic sites, VEGFR2 signaling is primarily required for the formation of blood islands (Shalaby et al., 1995), where vascular endothelial cells and hematopoietic cells differentiate to form primary plexuses. In the absence of VEGFR2 signaling, VEGFR2$^+$ progenitor cells fail to migrate to the extraembryonic sites (Shalaby et al., 1997). In the embryo proper, VEGFR2 signaling is required for endothelial specification of the vascular progenitor cells (Shalaby et al., 1997). Potential endothelial precursor cells are observed in the correct location where they would develop into embryonic blood vessels but fail to complete the pathway of differentiation. Recently, shear stress has been shown to induce differentiation of endothelial cells from progenitor cells (Yamamoto et al., 2003; Yamamoto et al., 2005), which is mediated through ligand-independent activation of VEGFR2 (Yamamoto et al., 2005). In embryoid body culture system in vitro, however, VEGFR2$^{-/-}$ ESCs still give rise to endothelial cells, though with low efficiency (Schuh et al., 1999). The endothelial differentiation observed in vitro may be caused by an effect of FGF-2, which was included in the culture medium (Schuh et al., 1999), because we previously found that FGF-2 supports endothelial differentiation of ESC-derived VEGFR2$^+$ cells to a modest extent (Kano et al., 2005). VEGFR2 signaling thus appears to be a pathway for endothelial specification of biological importance and high efficiency.

The roles of specific pathways downstream of VEGFR2 in mediating cell proliferation and migration have been elucidated. Phosphorylation of Y1175 of VEGFR2 leads to phospholipase C–γ activation, followed by PKC β–mediated Raf activation to induce cell proliferation (Takahashi et al., 2001). In contrast, phosphorylation of Y951 mediates signaling for cell migration and actin stress fiber organization through interaction with T cell–specific adaptor (Matsumoto et al., 2005). Phosphorylation of Y1214 is also implicated in actin stress fiber remodeling through the p38 pathway (Lamalice et al., 2004). However, which

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signaling pathway downstream of VEGFR2 is involved in endothelial specification has not been elucidated.

VEGF-A promotes the differentiation of endothelial cells from ESC-derived VEGFR2$^+$ cells, whereas PlGF, a specific ligand for VEGFR1, fails to induce endothelial differentiation (Yamashita et al., 2000). We have also reported that ectopically expressed VEGFR3 fails to transmit signal for endothelial differentiation of VEGFR2$^+$ progenitor cells (Suzuki et al., 2005). These findings suggest that VEGFR2 has unique features of signal transduction among VEGF receptor family members. In the present study, we unexpectedly found that Ras signaling downstream of VEGFR2 is involved in specifying endothelial differentiation of VEGFR2$^+$ vascular progenitor cells. We also found that the Raf–Erk pathway plays an important role downstream of Ras in endothelial specification. Interestingly, activation of Erk has been reported in blood islands of the E7.5 mouse embryo (Corson et al., 2003).

Ras signaling is known to act as a switch that determines cell fate in vulval formation in *Caenorhabditis elegans* (Sternberg and Han, 1998) and in photoreceptor development in *Drosophila melanogaster* (Wassarman et al., 1995). Ras is, however, activated by various extracellular stimuli in mammalian cells. ESC-derived VEGFR2$^+$ cells are differentiated into endothelial cells by VEGF-A, but not by PDGF-BB, although both ligands activate Ras in the cells. It will thus be important to determine how VEGFR2 transmits specific signals using an effector that is widely shared among different signaling pathways like Ras. In PC12 cells, EGF stimulation results in transient activation of Erk to induce cell proliferation, whereas NGF stimulation results in sustained activation of Erk to cause growth arrest and outgrowth of neurites (Marshall, 1995). Similarly, unique utilization of Ras by the VEGFR2 system likely accounts for the specific signaling to induce endothelial differentiation. In the present study, we found that Ras is specifically activated by VEGF-A around 6–9 h after stimulation. This delayed activation of Ras appears to transmit specific signaling for endothelial differentiation, which is consistent with the time course of FTI-277 sensitivity.

Usage of Ras by the VEGFR2 system differs in cells of various origins. In human aortic and umbilical vein endothelial cells as well as rat sinusoidal endothelial cells, activation of Ras by VEGF-A is modest. The PKC-dependent pathway, but not Ras, principally transmits the signal for Erk activation (Doanes et al., 1999; Takahashi et al., 1999; Yashima et al., 2001). In contrast, VEGF-A induces intense activation of Ras and Ras-mediated activation of Erk in HMECs (Yashima et al., 2001). These differential signaling properties may reflect the unique profiles of expression of signaling molecules in each type of cell. In our experiments using ESC-derived VEGFR2$^+$ progenitor cells, the PKC-dependent pathway appeared to be activated in the early phase because phosphorylation of Erk was notably increased but activation of Ras was modest 5 min after VEGF-A stimulation. In contrast, the Ras pathway was strongly activated to induce phosphorylation of Erk in the delayed phase (6–9 h after stimulation), a finding supported by the inhibition of Erk phosphorylation by FTI-277 (Fig. 5 D). The mechanism of this delayed activation of Ras remains to be elucidated in detail. It is possible that the activation is not direct and instead is mediated through transcriptional induction of certain signaling molecules. Notably, the delayed activation of Ras was not observed in mature endothelial cells, suggesting that it is not a common feature of VEGFR2 signaling.

In mature endothelial cells, Ras signaling appears to be involved in cell proliferation, tubule formation, and cell survival downstream of FGF receptor or integrin αv (Klint et al., 1999; Hood et al., 2003). However, the role of Ras downstream of VEGFR2 has been regarded as marginal (Shibuya and Claesson-Welsh, 2006). The present study is the first to suggest the crucial role of Ras–Erk signaling downstream of VEGFR2 in endothelial specification of vascular progenitor cells.

We examined vasculogenesis in allantoic explants obtained from E8.5 embryos and found reduced vascular formation in those from H-*ras*$^{-/-}$ (2 out of 12 embryos), whereas those from H-*ras*$^{+/+}$ (n = 16) or H-*ras*$^{+/-}$ (n = 26) embryos exhibited no such phenotype (unpublished data). We also examined vascular formation in H-*ras*$^{-/-}$ mice and found vascular aberration in the periphery of the brain of 73% of E9.5 H-*ras*$^{-/-}$ embryos. However, there was no obvious abnormality of E10.5 H-*ras*$^{-/-}$ embryos, which is consistent with the previous results that suggest H-*ras* knockout mice are born and grow normally (Ise et al., 2000; Esteban et al., 2001). These findings suggest that H-*ras*$^{-/-}$ embryos catch up for the delay in vascular formation in cephalic region until E10.5. One possibility is that expression of other members of the Ras family, N-Ras and K-Ras, is up-regulated and compensates for the loss of H-Ras as reported previously (Ise et al., 2000). Alternatively, reduction of endothelial differentiation in the absence of H-Ras may be permissive for embryonic development although quantitative assay in vitro exhibits substantial reduction (Fig. 1 B and Fig. 2 D). Compensatory growth of differentiated endothelial cells may offset reduction in endothelial differentiation. It appears likely that N-Ras and K-Ras are also involved in endothelial specification because treatment with FTI-277 that principally targets H-Ras or knockdown of H-Ras failed to completely inhibit endothelial specification induced by VEGF-A in the in vitro vascular differentiation assay.

In summary, we have demonstrated the involvement of Ras signaling in VEGFR2-mediated endothelial specification of vascular progenitor cells and provided novel insights into temporal aspects of signaling for cell lineage specification through widely shared effector molecules.

Materials and methods

Cells and cell culture

The CCE ESC line was obtained from M.J. Evans (University of Cambridge, Cambridge, UK) and MGZ5 and MGZRTcH ESC cells were obtained from H. Niwa (RIKEN Center for Developmental Biology, Kobe, Japan). Maintenance, differentiation, culture, and cell sorting of CCE, MGZ5, and MGZRTcH ESCs were performed as previously described (Yamashita et al., 2000). For in vitro differentiation, mouse ESCs were cultured on type IV collagen-coated dishes in the absence of leukemia inhibitory factor for 4 d. VEGFR2$^+$ vascular progenitor cells were then sorted and used for analysis of endothelial differentiation. We plated 2 × 10^4 ESC-derived VEGFR2$^+$ cells per well on type IV collagen-coated 8-well CultureSlides (IWAKI) for immunostaining or 0.6–1 × 10^3 cells per well on 1-well CultureSlides for limiting dilution assay. The cells were cultured for 2–4 d in α–minimum essential medium (Invitrogen) supplemented with 10% FBS in the presence of the following various ligands or inhibitors: VEGF-A (VEGF165; R&D Systems), Flt1-Fc chimera proteins (R&D Systems), PDGF-BB (PeproTech),

FGF-2 (R&D Systems), PlGF (R&D Systems), FTI-277 (EMD), and SU5614 (EMD). HMEC, an immortalized human dermal microvascular endothelial cell line, was obtained from T. Lawley (Emory University, Atlanta, GA) and was cultured in EGM-2 (Cambrex) containing 2% FBS and endothelial cell growth supplements (Clonetics).

Antibodies

For immunohistochemistry, monoclonal antibodies to murine VEGFR2 (AVAS12; BD Biosciences), PECAM1 (Mec13.3 [eBioscience] or 2H8 [Millipore]), VE-cadherin (11D4.1; BD Biosciences), CD34 (RAM34; BD Biosciences), endoglin (M17/18; BD Biosciences), αSMA (1A4; Sigma-Aldrich), and Ras (clone RAS10; Millipore) were used. Anti–β-catenin antibody was obtained from Santa Cruz Biotechnology, Inc. Secondary antibodies conjugated with Alexa 488, 594, or 647 anti–murine/rat IgG were obtained from Invitrogen, and HRP-conjugated anti–rat IgG and HRP-conjugated anti–Armenian hamster IgG were obtained from Invitrogen and Jackson ImmunoResearch Laboratories, respectively. TOTO3 iodide for nuclear staining was obtained from Invitrogen. For immunoblot analysis, antibodies to Ras (clone RAS10; Millipore), H-Ras (EPITOMICS), p-44/42 (Cell Signaling Technology), Erk (Millipore), p-Akt (Cell Signaling Technology), Akt (Cell Signaling Technology), and tubulin (Sigma-Aldrich) were used.

Immunohistochemistry

Whole-mount staining of embryos and yolk sacs was performed as described previously (Hogan et al., 1994), and microscopy was performed using a microscope (MZ6; Leica) with 5× objectives (Leica 10411589). Staining of culture cells was performed as described previously (Kano et al. 2005). For staining of AcLDL in endothelium, we used Alexa Fluor 594–conjugated AcLDL (Invitrogen) in accordance with the manufacturer's protocol. Stained cells were photographed using a confocal microscope (LSM510 META; Carl Zeiss, Inc.) with 10× objectives (Plan-Neofluar 0.3 NA) and LSM Image Browser (Carl Zeiss, Inc.). All images were taken at room temperature.

Ex vivo whole-embryo culture

Embryos were dissected out of the deciduum and placed in 500 µl DME containing 50% Rat IC serum (Charles River Laboratories), 5 mM of non-essential amino acids, 50 mM sodium pyruvate, and 27.5 mM 2-mercapto-ethanol, preequilibrated at 37°C with 5% CO_2. Embryos were cultured at 37°C with 5% CO_2 and analyzed. FTI-277 (dissolved in DMSO) was used at 10 µM. The concentration of DMSO was set at 0.1% in all cultures.

Mice

H-*ras*$^{+/-}$ mice (Ise et al., 2000) backcrossed into the C57BL/6J background were used. Mice were allowed to mate naturally at night. E0.5 was considered to be noon on the day the vaginal plug was observed. Embryos were genotyped by PCR analysis using yolk sacs as a DNA source as previously described (Ise et al., 2000). All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo.

Establishment and differentiation of ESC lines in which H-Ras is knocked down with inducible miRNA

We used the Block-iT Pol II miR RNAi expression system (Invitrogen) in MG-ZRTcH ESCs (Fig. S1 A; Masui et al., 2005). Stable ESC clones (Tc-miR-H-Ras) were established by transfecting pPthC-EmGFP-miRNA-H-Ras into MGZRTcH ESCs as described previously (Masui et al., 2005). Negative control cells (Tc-miR-NTC) were also established. For endothelial differentiation assay, ESCs were cultured in the absence of Tc for the last 2 d of in vitro differentiation to induce expression of miRNA. VEGFR2$^+$ cells were then sorted and used for limiting dilution assay. Results were confirmed in at least two independent cell lines.

Establishment of ESC lines inducibly expressing H-Ras [G12V], H-Ras [G12V, T35S], or H-Ras [G12V, Y40C]

Tc-H-Ras[G12V], Tc-H-Ras[G12V, T35S], Tc-H-Ras[G12V, Y40C], and Tc-empty cells were established as previously described (Masui et al., 2005; Mishima et al., 2007). cDNAs for H-Ras [G12V], H-Ras [G12V, T35S], and H-Ras [G12V, Y40C] mutants were described previously (Yoshida-Koide et al., 2004). Results were confirmed in at least two independent cell lines.

Three-dimensional culture

ESC-derived VEGFR2$^+$ cells were cultured for 12 h on Petri dishes with 1 µg/ml Tc and/or 30 ng/ml VEGF-A. Aggregates formed were suspended in type I collagen gel and cultured for 7 d in medium containing 1 µg/ml Tc and/or 30 ng/ml VEGF-A, followed by microscopic observation. In some of the samples, SU5614 was added. Collagen gels were photographed using microscopy (IX70; OLYMPUS) with 10× objectives (UPlanFl; 0.3 NA), at room temperature.

In vivo vascular formation assay

All ESCs were labeled with YFP retrovirus before in vivo vascular formation assay to distinguish cells of ESC origin and host origin. ESCs were cultured on type IV collagen-coated dishes in the absence of leukemia inhibitory factor for 4 d. Then 10^7 cells were pelleted and mixed with 100 µl PBS and 100 µl Matrigel and injected subcutaneously into the abdominal region of 4-wk-old male 129svJ mice. In vivo suppression of transgene was maintained by adding 1 µg/ml Tc in Matrigel and supplementing drinking water with 2 mg/ml doxycycline. The mice were killed on day 10, and the plaques were harvested and fixed with formalin. They were then frozen sectioned and stained with anti-PECAM1 and αSMA antibodies. Stained sections were photographed using a confocal microscope (LSM510 META) with 40× oil objectives (Plan-Neofluar; 1.3). All images were taken at room temperature.

Ras activation assay and immunoblot analysis

ESC-derived VEGFR2$^+$ cells (6×10^6) were stimulated with 30 ng/ml VEGF-A or 15 ng/ml PDGF-BB. Cells were harvested at the indicated time points and lysed. The cell lysates were subjected to pulldown assay using Raf-RBD (Ras activation assay kit; Millipore). The precipitated GTP-bound Ras was detected by anti-Ras antibody. Immunoblot analysis was performed as described previously (Suzuki et al., 2005). Image processing and storage (TIFF format) was performed using Photoshop software (Adobe).

RNA isolation, quantitative RT-PCR, and oligonucleotide microarray analysis

Culture of VEGFR2$^+$ cells with 10% FBS in the absence or presence of VEGF-A cells was used as a source of RNA. Total RNA was prepared with RNeasy (QIAGEN), according to the manufacturer's instructions, and reverse-transcribed with the SuperScript III first-strand synthesis system (Invitrogen). Expression of various markers of differentiation was compared by quantitative RT-PCR analysis. Primer sequences are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1). For oligonucleotide microarray analysis, GeneChip Mouse Genome 430 2.0 Arrays (Affymetrix) were used according to the manufacturer's instruction.

Online supplemental material

Fig. S1 shows Tc-regulated inducible expression of premiRNA in ESCs. Fig. S2 shows Tc-regulated inducible expression of H-Ras[G12V] in ESCs. Fig. S3 shows effects of pharmacological inhibitors on the induction of PECAM1$^+$ cells and tubule formation by H-Ras[G12V]. Fig. S4 shows Tc-regulated inducible expression of Ras effector mutants in ESCs. Fig. S5 shows time course of phosphorylation of Erk in ESC-derived VEGFR2$^+$ cells and HMECs after ligand stimulation. Table S1 shows genes induced by VEGF-A treatment of ESC-derived VEGFR2$^+$ cells for 48 h. Table S2 shows primers used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709127/DC1.

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Inhibition of endogenous TGF-β signaling enhances lymphangiogenesis

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Lymphangiogenesis is induced by various growth factors, including VEGF-C. Although TGF-β plays crucial roles in angiogenesis, the roles of TGF-β signaling in lymphangiogenesis are unknown. We show here that TGF-β transduced signals in human dermal lymphatic microvascular endothelial cells (HDLECs) and inhibited the proliferation, cord formation, and migration toward VEGF-C of HDLECs. Expression of lymphatic endothelial cell (LEC) markers, including LYVE-1 and Prox1 in HDLECs, as well as early lymph vessel development in mouse embryonic stem cells in the presence of VEGF-A and C, were repressed by TGF-β but were induced by TGF-β type I receptor (TβR-I) inhibitor. Moreover, inhibition of endogenous TGF-β signaling by TβR-I inhibitor accelerated lymphangiogenesis in a mouse model of chronic peritonitis. Lymphangiogenesis was also induced by TβR-I inhibitor in the presence of VEGF-C in pancreatic adenocarcinoma xenograft models inoculated in nude mice. These findings suggest that TGF-β transduces signals in LECs and plays an important role in the regulation of lymphangiogenesis in vivo. (Blood. 2008;111:4571-4579)

Introduction

Transforming growth factor-β (TGF-β) is a multifunctional cytokine, which regulates the growth, differentiation, migration, adhesion, and apoptosis of various types of cells. TGF-β binds to 2 different types of serine-threonine kinase receptors, known as type II (TβR-II) and type I (TβR-I).[1-3] Upon ligand binding, TβR-II transphosphorylates TβR-I, which in turn transmits specific intracellular signals. The type I receptors phosphorylate receptor-activated Smads (R-Smads), and induce complex formation between R-Smads and common-partner Smad (co-Smad). The R-Smad/co-Smad complexes accumulate in the nucleus, where they regulate transcription of target genes, including plasminogen activator inhibitor-1 (PAI-1) and Smad7, through interaction with various transcription factors and transcriptional coactivators. Smad2 and Smad3 are R-Smads activated by TβR-I, whereas Smad4 is the only co-Smad in mammals shared with the TGF-β family signaling pathways. Smad7 is an inhibitory Smad, which interacts with activated TβR-I and interferes with the phosphorylation of R-Smads by TβR-I.

TGF-β inhibits the growth and migration of blood vascular endothelial cells in vitro, whereas it induces angiogenesis in vivo.[4] Mice lacking certain components of TGF-β signaling (eg, TGF-β1, TβR-II, or TβR-I) exhibit abnormalities in blood vascular tissues.[5-7] We have recently shown that inhibition of TGF-β signaling by low-dose TβR-I inhibitor decreases the coverage of endothelium by pericytes, promoting leakiness of tumor blood vessels.[8] In tumor microenvironments, TGF-β signaling has also been reported to inhibit immune function and induce deposition of extracellular matrix proteins, inducing progression of tumors in advanced cancers.[9] However, the relationship between TGF-β signaling and lymphangiogenesis has not been determined.

Various growth factors have been reported to be involved in lymphangiogenesis. These include vascular endothelial growth factor (VEGF)-C and D, hepatocyte growth factor, and angiopoetin-1 and -2.[10-15] Neo-lymphangiogenesis has also been reported to be induced by receptor tyrosine kinases, for example, fibroblast growth factor receptor 3 (FGFR3) and platelet-derived growth factor receptor (PDGFR)-β.[13,16] Among members of the VEGF family, VEGF-A transmits signals through the tyrosine kinase receptors VEGF receptor-2 (VEGFR2) and VEGFR1, which mediate signals that are required for vasculogenesis and hematopoiesis.[17,18] VEGF-C and VEGF-D bind to VEGFR3 expressed on lymphatic vessels and mediate signals to LECs, although they also bind to VEGFR2.[19] Analysis of *Vegfc*-null mice has revealed that VEGF-C is essential for normal development of the lymphatic vessels.[20] Moreover, VEGF-C has been found to be expressed in various human cancers and to induce tumor metastasis through induction of angiogenesis and lymphangiogenesis.[21,22] VEGF-D also promotes metastatic spread of tumors through induction of lymphangiogenesis.[11]

Among transcription factors, Prox1, a homeobox transcription factor, has been well known as an important regulator of lymphangiogenesis. During embryonic development, LECs arise by sprouting from the cardinal veins and migrate toward VEGF-C to form the primary lymphatic plexus.[23] Prox1 is expressed in a subset of endothelial cells of the cardinal vein during embryonic development, and these cells sprout to form the primary lymphatic plexus.[24,25] In *Prox1*-null mice, migration of LECs from the veins is arrested, leading to a complete absence of the lymphatic vasculature. Prox1 induces the expression of various LEC markers, including VEGFR3, LYVE-1, podoplanin, and integrin α_9, and

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represses that of blood vascular endothelial cell markers (eg, VEGFR2 and VE-cadherin) in endothelial cells.[26-28]

In the present study, we show that TGF-β regulates the growth, migration, and cord formation of human dermal lymphatic microvascular endothelial cells (HDLECs) in vitro. In addition to inhibiting the proliferation and migration of HDLECs, TGF-β signaling suppressed the expression of LEC-related genes, including Prox1 and LYVE-1, in these cells. Moreover, inhibition of endogenous TGF-β signaling induced early lymph vessel development in mouse embryonic stem (ES) cells, and enhanced lymphangiogenesis in a mouse chronic peritonitis model and pancreatic cancer xenograft models. The present findings suggest that endogenous TGF-β signaling negatively regulates lymphangiogenesis in inflammatory tissues as well as in certain tumor tissues.

Methods

Cell culture and reagents

HDLECs were obtained from Cambrex (Walkersville, MD), and cultured in EGM2-MV medium containing endothelial cell growth supplements with 5% fetal bovine serum (FBS; Cambrex). The murine ES cell line R1 (a kind gift of A. Nagy, Mount Sinai Hospital, Toronto, ON)[29] was cultured on mitomycin C-arrested mouse embryonic fibroblasts (Chemicon International, Temecula, CA) in stem cell medium (Knock-out DMEM, Invitrogen, Carlsbad, CA) supplemented with 15% FBS, 2 mM of L-glutamine (Invitrogen), 0.1 mM of 2-mercaptoethanol (Invitrogen), 0.1 mM of MEM nonessential amino-acids (Invitrogen), 50 U/mL of penicillin-streptomycin (Invitrogen), and 1000 U/mL of leukemia inhibitory factor (Chemicon International), and passaged every 48 hours. Inflammatory macrophages were collected from ascites fluid in BALB/c mice 4 days after induction of peritonitis by intraperitoneal injection of thioglycollate (enriched thioglycollate medium, 2 mL; BD Biosciences, Franklin Lakes, NJ). BxPC3 and MIA PaCa-2 human pancreatic adenocarcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). BxPC3 cells were grown in RPMI 1640 supplemented with 10% FBS. MIA PaCa-2 cells were grown in DMEM with 10% FBS. Overexpression of VEGF-C in BxPC3 cells and that of TGF-β1 in MIA PaCa-2 cells were done using a lentiviral infection system (a kind gift from H. Miyoshi, RIKEN, Tsukuba, Japan). cDNA encoding VEGF-C,[30] active form of TGF-β1,[31] or green fluorescent protein (GFP) was inserted into the multicloning site of the lentiviral vector construct, pCSII-CMV-RfA, using pENTR according to standard protocol (Invitrogen). TβR-I inhibitors LY364947 and SB431542 were purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich (St Louis, MO), respectively. LY364947 was used as a TβR-I inhibitor, unless specifically described. TGF-β1 and TGF-β3 were purchased from R&D Systems (Minneapolis, MN). Cycloheximide was from Sigma-Aldrich. VEGF-A and VEGF-C were purchased from R&D Systems and Calbiochem, respectively.

Antibodies

Antibodies to LYVE-1, Prox1, and podoplanin were purchased from Abcam (Cambridge, United Kingdom), Chemicon International, and Research Diagnostic (Flanders, NJ), respectively. PECAM1 antibody and Mac-1 antibody were from BD PharMingen (Franklin Lakes, NJ). Rat anti–mouse LYVE-1 antibody was a kind gift from Y. Oike and T. Suda (Keio University, Tokyo, Japan). Antibody to phospho-Smad2 was a kind gift from A. Moustakas and C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). Antibodies to Smad2/3 and tubulin were from BD Transduction Laboratories (Franklin Lakes, NJ) and Sigma-Aldrich, respectively. Alexa488- and Alexa594-conjugated secondary antibodies and TOTO-3 were purchased from Invitrogen.

Immunoblotting

Cultured cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40 (Nacalai Tesque, Kyoto, Japan), 5 mM EDTA, 0.5% deoxycholic acid sodium salt-monohydrate (Nacalai Tesque), 0.1% sodium dodecyl sulfate (SDS, Nacalai Tesque), 1% aprotinin (Mitsubishi Pharma, Osaka, Japan), and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). The cell lysates were boiled in SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromophenol blue, 36% glycerol, 4% SDS, 10 mM dithiothreitol) and subjected to SDS-PAGE. Proteins were electrotransferred to PALL FLUOROTRANS W membranes (PALL, East Hills, NY), immunoblotted with antibodies, and detected using an ECL detection system (GE Healthcare, Little Chalfont, United Kingdom).

Immunostaining

Cultured cells were fixed in iced 1:1 acetone-methanol solution and incubated with antibody to Prox1 overnight at 4°C. Subsequently, cells were incubated with Alexa488-conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature and stained with TOTO-3 for nuclear staining. Frozen sections were briefly fixed with Mildform 10N (WAKO, Osaka, Japan), and incubated with anti-LYVE-1, anti-Prox1, or anti-podoplanin antibodies. Subsequently, samples were incubated with secondary antibodies, and stained with TOTO-3 for nuclear staining. Stained cells and frozen sections were observed with a confocal microscope (Model LSM510 META; Carl Zeiss MicroImaging, Thornwood, NJ). Images were imported into Adobe Photoshop and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Enzyme-linked immunosorbent assay

Expression levels of TGF-β1 protein were determined using TGF-β1 human ELISA kit Quantikine 2nd Generation (R&D Systems), according to manufacturer's protocol. TGF-β3 at 1 ng/mL was used as the exogenous TGF-β ligand to avoid complication in detection of TGF-β1 by ELISA. Induction of TGF-β1 in HDLECs was similar between TGF-β1 and TGF-β3 at 1 ng/mL.

RNA isolation and quantitative RT-PCR

Total RNAs from HDLECs were extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNAs were synthesized using the Quantitect Reverse Transcription kit (Qiagen) with random hexamer primers. Quantitative real-time reverse transcriptase–polymerase chain reaction (RT-PCR) analysis was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sequences used were as follows: human GAPDH: forward 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse 5′-GAAGATGGTGATGGGATTTC-3′, human Prox1: forward 5′-CCCAGGACAGTTTATTGACCG-3′, reverse 5′-GGTTGTAAGGAGTTTGGCCCA-3′, human LYVE-1: forward 5′-AGCCTGGTGTTGCTTCTCACT-3′, reverse 5′-GGTTCGCCTTTTTGCTCACA-3′, human Smad7: forward 5′-CCTTAGCCGACTCTGCGAACTA-3′, reverse 5′-CCAGATAATTCGTTCCCCCTGT-3′, human PAI-1: forward 5′-GGCTGACTTCACGAGTCTTTC-3′, reverse 5′-GCGGGCTGAGACTATGACA-3′, human TGF-β1: forward 5′-AGTGGACATCAACGGGTTCAG-3′, reverse 5′-CATGAGAAGCAGGAAAGGCC-3′, human TGF-β2: forward 5′-CTGTCCCTGCTGCACTTTTGTA-3′, reverse 5′-TGTGGAGGTGCCATCAATACCT-3′, human TGF-β3: forward 5′-TGGAAGTGGGTCCATGAAACCTA-3′, reverse 5′-GATGCTTCAGGGTTAAGAGTGTTG-3′, and human/mouse VEGF-C: forward 5′-TTCCTGCCGATGCATGTCTAA-3′, reverse 5′-TGTTCGCTGCCTGACACTGT-3′.

Cell growth and cord formation assays

HDLECs (2×10^3 cells) were seeded in 96-well plates, and cell growth was quantified for 2 days by WST-1 assay (Nacalai Tesque) according to manufacturer's protocol. Formation of cord-like structures was determined in 3-dimensional gel assays, where 10^4 cells were mixed with type I-A collagen gel (Nitta Gelatin, Osaka, Japan) and seeded onto culture slide.

The formation of cord-like structures was examined using video microscopy for 3 days. At the end of observation, the lengths of the cord-like structures were quantified in the 3-dimensional assay as follows: 4 microscopic fields with 5 Z-axis planes in each condition were photographed, and the total lengths of cord-like structures were quantified as the mean of the sum of the lengths in the 5 planes in each of the 4 microscopic fields. Long-running video microscopic analysis of living cells on the 8-well culture slides (BD Falcon) was performed using a Leica (Deerfield, IL) DM IRB microscope equipped with a hardware-controlled motor stage. The video images were analyzed using ImageJ software. For quantification of images, 8 to 10 fields were evaluated for each mouse.

Cell migration assay

Migration of HDLECs was determined using a Boyden chamber (8 μm pore size, BD Biosciences) with type I collagen coat. HDLECs (5×10^4) were seeded in serum-free medium containing 0.2% BSA in the upper chamber. VEGF-C (50 ng/mL) was added as the chemoattractant to the lower chamber, while TGF-β1 at 1 ng/mL or TβR-I inhibitor at 3 μM was added to the upper chamber. After a 6-hour incubation, cells in the upper chamber were carefully removed using cotton buds and cells at the bottom of the membrane were fixed and stained with 0.5% crystal violet in 20% methanol. Quantification was performed by counting the stained cells in triplicate.

Three-dimensional collagen assays of mouse ES cells

Three-dimensional collagen assays using mouse ES cells were performed as described previously.[32,33] Briefly, R1 ES cells were trypsinized, resuspended in stem cell medium without leukemia inhibitory factor supplemented with 30 ng/mL VEGF-A at day 0. The cells were then cultured in drops hanging. After 4 days, when ES cells aggregated to form embryoid bodies, drops were collected and embryoid bodies were seeded on a layer of solidified collagen type I solution (Nitta Gelatin), and a second layer of collagen solution was added on top. After 3 hours, medium with or without 30 ng/mL VEGF-A and 30 ng/mL VEGF-C was added. Medium containing VEGF-A and C was replaced every second day. TβR-I inhibitor at 3 μM or TGF-β1 at 1 ng/mL was added every second day for one week, started from one week after seeding. After culturing for 2 weeks, whole-mount samples were incubated with anti-LYVE-1, anti-PECAM1, anti-Prox1, and Alexa-conjugated secondary antibodies overnight. Samples were examined using a Zeiss LSM510 Meta confocal microscope for immunohistochemistry. Quantification of LYVE-1 stained areas was performed in 5 fields on 3 embryoid bodies.

Chronic peritonitis model

BALB/c mice of 4 to 5 weeks of age were obtained from Sankyo Laboratory (Tokyo, Japan). All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo. Chronic peritonitis was induced by 5% thioglycollate. Thioglycollate (2 mL) and TβR-I inhibitor (LY364947, 1 mg/kg) were administered intraperitoneally to BALB/c mice 3 times a week. After injections of these reagents into mice for 2 weeks, the mice were killed and the diaphragms were excised, fixed with Mildform 10N for 1 hour at room temperature and washed with sucrose buffer (dissolved in phosphate-buffered saline [PBS]). Whole-mount samples were subsequently incubated with anti-LYVE-1, anti-Mac-1, and Alexa594-conjugated secondary antibodies overnight. Samples were examined using a Zeiss LSM510 Meta confocal microscope for immunohistochemistry.

Cancer xenograft models

BALB/c nude mice 5 to 6 weeks of age were obtained from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory. Parental, or VEGF-C- or TGF-β1-expressing tumor cells (5×10^6) in 100 μL PBS were implanted subcutaneously into male nude mice and allowed to grow for 2 to 3 weeks to reach proliferative phase, before initiation of TβR-I inhibitor administration. TβR-I inhibitor LY364947, dissolved in 5 mg/mL in DMSO and diluted with 100 μL PBS, or the vehicle control, was injected intraperitoneally at 1 mg/kg, 3 times a week for 3 weeks. Excised samples were directly frozen in dry-iced acetone for immunohistochemistry. Frozen samples were further sectioned at 10-μm thickness in a cryostat and subsequently incubated with primary and secondary antibodies as described above. Samples were observed using a confocal microscope.

Results

TGF-β transduces signals in HDLECs

To study the effects of TGF-β on lymphangiogenesis, we first examined whether TGF-β transduces signals in HDLECs.[34] Specific small molecule inhibitors of TGF-β family type I receptor kinases ALK-4, -5, and -7 (TβR-I inhibitors, LY364947 and SB431542[35,36]) were used to suppress endogenous TGF-β family signaling. Immunoblot analysis using phospho-Smad2 antibody revealed weak phosphorylation of Smad2 in untreated HDLECs, and that 1 ng/mL of TGF-β1 enhanced the phosphorylation of Smad2 in these cells (Figure 1A). In contrast, 3 μM of LY364947 and SB431542 decreased the basal and TGF-β1-induced Smad2 phosphorylation.

The transcription induced by TGF-β in HDLECs was further examined by quantitative real-time (RT)-PCR analysis. TGF-β1 induced transcription of *SMAD7* and *PAI1*, and the TβR-I inhibitors strongly suppressed their transcription (Figure 1B,C), suggesting that TGF-β transduces signals in HDLECs and TβR-I inhibitors suppress the signals induced by endogenous TGF-β. We also examined the effects of TGF-β1 (1 ng/mL) and LY364947 and SB431542 (3 μM) on proliferation of HDLECs. TGF-β1 suppressed the growth of HDLECs, whereas TβR-I inhibitors enhanced their proliferation in the presence and absence of TGF-β1 (Figure 1D and data not shown). These findings indicate that HDLECs respond to TGF-β signals similarly to human umbilical vein endothelial cells (HUVECs; Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Next, production of TGF-β by HDLECs was examined at mRNA and protein levels. TGF-β induced the expression of TGF-β1 mRNA, whereas the TβR-I inhibitors suppressed it in the presence and absence of TGF-β1 (Figure 1E). Expression of TGF-β2 or TGF-β3 mRNA was not significantly suppressed by the TβR-I inhibitors (Figure S2). Moreover, ELISA revealed that HDLECs produce TGF-β1 protein at a level similar to that produced by HUVECs, and the secretion of TGF-β1 was stimulated and suppressed by exogenous TGF-β and TβR-I inhibitor, respectively (Figure 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 (Figure 2A,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 (Figure 2A). When the cells were treated with TGF-β1 for

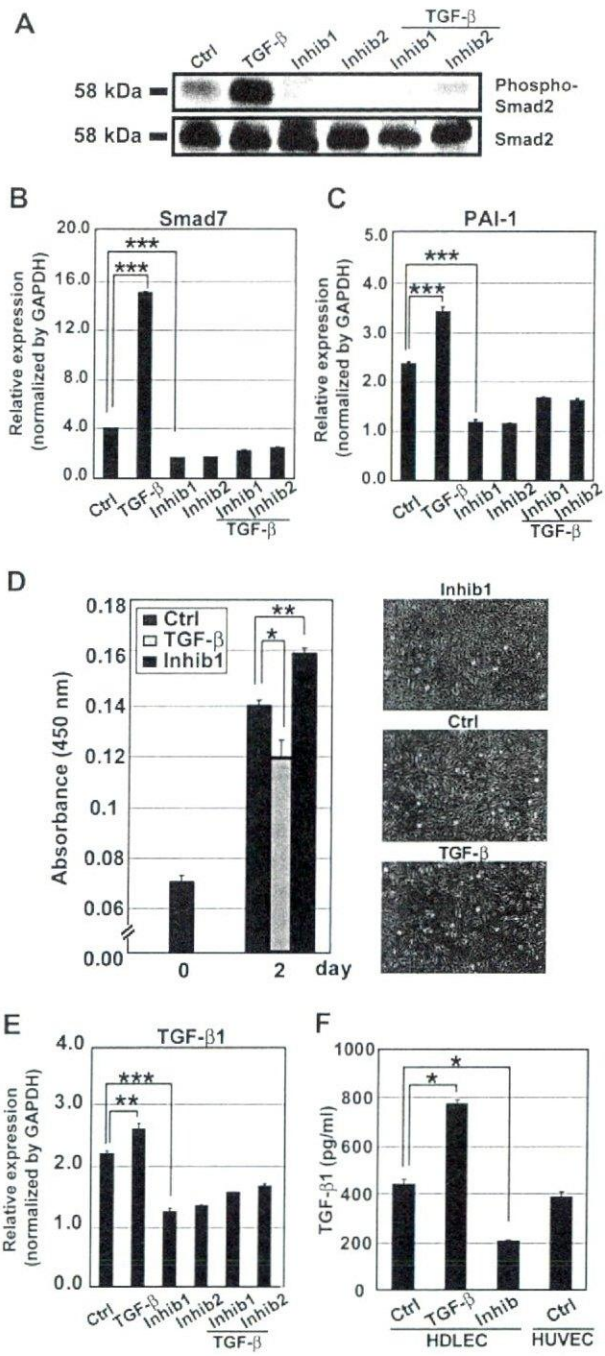


Figure 1. Transduction of TGF-β signals in HDLECs. (A) Immunoblotting of phospho-Smad2 after TGF-β or TβR-I inhibitor treatment. HDLECs were treated with TGF-β1 or with 2 kinds of TβR-I inhibitors (LY364947 or SB431542, shown as Inhib1 or Inhib2, respectively) in the presence and absence of TGF-β1 for 1 hour, and subjected to immunoblot analysis using phospho-Smad2 antibody (top panel) and Smad2/3 antibody (bottom panel). Ctrl indicates control. (B,C) Real-time PCR of Smad7 and PAI-1. HDLECs were treated as in panel A, and expression of Smad7 and PAI-1 mRNAs was determined at 1 hour and 24 hours after stimulation, respectively (***$P < .001$). (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 (*$P < .05$, **$P < .01$). (E,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 panel A was determined by real-time PCR (E). Production of TGF-β1 protein by HDLECs treated as in panel 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 (*$P < .05$, **$P < .01$, ***$P < .001$).

24 hours, 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 hours. Immunoblot analysis using anti-Prox1 antibody confirmed the results of immunocytochemistry (Figure 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 hours, and levels of expression of Prox1 and LYVE-1 mRNAs were determined by quantitative RT-PCR (Figure 2C,D). TGF-β1 suppressed the expression of Prox1 and LYVE-1, whereas LY364947 strongly induced their expression. Similar results were obtained by another TβR-I inhibitor SB431542 (Figure S3A,B). The induction of Prox1 and LYVE-1 by TβR-I inhibitor was abolished by suppression of protein synthesis with cycloheximide treatment of HDLECs, whereas that of PAI-1 was not suppressed (Figure 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 up-regulated (Figure S3C,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 (Figure 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 toward VEGF-C (50 ng/mL) was determined after 6-hour 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 (Figure 3B,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 (Figure 4A,B). Immunostaining of Prox1 in PECAM1-positive areas also increased with TβR-I inhibitor and decreased with TGF-β1 (Figure 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. Because chronic inflammation is

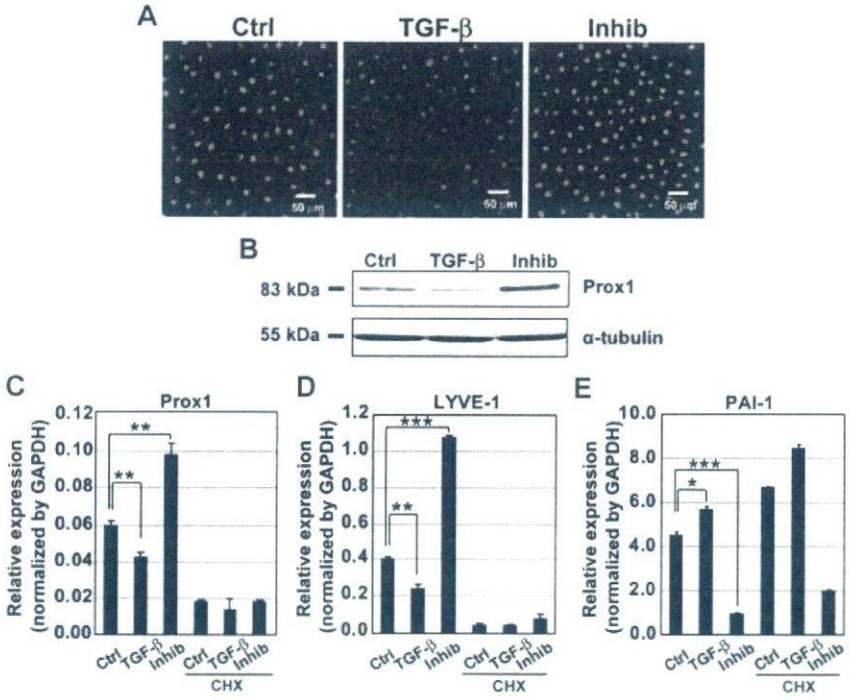


Figure 2. TGF-β signaling regulates the expression of LEC-related genes in HDLECs. (A) Expression levels of Prox1 determined by immunostaining in HDLECs. HDLECs were untreated (left) or treated with TGF-β1 (1 ng/mL; middle) or TβR-I inhibitor LY364947 (3 μM; right) for 24 hours and subjected to immunocytochemical examination. Bars represent 50 μm. (B) Expression levels of Prox1 in HDLECs treated as described in panel 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 hours after TGF-β1 or TβR-I inhibitor treatment. In the right 3 columns, cells were treated with 1 μM cycloheximide (CHX) for 24 hours before they were treated with TGF-β1 or TβR-I inhibitor for 24 hours. Values were normalized to amounts of GAPDH mRNA. Error bars represent SD (*$P < .05$, **$P < .01$, ***$P < .001$).

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) 3 times a week for 2 weeks. Lymphangiogenesis in the plaques of the diaphragms was then examined by immunostaining using LYVE-1 antibody. Formation of lymphatic plaques was observed after 2 weeks in the mice treated with thioglycollate, and TβR-I inhibitor significantly increased the LYVE-1-positive areas in the plaques (Figure 5A,B).

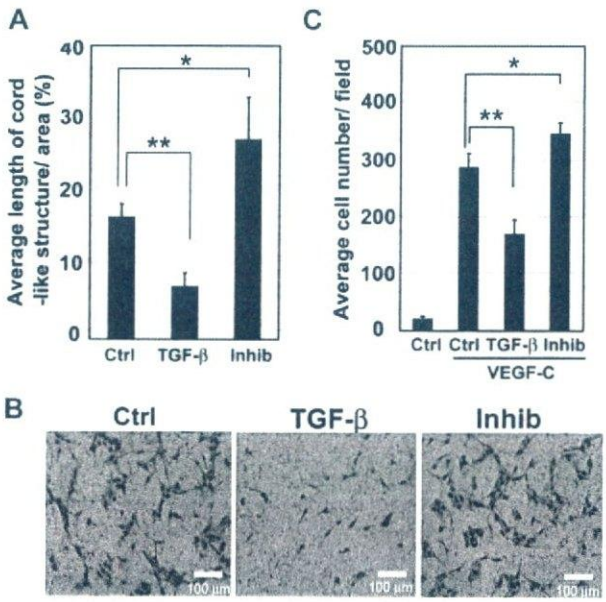


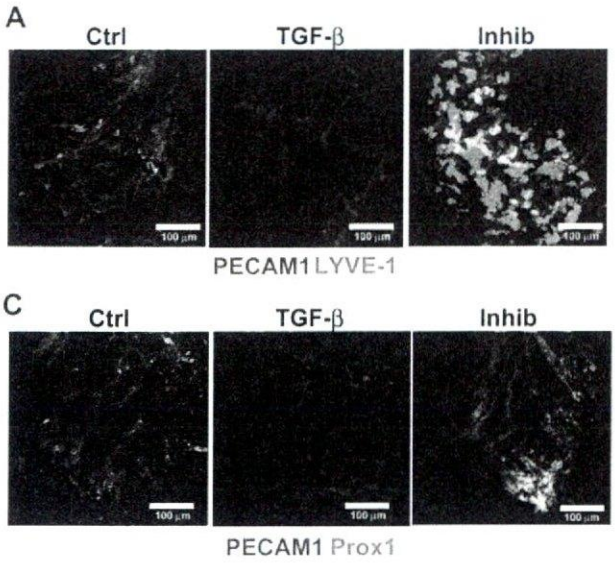
Figure 3. Formation of cord-like structures and migration of HDLECs are increased by inhibition of TGF-β signaling. (A) Total lengths of cord-like structures of HDLECs in three-dimensional culture were quantified. Cells were mixed with type I collagen gel at a density of 10^4 cells/well and seeded onto 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 SD (*$P < .05$, **$P < .01$). Effects of TGF-β signaling on migration of HDLECs were determined by Boyden chamber assay. Cells were seeded at 4×10^4 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, whereas 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 hours. Bars represent 100 μm. (C) Migration of HDLECs was quantified. Cells that had migrated to the lower chambers were counted after 6 hours in triplicate. Error bars represent SD (*$P < .05$, **$P < .01$).

Because 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 Figure 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 animal models of cancer

Because 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 TβR-I inhibitor LY364947 3 times a week for 3 weeks.

When the BxPC3 cells were mixed with or without 1 μg/mL 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 (Figure 6A,B). Z-stack



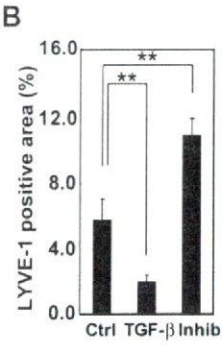


Figure 4. Enhancement of early lymph vessel development in ES cells cultured in 3-dimensional collagen by inhibition of TGF-β signaling. Mouse R1 ES cells were cultured in collagen gel with VEGF-A (30 ng/mL) for the first 4 days and subsequently with VEGF-C (30 ng/mL) and VEGF-A (30 ng/mL) for 14 days 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 represent 50 μm. Quantification of LYVE-1–stained areas was performed in 5 low-magnification microscopic fields on 3 embryoid bodies (B). Error bars represent SD (***$P < .001$). (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.

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). 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 costained by Prox1 and podoplanin antibodies (Figure 6C), whereas most PECAM1-positive cells were not stained by LYVE-1 or Prox1 antibodies (Figure 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 *Vegfc*, and an increase in the production of VEGF-C mRNA by the VEGF-C-lentivirus-infected cells was confirmed by quantitative RT-PCR (Figure 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 (Figure 6F).

The effects of TβR-I inhibitor on lymphangiogenesis were also examined using MIA PaCa-2 cells with or without 1 μg/mL VEGF-C protein. As shown in Figure 7, 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 (Figure S4).

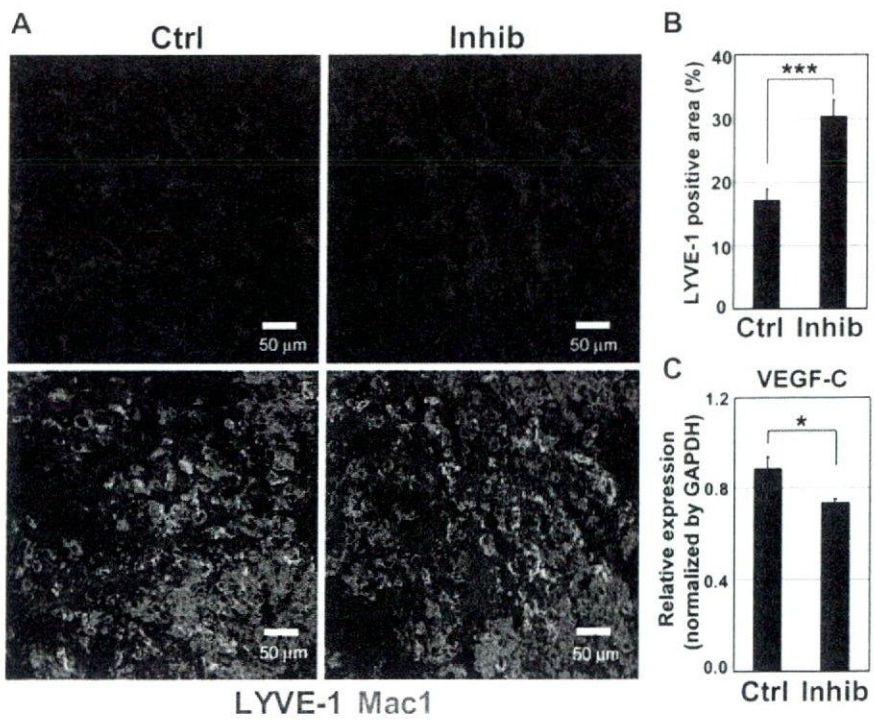


Figure 5. Enhancement of lymphangiogenesis by TβR-I inhibitor in a mouse model of chronic peritonitis. Mice were treated with 5% thioglycollate (2 mL) and TβR-I inhibitor (LY364947, 1 mg/kg) 3 times a week for 2 weeks. Their diaphragms were then examined for lymphangiogenesis in plaques. (A) LYVE-1 immunostaining (shown in red) of diaphragms treated without (control, left panels) or with TβR-I inhibitor (right panels). Sections were also stained for Mac1 (green) (bottom panels). Bars represent 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 SE (***$P < .001$). (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 4 days after induction of peritonitis by intraperitoneal injection of thioglycollate, seeded at 10^6, and treated with or without TβR-I inhibitor for 24 hours. Error bars represent SD (*$P < .05$).

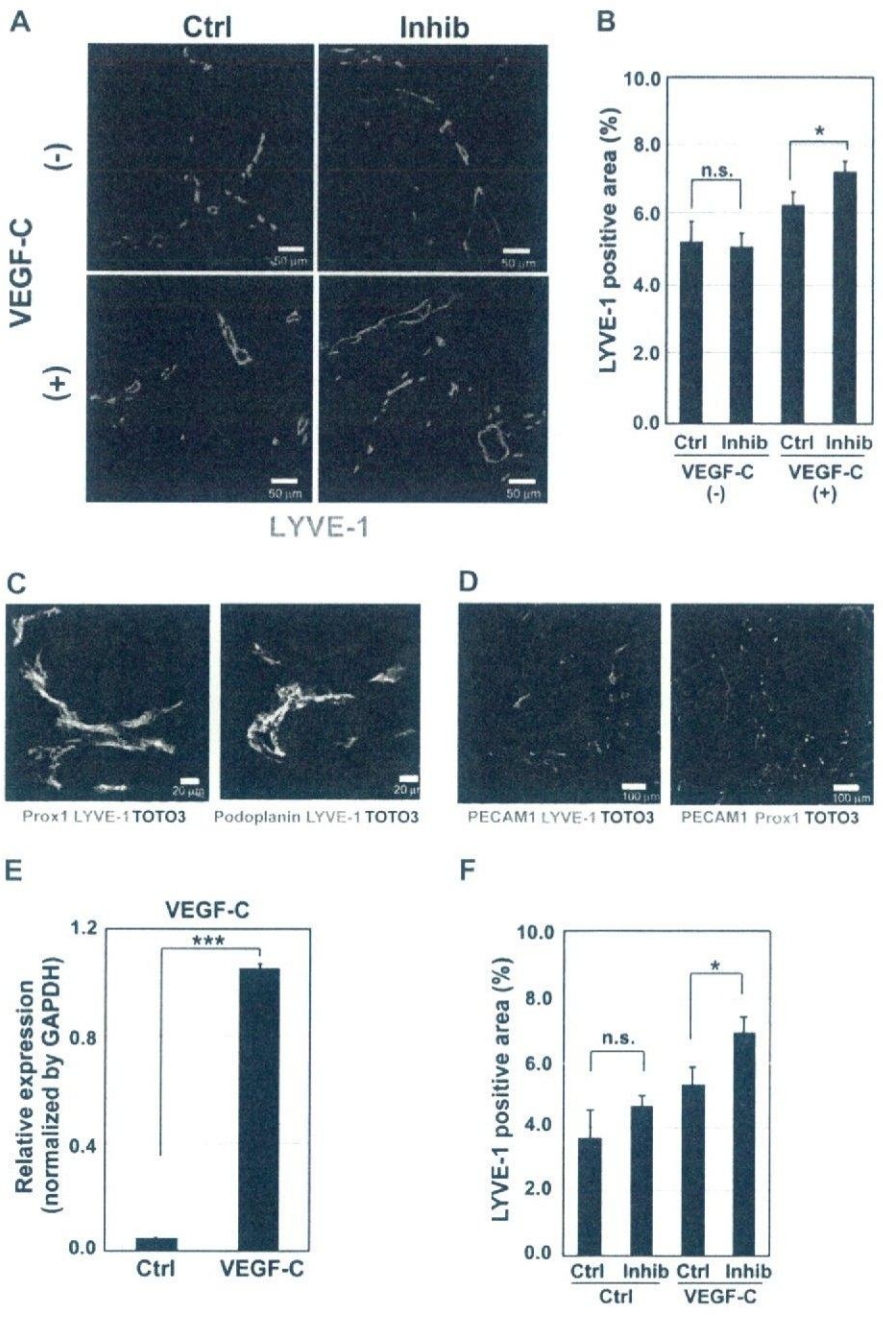


Figure 6. Lymphangiogenesis is increased by TβR-I inhibitor in pancreatic adenocarcinoma BxPC3 xenograft models. (A-D) Effects of TβR-I inhibitor on lymphangiogenesis were examined in a xenograft model using a human pancreatic cancer cell line, BxPC3. BxPC3 cells mixed with or without VEGF-C (1 μg/mL) 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) 3 times a week for 3 weeks. They were killed 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 represent 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 SE. n.s. indicates not significant (*$P < .05$). (C) Immunostaining of BxPC3 xenograft sections for Prox1 (left panel, red), podoplanin (right panel, red), and LYVE-1 (green). Bars represent 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 represent 100 μm. (E,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) Up-regulation 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 SE. n.s. indicates not significant (***$P < .001$).

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 toward 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 up-regulated 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 up-regulates the expression of various LEC-specific genes and

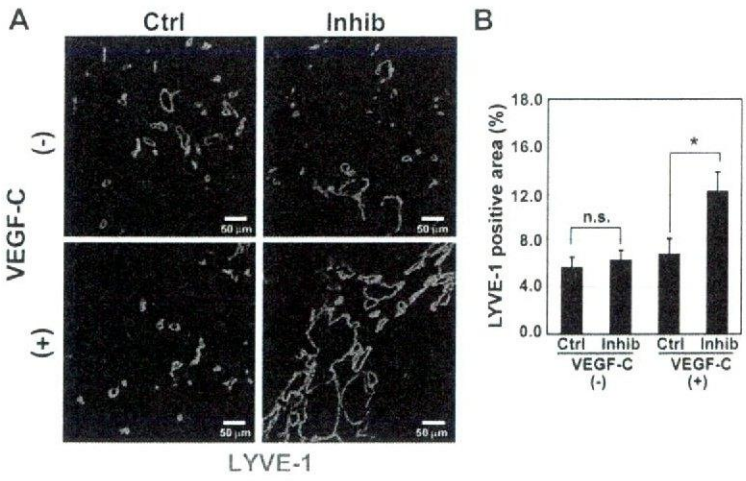


Figure 7. Induction of lymphangiogenesis by TβR-I inhibitor in MIA PaCa-2 xenograft models. (A,B) Effects of TβR-I inhibitor on lymphangiogenesis were examined in a xenograft model using a TGF-β–nonresponsive human pancreatic cancer cell line, MIA PaCa-2. MIA PaCa-2 cells mixed with or without VEGF-C (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 represent 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 SE. n.s. indicates not significant (*$P < .05$).

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] whereas interleukin-3 signaling has been reported to induce expression of Prox1 in dermal blood endothelial cells.[41] Infection by Kaposi 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 (eg, macrophages) and LECs could be observed in the plaques.[43] Some LECs were positive for Ki-67, 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 that 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 antitumor immune responses, are known to be functionally inhibited by TGF-β signaling.[45] 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 antitumor 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.

Acknowledgments

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