TGF-β receptor kinase inhibitor enhances growth and integrity of embryonic stem cell–derived endothelial cells

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Recent findings have shown that embryonic vascular progenitor cells are capable of differentiating into mural and endothelial cells. However, the molecular mechanisms that regulate their differentiation, proliferation, and endothelial sheet formation remain to be elucidated. Here, we show that members of the transforming growth factor (TGF)-β superfamily play important roles during differentiation of vascular progenitor cells derived from mouse embryonic stem cells (ESCs) and from 8.5–days postcoitum embryos. TGF-β and activin inhibited proliferation and sheet formation of endothelial cells. Interestingly, SB-431542, a synthetic molecule that inhibits the kinases of receptors for TGF-β and activin, facilitated proliferation and sheet formation of ESC-derived endothelial cells. Moreover, SB-431542 up-regulated the expression of claudin-5, an endothelial specific component of tight junctions. These results suggest that endogenous TGF-β/activin signals play important roles in regulating vascular growth and permeability.

Introduction

Embryonic vessels are thought to be formed by endothelial cells that arise from Flk1-expressing (Flk1+) mesoderm cells, surrounded by mural cells derived from mesoderm, neural crest, or epicardial cells. This embryonic paradigm, referred to as vasulogenesisis, contrasts with angiogenesis, the term applied to the post-natal neovascularization that results from the proliferation and remodelling of differentiated endothelial cells from preexisting capillaries (Risau, 1997). Recently, embryonic stem cell (ESC)–derived vascular progenitor cells have been shown to differentiate into both mural and endothelial cells (Yamashita et al., 2000; Marchetti et al., 2002; Ema et al., 2003). Moreover, bone marrow–derived vascular progenitor cells circulating in adult peripheral blood have been shown to include progenitor cells giving rise to both types of cells (Asahara et al., 1997; Sata et al., 2002), and to contribute to angiogenesis. Endothelial cell tyrosine kinase receptors for VEGFs have been implicated in the regulation of differentiation, growth, and integrity of endothelial cells. VEGF stimulates endothelial differentiation of vascular progenitor cells derived from ESC (Yamashita et al., 2000) and adult bone marrow (Asahara et al., 1997). VEGF also regulates growth of differentiated endothelial cells, and increases vascular permeability (Senger

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**Abbreviations used in this paper:** ALK, activin receptor-like kinase; BBB, blood-brain barrier; BMP, bone morphogenetic protein; dpc, days postcoitum; ESC, embryonic stem cell; PECAM1, platelet-endothelial cell adhesion molecule 1; R-Smad, receptor-regulated Smad; SMA, α-smooth muscle actin; TJ, tight junction.
et al., 1983). Although these properties of VEGF are of great importance in the processes of vasculogenesis and angiogenesis, VEGF may contribute to tissue edema because angiogenesis is accompanied by an increase in vascular permeability followed by vessel sprouting.

VEGF acts exclusively on endothelial cells; however, interaction between endothelial cells and mural cells (pericytes and vascular smooth muscle cells) is essential for development of vascular tissues and maintenance of their homeostasis in both embryonic and adult tissues (Folkman and D’Amore, 1996). TGF-β superfamily signaling has been implicated as one of cytokines that serve such interaction (Carmeliet, 2000).

Members of the TGF-β superfamily signal via heteromeric complexes of type II and type I serine/threonine kinase receptors. Upon ligand binding, the constitutively active type II receptor kinase phosphorylates the type I receptor which, in turn, activates the downstream signal transduction cascades, including Smad pathways. Activins and TGF-β bind to type I receptors known as activin receptor-like kinase (ALK)-4 and -5, respectively. The activated type I receptors phosphorylate receptor-regulated Smad (R-Smad) proteins. Smad2 and 3 transduce signals for TGF-βs and activins, whereas Smad1, 5, and 8 are specific for signaling of bone morphogenetic proteins (BMPs; Massague, 1998). As an exception, ALK-1, specifically expressed in endothelial cells, binds TGF-β and activates Smad1/5 pathways (Oh et al., 2000).

The roles of the TGF-β superfamily in vasculogenesis have been suggested by the findings that knockout mice deficient in various TGF-β superfamily signaling components exhibit defects in vascular tissues (Goumans and Mummery, 2000). Moreover, heterozygous mutations in human genes coding for endoglin, an accessory protein for the TGF-β receptor complex, and/or ALK-1 cause hereditary hemorrhagic telangiectasia (Marchuk, 1998). However, the lack of in vitro systems consisting of pure populations of these cellular lineages has hampered dissection of the roles of TGF-β superfamily signaling in vascular development.

To elucidate the roles of TGF-β superfamily signaling in vascular differentiation from their progenitors, we used in vitro differentiation systems derived from mouse ESC and 8.5–days postcoitum (dpc) embryos. We show that TGF-β and activin inhibit proliferation and sheet formation of ESC-derived endothelial cells. The effects of TGF-β on endothelial integrity are at least partly mediated by its down-regulation of the expression of claudin-5, an endothelial specific component of tight junctions (TJs). Interestingly, SB-431542, a synthetic molecule that inhibits the kinases of receptors for TGF-β and activin, facilitated proliferation and sheet formation of ESC-derived endothelial cells. Finally, we show that TGF-β down-regulated the growth and integrity of endothelial cells derived from 8.5-dpc embryos, whereas SB-431542 exhibited the opposite effects. These results suggest that endogenous TGF-β/activin signals play important roles in regulating vascular growth and permeability.

**Results**

**TGF-β and activin inhibit formation of ESC-derived endothelial sheets**

According to a previous report (Yamashita et al., 2000), undifferentiated mouse ESCs were cultured for 3 d on collagen IV–coated dishes with 10% FCS to induce Flk1⁺ cells, which were purified by cell sorting using anti-Flk1 antibody. After an additional 3 d of culture of Flk1⁺ cells with 10% FCS, we obtained cells positive for a mural cell marker, α-smooth muscle actin (SMA; Fig. 1 a). When 30 ng/ml VEGF was added, we were able to obtain platelet-endothelial cell adhesion molecule 1 (PECAM1)–positive sheets of endothelial cells. The remaining cells surrounding the sheets were positive for SMA.

To examine the effects of TGF-β superfamily signals on ESC-derived in vitro vasculogenesis, we studied the expres-
sion of various TGF-β superfamily signaling components by semi-quantitative RT-PCR analysis (Fig. 2). ESC-derived Flk1+ cells (Flk1+), mural cells (MC), and mixed populations of endothelial and mural cells (EC + MC) expressed transcripts for most components of TGF-β superfamily signaling pathways, suggesting that they are capable of responding to BMPs, TGF-βs, and activins. Next, we examined the effects of TGF-β superfamily proteins on the in vitro vascular differentiation of ESC-derived Flk1+ cells. Although BMP7 did not exhibit significant effects, TGF-β and activin led to the decrease in PECAM1+ sheets of endothelial cells (Fig. 1 a). Moreover, the effects of TGF-β and activin on PECAM1+ sheets of endothelial cells were reversed by the addition of anti –TGF-β neutralizing antibody and follistatin, a natural extracellular inhibitor of activin, respectively.

Goumans et al. (2002) showed that the response of mouse embryonic endothelial cells to TGF-β is biphasic, with ALK-1-mediated positive effects on growth and migration at low doses (0.25–0.5 ng/ml) and ALK-5-mediated negative effects at high doses (2.5 ng/ml and higher). To examine whether TGF-β exhibits biphasic effects in ESC-derived vascular cells, different doses of TGF-β were added to the culture of Flk1+ cells in the presence of VEGF (Fig. 1 b). The formation of endothelial sheet was significantly inhibited at 0.03 ng/ml, and its inhibition reached a maximum at 1 ng/ml, which remained at this level at higher doses (Fig. 1 b). These results suggest that TGF-β does not exhibit biphasic effects in the ESC-derived endothelial sheet formation.

**TGF-β receptor kinase inhibitors enhance growth and integrity of ESC-derived endothelial cells**

Next, we attempted to enhance formation of endothelium by inhibiting endogenous TGF-β and activin signaling. First, we added anti–TGF-β neutralizing antibody, follistatin or both of them to culture containing VEGF in the absence of exogenous TGF-β or activin. Neither of the extracellular inhibitors exhibited significant effects on the formation of endothelial sheets induced by VEGF (Fig. 3 a).

Synthetic inhibitors of signaling kinases have proven to be extremely useful for investigating signal transduction pathways and are potentially of use for the treatment of various human diseases in which growth factor signals play important roles. Recently, synthetic compounds, e.g., SB-431542 (compound 14), that specifically inhibit the kinases of ALK-4, -5, and -7 (a type I receptor for Nodal) have been identified (Callahan et al., 2002; Inman et al., 2002). They have no effects on the other, more divergent ALK family type I receptors that bind BMPs, or on other kinases including ERK, JNK, and p38 MAPK. We tested the specificity of SB-431542 for kinase activities of seven mammalian type I receptors for the TGF-β superfamily (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200305147/DC1), and confirmed that SB-431542 is a specific inhibitor for ALK-4, -5, and -7.

When SB-431542 was added to ESC-derived Flk1+ cells in the presence of VEGF and TGF-β, it reversed the inhibition of formation of endothelial sheets by TGF-β in a dose-dependent manner (Fig. 3 b), suggesting that SB-431542 is capable of inhibiting the ALK-5 kinase activity in these cells. Next, we added SB-431542 to the ESC-derived in vitro vascular differentiation culture containing VEGF in the absence of exogenous TGF-β. Interestingly, the endothelial sheets were significantly enlarged, and formed a fine cobblestone-like structure (Fig. 3, c–e), which was observed in a dose-dependent manner (Fig. 3 c). Similar results were obtained with another ALK-4/5/7 kinase inhibitor, compound 13 (Callahan et al., 2002; unpublished data). These results suggest that inhibition of ALK-4/5/7 kinase activities modifies the growth and sheet formation of endothelial cells.

**Quantitative analyses of the effects of TGF-β signals on proliferation, differentiation, and integrity of ESC-derived endothelial cells**

TGF-β has been shown to have strong growth inhibitory effects on endothelial cells (Baird and Durkin, 1986). To examine the effects of TGF-β on the proliferation of differentiating vascular progenitor cells, we added TGF-β or SB-431542 to the Flk1+ cells in the presence of 10% FCS and VEGF, and determined the change in cell number after 3 d (Fig. 3, c–e). TGF-β resulted in a reproducible decrease in cell proliferation, whereas SB-431542 induced an increase in proliferation, suggesting that TGF-β signals inhibit the growth of differentiating vascular progenitor cells. To examine whether this growth inhibitory effect of TGF-β signals is on endothelial cells or mural cells, we added TGF-β or SB-431542 to the Flk1+ cells in the presence of 10%
FCS without VEGF, and determined the change in cell number after 3 d (Fig. 4 b and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200305147/DC1). Addition of TGF-β/H9252 resulted in a reproducible decrease in the proliferation of mural cells, whereas SB-431542 induced an increase in proliferation, suggesting that TGF-β/H9252 signals inhibit the growth of ESC-derived endothelial and mural cells.

To further dissect the roles of TGF-β in the differentiation of Flk1+ cells into endothelial and mural cells, we performed quantitative analyses using a limiting dilution assay. When Flk1+ cells were plated at a lower density, they formed individual colonies, and the total number of colonies varied depending on the culture conditions. TGF-β decreased the number of colonies, whereas SB-431542 strongly increased it (Fig. 4 c).

Next, we evaluated the phenotypes of the colonies by immunohistochemical analysis. Culturing Flk1+ cells with 10% FCS resulted in colonies of a single type consisting of pure mural cells positive for SMA. VEGF induced four types of colonies emerging from single Flk1+ cells: PECAM1+ pure endothelial cells with or without sheet structure (EC-sheet and -scattered, respectively); pure mural cells (MC); and mixtures of both (Mix; Fig. 4 c). The frequency of pure endothelial cell colonies (EC-scattered and -sheet) was 25%. Addition of TGF-β or SB-431542 reproducibly decreased (22%) or increased (33%) this frequency, respectively, suggesting that TGF-β signals modify the balance of differentiation from vascular progenitor cells.

Although endothelial cells were observed in the presence and absence of TGF-β signals, formation of endothelial sheets was significantly affected (Fig. 4 c). The frequency of sheet formation among pure endothelial colonies was 60% when single Flk1+ cells were cultured in the presence of FCS and VEGF. When TGF-β was added, most endothelial colonies exhibited scattered phenotypes, whereas 86% of endothelial colonies formed sheet structures when SB-431542 was added (Fig. 4 c). In contrast, neither TGF-β or SB-431542 altered the integrity of the mural cell colonies (Fig. S2). Together with the finding that addition of SB-431542 increased the total number of vascular cells colonies, these results suggest that inhibition of endogenous TGF-β signals results in enhancement of endothelial sheet formation.
To examine the activity of endogenous TGF-β/H9252 ESC-derived vascular cells, we analyzed phosphorylation of Smads in ESC-derived primary endothelial and mural cells. Stimulation of mixed populations of endothelial and mural cells with TGF-β induced Smad2 phosphorylation at low doses (0.025–0.5 ng/ml) of TGF-β and reached a maximum at 0.25 ng/ml, which remained at this level at higher doses, whereas ALK-5 kinase activity is required for ALK-1–mediated phosphorylation of Smad1/5 in ESC-derived endothelial cells. Although TGF-β induced Smad2 phosphorylation in mural cells (Fig. 5 a, lane 10), it failed to phosphorylate Smad1/5. BMP led to phosphorylation of Smad1/5 in mural cells (Fig. 5 a, lane 11). These results suggest that TGF-β induced phosphorylation of both Smad2 and Smad1/5 only in ESC-derived endothelial cells.

To confirm that Smad1/5 as well as Smad2 is activated by TGF-β in ESC-derived endothelial cells, nuclear translocation of Smad proteins was studied using an antiphospho-Smad1/5 antibody and an anti-Smad2 antibody. TGF-β induced nuclear translocation of Smad2, whereas BMP failed to do it (Fig. 5 c). In agreement with the results of Western blot analysis, both TGF-β and BMP induced nuclear translocation of phosphorylated Smad1/5 in ESC-derived endothelial cells (Fig. 5 c).

When SB-431542 was added in the presence of TGF-β or activin, Smad2 phosphorylation was decreased in a dose-dependent manner because of its inhibitory effect on ALK-5 and -4, respectively (Fig. 5 a, lane 4 and 8, respectively; Fig. 5 d). We found that Smad1/5 phosphorylation induced by TGF-β was also decreased by SB-431542 (Fig. 5 a, lane 4). Because SB-431542 is not capable of inhibiting Smad phosphorylation by ALK-1 (Fig. S1), these results suggest that ALK-5 kinase activity is required for ALK-1–mediated phosphorylation of Smad1/5 in ESC-derived endothelial cells. Inhibition of Smad1/5 phosphorylation by SB-431542 is specific for ALK-1 because Smad1/5 phosphorylation by BMP was not decreased by SB-431542 (Fig. 5 a, lane 6).

Importantly, phosphorylation of Smad2 was weakly detected in the absence of exogenous ligands (Fig. 5 a, lane 1), suggesting that endogenous TGF-β or activin (Fig. 2)
Figure 5. TGF-β induced phosphorylation and nuclear translocation of Smad2 and Smad1/5 in ESC-derived endothelial cells. (a) Phosphorylation of R-Smads by TGF-β superfamily members in ESC-derived endothelial and mural cells. Flk1+ cells were cultured with 10% FCS and VEGF for 3 d to induce differentiation into endothelial and mural cells, and treated with TGF-β or BMP7 in the absence or presence of SB-431542 for 1 h. Mural cells differentiated from Flk1+ cells with 10% FCS were treated with TGF-β for 1 h. Cell lysates were directly subjected to immunoblotting using phospho-Smad2 (top), and phospho-Smad1/5 (bottom) antibodies. (b) Dose response of TGF-β and phospho-Smad1/5 (bottom) immunoblotting using phospho-Smad2 (top) antibodies. Flk1+ cells were untreated (left) or treated with TGF-β for 1 h. Cell lysates were directly subjected to immunoblotting using phospho-Smad2 antibodies. Flk1+ cells were cultured with 10% FCS and VEGF for 3 d to induce differentiation into endothelial and mural cells, and treated with different concentrations of TGF-β for 1 h. (c) Nuclear translocation of R-Smads by TGF-β and BMP in ESC-derived endothelial cells. Endothelial cells differentiated from Flk1+ cells by VEGF in serum-free culture (Yamashita et al., 2000) were untreated (left) or treated with TGF-β (middle) or BMP7 (right) for 1 h, and subjected to immunofluorescence staining using phospho-Smad2 (top) and phospho-Smad1/5 (bottom) antibodies (green) and observation by confocal microscopy. (Insets) Immunofluorescence staining of the same fields for PECAM1 (red). Bars, 200 μm. (d and e) Dose response effects of SB-431542 on Smad2 phosphorylation induced by exogenous or endogenous TGF-β. Flk1+ cells were cultured with 10% FCS and VEGF for 3 d to induce differentiation into endothelial and mural cells, and treated with different concentrations of SB-431542 for 1 h in the (d) presence or (e) absence of exogenous TGF-β.

TGF-β regulates integrity of ESC-derived endothelial cells through claudin-5 expression

Next, we attempted to elucidate the molecular mechanisms by which endogenous TGF-β signals regulate the integrity of endothelial sheets. Integrity of cell–cell contacts is vital for functioning of endothelial cells as barriers and fences between the blood and extravascular components. Endothelial cell–cell junctions such as TJJs are thought to control not only normal physiological conditions such as vascular permeability, leukocyte extravasation, and the formation and outgrowth of blood vessels, but also pathological conditions such as vasogenic edema (Morita et al., 1999).

Because TGF-β signals regulate ESC-derived endothelial sheet formation (Fig. 3 b), we searched for the molecular targets of ALK-5 signals. Recently, we conducted oligonucleotide microarray analysis to identify target genes of ALK-1 and -5 in human umbilical vein endothelial cells (Ota et al., 2002). One of the genes down-regulated by constitutively active ALK-5 was claudin-5, a component of TJ expressed exclusively in endothelial cells (Morita et al., 1999).

To study the effects of TGF-β signals on claudin-5 expression in ESC-derived endothelial cells, we performed quantitative RT-PCR analysis against mural cells obtained in the absence of VEGF, and mixed populations of endothelial and mural cells differentiated in the presence of VEGF (Fig. 6 a). Claudin-5 expression was detected in the populations containing endothelial cells. TGF-β suppressed the expression of claudin-5, whereas SB-431542 strongly enhanced its expression (Fig. 6 a). Interestingly, TGF-β signals did not alter the expression of other components of TJJs such as claudin-12 (Fig. 6 a; Tsukita et al., 2001), or VE-cadherin (a component of adherens junctions; unpublished data), suggesting that effects of TGF-β signals on endothelial integrity may be primarily mediated by transcriptional regulation of claudin-5.

The above results were confirmed by immunofluorescence staining of claudin-5 (Fig. 6 b). Claudin-5 was concentrated at cell–cell borders of PECAM1+ endothelial cells (Fig. 6 b, top). When these endothelial cells were treated with TGF-β, expression of claudin-5 was decreased in accordance with the disruption of endothelial sheet structure, and claudin-5 was not detected at cell–cell borders (Fig. 6 b, middle). When these endothelial cells were treated with SB-431542, claudin-5 expression was strongly increased with the enhancement of endothelial sheet formation (Fig. 6 b, bottom).

TGF-β regulates formation of endothelial sheets derived from 8.5-dpc mouse embryos

To examine the roles of TGF-β signals during vascular development in mice, we tested the effects of TGF-β and SB-431542 on the differentiation of Flk1+ cells obtained from 8.5-dpc mouse embryos. When embryo-derived Flk1+ cells were cultured with 10% FCS, >95% cells became positive for SMA (Fig. 7 a). When 100 ng/ml VEGF was added, we
were able to obtain PECAM1-positive sheets of endothelial cells. When TGF-β was added to the culture in the presence of VEGF, it inhibited the growth and integrity of endothelial cells. In contrast, SB-431542 strongly facilitated them. Subsequently, the effects of TGF-β signals on claudin-5 expression were examined by immunofluorescence staining (Fig. 7 b). Claudin-5 was concentrated at cell–cell borders of PECAM1+ endothelial cells. When these embryo-derived endothelial cells were treated with TGF-β, claudin-5 expression was decreased, whereas SB-431542 increased the expression of claudin-5, in accordance with alteration of endothelial sheet formation. These results strongly suggest that TGF-β signals play roles in vascular development in mouse embryos similar to those in ESC-derived vascular progenitor cells.

Discussion

Here, we showed that TGF-β superfamily signals play important roles during differentiation of vascular progenitor cells derived from ESC and embryos. TGF-β signals have two distinct effects on vascular formation. First, they decrease the number of vascular (endothelial and mural) cells differentiating from vascular progenitor cells. This inhibitory effect on cell proliferation was not specific to endothelial cells. In fact, addition of TGF-β decreased the number of mural cells in the presence or absence of VEGF (Fig. 4 b and unpublished data). The inhibitory effects of TGF-β signals on the growth (Fig. 4, a and b) and colony formation (Fig. 4 c) of Flk1+ cells could be the result of a decrease in proliferation rate, an increase in cell death, or a combination of these two as previously reported (Baird and Durkin, 1986; Pollman et al., 1999). Second, TGF-β decreased the integrity of endothelial sheets via down-regulation of claudin-5 expression. Interestingly, TGF-β selectively repressed the expression of claudin-5 but not that of other molecules involved in TJs (Fig. 6). These findings may help answer the question how TGF-β can function as both an inhibitor and a promoter of vasculogenesis and angiogenesis.

Endothelial cells express two types of TGF-β type I receptors, i.e., ALK-1 and -5, transducing distinct Smad path-

Figure 6. Effect of TGF-β signals on expression of claudin-5 in ESC-derived endothelial cells. (a) Levels of expression of claudin-5 and -12 in vascular cells derived from MGZ5 cells cultured in the absence or presence of VEGF in combination with TGF-β or SB-431542 analyzed by quantitative real-time RT-PCR. Error bars represent SD. (b) Claudin-5 (left, green) and PECAM1 (right, red) double immunofluorescence staining of differentiated Flk1+ cells with 10% FCS and VEGF alone (top), or in combination with TGF-β (middle) or SB-431542 (bottom). To obtain similar numbers of endothelial cells, different numbers of Flk1+ cells were plated depending on the culture conditions. Bars, 200 μm.

Figure 7. Effects of TGF-β signals on vascular differentiation of Flk1+ cells from 8.5-dpc embryos. (a) PECAM1 (purple) and SMA (brown) double immunostaining of differentiated Flk1+ cells derived from mouse embryos with 10% FCS in the absence or presence of VEGF in combination with TGF-β or SB-431542. (b) Claudin-5 (green) and PECAM1 (red) double immunofluorescence staining of differentiated embryo-derived Flk1+ cells with 10% FCS and VEGF alone, or in combination with TGF-β or SB-431542. To obtain similar numbers of endothelial cells, different numbers of Flk1+ cells were plated depending on the culture conditions. Bars: (a) 100 μm; (b) 200 μm.
ways. Goumans et al. (2002) showed that these distinct signaling pathways have opposite effects on endothelial cells, and that their balance contributes to both pro- and anti-angiogenic properties of TGF-β. The ALK-5–Smad2/3 pathway inhibits cell proliferation and migration, whereas the ALK-1–Smad1/5 pathway induces them (Goumans et al., 2002). Here, TGF-β and activin inhibited the proliferation and sheet formation of ESC-derived endothelial cells. Although TGF-β also activated ALK-1–Smad1/5 pathways in ESC-derived endothelial cells (Fig. 5), the biological effects observed in the present paper appear to be more dependent on the Smad2/3 pathways because similar results were obtained with activin, which activates only Smad2/3 pathways.

Phosphorylation of Smad2 was weakly detected in the absence of exogenous ligands (Fig. 5 a, lane 1), suggesting that endogenous TGF-β or activin acts on these cells. TGF-β3 is released from cells in latent forms consisting of the mature growth factors associated with amino-terminal propeptides and latent TGF-β–binding proteins (Annes et al., 2003). Endogenous activation of latent TGF-β has been shown to occur efficiently in cocultures of endothelial and smooth muscle cells through activation of plasmin on the cell surface, suggesting that endogenous TGF-β may regulate the functions of ESC-derived vascular cells.

Phosphorylation of Smad1/5 was also weakly detected in the absence of exogenous ligands (Fig. 5 a, lane 1). Although either endogenous TGF-β or BMPs can activate Smad1/5 through ALK-1 or BMP type I receptors, this activation appears to be mediated by BMP signals because SB-431542 could not inhibit it. BMP signals have been implicated in maintenance of vascular homeostasis by the findings that heterozygous mutations in BMP type II receptor gene cause human primary pulmonary hypertension (The International PPH Consortium, 2000; Nishihara et al., 2002). The roles of BMP signals in endothelial differentiation from ESC-derived vascular progenitor cells remain to be elucidated.

SB-431542, which inhibits ALK-4/5/5/-/7 kinase activities, stimulated proliferation, differentiation, and sheet formation of ESC-derived endothelial cells. All of these effects appear to improve the quantity and quality of endothelial sheets differentiated from vascular progenitor (Flk1+) cells. Vascular progenitor cells prepared from either ESC or peripheral blood cells are expected to be useful for therapeutic angiogenesis in various vascular diseases. However, they may differentiate into smooth muscle cells as well as endothelial cells, and may potentially aggravate vascular diseases such as atherosclerosis (Sata et al., 2002). TGF-β receptor kinase inhibitors might thus become invaluable for obtaining highly enriched, mature endothelial cells ex vivo. In addition, because VEGF increases vascular permeability (Senger et al., 1983), it may contribute to the progression of tissue edema if used in vascular regenerative medicine. Therefore, ALK-4/-5/-/7 kinase inhibitors might also be useful for regulation of vascular permeability, and maintenance of vascular integrity, in order to prevent tissue edema.

In brain endothelial cells, claudin-5 and -12 are major cell adhesion molecules of TJ (Nitta et al., 2003). Recently, claudin-5-deficient mice were generated, and their blood-brain barrier (BBB) was found to be selectively permissive for small molecules (<800 D), but not for larger ones (Nitta et al., 2003). This finding promised a new approach to deliver potential drugs across the BBB into the central nervous system. Our findings revealed that TGF-β signals specifically down-regulate the expression of claudin-5, and not that of claudin-12, resulting in inhibition of endothelial integrity. Thus, specific acceleration of TGF-β signaling in brain endothelial cells may size-selectively loosen the BBB to deliver the drugs of small molecular size into the central nervous system.

Materials and methods
Preparation of SB-431542
SB-431542 (compound 14) and compound 13 were synthesized as described previously (Callahan et al., 2002). A solution of SB-431542 in DMSO was prepared. This solution was used after diluting with medium for each assay.

Cells and cell culture
Maintenance, differentiation, culture, and cell sorting of CCE and MGZ5 ES cells (gifts from M.J. Evans [University of Cambridge, Cambridge, UK] and H. Niwa [RIKEN, CDB, Kobe, Japan], respectively) were performed as described previously (Yamashita et al., 2000). All experiments were performed using both ES cell lines and yielded essentially similar results. Flk1+ cells were also obtained from 8.5-dpc mouse embryos that were digested by dispase II (Roche), 30 ng/ml VEGF, 500 ng/ml BMP7, 3 ng/ml TGF-β, 100 ng/ml activin, and 1 μM SB-431542 were used in each experiment unless specifically described.

Immunohistochemistry and Western blot analysis
mAbs for PECAM1 (Mec13.3), SMA (1A4), and claudin-5 for immunohistochemistry were purchased from BD Biosciences, Sigma-Aldrich, and Zymed Laboratories, respectively. Staining of culture cells was performed as described previously (Yamashita et al., 2000). Stained cells were photographed using a phase-contrast microscope (model IX70; Olympus) or a confocal microscope (model LSM510 META; Carl Zeiss MicroImaging, Inc.). All images were imported into Adobe Photoshop as JPEGs for contrast manipulation and figure assembly. Antibodies for phospho-Smad2 and phospho-Smad1/5 for Western blot analysis and immunohistochemistry were obtained from Cell Signaling and Santa Cruz Biotechnologies, Inc., respectively. Western blot analysis was performed as described previously (Kawabata et al., 1998).

RNA isolation and RT-PCR
After 3 d of culture of Flk1+ cells with 10% FCS in the absence or presence of VEGF, TGF-β, or SB-431542, cells were used as a source of RNA. Total RNA was prepared with ISOGEN reagent (Nippongene) according to the manufacturer’s instructions, and reverse transcribed by oligo (dT) priming and PowerScript-Reverse Transcriptase (CLONTECH Laboratories, Inc.). Expression of various signaling components was compared by semi-quantitative RT-PCR analysis. PCR products were separated by electrophoresis in 1% agarose gel and visualized with ethidium bromide. Quantitative RT-PCR analysis was performed using the GeneAmp 5700 Sequence Detection System (Applied Biosystems). The primer sequences and expected sizes of PCR products are available online as indicated in Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200305147/DC1.

Online supplemental material
Fig. S1 shows the specificity of SB-431542 in the inhibition of TGF-β superfamily type I receptors. Fig. S2 shows the effects of TGF-β and SB-431542 on ESC-derived mural cell differentiation. Table S1 indicates the primer sequences and expected sizes of PCR products. The online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200305147/DC1.

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