BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo

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Summary

Members of the bone morphogenetic protein (BMP) family have been implicated in the development and maintenance of vascular systems. Whereas members of the BMP-2/4 and osteogenic protein-1 groups signal via activin receptor-like kinase (ALK)-2, ALK-3 and ALK-6, BMP-9 and BMP-10 have been reported to bind to ALK-1 in endothelial cells. However, the roles of BMP-9–ALK-1 signaling in the regulation of endothelial cells have not yet been fully elucidated. Here, using various systems, we examined the effects of BMP-9 on the proliferation of endothelial cells. Vascular-tube formation from ex vivo allantoic explants of mouse embryos was promoted by BMP-9. BMP-9, as well as BMP-4 and BMP-6, also induced the proliferation of in-vitro-cultured mouse embryonic-stem-cell-derived endothelial cells (MESECs) by inducing the expression of vascular endothelial growth factor receptor 2 and Tie2, a receptor for angiopoietin-1. A decrease in ALK-1 expression or expression of constitutively active ALK-1 in MESECs abrogated and mimicked the effects of BMP-9 on the proliferation of MESECs, respectively, suggesting that BMP-9 promotes the proliferation of these cells via ALK-1. Furthermore, in vivo angiogenesis was promoted by BMP-9 in a Matrigel plug assay and in a BxPC3 xenograft model of human pancreatic cancer. Consistent with these in vivo findings, BMP-9 enhanced the proliferation of in-vitro-cultured normal endothelial cells from dermal tissues of adult mice and of tumor-associated endothelial cells isolated from tumor xenografts in host mice. These findings suggest that BMP-9 signaling activates the endothelium tested in the present study via ALK-1.

Key words: Embryonic stem cell, Allantois, Tumor angiogenesis, Pancreatic cancer, VEGFR2, Tie2

Introduction

Blood vessels consist of a lining of endothelial cells surrounded by mural cells (pericytes and vascular smooth muscle cells), and play essential roles in the maintenance of tissue homeostasis by carrying oxygen and nutrients to distant organs (Carmeliet, 2005). Defects in vascular systems thus result in many types of human disease. Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu disease, is characterized by telangiectases in the nose, oral cavity and gastrointestinal tract, as well as arteriovenous malformations in multiple organs, including lung, liver and brain, which can result in severe ischemic injury or stroke (Shovlin et al., 2000). HHT is an autosomal dominant vascular disease, the majority of cases of which are caused by mutations in the gene for either activin receptor-like kinase (ALK)-1 (*ACVRL1*) or endoglin (*ENG*), which are a type-I receptor and co-receptor for members of the transforming growth factor (TGF)β family, respectively.

The TGFβ family consists of structurally related and multifunctional proteins, including TGFβs, activins, Nodal and bone morphogenetic proteins (BMPs) (Feng and Derynck, 2005). Members of the TGFβ family 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 downstream signal transduction cascades, including Smad pathways. Activins and TGFβs bind to type I receptors known as ALK-4 and ALK-5, respectively. BMPs bind three BMP type I receptors (ALK-2, ALK-3 and ALK-6). The activated type I receptors phosphorylate receptor-regulated Smad proteins (R-Smads). Smad2 and Smad3 transduce signals for TGFβs, activins and Nodal, whereas Smad1, Smad5 and Smad8 are specific to signaling of BMPs (Miyazono et al., 2010). An exception to this is ALK-1, which is preferentially expressed in endothelial cells. ALK-1 binds TGFβ and activates the Smad1 and Smad5 pathways (Oh et al., 2000). Goumans and colleagues showed that TGFβ activates ALK-5–Smad2/3 and ALK-1–Smad1/5/8 pathways, leading to inhibition and activation of endothelial cell migration and proliferation, respectively (Goumans et al., 2002).

BMP-family members are subdivided into separate subgroups on the basis of similarity of primary amino acid sequence. They include the BMP-2/4 group (BMP-2 and BMP-4), osteogenic protein-1 (OP-1) group (BMP-5, BMP-6, BMP-7 and BMP-8) and BMP-9/10 group (BMP-9 and BMP-10) (Miyazono et al., 2010). BMP-9 has been shown to be highly expressed in fetal liver (Celeste et al., 1994) and in the liver of adult mice (Miller et al., 2000). BMP-9 exhibits growth-stimulatory effects on primary rat hepatocytes (Song et al., 1995), and has also been implicated in osteogenesis, chondrogenesis (Majumdar et al., 2001), glucose and fatty-acid metabolism (Chen et al., 2003), iron homeostasis (Truksa et al., 2006) and the differentiation of cholinergic neurons (López-Coviella et al., 2000).

Recent studies have shown that BMP-9 binds with high affinity to ALK-1 and endoglin in endothelial cells, and induces phosphorylation of Smad1 and/or Smad5 (Brown et al., 2005). This signaling inhibits basic fibroblast growth factor (FGF)-stimulated proliferation and migration of bovine aortic endothelial cells and vascular endothelial growth factor (VEGF)-induced

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angiogenesis (Scharpfenecker et al., 2007). BMP-9 also inhibits the migration and growth of human dermal microvascular endothelial cells (HMVEC-ds) (David et al., 2007) and the DNA synthesis of human aortic endothelial cells (Upton et al., 2009) via ALK-1. Furthermore, BMP-9 is present in the circulating blood to maintain the maturation of blood vessels (David et al., 2008). These findings suggest that BMP-9 has inhibitory effects on endothelial cells, a conclusion that is not consistent with previous reports that TGFβ-mediated ALK-1 signaling activates endothelium (Goumans et al., 2002).

The development of the vascular system includes two distinct processes: vasculogenesis and angiogenesis (Coultas et al., 2005). During mouse embryogenesis, the initial development of vascular endothelium, termed vasculogenesis, occurs in the mesodermal layer of the yolk sac on embryonic day 7 (E7.0), yielding structures termed blood islands (E7.5-E8.5). The blood islands consist of endothelial progenitors termed angioblasts, which express VEGF receptor-2 (VEGFR2), which is also known as Flk1 (Kennedy et al., 1997; Nishikawa et al., 1998). In angiogenesis, new vessels sprout from the pre-existing vessels and are further remodeled to form mature blood vessels. In embryos, angiogenesis contributes to the establishment of hierarchical vascular trees after endothelial capillary networks have been formed by vasculogenesis. In adults, angiogenesis is essential for the repair and remodeling of tissues during wound healing and ischemia, and for the physiological female reproductive cycle. Neovascularization also plays a pivotal role in pathological processes such as tumor growth, chronic inflammation and diabetic vasculopathy.

Embryonic vascular differentiation has been recapitulated in an in-vitro-differentiation system from embryonic stem cells (ESCs) (Vittet et al., 1996; Hirashima et al., 1999; Yamashita et al., 2000). When VEGFR2-expressing (VEGFR2+) endothelial progenitors isolated from differentiation cultures are re-differentiated in the presence of VEGF, various endothelial markers are sequentially upregulated in a pattern similar to that observed in early embryos (Vittet et al., 1996). Using this system, we have previously shown that BMP-4 stimulates the proliferation and migration of endothelial cells via activation of VEGF–VEGFR2 and angiopoietin-1 (Ang-1)-Tie2 signaling in mouse embryonic-stem-cell-derived endothelial cells (MESECs) (Suzuki et al., 2008), whereas Kiyono and Shibuya (Kiyono and Shibuya, 2003) reported that BMP-4 induces apoptosis of capillary endothelial cells during rat pupillary-membrane regression. It is thus possible that BMP-9 has various effects depending on the cellular context.

In the present study, we examined the roles of BMP-9 in embryonic vascular development and adult angiogenesis. We found that the addition of BMP-9 to culture significantly promoted vasculogenesis of the allantois and induced proliferation of MESECs. Furthermore, we showed that BMP-9-induced proliferation was transduced via ALK-1. Finally, BMP-9 enhanced angiogenesis in Matrigel plug assays and pancreatic carcinoma xenografts in adult mice. These findings suggest that BMP-9 is capable of activating endothelium via ALK-1 and inducing vessel formation in vivo.

Results

Expression of components of the BMP-9 signaling pathway in E8.25 mouse embryos

We first examined whether the components of the BMP-9–ALK-1 signaling pathways are present in mouse embryos. The development of vascular systems in mouse embryos begins at E7.5, when VEGFR2+ endothelial progenitors initiate endothelial differentiation. At E8.5, the murine allantois, which lays down the umbilical vasculature that is critical for fetal survival and development, is formed and vascularizes intrinsically (Downs et al., 2001), rather than by angiogenesis involving the yolk sac or fetus. This tissue has therefore been used to study embryonic vessel formation.

E8.25 embryos were excised to obtain the allantois, which was subjected to semi-quantitative reverse transcriptase (RT)-PCR analyses. Endothelial and mural cells that differentiated from mouse ESCs (Yamashita et al., 2000; Watabe et al., 2003) were also examined for the expression of various BMP-9 signaling components. As shown in Fig. 1, expression of BMP-9 ligand was detected in allantoic, endothelial and mural cells. Furthermore, expression of type I receptors (ALK-2, ALK-3 and ALK-6) was also observed in all three samples (Fig. 1), whereas that of ALK-1 was restricted to the allantoic and endothelial cells, confirming endothelium-specific expression of ALK-1. Taken together with the findings that other BMP-related signaling components, including type II receptors (BMPR-II), R-Smads (Smad1, Smad5 and Smad8) and Co-Smad (Smad4), are expressed in the allantois (data not shown), these results suggest that signals mediated by BMP-9 are activated in the allantois of E8.25 embryos.

Induction of vessel formation by BMP-9 in ex vivo allantoic explants

Next, to examine the roles of BMP-9 in vasculogenesis and angiogenesis, we used an isolated allantois model (Downs et al., 2001). Excised allantoises from mouse E8.25 embryos were individually cultured in suspension for 2 days. When BMP-9 was added to the ex vivo culture, the PECAM1-positive area of allantoic explants was increased (Fig. 2A,B). However, the vessels formed in the presence of BMP-9 seemed disorganized and disconnected compared with the control (Fig. 2A). These findings suggest that BMP-9 promotes irregular formation of embryonic vascular vessels.

BMP-9 increases the number of MESECs

Because we observed pro-vasculogenic and -angiogenic effects of BMP-9 in an ex vivo culture system, we next examined the effects of BMP-9 signaling on embryonic endothelial cells with an in vitro

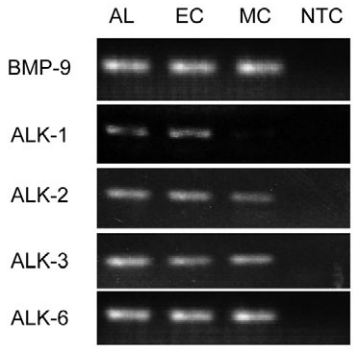

Fig. 1. Expression of BMP-related signaling components in allantoic explants. Allantoises (AL) were excised from E8.25 mouse embryos and subjected to semi-quantitative RT-PCR analyses for BMP-9, ALK-1, ALK-2, ALK-3 and ALK-6. Endothelial cells (EC) and mural cells (MC) were obtained by in vitro differentiation of CCE mouse ESCs and were also subjected to RT-PCR analysis for comparison of expression with that in allantois. NTC, no template control.

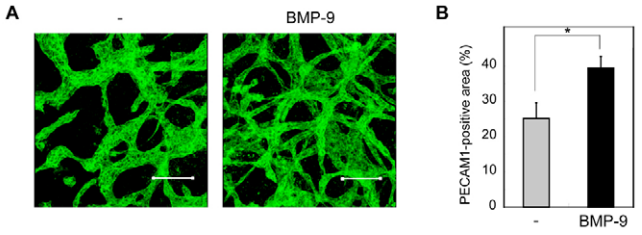

Fig. 2. Effects of BMP-9 on vessel formation from allantoic explants. (**A**) Allantoises were excised from E8.25 embryos and cultured in the absence (–) or presence of BMP-9 (4 ng/ml), followed by immunostaining for PECAM1 (green). Scale bars: 100 μm. (**B**) Percent PECAM1-positive area. Each value represents the mean of seven fields. Error bars, s.d. **P*<0.00004.

vascular differentiation system from mouse ESCs (Yamashita et al., 2000; Watabe et al., 2003). VEGFR2+ endothelial progenitor cells derived from ESCs differentiate predominantly into endothelial cells when cultured in serum-free SFO medium in the presence of VEGF. When BMP-9 was added to culture of VEGFR2+ cells, the number of endothelial cells obtained was significantly increased and their sheet formation was significantly inhibited (Fig. 3A,B), which is similar to our previous finding that BMP-4, a member of the BMP-2/4 group, significantly increases the number of MESECs (Suzuki et al., 2008) (Fig. 3B).

When BMP-6, a member of the OP-1 group, was tested in this proliferation assay, it also increased the number of MESECs (Fig. 3B). These findings suggest that members of all three BMP subfamilies are capable of promoting the proliferation of embryonic endothelial cells during differentiation from VEGFR2+ progenitor cells.

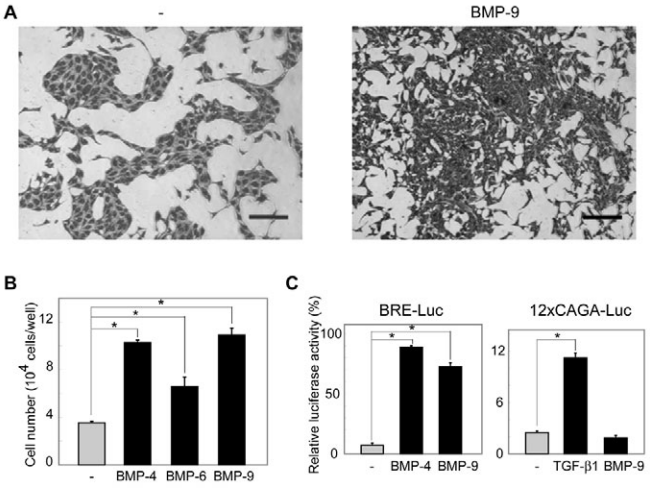

Fig. 3. Effects of BMP-9 on the number of MESECs. (**A**,**B**) VEGFR2+ cells (5×10^4/well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (–) or presence of BMP-4 (60 ng/ml), BMP-6 (360 ng/ml) or BMP-9 (1 ng/ml) for 2 days, followed by photography (A; '–' and BMP-9) and determination of cell numbers (B). Scale bars: 200 μm. (**C**) Luciferase promoter assays were carried out using MESECs to determine whether BMP-9 activates BMP-related (BRE-Luc) or TGFβ-related (12×CAGA-Luc) intracellular signaling pathways. BMP-4 (60 ng/ml) and TGFβ1 (1 ng/ml) were used as controls for BRE-Luc and 12×CAGA-Luc, respectively. Error bars, s.d. **P*<0.006.

BMPs activate the Smad1/5/8 signaling axis, whereas TGFβ, activins and Nodal activate the Smad2/3 signaling axis. To determine which intracellular Smad signaling pathways are activated by BMP-9 in MESECs, we carried out luciferase reporter assays using promoter reporter constructs that specifically respond to each signal. As shown in Fig. 3C, BMP-9 activated BMP–Smad1/5/8-specific (BRE-Luc) but not TGFβ–Smad2/3-specific (12×CAGA) reporter constructs, which were activated by BMP-4 and TGFβ, respectively. We also examined whether the concentrations of BMPs used in the proliferation assay activate the Smad1/5/8 signaling pathways at comparable levels. Activation of Smad1/5/8 signaling pathways can also be monitored by the expression level of Id1, a faithful target of BMP signaling (Valdimarsdottir et al., 2002). As shown in supplementary material Fig. S1, BMP-4, BMP-6 and BMP-9 induced Id1 expression to a similar relative extent as they enhanced the proliferation of MESECs (Fig. 3B). These findings suggest that activation of BMP-9 increases the number of MESECs by activating Smad1/5/8 signaling pathways.

Specific inhibition of the effects of BMP-9 on MESEC proliferation by soluble ALK-1

Although all members of the BMP family exhibit similar effects on endothelial cell proliferation, BMP-9 and BMP-10 have been reported to utilize ALK-1, whereas BMP-4 and BMP-6 bind ALK-2, ALK-3 and ALK-6 (David et al., 2007; Scharpfenecker et al., 2007). To examine whether the BMP-9 used in the assay preferentially binds to ALK-1 on MESECs, we used soluble chimeric receptors containing extracellular domains of ALK-1 and ALK-3 (ALK-1-Fc and ALK-3-Fc, respectively). As shown in Fig. 4A, ALK-1-Fc inhibited the BMP-9-mediated increase in number of MESECs, whereas control-Fc or ALK-3-Fc did not. These findings suggest that BMP-9 binds to ALK-1 with higher affinity than to ALK-3 in MESECs.

Increase of ALK-1 expression during endothelial differentiation

Although ALK-1 has been shown to be preferentially expressed in endothelial cells, its expression during endothelial differentiation from ESC-derived vascular progenitors has not been elucidated. We therefore examined the expression of ALK-1, ALK-3 and vascular markers over time during in vitro differentiation of ESC-derived VEGFR2+ cells. The expression of PECAM1, an endothelial marker, began to increase from 12 hours after stimulation with VEGF in the absence of serum (Fig. 4B) (Kawasaki et al., 2008). The level of expression of ALK-1 in VEGF-stimulated ESC-derived progenitor cells was similar to that of PECAM1, whereas that of endoglin increased earlier than those of PECAM1 and ALK-1. By contrast, ALK-3 expression was detected in the VEGFR2+ endothelial progenitors and decreased during endothelial differentiation. Although these findings need to be confirmed at protein levels, they suggest that ALK-1 signals mediated by BMP-9 are activated after endothelial cells are differentiated from ESC-derived VEGFR2+ cells, whereas ALK-3 signals mediated by BMP-4 can be activated in VEGFR2+ progenitor cells.

Essential role of ALK-1 in the BMP-9-mediated increase in number of MESECs

Although BMP-9 was shown to bind preferentially to ALK-1, it remains unclear whether the BMP-9-induced increase in the number

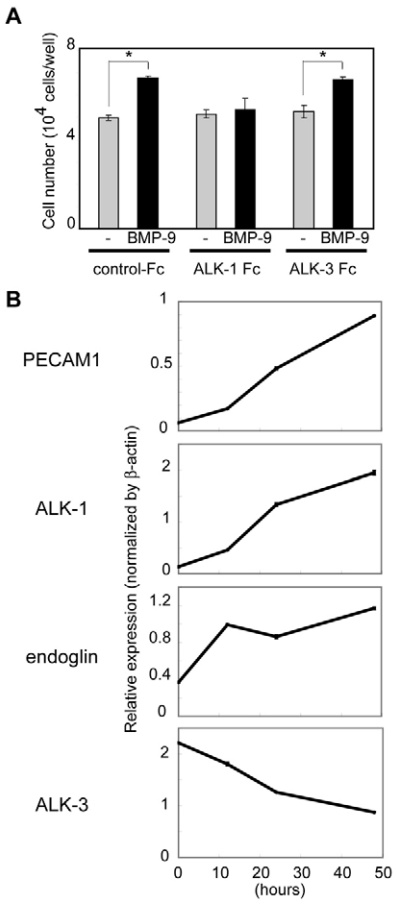

Fig. 4. Involvement of ALK-1 in the BMP-9-mediated increase in number of MESECs. (**A**) VEGFR2+ cells (5×10^4/well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (–) or presence of BMP-9 (1 ng/ml) in combination with control-Fc (30 ng/ml), ALK-1-Fc (30 ng/ml) or ALK-3-Fc (30 ng/ml) for 2 days, followed by cell-proliferation analysis. Each value represents the mean of three determinations; error bars, s.d. *P<0.004. (**B**) Expression of vascular markers and ALKs during in vitro differentiation of ESC-derived VEGFR2+ cells. Quantitative RT-PCR analysis of PECAM1, ALK-1, endoglin and ALK-3 of VEGFR2+ cells stimulated with 30 ng/ml of VEGF in the absence of serum. Error bars, s.d.

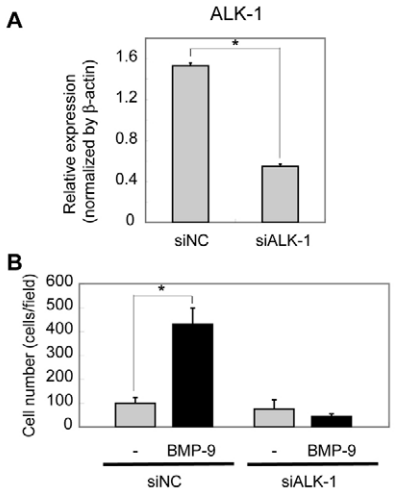

Fig. 5. Effects of decreased ALK-1 expression on MESEC proliferation. MGZ5 ESC-derived VEGFR2+ cells were transfected with siRNA for *ALK-1* (siALK-1) or negative control siRNA (siNC), and cultured in the absence (–) or presence of BMP-9 (1 ng/ml) in combination with VEGF (30 ng/ml) and serum for 4 days. (**A**) Expression of endogenous level of *ALK-1* was determined by quantitative RT-PCR analyses. Each value represents the mean of three determinations; error bars, s.d. *P<0.002. (**B**) Roles of ALK-1 on the proliferation of MESECs. In order to determine the number of endothelial cells, ESC-derived vascular cells containing both endothelial and mural cells were stained for PECAM1 and SMA, followed by counting the numbers of endothelial cells in each field. Each value represents the mean of five determinations; error bars, s.d. *P<0.00002.

of MESECs is mediated by ALK-1. We therefore knocked down ALK-1 expression in ESC-derived VEGFR2+ cells using siRNA during endothelial cell differentiation in the presence of serum. As shown in Fig. 5A, the level of transcripts encoding ALK-1 was significantly decreased by its specific siRNA.

Although the number of MESECs was not significantly altered by knocking down ALK-1 expression, BMP-9 failed to increase the number of MESECs only when ALK-1 expression was knocked down (Fig. 5B). These findings suggest that ALK-1 is required for BMP-9 to increase the number of MESECs.

Expression of constitutively active ALK-1 increases the number of MESECs

We next examined whether the expression of constitutively active ALK-1 (caALK-1) regulates the proliferation of MESECs in a fashion similar to the addition of BMP-9. Because we wished to induce the expression of caALK-1 in differentiating endothelial progenitor cells instead of undifferentiated ESCs, we established ESC lines carrying a tetracycline (Tc)-regulatable caALK-1 transgene (Tc–caALK-1) or no transgene (Tc-empty) (Masui et al., 2005; Mishima et al., 2007).

We differentiated the Tc-empty and Tc–caALK-1 ESCs into VEGFR2+ vascular progenitor cells in the presence of Tc, so that no transgene expression would be induced. Sorted VEGFR2+ cells were re-differentiated in the presence or absence of Tc. As shown in Fig. 6A, in endothelial cells derived from Tc–caALK-1 ESCs, expression of the caALK-1 transgene was induced only in the absence of Tc. When caALK-1 was expressed, the number of MESECs was significantly increased (Fig. 6B), consistent with the effects of addition of BMP-9. When caALK-1 transgene was expressed in MESECs, Id1 expression was significantly increased (Fig. 6C), a finding also observed when BMP-9 was added to culture of MESECs (Fig. 7). These findings suggest that activation of Smad1/5/8 signaling by expression of caALK-1 mimics the signaling induced by BMP-9.

Induction of endothelial cell proliferation by BMP-9–ALK-1 signaling via stimulation of VEGF-VEGFR2 and Ang-1–Tie2 signaling

We next attempted to identify the targets of BMP-9–ALK-1 signaling that induce endothelial cell proliferation. VEGF-VEGFR2 signaling pathways are known to stimulate endothelial proliferation, survival and migration. Ang-1 is an agonist for the Tie2 tyrosine kinase receptor, which inhibits apoptosis of endothelial cells (Maisonpierre et al., 1997; Holash et al., 1999). We previously showed that BMP-4 induces the expression of transcripts for

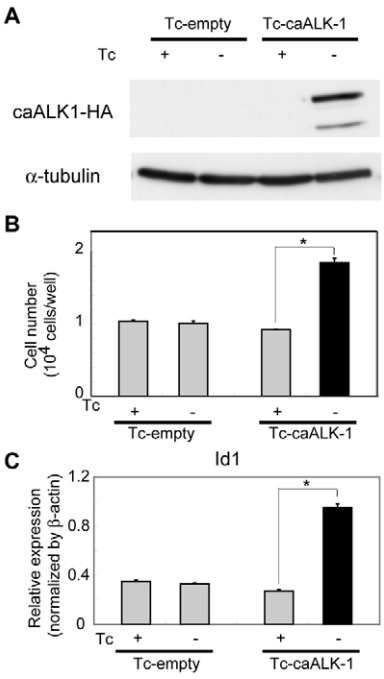

Fig. 6. Effects of constitutively active ALK-1 expression on MESEC proliferation. VEGFR2+ vascular progenitor cells derived from ESCs carrying a tetracycline (Tc)-regulated transgene encoding hemagglutinin (HA)-tagged constitutively active ALK-1 (Tc–caALK-1) or control transgene (Tc-empty) were cultured in the presence (+) or absence (–) of Tc. After 2 days of culture in the absence of serum, the MESECs obtained were subjected to immunoblotting for HA-epitope to determine the level of expression of *ALK-1* transgene (**A**), to cell-proliferation assay (**B**) or to quantitative RT-PCR analysis for Id1 expression (**C**). Error bars, s.d. **P*<0.003.

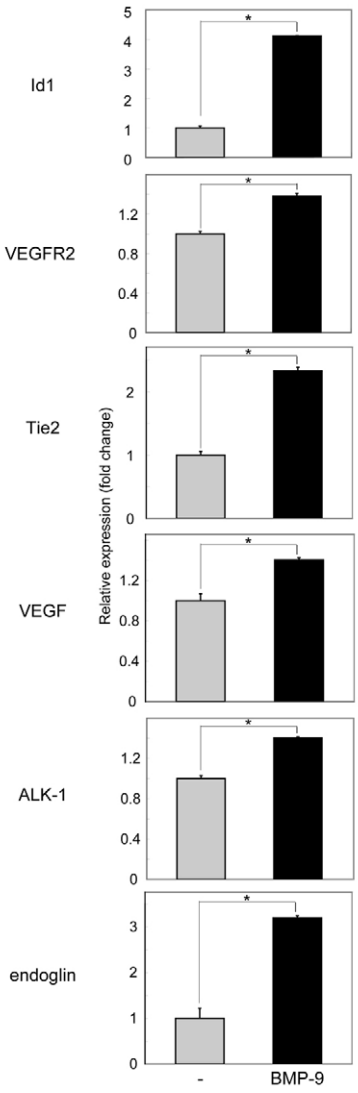

Fig. 7. Effects of BMP-9 on the expression of various angiogenesis-related factors. VEGFR2+ cells derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence (–) or presence of 1 ng/ml of BMP-9 for 2 days, and subjected to quantitative RT-PCR analyses for the expression of Id1, VEGFR2, Tie2, VEGF, ALK-1 and endoglin. Quantified mRNA values were normalized to the amount of β-actin mRNA, and results are given as fold change. Error bars, s.d. **P*<0.006.

VEGFR2 and Tie2, suggesting that activation of VEGF-VEGFR2 and Ang-1–Tie2 signaling by BMP-4 leads to the activation of MESECs (Suzuki et al., 2008).

In addition to increasing Id1 levels, BMP-9 increased the levels of transcripts for VEGFR2 and Tie2 (Fig. 7). Furthermore, we identified VEGF as a putative target of BMP-9–ALK-1 signaling in MESECs, suggesting that the BMP-9-mediated increase in the number of MESECs is promoted by the activation of VEGF-VEGFR2 and Ang-1–Tie2 signaling. In order to examine the roles of VEGF-VEGFR2 signals in the BMP-9-mediated promotion of angiogenesis, we treated allantoic explants with SU1498, a specific inhibitor of VEGFR2 kinase. As shown in supplementary material Fig. S2, the addition of SU1498 reduced the formation of blood vessels from allantoic explants, showing that blockade of VEGF signaling inhibits angiogenesis. Although BMP-9 enhanced blood-vessel formation in control explants, this augmentation was completely abrogated by SU1498. These results further suggested that VEGF-VEGFR2 signals play important roles in the promotion of angiogenesis by BMP-9.

Interestingly, expression of *ALK-1* transcripts increased upon the addition of BMP-9 to culture of MESECs, suggesting that BMP-9–ALK-1 signaling is self-activating as a result of a positive-feedback mechanisms. In addition, we found that the expression of endoglin is upregulated by BMP-9; this might modulate BMP-9–ALK-1 signaling.

BMP-9 enhances in vivo angiogenesis in Matrigel plug assays

We next attempted to examine whether present ex vivo and in vitro findings that BMP-9 activates the proliferation of endothelial cells can be applied to angiogenesis in adults. Adult angiogenesis includes not only neo-angiogenesis, which involves the proliferation of existing endothelial cells, but also vasculogenesis, which involves the differentiation of endothelial progenitor cells circulating in peripheral blood (Asahara and Kawamoto, 2004). As a model of angiogenesis in adults, we chose a Matrigel plug assay.

Matrigels mixed with FGF-2 in combination with BMP-9 were subcutaneously injected to Balb/c mice. One week later, Matrigel plugs were harvested, and resulting vasculature was examined by staining for PECAM1. As shown in Fig. 8A and B, a significantly

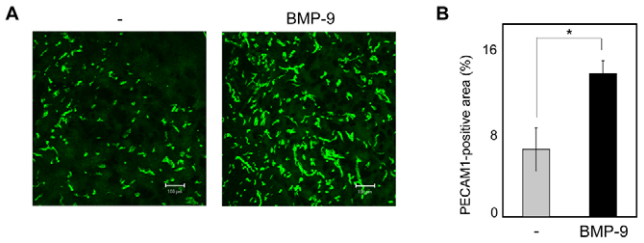

Fig. 8. Effects of BMP-9 on angiogenesis in a Matrigel plug assay in vivo. Matrigel plugs mixed with 1 μg/ml FGF-2 in the absence (–) or presence of 10 ng/ml of BMP-9 were subcutaneously injected in BALB/c mice. After 1 week, Matrigel plugs were harvested and examined for vascular density. (**A**) Immunostaining for PECAM1 (green) of sections obtained from both types of Matrigel plugs. Scale bars: 100 μm. (**B**) Percent PECAM1-positive area. Each value represents the mean of ten sections; error bars, s.d. **P*<0.005.

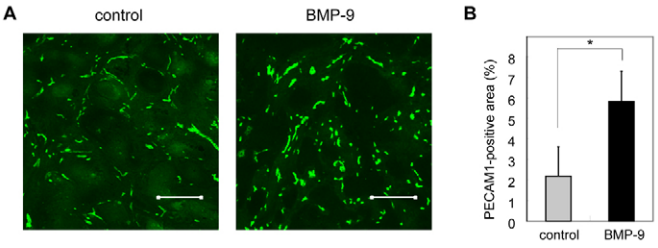

Fig. 9. Effects of BMP-9 on tumor angiogenesis in a mouse xenograft model of human pancreatic cancer. BxPC3 human pancreatic adenocarcinoma cells that were infected with lentiviruses encoding GFP (control) or BMP-9 were subcutaneously inoculated in BALB/c nude mice. After 5 weeks, tumors were excised and examined for vascular density. (**A**) Immunostaining for PECAM1 (green) of sections obtained from both types of tumors. Scale bars: 100 μm. (**B**) Percent PECAM1-positive area. Each value represents the mean of 15 sections; error bars, s.d. **P*<0.0000003.

higher density of microvessels was observed in the Matrigels that contained BMP-9 than in the controls. These findings suggest that BMP-9 promotes in vivo adult angiogenesis.

Promotion of tumor angiogenesis by BMP-9 in a human pancreatic-cancer xenograft model

We next tried to extend our finding that BMP-9 enhances angiogenesis in Matrigel plugs to another model of angiogenesis. Because previous studies showed that BMP-2 induced tumor angiogenesis in human lung- and breast-cancer xenograft models (Langenfeld and Langenfeld, 2004; Raida et al., 2005), we examined whether BMP-9 also plays any roles in tumor angiogenesis.

In order to avoid effects on tumor cells themselves of BMP-9 signaling, we established BxPC3 pancreatic adenocarcinoma cells that express green fluorescence protein (GFP; control) or *BMP-9* transgenes. Because BxPC3 cells exhibit a homozygous deletion of the *SMAD4* gene (Kleeff et al., 1999), BMP-9 secreted from BxPC3-BMP-9 tumor cells cannot activate the Smad pathways by itself. We confirmed that BMP-9 expression does not affect the rate of growth of tumor cells in vitro (data not shown). When conditioned medium prepared from BxPC3-BMP-9 cells was added to MDA-MB-231 cells, the expression of Id1 was significantly induced compared with when medium from BxPC3-GFP cells was added (data not shown).

Both types of BxPC3 cells were subcutaneously grafted to immunocompromised Balb/c nude mice to obtain tumors. Tumor vasculature was examined by staining for PECAM1. As shown in Fig. 9A and B, a significantly higher density of microvessels was observed in the tumors derived from BxPC3-BMP-9 cells than in the controls. These findings suggest that BMP-9 also promotes in vivo tumor angiogenesis.

BMP-9 promotes the proliferation of primary cultured normal and tumor-associated endothelial cells derived from adult mice

Present findings that BMP-9 promotes angiogenesis in Matrigels and tumors transplanted to mice prompted us to investigate whether BMP-9 induces the proliferation of endothelial cells directly or indirectly via activation of surrounding cells, including inflammatory cells. Recent findings have revealed that tumor-associated endothelial cells (TECs) are different from normal endothelial cells (NECs) in many respects (Hida et al., 2004; Hida et al., 2008; Akino et al., 2009). In order to examine the effects of BMP-9 on the proliferation of both types of primary cultured endothelial cells, we prepared TECs from renal carcinoma xenografts (OSRC-2) in nude mice and NECs from dermal tissues as a normal counterpart of TECs (see Materials and Methods). When NECs and TECs were cultured in the absence or presence of BMP-9, proliferation of both types of cell was significantly promoted by BMP-9 (Fig. 10). These results suggest that BMP-9 induces angiogenesis via direct activation of endothelial cells in normal and cancerous conditions.

Discussion

In the present study, we demonstrated for the first time that BMP-9 induces ex vivo allantois vascular formation, proliferation of in-vitro-cultured MESECs, and in vivo angiogenesis. Using in vitro systems, we showed that ALK-1 is necessary and sufficient for the BMP-9-induced proliferation of MESECs, and that BMP-9 induces the expression of various targets, including VEGFR2 and Tie2, involved in the proliferation of endothelial cells.

We previously reported that BMP-4 induces the proliferation of MESECs via activation of VEGFR2 and Tie2 signaling (Suzuki et al., 2008). Although in the present study we showed that members of all three BMP subfamilies (BMP-2/4, OP-1 and BMP-9/10) induce the proliferation of MESECs (Fig. 3B), their effects on MESECs seem to differ, given the differential onset of receptor expression and induction of other target genes. Because the

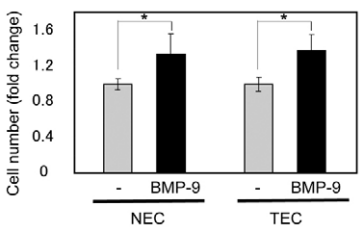

Fig. 10. Effects of BMP-9 on the number of normal and tumor-associated endothelial cells isolated from adult mice. Normal endothelial cells (NECs) prepared from dermal tissues and tumor-associated endothelial cells (TECs) prepared from renal carcinoma xenografts in nude mice were cultured in the absence (–) or presence of BMP-9 (5 ng/ml) for 3 days, followed by determination of cell numbers by MST assay. Each value represents the mean of five determinations; error bars, s.d. **P*<0.003.

expression of ALK-3, a receptor for BMP-4, begins before mesoderm induction, BMP-4–ALK-3 signaling is involved in mesoderm induction (Lawson et al., 1999; Winnier et al., 1995; Dunn et al., 1997; Mishina et al., 1995; Ahn et al., 2001) and formation of VEGFR2+ angioblasts (Park et al., 2004). By contrast, ALK-1 expression parallels that of PECAM1, further confirming that ALK-1 is an endothelial marker. These findings highlight the difference between the effects of BMP-4 and BMP-9: BMP-4 signaling occurs in the early stages during differentiation of progenitors cells, whereas BMP-9 signaling occurs only in fully differentiated endothelial cells.

In addition, we identified ALK-1 as a target of BMP-9 signaling. The BMP-9-mediated positive-feedback mechanisms involved in the regulation of ALK-1 expression seem to play important roles during embryonic vascular formation, because BMP-9–ALK-1 signaling enhances the proliferation of endothelial cells. We also found that endoglin expression is induced by BMP-9 in MESECs, consistent with our previous finding that adenovirally transduced caALK-1 induced the expression of endoglin in human umbilical-vein endothelial cells (Ota et al., 2002). Endoglin serves as a co-receptor for TGFβ-family signals (Yamashita et al., 1994). In vitro studies have shown that endoglin protein is able to form complexes with ALK-1 and to enhance the effects of ALK-1 in endothelial cells (Blanco et al., 2005; Goumans et al., 2002; Lebrin et al., 2004). The phenotype of endoglin-deficient mice strongly resembles that of ALK-1-deficient mice, suggesting that endoglin also plays a role in ALK-1 signaling in angiogenesis (Li et al., 1999). Although both ALK-1 and endoglin are involved in the pathogenesis of HHT, the roles of endoglin in MESECs remain to be elucidated.

BMP-9 has been reported to inhibit the proliferation and migration of endothelial cells (David at al., 2007; Scharpfenecker et al., 2007), a finding that is inconsistent with our results that BMP-9 inhibits the proliferation of three kinds of endothelial cells: MESECs, NECs and TECs. However, as previously reported (David et al., 2007), we also found that BMP-9 inhibits proliferation of HMVEC-ds (data not shown), suggesting that the effects of BMP-9 on endothelial cell proliferation are cell-type dependent. Identification of the components that determine whether BMP-9 induces or inhibits endothelial proliferation is of great interest.

Consistent with our in vitro data, we found that BMP-9 enhanced the angiogenesis in ex vivo culture of allantois and in vivo Matrigel plug assay. We also found that BMP-9 secreted by BxPC3 carcinoma cells affected the tumor microenvironment and promoted angiogenesis by directly activating endothelial cells, which is crucial for tumor growth. However, the tumors derived from BMP-9-expressing BxPC3 cells were smaller than control tumors (data not shown). Because Smad4 is defective in BxPC3 cells, the BMP-9 secreted from BxPC3-BMP-9 cells is unable to activate Smad signaling in the tumor cells, although we cannot exclude the possibility that BMP-9 activated Smad-independent signaling in tumor cells and thereby attenuated tumor growth, which we were not able to observe in vitro (data not shown). It is also noteworthy that the newly formed vasculature in BxPC3-BMP-9 tumor (Fig. 9) seemed to be immature and non-functional compared with that in the controls. We observed that pericyte coverage of the newly formed vessels was decreased in the tumors derived from BxPC3-BMP-9 cells (data not shown). These observations suggest that the in vivo growth of BxPC3 cells does not principally depend on newly formed vasculature, which typically is the case for pancreatic tumors. It will be of great interest to examine the effects of BMP-9 on the growth of other types of tumor cells.

Angiogenesis plays crucial roles in numerous pathological conditions such as inflammation and tumorigenesis. In addition, regenerative medicine for vascular systems has attracted much attention as a potential means of eliminating the vascular-system defects accompanying diabetes and other diseases. The present findings are thus useful in suggesting BMP-9 as a putative target or other type of resource in the treatment of inflammation, cancer and vascular dysfunction.

Materials and Methods

Cells and cell culture

The CCE ESC line was obtained from Michael J. Evans (University of Cambridge, UK). MGZ5 and MGZRTcH ESC lines were obtained from Hitoshi Niwa (RIKEN CDB, Kobe, Japan). Maintenance, differentiation, culture and cell sorting of ESCs were performed as previously described (Yamashita et al., 2000; Suzuki et al., 2008; Kokudo et al., 2008). Establishment of Tc-inducible ESC lines from parental MGZ5TcH2 cells was as described (Masui et al., 2005; Mishima et al., 2007). VEGF (30 ng/ml), BMP-4 (60 ng/ml), BMP-6 (360 ng/ml), BMP-9 (1 ng/ml), TGFβ1 (1 ng/ml), control-Fc (30 ng/ml), ALK-1-Fc (30 ng/ml) and ALK-3-Fc (30 ng/ml) were purchased from R&D Systems. The BxPC3 human pancreatic adenocarcinoma cell line was obtained from American Type Culture Collection (Manassas, VA). BxPC3 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS).

Isolation and culture of TECs and NECs

Endothelial cells were isolated as previously described (Ohga et al., 2009). In brief, TECs were isolated from renal carcinoma xenografts (OSRC-2) in nude mice aged 8-12 weeks (Sankyo Laboratory, Tokyo, Japan). NECs were isolated from the dermal tissue as a control. Both types of endothelial cells were isolated using a magnetic cell-sorting system (Miltenyi Biotec) according to the manufacturer's instructions using fluorescein isothiocyanate (FITC)-anti-PECAM1 antibody. PECAM1-positive cells were sorted and plated onto 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Clonetics) and 15% FBS. Diphtheria toxin (500 ng/ml; Calbiochem) was added to TEC subcultures to kill any remaining human tumor cells and to NECs to ensure technical consistency. The isolated endothelial cells were purified by a second round of purification, using FITC–Bandeirea Simplicifolia lectin 1-B4, and purity was determined by flow cytometry. Growth of TECs and NECs was quantified after 3 days of the culture in the absence or presence of BMP-9 by MTS assay using CellTiter 96 Aqueus One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol.

Cell-proliferation assays

The number of MESECs was counted in different ways depending on the conditions of differentiation from ESC-derived VEGFR2+ cells. When ESC-derived VEGFR2+ cells were re-differentiated in the absence of serum, we obtained only endothelial cells (Yamashita et al., 2000). Therefore, cell numbers were counted using a Coulter Counter (Yamato Kagaku). When ESC-derived VEGFR2+ cells were re-differentiated in the presence of serum, we obtained both endothelial cells and mural cells. Such mixed cell populations were immunostained for PECAM1, an endothelial cell marker, and smooth muscle α-actin (SMA), a mural cell marker, and subjected to manual counting of endothelial cells in the fields of photographs.

Luciferase assay

The transcriptional activation induced by BMPs and TGFβ1 was measured using BRE-Luc and 12×CAGA-Luc, which are luciferase reporter constructs containing BMP-responsive and TGFβ-responsive elements, respectively (Korchynskyi and ten Dijke, 2002; Dennler et al., 1998). ESC-derived VEGFR2+ vascular progenitor cells were seeded in 24-well plates and then transiently transfected with promoter-reporter constructs, followed by stimulation with ligands. Cell lysates were then prepared, and luciferase activities in the lysates were measured with the Dual-Luciferase reporter system (Promega) using a luminometer (MicroLumat Plus, Berthold). Values were normalized to *Renilla*-luciferase activity under control of cytomegalovirus promoter.

RNA interference

siRNAs for mouse *ALK-1* (Stealth RNAi Oligo ID MSS235821) and negative control (Stealth RNAi Negative Control Med GC) were purchased from Invitrogen, and were introduced into cells using HiPerFect reagent (QIAGEN) according to the manufacturer's instructions.

Ex vivo allantois assay

Explant culture of mouse allantois was carried out as previously described (Downs et al., 2001). Briefly, allantoises were excised from concepti of ICR mice at E8.25 in phosphate-buffered saline. Individual allantoises were cultured on eight-well culture slides in 250 μl of minimum essential medium (Invitrogen) supplemented with 10% FBS and β-mercaptoethanol. After 2 days of culture at 37°C in a 5% CO_2

incubator, allantois explants were subject to immunohistochemical examination. When SU1498 (Cosmobio) was added to the culture, dimethyl sulfoxide (DMSO) was used as vehicle control.

Matrigel plug assay

Balb/c male mice aged 5-6 weeks were obtained from CLEA Japan (Tokyo, Japan) and 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. Matrigel plug assays were carried out as previously described (Kano et al., 2005). Briefly, regular Matrigel (BD Biosciences) was mixed with FGF-2 (R&D Systems) and heparin (Aventis Pharma) in combination with 10 ng/ml BMP-9, and injected subcutaneously into the abdominal region of mice (n>5 mice per group). Matrigels were harvested 1 week after injection and were subject to immunohistochemistry for PECAM1.

Lentivirus production and infection

A lentiviral expression system was used to establish BMP-9-expressing BxPC3 cells (Shibuya et al., 2003). cDNA encoding human BMP-9 was cloned from liver cDNA of Multiple Tissue cDNA Panels (Clontech) using the following primers: forward, 5′-TTCCTTCAGAGCAAACAGCA-3′ and reverse, 5′-GTTGTGCTCAAATC-CCCATT-3′. *BMP-9* cDNA was subcloned into the pENTR vector and subsequently transferred into the pCS-EF-RfA lentiviral expression vector by the LR recombination reaction (Invitrogen). For production of lentiviral vectors, 293FT cells (Invitrogen) were transfected using Lipofectamine 2000 (Invitrogen) with three plasmids: vector construct, VSV-G- and Rev-expressing construct (pCMV–VSV-G–RSV–Rev) and packaging construct (pCAG-HIVgp). The culture supernatants were collected and viral particles were concentrated by centrifugation. For lentiviral infection, 1×10^5 BxPC3 cells were infected with lentivirus vectors in suspension and plated in six-well culture plates.

Balb/c nude mouse model of human pancreatic cancer

Balb/c nude male mice aged 5-6 weeks were obtained from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan). A total of 5×10^6 BxPC3 tumor cells in 100 µl phosphate-buffered saline (n>5 mice per group) were injected subcutaneously into the left flank of each mouse and allowed to grow for 5 weeks, at which point the major axis of tumors was approximately 10-mm long.

Immunohistochemistry and immunoblot analysis

Staining of cultured cells was performed as previously described (Watabe et al., 2003; Kokudo et al., 2008). Excised mouse tissue samples from Balb/c mice grafted with BxPC3 cells were snap-frozen in a dry-ice acetone bath for immunohistochemistry. Frozen samples were further sectioned at 10-µm thickness in a cryostat and subsequently incubated with primary and secondary antibodies. Monoclonal antibodies to PECAM1 (Mec13.3) and SMA (1A4) for immunohistochemistry were purchased from BD Pharmingen and Sigma, respectively. Stained specimens were examined using a phase-contrast microscope (Model IX70; Olympus) or an LSM 510 META confocal microscope (Carl Zeiss). All images were imported into Adobe Photoshop as JPEGs or TIFFs for figure assembly. Images were processed using ImageJ (NIH) to quantify PECAM1-positive areas. Immunoblot analysis was performed as described (Watabe et al., 2003). Antibodies to HA and α-tubulin for immunoblot analysis and immunohistochemistry were obtained from Sigma. The bound antibody was detected using a chemiluminescent substrate (ECL; Amersham) and a LAS-4000 Luminescent image analyzer (Fuji Photo Film).

RNA isolation and RT-PCR analysis

Total RNAs were extracted using the RNeasy Mini Kit (Qiagen). RNAs were reverse-transcribed by random hexamer priming using Superscript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR analysis was performed using the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) and Power SYBR Green (Applied Biosystems). All expression data were normalized to those for β-actin. The primer sequences are shown in supplementary material Table S1.

Statistical analysis

Results were statistically examined using the two-sided Student's t-test. Differences were considered significant at P<0.05.

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