Authorship

Contribution: M.O. performed the research and wrote the manuscript; C.I. collected data, contributed vital new reagents or analytical tools, and analyzed and interpreted the data; H.I.S. performed the research and collected the data; K.K. collected the data and contributed vital new reagents or analytical tools; Y.M. collected the data; T.W. contributed vital new reagents or analytical tools; A.K. collected the data; M.R.K. designed and performed the research, analyzed and interpreted the data, performed statistical analysis, and wrote the manuscript; K.M. designed the research, analyzed and interpreted the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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J. Biochem. **143,** 199–206 (2008)
doi:10.1093/jb/mvm215

BMPs Promote Proliferation and Migration of Endothelial Cells via Stimulation of VEGF-A/VEGFR2 and Angiopoietin-1/Tie2 Signalling

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Received September 18, 2007; accepted October 22, 2007; published online November 15, 2007

The differentiation, growth, and survival of endothelial cells (ECs) are regulated by multiple signalling pathways, such as vascular endothelial growth factors (VEGFs) and angiopoietins through their receptor tyrosine kinases, VEGF receptor (VEGFR) 2 and Tie2, respectively. Bone morphogenetic proteins (BMPs), members of the transforming growth factor (TGF)-β family, have been implicated in the development and maintenance of vascular systems. However, their effects on EC proliferation remain to be elucidated. In the present study, we show that BMPs induce the proliferation and migration of mouse embryonic stem cell (ESC)-derived endothelial cells (MESECs) and human microvascular endothelial cells (HMECs). Addition of BMP-4 to culture induced significant proliferation and migration of both types of ECs. BMP-4 also increased the expression and phosphorylation of VEGFR2 and Tie2. These findings suggest that BMP signalling activates endothelium via activation of VEGF/VEGFR2 and Angiopoietin/Tie2 signalling.

Key words: embryonic stem (ES) cell, Flk1, human microvascular endothelial cell (HMEC), Tie2, VEGFR2.

Abbreviations: BMP, bone morphogenetic protein; ECs, endothelial cells; ESC, embryonic stem cell; HMEC, human microvascular endothelial cell; MCs, mural cells; MESEC, mouse embryonic stem cell-derived endothelial cell; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Blood vessels consist of a lining of endothelial cells (ECs) surrounded by mural cells (MCs; pericytes and vascular smooth muscle cells), carry oxygen and nutrients to distant organs and are crucial for organ growth in the embryo and repair of wounded tissues in the adult. Defects in the formation and maintenance of blood vessels thus contribute to the pathogenesis of numerous disorders (*1*). Development of the vascular system occurs in two distinct processes, vasculogenesis and angiogenesis (*2*).

During mouse embryogenesis, the initial development of vascular endothelium, termed vasculogenesis, occurs in the mesodermal layer of the yolk sac, yielding structures, termed blood islands. Blood islands consist of endothelial progenitors called angioblasts, which express vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2), also known as Flk1 (*3*, *4*). This embryonic vascular differentiation has been recapitulated by an *in vitro* differentiation system from embryonic stem cells (ESCs) (*5–7*). When VEGFR2-expressing (VEGFR2+) endothelial progenitors, isolated from differentiation cultures, are re-differentiated in the presence of VEGF, various endothelial markers are sequentially up-regulated in a pattern that is similar to that observed in early embryos (*5*). Further analysis revealed that lineages of both ECs and MCs develop from a common ESC-derived endothelial progenitor cell type (*7*).

In angiogenesis, new vessels sprout from the pre-existing vasculature and are further remodelled 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 remodelling of tissues during wound healing and ischaemia, and for the physiological female reproductive cycle. Neovascularization also plays a pivotal role in pathological processes such as tumour growth, chronic inflammation and diabetic vasculopathy.

During vasculogenesis and angiogenesis, the differentiation, proliferation, and migration of ECs are regulated by a balance between positive and negative regulators. VEGF signalling through VEGFR tyrosine kinases has been implicated in regulation of the differentiation, growth, and integrity of ECs. VEGF stimulates endothelial differentiation of vascular progenitor cells derived from ESCs (*7*), and regulates the growth of differentiated ECs.

Angiopoietin (Ang)-1 binds and activates the Tie2 receptor tyrosine kinase, which is expressed almost exclusively on the surface of ECs (*8*). Ang-1 is required for maintenance of maximal interactions between ECs, MCs and the extracellular matrix (*9*). The Ang-1/Tie2 pathway plays important roles in embryonic

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development, physiological angiogenesis, maintenance of vascular integrity, inflammation and tumour growth (*10*). In the mature vasculature or cultured ECs, Ang-1 inhibits apoptosis (*11*) and promotes differentiation, sprouting and migration. In contrast, Ang-2 can act as either an agonist or an antagonist for Tie2, depending on the type of cell and the surrounding microenvironment (*12*). Ang-2 is expressed at sites of pericyte detachment and blood vessel remodelling in conjunction with VEGF, whereas in the absence of VEGF, Ang-2 activity leads to EC apoptosis. These findings strongly suggest that the members of the angiopoietin and VEGF families collaborate during different stages of angiogenesis (*13*).

In addition to VEGF/VEGFR2 and angiopoietins/Tie2 signalling, transforming growth factor (TGF)-β family proteins have been implicated in vascular development (*14*). The TGF-β family consists of structurally related and multifunctional proteins including TGF-βs, activins and bone morphogenetic proteins (BMPs). Members of the TGF-β 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 TGF-βs bind to type I receptors known as activin receptor-like kinase (ALK)-4 and -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 3 transduce signals for TGF-βs and activins, while Smad1, 5 and 8 are specific for signalling of BMPs (*15*). An exception to this is ALK-1, which is preferentially expressed in ECs, also binds TGF-β and activates the Smad1/5 pathways (*16*). Recently, BMP-9 and BMP-10 were shown to bind to ALK-1 and to activate the Smad1/5 pathways (*17*, *18*).

Perturbation of TGF-β family receptor signalling in humans leads to vascular disorders. Hereditary haemorrhagic telangiectasia is genetically linked to mutations of ALK-1 and endoglin, a co-receptor for TGF-β family members (*19*). Furthermore, loss-of-function mutations in the human BMP type II receptor (BMPR-II) are associated with the pathogenesis of familial primary pulmonary arterial hypertension (*20–22*). Studies of various mice knocked out for TGF-β family signalling components have also suggested their importance in vascular development (*23*). Mice lacking BMP-4 (*24*) or specific BMP receptors (*25*, *26*) exhibit abnormal development of the heart and vasculature. Mice lacking Smad1 or Smad5 die at embryonic day 10.5–11.5 due to defects in vascular development, with enlarged blood vessels surrounded by decreased numbers of vascular smooth muscle cells (*27*, *28*). The finding that mice deficient in BMPs, their receptors or their intracellular Smad effector proteins have impaired vascular development and the linkage of pulmonary arterial hypertension with mutations of BMPR-II suggest a role of the BMP/Smad signalling cascade in formation of the vascular system.

In vivo and *in vitro* gain-of-function analyses have also suggested a role of BMP signalling during vascular development. When CHO cells overexpressing BMP-4 were implanted in the avascular region of quail embryos, endothelial growth and capillary plexus formation were enhanced (*29*). Furthermore, *in vitro* experiments have shown that BMPs indeed modulate EC migration and capillary tube formation, an effect that can be antagonized by BMP antagonists (*29*, *30*). However, the effects of BMPs on the proliferation of cultured ECs, especially embryonic ECs, have not been fully determined.

In the present study, we examined the effects of BMP signalling on the proliferation and migration of mouse ESC-derived endothelial cells (MESECs) and human microvascular endothelial cells (HMECs). Addition of BMP-4 to culture significantly induced proliferation and migration of these cells. The expression and phosphorylation of VEGFR2 and Tie2 were up-regulated by BMP-4 in both types of cells. These findings suggest that BMP signalling activates endothelium via activation of VEGF/VEGFR2 and Ang/Tie2 signalling.

MATERIALS AND METHODS

Cells and Cell Culture—Maintenance, differentiation, culture, and cell sorting of CCE and MGZ5 mouse ES cells (gifts from Drs M.J. Evans and H. Niwa, respectively) were as described (*7*). All experiments were carried out using both ES cell lines and yielded essentially similar results. HMEC, an immortalized human dermal microvascular EC line, was obtained from Dr T. Lawley, and was cultured in EGM-2 (Cambrex) containing 2% fetal bovine serum (FBS) and EC growth supplements (Clonetics). VEGF and BMP-4 were purchased from R&D. Concentrations of BMP-4 used in this study (60 and 10 ng/ml for MESECs and HMECs, respectively) were verified by studying its dose-dependent effects on their proliferation (data not shown).

Cell Growth and BrdU Incorporation Assays—Cell number was counted using a Coulter Counter (Yamato Kagaku). The nucleotide BrdU is incorporated during the S phase of the cell cycle. In order to quantify the ratio of DNA synthesis, BrdU incorporation assays were performed according to the manufacturer's protocol (Roche). Briefly, BrdU was added to culture for 2 h during incubation. Fixed cells were incubated with anti-BrdU antibodies, followed by detection of immune complexes with a fluorescein isothiocyanate (FITC)-labelled anti-mouse antibody and nuclear staining with propidium iodide (PI). Confocal images were obtained by laser scanning/confocal microscopy (LSM 510 META; Carl Zeiss). The proportion of BrdU-positive cells was determined among at least 50 cells counted in three different fields chosen randomly.

TUNEL Assay—For detection of apoptosis, TUNEL assays were performed according to the manufacturer's protocol (Roche). Briefly, permeabilized ECs were subjected to terminal deoxynucleotidyltransferase (TdT) reaction with FITC-12-dUTP followed by nuclear staining with PI and was observed under a laser scanning/confocal microscope. The proportion of TUNEL-positive cells was determined among at least 100 cells counted in three different fields chosen randomly.

Video Time-Lapse Microscopy—Time-lapse imaging of migrating cells was performed on a Leica DM IRB

microscope equipped with a hardware-controlled motor stage over 24 h in serum-free medium at 37°C/5% CO_2. Images of HMECs or MESECs were obtained with a LEICA DC 350F CCD camera every 15 or 20 min, respectively, and analyzed using Image J software (National Institutes of Health, USA). Migration of each cell was analyzed by measuring the distance travelled by a cell nucleus over the 24-h time period (*31*). Average migration speed was calculated by analyzing at least 10 cells/group.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (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 GeneAmp 5700 system (Applied Biosystems) and SYBR Green (Applied Biosystems). All expression data were normalized to those for β-actin. The primer sequences are available online as indicated in Table S1.

Immunohistochemistry and Western Blot Analysis—Monoclonal antibodies to PECAM1 (Mec13.3) and SMA (1A4) for immunohistochemistry were purchased from BD Pharmingen and SIGMA, respectively. Staining of cultured cells was performed as described (*32*). Stained cells were photographed using a phase-contrast microscope (Model IX70; Olympus) or confocal microscope. All images were imported into Adobe Photoshop as JPEGs or TIFFs for contrast manipulation and figure assembly. Antibodies to FLAG and α-tubulin for Western blot analysis and immunohistochemistry were obtained from SIGMA. Antibodies to mouse VEGFR2, Tie2, human VEGFR2 and phospho-tyrosine for Western blot analysis and immunohistochemistry were obtained from eBioscience, RDI, Santa Cruz and Chemicon, respectively. Western blot analysis was performed as described (*33*).

RESULTS

BMP-4 Increases Endothelial Cell Number—To study the effects of BMP signalling on embryonic ECs, we utilized an *in vitro* vascular differentiation system from mouse ESCs (*7*). VEGFR2+ endothelial progenitor cells derived from ESCs differentiate predominantly into ECs when cultured in serum-free SFO medium in the presence of VEGF (Fig. 1A). We have previously shown that these MESECs are capable of transducing BMP signals (*34*). When BMP-4 was added to culture, cell number was significantly increased (Fig. 1A).

We also examined whether this effect of BMP-4 can be observed in mature ECs. We chose HMECs because they are capable of transducing both BMP and TGF-β signals as shown by western blot analysis for phosphorylation of R-Smads (Fig. S1), luciferase assays (Fig. S2) and quantitative RT-PCR analyses for target genes of BMP and TGF-β (Fig. S3A, B, respectively). As shown in Fig. 1B, number of HMECs was also significantly increased by the addition of BMP-4. These findings suggest that BMP-4 is capable of inducing proliferation of both embryonic and mature ECs.

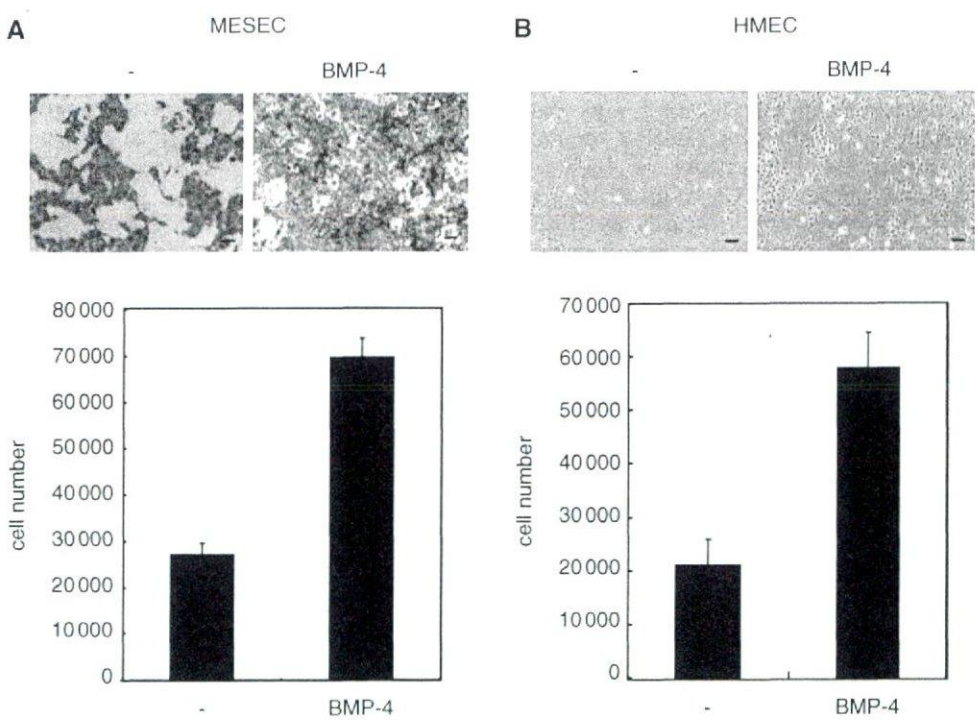

Fig. 1. **Effects of BMP-4 on number of ECs.** (A) VEGFR2+ cells (5×10^4/well) derived from CCE cells were cultured in serum-free SFO medium containing 30 ng/ml of VEGF in the absence or presence of 60 ng/ml of BMP-4, followed by PECAM1 (purple) immunostaining (top) and determination of cell number after 2 days (bottom). (B) HMECs (8×10^3/well) were cultured in serum-free EGM-2 medium in the absence or presence of 10 ng/ml of BMP-4, followed by photography (top) and determination of cell number after 6 days (bottom). Bars: 100 μm. Each value represents the mean of three determinations; Error bars, SD.

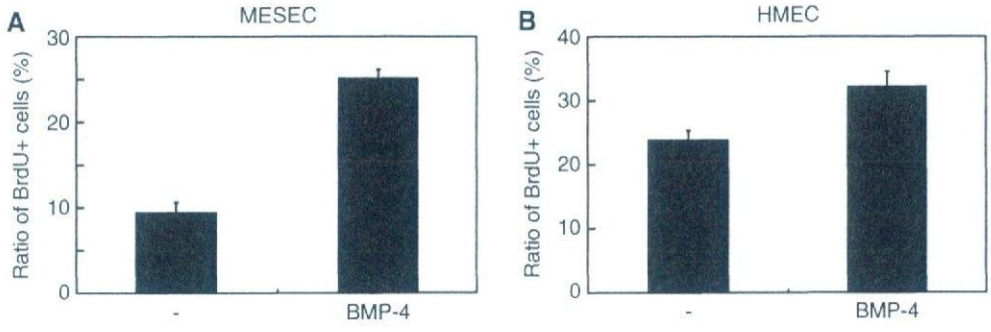

Fig. 2. **Effects of BMP-4 on DNA synthesis in ECs.** BrdU incorporation assays were carried out in MESECs (A) and HMECs (B) as described in MATERIALS AND METHODS. Each value represents the mean of eight determinations; Error bars, SE.

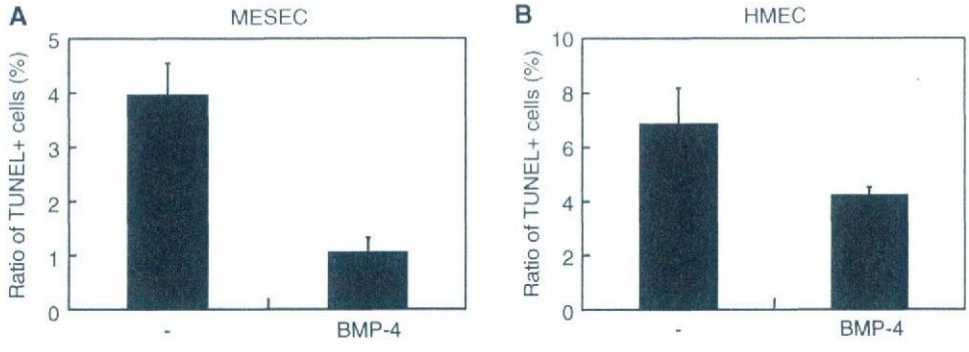

Fig. 3. **Effects of BMP-4 on apoptosis of ECs.** TUNEL assays were carried out in MESECs (A) and HMECs (B) as described in MATERIALS AND METHODS. Each value represents the mean of 3 (MESECs) and 10 (HMECs) determinations; Error bars, SE.

BMP-4 Promotes DNA Synthesis by ECs—Cell numbers are regulated by proliferation and apoptosis. To further examine the effects of BMP-4 on EC proliferation, we quantified the effects of BMP-4 on DNA synthesis in both types of ECs using BrdU incorporation assay. DNA synthesis was increased by BMP-4 in both types of ECs (Fig. 2), though to a more significant extent in MESECs.

BMP-4 Suppresses Apoptosis of ECs—We next examined the effect of BMP-4 on apoptosis of ECs by TUNEL assay. When BMP-4 was added, the ratio of TUNEL-positive cells was decreased in both MESECs and HMECs (Fig. 3). These findings suggest that BMP-4 increases EC numbers by induction of proliferation and inhibition of apoptosis.

BMP-4 Promotes Motility of ECs—Since induction of Id1 expression by BMP-6 is necessary and sufficient for BMP-induced activation of EC migration (*30*), we examined whether BMP-4 promotes the migration of ECs. Tracking of single ECs using video time-lapse microscopy showed that BMP-4 significantly increased the motility of MESECs (Fig. 4A) and HMECs (Fig. 4B). We further examined the effects of BMP-4 on Id1 expression in these ECs. As shown in Fig. 5A and B, BMP-4 significantly induced Id1 expression in MESECs and HMECs. These results provided further support for the previously reported finding that BMP-induced Id1 expression stimulates EC motility.

BMP-4 Induces the Expression and Phosphorylation of VEGFR2 Proteins—We next attempted to identify the mediators of BMP-4 inducing EC proliferation. Since VEGF/VEGFR2 signalling pathways stimulate the proliferation, survival and migration of ECs, we examined the effects of BMP-4 on the expression of VEGF and VEGFR2. As shown in Fig. 5C and D, BMP-4 induced the expression of transcripts for VEGFR2 in MESECs and HMECs. VEGFR2 is activated by phosphorylation of its tyrosine residues. We further examined the effects of BMP-4 on the synthesis and phosphorylation of VEGFR2 proteins by biochemical analyses. BMP-4 increased the levels of protein expression and phosphorylation of VEGFR2 in both MESECs and HMECs (Fig. 6A and B), suggesting that VEGFR2 signalling is activated when BMP-4 was added.

BMP-4 Induces the Expression and Phosphorylation of Tie2 Proteins—Ang-1 and -2 are agonist and antagonist, respectively, for the Tie2 tyrosine kinase receptor, which increases the integrity and inhibits apoptosis of ECs. We therefore studied if Ang/Tie2 signals are regulated by BMP signals in ECs. When BMP-4 was added to culture of both types of ECs, levels of transcripts for Tie2 (Fig. 5E and F) and Ang-2 (Fig. 5G and H) were increased and decreased, respectively, while those for Ang-1 were not altered (data not shown). Levels of Tie2 protein expression and phosphorylation of Tie2 were also increased in both types of ECs upon treatment with

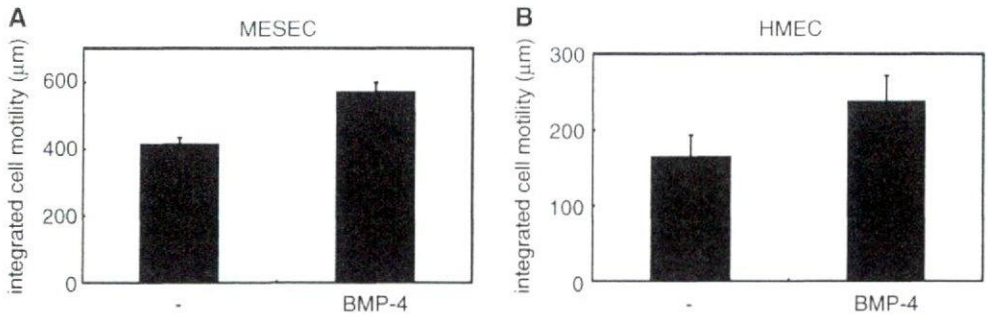

Fig. 4. **Effects of BMP-4 on endothelial cell migration.** Cell migration was measured by video-time lapse microscopy as described in MATERIALS AND METHODS. MESECs (A) and HMECs (B) were subjected to video microscopy for 24 h in the absence and presence of BMP-4. Results are integrated cell motility over 24 h. Each value represents the mean of 10 determinations; Error bars, SE.

BMP-4 (Fig. 6C and D), suggesting that Tie2 signalling is activated by BMP-4 stimuli.

DISCUSSION

While previous *in vitro* studies have shown that BMPs induce EC migration and capillary tube formation (*29*, *30*), the present finding that BMP-4 induces the proliferation of both embryonic and mature ECs (Fig. 1) reveals a novel mechanism by which BMP signalling contributes to vasculogenesis and angiogenesis.

We also found that BMP-4 induced the expression and phosphorylation of VEGFR2 tyrosine kinase (Figs. 5 and 6). When BMP-4-loaded beads are grafted into quail embryo lateral mesoderm, BMP-4 induces the expression of Quek1, the VEGFR2 homologue in quail (*35*), implicating similar molecular mechanisms in quail embryos. BMP-4 also induced the expression of VEGF in MESECs but not in HMECs (data not shown). Since VEGF/VEGFR2 signalling induces the proliferation and migration of ECs, the pro-angiogenic effects of BMP-4 appear to be mediated by activated VEGFR2 signals.

Furthermore, we found a novel relationship between BMP signalling and another pro-angiogenic signal. BMP-4 induced the phosphorylation of Tie2 receptor tyrosine kinase, possibly by induction of Tie2 expression and repression of Ang-2 expression in ECs (Figs. 5 and 6). While expression of Ang-1 was not altered in the ECs examined, BMP-4 induced Ang-1 expression in mouse ESC-derived MCs (data not shown), suggesting another role of BMPs in the activation of Tie2 signalling in blood vessels. Angiopoietin/Tie2 signalling elicits various effects including maturation of endothelial structures and survival of ECs. The roles of Tie2 activation during BMP-4-induced activation of ECs remain to be elucidated. In any case, since VEGF/VEGFR2 signalling is essential for the differentiation and proliferation of ECs, it is unlikely that BMP alone can induce proliferation of ECs in the absence of VEGF.

Vascular development during embryogenesis and adults requires the transition from the resolution to the activation phase and vice versa, which is determined by the balance between positive and negative regulators of ECs. Both pro- and anti-angiogenic properties have been ascribed to TGF-β by genetic studies of various knockout mice deficient for signalling components (*23*). Goumans and colleagues (*36*) showed that TGF-β can activate ALK-5/Smad2, 3 and ALK-1/Smad1, 5, 8 pathways, leading to inhibition and activation of cell migration and proliferation, respectively. We have also studied the roles of TGF-β signalling during endothelial differentiation from ESCs (*34*). TGF-β and activin inhibit EC proliferation, whereas inhibition of endogenous TGF-β and activin signalling by SB-431542, an inhibitor for ALK-4, 5, 7 kinases (*37*), facilitates their proliferation, suggesting that endogenous TGF-β and activin signalling predominantly suppresses embryonic endothelial growth. Taken together with the present finding, these results suggest that the transition between activation and resolution phases of ECs may be regulated by pro-angiogenic TGF-β, BMP/ALK-1, 2, 3, 6/Smad1, 5, 8 signalling and anti-angiogenic TGF-β/ALK-5/Smad2, 3 signalling.

In many types of cells, such as those of human breast and prostate carcinoma cell lines, BMPs induce the expression of $p21^{CIP1/WAF1}$, resulting in hypophosphorylation of pRb protein and growth arrest (*38–40*). During programmed capillary regression in the newborn rat eye, BMPs secreted by the lens induce apoptosis of ECs and inhibit endothelial tubulogenesis (*41*). These contradictory findings may be due to the characteristics of ECs (e.g. the profile of expression of BMP signalling components). Furthermore, other types of BMPs have also been shown to inhibit the growth of ECs. BMP-9 and 10 have been shown to inhibit the proliferation, migration, and tube formation of ECs via activation of ALK-1 (*17*, *18*). The effects of BMP-9 and 10 on the proliferation of MESECs and HMECs need to be elucidated in the future.

This growth-inhibitory effect of BMPs was previously found to participate in the pathogenesis of pulmonary arterial hypertension. Loss-of-function mutations in the BMPR-II gene have been found in patients with pulmonary arterial hypertension, which is characterized by abnormal intimal thickening and muscularization of pulmonary arterioles, which are believed to result from dysregulation of growth of endothelial and smooth muscle cells. While BMP signalling has been shown to inhibit the proliferation of smooth muscle cells (*42*), it has been shown to promote survival of pulmonary

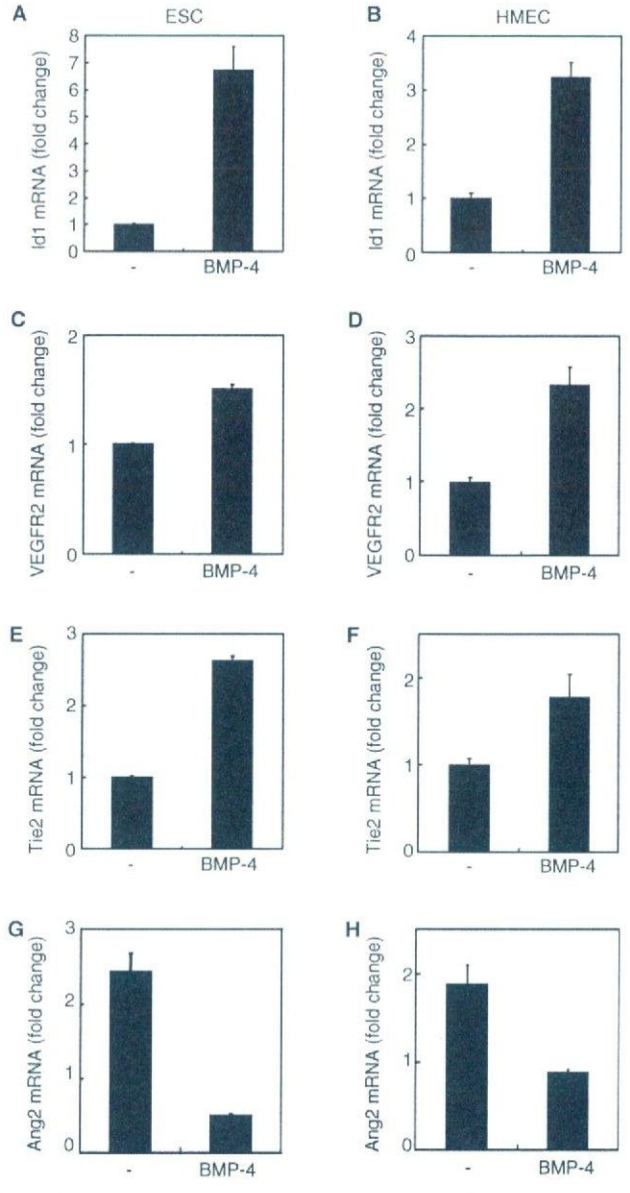

Fig. 5. **Effects of BMP-4 on expression of Id1, VEGFR2, Tie2, and Ang-2 in ECs.** MESECs (A, C, E, G) and HMECs (B, D, F, H) were cultured for 48 h in the absence or presence of BMP-4 and subjected to quantitative RT-PCR analyses for the expression of transcripts for Id1 (A, B), VEGFR2 (C, D), Tie2 (E, F) and Ang-2 (G, H). Quantitated mRNA values were normalized by the amounts of β-actin mRNA, and results are given as fold change. Each value represents the mean of three determinations; Error bars, SD.

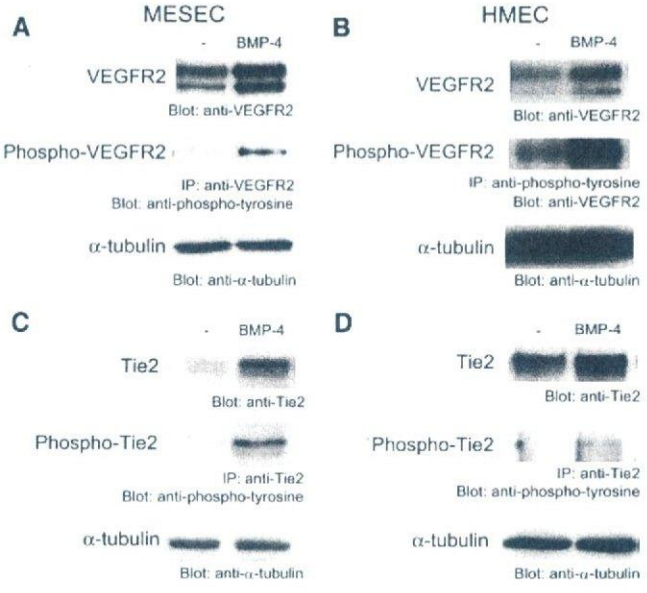

Fig. 6. **Effects of BMP-4 on expression and phosphorylation of VEGFR2 and Tie2 in ECs.** MESECs (A, C) and HMECs (B, D) were cultured for 48 and 24 h, respectively, in the absence or presence of BMP-4. Cell lysates were immunoblotted with anti-VEGFR2 (A, C) and anti-Tie2 (B, D) antibodies to examine the expression of VEGFR2 and Tie2 proteins, respectively (top panel). In order to determine levels of phosphorylation of VEGFR2 and Tie2 proteins, cell lysates were immunoprecipitated by anti-mouse VEGFR2 (A), anti-phospho-tyrosine (B), anti-mouse Tie2 (C) and anti-human Tie2 (D) antibodies, followed by immunoblotting by anti-phospho-tyrosine (A, C, D) and anti-human VEGFR2 (B) antibodies (middle panel). α-tubulin was used as a loading control (bottom panel).

artery ECs (*43*). Furthermore, the combination of apoptosis caused by inhibition of VEGFR2 with chronic hypoxia has been shown to result in selection of an apoptosis-resistant EC phenotype leading to increased proliferation of pulmonary artery ECs (*44*). Therefore, loss-of-BMPR-II-induced apoptosis in ECs may lead to the overproliferation of selected apoptosis-resistant cells, which may initiate the pathogenesis of pulmonary arterial hypertension.

Growth of solid tumours is highly dependent on sufficient blood supply by newly formed blood vessels. Many types of tumours including lung tumours and malignant melanomas express high levels of BMPs (*45*, *46*). In addition to their autocrine effects in promoting cell invasion and migration, a paracrine effect of BMPs on the vascular network has been reported. Under experimental conditions, recombinant BMP-2 produced large increases in size and number of tumour blood vessels, especially in the early phase of tumour growth (*45*, *46*). The findings of the present study suggest the possibility that targeting of BMP signalling may inhibit tumour angiogenesis and lead to tumour regression. Alternatively, activation of BMP signalling in endothelial and/or endothelial progenitor cells may be useful as a therapeutic strategy in regenerative medical treatment of ischaemia and vascular disorders.

Supplementary data are available at *JB* online.

We thank Dr Evans (University of Cambridge, Cambridge, United Kingdom) for CCE ES cells, Dr Niwa (CDB, Kobe, Japan) for MGZ5 ES cells, Dr Lawley (Emory University, Atlanta, USA) for providing the HMEC-1 cells, and Dr Maemura (University of Tokyo, Tokyo, Japan), Dr Hamaguchi (Keio University, Japan), Dr Soubrier (INSERM, France) and many of our colleagues for suggestions and discussion. This research was supported by

Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Science, Sports and Technology of Japan.

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