BMPER Is an Endothelial Cell Regulator and Controls Bone Morphogenetic Protein-4–Dependent Angiogenesis

Jennifer Heinke, Leonie Wehofsits, Qian Zhou, Christoph Zoeller, Kim-Miriam Baar, Thomas Helbing, Anna Laib, Hellmut Augustin, Christoph Bode, Cam Patterson, Martin Moser

Abstract—Bone morphogenetic proteins (BMPs) are involved in embryonic and adult blood vessel formation in health and disease. BMPER (BMP endothelial cell precursor–derived regulator) is a differentially expressed protein in embryonic endothelial precursor cells. In earlier work, we found that BMPER interacts with BMPs and when overexpressed antagonizes their function in embryonic axis formation. In contrast, in a BMPER-deficient zebrafish model, BMPER behaves as a BMP agonist. Furthermore, lack of BMPER induces a vascular phenotype in zebrafish that is driven by disarray of the intersomitic vasculature. Here, we investigate the impact of BMPER on endothelial cell function and signaling and elucidate its role in BMP-4 function in gain- and loss-of-function models. As shown by Western blotting and immunocytochemistry, BMPPER is an extracellular matrix protein expressed by endothelial cells in skin, heart, and lung. We show that BMPER is a downstream target of FoxO3a and consistently exerts activating effects on endothelial cell sprouting and migration in vitro and in vivo. Accordingly, when BMPER is depleted from endothelial cells, sprouting is impaired. In terms of BMPER related intracellular signaling, we show that BMPER is permissive and necessary for Smad 1/5 phosphorylation and induces Erk1/2 activation. Most interestingly, BMPER is necessary for BMP-4 to exert its activating role in endothelial function and to induce Smad 1/5 activation. Vice versa, BMP-4 is necessary for BMPER activity. Taken together, BMPER is a dose-dependent endothelial cell activator that plays a unique and pivotal role in fine-tuning BMP activity in angiogenesis. (Circ Res 2008;103:804-812.)

Key Words: BMPER ■ bone morphogenetic proteins ■ vascular biology ■ endothelial cell function ■ signaling

Angiogenesis is a basic biological event that is involved in embryonic development but also in adult physiological and pathological conditions, such as inflammation, tumor growth, atherosclerosis, or response to ischemia. This process depends on the orchestrated function of intra- and extracellular proteins, many of which are conserved from embryonic development through adulthood.1

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor (TGF)-β superfamily. Originally, they have been identified by their ability to induce ectopic bone formation and have been extensively studied during embryonic development, in which they control axis formation and organogenesis. Today, more than 20 BMP-related proteins and a number of BMP modulating proteins have been identified.2 A growing body of evidence suggests that they serve as important regulators in vascular development and disease.3 BMPs are extracellular proteins that signal through cell surface complexes of type I and type II serine/threonine kinase receptors. On activation, the receptors mediate intracellular signaling mainly through the Smad 1/5 transcription factors. BMP signaling is regulated at several levels: activity of R-Smads (1/5) is modulated by facilitating (eg, Smad 4) or inhibitory (eg, Smad 6) co-Smads.4 BMP receptors undergo regulation by clustering, and last, but not least, extracellular agonists such as BMP-4 are modulated in their function by extracellular binding proteins such as chordin,5 chordin-like 2 (CHL-2),6 noggin,7 drm/gremlin,8 twisted gastrulation (Tsg),9 and BMPER.10,11

BMPER was originally identified in a screen for differentially expressed proteins in embryonic endothelial precursor cells.10 BMPER is a secreted glycoprotein that contains 5 cysteine-rich domains, followed by a von Willebrand D domain and a trypsin inhibitor domain, and is, thus, the vertebrate homolog of drosophila crossveinless-2. BMPER binds directly to BMPs and modulates their function. So far, inconclusive data have been reported about BMPER and its modulating function on BMP-4. In gain-of-function assays anti-BMP activity of BMPER has been reported,10,12 whereas in loss-of-function models BMPER exerts pro-BMP functions.13-16

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From the Departments of Cardiology (J.H., L.W., Q.Z., C.Z., K.-M.B., T.H., C.B., M.M.) and Biology (J.H., K.-M.B.), University of Freiburg, Germany; German Center for Cancer Research (A.L., H.A.), Heidelberg, Germany; and Carolina Cardiovascular Biology Center (C.P.), University of North Carolina, Chapel Hill.

Correspondence to Martin Moser, University of Freiburg, Department of Cardiology, Hugstetter Strasse 55, 79106 Freiburg, Germany. E-mail Martin.Moser@uniklinik-freiburg.de © 2008 American Heart Association, Inc.

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Based on our findings in zebrafish, where loss of BMPER function results in a disarray of intersomitic blood vessels, here, we study the function of BMPER in endothelial cell biology and angiogenesis. Our data indicate that BMPER is necessary for endothelial cell sprouting and has a dose-dependent stimulating effect on sprouting and migration. To achieve these effects, both BMPER and BMP-4 are dependent on the presence of one another. We further show that BMPER is involved in Smad 1/5 and Erk1/2 signaling. In conclusion, BMPER has proangiogenic properties by modulating BMP-4 signaling.

Materials and Methods

Cell culture, immunocytochemistry, flow cytometry, adhesion assay, real-time PCR, Western blot analysis, human umbilical vein endothelial cell (HUVEC) transfection, chick chorioallantois membrane (CAM) assay, primer sequences, reagents, and antibodies are described in the online data supplement, available at http://circres.ahajournals.org.

RNA Interference

BMPER small interfering (si)RNAs were purchased from Ambion. BMP-4 and FoxO3a siRNAs were purchased from Invitrogen. Scrambled negative control Alexa Fluor 488 nm was purchased from Qiagen. The specific sequences are given in the online data supplement. For siRNA transfection, Lipofectamine RNAiMAX was used according to the protocol of the manufacturer (Invitrogen). Transfection efficiency was confirmed by quantitative real-time (quantitative) PCR. Functional cell culture assays were performed between 8 to 48 hours posttransfection.

Matrigel Sprouting Assay

Culture plates were coated with Matrigel (BD Biosciences) according to the instructions of the manufacturer. HUVECs were pretreated with basic fibroblast growth factor (40 ng/mL), BMP-4 (25 ng/mL), Noggin (100 ng/mL), or various concentrations of BMPER (all R&D) in 1% FBS/EBM for 24 to 18 hours. A total of 3 x 10^4 cells were cultured on Matrigel for 3 hours at 37°C. Cells were fixed with 4% paraformaldehyde and pictures were taken from 4 random microscopic fields. The cumulative sprout length and the number of branch points were quantified as described for the spheroid assay.

HUVEC Spheroid Sprouting Assay

HUVEC spheroids were generated as previously described. Briefly, HUVECs were grown as hanging drops of approximately 625 cells each for 24 hours in a cell culture incubator. For gel preparation spheroids were resuspended in carboxymethylcellulose containing 20% FBS, mixed with the same volume of collagen, adjusted to pH 7.4, rapidly aliquoted into a 24-well plate and incubated for 1 hour at 37°C for polymerization before sprouting was stimulated with 100 μL 0.5% BSA or growth factors in EBM for 24 hours in triplicates. To quantify in-gel angiogenesis the cumulative length of all capillary-like sprouts originating from the core of an individual spheroid was measured at 5x magnification using a digitized imaging system. At least 10 spheroids per condition were analyzed with AxioVision Rel. 4.6.
Migration Assay

To determine the migration of endothelial cells, HUVECs were labeled with 10 μmol/L CFDA-SE (Invitrogen) in PBS, detached with trypsin/Versene, harvested by centrifugation, resuspended in EBM with 0.5% BSA, placed in the upper chamber of a modified Boyden chamber (1x10⁵ cells per HTS FluoroBlok 24-well chamber; pore size 8 μm; BD Biosciences). The chambers were placed in 24-well culture dishes containing EBM with 0.5% BSA or growth factors. After incubation for 4 hours at 37°C, 5% CO₂, the cells were fixed with 4% paraformaldehyde and migrated cells were counted manually in 5 random microscopic fields using a fluorescent microscope.

Matrigel Plug Assay and Immunohistochemistry

Growth factor–reduced Matrigel (BD Biosciences) was thawed overnight at 4°C and mixed with heparin to a final concentration of 20 U/mL. BMPER was added to final concentrations of 20 up to 5000 ng/mL to a total volume of 500 μL of Matrigel. Basic fibroblast growth factor (150 ng/mL) was used as a positive control. Matrigel containing the respective growth factors or vehicle was injected subcutaneously in to the groins of female C57BL/6 mice (The Jackson Laboratory). After 9 days, plugs were isolated, fixed in 4% paraformaldehyde, and sectioned. For immunofluorescence staining, slides were blocked with 10% normal goat serum and incubated overnight with polyclonal primary antibody (anti-CD31) and secondary fluorescent antibody Cy-3. Blood vessel infiltration was analyzed in 10 random hematoxin/eosin-stained sections analyzed with Zeiss Axioplan2/Axiovision (version 4.6). Experiments were performed according to the Animals Scientific Procedures Act of 1986 and local ethics protocols.

Statistical Analysis and Quantification

Statistical analysis was performed using GraphPad Prism 4.0. Data are presented as means±SD, and comparisons were calculated by Student’s t test (2-sided, unpaired). Results were considered statistically significant when P<0.05. Densitometric analysis of Western blots was performed using Quantity One 1-D Analysis Software (version 4.4, Bio-Rad).

Results

BMPER Expression in Endothelial Cells

Based on our previous work, we hypothesized that BMPER may be expressed by mature endothelial cells. Indeed, BMPER was detectable in venous endothelial cells (HUVECs), as well as in human microvascular endothelial cells obtained from skin, heart, and lung (Figure 1A). BMPER is expressed in the extracellular space and at the surface of culture yolk sac endothelial cells as demonstrated by immunocytochemistry (Figure 1B). These data, taken together with our previous findings, indicate that BMPER is an extracellular protein expressed by endothelial cells.

BMPER Regulation by FoxO3a

FoxO transcription factors have been implicated in BMPER regulation in a mouse model lacking all 3 FoxOs. To test whether FoxO transcription factors regulate BMPER also in endothelial cells, we silenced FoxO3a using 2 different siRNAs in HUVECs. Indeed, BMPER RNA and protein was upregulated when FoxO3a was silenced (Figure 1D and 1E).

Figure 3. BMPER regulates endothelial cell sprouting. Serum-starved HUVECs were treated with or without BMPER at indicated concentrations or basic fibroblast growth factor (bFGF) (40 ng/mL) as a positive control for 16 to 18 hours before they were seeded onto Matrigel. A through C, Representative micrographs are shown. Scale bar=200 μm. D and F, Cumulative sprout length of capillary-like structures were measured after 3 hours. E and G, The number of branch points was counted in the same specimens as used for D and F. *P<0.05 vs control. F and G, HUVECs were transfected with either of 2 BMPER-specific siRNAs or scrambled siRNA control. Forty-eight hours posttransfection, the Matrigel assay was performed. Recombinant BMPER protein was added to the BMPER-depleted cells to demonstrate specific rescue of the siRNA effect. Cumulative sprout length and branch points of capillary-like structures were measured. Means±SD. D through G show the result of 1 of 3 independent experiments. *P<0.05 vs control; #P<0.01 vs siRNA alone.
suggesting that BMPER is a downstream target of FoxO3a. Accordingly, overexpression of a constitutively active variant of FoxO3a resulted in downregulation of BMPER mRNA (Figure 1F), indicating that FoxO3a is a suppressor of BMPER expression.

**BMPER Effect on Endothelial Cell Sprouting**

The function of BMPER in endothelial cells was investigated in gain- and loss-of-function models. Silencing of BMPER was effectively achieved at 24 and 48 hours after siRNA transfection, as determined by RT-PCR and Western blot analysis (Figure 2A through 2C). BMPER effect on endothelial cell sprouting was studied in the Matrigel tube-forming assay (Figure 3) and the HUVEC spheroid-sprouting assay providing collagen instead of Matrigel in a 3D matrix (Figure 4). In the Matrigel tube-forming assay, HUVEC sprouting was enhanced by up to 53% when BMPER was added at concentrations from 5 to 30 ng/mL, as quantified by assessment of total sprout length or the number of branch points (Figure 3A, 3B, 3D, and 3E). At high BMPER concentrations, HUVEC sprouting was less and less pronounced. When BMPER was depleted from HUVECs, cell sprouting and branching were inhibited, consistent with an activating role for BMPER at lower concentrations (Figure 3F and 3G). This effect could be rescued by adding BMPER to siBMPER silenced cells (Figure 3F and 3G). In the HUVEC spheroid-sprouting assay, we obtained very similar results. Low BMPER concentrations resulted in enhanced sprouting, whereas higher BMPER concentrations prevent the activation of HUVEC sprouting (Figure 4 through 4C). Taken together, these data indicate that BMPER is necessary for endothelial cell sprouting and that, independent from the assay system used, low BMPER concentrations enhance sprouting, whereas at higher concentrations endothelial cell sprouting is prevented.

**BMPER Effect on Endothelial Cell Migration**

To investigate the effect of BMPER on endothelial cell migration, we used a modified Boyden chamber system. Similar to the effects on endothelial cell sprouting, BMPER stimulated HUVECs to migrate faster at low concentrations, whereas migration is prevented at higher BMPER concentrations (Figure 4D). Thus, BMPER not only enhances endothelial cell sprouting but also stimulates endothelial cell migration in a dose-dependent manner.

**BMPER Increases Capillary Network Density in the CAM**

To investigate BMPER function in vivo, we performed the CAM assay in chick embryos. BMPER protein was applied to the CAM and differentiation of the chorionic capillary network was visualized by staining endothelial cells (Figure 5). The capillary network was denser, and the diameter of the capillaries was greater in the presence of BMPER, very similar to the effect obtained by addition of VEGF, indicating that endothelial cells are stimulated by BMPER. Consistent with our in vitro findings, high BMPER doses prevent endothelial cell activation in the CAM. Thus, these in vivo findings are consistent with increased endothelial cell migration and sprouting induced by BMPER observed in vitro.

**BMPER Induces Angiogenesis in the In Vivo Matrigel Plug Assay**

As a second in vivo model, we used the mouse subcutaneous Matrigel plug assay to investigate angiogenic activity of BMPER. Consistent with our observations in the CAM, we
found that increasing concentrations of BMPER enhanced the invasion of endothelial cells into the Matrigel plug in a dose-dependent manner (Figure 5B through 5D), but high BMPER doses prevented endothelial cell invasion. These data confirm in vivo that BMPER exerts proangiogenic characteristics.

**BMPER Effect on Endothelial Cell Adhesion**

Endothelial cell adhesion was examined in vitro by exposing HUVECs to protein matrices in the presence or absence of BMPER (Figure 6A). As expected, HUVECs adhered to different matrices but BMPER had no additional effect on endothelial cell adhesion. These data indicate that BMPER signaling is not involved in the initiation of endothelial cell adhesion. Nonetheless, we hypothesized that the quality of adhesion may be modulated by BMPER because BMPER induces endothelial cell sprouting and migration. Cell sprouting and migration is preceded by an “intermediate state of adhesion,” allowing for cell movement. To investigate adhesion in more detail, we visualized changes in cytoskeleton organization by fluorescent staining using phalloidin. Indeed, actin fibers change their confirmation, and cells seem to partially detach from the underlying matrix in a controlled manner, as suggested by the ring-shaped organization of actin fibers when BMPER is added (Figure 6B and 6C).

**BMPER Effect on Apoptosis and Intracellular Signaling**

Because we have observed the reversal of endothelial cell sprouting and migration at high BMPER doses, we asked whether apoptosis was involved. Therefore, we performed the annexin V assay on HUVECs incubated with increasing doses of BMPER (Figure 7A). Using staurosporine as a positive control, we did not observe apoptosis even at high BMPER concentrations, suggesting that apoptosis is not involved in the reversal of the BMPER effect at higher concentrations.

To investigate the signaling pathways involved in BMPER signaling in endothelial cells, we analyzed the Erk cascade and the Smad pathway, because both have been implicated in endothelial cell sprouting and migration (Figure 7B through 7D). Indeed, increasing amounts of BMPER resulted in increased Erk1/2 phosphorylation. In contrast, Smad 1/5 phosphorylation was enhanced at low BMPER concentrations and remained unchanged when the BMPER concentration was increased. In time course experiments, both Smad 1/5 and Erk1/2 phosphorylation reached a maximum at 20 minutes of BMPER exposure. Consistently, when BMPER was silenced, Erk1/2 and Smad 1/5 phosphorylation were blocked (Figure 7D). These data implicate that BMPER activates the Erk pathway and is permissive for Smad 1/5 phosphorylation.

**BMPER Controls BMP-4 Function in Endothelial Cells**

Previous work from our group indicates that BMPER interacts directly with BMP-4 and modulates its function. To assess whether BMPER is necessary for BMP-4 signaling, we analyzed Smad 1/5 phosphorylation and performed functional assays. BMP-4 induces phosphorylation of Smad 1/5 as a readout of the BMP pathway activity (Figure 8A). When BMPER was silenced, BMP-4 partly lost its ability to activate Smad 1/5. This signaling defect translated into an inhibition of functional BMP-4 response in endothelial cell sprouting and migration when BMPER was silenced (Figure 8B and 8C).
Here, we provide the first characterization of BMPER in angiogenic activity. The data presented here indicate that BMPER is necessary for BMP-4 activity, we used either 2 BMP-4–specific siRNAs or the natural BMP antagonists noggin or chordin (Figure 8D). These data indicate that BMPER is necessary for BMP-4 to stimulate angiogenesis and, on the other hand, a role for BMPER in the development of hemangiomas. In particular, FoxO3 has been implicated to be important in endothelial cell regulation. Depletion of FoxO3a from HUVECs is followed by enhanced sprouting and migration. In our experiments, depletion of FoxO3a from HUVECs led to upregulation of BMPER and overexpression of constitutively active FoxO3a to downregulation of BMPER (Figure 1), confirming that BMPER is a downstream target of FoxO3 in endothelial cells. Moreover, findings reported here suggest that BMPER may be the missing link to explain the effects of FoxO3a on endothelial cell function.

The most striking evidence that BMPER is involved in blood vessel formation and endothelial cell biology comes from our earlier work in zebrafish. In these experiments, BMPER is expressed at sites and at the time of vasculogenesis. Even more interesting, knock down of BMPER in zebrafish results in a vascular phenotype mainly driven by disturbed intersomitic blood vessel patterning. Therefore, we hypothesize that BMPER plays an important role in endothelial cell migration and sprouting.

BMPER Promotes Angiogenesis

First, we addressed the question of whether BMPER has an influence on endothelial cell sprouting. In the in vitro Matrigel sprouting assay, increasing concentrations of BMPER induced HUVEC sprouting, consistent with an activating role of BMPER (Figure 3). This finding holds true for both total sprout length and number of branch points. Similarly, BMPER induced sprouting in the 3D HUVEC spheroid assay providing collagen instead of Matrigel as a substrate (Figure 4). Consistent with an activating effect of BMPER, sprouting of HUVECs was significantly inhibited when BMPER was silenced in the Matrigel assay (Figure 3). Angiogenesis is not only dependent on endothelial cell sprouting but also on cell...
migration. Indeed, BMPER induces endothelial cell migration in vitro (Figure 4). In the in vivo CAM assay, which has been used before to characterize BMP pathway members,\textsuperscript{8} we observed enhanced endothelial cell activity in the chorionic capillary network when BMPER was added (Figure 5). As a second in vivo assay, we performed the subcutaneous Matrigel plug assay in mouse and also found increasing endothelial cell invasion induced by BMPER, confirming our in vitro and the CAM data. Obviously, in order for endothelial cells to proceed along the angiogenetic pathway, sprouting and migration has to be activated. However, at the same time, cells must detach in a controlled manner from the underlying matrix to allow for dislocation of the cell.\textsuperscript{1,21,22} Consistent with an activating role of BMPER on angiogenesis, endothelial cell adhesion was not increased, but cell motility was facilitated when BMPER was present (Figure 6). Interestingly, endothelial cell activity was prevented at higher BMPER concentrations in vitro and in vivo, suggesting a complex mechanism of regulation of angiogenesis by BMPER. Taken together, these findings support the notion that BMPER has proangiogenic capacity and modulates endothelial cell function in a concentration-dependent manner.

**BMPER and BMP-4 Interact Functionally**

Earlier work by us and others has demonstrated that BMPER interacts directly with BMP-4.\textsuperscript{10,12} Furthermore, BMPER and BMP-4 are at least partly coexpressed during embryonic development, as well as in adult organisms, and, in terms of angiogenesis, BMP-4 has reportedly very similar effects on endothelial cells compared with what we found for BMPER.\textsuperscript{17,23,24} We were interested to know whether BMPER and BMP-4 may act independently or whether they are dependent on the presence of one another. In loss-of-function experiments for BMPER, we found that BMP-4 cannot exert its proangiogenic response without BMPER. Vice versa, when BMP-4 was absent or blocked, endothelial cells were resistant to stimulation by BMPER (Figure 8). These observations indicate that both BMPER and BMP-4 are needed to create a pro-BMP signal in endothelial cells. During the preparation of this manuscript, the BMPER homolog crossveinless-2 has recently been shown in *Drosophila* to be a concentration-dependent regulator of BMP signaling, which is in line with our findings for the role of BMPER in endothelial cell activation.\textsuperscript{25}

To shed light on the downstream signaling events induced by BMP-4 and BMPER stimulation, we have analyzed Smad 1/5 and Erk1/2 signaling, because these cascades are involved in BMP signaling.\textsuperscript{17} Indeed, we found dose-dependent phosphorylation of Erk1/2, with increasing doses of BMPER (Figure 7). Quite differently, BMPER was permissive for Smad 1/5 phosphorylation but had no dose-dependent stimulatory effect, suggesting that the negative regulation of angiogenesis observed at higher BMPER doses is indepen-
BMP-4, whereas the dose-dependent Erk activation induced by BMPER may also be a BMP-independent effect.

**Model of BMPER–BMP-4 Interaction**

We have observed that the effect of BMPER is reversed at high concentrations. Similar results have been obtained by others for BMP-4. These investigators found that apoptosis was involved in the reversal of BMP-4 effects. In contrast, high concentrations of BMPER do not induce apoptosis (Figure 7). Taking into consideration these data and the controversial results that have been reported about BMPER function in embryogenesis, here, we propose a model for BMPER and BMP4 interaction (supplemental Figure II). BMPER supports a positive-feedback loop for BMP signals by presenting BMP-4 to its receptor. This effect helps to accumulate BMP-4 activity, as deduced from observations in *Drosophila*, in which the BMPER homolog crossveinless-2 contributes to BMP gradient formation and sharpening.

When either BMPER or BMPs are absent, pro-BMP signaling is inhibited and cellular function is impaired. Binding affinity of BMPs to BMPER equals the binding affinity of BMPs to their receptors. This may contribute to the net anti-BMP effect of BMPER at high concentrations, because BMPs then bind preferentially to BMPER and are not available for receptor binding.

In summary, functional BMPER experiments reveal an important concentration-dependent role of BMPER in controlling BMP-4 activity in vascular endothelial cells and, thereby, regulation of angiogenesis. In this context, BMPER is unique in terms of its dose-dependent pro- or anti–BMP-4 capacity, which contributes to locally fine-tuning BMP activity.

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**Disclosures**

None.

**References**


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**Materials and Methods**

**Cell Culture**

All experiments were performed according to the principles outlined in the Declaration of Helsinki for the use of human tissue. Human umbilical vein ECs (HUVECs) were freshly isolated from human umbilical veins of newborns by collagenase digestion and cultured in endothelial basal media supplemented with hydrocortisone, bovine brain extract, epidermal growth factor and 10% FBS (EBM-1) and used for experiments until the 6th passage. Human heart, lung and skin microvascular ECs (HMECs) were cultured in EBM-2 MV. C166 yolk sac endothelial precursor cells were cultured in DMEM (Gibco) with 10% FBS. Skin HMECs were kindly provided by V. Schacht, Freiburg, Germany. Other cells, cell culture media and reagents were purchased from Lonza Group Ltd, Switzerland.

**Transient transfection of HUVECs**

FoxO3a wildtype (pECE-HA FoxO3a_wt) and constitutively active Foxo3a A3 (pECE-HA FoxO3a_A3) expression constructs were generously provided by M. Potente, Frankfurt. For transient transfections, DNA plasmids were introduced into HUVECs using PromoFectin-HUVEC transfection reagent according to the manufacturer's instructions (Promocell).

**Immunocytochemistry**

C166 cells grown on glass coverslips were fixed in ice-cold Methanol/Acetone at -20°C for 10 minutes. Cells were blocked with 10% donkey serum for 30 minutes at room temperature and incubated with the polyclonal BMPER Antibody (1:50; R&D), donkey-anti goat-FITC (1:200; Dianova) and for nuclei-staining with DAPI (1:30000; Sigma).
All photographs were taken with Zeiss Axioplan2 and analyzed with Zeiss Axiovision Rel. 4.6.

For actin cytoskeleton staining HUVECs were grown for 24 h on Fibronectin coated glass coverslips in 2% FBS EBM-1 containing either BMPER (20 ng/ml) or BSA control. Cells were fixed in 4% paraformaldehyde for 15 minutes, blocked and permeabilized with 2% BSA and 1% Triton X-100 for 30 minutes and incubated with phalloidin-TRITC (1:800; Sigma). Images were acquired with a Zeiss 510 confocal microscope and analyzed with Zeiss Axiovision Rel. 4.6.

**RNA interference**

BMPER-siRNAs were purchased from Ambion. The sequences were siBMPER I:

forward: 5’-GCACCUUAGUCACAUACCCtt-3’, reverse 5’-

GGGAUGUGACUAAGGUGCtg-3’; siBMPER II: forward: 5’-

GCUGCCUCUUUCGAAGUGAAtt-3’, reverse: 5’-UCACUUCGAA AGAGGCAGCtc-

‘3. BMP-4 and FoxO3a siRNAs were purchased from Invitrogen. The sequences were:

siBMP-4 I: forward: 5’-AGAUCCACAGCACUGGUCUUGUA-3; reverse: 5’-

UACUCAAGACCAGUGCUGUGGAUCU-3; siBMP-4 II: forward: 5’-GGGCU UCCACCGUAUAAAACAUUUAU-3, reverse: 5’-

AUAAAUGUUAUACGGUGGAAGCC C-3, siFoxO3a I: forward: 5’-

CAGAAUGAGGGGAACUGGCAAGACCU-3, reverse: 5’-

AGCUCUUGCCAGUCCCUAUUCUG-3; siFoxO3a II: forward: 5’-

AGCAAGUUCUG AUUGACCAAACUUC-3, reverse: 5’-

GAAGUUUGGUAACAGACGUUGCU-3. Scrambled negative control-Alexa Fluor 488 nm was purchased from Qiagen. For siRNA transfection Lipofectamine RNAiMAX was used according to the manufacturer's protocol (Invitrogen).
Transfection efficiency was confirmed by quantitative real-time (q) PCR. Functional cell culture assay were performed between 8 to 48h post transfection.

**Real-Time PCR**

Quantitative real-time PCR analysis was performed using the real-time PCR detection system (Bio-Rad) with sequence-specific primer pairs for BMPER (forward primer: 5’-AGG ACA GTG CTG CCC CAA ATG-‘3 and reverse primer: 5’-TAC TGA CAC GTC CCC TGA AAG-‘3), BMP-4 (forward primer: 5’- CAC GAA GAA CAT CTG GAG AAC-‘3 and reverse primer: 5’- CCC TTG AGG TAA CGA TCG GCT-‘3), FoxO3a (forward primer: 5’-CTA CGA GTG GAT GGT GCG TTG C-‘3 and reverse primer: 5’-CGG CTC TTG GTA TAC TTG TTG C-‘3) and human RNA-Polymerase II (hRP) (forward primer: 5’-GCA CCA CGT CCA ATG ACA T-‘3 and reverse primer: 5’-GTG CGG CTG CTT CCA TAA-3’) Quantification was performed using MyiQ lightcycler software. Total RNA was extracted from HUVEC using the Aurum RNA Mini Kit (Bio-Rad). Reverse transcription was performed with iScript cDNA-Kit (Bio-Rad). Knockdown efficiency was calculated using the ∆∆C_T method. The housekeeping gene hRP was used for internal normalization.

**Apoptose Flow Cytometry Analysis**

HUVECs grown in 6-well culture dishes (Nunc) were stimulated for 24 h before FACS-Analysis with different concentrations of BMPER. As a positive control HUVECs were incubated for 4 h with Staurosporin (1 nmol/ml; Sigma). For staining the AnnexinV-Kit was used according to the protocol of the manufacturer (BD Biosciences).
**Adhesion Assay**

For adhesion experiments, 96-well ELISA plates were coated at 4°C overnight with 50 µg/ml collagen I, 10 µg/ml fibronectin, 10 µg/ml gelatine (all Sigma), 20 µg/ml fibrinogen (Calbiochem), or 2 µg/ml vitronectin (Promega). Following the coating, the wells were blocked with 1% BSA for 1 h at 25°C and washed 3 times with PBS. HUVECs were pre-treated as described for the matrigel sprouting assay. A cell suspension (1 × 10^5 cells) in EBM (0.5% BSA) was allowed to adhere for 1 h at 37°C in a cell culture incubator. Prior to analysis plates were washed 3 times with PBS (containing Ca^{2+} and Mg^{2+}) and permeabilization buffer (3 mg/ml phosphatase substrate (Sigma), 0.1% Triton X-100 and 100 mM Sodium acetate) was added to each well and incubated for at least 1 h at 37°C. Absorbance was read at 405 nm in a Spectramax plate reader.

**Western Blot Analysis**

Cells were washed twice with ice-cold phosphate buffered saline (PBS), lysed on ice in RIPA buffer and then centrifuged at 10,000 x g for 10 min at 4°C to remove insoluble material. The supernatant was collected and total cellular protein was quantified using Bradford protein assay (Bio-Rad). Equal amounts of protein were loaded and separated by a 12% SDS-PAGE. Electroblotting was performed to transfer proteins to nitrocellulose. After blocking with TBST supplemented with non-fat dried milk, the western blots were incubated overnight at 4°C with antibodies against hBMPER (1:2500, R&D), β-Tubulin (1:5000, R&D) p-Erk 1/2, Erk 1/2, pSmad 1/5 and Smad 5 (1:2000, Cell Signaling Technologies). Secondary Antibodies conjugated to horseradish peroxidase were from R&D and Dako. Visualisation was performed by an ECL system (Amersham Bioscience).
**Chicken embryo chorioallantoic membrane (CAM) assay**

Fertilized white Leghorn chicken eggs (Gallus gallus) (Tierzucht Bronner, Germany) were incubated in a humidified egg incubator at 37.8°C and 67% humidity. At day 3 of development (E3), a window was cut into the eggshell and sealed again with adhesive plaster (Durapore, 3M). At E9 BMPER (0.1µg, 0.5µg) or VEGF (0.3µg) was applied directly to the CAM within the central area of a Thermanox (Nunc) plastic ring. Membranes were harvested at E13, fixed with 4% PFA. Endothelium was stained by biotinylated Sambucus nigra lectin (Vector Laboratories), streptavidin-Alexa Fluor 546 conjugate (Invitrogen) and visualized in en face views by fluorescent microscopy. For analysis the application area within the ring was compared with the control area outside the ring within the same egg.

Reference

Figure Legends

**Online Figure I:** *Timeline for specific silencing of BMP-4 by siRNA in HUVECs and effect on endothelial sprouting on matrigel.* (A) BMP-4 mRNA expression after 24, 48 and 72 hours post-transfection with the siRNA BMP-4 I and II, respectively, compared to scrambled siRNA control. BMP-4 mRNA was quantified by real-time (q) PCR using specific primers for BMP-4 and hRP as internal control. Knock down efficiency was calculated using $\Delta\Delta C_T$ method. Data are presented as mean ± SD; n = 3. * = p < 0.005 versus control. (B) A representative western blot with the indicated antibodies blot is shown. (C) For matrigel assay BMP-4 siRNA silenced HUVECs or control cells (D) as well as with Noggin antagonized HUVECs were used. In addition to the results of cumulative sprout length in Figure 8E&G branch points were counted. Data are presented as mean ± SD result of 1 of 3 independent experiments are shown. * = p < 0.0001 versus control.

**Online Figure II:** *Working model for concentration-dependence of BMPER.* (A): Physiologic amounts of BMPER facilitate BMP-4 binding to its receptor. (B): High amounts of BMPER bind to extracellular BMP-4 and thereby prevent binding of BMP-4 to the receptor.
Online Figure I: Timeline for specific silencing of BMP-4 by siRNA in HUVECs and effect on endothelial sprouting on matrigel.
Online Figure II: Working model for concentration-dependence of BMPER