Fibroblast Growth Factor Signaling Pathway in Endothelial Cells Is Activated by BMPER to Promote Angiogenesis

Jennifer S. Esser, Susanne Rahner, Meike Deckler, Christoph Bode, Cam Patterson, Martin Moser

Objective—Previously, we have identified bone morphogenetic protein endothelial cell precursor–derived regulator (BMPER) to increase the angiogenic activity of endothelial cells in a concentration-dependent manner. In this project, we now investigate how BMPER acts in concert with key molecules of angiogenesis to promote blood vessel formation.

Approach and Results—To assess the effect of BMPER on angiogenesis-related signaling pathways, we performed an angiogenesis antibody array with BMPER-stimulated endothelial cells. We detected increased basic fibroblast growth factor (bFGF/FGF-2) expression after BMPER stimulation and decreased expression of thrombospondin-1. Additionally, FGF receptor-1 expression, phosphorylation, FGF signaling pathway activity, and cell survival were increased. Consistently, silencing of BMPER by small interfering RNA decreased bFGF and FGF receptor 1 expression and increased thrombospondin-1 expression and cell apoptosis. Next, we investigated the interaction of BMPER and the FGF signaling pathway in endothelial cell function. BMPER stimulation increased endothelial cell angiogenic activity in migration, Matrigel, and spheroid assays. To block FGF signaling, an anti-bFGF antibody was used, which effectively inhibited the proangiogenic BMPER effect. Accordingly, BMPER-silenced endothelial cells under bFGF stimulation showed decreased angiogenic activity compared with bFGF control. We confirmed these findings in vivo by subcutaneous Matrigel injections with and without bFGF in C57BL/6_Bmpper+− mice. Aortic ring assays of C57BL/6_Bmpper+− mice confirmed a specific effect for bFGF but not for vascular endothelial growth factor.

Conclusions—Taken together, the proangiogenic BMPER effect in endothelial cells is mediated by inhibition of antiangiogenic thrombospondin-1 and enhanced expression and activation of the FGF signaling pathway that is crucial in the promotion of angiogenesis. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304345.)

Key Words: angiogenesis ■ BMPER ■ endothelial cells ■ FGFR ■ TSP-1

To identify novel therapeutic approaches for adult pathological conditions, such as inflammatory disorders, cancer, retinopathy, atherosclerosis, or ischemic heart disease, it is essential to understand the molecular and cellular mechanism of blood vessel formation.1 During embryonic development, the process of angiogenesis, that is, the formation of new blood vessels from preexisting ones, is highly important and tightly regulated by a multitude of intra- and extracellular proteins.2 Of interest, some of the growth factors and signaling cascades that are active during embryonic vascular development have been shown to be reactivated during adult disease, as well as in regeneration processes, indicating their therapeutic potential.3,4

To achieve a complex, highly branched vasculature endothelial cell functions, such as migration, proliferation and sprout formation have to be fine-tuned by interacting pro- and antiangiogenic signals.5 For example, vascular endothelial growth factor (VEGF) signaling enhances expression of delta-like ligand 4 in endothelial tip cells, which in turn increases Notch cleavage in neighboring stalk cells. Notch cleavage leads to decreased VEGF receptor-2 expression and, subsequently, to the formation of a new capillary sprout.1 The bioavailability of the potent angiogenesis-inducer VEGF itself is regulated by antiangiogenic proteins, such as thrombospondin-1 (TSP-1), which is a nonstructural extracellular protein that acts on the cell surface together with other matricellular proteins to regulate cell interactions with the environment.6 Besides the VEGF pathway, the superfamily of fibroblast growth factors (FGFs) is well-known as potent inducer of neovascularization. In humans and mice, 22 FGF ligands and 4 tyrosine kinase high-affinity FGF receptors (FGFR1-4) have been identified; however, in endothelial cells, FGFR1 is the predominantly expressed FGFR.7,8 Regarding the cardiovascular system,
administration of basic FGF (bFGF) has been shown to exert a protective effect on cardiac myocytes and to act as survival factor on endothelial cells and vascular smooth muscle cells. Furthermore, clinical trials have revealed a positive but transient effect of bFGF on revascularization and angiogenesis. In addition to the FGF and VEGF growth factors, other growth factor families, such as the transforming growth factor-β (TGF-β) superfamily comprising the bone morphogenetic proteins (BMPs), contribute to the proper orchestration of blood vessel formation.

BMPs are extracellular proteins that signal through cell surface complexes of heterodimeric transmembrane serine/threonine kinase receptors. On activation of the receptor, small mothers against decapentaplegic 1/5 transcription factors become phosphorylated and translocate to the nucleus where they modulate gene expression. Besides this small mothers against decapentaplegic–dependent pathway, BMPs also phosphorylate other small mothers against decapentaplegic–independent signaling cascades, such as MAP kinases/Erk and phosphoinositide 3-kinase (PI3K)/Akt pathways. In line with a key role for BMPs in vascular development loss of function models of BMP ligands, the BMP receptors, or small mothers against decapentaplegic 1/5, show early embryonic lethality as a result of disturbed mesoderm development and thus reduced vasculature. BMP signaling is highly regulated in the extracellular space by BMP modulators, such as BMP endothelial cell precursor–derived regulator (BMPER). BMPER, the vertebrate homologue of Drosophila crossveinless-2, is a secreted glycoprotein that contains 5 cysteine-rich domains followed by a von Willebrand factor D domain and a trypsin inhibitor domain. For BMPER, either pro- or anti-BMP effects have been reported that are dependent on the concentration and availability of BMP ligands, as well as other BMP modulators, such as chordin or twisted gastrulation. Originally, BMPER was first identified in a screen for differentially expressed proteins in embryonic endothelial precursor cells, which overall emphasizes that BMPER plays a role in endothelial cell biology.

During the last few years, BMPER has emerged to be in the focus of interest in vascular biology, including endothelial cell inflammation, atherosclerosis, and angiogenesis. Regarding angiogenesis, we and others have previously shown that BMPER may enhance BMP signaling and the angiogenic response of endothelial cells in a concentration-dependent manner. Along the same line, BMPER was recently shown to be indispensable for normal coronary artery plexus formation during mouse embryonic development. Collectively, these data demonstrated that BMPER exerts an anti-inflammatory and a proangiogenic effect on endothelial cells. However, which angiogenic downstream target genes are regulated and activated by BMPER to facilitate enhanced endothelial cell activity has not been investigated to date. In this study, we now aimed to investigate how BMPER interacts with key angiogenesis-related proteins to activate endothelial cell function in angiogenesis. Therefore, we stimulated endothelial cells with proangiogenic BMPER concentrations and performed a Proteome Profiler Angiogenesis Antibody Array that revealed candidates for further analysis.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**BMPER Enhances bFGF and Reduces TSP-1 Expression in Endothelial Cells**

To assess the effects of BMPER on angiogenesis-related signaling pathways, human umbilical venous endothelial cells (HUVECs) were stimulated with recombinant BMPER protein for 24 hours, and afterward a Proteome Profiler™ human angiogenesis antibody array was performed. After BMPER stimulation, bFGF was the most upregulated protein among 55 angiogenesis-related proteins (Figure 1A). The proteome array results were confirmed by quantitative real-time PCR and Western blot analysis of HUVECs incubated with increasing BMPER concentrations. Consistently, bFGF expression was enhanced at the mRNA (126%) and protein (189%) levels after BMPER stimulation (Figure 1B and 1D). Of interest, expression of the antiangiogenic TSP-1 was reduced at the mRNA (74%) and protein (39%) levels (Figure 1C). Additionally, we used human lung microvascular endothelial cells to prove that the observed BMPER effect is not restricted to HUVECs. Indeed, we detected increased bFGF expression (Figure 1E; Figure IA and IC in the online-only Data Supplement) and decreased TSP-1 expression in human lung microvascular endothelial cells after BMPER stimulation (Figure 1E; Figure IB in the online-only Data Supplement). Together, these data indicate that BMPER increases the angiogenic potential of endothelial cells, on the one hand, via reduction of antiangiogenic TSP-1 expression and, on the other hand, by enhancing proangiogenic bFGF expression.

**BMPER-Induced Proangiogenic Endothelial Cell Function Is Dependent on FGF Signaling**

We hypothesized that BMPER–induced endothelial cell proangiogenic activity is dependent on bFGF. Therefore, we performed several functional cell culture assays and blocked bFGF with a neutralizing bFGF antibody (α-bFGF; Figure 2; Figure ID–IG in the online-only Data Supplement).
proper function of the neutralizing antibody. Similarly, addition of α-bFGF antibody to BMPER-stimulated HUVEC abolished the proangiogenic BMPER effect. To ascertain that the α-bFGF antibody effect is restricted to bFGF, we also stimulated HUVECs with VEGF alone or in combination with the α-bFGF antibody and observed no difference (Figure 2B; Figure ID and IE in the online-only Data Supplement). Furthermore, endothelial cell migration quantified by use of a modified Boyden chamber assay confirmed bFGF-dependence for the proangiogenic BMPER effect (Figure 2D). In the 3D spheroid sprouting assay, bFGF and BMPER stimulation significantly enhanced HUVEC sprout formation, and addition of the α-bFGF antibody reduced this effect (Figure 2C and 2E). Altogether, inhibition of bFGF by a neutralizing α-bFGF antibody inhibited BMPER-stimulated proangiogenic endothelial cell activity in functional endothelial cell culture assays.

**BMPER Activates the FGF Signaling Pathway**

Recently, experiments in zebrafish revealed that inhibition of transcription factors dlx3b/4b by morpholinos resulted in decreased BMPER and FGFR expression in the developing zebrafish embryo. Interestingly, coinjection of BMPER mRNA together with dlx3b/4b morpholinos restored FGFR expression, indicating that BMPER also regulates the expression of FGFR receptors. To investigate whether the same mechanism was also active in mammalian cells, we investigated FGFR1-4 expression in BMPER-stimulated endothelial cells (Figure IIA–IID in the online-only Data Supplement). By using quantitative real-time PCR, we detected significantly increased FGFR1 and FGFR2 expression (Figure IIA and IIB in the online-only Data Supplement), whereas FGFR3 expression was not regulated and FGFR4 expression showed only a tendency to rise in response to BMPER stimulation. As FGFR1 is the predominant FGF receptor in endothelial cells, we focused on FGFR1 protein expression. We confirmed increased FGFR1 protein expression in BMPER-stimulated HUVECs and human lung microvascular endothelial cells by western blot (Figure 3A; Figure IIG and IIH in the online-only Data Supplement). To ascertain whether BMPER promotes FGFR1 and bFGF expression by transcriptional regulation or by posttranscriptional stabilization, we blocked de novo transcription with actinomycin D (Figure IIIA–IIID in the online-only Data Supplement). Western blot and real-time PCR analysis revealed that BMPER failed to increase bFGF and FGFR1 expression if de novo transcription was inhibited, which clearly suggests a transcriptional regulation.

Given that BMPER enhanced bFGF and FGFR1 expression, we next investigated whether FGF signaling pathway activity was increased. Indeed, BMPER-stimulated HUVECs showed increased Akt and Erk1/2 phosphorylation compared with control (Figure 3B). Similarly, the in situ proximity ligation assay revealed more phosphorylated FGFR1 after bFGF or BMPER stimulation in the cytosol (Figure 3C). Of interest, Coleman et al have recently shown that stimulation of pancreatic stellate cells with bFGF led to increased nuclear bFGF/FGFR1

First of all, we performed the in vitro Matrigel capillary–like sprouting assay. HUVECs or human lung microvascular endothelial cells were stimulated with 30 ng/mL bFGF or BMPER, respectively, which resulted in enhanced endothelial cell sprouting and branch point formation (Figure 2A and 2B; Figure ID–IG in the online-only Data Supplement). Application of neutralizing α-bFGF antibody alone had no significant effect on HUVEC sprouting, but α-bFGF addition to bFGF effectively blocked sprouting, confirming...
Therefore, we next examined the localization of FGFR1 in endothelial cells after BMPER stimulation (Figure 3D; Figure IIIF in the online-only Data Supplement). At baseline conditions, a certain amount of FGFR1 is located in the nuclear region, whereas in the presence of the α-bFGF antibody, nuclear localization of FGFR1 is reduced. As positive control for FGFR1 activation, HUVECs were stimulated with bFGF, which resulted in increased nuclear FGFR1 localization. Along the same line, BMPER stimulation enhanced FGFR1 nuclear localization. Because BMPER increased FGF signaling activity, we next aimed to analyze whether downstream targets of the FGF signaling pathway, namely transcription factors Bcl-2 (B-cell lymphoma 2) antagonist of cell death (BAD) and Bcl-2, were regulated by BMPER in HUVECs. Apoptotic BAD expression is known to be downregulated by FGF signaling, whereas antiapoptotic Bcl-2 expression is upregulated.29,30 Along the same line as BMPER-stimulated HUVECs showed increased bFGF and FGFR1 expression, BAD expression is reduced (59%; Figure IIF in the online-only Data Supplement), whereas Bcl-2 expression is increased (139%; Figure 3E; Figure IIE in the online-only Data Supplement). Likewise, endothelial cell viability was increased (Figure 3F) and cell apoptosis showed a tendency to fall in response to BMPER stimulation (Figure IIIE in the online-only Data Supplement). Collectively, we found that BMPER increased the expression of FGFR1 and the activity of the FGF signaling pathway and along this line enhanced cell survival.

Silencing of BMPER Decreases FGF Signaling and Increases TSP-1 Expression and Cell Apoptosis

As BMPER-stimulated HUVECs showed upregulation of the FGF signaling pathway, we hypothesized that silencing of BMPER leads to bFGF and FGFR1 downregulation. Knockdown efficiency for the 2 BMPER-specific small interfering RNAs (B1 and B2) compared with scrambled control small interfering RNA were quantified 48 hours post transfection on mRNA and protein levels (Figure 4A). Indeed, in BMPER-deficient HUVECs, bFGF expression was decreased as detected in the angiogenesis proteome profiler array, as well as by Western blot analysis (Figure 4A and 4B, left). Accordingly, we detected reduced expression of FGFR1 on protein (Figure 4B, middle) and mRNA levels in BMPER-deficient HUVECs (Figure IVA in the online-only Data Supplement). Vice versa we found that BMPER-silenced HUVECs showed enhanced antiangiogenic TSP-1 protein expression (Figure 4B, right). Accordingly, FGF signaling pathway activity was reduced as shown by Western blot analysis for Akt and Erk1/2 phosphorylation of BMPER-deficient endothelial cells (Figure 4C). Consistent with downregulated bFGF and FGFR1 and upregulated TSP-1 that is known to induce apoptosis,31 we found decreased cell viability as well.
as increased cell apoptosis in BMPER-silenced endothelial cells (Figure 4D). Together, these data emphasize that lack of BMPER in endothelial cells induces an antiangiogenic expression profile by increasing antiangiogenic TSP-1, as well as reducing proangiogenic bFGF and FGFR1 expression.

**BMPER-Silenced Endothelial Cells Show Reduced Response to bFGF Stimulation**

Given that silencing BMPER in endothelial cells resulted in an antiangiogenic expression profile, we next asked whether BMPER-deficient HUVECs can respond to bFGF stimulation. Forty-eight hours post-small interfering RNA transfection, we performed the in vitro Matrigel capillary–like sprouting assay that confirmed reduced sprouting and branch point formation of BMPER-silenced endothelial cells compared with control cells (Figure 5A and 5B; Figure IVB in the online-only Data Supplement). As expected, bFGF stimulation of small interfering RNA control–transfected endothelial cells resulted in increased angiogenic sprout formation compared with basal conditions. However, BMPER-silenced endothelial cells only partially responded to bFGF stimulation. To further support this notion, we performed endothelial cell migration assays (Figure 5C) and 3D collagen gel spheroid sprouting assays (Figure 5D and 5E). Consistently, we obtained the same findings as in the Matrigel capillary-like sprouting assay. Taken together, these data demonstrate that BMPER-silenced...
endothelial cells do not fully respond to bFGF stimulation, indicating that the antiangiogenic state of BMPER-silenced endothelial cells is partly independent of bFGF availability, but is caused by reduced expression of FGFR1 and increased expression of TSP-1.

**FGF-Induced Angiogenesis Is Dependent on BMPER Ex Vivo and In Vivo**

To confirm our in vitro findings that bFGF-induced angiogenesis is decreased if BMPER expression is reduced, we next investigated the in vivo relevance in BMPER heterozygous C57BL/6 mice. Because BMPER−/− animals die at birth, we examined the differences of wild-type versus BMPER+/− mice in the ex vivo aortic ring assay (Figure 6A and 6B; Figure IVC in the online-only Data Supplement) and the in vivo Matrigel plug assay (Figure 6C and 6D). For the aortic ring assay, thoracic aortae from C57BL/6_BMPER+/− and wild-type littermate mice were removed, cut into rings, embedded into a collagen gel, and stimulated with or without bFGF or VEGF, respectively. Under basal conditions, few sprouts emerge from the aortic rings and we observed no difference between BMPER+/− and wild-type animals. In contrast, after bFGF stimulation, aortic ring sprout formation was reduced by >50% in BMPER+/− mice (Figure 6B). To prove that this effect was specific for the FGF signaling pathway, aortic rings were also stimulated with VEGF as a control.

Indeed, reduced BMPER expression had no effect on VEGF-induced aortic ring sprout formation. As a prototypic in vivo angiogenesis assay, we performed the mouse Matrigel plug assay. Mice were subcutaneously injected with pure Matrigel or Matrigel mixed with bFGF and VEGF, respectively. After 10 days, mice were euthanized, Matrigel plugs were embedded in paraffin, and histological analysis was performed. Consistent with our previous findings, endothelial cell ingrowth into the Matrigel plug was significantly reduced in BMPER+/− mice compared with wild-type littermates (Figure 6C and 6D). In line with our in vitro findings that BMPER-silenced endothelial cells only partially respond to bFGF stimulation (Figure 5), we observed only a partially increased endothelial cell ingrowth in bFGF Matrigel plugs of BMPER+/− mice compared with wild-type control bFGF Matrigel plugs. Again, reduced BMPER expression had no effect on VEGF-induced endothelial cell ingrowth. To confirm endothelial cell specificity of ingrowing cells, a CD31-Cy3 staining was performed. Indeed, cellular ingrowth into the Matrigel plug consisted mainly of CD31 (stained in red) and DAPI (nuclei in blue) double positive cells (Figure 6D; Figure IVD in the online-only Data Supplement). Collectively, these findings confirm that BMPER-deficiency impairs the proangiogenic FGF-signaling pathway, which results in reduced angiogenesis in vivo.
Discussion

In recent years, BMPER has moved into focus of increasing interest in endothelial cell biology. In regard to angiogenesis, we have previously shown that BMPER is necessary, as well as it augments endothelial cell function to form blood vessels in vitro and in vivo. The results of the present study now demonstrate that BMPER promotes proangiogenic endothelial cell behavior via upregulation of the FGF signaling pathway and by downregulation of anti-angiogenic TSP-1 expression (Figure 1–3). Consistently, BMPER-deficiency leads to decreased FGF pathway signaling and enhanced TSP-1 expression, resulting in reduced proangiogenic behavior of endothelial cells in vitro and in vivo (Figure 4–6).

During embryonic development, branched organs, such as the lung, the kidneys, large nerves, and the vasculature, are formed by patterning mechanisms that are activated by the same molecular pathways, including the FGF and the BMP signaling pathways. Regarding angiogenesis, FGFs as well as BMPs have repeatedly been shown to enhance endothelial cell proliferation, migration, sprouting, and ultimately, blood vessel formation. Vice versa, FGF-2 inhibition has been shown to inhibit coronary artery stem formation in quail hearts during embryonic development. Recently, a similar phenotype was reported for homozygous BMPER-deficient mice in coronary artery stem development. Therefore, it is tempting to speculate that because of these similarities in the phenotype, an interaction between BMPER and the FGF signaling pathway in branching morphogenesis might occur during angiogenesis. Indeed, the results of the present investigation reveal that BMPER enhances bFGF expression in endothelial cells and that the proangiogenic effect of BMPER is dependent on bFGF. Consistently, in BMPER-silenced endothelial cells, bFGF expression is decreased. Furthermore, the angiogenic activity of BMPER-silenced endothelial cells is reduced in vitro and in vivo, but stimulation with bFGF can partially rescue their proangiogenic properties. Taken together, our results support the notion that BMPER promotes bFGF pathway activity and, along this line, angiogenic behavior of endothelial cells.

Previously, the BMP family member BMP-2 was shown to enhance the expression of FGFR1 in PC12 cells and to augment FGF-induced differentiation of these cells. Therefore, it was concluded that BMP-2 and FGF act in concert to regulate...
cell differentiation in the nervous system. Along the same line, Esterberg and Fritz have shown that during zebrafish otic placode development, depletion of transcription factors dlx3b/4b result in decreased BMPER and FGFR expression. Interestingly, coinjection of BMPER mRNA together with dlx3b/4b morpholinos restores FGFR expression, indicating that BMPER acts upstream of FGFR receptor expression. In accordance, we detected increased FGFR1 expression in BMPER-stimulated endothelial cells and decreased FGFR1 expression in BMPER-depleted endothelial cells. Taken together, this is the first report demonstrating a connection between BMPER availability and FGFR1 expression in endothelial cells.

Given that BMPER upregulates bFGF and FGFR1 expression, we next investigated whether, accordingly, the FGF signaling pathway is activated. Therefore, we aimed to analyze that the FGF signaling pathway downstream targets transcription factor Bcl-2 and BAD, as well as the localization of FGFR1 in endothelial cells. We confirmed increased anti-apoptotic Bcl-2 expression and decreased proapoptotic BAD expression in HUVECs after stimulation with bFGF as well as with BMPER. These findings are in line with increased cell survival after bFGF treatment. Basic FGF can localize directly to the nucleus where it promotes proliferation, differentiation, and functional activation in a variety of cell types. Moreover, Coleman et al have recently shown that stimulation of pancreatic stellate cells with bFGF led to increased nuclear bFGF/FGFR1 colocalization, which was followed by enhanced proliferation and invasion. Similarly, we detected enhanced FGFR1 nuclear localization accompanied and increased phosphorylated FGFR in the cytosol of BMPER-stimulated HUVECs. Collectively, we found that BMPER promotes FGF signaling pathway activity by increased phosphorylation of FGFR1, downstream signaling pathway members Akt and Erk1/2, nuclear localization of FGFR1, regulation of Bcl-2 and BAD expression, and increased cell survival.

Besides positive regulators of angiogenesis, such as the FGF signaling pathway, inhibitors, such as TSP-1, play an important role in the regulation of angiogenesis. TSP-1 is
a modular protein and consists of several protein domains, including a von Willebrand factor C type and a procollagen domain. Because of its structure, TSP-1 belongs to the functional family of matricellular proteins that are defined as nonstructural extracellular proteins. Through its different domains, TSP-1 is able to interact simultaneously with different cell receptors, soluble cytokines and growth factors, extracellular matrix components, and proteases. It inhibits angiogenesis by suppressing cell proliferation and migration and induces apoptosis. Moreover, TSP-1 inhibits angiogenesis by suppressing cell proliferation and migration.

BMPER may be an option that should be explored in the future.

The disappointing results for Tsg and BMPER balance blood vessel formation. Tsg and BMPER regulate the proinflammatory phenotype of endothelium. BMPER regulates FGF-dependent angiogenesis at 2 levels. First, it controls expression of FGF pathway proteins, and second, it regulates an inhibitor of FGF signaling.

In the past application, overexpression of growth factors, such as FGF, has not proven to be clinically useful. The reasons for this disappointment are multifactorial, but obviously such a single growth factor is not sufficient to promote angiogenesis efficiently. Induction of proangiogenic pathways at several levels and in a more indirect fashion as we have shown this for BMPER may be an option that should be explored in the future.

Acknowledgments
We are indebted to Bianca Engert and Ute Wering for excellent technical assistance. We thank the staff of the confocal imaging facility for their support (Life Imaging Center [LIC], Center for Biological Systems Analysis Albert-Ludwig University of Freiburg).

Sources of Funding
Work in M. Moser's laboratory is supported by intramural funds and the German Research Foundation (DFG) DFG Mo793/6-1 and 7-1.

Disclosures
None.

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Significance

This study demonstrates that bone morphogenetic protein endothelial cell precursor-derived regulator (BMPER) promotes proangiogenic behavior of endothelial cells by downregulation of antiangiogenic thrombospondin-1 expression as well as upregulation of basic fibroblast growth factor (bFGF/FGF-2) and FGF receptor 1. Additionally, BMPER enhanced FGFR1 phosphorylation and FGF signaling pathway activity and increased B-cell lymphoma 2 expression to enhance cell survival. To demonstrate a functional interaction between BMPER and the FGF signaling pathway in endothelial cell function, bFGF was blocked with an anti-bFGF antibody that effectively inhibited proangiogenic BMPER effects. Accordingly, BMPER-silenced endothelial cell with bFGF stimulation showed decreased angiogenic activity compared with the control. These findings were confirmed by aortic ring assays and in vivo subcutaneous Matrigel injections with and without bFGF in C57BL/6 Bmpr1a−/− mice. Altogether, these findings show that BMPER plays a critical role in the regulation of FGF signaling pathway activity that is highly important for the promotion of angiogenesis.
Fibroblast Growth Factor Signaling Pathway in Endothelial Cells Is Activated by BMPER to Promote Angiogenesis

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Arteriosclerosis, Thrombosis, and Vascular Biology. Published online December 11, 2014; Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Materials and methods

Cell culture and reagents
All experiments were performed according to the principles outlined in the Declaration of Helsinki for the use of human tissue. Human umbilical vein endothelial cells (HUVECs) were freshly isolated from human umbilical veins of newborns by collagenase digestion and were cultured in enhanced endothelial cell growth medium (PELOBiotech GmbH, Martinsried, Germany). Human lung microvascular ECs (HMECs) were purchased and cultured in microvascular enhanced endothelial cell growth medium from PELOBiotech GmbH. Recombinant human BMPER, human VEGF-165 and human FGF basic 146 aa protein were reconstituted according to the manufacturer’s protocol (R&D Systems GmbH, Wiesbaden, Germany). Actinomycin D was reconstituted according to the manufacturer’s instructions (Sigma-Aldrich). For cell tracking CFDA-SE (green) was used (Life Technologies, Karlsruhe, Germany).

Proteome Profiler Human Angiogenesis Antibody Array
For the protein expression analysis array HUVECs were either stimulated with recombinant BMPER protein in 1%FBS/EBM (Lonza, Basel, Switzerland) for 24 h or transfected with BMPER siRNAs or control siRNA and analysed after 48 h, respectively. The proteome profiler human angiogenesis antibody array was performed following the manufacturer’s instructions (R&D Systems).

RNA interference
BMPER siRNAs (B1 and B2) were purchased from Life Technologies, Karlsruhe, Germany. Scrambled negative control Alexa Fluor-488 nm was purchased from Qiagen, Hilden, Germany. For transfection a final concentration of 100 nmol/L siRNA together with Lipofectamine RNAiMAX was used according to the manufacturer’s protocol (Life Technologies). Transfection efficiency was confirmed by quantitative real-time (q) PCR. Functional cell culture assays were performed between 8 to 48 h post transfection.

siRNA sequences:
siBMPER 1: forward: 5’-GCACCUUAGUCACAUCACCCTT-3’
    reverse: 5’-GGGUAUGUGACUAAGGUGCTG-3’
siBMPER 2: forward: 5’-GCUGCCUCUUUCGAAGUGATT-3’
    reverse: 5’-UCACUUCGAAAGGCCAGCTC-3’

RNA extraction and reverse transcription
DNA-free total RNA was extracted from HUVECs using the Aurum RNA Mini Kit (Bio-Rad, Munich, Germany). Reverse transcriptions were performed with iScript cDNA-Kit applying 1 µg RNA following the manufacturer’s protocol (Bio-Rad).

Quantitative real-time PCR
Quantitative real-time PCR analysis was performed using IQ SybrGreen 2xSupermix and the iCycler real-time PCR detection system (Bio-Rad). Quantification was performed using MyiQ lightcycler software (Bio-Rad). Differences in gene expression was calculated using the ∆∆CT method. The housekeeping gene human RNA polymerase II (hHRP) was used for internal normalization. Primers were purchased from Eurofins MWG Operon, Ebersberg, Germany.
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**Enzyme-linked immunosorbent assay (ELISA)**

The concentration of bFGF in supernatants was determined after stimulation of endothelial cell with recombinant BMPER protein in 1%FBS/EBM for 18 hours. The bFGF ELISA was performed following the manufacturer’s instructions (R&D Systems). The optical density of each well was detected by using SpectraMax microplate reader set to 450 nm with wavelength correction set to 540 nm and analyzed with SoftMaxPro software (Molecular Devices, Biberach an der Riss, Germany).

**Western blot analysis**

Western blot analysis was performed as previously described[^4]. Primary antibodies were incubated overnight at 4°C and secondary antibodies at room temperature for 1 h in 3% non-fat dried milk/TBST. Visualization was performed by an ECL system (GE Healthcare Europe GmbH, Freiburg, Germany) and a digital imaging system (ChemiDOC XRS and Image Lab 4.0 (Bio-Rad)).
### Primary antibodies

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<th>Host species</th>
<th>Catalog number</th>
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### Secondary antibodies

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**In situ Proximity ligation assay (PLA)**

To detect FGFR1 phosphorylation HUVECs were grown on glass coverslips, fixed with acetone for 20 min at -20°C and washed 3 times in PBS. Subsequently, the Duolink® PLA assay was performed following the manufacturer’s instructions (Sigma-Aldrich). In brief, rabbit anti-FGFR1 antibody and mouse anti-Phospho-Thyrosine antibody were incubated over night at 4°C. Afterwards, anti-rabbit and
anti-mouse oligonucleotides labeled secondary antibodies (PLA probes) were incubated, followed by a ligase and polymerase reaction to amplify the signal. Confocal images were taken by using a ZEISS LSM 5 Live DUO high speed confocal microscope at the Life Imaging Center, ZBSA, Freiburg, Germany.

**Immunocytofluorescence**

HUVECs grown on glass coverslips were fixed with acetone/methanol (1:1) for 20 min at -20°C, washed 3 times in PBS and then blocked with 10% goat serum for 1 h at room temperature. FGFR1 antibody (1:200) or control rabbit IgG were incubated in 5% goat serum overnight at 4°C, respectively. After 3 PBS washing steps, goat-anti rabbit-Cy2 (1:200) was incubated for 1 h at room temperature, followed by 3 washing steps and incubation of CD31 (1:20) or control mouse IgG in 5% rabbit serum overnight at 4°C, respectively. After 3 PBS washing steps rabbit-anti mouse-Alexa555 in 5% rabbit serum was incubated for 1 h at room temperature. For visualization of nuclei slides were treated with DAPI (1:30000; Sigma, Deisenhofen, Germany), 15 min at room temperature. Afterwards slides were mounted in MOWIOL (Sigma-Aldrich). All photographs were taken with an inverted ZEISS LSM 510 META UV confocal microscope at the Life Imaging Center, ZBSA, Freiburg, Germany.

**Matrigel capillary-like sprouting assay**

Matrigel sprouting assay was performed as described previously. Briefly, HUVECs were pre-treated with the indicated recombinant proteins and/or bFGF blocking antibody in 1% FBS/EBM for 16-18 h at 37°C, 5% CO₂. Duplets of 2 x 10⁴ cells per condition were cultured on phenol red-free Matrigel (356237, Corning BV, Amsterdam, The Netherlands) with 2% FBS and again recombinant proteins and/or bFGF blocking antibody for 3 h at 37°C, 5% CO₂. Cells were fixed with 4% paraformaldehyde (PFA) and pictures were taken from 4 random microscopic fields at 5x magnification using a digitized imaging system. The cumulative sprout length and the number of branch points were measured with AxioVision Rel. 4.8.

**HUVEC spheroid sprouting assay**

HUVEC spheroid sprouting assay was performed as previously described. Briefly, HUVECs were grown in 2% HUVEC medium with 20% carboxy-methylcellulose as hanging drops of approximately 625 cells each for 24 h in a cell culture incubator. For gel preparation spheroids were resuspended in carboxymethylcellulose containing 20% FBS, mixed with the same volume of culturex rat tail collagen (R&D Systems), adjusted to pH 7.4, rapidly aliquoted into a 24-well plate and incubated for 1 h at 37°C for polymerization before sprouting was stimulated with 100 µl of the indicated growth factors and/or bFGF neutralizing antibody in EBM for 24 h in triplicates. Spheroid gels were fixed with 4% PFA and 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**

Cell migration assay was performed as previously described. In brief, HUVECs were labelled with 10 µM CFDA-SE (Life Technologies), harvested by centrifugation, resuspended in migration medium (RPMI with 0.5% FBS, 0.1% BSA), counted and placed in the upper chamber of a modified Boyden chamber (1x10⁵ cells per HTS FluoroBlok 24-well chamber; pore size 8 µm; BD Biosciences, Heidelberg,
Germany). The chambers were placed in 24-well culture dishes containing migration medium and indicated recombinant proteins and/or bFGF blocking antibody. After incubation for 4 h at 37°C, 5% CO₂ the cells were fixed with 4% PFA and migrated cells were counted manually in 5 random microscopic fields using an Axiovert fluorescence microscope.

**Cell viability assay**

Cell viability was assessed by using the CellTiter-Fluor™ cell viability kit from Promega, Mannheim, Germany. CellTiter-Fluor™ measures a conserved and constitutive protease activity within live cells and therefore serves as a marker of cell viability. Briefly, endothelial cells were cultured for 48 h in 2% EC medium without bFGF, either stimulated with indicated proteins or transfected with siRNA. Cell viability assay was performed following the manufacturer’s instructions. The resulting fluorescence signal of each well was measured by using the Gemini fluorescence microplate reader set to 380 nm<sub>Ex</sub>/505 nm<sub>Em</sub> and SoftMaxPro software (Molecular Devices).

**Cell apoptosis assay**

Cell apoptosis was assessed by using the luminescence Caspase-Glo®3/7 assay kit from Promega. Caspase-Glo®3/7 is a proluminescent caspase-3/7 substrate, which is cleaved to release aminoluciferin, a substrate of luciferase used in the production of light. Briefly, endothelial cells were cultured for 48 h in 2% EC medium without bFGF, either stimulated with indicated proteins or or transfected with siRNA. Cell apoptosis assay was performed following the manufacturer’s instructions. The resulting luminescence signal of each well was measured by using the GloMax® 96 microplate luminometer (Promega).

**Aortic ring assay**

The aortic ring assay was performed as described by Baker et al. Thoracic aortae from seven 8-week old female C57BL/6_Bmper and wildtype littermate mice were dissected, cleaned, cut into rings and serum starved overnight in OptiMEM (Gibco, Life Technologies) in a cell culture incubator. Aortic rings were embedded into culturex rat tail collagen (R&D Systems) adjusted to pH 7.4, incubated for 30 min at 37°C for polymerisation before sprouting was stimulated with the indicated growth factors in 2.5% FBS/OptiMEM. Every 2-3 days the stimulation medium was renewed. After 9 days collagen gels were fixed with 4% PFA and aortic ring sprouts were counted by using a Zeiss Axiovert phase contrast light microscope. Afterwards aortic ring collagen gels were permeabilized with 0.25% Triton/PBS for 15 min and blocked with 5% BSA/PBS for 30 min at room temperature, before they were incubated with Tomato Lectin-FITC (1:200, FL-1171, Vector laboratories, Burlingame,US) over night at 4°C. Confocal images were taken by using a ZEISS LSM 5 Live DUO high speed confocal microscope at the Life Imaging Center, ZBSA, Freiburg, Germany.

**Mouse Matrigel plug assay and immunohistochemistry**

Experiments were performed according to the EU-Directive 2010/63/EU and local ethics protocols. Mouse Matrigel plug assay was performed as described previously. In brief, 500 µl of growth factor-reduced Matrigel (356231, Corning BV, Amsterdam, The Netherlands) was mixed with 10 U heparin alone, 10 U heparin and bFGF (c=200 ng/ml) or, 10 U heparin and VEGF (c=200 ng/ml) and injected subcutaneously into the abdominal flanks of female C57BL/6_Bmper heterozygous mice or wildtype littermates. After 10 days, plugs were isolated, fixed in 4% PFA,
embedded in paraffin and sectioned. Blood vessel infiltration was analyzed in 10 random hematotoxin/eosin (H&E) stained sections imaged with Zeiss Axioplan2/Axiovision Rel. 4.8. For immunohistofluorescence staining, slides were rehydrated in descending ethanol series, incubated for 20 min in boiling target retrieval solution (Dako, Hamburg, Germany), cool down for 30 min, washed with PBS and blocked with 10% normal donkey serum for 1 h at room temperature. Slides were incubated overnight with anti-CD31 (1:50) or rabbit IgG control, respectively. After several washing steps, secondary donkey-ant rabbit-alexa555 antibody (1:200) and Dapi (1:30000) was incubated at room temperature for 2 h, washed thrice and embedded in Mowiol 4-88 (Merck Millipore, Darmstadt, Germany). Slides were imaged with Zeiss Axioplan2/Axiovision Rel. 4.8.

**Statistical analysis and quantification**

Statistical analysis was performed using GraphPad Prism 5.0, La Jolla, USA. Data are presented as mean±SEM and comparisons were calculated by Student's t-test (2-sided, unpaired). All experiments were repeated at least three times in triplicates. Results were considered statistically significant for $P<0.05$.

Supplementary Online Figure Legends

**Supplemental Online Figure I**: FGF-dependency of BMPER stimulated proangiogenic endothelial cell function. (A-B) Quantification of western blot analysis from HMECs stimulated for 16-18 hours with indicated concentrations of BMPER for bFGF (A) and TSP-1 (B) expression. (C) Quantitative bFGF ELISA of HMEC supernatants. Values are means ± s.e.m.; n=3; *P<0.05 versus control. (D-G) Matrigel capillary-like sprouting assay. Serum-starved HUVECs (D-E) or HMECs (F-G) were untreated or treated with BMPER (30 ng/ml), with bFGF (30 ng/ml), with VEGF (50 ng/ml) or in combination with α-bFGF antibody (400 ng/ml) for 16–18 hours before they were seeded onto Matrigel with 2% FBS medium. (D) Representative phase-contrast micrographs of HUVEC control, α-bFGF and VEGF ± α-bFGF application are shown. Scale bars: 100 µm. (E) Branch points of HUVEC capillary-like structures were counted. Values are means ± s.e.m.; n=6; *P<0.01 versus control. (F) Quantification of cumulative sprout length of HMEC capillary-like structures after 3 hours. Values are means ± s.e.m.; n=3; *P<0.01 versus control. (G) Branch points of HMEC capillary-like structures were counted. Values are means ± s.e.m.; n=3; *P<0.02 versus control.

**Supplemental Online Figure II**: BMPER-stimulated endothelial cells showed increased expression of FGF signaling pathway members. (A-E) Quantitative real-time-PCR analysis of BMPER-stimulated HUVECs for FGFR1 (A), FGFR2 (B), FGFR3 (C), FGFR4 (D), Bcl-2 (E) and BAD (F) mRNA expression levels. Human RNA polymerase II serves as internal control. Values are means ± s.e.m.; n=3; *P<0.05 versus control. (G) Western blot analysis of HMECs stimulated for 16-18 hours with indicated concentrations of BMPER for FGFR1 and Bcl-2 expression. (H) Quantification of western blots for FGFR1 protein expression. Values are means ± s.e.m.; n=4; *P<0.05 versus control.

**Supplemental Online Figure III**: The transcription inhibitor actinomycin D abolished BMPER-induced bFGF and FGFR1 expression. (A-D) HUVECs were stimulated for 16-18 hours with indicated concentrations of BMPER in the presence of 5 µg/ml actinomycin D. (A) Western blot analysis of bFGF and FGFR1 protein expression. Tubulin serves as loading control. (B, left) Quantification of bFGF protein expression levels. (C, left) Quantification of FGFR1 protein expression levels. (B, right) Quantitative real-time-PCR analysis of bFGF and (C, right) FGFR1 mRNA expression levels. Human RNA polymerase II serves as internal control. Values are means ± s.e.m.; n=4. (D) Quantitative bFGF ELISA of HUVEC supernatants. Values are means ± s.e.m.; n=6. (E) Cell apoptosis assay of HUVECs stimulated with indicated proteins for 48 hours. Values are means ± s.e.m.; n=3. (F) HUVECs grown on culture slides were fixed and stained either with FGFR1 (green), CD31 antibodies (red) (left panel) or with primary control antibodies (right panel). Nuclei were stained with Dapi (blue). Scale bars: 10 µm.

**Supplemental Online Figure IV**: BMPER-silenced endothelial cells showed reduced response to bFGF stimulation in endothelial cell activity. (A-B) HUVECs were silenced for BMPER with either of two specific siRNAs or transfected with scrambled siRNA as control. (A) Quantitative real-time-PCR analysis of FGFR1 mRNA expression levels 48 hours post-transfection. Human RNA polymerase II serves as internal control. Values are means ± s.e.m.; n=4; *P<0.05 versus control. (B) Matrigel capillary-like sprouting assay was performed 48 hours post-transfection. Serum-
starved HUVECs were untreated or treated with bFGF, for 16–18 hours before they were seeded onto Matrigel with 2% FBS medium. Branch points of capillary-like structures were counted after 3 hours. Values are means ± s.e.m.; n=5; *P<0.05 versus control. (C) Aortic ring assay. Aortas from C57BL/6_Bmper+/- and wild-type (WT) mice were cut into rings, embedded in a collagen gel and stimulated with bFGF (50 ng/ml) or VEGF (50 ng/ml) for 9 days. Representative phase-contrast micrographs of aortic rings. Scale bars: 200 µm. (D) Heterozygous C57BL/6_Bmper deficient mice showed reduced endothelial cell proangiogenic activity after bFGF stimulation in the in vivo matrigel plug assay. Matrigel was injected subcutaneously into C57BL/6_Bmper+/- and wildtype mice. Matrigel plugs were harvested 10 days after implantation, fixed, sectioned and stained. Representative micrographs of C57BL/6 wildtype and Bmper+/- matrigel plugs stained with control IgG or CD31-Cy3 [red] to identify endothelial cells. Nuclei were stained with DAPI [blue]. Scale bar: 20 µm.

**Supplemental Online Figure V**: Schematic overview of BMPER and FGF signaling pathway interaction. (A) After stimulation with BMPER more bFGF, FGFR1 is expressed and consequently FGF signaling pathway is activated. Furthermore, anti-angiogenic TSP-1 expression is decreased. Taken together, this leads to enhanced endothelial cell proliferation, migration and survival. (B) If bFGF is blocked by a neutralizing antibody the pro-angiogenic BMPER effect is blocked. (C) If BMPER expression is blocked by RNA interference TSP-1 expression is increased, whereas bFGF and FGFR1 expression is decreased. Taken together, this leads to reduced angiogenic cell behavior and increased cell apoptosis.
Supplemental Online Figure I: FGF-dependency of BMPER stimulated proangiogenic endothelial cell function.
Supplemental Online Figure II: BMPER-stimulated endothelial cells showed increased expression of FGF signaling pathway members.
Supplemental Online Figure III: The transcription inhibitor actinomycin D abolished BMPER-induced bFGF and FGFR1 expression.
Supplemental Online Figure IV: BMPER-silenced endothelial cells showed reduced response to bFGF stimulation in endothelial cell activity.
Supplemental Online Figure V: Schematic overview of BMPER and FGF signaling pathway interaction.