Chapter 3

BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis

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Abstract

Genetic studies in mice and humans have shown that the transforming growth factor-β (TGF- β) type-I receptor activin receptor like kinase 1 (ALK1) and its co-receptor endoglin play an important role in vascular development and angiogenesis. Here, we demonstrate that ALK1 is a signalling receptor for bone morphogenetic protein-9 (BMP-9) in endothelial cells (ECs). BMP-9 bound with high affinity to ALK1 and endoglin, and weakly to the type-I receptor ALK2 and to the BMP type II receptor (BMPRII) and activin type II receptor (ActRII) in transfected COS cells. Binding of BMP-9 to ALK2 was greatly facilitated when BMPRII or ActRII were co-expressed. Whereas BMP-9 predominantly bound to ALK1 and BMPRII in ECs, it bounds to ALK2 and BMPRII in myoblasts. In addition, we observed binding of BMP-9 to ALK1 and endoglin in glioblastoma cells. BMP-9 activated Smad1 and/or Smad5, and induced ID1 protein and endoglin mRNA expression in ECs. Furthermore, BMP-9 was found to inhibit basic fibroblast growth factor (bFGF) stimulated proliferation and migration of bovine aortic ECs (BAECs) and to block vascular endothelial growth factor (VEGF)-induced angiogenesis. Taken together, these results suggest that BMP-9 is a physiological ALK1 ligand that plays an important role in the regulation of angiogenesis.

***Keywords*:** ALK1, Angiogenesis, bFGF, BMP, Endoglin, Signalling, Smad, VEGF

Introduction

Bone morphogenetic proteins (BMPs) belong to the TGF-β_superfamily and were originally identified for their ability to induce ectopic bone growth and cartilage formation (1, 2). Since then, substantial knowledge has been obtained about the multiple functions of these growth factors. BMPs regulate cell growth, differentiation andapoptosis of various cell types, and they are critically involved in the morphogenesis and differentiation of tissues and organs. In addition, they play an important role in adult tissue homeostasis (3, 4). BMP-9 is a secreted protein (5) that is expressed in the liver (6, 7). It has been associated with the regulation of genes involved in glucose and fatty acid metabolism (6), and it induces expression of hepcidin, which plays a key role in iron homeostasis (8). BMP-9 stimulates proliferation of non-endothelial cells (non-ECs) such as liver tumour cells, pre-adipocytes or myoblasts (6, 7). In addition, BMP-9 induces ectopic bone growth and hypertrophic chondrocyte formation and supports the differentiation of mesenchymal cells into cartilage (9). BMP-9 is also expressed in the septum and spinal cord of mouse embryos, and it is a differentiation factor for cholinergic neurons of the central nervous system (10).

In a recent study, BMP-9 was shown to bind with high affinity to the immobilised extracellular domain of activin receptor like kinase 1 (ACVL1, also known as, and hereafter referred to, as ALK1) in a BIAcore assay (5). The ALK1 receptor is mainly expressed in ECs (11), regulating EC proliferation and migration *in vitro* (12), and angiogenesis *in vivo* (13, 14). ALK1-deficient mice display impaired vessel remodelling, dilated blood vessels and defective recruitment of smooth muscle cells (13, 14). *In vitro* studies have shown that the co-receptor endoglin (ENG) is able to form complexes with ALK1 and to promote the effects of ALK1 on ECs (15, 12, 16). The phenotype of endoglin deficient mice is highly reminiscent of ALK1-deficient mice, thereby suggesting that endoglin also plays a role in ALK1 signalling in angiogenesis (17). Further evidence for the importance of ALK1 and endoglin in vessel formation and maintenance is derived from the vascular disorder hereditary haemorrhagic telangiectasia (HHT or Osler-Weber-Rendu disease). HHT is an autosomal-dominant vascular dysplasia that is characterised by recurrent epistaxis, telangiectases in mucosa and skin, gastrointestinal haemorrhage and arteriovenous malformations in brain, lung and liver. Mutations in the genes encoding ALK1 (responsible for HHT2) or endoglin (responsible for HHT1) have been identified to be the cause for the observed phenotype in most of the cases (18,19).

So far, TGF-β1 is the only described functional ALK1 ligand (20, 12, 13). Yet, ALK1 alone is not sufficient to transduce the TGF-β signal across the plasma membrane. TGF-β and also BMP signalling require a specific heteromeric complex of type I and type II serine/threonine kinase receptors (21, 22). Whereas TGF-β1 first binds to the type II receptor and subsequently recruits the type-I receptor, this order is reversed for some members of the BMP family (23, 24). Ligand-

induced receptor complex formation results in phosphorylation of the type-I receptor by the type II receptor kinase and in subsequent phosphorylation of downstream receptor-regulated Smads (R-Smads, i.e. Smad1, Smad2, Smad3, Smad5 and Smad8). Activated R-Smads then form heteromeric complexes with the common mediator (Co)-Smad4, which then translocate into the nucleus where they regulate specific gene transcriptional responses (21 - 23). In mammals, three BMP type II receptors (BMPRII, ActRIIA, ActRIIB) and three BMP type I receptors (ALK2, ALK3, ALK6) have been identified. Activation of BMP receptors leads to phosphorylation of Smad1, Smad5 and Smad8. In most cell types, TGF-β signals via the broadly expressed receptor type I receptor ALK5 thereby inducing phosphorylation of Smad2 and/or Smad3. The type I receptor ALK1 transduces TGF- β signals in ECs, which then leads to activation of Smad1, Smad5 and Smad8 (12, 13, 20).

The reported interaction of BMP-9 with ALK1 tempted us to investigate whether ALK1 is also a receptor for BMP-9 in ECs and how BMP-9 signalling influences EC function. Our results demonstrate that BMP-9 binds with high affinity to ALK1 and endoglin in ECs. In non-ECs, BMP-9 strongly bound to ALK2, thereby providing an explanation for the potent effects of BMP-9 in these cell types. BMP-9 was found to induce phosphorylation of Smad1 and/or Smad5 and to stimulate the expression of ALK1 target genes. Moreover, BMP-9 stimulated activation of a Smad1- and/or Smad5-responsive transcriptional reporter, which was blocked using small interference RNA (siRNA) targeting ALK1. The importance of BMP-9 signalling in ECs was shown by the inhibiting effect of BMP-9 on basic fibroblast growth factor (bFGF)-induced EC proliferation and by the abrogation of vascular endothelial growth factor (VEGF)-induced angiogenesis. Taken together these results show that BMP-9 signals via ALK1 and functions as a potent regulator of angiogenesis.

Results

BMP-9 binds with high affinity to ALK1 and endoglin in transfected COS-7 cells

To study BMP-9 receptor binding, we transfected COS-7 cells with cDNAs for ALK1 or ALK2 in combination with the type II receptors BMPRII or ActRIIB that have been shown to bind ALK1 in the cell-free BIAcore assay (5). Transfected cells were first affinity-labelled with [125I] BMP-9 and then ligand-receptor complexes were chemically crosslinked and subsequently immunoprecipitated with specific type I or type II antisera. Expression of ALK1, ALK2, BMPR-II and ActR-IIB after transfection was checked in parallel by western blotting (data not shown). Resolution of immunoprecipitated complexes by SDS-PAGE and detection of the radioactive signal on a phosphorimager screen showed that BMP-9 strongly binds to ALK1, but not to ALK2 when expressed without a type II receptor (Fig. 1A; lanes 1, 2). The two bands seen after

immunoprecipitation of ALK1 are probably caused by monomeric or dimeric BMP-9 crosslinked to the receptor. BMP-9 binding to the type II receptors BMPRII and ActRIIB alone was weak (Fig. 1A; lanes 3, 4). Binding of BMP-9 to ALK1 was slightly increased when ALK1 and BMPRII or ActRIIB were co-expressed (Fig. 1A; lanes 5, 6). By contrast, BMP-9 binding to ALK2 was greatly enhanced when the receptor was co-expressed with either BMPRII or ActRIIB (Fig. 1A; lanes 7, 8). Thus, whereas BMP-9 can bind to ALK1 alone, it can only interact with ALK2 in combination with BMPRII or ActRII.

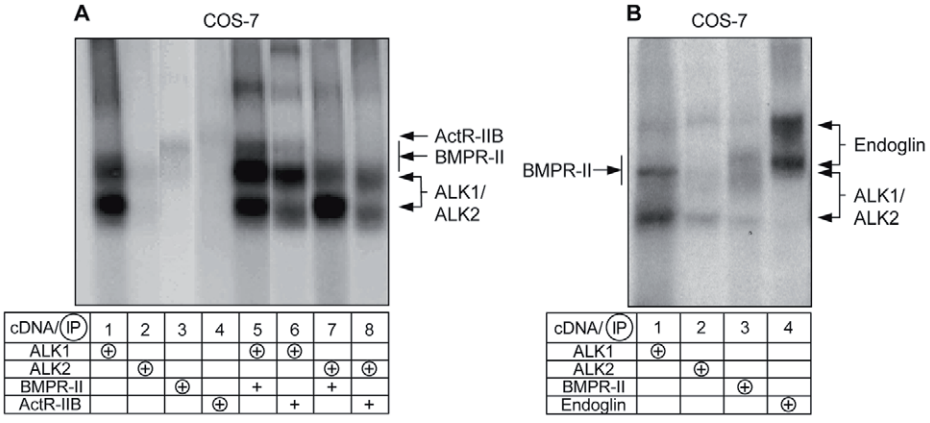

Fig. 1. *BMP-9 binds to ALK1 and endoglin in the absence of type II receptors. COS-7 cells were transiently transfected with cDNAs for ALK1, ALK2, BMPRII, ActRIIB or endoglin and affinity-labelled with [125I]BMP-9. Crosslinked complexes were immunoprecipitated with specific antisera (marked with a circle) and subjected to SDS-PAGE and autoradiography. (A) [125I]BMP-9 binds with high affinity to ALK1, but not to ALK2 in the absence of type II receptors. BMP-9 binding to ALK2 is strongly enhanced when BMPRII or ActRIIB are co-expressed. (B) [125I]BMP-9 binds to endoglin in the absence of type I or type II receptors.*

The accessory receptor endoglin has been described to form complexes with ALK1 and to promote ALK1 signalling (15, 16). To investigate whether BMP-9 binds to endoglin, we transiently transfected COS-7 cells with cDNAs encoding ALK1, ALK2, BMPRII or endoglin and incubated them with [125I]BMP-9. Fig. 1B shows that BMP-9 binds endoglin and ALK1 in the absence of type I or type II receptors.

BMP-9 binds to ALK1, ALK2, BMPRII, ActRII and endoglin in ECs

We then analysed the ability of BMP-9 to bind to endogenous endoglin, type I and type II receptors in primary ECs. Bovine aortic ECs (BAECs), human umbilical vein ECs (HUVECs) and human dermal microvascular ECs (HDMVECs) were incubated with [125I]BMP-9, and crosslinked receptor-ligand complexes were immunoprecipitated with antisera against endoglin, type II receptors and type-I receptors known to be involved in BMP signalling. BMP-9 strongly bound to ALK1 in all EC types investigated and it weakly bound to ALK2 in BAECs and HDMVECs (Fig. 2A, B and data not shown). In addition, in all EC types, BMP-9 bound to BMPR-II, ActR-IIA and endoglin. In HUVECs, BMP-9 was also found to bind ActRIIB. The observed co-immunoprecipitation of type I with type-II receptors (and vice versa) and of type I and type II receptors with endoglin (and vice versa) indicates that these receptors form heteromeric complexes with each other on the cell surface.

To verify that BMP-9 binding to ALK1 is specific, we competed [125I]BMP-9 binding to ALK1 in HUVECs with excess of unlabelled 'cold' BMP-9, activin, BMP-7 or TGF-β and immunoprecipitated receptor-ligand complexes with ALK1 antiserum (Fig. 2C). Only excess of cold BMP-9 competed with [125I]BMP-9-receptor binding, demonstrating the specificity of the BMP-9-receptor interactions. Moreover, incubation of BAECs with [125I]BMP-6 showed that BMP-6 does not bind to ALK1, but only to ALK2 and BMPRII (data not shown).

BMP-9 can bind ALK1 and ALK2 in non-ECs

We subsequently investigated BMP-9 binding to non-ECs. In C2C12 myoblasts, BMP-9 was found to bind to ALK2 and the type II receptors BMPRII and ActRIIA, but not to ALK1 (Fig. 3A). In XTH-1 breast cancer cells, BMP-9 bound to ALK2 and also to endoglin (Fig. 3B). By contrast, BMP-9 strongly bound to ALK1 and endoglin, and only weakly to ALK2 in T98G glioblastoma cells (Fig. 3C). These results suggest that BMP-9 signalling in non-ECs occurs via ALK1 and/or ALK2 and, thus, provides an explanation for the potent effects of BMP-9 on non-ECs described in the literature.

BMP-9 induces phosphorylation of Smad1 and/or Smad5, and stimulates expression of ID1 protein and endoglin mRNA in ECs

We then went on to investigate whether Smad1 and Smad5, which are downstream in the ALK1 signalling pathway, are activated by BMP-9. BAECs, HUVECs, and HDMVECs were stimulated for 45 minutes with different doses of BMP-9, lysed and subjected to SDS-PAGE and subsequent western blotting.

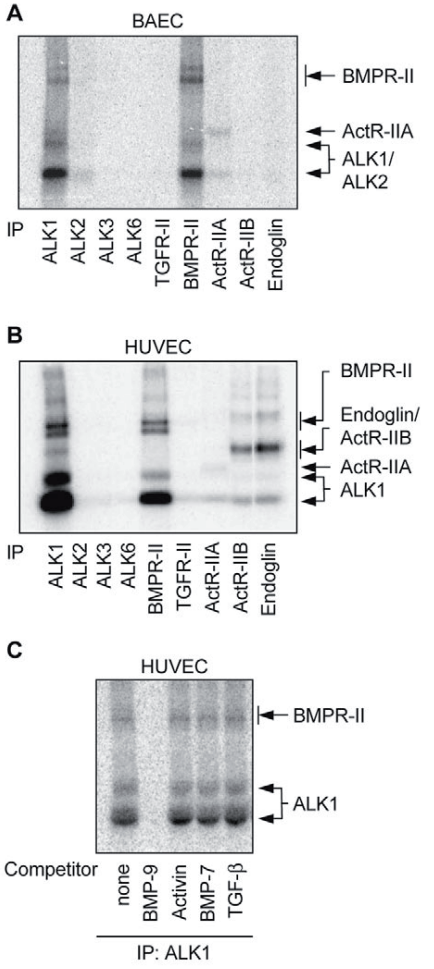

Fig. 2. *BMP-9 binds to endogenous receptors in endothelial cells. (A,B) BAEC (A) or HUVECs (B) were affinity-labelled with [125I]BMP-9 and crosslinked ligand-receptor complexes were immunoprecipitated with specific antisera as indicated. BMP-9 predominantly binds to ALK1 and BMPR-II, but also to ALK2, ActR-IIA, ActR-IIB and endoglin. (C) Competition of [125I]BMP-9 binding to ALK1 in HUVECs with either excess unlabelled (cold) BMP-9, activin, BMP-7 or TGF-β. Only cold BMP-9 can compete with [125I]BMP-9 binding to ALK1.*

Probing of membranes with an antibody that detects phosphorylated Smad1 and Smad5 demonstrated that BMP-9 dose-dependently induces phosphorylation of Smad1 and Smad5 in all cell types investigated (Fig. 4A-C). BMP-6, which is known to activate Smad1 and Smad5, was used as positive control. Equal loading was demonstrated by probing membranes with an anti-β-actin antibody. We also stimulated cells with BMP-6 and BMP-9, and analysed the expression

of *ID1*, described to be a direct target gene of Smad1 and/or Smad5 signalling (25). In HUVECs and HDMVECs, ID1 protein levels increased at the lowest BMP-9 concentration tested (Fig. 4B,C). In BAECs, upregulation of ID1 protein levels was seen at a concentration of 1 ng/ml BMP-9 (Fig. 4A).

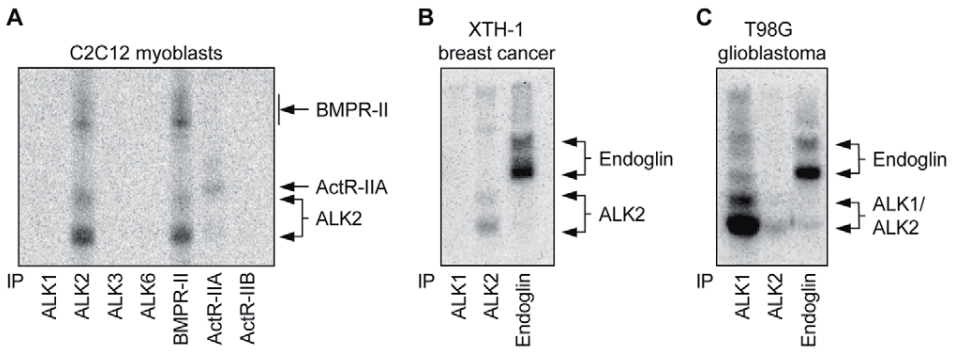

Fig. 3. *BMP-9 binds to endogenous receptors in non-endothelial cells. (A-C) Autoradiography of crosslinked complexes of [125I]BMP-9 with cell surface receptors in C2C12 myoblasts (A), XTH-1 breast cancer cells (B), and T98G glioblastoma cells (C). Ligand-receptor complexes were immunoprecipitated with specific antisera as indicated in the figure and subjected to SDS-PAGE. BMP-9 binds to ALK2 in all cell lines, to ALK1 in glioblastoma cells and to endoglin in breast cancer and glioblastoma cells.*

Endoglin has been shown to be induced by TGF-β (26) and by overexpression of constitutively active ALK1 (27, 28). Using quantitative PCR, we found that, similar to TGF-β, BMP-9 induces endoglin mRNA expression in BAECs (Fig. 4D). This suggests that BMP-9 regulates its own signalling pathway by inducing the expression of its co-receptor endoglin.

BMP-9 activates the Smad1- and Smad5-responsive BRE reporter via ALK1

The ID1-promoter-derived BMP reporter element (BRE)-luciferase reporter construct is a readout system transcriptional responses induced by Smad1 and/or Smad5. BAECs were transiently transfected with the BRE reporter and stimulated with TGF-β, BMP-6 or BMP-9 overnight. BMP-9 and BMP-6 but not TGF-β induced BRE activation (Fig. 5A). We then investigated whether addition of the Fc-ccoupled extracellular domain of ALK1 inhibits BMP-9-induced BRE activation. Incubation of BAECs with ALK1-Fc efficiently blocked BMP-9-induced BRE activity (Fig. 5B).

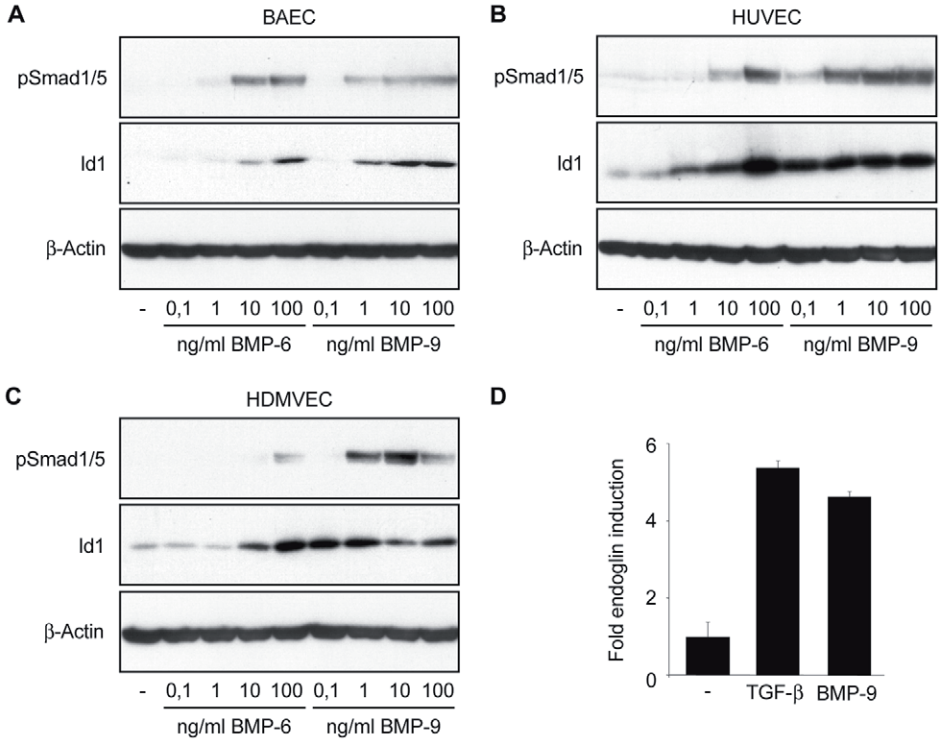

Fig. 4. *BMP-9 activates Smad1 and Smad5, and upregulates ID1 and endoglin in endothelial cells. (A-C) BAECs (A), HUVECs (B) or HDMVECs (C) were incubated with increasing doses of BMP-9 and BMP-6 as indicated in the figure. After 45 minutes, cells were lysed and samples subjected to SDS-PAGE and subsequent western blotting. Membranes were either probed with an antibody that specifically recognises phosphorylated Smad1 and Smad5 or with an antibody directed against ID1. An anti-β-actin antibody was used to confirm equal loading. (D) Effect of BMP-9 and TGF-β on endoglin mRNA expression in BAECs as measured by quantitative real-time PCR.*

To analyse whether BMP-9-mediated BRE activation occurs via ALK1 or ALK2, we designed short hairpin RNA (shRNA) expression constructs targeting ALK1 or ALK2. Transient transfection with ALK1 shRNA but not ALK2 shRNA inhibited BMP-9-induced reporter activity (Fig. 5C). By contrast, BMP-6-stimulated reporter activation was only affected by downregulation of ALK2 but not of ALK1 (Fig. 5D). To control the specificity of the shRNA constructs, we cotransfected BAECs with the ALK1 shRNA construct and with expression vectors for human ALK1 or ALK2. Inhibition of BMP-9-induced BRE activity by ALK1 shRNA was rescued by the ectopic expression of ALK1 but not of ALK2 (Fig. 5E). These experiments show that BMP-9 specifically activates the Smad1- and Smad5-responsive BRE reporter via ALK1 inBAECs.

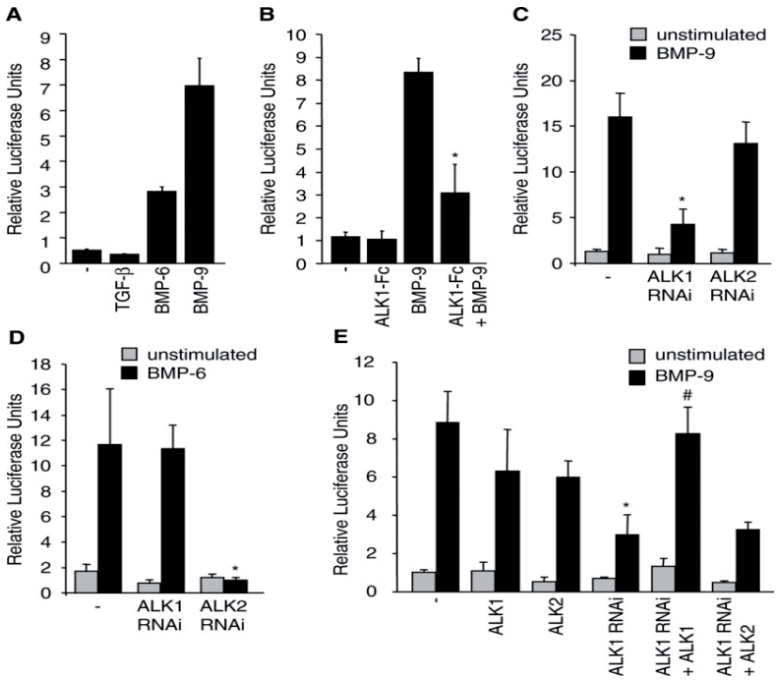

Fig. 5. *BMP-9 activates the Smad1- and Smad5-responsive BRE luciferase reporter through ALK1. (A-B) BAECs were transiently transfected with an ID1-promoter-derived luciferase reporter construct (BRE) and a construct for β-galactosidase as internal expression control. Before stimulation, cells were serum-starved for 8 hours and then incubated with the respective ligands overnight. (A) BRE activity is induced by BMP-9 (100 ng/ml) and BMP-6 (100 ng/ml) but not by TGF-β (5 ng/ml). (B) BMP-9 (5 ng/ml) stimulated BRE-induction can be inhibited by a 15-fold molar excess of ALK1-Fc. (C,D) shRNA constructs against ALK1 or ALK2 were cloned into the pSuper vector and co-transfected into BAECs. ALK1 shRNA blocks BMP-9 stimulated (5 ng/ml) (C), but not BMP-6-induced (25 ng/ml) BRE activation (D). (E) ALK1 shRNA-mediated reduction of BRE activity is rescued by overexpression of human ALK1, but not by human ALK2. *P<0.01 compared with BMP-9 stimulated control; #P<0.01 compared with ALK1 RNAi after BMP-9 stimulation.*

BMP-9 inhibits migration and proliferation of ECs

We next investigated the effect of BMP-9 on EC function. To study the effect of BMP-9 on migration, serum-starved monolayers of BAECs were wounded by scratching and stimulated with the ligands for 24 hours. BMP-9 weakly, albeit significantly, inhibited EC migration at a dose of 10 ng/ml (Fig. 6A; $P<0.01$).

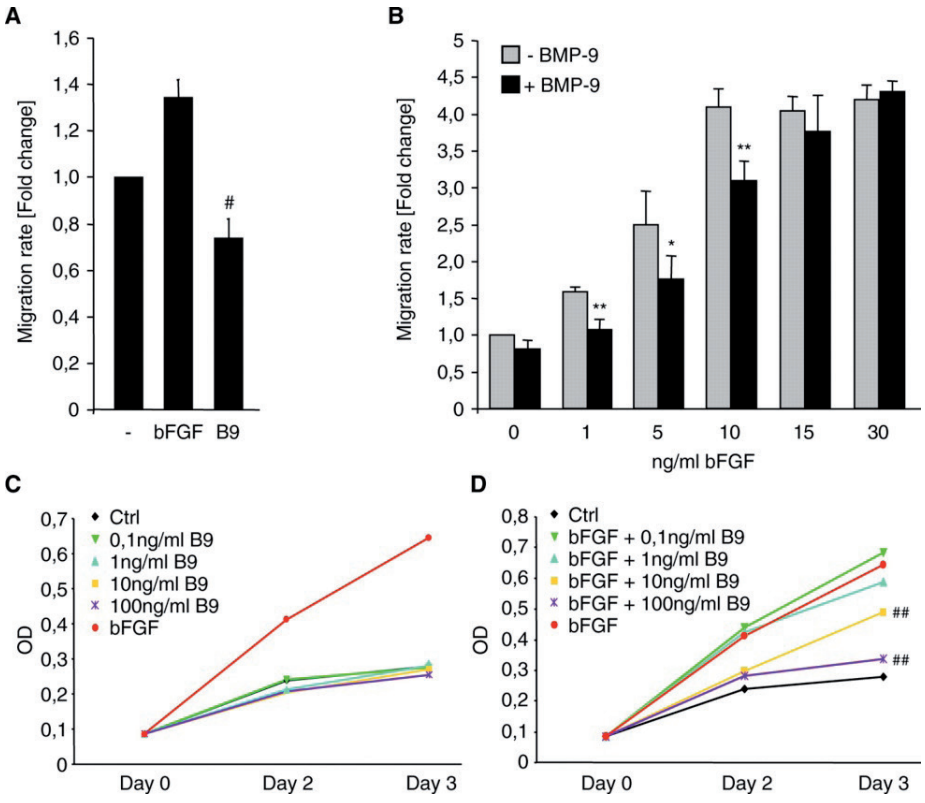

Fig. 6. *BMP-9 attenuates migration and inhibits proliferation of ECs. (A) BAECs were allowed to grow to confluence and serum-starved overnight. Monolayers were wounded and stimulated with either 30 ng/ml bFGF or 10 ng/ml BMP-9. Wound closure was measured after 24 hours using the Olympus Analysis software. (B) Scratched BAEC monolayers were incubated with 10 ng/ml BMP-9 and different concentrations of bFGF as indicated in the figure and cell migration was measured after 24 hours. (C, D) 3000 BAECs were seeded in 96-well plates and stimulated with different concentrations of BMP-9, 30 ng/ml bFGF or combinations of bFGF and BMP-9. Cell proliferation was determined after 2 and 3 days by adding the MTS reagent and measuring the absorbance at 490 nm. B9, BMP-9. ##P<0.001 compared with bFGF treatment; #P<0.01 compared with control; **P< 0.005 and *P<0.05 compared with the respective bFGF treatment without BMP-9*

We also studied the influence of BMP-9 on migration induced by bFGF and found that, in the presence of BMP-9, inhibition of bFGF-stimulated migration decreased with increasing concentrations (0-30 ng/ml) of bFGF (Fig. 6B). Furthermore, we analysed the effect of BMP-9 on cell growth and found that it potently inhibited bFGF-stimulated cell growth when applied at 10 and 100 ng/ml (Fig. 6D). However, BMP-9 alone had no effect on proliferation of BAECs (Fig. 6C).

BMP-9 inhibits vessel formation in vitro

Considering the data obtained from our functional studies and the established role for ALK1 in angiogenesis, we sought to elucidate the effect of BMP-9 on angiogenesis. Metatarsals of 17-day-old mouse embryos were used to study the effect of BMP-9 on EC outgrowth and vessel formation. Culturing of explanted bones leads to formation of a feeder layer of fibroblast-like cells, on which a tubular network of ECs is formed (29). Staining of the EC network using an anti-CD31 antibody showed that BMP-9 inhibits baseline sprouting of ECs and completely abolishes VEGF- induced formation of tube-like structures (Fig. 7). Of note, BMP-9 increased the size of the bone ends, which may reflect the reported stimulatory effect of BMP-9 on chondrogenesis (9). These results show that BMP-9 is a powerful inhibitor of angiogenesis *in vitro*.

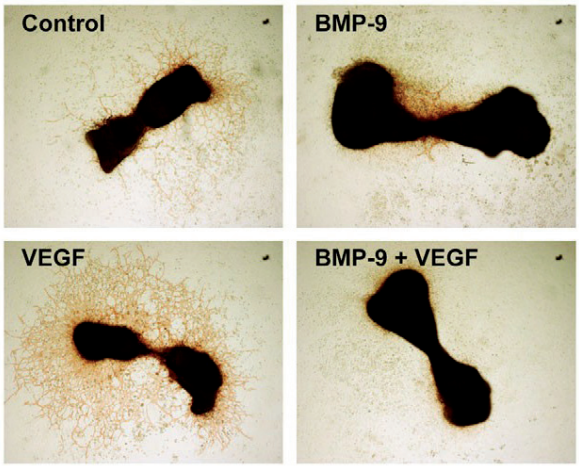

Fig. 7. *BMP-9 blocks endothelial network formation. Metatarsals of 17-day-old mouse foetuses were prepared, transferred to cell culture plates and allowed to adhere for 4 days. Medium was refreshed and bones were stimulated for 7 days with BMP-9 (100 ng/ml), VEGF (50 ng/ml) or both. Cultures were fixed and vessel-like structures were visualised by anti-CD31 staining. BMP-9 inhibits baseline formation of the endothelial network. Incubation with VEGF strongly stimulated the formation of vessel-like structures, which was completely abrogated by co-stimulation with BMP-9. Six bones were stimulated per experimental group and one representative picture of each group is shown.*

Discussion

The functional consequences of *Alk1* gene disruption in mice and loss-of-function mutations in humans have clearly demonstrated that ALK1 is crucial for angiogenesis. (13, 14, 18, 19,). The reminiscent phenotypes of TGF-β1-deficient and ALK1 deficient mice, together with the *in vitro* finding that TGF-β1 signals via ALK1, have suggested that TGF- β1 is the ALK1 ligand *in vivo* (30). In the present study, we demonstrate that BMP-9 is also a functional ligand of ALK1 in ECs. Moreover,

we show that BMP-9 inhibits bFGF-induced proliferation and VEGF- induced angiogenesis *in vitro*, suggesting that BMP-9 also plays a role in ALK1 signalling and vascular morphogenesis *in vivo*.

To study the binding pattern of BMP-9 in ECs, we performed crosslinking studies with 125I-labelled BMP-9 and found that BMP-9 binds to the type I receptors ALK1 and ALK2, and to the type-II receptors BMPRII, ActRIIA and ActRIIB. Compared with ALK2 binding, BMP-9 binding to ALK1 was strong even in the absence of a type II receptor. This finding is consistent with a study, in which BMP-9 was shown to bind with high affinity to immobilised Fc-coupled ALK1 in the cell-free BIAcore assay (5). However, BMP- 9 binding to ALK2 was enhanced when co- expressed with either BMPRII or ActRIIB. The affinity of TGF-β superfamily members for the type I receptor is low or absent, but is strongly increased after heteromeric complex formation with the type II receptor. BMP-9 binding to ALK2 resembles the binding pattern of BMP-6 and BMP-7 that bind only efficiently when both ALK2 and ActRII are present (24, 31). The high affinity of BMP-9 for ALK1 resembles more the binding pattern of BMP-2 and BMP-4 (24, 31). BMP-2 and BMP-4 strongly bind to the type I receptors ALK3 and ALK6, respectively, and show only low affinity for their type II receptor BMPRII.

In primary ECs, we identified ALK1 and BMPRII as the predominant BMP-9 receptors. Moreover, BMP-9 also bound to endoglin even in the absence of type II and type I receptors. So far, BMP-2, BMP-7, activin A, TGF-β1 and TGF-β3 have been described to bind endoglin. However, these molecules can only bind endoglin in the presence of either type I or type II receptors (33, 34). Together with our finding that BMP-9 potently induces endoglin mRNA expression; these results suggest an important role for endoglin in BMP-9 signalling.

BMP-9 has been described to have strong effects on non-ECs (6, 9, 35). We identified the type-I receptor ALK2 and the type II receptor BMPR-II as predominant BMP-9 receptors in myoblasts. In addition, BMP-9 bound to ALK2 and endoglin in XTH-1 breast cancer cells and to ALK1 and endoglin in T98G glioblastoma cells. These results indicate that BMP-9 elicits its broad effects on non-ECs by signalling via ALK2 and/or ALK1. In a recent study, BMP-9 has been shown to signal via ALK1 and the type II receptors BMPRII and ActRIIA in HDMVECs (36). We further extend these findings by showing that BMP-9 binds to these receptors in other EC types as well, that BMP-9 also binds to endoglin, ALK2 and ActRII-B in ECs, and that ALK1 and ALK2 are both receptors for BMP-9 in non-ECs.

We then sought to elucidate whether BMP-9 activates the signalling pathway downstream of ALK1. BMP-9 strongly induced phosphorylation of Smad1 and/or Smad5, and activated the Smad1- and Smad5-responsive BRE luciferase reporter in ECs. BMP-9-induced BRE activation was inhibited by ALK1-Fc. However, we were unable to inhibit BMP-9- stimulated BRE activity with a 500-fold higher concentration of the monomeric extracellular domain of ALK1 (data not shown). As proteins with Fc-fusion are artificially dimerised this suggests that ALK1 dimers are needed for high-affinity binding of BMP-9. Conclusive evidence for a role of ALK1 in BMP-9 signalling was

obtained by shRNA-mediated down-regulation of ALK1 or ALK2 in ECs. Decreased ALK1, but not ALK2 expression abrogated the BMP-9-induced BRE signal. By contrast, BMP-6 stimulated reporter activity was completely blocked by ALK2 shRNA but not by ALK1 shRNA, thereby proving the functionality of the shRNA constructs. Future studies will reveal whether ALK2 plays a role in BMP-9 signalling in ECs. Consistent with the BMP-9- induced activation of the *ID1*-promoter-derived BRE-luciferase reporter, we found an upregulation of ID1 protein after BMP-9 stimulation in all EC types investigated. *ID1* is one of the most important BMP target genes and has also been characterised as ALK1 target gene (27, 28). BMP-9 was also found to activate a Smad3- and Smad4-responsive transcriptional reporter, which could be blocked by ALK1 shRNA (P.t.D., unpublished observation). We are currently analysing the significance of this finding.

We further investigated the effect of BMP-9 on EC function. BMP-9 inhibited EC migration and significantly delayed bFGF-induced migration. Moreover, BMP-9 was found to inhibit proliferation of ECs stimulated with bFGF. Importantly, BMP-9 completely abrogated VEGF-induced formation of tubular EC structures in a bone-explant angiogenesis assay. The mechanism by which BMP-9 negatively interferes with bFGF- and/or VEGF-induced cellular responses remains to be elucidated. It is probable that BMP-9 exerts its anti-angiogenic activities by decreasing the expression of angiogenic factors and/or their receptors.

The observed negative effects of BMP-9 on angiogenesis are in contrast with other studies showing that several other BMP family members promote angiogenesis (37 - 41). BMP-9, however, is the first BMP family member described to signal through ALK1 and this may explain the discrepancy. As mentioned earlier, ALK1 and its accessory receptor endoglin are established as crucial regulators of angiogenesis *in vivo*. However, the question whether ALK1 plays a role in the activation or resolution phase of angiogenesis remains open. *In vitro* data show that expression of constitutive active ALK1 in ECs leads to positive as well as negative effects on EC migration and proliferation (21, 41). The lack of vessel sprouting in ALK1- or endoglin-deficient mice suggests that these receptors play a role in the activation phase of angiogenesis. However, these mice also have dilated, unstable vessels that lack smooth muscle cell coverage, which would point to a role in the resolution phase of angiogenesis. An explanation for all these contradictory findings could be that ALK1 and endoglin are involved in both the activation and in the resolution phase, and that a fine balance of receptor levels, receptor types (TβRII or BMPRII) and ligands (TGF-β or BMP-9) determines the effect on angiogenesis in the respective vascular bed.

Materials and Methods

Cell culture

BAECs were grown in low glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) with 10% foetal calf serum (FCS) and penicillin-streptomycin (PS). HUVECs were cultured in Medium 199 with Earle's salts and L-glutamine (Gibco), 10% FCS, heparin (LEO pharma), bovine pituitary extract (Gibco), and PS. HDMVECs were grown in EGM-2 MV (Clonetics). C2C12 mouse myoblast cells were grown in DMEM, 10% FCS, L-glutamine and PS. XTH-1 breast cancer cells (kind gift from Hartmut Beug, IMP, Vienna, Austria) were cultured in RPMI medium supplemented with 15% FCS, PS and 2.5 ng/ml TGF-β. T98G glioblastoma cells (42) were grown in DMEM with 10% FCS. All cells were cultured at 5% CO2, apart from BAECs which were cultured at 10% CO2.

RNA isolation and quantitative real-time PCR

Total DNA-free cellular RNA was extracted with the RNeasy kit (Macherery-Nagel). The oligonucleotide primers for PCR were designed using the Primer Express Software (Applied Biosystems). Expression of bovine endoglin and bovine hypoxanthine phosphoribosyltransferase (HPRT) were analysed using the following primers: endoglin forward, 5'-tcctcaactgggcgaatacg-3'; endoglin reverse, 5' gatgctttgcggcttgct-3'; HPRT forward, 5'-acgacagcactttgaggcatt-3'; HPRT reverse, 5'-agttaatactaccgaaacctactgaaacac-3'. Taqman PCR reactions were performed using the ABI prism HT7900 sequence-detection system (Applied Biosystems). All samples were plated in triplicate. Gene expression levels were determined with the comparative ΔCt method using *HPRT* as reference and the non-stimulated condition was set to 1.

Expression plasmids and shRNA constructs

Expression constructs for full-length human ALK1 and ALK2 have been cloned into the pcDNA3 vector as described previously (43). Vectors expressing short hairpin RNA (shRNA) targeting ALK1 and ALK2 were made by cloning bovine ALK1- and ALK2-derived constructs and complementary oligonucleotides into the pSuper vector (44). Experiments were performed with different shRNA constructs:

bovineALK1(5'-3') construct1,
gatccccGTGAGAGCGTAGCCGTCAAttcaagagaTTGACGGCTACGCTCTCACttttg gaaa;
bovine ALK1 (5'-3') construct 2
gatccccGACTTATTGTGAC- ATGAAAttcaagagaTTTCATGTCACAATAAGTCttttggaaa;
bovine ALK2 (5'-3') construct 1,
gatccccGATGAGAAGTCGTGGTTTAttcaagagaTAAACCACG- ACTTCTCATCttttggaaa.

The shRNA sequence (uppercase letters) was linked to its reverse complementary antisense shRNA sequence by a short spacer (lowercase letters). The shRNA sequence is flanked at the 3' end by a string of T residues that serve as a transcription termination site. Representative results of bovine ALK1 construct 1 and bovine ALK2 construct 1 were shown.

Recombinant proteins

Recombinant BMP-9 was obtained from Human Genome Sciences. The purity and specific activity of the preparation was accessed as previously described (5). Recombinant BMP-6 and BMP-7 were obtained from K. Sampath (Creative Biomolecules, Inc., Hoptinton, MA) and recombinant activin from Y. Eto (Ajinomoto Co, Kawasaki, Japan). Recombinant TGF-β3 was obtained from K. Iwata (OSI Pharmaceuticals, Inc., Boulder, CO). The VEGF165 isoform and ALK1- Fc were purchased from R&D systems and bFGF from Peprotech.

[125I]BMP-9 binding assay

Iodination of BMP-9 was performed according to the chloramine T method and cells were subsequently affinity-labelled with the radioactive ligand as described before (31, 45). In brief, cells were incubated on ice for 3 hours with the radioactive ligand. After incubation, cells were washed and crosslinking was performed using 54 mM disuccinimidyl suberate (DSS) and 3 mM bis(sulfosuccinimidyl)suberate (BS3, Pierce) for 15 minutes. Cells were washed, scraped and lysed. Lysates were incubated with the respective antisera overnight and immune complexes were precipitated by adding proteinA Sepharose (Amersham). Samples were washed, boiled in SDS sample buffer and subjected to SDS-PAGE. Gels were dried and scanned with the STORM imaging system (Amersham).

Transcriptional reporter assay

BAECs were seeded in 24-well plates and transiently transfected for 4 hours with the BRE-luciferase reporter construct (25) using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Expression vectors for human ALK1 or human ALK2 and shRNA expression constructs for bovine ALK1 or bovine ALK2 were co-transfected where indicated. An expression plasmid for β-galactosidase was co-transfected and used to correct for transfection efficiency. Cells were serum-starved the following the day (or two days after transfection for RNAi experiments) and stimulated overnight with the respective ligands. Cells were washed, lysed and luciferase and β-galactosidase activity were determined. Each transfection was carried out in triplicate and representative experiments are shown.

Western blot analysis

Cells were seeded in six-well plates and allowed to grow to 90% confluence. Cells were washed with PBS and serum-starved for 6 hours (HUVECs, HDMVECs) or overnight (BAECs). Cells were stimulated with different concentrations of BMP-6 and BMP-9 for 45 minutes, washed with PBS and lysed in SDS sample buffer. Samples were boiled for 10 minutes and subjected to SDS-PAGE and western blotting. Smad phosphorylation was detected with an antibody specifically recognising phosphorylated Smad1 and Smad5, which has been described previously (46). The anti-ID1 antibody was from Santa Cruz. Equal loading was shown with an anti-β-actin antibody (Sigma).

Cell growth and migration assay

BAECs were seeded at a density of 3000 cells/well in 96-well plates. The next day, medium was aspirated and replaced by fresh medium containing the respective ligands. Proliferation of cells was determined after 2 and 3 days by adding MTS solution (Promega), and by measuring the absorbance at 490 nm. To determine cell migration, BAECs were seeded in six-well plates and allowed to grow to confluence. After serum starvation, monolayers were wounded with three scratches and medium was replaced by fresh medium containing the ligands. Cell migration was measured in four areas per well directly after wounding and 24 hours later by automated image analysis using the Olympus Analysis software.

Metatarsal angiogenesis assay

Metatarsals from 17-day-old mouse foetuses were dissected as described earlier (47). Six bones per experimental group were transferred to 24-well tissue culture plates containing a.-MEM (Gibco), 10% FCS and PS and allowed to adhere for 4 days. Then, medium was replaced by fresh medium containing the ligands. Cultures were fixed 7 days after stimulation and vessel formation was visualised by anti-CD31 staining (29).

Statistical analysis

All results are expressed as the mean ± s.d. Statistical differences were examined by one-way ANOVA followed by Bonferroni's multiple comparison test. For statistical comparison of two samples, a two-tailed Student's *t*-test was used where applicable. $P<0.05$ was considered as statistically significant.

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