ERK signaling is a central regulator for BMP-4 dependent capillary sprouting

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Abstract

Objective: Bone Morphogenetic Protein-4 (BMP-4) and Extracellular-Signal Regulated Kinases (ERK) play crucial roles in vascular diseases. Here, we demonstrate that BMP-4 not only signals through the classical Smad cascade but also activates ERK phosphorylation as an alternative pathway in human umbilical vein endothelial cells (HUVEC) and that Smad and ERK pathways communicate through signal crosstalk.

Methods: HUVECs were treated with BMP-4 and/or MEK inhibitors. Smad 6 and constitutively active (ca) MEK1 were overexpressed. Loss of function of Smad 4 and Smad 6 was achieved by specific siRNA transfection. Cell lysates were analyzed by western blotting for Smad and ERK phosphorylation. HUVEC spheroids were generated for angiogenesis quantification.

Results: Treatment with BMP-4 results in a dose- and time-dependent activation of the MEK–ERK 1/2 pathway in addition to activation of the Smad pathway and is blocked by MEK inhibitors. Quantitative in-gel angiogenesis assays in the presence or absence of MEK inhibitors demonstrate that ERK signals are necessary for BMP-4 induced capillary sprouting. Furthermore sprouting is not blocked by inhibition of the Smad signaling pathway. Overexpression of the inhibitory Smad 6 inhibits ERK phosphorylation and ERK-induced capillary sprouting, whereas loss of function of Smad 4 has no effect.

Conclusions: We demonstrate that ERK1/2 functions as an alternative pathway in BMP-4 signaling in HUVECs. Capillary sprouting induced by BMP-4 is dependent on ERK phosphorylation. ERK is essential for efficient transduction of BMP signals and serves as a positive feedback mechanism. On the other hand, stimulation of Smad 6 inhibits ERK activation and thus results in a negative feedback loop to fine-tune BMP signaling in HUVECs.

Keywords: BMP-4; ERK 1/2; In-gel angiogenesis; HUVEC; Signal transduction

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily. They have originally been identified by their ability to induce ectopic bone and cartilage formation in rats [23]. Today more than 20 BMP-related proteins have been identified, and it becomes more and more apparent that BMPs are multifunctional proteins with a wide range of biological activities on various cell types. With respect to vascular development, BMP-2 and BMP-4 appear to be the most important family members [17].

BMPs signal through cell surface complexes of type I and type II serine/threonine kinase receptors [21]. The type II receptor kinase is constitutively active and phosphorylates the type I receptor kinase after ligand binding. Once activated, the type I and type II receptor kinases form heterodimers and mediate intracellular signaling through the central signal messengers — the Smad (small mother against decapentaplegic) proteins [8].
The Smad proteins form a group of transcription factors mediating TGF-β and BMP signaling [27]. They are subdivided into three classes, including receptor-regulated Smads (R-Smads), common-partner Smads (Co-Smads) and inhibitory Smads (I-Smads). To date, three R-Smads (Smad 1/5/8) are known to mediate BMP signaling upon phosphorylation by BMP type I receptors. After activation, R-Smads release rapidly from the receptor to form hetero-oligomeric complexes with the Co-Smad (Smad 4). The complex then translocates into the nucleus to regulate transcription of various target genes [3]. As a negative feedback loop, I-Smads (Smad 6 and 7) are activated. While Smad 6 inhibits BMP and TGF-β signaling with similar potency, Smad 7 inhibits TGF-β signaling more efficiently [9]. Smad 6 expression is induced by Smads 1 and 5 as well as other stimuli, e.g. fluid shear stress. It inhibits complex formation between Smad 1 and Smad 4. When overexpressed at high concentrations, Smad 6 also inhibits BMP signaling by interfering with the R-Smads [6].

Findings over the past few years have suggested that BMP responses result not only from activation of the Smad cascade, but from interactions among multiple signaling mechanisms [4]. Among these cascades, the MAPK (mitogen activated protein kinase) has been proposed to have important functions under physiological as well as pathological conditions [11].

The MAPK pathway refers to a cascade of protein kinases that are highly conserved in evolution. There are different isoforms, among which the Extracellular-Signal Regulated Kinase (ERK) is of great importance in regulating proliferation and differentiation [2]. ERK is activated by a variety of extracellular agents, including growth factors, hormones and neurotransmitters. Upon stimulation, adaptor proteins are recruited to the plasma membrane, where they induce the activation of small GTP-binding proteins (e.g. Ras). Signals are then transmitted to downstream proteins of the cascade, including Raf and MEK. The activated MEKs are dual specificity kinases with a unique selectivity towards ERK [1]. Depending on cell type- and target gene-specificity, activated ERK pathways have been reported to enhance [7] or inhibit [15] Smad activity.

Although both BMP-4 and ERK have been proposed to play crucial roles in the pathophysiology of vascular diseases [5], nothing is yet known about interactions of these pathways in endothelial cell lines. Here, we demonstrate that BMP-4 activates the ERK 1/2 cascade in HUVECs in parallel with the classical Smad pathway and that both signaling pathways communicate through signal crosstalk.

1. Materials and methods

1.1. Materials

BMP-4 was purchased from Chemicon International. All antibodies and the MEK inhibitors PD98059 and U0126 were purchased from Cell Signaling Technology. Reagents were dissolved as recommended by the manufacturer. Cell culture media and reagents were fromCambrex BioScience, Walkersville and PromCell, Heidelberg.

1.2. Cell culture

The investigation conforms with the principles outlined in the Declaration of Helsinki for the use of human tissues. HUVECs were kindly provided by H. Augustin, Freiburg, Germany. Cells were seeded in 75 cm² plastic culture dishes with 10 ml EBM containing EGF Single Quots. The medium was supplemented with hEGF, hydrocortisone, GA 1000, BBE and 10% fetal calf serum. Cells were incubated at 37 °C in an atmosphere containing 5% CO₂ and used for experiments before they reached passage 6. All experiments were performed in triplicate using two different isolates of HUVECs.

1.3. Transient transfection of cells

Smad 6 expression construct pcDNA3-Flag(N)-mSmad 6 was generously provided by T. Imamura, Tokyo, Japan [9]. For transient transfections, DNA plasmids were introduced into HUVECs using FuGene6 transfection reagent according to the manufacturer’s instructions (Roche Applied Science). Transfection was confirmed using primary antibody against the Flag tag (Stratagene).

Adenovirus coding for flag-tagged Smad 6 (AdSmad 6) was kindly provided by M.-J. Goumans, Amsterdam, Netherlands. Infection with Smad 6 or recombinant adenovirus encoding green fluorescent protein (AdGFP) as control was performed at 1×10⁹ total virus particles/ml in 2% FCS-containing medium. Infected cells were then cultured for an additional 24 h before in-vitro angiogenesis assay was performed. Infection efficacy was confirmed by fluorescent microscopy for GFP and by using primary antibody against the Flag tag (Stratagene).

Adenovirus coding for constitutively active MEK1 (caMEK1) and GFP under the control of a bicistronic promoter was kindly provided by K. Takimoto, USA [12]. Infection with caMEK1 or control virus was performed at 1×10⁹ total virus particles/ml in serum-free medium for 12 h. Infected cells were then cultured for 48 h in 2% FCS-containing medium. Successful infection of more than 85% of cells was confirmed by fluorescent microscopy for GFP.

1.4. Stimulation and inhibition experiments

Cells grown to 80% confluence in 35 mm dishes were serum starved for 2 h in OptiMEM. BMP-4 was dissolved as indicated by the manufacturer and was directly added to the cells. After gentle swirling, cells were incubated for 20 min, unless otherwise indicated. Controls were treated with buffer only. For inhibition experiments, appropriate concentrations of the MEK inhibitors were directly added to cells 30 min prior to the addition of BMP-4.
1.5. Western blotting

After treatment, cells were washed twice with ice-cold phosphate buffered saline (PBS), lysed on ice in RIPA buffer and then centrifuged at 1600 g for 1 min at 4 °C to remove insoluble material. The supernatant was collected and total cellular protein was quantified using Bradford protein assay. Equal amounts of protein (30–50 μg) were then loaded and separated by a 15% SDS-PAGE. Electrophoretic transfer was performed to transfer proteins to nitrocellulose. After blocking with TBST supplemented with non-fat dried milk, the membrane was incubated overnight at 4 °C with the appropriate primary antibody. Specific proteins were detected using horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako) and visualized by an ECL system (Amersham Bioscience).

1.6. In vitro angiogenesis assay

HUVEC spheroids were generated as published [13]. Briefly, HUVEC were grown as hanging drops of 500 cells each for 24 h in a cell culture incubator. Spheroids were placed into a collagen gel generated by the addition of pH 7.4 adjusted collagen extract containing carboxymethylcellulose at a ratio of 1:1 to prevent sedimentation of spheroids prior to polymerization of the collagen gel. After adding spheroids, the gel was rapidly aliquotted into wells of a 24 well plate and incubated at 37 °C for 24 h to polymerize. For stimulation or inhibition experiments BMP-4 protein or MEK inhibitors were added to the gel before polymerization started. To quantify in-gel angiogenesis the cumulative length of all capillary-like sprouts originating from the core plain of an individual spheroid was measured at 10× magnification using
a digitized imaging system. At least 10 spheroids per condition were analyzed.

1.7. Small interfering RNA (siRNA) transfection

Smad 4-siRNAs (siSmad4) Smad 6-siRNA (siSmad6) and their complementary RNA strands were purchased from Invitrogen. The sequences were siSmad4: (forward: 5'-GGA AUU GAU CUC UCA GGA UTT and reverse 5'-AUC CUG AGA GAU CAA UUC CTT), siSmad6: (forward: 5'-CCA CAU UGU CUU ACA UCC TT and reverse: 5'-UCC GUU UCA GUG UAA GAC AAU GUG G). siRNA transfection was performed according to the manufacturer’s protocol (Invitrogen). Transfection efficiency was confirmed by quantitative real-time PCR.

1.8. 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 Smad 4 (forward primer: 5'-CAC AGG ACA GAA GCC ATT GAG AGA and reverse primer: 5'-CGG TGA CAC TGA CGC AAA TCA AAG); Smad 6 (forward primer: 5'-CCC CCG GCT ACT CCATCA AGG TGT and reverse primer: 5'-GTC CGT GGG GGC TGT GTC TCT GG). Quantification was performed using lightcycler software. Total RNA was extracted from HUVEC using the RNA Mini Kit (Bio-Rad). Reverse transcription was performed with iScript cDNA-Kit (Bio-Rad). Knockdown efficiency was calculated using the ΔΔCt method. The housekeeping gene human RNA-Polymerase was used for internal normalization (Ambion).

1.9. Statistical analysis

All experiments were performed in triplicate. Western blots shown are representative for at least three independent experiments. Densitometric analysis was performed using Quantity One® 1-D Analysis Software Version 4.4, Bio-Rad. Relative densitometry results for phosphorylated MAP kinase and Smad signals were correlated for total MAP kinase or Smad signals (fold increase). Quantification of in-gel angiogenesis assay was performed using ImageJ 1.37v provided by the NIH. Data were expressed as mean±SEM and comparisons were calculated by Student’s t-test or Mann-Whitney U-Test respectively. Statistical analysis was performed using GraphPad Prism 4.0, USA. A p value of <0.05 indicated statistical significance.

2. Results

2.1. BMP-4 induces activation of the MEK–ERK pathway

In a search for non-Smad-dependent signaling pathways induced by BMP-4 that are required for maximal activation, we investigated the MAPK pathway in HUVEC. Incubated with increasing concentrations of BMP-4 for 20 min, we observed a dose-dependent phosphorylation of ERK 1/2 and its activator MEK (Fig. 1A). The amount of phospho ERK increased 5.6 fold after stimulation with 50 ng/ml BMP-4 compared to untreated cells. Concentrations of total ERK were not affected by BMP-4 stimulation. To investigate the time course of signal transduction after BMP-4 stimulation, we incubated the cells for different periods of time. As shown in Fig. 1B–D, both Smad 1/5 and ERK 1/2 phosphorylation reached a maximum at 20 min. At 10 min Smad phosphorylation attained already almost maximal concentrations, while ERK phosphorylation remained still relatively low. These data indicate that BMP-4 induces ERK phosphorylation in HUVECs in a dose- and time-dependent manner, with Smad phosphorylation occurring faster than ERK phosphorylation.

To specify the upstream mediator of BMP-4-induced ERK activation, HUVEC spheroids were readily responsive to stimulation by increasing doses of BMP-4 (Fig. 3B; F). Sprout length increased from 70 μm/spheroid under control conditions to 157 μm/spheroid after stimulation with 100 ng/ml BMP-4.
(p<0.05) and 262 μm/spheroid with 200 ng/ml BMP-4 (p<0.05). This effect was comparable to stimulation by VEGF (Fig. 3C; F), which was used as a positive control, and induced outgrowth to 280 μm/spheroid and 327 μm/spheroid at concentrations of 25 ng/ml and 50 ng/ml, respectively. These data indicate that BMP-4 exerts stimulative effects on
capillary sprouting in HUVEC that are similar to the effect induced by the angiogenic growth factor VEGF providing further support for the pro-angiogenic effect of BMP-4 in HUVECs.

2.3. ERK 1/2 is essential for capillary sprouting

Recent experiments have revealed the importance of ERK-MAPK signaling in angiogenesis. Given the observations that BMP-4 activates ERK 1/2 in HUVECs and is also capable to induce capillary sprouting, we sought to investigate the role of BMP-4 induced ERK 1/2 phosphorylation in mediating capillary sprouting. HUVEC spheroids were embedded in collagen gels with or without the MEK inhibitor U0126 and BMP-4. As shown in Fig. 3D and G, these spheroids show a significant reduction of total sprout length by 73.7% (p < 0.05) in comparison to spheroids incubated with BMP-4 alone. Thus endothelial cell sprouting is dependent on ERK signaling upon stimulation with BMP-4.

2.4. Smad 4 is not necessary for capillary sprouting

To investigate if endothelial cell sprouting is dependent on the Smad pathway, the expression of the Co-Smad Smad 4 was silenced in HUVECs employed for the sprouting assay. Smad 4 forms complexes with the R-Smads, and is necessary for nuclear translocation of the Smad complex, and thus for target gene transcription [6]. Consequently, loss of function of Smad 4 results in impairment of the Smad pathway. We achieved a knock-down efficacy of 82.7% as confirmed by quantitative real-time PCR. As shown in Fig. 3E and H, total sprout length of BMP-4 stimulated spheroids was not affected by specific silencing of Smad 4 compared to HUVEC treated with control siRNA. Thus, Smad 4 and downstream Smad signaling is not...
necessary for endothelial cell sprouting. These data support our findings that ERK signaling is the central mediator in BMP-4 induced HUVEC sprouting.

2.5. Smad 6 inhibits phosphorylation of ERK 1/2 and endothelial cell sprouting

As we found induction of both the Smad as well as the MAPK cascade after stimulation of HUVECs with BMP-4, we aimed to investigate potential interactions between these pathways. To inhibit the Smad pathway, we overexpressed Smad 6 — a well characterized inhibitory Smad. Recent results indicate that Smad 6 elicits its effect by inhibiting the complex formation of Smad 1 with Smad 4, resulting in reduced expression of Smad target genes [10]. It was also reported that when overexpressed at higher concentrations, Smad 6 prevents the activation of Smad 1 and Smad 5 by interacting with the type I BMP receptor [6,9]. To exclude this rather non-specific effect of Smad 6 on the BMP receptor level in our experiment, Smad 6 transfected cells were stimulated with BMP-4 and Smad 1/5 phosphorylation was detected by Western blotting. As shown in Fig. 4A, the BMP-4 induced Smad 1/5 phosphorylation in Smad 6 transfected cells was equal compared to HUVECs transfected with control vector. Thus, overexpression of Smad 6 did not affect Smad 1/5 phosphorylation, excluding an upstream effect of Smad 6 at the receptor level in our experiments. To investigate crosstalk between the Smad and ERK pathway, we analyzed ERK 1/2 phosphorylation in HUVECs upon stimulation with BMP-4, after blocking Smad signaling by overexpression of Smad 6 (Fig. 4A). As expected, incubation with BMP-4 increased ERK 1/2 phosphorylation in HUVEC. In contrast, ERK phosphorylation upon stimulation with BMP-4 was strongly inhibited in HUVECs overexpressing Smad 6. Even at baseline, without BMP-4 stimulation, ERK phosphorylation was lower in Smad 6 overexpressing cells. These results suggest an interaction between the Smad and the ERK pathway and indicate that Smad 6 not only inhibits the Smad pathway, but also elicits inhibitory effects on ERK signaling. To functionally characterize the crosstalk between the Smad and the ERK pathway, the effect of Smad 6 on capillary sprouting was investigated. HUVECs were transduced with AdSmad6 and then seeded into collagen gels prepared with BMP-4 or control. As shown in Fig. 4B–D, BMP-4 induced endothelial cell sprouting was blocked by Smad 6 overexpression down to the level of unstimulated spheroids. Along the same lines of evidence, loss of Smad 6 by siRNA knock down allows for enhanced endothelial cell sprouting (Fig. 4E). Together with the data presented above, this observation supports the hypothesis, that ERK 1/2 signaling is essential for capillary sprouting and that this pathway is negatively regulated by Smad 6.

2.6. Smad 6 does not inhibit MEK phosphorylation

To investigate at which step in the cascade Smad 6 interferes with the MEK/ERK cascade we analyzed the phosphorylation of MEK and ERK individually. As shown in Figs. 1 and 4 BMP-4 induces phosphorylation of both MEK and ERK 1/2. As MEK is the upstream mediator of ERK and ERK phosphorylation is inhibited by Smad 6 we asked if MEK phosphorylation was also affected by Smad 6. As shown in Fig. 4A Smad 6 does not interfere with MEK phosphorylation. These data indicate that Smad 6 differentially inhibits ERK but not MEK.

2.7. Smad 6 expression in HUVEC

We have used overexpression of Smad 6 as a tool to dissect crosstalk between ERK and Smad signaling and found that Smad 6 inhibits not only Smad but also ERK signaling. To investigate if endogenous Smad 6 is capable to regulate these pathways under physiologic conditions we analyzed the expression of Smad 6 in HUVEC cells. As detected by rT-PCR we found rather low levels of Smad 6 RNA in unstimulated HUVECs but after stimulation with BMP-4 Smad 6 transcription was upregulated (Supplemental Fig. 1). This indicates that sufficient amounts of Smad 6 are present in endothelial cells after stimulation with BMP-4 to regulate the crosstalk between the Smad and ERK pathways.

2.8. caMEK1 activates the phosphorylation of Smad 1/5

After determining that inhibition of Smad signaling has inhibitory effects on ERK phosphorylation, we sought to investigate the role of MAPK signaling on the Smad cascade in HUVEC. We overexpressed a constitutively active MEK1 mutant (caMEK1) in HUVECs and analyzed the effect of caMEK1 on Smad 1/5 phosphorylation. Transfection efficacy was confirmed by visualizing bicistronic GFP-expression of caMEK1-virus by fluorescent microscopy. Furthermore ERK 1/2 phosphorylation was detected as a positive control. As shown in Fig. 5, Smad 1/5 was activated in HUVECs overexpressing caMEK1, while no Smad 1/5 phosphorylation.
was detected in control cells. This indicates that caMEK1 is capable to activate Smad 1/5.

3. Discussion

Findings over the past few years show that BMP-4 has important functions during early embryogenesis and vascular development [17]. BMP-2, 4 and 7 have been demonstrated to inhibit aortic and pulmonary vascular smooth muscle cell proliferation [16,18], but the effect of BMP-4 on endothelial cells has not been well characterized, yet. BMP receptors are divided into type I and type II receptors, each comprising different receptor subtypes. Generally, the type I receptor is activated by the type II receptor after ligand binding and then initiates intracellular signaling by phosphorylation of specific receptor-regulated R-Smads, i.e. Smad 1/5 [21]. In the present study, we demonstrate in HUVECs that BMP-4 not only activates the classical Smad 1/5 pathway, but also activates ERK 1/2 in a dose- and time-dependent fashion. Although activation of Smad signaling occurs faster than activation of ERK, at 20 minutes both pathways are fully stimulated (Fig. 1). Furthermore the direct upstream mediator of ERK activation MEK is phosphorylated in parallel to ERK. Accordingly, BMP-4 stimulated ERK activation can be blocked by the MEK inhibitors PD98059 and U0126, confirming that MEK is an upstream mediator of ERK phosphorylation after stimulation with BMP-4 (Fig. 2).

BMP signaling has been implicated in important cellular functions such as endothelial cell sprouting [24]. To determine the functional impact of Smad and ERK mediated BMP-4 signaling in HUVEC, we have established a capillary sprouting assay. Quantitative analyses show that BMP-4 elicits pro-angiogenic effects, which are similar to the effects induced by VEGF. This effect was blocked when cells were pre-treated with the MEK inhibitor, indicating that ERK 1/2 is essential for capillary sprouting in HUVECs (Fig. 3). The importance of ERK 1/2 itself on HUVEC differentiation has been examined by Yang and colleagues. In a 3D gel matrix they show that inhibition of ERK by the MEK inhibitor PD98059 does not interfere with HUVEC differentiation [25]. This finding indicates that the anti-angiogenic effect in our experiment is not likely to be the result of ERK inhibition itself, but rather because ERK mediated activation of BMP-4 is inhibited. In order to inhibit Smad signaling we over-expressed the endogenous Smad inhibitor Smad 6. Most interestingly, we found that Smad 6 not only is an inhibitor of the Smad pathway, but also elicits inhibitory effects on the phosphorylation of ERK 1/2 (Fig. 4A). It is noteworthy that the BMP-4 induced phosphorylation of the direct upstream mediator MEK is not affected by Smad 6 overexpression indicating that Smad 6 interacts directly with ERK 1/2. To confirm that inhibition of ERK 1/2 by Smad 6 affects endothelial cell sprouting in the same manner as inhibition with MEK inhibiting peptides we performed the HUVEC spheroid sprouting assay using cells that overexpress Smad 6. As expected, in these experiments BMP-4 induced sprouting was effectively inhibited by Smad 6, indicating that Smad 6 when overexpressed connects the Smad and ERK signaling cascades (Fig. 3). To test whether Smad 6 may also serve as an endogenous regulator of Smad and ERK crosstalk we examined levels of Smad 6 transcription in HUVECs and found that Smad 6 RNA is indeed highly inducible upon stimulation with BMP-4 (Supplemental Fig. 1). Interestingly, knock down of Smad 6 results in increased spontaneous sprouting indicating that Smad 6 serves as a negative regulator of endothelial cell sprouting (Fig. 4E). To differentiate between the Smad and the ERK inhibiting effect of Smad 6 we then decided to block Smad signaling by loss of function of the ubiquitous Co-Smad Smad 4. Smad 4 has been shown earlier to be necessary for effective Smad signaling [4]. Transfection of HUVECs with siRNA specific for Smad 4 was highly effective in blocking Smad 4 transcription. In our experiments loss of function of Smad 4 in HUVECs used for the sprouting assay does not affect BMP-4 induced sprout length. Taken together this set of experiments indicates that ERK but not Smad signals are necessary for endothelial cell sprouting and that Smad 6 is a central regulator of Smad and ERK pathway crosstalk.

Recent data indicate that BMP signaling undergoes modulation by crosstalk with other pathways [19]. However, the effect of Smad and ERK interactions has been the subject of many controversies. In human mesangial cells a certain level of ERK activity is required for maximal induction of Smad activity by TGF-β [7], whereas other data suggest an inhibitory effect of ERK on the Smad pathway [14,15]. In a landmark paper, Imamura et al. have demonstrated inhibitory effects of Smad 6 in TGF-β signaling, based on its interference with the phosphorylation dependent heterodimerization of Smad 2 and Smad 4 [9]. Apart from the role of Smad 6 in Smad–Smad complex formation, Smad 6 interacts with type I receptors at high concentrations, forming stable complexes, and thus inhibits signaling by various TGF-β superfamily members non-selectively [6]. In our experiments, even high levels of Smad 6 do not interfere with Smad 1/5 phosphorylation upon stimulation with BMP-4, making it unlikely that Smad 6 acts as the receptor level in the experiments presented here (Fig. 4A). Instead, we show for the first time that Smad 6 acts as an inhibitor of ERK 1/2 phosphorylation in HUVECs. Thus, Smad 6 not only elicits an inhibitory effect in the Smad cascade, but also blocks ERK activity as part of a signaling pathway interaction. The central role of Smad 6 in linking these pathways is underlined by its upregulation upon pathway stimulation. Interestingly, this observation has also been confirmed in other cell systems [26]. Smad 6 is inducible by BMP-4 in VSMC in a very similar fashion as described in our manuscript in endothelial cells. Furthermore, BMP-4 activates the Smad 1, p38 and ERK 1/2 pathways in pulmonary arterial smooth muscle cells (PASMCs). However, while Smad signals are anti-proliferative in PASMCs, p38 and ERK result in cell proliferation and have anti-apoptotic activity in the same
cells, indicating that BMP-4 exerts complex effects in different cell systems.

Having shown that Smad signaling interferes with the ERK pathway, we also asked if the MAPK cascade may conversely interact with Smad signaling. Indeed MEK1 induces not only phosphorylation of ERK but also phosphorylation of Smad 1/5, which is the downstream effector of BMP signals (Fig. 5). Taking into consideration that Smad 1/5 was not suppressed in HUVECs pre-incubated with the MEK inhibitors our findings suggest that MEK is sufficient, but not necessary, for phosphorylation of Smad 1/5. This result is consistent with findings in osteoblastic cells, where collagen signals are transduced via activation of the ERK pathway, which in turn enhances the transcriptional activity of Smad 1 in response to BMP [6,22].

Taken together, bidirectional mechanisms of crosstalk between the Smad and MAPK pathways exist in HUVECs (Fig. 6). Our observations suggest that the BMP-4 induced activation of ERK 1/2 occurs in conjunction with phosphorylation of Smad 1/5 and supports the hypothesis that ERK 1/2 is part of a positive autoregulatory feedback loop in HUVECs to augment BMP-4 induced effects particularly necessary for endothelial cell sprouting. We found that not only Smad but also ERK 1/2 activation is necessary to mediate full activity of BMP-4 signaling. This notion is supported by published data indicating that ERK activity enhances TGF-β dependent responses in human mesangial cells [7,20,21]. Thus, additional phosphorylation of Smads by MEK/ERK signals is necessary for full activity of Smad signaling. In contrast, to control overshooting Smad signaling, inhibitory Smads such as Smad 6 are activated in parallel and interfere with Smad complex formation and signal transduction. In our case, we show an inhibitory effect of Smad 6 on ERK 1/2 phosphorylation. This antagonistic effect of Smad 6 on ERK signals contributes to a negative control feedback loop by inhibiting ERK 1/2’s activating role on Smad signals and thus helps to prevent excessive Smad signaling. We conclude that the balance between the inputs of Smad 6 and MEK/ERK 1/2 determine the level of transcriptional activity in the nucleus and therefore fine-tune BMP signaling in HUVECs. Smad 6 and MEK/ERK are parts of a central regulatory system in BMP-4 mediated signaling in HUVECs. MEK/ERK acts as an enhancer of BMP signals and is necessary for cell sprouting. Both, BMP signals and ERK 1/2 are controlled by Smad 6, an endogenous inhibitor that is upregulated upon BMP stimulation.

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All authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cardiores.2007.08.003.

References


