Transforming Growth Factor- β_1 Represses Bone Morphogenetic Protein–Mediated Smad Signaling in Pulmonary Artery Smooth Muscle Cells via Smad3

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Previous studies of pulmonary arterial hypertension (PAH) have implicated excessive transforming growth factor (TGF)- β_1 signaling and reduced bone morphogenetic protein (BMP) signaling in the disease pathogenesis. Reduced BMP signaling in pulmonary artery smooth muscle cells (PASMCs) from patients with heritable PAH is a consequence of germline mutations in the BMP type II receptor (BMPR-II). We sought to establish whether the TGF- β_1 and BMP4 pathways interact in PASMCs, and if this is altered in cells with BMPR-II mutations. Control PASMCs or from patients with PAH harboring BMPR-II mutations were treated with BMP4, TGF- β_1 , or cotreated with both ligands. Signaling was assessed by examination of Smad phosphorylation, luciferase reporters, and the transcription of BMP4 or TGF- β_1 -responsive genes. TGF- β_1 attenuated BMP4-mediated inhibitors of differentiation 1/2 induction and abolished the response in BMPR-II mutant PASMCs, whereas BMP4 did not alter TGF- β_1 -mediated transcription. Activin-like kinase 5 inhibition blocked this effect, whereas cycloheximide or pharmacological inhibitors of TGF- β -activated kinase 1, extracellular signal-regulated kinase 1/2, or p38 mitogen-activated protein kinase were ineffective. BMP4 and TGF- β_1 cotreatment did not alter the activation or nuclear translocation of their respective Smad signaling proteins. Small interfering RNA for Smad3, but not Smad2, Smad6, or Smad7, reversed the inhibition by TGF- β_1 . In addition, TGF- β -activated kinase 1 inhibition blocked Smad3 phosphorylation, implying that C-terminal Smad3 phosphorylation is not required for the inhibition of BMP4 signaling by TGF- β_1 . TGF- β_1 reduces BMP4 signaling in PASMCs, a response that is exacerbated on the background of reduced BMP responsiveness due to BMPR-II mutations. These data provide a rationale for therapeutic inhibition of TGF- β_1 signaling in PAH.

Keywords: pulmonary arterial hypertension; transforming growth factor beta; bone morphogenetic protein; inhibitor of differentiation

Transforming growth factor (TGF)- β_1 is a pleiotropic cytokine involved in embryonic development, inflammation, angiogenesis, immune cell function, and wound healing via the regulation

CLINICAL RELEVANCE

This research demonstrates that transforming growth factor (TGF)- β_1 directly inhibits bone morphogenetic protein signaling in pulmonary artery smooth muscle cells. This implicates pathogenic TGF signaling in the disease process and provides further rationale for anti-TGF therapies in pulmonary arterial hypertension.

of cell processes, such as growth, differentiation, adhesion, migration, and apoptosis (1). Excessive TGF- β_1 production is associated with fibrotic diseases of several organs, including the lung (2) and kidney (3). Dysregulated TGF- β_1 signaling is also emerging as a key pathological process in pulmonary arterial hypertension (PAH). We recently reported enhanced pulmonary vascular TGF- β_1 signaling in the monocrotaline (MCT) rat model of PAH (MCT-PAH) (4), and inhibitors of the TGF- β_1 type I receptor, activin-like kinase 5 (ALK5), attenuated disease progression in MCT-PAH, implicating TGF- β as a pathogenic cytokine in PAH (4–6). In man, most heritable PAH (HPAH) cases are associated with germline mutations in the gene encoding bone morphogenetic protein (BMP) receptor (BMPR)-II, a member of the TGF receptor superfamily (7). Furthermore, BMPR-II expression and downstream Smad signaling is reduced in the lungs of MCT-PAH rats (4). Therefore, dysregulated BMP and TGF- β_1 both contribute to the pathogenesis of PAH.

BMPs and TGF- β_1 signal via heteromeric complexes of type I and type II receptors. In smooth muscle cells, TGF- β_1 activates ALK5 and the TGF- β type II receptor. BMP4 primarily activates the BMP type I receptor, ALK3, in combination with BMPR-II. Activated TGF- β_1 and BMP receptors directly phosphorylate their canonical pathway substrate proteins, the receptor Smads, at C-terminal consensus motifs (8). TGF- β_1 receptors activate Smad2 and Smad3, whereas BMP4 receptors activate Smads1, -5, and -9 (also termed Smad8). Once activated, Smad2/3 or Smad1/5/9 form a complex with Smad4, the common Smad, translocate to the nucleus, and directly regulate the transcription of various target genes. BMP4 target genes include the dominant negative helix-loop-helix transcription factors, the inhibitors of differentiation (ID1–4). The Smad binding element (BRE) of the ID1 promoter has previously been characterized as Smad1/5 responsive, and is used for Smad reporter assays (9). Activated Smad3 recognizes different promoter sequences with a core AGAC motif, and target genes of TGF- β_1 signaling include plasminogen activator inhibitor-1 (PAI1) and connective tissue growth factor (CTGF). Whereas C-terminal phosphorylation activates Smads, Smad linker region phosphorylation may be inhibitory or activating, depending on the cellular compartment. Cytoplasmic phosphorylation of the linker region primes Smads for further phosphorylation, ubiquitination, and degradation, whereas nuclear Smad1 linker phosphorylation is required for efficient transcription (10–13). In addition to phosphorylation, Smad activities

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are negatively regulated through dephosphorylation by phosphatases, such as protein phosphatase 1A and small C-terminal phosphatases (14–17).

We reported previously that pulmonary artery smooth muscle cells (PASMCs) isolated from patients with HPAH exhibit reduced BMP4 responsiveness compared with cells from unaffected donors, regarding both Smad1/5 responses and ID gene transcription (18, 19). Furthermore, loss of BMPR-II renders PASMCs insensitive to growth inhibition by TGF- β_1 (20, 21). Based on our previous observations of enhanced TGF- β_1 signaling and reduced BMP signaling in MCT-PAH rats (4, 22), we questioned whether the TGF- β_1 and BMP signaling pathways interact in PASMCs. Here, we show that TGF- β_1 inhibits BMP4 responses in control PASMCs via targeting of BMP–Smad signaling and attenuation of ID1/2 transcriptional responses. Moreover, TGF- β_1 abolishes the ID1/2 responses completely in PASMCs harboring BMPR-II mutations. This inhibition is mediated via ALK5 and Smad3, but appears independent of Smad3 C-terminal phosphorylation. By identifying a direct repression of BMP signaling, this study provides further insight into the pathogenic role of TGF- β_1 in PAH, and reinforces the rationale for anti-TGF- β strategies in PAH.

MATERIALS AND METHODS

Cell Culture

Primary cultured human PASMCs were isolated from the peripheral arteries (< 2-mm external diameter) of explanted lungs from patients with HPAH ($n = 3$) with *BMPR2* mutations (W9X, C347R, and N903S) undergoing heart lung transplantation, or from unused donor tissue ($n = 3$ donors), as previously described (18). Ethical approval was obtained from the Papworth and Addenbrooke's Hospital Trust Ethics Committees, and subjects or relatives gave informed consent. Detailed methods are provided in the online supplement. Cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% FBS, penicillin, streptomycin, and amphotericin B.

For all experiments, PASMCs were seeded in six-well plates (1.5×10^5 cells/well) for RNA studies, or in 6-cm dishes (3.3×10^5 cells/dish) for protein extraction. Cells were grown for 2 days in 10% FBS, followed by serum restriction in 0.1% FBS for 16 hours, and then treatment with recombinant human BMP4, TGF- β_1 , or both ligands at the concentrations stated in the figure legends. For inhibitor studies, serum-restricted cells were pretreated with the inhibitor for 1 hour.

C2C12 myoblasts were kindly provided by Professor C. Shanahan (King's College London, London, UK), and mouse embryonic fibroblasts, generated from MF1 mice, were a kind gift from Dr. J. Nichols (Centre for Stem Cell Research, University of Cambridge, Cambridge, UK). C2C12 cells or mouse embryonic fibroblasts (MEFs) were seeded in six-well plates (2×10^5 cells/well) for RNA studies and grown to 90% confluence, serum-restricted for 16 hours, and then treated as described above.

RNA Extraction and Quantitative RT-PCR

For quantitative RT-PCR, confluent cells were treated directly or after siRNA transfection as described. Total RNA was prepared using an RNA extraction kit with on-column DNase digestion (Qiagen, Crawley, West Sussex, UK). Details of the quantitative RT-PCR protocol are provided in the online supplement.

Western Blotting

Serum-restricted PASMCs were treated with ligands as described in the figure legends. At specified time points, cells were snap frozen, lysed in 150 μ l of ice-cold lysis buffer (125 mM Tris-HCl [pH 7.4], 10% [vol/vol] glycerol, 2% [wt/vol] SDS containing an ethylenediaminetetraacetic acid-free protease inhibitor cocktail [Roche Diagnostics Ltd., Lewes, East Sussex, UK]), and sonicated. For nuclear and cytoplasmic fractionations, lysates were prepared using the NE-PER fractionation kit

(Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. Samples were analyzed by Western blotting. Antibody details are provided in the online supplement.

Plasmid Transfection and Luciferase Assays

PASMCs were transfected with either BRE-luciferase (luc) or CAGA₁₂-luc as previously described (21). C2C12 cells were transfected with BRE-luc and a *Renilla* luciferase control plasmid. Cells were treated for 16 hours with the ligands, followed by lysis and luciferase assay. For inhibitor studies, cells were pretreated for 1 hour. Detailed methods are provided in the online supplement.

Alkaline Phosphatase Staining and Assay

Alkaline phosphatase assays and staining of confluent C2C12 cells were performed as described in the online supplement.

RNA Interference

Control PASMCs were transfected with small interfering (si)RNAs, as described in the online supplement.

Statistical Analysis

Statistical analysis was performed using one-way repeated measures ANOVA for all gene expression data, or a paired Student's *t* test where stated in the figure legends.

RESULTS

TGF- β_1 and BMP4 Stimulate Different Gene Response Profiles in PASMCs

We first examined the time course and selectivity of the transcriptional responses of selected candidate BMP4- (ID1/2) or TGF- β_1 (CTGF, PAI1)-responsive genes (Figure 1A). BMP4 stimulated the expression of ID1 and ID2 at all time points up to 24 hours. Interestingly, TGF- β_1 transiently stimulated these ID genes at 1 hour, but repressed their expression at 4 and 24 hours. TGF- β_1 also stimulated the transcription of PAI1 and CGTF (Figure 1A), with increasing expression of both genes at 4 hours and sustained induction of PAI1, but not CTGF, at 24 hours. BMP4 did not alter PAI1 or CTGF expression in PASMCs. These responses were confirmed by Western blotting (Figure 1B) with densitometry data in Figure E1 in the online supplement.

TGF- β_1 Inhibits BMP4-Mediated Transcriptional Responses in PASMCs

To address a potential interaction between BMP4 and TGF- β_1 in PASMCs, we examined whether cotreatment altered the transcriptional responses of their target genes. Previous studies have suggested biphasic responses to low and high concentrations of TGF- β_1 , so we tested a range of TGF- β_1 concentrations (23). To avoid the confounding issue of the early ID1/2 response to TGF- β_1 at 1 hour, we examined these responses after 4 hours. TGF- β_1 inhibited the ID1 and ID2 (Figure 1C) responses to BMP4 at concentrations as low as 0.1 ng/ml, with greatest inhibition at 1 and 10 ng/ml TGF- β_1 . In contrast, BMP4 did not alter the induction of PAI1 or CTGF (Figure 1C) by TGF- β_1 , even when the BMP4 (10 ng/ml) was in excess (i.e., with 0.01 ng/ml TGF- β_1).

TGF- β_1 Abolishes BMP4 Responses in PASMC Harboring BMPR-II Mutations

We questioned whether BMP4 and TGF- β_1 cotreatment had similar effects in PASMCs from patients with HPAH harboring *BMPR2* mutations in comparison to donor PASMCs. In donor PASMCs, BMP4 robustly induced ID1 and ID2 expression, and

Figure 1. Transforming growth factor (TGF)- β_1 and bone morphogenetic protein (BMP) 4 signaling in pulmonary artery smooth muscle cells (PSMCs). (A) Control PSMCs were treated for 1, 4, or 24 hours with BMP4 (10 ng/ml) or TGF- β_1 (1 or 10 ng/ml) in 0.1% FBS. Target gene expression was determined using quantitative PCR (QPCR). Data are mean ($\pm$ SEM) of three separate experiments. (B) Total cell protein from parallel samples was immunoblotted for inhibitors of differentiation (ID) 1 or ID2. Blots are representative of three separate experiments. (C) Control PSMCs were treated for 4 hours with BMP4 (10 ng/ml) in 0.1% FBS alone, or with TGF- β_1 (0.01–10 ng/ml). Target gene was determined using QPCR. Data are means ($\pm$ SEM) for three separate experiments (* P < 0.05, ** P < 0.01, *** P < 0.001 compared with control [0]; one-way repeated measures ANOVA).

this was significantly attenuated by TGF- β_1 (Figure 2A). Consistent with our previous studies, PSMCs with *BMPR2* mutations exhibited reduced ID transcriptional responses to BMP4. TGF- β_1 still reduced the ID1 and ID2 response in these cells,

but as a consequence of the attenuated BMP signaling, TGF- β_1 abolished BMP4-mediated ID gene transcription (Figure 2A). In contrast, BMP4 did not alter the PAI1 or CTGF responses to TGF- β_1 , in either donor or *BMPR2*-mutation PSMCs. To

Figure 2. Attenuated BMP4 signaling in BMP type II receptor (BMPR)-II mutant cells is abolished by TGF- β 1. (A) Donor PASCs ($n = 3$) and PASCs harboring *BMPR2* mutations ($n = 3$; W9X, C347R, and N903S) were treated with TGF- β 1 (2 ng/ml) or BMP4 (10 ng/ml) alone, or combined, for 4 hours. Target gene expression was determined using QPCR. Data are means ($\pm$ SEM) for three separate experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with 0.1% FBS for each cell line; # $P < 0.05$, ## $P < 0.01$ relative to BMP4). (B) PASCs were transfected with the BRE-luciferase (BRE) or CAGA₁₂-luciferase (CAGA) luciferase reporter or lipofectamine 2,000 alone. Cells were treated with TGF- β 1 (2 ng/ml) or BMP4 (10 ng/ml) or both combined for 16 hours, then lysed and assayed for luciferase activity. Data are for a single experiment and are representative of three separate experiments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ relative to control; one-way repeated measures ANOVA).

establish whether TGF- β 1 was directly inhibiting BMP Smad signaling, donor PASCs were transfected with either the BMP-responsive BRE-luc reporter or the TGF- β 1-responsive CAGA₁₂-luc reporter (Figure 2B). Consistent with our expression data, TGF- β 1 abolished the BRE-luc response to BMP4, whereas BMP4 did not alter the TGF- β 1-mediated CAGA₁₂-luc response, indicating unidirectional inhibition. Furthermore, the BRE-luc plasmid contains a minimal Smad-responsive promoter element, so these data suggest that TGF- β 1 is directly targeting the activity of BMP Smads.

TGF- β 1 Inhibits BMP4 Signaling and Alkaline Phosphatase Activity in Mesenchymal Cells

To establish whether the TGF- β 1-mediated inhibition of BMP4 signaling was common to other mesenchymal cell types, we examined the impact of TGF- β 1 on the Id gene transcriptional responses to BMP4 in C2C12 myoblasts and murine embryonic fibroblasts (Figure 3A). In both cell types, TGF- β 1 reduced BMP4-mediated transcription of Id1, Id2, Id3, and Id4, whereas the PAI1 response to TGF- β 1 was unaffected by BMP4. To confirm our observations in PASCs, we also examined whether TGF- β 1 was directly inhibiting BMP Smad signaling in C2C12 cells transfected with the BRE-luc reporter. Similar to our data in PASCs, TGF- β 1 inhibited BMP4-mediated BRE-luc activity (Figure E2A) and also inhibited the responses to BMP2 and BMP6 (Figure E2B). These data suggest a mechanism that is common to other mesenchymal cells.

C2C12 cells undergo osteogenic differentiation in response to BMPs, with alkaline phosphatase activity providing a basic readout of this process (24). TGF- β 1 reduced the BMP4-mediated

induction of alkaline phosphatase activity in C2C12 cells, measured both by activity assay (Figure 3B) and histochemical staining (Figure 3C).

TGF- β 1 Inhibits BMP4 via ALK5, but Not TGF- β -Activated Kinase 1, and Does Not Alter BMP-Mediated Smad Phosphorylation

We first questioned whether TGF- β 1 was exerting a direct effect on Smad phosphorylation. PASCs were treated with TGF- β 1, BMP4, or both ligands combined for 1 and 4 hours, and the levels of C-terminal phosphorylated Smads, S206 phosphorylated Smad1, and total Smad1 levels examined (Figure 4A). BMP4 stimulated phosphorylation of the C-terminal and linker regions of Smad1/5, whereas TGF- β 1 stimulated Smad2 and Smad3 C-terminal phosphorylation. Cotreatment with both ligands did not alter Smad1/5 phosphorylation or the total levels of Smad1.

We assessed whether TGF- β 1 inhibited BMP4 signaling via ALK5. Pretreatment with the ALK5 inhibitor, SD208, reversed the inhibitory effect of TGF- β 1 on BMP4-mediated ID1 and ID2 transcription in PASCs (Figure 4B) and the BRE-luc response in C2C12 cells (Figure E2A). As expected, SD208 attenuated TGF- β 1-mediated PAI1 and CTGF transcription in PASCs (Figure 4B). We also considered the potential involvement of TGF- β -activated kinase (TAK) 1, which integrates multiple downstream kinase pathways of TGF- β 1. In initial studies, 4 μ M of the TAK1 inhibitor, (5Z)-7-oxozeanol (TAKI), abolished all the BMP- and TGF- β 1-mediated Smad phosphorylation and transcriptional responses (data not shown). We established that 0.5 μ M TAKI blocks both basal and TGF- β 1-stimulated p38 mitogen-activated protein kinase (MAPK) phosphorylation and

Figure 3. TGF- β_1 inhibits BMP4 responses in mesenchymal cells. (A) C2C12 myoblasts or mouse embryonic fibroblasts were treated with TGF- β_1 (2 ng/ml), BMP4 (10 ng/ml), or both combined for 4 hours. Target gene expression was determined using QPCR. Data are means ($\pm$ SEM) for four separate experiments (* P < 0.05, ** P < 0.01, *** P < 0.001 compared with BMP4, paired t test). (B and C) C2C12 cells were treated with BMP4 (10 ng/ml) alone, or with TGF- β_1 (2 or 5 ng/ml) in 0.25% FBS. (B) Cells treated for 72 hours were assayed for alkaline phosphatase activity. Data are means ($\pm$ SEM) and are representative of three separate experiments (*** P < 0.01 versus 0.25% serum, ### P < 0.01 versus BMP4; paired t test). (C) Cells were stained for alkaline phosphatase after a 4-day treatment. Scale bar, 50 μ m. pNP, p-nitrophenol.

TGF- β_1 -mediated Smad3 phosphorylation, with minimal effect on BMP-dependent Smad phosphorylation (Figure 4C). TGF- β_1 still inhibited BMP4-mediated transcription in PSMCs (Figure 4D) or C2C12 cells (Figure E2A) treated with the TAKi, suggesting that pathways downstream of TAK1 are not involved. Western blotting for MAPK activation demonstrated that TGF- β_1 induced extracellular signal-regulated kinase (ERK) 1/2 and p38 MAPK phosphorylation (Figure E3), whereas BMP4 did not activate these kinases at these time points (Figure E3). No differences were observed in the phosphorylation of these kinases in cells treated with combined BMP4 and TGF- β_1 compared with cells treated with the individual ligands (Figures E3). No SAPK/JNK response was observed (Figure E3A), the lower bands representing ERK1/2 cross-reactivity. Neither the MKK1 inhibitor, U0126, nor the p38 MAPK inhibitor, SB203580, altered the inhibitory effect of TGF- β_1 in PSMCs (Figures E4A and E4B) or C2C12 cells (Figure E2A). Also, JNK and IKK inhibitors did not reverse the inhibitory effect of TGF- β_1 in C2C12 cells (Figure E2A).

TGF- β_1 Does Not Alter BMP-Smad Localization or Degradation

Because Smad translocation into the nucleus is essential for transcription, we addressed whether TGF- β_1 was preventing BMP-Smad nuclear translocation. Initial studies of cytoplasmic and nuclear fractions isolated at 1 and 4 hours after treatment did not indicate altered distribution of pSmad1/5 (S463/465) or Smad1 with TGF- β_1 treatment (data not shown). Furthermore, no difference was observed in cells treated with leptomycin B, which blocks nuclear export of Smads (Figure 5A). Consistent with the early induction of ID1/2 expression by TGF- β_1 , we observed a transient nuclear pSmad1/5 (S463/465) signal at 1 hour (Figure 5A).

Because the contributions of the individual BMP-Smads to ID responses in PSMCs are unknown, we addressed this question using siRNAs for Smads 1, 5, and 9. Intriguingly, Smad5 siRNA consistently reduced the ID1/2 response to BMP4 (Figure 5B),

whereas Smad1 and Smad9 siRNAs had no effect, suggesting that Smad5 may be the major Smad driving ID responses in PSMCs. Therefore, we examined the nuclear localization of Smad5 and Smad4. Neither the C-terminal phospho-Smad5 (S463/465), nor the Smad5 distribution, were affected by TGF- β_1 (Figure 5C). Unlike Smad1, the Smad5 and phospho-Smad5 bands separated into a doublet of 57 kD and 59.5 kD in BMP4-treated cells, the 59.5 kD band potentially representing an additional modification. TGF- β_1 did not alter the intensity of this additional band (Figures E5A and E5B), so we did not pursue this effect in this study. TGF- β_1 differentially affected the Smad4 distribution in PSMCs. In the nuclear fraction, Smad4 resolved as a doublet of 60 and 64 kD, the higher mass band of which was consistently more intense in cells treated with TGF- β_1 (Figure E5C). While these studies were in progress, a study in epithelial cells reported that TGF- β_1 inhibits BMP4 signaling in a Smad4-independent manner (25). Therefore, we did not focus on Smad4 in this study, but the potential for TGF- β_1 -selective modifications of Smad4 warrants future investigation.

Reports have previously demonstrated that Smad activity can be reduced by degradation or by specific phosphatases. We examined this potential mechanism in PSMCs treated with BMP4 for 4 hours in the presence or absence of TGF- β_1 , after which the ALK2/3/6 inhibitor, dorsomorphin, was then added to block further BMP signaling. By Western blotting, we observed a decay of the C-terminal BMP Smad phosphorylation, which returned to baseline levels after 1 hour (Figure 5D). TGF- β_1 did not affect the rate of decay of the phosphorylation signal, or the levels of Smads 1, 5, or 4 proteins. The sustained TGF- β_1 activity was confirmed by the maintenance of the pSmad3 band with the pSmad3(S423/425) antibody, whereas the cross-reacting pSmad1/5 band disappeared. These data implied that altered Smad dephosphorylation or degradation were unlikely to contribute to the inhibitory effect of TGF- β_1 on BMP signaling. Furthermore, treatment of C2C12 cells with the phosphatase

Figure 4. TGF- β 1 inhibits BMP4 signaling via activin-like kinase 5 (ALK5) but does not reduce Smad signaling or act via TGF- β -activated kinase (TAK) 1. (A) Control PASCs were treated with BMP4 (10 ng/ml) or TGF- β 1 (2 ng/ml) alone, or combined, for 1 and 4 hours. Protein lysates were immunoblotted for phospho- and total BMP- and TGF- β 1 R-Smads. (B) Control PASCs were treated for 1 hour with SD208 (2 μ M) or dimethyl sulfoxide vehicle followed by treatment with BMP4 (10 ng/ml) or TGF- β 1 (2 ng/ml) alone, or combined for 4 hours. Target gene expression was determined using QPCR. Data are means ($\pm$ SEM) for five separate experiments. (C) Control PASCs were treated with (S)-7-oxozeaenol (TAKi; 0.5 μ M) for 1 hour, followed by treatment with TGF- β 1 (2 ng/ml) or BMP4 (10 ng/ml) for 1 hour. Protein lysates were immunoblotted for phospho-Smad1/3, Smad1, Smad3, phospho-p38, and total p38 mitogen-activated protein kinase (MAPK). (D) Control PASCs were pretreated with TAKi for 1 hour, followed by treatment with BMP4, TGF- β 1, or both combined for 4 hours. Target gene expression was determined using QPCR. Data are means ($\pm$ SEM) for three separate experiments (* P < 0.05, ** P < 0.01 versus BMP4; ## P < 0.01 versus TGF- β 1; one-way repeated measures ANOVA).

inhibitor, orthovanadate, did not alter the repression of the BMP4-mediated BRE response by TGF- β 1 (Figure E2A).

TGF- β 1 Does Not Inhibit BMP4 via I-Smads or *De Novo* Protein Synthesis

Based on a previous report implicating Smad6 as a mediator of the inhibition of osteogenic differentiation by TGF- β 1 (26), we examined roles for the inhibitory Smads, Smad6 and Smad7. BMP4 induced Smad6 (Figure 6A) and Smad7 (Figure 6B) in a time-dependent manner. TGF- β 1 did not affect Smad6 expression, and stimulated an early Smad7 induction, which returned to baseline at 24 hours. Notably, Smad6 mRNA expression was very low in PASCs, whereas Smad7 expression was relatively high. Smad6

siRNA transfected into PASCs did not alter the ID responses to BMP4 (Figure 6C) or the PAI1 or CTGF responses to TGF- β 1. Interestingly, Smad7 siRNA enhanced the basal and BMP4-mediated ID1 and ID2 responses, but did not alter the repression of the BMP4 response by TGF- β 1 (Figure 6D). These data suggest that Smad7 represents an endogenous repressor of BMP4 signaling in PASCs.

In epithelial cells, TGF- β 1 represses ID1 transcription via the induction of activating transcription factor 3 (ATF3), which then binds to the ID1 promoter with Smad3 to repress transcription. Pretreatment of PASCs with cycloheximide did not reverse the repression of BMP4-mediated ID1 or ID2 induction by TGF- β 1 (Figure E6). In addition, BMP4 and TGF- β 1 did not induce ATF3 in PASCs (data not shown), suggesting that this mechanism is not

Figure 5. TGF- β_1 does not reduce BMP4 signaling by altering smad nuclear translocation or Smad dephosphorylation. (A) PSMCs were treated with BMP4 (10 ng/ml) or TGF- β_1 (2 ng/ml) alone, or combined, for 1 or 4 hours, the presence of leptomycin B (10 ng/ml). PSMCs were lysed and nuclear and cytoplasmic fractions prepared. Lysates were immunoblotted for P-Smad1/5 or Smad1 and fractionation confirmed by blotting for α -tubulin (cytoplasmic) and ASF/SF2 (nuclear). Blots are representative of three separate experiments. (B) PSMCs were transfected with small interfering (si) RNAs for Smad1, Smad5, or Smad9. ID1 and ID2 expression were determined using QPCR. Data are means ($\pm$ SEM) from four separate experiments (* P < 0.05 versus 10 ng/ml BMP4-treated DH1 and siCP equivalents). Selective reduction of the Smads was confirmed by immunoblotting. (C) PSMCs were treated as described previously here, and nuclear and cytoplasmic fractions prepared. Lysates were immunoblotted for P-Smad5, Smad5, or Smad4, followed by reprobing for α -tubulin and ASF/SF2. Blots are representative of three separate experiments. (D) PSMCs were treated with BMP4 alone, or with TGF- β_1 for 4 hours. Dorsomorphin was then added to the cells which were lysed at the indicated time points. Lysates were immunoblotted for the specified phospho or total Smads, as indicated.

involved in PSMCs. Furthermore, the lack of effect of cycloheximide confirmed that I-Smad transcription does not contribute to the inhibitory effect of TGF- β_1 .

TGF- β_1 Inhibits ID2 and Partially Inhibits ID1 via Smad3

Our studies using the TAK1 inhibitor, which blocks Smad3 phosphorylation in PSMCs, suggested that the phospho-Smad3 pathway was unlikely to repress BMP-mediated ID signaling. However, phosphorylation of only one R-Smad in the heteromeric complex may be required for particular functions (27). Therefore, we addressed the potential role of Smad2 or Smad3 using siRNA (Figure 7). Loss of Smad2 did not alter the inhibition of BMP4 signaling by TGF- β_1 (Figure 7A), whereas reducing Smad3 expression by more than 80% reversed the inhibitory effects of TGF- β_1 on BMP4-mediated ID2 repression, as well as partially reversing the ID1 repression (Figure 7B). These data suggest a key role for Smad3 in the TGF- β_1 -mediated repression of BMP4 signaling in PSMCs. During the course of these experiments, a molecular mechanism for this process was reported in epithelial cells (25).

DISCUSSION

TGF- β_1 is implicated as a profibrotic cytokine in disorders of the airways (2) and kidney (3). Recent evidence supports a key role for pathogenic TGF- β_1 signaling in PAH, a disease associated with reduced BMPR-II expression and downstream BMP signaling (4, 5, 21, 28). As BMPs inhibit TGF- β_1 signaling in airway (29) and renal cells (30), we questioned whether the BMP and TGF- β_1 signaling pathways directly interact in PSMCs and the potential impact in the setting of reduced BMPR-II levels in PAH. Here, we report the novel finding that, contrary to the cell types mentioned above, TGF- β_1 inhibits BMP4-mediated ID1 and ID2 transcription in control and HPAH PSMCs with BMPR-II mutations. As BMP signaling is blunted in HPAH PSMCs, TGF- β_1 completely abolishes the Smad-mediated transcriptional responses in these cells compared with the partial inhibition observed in control PSMCs. TGF- β_1 mediates this repression via ALK5 and Smad3, yet this appears to be independent of Smad3 phosphorylation. These data support the hypothesis that TGF- β_1 is a pathogenic cytokine in HPAH, especially against a background of reduced BMPR-II function or expression.

Figure 6. TGF- β_1 does not reduce BMP4 signaling via inhibitory Smads. (A) Control PASCs were treated for 1, 4, or 24 hours with BMP4 (10 ng/ml) or TGF- β_1 (1 or 10 ng/ml) in 0.1% FBS. Smad6 and Smad7 expression were determined using QPCR. Control PASCs were transfected with siRNAs targeting either (B) Smad6 or (C) Smad7. PASCs were treated with BMP4, TGF- β_1 , or both ligands for 4 hours. ID1 and ID2 expression were determined using QPCR. Data are means ($\pm$ SEM) from four separate experiments (** $P < 0.01$, * $P < 0.05$ versus DH1 and siCP equivalents; one-way repeated measures ANOVA).

Most germline mutations in BMPR-II are predicted to cause haploinsufficiency which would be anticipated to reduce BMP signaling (7). We have shown that PASCs from patients with BMPR-II mutations exhibit reduced BMP4-mediated Smad phosphorylation and ID transcription (18, 19), and these cells exhibit altered functional responses to TGF- β_1 (20, 21). Moreover, studies in MCT-PAH rats have also shown enhanced TGF- β_1 and reduced BMP signaling (4, 22). From previous studies showing inhibition of TGF- β_1 by BMPs (29–31), we hypothesized that loss of BMP signaling might have a permissive effect on TGF- β_1 . Contrary to this hypothesis, TGF- β_1 reduced the BMP-mediated

Smad-dependent transcription in PASCs. Interestingly, TGF- β_1 does not abolish ID transcription in control cells, implying that, in PASCs with intact BMPR-II, TGF- β_1 tonically reduces BMP signaling rather than causing a complete loss. However, in HPAH PASCs with reduced BMPR-II and an attenuated BMP4-driven ID response (21), TGF- β_1 negates the BMP response. This suggests a potential pathogenic role of TGF- β_1 in HPAH via inhibition of the residual BMP signaling.

We addressed whether this inhibition was relevant to other mesenchymal cell types, namely C2C12 mouse myoblasts and MEFs. Indeed, TGF- β_1 reduced the BMP4-mediated transcription of

Figure 7. TGF- β_1 reduces BMP4 signaling via Smad3. (A) Control PSMCs were transfected with siRNAs targeting either (A) Smad2 or (B) Smad3. PSMCs were then treated with BMP4, TGF- β_1 , or both combined for 4 hours. ID1, ID2, plasminogen activator inhibitor (PAI) 1, and connective tissue growth factor (CTGF) expression were determined using QPCR. Data are means ($\pm$ SEM) from five separate experiments (* P < 0.05, ** P < 0.01 versus BMP4; # P < 0.05, versus TGF- β_1 ; one-way repeated measures ANOVA). Specific loss of Smad2 or Smad3 was confirmed by immunoblotting.

Id1–4 in C2C12 cells and MEFs, whereas the induction of PAI1 by TGF- β_1 was unaffected by BMP4. Our data, showing that TGF- β_1 robustly inhibits the alkaline phosphatase response to BMP4, are consistent with two recently published studies showing that TGF- β s inhibit BMP-mediated transcription (23, 25) and osteogenic differentiation (23) in C2C12 cells. Similar to our data, the authors demonstrate the inhibition of BRE-luc responses to BMPs by TGF- β isoforms (23, 25). Collectively, these data confirm that TGF- β s inhibit BMP-mediated Smad signaling in mesenchymal cells.

Consistent with studies in epithelial cells (16), TGF- β_1 stimulated ID1/2 transcription at 1 hour, followed by repression by 4 and 24 hours. In epithelial cells, Smad3 mediates the early ID response and also drives ATF3 transcription, which subsequently represses ID1 (32, 33). However, our data using cycloheximide

suggested that transcription is not required for the TGF- β_1 -mediated repression. Furthermore, our data using the BRE-luc plasmid implied a direct effect of BMP-Smad signaling rather than mediation via a separate regulatory element.

Several mechanisms have been identified for the attenuation of BMP signaling through ligand neutralization, receptor targeting, and alteration of R-Smad signaling. In PSMCs, TGF- β_1 did not alter Smad C-terminal or linker region phosphorylation, implying that the BMP4 was not being negated by ligand traps, receptor down-regulation, or Smad degradation. We have established, for the first time, that Smad5 is the key Smad driving ID transcription in PSMCs. Conversely, Smad1 is the major Smad mediating BRE responses in C2C12 cells (23), implying differing roles for Smad1 and Smad5 in different mesenchymal cell types. In PSMCs, the nuclear and cytoplasmic localization of Smad1,

Smad5, phospho-Smad1/5, and phospho-Smad5 were not altered by TGF- β_1 . Intriguingly, BMP4 treatment increased the mass of Smad5 (Figure 5C), implying that a significant modification occurs. This is not evident when immunoblotting for Smad1, suggesting that C-terminal phosphorylation is not sufficient to cause this shift. Therefore, our data suggest that Smad5 is phosphorylated at multiple sites, or that additional modifications occur.

Having examined Smad phosphorylation, we considered Smad dephosphorylation as a potential inhibitory mechanism. Small C-terminal peptidases can target Smads for dephosphorylation, but do not affect Smad1 protein or Id transcription (14, 15). protein phosphatase 1A can also drive Smad degradation independently of the C-terminal SSVS or linker region SMURF sites (15). In PSMCs, TGF- β_1 did not alter the rate of loss of the C-terminal Smad1/5 and Smad5 signals or the total Smad levels, including those of Smad4.

Using pharmacological inhibitors, we confirmed a role for ALK5 in both PSMCs and C2C12 cells. We also established that some of the core kinase pathways activated by TGF- β_1 , namely the TAK1, p38, and ERK1/2 pathways, were not mediating this inhibition. An interesting consequence of TAK inhibition was the blockade of Smad3 C-terminal phosphorylation, which initially led us to exclude Smad3 involvement. Based on a previous report suggesting that TGF- β_1 inhibits BMP-driven osteogenic differentiation via Smad6 in C2C12 cells (34), we examined the inhibitory Smads. Smad6 preferentially inhibits BMP signaling, whereas Smad7 is more promiscuous, as it inhibits signaling by TGF- β s, activins, and BMPs (35, 36). Smad6 is expressed at higher levels than Smad7 in C2C12 cells (26), whereas we found high levels of Smad7 expression in PSMCs and very low Smad6 expression. Our siRNA and cycloheximide experiments both indicate that inhibitory Smad transcription is not responsible for the inhibition of BMP signaling by TGF- β_1 . However, Smad7 siRNA increased ID1 and ID2 transcription in all conditions, implying that endogenous Smad7 negatively regulates BMP4- and TGF- β_1 -mediated ID responses and may contribute to other pathways that regulate BMP/TGF- β_1 signaling. This concurs with the reported inhibition of BMP4-mediated Id1 transcription by Smad7 overexpression in C2C12 cells (26).

Although our TAK1 inhibitor studies suggested that phosphorylated Smad3 was unlikely to contribute, we considered that TGF- β_1 , via Smad2/3, may modify BMP R-Smad signaling. In endothelial cells, TGF- β_1 -mediated Smad2/3 is repressed by TGF- β_1 -mediated Smad1/5 activation (37). Furthermore, TGF- β_1 is reported to repress muscle differentiation via Smad3 (10, 11). Collectively, our TAK1 inhibitor and Smad3 siRNA data imply a role for Smad3 that is independent of C-terminal phosphorylation. A recent report has demonstrated a similar mechanism in epithelial cells (25), whereby Smad3 represses BMP-mediated transcription by binding to Smad1/5 occupying the BRE-luc construct or to the BMP-response element in the ID1 or ID2 promoters. Moreover, the authors focused on ID2, because TGF- β_1 more robustly inhibited BMP7-dependent ID2 transcription compared with ID1 (25). This concurs with our data showing that Smad3 transfection completely reversed ID2 repression, but only partially reversed ID1 repression. With greater than 80% Smad3 knockdown, CTGF responses to TGF- β_1 were reduced, whereas PAI1 was affected less. This was surprising, as PAI1 is classically defined as Smad3 dependent (38), so we suggest that the small amount of residual Smad3 was sufficient to maintain PAI1 induction by TGF- β_1 . However, the Smad3 reduction was sufficient to abolish the repression of ID2 by TGF- β_1 . In the study by Gronroos and colleagues (25), C-terminal phosphorylated Smad3 and Smad1/5 could be coprecipitated using DNA pull-downs on biotinylated ID1, ID2, and BRE probes, implying that C-terminal Smad3 phosphorylation may be required, contrasting with our TAK inhibitor data. We cannot exclude the possibility

that other Smad3 modifications may be important for this association of Smad3 with the BMP-Smads. Acetylation of Smad3 is known to regulate its transcriptional capacity (39). In preliminary studies, we did not observe any alteration of the responses in C2C12 cells treated with the p300 inhibitor, C646 (P.D.U., unpublished data).

In conclusion, our data suggest that the potential pathogenic role of TGF- β_1 in HPAH may be, in part, due to a direct inhibition of the BMP-Smad pathway. This is particularly relevant on the background of reduced BMP signaling as a consequence of germline mutations in BMPR-II or potentially other factors that may lead to BMPR-II reduction. Our studies demonstrate how increased TGF- β_1 signaling can further impair BMP signaling in the setting of BMPR-II mutations, and support the potential therapeutic application of inhibitors of the ALK5/Smad3 pathways in PAH.

Author disclosures are available with the text of this article at www.atsjournals.org.

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