Endoglin Differentially Modulates Antagonistic Transforming Growth Factor-β1 and BMP-7 Signaling

Transformation growth factor-β1 (TGF-β1) and BMP-7 (bone morphogenetic protein-7; OP-1) play central, antagonistic roles in kidney fibrosis, a setting in which the expression of endoglin (CD105), an accessory TGF-β type III receptor, is increased. So far, endoglin is known as a negative regulator of TGF-β/ALK-5 signaling. Here we analyzed the effect of BMP-7 on TGF-β1 signaling and the role of endoglin for both pathways in endoglin-deficient LαEα cells. In this myoblastic cell line, TGF-β1 and BMPs are opposing cytokines, interfering with myogenic differentiation. Both induce specific target genes of which Id1 (for BMPs) and collagen I (for TGF-β1) are two examples. TGF-β1 activated two distinct type I receptors, ALK-5 and ALK-1, in these cells. Although the ALK-5/Smad3 signaling pathway mediated collagen I expression, ALK-1/Smad1/Smad5 signaling mediated a transient Id1 up-regulation. In contrast, BMP-7 exclusively activated Smad1/Smad5 resulting in a more prolonged Id1 expression. Although BMP-7 had no impact on collagen I abundance, it antagonized TGF-β1-induced collagen I expression and (CAGA)12-MLP-Luc activity, effects that are mediated by the ALK-5/Smad3 pathway. Finally, we found that the transient overexpression of endoglin, previously shown to inhibit TGF-β1-induced ALK-5/Smad3 signaling, enhanced the BMP-7/Smad1/Smad5 pathway.

TGF-β3 and BMP belong to the TGF-β superfamily of cytokines regulating a broad spectrum of cellular functions, including proliferation, apoptosis, and differentiation (1, 2). Mammals possess three different TGF-β isoforms (i.e. TGF-β1, TGF-β2, and TGF-β3) and more than 20 BMP-related proteins. Based on their structures and functions, BMPs can be subdivided into several groups (3). One group (BMP-2/4 group) contains BMP-2, BMP-4, and the Drosophila decapentaplegic (dpp) gene product. BMP-5, BMP-6, BMP-7 (also termed osteogenic protein-1, OP-1), BMP-8 (OP-2), and the Drosophila gbb-60A gene product form another subgroup (OP-1 group) (4). Signaling by TGF-β superfamily ligands has been analyzed in detail during myoblast differentiation in rat LαEα cells (5, 6). TGF-β1 inhibits myogenic differentiation that is morphologically characterized by cell fusion leading to multinucleated myotubes. In addition, biochemical differentiation measured by expression of muscle-specific genes (e.g. myogenin) is modulated when cells are cultured in the absence of mitogens or on collagen substrates (7–9). BMPs inhibit differentiation of mouse C2C12 myoblasts and induce osteogenic differentiation, which in contrast is not induced by TGF-β1 (10–12). This inhibition is mediated by Smad1 and/or Smad5 (13–14). Id1, a target gene of Smad1/Smad5, is induced by BMP ligands and inhibits myogenic differentiation in C2C12 cells by sequestration of MyoD and myogenin (10, 15–18).

In general, members of the TGF-β superfamily bind to heterotetrameric receptor complexes consisting of type I and type II receptors that contain serine/threonine kinase domains (19). Five type II receptors have been identified so far. They contain a constitutive active kinase domain, which in response to ligand binding trans-phosphorylate specific type I receptors at their glycine- and serine-rich domains (GS domain). Seven type I receptors, designated activin receptor-like kinases (ALK-1 to ALK-7), are presently known in mammals (20, 21). The activated type I receptors transfer the signals to intracellular mediators that are termed Smad proteins (3, 19, 22). The receptor-regulated Smads (R-Smads) include Smad2 and Smad3 (“TGF-β-Smads”) and Smad1, Smad5, and Smad8 (“BMP-Smads”). Once activated by phosphorylation at their carboxy-terminal SSXS motif, the R-Smads associate with the common mediator Smad4 (co-Smad), and this complex translocates into the nucleus regulating the expression of specific target genes. The I-Smads constitute negative feedback inhibitors that block TGF-β superfamily signaling in general (Smad7) or more specifically BMP signaling (Smad6) (22, 23).

In most cells, TGF-β binds to the ubiquitously expressed ALK-5 receptor that activates Smad2 and Smad3 (24). In a few other cells, TGF-β activates ALK-1 in the presence of functional ALK-5 resulting in phosphorylation of Smad1 and Smad5 (25–27). The decision which type I receptor is activated is determined by receptor expression and/or ligand concentration. ALK-1 activation triggers proliferation and migration, whereas ALK-5 opposes these responses (28). Members of the BMP-2/4 group bind and activate ALK-3 and ALK-6 (also termed BMPR-IA and BMPR-IB), whereas proteins of the OP-1 group preferentially bind to ALK-2 (ActR-1) and ALK-6 (29, 30) resulting in phosphorylation of Smad1/Smad5 and/or Smad8 (11, 31).
Beside these classical signaling receptors, two accessory type III receptors, i.e. betaglycan (TβRIII) and endoglin (CD105), are known (32, 33). Endoglin binds TGF-β1, TGF-β3, activin-A, BMP-2, and BMP-7 (34). The function of endoglin has been most thoroughly analyzed in endothelial cells and L<sub>e</sub>E<sub>0</sub> myoblasts (for review see Ref. 27). In endothelial cells, endoglin inhibits signaling via ALK-5 and promotes signaling via ALK-1 resulting in increased proliferation and migration (35). Additionally, endoglin counteracts TGF-β1 in mediating inhibition of proliferation (36, 37). Extracellular matrix genes (i.e. collagen, fibronectin, PAI-1, and connective tissue growth factor) are down-regulated when endoglin is overexpressed in L<sub>e</sub>E<sub>0</sub> cells reflecting blockade of Smad3 phosphorylation (36–38).

In this study, we analyzed the interdependence of BMP-7 and TGF-β1 signaling and the function of endoglin in L<sub>e</sub>E<sub>0</sub> cells. We show for the first time that TGF-β1 activates two different signaling pathways in this myogenic cell line. One activates matrix gene expression via Smad3, and the other inhibits myogenic differentiation by up-regulation of Id1 via the Smad1/Smad5 pathway. Additionally, we demonstrate that L<sub>e</sub>E<sub>0</sub> cells are highly responsive to BMP-7. BMP-7 inhibited TGF-β1-mediated (CAGA)<sub>12</sub>-MLP-Luc reporter gene activation and collagen I expression. Transient expression of endoglin resulted in the inhibition of TGF-β1/Smad3 responses, whereas signaling via the BMP-7/Smad1/5 pathway was enhanced.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—L<sub>e</sub>E<sub>0</sub> myoblasts were maintained in the undifferentiated state in growth medium (GM) containing HEPES-buffered DMEM (BioWhittaker, Verviers, Belgium) supplemented with 20% (v/v) fetal calf serum (FCS) (Perbio Science, Cramlington, UK), 4 mmol/liter l-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (all from Cambrex, Verviers, Belgium). To induce differentiation, cells were shifted to differentiation medium (DM) composed of HEPES-buffered DMEM supplemented with 0.5% (v/v) FCS. When indicated, the cells were incubated with 5 μM ALK5 inhibitor SB431542 (Tocris Bioscience, Ellisville, MO). Primary hepatic stellate cells (HSC) were isolated as described before and passaged once to obtain myofibroblasts (39). Parenchymal liver cells (PC, i.e. hepatocytes) were isolated following the collagenase method of Seglen (40). All cultures were kept in a humidified atmosphere containing 5% CO<sub>2</sub>.

**Plasmids**—The following plasmids have been described previously: CA-ALK-1 and CA-ALK-5 encoding constitutive active forms of the human TGF-β receptors ALK-1 and ALK-5 (24); the luciferase reporter constructs (CAGA)<sub>12</sub>-MLP-Luc (41) and (BRE)<sub>2</sub>-Luc (42); pcDNA-endoglin and pCMV-HA-tβRIII encoding rat endoglin (43); and rat betaglycan (33). Expression plasmids for rat Smad1 (IRBPp993A072D2) and mouse Id1 (IRAVp968B0979D) were purchased from the RZPD Resource Center (Berlin, Germany). The vector pEGFP-C1 was purchased from Clontech. For cloning pCMV-β-gal2 and pCMV-β-gal3, the coding regions of the respective β-gal2s were amplified by RT-PCR using the Expand High Fidelity PCR system (Roche Applied Science, Mannheim, Germany) and the primers listed in supplemental Table I. The cycling conditions were as follows: Smad3, initial denaturation for 2 min at 94 °C and then 35 cycles at 94 °C for 15 s, 54 °C for 30 s, 72 °C for 2.5 min, followed by a final elongation step at 72 °C for 10 min; Smad2, initial denaturation for 2 min at 94 °C and then 35 cycles at 94 °C for 15 s, 52 °C for 30 s, 72 °C for 2 min, followed by a final elongation at 72 °C for 10 min. The resulting PCR fragments were first cloned into the pGEM<sup>T</sup>-T Easy vector (Promega, Mannheim, Germany), verified by sequence analysis, and subsequently cloned into pCDNA3 (Invitrogen, Karlsruhe, Germany).

**Transient Transfections**—For transfection, cells were plated in 6-well dishes at a density of 2.5 × 10<sup>5</sup> cells/well and transfected with 2 μg of DNA per well of indicated expression plasmids using the FuGENE<sup>®</sup> transfection reagent (Roche Applied Science). After 24 h, the medium was renewed, and cells were harvested after 48 h.

**Stimulation Experiments**—For stimulation, cells were plated in 6-well plates at a density of 2.5 × 10<sup>5</sup> cells/well and transfected with 1 μg of (CAGA)<sub>12</sub>-MLP-Luc and (BRE)<sub>2</sub>-Luc or 2 μg of CA-ALK-1, CA-ALK-5, and pcDNA-endoglin using the Lipofectamine Plus reagent (Invitrogen). Briefly, transfections were carried out in DMEM without supplements, and the medium was replaced by normal growth medium 6 h post-transfection. After a further 12-h incubation period, the medium was renewed, and 24 h later the cells were starved for 16 h in medium containing 0.5% FCS. Thereafter, stimulation with BMP-7 and TGF-β1 was carried out for indicated times in medium containing 0.2% FCS. Cell extracts were prepared in 350 μl of passive lysis buffer (Promega), and 20 μl were applied to luciferase measurements. All experiments were done in triplicate and normalized to the protein content, and the relative luciferase activities (± S.D.) are given.

**Western Blot Analysis**—For Western blot analysis, cells were washed in ice-cold Hanks’ buffered saline solution and extracted in lysis buffer containing protease inhibitors and phosphatase inhibitors. Equal amounts of protein lysates were diluted with nonreducing NuPAGE lithium dodecyl sulfate-electrophoresis sample buffer (Invitrogen), heated at 75 °C for 10 min, and separated on 10 or 4–12% BisTris gels (Invitrogen) using MOPS-SDS running buffer (50 mmol/liter MOPS, 50 mmol/liter Tris-HCl (pH 7.7), 3.47 mmol/liter SDS, and 1.025 mmol/liter EDTA) or MES-SDS running buffer (50 mmol/liter MES, 50 mmol/liter Tris-HCl (pH 7.3), 3.47 mmol/liter SDS, and 1.025 mmol/liter EDTA), respectively. The analysis of collagen I expression was done on 3–8% Tris acetate gels (Invitrogen) in...
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MOPS-SDS running buffer. Proteins were electrophoretically using NuPAGE transfer buffer (Invitrogen) onto nitrocellulose membranes (Schleicher & Schuell), and equal protein loading was monitored in Ponceau S stain. Unspecific binding sites were blocked in TBST (10 mm Tris-HCl, 150 mm NaCl, 0.1% (v/v) Tween 20 (pH 7.6)) containing 5% (w/v) nonfat milk powder. Primary antibodies used are listed in supplemental Table 2. The antibody PPabE1 was raised by order against keyhole limpet hemocyanin-coupled peptide LALHPSTLSQEVY corresponding to amino acids 548–560 of rat endoglin (Davids Biotechnologie, Regensburg, Germany). Primary antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) using the SuperSignal West Dura Extended Duration substrate (Perbio Science). In some cases the signals were enhanced with a biotinylated secondary antibody prior to incubation with a streptavidin-horseradish peroxidase conjugate. Signal quantification and normalization were done in a Lumi-Imager™ (Roche Applied Science) using the LumiAnalyst 3.0 software.

Affinity Labeling of TGF-β Receptors and Immunoprecipitation—125I-TGF-β1 affinity labeling and cross-linking experiments were performed as described previously (43). Briefly, confluent monolayers of L6E9 cells were washed and incubated in binding buffer (50 mmol/liter HEPES (pH 7.4), 128 mmol/liter NaCl, 5 mmol/liter KCl, 5 mmol/liter MgSO4, 13 mmol/liter CaCl2, 0.5% (v/v) bovine serum albumin) at 37 °C for 0.5 h. Thereafter, the cells were washed in ice-cold binding buffer, and ligand binding was performed for 3 h at 4 °C using 2.8 ng of 125I-TGF-β1/ml with a activity of 1621 Ci/ml with a specific activity of 3 and 3.6 kb in size, whereas no endoglin signals were obtained in L6E9 cells (not shown). The deficiency for endoglin was further confirmed by RT-PCR (Fig. 2A) and in cross-linking experiments (Fig. 2B) when L6E9 cells were stimulated with TGF-β1 (1 ng/ml), BMP-2 (25 ng/ml), or BMP-7 (25 ng/ml) for 1 h, and cell lysates (20 μg) were analyzed by Western blot for phosphorylated Smads using antibodies specific for BMP-Smads (Smad1/Smad5) or TGF-β1-Smad (Smad2). The membrane was re-probed with a β-actin antibody.

RESULTS

L6E9 cells are Deficient for Endoglin and Mediate Smad1/Smad5 Phosphorylation in Response to BMP-7 and TGF-β1—To determine the function of endoglin in BMP-7 and TGF-β1 signaling in L6E9 cells, we first re-evaluated the reported endoglin deficiency at both the RNA and protein level. In rat hepatic stellate cells, we found two specific endoglin transcripts of 3 and 3.6 kb in size, whereas no endoglin signals were obtained in L6E9 cells (not shown). The deficiency for endoglin was further confirmed by RT-PCR (not shown) and Western blot analysis (Fig. 1A). In addition, affinity labeling experiments with iodinated TGF-β1 followed by immunoprecipitation reconfirmed the absence of functional endoglin in L6E9 cells (see below).

To focus on the Smad activation pattern in L6E9 cells, we next performed stimulation experiments with TGF-β1 and BMP-7 and analyzed the respective protein extracts with antibodies that exclusively detect carboxyl-terminal Smad phosphorylation (induced by the type I receptor kinase) and not those of the Smad linker regions (induced by mitogen-activated protein kinases). When cells were stimulated with BMP-2 or BMP-7, a strong phosphorylation of Smad1/Smad5 was provoked (Fig. 1B). When L6E9 cells were stimulated with TGF-β1, we observed phosphorylation of Smad2 and Smad1/Smad5 indicating that TGF-β1 activates both Smad signaling pathways.

TGF-β1 Dose-dependently Activates the ALK-5 and ALK-1 Pathways—The presence of ALK-1 in L6E9 cells was analyzed by RT-PCR (Fig. 2A) and in cross-linking experiments (Fig. 2B). The cross-linking experiments further confirmed the already mentioned absence of functional endoglin in L6E9 cells.
Stimulation of L6E9 cells with increasing amounts of TGF-β1 revealed that Smad1/Smad5 and Smad3 were dose-dependently phosphorylated (Fig. 2C). Both signals were abrogated in the presence of the synthetic ALK-5 inhibitor SB431542. Consistent with this finding, we observed a time-dependent accumulation of PAI-1 in supernatants of TGF-β1-stimulated L6E9 cells and a strong activation of the pSmad3 reporter (CAGA)12-MLP-Luc upon TGF-β1 stimulation (Fig. 2, D and E), which was prevented in the presence of SB431542 (data not shown). Similarly, a strong stimulation was induced by a constitutive active ALK-5 receptor (CA-ALK-5) (Fig. 2F) that induces Smad2 and Smad3 phosphorylation. In conclusion, this set of experiments revealed that TGF-β1 was sufficient to activate Smad3 and Smad1/Smad5. Interestingly, both phosphorylation of Smad3 and Smad1/5 was blocked in the presence of SB431542 (cf. Fig. 2C). Because ALK-1 is insensitive toward this inhibitor (46), this finding indicated that ALK-1 signaling itself is ALK-5-dependent in L6E9 cells.

**BMP-7 Signaling and Receptors in L6E9 Cells**—To examine BMP-7 signaling in L6E9 cells, we first analyzed the expression of potential BMP-7 membrane receptors, i.e. ActR-I (ALK-2), BMPR-IA (ALK-3), or BMPR-IB (ALK-6) by RT-PCR. Under the experimental setting, we were able to amplify fragments specific for ALK-3 (677 bp) and to a much lower content ALK-6 (620 bp), but we failed to detect ALK-2 mRNA expression in L6E9 cells (Fig. 3A). In addition, the analysis revealed that the putative type II receptors BMPR-II (682 bp), ActR-IIb (678 bp), and ActR-II (700 bp) were expressed in L6E9 cells. In contrast, BMP-7 transcripts were not found. As positive controls for BMP-7 (507 bp) and ALK-2 (1560 bp), we analyzed cDNAs from rat hepatocytes (PC) or rat HSC that express high contents of corresponding genes. Having shown that the necessary receptors for BMP-7 are expressed, we next examined the activation of the downstream effectors Smad1/Smad5 by BMP-7 in Western blot (Fig. 3B). In agreement with Smad1/Smad5 activation, the (BRE)2-Luc reporter harboring a Smad1/Smad5-dependent response element of the Id1 promoter was dose-dependently stimulated by BMP-7 (Fig. 3C), whereas the (CAGA)12-MLP-Luc reporter was left unaffected (data not shown). In contrast to the comparable activation potency of the (CAGA)12-MLP-Luc construct by CA-ALK-5 and TGF-β1 (cf. Fig. 2F), the (BRE)2-Luc reporter was only weakly stimulated by a constitutive active ALK-1 receptor (CA-ALK-1) (Fig. 3D) that potently induced the phosphorylation of Smads involved in BMP signaling.

**BMP-7 Blocks TGF-β1 Signaling in L6E9 Cells**—To analyze the interdependence of BMP-7 and TGF-β1 signaling, we performed advanced reporter assays. TGF-β1 alone strongly activated the (CAGA)12-MLP-Luc-reporter, but when BMP-7 was simultaneously applied, the reporter activity was significantly reduced (Fig. 4A). Consistently, the amount of pSmad3 was lower in the presence of BMP-7 compared with samples that

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4. O. Scherner, S. K. Meurer, L. Tihaa, A. M. Gressner, and R. Weiskirchen, unpublished observations.
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**A**

![Image](374x26 to 402x38)

**B**

![Image](60x281 to 288x733)

**C**

![Image](200x116)

**D**

![Image](200x116)

FIGURE 3. BMP-7 provokes a strong Smad response in L6E9 cells. A, expression of the activin and BMP type I and type II receptors was examined by RT-PCR using cDNA from L6E9 cells and primers listed in supplemental Table I. The sizes of molecular weight markers (GeneRuler™, Fermentas) are depicted in the left margin. r, rat. Cycle numbers for PCR were as follows: 40 cycles for BMPR-II, rALK-3, rBMP-7, and rALK-2; 45 cycles for all others. B, L6E9 cells were stimulated with BMP-7 or left untreated (control) for 1 h and analyzed for phosphorylated Smad1/5 by Western blot. Equal protein loading was demonstrated with a β-actin antibody. C, cells were transfected with the (BRE)2-Luc reporter and the relative luciferase activities were measured after stimulation with the indicated concentrations of BMP-7 for 1 h and analyzed for phosphorylated Smad1/Smad5 by Western blot. D, L6E9 cells were transfected with the (BRE)2-Luc reporter alone or in combination with a constitutive active ALK-1 expression plasmid. Cells were then stimulated with BMP-7 (50 ng/ml) or left untreated (control). The relative luciferase activities were measured. Both the stimulation with BMP-7 and the co-transfection with the constitutive active ALK-1 receptor resulted in highly significant induction of luciferase activities. ** indicates highly significant p values.

were only treated with TGF-β1 (Fig. 4B). Conversely, BMP-7 induced a strong activation of the (BRE)2-Luc-reporter that was decreased when TGF-β1 was present (Fig. 4C). Likewise, application of TGF-β1 in the presence of BMP-7 resulted in reduced Smad1 and/or Smad5 phosphorylation. In addition, Smad3 phosphorylation was only marginally increased by TGF-β1 in the presence of BMP-7 (Fig. 4D).

**Differential Effects of BMP-7 and TGF-β1 on Id1 and Collagen I Expression**—To further examine BMP-7 and TGF-β1 downstream signaling, we evaluated Smad-induced transcription responses of endogenous target genes, i.e. Id1 and collagen I. Therefore, we first analyzed the expression of Id genes by RT-PCR in cells cultured in growth medium. We were able to amplify a prominent Id1 fragment (510 bp), whereas Id2 (520 bp) and Id3 (553 bp) were nearly undetectable (Fig. 5A). As positive controls for Id2 and Id3, we analyzed cDNAs from rat myofibroblasts or total rat liver that express the respective genes. Next we analyzed the expression of Id1 in response to TGF-β1 stimulation, and we found that in contrast to the (BRE)2-Luc reporter (data not shown) Id1 protein expression was dose-dependently induced by TGF-β1 (Fig. 5B). This increase was abrogated when SB431542 was present during stimulation. A further detailed analysis revealed that the increase of Id1 expression was only transient (Fig. 5C). In contrast, when the cells were stimulated with BMP-7, the observed induction of Id1 expression was more sustained (Fig. 5D). In respective control cells, Id1 expression was very low after 12 h and decreased further after 24 h in accordance to previous reports showing that Id1 expression is down-regulated during myoblastic differentiation (see below).

In contrast to Id1, which is influenced by both cytokines, collagen I expression was only induced by TGF-β1 (Fig. 5F). In accordance with the (CAGA)12-MLP-Luc reporter assay, BMP-7 was also able to abrogate the TGF-β1-induced collagen I expression.

**BMP-7 Inhibits TGF-β1-induced Collagen I Expression in Differentiating L6E9 Cells**—The reporter gene analysis (cf. Fig. 4A) implicated that BMP-7 antagonized TGF-β1-induced ALK-5/Smad3 signaling in L6E9 cells. This inhibitory effect was confirmed upon short term stimulation, showing that TGF-β1 leads to increased collagen I expression that was blocked in the presence of BMP-7 (cf. Fig. 5F). To transfer this finding into a more physiological process, we examined the interaction between TGF-β1 and BMP-7 in myogenic differentiation. Cultivation of L6E9 cells under low serum conditions induced differentiation to multinucleated myotubes (Fig. 6A, DM). In parallel, the expression of collagen I and Id1 was down-regulated and, on the contrary, the muscle regulatory factor myogenin was up-regulated (Fig. 6, B and C, lanes 1 and 2). TGF-β1 at a concentration higher than 0.1 ng/ml inhibited the process of fusion (Fig. 6A, TGF-β1) and induced collagen I and Id1 expression (Fig. 6, B, lane 5, and C, lanes 4 and 5). Concomitantly, we found a strong suppression of myogenin. In contrast, 0.1 ng/ml BMP-7 failed to interfere with myoblast fusion, Id1, and myogenin expression (Fig. 6B, lane 4). In addition, there was only a marginal offset in collagen I expression (Fig. 6C, lane 3). BMP-7 blocked fusion of L6E9 cells (Fig. 6A, BMP-7), increased Id1, and abolished myogenin expression (Fig. 6B, lanes 6 and 7). As observed previously for the Smad activation (Fig. 2C), the effects of TGF-β1 but not BMP-7 were completely blocked by application of inhibitor SB431542 (data not shown). In contrast to TGF-β1, BMP-7 had no impact on collagen I expression (Fig.
Antagonism of TGF-β1 and BMP-7 signaling in L6E9 cells. A. L6E9 cells were transfected with the (CAGA)12-MLP-Luc reporter plasmid and stimulated with TGF-β1 (0.5 ng/ml), TGF-β1 (0.5 ng/ml)/BMP-7 (50 ng/ml) or were left untreated. The cells were extracted and the relative luciferase activities determined. ** indicates highly significant p values. B. L6E9 cells were stimulated (1 h) with TGF-β1 (1 ng/ml) and increasing concentrations of BMP-7 (5, 10, 25, and 100 ng/ml). Respective lysates (20 μg) were analyzed by Western blot using an antibody recognizing phosphorylated BMP-Smads1/5 (upper band) and Smad3 (lower band). To monitor equal loading, the membrane was reprobed with an antibody directed against total Smad2/Smad3. C. L6E9 cells were transfected with the (BRE)2-Luc reporter plasmid and stimulated with BMP-7 (50 ng/ml), BMP-7 (50 ng/ml)/TGF-β1 (0.5 ng/ml) or left untreated. The cells were extracted, and the relative luciferase activities were determined. * indicates significant p values, and ** indicates highly significant p values. D. L6E9 cells were stimulated (1 h) with a constant concentration of BMP-7 (25 ng/ml) and increasing concentrations of TGF-β1 (0.01, 0.1, 1, and 10 ng/ml). Respective lysates (20 μg) were analyzed by Western blot using an antibody recognizing phosphorylated BMP-Smads1/5 (upper band) and Smad3 (lower band). To monitor equal loading, the membrane was reprobed with an antibody directed against total Smad2/Smad3.

Endoglin Inhibits TGF-β1/Smad3 and Enhances BMP-7/Smad1/5 Signaling—To unravel the function of endoglin in the mentioned signaling pathways, we next performed transient transfection experiments. The typical transfection efficacy of L6E9 cells in our experiments was 30% (not shown). To evaluate if this efficiency was sufficient to provoke functional consequences, we first monitored the expression of PAI-1 upon TGF-β1 stimulation. Although the PAI-1 expression was dose-dependently increased by TGF-β1, transfection with the endoglin expression vector provoked no significant changes in PAI-1 expression. Likewise, we found no change in Smad3 phosphorylation when endoglin was transiently expressed (not shown). In addition, the stimulation of (BRE)2-Luc reporter-transfected cells with 0.5 ng/ml TGF-β1 only resulted in a marginal (~2-fold) increase in luciferase activity that was not significantly altered when endoglin was present (Fig. 7A). In contrast, endoglin drastically suppressed the (CAGA)12-Luc reporter activity, irrespective of whether a low (0.05 ng/ml) or high (0.5 ng/ml) concentration of TGF-β1 was used in stimulation (Fig. 7B). On the other hand, BMP-7 caused a strong induction of the (BRE)2-Luc reporter that was enhanced by endoglin (Fig. 7C). In addition, the overexpression of Smad3 in (BRE)2-Luc reporter assay mimicked the effect imposed by coinubation of TGF-β1 and BMP-7 (not shown).

**DISCUSSION**

To delineate the interdependence of “profibrogenic” TGF-β1 and “antifibrogenic” BMP-7 signaling and to clarify the function of endoglin in this two interconnected pathways, we used an established model system reported to be highly responsive to TGF-β1 that lacks endoglin (Figs. 1 and 2) and is responsive to BMP-7 (Figs. 1 and 3). The phenotypic alterations and the effects of TGF-β1 on target gene expression are well described and attributed to the classical signaling pathway encompassing the type I receptor ALK-5, Smad2, and Smad3. In contrast, BMP signaling that has been thoroughly analyzed in the mouse myoblast cell line C2C12 (47–49) was not studied yet in L6E9 cells.

Here we demonstrated that L6E9 cells express activin and BMP type I and II receptors (Fig. 3A) and show responsiveness toward BMP-7 (Figs. 1B and Fig. 3, B and C). Moreover, the BMP-Smads and the BMP-Smad reporter (BRE)2-Luc were dose-dependently activated by BMP-7. In addition, we showed that BMP-7 induces Id1 expression in these cells (Fig. 5, D and 6C, lanes 6 and 7). In line with the reporter experiments (Fig. 4) and the short term stimulation (Fig. 5), BMP-7 reduced the effects of TGF-β1 on collagen I expression confirming the inhibitory effect of BMP-7 on ALK-5/Smad3 (Fig. 6C, lanes 8–10). Thus, we conclude that BMP-7 specifically interferes with TGF-β1-mediated ALK-5/Smad3 signaling.
The respective protein expression kinetics in response to BMP-7 resembles that of the mRNA expression observed in C2C12 cells stimulated with BMP-2 and is characterized by a profound increase that becomes already apparent after a 1-h stimulation period and fades out after prolonged stimulation times (10). On the other hand, collagen I expression is not influenced by BMP-7 (Fig. 5). Although the Id1 that mediates inhibition of myogenesis has been described as a typical BMP target gene (50), we found that TGF-β1 dose-dependently induced Id1 expression. However, there is a conspicuous difference in the protein expression kinetics. In contrast to BMP-7, TGF-β1 led to a transient increase of Id1 peaking at 2 h and returning to basal level at 4 h. In general, the finding that TGF-β1 induced Id1 expression is not surprising, and similar results were observed in endothelial cells and hepatic stellate cells (28, 51). The underlying pathway is most likely based on the expression of an alternative type I receptor ALK-1 activating Smad1 and Smad5 (52). In line, we here demonstrated that L6E9 cells express ALK-1. However, TGF-β1 was unable to induce Id1 expression in C2C12 cells implying that these cells do not activate BMP-Smads in response to TGF-β1 (10). In terms of myogenic differentiation, it is most interesting that Id1 expression is increased by TGF-β1. So far the mechanisms by which TGF-β1 inhibits terminal myogenic differentiation are not solved entirely. It has been shown that the muscle regulatory factors MyoD, Myf5, Mrf4, and myogenin are key factors involved in this regulation (53). The degradation of myogenin as well as the impairment of myogenin to bind to DNA are regulated by Id1 (54), and therefore, one potential mechanism by which TGF-β1 regulates myogenic differentiation is the transient induction of Id1 expression.

Unexpectedly, the (BRE)2-Luc reporter was not activated by TGF-β1 in our experiments. However, in similar experiments performed in endothelial or hepatic stellate cells, the activation of the ALK-1 signaling pathway by TGF-β1 was demonstrated (28, 51). Noteworthy, the artificial (BRE)2-Luc reporter only directs binding of Smad5, whereas the endogenous Id1 gene is regulated by Smad1 and Smad5 (42, 50). In our experiments we could not differentiate between activation of Smad1 and/or Smad5, because the antibodies used in this study recognize both BMP-Smads. Previous reports have undoubtedly demonstrated that ALK-1 activates both Smads (13). Therefore, it is unlikely that differential Smad activation is the basis underlying the failure of TGF-β1 inactivating the respective reporter. Currently, we are evalu-
One feature in ALK-1 signaling is the strict dependence on ALK-5 activity that was previously observed in ALK-5-deficient endothelial cells (25). We confirmed this characteristic because TGF-β-dependent Smad3 and Smad1/5 phosphorylation was completely blocked in the presence of the ALK-5 inhibitor SB431542 (Fig. 2C) that does not interfere with ALK-1 group receptors (46). In addition, parallel application of TGF-β1 and this ALK-5 inhibitor led to a higher fusion rate (data not shown) than observed for TGF-β1 alone that blocks morphological differentiation (Fig. 6A). This finding was further confirmed by analysis of target genes. TGF-β1 (at concentrations higher than 0.1 ng/ml) induced collagen I and Id1 expression and inhibited myogenin gene activity (Fig. 6, B and C). In the presence of SB431542, collagen I and Id1 up-regulation was suppressed, whereas the expression of myogenin was restored (data not shown). BMP-7 signaling on the other hand was found to be completely independent from the SB431542 compound (data not shown). In summary, these observations corroborate that ALK-1 signaling is ALK-5-dependent, and TGF-β1-mediated collagen I and Id1 up-regulation are triggered by Smad activation that was previously shown by application of Smad7 (55). Furthermore, the outlined experiments reveal that BMP-7 signaling is mediated by a classical activin or BMP type I receptor that is insensitive toward SB431542. We are currently not able to exclude any functional involvement of ALK-1 in BMP-7 signaling as recently reported for BMP-9 and BMP-10 (56). To address this issue in more detail, studies with short interfering RNA targeting individual components of the ALK-1 pathway will be performed.

The fact that BMP-7 inhibits the (CAGA)12-Luc reporter (Fig. 4A) implies that BMP-7 interferes with the activation of the profibrogenic Smad3. In a recent report it has been shown that Smad5 is primarily activated by BMP-7 in mesangial cells. This in turn leads to a reduced nuclear level of Smad3 and concom-

![FIGURE 6. TGF-β1 and BMP-7 both inhibit L6/myogenic differentiation but differentially affect target gene expression and terminal differentiation. A, L6 cells were cultured for 24 h in GM containing 2% FCS. Thereafter, the cells were further cultured for 3 days in GM, DM, or in DM containing TGF-β1 (1 ng/ml), BMP-7 (50 ng/ml), or a combination of both cytokines (TGF-β1/BMP-7). High frequent formation of myotubes (black arrows) was only observed when cells were cultured in DM without addition of cytokines. TGF-β1-treated cells acquired a fibroblast-like phenotype, whereas BMP-7-treated cells evolved an overall spheric appearance. B, L6 cells were cultured for 24 h in GM and further 3 days in GM (lane 1), DM (lane 2), or in DM in the presence of 150 ng/ml insulin-like growth factor-1 (lane 3), 0.1 ng/ml TGF-β1 (lane 4), 1 ng/ml TGF-β1(lane 5), 50 ng/ml BMP-7 (lane 6), 100 ng/ml BMP-7 (lane 7), or a combination of both cytokines (1 ng/ml TGF-β1/50 ng/ml BMP-7 (lane 8) or 1 ng/ml TGF-β1/100 ng/ml BMP-7 (lane 9)). C, L6 cells were cultured for 24 h in GM and for further 3 days in GM (lane 1), DM (lane 2), or in DM in the presence of 0.1 ng/ml TGF-β1 (lane 3), 1 ng/ml TGF-β1 (lane 4), 5 ng/ml TGF-β1 (lane 5), 50 ng/ml BMP-7 (lane 6), 100 ng/ml BMP-7 (lane 7), or a combination of both cytokines (0.1 ng/ml TGF-β1/50 ng/ml BMP-7 (lane 8); 1 ng/ml TGF-β1/50 ng/ml BMP-7 (lane 9); 5 ng/ml TGF-β1/50 ng/ml BMP-7 (lane 10)). Extracts were tested for expression of collagen I, Id1, and myogenin by Western blot. As a control for equal gel loading, we probed the blots with antibodies directed against α-tubulin or β-actin. For densitometric analysis, the relative intensities for Id1 (B) and collagen I (C) were normalized to the respective loading controls and set as 100%. One representative experiment of three independent experiments is depicted.
Endoglin in BMP-7 Signaling

FIGURE 7. Endoglin abrogates TGF-β1-induced ALK-5/Smad3 responses and promotes BMP-7/Smad1/Smad5 signaling. A, L6E9 cells were transfected with combinations of reporter plasmid (BRE)2-Luc and pcDNA (white bars) or pcDNA-endoglin (black bars). The cells were then stimulated with indicated concentrations of TGF-β1, and the luciferase activities were measured. B, L6E9 cells were transfected with combinations of reporter plasmid (CAGA)12-MLP-Luc and pcDNA (white bars) or pcDNA-endoglin (black bars). The cells were then stimulated with indicated concentrations of TGF-β1, and the luciferase activities were measured. C, L6E9 cells were transfected with combinations of plasmid (BRE)2-Luc and pcDNA (white bars) or pcDNA-endoglin (black bars). The cells were then stimulated with indicated concentrations of BMP-7, and the luciferase activities were measured. * indicates significant p values, and ** indicates highly significant p values (in B and C).

Itant up-regulation of Smad6. As a consequence, the transient overexpression of Smad5 or Smad6 suppresses ALK-5/Smad3-mediated responses (57). In addition to the reported change in translocation (57), we also observed a reduction in Smad phosphorylation (Fig. 4, B and D). In addition to the reduced reporter activities, we demonstrated that the co-stimulation of L6E9 cells with TGF-β1 and BMP-7 decreased collagen I expression (Figs. 5F and 6C). Moreover, the morphology of the cells stimulated by both cytokines resembled more the phenotype of BMP-7-stimulated cells (Fig. 6A) suggesting that BMP-7 inhibited myogenic differentiation and switched on the osteogenic program in a manner independent from TGF-β1. If the inhibition of BMP-7 on collagen I expression is a direct or indirect consequence is presently unclear. Recently, a similar observation showing that BMP-7 antagonizes TGF-β1-induced collagen I luciferase reporter activity and up-regulates the expression of Id2 in pulmonary myofibroblasts has been reported (58). In line, overexpression of Id2 decreases the TGF-β1-induced collagen I reporter activity and the amount of secreted collagen I (58).

On the other hand, TGF-β1 inhibited the activity of the (BRE)2-Luc reporter. This inhibition was mimicked when Smad3 was overexpressed and even strengthened when TGF-β1 was additionally applied (data not shown).

With respect to the functional role of endoglin, much of the reported data have been generated in endothelial cells, because it was recognized that the human disease HHT-1 (hereditary hemorrhagic telangiectasia-1) is the consequence of defects in the type III receptor endoglin (59). Because HHT-2 that is based upon defects in ALK-1 had a very similar phenotype, it was postulated that these two receptors are involved in the same signaling cascade (59, 60). This hypothesis was substantiated by corresponding mouse models, which are deficient for endoglin or ALK-1 (26, 61, 62). Although endoglin inhibited TGF-β-mediated responses in several systems via ALK-5/Smad3, TGF-β was also found to induce signaling via the ALK-1/Smad1/Smad5 pathway (35). The inhibitory role of endoglin on the ALK-5 pathway was confirmed in our analysis (Fig. 7B). However, we were unable to demonstrate the promoting effect of endoglin on the ALK-1 pathway in L6E9 cells (Fig. 7A).

The most spectacular finding concerning the function of endoglin was the promoting effect on BMP-7 downstream signaling (Fig. 7C). So far it was demonstrated that endoglin is able to bind BMP-2 and BMP-7 (34), but except for the data presented here, there was no functional link of endoglin and BMP-7 signaling. There is only one recent paper describing the enhancement of BMP-9 signaling by endoglin (56). In the light of fibrogenic processes, the finding that endoglin is a modifier of BMP-7 signaling is very interesting. In experimental models of kidney fibrosis and liver fibrosis, the administration of BMP-7 resulted in resolution of fibrosis (63, 64). BMP-7 targets mesangial cells in kidney and hepatic stellate cells in liver that are key mediator cells during fibrogenesis. Moreover, it has been shown in both cell types that BMP-7 directly interferes with profibrogenic TGF-β1 signaling (57, 64). Because endoglin is also expressed by mesangial and hepatic stellate cells (43, 65), it is tempting to speculate that endoglin is a critical modifier of fibrogenic responses.

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