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of Xvent-1 and Xvent-2 transcription and dorsalization of mesoderm (5); and 2) in transgenic Xenopus embryos expressing the green fluorescence protein reporter gene under the regulation of the Xvent-2 promoter, mutation of the OAS in the Xvent-2 promoter leads to misexpression of green fluorescence protein (9). The human and mouse orthologs of OAZ are expressed in embryonic tissues responsive to BMP, suggesting that OAZ might be involved in BMP responses during early embryogenesis in mammals. However, a mammalian gene regulated by BMP through OAZ has not been identified.

Recently, a protein structurally similar to OAZ, named Early Hematopoietic Zinc Finger protein (EHZF) (also known as Ev3 or ZNF521), has been identified (10–12). Both OAZ and EHZF contain 30 Kru¨ppel-topoietic Zinc Finger protein (EHZF) (also known as Evi3 or ZNF521), but EHZF is expressed highly in hematopoietic cells in early embryogenesis in mammals. However, a mammalian gene regulated by BMP through EHZF has not been identified.

The mouse Smad6 gene was characterized as a target gene of the BMP signaling pathway (13, 14). With Smad7, Smad6 belongs to a distinct subclass of Smads (Inhibitory Smads or I-Smads) that antagonize BMP signaling pathway (13, 14). With Smad7, Smad6 was detected with rabbit polyclonal antisera raised against synthetic peptides (Upstate Biotechnology and Zymed Laboratories Inc.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with mouse anti-GAPDH monoclonal antibody (clone 1D4, COVANCE).

Plasmid Constructs—A reporter containing three copies of the BRE of the mouse Smad6 promoter was generated by inserting the oligonucleotides containing the wild type or mutated BRE sequence into the pE1b-Luc vector (5). The wild type and mutant BRE sequences are: 5’-CGGGCCGCGCCGCTCCAGGGCAGGCGGCTTTA-3’ (WT), 5’-GGTCCGCTCCGATCGGGCAGGGCGGCGCCCTT- AA-3’ (MUT1), and 5’-CGGGCCGCGCCGCAATCCAGGGCAGGAGCGCCCTTTA-3’ (MUT2).

Reverse Transcription (RT)-PCR Assay—Cells were treated with 200 ng/ml BMP4 (R&D Systems) in 0.2% FCS and DMEM. Total RNA was extracted by TRIzol (Invitrogen), and 5 μg of total RNA was subjected to RT using a first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. One-tenth of the reaction mixture was used as template for each PCR. The products of semiquantitative PCR were separated on a SDS-PAGE. The quantitative analysis of the change in expression levels was calculated by real time PCR (Bio-Rad) following the manufacturer’s protocol. Primers used were: 5’-CCACTTGGGAGAAGTCTCTTCTCTTCCTCG-3’ and 5’-ATGTTAGGCGCATCCTGTTTCG-CG-3’ for mouse Smad6, 5’-GTCGACGAGGAGGCTCTTTA-3’ and 5’-AGCTCTCTTGTCTTCCTGGAG-3’ for mouse Id3, and 5’-CACC CGACGCTGTCGCGAAGGC-3’ and 5’-CTCCACAGAGGGCGCGTGGAGTCGGG-3’ for mouse OAZ. Human GAPDH primers and mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT) primers were designed before (5, 22).

RNA Interference—Synthetic small interference RNA (siRNA) was purified and annealed according to the manufacturer’s instructions (Dharmacon). The siRNA sequence targeting OAZ corresponds to nucleotides 17–37 after the start codon. siRNA with a nontargeting sequence (Dharmacon) was used as a negative control. The siRNAs were transfected by OligofectAMINE (Invitrogen) according to the manufacturer’s instructions. 48 h after transfection, cells were treated with BMP4 and harvested. Transfection efficiencies as determined by fluorescent-labeled siRNA (Dharmacon) were in the range of 80–90%. Specific silencing of OAZ was confirmed by Western blotting using anti-Smad1 (Zymed Laboratories Inc.), anti-lamin A, and anti-vimentin antibodies as described previously (23).

Luciferase Assay—After transfection, the cells were seeded onto 12-well plates and treated with 200 ng/ml BMP4 for 20 h in 0.2% FCS and DMEM. Luciferase assays were carried out essentially as described previously (5).

Immunoblot Assay—Cells were treated with 200 ng/ml BMP4 and lysed in TNE buffer (1% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl). Proteins were separated on a SDS-PAGE, transferred to polyvinylidene difluoride membranes, immunoblotted with antibodies, and visualized using an enhanced chemiluminescence detection system (Amersham Biosciences).

Constructions of Recombinant Adenoviruses—Recombinant adenoviruses were constructed as described previously (24). Briefly, FLAG-epitope tagged OAZ and Smad6 cDNAs were subcloned into the pShuttle-CMV vector. The recombinant adenoviruses generated by homologous recombination were isolated, and high titer stocks of recombinant adenoviruses were grown in 293 cells and purified. Infection of recombinant adenoviruses was performed at a multiplicity of infection of <8 × 10^3 plaque-forming units/cell. As a control, adenovirus driving β-galactosidase (LacZ) expression was used (Vector Biolabs, Inc.)
Chromatin Immunoprecipitation Assay—The chromatin immunoprecipitation assay was performed as described previously (25, 26). Chromatin was sheared to an average length of 400 bp. We used a set of PCR primers for the detection of the mSmad6 BRE (−901/−832) and a control set corresponding to the 5′-upstream region of mSmad6 (−2031/−1853).

Alkaline Phosphatase Assay—Histochemical analysis of alkaline phosphatase (ALP) activity was performed in 12-well plates as described previously (27, 28). After histochemical ALP analysis by phase contrast microscopy, the ALP activity was quantified by measuring the absorbance with the image documentation system Imagestore 7500 (Packard).

Apopotosis Assay—Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed following manufacturer’s instructions. Briefly, PASMCs were cultured in serum-free DMEM for 48 h, followed by stimulation with 200 ng/ml BMP4 for 48 h. Cells were then subjected to TUNEL assay using a Cell Death Detection kit (Roche Applied Science).

Statistical Analysis—Statistical significance was determined by analysis of variance and Fisher’s least significant difference, or by Student’s t test analysis (p < 0.05) as appropriate. All data are plotted as the mean ± S.E.

RESULTS

A Putative OAZ Binding Sequence Is Conserved in the Mouse and Human Smad6 Promoters—Smad6 is a known direct BMP target gene in vertebrates. The BMP receptor-specific Smads, Smad1 and Smad5, as a complex with Smad4 (13, 14), induce Smad6 expression. To identify a conserved region of the Smad6 promoter potentially involved in BMP-mediated transcriptional regulation, we aligned the mouse Smad6 promoter with the promoter of its human ortholog (Fig. 1A). A 57-bp region (−891/−834) of the mouse Smad6 promoter is highly conserved in the human Smad6 promoter (−1062/−1003) (Fig. 1A, sequence highlighted). A 28-bp sequence (−874/−847) within this region (Fig. 1A, underlined) has been identified previously as a region critical for BMP-mediated activation of the mouse Smad6 promoter and has been named BMP response element (13). We found two head-to-head repeats of a putative OBS, which was identified previously in the Xenopus BMP target genes Vent-2 and Vent-1B, in both the mouse and human Smad6 promoter (Fig. 1A, shown with arrows, and Fig. 1B, black boxes) (5). This observation suggests that OAZ might be involved in the transcriptional activation of Smad6 by the BMP signaling pathway. Furthermore, two sequences resembling the BREs found in the Xvent genes, comprising an OBS-like sequence adjacent to an OBS, were recently found in the Xenopus Id3 promoter by genome-wide in silico sequence analysis (Fig. 1B) (9, 29). Id3 is transcriptionally regulated by the BMP signaling pathway both in Xenopus embryos and P19 cells (29). Therefore, it is possible that Id3 might be regulated by BMP through the BRE in an OAZ-dependent manner.

The Putative OAZ Binding Sequence Is Essential for Activation of Smad6 by BMP4—To test the functional significance of a putative OBS in the Smad6 promoter, we transfected a luciferase reporter construct containing three copies of the wild type Smad6 BRE sequence (−874/−847) from the mSmad6 promoter together with increasing amounts of an OAZ expression plasmid into the mouse embryonic carcinoma cell line P19 (Fig. 2A). P19 cells express all known BMP signaling molecules including OAZ (5). Overexpression of OAZ weakly augmented the basal activity of the reporter, but the induction by BMP4 stimulation was strongly increased by OAZ in a dose-dependent manner (Fig. 2A, WT). In agreement with previous observations (13), the reporter completely lost its responsiveness to BMP4 when both the upstream OBS and the 5′-GC-rich sequence of the BRE were mutated (Fig. 2A, MUT1) (13). Overexpression of OAZ did not alter its activity (Fig. 2A, MUT1). Similar results were obtained when mutations were introduced only in the upstream OBS (Fig. 2A, MUT2). Mutations at the 3′-end of the BRE did not alter its response to BMP4 and exogenous OAZ (Fig. 2A, MUT3) (13). These results demonstrate that the upstream OBS in the SBE sequence is necessary for the BMP-OAZ-mediated activation of Smad6. We did not investigate the potential function of the downstream, inverted OBS; however, our results indicate that it is not sufficient to mediate OAZ or BMP activation of the reporter construct (Fig. 2A, MUT2).

Recruitment of OAZ to OBS in Response to BMP4—Recruitment of OAZ to the BRE of the Smad6 promoter was confirmed in vivo by chromatin immunoprecipitation assay. Soluble chromatin was prepared after formaldehyde treatment of cells treated with BMP4 for 2 h. Antibodies against Smad1, Smad4, and OAZ were used to immunoprecipitate Smad1-, Smad4-, or OAZ-bound genomic DNA fragments. These genomic regions were analyzed by PCR using specific pairs of primers spanning the BRE of Smad6 (Fig. 2B, BRE). The negative control was provided by PCR primers corresponding to a 5′-upstream region of the BRE (−2031/−1853) which is not involved in the BMP-dependent regulation of mSmad6 and has no sequence homology with human Smad6 (Fig. 2B, control). No PCR signal was detected in BRE or control primers samples in the absence of BMP stimulation. However, in BMP4-treated cells, Smad1, Smad4, and OAZ were recruited to the BRE, but not to the 5′-control region (Fig. 2B). DNA fragments immunoprecipitated by nonspecific IgGs and amplified with the BRE primers yielded no signal (Fig. 2B), suggesting that recruitment of Smad1, Smad4, and OAZ to the BRE is specific. These results demonstrate that activation of Smad6 by BMP4 correlates with the recruitment of Smads and OAZ to the BRE. Thus, Smad6 is the first mammalian gene regulated directly by OAZ.

OAZ Is Essential for the Activation of the Smad6 Gene in P19 Cells—To test whether OAZ is necessary for Smad6 induction by BMP4 in vivo, we decreased the expression of endogenous OAZ in P19 cells by RNA interference. A synthetic double-stranded siRNA complementary to the OAZ mRNA specifically down-regulated the expression of OAZ, but not of the control proteins Smad1, lamin A, and vimentin (Fig. 2C, left panel). We then measured by RT-PCR the mRNA level of Smad6 before and after BMP stimulation (Fig. 2C, right panel). In P19 cells treated with a control nontargeting siRNA (Fig. 2C, right panel, control), OAZ expression was unchanged, and Smad6 mRNA was strongly induced by BMP4 treatment. In OAZ siRNA-treated cells, OAZ expression was blocked, and Smad6 induction by BMP4 was abolished (Fig. 2C, right panel, OAZ siRNA). This result suggests that OAZ is essential for the activation of Smad6 by BMP4.

Extended Duration of BMP Signal in P19 Cells by Down-regulation of OAZ—To assess the effect of OAZ-mediated regulation of Smad6 on the BMP signaling pathway, P19 cells were transiently transfected with control siRNA or OAZ-specific siRNA to down-regulate endogenous expression of OAZ, stimulated with BMP4, and subjected to anti-phospho-Smad1/5/8 Western blot. Cells treated with control siRNA showed a robust increase of phosphorylation of Smad1/5/8 between 30 min and 2 h after BMP treatment. However, the level of phospho-Smad1/5/8 was dramatically reduced after 4 h of treatment (Fig. 2D). The same membrane blotted with anti-Smad1 polyclonal antibodies showed that the total amount of Smad1 was not changed by BMP4 treatment. As observed previously, the Smad6 protein level increased and reached its

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3 M. Ku and A. Hata, unpublished observation.
FIGURE 1. Identification of putative OAZ binding sites in the Smad6 promoter. A, mouse Smad6 and the human ortholog of Smad6 share two highly conserved regions (shown by gray bars) ~1 kb upstream of transcription initiation site (nucleotide sequence highlighted) and just upstream of the transcription initiation site. The underlined sequence was identified previously as a region responsible for BMP-mediated activation of the Smad6 promoter. Two inverted repeated putative OBS are shown with arrows. B, alignment of the mouse Smad6 promoter with the opposite strand of the Xvent-2, Xvent-1B, and XId3 promoter. Putative OBS are shown by black boxes. Note that mSmad6 does not contain a canonical SBE, indicated by a gray box, unlike Xvent-2, Xvent-1B, and XId3. The XId3 promoter contains two sets of OBS and SBE in the distal region (−2361/−2316) (indicated as XId3 (D)) and the proximal region (−718/−673) (indicated as XId3 (P)) (29).
FIGURE 2. OAZ is essential for the transcriptional activation of Smad6 by BMP4. A, overexpression of OAZ increases the BMP-dependent activation of the Smad6 promoter. P19 cells were transfected with the Smad6 BRE-reporter construct (WT) or BRE with three different mutations (MUT1–MUT3) with increasing amounts of OAZ expression vector (0, 500, and 750 ng/well). Cells were then treated with or without 200 ng/ml BMP4 for 20 h, and luciferase activity was measured. OBS is indicated with black boxes.

B, P19 cells, with or without BMP4 stimulation, were treated with formaldehyde to cross-link the DNA-protein complex. Genomic DNA was extracted and fragmented by sonication. The genome fragments bound by OAZ, Smad1, or Smad4 were isolated by anti-OAZ, anti-Smad1, or anti-Smad4 antibodies. As a negative control of immunoprecipitation, nonspecific IgGs were used. Precipitated genomic fragments were subjected to PCR using primers to mSmad6 (–901/–832) (BRE). As a negative control, primers corresponding to mSmad6 upstream of the BRE (–2031/–1853) (control) were used. The total genomic DNA (input) was subjected to PCR as a positive control of both PCR primers. C, down-regulation of OAZ abolishes induction of Smad6 mRNA by BMP4. P19 cells, transfected with scrambled siRNA (control) or OAZ siRNA-transfected, were lysed and subjected to immunoblot analysis using antibodies against OAZ. Immunoblot analyses using antibodies against Smad1, lamin A, and vimentin are shown as controls (Immunoblot). Total RNAs from these cells were subjected to RT-PCR analysis using primers to Smad6, OAZ, and HPRT (as a loading control) (RT-PCR). D, duration of the BMP signal is OAZ-dependent. P19 cells were transfected with scrambled siRNA (Control) or OAZ siRNA. Cells were then treated with BMP4 for various lengths of time, as indicated. Total cell lysates were prepared from these cells, run by SDS-PAGE, and subjected to immunoblot analysis using anti-phospho-Smad1, anti-Smad6, and anti-Smad1 antibodies.
maximum level 4 h after BMP4 stimulation (Fig. 2D). This result suggests that the attenuation of phosphorylation of Smad1, 5, or 8 might be caused, at least in part, by the concurrent accumulation of the BMP-signal antagonist Smad6. In cells transfected with OAZ siRNA, induction of phosphorylation of Smad1/5/8 was observed as early as 0.5 h after BMP stimulation, as seen in control cells. However, the intensity of phosphorylation at the early time points was reduced significantly compared with control siRNA-transfected cells (Fig. 2D, 0.5 and 2 h, aPhospho-Smad1). Interestingly, unlike control siRNA-treated cells, in OAZ-depleted cells phosphorylation of Smad1/5/8 was detectable for up to 6 h.
16 h after stimulation (Fig. 2D, αPhospho-Smad1). The level of phosphorylated Smad1/5/8 proteins in OAZ siRNA-treated cells was low throughout the time course compared with that seen at 0.5 or 2 h after BMP stimulation in control siRNA-treated cells (Fig. 2D). The Smad6 protein level did not change upon BMP4 treatment in OAZ-depleted cells (Fig. 2D). This is consistent with the observation that there is no robust reduction of phosphorylation of Smad1 in OAZ-depleted cells (Fig. 2D). These results demonstrate that OAZ-mediated transcriptional activation of Smad6 upon BMP4 stimulation leads to accumulation of Smad6, which in turn might play a role in the attenuation of the BMP4 signaling pathway in P19 cells.

Overexpression of OAZ Elevates Smad6 Expression in C2C12 Cells—OAZ is ubiquitously expressed in early vertebrate embryos, but OAZ mRNA is detected only in brain, heart, and lung tissues in adults (5). It has been shown previously that cell lines of embryonic origin, such as P19 and C3H10T1/2, express OAZ, respond to BMP stimulation, and differentiate into various cell types (5). We compared the expression levels of OAZ mRNA in P19 and mouse myoblast C2C12 cells by semi-quantitative RT-PCR analysis. In agreement with our previous observation (5), P19 cells express a higher level of OAZ (16-fold) than C2C12 cells compared with endogenous HPRT levels (Fig. 3A). This is consistent with the observation that the OAZ-dependent reporter construct Xvent-2 BRE-Luciferase (Vent-2-Luc) does not respond to BMP in C2C12 cells as much as in P19 cells (5).

To examine the effect of exogenously expressed OAZ on the Smad6 promoter, C2C12 cells were transfected with the wild type or the mutant (MUT2; see Fig. 2A) Smad6-Luc reporter constructs in the absence or presence of OAZ expression plasmid (Fig. 3B). Despite the low level of expression of OAZ in C2C12 cells, the Smad6-Luc reporter was induced by BMP4 treatment about 3-fold in C2C12 cells (Fig. 3B, left panel, WT) (13). Expression of exogenous OAZ further increased the BMP-induced reporter activity, similarly to the effect observed in P19 cells (Fig. 2A, WT). Mutation of the OBS completely abolished responsiveness to BMP4 and to exogenous OAZ, suggesting that the low level of OAZ in C2C12 cells might be sufficient to activate Smad6 BRE (Fig. 3B, left panel, MUT2). To examine this possibility, we measured the Smad6-Luc reporter activity in C2C12 cells in which the endogenous OAZ expression was down-regulated by OAZ siRNA. When the OAZ level was reduced to about 20% of the endogenous expression level, BMP4-mediated activation of the Smad6-Luc reporter was completely abolished, demonstrating that a low amount of OAZ mediates the weak activation of Smad6 BRE in C2C12 cells (Fig. 3B, right panel).

To study whether the low level of OAZ is a limiting factor in the inducibility of endogenous Smad6 by BMP in C2C12 cells, the mRNA level of Smad6 was examined in C2C12 cells by RT-PCR analysis in the absence or presence of exogenously expressed OAZ (Fig. 3D). C2C12 cells were infected with recombinant adenovirus expressing either
FLAG epitope-tagged OAZ or β-galactosidase (control) and then stimulated with BMP4 for various periods of time. Expression of adenovirus-derived protein was examined by immunoblot analysis with anti-FLAG antibody and anti-GAPDH antibodies as a loading control (Fig. 3C). Smad6 mRNA levels were quantified by real-time PCR (Fig. 3D). 24 h after infection, C2C12 cells infected with adenovirus carrying OAZ expressed a high level of OAZ protein (Fig. 3C). Smad6 mRNA was weakly induced within 2 h after BMP4 stimulation in C2C12 cells infected with control virus (Fig. 3D, open bars). This is consistent with the result obtained with the Smad6 (BRE)-Luc reporter construct and supports that a low level of OAZ in C2C12 cells is sufficient for the weak activation of the Smad6 promoter by BMP (see Fig. 3B). When cells were infected with OAZ virus, the level of Smad6 mRNA after 2 h of BMP4 stimulation was 2.5-fold higher than in control virus-infected cells (Fig. 3D, 2 h). These data demonstrate that exogenous OAZ leads to increased expression of Smad6 in C2C12 cells. Because the Xld3 promoter contains two sequences similar to the BRE of the Xvent-2 promoter (see Fig. 1B), the expression of endogenous Id3 in the presence or absence of exogenous OAZ was examined in C2C12 cells to test whether BMP/OAZ can regulate the Id3 gene. Id3 expression was augmented 5.7-fold after a 2-h stimulation with BMP4 in control virus-infected C2C12 cells (Fig. 3E, open bars). When exogenous OAZ was expressed, Id3 expression was induced further (11.6-fold) within 0.5 h after BMP4 stimulation, suggesting that Id3 is likely regulated by OAZ (Fig. 3E, solid bars).

Overexpression of OAZ Reduces Osteoblastic Differentiation of C2C12 Cells—Smad6 is an antagonist of BMP signaling. When overexpressed, Smad6 blocks the BMP-Smad signaling pathway (19, 21, 30). Therefore, we tested whether expression of exogenous OAZ in C2C12 cells would mediate a reduction of the BMP response via induction of Smad6. As readout of an active BMP pathway, we employed the phosphorylation status of BMP-specific Smads using an anti-phospho-Smad1/5/8 antibody (Fig. 3F). A robust induction of BMP receptor kinase-mediated phosphorylation of Smad1/5/8 was observed within 30 min after BMP4 stimulation in cells infected with control (β-galactosidase) virus (Fig. 3F) and uninfected cells (data not shown). After 3 h, the amount of phospho-Smad1/5/8 gradually decreased (Fig. 3F). However, unlike the P19 cells shown in Fig. 2D, the levels of phospho-Smad1/5/8 after 7 and 28 h of BMP treatment were significantly higher than unstimulated cells (0 h), suggesting that the duration of the BMP signal might be longer in C2C12 cells (Fig. 3F). Furthermore, there was little accumulation of Smad6 after BMP4 treatment in C2C12 cells infected with control virus (Fig. 3F). A longer duration of BMP signaling and lack of induction of Smad6 were also observed in P19 cells transfected with OAZ siRNA (Fig. 2D, OAZ siRNA) and are consistent with the fact that C2C12 cells express a low level of OAZ (see Fig. 3A). In contrast, C2C12 cells expressing exogenous OAZ demonstrated a decreased level of phospho-Smad1/5/8 at early time points (0.5 and 1.5 h) compared with control C2C12 cells. Consistent with the reduced phosphorylation of Smad1/5/8, an increase in the expression of Smad6 was observed 3–28 h after BMP4 stimulation in OAZ virus-infected cells (Fig. 3F). These results indicate that exogenous OAZ mediates increased expression of Smad6 and down-regulates the intensity of the BMP signal in C2C12 cells. Unlike P19 cells, complete attenuation of the BMP signal was not observed up to 28 h after BMP4 stimulation in C2C12 cells expressing exogenous OAZ (Fig. 3F). This might be because of a lack of cofactors or the inability of Smad6 protein to accumulate to a level sufficient to inhibit completely the BMP signal in C2C12 cells expressing exogenous OAZ.

Next, we examined whether the decrease of intensity of the BMP signal as a result of overexpression of OAZ has any effect on BMP-mediated cellular events, such as differentiation. C2C12 cells can differentiate into an osteoblast-like, ALP-positive cell type in response to stimulation of the BMP-Smad signaling pathway (28). C2C12 cells infected with adenoviruses expressing OAZ, Smad6, or β-galactosidase (control) were treated with 200 ng/ml BMP4 for 3 days and subsequently subjected to an assay to detect ALP activity. BMP4 treatment of both uninfected C2C12 cells (not shown) and cells infected with control virus strongly induced ALP activity, indicating differentiation of these cells into osteoblasts (Fig. 3G). However, cells infected with Smad6 virus showed marked decrease in ALP activity compared with control virus-infected cells (100% versus 49%, p < 0.00001), showing that overexpression of Smad6 prevents osteoblastic differentiation by BMP4 (Fig. 3G). Interestingly, cells infected with OAZ virus also showed decrease in ALP activity compared with control virus-infected cells (100% versus 75%, p = 0.007) (Fig. 3D). This result is consistent with the observation that OAZ virus-infected cells show a decrease in the BMP signal as shown by the phosphorylation status of Smad1/5/8 (Fig. 3F).

Exogenous OAZ Blocks BMP4-mediated Induction of Apoptosis in Pulmonary Smooth Muscle Cells—Human PASMCs express all of the signal transducers essential for BMP signaling (22), and BMP treatment of serum-starved PASMCs induces apoptosis (22). RT-PCR analysis indicated that expression of OAZ mRNA is undetectable in PASMCs (Fig. 4A). Consistently, endogenous Smad6 mRNA was not induced upon BMP4 stimulation (Fig. 4A). The OAZ-dependent reporter construct Vent-2-Luc did not respond to BMP4 treatment in PASMCs; however, exogenous OAZ (but not exogenous Smad6) rescued the BMP responsiveness of the Vent-2-Luc reporter (Fig. 4B). These results suggest that OAZ is essential for BMP4-mediated induction of Smad6 and Vent-2 in PASMCs.

Finally, the effect of exogenous OAZ or Smad6 on BMP-mediated apoptosis was examined by the TUNEL assay. After a 48-h treatment with 1 and 10 nM BMP4, 17 ± 1.8% and 85 ± 3.2% of the cells were TUNEL-positive, respectively. This result indicates that BMP4 efficiently induces cell death in PASMCs (Fig. 4C) as reported previously (22). We were able to observe condensation and/or breakage of the nuclei by 4′,6-diamidino-2-phenylindole (DAPI) staining in BMP4-treated cells (data not shown). PASMCs transiently transfected with transfection tracer alone (a vector expressing red fluorescence protein (RFP)), or either Smad6 or OAZ expression plasmids together with the transfection tracer, were subjected to the TUNEL assay. 90 ± 2.0% of PASMCs transfected with RFP vector alone (control) were positive in TUNEL staining upon 10 nM BMP4 treatment (Fig. 4D). The percentage of TUNEL-positive cells decreased to 57 ± 5.2% in cells transfected with Smad6, suggesting that overexpression of Smad6 in PASMCs reduces BMP-mediated apoptosis (Fig. 4D). Similarly to Smad6-overexpressing PASMCs, only 50.8 ± 3.7% of PASMCs overexpressing OAZ were TUNEL-positive (Fig. 4D). The difference in the percentage of TUNEL-positive cells between control cells and cells expressing OAZ or Smad6 was highly significant (p < 1 × 10^{-5} or p < 1 × 10^{-4}, respectively). Samples not treated with BMP4, whether transfected with control vector or with plasmids driving the expression of OAZ or Smad6, had few or no TUNEL-positive cells (Fig. 4D). Immunoblot analysis showed that the Smad6 protein level was not changed upon BMP4 stimulation in PASMCs infected with control (β-galactosidase) virus (Fig. 4E). This finding is consistent with the fact that PASMCs express a negligible amount of OAZ compared with P19 cells (Fig. 4A). However, when PASMCs were infected with OAZ-expressing virus, a BMP4-dependent increase of Smad6 protein was observed (Fig. 4F). These results together suggest that overexpression of OAZ in PASMCs mediates increased apoptosis.
activation of Smad6 by BMP, which leads to premature attenuation of the BMP signal and inhibition of BMP-mediated apoptosis.

**DISCUSSION**

**OAZ as a Positive Transcriptional Regulator of Smad6**—OAZ was first identified as a cofactor of BMP-activated Smads for the BMP target gene Vent-2 in *Xenopus* embryos. In the Vent-2 promoter, both the SBE and OBS sequences are strictly required for correct developmental expression (9). Because human and mouse OAZ proteins share high sequence homology with *Xenopus* OAZ (89% amino acid identity between human and *Xenopus*), and are highly expressed in embryos, we speculated that mammalian OAZ might play an important role during.

**FIGURE 4.** Overexpression of OAZ inhibits BMP4-mediated apoptosis in pulmonary smooth muscle cells. A, PASMCs and P19 cells were treated with or without 200 ng/ml BMP4 for 2 h. Total RNA was extracted from cells and subjected to RT-PCR analysis using primers to OAZ, Smad6, and GAPDH (as a loading control). B, PASMCs were transfected with the OAZ-dependent luciferase reporter construct (Vent-2-Luc, 1 μg) alone or together with RFP (control), OAZ, or Smad6 expression vectors (300 ng/well). Cells were then treated with or without 200 ng/ml BMP4, and luciferase activity was measured. C, PASMCs were serum starved for 48 h and then treated with 1 or 10 nM BMP4 for 48 h. Cells undergoing apoptosis were stained by TUNEL assay (green). All cells are stained by phallolidin (red). D, PASMCs were transfected with control vector (100 ng/well RFP) alone or together with OAZ or Smad6 expression plasmids (300 ng/well). 24 h after transfection, cells were cultured in low serum medium (DMEM 0.1% FCS) for 48 h prior to 10 nM BMP4 treatment for 48 h, followed by TUNEL staining (Roche Applied Science). The number of RFP/TUNEL-double positive cells was divided by the total number of RFP-positive cells and indicated as percent TUNEL-positive staining. The difference between the percentage of TUNEL-positive cells transfected with control (RFP) plasmid and OAZ or Smad6 expression plasmids is statistically significant (t test of the arcsin values: *, p < 1 × 10⁻⁴ and **, p < 1 × 10⁻⁵). E, Total cell lysates were prepared from PASMCs infected with adenovirus expressing β-galactosidase (control), OAZ, or Smad6, run by SDS-PAGE, and subjected to immunoblot analysis using anti-Smad6 or anti-OAZ antibodies.
mammalian embryogenesis by regulating genes in BMP-responsive tissues. A previous study by Ishida and colleagues (13) demonstrated that a 28-bp region of the *mSmad6* promoter (−874/−847) is sufficient to mediate response to the BMP signal and binds Smad1 (or Smad5) and Smad4 (13). The authors identified four overlapping copies of a GC-rich sequence (GCCGC/GnnC) in this region, which is similar to a sequence identified as the binding site of the *Drosophila* Smad homologs Mad and Medea (GCCGnCGG). Therefore, it was speculated that the Smad protein complex might be sufficient to bind directly and activate the Smad6 BRE. Our data suggest that OAZ and its binding sequence are essential for the activation of Smad6 in embryonic P19 cells. Smad6 is thus the first mammalian gene characterized as a target of OAZ. In C2C12 cells, both endogenous Smad6 and the Smad6-BRE reporter were induced by BMP4 treatment despite low expression of OAZ. The low expression of OAZ is sufficient to mediate activation of Smad6 in these cells because further reduction of OAZ expression through RNA interference abolishes BMP induction. Furthermore, forced expression of OAZ is able to potentiate Smad6 induction by BMP strongly. In PASMCs, OAZ transcripts are undetectable, and BMP is completely unable to activate an OAZ-dependent reporter or the endogenous Smad6 gene. Exogenous OAZ expression rescues both responses. Thus, the level of expression of OAZ in different cell types correlates with the ability of BMP to induce Smad6.

**Do Other Mammalian Genes Require OAZ for BMP Induction?**—Recently, *Xvent-2*, the human ortholog of *Xenopus Vent-2*, has been identified. The *hXVENT2* promoter (−227/−196) contains a sequence similar to the BRE of *Xvent-2* (−173/−153), including the OAZ and Smad binding sequences (OBS and SBE) (9, 29). It is thus possible that *hXVENX*2 be regulated through OAZ and Smad proteins upon BMP stimulation in human cells. We were unable to identify a murine ortholog of *Vent-2*/XVENT2 in the current mouse genome data base. Therefore, the possible role of OAZ in the regulation of a murine homolog of *Vent-2*/XVENT2 has not been tested in P19 cells.

An *in silico* sequence comparison among the promoter sequences of *Xenopus*, human, and mouse *ld3* genes revealed two highly conserved regions (−2340/−2323 and −718/−670 of the amphibian sequence), both containing a combination of OBS and SBE (9, 29). In *Xenopus*, deletion of the OBS from the proximal BRE-like sequence (−718/−670) of the *ld3* promoter abolishes its BMP-responsiveness, suggesting that OBS is critical for BMP-dependent *ld3* activation (31). In this study, we show that overexpression of OAZ in cells in which OAZ is expressed at a low level strongly induces *ld3* expression in a BMP-dependent manner. In *Xenopus*, *ld3* expression begins at blastula stage around the same stage when *Xvent-2* is first expressed (32). *ld3* is a member of the Id (inhibitor of differentiation or inhibitor of DNA binding) family of proteins, which is composed of Id1 through Id4. Id proteins physically interact with ubiquitous basic helix-loop-helix (bHLH) proteins and function as their dominant-negative antagonists. Because many bHLH transcription factors induce differentiation in various tissues and cells, Id proteins generally act as inhibitors of differentiation. BMPs are among the strongest inducers of Id1, Id2, and Id3 synthesis (33, 34).

During early embryogenesis in *Xenopus*, BMP antagonists induce formation of neural tissues, suggesting that BMPs inhibit neurogenesis in vivo. Also in mammals, BMPs alter the developmental fate of fetal mouse brain cells and inhibit neurogenesis (35). The mechanism by which BMPs blocks neurogenesis is unclear, but it is known that bHLH transcription factors such as Mash1, neurogenin, and NeuroD play important roles in neurogenesis. BMP-2 induces expression of Id1 and Id3 in neuroepithelial cells, and both Id1 and Id3 repress the action of neurogenic bHLH transcription factors and inhibit neurogenesis. Therefore, it is likely that BMPs exert their antineurogenic effects, at least in part, by promoting the accumulation of Id proteins. Because OAZ is highly expressed in the neural tissue, the role of OAZ during brain development and neurogenesis deserves further *in vivo* studies. It is also important to investigate which BMP-regulated biological effects, besides neurogenesis, are propagated by Id proteins.

**Different Levels of OAZ Alter Intensity and/or Duration of the BMP Signaling Pathway**—Growth factors of the TGFβ family can act as morphogens, inducing distinct cell fates along a concentration gradient during embryogenesis. Consistently, differential TGFβ family receptor occupancy by the ligand leads to different levels of phosphorylation and nuclear localization of Smad proteins and induces different sets of target genes (36, 37). It can be speculated that the level of nuclear Smad complexes will select different transcription cofactors with differential affinities to Smads, which in turn dictate which genes are induced or suppressed in response to a given strength of signal. Because the phosphorylation status controls the time that active Smad complexes remain in the nucleus (38), the duration of Smad phosphorylation is a critical determinant of signaling specificity. For example, some pancreatic tumor cells demonstrate transient Smad phosphorylation and a short duration of nuclear residence of Smad proteins. These cells can evade TGFβ-induced growth arrest because they fail to induce p21Cip1/WAF1 upon TGFβ stimulation (39). Therefore, for cells to receive a specific signal and regulate specific sets of target genes, both the intensity and the duration of phosphorylation of R-Smads must be tightly regulated.

Receptor-mediated phosphorylation of Smads can be regulated by various mechanisms. In the BMP signaling pathway, overexpressed Smad6 interacts with the activated type I BMP receptor, inhibits phosphorylation of R-Smads, and blocks signaling. It is also known that Smad6 recruits the E3 ubiquitin ligase Smurf-1 to the activated type I BMP receptor, resulting in receptor ubiquitination, degradation, and termination of signaling (40). A similar mechanism has been proposed for the regulation of the TGFβ signaling pathway by Smad7 (38). Our data suggest that different levels of OAZ are able to modulate intensity and/or duration of phosphorylation of Smad1/5/8 by inducing Smad6 at different levels or at a different time interval after stimulation. As a result of a change in signal intensity and duration, the response of cells to the BMP signaling pathway can be altered, resulting in altered gene expression and cellular responses.

**BMP Signaling Pathway and Pulmonary Hypertension**—Because BMPs are synthesized and secreted from pulmonary vascular smooth muscle and endothelial cells, it has been speculated that the BMP signal might play a role in maintaining the homeostasis of the pulmonary vascular structure. Recently, germ line mutations in the gene encoding the BMP type II receptor (BMPRII) have been found in primary or idiopathic pulmonary hypertension (PPH or IPH) patients (41–43). Typical cell morphological changes among PPH patients include increased muscularization of small arteries and thickening of the intima (44, 45). It has been reported that BMP2-induced apoptosis is significantly inhibited in PASMCs from PPH patients (22). Disruption of BMP signaling as a consequence of a *BMPRII* mutation in PPH might contribute to the increase in the number of smooth muscle cells observed in the pulmonary arteries seen in PPH patients. It is plausible, but yet untested, that up-regulation of OAZ in the lung vasculature might contribute to the development of pulmonary hypertension via inhibition of BMP-mediated apoptosis.

Smad1-null or Smad5-null mice exhibit enlarged and dilated blood vessels (46–48), which are reminiscent of the vascular abnormalities observed in Id1-Id3 double knock-out mice (49). This coincidence may
indicate a functional link between the BMP-Smad pathway and Id proteins in endothelial cells, with the induction of Id proteins by BMPs playing a significant role in the development and homeostasis of vascular tissues. Therefore, the possibility that mutation or abnormal expression of OAZ and its target genes, such as Smad6 or Id, might contribute to the pathogenesis of PPH should be investigated in the future.

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