Bone morphogenetic protein (BMP) signals regulate the growth and differentiation of diverse lineages. The association of mutations in the BMP type II receptor (BMPRII) with idiopathic pulmonary arterial hypertension suggests an important role of this receptor in vascular remodeling. Pulmonary artery smooth muscle cells lacking BMPRII can transduce BMP signals using ActRIIA (Actinin type II receptor). We investigated whether or not BMP signaling via the two receptors leads to differential effects on vascular smooth muscle cells. BMP4, but not BMP7, inhibited platelet-derived growth factor-activated proliferation in wild-type pulmonary artery smooth muscle cells, whereas neither ligand inhibited the growth of BMPRII-deficient cells. Adenoviral gene transfer of BMPRII enabled BMP4, as well as BMP7, to inhibit proliferation in BMPRII-deficient cells. BMP-mediated growth inhibition was also reconstituted by the BMPRII short isoform, lacking the C-terminal domain present in the long form. BMP4, but not BMP7, induced the expression of osteoblast markers in wild-type cells, whereas neither ligand induced these markers in BMPRII-deficient cells. Overexpression of short or long forms of BMPRII in BMPRII-deficient cells enabled BMP4 and BMP7 to induce osteogenic differentiation. Although signaling via BMPRII or ActRIIA transiently activated SMAD1/5/8, only BMPRII signaling led to persistent SMAD1/5/8 activation and sustained increases in Id1 mRNA and protein expression. Pharmacologic blockade of BMP type I receptor function within 24 h after BMP stimulation abrogated differentiation. These data suggest that sustained BMP pathway activation, such as that mediated by BMPRII, is necessary for growth and differentiation control in vascular smooth muscle.

Bone morphogenetic proteins (BMPs)\(^2\) are members of the TGF-\(\beta\) family that play essential roles in early embryonic patterning, gastrulation, and organogenesis as well as in the remodeling of mature tissues (1). BMP signals are involved in the commitment of vascular precursor cells in vasculogenesis and regulate the growth, differentiation, and turnover of vascular cell populations (2–4). There is increasing evidence that BMP signals contribute to vascular calcific disease of the intima and tunica media (5). Further underscoring the importance of BMP signaling in vascular tissues, defects in the BMP signaling apparatus have been linked to two genetic vascular syndromes, hereditary hemorrhagic telangiectasia syndrome and idiopathic pulmonary arterial hypertension (4). In idiopathic pulmonary arterial hypertension, diverse loss-of-function mutations in the canonical BMP type II receptor (BMPRII) are associated with the majority of familial and a subset of sporadic cases (6–9). Advanced idiopathic pulmonary arterial hypertension is marked by an obstructive and proliferative vasculopathy involving endothelial and myofibroblast lineages (10). Although it has been suggested that defective BMP-mediated regulation of cell differentiation and turnover could contribute to abnormal vascular remodeling, the precise contribution of BMPRII to these functions is incompletely understood.

Signaling by BMP ligands is accomplished by at least three type II receptors, BMPRII, ActRIIa, and ActRIIB, and three type I receptors, ALK2, ALK3, and ALK6 (11–19). At least 25 different BMP ligands can facilitate the assembly of type II and type I receptor heteromers to cause phosphorylation and activation of type I receptor kinases by constitutively active type II receptor kinases. Activated type I receptors phosphorylate BMP-responsive SMAD (BR-SMAD) intracellular effectors (SMAD1, -5, and -8). Downstream effects of BR-SMAD activation are mediated in part by the transcription of the dominant negative bHLH Inhibitor of differentiation (Id) proteins, as well as by
targets of the Notch signaling pathway (20, 21). BMP signals may also activate MAPKs, such as p38 or extracellular signal-regulated kinase (22–25), via non-SMAD mechanisms. BMPRII is expressed in both short and long isoforms. The long form regulates kinase (22–25), via non-SMAD mechanisms. BMPRII may also activate MAPKs, such as p38 or extracellular signal-regulated kinase (22–25), via non-SMAD mechanisms. BMP signals may also activate MAPKs, such as p38 or extracellular signal-regulated kinase (22–25), via non-SMAD mechanisms. BMPRII may also activate MAPKs, such as p38 or extracellular signal-regulated kinase (22–25), via non-SMAD mechanisms. BMP signals may also activate MAPKs, such as p38 or extracellular signal-regulated kinase (22–25), via non-SMAD mechanisms.

**EXPERIMENTAL PROCEDURES**

Isolation of PASMC from Mice with Floxed BMPRII Alleles—Mice possessing BMPRII alleles modified to contain floxP sites flanking exons 4 and 5 (encoding the transmembrane and a portion of the kinase domain) were generated by gene targeting in embryonic stem cells (52). Pulmonary artery explants were harvested from mice homozygous for the conditional knock-out allele (BMPRII<sup>flox/flox</sup> on a hybrid C57BL/6 and Sv129 background. Intima and adventitia were dissected from media, which was cultured to yield PASMC, whose phenotype was confirmed by typical morphology and immunohistochemical staining for α-smooth muscle actin. Six separate isolates of PASMC were obtained and characterized, each derived from individual BMPRII<sup>flox/flox</sup> mice.

Adenovirus Infection—To disrupt the BMPRII gene in PASMC isolated from BMPRII<sup>flox/flox</sup> mice, cells were infected with adenoviruses expressing Cre (Ad.Cre) or GFP (Ad.GFP) as a control. Virus titers were determined by the plaque titer method (53), and PASMC were infected at a multiplicity of infection of 25 unless otherwise noted. After infection, cells were cultured for 5–7 days and passaged, and the expression of BMPRII in the resulting BMPRII<sup>del/del</sup> cells was measured by quantitative RT-PCR and immunoblotting to confirm >99% recombination (15). In order to reconstitute BMPRII expression in BMPRII-deficient cells, cells were infected with adenoviruses expressing either BMPRII long isoform (Ad.LF) or short isoform (Ad.SF) cDNA sequences (kindly provided by Dr. Akiko Hata (New England Medical Center)) (40) or Ad.GFP as a control.

siRNA Inhibition of BMP Type I and Type II Receptor Expression—Previously validated siRNA duplexes specific for BMPRII, ActRIIa, ALK2, ALK3, and ALK6 were transfected into PASMC using Oligofectamine (Invitrogen) as previously described (15).

Measurement of Gene Expression by Quantitative RT-PCR—Total RNA and cDNA were produced from cultured PASMC as previously described (15). Target gene sequences were amplified from cDNA by PCR and quantified using the ABI Prism 7000 (Applied Biosystems, Inc., Foster City, CA) and primers specific for BMPRII (exons 4 and 5), 18 S ribosomal RNA (18 S), alkaline phosphatase, osteopontin, osteoprotegerin, and receptor-associated activator of NF-κ ligand (RANKL) cDNA sequences (Table 1) using SYBR Green PCR Master Mix (Applied Biosystems, Inc.). Id1 and Id3 sequences were amplified using Taqman primer sets with Taqman PCR Master Mix.
BMPRII Regulates PASMC Growth and Osteogenic Differentiation

Disrupting BMPRII Abrogates Growth Inhibition Mediated by BMP4—PASMC from BMPRIIflox/flox mice were infected with Ad.Cre to generate BMPRdel/del (KO) cells, and BMPRIIflox/flox PASMC after infection with Ad.GFP were used as wild-type (WT) controls (15). BMPRI KO cells exhibited <1% residual wild-type BMPRII mRNA expression compared with WT cells and undetectable levels of WT BMPRII protein by Western blotting (15). WT and KO PASMC proliferated equally in response to varying concentrations of PDGF-BB, as assayed by [3H]thymidine incorporation (Fig. 1A). WT and KO cells also responded equally to other mitogens, such as bFGF (Fig. 1B) and serum (not shown). Quiescent PASMC exhibited a very low basal rate of proliferation, which was not impacted by treatment with BMP ligands alone (Fig. 1B). However, BMP4 inhibited the PDGF-induced proliferation of WT cells in a dose-dependent fashion (Fig. 1C). We have previously shown that BMPRII KO cells retain the capacity to transduce BMP signals and activate SMAD1/5/8 via the activity of ActRIIa (15). However, despite persistent BMP signaling, the antiproliferative effects of BMP4 were essentially abolished in KO cells. In WT cells, BMP7 did not inhibit PDGF-induced proliferation (Fig. 1D). BMP7, despite potently activating SMAD1/5/8 and MAPK p38 in KO cells (15), failed to inhibit the proliferation of KO cells in a dose-dependent fashion. Thus, only BMP4 inhibited mitogen-induced proliferation and did so only in the presence of BMPRII. Similar results were obtained using serum and bFGF as mitogenic stimuli (data not shown). Consistent results were obtained in replicate experiments performed using six pairs of WT and KO PASMC isolates derived from six individual mice.

The effect of BMPs on the proliferation of cultured VSMC is reported to vary with passage (41). Consistent with these observations, sequential passaging of WT PASMC led to a decrease in the ability of BMP4 to inhibit mitogen-induced proliferation
Passage-dependent effects were minimized in present experiments by using cells with low passage numbers (≤6).

Reconstitution of BMPRII in BMPRII-deficient PASMC Is Sufficient to Restore BMP-mediated Growth Inhibition—To confirm the role of BMPRII and to help rule out the possibility that BMPRII KO cells lost BMP-induced growth arrest due to extensive passaging or Cre-mediated toxicity (56), the effect of restoring BMPRII expression by gene transfer was tested. Although control virus expressing GFP did not impact BMP function, infection with adenovirus expressing short or long forms of BMPRII (results with short form, Ad.SF, shown in Fig. 1F) restored the ability of BMP4 to inhibit PDGF-mediated proliferation in a multiplicity of infection-dependent manner. Moreover, infection with higher concentrations of Ad.SF enabled BMP7 to inhibit proliferation of PDGF-stimulated KO PASMC. Short and long forms of BMPRII did not differ in their capacity to restore antiproliferative functions of BMP ligands (data not shown). Thus, overexpression of BMPRII restored antiproliferative effects of BMP4 in KO cells but also enabled growth inhibition by BMP7, a ligand that did not effect inhibition of proliferation in WT cells.

BMP signals are reported to regulate apoptosis in a variety of cell types (12, 57, 58). As a potential explanation for their antiproliferative effects, it was tested whether or not BMP ligands induce apoptosis in PASMC. Both WT and KO cells exhibited a low basal rate (4–10%) of apoptosis in response to serum withdrawal for 72 h (Supplemental Fig. 1), as measured by nuclear condensation via Hoechst 33258 staining. In WT or KO cells, neither BMP4 nor BMP7 increased the frequency of apoptotic cells. Thus, in murine PASMC, BMP-mediated growth inhibition could not be attributed to the induction of apoptosis.

Disrupting BMPRII Abrogates BMP-mediated Differentiation of
Cultured PASMC—In addition to regulating cell proliferation and apoptosis, BMPs are reported to induce differentiation in multipotent mesenchyme-derived cell populations (16, 59). The specific contribution of BMPRII-mediated signaling to cell differentiation was tested. BMP4 induced the expression of alkaline phosphatase activity, an early marker of osteogenic differentiation, in WT PASMC after 5 days of culture (Fig. 2A). Untreated WT PASMC expressed alkaline phosphatase activity at a frequency of 6 ± 8% (mean ± S.D.) of cells, whereas after BMP4 treatment, the frequency increased to 46 ± 9%, measured in three independent cell isolates. Using a colorimetric assay, BMP4 induced alkaline phosphatase activity more than 5-fold in WT cells, whereas BMP7 did not have an effect (Fig. 2B). In contrast, despite inducing BR-SMAD activation and Id1 gene expression (15), neither BMP4 nor BMP7 induced alkaline phosphatase activity in BMPRII KO cells. These findings, representative of results obtained with four pairs of WT and KO PASMC isolates derived from four individual mice, suggest that BMPRII-mediated signaling is necessary for BMP-induced alkaline phosphatase activity.

Reconstitution of BMPRII in BMPRII-deficient PASMC Is Sufficient to Restore BMP-induced Differentiation—To confirm the role of BMPRII in mediating alkaline phosphatase induction, the ability of BMPRII gene transfer to restore alkaline phosphatase induction in KO cells was tested. Infection with adenoviruses expressing short or long BMPRII isoforms (Ad.SF or Ad.LF, respectively) increased the ability of BMP4 to induce Id1 promoter activity (BRE-Luc) in WT PASMC treated with a 25 multiplicity of infection of Ad.GFP, Ad.SF, or Ad.LF. KO cells infected with Ad.SF or Ad.LF exhibited enhanced BRE-Luc activity as compared with cells infected with Ad.GFP. D, by histochemical staining for alkaline phosphatase activity, WT PASMC treated with BMP4 (10 ng/ml) showed increased alkaline phosphatase activity after 5 days, whether they were treated with control virus (Ad.GFP) or virus expressing short or long isoforms of BMPRII (Ad.SF or Ad.LF). BMPRII KO cells infected with Ad.GFP and treated with BMP4 did not express alkaline phosphatase activity, but BMP-induced alkaline phosphatase activity was restored if KO cells were infected with BMPRII-expressing adenoviruses (Ad.SF or Ad.LF). E, by histochemical staining for alkaline phosphatase activity, BMP4 but not BMP7 (both at 10 ng/ml) induced alkaline phosphatase activity in WT PASMC. With progressively higher multiplicity of infection (12.5, 25, and 50) of Ad.SF infection, BMPRII KO cells exhibited both BMP4- and BMP7-induced alkaline phosphatase activity.

FIGURE 2. BMPRII is required for BMP-induced expression of alkaline phosphatase activity PASMC. A, WT PASMC treated with BMP4 (10 ng/ml) in culture for 5 days exhibited alkaline phosphatase (Alk Phos) activity by histochemical staining at high frequency compared with untreated cells (left-hand panels). Cells were stained with SYBR green (right-hand panels). B, by colorimetric assay, WT but not KO cells treated with BMP4 (10 ng/ml) exhibit increased alkaline phosphatase activity. BMP7 failed to induce alkaline phosphatase activity in either WT or KO cells. C, BMP4 (10 ng/ml) induced Id1 promoter activity (BRE-Luc) in WT PASMC treated with a 25 multiplicity of infection of Ad.GFP, Ad.SF, or Ad.LF. KO cells infected with Ad.SF or Ad.LF exhibited enhanced BRE-Luc activity as compared with cells infected with Ad.GFP. D, by histochemical staining for alkaline phosphatase activity, WT PASMC treated with BMP4 (10 ng/ml) showed increased alkaline phosphatase activity after 5 days, whether they were treated with control virus (Ad.GFP) or virus expressing short or long isoforms of BMPRII (Ad.SF or Ad.LF). BMPRII KO cells infected with Ad.GFP and treated with BMP4 did not express alkaline phosphatase activity, but BMP-induced alkaline phosphatase activity was restored if KO cells were infected with BMPRII-expressing adenoviruses (Ad.SF or Ad.LF). E, by histochemical staining for alkaline phosphatase activity, BMP4 but not BMP7 (both at 10 ng/ml) induced alkaline phosphatase activity in WT PASMC. With progressively higher multiplicity of infection (12.5, 25, and 50) of Ad.SF infection, BMPRII KO cells exhibited both BMP4- and BMP7-induced alkaline phosphatase activity.
BMPRII is required for BMP-induced alkaline phosphatase activity.

BMP-mediated Differentiation in PASMC Is Mediated by BMPRII and ALK3—To determine the type II and type I receptors that mediate BMP-induced alkaline phosphatase expression, WT PASMC were treated with validated siRNAs specific for BMPRII, ActRIIa, ALK2, and ALK3 for 24 h prior to BMP stimulation. Since WT PASMC were previously not found to express significant levels of ALK6, siRNA specific for ALK6 was used as a transfection control. Only treatment with siRNA specific for BMPRII and ALK3 significantly inhibited BMP4-induced expression of alkaline phosphatase, consistent with their roles in mediating BMP4 signaling in WT cells (Fig. 3A). Targeting of ALK2, ALK6, and ActRIIa had minimal effect on alkaline phosphatase activity with siRNA targeting ALK2, ALK6, and ActRIIa. By colorimetric assay, alkaline phosphatase activity was induced in WT cells only after treatment with BMP4 (20 ng/ml) for 5 days. siRNA targeting BMPRII or ALK3, but not ALK2, ALK6, and ActRIIa, inhibited the BMP-induced expression of alkaline phosphatase.

BMPRII Regulates Expression of Alkaline Phosphatase, Osteoprotegerin, and RANK Ligand in PASMC—To determine whether or not the induction of alkaline phosphatase activity by BMPRII-dependent signaling correlated with other markers of osteogenic differentiation, the expression of RANKL, osteoprotegerin (OPG), and osteopontin (OPN) as well as the tissue-nonspecific isoform of alkaline phosphatase (TNS-ALP) was measured in WT and KO cells. Consistent with measurements of alkaline phosphatase activity, BMP4 increased TNS-ALP mRNA expression in WT cells by more than 12-fold at 24 h, whereas BMP7 did not have an effect (Fig. 4). Neither BMP4 nor BMP7 induced TNS-ALP expression in BMPRII KO cells. Similarly, BMP4, but not BMP7, induced the expression of RANKL and OPG (5- and 2.8-fold, respectively) in WT cells, but this activity was lost in KO cells. In contrast to RANKL and OPG, OPN expression did not correlate with alkaline phosphatase activity in WT cells, being modestly induced by BMP7 only. However, in BMPRII KO cells, both BMP4 and BMP7 induced OPN expression by 10-fold. These findings suggest that expression of the osteoblast lineage markers TNS-ALP, RANKL, and OPG in response to BMP4, whereas BMPRII KO cells express OPN in response to BMP4 and BMP7. By quantitative RT-PCR measurements, BMP4 but not BMP7 (both 10 ng/ml) increased levels of tissue nonspecific alkaline phosphatase mRNA in WT cells after 24 h of treatment. Similarly, BMP4 but not BMP7 induced the expression of RANKL and OPG in WT cells after 24 h. Neither BMP4 nor BMP7 modulated the expression of these markers in KO cells. BMP7 but not BMP4 modestly induced expression of OPN in WT cells, whereas in KO cells, both BMP4 and BMP7 induced a ~10-fold increase in OPN mRNA expression.

BMP-mediated Growth Inhibition in PASMC Does Not Require MAPK p38 or Notch-mediated Signaling—Several signaling pathways by which BMPRII-mediated signaling might
regulate cell proliferation or osteogenic differentiation were considered. The potential contribution of MAPK p38 activation to BMP function in murine PASMC was tested by pharmacologic inhibition. Cotreatment of cells with SB239063, a specific inhibitor of MAPK p38 activity, did not impact PDGF-induced proliferation or the ability of BMP4 to inhibit PDGF-induced cell proliferation (Fig. 5A). Similarly, treatment of cells with SB239063 did not affect the ability of BMP4 to induce alkaline phosphatase activity in WT PASMC (data not shown). Taken together, these results suggest that BMP4-mediated growth arrest and osteogenic differentiation cannot be attributed to MAPK p38 activation.

Notch-mediated signaling has been reported to modulate BMP functions positively or negatively in a variety of cell lineages (20, 21, 60–62). Expression of Hes1 mRNA was efficiently induced by BMP signaling in PASMC within 1 h (Supplemental Fig. 2), consistent with the previously reported BMP-mediated, Notch-dependent transcription of Hes1 in C2C12 cells (61). To evaluate the role of Notch-signaling in BMP-mediated growth arrest and differentiation, WT and KO PASMC were pretreated for 12 h with γ-secretase inhibitors L685,458 and DAPT, which inhibit Notch intracellular domain cleavage and downstream signaling. Neither L685,458 nor DAPT impacted the ability of PDGF to stimulate proliferation of PASMC; nor did they affect the ability of BMP4 to antagonize PDGF-induced proliferation (Fig. 5, B and C). Both L685,458 and DAPT were able to selectively inhibit Hes1 mRNA expression without affecting Id1 expression under these conditions (Fig. 5, D and E). Moreover, the addition of L685,458 or DAPT did not interfere with the ability of BMP4 to induce alkaline phosphatase activity in PASMC (data not shown). Based on these results, Notch-dependent signaling does not appear to be essential for BMP-mediated growth arrest or differentiation of PASMC.

BMP-mediated Signaling in PASMC Activates SMAD1—Although BMPs induce the activation of BR-SMADs in both BMPRII WT and KO cells (Fig. 6A), it is not known whether or not different BR-SMADs are activated in response to BMPRII or ActRIIa signaling. To analyze the activation of specific BR-SMADs, extracts of BMP-treated WT and KO cells were reacted with anti-phospho-SMAD1/5/8 antibody and precipitated. The precipitated phospho-SMADs were identified by immunoblot with SMAD1-, SMAD5-, or SMAD8-specific antibodies. SMAD5 and SMAD8 were not detected in these immunoprecipitates; however, SMAD1 was detected in immunoprecipitates from BMP-treated WT and KO cells (Fig. 6B). The levels of activated SMAD1 detected by this approach were proportional to levels of total activated SMAD1/5/8, with similar results replicated in two pairs of WT and KO cell isolates. Since BMP4 and BMP7 both activated SMAD1/5/8, with similar results replicated in two pairs of WT and KO cell isolates, differences in BMP function could not be ascribed to activation of different BR-SMADs.

BMPRII-mediated Signaling Is Necessary and Sufficient for Sustained Activation of SMAD1/5/8 in PASMC—Although BMPRII KO and WT cells both transduce BMP signals to activate SMAD1, it was not known if the kinetics of SMAD pathway activation differ between BMPRII- and ActRIIa-mediated signaling. We therefore tested whether or not functional differences in WT and KO cells correlated with altered kinetics of BMP pathway activation. BMP4 and BMP7 both elicited activation of BR-SMADs in WT and KO cells at 30 min (Fig. 6A); however, only BMP4 elicited sustained activation of BR-SMADs in WT cells at 12, 24, and 48 h (Fig. 6C). BMP7 induced modest activation of BR-SMADs in WT cells at 12, 24, and 48 h, whereas BMP4 and BMP7 elicited very weak activation of BR-SMADs in KO cells at these time points. During this time, no changes in the levels of total SMAD1 or the SMAD phosphatase PPM1a were observed. These findings, replicated in two pairs of WT and KO isolates, suggest that signaling via BMPRII and ActRIIa
activate SMAD1 with distinct kinetics and that signaling via BMPRII is required to achieve sustained activation of SMAD1 in PASMC.

Although BMPRII KO cells did not maintain sustained activation of SMAD1/5/8 in response to BMP4 at 24 and 48 h, gene transfer of either BMPRII short or long isoforms was able to reconstitute sustained activation of SMAD1/5/8 (Fig. 6D) and 48 h (not shown). These results suggested that BMPRII expression is sufficient to facilitate sustained SMAD1/5/8 activation in response to BMP ligands.

The expression levels of SMAD6 and -7 were examined to determine whether or not differences in the levels of inhibitory SMADs could explain the distinct kinetics of BR-SMAD activation in WT and BMPRII KO cells. By quantitative RT-PCR, SMAD6 levels were seen to increase 20-fold 2 h following BMP4 stimulation in WT or KO cells (Supplemental Fig. 3). BMP7 induced the expression of SMAD6 to comparable levels in KO cells but had minimal impact in WT cells. These data suggest that differential regulation of inhibitory SMADs could not account for the prolonged activation of BR-SMAD by BMP4 in WT cells or the transient activation of BR-SMAD by BMP7 in WT or KO cells.
BMPRII Regulates PASMC Growth and Osteogenic Differentiation

BMPRII-mediated Signaling Is Necessary for Sustained Id1 mRNA and Protein Expression in PASMC—The Id family of bHLH transcription factors, whose expression is rapidly induced by BMP ligands, is pivotal in regulating growth and phenotype modulation of VSMC via their dominant negative interactions with key transcriptional factors (63). We tested the hypothesis that disruption of BMPRII alters BMP-mediated expression of Id genes and gene products. BMP4 treatment of WT cells elicited a 24-fold increase in Id1 mRNA levels within 1 h, which was sustained at a level 7-fold higher than base-line levels at 24 h (Fig. 6E) and 48 h (Fig. 6F). BMP4 stimulation in KO cells elicited a 22-fold increase in Id1 mRNA levels within 1 h, but this response normalized to nearly base-line levels within 4 h. In WT cells, BMP7 elicited a 5-fold increase in Id1 mRNA levels within 1 h, which also decreased to nearly base-line levels within 4 h. In KO cells, BMP7 elicited a 31-fold increase in Id1 mRNA levels within 1 h; however, Id1 mRNA levels diminished substantially by 24 h (Fig. 6, E and F). Similar results were observed for the BMP-induced expression of Id3 (Supplemental Fig. 3). Consistent with sustained expression of Id1 mRNA in response to BMP4, increased Id1 protein levels were detected in WT PASMC incubated with BMP4 for 24 and 48 h (Fig. 6G). BMP7 transiently increased Id1 protein levels in WT cells, but these levels were reduced at 24 and 48 h. Despite transiently activating SMAD1 and transiently up-regulating Id1 mRNA levels in KO cells, neither BMP4 nor BMP7 increased Id1 protein levels in KO cells at 12 h or beyond. These data suggested a requirement for BMPRII-mediated signaling to induce sustained increases in Id1 mRNA and protein expression.

Sustained Activation of SMAD1/5/8 Is Necessary for BMP4-induced Differentiation of PASMC—To confirm whether sustained activation of SMAD1/5/8 is necessary for BMP4-induced differentiation in cultured PASMC, we used a recently described selective inhibitor of BMP type I receptor kinases ALK2, ALK3, and ALK6, dorsomorphin (64), to curtail SMAD1/5/8 activation before and after BMP4 stimulation. BMP4 stimulation of WT PASMC induced alkaline phosphatase expression at 5 days (Fig. 7). Pretreatment of cells with dorsomorphin abrogated the induction of alkaline phosphatase expression, as did treatment of cells starting at 2–24 h following BMP stimulation. However, dorsomorphin treatment at 48 and 72 h resulted in progressively less inhibition of alkaline phosphatase expression. Thus, the inhibition of BMP type I receptor activity during the first 24 h but not after 24 h abrogated BMP4-induced differentiation, consistent with the requirement for sustained SMAD1/5/8 activation for at least 24 h for efficient BMP-mediated differentiation.

DISCUSSION

Using targeted disruption and gene transfer approaches, BMPRII was found to be necessary for BMP-induced growth inhibition and differentiation in PASMC. Although BMPRII KO cells transduce BMP signals via ActRIIa (15), ActRIIa signaling in these cells does not appear to elicit the same functions as those mediated by BMPRII. Gene transfer of BMPRII restored BMP-induced growth inhibition and differentiation in KO cells. Although ActRIIa signaling transiently induced SMAD1 activation and Id1 mRNA expression, the ability of BMPRII signaling to elicit these functional endpoints correlated with sustained activation of SMAD1 and sustained expression of Id1 mRNA and protein at 24 and 48 h. Consistent with a requirement for sustained pathway activation, pharmacologic termination of BMP signaling abrogated the ability of BMPs to induce differentiation. The inability of BMPRII KO cells to achieve these functions was not explained by differential induction of inhibitory SMAD6 or -7, differential degradation of BR-SMADs (i.e. via activity of SMAD ubiquitin ligases), or altered SMAD phosphatase expression in WT versus KO cells. Rather, these data suggest intrinsic differences in the nature of BMPRII-mediated versus ActRIIa-mediated signaling.

Although functional differences between BMPRII and ActRIIa signaling correlated with the distinct kinetics of BMP signaling by these receptors, alternate explanations for sustained BMP signaling in WT cells and transient BMP signaling in KO cells were considered. Arguing against a simple decrease in ligand sensitivity in KO cells, BMP ligands at lower concentrations (1–10 ng/ml) activated SMAD1/5/8 in KO cells to levels comparable with those observed in WT cells (15). Moreover, BMPRII KO cells did not exhibit growth inhibition even with high BMP4 or BMP7 concentrations (up to 200 ng/ml; not shown). Thus, the lack of BMP-induced growth arrest in
BMPRII KO cells was not explained by a decrease in signaling efficiency that could be compensated for by providing excess ligand. Conversely, sustained SMAD1/5/8 activation in WT cells could result from autocrine positive feedback induction of BMP synthesis subsequent to BMPRII signaling. However, no substantial or sustained increase (>1.5-fold) in BMP2, BMP4, or BMP7 expression was observed by quantitative RT-PCR in WT or KO PASMC within 24 h after ligand stimulation (data not shown). Alternatively, the differential activity of BMP4 and BMP7 in eliciting growth inhibition or differentiation might be explained by differential stability of these ligands. However, modest increases in phospho-SMAD1/5/8 were observed at 12, 24, and 48 h in WT cells exposed to BMP7, consistent with persistent activity of BMP7 in culture (Fig. 6B). In contrast, BMP4 elicited only modest increases in phospho-SMAD1/5/8 in KO cells at these time intervals, despite having persistent activity in WT cells. In differentiation assays, end points were not altered by the daily addition of BMP ligands, lessening the likelihood that decreased BMP ligand stability accounted for transient signaling and disrupted function. Taken together, these results suggest that the ability of BMPRII signaling to regulate growth and differentiation is likely attributable to the manner by which BMPRII, in conjunction with its preferred ligands and coreceptors, recruits and activates downstream effectors.

As further evidence that growth arrest and differentiation are functions associated with BMPRII, overexpression of BMPRII enabled these functions for BMP7, a ligand that did not achieve these end points in WT cells. This finding suggests that BMP ligands typically recognized by other receptors, such as ActIIa (65), can induce growth inhibition and differentiation when transduced via BMPRII. This observation has practical and theoretical consequences. One implication is that systems that rely on the overexpression of BMP receptors may not accurately model the endogenous receptor utilization of various ligands, since differences in receptor affinities for ligand might be made irrelevant with high levels of receptor expression. These findings also suggest that distinct cell populations and tissues might express BMPRII at varying levels in order to modulate the sensitivity to specific BMP ligands for growth arrest or differentiation or to modulate the threshold at which these cellular end points occur. Conversely, various tissues might express ActIIa at varying levels in order to regulate functions specific to ActIIa, in contrast to BMPRII.

Only BMPRII-mediated signaling led to sustained SMAD1 activation, Id1 mRNA expression, and importantly, Id1 protein accumulation. With respect to growth inhibition and differentiation, Id expression may be a surrogate marker for other downstream effects of persistent BMP signaling pathway activation or could itself be a causal factor. The notion that sustained Id1 expression contributes directly to BMP-mediated growth inhibition and differentiation would be consistent with the known potent effects of Id1 and its bHLH binding partners upon cell cycle control and cell fate (66).

The apparent requirement for sustained SMAD1 activation and Id1 expression for growth arrest and differentiation resembles the requirement for persistent SMAD2/3 activation required for TGF-β-mediated growth arrest (67, 68). Cellular defects that diminish the amplitude or duration of TGF-β-mediated SMAD2/3 activation, such as those found in a number of pancreatic tumor lines, abolish the ability of TGF-β to inhibit cell proliferation (68). In those studies, the duration of continuous TGF-β-mediated signaling required for growth arrest was found to be ~12 h. In the present studies, BMPRII- but not ActIIa-mediated BR-SMAD signaling persisted for >12 h, and only BMPRII signaling led to growth inhibition. Due to the parallel function and homology of TGF-β and BMP signaling pathways, it would not be surprising that prolonged BR-SMAD activation is required for BMP functions, including growth inhibition. It is thought that decreased expression of TGF-β signaling components, including type II or type I receptors, or SMAD4, may contribute to loss of growth control and tumorigenesis. Similarly, the loss of BMPRII expression observed in the vasculoproliferative lesions of idiopathic pulmonary arterial hypertension and in certain prostate and renal cell carcinomas (69–72) may directly contribute to the loss of BMP-mediated growth inhibition and differentiation. It is observed that prolonged SMAD2/3 activation requires persistent activity of TGF-β receptor-ligand complexes (67) and that enhanced and persistent SMAD2/3 activation occurs via the activity of clathrin-dependent TGF-β ligand-receptor endosomes (73). BMPRII signaling that results in alkaline phosphatase induction is also thought to occur via a clathrin-mediated endocytic pathway (74, 75), and analogously, this signaling pathway may be required for BMPRII-mediated effects upon cell proliferation and differentiation.

The long C-terminal domain of the BMPRII long isoform is unique among type II receptors and is postulated to regulate cytoplasmic signaling functions and intracellular trafficking of BMPRII (28–30). In present studies, both short and long isoforms of BMPRII were found to restore BMP-mediated growth inhibition and differentiation in KO cells, indicating that the C-terminal domain may be dispensable for these BMP functions, whereas the ligand-binding and kinase domains together are sufficient. Cytoplasmic binding partners proposed to interact with the long BMPRII C-terminal domain may thus not be critical for BMP-mediated growth arrest or differentiation in PASMC. However, cytoplasmic proteins that specifically interact with the BMPRII serine-threonine kinase domain, such as the recently characterized RACK1 (receptor for activated protein kinase C1) (76), might augment BR-SMAD activation and thus enhance signaling mediated by either BMPRII short or long isoforms.

BMPs have been reported to antagonize the proliferation of VSMC while inducing apoptosis or a quiescent smooth muscle cell phenotype (12, 19, 40). Here, it is shown that BMP4 induces WT PASMC to express a limited number of osteoblast-like markers while maintaining expression of two markers of smooth muscle phenotype, SM22-α and SM α-actin. Although this property of PASMC resembles the activity of calcifying vascular cells (49), murine PASMC do not up-regulate other products associated with osteogenesis (Matrix Gla protein, basic Gla protein, procollagen COL1A1 and COL1A2); nor do they form calcifications in the presence of high concentrations of phosphate (data not shown). BMP4 stimulates WT PASMC to express RANKL and OPG, which coordinate osteoclast dif-
BMPRII Regulates PASMC Growth and Osteogenic Differentiation

In summary, two key functions of BMPs in the vascular smooth muscle, inhibition of mitogen-induced proliferation and osteogenic differentiation, depend critically on BMPRII and correlate with the ability of BMPRII to activate SMAD1 signaling and Id1 expression in a sustained fashion (Fig. 8). Regulated expression of BMPRII may serve to modulate these BMP-mediated functions in a cell type-specific manner. The apparently critical nature of BMPRII in maintaining BR-SMAD activation parallels the importance of other TGF-β signaling components for growth inhibition. Analogous to defects in TGF-β signaling, altered BMP signaling kinetics seen with BMPRII disruption may contribute to the pathogenesis of tumorigenesis and vasculoproliferative disease via dysregulation of cell growth and differentiation control.

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