Bone morphogenetic protein (BMP) ligands signal by binding the BMP type II receptor (BMPR2) or the activin type II receptors (ActRIIA and ActRIIB) in conjunction with type I receptors to activate SMADs 1, 5, and 8, as well as members of the mitogen-activated protein kinase family. Loss-of-function mutations in Bmpr2 have been implicated in tumorigenesis and in the etiology of primary pulmonary hypertension. Because several different type II receptors are known to recognize BMP ligands, the specific contribution of BMPR2 to BMP signaling is not defined. Here we report that the ablation of Bmpr2 in pulmonary artery smooth muscle cells, using an ex vivo conditional knock-out (Cre-lox) approach, as well as small interfering RNA specific for Bmpr2, does not abolish BMP signaling. Disruption of Bmpr2 leads to diminished signaling by BMP2 and BMP4 and augmented signaling by BMP6 and BMP7. Using small interfering RNAs to inhibit the expression of other BMP receptors, we found that wild-type cells transduce BMP signals via BMPR2, whereas BMPR2-deficient cells transduce BMP signals via ActRIIA in conjunction with a set of type I receptors distinct from those utilized by BMPR2. These findings suggest that disruption of Bmpr2 leads to the net gain of signaling by some, but not all, BMP ligands via the activation of ActRIIA.

Bone morphogenetic protein (BMP) signals regulate embryonic tissue patterning and organogenesis, as well as the remodeling of mature tissues (1). BMPs, like other transforming growth factor β superfamily ligands, induce apposition of type I and type II receptors to cause phosphorylation of type I receptors. Activated BMP type I receptors phosphorylate the BMP-responsive SMAD proteins 1, 5, and 8, leading to their nuclear translocation and the regulation of target gene transcription. The Id (inhibitor of differentiation) gene family is an important target of BMP signals (2–5), serving to regulate the differentiation and proliferation of a variety of mature and embryonic cell lineages, including vascular smooth muscle and endothelium (2, 6–8). BMP ligands may also trigger the activation of MAP kinases via SMAD-independent signaling pathways (9–11).

Three type II receptors, BMPR2 and the activin type II receptors ActRIIA and ActRIIB (12–17), can pair with three different type I receptors, ActRIIA/ALK2, BMPRIa/ALK3, and BMPRIIB/ALK6 (13, 18–23), to transduce BMP signals. These various BMP receptors have distinct temporal and spatial expression in tissues and have varying affinities for each of more than 15 known BMP molecules (1, 24). Although BMPR2, ActRIIA, and ActRIIB can each bind BMP ligands (12–15, 21, 22, 25), the preferred ligand-receptor complexes used by cells for BMP signaling are not known.

In this study, we examined the signaling mediated by two structurally distinct classes of BMP ligands that are known to be expressed in vascular smooth muscle and endothelial cells and that regulate their function (6, 26–30). The first class of BMP ligands includes the 92% homologous BMP2 and BMP4, considered prototype BMPs in that they possess greater affinity for type I than type II receptors (19, 31, 32). The second class of BMP ligands we studied consists of the closely related BMP5, BMP6, BMP7, and BMP8, which are ~60% homologous to BMP2 or BMP4 and share with activin ligands a greater affinity for their corresponding type II receptors (15, 21, 22, 25).

To define the contribution of BMPR2 to signaling by these two classes of BMP ligands, we tested the effect of ablating Bmpr2 in pulmonary arterial smooth muscle cells (PASMC). The Bmpr2 gene was disrupted in PASMC isolated from genetically modified mice possessing Bmpr2 alleles with internal loxP sites, using an adeno virus specifying Cre recombinase. In a complimentary approach, BMPR2 expression was inhibited with high efficiency by specific small interfering RNA (siRNA) duplexes. Remarkably, the loss of BMPR2 did not uniformly reduce BMP signal transduction but instead increased the activation of SMAD and p38 by BMP6 and BMP7. In BMPR2-deficient cells, BMP signals were transduced by receptor complexes consisting of the ActRIIA receptor and a set of type I co-receptors distinct from those utilized by BMPR2, resulting in the gain of signaling for certain BMP ligands.

MATERIALS AND METHODS

Mice with Genetically Modified BMPR2 Alleles—Mice heterozygous for a Bmpr2 mutant allele (Bmpr2<sup>−/−</sup>) were generated as previously described (33, 34) and backcrossed for 10 generations to inbred C57BL/6 (wild-type) mice. The generation of mice with a conditionally disrupted Bmpr2 allele was described by Beppu et al. (35). Briefly, a construct containing exons 4 and 5 (which encode the transmembrane and a portion of the kinase domain) of the Bmpr2 gene flanked by two loxP sites and a neomycin selection cassette (pgk-neo) with a third loxP site...
was used to target BMPr2 in embryonic stem cells. Mice homozygous for the conditional knock-out allele (Bmpr2\textsuperscript{fl/fox}) on a hybrid C57BL/6 and Sv129 background were viable and appeared normal.

Isolation of Pulmonary Artery Smooth Muscle Cells—Explants of murine pulmonary arteries were cultured as described previously (36) to yield PASMCs whose phenotype was confirmed by immunohistochemical staining for a-smooth muscle actin. Three separate isolates of PASMCs were obtained, each derived from an individual Bmpr2\textsuperscript{fl/fox} mouse. PASMCs were also isolated from wild-type and Bmpr2\textsuperscript{-/-} mice.

Disruption of the BMPR2 Gene in PASMC—To disrupt the Bmpr2 gene, PASMCs isolated from Bmpr2\textsuperscript{fl/fox} mice were infected with an adenovirus specifying Cre recombinase (Ad.Cre) or an adenovirus specifying green fluorescent protein (Ad.GFP) as a control, each at a multiplicity of infection of 150. Cultured cells were allowed to recover for 7 days after infection and then passaged twice to ensure depletion of BMPR2 protein.

Measurement of BMPR2 Protein Expression—Proteins were extracted from PASMCs in SDS-s Mitsuyasu buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. BMPR2 was detected using a monoclonal antibody directed against the C-terminal domain (BD Biosciences). Bound antibody was detected with an horseradish peroxidase-linked antibody directed against mouse IgG and was visualized using chemiluminescence with ECL Plus (Amersham Biosciences).

Measurement of Gene Expression—Total RNA was extracted from PASMCs using TRIzol reagent (Invitrogen) and cDNA was synthesized by the reverse transcriptase reaction. Gene sequences were amplified from cDNA by PCR and quantitated using an ABI Prism 7000 (Applied Biosystems, Foster City, CA) with the following primers. To detect Bmp2 cDNA, the forward primer 5'-GAAACGATAATCATTGTTTG-GC-3' corresponding to a sequence in exon 4 and reverse primer 5'-CCGCTGCTCTCTGT-3' corresponding to a sequence in exon 5 were used. To detect Alk2, Alk3, Alk6, ActRIIB, ActRIIB, Id1, Smad6, Smad7, and 18 S ribosomal cDNA sequences, TaqMan primer sets supplied by Applied Biosystems were used. Changes in the relative gene expression normalized to 18 S rRNA levels were determined using the relative C\textsubscript{t} method (37, 38).

Measurement of SMAD1/5/8 and p38 Phosphorylation—After serum starvation in RPMI medium (Invitrogen) for 24 h, PASMCs were incubated with BMPs 2, 4, 6, or 7 (R&D Systems, Inc., Minneapolis, MN). Cultured cell lysates (15 \textmu g/lane) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Amersham Biosciences), and probed with antibodies specific for phosphorylated (\textit{p}) SMAD1/5/8, Alk2, Alk3, Alk6, ActRIIB, ActRIIB, Id1, Smad6, Smad7, and 18 S ribosomal cDNA sequences, TaqMan primer sets supplied by Applied Biosystems were used. Changes in the relative gene expression normalized to 18 S rRNA levels were determined using the relative C\textsubscript{t} method (37, 38).

RESULTS

Disruption of the BMPR2 Gene in PASMCs—PASMCs isolated from Bmpr2\textsuperscript{fl/fox} mice were infected with Ad.Cre to excise the genomic fragment containing exons 4 and 5 of the Bmpr2 gene, yielding Bmp2\textsuperscript{gld/gld} cells. Cre-mediated recombination was confirmed first by PCR analysis of genomic DNA using primers specific for sequences in introns 3 and 5, which yielded a product consistent with excision of exons 4 and 5 (35). Second, RNA extracted from Bmp2\textsuperscript{gld/gld} cells was amplified by RT-PCR with primers specific for sequences in exons 3 and 8, and the product was sequenced to reveal direct splicing of exon 3 to exon 6, with stop codons in exon 6 resulting from a frameshift (35). Third, quantitative RT-PCR performed on RNA from each isolate of Bmp2\textsuperscript{gld/gld} cells using primers specific for exons 4 and 5 demonstrated levels of wild-type Bmp2\textsuperscript{e} mRNA that were 0.05 to 2% of that found in Bmp2\textsuperscript{fl/fox} cells infected with Ad.GFP (Fig. 1A). In comparison, PASMCs from Bmp2\textsuperscript{gld/gld} cells did not express detectable wild-type BMPR2 protein, in contrast to Bmp2\textsuperscript{fl/fox} cells infected with Ad.GFP (Fig. 1C).

SMAD1/5/8 Activation Is Attenuated in Bmp2\textsuperscript{-/-} PASMCs—We previously observed that the ability of Bmp2\textsuperscript{-/-} cells to activate SMAD1/5/8 in response to BMP2 was diminished compared with wild-type cells (34). To determine whether SMAD signaling in Bmp2\textsuperscript{-/-} cells was diminished in response to other BMP ligands, cells were stimulated with BMPs 4, 6, or 7 for 30 min. For each of these BMP ligands,
the phosphorylation of SMAD1/5/8 was decreased in Bmpr2+/− cells to levels 40–60% of that observed in wild-type cells (results for BMP7 shown in Fig. 2, A and B).

**SMAD1/5/8 Activation Is Altered in BMPR2-deficient PASMCs**—SMAD1/5/8 activation was measured in Bmpr2+/− cells to determine whether the ablation of Bmpr2 would further attenuate BMP signaling in comparison to the reduction in

signaling observed in Bmpr2+/− cells. Bmpr2+/− cells retained the ability to phosphorylate SMAD1/5/8 in response to BMP4, at levels that were 30–50% of those in Bmpr2+/− cells (Fig. 2, C and D). In response to BMP7, however, Bmpr2+/− cells phosphorylated SMAD1/5/8 at levels 4–5-fold greater than in Bmpr2+/− cells.

The signaling mediated by other BMP ligands was tested to

**BMPR2-deficient Cells Gain BMP Signaling Function**

![Diagram](http://www.jbc.org/)

**Fig. 2.** BMP-induced SMAD1/5/8 activation is attenuated in Bmpr2+/− PASMCs, whereas that of Bmpr2+/− PASMCs is attenuated for BMP2 and BMP4 and augmented for BMP6 and BMP7. A, phospho-SMAD1/5/8 (p-SMAD1/5/8) and total SMAD1 were measured in PASMCs from Bmpr2 wild-type (+/+) or heterozygote (+/−) mice after incubation with varying concentrations of BMP7 for 30 min. B, relative SMAD1/5/8 levels were quantitated by dividing the phospho-SMAD1/5/8 immunoreactivity by total SMAD1 immunoreactivity, measured by scanning fluorimetry. BMP-induced activation of SMAD1/5/8 in Bmpr2+/− PASMCs was approximately half of that in wild-type cells. Results show that the experiments performed on three separate isolates each of Bmpr2+/− and wild-type cells. C, phospho-SMAD1/5/8 and SMAD1 were measured in the lysates of Bmpr2+/− cells treated with Ad.GFP (flox/flox) and Bmpr2+/− cells treated with Ad.Cre (del/del) after incubation with varying concentrations of BMP4 or BMP7 for 30 min. In Bmpr2+/− cells, BMP4-induced SMAD1/5/8 activation was attenuated compared with Bmpr2+/− cells, whereas BMP3-induced SMAD1/5/8 activation was increased. D, relative SMAD1/5/8 levels were determined by dividing phosphorylated SMAD1/5/8 immunoreactivity by the total SMAD1 immunoreactivity, measured by scanning fluorimetry. These results are representative of experiments performed upon PASMCs derived from three separate isolates obtained from individual Bmpr2+/− mice. E, SMAD1/5/8 and p38 activation in Bmpr2+/− and Bmpr2+/− PASMCs. Phospho-SMAD1/5/8 and p38 were measured by immunoblot in Bmpr2+/− and Bmpr2+/− PASMCs incubated with BMP ligands (10 ng/ml) for 15 and 60 min. Equivalent loading of samples was confirmed by measuring total SMAD1 immunoreactivity (not shown). The addition of BMP ligands activated SMAD1/5/8 and p38 in all PASMCs examined, and the activation of p38 was delayed compared with the activation of SMAD1/5/8. Bmpr2+/− cells were more sensitive to BMP2 and BMP4 in activating SMAD1/5/8 and p38, whereas Bmpr2+/− cells were more sensitive to the effects of BMP6 and BMP7. The data presented are representative of experiments performed with PASMCs derived from three separate isolates obtained from individual Bmpr2+/− mice.
BMPR2-deficient Cells Gain BMP Signaling Function

Fig. 3. Role of BMPR2 in BMP-induced Id1 gene transcription. Left panel, Bmpr2fl/fl-box and Bmpr2del/del PASMCs were transfected with a firefly luciferase reporter construct under the control of the Id1 gene promoter and with a Renilla luciferase reporter construct as a transfection control and were incubated with BMP4 or BMP7 (10 ng/ml each) for 12 h in the absence of serum. Id1 promoter activity was determined by dividing firefly luciferase activity by Renilla luciferase activity and was normalized to promoter activity in PASMCs not exposed to BMP ligand. Values shown are mean ± S.D. of triplicate measurements. BMP4 and BMP7 activated Id1 promoter activity in Bmpr2fl/fl-box and Bmpr2del/del cells. Compared with Bmpr2fl/fl-box cells, the response to BMP4 in Bmpr2del/del cells was attenuated by 70%, whereas the response to BMP7 was increased 5-fold (*, p < 0.05 for both). The data presented are representative of experiments performed with cultured PASMCs derived from three separate isolates obtained from individual Bmpr2fl/fl-box mice. Right panel, Id1 gene expression was measured by quantitative RT-PCR in Bmpr2fl/fl-box and Bmpr2del/del PASMCs 1 h after stimulation with BMP7 (10 ng/ml). Values shown are the mean ± S.D. of triplicate measurements. Bmpr2fl/fl-box and Bmpr2del/del cells had comparable levels of Id1 mRNA at baseline. The BMP7-mediated increase in Id1 mRNA was greater in Bmpr2fl/fl-box versus Bmpr2del/del cells (*, p < 0.01). This result is representative of experiments performed with PASMC cultures derived from three separate isolates obtained from individual Bmpr2fl/fl-box mice.

BMPR2-deficient cells could be generalized to structurally similar BMPs. Bmpr2del/del cells exhibited attenuated SMAD1/5/8 activation in response to both BMP2 and BMP4 compared with Bmpr2fl/fl-box cells (Fig. 2E). Bmpr2del/del cells exhibited increased SMAD1/5/8 activation in response to both BMP6 and BMP7 compared with Bmpr2fl/fl-box cells. The increased ability of Bmpr2del/del cells to activate SMAD1/5/8 was more marked for BMP7 than for BMP6. These changes in the sensitivity to different BMP ligands were consistently observed in Ad-Cre-infected PASMC isolated from three individual Bmpr2fl/fl-box mice.

p38 MAP Kinase Activation Is Altered in BMPR2-deficient PASMC—It has been previously reported that BMPs activate p38 MAP kinase in several cell types independently of SMAD activation (9–11). To determine whether the BMP signaling changes observed in Bmpr2del/del cells might be reflected in the activation of effectors other than SMADs, the BMP-induced phosphorylation of p38 was examined in Bmpr2fl/fl-box and Bmpr2del/del PASMCs (Fig. 2E). In Bmpr2fl/fl-box cells, stimulation with all BMP ligands induced the activation of p38 by 60 min, which was delayed compared with the activation of SMAD1/5/8. The activation of p38 by BMP2 and BMP4 was decreased in Bmpr2del/del cells compared with Bmpr2fl/fl-box cells, consistent with decreases in SMAD1/5/8 activation for those ligands. The activation of p38 by BMP6 and BMP7 was increased in Bmpr2del/del cells compared with Bmpr2fl/fl-box cells, also consistent with increases in SMAD1/5/8 activation for those ligands.

BMP-induced Id1 Gene Transcription in BMPR2-deficient Cells—The BMP-induced transcription of Id1 gene family is a primary means by which BMP ligands achieve effects in target cells (2–5). To determine whether alterations in the ability of BMPR2-deficient cells to activate SMAD1/5/8 were reflected in the ability to transcribe BMP-responsive genes, Id1 gene transcriptional activity was measured using a luciferase reporter gene under the control of the BMP-responsive Id1 gene promoter (Fig. 3, left panel). At baseline, Bmpr2fl/fl-box and Bmpr2del/del PASMCs had equivalent Id1 transcriptional activity. After stimulation with BMP4, Bmpr2del/del cells induced Id1 promoter activity >20-fold. BMP4 also induced Id1 promoter activity in Bmpr2del/del cells but 70% less than that observed in Bmpr2fl/fl-box cells. After stimulation with BMP7, Bmpr2del/del cells had only modest induction of Id1 promoter activity, whereas Bmpr2fl/fl-box cells had 5-fold greater induction of Id1 promoter activity in response to this ligand than did Bmpr2del/del cells. Consistent with the promoter assay, quantitative RT-PCR measurements revealed that incubation with BMP7 for 1 h increased Id1 mRNA levels to a greater extent in Bmpr2del/del PASMC than in Bmpr2fl/fl-box cells (Fig. 3, right panel). These findings suggest that the ability of Bmpr2fl/fl-box and Bmpr2del/del cells to activate SMAD1/5/8 correlated closely with the transcriptional activity of a key BMP-responsive gene.

BMPR2 Gene Disruption Does Not Alter the Expression of Other BMP Receptors or Inhibitory SMADs—ActRIIa, ActRIIb, Alk2, and Alk3 receptor sequences were readily detected by quantitative RT-PCR of mRNA from each of the PASMC isolates examined (≥26 cycles of PCR amplification from 200 ng of RNA). The expression levels of ActRIIa, ActRIIb, Alk2, and Alk3 receptors were not altered in Bmpr2del/del cells as compared with Bmpr2fl/fl-box (shown) or wild-type (data not shown) cells (Fig. 1A). Very little Alk6 cDNA was detected in the PASMC isolates tested (requiring >35 cycles of amplification), consistent with previous reports of low levels of Alk6 mRNA in normal human PASMCs (39). When measured by quantitative RT-PCR, the expression levels of the inhibitory SMADs, Smad6 and Smad7, were not significantly different in Bmpr2del/del and Bmpr2fl/fl-box cells (data not shown).

Inhibition of BMPR2 Expression by Specific siRNA Attenuates BMP4 and Enhances BMP7 Response—BMPR2 expression was inhibited by transfecting wild-type PASMCs with a gene-specific siRNA duplex for 48 h. This siRNA reduced BMPR2 protein expression by >90% in wild-type PASMCs, whereas transfection with siRNAs specific for Alk6 did not alter BMPR2 protein levels (Fig. 4). Treatment with siRNAs did not affect the expression of total SMAD1 protein. Treatment with siRNA specific for Alk6 did not alter the phosphorylation of SMAD1/5/8 induced by BMP4 or BMP7, consistent with low levels of Alk6 expression observed in PASMCs. Treatment with siRNA specific for Bmpr2 attenuated the phosphorylation of SMAD1/5/8 induced by BMP4 while increasing...
that induced by BMP7. Changes in BMP signaling resulting from the efficient RNA interference-mediated inhibition of BMPR2 expression in wild-type PASMCS were thus consistent with the ligand-specific changes in BMP signaling observed in Bmpr2del/del cells.

Receptors That Transduce BMP Signals in the Presence or Absence of BMPR2—To identify the type II receptors responsible for BMP signaling in Bmpr2fllox/fllox and Bmpr2del/del PASCMS, siRNA-mediated inhibition of receptor expression was employed. 48 h after transfecting cells with siRNAs specific for the type II receptors ActRIIA or ActRIIB, the expression of the target gene relative to 18 S ribosomal RNA expression was measured by quantitative RT-PCR (Fig. 5A). siRNAs specific for either ActRIIA or ActRIIB inhibited their expression (70 and 45%, respectively) in a specific manner with minimal effect on the expression of the homologous receptor. After pretreatment with siRNAs, cells were exposed to BMP ligands for 30 min and the activation of SMAD1/5/8 was assessed (Fig. 5, B–D). The inhibition of ActRIIA or ActRIIB expression in Bmpr2fllox/fllox cells did not affect the activation of SMAD1/5/8 induced by BMP4 or BMP7 (Fig. 5, B and C). In contrast, the inhibition of ActRIIA in Bmpr2del/del cells impaired the activation of SMAD1/5/8 by BMP4 or BMP7 (Fig. 5, B and D). The inhibition of ActRIIB had no effect under these conditions. These results suggest that BMPR2 is the principal type II receptor transducing BMP signals when it is expressed and that ActRIIA is the principal type II receptor transducing BMP signals when BMPR2 is not expressed.

To identify the type I receptors that transduce BMP signals in Bmpr2fllox/fllox and Bmpr2del/del PASCMS, siRNA specific for Alk2, Alk3, and Alk6 were used. siRNAs for Alk2 and Alk3 inhibited the expression of these type I receptors (60 and 75%, respectively) in a specific manner with minimal effects on the expression of other type I receptors (Fig. 6A). In Bmpr2fllox/fllox cells, BMP4-mediated SMAD1/5/8 signaling was inhibited only by siRNA specific for Alk3, whereas BMP7-mediated signaling was inhibited by siRNA specific for Alk2 (Fig. 6, B and C). In Bmpr2del/del cells, BMP4-mediated signaling was inhibited by siRNA specific for Alk2 or Alk3, whereas BMP7-mediated signaling was inhibited only by siRNA specific for Alk2 (Fig. 6, B and D). Inhibition of Alk6 expression did not have any effect upon BMP signaling in Bmpr2fllox/fllox or Bmpr2del/del cells (Fig. 6, B–D). The disruption of Bmpr2 thus altered the utilization of type I, as well as type II, receptors in BMP signaling.

The objective of this study was to examine the impact of BMPR2 deficiency on BMP signaling. Because conventional gene targeting of both Bmpr2 alleles in mice results in embryonic lethality (33), both Bmpr2 alleles were disrupted in PASMCS ex vivo using the Cre-lox system. Based on the attenuated BMP signaling previously observed in Bmpr2+/− cells (34), it was expected that the disruption of both Bmpr2 alleles might further impair BMP signaling. Despite the efficient ablation of Bmpr2 in Bmpr2del/del PASCMS, signaling by the class of ligands represented by BMP2 and BMP4 was preserved but attenuated compared with wild-type cells. Paradoxically, the ablation of Bmpr2 augmented the activation of SMAD1/5/8 by the class of ligands represented by BMP6 and BMP7. These findings were not because of nonspecific effects resulting from expression of a conditional knock-out gene or adenovirus-mediated Cre recombinase gene transfer, as the alteration in BMP signaling was recapitulated by the targeting of BMPR2 expression with a high efficiency siRNA duplex in wild-type PASMCS.

Importantly, the perturbation of SMAD1/5/8 activation observed in BMPR2-deficient cells resulted in commensurate changes in the induction of Id1 gene transcription. The induction of Id1 gene expression by BMP ligands is necessary and sufficient to induce in vitro tube formation by vascular endothelial cells, supporting the role of BMP signaling in normal and pathologic angiogenesis (2, 5–7, 40, 41). In vascular smooth muscle cells, Id gene family expression is necessary for angiogenesis II-mediated cell growth (8) and plays pivotal roles in regulating smooth muscle cell differentiation, proliferation, and the expression of α-smooth muscle cell actin (42, 43). Alterations in BMP-induced Id gene transcription observed after disruption of Bmpr2 in vascular smooth muscle cells would be predicted to alter BMP-modulated vascular cell functions.

Although SMADs 1, 5, and 8 are the principal effectors of BMP signals, additional effectors including MAP kinase and STAT pathways have also been implicated (44–47). We found that PASMCS efficiently phosphorylated p38 MAP kinase in response to BMP ligands. Changes in p38 activation after disruption of Bmpr2 correlated closely with changes in SMAD1/5/8 activation, suggesting a common upstream origin of these two effectors, possibly in the type I receptor. The delayed kinetics of p38 compared with SMAD1/5/8 phosphorylation in PASMCS suggests the role of intermediary signaling molecules such as TAK1 that have been previously implicated in BMP-mediated p38 signaling in other cell types (11, 46).

The preserved BMP signaling in Bmpr2del/del cells was unlikely to be attributable to the very low residual levels of BMPR2 expression, as BMP signaling by BMP2 and BMP4 persisted at levels similar to those observed in Bmpr2+/− cells rather than being further attenuated. Altered BMP signaling observed in Bmpr2del/del cells was also not likely to be because of signaling via mutant BMPR2 receptors encoded by the conditional knock-out gene: the allele resulting from the excision of Bmpr2 exons 4 and 5 specifies a mutant mRNA that, if transcribed, would result in a product truncated after exon 3 that would lack both the transmembrane domain as well as the kinase domain necessary to activate type I receptors (33, 35). It is possible that an N-terminal product of the mutant Bmpr2 allele, if produced, might increase BMP signaling (potentially in a ligand-specific manner) by providing inactive targets for constitutively expressed inhibitors. This possibility is made less probable by the observation that Bmpr2+/− cells, which possess one copy of the same mutant allele (33, 34), do not exhibit augmented signaling in response to BMP6 or BMP7. Moreover, siRNA-mediated inhibition of BMPR2 expression replicated findings observed in Bmpr2del/del cells. The increased

**DISCUSSION**

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FIG. 5. A, specificity and efficacy of siRNA targeting type II receptor expression. siRNA derived from sequences of Ac-
trR1Ia and ActRIIb were employed to decrease their expression in Bmpr2flox/flox PASMCs. Receptor mRNA levels were measured 48 h after cells were transfected with specific siRNA by quantitative RT-PCR, were normalized to 18 S rRNA levels, and are expressed as a fraction of values from cells not treated with siRNA. Values shown are the mean of triplicate measurements ± S.D. ActRIIa and Act-
trRIIb-specific siRNA reduced the expression of those genes by 70 and 45%, respectively. B, impact of siRNA targeting of ActRIIa and ActRIIb on BMP signaling. Bmpr2flox/flox and Bmpr2del/del PASMCs were transfected with siRNA specific for ActRIIa or ActRIIb for 48 h prior to stimulation, whereupon the ability of BMP4 or BMP7 to activate SMAD1/5/8 was measured by immunoblot techniques. Total SMAD1 were assayed to confirm equivalent loading of gels (data not shown). Ex-
periments were performed in duplicate. C and D, immunoblots in panel B were quantitated by scanning fluorimetry and averaged. Transfection of Bmpr2flox/flox cells with siRNA specific for ActRIIa or ActRIIb did not modulate the activation of SMAD1/5/8 by BMP4 or BMP7. Transfec-
tion of Bmpr2del/del cells with siRNA specific for ActRIIa, but not ActRIIb, decreased p-SMAD1/5/8 immunoreactivity at baseline and after stimulation with BMP4 or BMP7. This experiment is rep-
resentative of experiments performed with PASMCs derived from three separate isolates of PASMCs from individual Bmpr2flox/flox mice.

The changes in BMP signaling observed in Bmpr2del/del versus Bmpr2flox/flox cells are consistent with the use of alternate BMP receptor signaling complexes in the absence of the BMPR2 receptor. In the presence of BMPR2, BMPR2 was the principal type II receptor for both classes of BMP ligands, transducing BMP4 signals through ALK3 and transducing BMP7 signals through either ALK2 or ALK3. In the absence of BMPR2, ActRIIa was the principal type II receptor for both classes of BMP ligand, transducing BMP4 signals through either ALK2 or ALK3 and transducing BMP7 signals potently through ALK2. The impact of ablating Bmpr2 on BMP receptor utilization is pictured schematically in Fig. 7. The apparent exclusive function of BMPR2 in wild-type cells and of ActRIIa in BMPR2-deficient cells may explain the striking differences in BMP signaling. ActRIIa differs from BMPR2 both in its preferences for type I receptors and in its ligand specificities (24). Increased BMP6 and BMP7 signaling in BMPR2-deficient cells is consistent with the finding that ActRIIa has higher monovalent affinity for this class of BMP ligands than for BMP2 and BMP4 (25) and that ActRIIa-ALK2 complexes have high avidity for BMP7 (21, 25). The observation that wild-type cells do not transduce BMP signals through ActRIIa, despite its expression, indicates that expression of BMPR2 suppresses BMP signaling via ActRIIa. Such inhibition would potentially explain the finding that Bmpr2+/+ cells possessing half-normal BMPR2 levels do not exhibit gain of signaling for BMP6 or BMP7. BMPR2 may inhibit BMP signaling via ActRIIa by preventing assembly of BMP-ActRIIa type I receptor com-
plexes. Although ActRIIa was expressed in PASMCs, a role for this receptor in BMP signaling was not identified. This finding may be because of relatively higher levels of ActRIIa expression in these cells as was suggested by quantitative RT-PCR (data not shown) or because the siRNA-mediated inhibition of ActRIIa expression was not sufficiently effective.

Although the fact that Activin type II receptors may trans-
duce BMP signals might suggest that BMPR2 is dispensable for BMP signaling, the finding that embryonic mice lacking Bmpr2 are arrested in early gastrulation (33) clearly demon-
strates that BMPR2 provides an essential function in developing tissues. There are several explanations for the discrepancy between the necessity of BMPR2 for development and the ap-
parent redundancy in BMP signaling. First, the distinct spatio-
temporal expression of BMPR2, ActRIIa, and each of the type I receptors suggests that in the absence of BMPR2 the full com-
plement of receptors required for the transduction of BMP signals might not be expressed in all relevant cells. Alterna-
tively, BMPR2 may provide its function not only by transduc-
ing BMP signals but also by regulating the activity of other receptors such as ActRIIa.

The regulation of BMP signaling by BMPR2 may be relevant to a number of human diseases. Loss-of-function mutations in Bmpr2 may contribute to tumorigenesis, as receptor expression is frequently lost in renal cell carcinoma and receptor expres-
sion is more frequently lost in higher grade prostate carci-
nosas (50–53). The impact of loss of BMPR2 could be either diminution or gain of signaling, depending on the identity of
BMPR2-deficient Cells Gain BMP Signaling Function

Fig. 6. A, specificity and efficacy of siRNA targeting BMP type I receptor expression. siRNA specific for type I receptors Alk2 and Alk3 reduced the expression of those genes by 60 and 70%, respectively. siRNA treatment had minimal effect on the expression of non-targeted genes. B, impact of siRNA targeting of Alk2 and Alk3 on BMP signaling. Bmpr2<sup>floxflox</sup> and Bmpr2<sup>del/del</sup> PASMcs were transfected with siRNA specific for type I BMP receptors for 48 h prior to stimulation with BMP ligands, whereupon the ability of BMP4 or BMP7 to activate SMAD1/5/8 was measured by immunoblot techniques. Total SMAD1 and total protein were assayed to confirm equivalent loading of gels (data not shown). C and D, the immunoblots presented in panel A were quantitated by scanning fluorimetry and averaged. Transfection of Bmpr2<sup>del/del</sup> cells with siRNA specific for Alk2 or Alk3, but not Alk2 or Alk6, decreased p-SMAD1/5/8 immunoreactivity after stimulation with BMP4. Transfection of Bmpr2<sup>del/del</sup> cells with siRNA specific for Alk2 or Alk3, but not Alk6, decreased p-SMAD1/5/8 immunoreactivity after stimulation with BMP7. Transfection of Bmpr2<sup>del/del</sup> cells with siRNA specific for Alk2, but not Alk3 or Alk6, decreased p-SMAD1/5/8 immunoreactivity after stimulation with BMP4. Transfection of Bmpr2<sup>del/del</sup> cells with siRNA specific for Alk2, but not Alk3 or Alk6, decreased p-SMAD1/5/8 immunoreactivity after stimulation with BMP7. The lack of effect of Alk6 siRNA transfection was consistent with low levels of Alk6 expression in PASMcs. This experiment is representative of experiments performed with PASMcs derived from three separate isolates of PASMcs from individual Bmpr2<sup>del/del</sup> mice.

Fig. 7. A model for BMP receptor signal transduction in wild-type versus BMPR2-deficient PASMcs. The known type II and type I BMP receptors in PASMcs are shown. ALK6 is shown with a strikethrough to reflect the very low levels of expression of this receptor. Left panel, BMP4 signaling. Bmpr2 and ALK3 are the principal receptors for BMP4 in Bmpr2<sup>del/del</sup> cells. ActRIIA and either ALK2 or ALK3 mediate the transduction of BMP4 signals in Bmpr2<sup>del/del</sup> cells. Right panel, BMP7 signaling. Bmpr2 pairs with either ALK2 or ALK3 to transduce BMP7 signals in Bmpr2<sup>del/del</sup> cells, whereas ActRIIA pairs only with ALK2 to transduce BMP7 signals in Bmpr2<sup>del/del</sup> cells.

Because the disease has only 20% penetrance in individuals harboring the mutant alleles. It has been proposed that mutations in Bmpr2 might contribute to the pathogenesis of disease by decreasing transmission of BMP signals in relevant vascular tissues (52, 54, 55, 58), a notion supported by the decrease in BMP-mediated SMAD1/5/8 activation observed in Bmpr2<sup>−/−</sup> PASMcs (34). If the expression of the Bmpr2 receptor was severely diminished or lost, as was reported in one study examining Bmpr2 expression in PPH pulmonary vascular lesions (59), our findings suggest that augmented BMP signaling mediated by ActRIIA, such as that observed in Bmpr2-deficient cells, might play a pathogenetic role.

In summary, we found that disruption of Bmpr2 in pulmonary artery smooth muscle cells caused BMP signals to be transduced by an alternate receptor, ActRIIA, a function that appears to be suppressed by Bmpr2 in wild-type cells. ActRIIA and its co-receptors transduce BMP signals with altered characteristics compared with Bmpr2 and its co-receptors, and such changes in BMP signal transduction may contribute to conditions associated with the loss of Bmpr2 function.

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