Loss-of-function mutations in bone morphogenetic protein receptor II (BMP-RII) are linked to pulmonary arterial hypertension (PAH); the ligand for BMP-RII, BMP-2, is a negative regulator of SMC growth. Here, we report an interplay between PPARγ and its transcriptional target apoE downstream of BMP-2 signaling. BMP-2/BMP-RII signaling prevented PDGF-BB–induced proliferation of human and murine pulmonary artery SMCs (PASMCs) by decreasing nuclear phospho-ERK and inducing DNA binding of PPARγ that is independent of Smad1/5/8 phosphorylation. Both BMP-2 and a PPARγ agonist stimulated production and secretion of apoE by SMCs. Using a variety of methods, including short hairpin RNAi in human PASMCs, PAH patient–derived BMP-RII mutant PASMCs, a PPARγ antagonist, and PASMCs isolated from PPARγ– and apoE-deficient mice, we demonstrated that the antiproliferative effect of BMP-2 was BMP-RII, PPARγ, and apoE dependent. Furthermore, we created mice with targeted deletion of PPARγ in SMCs and showed that they spontaneously developed PAH, as indicated by elevated RV systolic pressure, RV hypertrophy, and increased muscularization of the distal pulmonary arteries. Thus, PPARγ-mediated events could protect against PAH, and PPARγ agonists may reverse PAH in patients with or without BMP-RII dysfunction.

Introduction
Bone morphogenetic protein 2 (BMP-2) is a negative regulator of SMC growth, but the mechanism by which it counteracts proliferation induced by growth factors (i.e., PDGF-BB, EGF) associated with pulmonary arterial hypertension (PAH) (1, 2) remains to be characterized. Loss-of-function-mutations in the BMP receptor II (BMP-RII) gene occur in 50%–60% of patients with familial PAH (FPAH) (3–5), 10%–20% of patients with idiopathic PAH (IPAH), and 6%–9% of patients with secondary forms of PAH associated with anorexie drug use (fenfluramine derivatives) or congenital heart defects (APAH) (6, 7). However, independent of a mutation, patients with IPAH/FPAH (formerly called “primary PH”), and even those with APAH (formerly called “secondary” PAH), albeit to a lesser extent, have reduced pulmonary expression of BMP-RII (8). Thus, there are likely environmental modifiers and additional genetic factors that contribute to the decreased expression and function of BMP-RII in association with the development of PAH. This would suggest that it might be possible to rescue the adverse sequelae of reduced expression and antimitogenic signaling of BMP-RII by manipulating its downstream effectors to advantage.

Two potential downstream effectors of BMP-RII signaling are the transcription factor PPARγ and its putative target apoE (9). Interestingly, mRNA expression of both factors, in addition to BMP-2, is decreased in lung tissues from PAH patients (8, 10, 11). PPARGs are ligand-activated transcription factors belonging to the nuclear receptor superfamily. Upon ligand activation, PPARs heterodimerize with the retinoid X receptor (RXR) and bind to PPAR response elements (PPREs) in regulatory promoter regions of their target genes (12, 13). PPARs can also interact with signaling molecules to regulate gene expression independent of DNA binding (13). For example, PPARγ impairs phosphorylation (i.e., activation) of ERK (14, 15), a MAPK downstream of PDGF-BB/PDGF-β signaling implicated in SMC proliferation and migration (12).

There is supporting evidence that links PPARγ with transcription of apoE. A functional PPARγ response element is present in the apoE promoter (9), and conditional disruption of the PPARγ gene (Pparg) in mice results in decreased apoE expression in macrophages (16), and PPARγ activation leads to apoE mRNA expression and protein secretion in an adipocyte cell line (17). apoE inhibits PDGF-BB–mediated SMC proliferation and migration (18, 19) by binding to LDL receptor–related protein (LRP) and internalizing the PDGFR-β (20, 21). Heightened arterial PDGF-BB/MAPK signaling is not only evident in apoE–/– mice (22), but is also a key clinical feature of pulmonary vascular disease underlying PAH (2, 23, 24).

We have recently shown that insulin-resistant apoE-deficient (apoE–/–) mice on a high-fat diet develop PAH. However, the fact that a PPARγ agonist reversed PAH in this model (25) suggests that PPARγ targets independent of apoE are also important in suppressing pulmonary vascular remodeling. The antidiabetic drugs rosiglitazone and pioglitazone, both PPARs ligands of the thiazoli-dinedione (TZD) class, inhibit PDGF-BB–induced SMC proliferation and migration in culture and in animal models of systemic cardiovascular disease (reviewed in ref. 12). Because of these and additional antiinflammatory and proapoptotic effects of PPARγ activation (reviewed in ref. 12), PPARγ agonists may be useful in the future treatment of PAH.
Here, we report for the first time to our knowledge that both PPARγ and apoE act downstream of BMP-2/BMP-RII in primary cells (human and murine pulmonary artery SMCs [PASMCs]) and prevent SMC proliferation in response to PDGF-BB. BMP-2–mediated PPARγ activation occurs earlier than Smad1/5/8 phosphorylation and therefore appears to be independent of this established signaling axis downstream of BMP-RII. BMP-2 induces a decrease in nuclear phospho-ERK, and rapid nuclear shuttling and DNA binding of PPARγ, whereas PDGF-BB has the opposite effects. Both BMP-2 and the PPARγ agonist rosiglitazone stimulate production and secretion of apoE in PASMCs. Using short hairpin RNAi in human PASMCs (HPASMCs), PASMCs from a patient with PPAH and a mutation in BMP-RII (W9X), a PPARγ antagonist, and PASMCs lacking PPARγ or apoE, we demonstrate that the antiproliferative effect of BMP-2 is BMP-RII, PPARγ, and apoE dependent. Consistent with these data, we show that mice with deletion of PPARγ1 in SMCs (SM22α Cre PPARγ1−/−/floxflox mice) spontaneously develop PAH. Taken together, our results reveal a novel PPARγ/apoE axis downstream of BMP-2 signaling that could explain the antiproliferative effect of BMP-RII activation in HPASMCs. Our data also suggest that PPARγ agonists might reverse SMC proliferation and vascular remodeling in PAH patients with or without BMP-RII dysfunction.

**Results**

Additional results are provided in the supplemental material (available online with this article; doi:10.1172/JCI32503DS1).

**BMP-2–mediated inhibition of HPASMC proliferation requires BMP-RII, PPARγ, and apoE.** For long-term gene silencing of human BMP-RII, we constructed a pLentivirus 6 with an integrated short hairpin oligonucleotide directed against the mRNA of human BMP-RII (shRNAi). We confirmed, by quantitative RT-PCR, an 85% stable knockdown of BMP-RII mRNA in shBMP-RII versus shLacZi (control) transfected HPASMCs (Supplemental Figure 1). Reconstituted BMP-2 (10 ng/ml) inhibited PDGF-BB–induced proliferation in LacZi control but not in shBMR-RII HPASMCs as judged by cell counts (Figure 1). Results of MTT proliferation assays shown in Supplemental Figure 2 are consistent with cell counts. We reproduced the growth-inhibitory effect of BMP-2, with the same low concentration (10 ng/ml) of BMP-4 and -7, although BMP-7 appeared to have a weaker effect than BMP-2 and -4. Furthermore, with siBMP-RII (knockdown), there was less growth inhibition in response to BMP-2, -4, and -7 (Supplemental Figure 3). We also confirmed that shBMP-RII abolished BMP-2–induced phosphorylation of Smad1/5/8 (Supplemental Figure 4).

We then showed that the BMP-2–mediated inhibition of PDGF-BB–induced HPASMC proliferation requires not only BMP-RII, but also PPARγ. First, the antitrophic effect of BMP-2 could be reproduced by the PPARγ agonist rosiglitazone (1 μM) (Figure 1B). Second, the antiproliferative effect of BMP-2 was lost in the presence of the irreversible PPARγ antagonist GW9662 (Figure 1C). Finally, BMP-2–mediated inhibition of PDGF-BB–induced cell proliferation was not observed in murine PASMCs with deletion of PPARγ but was found in PASMCs from littermate controls (Figure 1D). To address whether the effect of PPARγ could be mediated by induction of apoE, we first established that a physiological dose of recombinant apoE (10 μM) completely blocked PDGF-BB–induced proliferation of HPASMCs (Figure 1E). Moreover, the growth-inhibitory effect of BMP-2 on PDGF-BB–induced cell proliferation was lost in PASMCs from apoE−/− mice (Figure 1F). Taken together, these data support the presence of a novel antiproliferative axis downstream of BMP-2 that requires BMP-RII signaling, PPARγ activation, and production of apoE, a lipoprotein not previously known to be synthesized by SMC. Documentation of apoE production and secretion in HPASMCs in response to BMP-2 and rosiglitazone is described below.

**Opposing effects of PDGF-BB and BMP-2 on phospho-ERK and PPARγ activation in HPASMCs.** We next determined whether BMP-2 and PDGF-BB might have opposing effects on the subcellular localization of phospho-ERK and PPARγ that would explain their functional antagonism in PASMCs. PPARγ has been shown to activate phosphatases and prevent ERK phosphorylation in vascular SMCs (14, 15). In addition, PPARγ activation can directly inhibit PDGF-BB–mediated phospho-ERK activity (26) by blocking its nuclear translocation (27). Conversely, PDGF-BB/PDGFR-β–mediated phosphorylation of ERK leads to phosphorylation and thereby inactivation of PPARγ at its N terminus (28).

PDGF-BB stimulated a 3- to 5-fold increase in phospho-ERK1/2 in nuclear extracts and a 4-fold rise in phospho-ERK1 in cytoplasmic extracts (Figure 2A). BMP-2, however, led to a rapid decrease in phospho-ERK1/2 in nuclear extracts (Figure 2B) and significantly reduced phospho-ERK2 in cytoplasmic extracts (Figure 2B). PDGF-BB rapidly and transiently decreased nuclear protein levels and DNA binding of PPARγ. This decrease in PPARγ DNA binding (Figure 2C, upper panel) temporally coincided with the rapid appearance of phospho-ERK1/2 in the nucleus upon PDGF-BB stimulation (maximum at 5–10 min; Figure 2A). There was no significant change in PPARγ levels in cytoplasmic extracts (Figure 2C). In contrast to PDGF-BB, BMP-2 induced a rapid and marked increase in PPARγ DNA binding (Figure 2D) associated with elevated levels of PPARγ protein in nuclear extracts. This could represent stabilization of PPARγ, but since PPARγ tended to be concomitantly lower in cytoplasmic extracts, transient nuclear shuttling of PPARγ is also likely (Figure 2D). Of note, BMP-2–mediated PPARγ activation in HPASMCs (Figure 2, B and D) occurred earlier than phosphorylation of Smad1/5/8 (Supplemental Figure 4). Therefore, phospho-Smad1/5/8 does not appear to mediate DNA binding of PPARγ.

Interestingly, when we prepared total cell lysates containing the cytoplasmic membrane fraction, we found that BMP-2 induces rapid ERK1/2 phosphorylation (Supplemental Figure 5A). This fraction is absent in nuclear and cytoplasmic extract preparations due to high spin steps. We showed by immunohistochemistry that concomitant with the rapid decrease in phospho-ERK1/2 in the nucleus (shown by Western immunoblot in Figure 2B), BMP-2 led to strong phospho-ERK1/2 staining at the cytoplasmic membrane (Supplemental Figure 5B). It has been previously demonstrated in other cell types that phospho-ERK binds to cytoplasmic membrane proteins such as the receptor for advanced glycation end products (29).

**BMP-2 and a PPARγ agonist inhibit PDGF-BB signaling in HPASMCs.** We next determined whether BMP-2 and PPARγ activation inhibit PDGF-BB–induced MAPK pathways (i.e., phospho-ERK1/2). BMP-2 inhibited PDGF-BB–induced nuclear and cytoplasmic ERK phosphorylation (Figure 3A). BMP-2 also prevented PDGF-BB–mediated inhibition of PPARγ DNA binding. In fact an increase in PPARγ DNA binding was observed with BMP-2 despite concomitant PDGF-BB stimulation (Figure 3B). Moreover, 24-hour preincubation with the PPARγ agonist rosiglitazone significantly reduced and delayed PDGF-BB–induced ERK phosphorylation in total cell lysates (Figure 3C). Hence, BMP-2 and the PPARγ agonist rosiglitazone act as functional antagonists of PDGF-BB signaling by inhibiting ERK1/2 phosphorylation.
Rosiglitazone blocks PDGF-BB–induced proliferation of BMP-RII mutant HPASMCs. We next investigated whether PPARγ activation could inhibit PDGF-BB–induced proliferation of HPASMCs with a loss-of-function mutation in the BMP-RII. Therefore, we isolated PASMCs from the explanted lung of a patient with FPAH known to harbor a frameshift mutation in BMP-RII. BMP-2 inhibited PDGF-BB–induced proliferation in WT but not BMP-RII mutant HPASMCs (Figure 4). In contrast, the PPARγ agonist rosiglitazone blocked PDGF-BB–induced proliferation in both WT and BMP-RII mutant cells so that cell numbers were similar to those in unstimulated controls.

Figure 1
Antiproliferative effects of BMP-2 (A, C, D, and F), the PPARγ agonist rosiglitazone (Rosi; B), and apoE (E) on PDGF-BB–induced proliferation of human (A, B, C, and E) and murine (D and F) PASMCs. PASMCs were seeded at 2.5 × 10^4 cells per well of a 24-well plate in 500 μl of growth medium and allowed to adhere overnight. The cells were washed with PBS prior to the addition of starvation media (0.1% FBS) and incubated for 24 hours (murine PASMCs) or 48 hours (HPASMCs) and then stimulated with PDGF-BB (20 ng/ml) for 72 hours. BMP-2 (10 ng/ml), rosiglitazone (1 μM), and recombinant human apoE (1–10 μM) were added to quiescent cells 30 minutes prior to PDGF-BB stimulation. The PPARγ antagonist GW9662 (GW; 1 μM) was added 24 hours prior to the addition of BMP-2. Cells were finally washed twice with PBS, trypsinized, and counted in a hemacytometer (4 counts per well). Cell numbers in controls at time points 0 (CON) and 72 hours were not significantly different. A: shLacZi, HPASMCs transfected with short hairpin LacZi pLentivirus 6 (control); shBMP-RIIi, HPASMCs transfected with short hairpin pLentivirus 6 BMP-RIIi (i.e., BMP-RII–deficient PASMCs). D: Littermates, littermate control PASMCs; SMC PPARγ–/–, PASMCs isolated from SM22α Cre PPARγflox/flox mice. F: C57BL/6, control murine PASMCs; apoE–/–, PASMCs isolated from apoE-deficient mice. Bars represent mean ± SEM (n = 3 in A, D, and F; n = 4 in B and C; n = 6 in E; n = 12 in controls of A). *P < 0.05; **P < 0.01; ***P < 0.001 as indicated; ANOVA with Bonferroni’s multiple comparison test.
BMP-2 and rosiglitazone, in the (low) concentrations used, had no significant effect on the basal cell proliferation rate (Figure 4). Thus, PPARγ agonists have the potential to rescue the growth-inhibitory effect of BMP-2 in PASMCs with BMP-RII dysfunction.

BMP-2 and rosiglitazone induce apoE expression and secretion in HPASMCs. Since the growth-inhibitory effect of BMP-2 is absent in apoE-deficient PASMCs (Figure 1F), we hypothesized that apoE might be a transcriptional target of BMP-2–activated PPARγ in SMCs. Indeed, both BMP-2 and rosiglitazone induced apoE protein expression (cell lysates) and secretion (supernatant) in HPASMCs (Figure 5A). Moreover, the BMP-2–mediated upregulation of apoE protein was reduced by half in PASMCs harvested from SM22α Cre PPARγ flox/flox mice (Figure 5B). This suggests that the induction of apoE expression by BMP-2 is to a great extent PPARγ dependent.

Creation of mice with targeted deletion of PPARγ in arterial SMCs (SM22α Cre PPARγ flox/flox). To explore the vasoprotective role of PPARγ in preventing the development of PAH in an intact animal, we investigated a transgenic mouse with targeted deletion of PPARγ in arterial SMCs (SM22α Cre PPARγ flox/flox). We documented, by PCR, gain of a new knockout transcript (300 bp) and almost complete loss of the 700-bp wild-type transcript in PASMCs and aorta isolated from SM22α Cre PPARγ flox/flox mice (Figure 6A). Both the wild-type and the knockout transcript were found in lungs from SM22α Cre PPARγ flox/flox mice, since the tissue contains several cell types besides SMCs. In contrast, only the wild-type transcript was detected in lung
tissue from littermate control mice (Figure 6A). We also confirmed knockout of PPARγ protein in PASMCs from SM22α Cre PPARγfl/fl mice (Figure 6B). BMP-2 stimulation of these murine PPARγ-deficient PASMCs revealed intact phospho-Smad1/5/8 signaling that occurred earlier (5–10 minutes; Figure 6C) than in human control PASMCs, where it was observed at 30 minutes (Supplemental Figure 4). Hence, the established BMP-2/phospho-Smad1/5/8 signaling pathway appears to be independent of PPARγ, since it occurs in PASMCs with deletion of PPARγ (Figure 6C).

Mice with targeted deletion of PPARγ in arterial SMCs (SM22α Cre PPARγfl/fl) have PAH. SM22α Cre PPARγfl/fl mice had elevated RV systolic pressure (RVSP) in room air when compared with controls (29.0 versus 21.5 mmHg; P < 0.001; Figure 7A). Systemic blood pressure, RV function (RV dP/dt maximum and minimum) and LV function (fractional shortening, ejection fraction), and cardiac output were not significantly different when comparing the 2 groups (Table 1). In association with elevated RVSP as a measure of PAH, SM22α Cre PPARγfl/fl mice also developed RV hypertrophy (RVH), as judged by the ratio of RV weight to that of the LV and septum (0.46 versus 0.26; P < 0.001; Figure 7A) and the ratio of RV to body weight (P < 0.001; Table 1). SM22α Cre PPARγfl/fl mice had a similar number of pulmonary arteries per 100 alveoli (Table 1) and per surface area (data not shown) but showed more muscularized pulmonary arteries at the alveolar wall level, when compared with littermate controls (Figure 7, C–E). The muscular thickening in small pulmonary arteries seen in lung sections from SMC PPARγ-deficient mice (Movat staining; Figure 7, D and E) was confirmed by immunohistochemistry with specific antibodies for α-SMA (Figure 7, F and G) and associated with an enhanced signal for proliferating cell nuclear antigen (PCNA; Figure 7, H and I) in PASMCs. LV end-diastolic inner diameter (LVIDD), LV end-diastolic posterior wall thickness (LVPWd), and end-diastolic interventricular septum thickness (IVSd) as measures of LV dilatation and LV hypertrophy (LVH) were not different between the 2 genotypes (Table 1). Thus, LV dysfunction does not account for the PAH in SM22α Cre PPARγfl/fl mice. SM22α Cre PPARγfl/fl mice had similar hematocrit and glucose values but slightly higher wbc counts than controls (Table 1).

**Discussion**

This report is the first indication to our knowledge that the anti-proliferative effects of BMP-2/BMP-RII signaling in primary cells (i.e., PASMCs) can be attributed to activation of PPARγ and its
putative transcription target apoE, a protein not previously known to be synthesized and secreted by SMCs (Figure 8A). Furthermore, we establish that endogenous expression of PPARγ in SMCs can protect against the spontaneous development of PAH. Our experiments using a PPARγ antagonist and PPARγ-deficient PASMCs further demonstrate that PPARγ is required for BMP-2-mediated inhibition of PASMC proliferation induced by PDGF-BB. By using RNAi and PASMCs with a known loss-of-function mutation of BMP-RII, we show that BMP-2 requires BMP-RII to block SMC proliferation and provide evidence that BMP-RII dysfunction that occurs with or without BMP-RII mutations (3, 4) could lead to unopposed mitogenic SMC stimulation by PDGF-BB and other growth factors (Figure 8B). BMP-RII dysfunction may, however, be rescued by PPARγ agonists such as pioglitazone or rosiglitazone (Figure 8C), as we have demonstrated in PDGF-BB–stimulated BMP-RII mutant HPASMCs.

In this study, we investigated whether BMP-2 and PDGF-BB might have opposing effects on the growth-inhibitory transcrip-
ation factor PPARγ and the growth-promoting MAPK nuclear phospho-ERK (30). We observed that BMP-2 activation of PPARγ in HPASMCs was independent of the phospho-Smad1/5/8 pathway but correlated with reduced nuclear phospho-ERK expression, presumably due to PPARγ activation of phosphatases (14, 15) or inhibition of phospho-ERK nuclear translocation (27). Conversely, PDGF-BB/PDGFR-β-mediated induction of nuclear phospho-ERK was associated with reduced PPARγ DNA binding, probably due to phosphorylation and inactivation of PPARγ at its N terminus (28) and/or enhancement of nuclear export (31) or ubiquitin/proteasome-mediated degradation and rapid turnover of PPARγ (32). Thus, it may be that continuous endogenous BMP-2/BMP-RII signaling is necessary as a gatekeeper to prevent inactivation of PPARγ and nuclear translocation of phospho-ERK in response to PDGF-BB/PDGFR-β stimulation.

Low-dose rosiglitazone and a physiological dose of recombinant apoE completely blocked PDGF-BB–induced proliferation of HPASMCs, consistent with previous work in systemic SMCs (12, 33). Since we showed that both BMP-2 and rosiglitazone induce apoE protein synthesis and secretion in HPASMCs, we reason that, in addition to lowering phospho-ERK in the nucleus, PPARγ-mediated induction of apoE inhibits PDGF-BB/PDGFR-β signaling (20, 21). The fact that some upregulation of apoE by BMP-2 occurs even in PPARγ-deficient SMCs indicates that apoE also can be regulated by a PPARγ-independent pathway. Further studies using apoE promoter-reporter assays would delineate the nature of PPARγ-mediated transcriptional activation of this target gene.

The spontaneous development of PAH in the SM22α Cre PPARγfloxFlox mice is in contrast to our observations that apoE−/− mice at similar age develop PAH only when fed a high-fat diet leading to insulin resistance (25). Since we found that the PPARγ agonist rosiglitazone can completely reverse PAH in the apoE−/− mouse, multiple other PPARγ-dependent mechanisms in addition to apoE induction may prevent PASMC proliferation and PAH in response to growth factors. In our previous study, we attributed the rescue effect of PPARγ activation to enhanced production of adiponectin, an adipocytokine that sequesters the ligand PDGF-BB, thereby inhibiting SMC proliferation and survival (34). However, we have not been able to detect adiponectin mRNA or protein expression in HPASMCs. Nonetheless, activated PPARγ can induce multiple other growth-inhibitory and proapoptotic gene products and repress growth-promoting factors in vascular cells (Figure 8C). For example, PPARγ activation blocks PDGF gene expression (35) and induces the expression of LRP (36), the receptor necessary for apoE-mediated suppression of PDGF-BB signaling (20, 21) (Figure 8, A and C). PPARγ activation also reduces levels of endothelin-1 (ET-1) (37) and the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) (38, 39), factors that are implicated in the pathobiology of PAH (39). Moreover, activated PPARγ stabilizes the cyclin-dependent kinase inhibitor p27KIP1 (40) and inhibits telomerase activity (41), retinoblastoma protein phosphorylation (40), and ultimately G1 to S phase transition, cell-cycle progression, and vascular SMC proliferation (40). By blocking important survival pathways downstream of activated PDGFR-β, i.e., PI3K (42),

Figure 6
Mice with targeted deletion of PPARγ in SMCs maintain BMP-2–induced pSmad1/5/8 signaling. (A and B) Genotyping of mice with targeted deletion of PPARγ in SMCs. (A) PCR reactions showing gain of a 300-bp knockout transcript and almost complete loss of the 700-bp wild-type transcript in PASMCs and aorta from SM22α Cre PPARγfloxFlox mice. In the lung, which contains SMCs but also many other cell types, both transcripts are found in SM22α Cre PPARγfloxFlox mice, whereas only the wild-type transcript is detected in littermate control mice. (B) Western immunoblotting of PASMC lysates isolated from both littermate and SM22α Cre PPARγfloxFlox (SMC PPARγ−/−) mice (n = 2 each) showed no detectable PPARγ protein expression when compared with control cells. (C) Both littermate control and SMC PPARγ−/− PASMCs were stimulated with BMP-2 (10 ng/ml) for 5–60 minutes as described in the legend for Figure 1, and phospho-Smad 1/5/8 protein expression was detected by immunoblotting as described in Methods (densitometric values were corrected for equal loading using α-tubulin). Data for 1 of 2 representative experiments with similar results are shown.
PPARγ agonists also lead to apoptosis of proliferating vascular cells (12, 43). In addition, it is known that PPARγ ligands impair production of matrix metalloproteinases (44) that can be activated by elastase (45). Our group has shown that inhibition of this proteolytic cascade not only prevents but also reverses advanced fatal PAH in rats (46).

Previous studies have shown beneficial effects of BMP-2 (47), PPARγ activation (reviewed in ref. 12), and apoE (18, 19) in preventing systemic vascular pathology, but our observations are the first indication to our knowledge that all 3 factors are linked. More recently, a connection between PPARγ and apoE has been made in patients with Alzheimer disease, in that the improvement of cognitive function with rosiglitazone is not apparent in patients who carry the APOE epsilon 4 allele (48). Hence, the novel axis we describe may be relevant in addressing mechanisms that underlie many different pathologic processes.

In summary, our data reveal a novel PPARγ/apoE axis downstream of BMP-2 signaling in HPASMCs. Failure to activate PPARγ in response to BMP-2 when there is BMP-RII dysfunction could place a patient at risk for the development or progression of PAH. We suggest that PPARγ agonists might rescue BMP-RII dysfunction and reverse SMC proliferation and vascular remodeling in PAH patients and may be useful antiproliferative agents even in those patients without BMP-RII dysfunction.

Methods
Additional and more detailed methods are provided in the supplemental materials.

Creation of mice with targeted deletion of PPARγ in arterial SMCs using the Cre-loxP system. We cross-bred SM22α promoter–driven Cre-transgenic mice with PPARγ homozygous floxed mice. Both strains were obtained from the Jackson Laboratory, and the cross resulted in SM22α Cre PPARγ<sup>flox/flox</sup> (SMC PPARγ<sup>−/−</sup>) mice. The offspring genotypes were determined by PCR (see Supplemental Methods). PCR conditions and primer information are available from the Jackson Laboratory. For the experiments involving PASMC isolation and subculture described below, apoE-deficient (B6.129P2-Apoetm1Unc/J) and C57BL/6 control mice were purchased from the Jackson Laboratory.

Genotyping/RT-PCR analysis. To detect the deletion of PPARγ exon 1 and exon 2, two primers were designed and located in exon A1 and exon B.
4 of the Ppar1 gene for RT-PCR to recognize the full-length (700 bp) and recombined mRNA (300 bp), as previously described (49) (for primers and PCR protocol, see Supplemental Methods). Total RNA was extracted from PASMCs, aorta, and lung with TRIzol reagent (Invitrogen). PASMCs were obtained from pulmonary arteries of Sm22α Cre PPARγflox/flox mice and littermate control mice and cultured for 10 days. Then RNA samples from the cells were reverse transcribed using the Superscript III Reverse Transcriptase kit (Invitrogen). PCR was continued with further experiments.

**Table 1**

<table>
<thead>
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<th>Hemodynamics</th>
<th>Littermate control</th>
<th>SMC PPARγflox/flox</th>
<th>P</th>
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<td>RVSP (mmHg)</td>
<td>21.5 ± 0.6</td>
<td>29.0 ± 0.6</td>
<td>P &lt; 0.001</td>
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<td>RV dP/dt max (mmHg/s)</td>
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<td>RV dP/dt min (mmHg/s)</td>
<td>−1,228 ± 67</td>
<td>−1,405 ± 63</td>
<td>7–8</td>
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<tr>
<td>Systolic BP (mmHg)</td>
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<td>97 ± 3.1</td>
<td>9–10</td>
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<tr>
<td>MAP (mmHg)</td>
<td>85 ± 2.0</td>
<td>78 ± 3.1</td>
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<td>Diastolic BP (mmHg)</td>
<td>74 ± 1.9</td>
<td>68 ± 3.1</td>
<td>9–10</td>
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</table>

**Echocardiography**

- Heart rate (bpm): 423 ± 22 vs. 411 ± 20 (P < 0.0001), 9–10
- Ejection fraction (%): 72.7 ± 2.2 vs. 76.8 ± 1.2 (9–10)
- Fractional shortening (%): 36.5 ± 1.8 vs. 39.8 ± 1.1 (9–10)
- Cardiac output (ml/min): 35.3 ± 2.7 vs. 38.7 ± 3.6 (9–10)
- LV end-diastolic inner diameter (LVDD, mm): 3.58 ± 0.05 vs. 3.62 ± 0.10 (9–10)
- LV end-systolic inner diameter (LVISD, mm): 2.26 ± 0.09 vs. 2.19 ± 0.09 (9–10)
- LV posterior wall thickness (LVPWd, mm): 0.60 ± 0.04 vs. 0.58 ± 0.03 (9–10)
- IVSd (mm): 0.56 ± 0.02 vs. 0.55 ± 0.02 (9–10)

**Hemodynamics**

- RV/LV+S (0.26 ± 0.01 vs. 0.46 ± 0.02, P < 0.0001, 8–10)
- Heart weight (RV/body weight ×103): 0.26 ± 0.01 vs. 0.46 ± 0.02 (P < 0.0001, 8–10)

**Number and muscularization of pulmonary arteries**

- No. of arteries/alveoli (%): 2.2 ± 0.2 vs. 2.3 ± 0.2 (5–6)
- Musc. arteries, alv. wall (%): 6.5 ± 3.1 vs. 20.6 ± 1.2 (5–6)

**Blood**

- HCT (%): 48.7 ± 0.8 vs. 49.3 ± 1.0 (P = 0.0014, 5–6)
- wbc count (×109 cells/μl): 5.2 ± 0.8 vs. 7.4 ± 0.4 (P = 0.0168, 8–10)
- Glucose (mg/dl): 126.6 ± 6.2 vs. 122.8 ± 4.2 (P < 0.05, 8–10)

**Cell culture**

Primary murine PASMCs were isolated from 13- to 15-week-old apoE−/− and C57BL/6 mice, as well as Sm22α Cre PPARγflox/flox and littermate control mice, using a modified elastase/collagenase digestion protocol as previously described (50). Primary HPASMCs were purchased from Cascade Biologics. Moreover, control PASMCs were isolated from surgical resection specimens derived from a patient undergoing lobectomy or pneumonectomy for suspected lung tumor. Additional PASMCs were obtained from a patient undergoing heart-lung transplantation for FPAH and known to harbor a mutation in BMP-RII (W9X), as previously described (51). The nature of the BMP-RII mutation, cell isolation, and culture techniques are described in Supplemental Methods.

**Cell proliferation assays.** For determination of cell number, PASMCs were seeded at 2.5 × 104 cells per well of a 24-well plate in 500 μl of growth medium and allowed to adhere overnight. The medium was removed and the cells washed 3 times with PBS prior to the addition of starvation media (DMEM, 0.1% FBS, penicillin/streptomycin) and incubated at 37°C, 5% CO2 for 24 hours (murine PASMCs) or 48 hours (HPASMCs) prior to PDGF-BB stimulation (20 ng/ml) for 72 hours (treatments and concentrations are given in the figure legends). The media with or without growth factors and/or inhibitors was changed every 24 hours. Cells were washed twice with PBS and trypsinized in 150 μl of trypsin/EDTA for 7 minutes, followed by the addi-
tion of 150 μl trypsin neutralizer (Cascade Biologics). The cells were then resuspended and counted in a hemacytometer (3–6 wells per condition, 4 counts per well). The biochemical MTT cell proliferation assay (ATCC) is described in Supplemental Methods. In cell proliferation studies, BMP-2 (10 ng/ml; Sigma-Aldrich), rosiglitazone (1 μM; Alexis), or recombinant human apoE (1–10 μM; Chemicon) were added to quiescent cells 30 min-utes prior to mitogenic stimulation with PDGF-BB (human, 20 ng/ml; R&D Systems) for 72 hours. The PPARγ antagonist GW9662 (1 μM; Cayman) was added 24 hours prior to the addition of BMP-2. The media with or without growth factors and/or inhibitors was changed every 24 hours.

Protein expression and compartmental localization. Murine and human PASMCs (wild-type, shLacZi control, or shBMP-RII) were grown to 70% confluence and cultured in starvation media (DMEM, 0.1% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin; Gibco; Invitrogen) for 24 and 48 hours, respectively. PDGF-BB, BMP-2, apoE, or rosiglitazone was added to quiescent cells for 5–60 minutes and for 24 hours (treatments and concentrations are stated in the figure legends). In addition to total cell lysates, subcellular fractions (nuclear matrix, nuclear extract, cytoplasmic extract) were prepared using a modified low-salt/high-salt protocol as previously described (52). For details, see Supplemental Methods.

apoE protein secretion. Quiescent HPASMCs were cultured in T75 cell culture flasks (75 cm²) covered with minimal media (1 ml) and were then stimulated with BMP-2 (10 ng/ml) or rosiglitazone (1 μM) for 24 hours. The supernatant media was collected from 3 cell culture flasks per con-
tion, pooled, and concentrated using an Amicon-4 Centriprep device (Millipore). Protein extracts were then prepared as described in Supplemental Methods, and 10 μg protein per sample was loaded for SDS-PAGE immunoblotting (Invitrogen).

Western immunoblotting. Preparation of subcellular fractions (nuclear matrix, nuclear extract, cytoplasmic extract) and whole-cell lysates (protein extracts) as well as immunoblotting techniques are described in Supplemental Methods. Primary antibodies against phospho-ERK1/2, ERK 1/2, phospho-Smad1/5/8, Smad1 (all Cell Signaling Technology), PPARY (Santa Cruz Biotechnology Inc.), apoE (Abcam), BMP-RII (BD Biosciences – Pharmingen), and α-tubulin (Sigma-Aldrich) were used.

PPARY DNA binding assay. A multiplex transcription factor DNA binding assay (Merklinger, S.L., Jones, P.L., Martinez, E.C., and Rabinovitch, M. 2005. Epidermal growth factor receptor blockade mediates smooth muscle cell apoptosis and improves survival in rats with pulmonary hypertension. Circulation. 112:423–431). Lung parallel to the hilum and embedded it in the same manner. Pulmonary artery muscularization was assessed at x400 magnification by calculating the proportion of fully and partially muscularized peripheral (alveolar wall) pulmonary arteries to total peripheral pulmonary arteries in 5 random fields (1 field = x200 magnification). The total number of alveolar wall and duct arteries was expressed as both the ratio of number of pulmonary arteries per 100 alveoli and number of pulmonary arteries per surface area (5 random fields at x200 magnification). Approximately 1,000 alveoli were counted per animal. All measurements were carried out by investigators blinded to genotype and condition.

Immunohistochemistry. Paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded alcohol. Antigen retrieval was performed using a heat-mediated epitope retrieval method by heating the sections in citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) for 10 minutes at 95°C and then allowing the sections to cool to room temperature. Sections were then incubated with primary antibodies specific for PCNA and α-SMA (Abcam) overnight at 4°C. Staining was then continued using the Vectastain Elite ABC Kit (Vector Laboratories) according to the manufacturer’s instructions, using 3,3-diaminobenzidine as a substrate for peroxidase, and counterstained with hematoxylin.

Fasting whole-blood measurements. Tail vein puncture was performed in nonanesthetized, overnight-starved mice, followed by immediate, duplicate whole-blood glucose measurements with a glucometer (FreeStyle; Abbott), to rule out any influence of the SMC-targeted PPARY knockout on glucose hemostasis. Additional blood was obtained by cardiac puncture after the hemodynamic measurements. White blood cell count and hematocrit were assessed by the Stanford Animal Facility Laboratories (see Supplemental Methods).

Statistics. Values from multiple experiments are expressed as mean ± SEM. Statistical significance was determined using 1-way ANOVA. When only 2 groups were compared, statistical differences were assessed with the unpaired 2-tailed t-test. A P value of less than 0.05 was considered as significant. The number of samples or animals in each group is indicated in the figure legends.

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Address correspondence to: Marlene Rabinovitch, Vera Moulton Wall Center for Pulmonary Vascular Disease, Stanford University School of Medicine, CCSR Building, Room 2245B, 269 Campus Drive, Stanford, California 94305-5162, USA. Phone: (650) 723-8239; Fax: (650) 723-6700; E-mail: marlener@stanford.edu.

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