Bone Morphogenetic Protein Receptor-2 Signaling Promotes Pulmonary Arterial Endothelial Cell Survival

Implications for Loss-of-Function Mutations in the Pathogenesis of Pulmonary Hypertension

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Abstract— Mutations in the bone morphogenetic protein (BMP) receptor-2 (BMPR2) have been found in patients with idiopathic pulmonary arterial hypertension (IPAH); however, the mechanistic link between loss of BMPR2 signaling and the development of pulmonary arterial hypertension is unclear. We hypothesized that, contrary to smooth muscle cells, this pathway promotes survival in pulmonary artery endothelial cells (ECs) and loss of BMPR2 signaling will predispose to EC apoptosis. ECs were treated with BMP-2 or BMP-7 (200 ng/mL) for 24 hours in regular or serum-free (SF) medium, with and without addition of tumor necrosis factor α, and apoptosis was assessed by flow cytometry (Annexin V), TUNEL, or caspase-3 activity. Treatment for 24 hours in SF medium increased apoptosis, and both BMP-2 and BMP-7 significantly reduced apoptosis in response to serum deprivation to levels not different from serum controls. Transfection with 5 μg of small interfering RNAs for BMPR2 produced specific gene silencing assessed by RT-PCR and Western blot analysis. BMPR2 gene silencing increased apoptosis almost 3-fold (P=0.0027), even in the presence of serum. Circulating endothelial progenitor cells (EPCs) isolated from normal subjects or patients with IPAH were differentiated in culture for 7 days and apoptosis was determined in the presence and absence of BMPs. BMP-2 reduced apoptosis induced by serum withdrawal in EPCs from normal subjects but not in EPCs isolated from patients with IPAH. These results support the hypothesis that loss-of-function mutations in BMPR2 could lead to increased pulmonary EC apoptosis, representing a possible initiating mechanism in the pathogenesis of pulmonary arterial hypertension. (Circ Res. 2006;98:209-217.)

Key Words: bone morphogenetic proteins ■ bone morphogenetic receptor-2 ■ pulmonary arterial hypertension ■ endothelial cells ■ endothelial progenitor cells ■ apoptosis

Idiopathic pulmonary arterial hypertension (IPAH) is a rare and fatal disorder, with an estimated incidence of 1 to 2 per million cases per year and a median survival from diagnosis of only 2.8 years. Pulmonary arterial hypertension (PAH) is characterized by increased pulmonary vascular resistance (PVR) caused by vasoconstriction and excessive remodeling of small pulmonary arteries, resulting in widespread microvascular narrowing and obliteration. Clinically, the pulmonary pathology in advanced PAH is characterized by abnormal muscularization of pulmonary arterioles, intimal thickening, and fibrosis, as well as the presence of plexiform lesions, which are believed to result from dysregulation of endothelial cell (EC) growth.

Although increased EC growth may be a feature of late stages of disease, there is increasing evidence that other mechanisms may predominate in early phases of PAH. In experimental models, blockade of EC growth factor receptors resulted in the potentiation of PAH and marked worsening of the pathological vascular remodeling, even reproducing some of the “angioproliferative” features typical of advanced PAH. Interestingly, this effect could be reversed by inhibitors of apoptosis, suggesting that increased apoptosis of ECs in response to loss of survival signaling created conditions favoring the emergence of apoptosis-resistant cells with increased growth potential. Moreover, we have shown that overexpression of EC growth and survival factors, such as...
vascular endothelial growth factor (VEGF) and angiopoietin-1, prevented the development of monocrotaline-induced PAH, an effect that was associated with reduced EC apoptosis. Together, the findings implicate EC apoptosis as a central mechanism in the initiation of PAH and suggest that pulmonary microvascular endothelium is dependent on tonic survival signaling, possibly more so than for other circulations.

The most significant advance in the understanding of the pathogenesis of PAH has been the recent demonstration of germline mutations in the familial form of this disease that have been mapped to a single locus on chromosome 2q31-32. Mutations in the open reading frame of the bone morphogenetic protein (BMP) receptor-2 (BMPR2) gene were identified in 40% of familial PAH and in up to 15% of patients with no family history (ie, IPAH). BMPR2 is a member of the transforming growth factor (TGF)-β superfamily of transmembrane serine/threonine kinase receptors. More than 46 different mutations of BMPR2 have already been identified, some of which have been demonstrated to cause loss of the receptor function. However, how haploinsufficiency in this BMPR2 leads to pulmonary hypertension is unknown.

The ligands for the BMPR2 receptor represent a family of secreted growth factors known as the BMPs. Signal transduction through this pathway involves heterodimerization of BMPR2 with BMPR1, which results in phosphorylation of BMPR1, initiating activation of signal transduction. BMPs can have pleiotropic effects depending on the cell type, the specific ligand, and the environmental context. For example, BMPs can inhibit proliferation and induce apoptosis in human pulmonary artery smooth muscle cells; however, in other cell types, including cardiomyocytes and epithelial cells, signaling via this pathway can have an opposite effect and promote cell survival. Therefore, the role of BMPR2 in the normal lung may be complex, with different biological actions on different vascular cells, and its effect, particularly on pulmonary ECs and endothelial progenitor cells (EPCs) from patients with IPAH, has yet to be clarified.

The aim of the present study was to elucidate the effect of BMPR2 signaling on survival of pulmonary arterial ECs and EPCs isolated from normal subjects or patients with IPAH. We now show for the first time that BMP-2 and BMP-7 promoted survival of human pulmonary arterial ECs as well as EPCs isolated from normal subjects, consistent with a role of the BMPR2 signaling pathway in protecting pulmonary microvascular EC from apoptosis. In contrast, in EPCs isolated from IPAH patients, there was either reduced survival or even an accentuation in apoptosis in response to BMPs. These results suggest a new paradigm for loss-of-function BMPR2 mutations in the pathogenesis of PAH, contributing to the initial endothelial microvascular loss, as well as potentiating reactive arterial remodeling in the remaining arteriolar and arterial bed.

### Materials and Methods

#### Cell Culture and Experimental Design

Human pulmonary artery ECs (HPAECs) were obtained from (Cambrex) and grown in EBM-2 (SingleQuot Media BulletKit, Cambrex) supplemented with 2% fetal bovine serum. All experiments were performed using subconfluent cultures (~80%), of the same batch, derived from pooled donors, and used only between passages 3 to 4. Culture conditions for other cell types are described in the online data supplement available at http://circres.ahajournals.org. To induce apoptosis, cells were exposed to tumor necrosis factor (TNF)-α (20 ng/mL; R&D) or serum withdrawal for 24 hours in the presence or absence of BMP-2 or BMP-7 (200 ng/mL; R&D).

Cells were pretreated with BMPs for 2 hours before exposure to TNF. After treatment, cells were either suspended for flow cytometry.
Apoptosis Assays
Apoptosis was assessed by 3 independent methods.

Flow Cytometry
Apoptotic and necrotic cell death was assessed in HPAECs by flow cytometric analysis (Annexin V-FLUOS Staining Kit, Roche) under control or apoptosis-inducing conditions, in the presence or absence of BMPs. At the end of the treatment period, cells were suspended by a brief trypsinization (0.05% with EDTA) and washed twice with cold PBS. Cells were then resuspended in 100 μL of 1× binding buffer with 2 μL of Annexin V–fluorescein isothiocyanate (FITC) and 2 μL of propidium iodide (PI). The cells were gently mixed and incubated for 15 minutes at room temperature in the dark, and then 400 μL of 1× binding buffer was added to each tube before analysis. Fluorescence was induced with the 488-nm argon laser and monitored at 512 nm (FL1) and 543 nm (FL3) on a Beckman Coulter Cytomics FC500 analyzer.

TUNEL Staining
After the various treatments, cells were fixed in 2% paraformaldehyde in PBS for 10 minutes and washed 3 times with PBS, permeabilized with 0.2% Triton-X, and stained using the Dead/End Fluorometric apoptosis detection system (Promega), following the instructions of the manufacturer, producing fluorescein TUNEL staining and PI nuclear counterstaining. Merged images were generated by dual scanning at 488 nm and 543 nm with a confocal microscope (Bio-Rad, Radiance 2100) and TUNEL-positive or -negative cells were counted in 6 random fields per well in a blinded fashion.

Caspase-3 Activity
A fluorometric assay kit (Promega) was used for detection of activated caspase-3. Freeze/thaw cytosolic extracts were incubated at 30°C with the fluorogenic substrate, 7-amino-4methyl coumarin (Ac-DEVD-AMC) in 96-well microliter plates, and emission fluorescence was detected at 460 nm after excitation at 360 nm. Results were calculated from a standard curve per the instructions of the manufacturer. Activated caspase-3 was also assessed by Western analysis as described in the online data supplement.

Silencing of BMPR2 mRNA
Gene silencing of BMPR2 was achieved using small interfering RNA (siRNA) as described by Elbashir and colleagues (see online data supplement). Forty-eight to 72 hours after transfection, HPAECs were lysed with 100 μL of sodium dodecyl sulfate (SDS) sample buffer (10 mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.4% dithiothreitol, 1 mmol/L orthovanadate), and BMPR2 expression was assessed by semiquantitative RT-PCR and Western blot analysis, as described in the online data supplement.
Isolation and Differentiation of EPCs
All use of human material was approved by the Research Ethics Boards at St. Michael’s Hospital and the University Health Network. EPCs were isolated from peripheral blood as described previously.21 Briefly, peripheral venous blood was obtained from 16 patients with IPAH recruited from the Pulmonary Hypertension clinic (age 45±13 years, 15 women; see Table) or 5 normal subjects (age 38±11 years, 4 women) after written informed consent. The mononuclear cell fraction was isolated by Ficoll–Paque density gradient (Becton Dickinson) centrifugation and washed 3 times with PBS, and cells were plated at a density of 1.5±10^6 mononuclear cells/mL on fibronectin-coated culture slides (Becton Dickinson) in basal medium-2 (EBM-2; Cambrex) supplemented with 5% FBS, with human VEGF-A, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, and ascorbic acid (EGM-2 MV SingleQuot Media BulletKit; Cambrex). Cells were grown for 7 days with culture media changes every 48 hours and then characterized as described in the online data supplement. EPCs were treated with BMP-2 (200 ng/mL) for 24 hours in EGM-2 medium in the presence or absence of 5% FBS, and the number of cells undergoing apoptosis was determined by flow cytometry.

Statistical Analysis
Data are presented as mean±SEM. Significance of differences was assessed using ANOVA followed by post hoc unpaired t test, unless otherwise specified. The significance of relationships between variables was assessed using linear regression. A value of P<0.05 was considered statistically significant. All analyses were conducted using InStat (Prism) software.

Results
Effect of BMPR2 Ligands on EC Survival
Exposure of HPAECs for 24 hours to SF medium significantly increased apoptosis (Figure 1A and 1B, a and b). Addition of BMP-2 or -7 (200 ng/mL) markedly reduced the number of the cells undergoing apoptosis in response to serum withdrawal (c and d). Figure 1C presents summary data of the results of 5 experiments showing that serum withdrawal produced a significant increase in Annexin V staining in cultured HPAECs, which could be largely prevented by the addition of either BMP-2 or -7 for 24 hours. Consistent with these results, we also observed that BMP-2 protected against apoptosis induced by TNF-α assessed by TUNEL staining (Figure 2A and 2B). In separate experiments, treatment with BMP-7 also significantly reduced apoptosis in response to serum deprivation from 23.85±1.20% to 1.98±0.54% (P<0.05). Similarly, caspase-3 activity in response to serum deprivation was significantly reduced in cells treated with both BMP-2 and -7 compared with control cells in SF conditions (Figure 2C). Moreover active caspase-3, assessed by the presence of cleaved 19 and 17 kDa subunits by Western blot analysis, was reduced by BMP-2 and -7 treatment compared with control cells in SF conditions, as show on representative blot (Figure 3A) and summary data (Figure 3B), again confirming that treatment with BMP-2 or -7 for 24 hours markedly reduced apoptosis. A similar protective effect in response to BMP-2 and BMP-7 was seen in human arterial endothelial cells (HAEC) (supplemental Figure I).

Effect of Reduced BMPR2 Expression on EC Survival
To explore whether the protective effects of BMPs were indeed mediated through BMPR2 signaling, we used specific
silencing siRNA to knockdown BMPR2 expression (Figure 4). Forty-eight hours after transfection with siRNA, a \( >50\% \) knockdown of BMPR2 protein expression relative to \( \beta\)-actin was observed by Western analysis, whereas there was no effect of the nonsilencing siRNA (data not shown). Inhibition of BMPR2 receptor expression using specific siRNA significantly increased apoptosis in HPAECs cultured in the presence of serum (Figure 5), confirming the important role of this pathway in mediating EC survival signaling under basal conditions.

**Effect of BMPs on Endothelial Progenitor Cells**

In the next series of experiments, we examined the functional significance of these findings using EPCs isolated from the circulating blood of patients with IPAH. EPCs represent an accessible source of cells that can be differentiated to an endothelial phenotype and thus can provide a useful surrogate for somatic human endothelial cells. After 7 days in differential culture, EPCs exhibited a typical endothelial-like morphology (Figure 6A, a), and \( >80\% \) of cells were positive for EC markers including Tie2, VEGFR2, and UEA-1 lectin (Figure 6, b and d, respectively). EPCs from patients were qualitatively similar to those from normal controls, and neither showed evidence of differentiation to myofibroblast lineage under the conditions studied (supplemental Figure II).

There was no significant difference in basal rates of apoptosis in EPCs from patients with IPAH versus normal controls 24 hours of serum withdrawal (21.06 ± 2.89\% versus 14.58 ± 3.05\%, respectively), although there was a tendency toward slightly higher rates in IPAH cells when grown in the presence of serum (7.69 ± 2.04\% versus 3.18 ± 0.71\%, respectively). However, with the addition of BMP-2, EPCs from control subjects showed a highly significant reduction in the rate of apoptosis (\( \approx 50\% \) inhibition, Figure 6B) consistent with a protective effect of BMP/BMPR2 signaling in a manner analogous the normal HPAECs. In contrast, the protective effect of BMP-2 was markedly altered in EPCs derived from IPAH patients compared with those isolated from normal subjects. Although some patients exhibited weak protection, the majority demonstrated a paradoxical increase in apoptosis in response to BMP-2, and overall this response...
was significantly different from cells derived from normal subjects \( (P=0.02) \). Interestingly, the magnitude of response to BMP-2 was associated with the hemodynamic severity the PAH \( (P<0.05) \), such that the patients with the highest PAP exhibited the most dramatic increase in apoptosis (Figure 6C).

Expression of BMPR2 Receptor by Human EPCs
We assessed BMPR2 receptor expression to determine whether the heterogeneous response to BMP-2 in EPCs from patients with PAH might be attributable to varying levels of receptor expression. Cells from both control subjects and patients with PAH showed similar patterns of BMPR2 expression by immunostaining (Figure 7A). Moreover, there were no significant differences in mean BMPR2 mRNA levels assessed by quantitative RT-PCR between EPCs from PAH patients and controls (Figure 7B), although there was considerable individual variation, with some patients exhibiting reduced expression and others exhibited levels up to 2- to 3-fold higher than the mean. However, when BMPR2 mRNA expression was plotted against magnitude of apoptosis in response to BMP-2, no relationship was seen (Figure 7C).

Discussion
This is the first demonstration that the BMP/BMPR2 pathway protects against EC apoptosis, which is in sharp contrast to its well-established proapoptotic and growth inhibitory effects in smooth muscle cell (SMC) lines. In this report, we show that BMPs decreased apoptosis in response to serum deprivation and TNF-\( \alpha \), whereas knockdown of the BMPR2 using siRNA increased the basal level of apoptosis in normal HPAECs. Moreover, defects in this pathway may be relevant clinically, because we also show altered response to BMP-2 in EPCs isolated from patients with IPAH compared with those from normal subjects. These observations may have important implications regarding the link between mutations in the BMPR2 gene and the development of disease and suggest that, in addition to dysregulated SMC growth, patients with this mutation may be more susceptible to EC loss, which has now been implicated as an initiating event in the pathogenesis of PAH in various experimental models.4,22

The recent identification of the association between mutations of the BMPR2 receptor and idiopathic PAH,8,11,23,24 both familial and sporadic, represents a major advance toward an understanding of the complex pathogenic mechanisms that underlie this fatal disease. Although BMPR2 is expressed by both pulmonary artery endothelial and SMCs, most investigations have focused on the potential importance of BMPR2 mutations on SMCs25 and a clear picture has emerged that BMP signaling represents an inhibitory pathway, which prevents excessive pulmonary arterial muscularization by reducing SMC growth and increasing apoptosis.15,16 However, if anything BMPR2 is found to a greater extent on the pulmonary vascular endothelium than in the surrounding SMCs.26 Moreover, lung vascular endothelium has been recently reported to exhibit high levels of activation of
downstream signaling molecules (ie, SMADs),27 further implicating the endothelial BMPR2 system in this disease. PAH has also been described in patients harboring mutations in endothelial restricted gene, the ALK-1, another member of the TGF family of receptors.28–30 These mutations that are well known to be associated with arteriovenous malformations that are characteristic of hereditary hemorrhagic telangiectasia (HHT).28,29 The fact that mutations in an endothelial-restricted gene have now been linked to IPAH further implicates the endothelium as a critical target in the molecular pathogenesis of this disease. Therefore, the elucidation of the functional importance of BMP signaling in ECs as opposed to SMCs may provide important insight into the mechanisms whereby mutations of this receptor result in predisposition for PAH.

Our results strongly suggest that BMPs protect against apoptosis in HPAECs, which support a role for the BMPR2 pathway in survival signaling in normal human pulmonary endothelium. Therefore, mutations in the BMPR2 receptor, and possibly other related pathways, may lead to increased EC loss in response to environmental triggers. Loss of microvascular endothelium, particularly at the level of the fragile precapillary arterioles, which consist of little more than endothelial tubes, could result loss of continuity of distal arterioles, thus progressively excluding portions of the microvasculature from the pulmonary circulation.6 Indeed, we have shown that loss of precapillary arteriolar continuity precedes the development of PAH in the monocrotaline model and that gene transfer of EC survival factors such as VEGF and angiopoietin-1, prevented the development pulmonary vascular disease.5,6 Similarly, inhibition of VEGF signaling was reported to result in marked potentiation of PAH in the chronic hypoxic model, associated with exaggerated vascular remodeling and evidence of angioproliferative lesions.4 Interestingly, this was completely rescued by the use of the caspase inhibitor, Z-Asp,4,22 again suggesting that EC apoptosis plays a central role in this model.

In this report, we also examined the effect of BMPs on survival of EPCs harvested from the circulating blood of patients with IPAH compared with normal subjects. Increasingly, it is recognized that these bone marrow–derived cells circulate postnatally in the peripheral blood and are believed to home to regions of the vasculature with injured endothelial lining, and a variety of diseases are characterized by alterations in the number or quality of circulating EPCs.31–34 The present data show that EPCs from patients with IPAH are abnormal in terms of their response to BMPs. Although the mutation status of our PAH cohort is not known, it is likely that the majority of these patients with sporadic disease would not harbor a mutation in the BMPR2 gene.35
reduction in the survival in response to BMP-2 is consistent with a possible downregulation of receptor expression, as has been previously reported in the pulmonary vasculature of patients with IPAH. However, we saw no overall reduction in BMPR2 expression in EPCs from patients with IPAH compared with normal controls. Rather, the increase in apoptosis, which was seen in cells from the majority of patients, is consistent with abnormal signaling in response to BMPs, as was described in SMCs of patients with IPAH.

Of note, there was a significant correlation between the degree of EPC apoptosis induced by BMP and the severity of hemodynamic abnormality in the pulmonary bed. Although this might indicate that patients exhibiting this response were predisposed to develop severe PAH, we cannot exclude a confounding effect of hemodynamic factors (ie, increased shear forces) or concomitant drug therapy (ie, epoprostenol).

Nevertheless, the present results suggest that mutations of BMPR2, and possibly related genes as well, could lead to diametrically opposite consequences in ECs and SMCs, which may both contribute importantly to the development of PAH (supplemental Figure III). In pulmonary endothelium, loss of BMPR2 signaling may increase the susceptibility to programmed cell death in response to injurious environmental stress, particularly at the level of the distal “precapillary” arterioles. EC apoptosis could be an initiating mechanism for IPAH, leading directly to microvascular obliteration, as a result of degeneration of these fragile endothelial structures. Alternatively, repeated waves of EC loss may indirectly result in a reactive increase in proliferation of the remaining endothelium and provide conditions favoring the emergence of apoptosis-resistant, hyperproliferative ECs, which is characteristic of the later stages of this disease. In contrast, the same mutation in pulmonary SMCs will result in the loss of inhibitory regulation of cell growth, thus amplifying medial hypertrophy occurring in response to abnormal pulmonary hemodynamics and increased vasoconstrictor/growth factor expression (ie, serotonin and endothelin). Taken together, this paradigm may help explain why the lung is uniquely susceptible to extreme vascular remodeling resulting from haploinsufficiency in a growth and differentiation receptor that is ubiquitously expressed in all tissues throughout the body.

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Detailed Methods

Cell culture: Human aortic ECs (HAEC), lung microvascular ECs (HMVEC-L), dermal fibroblast (NHDF-Ad) and pulmonary artery smooth muscle cells (PASMC) were cultured in media as recommended by the supplier (Singlequot Media Bullet Kit; Cambrex: EBM-2; EGM-2MV; FGM-2; SmGM-2; respectively).

Silencing of BMPR2 mRNA: Four siRNAs (synthesized by Qiagen) designed from the human BMPR2 target cDNA sequence (accession NM_001204) were tested, maximal inhibition was attained with the a duplex based on the following sequence AACCCTCTCTTGATCTAGATA as follows: r(CCCUCUCUUGAUCUAGAUA)d(TT) and r(UAUCUAGAUCAAGAGGG)d(TT), and this duplex was used for all subsequent experiments. HPAECs were seeded at density 3-3.5x10⁵ cells/well in 6-well plates and transfected with 5µg of annealed double strand siRNA using 15µl of RNAiFect Transfection Reagent (Qiagen). After 12 hours, complexes were removed and gene silencing was monitored after 48 and 72 hr by measuring mRNA and protein levels as described below. Nonsilencing siRNA (NS), with no known homology with any mammalian genes was used as a negative control to eliminate the possibility of nonspecific silencing effects, and siRNA directed against the human lamin A/C gene (Qiagen) was used as a positive control.

Caspase-3 Western Blotting: After 24 hr of treatment with BMP-2 or BMP-7 cells were lysed with 100µl of SDS sample buffer and subjected to SDS-polyacrylamide gel
electrophoresis (PAGE) in 14% Tris-Glycine gels (Invitrogen), as described below. The membranes were then incubated overnight with a rabbit polyclonal antibody to cleaved caspase-3 (Asp 175, Cell Signaling) at a dilution of 1:1000, in TBS with 5% skim milk, followed by an anti-rabbit IgG secondary antibody conjugated to horse-radish peroxidase (1:2500, 1 hr; Promega). Bands of 17/19kDa represented activated caspase-3 resulting from cleavage adjacent to Asp175 were visualized using enhanced chemiluminescence substrate system ECL. Total level of full length caspase-3 (35kDa) was detected with caspase-3 antibody (1:1000, Cell Signaling). Densitometry was performed as described below.

**Evaluation of BMPR2 by Western blot analysis**: HPAEC were lysed with 100µl of SDS sample buffer (10mmol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.4% dithiothreitol, 1mmol/L orthovanadate) and 50 µg of total protein per lane and 50ng of recombinant human BMPR2/Fc chimera (positive control 70kDa) were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in 4-20% Tris-Glycine gels (Invitrogen), transferred to nitrocellulose membranes and blocked in 5% BSA in TBST buffer (10mM/L Tris (pH 7.5) 150 mM/L NaCl, pH 7.5) and 0.1 % Tween 20. The membranes were then incubated overnight with goat anti-human polyclonal antibodies to BMPR2 (R&D) at a dilution of 1:500, in TBS with 3% BSA, followed by an anti-goat IgG secondary antibody conjugated to horse-radish peroxidase (1:2500, 1 hr; Promega). The BMPR2-specific band (115 kDa) was visualized using an enhanced chemiluminescence substrate system ECL (Amersham Pharmacia Biotech). The blots were then stripped and reprobed with monoclonal anti β-actin for in TBST with 5% skim milk (1: 8000; Sigma).
In some experiments, a mouse monoclonal antibody directed against the lamin A/C gene product (NovoCastra) was used as positive control. Densitometry was performed and the intensity of each band was analyzed using the Molecular Analyst software (Imaging Densitometer, BioRad).

**Semi-quantitative RT-PCR:** RNA was isolated using the GenElute mammalian total RNA kit (Sigma). 2 µg of total RNA from HPAEC after BMPR2 silencing, and 200ng of RNA from human EPCs (control subjects and patients with PAH) were reverse transcribed in 20 µl volume using the Omniscript RT kit (Qiagen) with 1 µg random primers (Invitrogen). Subsequently, RTs were used for semi-quantitative or real-time quantitative RT-PCR respectively. For each RT product, aliquots (2-10 µl) of the final reaction volume was amplified in 2 parallel PCR reactions using Taq polymerase (Pharmacia Biotech Amersham) and the following primers:

**BMPR2**

forward: 5’-TAACACTCAAACCTCACC-3’; reverse: 5’-CACCAGTCTATTTCCAGTC-3’;

**GAPDH**

forward:5’-CTCTAAGGCTGTGGGCAAGGTCT-3’; reverse: 5’-GAGATCCACCACCCCTG-TTGCTGTA-3’.

The reaction was carried out for 25 cycles with an annealing temperature of 51.50 C and 600 C respectively. The amplification products were separated on 2% agarose gels and visualized by ethidium bromide staining. The expected size of PCR products for BMPR2, and GAPDH were 791bp and 343 bp, respectively. Quantification of RT-PCR products
was performed by densitometry. Results are presented as the ratio between the target genes and GAPDH.

**Real-time quantitative RT-PCR to evaluate expression level of BMPR2 in human EPC from patients with PAH:** Quantitative PCR was performed using the ABI prism 7700 sequence detection system (ABI, Foster City, CA). 1 µl of each RT was added to the real-time PCR amplification mixture (final volume, 20 µl) containing 10 µl of TagMan Universal PCR Master Mix and 1 µl TaqMan gene expression assay mix (predesigned gene-specific primers and a 6-FAM dye-labeled TaqMan MGB minor groove binder probe, Applied Biosystem). GAPDH, as endogenous control was amplified in parallel with the genes of interest using primers from Applied Biosystems. The reaction conditions were as follows: initial activation of hot-start TagMan probe at 95°C for 10 min, followed by 40 cycles consisting of 15 sec. at 95°C for denaturing and 1 min at 62°C for annealing and extension. Data were analysed using SDS 2.1 (Applied Biosystems) to obtain the threshold cycle (Ct) values. Relative quantification of mRNA expression was calculated using the software tool, RESTO, provided by Pfaffl et al. Results were calculated as the expression difference between samples comparing the normal controls, normalized to the gene of reference (GAPDH). We used the $2^{-\Delta\Delta Ct}$ method to determine the relative gene expression, as described by Livak KJ and Schmittgen TD.

**EPC Characterization:** Cells were plated at 1.5 x 10^6 cells/ml in 2-well fibronectin-coated chamber slides and grown for 7 days in EGM-2MV as described in Methods. Cells were fixed with 2% paraformaldehyde in PBS for 10 minutes and separate wells
stained with various EC specific markers: EC-lectin (UEA-1, FITC-conjugated, Sigma Aldrich), rabbit anti-human VEGFR-2 (KDR/Flk-1, Calbiochem) and mouse anti-human Tie2 (Clone AB33, Upstate Biotechnology), followed by exposure to a FITC conjugated secondary antibody (either anti- goat, anti rabbit or anti-mouse; Vector Laboratories). BMPR2 receptor was visualized in by immunostaining with anti-human polyclonal BMPR2 (R&D) follow by Alexa Fluor 488 anti-goat secondary antibody (Invitrogen).

**Immunostaining for smooth muscle alpha-actin (SMA):** To eliminate the possibility of differentiation to SMC or myofibroblast, EPCs cultures were stained for smooth muscle alpha-actin (SMA), using a mouse monoclonal anti-human SMA antibody (DAKO, Clone 1A4), followed secondary antibody for detection goat anti-mouse IgG conjugated to AlexaFluor 546 (Molecular Probes).

Human SMCs and fibroblasts were used as positive controls for SMA expression. Actin filaments in all cells were visualized with AlexaFluor 546-conjugated phalloidin (Molecular Probes, 0.25U/ml). Cells were counterstained with TO-PRO-3 as a nuclear marker (Molecular Probes) and mounted in Vectashield mounting medium (Vector Laboratories). Cells were then visualized by confocal microscopy (BioRad Radiance 2100) and the percentage of cells expressing, or absence of SMA, was recorded.
Results

Effect of BMPs on HAEC survival: Consistent with results with HPAEC, exposure of not pulmonary EC (HAECs) for 24hr to serum free (SF) medium significantly increased apoptosis (Figure WS-1). Similarly, addition of BMP-2 or -7 (200 ng/ml) markedly reduced the number of the cells undergoing apoptosis in response to serum withdrawal (Figure WS-1). Similar results were obtained with human lung microvascular endothelial cells (data not shown).

Absence of myofibroblast in EPC cultures: Cultures of HPASMC, human dermal FBs and EPC from patients with PAH were stained with SMA Figure WS-2 (panel A, C and E; respectively) or with phalloidin (panel B, D and F; respectively). EPCs from patients with PAH did not show any staining for SMA, unlike HPASMCs and FBs. As well, while the other cells which showed well organized stress fiber, human EPCs had little cytoplasm and exhibited a lower levels of stress fiber.

Figure legends

Figure WS-1. Effect of BMPs of serum deprivation-induced apoptosis in HAEC. HAECs were grown in EBM-2 medium supplemented with 2% FBS and growth factors or in serum free (SF) medium to induce apoptosis in the presence or absence of BMP-2 or BMP-7. Cells were labeled with Annexin V-FITC and propidium iodide (PI) and apoptosis was assessed by Flow Cytometry (FACS). Summary data of 3 experiments showing the effect of BMP-2 or BMP-7 for 24hr in serum free medium. * = p<0.05 vs. FCS; † = p< 0.05 vs. SF
Figure WS-2. Immunofluorescence microscopy of human PASMC (A, B) human FB (C, D) and human EPC (E, F) stained for smooth muscle cell marker (SM α actin, red) and (phalloidin, green). Nuclei were counterstained with TO-PRO (blue).

Figure WS-3. “Double Jeopardy” hypothesis of the role of BMPR2 mutations in the pathogenesis of IPAH. Loss-of-function mutations in the BMPR2 gene lead to opposite consequences in pulmonary endothelial cells (ECs) and smooth muscle cells (SMCs), these opposing responses interact synergistically to produce the phenotypic features of IPAH. In SMCs, this pathway mediates inhibition of cell growth, and therefore it is thought that BMPR2 mutations contribute to marked medial hypertrophy of pulmonary arteries a characteristic of advanced IPAH. However, the same pathway promotes survival of ECs, and thus mutations would be expected to lead to increased susceptibility to pulmonary EC apoptosis in response to environmental triggers, possibly directly contributing to damage of the fragile precapillary pulmonary microvessels as an initiating mechanism of IPAH. Together, these differential effects might explain why BMPR2 mutations result in a unique pulmonary vascular phenotype.

Reference List

(1) Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002;1;30:e36.

% of Annexin V positive cells

Serum | SF | BMP-2 | BMP-7

# p<0.05 vs. serum
* p<0.05 vs. NS

WS-1