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Primary Pulmonary Hypertension Is Associated With Reduced Pulmonary Vascular Expression of Type II Bone Morphogenetic Protein Receptor

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Background—Mutations in the type II receptor for bone morphogenetic protein (BMPR-II), a receptor member of the transforming growth factor-β (TGF-β) superfamily, underlie many familial and sporadic cases of primary pulmonary hypertension (PPH).

Methods and Results—Because the sites of expression of BMPR-II in the normal and hypertensive lung are unknown, we studied the cellular localization of BMPR-II and the related type I and II receptors for TGF-β by immunohistochemistry in lung sections from patients undergoing heart-lung transplantation for PPH (n=11, including 3 familial cases) or secondary pulmonary hypertension (n=6) and from unused donor lungs (n=4). In situ hybridization was performed for BMPR-II mRNA. Patients were screened for the presence of mutations in *BMPR2*. In normal lungs, BMPR-II expression was prominent on vascular endothelium, with minimal expression in airway and arterial smooth muscle. In pulmonary hypertension cases, the intensity of BMPR-II immunostaining varied between lesions but involved endothelial and myofibroblast components. Image analysis confirmed that expression of BMPR-II was markedly reduced in the peripheral lung of PPH patients, especially in those harboring heterozygous *BMPR2* mutations. A less marked reduction was also observed in patients with secondary pulmonary hypertension. In contrast, there was no difference in level of staining for TGF-βRII or the endothelial marker CD31.

Conclusions—The cellular localization of BMPR-II is consistent with a role in the formation of pulmonary vascular lesions in PPH, and reduced BMPR-II expression may contribute to the process of vascular obliteration in severe pulmonary hypertension. **(*Circulation*. 2002;105:1672-1678.)**

Key Words: hypertension, pulmonary ■ immunohistochemistry ■ receptors

Primary pulmonary hypertension (PPH) is a rare disorder, with an estimated incidence of 1 to 2 per million per year.[1] The disease is characterized by vascular cell proliferation and obliteration of small pulmonary arteries,[2] which leads to severe pulmonary hypertension (PH) and right ventricular failure. Typical morphological appearances include increased muscularization of small arteries and thickening or fibrosis of the intima.[3] The term plexogenic arteriopathy is used because of the existence of plexiform lesions (200 to 400 μm in diameter), which are capillary-like channels adjacent to small pulmonary arteries.[4]

Recently, heterozygous germline mutations that involve the gene encoding the type II bone morphogenetic protein receptor (*BMPR2*), a member of the transforming growth factor-β (TGF-β) receptor superfamily, have been found to underlie many cases of familial[5,6] and sporadic[7] PPH. The precise molecular mechanisms of disease pathogenesis remain to be elucidated but are likely to involve altered BMPR-II function.[8] In support of this, we recently found that compared with cells from control subjects or patients with secondary PH, pulmonary artery smooth muscle cells from patients with PPH fail to respond to the growth-suppressive effects of bone morphogenetic proteins (BMPs).[9]

Further insight into the role of mutant BMPR-II in the pathogenesis of PPH would be gained by detailed knowledge of the cellular distribution of this receptor in the normal and PH lung. This information might also be useful to identify the key cell types involved in initiation of the disease. The aim of the present investigation was to determine the pattern of expression and cellular localization of BMPR-II in the human lung. Considerably more is known regarding the expression of TGF-β receptors in the normal and diseased systemic vasculature, where abnormal TGF-β signaling contributes to the pathogenesis of atherosclerosis[10] and restenosis after

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Clinical Data for Patients With PH

Patient	Sex/Age, y	Mean PAP, mm Hg	Cardiac Output, L/min	Underlying Condition	Final Diagnosis	*BMPR2* Mutation Analysis
1	M/41	79	3.2	None	PPH	ND
2	F/25	95	3.1	None	PPH	Partial deletion
3	M/48	56	NA	None	FPPH	Partial deletion
4	M/23	88	3.5	None	PPH	ND
5	F/50	90	3.0	None	PPH	ND
6	F/19	69	2.7	None	PPH	ND
7	F/28	70	3.5	None	FPPH	2q33 Allele sharing
8	F/59	67	2.6	None	FPPH	355delA
9	F/36	73	3.7	None	PPH	ND
10	M/46	NA	NA	None	PPH	ND
11	F/41	60	NA	None	PPH	ND
12	F/42	NA	NA	VSD	SPH	ND
13	F/47	NA	NA	VSD	SPH	ND
14	F/30	NA	NA	VSD	SPH	ND
15	F/31	82	NA	VSD	SPH	ND
16	M/35	97	2.6	CTE	SPH	ND
17	F/33	54	NA	Scleroderma	SPH	ND

PAP indicates pulmonary arterial pressure; NA, not available; FPPH, familial PPH; ND, not detected; VSD, ventricular septal defect; SPH, secondary PH; and CTE, chronic thromboembolism.

injury.[11] It has been suggested that an additional acquired somatic mutation may be necessary for the clinical manifestation of PPH in a genetically susceptible individual.[5] This hypothesis remains attractive, because somatic mutations in the TGF-β signaling pathway are relatively common and may contribute to the pathogenesis of certain tumors[12] and atherosclerosis.[13]

In the present study, we demonstrate that BMPR-II mRNA and protein expression is mainly localized to the endothelium in the normal pulmonary circulation, although it is also expressed by myofibroblasts comprising concentric intimal lesions in severe PH. In addition, we report that pulmonary vascular BMPR-II expression is reduced in PPH cases, most markedly in those PPH cases with an underlying mutation in the *BMPR2* gene.

Methods

Lung Tissue Preparation

The PH lung tissue used in the present study comprised material from patients undergoing heart-lung transplantation for PPH (n=11) or secondary PH (n=6) at Papworth Hospital, Cambridgeshire, UK. The Huntingdon Local Research Ethics Committee approved this study. Material was fixed in 10% phosphate-buffered formalin by airway perfusion immediately after resection, then processed for sectioning (3 μm). Samples were selected from specimens that showed features of excellent tissue preservation and adequate lung inflation. Adult control lung was obtained from unused donor lungs for heart-lung transplantation.

Immunohistochemistry

Paraffin-embedded lung sections were processed by an antigen retrieval technique. Endogenous peroxidase was quenched by treatment with hydrogen peroxide (Dako). Sections were microwaved (Euroserv 750-W microwave) on full power for 10 minutes in 0.01 mol/L sodium citrate solution (pH 6). Anti-BMPR-II polyclonal antibody designated SMN (a gift from Dr K. Miyazono, Tokyo, Japan[14]; dilution 1:30) was raised against a peptide corresponding to amino acid residues 185 to 202 of the 1038–amino acid BMPR-II sequence. Polyclonal antibodies raised against the human TGF-β type I receptor (TGF-βRI; 10 μg/mL) and TGF-β type II receptor (TGF-βRII; 10 μg/mL) were obtained from Research Diagnostics, Inc. To identify cells of endothelial lineage in serial sections, the monoclonal antiendothelial cell markers (all from Dako) anti-CD31 (1:80), anti-von Willebrand factor (1:20), anti-CD34 (1:400) were used. The monoclonal anti-α-smooth muscle actin (Dako) identified cells of smooth muscle lineage, and anti-CD68 (1:400) (Dako) identified macrophage cells. Primary antibodies were incubated for 1 hour at room temperature. Antigen was visualized with a labeled streptavidin biotin peroxidase technique (Dako Chemate LSAB, Dako) with diaminobenzine substrate. Control and PH cases were batched (25 to 50 sections) and processed together in a semiautomated protocol (Dako Techmate 500 autostainer). The specificity of immunostaining was demonstrated by the absence of signal in sections incubated with antibody after absorption with the peptide against which the antibody was raised and after omission of the primary antibody. Sections were then counterstained with hematoxylin and examined by light microscopy.

Image Analysis

Two methods were used to semiquantify the results of immunohistochemistry. First, PH sections were examined by 2 observers unaware of the underlying diagnosis (C.A. and N.W.M.). The observers identified lesions and classified them as concentric intimal lesions or plexiform lesions. Immunostaining for BMPR-II, TGF-βRII, and CD31 was classified as present or absent and the number of positive lesions expressed as a percentage of the total number of lesions in each case. Second, images were acquired from 5 randomly selected fields and stored as digital image files. Images were imported into an image-analysis software package (Aeqatis). This allowed calculation of the total tissue area and the area of positive immunostaining, which was then expressed as a percentage.

In Situ Hybridization

A 424-bp cDNA fragment (nucleotides 803 to 1226) of human BMPR-II cDNA was ligated into the *Pst*I/*Xho*I site of the plasmid

vector pBluescriptKS(+). In vitro transcription from the T3 and T7 promoters yielded antisense and sense probes, respectively. Transcripts were labeled with sulfur-35-uridine triphosphate (^{35}S-UTP; $>$1200 Ci $\cdot$ mmol^{-1} $\cdot$ L^{-1}; New England Nuclear). After hybridization with cRNA probe, slides were processed as described previously.[15] Slides were exposed for up to 10 weeks at 4°C. After development of the photographic emulsion, slides were stained with hematoxylin and eosin. Silver grains were visualized by light- and dark-field microscopy.

Mutation Analysis of the *BMPR2* Gene

The entire protein-coding region and intron/exon boundaries of the *BMPR*2 gene were amplified by polymerase chain reaction, as described previously.[5] Amplified fragments were sequenced with a dye-terminator cycle-sequencing system (ABI PRISM 377, Perkin-Elmer Applied Biosystems). In addition, gene dosage analysis of *BMPR2* exons 1 and 12 was performed to detect partial gene deletions, as described previously.[8] In 1 individual with familial PPH in whom no *BMPR2* mutations were identified by these methods, allele-sharing studies of the *BMPR2* locus were performed, as described previously.[16]

Results

Clinical Data

Clinical data for patients with PPH and secondary PH are shown in the Table.

Mutation Analysis of the *BMPR2* Gene

A total of 3 germline *BMPR2* mutations were detected among the 11 PPH patients studied. Direct DNA sequencing was used to identify an exon 3 frameshift mutation (355delA) in 1 patient with familial PPH (patient 8) that created a premature truncating codon, which, if translated, may fail to reach the cell surface.[5] *BMPR2* gene dosage studies determined heterozygous partial gene deletions in 1 patient with familial PPH (patient 3) and 1 with sporadic PPH (patient 2), the extent of which remains to be determined.[8] In the remaining patient with familial PPH (patient 7), no *BMPR2* mutations were detected; however, allele-sharing studies in this family were consistent with linkage to the *BMPR2* locus at 2q33. No mutations in *BMPR2* were identified in patients with secondary PH.

Cell Types Comprising Vascular Lesions

We performed immunohistochemical studies to determine the main cell types present within vascular lesions in PH. Expression of endothelial cell markers was confined to the monolayer of cells lining vascular channels (Figure 1). The remaining cell layers comprising concentric lesions stained heavily for α-smooth muscle actin (Figure 1). Although the vascular channels of plexiform lesions stained for endothelial cell markers, the stroma surrounding these channels also stained for α-smooth muscle actin. Staining with anti-CD68 demonstrated the presence of macrophages in many lesions (Figure 1).

Expression of BMPR-II mRNA and Protein

In normal lungs, hybridization to BMPR-II mRNA was predominantly observed on vascular endothelial cells, with focal expression in the underlying smooth muscle and interstitial cells, which appeared to be macrophages (Figure 2). The signal was similar in endothelial cells that lined larger

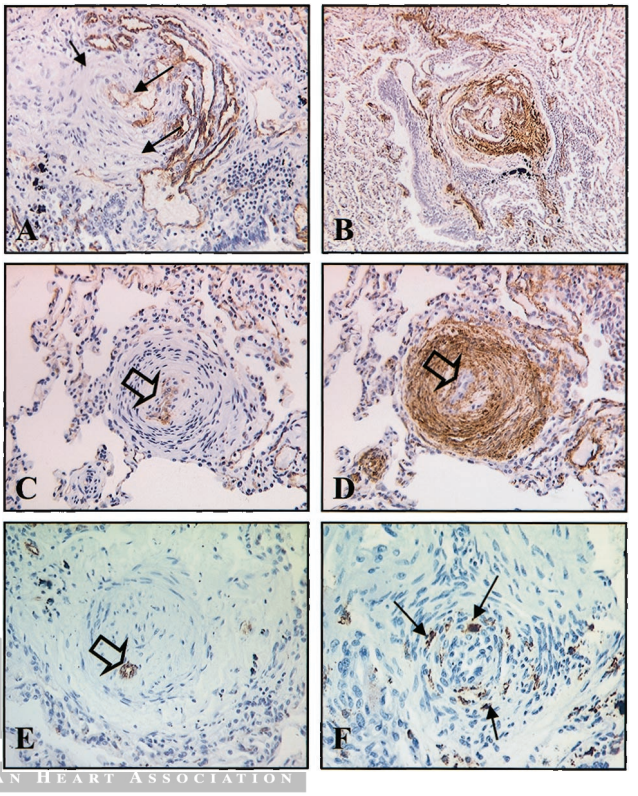

Figure 1. Photomicrographs of lung sections from patients with PPH demonstrating cellular composition of vascular lesions. Plexiform lesions demonstrate positive immunostaining of endothelial marker CD31 (A) on single layer of cells lining endothelial channels (arrowed), whereas supporting stroma of plexiform lesion stains positively for α-smooth muscle actin (B). In concentric intimal lesions, single layer of cells adjacent to vascular lumen (arrow) stains for endothelial markers CD31 (C) or von Willebrand factor (E). Concentric layers of cells comprising vascular wall stain for α-smooth muscle actin (D). Numerous macrophages stained with anti-CD68 are present within many lesions (arrowed; F). Approximate magnification $\times$200.

(200- to 500-μm diameter) and small ($<$200 μm-diameter) pulmonary arteries and alveolar capillary endothelium. Despite repeated attempts, specific hybridization to BMPR-II mRNA was low in PH cases (not shown). Immunohistochemistry confirmed the predominantly endothelial localization of BMPR-II protein in normal lung (Figure 3) but also suggested some focal weaker expression by vascular smooth muscle. BMPR-II immunostaining was also demonstrated in macrophages. In PH cases, BMPR-II protein expression was observed on endothelial cells and myofibroblasts comprising intimal lesions (Figure 3). Endothelial cells lining the vascular channels of plexiform lesions also expressed BMPR-II (Figure 3).

Immunolocalization of TGF-β Receptors

In normal lungs, TGF-βRI and TGF-βRII colocalized predominantly to vascular endothelium, with focal staining of the underlying smooth muscle (Figure 4). However, airway epithelial staining was also prominent. In PPH and secondary PH, TGF-βRI and TGF-βRII immunostaining remained prominent on endothelial cells, including those of plexiform lesions, as well as on myofibroblast cells comprising intimal lesions.

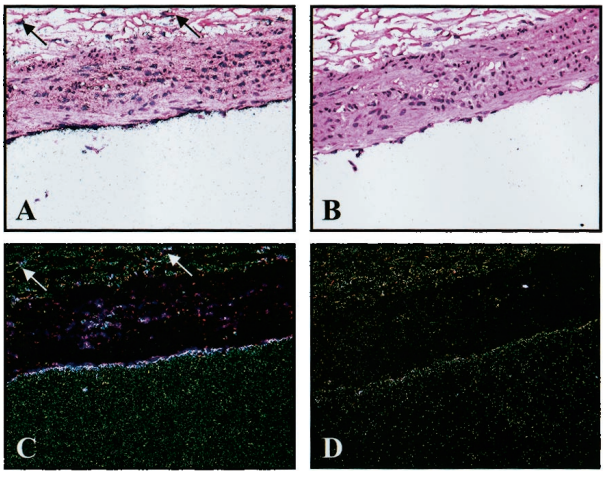

Figure 2. Light-field (A and B) and dark-field (C and D) photomicrographs of in situ hybridization for BMPR-II mRNA expression in normal pulmonary artery. Antisense probe (A and C) demonstrated BMPR-II mRNA expression predominantly in endothelial cells lining vascular lumen, with focal expression by underlying medial smooth muscle cells. Incubation of sections with control (sense) probe (B and D) showed no hybridization. Approximate magnification ×200.

Semiquantification of Immunostaining

Comparison of the staining intensity of endothelial cells for BMPR-II revealed a generally reduced level of BMPR-II

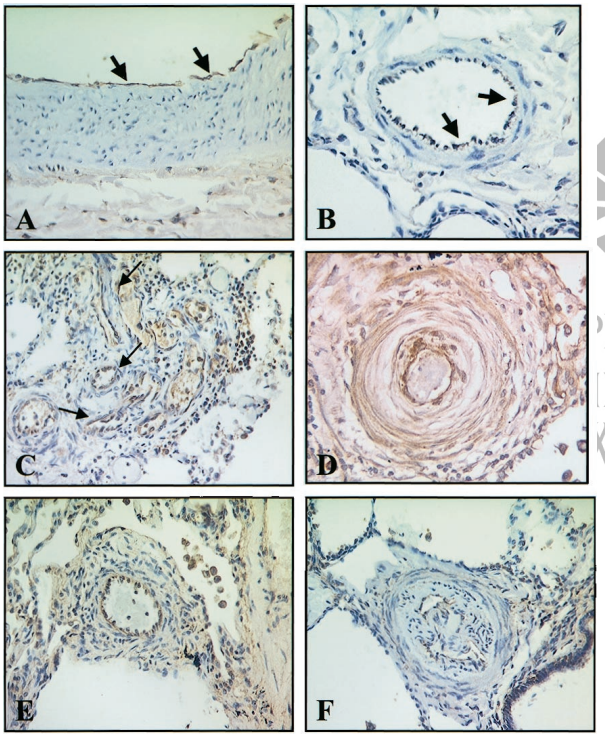

Figure 3. Photomicrographs of lung immunostained for BMPR-II. In normal arteries (A and B), BMPR-II immunostaining predominates in endothelium (arrowed). In vascular lesions of PPH (C through F) and secondary PH (D and E), endothelial staining is again prominent, including cells lining vascular channels of plexiform lesions (C, arrowed) and endothelial cells lining concentric lesions (D through F). In addition, BMPR-II expression is noted on myofibroblast cells comprising intimal lesions (D). Approximate magnification ×200.

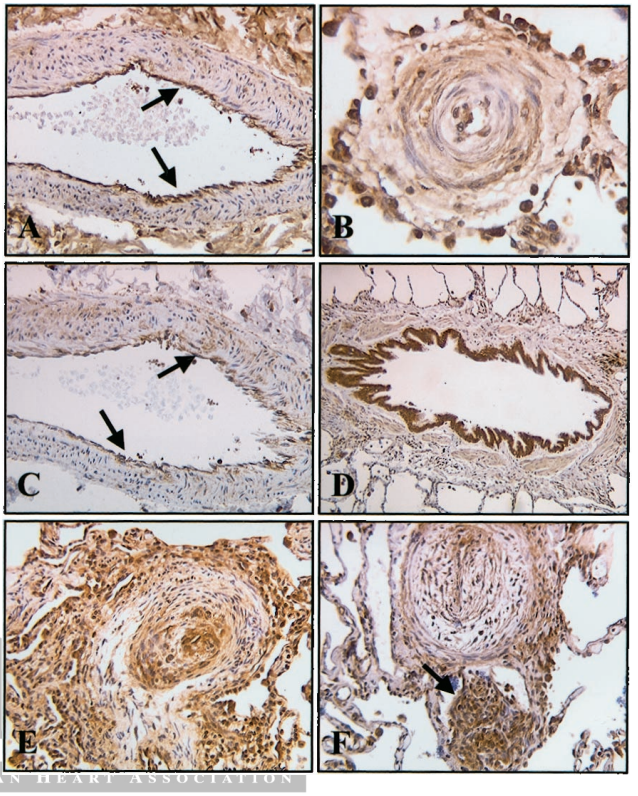

Figure 4. Photomicrographs of lung immunostained with anti-TGF-βRI (A and B) or anti-TGF-βRII (C through F). A, TGF-βRI immunostaining is prominent on endothelial cells lining normal pulmonary artery, with focal staining of underlying smooth muscle. B, Within intimal lesions, TGF-βRI is found on endothelium and on underlying myofibroblasts within lesion. TGF-βRII has similar distribution in normal artery (C) and is also prominent on airway epithelial cells (D). In concentric intimal lesions, endothelial and underlying myofibroblast expression of TGF-βRII is observed (E and F). The majority of plexiform lesions also expressed abundant TGF-βRII (F, arrowed). Approximate magnification ×100 (D), ×200 (A,C,E,F), and ×400 (B).

protein expression in PPH cases compared with control or secondary PH cases (Figure 5). Semiquantification of the level of immunostaining by image analysis revealed that staining was significantly decreased in all PH cases, with the most marked reductions observed in PPH cases that harbored mutations predicted to cause loss of protein expression or linkage to the *BMPR2* locus (Figure 6). In contrast, the area of tissue stained by anti-CD31 and anti-TGF-βRII was similar between groups (Figure 6).

Because some vascular lesions in PH cases appeared not to stain for BMPR-II, we performed a further analysis to compare the proportion of vascular lesions that were positive for BMPR-II and TGF-βRII in each PH group. Between 5 and 12 lesions were examined per case. The proportion of positive stained lesions was reduced in PPH cases that harbored a mutation or demonstrated linkage to 2q33 (Figure 7). In contrast, similar proportions of lesions stained positively for TGF-βRII between groups (Figure 7).

Discussion

This study has demonstrated the cellular distribution of BMPR-II in the normal and hypertensive lung. Our observa-

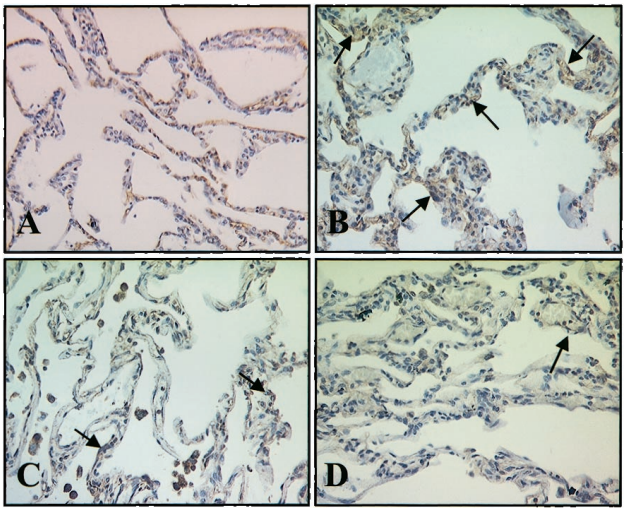

Figure 5. Photomicrographs of peripheral lung stained for BMPR-II from control subject (A) and patients with secondary PH (patient 12; B), PPH in which no *BMPR2* mutation was identified (patient 4; C), and PPH in which truncating mutation in BMPR2 was identified (patient 8; D). Note reduced alveolar capillary staining for BMPR-II (arrowed) compared with control, particularly in PPH cases. Approximate magnification ×200.

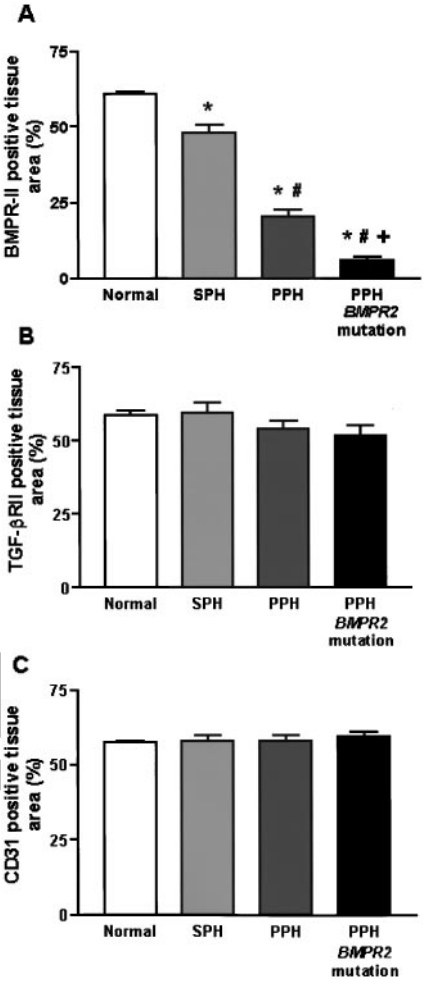

Figure 6. Semiquantification of lung tissue area positively stained for BMPR-II (A), TGF-βRII (B), and CD31 (C) in controls and patient groups. SPH indicates secondary PH. *$P<0.05$ vs control, #$P<0.05$ vs SPH, +$P<0.05$ vs PPH, by ANOVA and Tukey multiple comparison test.

tions suggest that this receptor is predominantly expressed by endothelial cells in the pulmonary circulation, with a lower level of expression in vascular smooth muscle. In addition, BMPR-II is expressed within the characteristic lesions found in PPH, specifically by endothelial cells of plexiform lesions and by endothelial and myofibroblast cells in the intimal lesions. The cellular localization of BMPR-II to key cell types implicated in the vascular remodeling of PPH supports the suggestion from genetic studies that mutations in this receptor play a causal role in disease pathogenesis. Furthermore, the observation that BMPR-II protein expression is reduced in the lungs of patients with severe PH, most markedly in patients harboring heterozygous germline mutations in *BMPR2*, suggests that reduced BMPR-II signaling may be implicated not only in the in the pathogenesis of PPH in which *BMPR2* mutations have been identified but also in other mutation-negative cases of PPH, and possibly in secondary forms of PH.

To the best of our knowledge, this is the first study to examine the cellular localization of BMPR-II expression in human lung. Northern blots have revealed that BMPR-II is widely expressed in various human tissues, with a high level of expression in lung tissue.[14] Although BMPR-II mRNA was readily demonstrated in normal lungs, we could not reliably detect hybridization in diseased lungs. In light of our immunohistochemical findings, it may be that reduced expression of BMPR-II protein in severe PH is, at least in part, transcriptionally regulated.

We performed mutation analysis of genomic DNA from all patients with severe PH. Three germline mutations were identified (1 frameshift and 2 partial deletions) that were predicted to cause reduced expression of BMPR-II protein.[8] In another familial PPH case, no mutation was identified, but linkage to the *BMPR2* locus at 2q33 was demonstrated. Comparison of the level of immunostaining in known mutants with that in other PPH and secondary PH cases revealed an obvious reduction in the level of BMPR-II protein expression, with absent expression in some lesions. The complete lack of staining within some vascular lesions was most evident in the PPH cases with *BMPR2* mutation or evidence of linkage. Further semiquantification of the area of peripheral lung tissue stained positively for BMPR-II revealed that BMPR-II protein expression was dramatically reduced in PPH cases with *BMPR2* mutation or linkage. However, there was also a marked reduction in the level of BMPR-II immunostaining in PPH cases in which no mutation was identified. In these cases, the endothelium remained intact, as judged by a normal level of staining for CD31. Moreover, the observation appeared specific for BMPR-II in that the level of immunostaining for a related receptor, TGF-βRII, was similar between groups. Interestingly, a less marked but significant reduction in the level of BMPR-II immunostaining was also observed in secondary PH. These results suggest that reduced expression of BMPR-II may contribute not only to the pathogenesis of PPH but also to other forms of severe PH. Similar reductions in the level of expressed protein have been

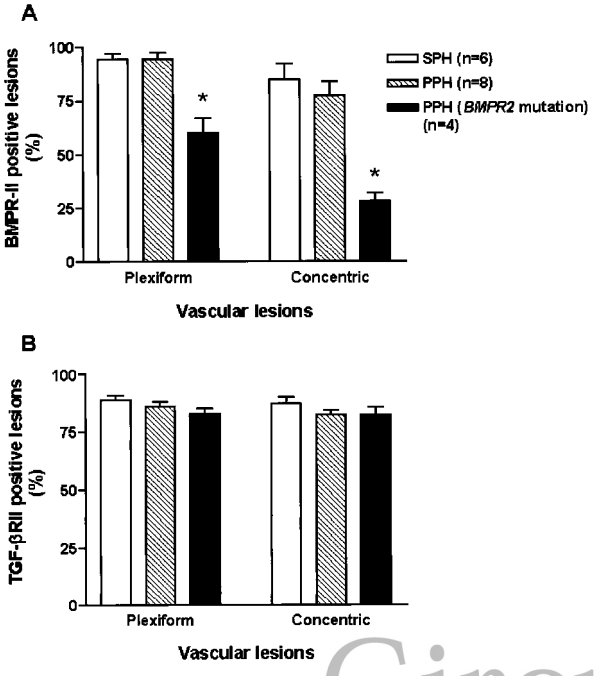

Figure 7. Analysis of frequency of lesions stained positively for BMPR-II (A) and TGF-βRII (B) in patient groups. *$P<0.05$ compared with PPH or secondary PH groups, by ANOVA and Tukey multiple comparison test. SPH indicates secondary PH.

noted in hereditary hemorrhagic telangiectasia, with an underlying heterozygous null mutation in the gene encoding endoglin.[17] An intriguing possibility is that additional loss of the wild-type *BMPR2* allele or reduction in BMPR2 transcription may be necessary to trigger the formation of vascular lesions in patients harboring a germline mutation. This "second hit" might explain the low penetrance of the condition within families.[5] Additional molecular analysis of individual lesions will be necessary to determine whether such a mechanism occurs. Analysis of tissue from the systemic circulation of PPH patients would be necessary to determine whether the marked reduction in BMPR-II expression is restricted to the lung, which may in part explain the lung-specific nature of this disease.

A recent report identified an increased frequency of somatic mutations that arose as a result of microsatellite instability in the *TGF-βRII* gene[18] in plexiform lesions of PPH patients. The present study found that ≈20% of vascular lesions in severe PH demonstrated absent expression of TGF-βRII but no difference in the frequency of negative lesions between PPH and secondary PH groups. However, it remains conceivable that germline mutations in *BMPR2* and somatic mutation in *TGF-βRII* may have similar and/or additive effects, removing the growth-inhibitory effect of TGF-β superfamily members and allowing clonal expansion of vascular cells, leading to occlusion of small pulmonary arteries.

Although a limited number of studies have suggested a role for TGF-β in hypertensive pulmonary vascular remodeling,[19] its precise role is poorly defined. Immunostaining for TGF-β isoforms, especially TGF-β2 and TGF-β3, appears to be increased in actively remodeling hypertensive pulmonary arteries in patients with PPH compared with controls.[19] However, TGF-β is not a known ligand for BMPR-II, and the cellular localization of BMP expression in the lung is yet to be determined.

We have recently reported that pulmonary artery smooth muscle cells from patients with PPH exhibit altered growth responses to BMPs in that BMPs suppress proliferation of cells from normal controls and secondary PH patients but not PPH patients.[9] We speculate that the failure of BMP-induced growth suppression may contribute to abnormal proliferation of vascular cells in PPH. Interestingly, in that study,[9] cells from all PPH patients behaved similarly, whether a mutation in *BMPR2* was present or not. The findings of the present study provide a possible explanation for this in that markedly reduced BMPR-II expression was a feature of all PPH cases, whether or not *BMPR2* mutations were present. Thus, reduced expression of BMPR-II protein, either as a result of underlying mutation or transcriptional repression of *BMPR2*, may contribute to the abnormal vascular cell responses to BMPs in PPH.

The nature of the cell type responsible for the intimal obliteration of small pulmonary arteries in PPH has been the subject of considerable debate.[3,18,20] The evidence, supported by our findings, suggests that the intimal lesion is composed of myofibroblast-like cells, which stain positively for α-smooth muscle actin and vimentin, and that the expression of endothelium-specific markers is confined to cells lining the vascular lumen.[3,21] The plexiform lesion would appear to consist of a proliferation of endothelial cells supported by a myofibroblast stroma.[3,4,22,23]

In summary, the present study identifies predominantly endothelial cells but also myofibroblast cells comprising intimal lesions as the sites of pulmonary vascular expression of BMPR-II in PPH and secondary PH. The reduced or absent expression of BMPR-II in vascular lesions of PPH patients, especially in those harboring an underlying mutation in the *BMPR2* gene, supports the hypothesis that loss of BMPR-II function contributes to the formation of lesions.

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