ORIGINAL ARTICLE

RNA Sequencing Analysis Detection of a Novel Pathway of Endothelial Dysfunction in Pulmonary Arterial Hypertension

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Abstract

Rationale: Pulmonary arterial hypertension is characterized by endothelial dysregulation, but global changes in gene expression have not been related to perturbations in function.

Objectives: RNA sequencing was used to discriminate changes in transcriptomes of endothelial cells cultured from lungs of patients with idiopathic pulmonary arterial hypertension versus control subjects and to assess the functional significance of major differentially expressed transcripts.

Methods: The endothelial transcriptomes from the lungs of seven control subjects and six patients with idiopathic pulmonary arterial hypertension were analyzed. Differentially expressed genes were related to bone morphogenetic protein type 2 receptor (BMPR2) signaling. Those down-regulated were assessed for function in cultured cells and in a transgenic mouse.

Measurements and Main Results: Fold differences in 10 genes were significant ($P < 0.05$), four increased and six decreased in patients versus control subjects. No patient was mutant for *BMPR2*. However, knockdown of BMPR2 by siRNA in control pulmonary arterial endothelial cells recapitulated 6 of 10 patient-related gene changes, including decreased collagen IV (COL4A1, COL4A2) and ephrinA1 (EFNA1). Reduction of BMPR2-regulated transcripts was related to decreased β-catenin. Reducing COL4A1, COL4A2, and EFNA1 by siRNA inhibited pulmonary endothelial adhesion, migration, and tube formation. In mice null for the EFNA1 receptor, EphA2, versus control animals, vascular endothelial growth factor receptor blockade and hypoxia caused more severe pulmonary hypertension, judged by elevated right ventricular systolic pressure, right ventricular hypertrophy, and loss of small arteries.

Conclusions: The novel relationship between BMPR2 dysfunction and reduced expression of endothelial COL4 and EFNA1 may underlie vulnerability to injury in pulmonary arterial hypertension.

Keywords: pulmonary hypertension; vascular endothelium; transcriptome; ephrin; collagen IV

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At a Glance Commentary

Scientific Knowledge on the Subject: The endothelium is an early site of injury in the pathogenesis of pulmonary arterial hypertension, and loss of endothelial control of pulmonary vascular tone and structure contributes to the adverse remodeling and to the increased pulmonary artery pressure and resistance that define the disease. Transcriptomic approaches allow for broad comparisons of the gene expression profiles of diseased and healthy tissues and shed light on novel pathways that are relevant to pathogenesis.

What This Study Adds to the Field: This is the first RNAseq transcriptome comparison of pulmonary arterial endothelial cells from patients with idiopathic pulmonary arterial hypertension versus controls, and a novel set of genes is now implicated in disease pathogenesis. Although the search was unbiased, it is particularly of interest that most of those genes differentially expressed could be reproduced in control cells by reducing levels of bone morphogenetic protein receptor 2, which is mutated in familial and dysfunctional in other forms of pulmonary arterial hypertension. The down-regulated genes further pursued, collagen IV (A1 and A2) and ephrin A1, support cell adhesion and angiogenesis and are implicated in restoration and regeneration of damaged blood vessels. Thus, reduced levels of these genes may underlie the inability of the pulmonary vasculature to respond to and repair the damage observed in pulmonary hypertension. Consistent with observations in cultured cells, we also provide evidence that mice lacking the main receptor for ephrin A1 (EphA2) develop more severe pulmonary hypertension under conditions where there is endothelial vulnerability to apoptosis.

Idiopathic pulmonary arterial hypertension (IPAH) is a rare but debilitating disease characterized by endothelial cell (EC) dysfunction and pulmonary vascular obliteration and loss (for details, *see* Reference 1). These features cause a progressive increase in resistance to flow that can culminate in right heart failure and in the patient's demise. Several lines of evidence pinpoint the pulmonary arterial (PA) endothelium as playing a critical role in the pathogenesis of IPAH and pulmonary arterial hypertension (PAH) associated with other primary conditions (APAH). Pulmonary arterial ECs (PAEC) cultured from patients with PAH display impaired angiogenesis (2–5), altered bioenergetics (6), and chromosomal/genetic instability not present in other cell types (7). These features influence PAH pathogenesis and the potential for reversibility of this condition. Moreover, PAECs are the major site of expression of bone morphogenetic protein type 2 receptor (BMPR2) in the lung (8), and mutations in *BMPR2* underlie most heritable cases and a small proportion of sporadic cases of IPAH (9, 10). Reduced BMPR2 expression and function is also described in patients with IPAH or with APAH who do not have a mutation (8). Loss of BMPR2 impairs PAEC survival (2–5) and barrier function (11). Our previous studies demonstrated that BMPR2 signaling activates β-catenin/peroxisome proliferator-activated receptor (PPAR) γ–mediated transcription of PAEC genes, such as apelin (3, 4). In PAH PAECs, we have shown that BMPR2 deficiency and the resultant reduction in apelin can lead to apoptosis, and an inability to form vascular networks and to control smooth muscle cell proliferation (4, 5).

We reasoned that a comprehensive search for genes that are dysregulated in PAH ECs could improve our understanding of the mechanisms underlying the dysfunction of these cells and might facilitate development of targeted therapies. Such unbiased approaches to gene expression analysis in PAH have been performed on peripheral blood mononuclear cells (12), whole lung tissue (13), and microdissected vessels (14). A recent study investigated proteomic changes in blood outgrowth ECs from four control subjects and four heritable patients with PAH (15) and another compared metabolomic and transcriptomic changes in PAECs engineered with a *BMPR2* mutation (16), but no studies to date have specifically addressed changes in gene expression and the functional significance of those features in PAECs derived from patients with IPAH versus those from control lungs. This opportunity has been made possible through the Pulmonary Hypertension Breakthrough Initiative Network, which collects lung tissue from patients with IPAH and APAH and from unused donor control lungs (*see* the online supplement).

Moreover, a new opportunity to sensitively detect specific changes in gene expression is available through RNA-sequencing analysis (RNAseq) (17). We therefore applied RNAseq to compare gene expression profiles of ECs derived from the lungs of patients with IPAH with those obtained from unused donor lungs as control subjects. Significant changes in gene expression were related to reduced BMPR2 and a downstream effector, β-catenin. The functional significance of three transcripts that were highly down-regulated in IPAH PAECs (collagen IV-A1 and A2 [COL4A1, COL4A2] and ephrinA1 [EFNA1]) were investigated in cultured cells, and one (EFNA1) was pursued in a transgenic mouse with loss of the cognate receptor EphA2.

Methods

An expanded section is included in the online supplement.

Cell Culture

PAECs from patients with IPAH and unused donor control PAECs were harvested and cultured according to previous protocols (4) from lungs obtained through the Pulmonary Hypertension Breakthrough Initiative Network of transplant centers described in the Acknowledgment section. Demographic information regarding patients and unused donor control lungs is provided in Table 1.

Commercially available human PAECs (PromoCell, Heidelberg, Germany) were used for studies not involving patient-derived cell lines. PAECs were cultured in EC medium containing growth factors, 5% fetal bovine serum, and penicillin/streptomycin (ScienCell, Carlsbad, CA).

RNAseq Analysis of PAECs

We used PAECs from control subjects and subjects with IPAH at similar passage 3–5 for RNAseq and reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) experiments. RNA was

Table 1. Characteristics of Patients with IPAH and Unused Donor Control PAECs Used in RNAseq Experiments

Patient ID	Age/Sex	Race	Cell Type	Diagnosis/Cause of Death	PAP S/D/M (*mm Hg*)	PVR (*Wood Units*)	Total PAH Medications
Cells used in RNAseq experiment 1							
Con-1	57/F	White	MV	Intracranial hemorrhage/ stroke			
Con-2	41/F	White	MV	Grade IV subarachnoid hemorrhage, ruptured anterior cerebral artery aneurysm			
Con-3	19/M	White	MV	Brain anoxia			
PAH-1	56/F	White	MV	FPAH, no BMPR2 mutation	110/55/75	29.6	Epoprostenol, bosentan, ambrisentan, sildenafil
PAH-2	49/F	White	MV	IPAH	100/50/75	16.8	Ambrisentan, sildenafil, epoprostenol
PAH-3	55/F	African American	MV	IPAH	89/41/53	12.3	Sildenafil, bosentan, epoprostenol
Cells used in RNAseq experiment 2							
Con-4	40/F	White	MV	Motor vehicle accident: extensive subarachnoid hemorrhage			
Con-5	52/F	White	MV	Hypoxic brain death			
Con-6	55/M	White	MV	Anoxia			
Con-7	14/M	White	MV	Gunshot wound to head			
PAH-4	54/F	White	MV	IPAH	100/45/60	12.0	Sildenafil, epoprostenol, ambrisentan, bosentan
PAH-5	56/F	White	MV	IPAH	83/39/57	11.4	Sildenafil, ambrisentan, treprostinil
PAH-6	25/M	White	SPA	IPAH	65/15/36	9.0	Epoprostenol, sildenafil, treprostinil

Definition of abbreviations: BMPR2 = bone morphogenetic protein type 2 receptor; Con = control; FPAH = familial pulmonary arterial hypertension; ID = identification; IPAH = idiopathic pulmonary arterial hypertension; MV = microvasculature; PAEC = pulmonary arterial endothelial cell; PAH = pulmonary arterial hypertension; PAP = pulmonary artery pressure; PVR = pulmonary vascular resistance; S/D/M = systolic/diastolic/mean; SPA = small pulmonary arteries <1 mm by dissection.
PAH medications are listed according to total drug exposure during treatment period of patient, not necessarily in combination.

extracted from 90–100% confluent PAECs in growth medium and RNAseq libraries were prepared. The libraries were sequenced on 1–2 HiSeq 2000 lanes to obtain an average of approximately 100 and 150 million uniquely mapped reads for control and IPAH groups, respectively.

For all control and IPAH samples, raw expression (RPKM) data were filtered to exclude any genes that did not meet the prespecified criteria of more than five RPKM. To allow comparisons across samples, the RPKM data distributions were quantile-normalized using the R package "preprocessCore" (Benjamin Milo Bolstad Preprocess Core: A collection of pre-processing functions, v1.18.0).

Transcriptome data (FASTQ files) are being submitted to the NIH database of Genotypes and Phenotypes (dbGaP).

Quantitative PCR

RNA was extracted using spin column-based kits and RT-PCR performed as per the manufacturer's guidelines. qPCR was performed with primer sequences, designed using National Center for Biotechnology (NCBI) (Bethesda, MD) Primer-BLAST function or taken from Reference 18 (*see* Table E1 in the online supplement). Identification of specific MiRs and housekeeping MiRs were from Life Technologies (Carlsbad, CA), indicated in the online supplement.

Immunoblotting

Nitrocellulose membranes were incubated with rabbit anti-COL4, rabbit anti-EFNA1, or mouse anti–β-actin overnight at 4°C, then incubated with secondary antibodies and imaged using enhanced chemiluminescence reagent.

siRNA and DNA Transfections

Control, BMPR2, EFNA1, COL4A1, COL4A2, β-catenin, PPARγ, ID1 siRNA, and SMAD1 and SMAD3 mutant constructs were transfected into subconfluent PAECs.

Modified Boyden Chamber PAEC Migration Assay

Migration was assessed 8 hours after seeding the cells using the modified Boyden Chamber Assay previously described (4).

Cell Adhesion and Tube Formation Assays

Cell adhesion to gelatin or collagen was assessed at 40 minutes, and tube formation assays in Matrigel were carried out as previously described (19).

Studies in Transgenic Mice

The Animal Care Committee at Stanford University approved all the experimental protocols used in this study. Wild-type C57BL/6J and *EphA2* knockout mice

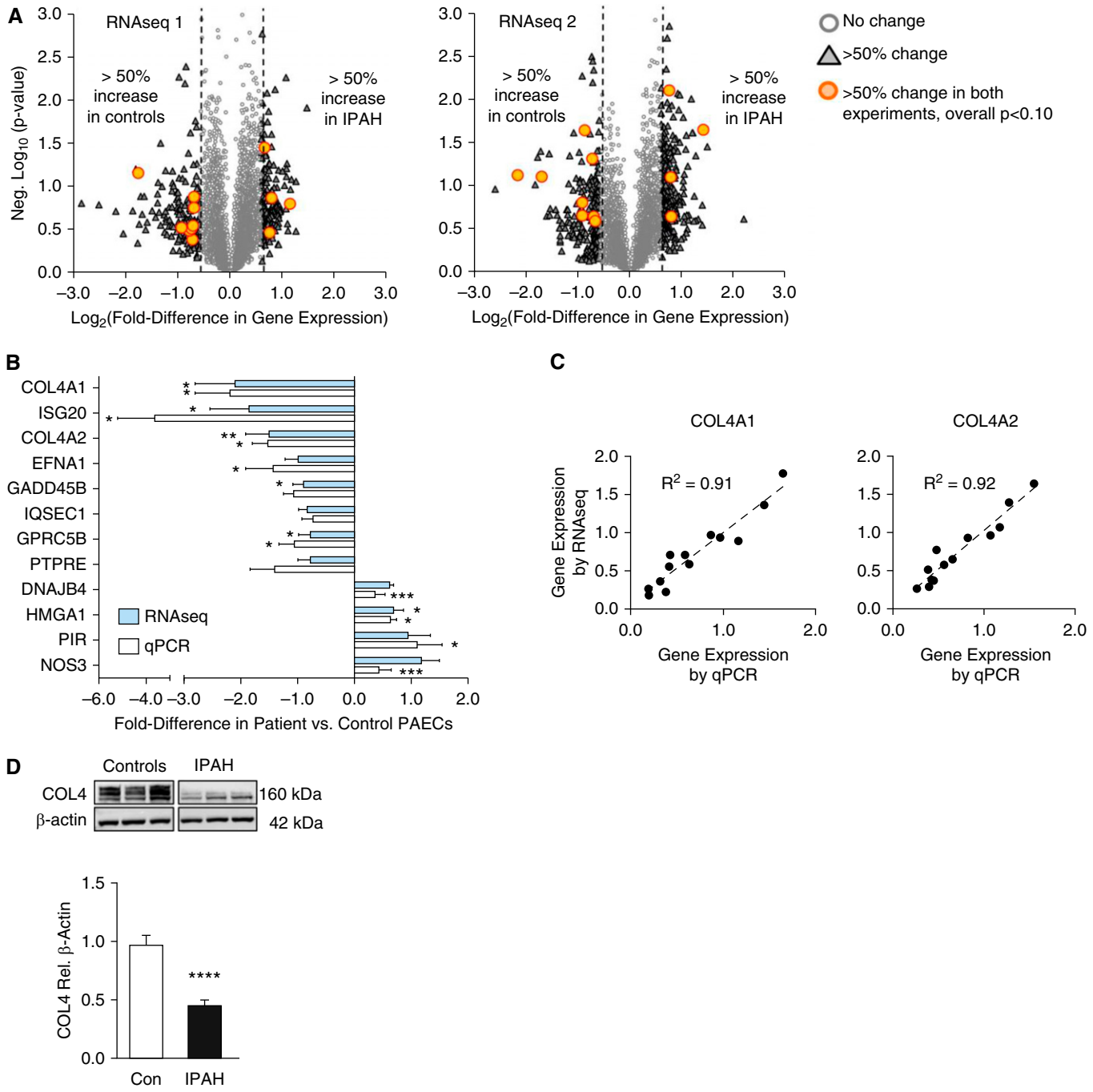

Figure 1. RNAseq analysis of pulmonary arterial endothelial cells (PAECs) from patients with idiopathic pulmonary arterial hypertension (IPAH) and unused donor controls (Con) reveals differences in gene expression. (*A*) Volcano plots illustrate fold differences in individual gene expression in IPAH versus control subjects, and associated *P* values (negative log10), in two RNAseq experiments. (*B*) Mean ± SEM fold differences (e.g., +1 = 100% higher, +0.5 = 50% higher) in gene candidates as measured by quantitative polymerase chain reaction (qPCR) and RNAseq. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control value, by *t* test. (*C*) Comparison of RNAseq and qPCR measurements in individual IPAH and control cells for COL4A1 and COL4A2. Data are arbitrary units normalized to the mean value in each experiment. (*D*) Representative immunoblot for COL4 protein in PAEC lysates, with β-actin as a loading control, and densitometry analysis below. *Bars* represent mean ± SEM of n = 3 with $^{****}P < 0.0001$. COL4A = collagen IV-A.

(Jackson Laboratory, Bar Harbor, ME) were given a subcutaneous injection of the vascular endothelial growth factor (VEGF) receptor blocker Sugen 5416 (20 mg/kg) followed by 4-week exposure to chronic hypoxia (air at 10% O_2). Cardiac function and output were assessed by echocardiography and right ventricular (RV) systolic pressure was measured by closed chest technique (20). RV hypertrophy was assessed as the weight of the RV relative to left ventricle (LV) plus

Table 2. Patients with IPAH versus Control Subjects: Gene Expression Fold Differences by RNAseq and PCR

Gene	RNAseq1	RNAseq2	Combined Data	*P* Value	PCR	*P* Value
Genes increased in IPAH PAECs in both experiments						
DNAJB4	+0.57	+0.67	+0.62	0.0001	+0.37	0.08
NOS3	+0.73	+1.63	+1.18	0.012	+1.50	0.006
HMGA1	+0.69	+0.71	+0.70	0.04	+0.63	0.020
PIR	+1.20	+0.72	+0.96	0.055	+1.10	0.023
PTGR1	+0.57	+0.55	+0.56	0.13		
MT1E	+0.89	+0.61	+0.75	0.16		
MT2A	+0.69	+0.73	+0.71	0.19		
Genes decreased in IPAH PAECs in both experiments						
COL4A1	−0.62	−3.47	−1.37	0.016	−1.65	0.010
COL4A2	−0.62	−2.23	−1.16	0.016	−1.39	0.004
ISG20	−2.40	−0.59	−1.17	0.016	−2.19	0.016
EFNA1	−0.91	−0.83	−0.87	0.023	−1.06	0.061
GPRC5B	−0.68	−0.65	−0.66	0.026	−0.88	0.025
GADD45B	−0.72	−0.90	−0.80	0.050	−1.01	0.011
PTPRE	−0.64	−0.62	−0.63	0.056	−1.00	0.061
IQSEC1	−0.65	−0.89	−0.76	0.091	−0.59	0.189
EPHX1	−0.53	−0.63	−0.58	0.12		
SOCS3	−0.52	−0.86	−0.67	0.12		
JUNB	−2.06	−0.65	−1.14	0.14		
TIMP1	−1.17	−1.03	−1.10	0.16		
VWF	−0.51	−0.57	−0.54	0.17		
CCL2	−0.57	−0.57	−0.57	0.19		
LOX	−0.78	−1.54	−1.09	0.20		
TGFBI	−0.87	−0.86	−0.87	0.21		

Definition of abbreviations: IPAH = idiopathic pulmonary arterial hypertension; PAEC = pulmonary arterial endothelial cell; PCR = polymerase chain reaction.
Genes with the most consistent differences in combined data ($P < 0.1$) were selected for further study by quantitative PCR analysis. Fold differences in quantitative PCR are normalized to β-actin expression.

septum. Lungs were perfused with normal saline and fixed for routine histology, and morphometric analysis performed as previously described (5). Mouse lung tissue sections were stained with fluorescent antibodies for von Willebrand factor and α-smooth muscle actin as previously described (20).

Statistical Analysis

Genes analyzed by RNAseq and qPCR were compared in control and IPAH cells by expression level using average fold differences and an unpaired *t* test to establish significance. Correlations were assessed by Spearman rank test. Comparisons between two cohorts were assessed by *t* test; between three or more cohorts by one-way analysis of variance with Dunnett; and when combining more than one condition, a two-way analysis of variance with Bonferroni-corrected *post hoc* testing was used. All cell culture experiments were conducted three or more times with two to four replicates per condition.

Results

RNAseq Analysis of PAECs from Patients with IPAH

RNAseq transcriptomic profiling was performed on RNA isolates from PAECs from two separate groups of control subjects (unused donor, n = 3 in RNAseq1 and n = 4 in RNAseq2) and patients (IPAH, n = 3 in RNAseq1 and n = 3 in RNAseq2) (Table 1, Figure 1A). Genes with average fold differences greater than or equal to 50% were selected for comparison. When combining the two sets of control and IPAH PAECs, 23 genes were differentially expressed by greater than or equal to 50% (Table 2). In 12 of these, the difference between IPAH and donor control gene expression was *P* less than 0.1, and in 9 of 12 *P* less than 0.05. These gene expression differences were selected for further investigation.

qPCR analysis of these 12 genes confirmed similar fold changes detected by RNAseq analysis (Spearman $\rho = 0.97$; $P < 0.0001$) (Figure 1B). In 10 genes we established a significant difference ($P < 0.05$) between IPAH and control PAECs, either by RNAseq and/or by qPCR. Correlations for the two alpha chains of collagen IV (COL4A1 and A2) between RNAseq and qPCR further illustrate the close agreement using RNAseq and qPCR to measure gene expression (Figure 1C). Immunoblot analysis confirmed that COL4 protein levels were decreased in cell lysates obtained from IPAH versus control PAECs ($P < 0.0001$) (Figure 1D). Both collagen chains were also down-regulated along with BMPR2 expression in a separate cohort of six patients and five control subjects (*see* Figure E1). Levels of EFNA1 also tended to be lower in these patients but did not reach statistical significance ($P = 0.059$) (*see* Figure E2).

Reduced BMP Signaling and Gene Expression Changes in IPAH PAECs

Dysfunction in signaling of BMPR2 underlies most heritable cases of PAH and also has been demonstrated in patients with IPAH lacking a mutation (8). To investigate the relevance of BMPR2 dysfunction to the gene expression changes detected in our RNAseq analyses, we reduced levels of BMPR2 with targeted siRNA in commercially available control large PAECs (*see* Figure E2). We reproduced the same significant changes in expression in 6 of 10 genes seen in IPAH versus control PAECs by qPCR or RNAseq (Figure 2A). That is, with BMPR2 siRNA versus control siRNA, we recapitulated changes in three of four genes that were significantly up-regulated and three of six that were down-regulated in IPAH versus control PAECs. EFNA1 can regulate the release of COL4 from ECs (21) and therefore was selected along with COL4 for further functional analysis.

Protein levels of both COL4A1/A2 and EFNA1 were reduced in the media of PAECs transfected with BMPR2 targeting siRNA (both $P < 0.0001$) (Figure 2B). In contrast to down-regulation of the receptor, BMP9 stimulation of PAECs up-regulated COL4A1 and COL4A2, in association with increasing mRNA expression levels of BMPR2, ALK3, and ALK6. However, BMP9 stimulation actually reduced mRNA expression of

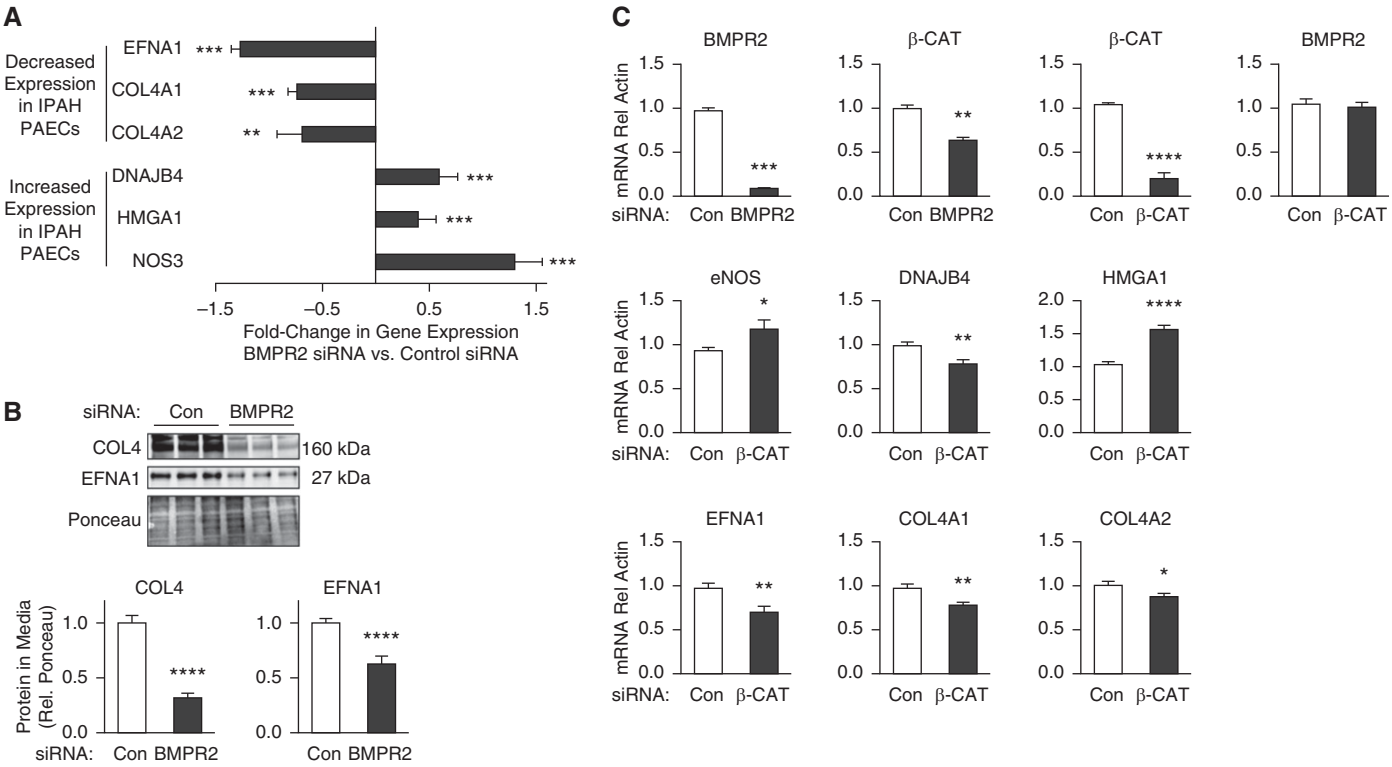

Figure 2. Reduced bone morphogenetic protein (BMP) signaling recapitulates many of the changes in transcripts identified by RNAseq analysis in idiopathic pulmonary arterial hypertension (IPAH) versus control pulmonary arterial endothelial cells (PAECs). (*A*) Fold change in gene expression of siBMPR2-transfected PAECs versus scrambled small interfering RNA (siRNA)-transfected cells calculated as in Figure 1B. Bars represent mean ± SEM of n = 3, $^{**}P < 0.01$ and $^{***}P < 0.001$ by *t* test. (*B*) Immunoblot analysis of collagen IV (COL4) and ephrinA1 (EFNA1) protein in media from PAECs transfected with scrambled (Con) or BMPR2 targeting siRNA. Ponceau indicates loading. Bars represent mean ± SEM. $^{****}P < 0.0001$ by *t* test versus control siRNA. (*C*) Quantitative polymerase chain reaction of transcripts altered by BMPR2 siRNA or β-catenin (β-CAT) siRNA versus nontargeting control siRNA. Bars represent mean ± SEM of n = 3 different PAEC harvests. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one-way analysis of variance and *post hoc* Bonferroni tests. BMPR2 = bone morphogenetic protein type 2 receptor; eNOS = endothelial nitric oxide synthase; HMGA1 = high-mobility group AT-hook 1; mRNA = messenger RNA.

EFNA1 and ALK1 in a manner similar to loss of BMPR2 (*see* Figure E3).

To address the mechanism leading to the reduction in gene and protein expression of COL4A1/A2 and EFNA1 observed with loss of BMPR2, we monitored levels of microRNAs that have been implicated in PAH, BMPR2 function, or regulation of COL4A1/A2; miRs-145 (22), -130a (23), -21 (24), -29a (25), -137 (26), and -9 (27) in PAECs from control subjects and patients, but no differences were found that could explain the changes in transcripts identified by loss of BMPR2 (data not shown). We also transfected control PAECs with dominant negative constructs of downstream effectors of BMPR2 signaling, SMAD1 or SMAD3 (3), or siRNA against ID1 (a downstream effector of pSMAD1/5) (28) but suppressing this axis could not reproduce any of the changes in the transcripts differentially expressed by loss of BMPR2 (*see* Figure E4).

Having shown in PAEC that BMPR2 activation induces a transcriptionally active complex between PPARγ and β-catenin and that these factors independently regulate genes downstream of BMPR2 signaling (4), we assessed gene expression in PAECs following knockdown of PPARγ or β-catenin. COL4A2 was down-regulated by both PPARγ and β-catenin siRNAs as it was by BMPR2 siRNA. However, all of the other differentially expressed genes were altered only by β-catenin siRNA in a manner similar to loss of BMPR2 (Figure 2C). Loss of BMPR2 and loss of β-catenin similarly down-regulated DNAJB4 (Figure 2), in contrast to the up-regulation of DNAJB4 observed in IPAH PAECs.

Functional Effects of Loss of COL4 and EFNA1 Expression on PAECs

COL4A1/2 and EFNA1 increase adhesion and migration of systemic ECs (21), therefore we evaluated these properties in PAECs using siRNA. In PAECs, COL4A1/2 and EFNA1 siRNA reduced levels of the proteins more than 50% (*see* Figure E5) and this resulted in a similar inhibition of both adhesion and migration of PAECs (all $P <$ 0.001) (Figures 3A and 3B). Combined knockdown of EFNA1 and COL4A1/A2 did not result in a further decrease in adhesion or migration when compared with reducing levels of the individual transcripts, suggesting that these proteins may interact through the same mechanism. Coating wells with collagen IV significantly improved the adhesion of PAECs in cells transfected with siRNA targeting EFNA1 or COL4 to levels observed in control cells on gelatin (Figure 3A). However, there was still reduced adhesion when compared with PAECs transfected with scrambled siRNA on collagen-coated wells. This suggested that endogenous production of COL4 is necessary for restoration of the normal phenotype.

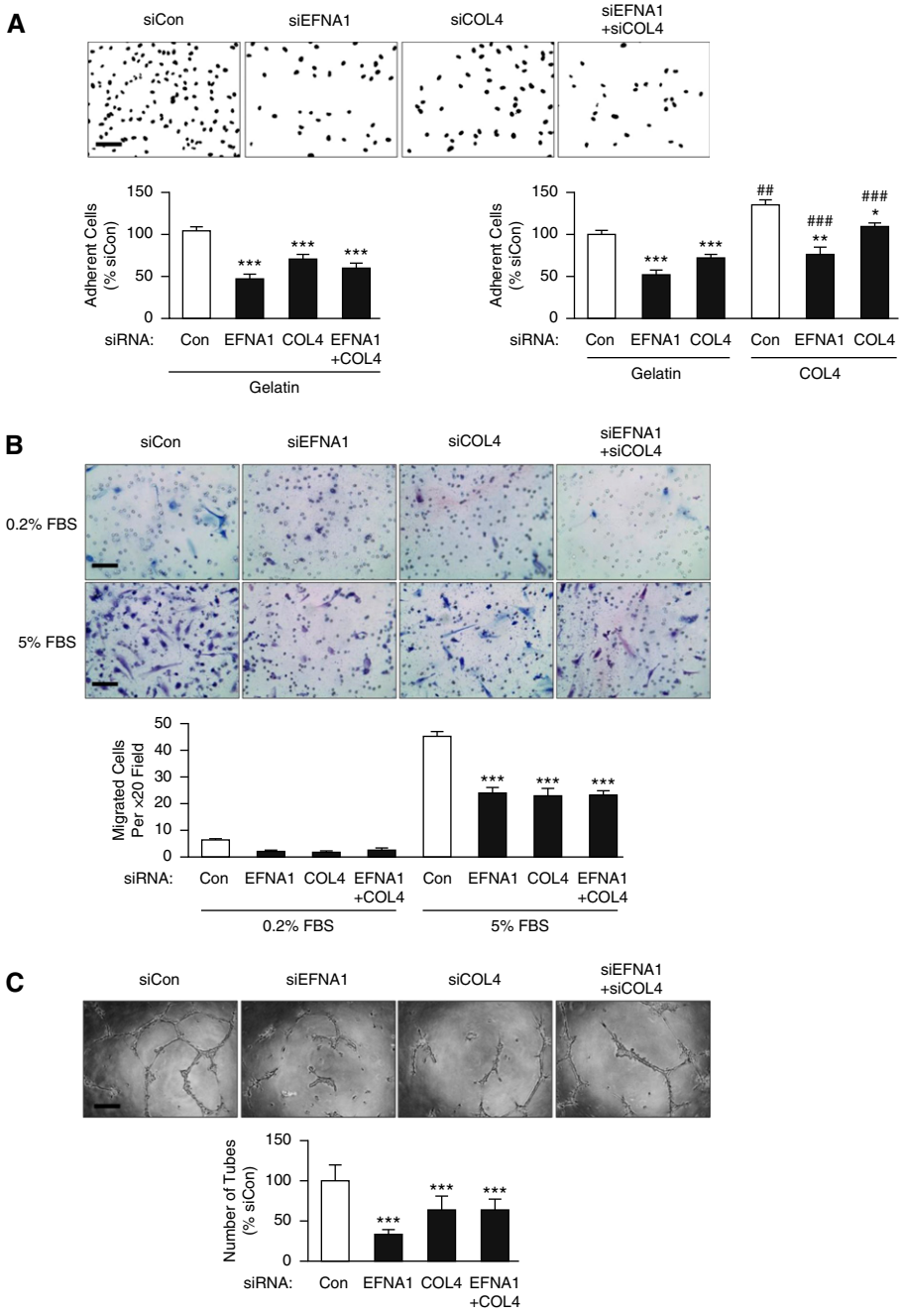

Figure 3. The functional consequences of loss of collagen IV (COL4) and ephrinA1 (EFNA1) expression in pulmonary arterial endothelial cells (PAECs). Representative fields are shown *above* and quantitative data *below*. (*A*) PAECs transfected with EFNA1 and/or COL4 small interfering RNA (siRNA), normalized to scrambled siRNA-transfected cells (Con) were adherent to gelatin or collagen-IV-coated wells after 40 minutes, assessed by semiautomated counting of 4′6-diamidino-2-phenylindole–positive nuclei. (*B*) PAECs transfected with EFNA1 and/or COL4 siRNA versus control siRNA and migration assessed at 8 hours by modified Boyden Chamber assay. (*C*) Matrigel assays in cells transfected with EFNA1, COL4 siRNA, or both versus scrambled (Con) siRNA, over 19 hours, in response to 0.2% FBS + 25 ng/ml vascular endothelial growth factor. *Scale bars* = 100 μm. *Bars* represent mean ± SEM of n = 3. $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ versus Con siRNA, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus gelatin coated condition by one-way analysis of variance or two-way analysis of variance when comparing collagen with gelatin and siRNA condition, and *post hoc* Bonferroni test. FBS = fetal bovine serum.

Tube formation on reduced growth factor Matrigel, induced by 25 ng/ml VEGF in 0.2% fetal bovine serum, was significantly decreased by loss of either EFNA1 or COL4A1/2 or both (all $P < 0.001$) (Figure 3C). Coating wells or inserts with collagen IV also significantly improved adhesion ($P < 0.05$) and migration ($P < 0.001$) of PAECs from patients with IPAH (*see* Figure E6).

EphA2 Knockout Mice versus Control Animals and Severity of Pulmonary Hypertension

Because EFNA1 and COL4 seem to act through the same pathway in promoting adhesion or migration of PAECs, we investigated whether reducing EFNA1 signaling in mice lacking the EFNA1 receptor ($EphA2^{-/-}$) would increase endothelial dysfunction and augment pulmonary hypertension during hypoxia by promoting a greater loss of small arteries. There were no differences at baseline in normoxia when comparing pulmonary hemodynamic data in $EphA2^{-/-}$ mice versus control animals, and no differences in the response to a 3-week period of hypoxia. However, the addition of the VEGF receptor blocker Sugen 5416 by subcutaneous injection followed by a 4-week period of hypoxia (10% oxygen) elicited more severe pulmonary hypertension in $EphA2^{-/-}$ when compared with control mice. $EphA2^{-/-}$ mice demonstrated greater elevation in RV systolic pressure ($P < 0.001$) and RV hypertrophy judged by the weight of the RV/LV and septum (LV+S) ($P < 0.01$) (Figures 4A and 4B). Cardiac output and muscularization of distal vessels was similar (Figures 4C and 4D) but morphometric analyses revealed a greater loss of small arteries relative to alveoli in $EphA2^{-/-}$ mice when compared with wild-type control animals ($P < 0.0001$) (Figure 4E). The number of alveoli per square millimeter was similar in the $EphA2^{-/-}$ and wild-type mice (Figure 4F).

Discussion

Endothelial dysfunction is a key feature in the pathogenesis of PAH. In this study, we identified disease-related differences in gene expression not previously reported, including reduced COL4 and EFNA1. The latter have important functional consequences on

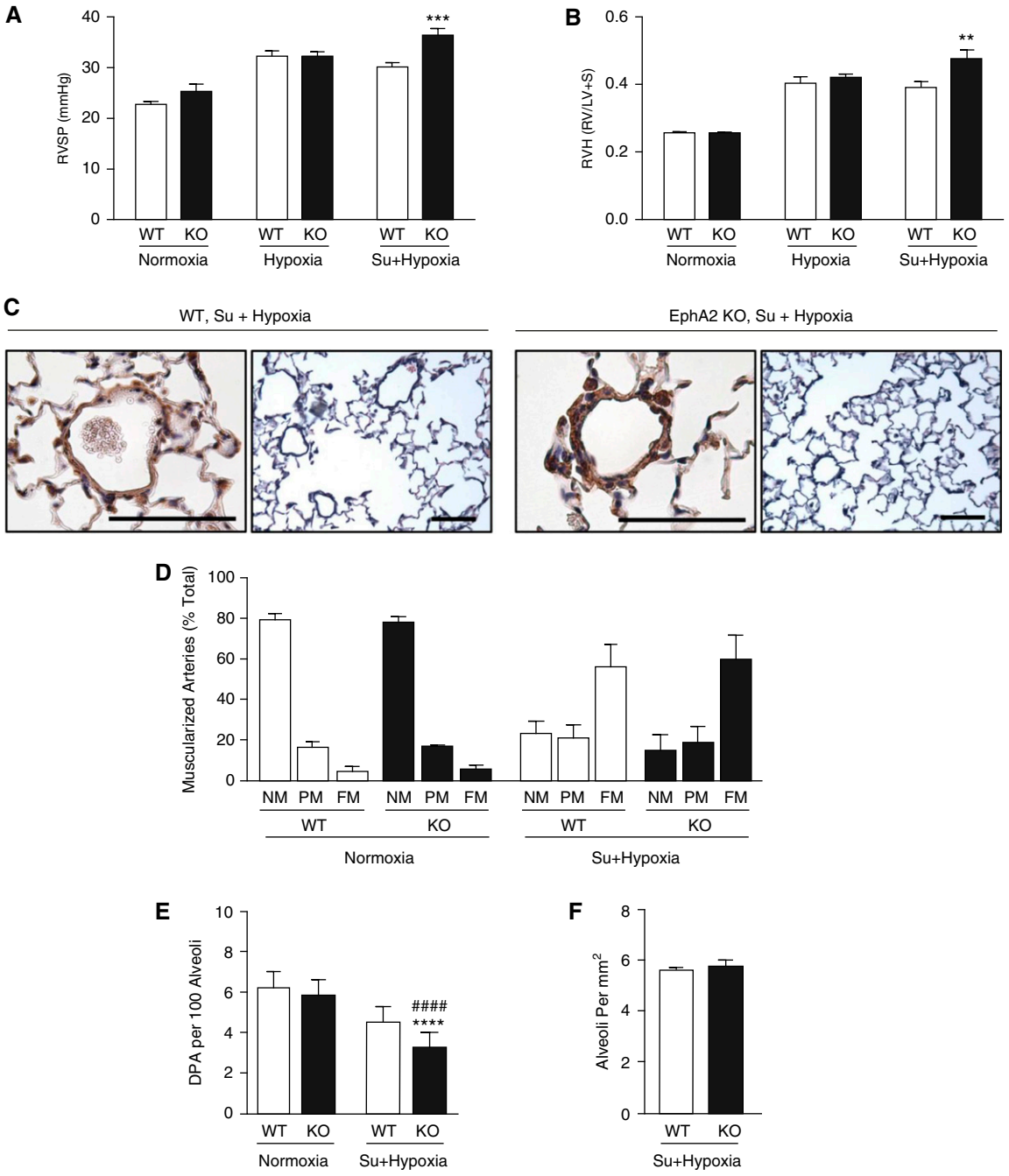

Figure 4. Mice with deletion of EphA2 develop exaggerated pulmonary hypertension (PH). *EphA2*$^{-/-}$ (KO) and wild-type (WT) mice were exposed to room air (normoxia), 10% O_2 (hypoxia), or subcutaneous injection of the vascular endothelial growth factor receptor blocker Sugen 5416 (Su) in addition to hypoxia for 4 weeks and development of PH assessed. n = 5 (3 males, 2 females) for each group. *Bars* indicate mean ± SEM. (*A*) Right ventricular systolic pressure (RVSP). (*B*) Right ventricular hypertrophy (RVH) given by the Fulton index (weight of right ventricle [RV]/left ventricle [LV] and septum [S]). $^{**}P < 0.01$, $^{***}P < 0.001$ versus WT by two-way analysis of variance with Bonferroni post test. (*C*) Representative histology of distal pulmonary arteries (DPA) from mice exposed to hypoxia following Sugen injection; *scale bars* = 50 μm. The photomicrographs show similar muscularization of DPAs in the two genotypes but a greater reduction in the number of DPAs in the *EphA2*$^{-/-}$ versus WT. *Left* images show high magnification of α-smooth muscle actin–immunostained vessels, and *right* images lower-magnification hematoxylin and eosin–stained tissue sections. (*D*) Muscularization of distal pulmonary arteries. FM = fully muscularized; NM = nonmuscularized; PM = partially muscularized. (*E*) Number of vessels (DPA) per 100 alveoli. (*F*) Alveoli per square millimeter. *Bars* represent mean ± SEM, $^{****}P < 0.0001$ versus normoxia, $^{\#\#\#\#}P < 0.0001$ versus WT, by two-way analysis of variance and *post hoc* Bonferroni test.

angiogenesis and on the PA response to injury that relate directly to the development of PAH. We focused on COL4 and EFNA1 in particular, because both genes were down-regulated in the RNAseq analysis, and both have important, intertwining roles in endothelial biology; COL4 is a major component of vascular basement membranes and EFNA1 is an EC guidance molecule (29) that induces the release of COL4 from these cells (21). Loss of BMPR2 signaling also impaired the ability of PAECs to produce COL4 and EFNA1 by a mechanism that was reproduced by reducing β-catenin, a previously reported downstream effector of BMPR2 gene regulation (4). Repressing the expression of these proteins had negative effects on PAEC adhesion and migration, with a loss of the receptor for EFNA1 in transgenic mice worsening the severity of pulmonary hypertension in association with a greater loss of distal arteries.

In addition to the reduction in EFNA1 and COL4 expression, RNAseq analyses identified IQSEC1 and ISG20 as transcripts decreased in patients with IPAH versus control subjects. IQSEC1 (IQ motif and Sec7 domain 1) is a guanidine nucleotide exchange protein recruited by EGFR/VEGFR2. It stimulates angiogenesis and is important in cell adhesion by controlling the turnover of β-integrins (30). Dominant-negative ISG20 (interferon-stimulated exonuclease gene 20 kD) inhibits angiogenic function in human umbilical vein ECs by a less well-defined mechanism (31). Other transcripts that were reduced included GADD45B, the DNA repair protein (reviewed in Reference 32); GPCR5B, best studied in differentiation of macrophages (33); and PTPRE, a phosphatase that can up-regulate EGF receptor signaling (34). Transcripts that were increased in IPAH versus control PAECs by RNAseq include high-mobility group AT-hook 1 (HMGA1) and endothelial nitric oxide synthase (NOS3). HMGA1 has been extensively studied as a transforming factor linking viral infection and other perturbations to cancer (reviewed in Reference 35) and HMGA1 is also highly proinflammatory in ECs in response to viral infection (36). The increase in NOS3 expression may reflect uncoupling of NO and peroxynitrite production seen with elevated thromboxane (37) and with inflammation (38). Up-regulation of the NOS3 transcript may represent an attempt to make up for reduced levels of the NOS3 protein in PAH as has been recently shown (39). DNAJB4 and PIR were also increased in IPAH versus control PAECs. DNAJB4 is a chaperone protein induced by DNA damage that stabilizes E-cadherin (40). PIR is an iron-binding protein driven by the antioxidant transcription factor NRF2 that could modulate the proinflammatory effects of nuclear factor-κB (41). Interestingly, none of these transcripts matched proteins identified in blood outgrowth ECs previously studied in patients with heritable PAH, perhaps related to differences in cell type and patient cohort (15).

RNAseq was chosen as a methodology in this analysis because it is highly sensitive and enables precise measurement of thousands of distinct RNAs. For example, in our study around 6,000 RNAs met the strict inclusion criteria of detection above five RPKM, although it is likely that thresholds lower than this will produce useful data in the future as total number of reads per lane increases. The cell lines analyzed are obtained from patients undergoing transplantation and therefore represent the most severe cases of end-stage disease. Because in our experience it is technically difficult to separate PAECs from neointimal cells in PAH arteries for laser capture microscopy, prospective studies using single-cell transcriptomic analysis may hold promise in distinguishing the subset of PAECs that express low COL4A1/2 and EFNA1 (42). It would be of great interest to see if these changes were replicated in IPAH ECs at disease initiation, or whether they represented changes observed in endothelial progenitor cells or in ECs derived from induced pluripotent stem cells.

The three transcripts significantly increased in response to BMPR2 siRNA were HMGA1, NOS3, and DNAJB4. Those down-regulated in response to reduction of BMPR2 by siRNA included COL4A1, COL4A2, and EFNA1. Interestingly, dasatinib, a SRC inhibitor recently linked to the development of PAH (43), reduces the phosphorylation state of the receptor EphA2 (44); it is possible that through this mechanism dasatinib mimics loss of EFNA1 and its ability to regulate COL4 production, thus disturbing the function of PAECs and leading to the development of pulmonary vascular disease (43). It is intriguing that BMP9 can up-regulate COL4 in association with an increase in expression of BMPR2, and its coreceptors ALK3 and ALK6. BMP9 causes a reduction in EFNA1 in association with a reduction in ALK1, indicating that the loss of the BMPR2 coreceptor ALK1 may be as important as the loss of BMPR2 in regulating EFNA1 expression. This pathway may therefore be especially relevant in patients who develop PAH related to ALK1 mutations, with or without associated hereditary hemorrhagic telangiectasia (45).

Concomitant knockdown of both EFNA1 and COL4 had no additional inhibitory effect on the ability of PAECs to adhere or migrate, suggesting that they function by activating the same downstream pathway. For example, COL4 is required to activate focal adhesion kinase (FAK) in ECs; EphA2, the preferred receptor for EFNA1, interacts directly with FAK (46). We have observed that knockdown of either EFNA1 or COL4 leads to increased expression of both activated and total FAK (data not shown). Because EFNA1 can down-regulate its receptor and FAK, it is possible that loss of the ligand up-regulates the receptor and FAK, in concert with $p130^{cas}$ (47), but the absence of EFNA1 or COL4 precludes an effective signal.

Mice lacking expression of COL4 die with vascular abnormalities at E10.5–11.5 (48). Mice lacking expression of EFNA1 develop abnormal heart valves (49) but their vasculature has not been studied. Mice lacking the receptor crucial for the response of PAECs to EFNA1, EphA2, develop normally with no overt phenotype, but are protected from tumor development because of impaired angiogenesis (50). This emphasizes the importance of both COL4 and EFNA1 in vascular development and homeostasis in the intact animal. We were able to elicit a greater loss of distal PAs associated with more severe pulmonary hypertension in mice with loss of *EphA2* following VEGF receptor blockade and chronic hypoxia.

In conclusion, we have applied RNAseq for the first time to cohorts of PAECs derived from patients with IPAH and control unused donor lungs, and have identified a pathway related to reduced BMPR2 in patients without mutations that indicates additional ways in which the endothelium becomes dysfunctional. The new data suggest that optimizing BMPR2 receptor function (5, 20) may allow for normalization of a wide range of targets. ■

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