Identification of a common Wnt-associated genetic signature across multiple cell types in pulmonary arterial hypertension

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PULMONARY ARTERIAL HYPERTENSION (PAH) is characterized by vascular remodeling, including endothelial cell (EC) dysfunction and occlusion or rarefaction of the peripheral pulmonary microvasculature. More recently, the contribution of multipotent mesenchymal stromal cells (MSC) to muscularization of microvessels has been described (13). The interactions between the lung microenvironment, vascular EC, and MSC during remodeling in PAH remain unclear. All forms of PAH have a high mortality rate, despite current therapeutic options.

Deregulated bone morphogenetic protein (BMP) receptor type II (BMPR2) signaling is strongly associated with the development of PAH in both heritable (BMPR2mut) and Cav1.2mut) and idiopathic cases, although the molecular mechanisms through which BMPR2 derangement promotes PAH are unknown. Unfortunately, most rodent models of PAH do not precisely recapitulate the disease pathology; these models display less substantial pulmonary vascular remodeling in both proximal arteries and distal microvasculature, significantly slowing drug discovery efforts. Current in vitro models over-expressing mutant BMPR2 in cell types of interest are complicated by persistent retention of wild-type (WT) signaling. Moreover, human PAH tissue is limited in quantity, and specimens are typically obtained posttransplant or at autopsy, which limits conclusions about disease initiation and propagation. Previous global gene expression analyses using patient samples to identify risk factors for PAH have had two fundamental caveats. First, samples isolated from end-stage disease tissue were likely compromised by effects of end-stage stress and drugs. We have overcome this difficulty by analyzing cultured lymphocytes, based on the rationale that the molecular lesions that cause PAH are present in all cell types evaluated, regardless of origin, and that stimulation of the Wnt signaling pathway was a common molecular defect in both heritable and idiopathic PAH.

The interactions between the lung microenvironment, vascular EC, and MSC during remodeling in PAH remain unclear. All forms of PAH have a high mortality rate, despite current therapeutic options.
ever, the use of lymphocytes exemplifies a second fundamental problem: since lymphocytes are nonadherent, any pathway relevant to cell-cell contact, matrix interactions, and polarity is not represented. Taken together, the underlying mechanisms of vascular dysfunction remain unclear, despite known genetic mutations that affect the BMPR2 signaling pathway.

The evaluation of early molecular events in the cells of PAH patients has been limited, because vascular-specific cells can only be studied at a late stage in the disease process (at lung transplant or postmortem evaluation), at the time of severe pulmonary vascular abnormality. However, the use of induced pluripotent stem (iPS) cells derived from PAH patients confers the ability to study early, initiating cellular events in the pathogenesis of PAH in relevant cell types. With embryonic stem cells and developmental differentiation used as a road map, these iPS cells may be differentiated to specific affected cell lineages (44, 53). Therefore, to address the aforementioned limitations and to investigate molecular pathways affected by dysregulated BMPR2 signaling, we engineered iPS cells and derived vascular multipotent mesenchymal stromal cells (MSC) and endothelium. We used this approach to study altered gene expression profiles during differentiation across mesenchymal cells (MC) and endothelial cells (EC), as well as skin fibroblasts from control, heritable PAH (HPAH), and idiopathic PAH (IPAH) patients. These findings were validated in vitro using primary patient cell lines. Here we address the hypothesis that altered human BMPR2 signaling results in a genetic signature common across multiple cell types, culminating in the pathological processes recognized as PAH. Taken together, our results suggest that altered Wnt signaling is inherent to the cells of PAH patients and is likely due to decreased BMPR2 signaling.

**METHODS**

**Isolation of patient skin fibroblasts and identification and characterization of the BMPR2 mutation.** The subjects were recruited via the Vanderbilt Pulmonary Hypertension Center. The Vanderbilt University Medical Center Institutional Review Board approved all study protocols (Vanderbilt University Institutional Review Board Protocol 9401). All participants gave informed written consent to participate in genetic and clinical studies and underwent genetic counseling in accordance with the guidelines of the American College of Chest Physicians (47). The PAH phenotype was defined according to accepted international standards of diagnosis. Specifically, PAH was defined diagnostically by autopsy results showing plexiform pulmonary arteriopathy in the absence of alternative causes, such as congenital heart disease, or by clinical and cardiac catheterization criteria. These criteria included a mean pulmonary arterial pressure of >25 mmHg with a pulmonary capillary or left atrial pressure of <15 mmHg and exclusion of other causes of pulmonary hypertension, in accordance with accepted international diagnostic criteria (45, 59).

Skin biopsy specimens were obtained via a sterile 3-mm punch skin biopsy. Primary skin fibroblasts were cultured using standardized measures. All cell lines were grown in the same manner using DMEM (with 4.5 g/l glucose, l-glutamine, and sodium pyruvate) (Mediatech, Manassas, VA) with 20% FBS (Invitrogen, Carlsbad, CA). For identification of BMPR2 gene mutations, genomic DNA was isolated from whole blood using Puregene DNA Purification Kits (Gentra, Minneapolis, MN) according to the manufacturer’s protocol. BMPR2 gene mutation was detected by sequencing exons and exon-intron boundaries of genomic DNA and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis (14).

**Summary of cell lines.** PAH iPS and control cell lines (2 each) were differentiated into MC and subsequent EC-like (ECL) cells (twice independently). Three pulmonary artery EC (PAEC) control and three PAEC IPAH primary cell lines were obtained through the Pulmonary Hypertension Breakthrough Initiative (PHBI).

**Karyotyping of skin fibroblasts and iPS cells.** Karyotyping was performed prior to reprogramming on skin fibroblast lines and following transgene removal to confirm normal chromosome complement and banding. Cultured cells were incubated for 4 h with colcemid (0.05 μg/ml) to enhance mitotic index, trypsinized, and collected into a centrifuge tube. This process was followed by a 12-min incubation in hypotonic solution (0.075 M KCl) at 37°C. Cells were then fixed using 3:1 methanol-acetic acid. Slides of metaphase cells were prepared using a standard air-dry procedure. G1 banding was performed in 5-day-old slides. Briefly, cells were digested in trypsin for 30–40 s and incubated for 5 min in Leishman’s stain. Karyotyping was carried out using BandView software (Applied Spectral Imaging). A total of 100 metaphases were screened per specimen for calculation of ploidy, and karyotyping was performed in ≥10 metaphases. A characterization of patient skin fibroblast samples is presented in Table 1. Western blot analysis was performed to evaluate the levels of BMPR2 protein expression in cells exposed to the primary antibody AF811 (R & D Systems, Minneapolis, MN) for 1 h and the secondary antibody 111-035-003 (Jackson Immunoresearch, West Grove, PA).

**iPS cell reprogramming and characterization.** To study the primary effects of BMPR2 mutation without concern for the pressure- or flow-mediated changes in vascular cell function (or other in vivo milieu variables), transgene-free iPS cells were generated from a control patient with no known BMPR2 mutation (WT) and a HPAH patient with a known BMPR2 mutation (BMPR2mut iPS) using the excisable polycistronic lentiviral vector (EFla-hiSTEM-CCA-loxP) encoding the four reprogramming factors (Oct3/4, Sox2, Nanog, and c-Myc), as described elsewhere (60). Briefly, iPS cell clones containing a single integrated copy of the vector were exposed to transient Cre recombinase to excise the floxed STEM-CCA vector to produce iPS cell lines free of exogenous reprogramming transgenes (60). Karyotyping was performed prior to reprogramming and following transgene removal (not shown). Sequencing of genomic DNA confirmed the retention of mutation in the BMP3ACr1 iPS cell line. iPS cells exhibited an embryonic stem cell-like morphology and displayed functional pluripotency in standard in vivo teratoma assays in nude mice. iPS cells (1 × 10⁶) were mixed with 100 μl of Matrigel (catalog no. 356237, Becton Dickinson, San Jose, CA) and injected subcutaneously into the flank of 6–wk-old severe combined immunodeficiency (SCID) mice (Jackson Laboratory, Bar Harbor, ME). The animals were monitored for 2 mo for tumor formation. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Principal component analysis (PCA) was performed to compare two independent patient control iPS cell clones with two BMPR2mut iPS clones and confirmed that the clones segregated based on the presence of BMPR2 mutation (not shown). This segregation illustrates that the mutant clones are more similar to each other than to controls.

**Passage and expansion of iPS cells.** Human dermal fibroblasts (Invitrogen) were expanded and mitomycin C was inactivated in the medium, as described elsewhere (9, 60). iPS colonies were grown on this feeder layer in 5% CO₂ and routinely passaged every 5–6 days after disaggregation with collagenase type IV (Invitrogen) at a ratio of 1:4–1:6, depending on colony density, onto a fresh feeder layer with...
### Table 1. Characterization of patient samples

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<th>PAP, mmHg</th>
<th>6-Minute Walk, m</th>
<th>PVR, Wood unit</th>
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PAP, pulmonary arterial pressure; PVR, pulmonary vascular resistance; HPAH, heritable pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; WT, wild-type; BMPR2, bone morphogenetic protein receptor type II; PAEC, pulmonary artery endothelial cell; PDE5, phosphodiesterase 5; RHC, right heart catheterization; NA, not available; NR, not reported.

the Rho-associated protein kinase inhibitor Y-27632 (30 μM) (60). Medium was replaced every other day. To separate iPS cells colonies from the feeder layer, plates were digested using collagenase type IV, transferred to a 0.1% gelatin (Sigma, St. Louis, MO)-coated tissue culture plate, and incubated for 1 h, allowing for fibroblast adherence. The cells and supernatant were then collected and plated on Matrigel-coated plates with Rho-associated protein kinase inhibitor (10 μM). iPS cells were cultured in this feeder-free system by switching to MTeSR1 medium.

**Directed differentiation of iPS cells and phenotyping.** MSC differentiation of iPS cells was performed using defined medium (knockout DMEM supplemented with 10% serum replacement medium, 10 ng/ml basic fibroblast growth factor, 10 ng/ml platelet-derived growth factor AB, and 10 ng/ml epidermal growth factor), as previously described by Lian et al. (43). iPS cells cultured in a feeder-free system using Matrigel were exposed to the mesenchymal medium until they became confluent for passage at 14 days (Fig. 1). Upon passage, cells were cultured on plastic using α-MEM supplemented with 20% fetal calf serum. Differentiation of WT and BMPR2mut iPS cells into iP-SMSC was demonstrated by surface marker expression of CD73, Stro-1, CD29, and CD105 and lack of the hematopoietic markers CD45, CD14, CD3, and c-kit. Multilineage differentiation to mesenchymal lineages was also performed (not shown), meeting the established criteria for MSC (20) (Fig. 2, A and B). Differentiation to MSC was performed twice per clone, and characterization was performed on each line.

MSC at passage 2 were plated onto collagen type I, and differentiation to EC was performed using the EGM-2 Bullet kit (Lonza/Clonetics, San Diego, CA). When cells reached confluence (2 wk), they were incubated with acetylated DiLDL labeled with Alexa 488 (10 μg/ml; Invitrogen) in culture medium for 2 h. Cells were photographed and RNA was collected for array analysis, or cells were trypsinized to form a single cell suspension for sorting by flow cytometry using a MOFlow sorter (Dako Cytomation, Ft. Collins, CO) and Cell Quest software. DiLDL-enriched iPS-ECL cells were expanded and, after up to two passages continuing EC differentiation conditions, trypsinized to form a single cell suspension for sorting by flow cytometry using a MOFlow sorter (Dako Cytomation, Ft. Collins, CO) and Cell Quest software. DiLDL-enriched iPS-ECL cells were expanded and, after up to two passages continuing EC differentiation conditions, trypsinized to form a single cell suspension for sorting by flow cytometry using a MOFlow sorter (Dako Cytomation, Ft. Collins, CO) and Cell Quest software.
sion by MSC and EC was analyzed by incubation of primary antibodies directly conjugated to phycoerythrin (PE), FITC, or allophycocyanin (APC) (see Supplemental Table S2 in Supplemental Material for this article available online at the Journal website) with $1 \times 10^5$ cells for 10 min on ice. The cells were then washed and resuspended for analysis in cold Hanks’ solution containing 2% fetal calf serum with DAPI to exclude dead cells. Flow cytometry to detect staining was performed using a Beckman Coulter Cyan analyzer and Cell Quest software. Gates were set using a known positive and negative for each color. Patient PAEC

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**Diagram A**

- **T=0**
  - iPS
  - Early MSC
  - Msx2
  - FrzB

- **T=14 days**
  - Late MSC
  - CD45-
  - CD73+
  - Stro1+
  - CD29+
  - CD105+

- **T=24**
  - Early MSC
  - + Matrigel
  - +bFGF
  - +PDGFAB
  - +EGF

- **T=28 days**
  - EC-like
  - VE-cadherin (CD144)+
  - Flt-1
  - VEGFA
  - Endoglin (ENG)
  - Cyp1B1
  - Flk/KDR
  - Fzd4

- **T=45+ days**
  - EC-like
  - + IG-F-1
  - + ascorbic acid
  - + VEGF

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**Images**

- **B** WT iPS-MSC
- **C** BMPR2 mut iPS-MSC
- **D** WT iPS-ECL
- **E** BMPR2 mut iPS-ECL
- **F** WT iPS
- **G** BMPR2 mut iPS
- **H** AcDiLDL
- **I** WT iPS-ECL
- **J** BMPR2 mut iPS-ECL
- **K** FLT1
- **L** FLT1
- **M** WT iPS
- **N** BMPR2 mut iPS
were obtained from <1-mm-diameter vessels through the PHBI network. Patient characteristics are summarized in Table 1. BMPR2 expression levels were analyzed by Western blotting (not shown).

Phenotyping assays. Cells were plated in triplicate at a density of \(5 \times 10^4\) cells per well in a six-well plate and harvested at 0, 24, 48, and 72 h. Cell numbers and viability were analyzed using a Countess counter (Invitrogen). Apoptosis was determined by flow cytometry using the YoPro propidium iodide kit (Invitrogen). Flow cytometry to detect staining was performed using a Beckman Coulter Cyan analyzer and Cell Quest software. Oxidative stress was measured using the GSH-to-GSSG ratio in cell extracts according to the suggested manufacturer’s protocol for the Glutathione One Fluorescent Detection Kit (catalog no. K006-F1, Arbor Assays, Ann Arbor, MI). All assays were performed twice independently. For Flexcell tension assays, cells were plated on collagen I plates (Flexcell International, Hillshboro, NC) and harvested at 0, 24, and 72 h. Cells were exposed to 10% elongation/deformation at 1 Hz to represent approximately one heartbeat and moderate distension. Control groups included unstretched cells. Two independent biological replicates were performed for each sample, and PCR analysis was performed in triplicate. iPS cell-derived MC from two control and two PAH patients were analyzed. Tube-forming ability was determined by using tissue culture-treated 24-well plates that were prechilled and coated with 200 \(\mu\)l of Matrigel, which was allowed to harden at 37°C for 45 min. Concurrently, cells from both lines were trypsinized, filtered, and centrifuged to be resuspended at 2 \(\times\) 10^5 cells/ml in growth medium. A volume of 0.5 ml was added to each well. Plates were incubated at 37°C in 5% CO₂, and tube formation was documented at 2–10 h after plating.

Isolation of primary human lung MSC. Human lung fibroblasts were isolated from human lung tissue postautopsy or at transplant by collagenase digestion of lung tissue explants. After expansion in culture under ambient conditions in α-MEM with 20% FBS, the cells were digested to form a single cell suspension. The cells were stained with antibody to detect and sort CD45^-/^-ABCG2^-/^- cells (lung MSC). The compensation controls were established as cells only, cells + DAPI, cells + APC-CD45 antibody, and cells + PE-ABCG2/ABCG2 antibody, and the sort sample consisted of cells + DAPI + APC-CD45 antibody + PE-ABCG2 antibody. Each sample was mixed well and incubated for 20 min at room temperature. DAPI was used to exclude dead cells. After expansion, cells were analyzed by flow cytometry to confirm the presence of CD105, CD106, CD73, and ScaI, as well as the absence of c-kit, CD14, and CD45.

Western blot analysis. Protein extracts were made by scraping cells in RIPA buffer (catalog no. 9806S, Cell Signaling, Boston, MA) containing protease and phosphatase inhibitors (catalog no. 78444, ThermoFisher Scientific, Waltham, MA). After determination of protein concentrations and standardization, cell lysates were mixed with an equal volume of Laemmli-SDS loading buffer, resolved on 10% polyacrylamide-SDS gels, and transferred to PVDF membranes. The blots were blocked with phosphate-buffed saline containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that detect the target proteins overnight at 4°C (see Supplemental Table S2). The blots were washed and subsequently treated with appropriate secondary antibodies conjugated to horseradish peroxidase. After the blots are washed, specific immune complexes were visualized with SuperSignal West Pico Chemiluminescent Substrate. Secreted frizzled-related protein 2 (Sfrp-2) band size is \( \sim 33 \) kDa.

Transcriptome analysis. Total RNA was prepared with RNA Isolation Kit reagents (Qiagen, Valencia, CA). RNA was isolated from 20 patient skin fibroblast cultures, 2 independently isolated control iPS cell cultures, and 2 independent HPAH iPS cell clones. For developmental stage analyses, RNA was collected from two independently generated clones, and chips were run in duplicate. cDNA generated from amplified RNA was hybridized to duplicate Affymetrix (Santa Clara, CA) human gene 1.1 or 1.0 ST chips. Gene ontology groups were analyzed and compiled using Webgestalt (Vanderbilt University), heat maps using JMP 9, and correlation plots using Microsoft Excel, with statistics performed using JMP 9. Array analysis and quantitative RT-PCR validation was performed as described elsewhere (13, 37). Quantitative RT-PCR assays were performed in triplicate, and levels of analyzed genes were normalized to hypoxanthine phosphoribosyltransferase abundance (see primer list in Supplemental Table S1).

Dual luciferase assay to detect Wnt signaling activity. Cells (5 \(\times\) 10^4 per well) were plated on a 12-well plate. Five microliters of TCF/LEF reporter plasmid (100 ng/\(\mu\)l stock; Cignal Reporter Assay, Qiagen) were diluted in 50 \(\mu\)l of Opti-MEM (Invitrogen), and 1 ml of Lipofectamine 2000 (1 mg/ml stock; Invitrogen) was diluted in 50 \(\mu\)g/ml Opti-MEM (Invitrogen) according to the manufacturers’ instructions. The TCF/LEF-responsive construct encodes the firefly luciferase reporter gene under the control of a minimal CMV promoter and tandem repeats of the TCF/LEF transcriptional response element. Diluted reporter plasmid and Lipofectamine were combined, and the cells were incubated at room temperature for 30 min. Cells were then rinsed with Opti-MEM, and transfection reagents were added. After 10 h of transfection, the medium was replaced with culture medium. LiCl (10 mM), a positive regulator of Wnt signaling, was added to the wells as a positive assay control (not shown). Cells were harvested at 24 h, 48 h (inhibitor studies), or 72 h using the Dual Luciferase Reporter Kit (Promega, Madison, WI), and dual luciferase activity was quantitated using a luminometer. These experiments were repeated twice independently. Transfection efficiency was standardized to Renilla luciferase.

Detection of Sfrp-2 in human PAH specimens. Human tissue was obtained from postautopsy specimens from PAH patients (2 control and 3 PAH with different mutations) after approval from the Vanderbilt University Institutional Review Boards. Sections of patient lung tissue were evaluated by antibody staining for the presence of the secreted Wnt inhibitor Sfrp-2 (catalog no. 92667, Abcam) using diaminobenzidine detection. Images were captured using a Nikon Eclipse 90i/DSFi-1 microscope with NIS Elements...
software. ELISAs to detect protein levels in conditioned medium from iPS and primary cells in culture and plasma were performed according to the manufacturer’s instructions (MyBioSource, San Diego, CA).

**Statistical analysis.** Data were analyzed by one-way ANOVA followed by Tukey’s honestly significant difference post hoc test using JMP 9. Significance was defined as \( P < 0.05 \).

**RESULTS**

iPS cell-derived PAH cell lineages show subtle, but significant, differences in morphology and differentiation potential. We employed iPS cell technology to study vascular-associated MSC and ECL cell lineages that may actively participate in the cell-based pathology of PAH. This allows us to avoid the complication of consequences, rather than causes, of disease found in cells directly obtained from patient explants. It also allowed the derivation of multiple cell lineages from a single patient, which allows examination of differentiation state-dependent effects of dysregulated BMPR2 due to mutation. Transgene-free iPS cells were generated from WT skin fibroblasts or skin fibroblasts with known BMPR2 mutation and directed to differentiate toward multipotent mesenchymal (20, 43) (iPS-MSC) and, subsequently, ECL (iPS-ECL) cell lineages (Figs. 1 and 2). This direction for differentiation and cell types to study was selected, because, developmentally, distal pulmonary microvasculature is thought to be of mesenchymal origin (3). iPS-MSC exhibited characteristic phenotypes (Fig. 1, B–G) and formed mesenchymal structures in vitro (27). Visible differences in cell organization, including a more elongated morphology, were noted. However, both WT and BMPR2mut iPS-MSC formed characteristic two-dimensional organizational patterns and ridges of high density (Fig. 1, F and G) (15, 27). Acetylated DiLDL uptake was employed to enrich putative ECL cells and also demonstrated that BMPR2 mutation decreased the efficiency of differentiation: 79.3% for WT and 69.8% for BMPR2mut (Fig. 1, H–K, and Fig. 2D). ECL cell differentiation was demonstrated by the appearance of a characteristic morphology and expression of Flt-1 [VEGF receptor 1 (VEGFR1)], as well as angiogenic tube-forming ability (Fig. 1, K–N). At the level of gene expression, the BMPR2mut iPS-ECL cells demonstrated decreased expression of the differentiated markers VEGF-A, Tie-2/TEK, and Flk1/KDR (Fig. 2E). The mutant MC exhibited lower rates of apoptosis and increased oxidant stress in response to hypoxia (Fig. 2, F and G). The mutant iPS-derived MC and primary lung MSC did not demonstrate a basal difference in proliferation rate (Fig. 2, H and I), similar to previous observations of pulmonary artery smooth muscle (51). We have shown that BMPR2mut iPS cells can be differentiated to MSC and ECL cell lineages in vitro. These lineages exhibit characteristic of PAH cells, and they are therefore an effective model to understand the molecular consequences of altered BMPR2 signaling in cell function in the context of PAH. Taken together, these results confirm that reprogramming of the primary patient cells did not affect their ability to demonstrate a “PAH phenotype.”

**BMPR2 mutation alters gene expression dependent on, and independent of, cell lineage differentiation state.** We next performed global gene expression analysis of these validated iPS-derived cells to determine how decreased BMPR2 signaling affects gene expression at various stages of differentiation. We used two arrays per differentiation stage, with four differentiation stages, and both WT and BMPR2 mutant cells, for a total of 16 arrays. As shown in Fig. 1, differentiation stages included early MSC, late MSC, and ECL cells at passages 4 and 5.

For initial analysis of the resulting data, we used PCA. PCA is a powerful approach to circumvent the dimensionality problem in array data; tens of thousands of probe sets can be projected onto a small number of principal components that accurately reflect the variability in the data set (8). After preprocessing to remove control probe sets and probe sets below the noise threshold, the remaining 13,062 probe sets were subjected to PCA. PCA found that differentiation state accounted for 42% and mutation status for 15% of the variability between the 16 arrays (Fig. 3A). Gene expression in passage 4 and 5 ECL cells was very similar within genotype, suggesting stable molecular phenotype. Progress along the differentiation axis involved similar gene expression changes in WT and BMPR2mut cells. Between early MSC and ECL cells, 826 probe sets changed more than fourfold; 200 of these probe sets, which are depicted in the heat map in Fig. 3B, consisted of waves of upregulation of developmental, cell cycle, and angiogenesis-related genes (see Supplemental Tables S3–S5), ending in upregulation of cell adhesion molecules associated with endothelial differentiation (\( P = 4.8 \times 10^{-2} \) for overrepresentation), including VCAM1, ICAM1, CERCAM, and ITGBL1, which correlate with the flow cytometry data (Fig. 3C and Fig. 2, C–E).

In addition to the above-mentioned genes, which are developmentally regulated but not different between control and mutant cells, several categories of genes distinguished the PAH-derived cells from WT cells. These include genes that are over- or underexpressed in BMPR2 mut cells at every differentiation stage (Fig. 3, D and E) and genes that are only differentially expressed in differentiated cell types (Fig. 4). There were 271 probe sets at least 50% more strongly expressed in HPAH than WT at every stage (Fig. 3D; see Supplemental Table S6). These map to 220 unique genes, of which 85 are developmental (\( P = 5.0 \times 10^{-4} \) for overrepre...
sentation). These include transforming growth factor-β (TGF-β) pathway genes such as endoglin (ENG) and the repressor latent TGF-β-binding protein 2 (LTBP2); numerous homeobox genes, including DLX1/2, MEIS2, MSX2, PBX1, and SIX2; three semaphorins (SEMA3C, SEMA3F, and SEMA7A); and the Wnt pathway decoy receptor FRZB (see examples in Fig. 3E). The presence of CYP1B1 (Fig. 3E) in this group sounds a cautionary note about this approach. We and others previously showed that CYP1B1 is a powerful modifier gene; expression levels as measured in lymphoblastoid cells and functionally in patient urine correlate with disease penetrance, rather than BMPR2 expression levels (4, 65).

The final group of genes examined were those that were only differentially regulated by BMPR2 in the context of differentiated cells. Using criteria of no significant difference in early MC, but a raw P < 0.05 of a 1.5-fold difference in ECL cells, we found 190 probe sets representing 164 unique Entrez IDs that fit this category (Fig. 4A; see Supplemental Table S7). Overrepresented gene ontology groups [Benjamini and Hochberg (6) multiple test adjusted P < 0.01] included cell adhesion (22 genes), cell death (36 genes), proliferation (31 genes), stimulus response (85 genes), cell surface receptor signaling (41 genes), and developmental (54 genes). The largest set consisted of 93 probes representing 72 genes that were specifically upregulated in BMPR2 mutants, but not controls, during cell differentiation (group I in Fig. 4A). These included 33 developmental genes and 18 genes related to cell death. Of particular note, these included a large number of cell surface and secreted Wnt receptors and Wnt pathway target genes (Fig. 4B). Upregulation of the Wnt receptors Fzd4 and Fzd5 and secreted modulators Srfp1 and Sfrp2, as well as Msx2, Tie2/TEK, Cyp1B1, and Tsp2, was confirmed by quantitative RT-PCR, which correlated strongly to array results (Fig. 4, C and D, and results not shown).

In summary, we have shown that there are genes that are changed by differentiation state, but not by mutation (Fig. 3, B and C), genes that are always changed by mutation, regardless of differentiation state (Fig. 3, D and E), and genes that are changed by mutation only in differentiated cells (Fig. 4A). One of the largest groups of genes that are changed by mutation
included Wnt signaling molecules (Fig. 4B). Confirmation by RT-PCR correlates well with array results (Fig. 4, C and D, and results not shown).

**PAH-dependent changes in the Wnt pathway are a function of differentiation, per se, and not a particular somatic lineage.** The comparison of differentiation states of vascular cells derived from iPS cells highlighted genetic signatures conserved across differentiation state. However, because the iPS cell lines were derived from outbred individuals, it is not possible a priori to say which differences between them result from deregulated BMPR2 signaling and which derive from other individual-to-individual differences. The most feasible approach to resolving this limitation was to determine the universality of the specific differences identified across additional PAH patients. Therefore, using 21 fibroblast lines derived from healthy control, HPAH, or IPAH patient skin (see clinical characteristics in Table 1), we performed global gene expression profiling. The HPAH patients included both BMPR2 and caveolin-1 mutations. Hierarchical clustering of samples showed that, in general, HPAH samples clustered together, iPSAH samples clustered together, and controls clustered together (Fig. 5A). We found 409 probe sets representing 279 unique Entrez IDs with average differences of at least twofold between controls and either HPAH or IPAH samples (see Supplemental Table S8). Analysis of statistically overrepresented gene ontology groups showed that pathways differentially regulated in fibroblast lines were, for the most part, similar to pathways we previously reported to be dysregulated in cultured patient lymphocytes (5). These included 134 of the 279 genes related to altered metabolism, 25 cell adhesion genes \( P = 0.013 \) for overrepresentation of gene ontology group, by hypergeometric test, with Benjamini and Hochberg (6) multiple comparisons adjustment, 16 circulatory system process genes \( P = 0.0002 \), and 34 chemical stimulus response genes \( P = 0.0022 \), including 10 oxygen-level response genes \( P = 0.008 \).

To explicitly test the hypothesis that genes identified in the iPS cells were common to other PAH patients, we examined expression levels of all 164 unique genes identified in Fig. 4A. Of these, 154 were also expressed in fibroblasts and 117 showed concordant differential regulation between iPS cell-derived ECL cells and fibroblasts (e.g., genes upregulated in ECL cells were also upregulated in fibroblasts, \( P = 0.0013 \) by \( \chi^2 \) test). Correlation in fold change between BMPR2 mutant and controls and iPS cell-derived ECL cells and fibroblasts was 0.50, with correlation \( z \)-test \( P < 0.0001 \) (Fig. 5B). These results indicate that while cell type-specific changes do exist, the changes identified in our iPS cell-derived ECL cells are broadly conserved across differentiated cell types and across patients. HPAH and IPAH patients also had upregulation of Wnt pathway genes. Eight of 10 HPAH patients had upregulation of the secreted Wnt receptor SFRP1 compared with controls and 10 of 10 had upregulation of SFRP2 and the Wnt target genes PRICKLE2 and WISP2 (Fig. 5C). These differentially regulated Wnt genes were also detected in IPAH patients (Fig. 5D). That Wnt pathway genes are upregulated in skin fibroblasts from every patient, not just on average, demonstrates that our finding of upregulated Wnt genes in iPS-derived cells is correlated to disease status, rather than individual variation. Taken together, these data show that gene expression changes in both HPAH and IPAH are detectable in multiple differentiated cell types, are true across individuals,
are likely independent of the state of disease progression, and are consistent with our previously reported findings.

**Decreased BMPR2 signaling activity deregulates Wnt signaling in MSC.** Dysregulation of Wnt signaling has previously been noted in group I PAH, but in a context in which it was not clear whether it was a consequence of end-stage disease (39), a modifier (18), or a direct consequence of known mutation. Our identification of Wnt pathway upregulation in our iPS cells and as one of the most powerful common factors across 21 patient fibroblast lines suggests that it is a direct effect of deregulated BMPR2 signaling.

We next performed functional analyses to quantify canonical Wnt signaling activity in BMPR2mut iPS-MSC in vitro. First, Wnt activity was measured indirectly via TCF/LEF luciferase activity and normalized to Renilla luciferase activity following transfection (Fig. 6A). At 72 h, Wnt signaling was significantly increased in BMPR2mut iPS-MSC compared with WT iPS-MSC. Second, to show that this result was specific to decreased BMPR2 signaling, we took advantage of a group of small-molecule kinase inhibitors with differential effects on BMPR2 and decreased BMP signaling in WT cells (32). BMPR2, a type II serine/threonine kinase receptor, transduces signals through heterotrimeric complexes with BMPR type I receptors (BMPR1). Dorsomorphin (DM) is a prototype inhibitor that targets BMPR1 and BMPR2 (IC$_{50}$ = 74 nM for BMPR2 (31, 34, 67)), whereas LDN-193189 (LDN) and DM homolog 1 (DMH1) are selective inhibitors of BMPR1 and are less active against BMPR2 (IC$_{50}$ = 3,845 and >100,000 nM, respectively). We tested canonical Wnt signaling activity in WT iPS-MSC in the presence or absence of DM and the BMPR1 selective inhibitors DMH1 and LDN (Fig. 6B). BMPR2 signaling inhibition by DM significantly increased Wnt signaling at 24 h. DMH1 and LDN treatment slightly increased Wnt signaling in WT iPS-MSC at 48 h (results not shown).

In both iPS-derived MSC and ECL cells, as well as skin fibroblasts, $Sfrp2$ expression was strongly upregulated. To validate these findings, we evaluated expression of $Sfrp2$ on a protein level using both iPS cell-derived and primary patient cells. Using supernatant from iPS-MSC and iPS-ECL cells, we

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**Fig. 5.** Differential regulation of genes by deregulated BMPR2 signaling in iPS-ECL cells is strongly correlated to that in skin fibroblasts from PAH patients. Gene expression arrays were performed using RNA from 21 fibroblast lines derived from HPAH ($n$ = 10), idiopathic pulmonary arterial hypertension (IPAH, $n$ = 7), or control ($n$ = 4) patients applied to Affymetrix Human Genome ST 1 chips. From 54,675 initial probe sets, 13,062 had a range of $>$0.4 and at least 1 sample with an expression $>$7 in log base 2 units. Restriction of analysis to these genes prevents inclusion of noise. A: heat map of 409 probe sets representing 255 unique Entrez IDs shows average changes of $\pm$2-fold between controls and either HPAH or IPAH. B: differential regulation of genes by deregulated BMPR2 signaling in iPS-ECL cells is strongly correlated to average differential regulation of genes in skin fibroblasts from PAH patients (correlation = 0.50, $P < 0.0001$ by correlation $z$-test). Each circle represents 1 gene. C: upregulation of Wnt pathway and target genes in skin fibroblasts from HPAH patients compared with controls. Each symbol represents gene expression in 1 patient, normalized to average of controls. D: analyses of developmental pathways with altered gene expression confirm alterations in Wnt signaling, including secreted modulators $Sfrp2$ and $WISP2$, and included the Notch pathways in both cells from IPAH and HPAH patients relative to control.
Deregulated BMPR alters the Wnt signaling pathway and Sfrp-2 expression. A: Wnt activity by WT or BMPR2mut iPS-MSC was measured at 72 h using a luciferase reporter assay. LiCl was used as a positive control for Wnt activation. B: Wnt activity by WT iPS-MSC in the presence of BMPR2 and BMPR1 signaling inhibitors was measured using the Wnt-luciferase reporter assay. Values represent mean fold change over WT or vehicle control at 24 h. DM, dorsomorphin; DMH, DM homolog; LDN, LDN-193189. C and D: ELISA and Western blot analysis of secreted and cell-associated expression levels of Sfrp-2 protein by WT and BMPR2mut iPS-MSC and iPS-ECL cells. C: ELISA of secreted Sfrp-2 protein using cell conditioned medium, performed in triplicate. D and E: representative Western blots of Sfrp-2 and β-actin, repeated twice independently and normalized to actin. Primary PAEC: n = 2 control and 3 PAH. Primary human lung MSC: n = 1 WT, 3 non-PAH, and 2 PAH. F: qRT-PCR was performed to detect the effect of decreased BMPR signaling on Sfrp-2 expression. Values are shown as mean fold change compared with vehicle (Veh) controls at 24 h; n = 2 control independent patient iPS-MSC lines. G: effects of mechanical stretch on Sfrp-2 expression in control vs. PAH iPS-MSC was analyzed by qRT-PCR. Cells were plated on collagen-coated plates and separated into unstretched (UT) and stretched groups. Values are shown as mean change normalized to hypoxanthine phosphoribosyltransferase (HPRT) at 72 h; n = 2 control and 2 PAH independent patient iPS-MSC lines. *P < 0.05; **P < 0.01; ***P < 0.001.
performed ELISA to measure levels of secreted Sfrp-2. We found that, in both cell types, BMPR2 mutant patient-derived cells displayed significantly higher levels of secreted Sfrp-2 (Fig. 6C). We then investigated Sfrp-2 secretion in primary PAEC cultured from explanted IPAH patient or failed donor control lungs \( (n = 3–4 \text{ for each}) \) and found significantly higher secreted Sfrp-2 in small vessel-derived EC lines (Fig. 6C). By Western blot analysis of whole cell lysates from all these cell types, we found that, as we expected, the PAH EC that secreted higher levels of Sfrp-2 had less cell-associated Sfrp-2 protein (Fig. 6D) than primary human lung MSC, which had increased levels of Sfrp-2 protein (Fig. 6E). To directly link decreased BMPR signaling to regulation of Sfrp-2 expression, we used DM to inhibit BMPR signaling in control iPS-MSC (Fig. 6F). Inhibition of BMPR signaling resulted in significantly increased expression of Sfrp-2 message. Because we are studying a simple system in the absence of physiological influence, we next evaluated the effect mechanical forces might have on Sfrp-2 gene expression in control vs. PAH iPS-MSC (Fig. 6G). Cells were exposed to the deformation of approximately one heartbeat and moderate distension. At 72 h, baseline expression of Sfrp-2 transcript was greater in PAH iPS-MSC than controls. Interestingly, stretch did not significantly affect the control iPS-MSC; however, the PAH cells decreased their expression levels by approximately twofold. The decrease in PAH expression rendered the levels similar to controls. No significant difference in gene expression was observed at 24 h. Taken together, our data imply that PAH patients produce more Sfrp-2 and the expression of Sfrp-2 transcript may be regulated by vascular tone.

To validate these findings in human PAH tissue specimens, we performed immunohistochemistry to detect Sfrp-2 (Fig. 7). Interestingly, Sfrp-2 localized to endothelium, parenchyma, and smooth muscle cells in control and PAH tissue. However, the intensity of Sfrp-2 staining was significantly greater in PAH tissue, in areas of remodeling and in smooth muscle cells, than in control specimens (Fig. 7, A–F). ELISA was performed to analyze levels of Sfrp-2 in PAH patient plasma relative to controls (Fig. 7G). We did not detect a significant difference between control, HPAH, and IPAH plasma samples, suggesting that Sfrp-2 is likely retained locally in the lung. This theory was confirmed by Western blotting to detect levels of Sfrp-2 protein in vivo using murine lungs, in the absence or presence of the R899x BMPR2 mutation (Fig. 7H). As antic-
ipated, Sfrp-2 protein levels were significantly increased in the mutant mouse lungs. Our results suggest that abnormal BMPR2 signaling regulates Wnt signaling during PAH and may be, in part, due to Sfrp-2.

DISCUSSION

The relationship between the BMPR2 signaling pathway and Wnt signaling pathways has been delineated during development with regard to body axis patterning; however, their relationship during adult PAH is incompletely understood. In the present study, we used patient tissue samples and primary patient cell lines (iPS cell-derived, IPAH and HPAH) to demonstrate that BMPR2 dysfunction alters Wnt signaling and yields a common molecular phenotype at various stages of vascular cell development, as well as in adult somatic cells. We used iPS cell-derived MSC and ECL cell lines to identify molecular signatures of PAH without concern for confounding by secondary effects of end-stage disease, drug therapy, or altered local milieu from elevated pressures. These studies, as well as previous data from our murine modeling systems, suggest that decreased BMPR2 signaling increases canonical Wnt signaling.

iPS cell-derived and primary patient MSC were used in this study, because mesenchyme is a source of multipotent vascular precursors during development, as well as in the adult lung, and their role in PAH has not been delineated (1, 2, 13, 37, 46). The intimacy of the relationship between mesenchyme and epithelium/endothelium persists into the adult tissue and is recapitulated during organ repair and remodeling. Using this method, we identified cell and developmental stage-specific signatures through comparative analyses (1, 2, iPS-MSC and iPS-ECL cells demonstrated consistent expression of BMPR2, thus providing the opportunity to model deregulated BMPR2 and Wnt signaling in vitro. Here we demonstrate that the iPS cell-derived cell lineages retain characteristics of PAH, including decreased expression of Tie2, VEGF-A, Flk-1/KDR, and BMP-4. Although characteristic surface markers of MC and EC were demonstrated, there were significant differences in differentiation in ECL cells, as well as gene expression profiles and Wnt signaling between the WT and PAH-derived cells, including VEGF-A, Tie2/TEK, and Flk1/KDR, all factors that regulate vascular stability. These genes lack identified SMAD-binding sites within their promoters, which suggests an indirect regulation of expression, not direct regulation by BMP SMAD signaling. It is likely that a derangement of BMPR2 signaling pathways in the differentiated cells resulted in alteration of additional signaling pathways, affecting cell self-renewal, cell proliferation, and cell fate determination (25, 68).

Apoptosis followed by proliferation of apoptosis-resistant EC is a paradigm to explain vascular remodeling in PAH (21, 41, 61, 62). Our studies expand this paradigm and show that while PAH iPS cell-derived and primary MC did not have significantly different rates of proliferation relative to control lines, they were also less likely to undergo apoptosis. Also interesting was the finding that intracellular oxidative stress was not increased in BMPR2 mutant MC under ambient culture conditions, which is consistent with previous reports from studies using cells with BMPR2 mutations (38). However, we showed that when placed in low oxygen over time, BMPR2 mutant MC had a significant increase in intracellular accumulation of GSSH, decreasing the ratio of GSH to GSSG, indicative of oxidative stress. This may be important in areas of tissue hypoxia that increase with the progression of disease.

We exploited the use of global gene expression analysis to identify common molecular pathways affected by deregulated BMPR2 signaling. Analysis of multiple stages of differentiation from MSC to ECL cells, as well as dermal fibroblasts (including control, HPAH, and IPAH samples), demonstrated consistent increases or decreases in expression levels of Wnt signaling pathway members, including modulators or inhibitors, as well as receptors. The Wnt signaling pathway influences cell-cell communication, adult tissue maintenance, and gene expression. BMPR2 signaling may regulate both canonical and noncanonical Wnt pathways in EC and MC to influence proliferation, survival, and motility during angiogenesis and remodeling of the pulmonary circulation (3, 19, 39). While the relationship of BMPR2 and Wnt pathways has been defined during development, the regulatory targets of Wnt signaling, common across multiple cell types, in BMPR2-associated PAH are unknown.

On the basis of these data, we evaluated Wnt signaling in PAH-susceptible MC. In our iPS-MSC model, decreased BMP signaling via BMPR2 mutation or soluble inhibitor specific to BMPR2 (31, 34, 67) resulted in increased Wnt signaling activity. We used this approach, since modulators of the Wnt canonical and noncanonical planar cell polarity signaling pathways were previously shown to have increased expression in patients with PAH (39) and because proper BMP signaling regulates both canonical and planar cell polarity pathways in the endothelium and smooth muscle to influence cell proliferation, survival, and motility in the pulmonary circulation (19). Increased Wnt signaling in adult lung MSC has been correlated with their transition to a contractile cell that participates in vascular remodeling during PAH (13).

Expression analyses across multiple cell types identified Sfrp-2 as differentially regulated in PAH cell lines vs. control. It was not surprising that Akt (protein kinase B), a critical component of vascular remodeling in PAH (35), is a key mediator of Sfrp-2 expression (49). Here we directly link decreased BMP signaling and the mechanical properties of the vasculature to regulation of Sfrp-2 transcript expression. Furthermore, both PAH patient lung tissue and BMPR2 mutant mouse lungs expressed higher levels of the protein. Sfrp-2 was initially identified as a Wnt antagonist, typically expressed during lung morphogenesis to promote alveolarization (26). Sfrp proteins are required for Wnt diffusion, activation, canonical signaling, and proper tissue differentiation; their effects on Wnt signaling are dependent on their concentration or Wnt ligands present in tissue (23, 26, 42, 63a). For example, Sfrp-2 can enhance activation of the canonical Wnt pathway (17, 64, 66) while inhibiting the noncanonical pathway, resulting in abnormal cell alignment and shape (12). Sfrp-2 can inhibit BMP-4 expression and prevent programmed cell death (22). Interestingly, the BMPR2 mutant iPS EC had decreased levels of BMP-4 expression relative to WT. Sfrp-2 is also prosangiogenic, inhibits cell apoptosis, and increases migration (17, 49) and, therefore, has the potential to play a role in the pathology of disease. Sfrp-2 is also known to decrease bone formation via regulation of BMP (54). This is likely due to the inhibitory effect of Sfrp-2 on BMP-1 and other tolloid proteases necessary for cleavage and inactivation of BMP antagonists, includ-
integrating viruses (29), which may mask the function of the formation and proper differentiation of the pluripotent epiblast used. This limitation is most likely due to the importance of deriving iPS cells from HPAH patients with BMPR2 mutation efficiency of reprogramming observed, a major obstacle to limitations to this approach remain.

Prior etiological interpretation of previous expression array studies using patient-derived samples may have been complicated by the overwhelming signal induced by end-stage disease and treatment. In one study of PAH lung tissue, of ~14,000 genes with measurable expression, 13,889 were altered (57). Among studies of PAH patient-derived samples, there have been three studies of lung tissue specimens (28, 39, 57), four studies of freshly isolated circulating cells (11, 30, 56, 63), and three studies of cells cultured from PAH patients (5, 24, 65). While the overall results have been recently reviewed (48), we reexamined these data to determine whether they also identified alterations in expression of Wnt signaling molecules as a disease-associated signaling pathway (Table 2). Wnt pathway signal, aside from increased TCF expression, was not apparent in any of the studies relying on fresh or cultured peripheral blood mononuclear cells (PBMC). On the basis of our own published arrays, this is likely because cell-specific Wnt pathway components are not significantly altered in PBMC (5, 65). Thus PBMC are likely not the ideal candidate cell type relative to adherent/polar vascular cells in which to assay this pathway. Of the four remaining studies, the most recent three showed a strong indication of increased Wnt signaling. In both normotensive and hypertensive pulmonary artery arterioles from idiopathic pulmonary fibrosis patients, Patel et al. (55) demonstrated that gene expression indicative of activated Wnt signaling was increased, which was characteristic of abnormal proliferation, apoptosis, and adverse remodeling.

Our studies show that global gene expression data obtained using multiple adherent somatic cell types from IPAH and HPAH patients could be utilized to identify common pathways affected in PAH, specifically the canonical Wnt pathway. We recognize that while iPS cells and patient primary cells provide a powerful model to understand PAH at the cellular level, limitations to this approach remain. 1) On the basis of the low efficiency of reprogramming observed, a major obstacle to deriving iPS cells from HPAH patients with BMPR2 mutation clearly exists when nonintegrating or excisable technology is used. This limitation is most likely due to the importance of intact coordinated BMP and Wnt signaling required for the formation and proper differentiation of the pluripotent epiblast (7, 58). These newer technologies are preferred over multiple integrating viruses (29), which may mask the function of the mutation, induce their own mutant behavior, and obscure true penetrance via the creation of virus-dependent genetic alterations (52, 60). However, with the rapid simplification of accessible technology, these limitations are being overcome. 2) The iPS cell model system lacks the capacity to model complex in vivo events and alterations in the physiological milieu. 3) It is possible that the cellular ramifications of a BMPR2 mutation are not uniform across mutation type. However, in contrast to in vitro models that ectopically express BMPR2 mutation in WT cells while retaining WT BMPR2 signaling, the iPS and primary patient cells have allowed us to preserve the disease-specific regulation of two key signaling pathways involved in PAH. We have been successful in identifying the cell-specific changes in Wnt signaling, including Sfrp-2, that have been linked in development but have not been studied in the context of adult disease. Ongoing investigation in our laboratories is focused on understanding the regulation and role of Sfrp-2 during vascular lesion formation in PAH, as well as correction of BMPR2 mutation in our HPAH line using CRISPR gene-editing technology to elucidate the direct effects of BMPR2 on this system.

Our studies linked deregulated developmental pathways with adult disease over multiple cell types and differentiation states. We show that decreased BMPR2 signaling results in a genetic signature common across multiple cell types, culminating in the pathological processes recognized as PAH. Taken together, our results suggest that increased Wnt signaling is inherent to the cells of PAH patients and is likely due to decreased BMPR2 signaling. This combination of iPS and primary patient cell modeling may ultimately enable the identification of cellular defects that lead to the clinical manifestations of PAH and provide access to multiple renewable cell types in which to test potential therapies.

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