Detailed Materials and Methods

Breeding, Hypoxia, Hemodynamic and Morphometric Studies in Mice

The Animal Care Committee at Stanford University approved all the experimental protocols used in this study in accordance with the guidelines of the American Physiological Society. *Bmpr1a*$^{(+/flox)}$ mice[1, 2] and *Bmpr2*$^{(+/flox)}$ mice[3] were each crossed with inducible ACTB-Cre^{ER} mice (The Jackson Laboratory, Stock no: 003376). The offspring were given an intraperitoneal injection of tamoxifen for 10 days to induce deletion of target genes and generate *Bmpr1a*$^{+/-}$ and *Bmpr2*$^{+/-}$ mice (F1). Further breeding of the offspring generated *Bmpr2/1a* compound heterozygous progeny. The mice were initially on a mixed C57 BL/6J, SV129 and FVB background but were back-crossed for more than six generations onto a C57BL/6J background. To avoid gender bias, mice of both genders were used at 8-10 weeks of age. For hemodynamic studies that included VEGF receptor blockade, Sugen 5416 (Sigma, St. Louis, MO) was given at 20mg/kg, by subcutaneous injection once weekly at the beginning of each week for three weeks[4] during which the mice were placed in a hypoxia chamber and exposed to 10% inspired O_2 with access to food and water ad libitum. Echocardiographic measurements of cardiac function included pulmonary artery acceleration time[5] carried out under isoflurane anesthesia (1.5% - 2.5% in 2L O_2/min). Assessment of right ventricular systolic pressure (RVSP) was carried out under isoflurane anesthesia (1.5% - 2.5% in 2L O_2/min) in unventilated mice using a closed-chest technique as previously described[6]. Right ventricular hypertrophy (RV) was assessed as the weight of the RV relative to left ventricle (LV) plus septum. Lungs were perfused with normal saline and fixed for routine histology and morphometric analyses carried out as previously described[3].

Morphometric Evaluation of Main Pulmonary Artery (PA)

The autofluorescence of elastin permits visualization of the internal elastic lamina and the fenestrations that perforate it using confocal microscopy[7, 8]. The size of the fenestrations in the internal elastic lamina was assessed before and after elastase or vehicle treatment as a measure of susceptibility of the elastin to degradation[9]. For these studies, *Bmpr2/1a* compound heterozygote (Het) and wild type (WT) mice (n=5 per group) were euthanized with 120 mg/kg of pentobarbital and the heart and lungs isolated *en bloc*. The lungs were either flushed slowly with PBS and perfused with PBS (controls), or flushed with PBS and perfused with PBS containing 5μg/mL porcine pancreatic elastase (Sigma, St. Louis, MO) and then immersed in the same solution for 20min at 37°C. A second cohort of WT and Het murine lungs were flushed and perfused with saline at the end of the Sugen 5416+Hypoxia treatment. The pulmonary vasculature was fixed by perfusion using 4% paraformaldehyde at room temperature for five minutes, and immersion-fixed for one additional hour. The main PAs were removed, mounted lumen side down in glycerol on glass slides, and visualized with an Olympus IX81 microscope using a FV1000 confocal scanning system. Autofluorescence of elastin was excited at 488nm, and emission was detected at 500-560nm wavelengths. Serial optical sections (z step=0.81μm) from the lumen to adventitia were captured with a ×63 oil objective and superimposed as nine images with the best visualization of the internal elastic lamina. For each PA, quantification of the number and size of fenestrations in the elastic lamina was assessed in six separate fields per sample, at ×400 magnification using ImageJ.

Lung tissues from IPAH and HPAH Patients with a *BMPR2* mutation, and Controls

Lung tissues from IPAH and HPAH patients with a *BMPR2* mutation who underwent lung transplantation, and from unused donor lungs as controls were obtained through the Pulmonary Hypertension Breakthrough Initiative (PHBI) Network, which is funded by the NIH/NHLBI and

the Cardiovascular Medical Research and Education Fund (CMREF). The tissues were procured at the Transplant Procurement Centers at Allegheny General Hospital, Baylor University, Cleveland Clinic, Duke University, Stanford University, the University of California, San Diego, University of Alabama at Birmingham, and Vanderbilt University, and de-identified patient data were obtained via the Data Coordinating Center at the University of Michigan. Lung tissues were kept in RPMI 1640 media supplemented with antibiotics for up to 24h during transportation from the transplant procurement centers. Small pieces of the lung tissue were fixed in 10% formaldehyde overnight for immunohistochemistry. Procurement of the tissues from human subjects is approved by the Administrative Panel on Human Subjects in Medical Research at Stanford University (IRB #350, Panel 6), and at the other procuring centers.

Cell Culture

Pulmonary artery (PA) smooth muscle cells (SMC) fibroblasts (PAF) from patients with IPAH and HPAH with a *BMPR2* mutation and unused donor controls were harvested and cultured from lungs obtained through the PHBI according to previous protocols[10]. Demographic information regarding patients and unused donor control lungs is provided in Table 1.

PA SMC and PAF were grown in PA SMC growth medium (Lonza, Allendale, NJ) containing growth factors, 10% fetal bovine serum, and penicillin/streptomycin; subcultured at a 1:4 ratio in 100mm dishes (Corning, Lowell, MA) or Nunc™ Lab-Tek™ II Chamber Slide™ (Thermo Fisher Scientific, Waltham, MA) and used between passages 3-6. Cells were starved in PA SMC starvation medium (0.1% FBS) for 24h before adding BMP4 (10ng/ml, B2680, Sigma, St. Louis, MO), TGFβ1 (2ng/ml, ab50036, Abcam, Cambridge, MA) or vehicle (growth factor re-suspension media: 4mM HCL containing 0.1% human serum albumin). For assessment of elastic fiber formation, 30,000 PAF were seeded in Nunc™ Lab-Tek™ II Chamber Slide™ (Thermo Fisher Scientific, Waltham, MA) and allowed to grow for four days until confluence. Following starvation for 24h, cells were stimulated as indicated every other day, for seven days. Cells then were rinsed once with PBS and fixed with 4% paraformaldehyde. Elastic fiber and fibrilin-1 staining were visualized using elastin (1:500, sc17580, Santa Cruz Technologies) and fibrilin-1 (1:500, Elastin Products Company) antibodies, respectively. In siRNA experiments, cells were treated with siRNA and allowed 24h for recovery before starvation and stimulation. Elastic fibers and fibrilin-1 staining were quantified by Image J software and normalized to cell number assessed by nuclei DAPI staining, in six images per condition.

For the conditioned media experiments, SMC and PAF were grown as previously described. After cells were stimulated for 24h, the media produced by PAF was recovered and added to SMC cells; concurrently the media from SMC was added to PAF. An internal control was used where media from PAF cells was added to PAF and media from SMC cells was added to SMC cells. In siRNA experiments, cells were treated with siRNA and allowed 24h for recovery before starvation and stimulation. In the decorin experiments cells were grown and stimulated as previously described, and cells were treated with decorin siRNA or decorin peptide (100μM, ab71694, Abcam) for seven additional days. Elastic fibers were quantified as the percentage of fluorescence per field using ImageJ, in six images per condition.

Quantitative PCR

RNA was extracted using spin column-based kits (Zymo Research, Irvine, CA) and RT-PCR performed following the manufacturer's guidelines. qPCR was performed with primer sequences designed using the National Center for Biotechnology (NCBI) (Bethesda, MD) Primer-BLAST function.

Primers Used for qPCR Analysis:

Gene	Forward Sequence	Reverse Sequence
Emilin-1 *(EMILIN)*	GGGAGTCAGAGAAGGTGCAG	AGCTGGTGCTGGATCTCATT
Fibrillin-1 *(FBN1)*	ACCGTGCTTTTAGCGTCCTA	GGCAAATGGGGACAATACAC
Fibulin-5 *(FBLN5)*	TATTGATGAATGCCGAACCA	ACCTGAGTAGGGGGTCGAGT
Lysyl oxidase *(LOX)*	CGACCCTTACAACCCCTACA	AAATCTGAGCAGCACCCTGT
MAGP-1 *(MFAP2)*	CGGAGGCTGTAGAAGCAGAC	ACCAGATCGACAACCCAGAC
MAGP-2 *(MFAP5)*	ACTCGGTGGAAAGAGCAGAA	GCCAAAACAGCCAAAACTGT
Tropoelastin *(ELN)*	GGCTGAGGAACCACCGCACC	TGGGGCCTGGAGGCAAACCT

Western Immunoblot Analysis

For protein expression analysis, PA SMC or PAF were washed with ice-cold PBS, and lysates prepared by adding boiling lysis buffer (10mM Tris HCl pH7.5, 1% SDS) containing protease and phosphatase inhibitors (Thermo Scientific, Waltham, MA). Lysates were scraped into a 1.5-mL microcentrifuge tube and boiled for 10min before centrifugation. Supernatants were transferred to fresh microcentrifuge tubes and stored at −80°C. Conditioned medium was concentrated using Amicon Ultra-15 Centrifugal Filter Units, per the manufacturer's protocol (Millipore, Billerica, MA). Protein concentration was determined by the Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA). Equal amounts of protein were loaded onto each lane of a 4–12% Bis-Tris gel and subjected to electrophoresis under reducing conditions. After blotting, polyvinylidene difluoride membranes were blocked for 1h (5% milk powder in PBS/0.1% Tween) and incubated with primary antibodies overnight at 4°C. The binding of secondary HRP-conjugated antibodies was visualized by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA). Normalizing for total protein was performed by reprobing the membrane with a mouse monoclonal antibody against β-actin for elastin or by Ponceau staining for fibrillin-1. Densitometric analysis of the bands was then performed.

The antibody dilutions used were: elastin (1:500, sc17580, Santa Cruz Technologies, Santa Cruz, CA), fibrillin-1 (1:1000, Elastin Products Company, Owensville, MO), β-actin (1:5000, sc47778, Santa Cruz Technology, Santa Cruz, CA), BMPR2 (1:250, 612292, BD Transduction, San Jose, CA), fibronectin (1:2000, F3648, Sigma-Aldrich, St Louis, MO). Secondary antibodies were used with dilutions of 1:2000 to 1:5000.

2D DIGE Gel

Cells were stimulated for 48 hours in starvation media as previously described, and the conditioned media collected. After spinning at 14000 rpm for 30 min the supernatant was collected and concentrated using Amicon Ultra-15 Centrifugal Filter Units, per the manufacturer's protocol (Millipore, Billerica, MA). A minimum of 0.3 mg of total protein was recovered and sent to Applied Biomics (Hayward, CA) for 2D DIGE analysis. The 2D gel was run as follows: Cy2: Internal Standard (equally mixed samples); Cy3: SMC; Cy5: PAF. The data was analyzed using ImageQuant software and DeCyder 2D software to find significantly

changed spots (analysis performed by Applied Biomics). Following this step the images were analyzed and 20 spots were picked for mass-spectrometry identification. Protein identification is based on peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data). Applied Biomics uses the MASCOT search engine to identify proteins from primary sequence databases.

Immunofluorescence

Sections from formaldehyde-fixed and paraffin-embedded human and mouse lung tissues were deparaffinized and rehydrated. Epitope retrieval was performed by boiling the sections in 0.25mM EDTA. Sections were reacted with hydrogen peroxide to block endogenous peroxidase, washed, and blocked with Sea Block (ThermoFisher Scientific, Wlatham, MA). Sections were then incubated with the primary antibodies against elastin (1:1000, sc17580, Santa Cruz Technologies) or fibrillin-1 (1:1000, Elastin Products Company) overnight at 4°C. The rabbit polyclonal antibody (pAb 9543, 1:400) produced against a human fibrillin-1 immunogen (recombinant fibrillin polypeptide rF11, which spans the N-terminal half of fibrillin-1) has been characterized and cross-reacts with mouse fibrillin-1[11], and was kindly provided by Dr. Lynn Sakai. Fragmentation or discontinuity of elastin that was not attributable to sectioning artifact was quantified as the numbers of breaks per pulmonary artery. Five arteries were quantified for every section and controlled for similar arterial size in WT and transgenic mice.

siRNA Transfection

siRNAs for *BMPR2* and *FBN1* (SMARTpool: ON-TARGETplus, L-005309-00 and L-011034-00-0005, respectively, Thermo Fisher Scientific, Waltham, MA) were transfected into PAF using RNAiMax (Invitrogen, Carlsbad, CA). siRNA for DCN (SMARTpool: ON-TARGETplus, L-021491-00-0005, Thermo Fisher Scientific, Waltham, MA) were transfected into SMC. siRNAs were transfected as described previously[3]. The knockdown efficiency for both BMPR2 and Fibrillin-1 were determined by western immunoblotting. Nontargeting siRNA (D-001810-10, Dharmacon, Thermo Fisher Scientific, Waltham, MA) was used as a control.

Statistical Analysis

The number of samples or animals studied per experiment is indicated in the figure legends. Values from multiple experiments are expressed as mean±SEM. Data were consistent with normal variance. Statistical significance was determined using unpaired *t*-test for two comparisons only or one- or two-way ANOVA followed by Bonferroni multiple comparison tests. A *p*-value of <0.05 was considered significant.

Materials and Methods References

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