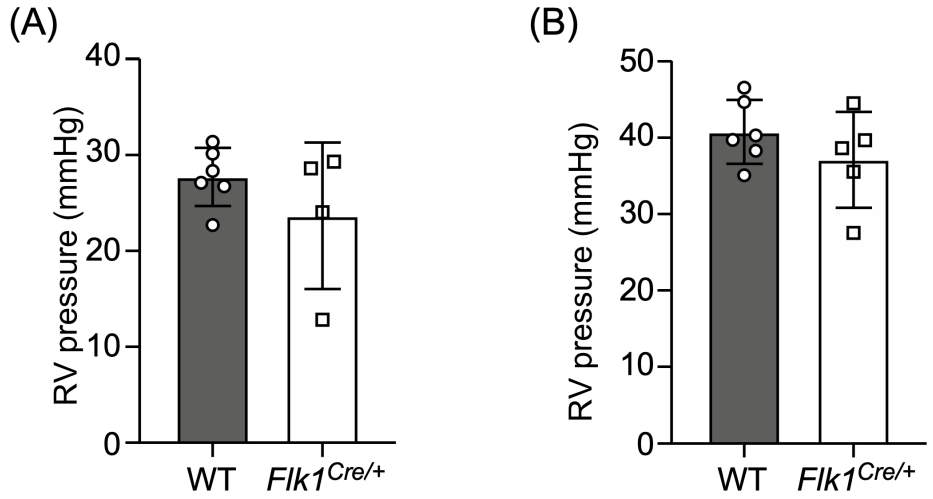
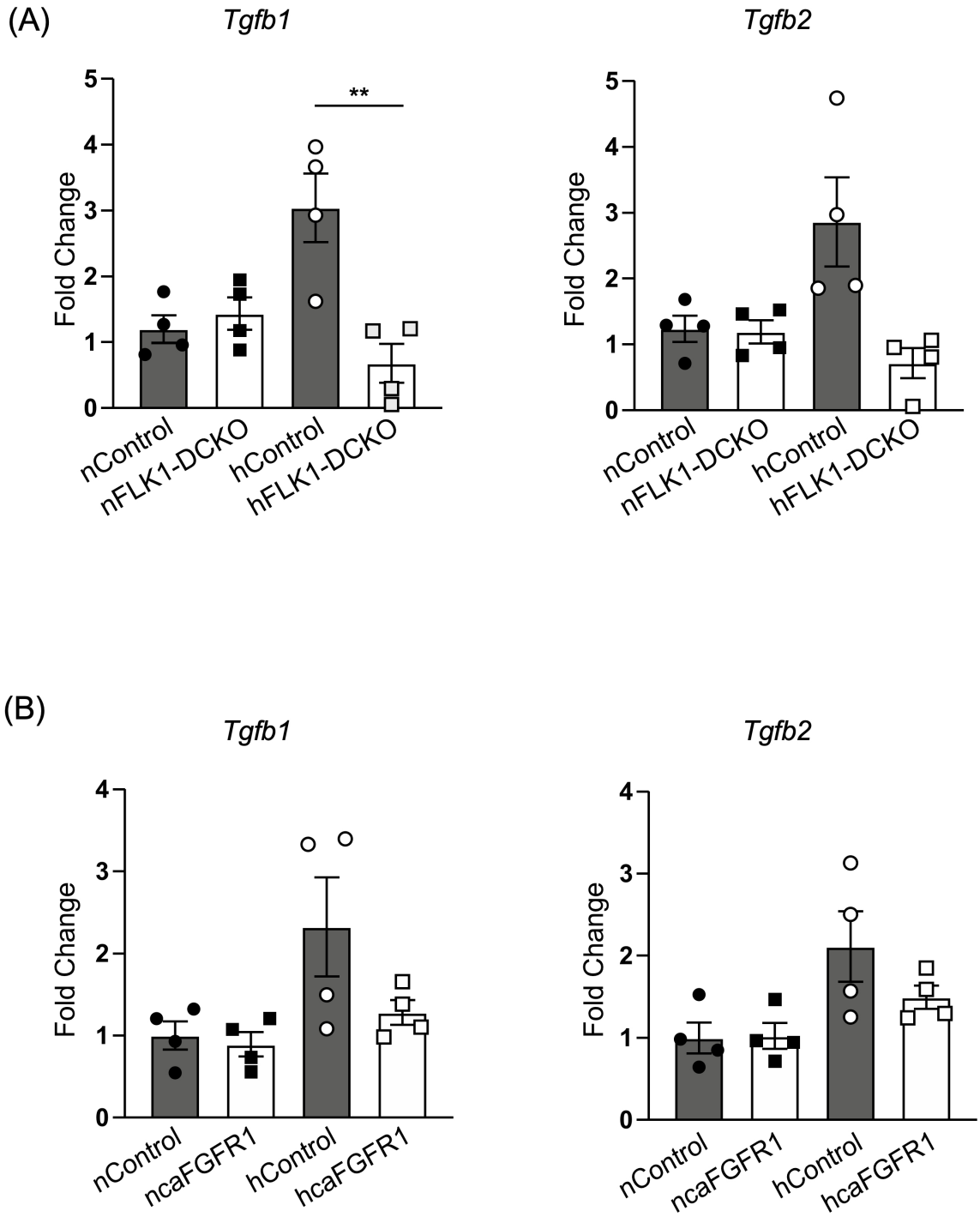
Supplemental Figure 1



Supplemental Figure 1. RV pressure in wild type and Flk1^{Cre} mice. RV pressures were determined by cardiac catheterization of hypoxia-challenged *Flk1^{Cre}* and *Fgfr1$^{f/f}$; Fgfr2$^{f/f}$* mice under (A) normoxia and (B) hypoxia conditions. Statistical significance was determined by two-tailed, unpaired Student's *t* test. All data are shown as the mean ± SEM.

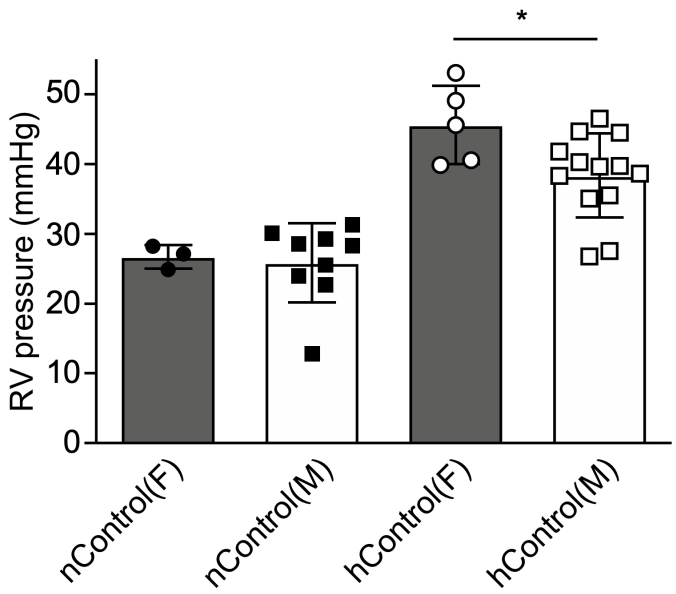
Supplemental Figure 2



Supplemental Figure 2. Effect of hypoxia and FGFR signaling on expression of TGFβ ligands. Expression of TGFβ1 and TGFβ2 in hypoxia-exposed (A) FLK1-DCKO and (B)

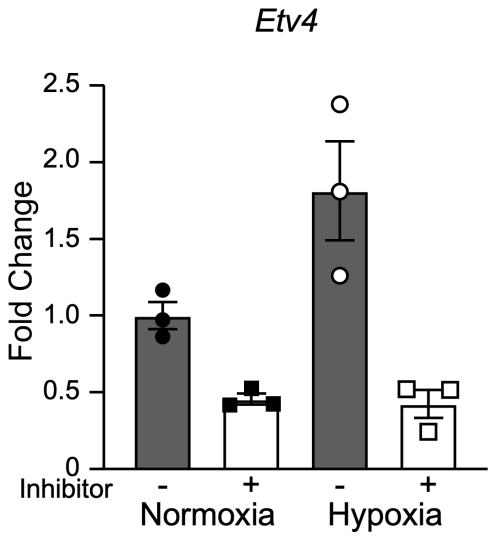
caFGFR1 mouse lungs, respectively, n = 4. Statistical significance was determined by two-way ANOVA with Holm-Šídák's multiple comparisons. All data are shown as the mean ± SEM. ** $p<0.01$. Closed circle, control mice in normoxia (nControl); Open circle, control mice in hypoxia (hControl); Closed square, *Flk1*^{Cre}*; Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$ mice in normoxia (nFLK1-DCKO) or caFGFR1 mice in normoxia (ncaFGFR1); Open square, *Flk1*^{Cre}*; Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$ mice in hypoxia (hFLK1-DCKO) or caFGFR1 mice in hypoxia (hcaFGFR1).

Supplemental Figure 3



Supplemental Figure 3. RV pressure in hypoxia exposed male and female mice. RV pressures were determined by cardiac catheterization of hypoxia-challenged female (F) and male (M) *Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$ (Control) mice. Statistical significance was determined by two-way ANOVA with Holm-Šídák's multiple comparisons. All data are shown as the mean ± SEM.

Supplemental Figure 4



Supplemental Figure 4. Effect of FGFR inhibition on *Etv4*. Relative RNA expression of *Etv4* in HPAECs exposed to14 days of hypoxia compared to controls in normoxia, with or without FGFR inhibitor, BGJ398, n = 3. Statistical significance was determined by two-way ANOVA with Holm-Šídák's multiple comparisons. Closed circle, HPAECs in normoxia treated with vehicle; Closed square, HPAECs in normoxia treated with BGJ398; Open circle, HPAECs in hypoxia treated with vehicle; Open square, HPAECs in hypoxia treated with BGJ398. All data are shown as the mean ± SEM.

Diagnosis	Avg Age(yr)	Females(n=)	Males(n=)	Avg mPAP(mmHg)	Avg RAP(mmHg)	Avg PVR(Woods units)	Avg CI(L/min/m2)
Combined Pulmonary Fibrosis with Emphysema	67 (1.4)	1	3	31.5(5.8)	7.8(2.6)	3.8(0.3)	2.6(0.2)
Controls	42.2 (21.7)	2	3	-	-	-	-

Table 1. Characteristics of Patients

Supplemental Table 1. Patient Characteristics

Supplemental Methods

Mouse models

All mouse lines have been previously described: *Fgfr1*$^{f/f}$ (83), *Fgfr2*$^{f/f}$ (84), *Flk1*^{Cre} (85), Tie2-Cre (85), *ROSA*^{rtTA} (86), TRE-caFgfr1 (44), Cdh5-CreERT2 (87). To create a conditional inactivation of *Fgfr1* and *Fgfr2* in ECs, *Flk1*^{Cre} knock-in mice (85) were crossed with mice with both *Fgfr1* and *Fgfr2* genes flanked by loxP sites (*Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$). This resulted in EC-specific ablation of FGFR1 and FGFR2 (*Flk1*^{Cre}; *Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$). These mice are referred to as FLK1-DCKO mice. Controls for these experiments include *Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$ double flox mice and *Flk1*^{Cre} mice (Control). No differences were observed between either control groups for any outcomes, so the presented data include a combination of both types of controls. Cdh5-CreERT2 mice were bred into a background of animals carrying *Fgfr1*$^{f/f}$ and *Fgfr2*$^{f/f}$ alleles. These mice are referred to as Cdh5-DCKO mice. At p21, Cdh5-DCKO and control mice were given three intraperitoneal injections of tamoxifen (no. T5648, Millipore-Sigma) dissolved in corn oil (no. C8267, Millipore-Sigma) at 200 µg/gram bodyweight on alternate days.

We previously generated a tetracycline response element TRE-Fgfr31c(R248C) transgene that expresses a chimeric receptor consisting of the FGFR3c(R248C) mutant extracellular and transmembrane domain fused to the FGFR1 tyrosine kinase domain (caFGFR1) (44, 88). The FGFR3c(R248C) mutation confers ligand-independent dimerization (88), and thus produced a constitutively active FGFR1 intracellular tyrosine kinase domain. To create a mouse with conditional activation of FGFR1 in ECs we utilized the doxycycline (DOX)-inducible TET-on system and generated Tie2-Cre, *ROSA*^{rtTA}, TRE-Fgfr31c(R248C) mice (44), referred to as TRE-caFGFR1 mice. At age 4 weeks, TRE-caFGFR1 and control mice were given doxycycline chow (200 mg/kg of doxycycline; no. S3888, Bio-Serv) and kept on the diet until the end of the experiment. Ikeda et al. reported genetic background specific responses to hypoxia (89). To mitigate a potential genetic background effect, all experimental mice were maintained on a mixed C57BL/6J and 129X1/SvJ genetic background.

Chronic hypoxia model

Adult male and female mice at 6–8 weeks of age were randomly divided into two experimental groups based on exposure to normoxia and hypoxia. Hypoxic groups were kept in a normobaric ventilated hypoxic cabinet (Coy Laboratory Products, Grass Lake, Michigan) for 14 days with fraction of oxygen in the inspired air (FiO2) set at 10%. Normoxic groups were kept in room air for 14 days. Routine husbandry was performed within the chamber with no interruption of oxygen levels. At the end of the hypoxia exposure period, the adult FLK1-DCKO hypoxia and normoxia groups were analyzed by echocardiography. The adult hypoxic mice were placed back in the hypoxia chamber for at least 2 hours after echocardiography before right heart catherization for RV pressure. Cdh5-DCKO and TRE-caFGFR1 mice were analyzed by right heart catheterization for RV pressure after 14 days of hypoxia exposure.

Echocardiography

Transthoracic 2D M-mode and Doppler imaging were performed in the Washington University Mouse Cardiovascular Phenotyping Core facility (https://mcpc.wustl.edu/) using a VisualSonics Vevo 2100 In Vivo Imaging System (Visual Sonics, Toronto, ON) according to the guidelines of the American Society of Echocardiography. Mice were anesthetized with Avertin (2,2,2-tribromoethanol, 100 mg/kg, i.p.). Avertin was chosen due to its lack of cardio-depressive effects at the doses administered in this study. Pulmonary hemodynamics were assessed through

images acquired from the left parasternal short-axis view. Pulse-wave Doppler was used with sample volume placed in the center of the color Doppler of the main PA and interrogated along the line of flow. To interrogate pulmonary artery flow and RV outflow tract systolic time intervals, PAAT was measured from the interval between the onset of systolic ejection and the peak flow velocity. RV ejection time (RVET) was also measured from the interval between the onset of RV ejection to the point of systolic pulmonary arterial flow cessation.(90) To account for heart rate variability, PAAT was adjusted to RVET and presented as PAAT:RVET. PAAT and PAAT:RVET are non-invasive measure of RV afterload that provide an accurate estimate of invasive PVR, PA pressure, and PA compliance in children with PH (91) and in hypoxia PH mouse models (92).

Cardiac catheterization

Adult mice were anesthetized with Isoflurane (2% maintenance) + pancuronium (1 mg/kg given once). This anesthesia produces a near physiologic heart rate of 500 beats/min, while still allowing for a surgical plane of anesthesia. The mice were intubated and ventilated with a Harvard ventilator set at 200-400 µl. The right external jugular vein was identified in the region of the neck and cannulated with a 1.2 French high fidelity micromanometer pressure only catheter (SciSense Advantage System, Transonic, London, Ontario, Canada). The catheter was advanced retrograde into the right atrium and through the tricuspid valve into the RV to assess pressures. Continuous RV systolic and diastolic pressures were recorded and analyzed with SciSense analysis software by a double-blinded observer.

Tissue processing

Mice were euthanized with a lethal dose of ketamine and xylazine and exsanguinated by cutting the abdominal aorta. The trachea was dissected and cannulated, lungs were fixed via intra-tracheal inflation with 4% paraformaldehyde at a pressure of 20 cm H_2O for 10 min. Heart and lungs were next immersed in fresh fixative overnight at room temperature. Lung samples were dehydrated in ethanol and xylene, embedded in paraffin, cut in 6 µm sections, and stained with hematoxylin and eosin (H&E). Generally, the right lobes were used for immunohistochemical analyses and the left upper lobe was used for RNA analyses. Samples for RNA analyses were stored at −80°C until use.

Measurement of ventricular weights

Atria and outflow tracts were removed from the hearts and the RV was carefully excised following their septal borders by a blinded observer. The weight mass of the RV, and the remainder of the heart, left ventricle (LV) + septum(S), were recorded. RV hypertrophy was measured by calculating the RV mass relative to the LV+S and the RV or LV+S relative to the animal's body weight.

Histological assessment of pulmonary hypertension

For assessment of pulmonary vascular muscularization, lung tissue sections were immunostained for alpha-smooth muscle actin, and 40 vessels (25-50 µm and 50-100 µm in diameter from each lung) were outlined by a double-blinded observer. The degree of medial wall thickening was expressed as a ratio of medial area to cross sectional area. To assess the number of vessels with increased muscularization, a double-blinded observer identified 40 intra-acinar vessels 20-50 µm in diameter (from each mouse) and categorized them as non-muscular (no evidence of vessel wall muscularization), partially muscular (VSM cells identifiable in less than three-fourths of the vessel circumference), or muscular (VSM cells in more than three-fourths of the vessel circumference) (93). The results were expressed as the ratio of non-, partially-, fully-

muscularized vessels to the total number of vessels. Muscularization was defined as the presence of VSM cells immunostained for αSMA (ACTA2). To obtain the percentage of pulmonary vessels in each muscularization category, the number of vessels in that category was divided by the total number counted in the same experimental group.

Immunohistochemistry and Immunofluorescence

Histological sections (6 μm) were prepared from paraffin-embedded tissues. Sections were deparaffinized and re-hydrated. Antigen retrieval was performed using a pressure cooker and citrate buffer (pH 6.0). Tissues were blocked with 5% goat or donkey serum. Primary antibody for αSMA (1:200, Dako North America, M0851) was added to blocking buffer and slides were incubated overnight at 4 °C. Colorimetric reaction was performed using DAB staining (Vector Labs, Burlingame, CA) and sections were counterstained with hematoxylin. For immunofluorescence, the following antibodies were used: PECAM1 (CD31, 1:50, Dianova, Dia310),rabbit anti-pSMAD2/3 (1:100, Abcam, ab272332), rat anti-tdTomato (1:50 Kerafast, EST203), FITC-conjugated mouse anti-αSMA (1:100, MilliporeSigma, F3777), mouse anti-αSMA (1:100, Agilent Dako, M0851), goat anti-VE-cadherin (1:200, Santa Cruz, sc-9989), rabbit anti-ETV5 (1:100, Abcam, ab102010), mouse anti-FGF2 (1:200, Santa Cruz, sc-365106) and Alexa Fluor-conjugated secondary antibodies (1:200, ThermoFisher) and Image J. Autofluorescence in human lung tissue samples were quenched using ReadyProbes Tissue Autofluorescence Quenching Kit (Invitrogen, R37630).

Imaging

Immunofluorescent imaging was performed using a Zeiss Apotome II and image processing was performed using Zeiss Axioplan software. Whole-lobe images were processed using NanoZoomer Digital Pathology (NDP.view) software or ImageJ (Fiji). Digital scanning of whole slides was performed using a Nanozoomer 2.0 HT digital slide scanner (Hamamatsu, Bridgewater, NJ), available through the Washington University Hope Center Alafi Neuroimaging Lab (NIH Shared Instrumentation Grant (S10 RR027552)). Images were analyzed using NDP.view2 software. For imaging of lineage tracing with double staining of endothelial and smooth muscle actin markers, settings for αSMA staining were used to set the exposure for all samples in the same experiment.

Quantitative RT-PCR

RNA was isolated from mouse lungs with TRIzol Reagent (Thermo Fisher, #15596026). RNA from HPAECs were isolated with RNeasy Kit (Qiagen, #74104). cDNA was synthesized from lung tissue and HPAEC RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, #170-8841) and quantified using TaqMan Fast Advanced Master Mix (Life Technologies, 4 444 557). Rpl32 was used as normalization control. Results were analyzed by the $\Delta\Delta$CT method (normalized to Rpl32, then normalized to normoxia control).

Taqman assay probes

Taqman assay probes used were (mouse) *Rpl32* (Thermo Fisher, Mm02528467_g1), *Fgf2* (Thermo Fisher, Mm00433287_m1), *Fgfr1* (Thermo Fisher, Mm00438930_m1), *Fgfr2* (Thermo Fisher, mm01269930_m1), *Tgfbr2* (Thermo Fisher, Mm03024091_m1), *Snai1* (Thermo Fisher, Mm00441533_g1), *Snai2* (Thermo Fisher, Mm00441531_m1), *Twist1* (Thermo Fisher, Mm00442036), *Pecam1* (Thermo Fisher, Mm01242576_m1), *Acta2* (Thermo Fisher,

Mm00725412_m1); (human) *Rpl32* (Thermo Fisher, Hs00851655_g1), *Fgf2* (Thermo Fisher, Hs00266645_m1), *Etv4* (Thermo Fisher, Hs00385910_m1), *Etv5* (Thermo Fisher, Hs00927557_m1), *Dusp6* (Thermo Fisher, Hs04329643_s1), *Tgfb2* (Thermo Fisher, Hs00234244_m1), *Tgfb3* (Thermo Fisher, Hs00234245_m1), *Tgfbr2* (Thermo Fisher, Hs00234253_m1), *Snai2* (Thermo Fisher, Hs00161904_m1), *Twist1* (Thermo Fisher, Hs04989912_s1), *Cdh5* (Thermo Fisher, Hs00901465_m1), *Vim* (Thermo Fisher, Hs00958111_m1).

In vivo EndoMT quantitation

Flk1^{Cre}*; Fgfr1*$^{f/f}$*; Fgfr2*$^{f/f}$ *; ROSA*^{tdTomato} and Tie2-Cre; *ROSA*^{rtTA}; TRE-caFgfr1; *ROSA*^{tdTomato} mice were exposed to hypoxia or normoxia for 14days. Lung sections were prepared for histology and immunofluorescence as described above. Slides were incubated with 5% goat serum blocking solution for 1 hour, then with rat anti-tdTomato (1:50 Kerafast, EST203) and FITC-conjugated mouse anti-αSMA (1:100, MilliporeSigma, F3777) in blocking solution overnight at 4°C. Slides were washed with PBSx3 then incubated with Cy3-conjugated goat anti-rat secondary antibody (1:200, Jackson ImmunoResearch, 112-165-003) for 1 hour at room temperature. The slides were washed in PBS and mounted with Vectashield Antiface Mounting Medium with DAPI (Vector Laboratories, H-1200). Vessels were identified by morphology and presence of αSMA-positive vessel wall. Twenty images were taken per mouse and the number of αSMA-positive cells that were also TdTomato-positive were recorded.

Cell culture

Human pulmonary endothelial cells (HPAECs) were purchased from Promocell (C-12241, PromoCell GmbH, Sickingenstr. 63/65 69126 Heidelberg, Germany). HPAECs were cultured on gelatin-coated dishes using Endothelial Cell Growth Medium BulletKit (EGM-2, Lonza, Allendale, NJ) containing 2% FBS and FGF2, passaged and used for experiments at either passage 4 or 5. Experiments were repeated in triplicate and performed with 2 different lots of HPAECs. Infagratinib (BGJ398) was a gift from MedChemExpress (HY-13311, Monmouth Junction, NJ, USA).

In vitro hypoxia

HPAECs were cultured in a dedicated hypoxia cell culture incubator (IncuSafe Multigas Incubators, Panasonic Healthcare, Wood Dale, IL, USA) at 5% O_2 and 5% CO_2 for 48 hours or 14 days. Cells were briefly removed from the incubator for media change every 2 days. Control cells were cultured in a separate cell culture incubator at 20% O_2, 5% CO_2 for 14 days.

Western blot

Cells were lysed in RIPA Lysis Buffer (MilliporeSigma, #R0278) with ProBlock Mammalian Protease Inhibitor Cocktail (Gold Biotechnology, #GB-331-1) and Simple Stop 2 Phosphatase Inhibitor Cocktail (Gold Biotechnology, #GB-451). Lysates were then placed on ice for 30 min followed by centrifugation at 13,200 rcf at 4ºC after which supernatant was collected. BCA assay (Thermo Fisher Scientific, #23227) was used to quantify protein before denaturation in LDS Sample Buffer (Life Technologies Corporation, #NP0007) and Sample Reducing Buffer (Life Technologies Corporation, #NP0009) at 70ºC for 10mins then stored at -80ºC. Protein concentrations were determined with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific,

23225). Lysates were run at 160V for 1.5 h on ice at 4ºC using NuPage Bis-Tris 8% gel (Thermo Fisher Scientific, #NP0302BOX) in MOPS buffer (Thermo Fisher Scientific, #NP0001). iBlot System (Thermo Fisher Scientific, #IB1001) was used to transfer protein onto PVDF membrane, according to manufacturer protocol. The membrane was then incubated in TBS-Tween 0.05% (TBS-T) for 10 min before blocking at room temperature for 1 hour using TBS-T with 5% (w/v) non-fat milk (TBS-TM). Membranes were then incubated overnight at 4ºC with the rabbit anti-pSMAD2/3 (1:100, Abcam, Cambridge, UK, ab272332) in with TBS-TM. Following wash (3x10 min with TBS-T), blots were incubated for 1 hour at room temperature in HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, #SC-2301), washed 3x10 min with TBS-T, then incubated in ECL (Bio-Rad Laboratories, #1705061) followed by imaging. Blots were incubated with stripping buffer and incubated with rabbit anti-Smad2/3 (catalog no. 610842; BD Pharmingen) as indicated. Western blot membranes were imaged and quantified using Biorad Chemidoc (Bio-Rad Laboratories) and Image Lab Software (Bio-Rad Laboratories). Densitometry analyses were performed using ImageJ.

Flow cytometry

Mice were sacrificed with an overdose of a cocktail containing ketamine and xylazine, perfused with PBS through the right ventricle and the lungs were inflated with a digest media containing 50 units/ml Dispase (Beckton Dickinson, 354235) and 2 mg/ml Collagenase Type 2 (Worthington Biochemicals, Cat# 4177). Lungs were minced into small pieces with razor blades and digested for 30 min at 37°C with gentle agitation. Digestion enzymes were inactivated with FACS buffer (PBS+10% FBS+1 mM EDTA), samples were filtered through a 100 µm cell strainer and then a 40 µm cell strainer. Red blood cells were lysed with ACK lysis buffer (Thermo Fisher, A1049201) and cells were resuspended in FACS buffer.

HPAEC quantitation

HPAECs were cultured on gelatin coated Nunc Lab-Tek II 4-well Chamber Slide (Thermo Fisher, 154526) in hypoxia or normoxia for 14 days. Cells were fixed in fresh cold 4% paraformaldehyde on ice for 10 minutes and washed in PBS x3. Blocking was performed with 5% donkey serum for 1 hour at room temperature. HPAECs were incubated overnight in 4°C with mouse anti-αSMA (1:100, Agilent Dako, M0851) and goat anti-VE-cadherin (1:200, Santa Cruz, sc-9989) in blocking solution. Cells were washed with PBSx3 and incubated with fluorescent secondary antibodies AlexaFluor 594-conjugated donkey anti-goat (Thermo Fisher, #A-11058) and AlexaFluor 488-conjugated donkey anti-mouse (Thermo Fisher, #A-21202) for 1 hour at room temperature. Finally, HPAECs were washed in PBSx3, chambers removed and coated in Vectashield Antiface Mounting Medium with DAPI (Vector Laboratories, H-1200) and coverslipped. Immunofluorescent imaging was performed using a Zeiss Apotome II and image processing was performed using Zeiss Axioplan software. Each well was visually scanned entirely and all colonies of αSMA expressing cells were imaged. The total number of colonies per well are plotted as one datapoint.

Studies of human lung tissue samples

Human lung tissue samples were obtained from 4 patients (3 males and 1 female) with combined pulmonary fibrosis with emphysema (CPFE) at the time of lung transplant surgery at Barnes-Jewish Hospital (St. Louis, MO) and from 5 (3 male and 2 female) non-transplantable donors between 2008-2015. In the patients with CPFE, mean pulmonary artery pressure was 31.5$\pm$5.8 mmHg (range, 27 to 40 mmHg), mean pulmonary vascular resistance was 3.8$\pm$0.3

Woods units (range, 3.52 to 4.17 Woods units) and mean cardiac index 2.6±0.2 $L \bullet min^{-1} \bullet m^{-2}$ (range, 2.32 to 2.94 $L \bullet min^{-1} \bullet m^{-2}$) (Table 1).

Statistics

Data were analyzed using the unpaired Student's *t* test to compare two samples with equal variance. Two-way ANOVA with Holm-Šídák's multiple comparisons was used to compare multiple conditions and genotypes. All data was analyzed and plotted using GraphPad Prism software (ver. 9.0; GraphPad, La Jolla, CA). Differences with $P < 0.05$ were considered significant. All values are presented as mean ± SEM. *P<0.05; **P<0.01. All figures were made using Canvas X (Canvas GFX) software.

Full Western Blots for Figure 11