Supplemental methods

Animals

Adult male C57BL/6J mice, 20–22 g, age 3-4 months, were obtained from Charles River Laboratories, Sulzfeld, Germany. Animals were housed under controlled conditions with a 12 hr light/dark cycle and food and water supply *ad libitum*. All animal experiments were approved by the governmental authorities (Regierungspräsidium Gießen).

Tobacco smoke exposure and riociguat treatment in mice

Mice were exposed to tobacco smoke of 3R4F cigarettes (Lexington, KY, USA) at 140 mg particulate matter/m^3 for 6 hr/day, 5 days/week during 8 months (1). Age-matched controls were kept under identical conditions but without smoke exposure. Animals were sacrificed at 8 (control and smoke-exposed) or 11 (curative approach) months. In the curative approach, animals were exposed to tobacco smoke for 8 months and tobacco smoke exposure was discontinued in the last 3 months treatment period. Riociguat was administered once per day over period of 3 months via gavage, dissolved in 0.2 ml 2% methylcellulose at a dosage of 3mg/kg or 10mg/kg. As controls, tobacco smoke-exposed and -unexposed animals treated with placebo were used. In the placebo treated groups 0.2 ml 2% methylcellulose was administered as placebo. Riociguat and placebo solutions were prepared freshly every day.

Echocardiography

Echocardiography was performed one day prior to the final measurements under isoflurane inhalation anaesthesia as previously described (2). An experienced observer who was blind to the results of blood pressure measurements performed echocardiographic analyses.

FMT-CT

FMT-CT was performed 7 days before sacrificing animals under isoflurane inhalation anaesthesia. The MMP probe (MMPSense 750 FAST; Cat# NEV10168; Perkin Elmer, Waltham, USA) was administered via the tail vein. Six hours after MMP probe injection, mice were anesthetized with isoflurane, and micro-CT (SkyScan 1178, Belgium) followed by FMT (VisEn Medical, Bedford, USA) were performed as described (3).

In vivo measurements of lung function and hemodynamics

Experiments were performed as described previously (1). Animals received IP injections of anaesthesia at a dosage of 100 mg/kg ketamine (Ursotamin, Serumwerk) and 12 mg/kg Xylazine (Ceva). After tracheotomy, the animals were intubated with a 18G metal tube (SCIREQ Scientific Respiratory Equipment Inc.). The trachea was fixed with a thread and ventilated, using a flexiVent system (SCIREQ Scientific Respiratory Equipment Inc.) at a frequency of 150 breaths/minute and a tidal volume of 5 ml/kg.

Lung function tests were performed using the flexiVent predetermined script at a PEEP of 3 cm H_2O, with consistent perturbation order, following the manufacturer's recommendations. The main parameter for the evaluation of lung function was dynamic compliance. The results are presented as an average of measurements with the coefficient of determination above 0.95. Pressure-volume curves were generated using a stepwise pressure-driven perturbation (PVs-P).

For right ventricular systolic pressure (RVSP) measurements, the jugular vein was catheterized by a micro-tip catheter (Millar Instruments, SPR 671, REF 8406719) forwarded into the right ventricle. Systemic arterial and left ventricular systolic pressures were measured by a catheter inserted through the carotid artery. After finishing measurements, heparin (Ratiopharm) solution was injected IP at a dosage of 1000U/kg. The animals were sacrificed by exsanguination.

Lung fixation and organ harvest

The lungs were perfused through the pulmonary artery with saline solution under 22 cmH_2O pressure during continuous ventilation (Minivent, Hugo Sachs Electronic). Right lung lobes were collected and snap-frozen in liquid nitrogen for further analysis. Left lung lobes were either fixed with formalin for paraffin embedding (n=10) or cryopreserved (n=8). Fixation was done under simultaneous vascular perfusion (22 cmH_2O) and inflation (12 cmH_2O) with formalin (Otto Fischar GmbH). For cryopreservation, Tissue-Tek (Sakura Finetek) was applied gently through the trachea, using a 1 ml syringe until the left lung lobe was properly inflated. The lung was then removed, placed in a cassette (Tissue-Tek Cryomold, Sakura Finetek) containing Tissue-Tec, snap-frozen in liquid nitrogen and stored at –80°C until further analysis.

For right-heart hypertrophy assessment, the right ventricle (RV) was separated from the left ventricle and septum (LV+S), and the weight was measured.

Lung volume of paraffin fixed left lung lobes was measured via `water displacement` (Archimedes principle) method as previously described (4). Lungs were further put in agarose, cut in 3 mm thick sections, dehydrated and embedded in paraffin.

Histology

For histological analysis, paraffin blocks were cut in 3µm sections, de-paraffinized, rehydrated and stained following routine protocols. Airspace percentage, septal wall thickness and mean linear intercept (MLI) were assessed on H&E stained slides using uniform random sampling and alveolar morphometry software. The degree of vessel muscularization was determined from stained lung sections as described previously (1). For estimation of the alveoli number, uniform random sampling and the physical dissector method were used as described previously (4).

Nitrotyrosine formation in the lung was assessed by immunohistochemistry staining against nitrotyrosine on 3µm paraffin-embedded lung sections. For staining, a 1:250 dilution of anti-nitrotyrosine antibody (rabbit anti-nitrotyrosine; Sigma-Aldrich, Munich, Germany) was used. Subsequently the immune complexes were visualized with a peroxidase-conjugated secondary antibody (Vector labs, LINARIS, Wertheim-Bettingen, Germany). An additional methyl green counterstaining of the sections was performed.

Laser assisted microdissection

Cryosections (5 µm) of Tissue Tek-embedded (Sakura Finetek, Staufen, Germany) lung tissue were mounted on membrane-coated glass slides. After hemalaun staining for 45 s, mouse lung sections were subsequently immersed in water, 70% ethanol, 96% ethanol, and then stored in 100% ethanol until use. Intrapulmonary arteries with a diameter of 50–100 µm, alveolar septa, and bronchi (diameter 140–300 µm) were selected and microdissected under optical control using the laser-microdissection device LMD6000 (Leica, Wetzlar, Germany). Afterwards, the microdissected material was collected in Eppendorf tubes filled with RNA lysis buffer (Qiagen) containing β-mercaptoethanol (Sigma). After collection, the samples were directly frozen and stored in liquid nitrogen until analysis.

Transcriptome analysis via microarray

Purified total RNA from microdissected vessels, septa and bronchi was amplified using the Ovation PicoSL WTA System V2 kit (Cat# 3312-24; NuGEN Technologies, Bemmel, Netherlands). Per sample, 2 µg amplified cDNA was Cy-labeled using the SureTag DNA Labeling Kit (Cat# 5190-3400; Agilent, Waldbronn, Germany). Hybridization to 8x60K 60mer oligonucleotide spotted microarray slides (mouse genome 8x60K, Agilent Technologies, Design ID 028005) and subsequent washing and drying of the slides was performed following the Agilent hybridization protocol in Agilent hybridization chambers, with the following

modifications: 2 µg of the labeled cDNA were hybridized for 22 h at 65°C. The cDNA was not fragmented before hybridization.

The dried slides were scanned at 2 µm/pixel resolution using the InnoScan is900 (Innopsys, Carbonne, France). Image analysis was performed with Mapix 6.5.0 software (Innopsys; RRID: SCR_002723), and calculated values for all spots were saved as GenePix results files. Stored data were evaluated using R software (The R Foundation; RRID: SCR_001905) and the limma package from BioConductor (RRID: SCR_010943) (5). Log2 mean spot signals were taken for further analysis. Data were background corrected using the NormExp procedure on the negative control spots and quantile-normalized before averaging (6). Log2 signals of replicate spots were averaged, and from several different probes addressing the same gene only the probe with the highest average signal was used. Genes were ranked for differential expression using a moderated t-statistic. Pathway analyses were done using gene set tests on the ranks of the t-values (7). Pathways were taken from the KEGG database (http://www.genome.jp/kegg/pathway.html). Heatmaps were generated from the normalized log2 spot intensities (I) and show the gene-wise z-values.

RNA Isolation, cDNA Synthesis, and Real-Time PCR

Total messenger RNA was extracted from murine lung tissue using an RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCR with reverse transcription was performed with 1µg of RNA each, using the iScript cDNA Synthesis Kit (BioRad, Munich, Germany). The condition for the reverse transcription was as follows: 1 cycle at 25°C for 5 min; 1 cycle at 42°C for 30 min; 1 cycle at 85°C for 5 min.

Real-time PCR was performed with the iQ SYBR Green Supermix according to the manufacturer's instructions (BioRad, Munich, Germany). The intron-spanning primers were

designed by using sequence information from the NCBI database. The Ct values were normalized to the endogenous control (Porphobilinogen deaminase, PBGD).

Nitrotyrosine quantification

Proteins were isolated from lung homogenate and protein concentration was quantified using a Bradford assay. For each sample 50µg of protein was loaded per well and nitrotyrosine formation was quantified using a nitrotyrosine ELISA kit (17-376RF, Millipore) following the manufacturer´s protocol.

PH-COPD patients MMP9 levels measurement

Citrate plasma samples were obtained from blood collected in pulmonary artery during right heart catheterizations. MMP9 levels were measured using a MMP9 ELISA kit (BMS2016-2, Thermo Fisher Scientific) following the manufacturer´s protocol. Calculation of the concentration was done using a five parameter standard curve.

MMP activity measurement in A459 cells

MMP activity in lung homogenates and in A549 cell lysates was measured according the manual of the MMP Activity Assay Kit (Abcam, Cambridge, UK). Briefly, lungs and cells were homogenized in Hanks' Balanced Salt solution (HBSS) by Precelly®24 Homogeniser (Peqlab, Erlangen, Germany). Fluorescence recovery was measured by multimode plate reader Infinite m200 (Tecan Group Ltd, Männedorf, Switzerland) after 20 min of incubation with FRET peptide. MMP activity in lung homogenates and cell lysates was normalized to protein concentration measured by BioRad protein assay.

Proliferation of A549 cells

For assessment of proliferation, A549 were seeded in 48-well plates at a density of 3×10^5 cells/well in 10% FCS/DMEM medium (Thermo Fisher Scientific, Waltham, USA). Cells

were rendered quiescent by incubation in 0.1% FCS for 24 hours. Subsequently, they were stimulated with 10% FCS/human medium to induce cell cycle re-entry. BrdU (Bromodeoxyuridine / 5-bromo-2'-deoxyuridine) incorporation was evaluated according the cell proliferation ELISA, BrdU kit (Sigma-Aldrich, St. Louis, USA). 6h before adding 10% FCS/human medium with 10µM BrDU, different doses of riociguat or 8-bromoguanosine 3′,5′-cGMP (8-Br-cGMP) were added to the cells.

Supplementary Table

Echocardiography and Lung function in COPD Patients (n=3)

pO_2: partial oxygen pressure, pCO_2: partial carbon dioxide pressure, O_2-suppl: oxygen supplementation, Rtot: total airway resistance, FEV1: forced expiratory volume in 1 second, VC: vital capacity, DLCO/SB: diffusion capacity for carbon monoxide, sPAP: systolic pulmonary arterial pressure, TAPSE: tricuspid annular plane systolic excurstion.

*compared to pre-treatment. #compared to first time point post-treatment. All p-values are provided for comparison to pre-treatment groups unless otherwise labelled.

	Pre-Treatment	Post-Treatment (4-7 month after start of riociguat	Post-Treatment (21-29 months after start of riociguat)
Blood gases			
pO_2 (mmHg)	67 ± 6	74 ± 4 (p=0.939)	75 ± 9 (p=0.927)
pCO_2 (mmHg)	38 ± 7	39 ± 3 (p=0.997)	40 ± 4 (p=0.990)
O_2-suppl. (l/min)	2.00 ± 2.00	2.00 ± 2.00 (p=1.000)	2.00 ± 2.00 (p=1.000)
Lung function parameters			
Rtot%	225 ± 17	174 ± 37* (p=0.039)	240 ± 55 (p=0.737) [#] (p=0.005)
FEV1%	56 ± 3	63 ± 7 (p=0.956)	59 ± 3 (p=0.997)

FEV1/VC%	59 ± 3	60 ± 5 (p=0.999)	52 ± 3 (p=0.945)
RV/TLC%	56 ± 3	53 ± 5 (p=0.973)	52 ± 6 (p=0.954)
DLCO/SB%	31 ± 8	40 ± 10 (p=0.895)	41 ± 11 (p=0.888)
Echocardiographic parameters			
sPAP (mmHg)	69 ± 6	63 ± 5 (p=0.943)	61 ± 10 (p=0.920)
TAPSE (mm)	16 ± 2	18 ± 3 (p=0.998)	20 ± 2 (p=0.986)

Literature

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