Materials and methods:

Cell culture and Reagents. HEK cells and human lung fibroblasts were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing L-glutamine, penicillin, streptomycin, and 10% (v/v) fetal bovine serum. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen) as described previously. Human pulmonary artery endothelial cells (HPAEC), human pulmonary artery vascular smooth muscle cells (HPAVSMC) were purchased from Lonza, and were grown in Endothelial Growth Medium-2-Microvessel (EGM-2MV) or smooth muscle growth media (SmGM) consisting of defined growth factors and supplemented with additional FBS up to 5% final concentration (Lonza). Cells were grown at 37 °C in 5% CO2 incubator and used from passage 2–6. All chemicals were purchased from Sigma unless indicated otherwise.

DNA/adenoviral constructs. Plasmid DNA encoding Nox5β (AF325189), Nox1 and Nox4 have been described previously[1, 2]. A Nox4 adenovirus was generated using the Invitrogen Virapower system as described previously for Nox5[3].

Cell Proliferation and Migration. Human lung fibroblasts were cultured in a 8W10E array. Control (LacZ) or Nox4 adenovirus (MOI 30) were added at 20h and resistance was measured using the ECIS Zθ (Applied Biophysics) and normalized to the value of each well at 0h. Alternatively, fibroblasts were exposed to the indicated amounts of adenovirus and cell number determined at 48h by manual count or MMT assay. In brief, cells were incubated with CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent (Promega) for 1 h at 37°C. Absorbance at 495nm was measured BMG Polarstar plate reader. Cell migration was determined in fibroblasts exposed to control or Nox4 adenovirus (MOI 30) and determined using the Oris™ Cell Migration Assay (Platypus Technologies).

*Rat models of PH***.** Three rat models of pulmonary hypertension (PH) were employed. The monocrotaline (MCT)-model was induced by a single i.p. injection of MCT (60/mg/kg), which produces a progressive and severe PH after 4 weeks of MCT exposure [4]. The Sugen/Hypoxia (SU/HYP) model of PH results from injection of the VEGF receptor antagonist SU-5416 (20mg/kg,SQ) followed by 3 weeks of hypoxia (10% O_2) and 11 weeks of normoxia (21% O_2) as previously described[5]. The Fawn-hooded rat (FHR), a genetic model of PH spontaneously develops PH after 20 weeks of age [6]. Adult age-matched male Sprague-Dawley (SDR, 250-300g) rats were used as controls for all rat models of PH. The Animal Care and Use Committee at Georgia Regents University approved all procedures and protocols, and this study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). All groups of rats were housed under temperature-controlled conditions (21-23°C), maintained on standard rat chow, allowed free access to food and water, and exposed to a 12:12-h light-dark cycle.

Murine model of PH. C57BJ6 mice were exposed to room air (normoxia) or 10% oxygen for 3 weeks as previously described[7].

*Nox4 Inhibitor treatments for MCT-induced PH***.** Three different small molecule Nox4 inhibitors were administered to MCT-treated rats: VCC588646 (2-[N'-(3,4-Dihydroxy-benzylidene)-hydrazino]-N-(3-nitro-phenyl)-2-oxo-acetamide; Inhibitor '**A**'), VCC202273 (N^4-(4-Amino-phenyl)-[1]benzothieno[3,2-d]pyrimidine-4,8-diamine; Inhibitor '**C**'), both from Vichem-Chemie, LTD [8], or GKT136901 (2-(2-Chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo-[4,3-c]pyridine-3,6(2H,5H)-dione; Inhibitor '**G**') [9]. To assess prevention, the Nox4 inhibitors ('A', 'C',

or 'G'), were dissolved in DMSO and administered via i.v. injection daily at a dose of 1mg/kg/day, commencing on the day of the single MCT injection (day 1), for a duration of 28 days. To assess whether Nox4 inhibition could halt the progression of PH (reversal protocol), Nox4 inhibitor VCC202273 (C) was administered (1mg/kg/day) daily to MCT-treated rats upon the first detectable increase in right ventricle (RV) thickness, which occurred at week 3 post MCT administration. Inhibitors or vehicle (DMSO) were given daily for 21 days and cardiopulmonary indices were monitored on a weekly basis for three weeks corresponding to weeks 4, 5, and 6 of MCT exposure.

Assessment of RV function. Rats were anesthetized (pentobarbital, 50 mg/kg, i.p.) and the trachea intubated. The diaphragm was surgically exposed through the abdomen, and a 25 gauge needle connected to a pressure transducer (AD instruments) was inserted into the right ventricle (RV) through the diaphragm, and RV pressure was continuously monitored for 10-15 minutes. Indices of RV function (RVSP; RV max dp/dt) were recorded using a PowerLab data acquisition system (AD Instruments). With this approach, the diaphragm remains intact without opening the chest. We have previously established that measurements of right ventricular systolic pressure (RVSP) are comparable to measurements obtained using the right jugular vein[7].

Non-invasive measurement of cardiopulmonary parameters. Rats were temporally anesthetized (1-4% inhaled isoflurane), and RV hypertrophy and functional parameters of PA remodeling (velocity time integral (VTI), pulmonary ejection time (PET) and the pulmonary artery acceleration time (PAAT) were measured using the VEVO 2100 digital ultrasound micro-imaging system (VisualSonics).

Histological analysis. Post hemodynamic measurements, rats were euthanized by thoracotomy. Blood in the pulmonary vasculature was removed by PBS infusion through the pulmonary artery and the heart and lungs removed en bloc. The free wall of the RV, left ventricle (LV), and septum (S) were carefully dissected free and weighed individually to calculate the RV/LV+S ratio (Fulton index) as an index of RV hypertrophy. The right lungs were removed and snap frozen in liquid nitrogen for preparation of homogenates, and the left lungs were filled with 4% PFA solution with 0.5% agarose at 25 cm H_2O and fixed in 4% PFA for 24 hours. The fixed lungs were then sliced mid-sagittally and embedded in paraffin. The slides (7μm thickness) were stained with hematoxylin and eosin for morphometric analysis and were examined with an Olympus BX41 microscope. An Olympus DP72 digital camera and ImageJ software (http://rsbweb.nih.gov/ij/) were used to analyze slides. A minimum of 10 microscopic fields were examined for each slide. To quantitate pulmonary arterial wall thickness, the lumen area at the level of the basement membrane and total vascular area at the adventitial border in 20 muscular arteries with diameters of 50–100 μm per lung section were outlined, and area sizes were measured using ImageJ. The vascular wall thickness was calculated as follows: wall thickness = (total vascular area − lumen area)/ total vascular area.

Confocal microscopy. To determine the location of specific cellular markers in blood vessels, both normotensive and PH lung sections were stained with α-actin (Abcam; 1:700 dilution) for 30 minutes before being double-stained with antibodies against either Nox4 (Abcam (ab116534); or Epitomics; 1:1000 dilution), CD90 (Abgent; 1:1000 dilution), FAP (Santa Cruz; 1:500 dilution), cellFN1 (SCBT; 1:500 dilution), CD11b (BD Pharmingen; 1:1000 dilution), Tenascin-N (Santa Cruz; 1:250 dilution), Periostin (Novus; 1:500 dilution), Vimentin (Oncogene; 1:1000 dilution) and the marker of ROS production, 8-hydroxydeoxyguanosine, (Thermo Scientific; 1:200 dilution) for 30 minutes. For (negative) control IgG images, human lung sections were incubated first with non-immune rabbit IgG and mouse IgG (dilution 1:100)

overnight and then with goat anti-rabbit IgG Alexa Fuor 488 and goat anti-mouse IgG Alexa Fuor 594 for 2 hrs. All fluorescence-labeled lung sections were examined using a Zeiss LSM 510 laser scanning confocal microscope.

Analysis of Gene expression - Pulmonary arteries (down to 4th order) were dissected from the surrounding pulmonary parenchyma, snap frozen in liquid nitrogen, pulverized and RNA extracted using TRIZOL or proteins solubilized in 2x Laemmli buffer. cDNA was synthesized using the iScript cDNA Synthesis Kit (Biorad) and used to assess relative gene expression using real time RT-PCR (Bio Rad iQ SYBR Green). Western blot experiments were performed as described [2] and relative densitometry determined using ImageJ software (NIH).

Nox activity – Nox4 activity was determined by the Amplex Red assay in HEK293 cells stably expressing Nox4 as described [1]. The relative activity of Nox1 and Nox5 was determined using L-012 as described [1, 2].

Statistical analysis - Statistical analysis was performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA). The mean ± SEM was calculated in all experiments. Data sets were assessed for normal distribution and statistical significance determined either by the unpaired t-test (for 2 groups) or ANOVA (for $\geq$ 3 groups). For the ANOVA analyses, Newman-Kuels post-hoc testing was employed. A value of $p < 0.05$ was considered significant.

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