Online Supplemental Methods and Data

Monocrotaline-induced Pulmonary Hypertension Involves Downregulation of Anti-aging Protein Klotho and eNOS activity

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Running title: Klotho and Pulmonary Hypertension

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Online Supplemental Methods

Construction and packaging of lentiviral vector overexpressing mSKL

To construct a lentiviral vector overexpressing the mouse SKL (mSKL) gene, the SR-CMV promoter from the pSR-CMV-SV-N2A-GFP-polyA (PMID: 20410313) plasmid was PCR amplified with the primers (F) 5'-TTCTAGACGCGTGGCCTGAAATAACCTCT-3' and (R) 5'-CCTCGAGGATCAGATCGGAATTCCGGCGCCT-3'. The PCR product was digested with *Xba* I and *Xho* I and then ligated into the backbone plasmid pHR-cPPT-hB.7-SIN[1] digested with the same restriction enzymes. The resulting plasmid was first completely digested with *BamH* I and then partially digested with *Nde* I. A fragment containing part of the CMV promoter and the coding sequence of the mSKL gene was released from the pAAV-mSKL plasmid (previously constructed in our lab)[2] by first completely digesting with *Nde* I and then partially digesting with *Bgl* II. Digested DNAs were separated through agarose gel electrophoresis, extracted, and ligated. The desired recombinant plasmids were then identified through colony PCR using primers specific for the mSKL gene. Extra-pure plasmid DNA was extracted using the E.Z.N.A.® Plasmid Mini Kit I (Omega Bio-Tek) and used for packaging of infectious lentiviral vectors through a transient co-transfection method as previously described.[3] Passage 3–4 mouse MSCs (Invitrogen) were subsequently transduced at a multiplicity of infection (MOI) of 10–20.

Animal studies

The experimental protocol was approved by the Institutional Animal Care and Usage Committee of the University of Oklahoma Health Sciences Center. Six- to eight-week-old male Sprague Dawley rats (Harlan Laboratories) were acclimatized for a week at the animal facility. Five groups of rats (six rats per group) were established for the study: Saline, MCT, MCT + MSC, MCT + MSC-GFP and MCT + MSC-SKL-GFP. Four groups were given monocrotaline (MCT) daily (60 mg/kg) *via* subcutaneous injections, while one group was given saline and served as a control. After 3 days of MCT injections, three MCT-treated groups were administered MSCs, MSCs carrying eGFP (MSC-GFP), and MSCs carrying eGFP-SKL (MSC-SKL-GFP), respectively, *via* injection into the right jugular vein (3.5×10^6 cells/rat), while one MCT-treated group received no treatment and served as a control. Twenty-one days post MCT injection, RV pressures were measured under anesthesia before the animals were euthanized for tissue collection. The scheme of the experimental protocol is shown in Supplemental Figure 1.

Measurement of right ventricular systolic pressure (RVSP)

Twenty-one days post MCT injection, rats were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). An incision approximately 3 cm in length

was made, and the trachea was exposed. PE 240 tubing was guided into the trachea and connected to a ventilator (Harvard Apparatus, MA) to control respiration. Animals were placed on a heating pad for maintaining body temperature at 39°C. The chest cavity was opened to expose the heart, and the apex of the right ventricle was localized and held firmly by a pair of blunt forceps. A PA-C40 transmitter (Data Sciences International, MN) was carefully introduced into the right ventricle using a 25-gauge needle. After a stable pressure was obtained and recorded, the rats were euthanized, and PA, heart, and lungs were collected for further analysis as described below.

Pulmonary vascular responses to vasodilators

Small intralobar 3rd-order PA rings (2 mm in length) were isolated from euthanized rats and mounted on a wire myograph system (DMT, MI). The PA rings were incubated in physiological saline solution and aerated with 5% CO_2 and 95% O_2. The vessels were normalized to a resting tension equivalent to 20–29 mmHg, equilibrated for at least 60 minutes, and challenged three times with 60 mM K^+ solution before starting experiments for testing relaxation responses to vasodilators. PA rings were pre-constricted with phenylephrine before measuring PA relaxation in response to cumulative doses of acetylcholine (ACh) or sodium nitroprusside (SNP). Data is presented as the percentage relaxation of phenylephrine-induced pre-constriction.

Determination of RV hypertrophy

Hearts were weighed before RVs were dissected from left ventricles and septa (LV+S). RV and LV+S weights were measured separately, and RV hypertrophy was assessed by the ratio of RV/LV+S per g body weight. Some of the animals were perfused with saline, and hearts were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, sectioned at a 5-µM thickness, and stained with H&E. The width of the right ventricular wall was measured at the thickest point in each transverse section and used as a measure of RV hypertrophy.

Immunohistochemical (IHC) analysis of the lungs

The IHC was performed as described recently.[4-6] Lungs from saline-perfused rats were isolated and fixed in 4% PFA or in optimal cutting temperature (OCT) embedding compound (Tissue-Tek, CA). To look at innate eGFP fluorescence in lung tissue, 10-µm-thick cryo-sections of OCT-embedded lungs were thawed at room temperature (RT) for 10 minutes, fixed in 10% neutral buffered formalin (NBF) for 20 minutes, stained with DAPI mounting solution (Santa-Cruz), and observed under a fluorescence microscope (Olympus IX73). For immunofluorescence labeling with epithelial, smooth muscle cells (SMCs) and EC antibodies, NBF-fixed cryo-sections were incubated in 1% SDS for 5 minutes for antigen retrieval and in 3% H_2O_2 for 15 minutes. After blocking for 1 hour at RT with 5% BSA, the following primary

antibodies were used: EC marker vWF (Abcam, 1:100), SMC marker α-SMA (Abcam, 1:100), alveolar epithelial type I cell marker P_2X_7 (Santa Cruz), alveolar epithelial type II cell marker LB-180 (BioLegend), GFP antibodies, and Rabbit Alexa Fluor 488 (Life Technologies 1:100), and mouse IgG (Santa Cruz, 1:100). Sections (5-μM thick) from paraffin-embedded lung tissues were used for H&E staining for morphometric analysis of PA medial thickness and lumen area and for IHC staining of CD68 (Abcam, 1:100) and α-SMA (Abcam, 1:500).

Western blot for SKL, SIRT1, eNOS, and p-eNOS expression in serum and lung lysates

Western blotting was performed as we described previously.[7, 8-11] Lungs were lysed in RIPA buffer containing protease inhibitors. Protein concentrations were measured using a bicinchoninic acid (BCA) assay. Serum samples were diluted 20 fold before adding loading buffer and reducing agents. An equal amount of total protein was loaded for each sample. Primary antibodies were used against SKL (R&D, 1:200), SIRT1 (Abcam, 1:500), eNOS (BD Biosciences, 1:1000), p-eNOS (Millipore, 1:500), and β-actin (Abcam, 1:10000) as loading control. Ponceau-stained membrane was used to quantify total protein as a loading control for serum samples.

Cell culture experiments

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza, and passage 3–5 cells were used for viability assays using MTT (Roche). HUVECs were cultured in 2% serum medium EGM-2 (Lonza), seeded at a density of 5000 cells per well in a 96-well plate, and allowed to attach overnight. Various concentrations of MCT were added to see dose-dependent effect on HUVEC viability, and 1 mg/ml MCT and 0.5 μg/ml SKL were added at the same time to see the protective effect of SKL on MCT-induced cell death. Five replicates were assayed for each experimental condition. Two days after adding MCT and SKL, MTT reagent was added, and the viability assay was performed following the manufacturer's instructions.

The same experimental conditions were used for the DAF-2DA assay for NO production in HUVECs (see below).

DAF-2DA assay for NO production

The concentrations used for MCT and SKL were 5 μg/ml and 2 μg/ml, respectively. Both MCT and SKL were added at the same time. After 4 days, cells were washed and incubated in serum-free EBM medium (Lonza) for 1 hour and in 1 μM DAF-2DA for 15 minutes at room temperature. Following this procedure, cells were washed twice with PBS and then fresh EBM medium was added. Mean fluorescence intensity at 515-nm excitation wavelength was measured using a Biotek microplate reader 60 minutes after removing DAF-2DA from the

cells. After measuring fluorescence, images were acquired using an Olympus iX73 inverted fluorescence microscope. To confirm eNOS-specific NO production, 100 µM L-NAME (Sigma-Aldrich) was added along with MCT and SKL for 4 days, followed by incubation with DAF-2DA for 15 minutes.

References

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Online Supplemental Data

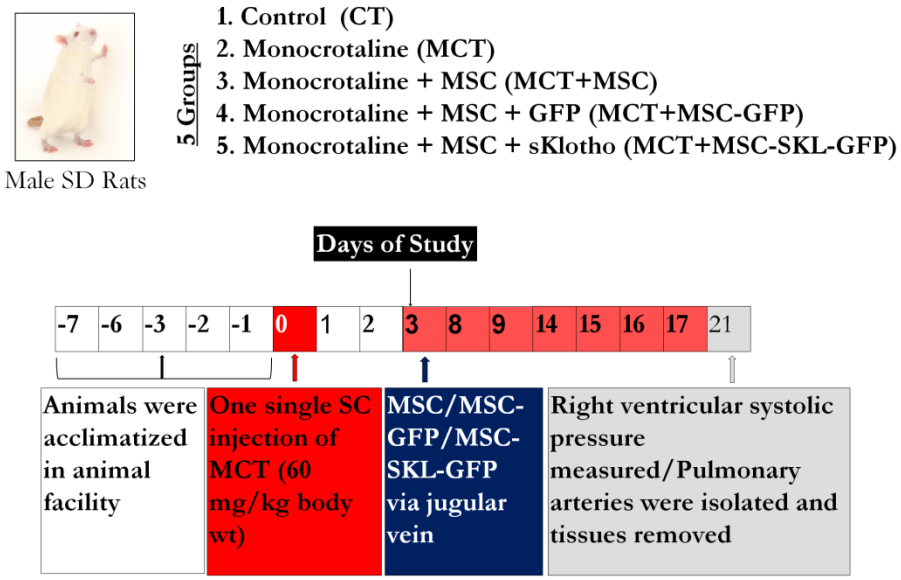

Figure S1. Experimental design.

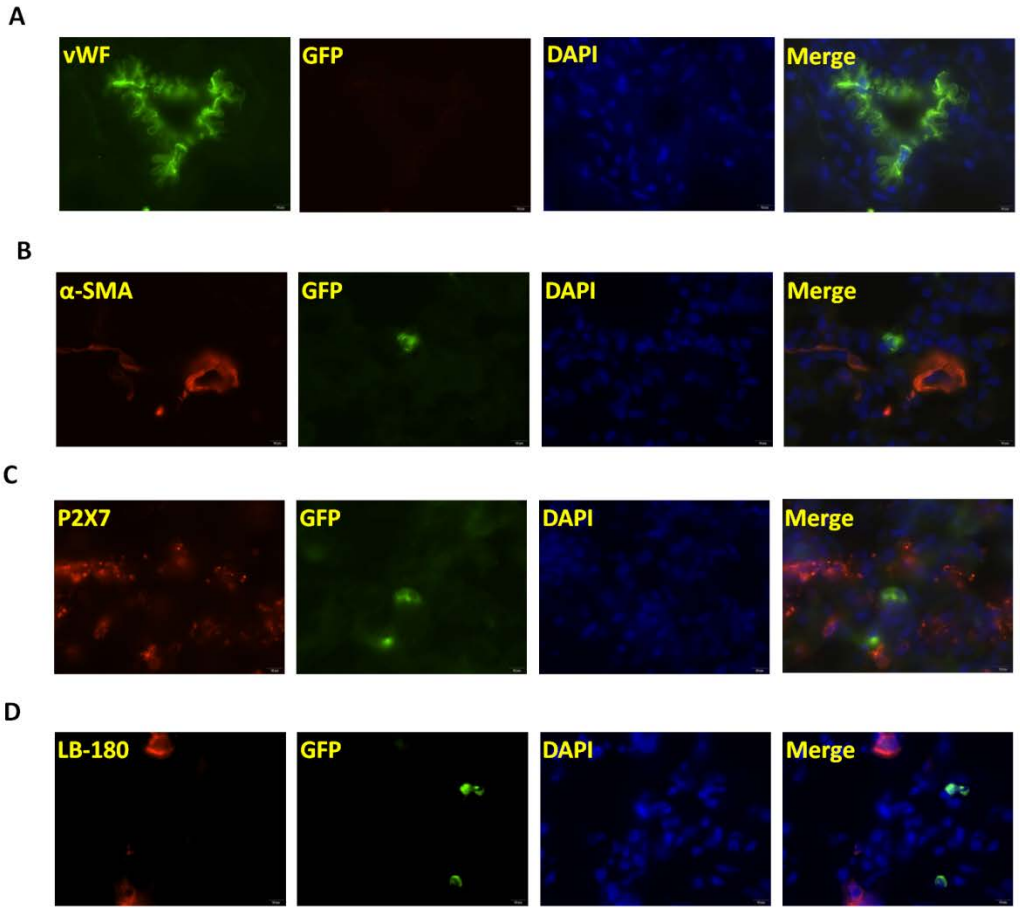

Figure S2. SKL-GFP-transfected MSCs do not differentiate into epithelial, endothelial, or smooth muscle cell types *in vivo* in the lungs of MCT + MSC-SKL-GFP rats.
A) Immunofluorescent staining with endothelial marker vWF (green) and GFP (red) antibodies. **B)** Immunofluorescent staining with smooth muscle cell marker α-SMA (red) and GFP (green) antibodies. **C)** Immunofluorescent staining with the alveolar epithelial type I cell marker P2X7 (red) and GFP (green) antibody. **D)** Immunofluorescent staining with alveolar epithelial type II cell marker LB-180 (red) and GFP (green) antibodies.

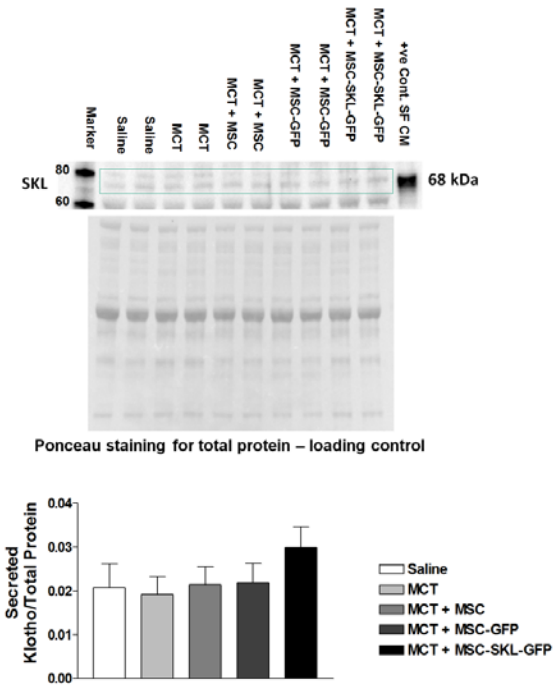

Figure S3. Western blot analysis of SKL levels in serum.

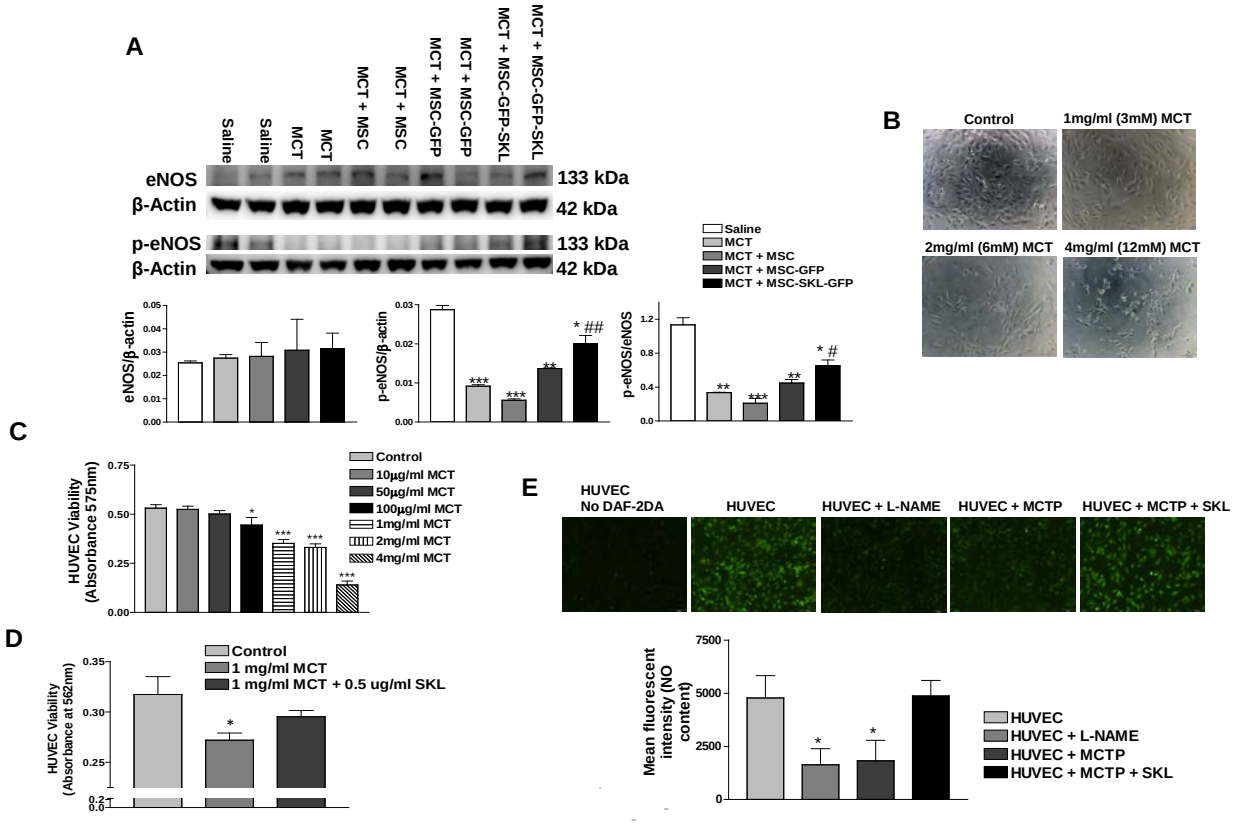

Figure S4. SKL prevented MCT-induced downregulation of eNOS activity in the lungs and nitric oxide bioavailability in HUVECs.

A) Western blot analysis of eNOS and p-eNOS in lung lysates. *p<0.05, **p<0.01, ***p<0.001 vs. Saline; #p<0.05 *vs.* MCT; n=6. Data = means±SEM. **B)** Photomicrographs of HUVECs under different MCT concentrations. **C)** MTT assay for HUVEC viability under different MCT concentrations. **p<0.05, **p<0.001 *vs.* Control. **D)** MTT assay for HUVEC viability under treatment with MCT and MCT plus SKL. *p<0.05 *vs.* Control. **E)** DAF-2DA assay for NO production in HUVECs under treatment with MCT and SKL. **p<0.05 *vs.* HUVEC. Triplicates were used in each experiment, and each experiment was repeated three times. Data = means±SEM.