SUPPLEMENTAL MATERIAL

Supplemental Methods:

Cell proliferation. Proliferation of PASMCs and PAECs was measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation for 48 h using the Cell Proliferation ELISA, BrdU (colorimetric) assay (Roche, Indianapolis, IN), according to the manufacturer's instructions.[1]

Cell Migration. Migration of PASMC and PAEC was assessed using a micro Boyden Chamber QCM™ 24-Well Colorimetric Cell Migration assay (ECDM 508, Millipore, Billerica, MA) according to the manufacturer's instructions.[1] Migrated cells were quantified using a microplate reader at 560 nm by colorimetric assay. All experiments were performed in triplicate and data is expressed as % migrated cells/βgal-infected cells.

Western immunoblotting. Protein lysates (30 μg) were size-fractionated electrophoretically using SDS-PAGE, transferred onto a nitrocellulose membrane, blocked, and incubated with primary antibodies overnight at 4°C. The membranes were incubated with anti-SERCA2a (21st Century Biochemicals, Marlborough, MA), anti-phospho-eNOS Ser1177 and total eNOS (BD Biosciences, San Jose, CA), anti-Cyclin D1 (BD Biosciences, San Jose, CA), anti-phospho-STAT3, anti-STAT3 (Cell Signaling, Beverly, MA), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma, St. Louis, MO), or anti-PP2B (Santa Cruz, Dallas, TX). Levels of proteins and phosphoproteins were detected by using horseradish peroxide-linked secondary antibodies (Cell Signaling, Beverly, MA) and the ECL System (Thermo Scientific, Rockford, IL) or the Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE). GAPDH protein expression was used as the loading control.

Lung tissue histology. Rat lungs were inflated with OCT/PBS (50/50) at a pressure of 20 cm H_2O injected through the trachea prior to tissue harvest. The lungs were then frozen, embedded in OCT, sectioned, and 8 μm sections were fixed with ice cold acetone. Sections were stained using hematoxylin and eosin and examined by light microscopy. Smooth muscle α-actin immunohistochemical staining was performed as described previously.[2] Pulmonary arterioles located distal to terminal bronchioles were identified. The external diameter and the cross-sectional medial wall thickness were measured in 30 muscular arteries per rat ranging in size from 25-50, 51-100, 101-150 μm and up to 150 μm in external diameter. Percent wall thickness was calculated as [(medial thickness x 2)/external diameter] x100. Fibrosis and collagen deposition was examined in lung tissue frozen sections (8μm) that were fixed in 1% paraformaldehyde and stained with Masson's trichrome stain. Sections were visualized and collagen deposition was quantified using ImageJ software (http://rsbweb.nih.gov/ij/).

NFAT-reporter gene assay. Cells were transfected with a NFAT-promoter-luciferase construct using FuGENE 6 as the transfection reagent (Roche, Indianapolis, IN). After 24 h, luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI). Results were normalized to total protein, and expressed as % control non-infected cells in relative luciferase units (RLU).

Right ventricular weight and right and left ventricular histology. The heart was dissected immediately after sacrifice and weighed. The weight ratio of the right ventricle

(RV) to the left ventricle (LV) plus septum [RV/(LV+S)] was calculated as an index of right ventricular hypertrophy (RVH). The RV and LV sections were then fixed with ice-cold acetone, embedded in OCT, and hematoxylin and eosin staining was performed on 8 μm–thick sections that were subsequently examined using light microscopy. Fibrosis and collagen deposition was examined in frozen sections (8μm) that were fixed in 1% paraformaldehyde and stained with Masson's trichrome stain. Sections were visualized and collagen deposition was quantified using ImageJ software. Cardiomyocyte cross-sectional area was measured using fluorescence-tagged wheat germ agglutinin (Invitrogen) that binds to saccharides of cellular membranes. Images of RV cardiomyocyte cell membranes were captured digitally and analyzed by image analysis using ImageJ software

X-gal staining. The efficiency of intratracheal AAV1.gene transfer was determined by X-Gal staining. Lung tissue sections transduced with AAV1.Adβgal were fixed for 30 min at 4°C in 2% formaldehyde/0.2% glutaraldehyde in PBS, pH 7.4. An X-Gal solution (Thermo Scientific, Pittsburgh PA) was added to the tissue sections and incubated at 37°C for 2 h.

Quantitative real-time PCR. Total RNA was isolated from the lung and RV using Trizol reagent and the RNeasy Protect Mini kit (Qiagen, Valencia, CA). First-strand cDNA was reverse-transcribed from 2.0 μg of total RNA using a high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using an ABI PRISM Sequence Detector System 7500 (Applied Biosystems, Foster City, CA) with SYBR Green (BioRad, Hercules, CA) as the fluorophore and ROX (Takara, Otsu, Japan) as a passive reference dye. To determine exogenous human SERCA2a mRNA expression, the following primer was used: forward 5'-AGACCCAAGCTGGCTAGCGTTTA-3', reverse 5'-TTCTTCAGCCGGTAACTCGTTGGA-3'. The primers 18S-F, 5′-TGCGGAAGGATCATTAACGGA-3 and 18S-R, 5′-AGTAGGAGAGGAGCGAGCGACC-3′ was used as an endogenous loading control. Fold changes in gene expression were determined using the relative comparison method with normalization to 18S rRNA.

Immunohistochemistry. Frozen sections (8 μm) were incubated in 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Sections were blocked with 10% normal goat serum and incubated overnight at 4°C with a rabbit antibody against SERCA2 (1:100; Thermo Scientific, Pittsburgh, PA) or CD68 (1:50; BD Bioscience, San Jose, CA). Sections were then incubated with a biotinylated goat anti-rabbit IgG (Dako, Japan) for 30 min, followed by peroxidase labeling with streptavidin (LSAB kit, Dako, Japan) for an additional 20 min at 25°C. Protein expression or presence of macrophages/monocytes was quantified using Image J software.

***In vivo* proliferation and apoptosis..** Apoptosis was determined by TUNEL staining of lung tissue sections fixed in 1% paraformaldehyde using the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI) according to manufacturer's instructions. Images were visualized on a Nikon Eclipse TE2000-U microscope at 20X and images captured using Openlab software (Improvision, Waltham, MA). Results were quantitated

by determining % TUNEL positive nuclei per vessel for 10 vessels per section, 2 sections per rat, and 3 rats per treatment. To measure proliferation, BrdU (100 mg/kg) was delivered by intraperitoneal injection before sacrifice, and proliferation was assessed using an anti- BrdU antibody (Abcam, Cambridge, MA) according to the manufacturer's instructions. The number of TUNEL- or BrdU-positive PASMCs was divided per the total number of DAPI-positive nuclei and expressed as a percentage.

Supplemental References:

1. Bobe R, Hadri L, Lopez JJ, Sassi Y, Atassi F, Karakikes I, Liang L, Limon I, Lompre AM, Hatem SN, Hajjar RJ, Lipskaia L. SERCA2a controls the mode of agonist-induced intracellular Ca2+ signal, transcription factor NFAT and proliferation in human vascular smooth muscle cells. *J Mol Cell Cardiol*. 2011;50:621-633.

2. Maron BA, Zhang YY, White K, Chan SY, Handy DE, Mahoney CE, Loscalzo J, Leopold JA. Aldosterone inactivates the endothelin-B receptor via a cysteinyl thiol redox switch to decrease pulmonary endothelial nitric oxide levels and modulate pulmonary arterial hypertension. *Circulation*. 2012;126:963-974.

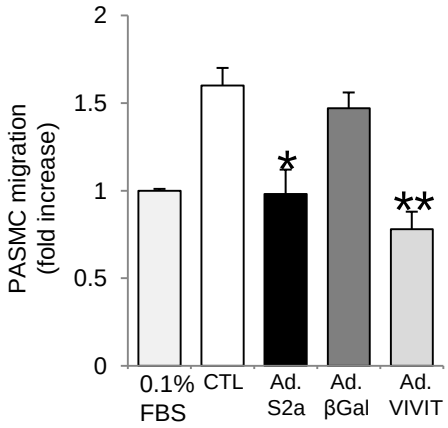
Supplemental Table 1. PAH patient pulmonary hemodynamics pre-lung transplant

	Sex	Diagnosis	Date of LTx	Age at LTx	Pre-LTx TPR (Wood units)	Pre-LTx mPAP (mmHg)	Pre-LTx CI (l/min/m²)
Patient 1	female	IPAH	05/19/2011	31.3	14.5	69	2.45
Patient 2	male	IPAH	08/20/2010	49.7	13	43	3.23
Patient 3	female	IPAH	01/29/2011	59.1	9.4	53	2.85
Patient 4	female	HPAH	01/20/2012	51.8	11.2	64	2.6
Patient 5	female	IPAH	12/04/2011	31.2	14	61	1.6
Patient 6	female	IPAH	07/29/2011	18.9	26.4	100	1.7

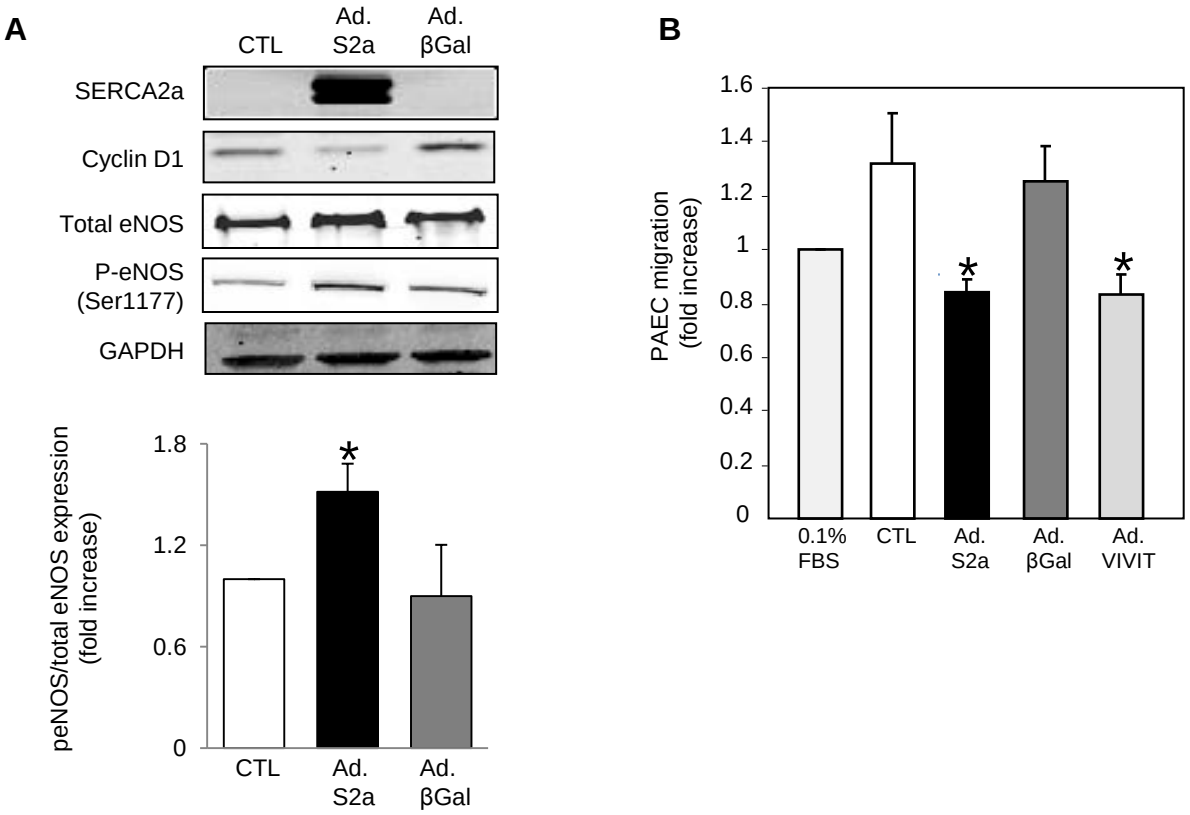
Lung Transplantation (LTx) ; pulmonary arterial pressure (PAP); Total pulmonary vascular resistance (TPR); Cardiac index (CI)

Supplemental Table 2. Control subjects diagnosis at time of surgery

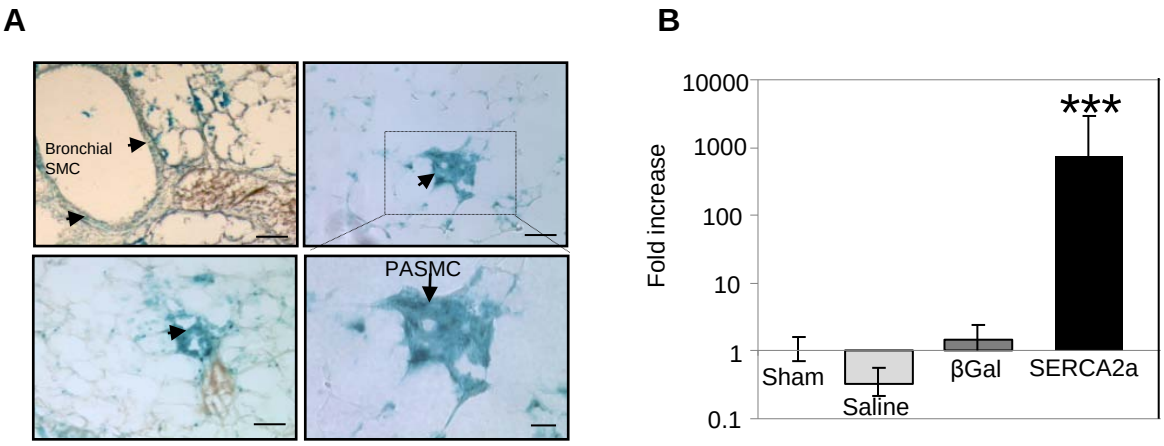
	Sex	Diagnosis
Patient 1	male	squamous cell carcinoma
Patient 2	male	adenocarcinoma
Patient 3	male	adenocarcinoma
Patient 4	male	squamous cell carcinoma
Patient 5	male	adenocarcinoma



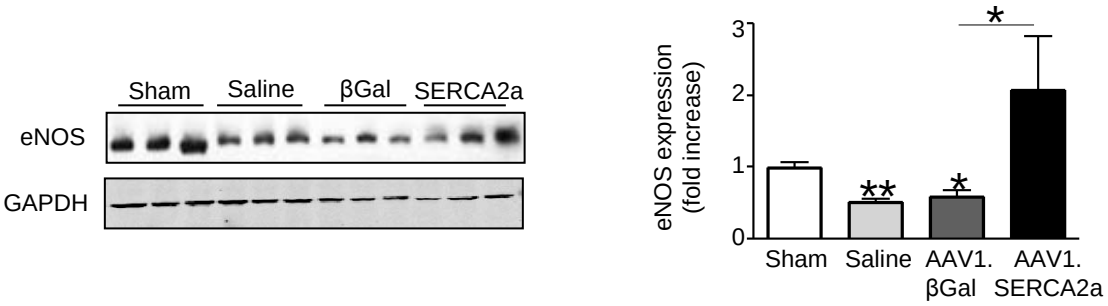
Supplemental Figure 1. SERCA2a gene transfer inhibits serum-stimulated PASMC migration. PASMCs were infected for 48 h with Ad.SERCA2a, Ad.βgal, or Ad.VIVIT and then cultured for 48 h in virus-free medium. Cell migration was determined using a micro-Boyden chamber assay. Cells were stimulated by supplementing the medium with 5% FBS. Non-infected PASMCs and PASMCs cultured with 0.1% FBS to maintain quiescence cultured under the same conditions served as controls (n=3). *$p<0.03$ vs. CTL. **$p<0.005$ vs CTL.



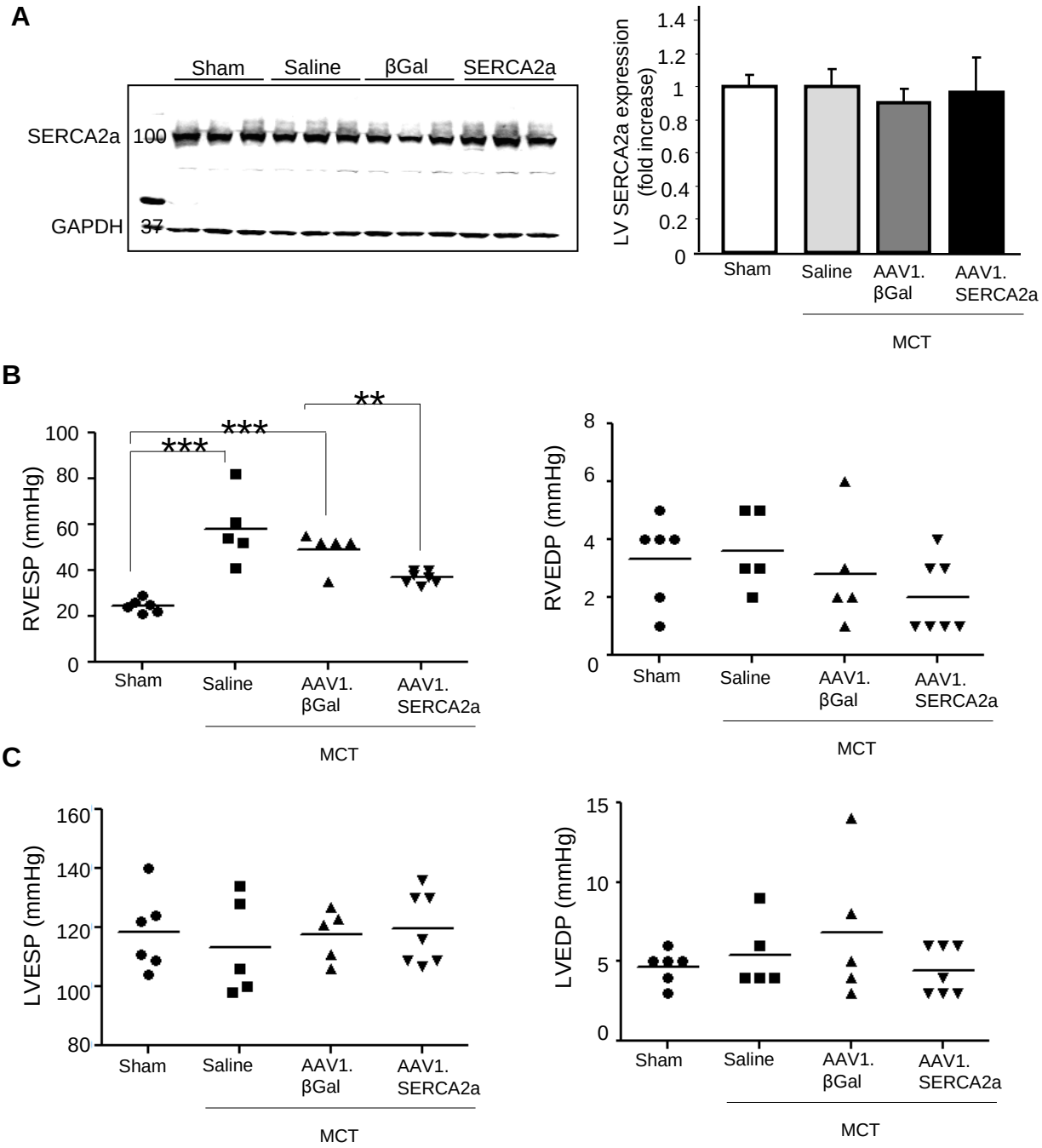
Supplemental Figure 2. SERCA2a gene transfer increases PAEC eNOS activation and inhibits serum-stimulated cell migration. (**A**) Representative immunoblot of SERCA2a, Cyclin D1, and phosphorylated and total eNOS in PAECs infected with Ad.βgal or Ad.SERCA2a. The relative ratio of phosphorylated eNOS at Ser1177was normalized to total eNOS (n=3). GAPDH was used as a loading control. Representative blots are shown. *p<0.03 vs. CTL. (**B**) Effect of SERCA2a overexpression on PAEC migration was assessed using a micro-Boyden chamber assay. Cells were stimulated with 5% FBS. *p<0.02 vs. CTL. CTL, control; FBS, fetal bovine serum.



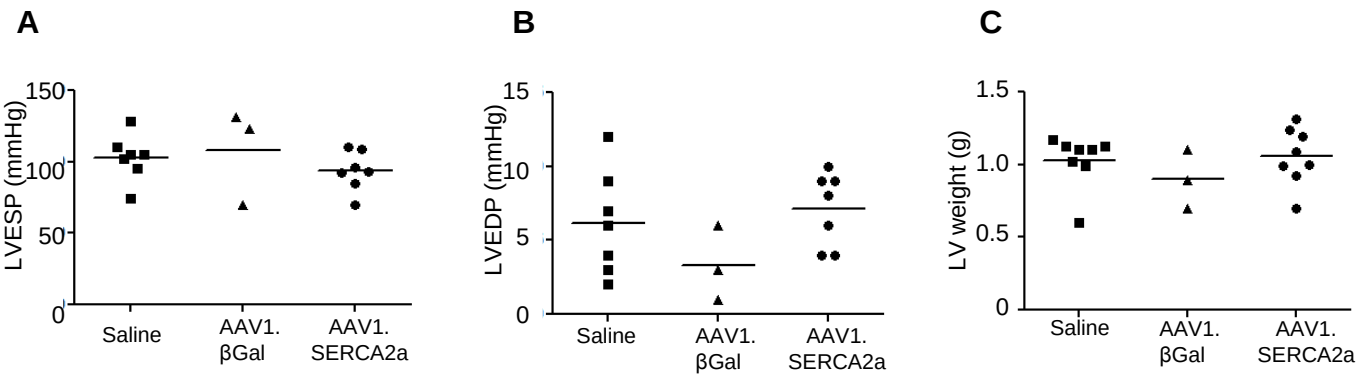
Supplemental Figure 3. Efficiency of intratracheal targeted delivery of AAV vectors and SERCA2a gene transfer to the lung vasculature. (**A**) X-Gal staining of lung tissue sections was performed 30 days after intratracheal delivery of AAV1.βgal in MCT–treated animals. Arrows indicate the localization of βgal protein in a pulmonary arteriole (right) and evidence of X-Gal staining in bronchial smooth muscle cells (left). Scale bar=50 μm for the upper panels, 20 μm for the lower panels. Representative photomicrographs are shown. (**B**) To demonstrate transduction of the lungs with AAV1.SERCA2a, 15 days after MCT injection, rats were randomized to receive intratracheal administration of AAV1-SERCA2a, AAV1.βgal or saline, and total mRNA was isolated from lung samples. Real-time PCR using primers selective for exogenous human SERCA2a was performed. Results are expressed as average fold change in gene expression levels relative to sham; ***P < 0.001 vs. Sham, Saline, AAV1.βgal.



Supplemental Figure 4. AAV1.SERCA2a gene transfer increases pulmonary vascular eNOS expression. Expression of eNOS in lung homogenates from Sham (n=6) and MCT-PAH rats treated with aerosolized saline (n=5), AAV1.βgal (n=5), or AAV1.SERCA2a (n=7). GAPDH was used as a loading control. A representative blot (n=3) is shown. *p<0.03 vs. Sham, AAV1.SERCA2a, **p=0.003 vs. Sham, AAV1.SERCA2a.



Supplemental Figure 5. SERCA2a expression in the heart and the effect of AAV1.SERCA2a on cardiac hemodynamics. (**A**) SERCA2a expression was examined in LV homogenates. A representative blot is shown (n=3). GAPDH was used as a loading control. (**B**) Effect of AAV1.SERCA2a treatment on right ventricular systolic (RVSP) and end diastolic pressure (RVDP) in sham and MCT-injected rats treated with saline, AAV1.βgal or AAV1.SERCA2a. (**C**) Left ventricular end-systolic (LVSP) and end-diastolic (LVEDP) pressures in Sham and Saline-, AAV1.βgal- and AAV1.SERCA2a-treated rats. **p<0.005, ***p<0.001.



Supplemental Figure 6. AAV1.SERCA2a did not influence LV hemodynamics in the prevention protocol. On day 0, rats were injected with MCT and co-administered aerosolized saline (n=15), AAV1.βgal (n=6), or AAV1.SERCA2a (n=15). LV hemodynamics were measured invasively. (**A**) LV end-systolic pressure (LVESP), (**B**) LV end-diastolic pressure (LVEDP), and (**C**) LV weight were determined.