ONLINE DATA SUPPLEMENT

Copper-dependence of angioproliferation in pulmonary arterial hypertension in rats and humans

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Supplemental Methods

Protein expression

One lobe of the right lung was homogenized in RIPA (Radio-Immunoprecipitation Assay) buffer (0.5g tissue/ml RIPA) for protein isolation. After 30 minutes of incubation at 4˚C, homogenates were centrifuged at 13000 RPMI for 15 minutes. Protein concentrations were determined with BioRad Protein D_C Protein Assay (BioRad, Hercules, CA). Protein lysates were incubated for 10 minutes at 70˚C in 2x Electrophoresis Sample Buffer (Santa Cruz, Santa Cruz, CA) and 50µg protein were loaded per lane on SDS- page. After electrophoresis, proteins were blotted on PVDF- membrane (BioRad), incubated with blocking buffer (5% non- fat dry milk/PBS-0.1% Tween-20, BioRad) for 1 hour at room temperature. Membranes were incubated with primary antibodies for PCNA, cleaved caspase 3, AIF, integrins αv and β3, Bim, survivin (all 1:1000, Cell Signaling Technology Inc., Danvers, MA) or β- actin (loading control, 1:10000, Sigma- Aldrich, St. Louis, MO) in blocking buffer overnight at 4˚C. Secondary antibodies (anti- rabbit HRP conjugated (1:1000, Cell Signaling) or anti-mouse HRP conjugated (1:500, Pierce, Thermo Scientific, Rockford, IL)) were applied for 1 hour at room temperature in blocking buffer. Blots were developed with ECL (PerkinElmer, Waltham, MA) on GeneMate Blue Basic Autorad Films (BioExpress, Kaysville, UT). Blots were scanned and densitometry analysis was done with ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2011). Ceramide and other sphingolipid metabolites were quantified using Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometry, as described previously[1].

Histology and Immunohistochemistry

The left lung was inflated with 0.5% agarose at a pressure of 20cm H_2O, fixed for 24 hours and paraffin embedded. 5µm thick transversal lung slides were stained for H&E and Movat-pentachrome. Transversal lung slides (3µm) were deparaffinized and rehydrated. Antigen retrieval was carried out by boiling in 0.01M citrate buffer pH6.0 for 20 minutes or enzymatic digestion with proteinase K 1:50 (Dako, Carpinteria, CA) for vWF was performed.. Endogenous peroxidase was blocked with 3% H_2O_2/PBS for 5 minutes, and then slides were blocked with 1% Normal Swine Serum (NSS) for 15 minutes. Primary antibody for vWF (1:5000), α-SMA (1:100, both Dako) and SOD1 (1:500, Abcam, Cambridge, MA) was incubated overnight at 4˚C in 1%NSS, followed by an incubation step with secondary, anti-rabbit- or anti mouse biotin conjugated antibody (both 1:1500, Millipore, Billerica, MA) in 1%NSS for 1 hour at room temperature. An amplification step with Streptavidin/HRP (1:200, Vector Laboratories, Inc., Burlingame, CA) was performed for 45 minutes in 1%NSS at room temperature. Sections were developed with 3,3′-diaminobenzidine (DAB) chromogen substrate (Sigma- Aldrich) and counterstaining was performed with Mayer's hematoxylin. The ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA) was used for TUNEL staining according to manufacturer's instructions. For double immunofluorescence, slides were steamed for 20 minutes in 0.01M citrate buffer pH6.0 and blocked with 1%NSS for 15 minutes. Primary antibody no. 1, PCNA (1:200) or Bim (1:25, both Cell Signaling) was applied overnight at 4°C in 1%NSS. Secondary antibody no. 1, anti-rabbit- or anti-mouse Alexa Fluor488 (both 1:100, Invitrogen, Carlsbad, CA) was incubated for 4 hours at room temperature in PBS. Sections were incubated with primary antibody no. 2, anti-mouse vWF (1:20, LifeSpan Biosciences, Inc., Seattle, WA) for Bim/vWF, anti- rabbit vWF (1:500, Dako) for PCNA/vWF or direct labeled anti- mouse α-SMA Cy3 (1:500, Sigma-Aldrich) overnight at 4°C in PBS, then secondary antibody no. 2, anti-mouse or -rabbit Alexa Fluor 594 (both 1:100, Invitrogen) was applied for 4 hours at room temperature in PBS.

Slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 1:20,000 and mounted with SlowFade® Antifade (both Invitrogen). Negative controls with unspecific IgG were run in parallel. For TUNEL/vWF and TUNEL/α-SMA, the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Applied Science, Indianapolis, IN) was used. Antigen retrieval was carried out with 0.01M citrate buffer pH6.0 prior to TUNEL according to manufactures instruction. Primary antibody for vWF (1:500) or α-SMA (1:100, both Dako) were applied overnight, followed by secondary anti- rabbit- or anti- mouse Alexa Fluor 594 (both 1:100, Invitrogen). Negative control (terminal transferase omitted) and positive control (DNAse treated section) were run in parallel. Light microscope images were acquired with an Axioscope AX10 microscope and Axioviosion 4.6 software (both from Carl Zeiss) and immunofluorescence images were taken with Olympus IX70 microscope and cellSens Dimension 1.5 software (both from Olympus). Right ventricular fibrosis and capillary rarefaction were determined as described previously.

Assessment of angioproliferative vascular lesions

A quantitative analysis of luminal obstruction was performed by counting at least 200 small pulmonary arteries (OD, <50 um) per lung section from each rat in the 2 groups by two investigators blinded to the treatment group. Vessels were assessed for occlusive lesions on hematoxylin/eosin slides from two random left lung slices and scored as: no evidence of neointimal formation (patent); partially patent (<50%); and full-luminal occlusion (fully obliterated).

Reference List

E1. Takabe, K., R. H. Kim, J. C. Allegood, P. Mitra, S. Ramachandran, M. Nagahashi, K. B. Harikumar, N. C. Hait, S. Milstien, and S. Spiegel. 2010. Estradiol induces export of sphingosine 1-phosphate from breast cancer cells via ABCC1 and ABCG2. *J.Biol.Chem.* 2010; 285:10477-10486.

Legends to supplemental figures

Figure E1 *Movat stain. SuHx lungs exhibit occlusive vascular lesions with red intraluminal cells suggestive of a myofibroblast origin. (panels A and B: magnification 100x, 10x objective, scale bar 100um; panel C is an enlargement of the insert in panel B; magnification 400x, 40x objective, scale bar 25um. Intraluminal cells are removed with Tetrathiomolybdate (TTM) treatment (panels D-F).*

Figure E2 *Tetrathiomolybdate (TTM) treatment of SuHx rats mitigates right ventricular (RV) hypertrophy (panel A) but has no significant effects on RV fibrosis (panel B; determined in Masson's trichrome stain) or capillary rarefaction (panel C; caveolin-1 stain).*

Figure E3 *Pulmonary vascular remodeling in the SuHx model is associated with high rates of cell death, as shown by TUNEL staining. Pictures are taken with a 40x objective (400x magnification); the scale bar denotes 25um. Before tetrathiomolybdate (TTM) treatment, TUNEL positivity is almost exclusively found in cells around occlusive lesions, whereas after treatment TUNEL positive intraluminal cells are found as well.*

Figure E4 *Cessation of tetrathiomolybdate (TTM) treatment results two weeks later in recurrence of pulmonary vascular lesions in SuHx rats, as shown by an increase in mean pulmonary artery pressure (mPAP; panel A), an increase in right ventricular hypertrophy (RV/LV+S is right ventricular weight divided by left ventricular plus septal weight; panel B) and an increase in lumen occlusions (panels C and D; arrows denote occluded vessels; H&E stains).*

Figure E1

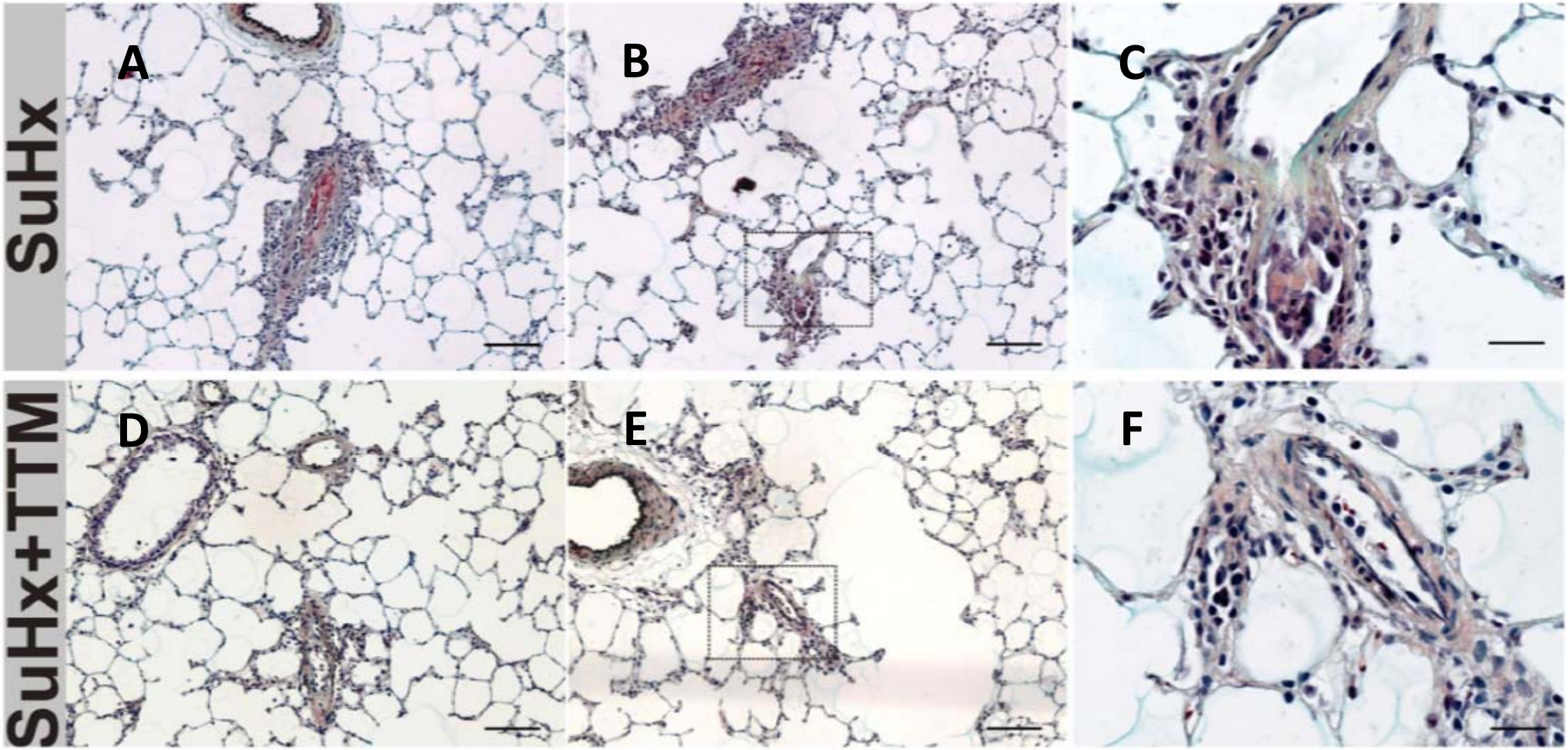


Figure E2

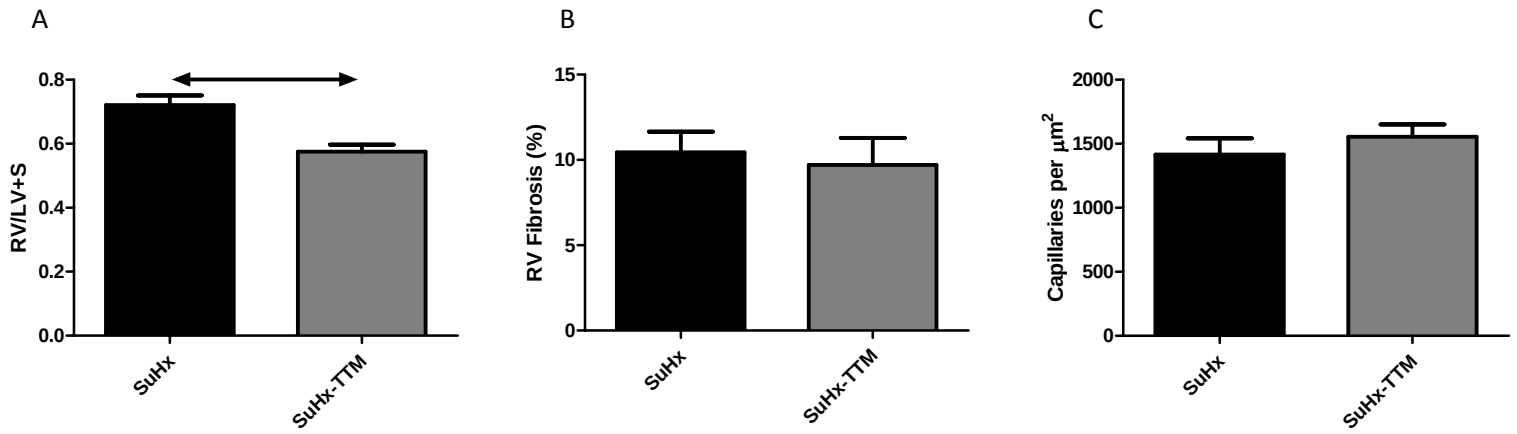

A
RV/LV+S
0.8
0.6
0.4
0.2
0.0
SuHx
SuHx-TTM
B
RV Fibrosis (%)
15
10
5
0
SuHx
SuHx-TTM
C
Capillaries per µm2
2000
1500
1000
500
0
SuHx
SuHx-TTM

Figure E3

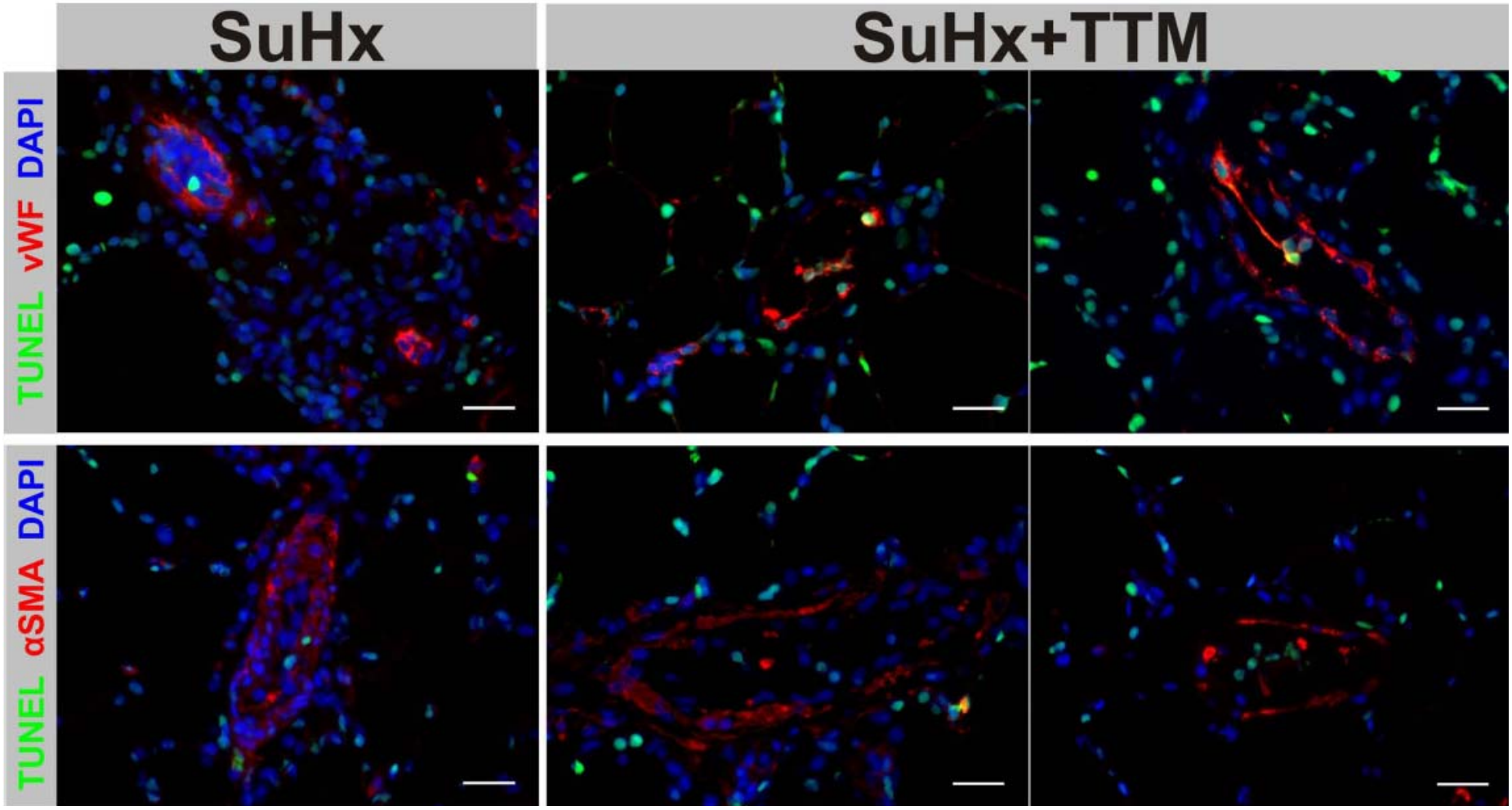
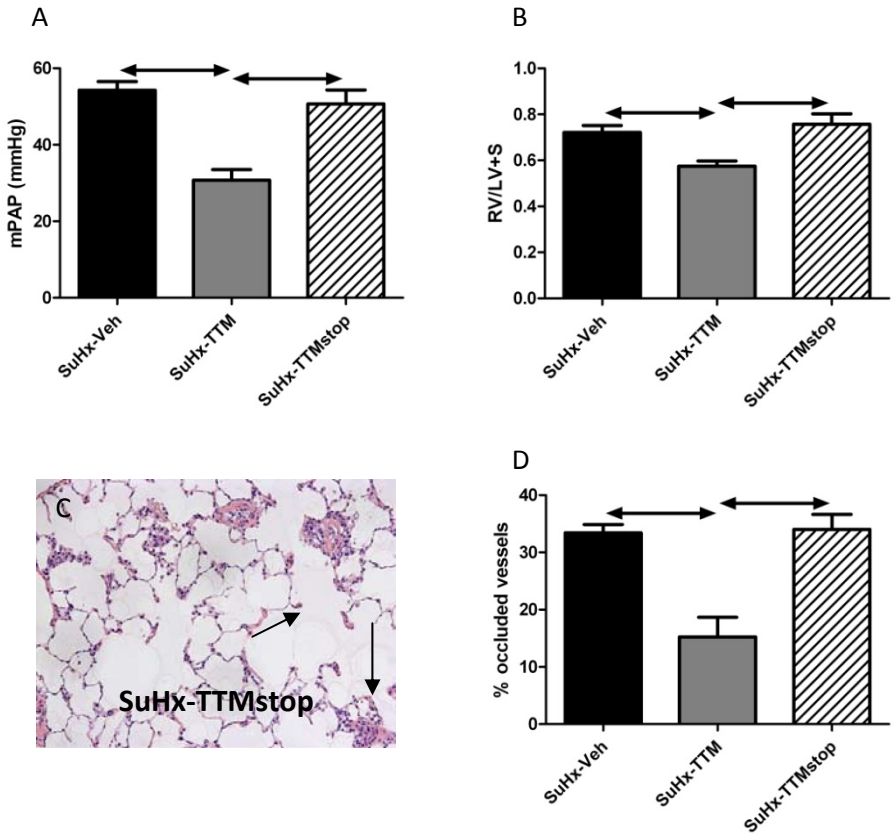


Figure E4


A
mPAP (mmHg)
60
40
20
0
SuHx-Veh
SuHx-TTM
SuHx-TTMstop
B
RV/LV+S
1.0
0.8
0.6
0.4
0.2
0.0
SuHx-Veh
SuHx-TTM
SuHx-TTMstop
C
SuHx-TTMstop
D
% occluded vessels
40
30
20
10
0
SuHx-Veh
SuHx-TTM
SuHx-TTMstop