Endothelial-to-Mesenchymal Transition and Inflammation Play Key Roles in Cyclophilin A-Induced Pulmonary Arterial Hypertension

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Short title: EndMT and inflammation in CypA-induced PAH

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Detailed Methods

Generation of mice

All animal experiments were conducted in accordance with the experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester. We utilized a Cre/LoxP strategy to prepare CypA transgenic mice. In brief, a LacZ^{flox}-CypA construct was prepared using the pZ/EG vector. The pZ/EG double reporter construct was a kind gift from the Nagy lab. This vector contains LacZ floxed by two loxP sites, driven by the chicken β-actin promoter and a cytomegalovirus (CMV) enhancer with enhanced green fluorescent protein (EGFP) downstream. We replaced EGFP with full-length wild type mouse CypA carrying a Flag tag to make the LacZ*flox*-Flag-CypA construct. ES cells transfected by electroporation with linearized LacZ^{flox}-Flag-CypA cDNA were screened by neomycin resistance and LacZ expression. ES clones with a single copy by Southern blotting were used to generate chimeric mice by ES cell – embryo aggregation. The chimeric mice were bred to C57BL/6J mice to produce hemizygous transgenic offspring. Hemizygous offspring with germline transmission were identified by PCR of DNA harvested from tail snippets of weaned offspring. We obtained 9 germline mice from the 2A3 ES cell clone and 8 from the 3H9 ES cell clone. Transgenic mice were backcrossed to C57BL/6J mice for 7 generations to establish experimental lines. To generate EC specific overexpression of CypA transgenic mice, the LacZ^{flox}-CypA transgenic mice and Cdh5-Cre mice (C57BL/6J background) were crossed. These EC specific CypA overexpression mice were then crossed with the B6.129(Cg)- *Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J mice (The Jackson Laboratory, Stock No. 007676) to generate EC-specific reporter mice.

Human tissue harvest

All protocols using human specimens were approved by the Institutional Review Board at the University of Rochester. Informed consent was obtained from all subjects. Patients died directly as a result of advanced PAH were autopsied at the University of Rochester Medical Center. After autopsy, samples from each lobe of formalin fixed lungs were cut and then sectioned at 5 microns before staining with hematoxylin/eosin. A separate modified trichrome stain to emphasize elastin and collagen in the vascular structures was also performed.

Cell isolation and culture

Animals (8–12 weeks old) were anaesthetized with a single intraperitoneal injection of ketamine (130mg/kg) and xylazine (8.8mg/kg). Saline perfusion was performed to eliminate the blood cells. Lungs were harvested and mouse pulmonary microvascular endothelial cells (MPMECs) were isolated following the protocol from Dr. Simons Lab at Dartmouth Medical College. MPMECs were maintained in Dulbecco's modified eagle medium (DMEM) containing 20% fetal bovine serum (FBS), 10mg/L Endothelial Cell Growth Factor ECGF (BioMedical Technologies Inc), 40mg/L heparin and 1X

Pen/Strep. Human pulmonary microvascular endothelial cells (HPMECs) were purchased from ScienCell and maintained in Endothelial Cell Medium purchased from ScienCell (Cat No. 1001).

In vitro acetylation assay

Reactions (20μL) containing rhCypA (R&D System and Sigma-Aldrich) 50 nmol/L, 1.2 mmol/L acetyl-CoA (Sigma) and 1μg p300 protein (Millipore) in HAT buffer (50 mmol/L Tris HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L PMSF, 1 mmol/L DTT, 10 mmol/L sodium Butyrate and 10% Glycerol) were incubated at 30℃ for 45 minutes and reaction was stopped by incubation on ice.

Western Blot

Cells were harvested and lysed in ice-cold 1x lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by Bradford protein assay (Bio-Rad). Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred onto nitrocellulose membranes, and were subsequently blocked in 5% milk in PBST (Phosphate-buffered saline containing 0.1% Tween 20) for 1 hour. Then the blots were incubated overnight at 4℃ with appropriate primary antibodies. The following primary antibodies were used: VE-Cadherin (1:1000, Santa Cruz), PECAM-1 (1:1000, Santa Cruz), claudin-5 (1:500, GeneTex), αSMA (1:500, Sigma-Aldrich), phospho-vimentin (1:500, Cell Signaling), Snail (1:1000, Cell Signaling), Slug (1:1000, Cell Signaling), phospho-Smad2 (1:1000, Cell Signaling), phospho-Smad3 (1:1000, Cell Signaling), Smad2 (1:1000, Cell Signaling), Smad3 (1:1000, Cell Signaling), phospho-TAK1 (1:500, GeneTex) and GAPDH (1:5000, Millipore). After being washed 3 times with PBST, membranes were incubated with horseradish peroxidase-coupled goat anti-rabbit, goat anti-mouse or horse anti-goat IgG(1:10000, Amersham) for 1 hour. Visualization of signals were by chemiluminescence (ECL Reagent, Millipore). Densitometric analysis of films was performed using ImageJ software.

Immunofluorescence

Sections were treated with 3% H_2O_2 for 10 minutes at room temperature followed by antigen retrieval for 20 minutes with steam using 1X Citrate buffer, pH=6.0 (Millipore). Sections were blocked in 10% goat serum in PBS for 1 hour at room temperature followed by overnight incubation at 4℃ with primary antibodies; SM22α (1:200, Abcam), vimentin (1:200, Abcam), αSMA (1:500, Dako) or GFP (1:200, Abcam) in 10% goat serum in PBS. Fluorescence-conjugated secondary antibodies were incubated for 1 hour at room temperature and followed by three washes with PBS. For cultured cell staining, cells seeded on coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The fixed cells were blocked with 10% goat serum in PBS for 1 hour at room temperature followed by overnight incubation at 4℃

with primary antibodies PECAM-1 (1:100, Abcam for MPMEC; 1:100, Dako for HPMEC), claudin-5 (1:100, GeneTex), αSMA (1:500, Sigma-Aldrich) or FLAG (1:100, Sigma) overnight at 4℃. Fluorescence-conjugated secondary antibodies were incubated for 1 hour at room temperature and followed by three times washing with PBS. Images captured by a confocal microscope (Olympus IX81).

Immunohistochemistry

Sections were treated with 3% H_2O_2 for 10 minutes at room temperature followed by antigen retrieval for 20 minutes with steam using 1X Citrate buffer, pH=6.0 (Millipore). Sections were blocked in 10% goat serum in PBS for 1 hour at room temperature followed by overnight incubation at 4°C with primary antibodies in 10% goat serum in PBS. The following primary antibodies were used: CypA (1:1000, Enzo), αSMA (1:500, Dako), IL-6 (1:500, Abcam) and MCP-1 (1:1000, Abcam). Sections were then incubated with secondary antibodies in 10% goat serum in PBS for 1 hour at room temperature. The following secondary antibodies were used: biotinylated goat anti-rabbit IgG (1:500, Vector) and biotinylated goat anti-mouse IgG (1:500, Vector). ABC-HRP conjugate (Vector PK-6100) was used in conjunction with the chromogenic HRP substrate/reagent DAB (Dako).

ELISA

96 well Polysorp ELISA plates (Thermo-Nunc) were coated with 1µg/ml mouse monoclonal anti-CypA (Santa Cruz; sc134310) in 0.2M bicarbonate buffer pH 9.4 for 2 hours at room temperature. Following washing, blocking was performed with PBST/1%BSA for 1 hour at room temperature. Serial dilutions of rat plasma were incubated in the plates at room temperature for 1 hour followed by extensive washing with PBST. Immunoadsorbed CypA was detected using 2.5µg/ml rabbit polyclonal anti-CypA (Enzo Biosciences; BML-SA296-0100). Our custom peptide specific AcK-CypA antibody specifically detects Ack-CypA and was used at 10µg/ml. Both incubations were at room temperature for 2 hours. Following extensive washing in PBST reactivity was detected with 50ng/ml goat anti rabbit-HRP for 1 hour at room temperature (Jackson ImmunoResearch) and Super-Signal Pico Chemiluminescent reagent (Thermo-Pierce). Luminescence was measured on a Fluostar Optima plate reader (BMG LabTech).
MCP-1 (Biolegend, 432704) and IL-6 (Biolegend; 431304) ELISA were performed according to the manufacturer's instructions.

Cytokine array

The culture medium was collected from indicated cells. Cytokines from HPMEC and MPMEC were analyzed using Human Cytokine Antibody Array (Abcam, ab133997) or Mouse Inflammation Antibody Array (Abcam, ab133999) respectively, according to the manufacturer's instructions.

Scratch wound assay

MPMECs were seeded in 12-well plates in EC growth medium (20%FBS) described above. Medium was changed to DMEM without FBS and starved for 24 hours. Confluent cells were scratched with a pipette tip and medium was replaced with DMEM with 0.1%FBS. Cells were then treated with vehicle, CypA or AcK-CypA and allowed to grow for 72 hours. For migration assay, cells were pretreated with anisomycin (10μmol/L, Sigma-Aldrich) for 2 hours to block protein synthesis (cell proliferation) before scratch. After scratch, medium was replaced with EC growth medium (20%FBS). Cells were then treated with vehicle, CypA or AcK-CypA and allowed to migrate for 72 hours.

BrdU incorporation assay

MPMECs were seeded on coverslips in EC growth medium (20%FBS) mentioned above at a very low density and starved for 24 hours in DMEM without FBS. Cells were then treated with vehicle, CypA or AcK-CypA in DMEM without FBS containing 10μmol/L BrdU (Thermo Fisher Scientific) at 37℃ for 2.5 hours and followed by fixation with 2% paraformaldehyde and permeabilization using 0.1% Triton X-100. Cells were then incubated with 1mol/L HCL for 10 minutes at room temperature for DNA hydrolysis and neutralized with 0.1mol/L sodium borate buffer pH 8.5 for 10 minutes at room temperature. The fixed cells were blocked with 10% goat serum in PBS for 1 hour at room temperature followed by overnight incubation at 4℃ with primary antibodies BrdU (1:200, Dako) in PBS with 10% goat serum. Fluorescence-conjugated secondary antibodies were incubated for 1 hour at room temperature and followed by three times washing with PBS. Images were captured using a BX51 Epi-Fluorescence Microscope (Olympus).

Mitotracker staining

Cells were incubated with 500nmol/L of MitoTracker™ Red CMXRos (Thermo Fisher Scientific) in DMEM without FBS for 30 minutes at 37℃ in dark followed by fixation with 2% paraformaldehyde and permeabilization using 0.1% Triton X-100. Images were then captured using a BX51 Epi-Fluorescence Microscope (Olympus) or a confocal microscope (Olympus IX81).

Mitochondria membrane potential assay

Cells seeded in dark 96-well microplate were incubated with 10μmol/L JC-1 (Abcam, ab113850) for 10 minutes at 37℃ in the dark according to the manufacturer's instructions. Cells were then treated with vehicle, CypA or AcK-CypA for 24 hours. Fluorescence counts for aggregate form was read using BMG LABTECH microplate reader with excitation wavelength set at 570nm and emission wavelength at 620nm. For monomer form, excitation wavelength was set at 485nm and excitation wavelength

at 520nm. To avoid background caused by phenol red, Opti-MEM with 20% FBS was used as the growth medium for MPMEC in this experiment.

Statistical analysis

Data are means ± SEM of at least three independent experiments. The significance between samples was determined by Student's t-test for two group comparisons or analysis of variance for more than two groups using the Graphpad Prism software. $p<0.05$ was considered statistically significant.

Patients	Age	Sex	PAH subtype
Patient 1	43	Female	Idiopathic PAH
Patient 2	53	Female	Idiopathic PAH
Patient 3	45	Female	Scleroderma PAH

Table S1. Information about age, sex and PAH subtypes of the PAH patients.

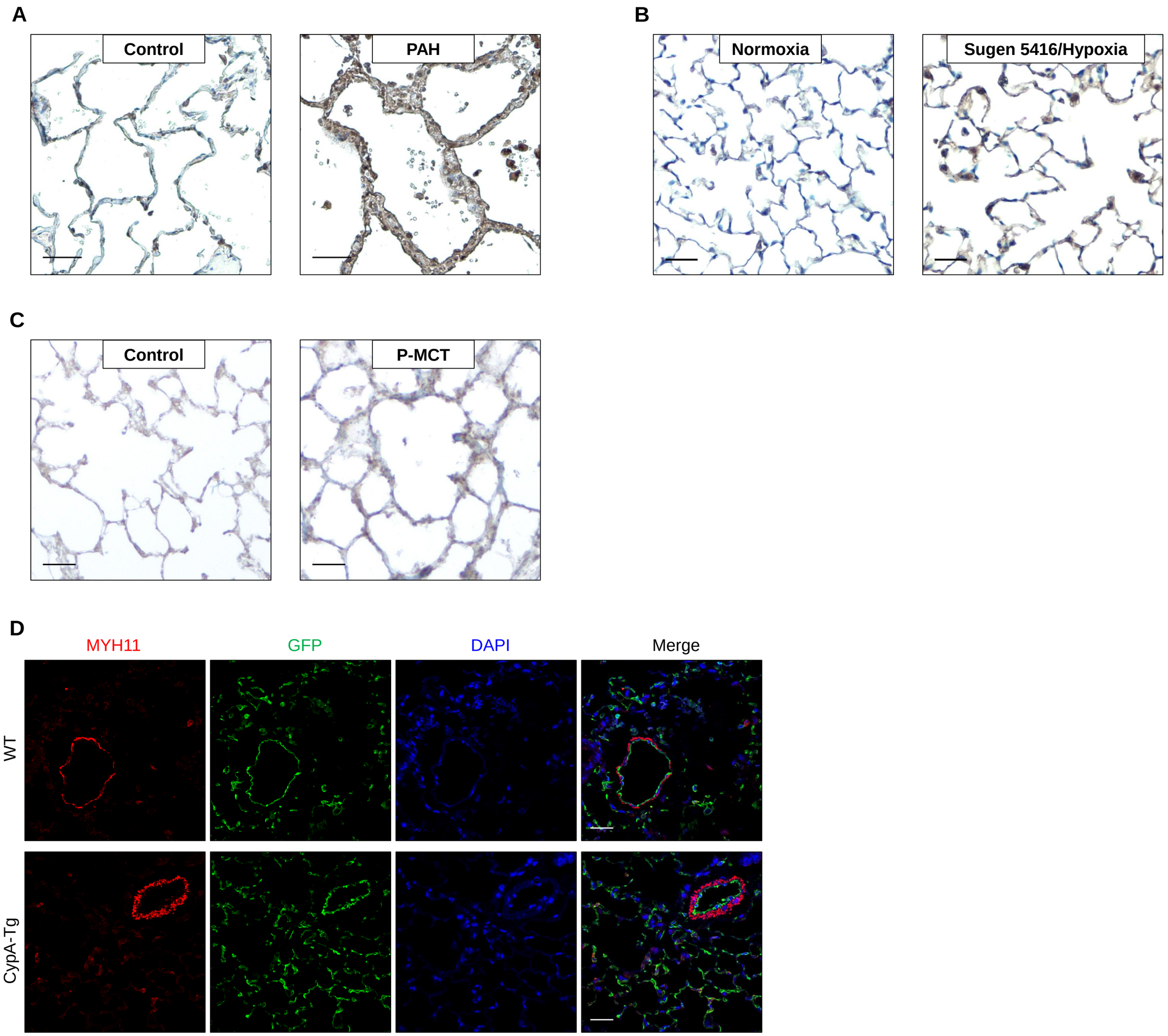
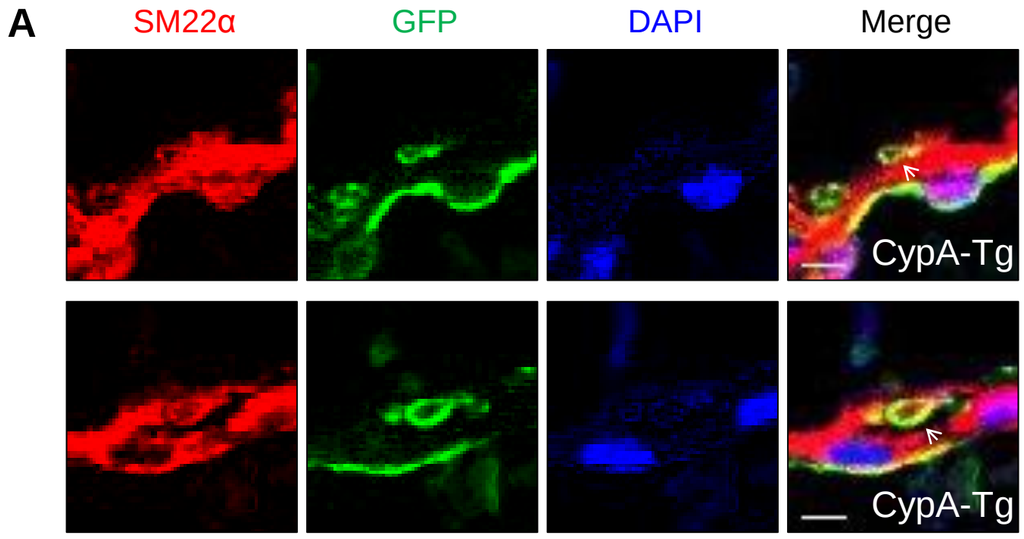
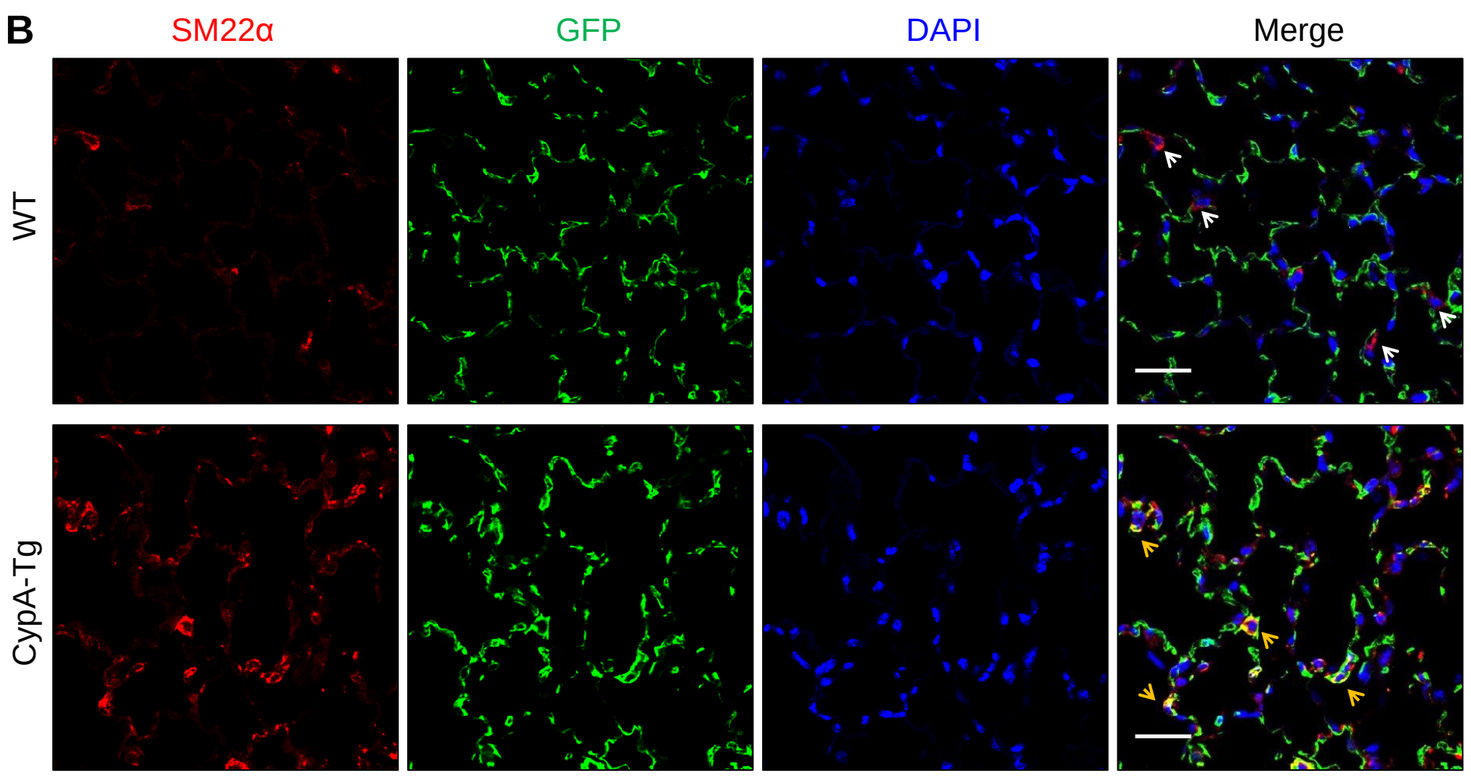
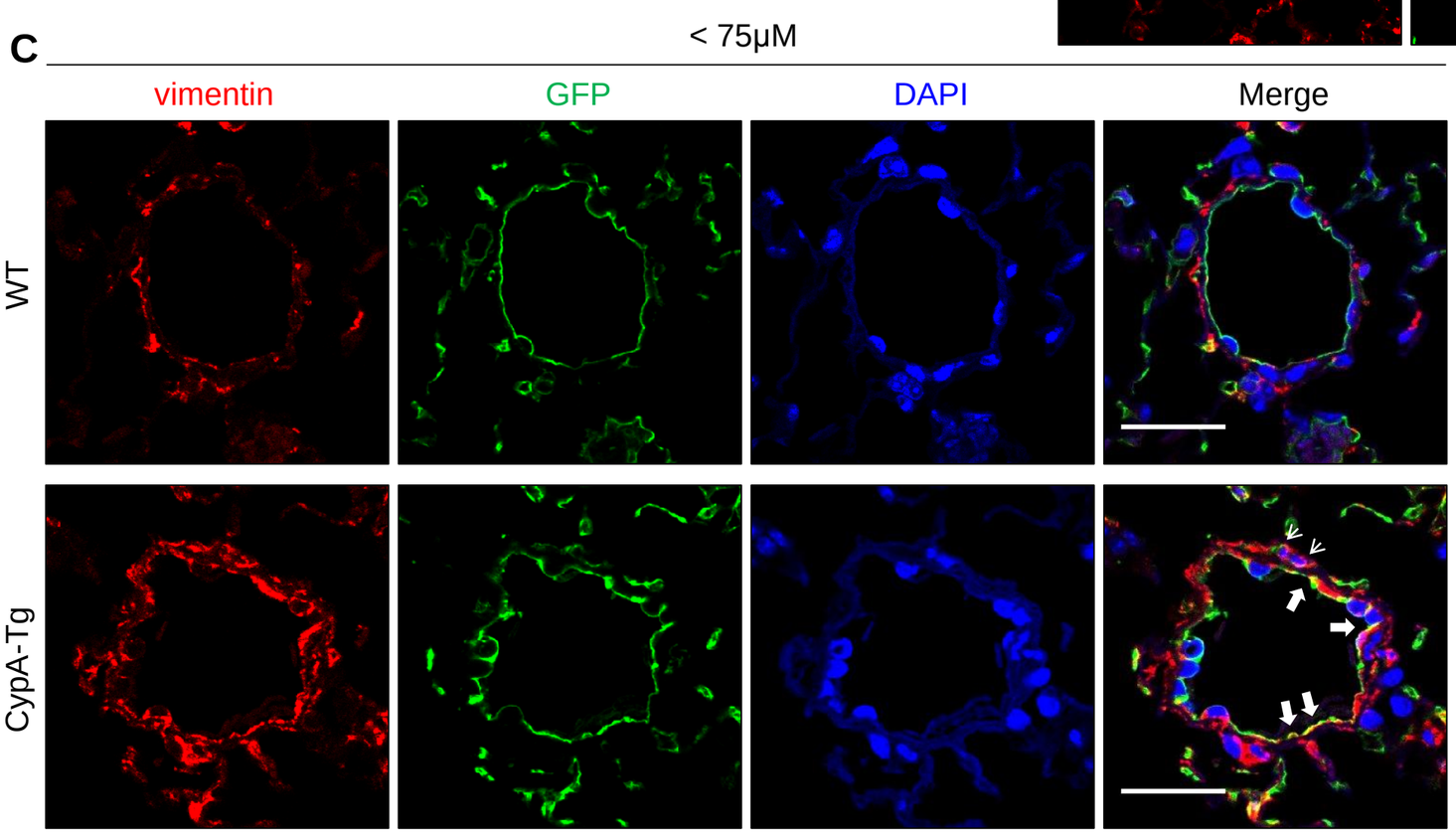
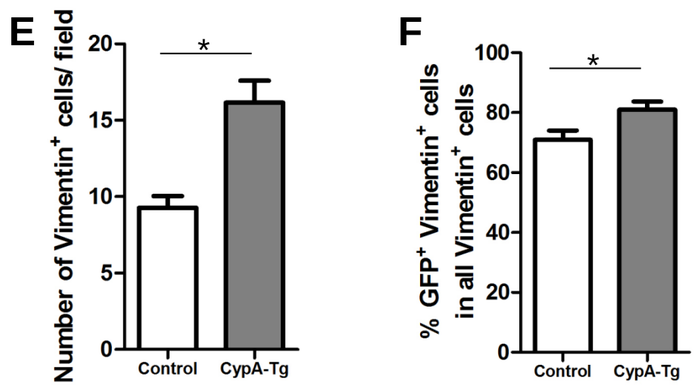


Figure S1. A-C, Representative lung IHC for CypA. **A**, controls (n=5) versus PAH patients (n=3). **B**, normoxia and Sugen 5416/hypoxia mice (n=4 each). **C**, controls (n=3) and P-MCT rats (n=5). Scale bar, 50μm (**A** and **C**) and 30 μm (**B**). **D,** Representative immunofluoresence images for MYH11 (red), GFP (green) and DAPI (blue) of pulmonary arteries and alveoli in lung sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 25μm.









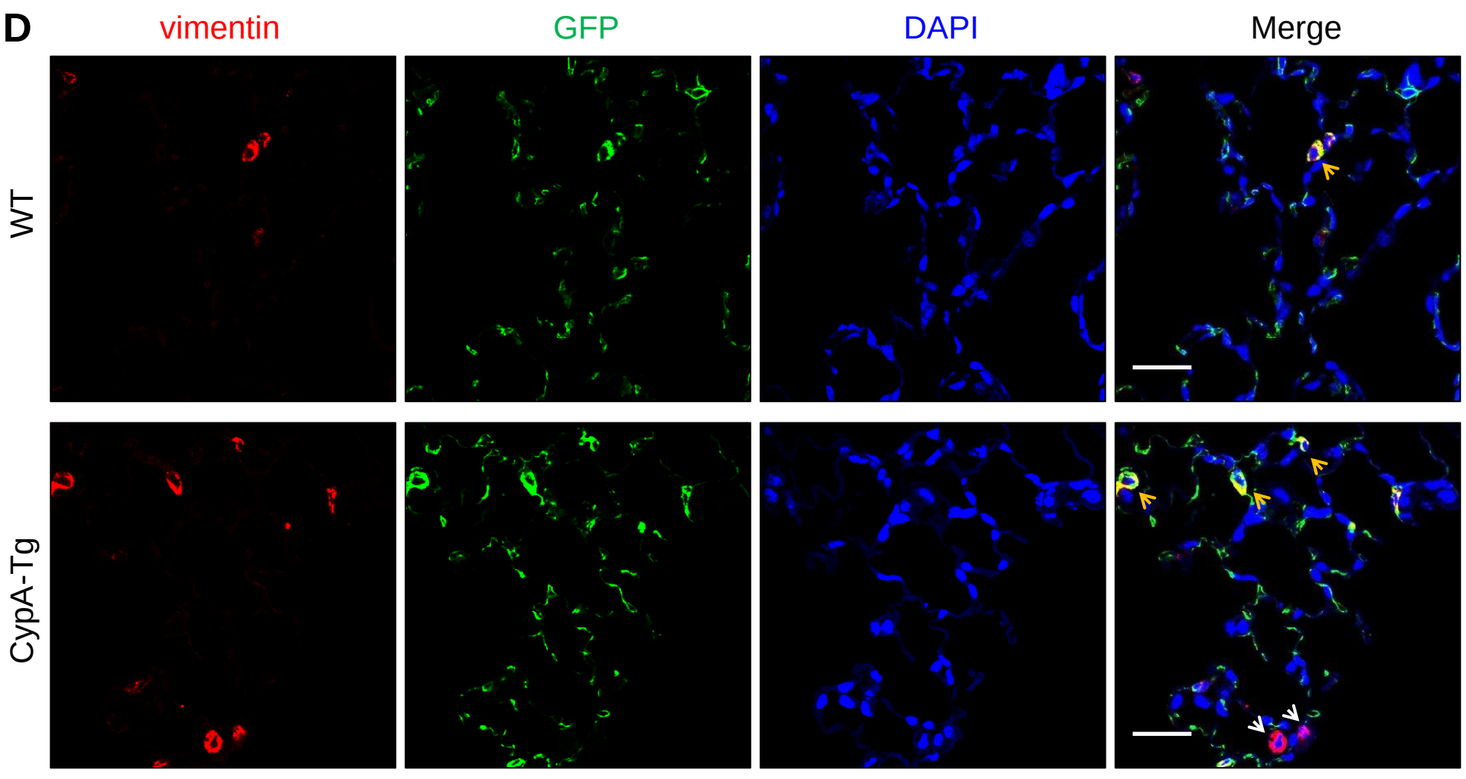


Figure S2. A, Magnification of white boxes indicated in **Fig 2B**. Immunofluoresence images for SM22α (red), GFP (green) and DAPI (blue) of SM22α+ GFP+ cells in pulmonary arteries in lung sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 5μm. **B,** Representative immunofluorescence images for SM22α (red), GFP (green) and DAPI (blue) of alveoli in lung sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 25μm. **C,** Representative immunofluoresence images for vimentin (red), GFP (green) and DAPI (blue) in pulmonary arteries in lung sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 25μm. **D,** Representative immunofluoresence images for vimentin (red), GFP (green) and DAPI (blue) of alveoli in lung sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 25μm. **E-F,** Quantification of the total number of vimentin$^+$ cells per field (**E**) and the percentage of vimentin$^+$ cells that were GFP$^+$ in all vimentin$^+$ cells (**F**) as shown in **D**. Data are mean±SEM. *P<0.05.

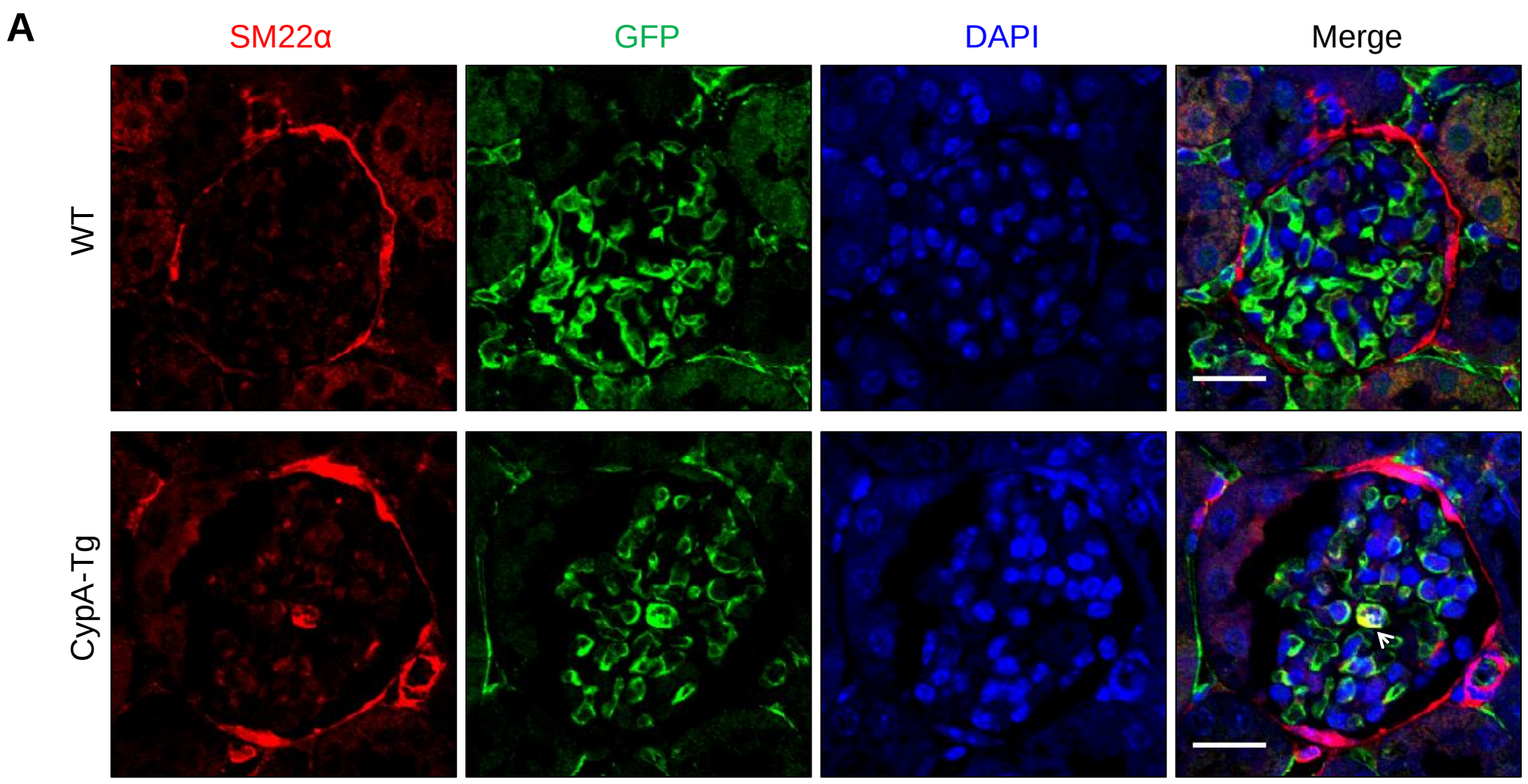


Figure S3. A, Representative immunofluoresence images for SM22α (red), GFP (green) and DAPI (blue) of glomeruli in kidney sections from WT;mTmG and Cdh5-CypA;mTmG mice. **B,** Representative immunofluoresence images for vimentin (red), GFP (green) and DAPI (blue) of glomeruli in kidney sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 20μm for all images.

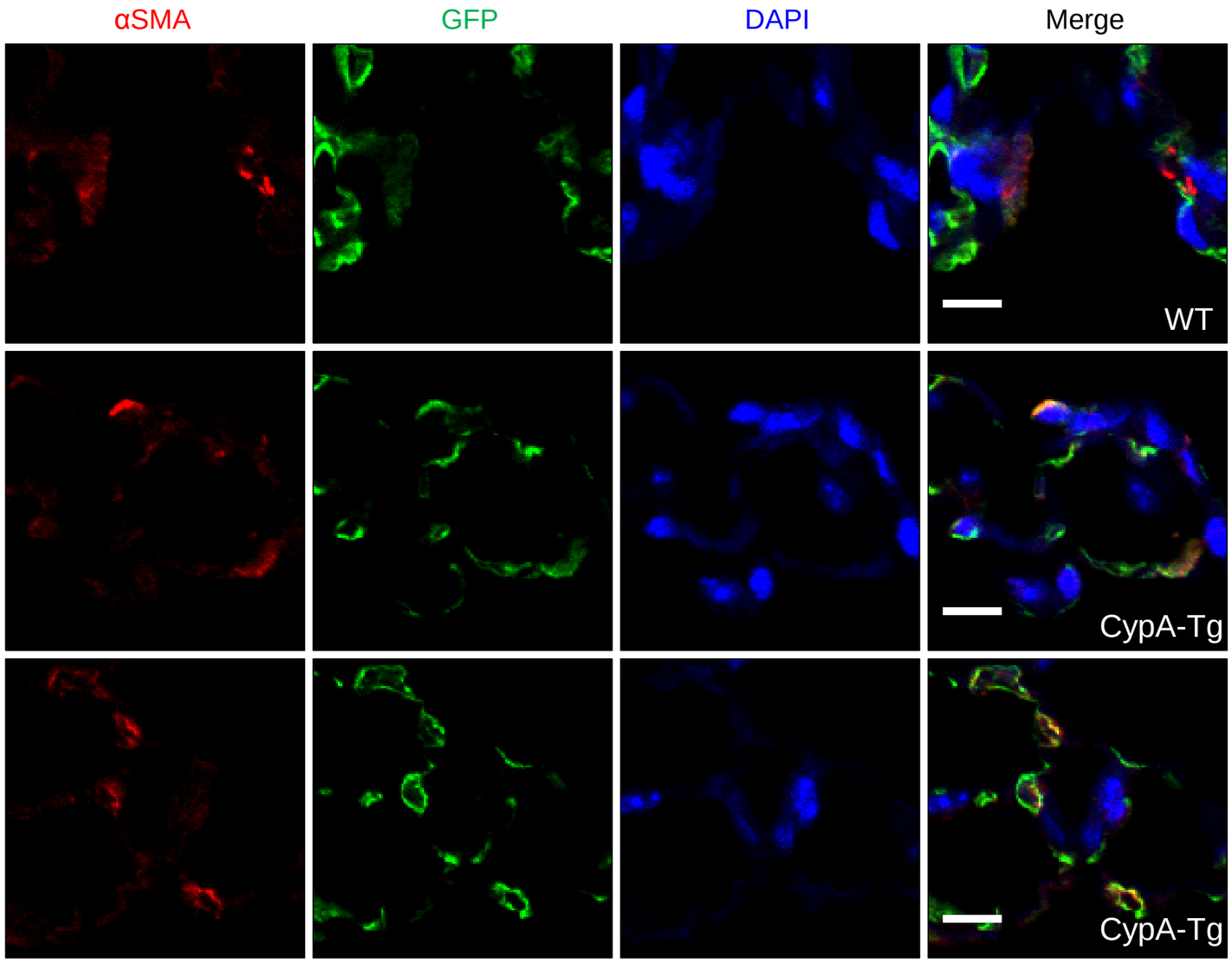
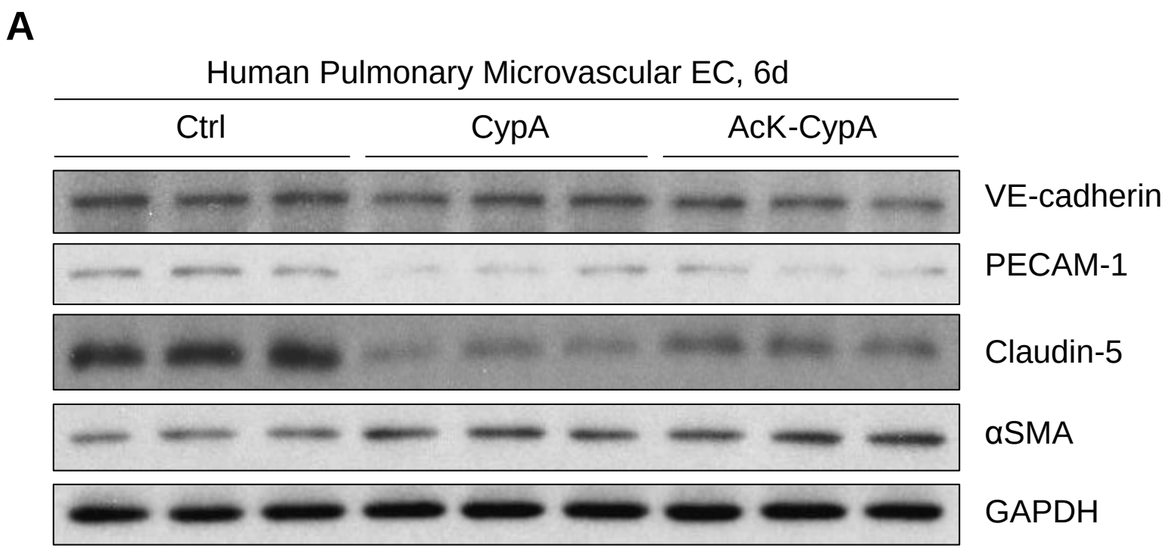
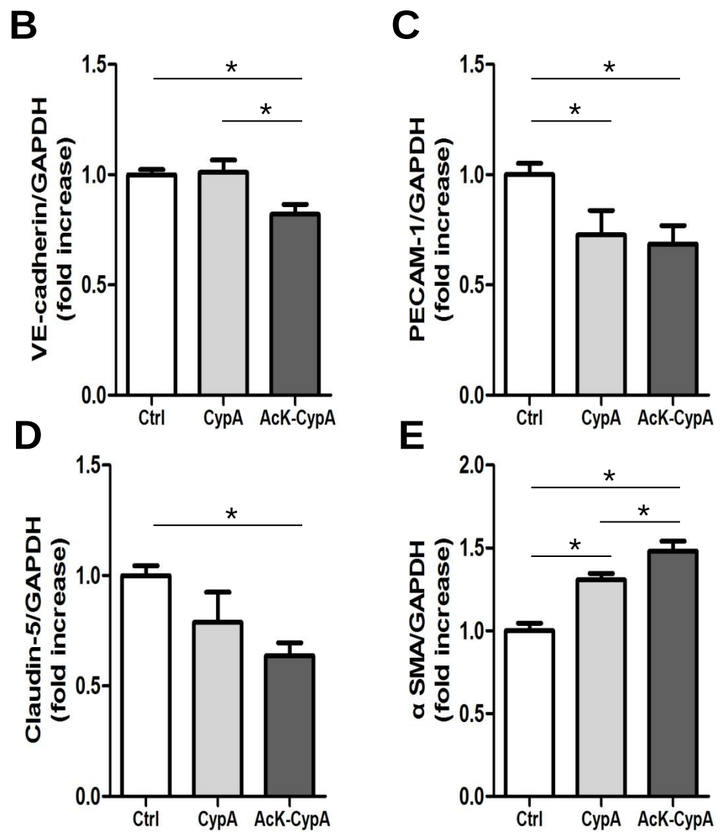


Figure S4. A, Representative immunofluoresence images for αSMA (red), GFP (green) and DAPI (blue) of pulmonary arteries and alveoli in lung sections from WT;mTmG and Cdh5-CypA;mTmG mice. Scale bar, 25μm.





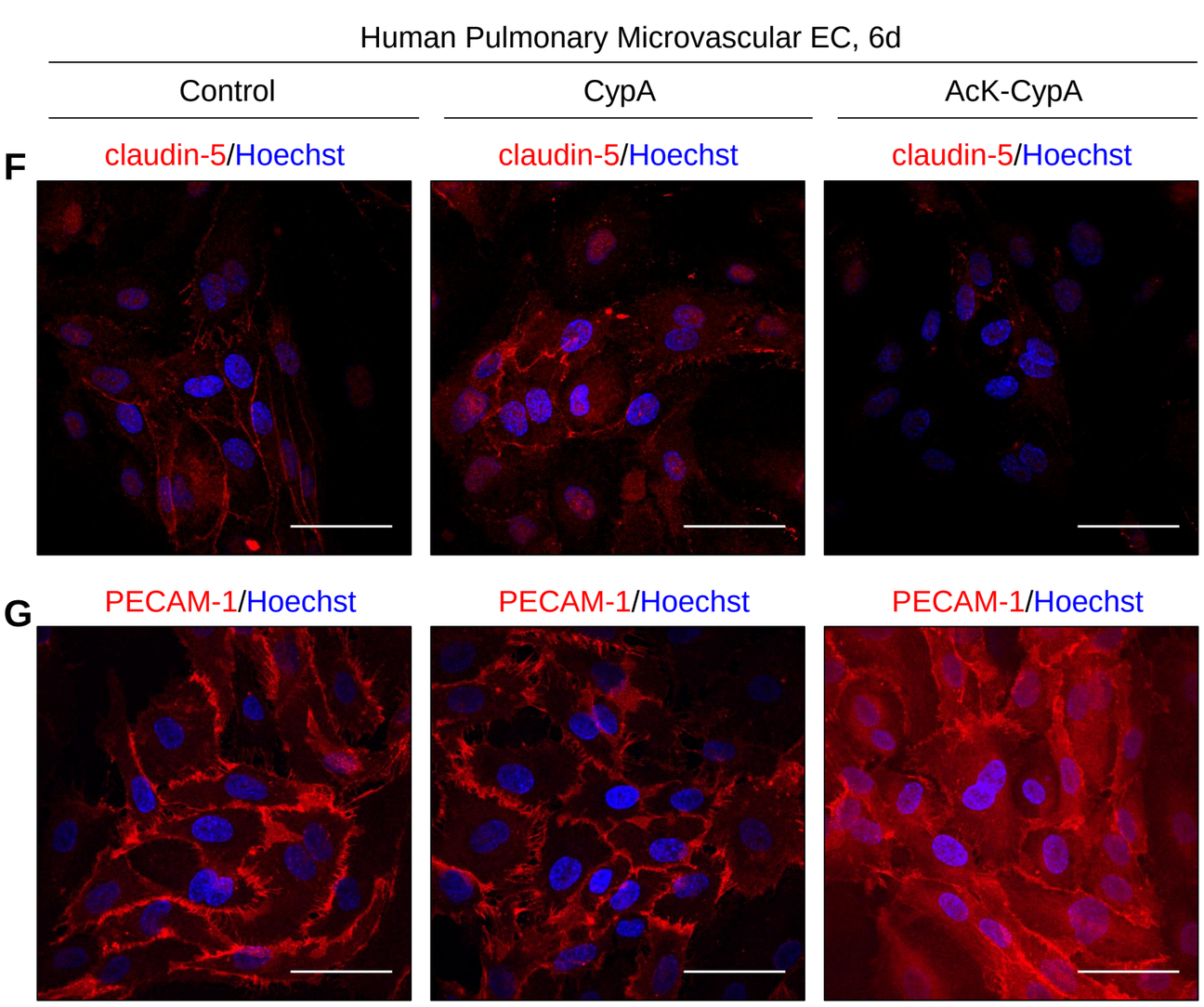


Figure S5. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A,** Expression of endothelial and mesenchymal markers in HPMEC treated with CypA or AcK-CypA. **B-E,** Quantification of protein expression shown in **A**. Data are mean±SEM. *P<0.05. **F-G,** Representative immunofluoresence images of HPMEC treated with CypA or AcK-CypA staining for Claudin-5(**F**) and PECAM-1(**G**). Scale bar, 50μm.

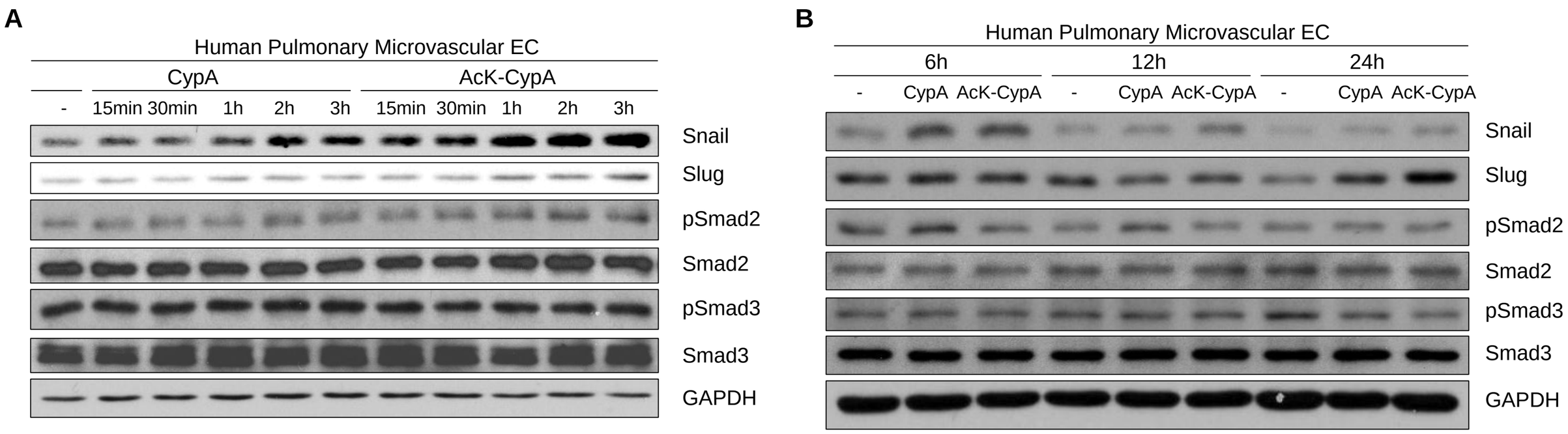
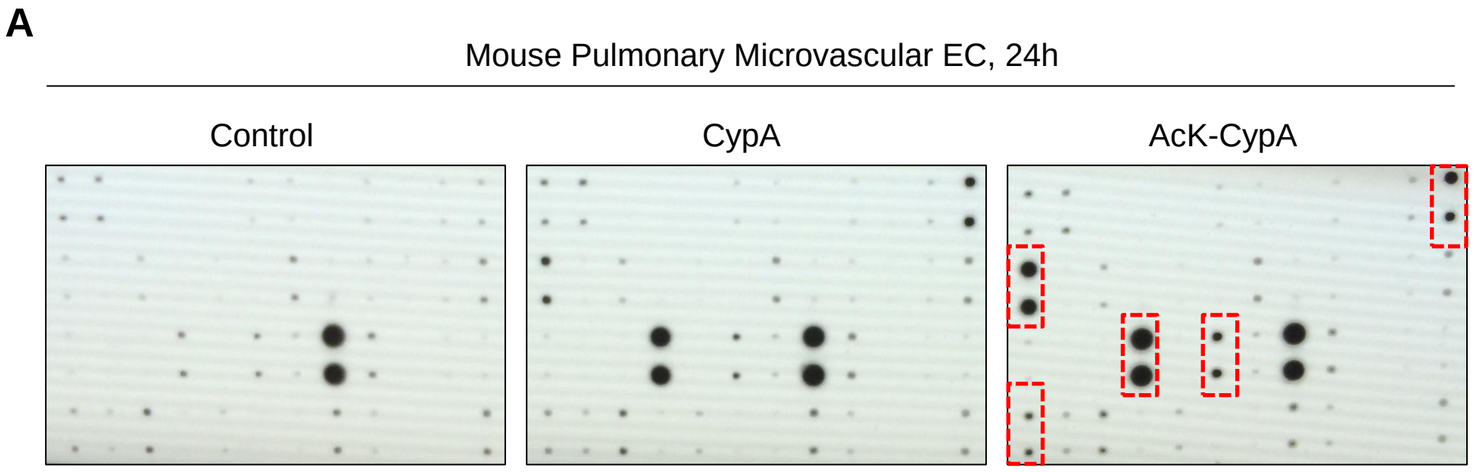
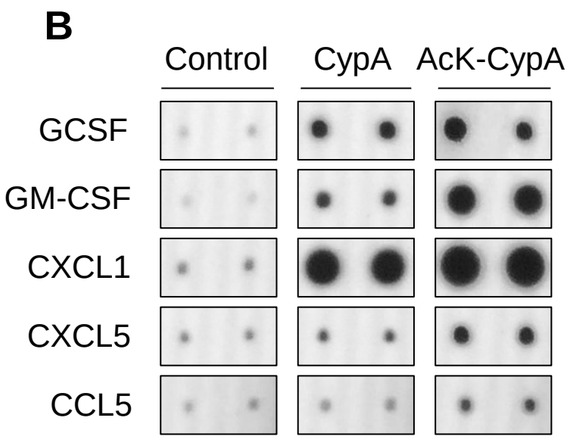
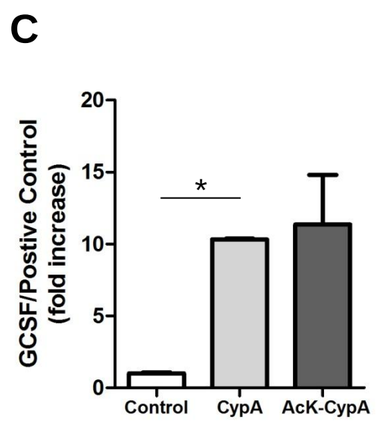
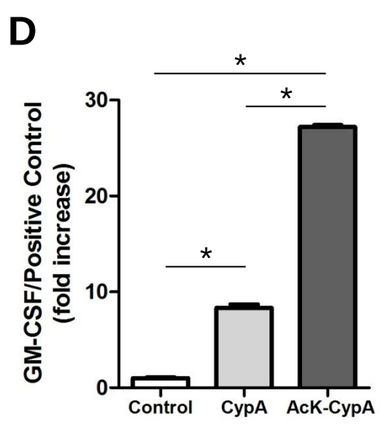


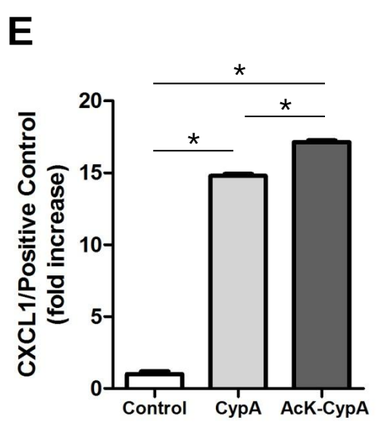
Figure S6. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A,** Time course of Snail and Slug expression and Smad2/3 activation in HPMEC over 3 hours. **B,** Snail and Slug expression and Smad2/3 activation in HPMEC treated with CypA or AcK-CypA for 6, 12 and 24 hours.

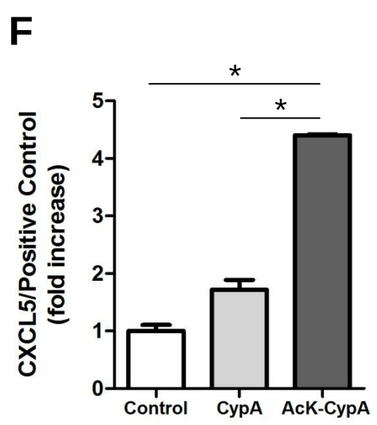


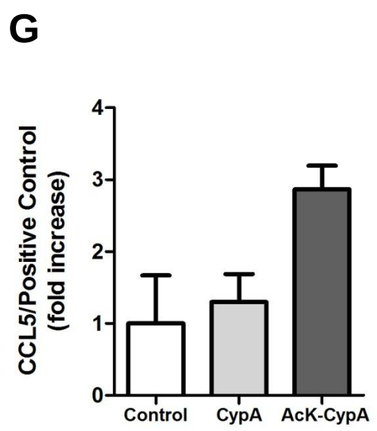


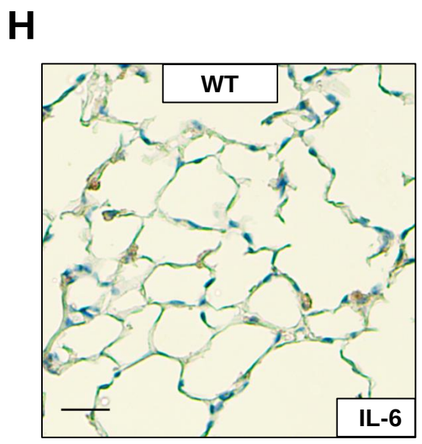


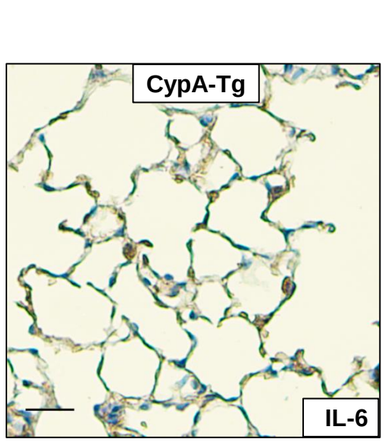


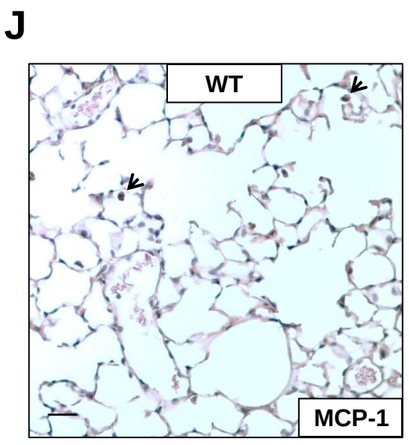


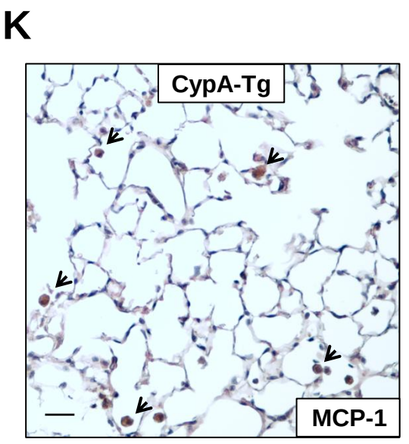


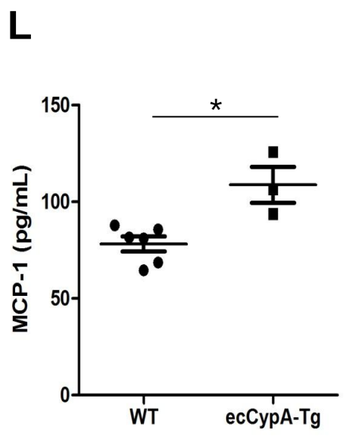












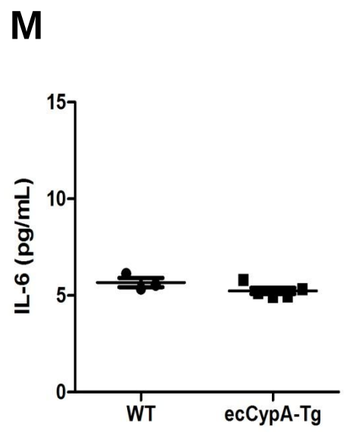


Figure S7. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A,** Representative images of cytokine array using conditioned medium (CM) of MPMEC after 24 hours of CypA or AcK-CypA treatment. Red boxes indicate the cytokines with increased levels in CypA or AcK-CypA treated CM compared to control CM. **B,** Levels of cytokines that are indicated by red boxes in **A. C-G,** Quantification of the selected cytokines from the cytokine array studies shown in **B**. Data are mean±SEM. *P<0.05. **H-K,** Representative lung IHC for IL-6 (**H-I**) and MCP-1 (**J-K**) from WT and ecCypA-Tg mice. Scale bar, 25μm for all images. **L-M,** Levels of MCP-1 (**M**) and IL-6 (**N**) in the plasma of control and ecCypA-Tg mice. Data are mean±SEM. *P<0.05.

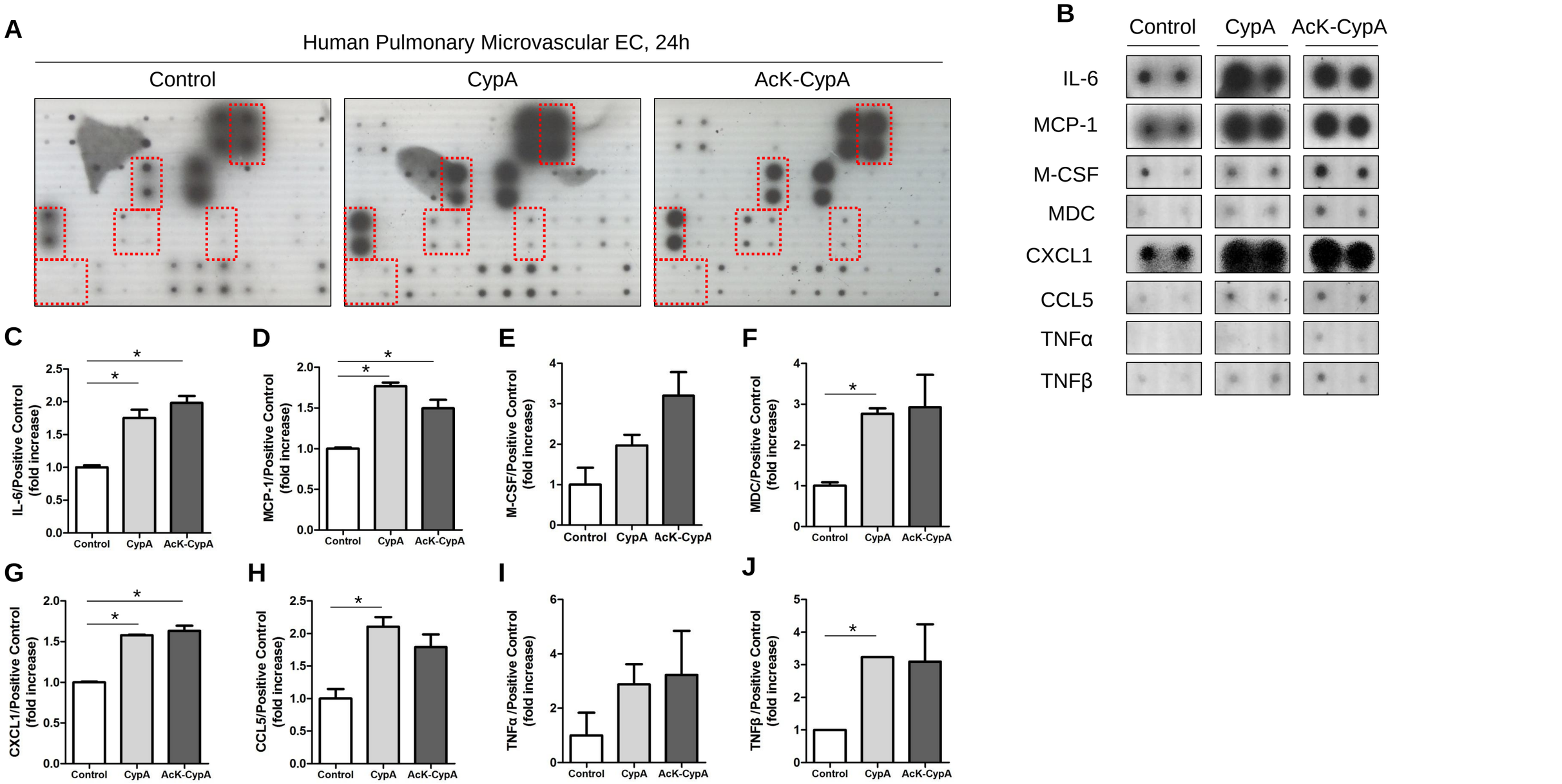


Figure S8. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A,** Representative images of cytokine array using CM of HPMEC after 24 hours of CypA or AcK-CypA treatment. Red boxes indicate the cytokines with increased levels in CypA or AcK-CypA treated CM compared to control CM. **B,** Levels of cytokines that are indicated by red boxes in **A. C-J,** Quantification of the selected cytokines from the cytokine array studies shown in **B**. Data are mean±SEM.

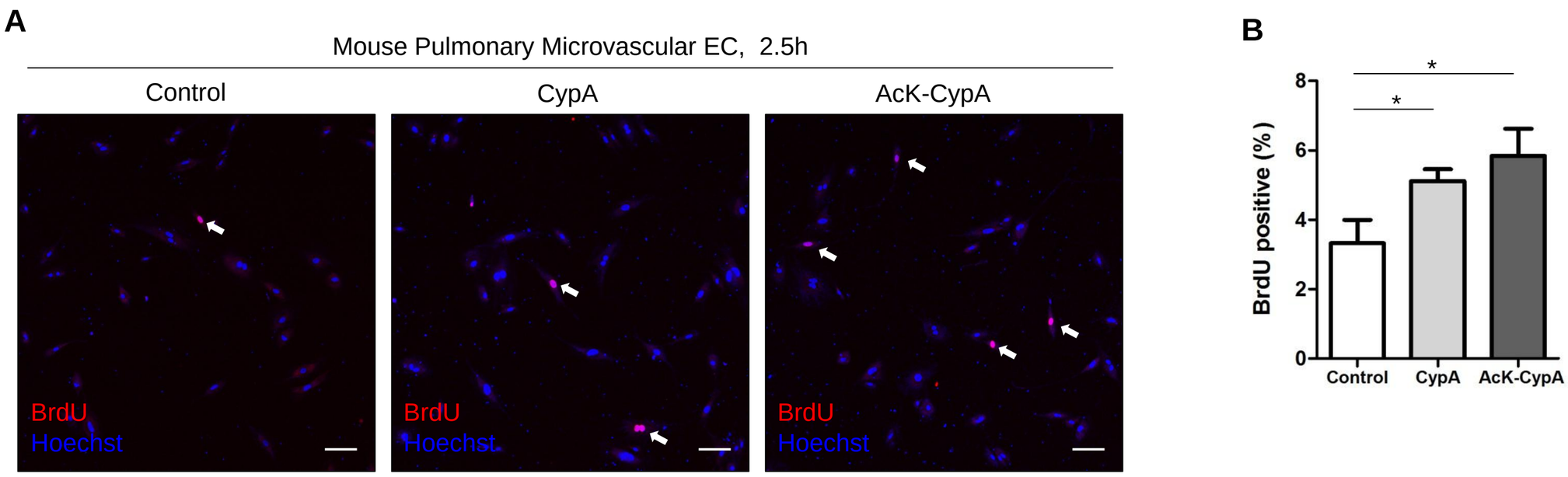


Figure S9. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A,** Representative immunofluoresence image of BrdU staining in MPMEC. Extremely low-density cultures of MPMECs starved in 0% serum medium for 24 hours were followed by CypA or AcK-CypA treatment for 2.5 hours. Scale bar, 100μm. **B,** Quantification of the percentage of BrdU positive cells from **A**. Data are mean±SEM. *P<0.05.

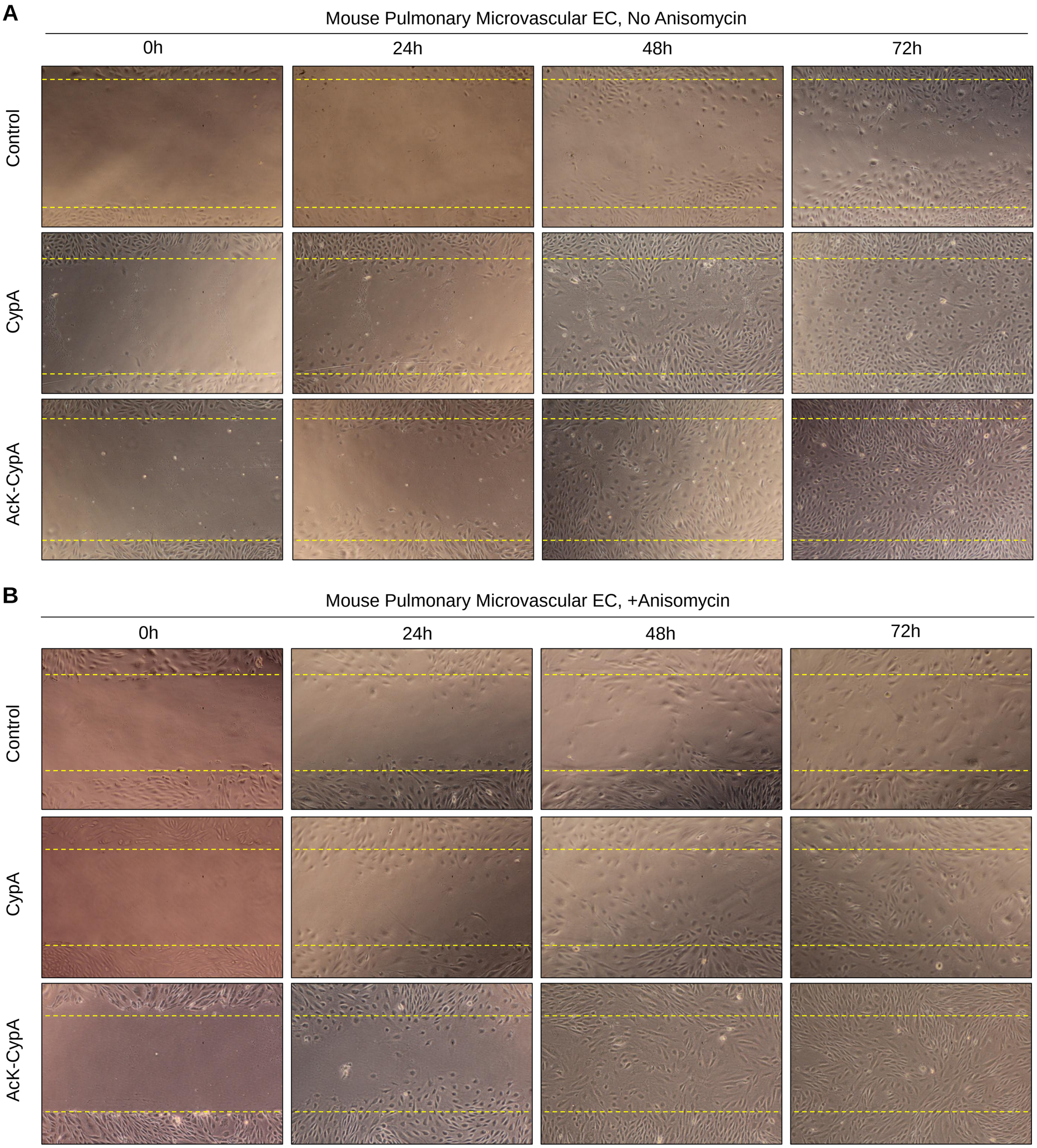
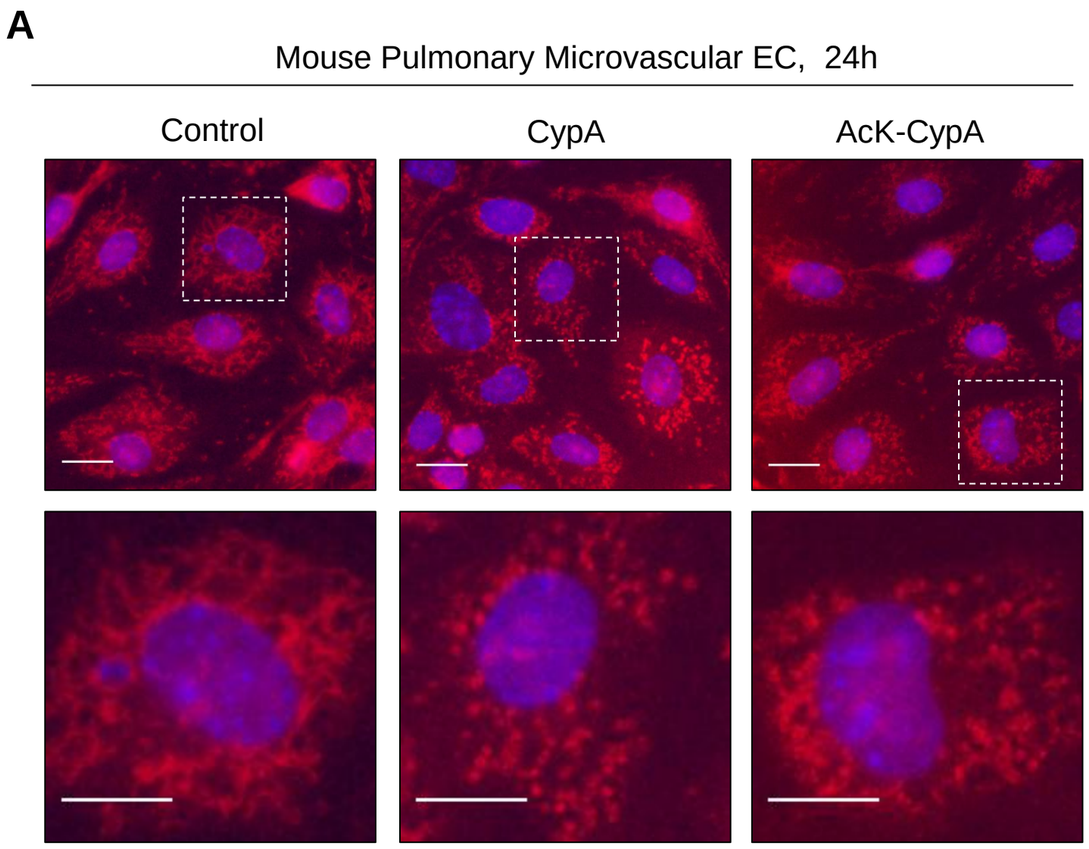
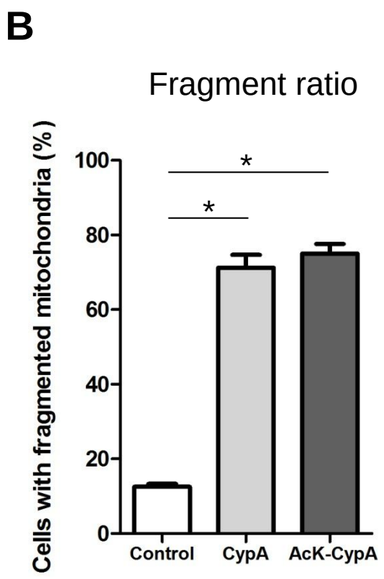
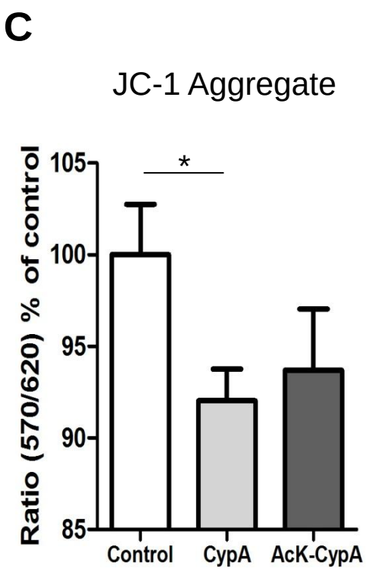
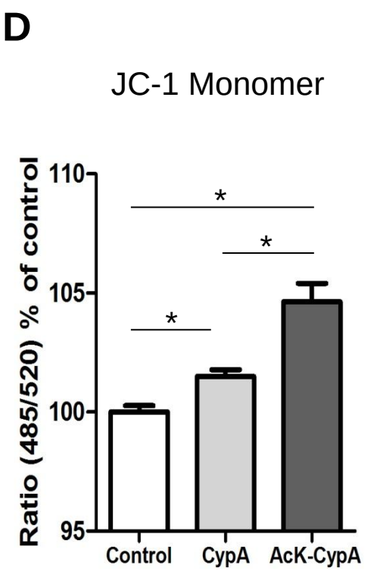


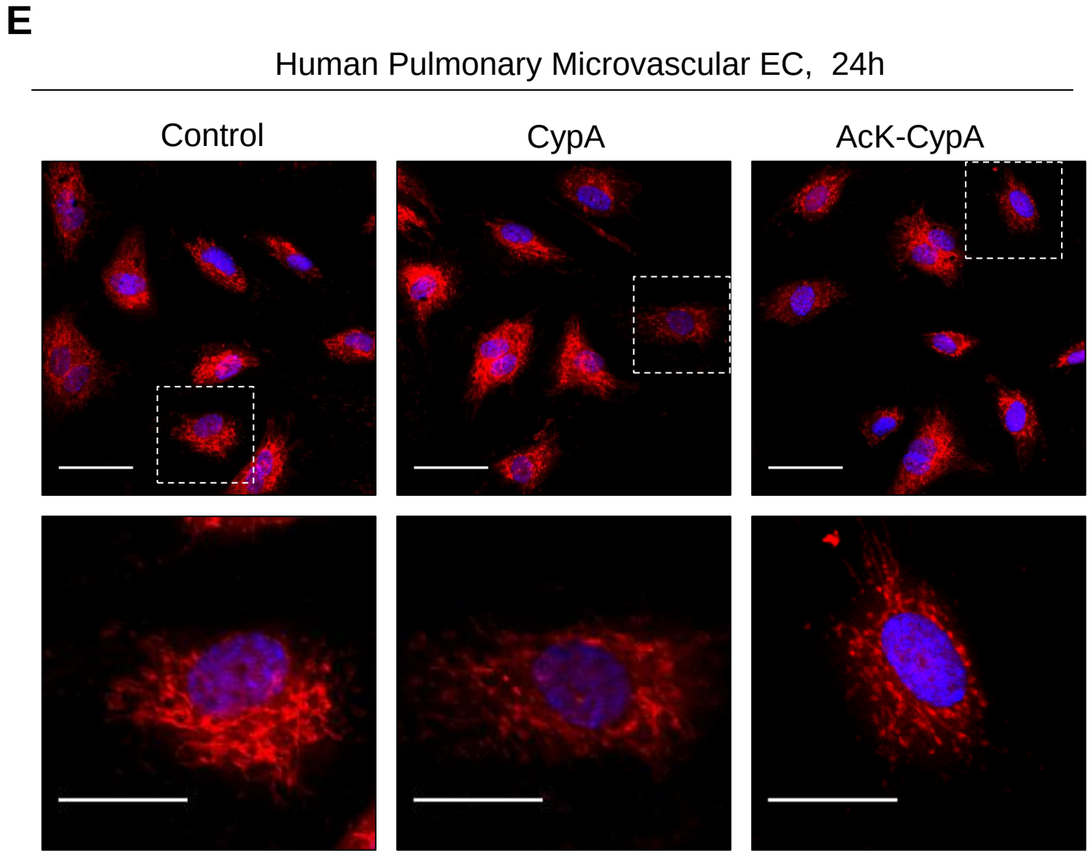
Figure S10. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A-B,** Representative light microscopy images of 72-hour scratch wound assay using MPMEC treated with CypA or AcK-CypA without (**A**) or with (**B**) anisomycin pre-treatment. **C-D,** Quantification of the 72-hour scratch wound assay (represented as percentages of scratch closure) using MPMEC treated with CypA or AcK-CypA without (**C**) or with (**D**) anisomycin pre-treatment.

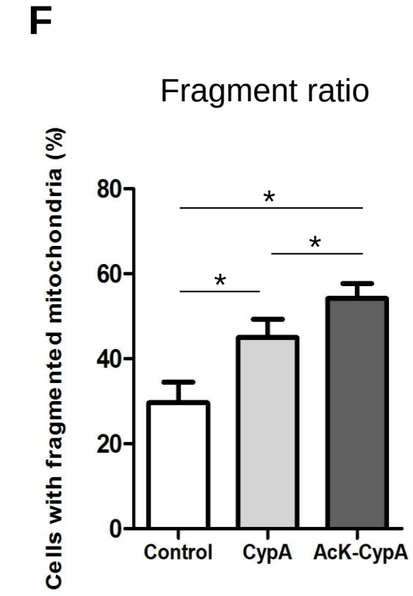


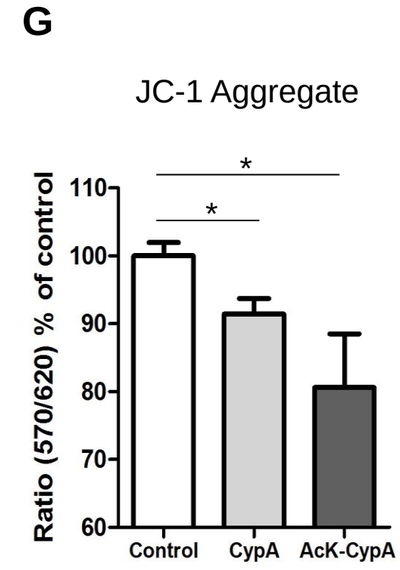












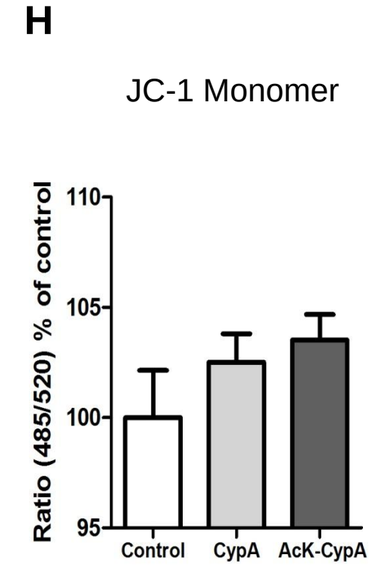


Figure S11. For all experiments CypA and AcK-CypA were used at 50nmol/L. For all experiments CypA and AcK-CypA were used at 50nmol/L. **A,** Representative immunofluoresence images of MPMEC treated with CypA or AcK-CypA and addition of 500nmol/L MitoTracker™ Red CMXRos (Red) and Hoechst (Blue). Scale bar, 15μm (upper panel) and 10μm (lower panel). **B,** Quantitative analysis of the percentage of cells with fragmented mitochondria. Data are mean±SEM. *P<0.05. **C-D,** Quantification of relative fluorescence counts of aggregate(**C**) or monomeric(**D**) form of JC-1 in MPMEC treated with CypA or AcK-CypA for 24 hours. **E,** Representative immunofluoresence images of HPMEC treated with CypA or AcK-CypA and addition of 500nmol/L MitoTracker™ Red CMXRos (Red) and Hoechst (Blue). Scale bar, 50μm (upper panel) and 25μm (lower panel). **F,** Quantitative analysis of the percentage of cells with fragmented mitochondria. Data are mean±SEM. *P<0.05. **G-H,** Quantification of relative fluorescence counts of aggregate(**G**) or monomeric(**H**) form of JC-1 in HPMEC treated with CypA or AcK-CypA for 24 hours.