**SUPPLEMENTAL MATERIAL**

# Second Heart Field-derived Cells Contribute to Angiotensin II-mediated Ascending Aortopathies

Hisashi Sawada[1,2,3], Yuriko Katsumata[4,5], Hideyuki Higashi[6], Chen Zhang[7,8], Yanming Li[7,8], Stephanie Morgan[6], Lang H. Lee[6], Sasha A. Singh[6], Jeff Z. Chen[1,3], Michael K. Franklin[1], Jessica J. Moorleghen[1], Deborah A. Howatt[1], Debra L. Rateri[1], Ying H. Shen[7,8], Scott A. LeMaire[7,8], Masanori Aikawa[6], Mark W. Majesky[9], Hong S. Lu[1,2,3], Alan Daugherty[1,2,3]

1. Saha Cardiovascular Research Center, College of Medicine, University of Kentucky, KY
2. Saha Aortic Center, College of Medicine, University of Kentucky, KY
3. Department of Physiology, College of Medicine, University of Kentucky, KY
4. Department of Biostatistics, College of Public Health, University of Kentucky, KY
5. Sanders-Brown Center on Aging, University of Kentucky, KY
6. Center for Interdisciplinary Cardiovascular Sciences, Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA
7. Division of Cardiothoracic Surgery, Michael E. DeBakey Department of Surgery, Baylor College of Medicine, Houston, TX
8. Department of Cardiovascular Surgery, Texas Heart Institute, Houston, TX
9. Center for Developmental Biology & Regenerative Medicine, Seattle Children's Research Institute, Departments of Pediatrics and Pathology, University of Washington, Seattle, WA

**Running title:** SHF-derived cells and thoracic aortopathy

**Corresponding authors:**
Hisashi Sawada, MD, PhD
Saha Cardiovascular Research Center, University of Kentucky
741 South Limestone Street
BBSRB, B251
Lexington, KY, 40536, USA
Tel: +1-859-218-1705
E-mail: hisashi.sawada@uky.edu

Alan Daugherty, PhD, DSc
Saha Cardiovascular Research Center, University of Kentucky
741 South Limestone Street
BBSRB, B243
Lexington, KY, 40536, USA
Tel: +1-859-323-3512
E-mail: alan.daugherty@uky.edu

## Supplemental Methods

*Mice*

Mice were housed in ventilated cages with negative air pressure (Allentown Inc). Mouse housing conditions are described in **Supplemental Table II**. Briefly, Aspen hardwood chips were used as bedding (#7090A, Envigo). Mice were fed a normal laboratory rodent diet (#2918, Envigo) and provided with drinking water from a reverse osmosis system (pH 6.0 - 6.2) ad libitum. Ambient temperature ranged from 68 to 74°F and humidity was 50 to 60%. The room's light:dark cycle was 14:10 hour. For mice expiring before the study endpoint, necropsy was performed to determine cause of death. Genotypes were confirmed at termination using DNA isolated from tails or brains using Maxwell tail DNA purification kits (#AS1120, Promega) and PCR with primers shown in **Supplemental Table III**.

*Embryonic Study*

To investigate aortic malformation during the prenatal phase, fetuses were harvested from pregnant females on either E11.5 or E12.5. The morning after detection of a vaginal plug in mated females was defined as E0.5 of gestation. Gravid female mice were euthanized using a ketamine/xylazine cocktail (90 and 10 mg/kg, respectively), and saline (8 ml) was perfused into the left ventricle. The abdominal cavity of females was opened and fetuses were dissected free. Gross appearance of fetuses was recorded using a dissection microscope with a high-resolution camera (#SMZ800, #DS-Ri1, Nikon) and cranial tissue was retrieved for genotyping. Embryos were immersed in buffered formalin (10% wt/vol). Twenty-four hours later, chest wall, pericardium, and atriums were removed gently, and gross appearance of the outflow tract was imaged using a dissection microscope coupled to a high-resolution camera. Diameters of the outflow tract were measured at 300-400 μm distal to the aortic root in viable fetuses at termination.

*Aortic Tissue Processing for Western Blot and Histological Analyses*

Aortic tissue was harvested after either 3 days or 4 weeks of infusion for Western blot and histological analyses. Mice were euthanized using a ketamine/xylazine cocktail. The thoracic cavity was cut open and saline (8 ml) was perfused through the left ventricle. For Western blot analysis, aortic samples were harvested and snap-frozen in liquid nitrogen. For image acquisition, a black thin plastic sheet was placed beneath the heart and ascending aorta in situ. Hearts and thoracic aortas were dissected free, and immersed either in paraformaldehyde (PFA, 4% wt/vol) for gross tissue histology or placed in OCT for sectioning. For frozen sectioning, serial cross-sections (10 μm) were collected starting at aortic valves and ending at the innominate artery.

*Histological Analyses*

To detect β-galactosidase activity, whole tissues and fresh frozen sections were fixed with PFA for either 1 hour at 4°C or 10 minutes at room temperature, respectively. PFA-fixed tissues were incubated in buffer containing sodium phosphate (100 mM, pH 7.3), $MgCl_2$ (2 mM), sodium deoxycholate (0.01% wt/vol) and NP40 (0.02% wt/vol). X-gal (1 mg/ml, V394A, Promega), potassium ferricyanide (5 mM), and potassium ferrocyanide (5 mM) were added to buffer and samples were incubated overnight at room

temperature. Whole tissues were subsequently immersed in formalin (10% wt/vol). Tissue sections on slides were rinsed to remove X-gal, and incubated with eosin (1% wt/vol) for 2 minutes, and coverslipped using glycerol gelatin (GG1, MilliporeSigma). Movat's pentachrome and Verhoeff's iron hematoxylin stains were performed to visualize elastin fibers as described previously.[58] For immunostaining, unfixed frozen sections were incubated with acetone for 10 minutes at −20°C. Paraffin-embedded sections (5 μm) were deparaffinized using limonene (#183164, MilliporeSigma). Sections were incubated subsequently with goat serum for 1 hour at 40°C. Rabbit anti-LRP1 (0.5 μg/ml, 15 min, #ab92544, abcam), rabbit anti-α-smooth muscle actin (2 μg/ml, 30 min, #ab5694, abcam), or rabbit anti-PAI1 (for mouse: 0.2 μg/ml, 30 min, #ab222754, abcam; for human: 5 μg/ml, 12 hours, #ab66705, abcam) antibodies were used as primary antibody. Detection of primary antibodies was facilitated with a goat anti rabbit IgG antibody (3 μg/ml, 15 min, BA-1000, Vector) and VECTASTAIN® Elite ABC-HRP Kit (PK-6100, Vector) or ImmPRESS® HRP Goat Anti-Rabbit IgG Polymer Detection Kit (30 min, MP-7451, Vector). ImmPACT AEC (ZH0406, Vector) was used as a chromogen. Isotype matched non-immune rabbit IgG antibody (0.5, 2, or 5 μg/ml, I8140, MilliporeSigma) was used as negative control. Slides were cover slipped with glycerol gelatin (GG1, MilliporeSigma). Antibodies used are described in **Supplemental Table IV**.

Histological images were captured using either Nikon E600 microscope or ZEISS Axio Scan Z1. αSMA or PAI1 positive areas, collagen deposition, and X-gal stained areas were quantified in 40x images (8 bit) using NIS-Elements AR software. Since elastic fibers have autofluorescence illuminated by the FITC (fluorescein isothiocyanate) channel, FITC images of X-gal staining were used to evaluate medial thickening. Medial and adventitial areas were traced using 40x FITC images. X-gal positive area was then assessed in the green channel after subtraction of white color. Elastin fragmentation was defined as the presence of discernable breaks of elastic lamina and counted in the aorta of three serial Movat's stained sections (100 μm apart). Measurements were verified by an independent investigator who was blinded to study groups.

*Western Blot Analyses*

Ascending aortic tissue was harvested from mice and endothelial cells were removed using a cotton swab. Aortic tissues were homogenized in RIPA buffer (#9803, Cell Signaling Technology) with protease inhibitor (#P8340, MilliporeSigma) using Kimble Kontes disposable pellet pestles (#Z359971, DWK Life Science LLC.). For the verification of SHF-specific LRP1 deletion, after the removal of endothelial cells, aortic tissues were incubated with collagenase type I (#SCR13, MilliporeSigma) at 37°C for 12 minutes. Subsequently, adventitia was removed carefully using forceps. Cell lysis buffer (#9803, Cell Signaling Technology) and the protease inhibitor were used for the homogenization. Protein concentrations were determined using DC assay kits (#5000111, Bio-Rad). Equal amounts of protein samples (5 μg) were resolved by SDS-PAGE (10% wt/vol) and transferred electrophoretically to PVDF membranes (#1704273, Bio-Rad). After blocking, antibodies against the following proteins were used to probe membranes: LRP1 (0.4 μg/ml, ab92544, abcam), PAI1 (0.6 μg/ml, ab222754, abcam), and β-actin (1:3000, A5441, MilliporeSigma). Membranes were incubated with either

goat anti-rabbit (0.3 µg/ml, #PI-1000, Vector Laboratories) or goat anti-mouse secondary antibodies (1:3000, #A2554, MilliporeSigma). Immune complexes were visualized by chemiluminescence (#34080, Thermo Scientific) and quantified using a ChemiDoc MP Imaging system (#12003154, Bio-Rad).

*Ultrasonography*
The ascending aorta was imaged in vivo using a Vevo 2100 ultrasound system with a MicroScan MS550 transducer (40 MHz, FUJIFILM VisualSonics Inc) as described previously.[59, 60] Briefly, mice were anesthetized using isoflurane (1.0-2.5% vol/vol) and heart rate was adjusted to 400-550 beats per minute during ultrasonography. Aortic luminal diameter was measured between the inner edge to inner edge of the vessel at the end diastole from three separate heart beats. Aortic measurements were verified by an independent investigator who was blinded to study groups.

*Systolic Blood Pressure Measurements*
Systolic blood pressure was measured by a non-invasive tail cuff system (Coda 8, Kent Scientific) as described previously.[61] Conscious mice were restrained in a holder and put on a heated platform. Blood pressure was measured 20 times at the same time each day for three consecutive days. Data showing <60 or >250 mmHg, standard deviation >30 mmHg, or collected cycles <5 of 20 were excluded.

*Aortic Tissue Proteolysis for Mass Spectrometry Assisted Proteomics*
Aortic tissues were harvested after 3 days of either saline or AngII infusion, and minced before submersion in RIPA buffer (#9806, Cell Signaling Technology) supplemented with protease inhibitor cocktail (#P8340, MilliporeSigma). Tissue pieces were placed in a Precellys CK14 homogenizing tube with RIPA buffer and ceramic beads (1.4 mm; Bertin Instruments). Samples were homogenized using a Precellys 24 tissue homogenizer using three 10 second cycles at 5,000 rpm. Debris were removed by centrifugation for 10 minutes at 4°C and protein concentrations of supernatant samples were measured using the Pierce BCA Protein Assay (#23225, Thermo Fisher). Equal amounts of protein (10 µg) for each aortic segment were processed using the PreOmics iST in solution trypsinization kit (#00027, PreOmics) according to the manufacturer's recommended protocols. The final peptide precipitate was dissolved in sample buffer (40 µl, 5% wt/vol acetonitrile, 0.5% wt/vol formic acid in mass spectrometry grade water).

*Mass Spectrometry (MS)*
Peptides were diluted [1/2] and analyzed using the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) with an Easy-Spray ion source and Easy-nLC1000 HPLC pump (Thermo Fisher Scientific). Peptides were resolved using a dual column set-up: an Acclaim PepMap RSLC C18 trap column, 75 µm x 20 mm; and an EASY-Spray LC heated (45°C) column, 75 µm x 250 mm (Thermo Fisher Scientific). An aqueous to organic gradient (solvent A, 0.1% formic acid in MS-grade water mixed with solvent B, 0.1% formic acid in MS-grade acetonitrile) was generated with a flow rate of 300 nl/min from 5 to 21% solvent B for 75 minutes, 21 to 30% vol/vol solvent B for 15 minutes, followed by ten minutes of a 'jigsaw wash', alternating between 5 and 95%

vol/vol solvent B. The instrument was set to 120 K resolution, and the top N precursor ions in a 3 second cycle time (within a scan range of 400-1500 m/z; isolation window, 1.6 m/z; ion trap scan rate, normal) were subjected to collision induced dissociation (collision energy 30%) for peptide sequencing (or MS/MS). Dynamic exclusion was enabled (60 seconds).

*MS/MS Data Analysis*
The MS/MS data were queried against the mouse UniProt database (downloaded on August 1, 2014) using the SEQUEST search algorithm, via the Proteome Discoverer (PD) Package (version 2.2, Thermo Fisher Scientific), using a 10 ppm tolerance window in the MS1 search space, and a 0.6 Da fragment tolerance window for CID. N-terminal acetylation and methionine oxidation were set as a variable modification, and carbamidomethylation of cysteine residues was set as a fixed modification. In order to quantify peptide precursors detected in the MS1 but not sequenced from sample to sample, we enabled the 'Feature Mapper' node. Chromatographic alignment was done with a maximum retention time (RT) shift of 10 minutes and a mass tolerance of 10 ppm. Feature linking and mapping settings were, RT tolerance minimum of 0 minutes, mass tolerance of 10 ppm and signal-to-noise minimum of five. Precursor peptide abundances were based on their chromatographic intensities and total peptide amount was used for normalization. Peptides assigned to a given protein group, and not present in any other protein group, were considered as unique. Consequently, each protein group is represented by a single master protein (PD Grouping feature). We used unique and razor peptides per protein for quantification.

*Aortic Cell Suspension for Single Cell RNA Sequencing (scRNAseq)*
Ascending aortic samples were harvested from Mef2c-*Cre* ROSA26R$^{mT/mG}$ male mice (n=5) at baseline and after 3 days of AngII infusion (1,000 ng/kg/min, H-1705, Bachem, n=4). Aortic samples were pooled in Hanks' Balanced Salt Solution (HBSS, #14175095, Thermo Fisher Scientific) with fetal bovine serum (10% vol/vol). Periaortic tissues were removed and aortic tissues were cut into small pieces. Aortic samples were subsequently digested with enzyme cocktail (**Supplemental Table V**) in Ca/Mg contained-HBSS (#14025092, Thermo Fisher Scientific) for 60 minutes at 37°C. Cell suspensions were filtered through a 40 μm cell strainer (CLS431750-50EA, MilliporeSigma), centrifuged at 300 g for 10 minutes, and resuspended using cold HBSS (#14175095) with fetal bovine serum (5% vol/vol). Cells were stained with DAPI and sorted to select viable cells (≥ 95% viability) by flow cytometry (FACS Aria III, BD Biosciences). Cells were also sorted based on mTomato and mGFP signals.

*scRNAseq*
mGFP positive cells were dispensed onto the Chromium Controller (10x Genomics) and indexed single cell libraries were constructed by a Chromium Single Cell 3' v3 Reagent Kit (10x Genomics). cDNA libraries were sequenced in a pair-end fashion on an Illumina NovaSeq 6000. Raw FASTQ data were aligned to the Genome Reference Consortium Mouse Build 38 (GRCm38/mm10) reference and gene expressions were quantified using Cell Ranger 3.0 (baseline) or 5.0.1 (AngII).

*Experimental Design*
*Randomization:* Each experimental mouse had a unique number generated by the "RAND" function in Excel, and mice were divided into the study groups in numerical order of the unique number.
*Blinding:* All experimental data were verified by an independent investigator blinded to the study group information. For proteomic analyses, the identities of all samples were blinded to the operator.
*Number of replicates:* All experiments included biological replicates. The number of samples in each experiment is described in each figure legend.
*Others:* For all experiments, control data were acquired concurrently with data in which statistical comparisons were performed.

**Supplemental Table I. Mouse strains**

| Strain | Vendor or Source | Stock # |
|---|---|---|
| ROSA26R$^{LacZ}$ | The Jackson Laboratory | 003474 |
| ROSA26R$^{mT/mG}$ | The Jackson Laboratory | 007676 |
| *Lrp1* floxed | The Jackson Laboratory | 012604 |
| *Tgfbr2* floxed | The Jackson Laboratory | 012603 |
| Wnt1-*Cre* | The Jackson Laboratory | 022501 |
| Mef2c-*Cre* | Mutant Mouse Resource and Research Center | 030262 |

**Supplemental Table II. Mouse housing conditions**

| | Mouse Housing Condition | Note |
|---|---|---|
| Set temperature range | 68-74°F (20-23°C) | |
| Set humidity | 50% | |
| Light cycle (light:dark) | 14:10 hours | |
| Water | Reverse osmosis water | ad libitum |
| Feed | 18% Protein Rodent Diet, #2918, Teklad Irradiated Global (Envigo) | ad libitum |
| Bedding | Aspen hardwood chips, #7090A, Harlan Teklad Global (Envigo) | |
| SPF (Specific pathogen free) | Yes | |

**Supplemental Table III. Primer sequences for genotyping**

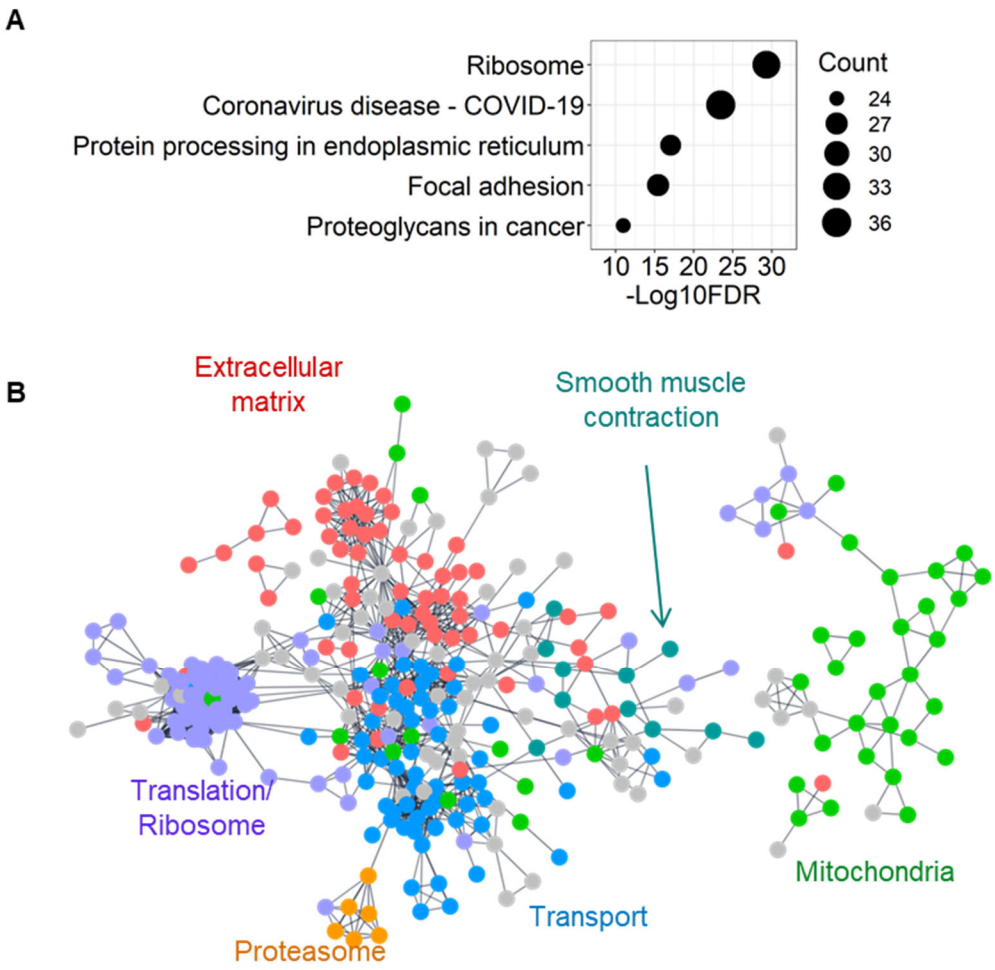
| Gene | | Primer Sequence |
|---|---|---|
| *Cre* | Forward | ACCTGAAGATGTTCGCGATT |
| | Reverse | CGGCATCAACGTTTTCTTTT |
| *IL-2* | Forward | CTAGGCCACAGAATTGAAAGATCT |
| | Reverse | GTAGGTGGAAATTCTAGCATCATCC |
| *Lrp1 flox* | Forward | CATACCCTCTTCAAACCCCTTCCTG |
| | Reverse | GCAAGCTCTCCTGCTCAGACCTGGA |
| *Lrp1 delta flox* | Forward | AAAGAGGCACTAGAGCGCAG |
| | Reverse | CCTCTGGCTGCAAAAATGCAC |
| *Tgfbr2 flox* | Forward | TATGGACTGGCTGCTTTTGTATTC |
| | Reverse | TGGGGATAGAGGTAGAAAGACATA |

**Supplemental Table IV. Antibodies**

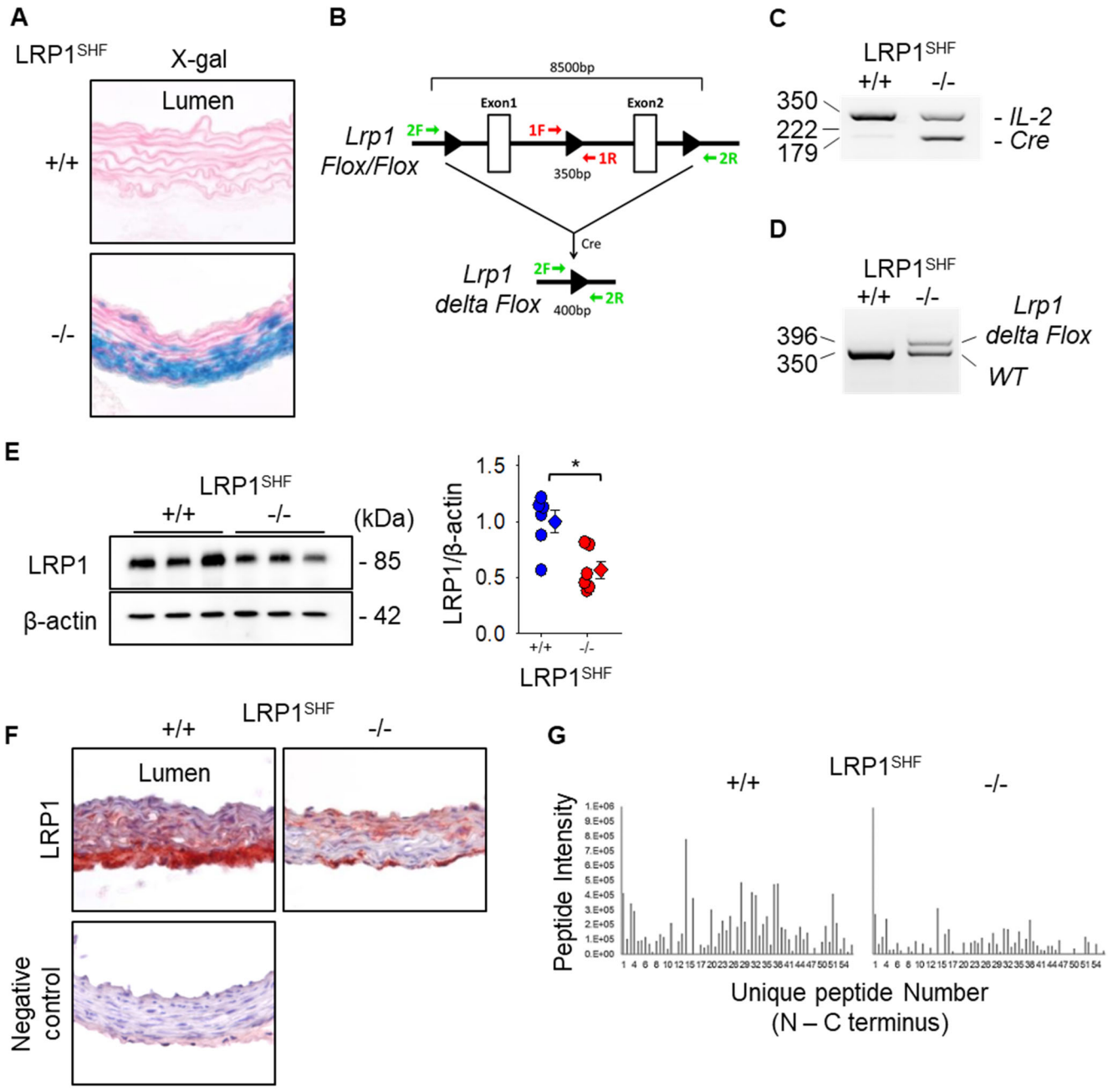
| Target antigen | Vendor | Catalog # | Working Concentration |
|---|---|---|---|
| Rabbit anti LRP1 | abcam | ab92544 | 0.4 μg/ml for Western blot<br>0.5 μg/ml for immunostaining |
| Rabbit anti α-SMA | abcam | ab5694 | 2 μg/ml |
| Rabbit anti PAI1 | abcam | ab222754 | 0.6 μg/ml for Western blot<br>0.2 μg/ml for immunostaining in mouse aortas |
| Rabbit anti PAI1 | abcam | ab66705 | 5 μg/ml for immunostaining in human TAA tissues |
| Mouse anti mouse β-actin | MilliporeSigma | A5441 | 1:3000 |
| Goat anti-rabbit IgG | Vector Laboratories | PI-1000 | 3 μg/ml |
| | | MP-7451 | Ready to use |
| Goat anti-mouse IgG | MilliporeSigma | A2554 | 1:3000 |
| Non-immune rabbit IgG | MilliporeSigma | I8140 | 0.5 μg/ml for LRP1<br>2 μg/ml for α-SMA<br>5 μg/ml for PAI1 |

**Supplemental Table V. Enzyme cocktail for single cell suspension**

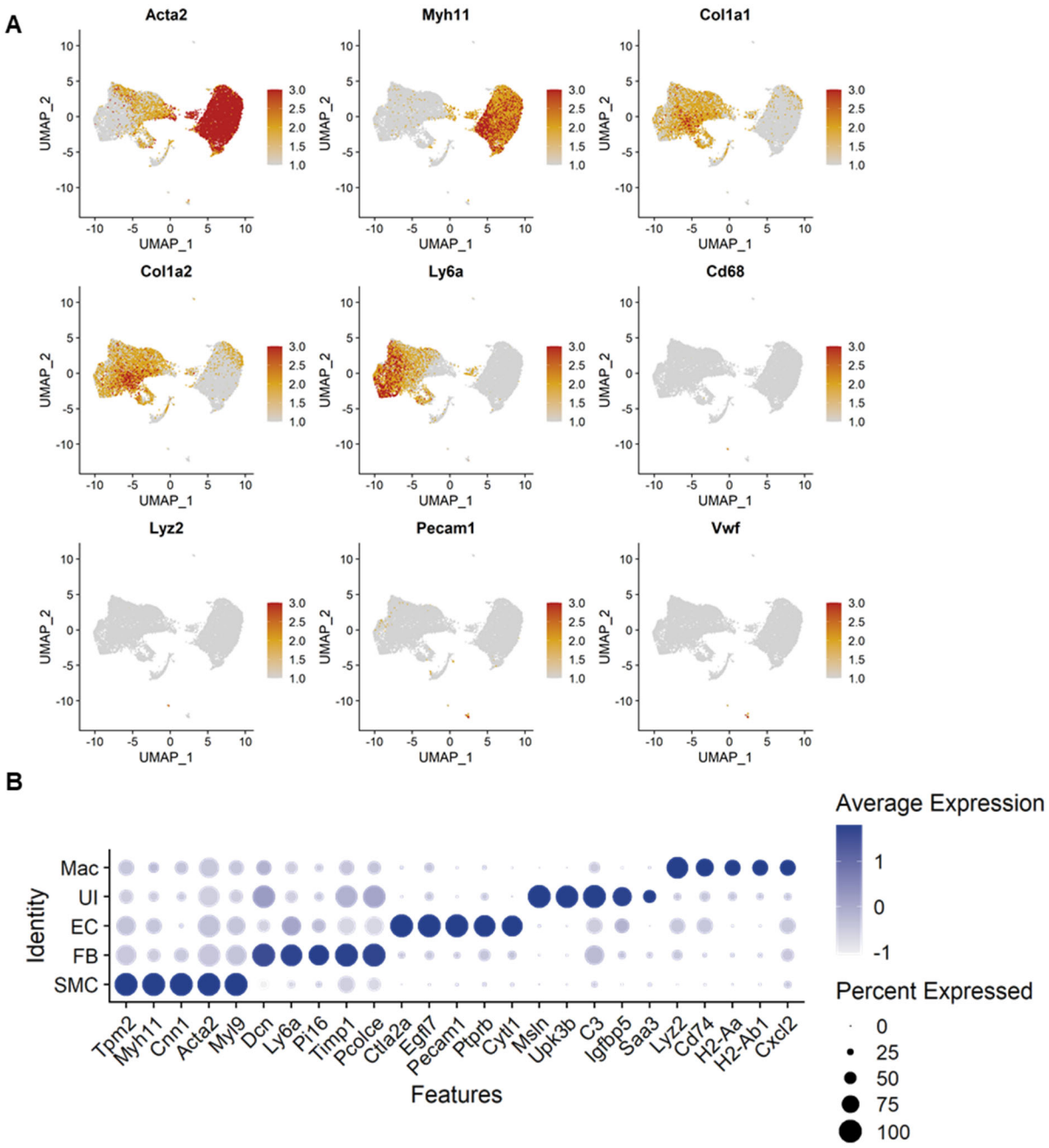
| Reagent | Vendor | Catalog # | Working Concentration |
|---|---|---|---|
| Collagenase type II | Worthington | LS004176 | 3 mg/ml |
| Collagenase type XI | MilliporeSigma | C7657 | 0.15 mg/ml |
| Hyaluronidase type I | MilliporeSigma | H3506 | 0.24 mg/ml |
| Elastase | Worthington | LS002290 | 0.19 mg/ml |
| HEPES | MilliporeSigma | H4034 | 2.38 mg/ml |



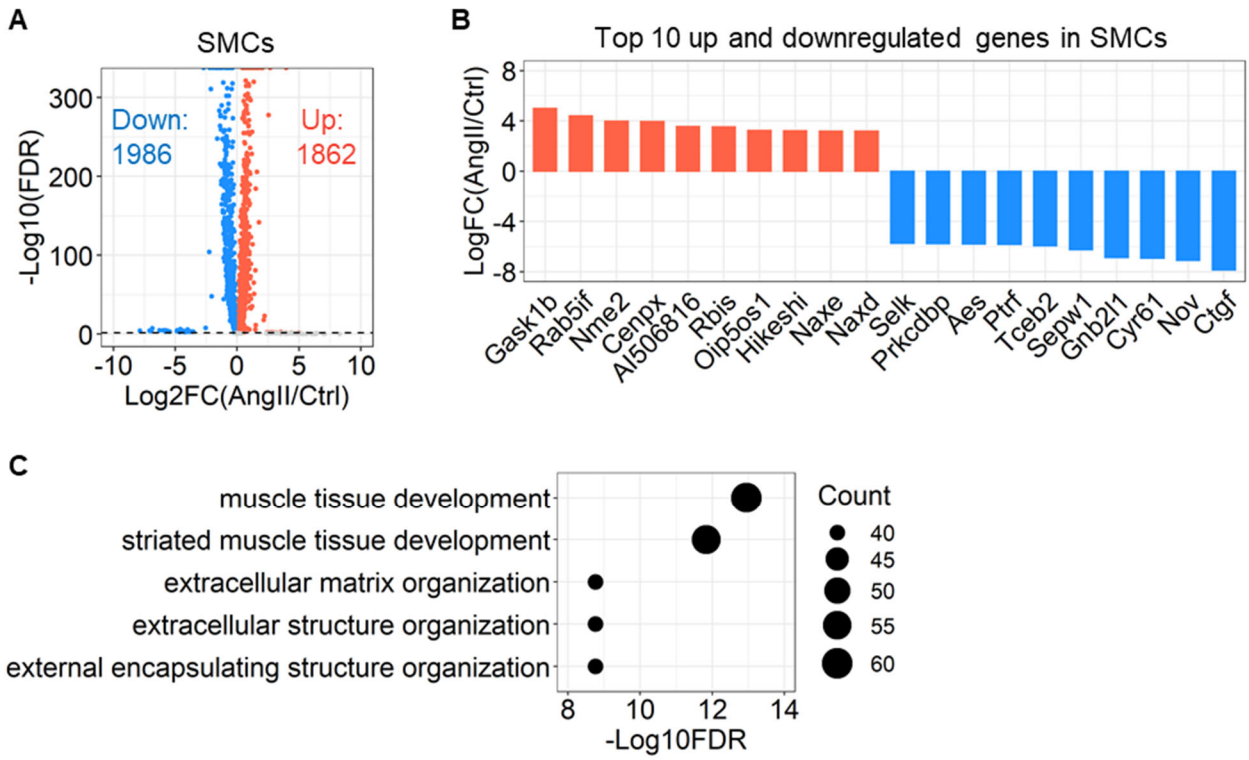

**Supplemental Figure I. Characteristics of DEGs in the ascending aorta of AngII-infused mice. (A)** Top 5 significant annotations in enrichment analysis for KEGG pathway of altered molecules detected by the proteomics analysis. **(B)** Protein-protein interaction of 596 altered proteins. Disconnected nodes are not shown.



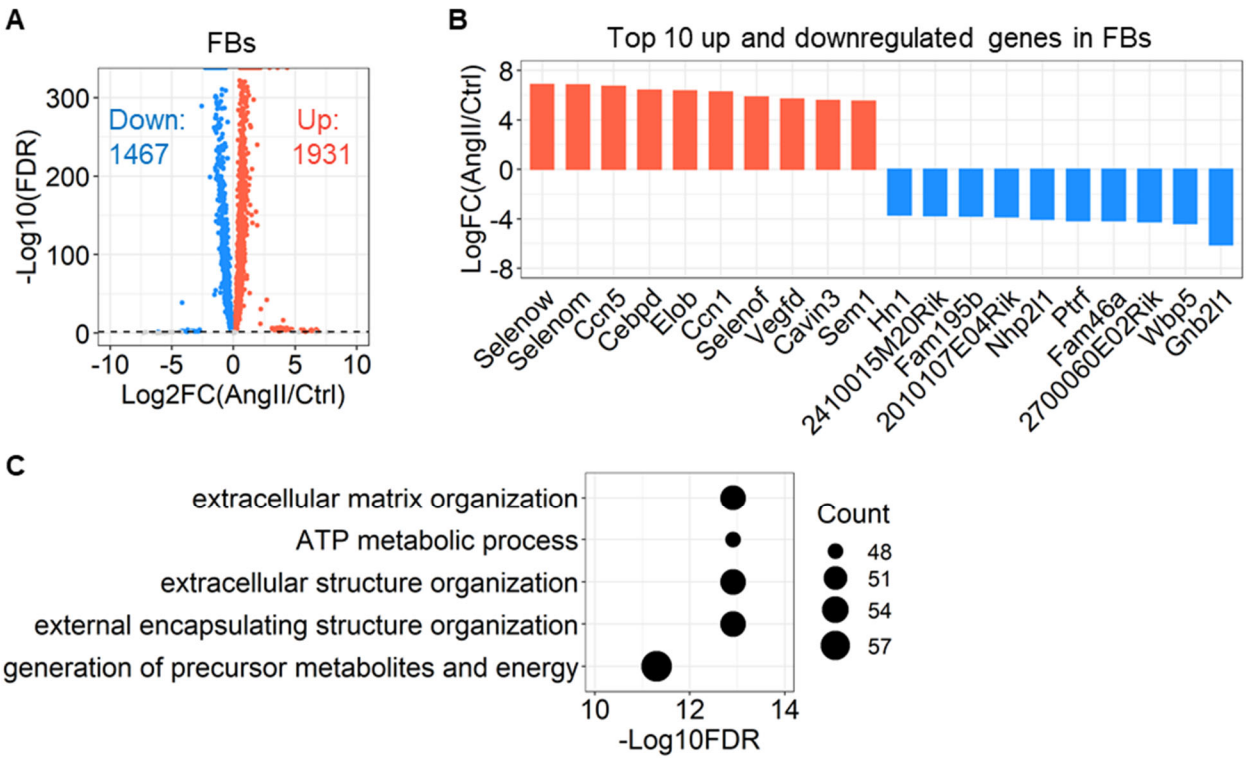

**Supplemental Figure II. Verification of LRP1 deletion in SHF-derived cells in Mef2c-Cre +/0 *Lrp1* floxed mice.** Aortic tissues were harvested from SHF-specific LRP1-deleted mice and wild type littermates at 10 to 12 weeks of age (male, n=3 to 6 per group). **(A)** Representative aortic images of X-gal staining. **(B)** Primer design for PCR of *Lrp1 Flox* sequence. Red and green primers were used for PCR. Representative PCR results of **(C)** *Cre* and **(D)** *Lrp1 delta Flox* sequences. **(E)** Western blot for LRP1 and β-actin and **(F)** immunostaining for LRP1 in the ascending aorta of wild type or SHF-specific LRP1 deleted mice. As negative control, sections were incubated with non-immune rabbit IgG antibody. *P=0.006 by two-tailed Student's t-test. **(G)** Normalized peptide intensities of LRP1 were exported from Proteome Discoverer 2.2. Peptide intensities were plotted from N to C-terminus with the numbering conserved in the ascending aorta of SHF-specific LRP1-deleted mice and wild type littermates (n=3 per group).



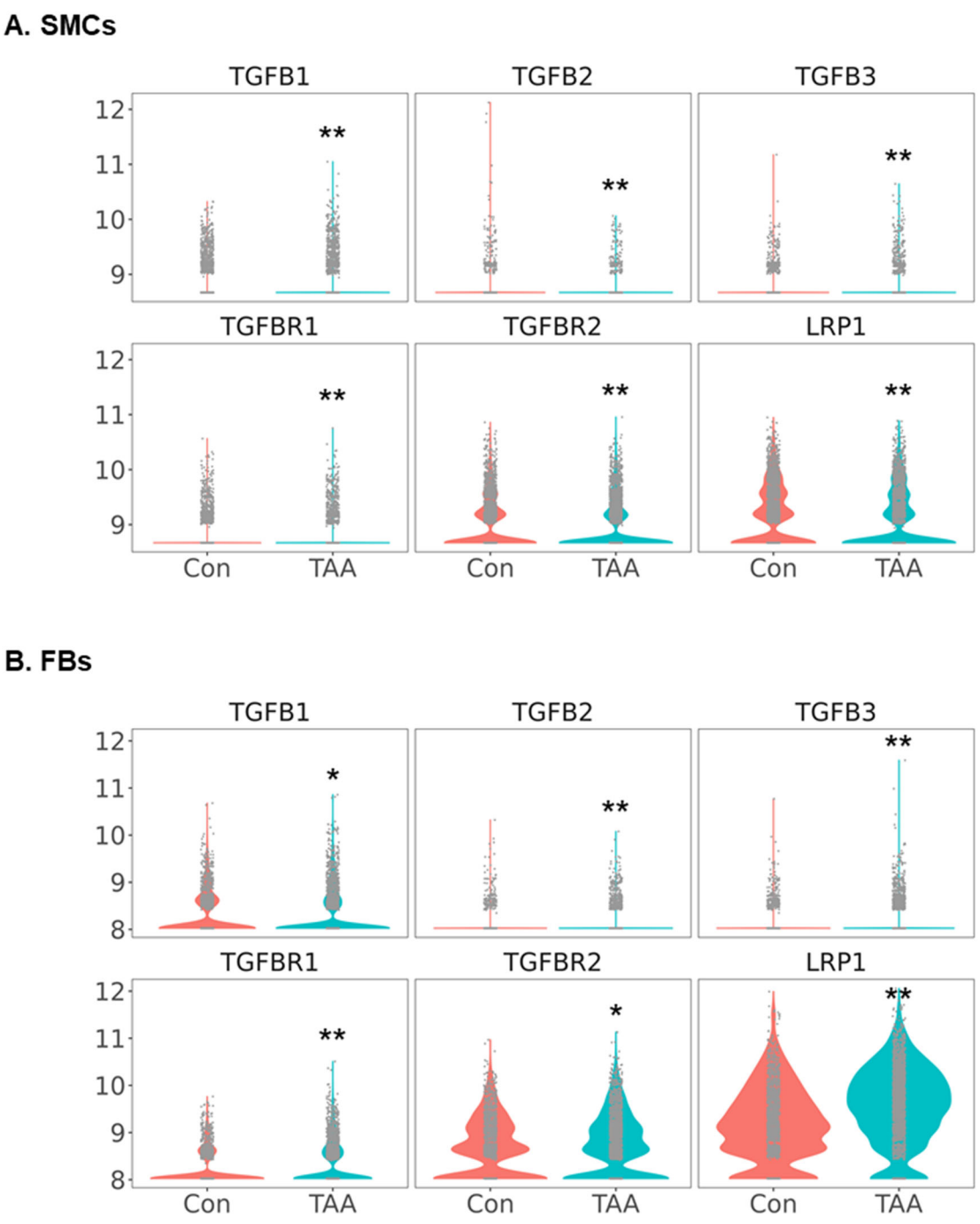

**Supplemental Figure III. scRNAseq identified multiple cell type clusters in mouse aortas at baseline and after 3 days of AngII infusion. (A)** Featured plots for cell marker genes in the UMAP plot. **(B)** Dot plots for highly abundance genes in each cell type cluster. SMC indicates smooth muscle cell; FB, fibroblast; EC, endothelial cell; UI, unidentified cell; Mac, macrophage.



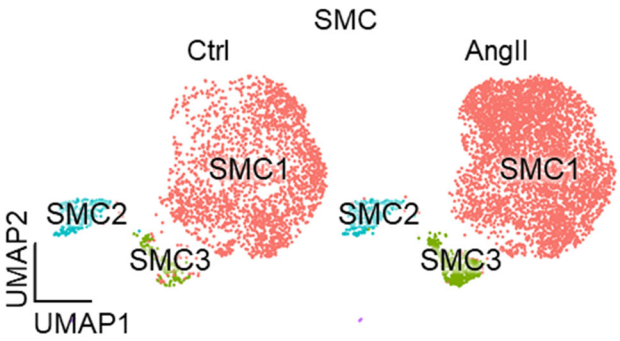

**Supplemental Figure IV. Transcriptomic alteration by AngII infusion in SHF-derived SMCs. (A)** Volcano plot of DEG analysis, **(B)** top 10 altered genes by AngII infusion, and **(C)** top 5 terms of gene ontology enrichment analysis in the SMC cluster.




**Supplemental Figure V. Transcriptomic alteration by AngII infusion in SHF-derived FBs. (A)** Volcano plot of DEG analysis, **(B)** top 10 altered genes by AngII infusion, and **(C)** top 5 terms of gene ontology enrichment analysis in the FB cluster.



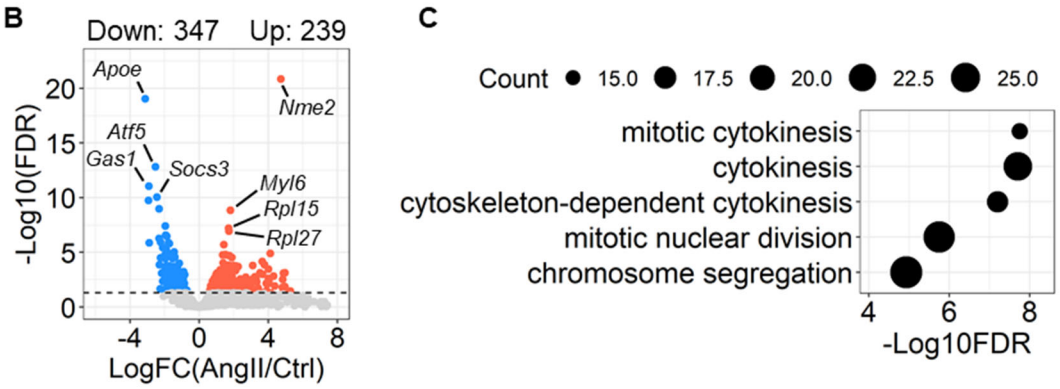

**Supplemental Figure VI. Transcriptomic alteration of TGFβ ligands and receptors in SMCs and FBs of TAAs.** Violin plots for TGFβ ligands and receptors in **(A)** SMCs and **(B)** FBs of control and aneurysmal ascending aortas. scRNAseq data were obtained from the GEO (GSE155468). *P<0.001, **P<0.0001 analyzed using the Hurdle model adjested for age implemented.



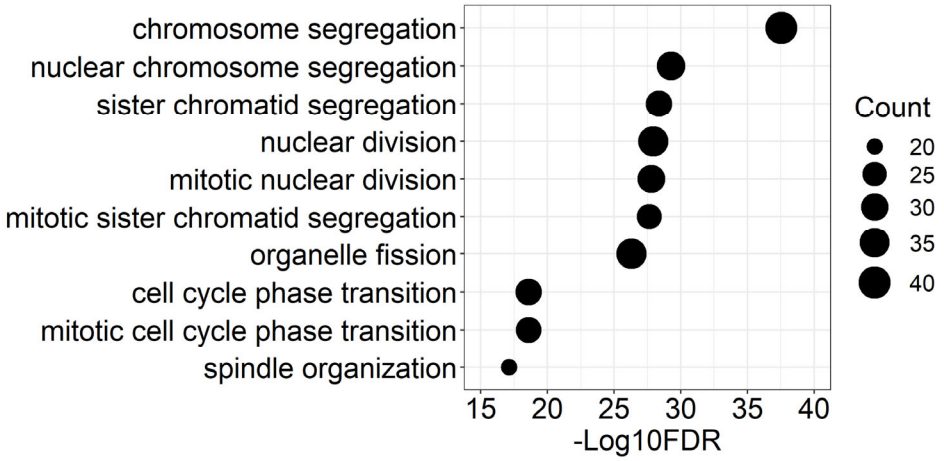

**Supplemental Figure VII. Infusion of AngII did not alter transcriptomic distributions of SMC sub-clusters.** UMAP plots at baseline (Ctrl) and after 3 days of AngII infusion in SHF-derived SMCs.

A

| | Ctrl (%) | AngII (%) |
|---|---|---|
| FB1 | 1765 (31) | 1290 (33) |
| FB2 | 3462 (61) | 2062 (52) |
| FB3 | 448 (8) | 58 (1) |
| FB4 | 10 (0.2) | 544 (14) |
| Total | 5685 (100) | 3954 (100) |




**Supplemental Figure VIII. Impact of AngII infusion on the transcriptome of FB4 sub-cluster. (A)** Number of cells in each FB sub-cluster. **(B)** A volcano plot for DEGs between Ctrl and AngII in FB4 sub-cluster. **(C)** Top 5 significant annotations in enrichment analysis for gene ontology (biological process) using the DEGs in comparing between Ctrl and AngII in FB4 sub-cluster.




**Supplemental Figure IX. The feature of FB4 sub-cluster.** Enrichment analysis for biological process using feature genes expressing in fibroblasts of FB4 sub-cluster.