Online Supplemental Methods

Functional Role of Second Heart Field-derived Cells in Thoracic Aortopathies

Hisashi Sawada[1], Hideyuki Higashi[2], Debra L. Rateri[1],
Jeff Z. Chen[1, 3], Jessica J. Moorleghen[1], Deborah A. Howatt[1], Hong S. Lu[1, 3],
Stephanie Morgan[2], Lang H. Lee[2], Sasha A. Singh[2],
Masanori Aikawa[2], Mark W. Majesky[4], Alan Daugherty[1,3]

1. Saha Cardiovascular Research Center, University of Kentucky, KY
2. Center for Interdisciplinary Cardiovascular Sciences, Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA
3. Department of Physiology, University of Kentucky, KY
4. 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 impact TAA

Corresponding author:
Alan Daugherty
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

Methods

All raw data and analytical methods are available from the corresponding author upon appropriate request.

Mice

The following mice were purchased from The Jackson Laboratory **(Table I)**: ROSA26R^{LacZ} (#003474), *Lrp1* floxed (#012604), *Tgfbr2* floxed (#012603), Wnt1-*Cre* [#022501 (C57BL/6J background, also known as B6-Wnt1-*Cre*2), #022137 (Wnt1-*Cre*2 on a 129S4 background)]. Mef2c-*Cre* mice (#030262) were purchased from the Mutant Mouse Resource and Research Center. For cell tracking studies of the cardiac neural crest (CNC) and second heart field (SHF), either Wnt1-*Cre* (#022501) or Mef2c-*Cre* male mice were bred to ROSA26R^{LacZ} female mice, respectively. To delete LRP1 in CNC-derived cells, Wnt1-*Cre* mice were bred with *Lrp1* floxed female mice. Embryonic lethality, a phenotype of LRP1 deletion in CNC-derived cells, was confirmed using 129S4 background Wnt1-*Cre* strain. Embryos with CNC-specific LRP1-deletion were harvested from *Lrp1* floxed female mice bred with Wnt1-*Cre* (#22137) males. To delete LRP1 in SHF-derived cells, Mef2c-*Cre* male mice were crossbred to *Lrp1* floxed female. Wnt1-*Cre* and Mef2c-*Cre* male mice were used for lineage-specific deletion of TGFBR2 in CNC and SHF-derived cells, respectively. Because of low incidence of AngII-induced TAA in female mice, only male mice were studied (1). Mice were housed in ventilated cages with negative air pressure (Allentown Inc). Mouse housing conditions are described in **Table II**. Briefly, Aspen hardwood chips were used as bedding (#7090A, Harlan Teklad Global). Mice were fed a normal laboratory rodent diet (#2918, Harlan

Teklad Global) 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 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 **Table III**. All procedures were approved by the University of Kentucky's IACUC.

Pump implantation

Saline or AngII (1,000 ng/kg/min, H-1705, Bachem) was infused via a subcutaneously implanted osmotic pump (either Alzet model 2001 for 3 days for experiments using aortic tissues prior to overt pathology, or model 2004 for 28 days, Durect) into male mice at 10 - 14 weeks of age, as described previously (2). Surgical staples were used to close incision sites and were removed 7 days after surgery. Post-operative pain was alleviated by application of topical lidocaine cream (4% wt/wt, LMX4, #0496-0882-15, Eloquest Healthcare, Inc).

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. Female breeders 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 female

breeders was opened and fetuses were dissected free. Gross appearance of fetuses were 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 of the root in fetuses that survived at the termination.

Aortic Tissue Processing

Aortic tissue was harvested at either 3 days of infusion for proteomics analyses or at 4 weeks of infusion for other experiments. Mice were euthanized using a ketamine/xylazine cocktail. The thoracic cavity was cut open and saline (8 ml) was perfused through the left ventricle. The following organs and tissues were gently removed: adipose, adrenal gland, bladder, bone, brain, esophagus, kidney, intestine, liver, lung, muscle, pancreas, skin, superior mesenteric artery, spleen, stomach, testis, thymus, and trachea. For gross specimen photography, 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 a mold of OCT for frozen 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 (10 µm) were fixed with PFA for either 1 hour at 4°C or 10 minutes at room temperature, respectively. PFA-fixed tissues were preincubated 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 post-fixed with buffered formalin (10% wt/vol). Tissue sections on slides were rinsed free of X-gal, and incubated with eosin (1% wt/vol) for 2 minutes, and cover slipped using glycerol gelatin (GG1, Sigma-Aldrich). Elastin fibers were visualized by illuminating X-gal stained sections using a FITC channel.

Movat's pentachrome and Verhoeff's iron hematoxylin stains were performed to visualize elastin fibers as described previously (3). Hematoxylin and eosin stain was performed to examine structure of the outflow tract in mouse fetuses.

For immunostaining, fresh frozen sections (10 µm) were fixed with acetone for 10 minutes at −20 °C. Sections were incubated subsequently with goat serum for 1 hour at 40 °C. LRP1 antibody (0.5 µg/ml, #92544, abcam, **Table IV**) was used as primary antibody. Detection of primary antibodies was visualized with a goat anti rabbit IgG antibody (3 ng/ml, BA-1000, Vector). Isotype matched non-immune rabbit IgG antibody (0.5 µg/ml, I8140, Sigma-Aldrich) was used as negative control. Slides were cover slipped with glycerol gelatin (GG1, Sigma-Aldrich). Images were obtained with a Nikon E600 microscope and analyses were performed using NIS-Elements AR software.

Western Blot Analyses

Ascending aortic tissue was harvested from mice and endothelial cells were removed using a cotton swab. Aortic tissues were incubated with collagenase type I (#SCR13, Sigma-Aldrich) at 37 °C for 12 minutes. Subsequently, adventitia was removed carefully using forceps. Aortic tissues were homogenized in cell lysis buffer (#9803, Cell Signaling Technology) and protease inhibitor (#P8340, Sigma-Aldrich) using Kimble Kontes disposable Pellet Pestles (#Z359971, DWK Life Science LLC.,). Protein concentrations were measured using DC assay kits (#5000111, Bio-Rad). Equal amounts of protein samples (3 µg) were resolved by SDS-PAGE (10% wt/vol) and transferred electrophoretically to PVDF membranes. After blocking, antibodies against the following proteins were used to probe membranes: LRP1 (0.4 µg/ml, ab92544, abcam) and β-actin (1:1000, A5441, Sigma-Aldrich). Membranes were incubated with either goat anti-rabbit (1 µg/ml, #PI-1000, Vector Laboratories) or goat anti-mouse secondary antibodies (1:1000, #A2554, Sigma-Aldrich) Immune complexes were visualized by chemiluminescence (#34080, Thermo Scientific) and quantified using a Thermo Scientific myECL Imager.

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 (4, 5). Briefly, mice were anesthetized using isoflurane (1.0 - 2.5% vol/vol) and heart rate was adjusted between 400 - 550 beats per minutes during

ultrasonography. Aortic luminal diameter was measured at the end diastole in three separate heart beats between the inner edge to inner edge.

Systolic blood pressure measurements

Systolic blood pressure was measured by a non-invasive tail cuff system (#20269, Kent Scientific) as described previously (6). 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, SD > 30 mmHg, or available cycles < 5 of 20 were excluded.

Mass spectrometry assisted proteomics

Mouse aortic tissue proteolysis: Aortic tissues harvested after 3 days of either saline or AngII infusion were minced before submersion in RIPA buffer (#9806, Cell Signaling Technology) supplemented with protease inhibitor cocktail (#P8340, Sigma-Aldrich). Tissue pieces were placed in a Precellys CK14 homogenizing tube with RIPA buffer and ceramic beads (1.4 mm; Bertin Instruments). Samples were homogenized on 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 manufacturer's recommended protocols. The final peptide precipitate was

dissolved in sample buffer (40 µl, 5% acetonitrile, 0.5% formic acid in mass spectrometry grade water).

Mass spectrometry (MS): Data-dependent acquisition (DDA, unbiased peptide sampling) - the peptides were diluted [1/2] and analyzed using the Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) fronted with an Easy-Spray ion source, and coupled to an Easy-nLC1000 HPLC pump (Thermo Fisher Scientific). The peptides were separated 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 modifications, 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.

Experimental design

Randomization: Each experimental mouse had a unique number generated by the RAND function in Excel, and mice are 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 include biological replicates. The number of samples in each experiment is described in each figure legend. For all experiments, control data were acquired concurrently with data in which statistical comparisons were performed.

Statistics:

Statistical analyses were performed using SigmaPlot version 14.0 (SYSTAT Software Inc.). Normality and equal variance were assessed by Shapiro-Wilk test and Brown-Forsythe test, respectively. For data comparison between two groups of equivalent variance and normal distribution, two-sided Student's t-test was performed. To compare multiple groups that passed normality and equal variance tests, two Way ANOVA with Holm-Sidak test was used. Kruskal-Wallis followed by Dunn's test was used for data that failed normality or equal variance test. Logrank test was used to compare the survival rate. Mice died due abdominal aortic rupture were excluded. $P < 0.05$ was considered statistically significant.

Statistical analyses for proteomics data were performed by Qlucore Omics Explorer 3.5 (Qlucore). For a principal component analysis (PCA) plot and heat maps, proteins were filtered by their q-value of two-group or multiple group comparisons. The q-value was calculated using Qlucore and $q = 0.1$ was used as a threshold. Protein clustering in the PCA plot was performed by k-means clustering in Qlucore.

Protein-protein interaction networks were constructed by the STRING database.(7) Interactions were acquired using the following thresholds (confidence interaction scores ≥ 150, active interaction sources = text mining, experiments, databases, co-expression, neighborhood, gene fusion, co-occurrence). For the interaction of LRP1 in Supplemental Figure 1b, the STRING program was launched using detected 42 proteins with LRP1. For the interaction of TGFBR2 in Fig 2h, the STRING program was performed using detected 7 proteins and genes related to TGF-β signaling pathway which were cited from the KEGG PATHWAY Database (#04350).

Protein interaction figures were modified using Photoshop CC 2019 (Adobe Systems Incorporated).

Table I. Mouse Strains

Strain	Company	Stock #
ROSA26R^{LacZ}	The Jackson Laboratory	#003474
Lrp1 floxed	The Jackson Laboratory	#012604
Tgfbr2 floxed	The Jackson Laboratory	#012603
Wnt1-*Cre* (C57BL/6J)	The Jackson Laboratory	#022501
Wnt1-*Cre* (129S4)	The Jackson Laboratory	#022137
Mef2c-*Cre*	Mutant Mouse Resource and Research Center	#030262

Table II. Mouse Housing Conditions

	Mouse Housing Conditions	Note
Set temperature range	68 - 74°F (20 - 23°C)	
Set humidity	50%	
Light cycle (light:dark)	14:10 hours	
Water	RO Water	ad libitum
Feed	18% Protein Rodent Diet, # 2918, Teklad Irradiated Global	ad libitum
Bedding	Aspen hardwood chips, #7090A, Harlan Teklad Global	
SPF	(+)	

Table III. Primer Sequences for Genotyping PCR

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

Table IV. Antibodies

Target antigen	Vendor	Catalog #	Working Concentrations
Rabbit LRP1	abcam	Ab92544	0.4 µg/ml for Western blot 0.5 µg/ml for Immunostaining
Mouse β-actin	Sigma-Aldrich	A5441	1:1000
Goat anti-rabbit IgG	Vector	PI-1000	1 µg/ml
Goat anti-mouse IgG	Sigma-Aldrich	A2554	1:1000
Non-immune rabbit IgG	Sigma-Aldrich	I8140	0.5 µg/ml

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