CONCISE COMMUNICATION

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

Current affiliation:
HH – New Drug Research Laboratories, Kowa Company, Ltd., Tokyo, Japan
SM – Anokion, Cambridge, MA
LHL – Novartis Institute for Biomedical Research, Cambridge, MA

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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

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The following authors have declared that no conflict of interest exists: HS, DLR, JZC, JJM, DAH, HSL, SAS, MA, MWM, AD. HH is an employee of Kowa Company, Ltd. (Nagoya, Japan) and was a visiting scientist at Brigham and Women's Hospital when the study was conducted.

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Abstract

The ascending aorta is a common location for thoracic aortopathies. Pathology predominates in the aortic media with disease severity being most apparent in outer laminar layers. In the ascending aorta, smooth muscle cells are derived from two different embryonic origins: cardiac neural crest and second heart field. Cells of these two origins have distinct distributions, and the localization of second heart field is coincident with the regional specificity of some forms of thoracic aortopathies. However, the role of second heart field-derived cells in the pathophysiology of thoracic aortopathies has been unclear. This study demonstrated a functional role of second heart field-derived smooth muscle cells using angiotensin II-induced aortopathy in mice. Unbiased proteomic analyses discerned potential roles of low density lipoprotein receptor-related protein 1 and TGF-β pathways on the aortic integrity. Functional ablation of either these pathways in cells derived from the second heart field augmented aortopathies including aneurysm and rupture in the ascending region. These results indicated a functional role of cells derived from the second heart field on the integrity of the aortic wall.

Introduction

Thoracic aortopathies are a spectrum of diseases associated with aneurysms, dissection, and rupture (1, 2). Despite the devastating consequences of these diseases, current medical therapy has limited efficacy. Therefore, there is an urgent need to investigate mechanisms of thoracic aortopathies to facilitate development of novel therapeutics.

Aortopathies can occur throughout the thoracic aorta with the ascending region being a frequent site for aortopathy formation (3, 4). Medial thickening is a histological feature of thoracic aortopathies and this pathology shows a gradient across the media that are detected predominantly in outer laminar layers (5-10). Therefore, thoracic aortopathies have regional and transmural specificities with outer medial layers in the ascending aorta being a disease-prone location. However, the mechanism by which aortopathy is restricted in the outer layers of the ascending aorta remains unclear.

The major cellular component of the aortic media is smooth muscle cells (SMCs) that exerts a pivotal role in the pathogenesis of thoracic aortopathies (11). SMCs in the ascending aorta are derived from two different embryonic origins: cardiac neural crest (CNC) and second heart field (SHF) (12). Although there is no mode of grossly discerning the origin of aortic SMCs, lineage tracking studies demonstrated that CNC- and SHF-derived SMCs occupy inner and outer layers, respectively, of the ascending aorta (13). The region specific distribution of thoracic aortopathies appears to coincide with the distribution of SMCs from the SHF rather than CNC. Therefore, differences of embryonic origins may lead to functional differences that have pathological consequences. However, there is scant evidence to test this hypothesis. In this study, we determined whether SMCs derived from the SHF are colocalized with the evolution

of aortic pathology produced by chronic angiotensin II (AngII) infusion (6, 14, 15), and investigated the molecular basis for the regional specificity of thoracic aortopathies using a proteomic approach.

Results and Discussion

Region-specific alteration of aortic protein profile by AngII.

Mass spectrometry-assisted proteomics was performed to investigate mechanisms driving regional specificity of TAAs. AngII was used to induce TAAs in mice, (14) and aortic tissues were harvested prior to overt pathology. Saline infusion and the aneurysm-resistant descending aorta were included as negative controls. Proteomics analysis identified 2644 proteins among all aortic samples. In principal component analyses using the unfiltered proteome, protein profiles of ascending and descending aortas in saline-infused mice were not significantly different. However, AngII infusion altered these profiles compared to saline infusion, and the profiles were significantly different between the ascending and descending thoracic regions (40% variation along PC1, **Figure 1A**). In the ascending aorta, AngII infusion differentially regulated 864 protein abundances compared to the saline-infused group (**Figure 1B**). Within AngII-infused mice, abundances of 77 proteins were different between ascending and descending aortas (**Figure 1B**). Forty two proteins were common between these two subsets, indicating that these molecules may be specific targets for TAA formation (**Figure 1B, C**). Accumulating literature supports that low density lipoprotein receptor-related protein 1 (LRP1), a multi-functional protein expressed in SMCs, contributes to aortic wall integrity.(16-18) Thus, protein-protein interaction of LRP1 and 42 overlapped molecules were investigated. LRP1 interacted with 10 proteins including several LRP1 ligands: plasminogen activator inhibitor 1 (PAI1), thrombospondin 1, and matrix metalloproteinase 2. AngII altered abundance of these molecules in the ascending aorta (**Figure 1D, E**).

Susceptibility of SHF-derived cells to AngII-induced medial pathology.

We next examined interactions between SMC origins and AngII-induced aortic pathology. CNC- and SHF-derived cells were tracked using Wnt1- or Mef2c-*Cre* ROSA26R^{LacZ} mice infused with AngII. Consistent with our previous study (13), the ascending aorta was composed of both CNC- and SHF-derived SMCs with neither extending beyond the aortic arch (**Figure 2A**). AngII infusion did not alter their distribution, whereas medial thickening occurred in outer layers where SMCs derive from the SHF (**Figure 2B)**. These data are consistent with SHF-derived SMCs exerting a critical role in AngII-induced aortic pathologies.

Augmentation of thoracic aortopathies by LRP1 deletion in SHF-derived cells.

Deletion of LRP1 in all SMCs causes multiple aortic pathologies including aneurysm and tortuosity (16-18), but it was unclear whether LRP1 deletion in a specific SMC origin affects TAA formation. Therefore, we deleted LRP1 in either CNC or SHF-derived SMCs. CNC-specific deletion of LRP1 using the Wnt1 promoter to drive *Cre* expression was embryonically lethal between E11.5 and E12.5 (**Supplemental Figure 1A**). Gross appearance was comparable between genotypes at E11.5 with no obvious vascular malformation (**Supplemental Figure 1B**). Since this promoter is not restricted to the aorta (**Supplemental Figure 2**), extra-aortic LRP1 deletion may have contributed to embryonic lethality. Next, LRP1 was deleted in SHF-derived SMCs using a Mef2c promoter driven *Cre*. In Mef2c-*Cre*-expressing mice, *Cre* activity was observed mainly in the outer media (**Supplemental Figure 3A**). *Lrp1 delta flox* fragment was observed in addition to a band of native *Lrp1* (**Supplemental Figure 3B-D**). LRP1 abundance was reduced by 37% in the ascending aortic media of SHF-specific LRP1 deleted mice

compared to *Cre* negative littermates, and LRP1 deletion was restricted in the outer media (**Supplemental Figure 3E-F**). SHF-specific LRP1 deletion did not affect growth or development of mice. To promote TAAs, AngII was infused subcutaneously for 4 weeks. LRP1 deletion in SHF-derived cells augmented AngII-induced aortic dilation in the ascending aorta as demonstrated by both ultrasound and in situ imaging (**Figure 3A, B**). Aortic pathology was not observed in the descending aorta (**Figure 3A**). SHF-specific LRP1 deletion also exacerbated AngII-induced ascending aortic rupture and elastin fragmentation (**Figure 3C-E**). Systolic blood pressure was not different between genotypes in response to AngII infusion (**Figure 3F**), demonstrating augmented aortopathy was independent of systolic blood pressure. To explore molecular mechanisms behind augmentation of TAAs by LRP1 deletion in SHF-derived cells, proteomics analyses were performed using aortas harvested from wild type and SHF-specific LRP1 deleted mice. Analyses were performed under basal conditions and after a brief interval of AngII infusion that did not cause overt pathology. Deletion of LRP1 in SHF-derived cells was verified by the reduction of peptide intensity in the ascending aorta of Mef2c-*Cre* expressing mice (**Supplemental Figure 4**). Based on $q < 0.1$ threshold, deletion of LRP1 in SHF-derived cells altered abundances of seven proteins (**Figure 3G**). Of note, six of these proteins linked to transforming growth factor β (TGF-β) signaling molecules (**Figure 3H**). These results indicate that the protective function of LRP1 in SHF-derived SMCs against AngII-induced TAA associates with alterations of TGF-β signaling-related proteins.

Prenatal thoracic aortopathy induced by TGF-β receptor 2 deletion in SHF-derived cells.

Genetic mutations of TGF-β type 2 receptor (TGFBR2) cause Loeys-Dietz

syndrome (LDS) with probands predisposed to aneurysms (19-21). In mice, postnatal deletion of TGFBR2 in SMCs promotes severe TAA formation with pathologies localized to outer medial layers of the ascending aorta (7, 22). Since regional and transmural location of pathologies in LDS corresponded to distribution of SHF-derived SMCs, we investigated the role of TGFBR2 in SHF-derived cells on aortic structure. SHF-specific TGFBR2 depletion led to embryonic lethality with peritoneal hemorrhage beginning at E12.5 (**Figure 4A**). In addition, the outflow tract was dilated significantly in fetuses with SHF-specific TGFBR2 deletion compared to wild type littermates (**Figure 4A, B**). These data, alongside our findings in SHF-derived LRP1, support the premise that SHF-derived SMCs exert a critical role in the structural integrity and aneurysm development of the ascending aorta.

Our study demonstrated that AngII infusion induced region-specific alterations in proteins in the thoracic aorta and susceptibility of SHF-derived cells to AngII-induced medial pathology. Gene manipulation studies using mice with SHF-specific LRP1 or TGFBR2 deletion demonstrated that SHF-specific SMCs play an important role in the pathophysiology of thoracic aortopathies.

Multiple studies have shown an important role for TGF-β signaling in maintaining aortic wall integrity (7, 19-21). Both LRP1 and TGFBR2 are related to TGF-β signaling pathways (19, 23), and genetic deletions of these molecule in pan-SMCs promotes TAA formation in mice (7, 10, 22). The present study demonstrated thoracic aortopathy formation by functional deletion of these proteins only in SHF-derived cells. These data are consistent with the outer SMC sleeve derived from the SHF being crucial for development of thoracic aortopathies related to TGF-β signaling. A recent study using mice expressing a fibrillin1 mutation ($Fbn1^{C1041G/+}$) reported that heterozygous deletion

of Notch1 in SHF-derived cells had a trend for augmentation of luminal dilatation of the aortic sinus (24). In contrast, another recent study demonstrated that Smad2 deletion in CNC-derived cells reduced dilation of the aortic sinus in a mouse model of LDS (25). Since there are aortic region-specific responses to TGF-β (26), these two developmental origins may have divergent pathophysiological functions in different aortic regions.

In the present study, LRP1 deletion in CNC-derived cells did not lead to vascular malformation at the prenatal phase, but these mice were embryonic lethal. Previous studies have reported that prenatal deletion of TGFBR2 in CNC-derived cells show cleft palate and a congenital heart defect, persistent truncus arteriosus (27, 28). These mice die in the immediate postnatal period. Therefore, the postnatal effects of LRP1 and TGFBR2 in CNC-specific cells are unclear. Because the Wnt1 gene is not expressed in the postpartum phase (29), mouse experiments using inducible gene deletion are not feasible. Further studies are needed to overcome this barrier to compare the role of two origins on development of thoracic aortopathies.

In conclusion, our study highlights the functional importance of SHF-derived cells in the integrity of the ascending aortic wall using multiple TAA mouse models. This study provides strong evidence that SHF-derived cells exert a protective role during the development of TAA. Heterogeneity of SMC origins contributes to complex mechanisms of TAA formation, which should be taken into consideration when investigating the pathophysiology of TAAs.

Methods

Detailed methods are presented in the Supplemental Material. All raw data and analytical methods are available from the corresponding author upon appropriate request.

Study approval: All animal studies and experimental procedures were approved by the University of Kentucky's IACUC (#2006-0009, #2019-2967).

Author Contributions

Study design: HS, HH, DLR, JZC, HSL, SM, SAS, MA, MWM, AD.

Implementation of animal experiments: HS, DLR, JJM, DAH.

Data analyses: HS, DLR, JZC, HSL.

Proteomics and associated informatics: HH, SM, LHL, SAS.

Supervising and data verification: MA, MWM, AD.

Writing the manuscript: all authors.

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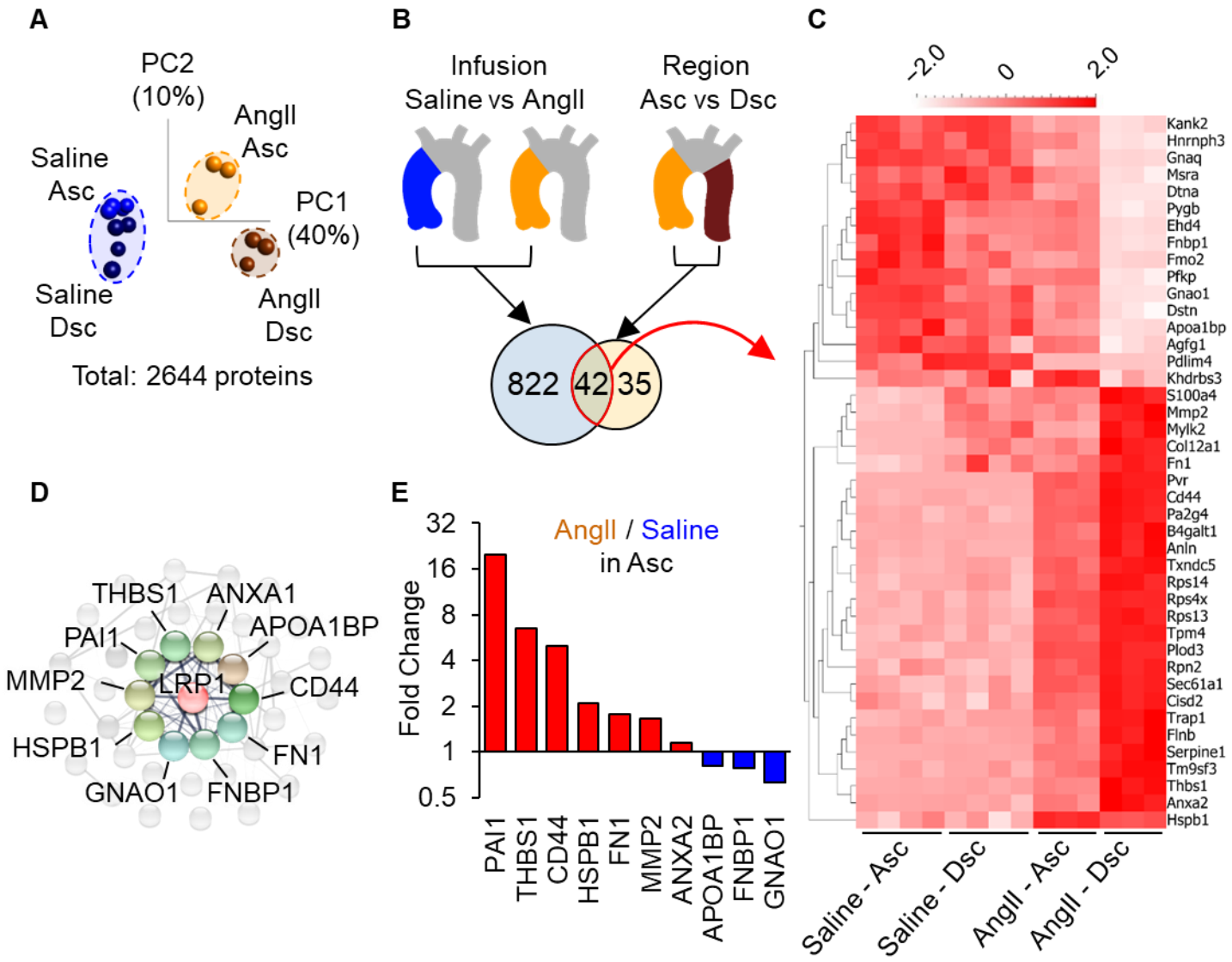


Figure 1. AngII differentially altered the protein profile of the ascending and descending aortas related to LRP1. (A) Principal component analysis of the unfiltered proteome. Asc = ascending aorta, Dsc = descending aorta (n = 3 - 4/group). **(B)** Venn diagram of differentiated proteins (q < 0.1). **(C)** Heat map with Z score coloring for overlapped 42 proteins. **(D)** Protein-protein interaction between overlapped 42 proteins and LRP1. **(E)** Fold change of protein intensity related to LRP1 in overlapped 42 proteins compared to ascending aortas from either saline- or AngII-infused mice.

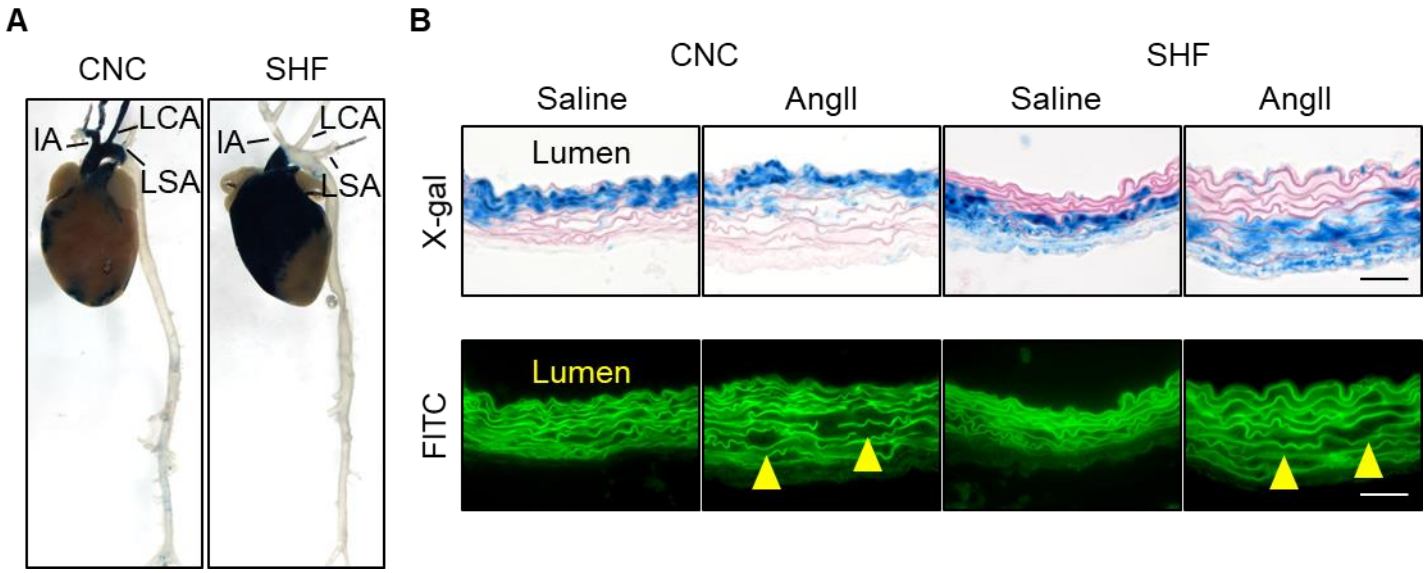


Figure 2. AngII-induced medial disruption that was predominantly in outer medial layers derived from the SHF. (A) Representative images of X-gal stained aortas from Wnt1- and Mef2c-*Cre* ROSA26R^{LacZ} mice (n = 3/group). IA = innominate artery, LCA = left common carotid artery, LSA = left subclavian artery. **(B)** Representative X-gal staining and FITC images of aortas from either saline or AngII-infused Wnt1- and Mef2c-*Cre* ROSA26R^{LacZ} mice (n = 5 - 7/group). Yellow triangles indicate medial thickening. Scale bar = 50 µm.

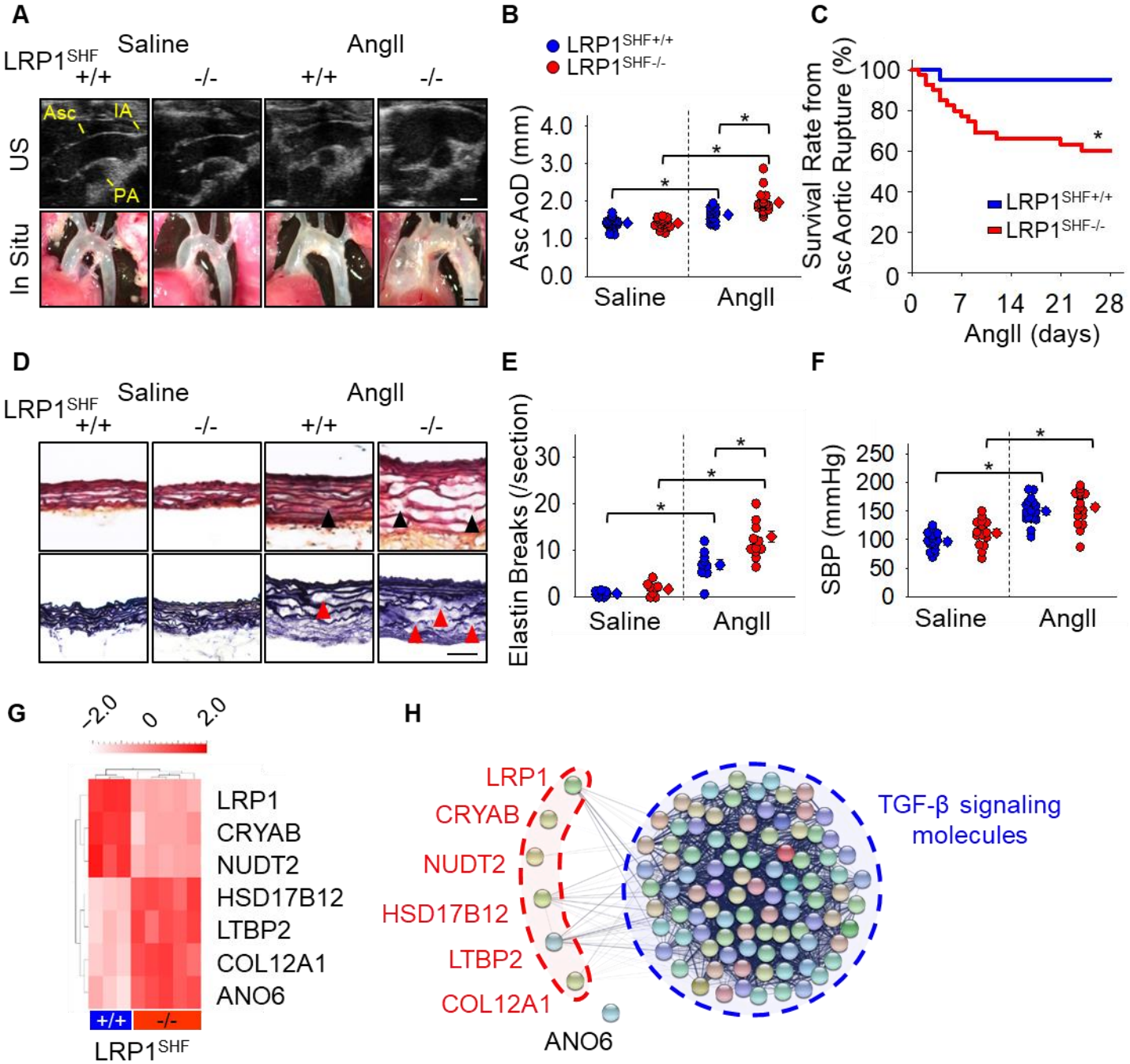


Figure 3. LRP1 deletion in SHF-derived SMCs augmented AngII-induced TAA formation and aortic rupture. (A) Representative ultrasound (US) images and in situ gross appearances of ascending aortas. n = 21 - 40. Asc = ascending aorta, IA = innominate artery, PA = pulmonary artery. Scale bar = 1 mm. **(B)** Ascending aortic diameter measured by ultrasonography. **(C)** Survival rate from ascending aortic rupture. **(D)** Representative Movat's and Verhoeff iron hematoxylin staining. Scale bar = 50 µm. **(E)** Elastin break counts in Movat's stained sections. **(F)** Systolic blood pressure. **(G)** Heat map with Z score coloring for differentiated proteins in comparison between wild type and SHF-specific LRP1 deleted ascending aortas ($q < 0.1$, n = 3 - 5/group). **(H)** Protein-protein interaction between 7 identified proteins and TGF-β signaling molecules. Red font indicates proteins related to TGF-β signaling. * = $p < 0.001$ by two-way ANOVA followed by Holm-Sidak test, or Log rank test.

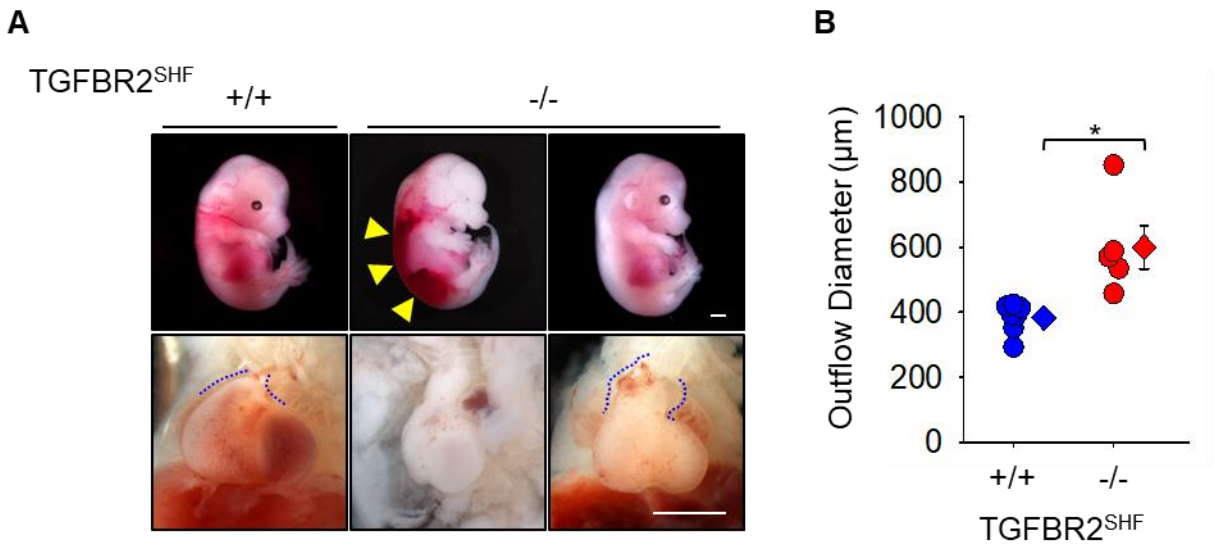
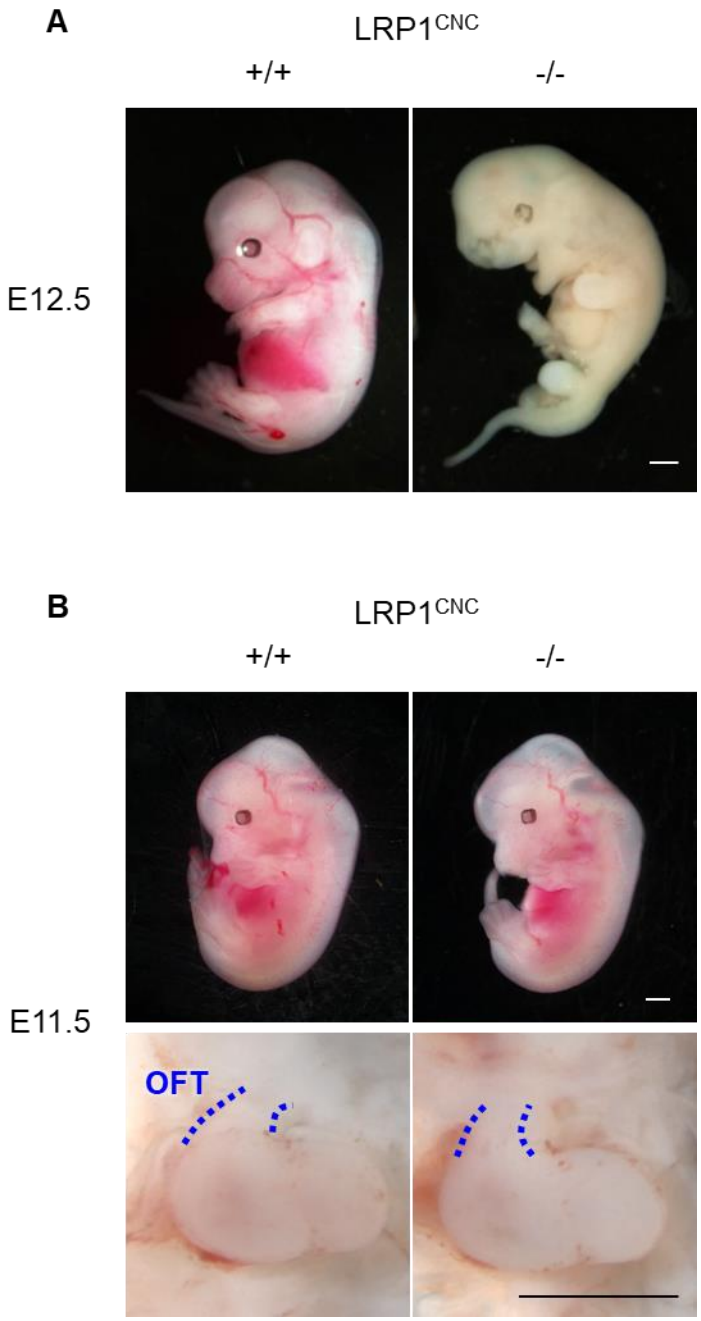
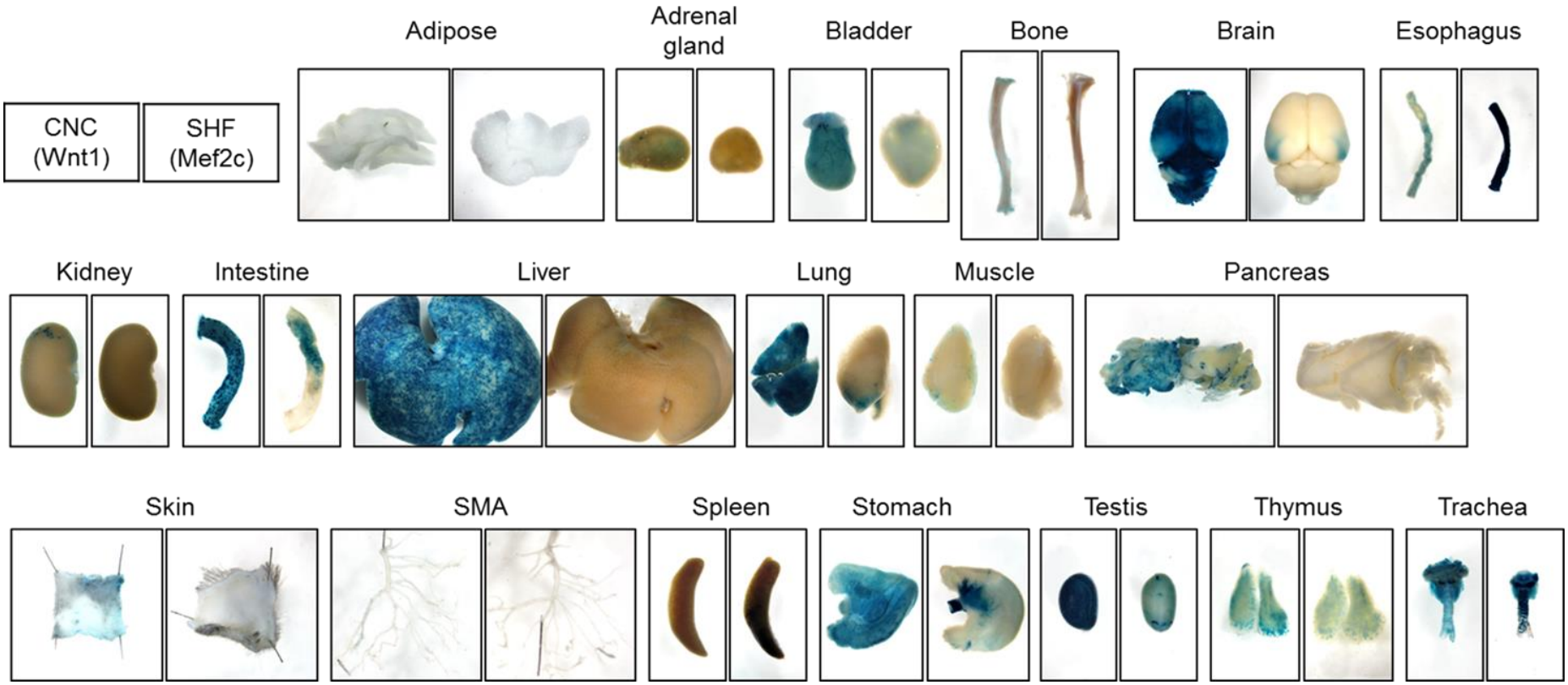


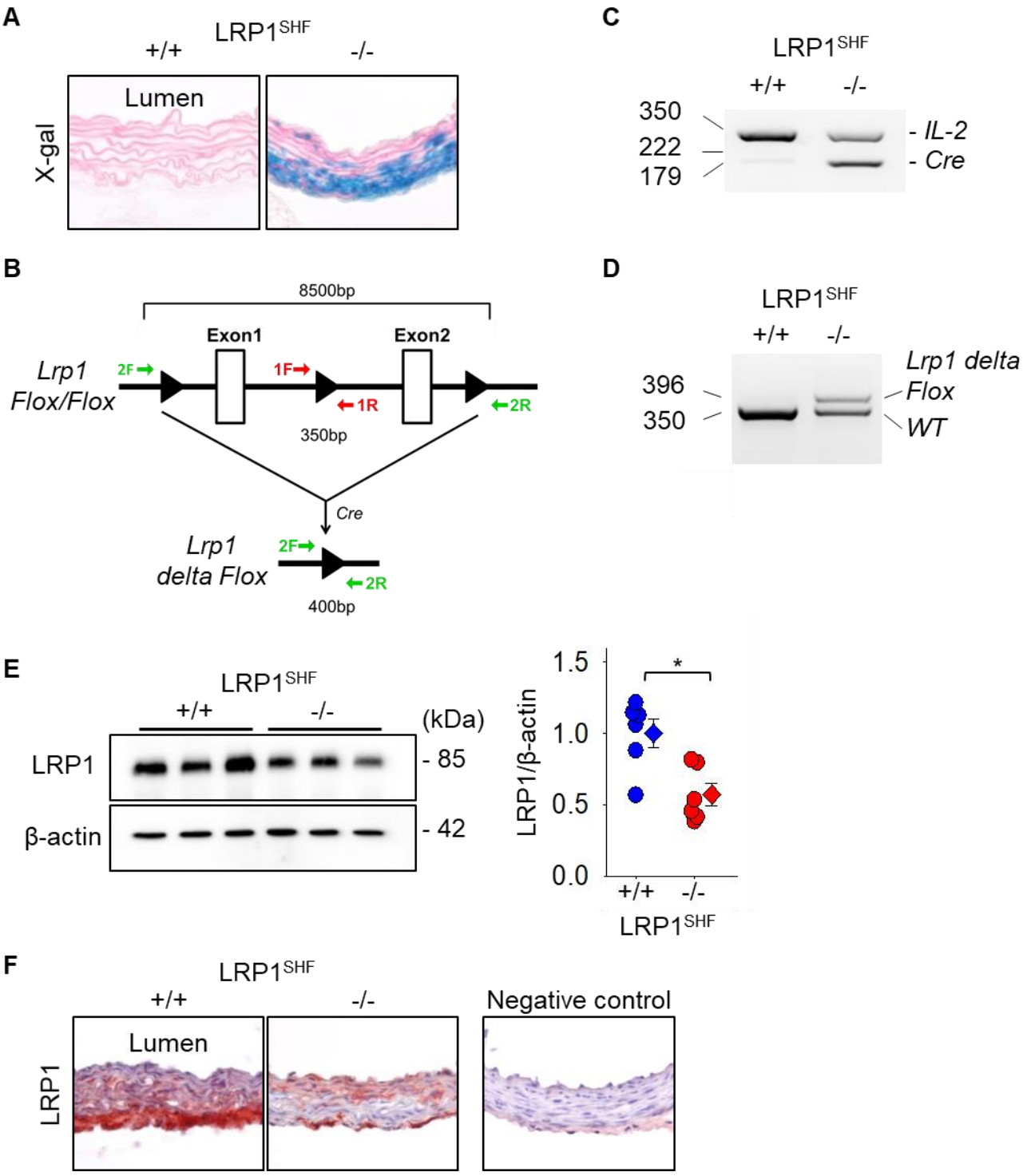
Figure 4. TGF-β receptor 2 deletion in SHF-derived cells provoked embryonic lethality and thoracic aortopathy at the embryonic stage. (A) Representative images of gross appearance and outflow tract of wild type littermates (left), dead (middle), and survived (right) fetuses with SHF-specific TGFBR2 deletion at E12.5. Blue dotted lines indicate the edge of the outflow tract. **(B)** Outflow diameter was measured at 300 - 400 μm distal of the root of fetuses that survived at the termination. n = 5 - 7/group. Yellow triangles indicate peritoneal hemorrhage. Scale bar = 1 mm. * = $p < 0.001$ by two-tailed Student's t test.



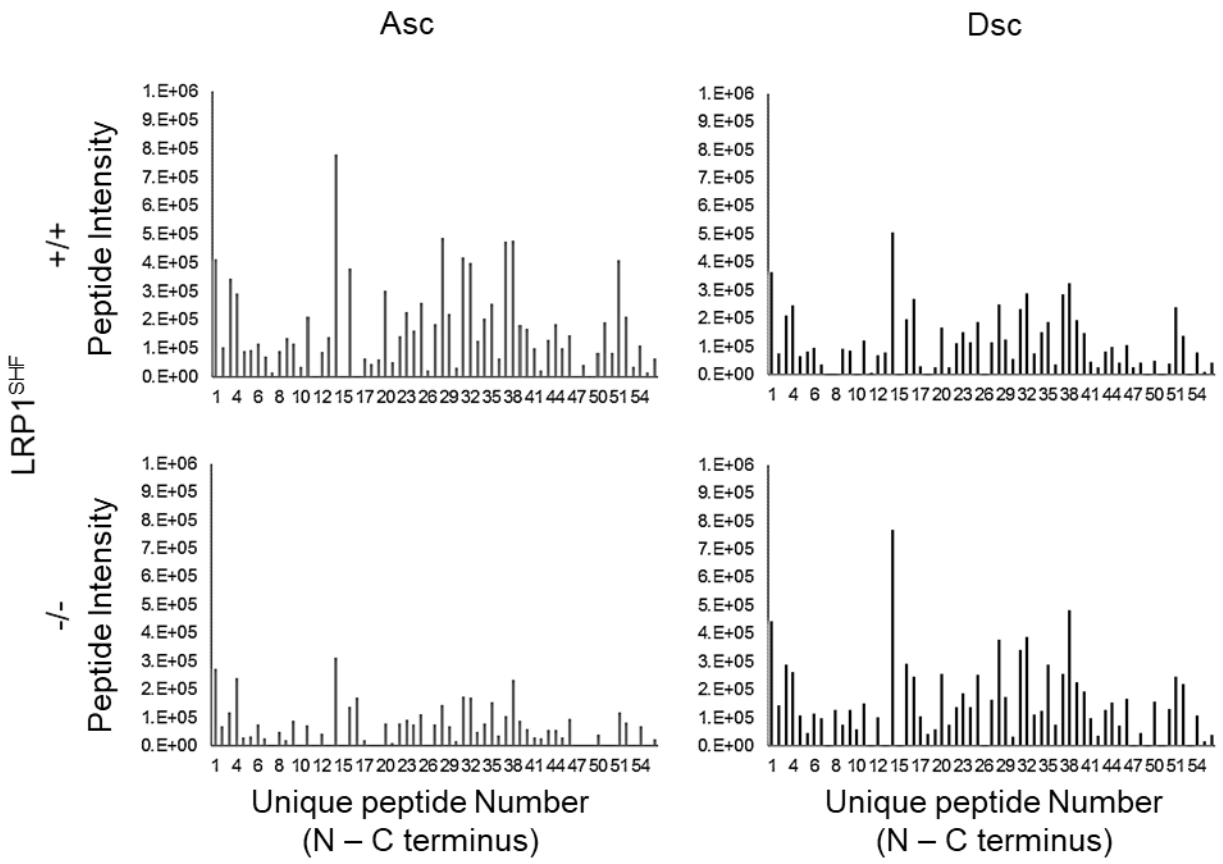
Supplemental Figure 1. Embryonic lethality provoked by LRP1 deletion in CNC-derived cells. (A) Gross appearance of wild type and CNC-specific LRP1-deleted fetuses at E12.5. n = 5 - 7/group **(B)** Gross appearance of fetuses and outflow tract in wild type and CNC-specific LRP1-deleted fetuses at E11.5. n = 5/group. Scale bar = 1 mm. Blue dotted lines = outflow tract (OFT).



Supplemental Figure 2. Representative X-gal staining images of major organs from Wnt1- or Mef2c-*Cre* ROSA26R^{LacZ} male mice. CNC = cardiac neural crest, SHF = second heart field, SMA = superior mesenteric artery.



Supplemental Figure 3. 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 - 12 weeks of age (male, n = 3 - 6/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. Representative images of **(E)** Western blot of LRP1 and β-actin. **(F)** Immunostaining for LRP1. As negative control, sections were incubated with non-immune rabbit IgG antibody. * = $p < 0.01$ by two-tailed Student's t test.



Supplemental Figure 4. Verification of LRP1 deletion in SHF-derived cells by proteomics analysis. The normalized peptide intensities of LRP1 were exported from Proteome Discoverer 2.2. The peptide intensities were plotted from N to C-terminus with the numbering conserved across the ascending (Asc) and descending (Dsc) aortas of SHF-specific LRP1-deleted mice and wild type littermates. n = 3/group.