Endocardium Minimally Contributes to Coronary Endothelium in the Embryonic Ventricular Free Walls

Hui Zhang, Wenjuan Pu, Guang Li, Xiuzhen Huang, Lingjuan He, Xueying Tian, Qiaozhen Liu, Libo Zhang, Sean M. Wu, Henry M. Sucov, Bin Zhou

Rationale: There is persistent uncertainty regarding the developmental origins of coronary vessels, with 2 principal sources suggested as ventricular endocardium or sinus venosus (SV). These 2 proposed origins implicate fundamentally distinct mechanisms of vessel formation. Resolution of this controversy is critical for deciphering the programs that result in the formation of coronary vessels and has implications for research on therapeutic angiogenesis.

Objective: To resolve the controversy over the developmental origin of coronary vessels.

Methods and Results: We first generated nuclear factor of activated T cells (Nfatc1)-Cre and Nfatc1-Dre lineage tracers for endocardium labeling. We found that Nfat1 recombinases also label a significant portion of SV endothelial cells in addition to endocardium. Therefore, restricted endocardial lineage tracing requires a specific marker that distinguishes endocardium from SV. By single-cell gene expression analysis, we identified a novel endocardial gene natriuretic peptide receptor 3 (Npr3). Npr3 is expressed in the entirety of the endocardium but not in the SV. Genetic lineage tracing based on Npr3-CreER showed that endocardium contributes to a minority of coronary vessels in the free walls of embryonic heart. Intersectional genetic lineage tracing experiments demonstrated that endocardium minimally contributes to coronary endothelium in the embryonic ventricular free walls.

Conclusions: Our study suggested that SV, but not endocardium, is the major origin for coronary endothelium in the embryonic ventricular free walls. This work thus resolves the recent controversy over the developmental origin of coronary endothelium, providing the basis for studying coronary vessel formation and regeneration after injury. (Circ Res. 2016;118:1880-1893. DOI: 10.1161/CIRCRESAHA.116.308749.)

Key Words: blood vessel ■ coronary ■ coronary artery ■ coronary development ■ coronary origin ■ lineage tracing

A long-standing mystery in the cardiovascular field is the developmental origin of coronary arteries.1–4 There is consensus that coronary veins arise from the venous endothelium of the sinus venosus (SV; the common vein through which blood passes before entry into the right atrium), which sends nascent sprouts to the heart that assemble into a subepicardial plexus before further maturation. Coronary veins remain in a subepicardial location on the heart surface. Studies of the origin of coronary arterial endothelium implicated 2 major candidate sources: ventricular endocardium (VE) and SV, but there is ongoing controversy over their relative contribution.5–11 Resolving this issue is important because these 2 proposed origins represent 2 completely different development mechanisms for coronary vessel formation. An SV source implies that cells that were originally venous endothelium become reprogrammed to an arterial fate and subsequently invade the ventricle wall in an epicardium-to-endocardium direction to form coronary arteries, which are found in an intramyocardial location in the heart wall. In this model, coronary arteries and veins share a common developmental origin from the SV (model 1, Online Figure IA).5,6 In contrast, a VE source supports a model in which ventricular endocardial cells invade the ventricle wall in endocardium-to-epicardium direction to form coronary arteries, which, therefore, have a developmental origin distinct from subepicardial veins (model 2, Online Figure IA).7 There is consensus

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From the Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences (H.Z., W.P., X.H., L.H., X.T., Q.L., L.Z., B.Z.) and Institute of Neuroscience, State Key Laboratory of Neuroscience, CAS Center for Excellence in Brain Science and Intelligence Technology, Shanghai Institutes for Biological Sciences (B.Z.), Chinese Academy of Sciences, Shanghai, China; Cardiovascular Institute, Division of Cardiovascular Medicine, Department of Medicine, and Institute for Stem Cell Biology and Regenerative Medicine, Stanford University School of Medicine, CA (G.L., S.M.W.); Broad CIRM Center and Department of Stem Cell Biology and Regenerative Medicine, University of Southern California, Los Angeles (H.M.S.); and School of Life Science and Technology, ShanghaiTech University, Shanghai, China (B.Z.).

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that both models are involved in coronary arterial formation; however, quantification by different groups reached vastly different estimates of the relative contribution of these 2 sources.\textsuperscript{3,7,10,12} This discrepancy engenders an urgent need for resolution of this controversial issue, not only to reveal the developmental sources of the coronary endothelium, but also more importantly to unravel the distinct developmental mechanisms responsible for coronary vessel formation and to aid in designing new strategies for therapeutic cardiac revascularization.

A recent study used nuclear factor of activated T cells (Nfatc1)-Cre lineage tracing to show that Nfatc1-derived cells constitute the majority of coronary arteries in the developing heart.\textsuperscript{7} In this study, Nfatc1-Cre was asserted to recombine in VE but not in SV endothelium, and therefore, these data were interpreted to conclude that the VE, but not the SV, gives rise to most coronary arteries. However, the extent to which Nfatc1-Cre labels the SV was not fully investigated in that study. Furthermore, ectopic expression of Nfatc1 in coronary vessels might undermine the interpretation of those results, which were based on a constitutively active Cre line.\textsuperscript{11} The major conclusion of the Wu et al study contradicts an earlier clonal analysis that implicated the SV as the major source for coronary arteries.\textsuperscript{3} In addition, a recent lineage tracing based on Apln-CreER showed that subepicardial vessels invade myocardium to generate most coronary arteries in ventricular free walls,\textsuperscript{9} and recent molecular studies of coronary vessel formation also support the SV as the primary origin for coronary arteries in the embryonic ventricular free walls.\textsuperscript{14} These observations challenge the VE as the origin of most coronary arteries in the developing heart. However, direct evidence to either support or refute this conclusion has been lacking. Here, we generated new mouse genetic tools and revisited this controversial question with fate mapping and intersectional lineage tracing studies. Our analysis demonstrated that ventricle endocardium minimally contributes to coronary endothelium in the embryonic ventricular free walls (model 2, Online Figure 1A), suggesting that the SV is the major source of coronary endothelium in the developing heart (model 1, Online Figure 1A).

## Methods

Detailed Materials and Methods are provided in the Online Data Supplement. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. Immunostaining and in situ hybridization were performed according to protocol described previously.\textsuperscript{13} The

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### Nonstandard Abbreviations and Acronyms

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<th>Abbreviation</th>
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<tr>
<td>CDH5</td>
<td>cadherin 5</td>
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<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>ESR</td>
<td>estrogen receptor</td>
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<tr>
<td>Fabp4</td>
<td>fatty acid binding protein 4</td>
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<tr>
<td>Nfatc1</td>
<td>nuclear factor of activated T cells</td>
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<tr>
<td>Npr3</td>
<td>natriuretic peptide receptor 3</td>
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<tr>
<td>Pecam</td>
<td>platelet/endothelial cell adhesion molecule 1</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<tr>
<td>SV</td>
<td>sinus venosus</td>
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<td>VE</td>
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\textsuperscript{5} In addition, a recent lineage tracing based on Apln-CreER showed that subepicardial vessels invade myocardium to generate most coronary arteries in the developing heart.\textsuperscript{14} These observations challenge the VE as the origin of most coronary arteries in the developing heart. However, direct evidence to either support or refute this conclusion has been lacking. Here, we generated new mouse genetic tools and revisited this controversial question with fate mapping and intersectional lineage tracing studies. Our analysis demonstrated that ventricle endocardium minimally contributes to coronary endothelium in the embryonic ventricular free walls (model 2, Online Figure 1A), suggesting that the SV is the major source of coronary endothelium in the developing heart (model 1, Online Figure 1A).

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### Results

**Nfatc1 Constitutive Recombinases Label Both VE and SV**

To evaluate the lineage and fate of Nfatc1+ cells, we first generated \( \text{Nfatc1-Cre} \) by knockin of IRES-Cre into the Nfatc1 3′ untranslated region (Figure 1A), which targets exactly the same gene location as the mouse line reported previously.\textsuperscript{7} We crossed \( \text{Nfatc1-Cre} \) with reporter line \( \text{R26-tdTomato} \) to permanently label Nfatc1-expressing cells and their descendants (Figure 1B). We collected E9.5 to E15.5 \( \text{Nfatc1-Cre;R26-tdTomato} \) embryos (Figure 1C) and performed immunostaining for genetic lineage marker tdTomato and endothelial cell (EC) marker VE-cadherin (CDH5). The tdTomato+ cells, derived from the Nfatc1+ precursors, are detected in most SV ECs as well as VE at E9.5 and E11.5 (Figure 1D and 1E). By staining for coronary EC-specific marker fatty acid binding protein 4 (FABP4), we found that Nfatc1-derived cells (tdTomato+) contribute to the majority of FABP4+ subepicardial ECs as well as intramyocardial coronary vessels in E13.5 and E15.5 embryonic heart (Figure 1F and 1G; Online Figure 1B and 1C). These data demonstrated that \( \text{Nfatc1-Cre} \) line labels SV notably, in addition to VE in the developing heart.

To confirm the above lineage tracing data, we generated another recombinase line, \( \text{Nfatc1-Dre} \), for lineage tracing of Nfatc1+ cells.\textsuperscript{15} We crossed \( \text{Nfatc1-Dre} \) with its specific reporter line \( \text{Rosa26-Rox-Stop-Rox-tdTomato} \) to perform lineage tracing of Nfatc1+ cells in the developing heart (Figure 2A). In E10.5 and E11.5 \( \text{Nfatc1-Dre;R26-RSR-tdTomato} \) hearts, we found that Nfatc1-Dre labels SV cells as well as ventricular endocardial cells (Figure 2B–2D). Nfatc1-derived cells contribute to subepicardial ECs at E12.5 (Figure 2E) and to the majority of intramyocardial coronary ECs at E15.5 (Figure 2F). Quantification of the number of labeled subepicardial vessels and intramyocardial vessels at E15.5 showed that there was no significant difference in the labeling efficiency of these 2 populations (Figure 2G). These data suggest that Nfatc1+ cells contribute equally to subepicardial vessels and intramyocardial arteries. Immunostaining for the arterial endothelial marker connexin 40 confirmed that coronary arteries in the ventricular free walls of the embryonic heart express the Nfatc1 lineage marker (Figure 2H). In sum, our Nfatc1 recombinase lines consistently showed that Nfatc1+ cells constitute the SV in addition to the VE. Because both \( \text{Nfatc1-Cre} \) and \( \text{Nfatc1-Dre} \) efficiently label the SV, the interpretation of the observation that the coronary arteries are Nfatc1-derived cannot be attributed to only a VE source.

**Nfatc1 Is Expressed in the SV and Coronary Vessels**

We next studied the reason why Nfatc1-Dre labels the SV because previous work implied that Nfatc1 mRNA and protein are not expressed in the SV.\textsuperscript{7} It should be noted that this negative expression was based on observations of E9.5 and
E10.5 embryos. Because Nfatc1 constitutive Cre was used for fate mapping, it is critical to examine other time points to fully exclude the possibility of unrecognized Nfatc1 expression. To examine Nfatc1 expression at earlier time point (eg, E8.0–E8.5), we used an Nfatc1-GFP knockin mouse line, in which the Nfatc1 coding region is followed by GFP with a 2A linking peptide. By whole-mount immunostaining of GFP as a surrogate for Nfatc1 expression, we detected GFP expression in the 2 horns of the SV at E8.0 to E8.5 (Online Figure IIA). In sections, it was evident that Nfatc1 (as visualized by Figure 1. Nfatc1+ cells contribute to both SV and ventricular endocardium. A–C, Schematic showing the knockin strategy of Nfatc1-Cre allele, lineage tracing principle, and experimental procedures. D and E, Immunostaining for tdTomato and CDH5 on sections of E9.5 and E11.5 Nfatc1-Cre;R26-tdTomato embryos showed Nfatc1+ cells contribute to endothelial cells in SV (tdTomato+CDH5+, arrowheads) and in ventricle (V) and atrium (A). F, Whole-mount fluorescence view of E15.5 embryos showed labeling of coronary vessels. G, Immunostaining for tdTomato and coronary vascular endothelial cell marker FABP4 on heart sections of Nfatc1-Cre;R26-tdTomato embryo showed Nfatc1+ cells contribute to the majority of coronary vascular endothelial cells (arrowheads) in the compact myocardium. Scale bars, 100 μm in D and E; 500 μm in F and G. A indicates atrium; V, ventricle; SV, sinus venosus; and UTR, untranslated region.
Figure 2. Nfatc1\(^+\) cells contribute significantly to both SV and subepicardial vessels. 

A, Schematic showing the strategy for lineage tracing of Nfatc1\(^+\) cells by Dre-Rox system. 

B and C, Immunostaining for tdTomato and PECAM on Nfatc1-Dre;R26-RSR-tdTomato embryonic sections. Boxed region is magnification of SV. Nfatc1\(^+\) cells contribute to a substantial number of SV endothelial cells (arrowheads). 

D, Cartoon figure showing atrium, ventricle, and sinus venosus are labeled by Nfatc1-Dre. 

E, Immunostaining for tdTomato and PECAM on E12.5 heart section shows that Nfatc1-Dre labels subepicardial vessels (arrowheads). 

F, Immunostaining for tdTomato and PECAM on E15.5 heart sections shows Nfatc1\(^+\) cells contribute to subepicardial vessels (arrowheads) and intramyocardial vessels. Dotted line indicates the border between compact myocardium and trabecular myocardium (Trab.). 

G, Quantification of the percentage of tdTomato\(^+\) coronary vessels shows there is no significance (n.s.) in percentage between intramyocardial vessels and subepicardial vessels. The percentage of tdTomato\(^+\) cells in coronary vessels was averaged from 4 E15.5 hearts. 

H, Immunostaining for tdTomato, PECAM with arterial marker connexin 40 (CX40) shows Nfatc1-derived cells contribute to arterial endothelial cells (arrowheads). Scale bars, yellow 500 μm; white 100 μm. A indicates atrium; LV, left ventricle; PECAM, platelet/endothelial cell adhesion molecule 1; RV, right ventricle; SV, sinus venosus; and V, ventricle.
Nfatc1 was expressed in SV endothelium rather than Nkx2.5+ or Tnni3+ myocardium in the early developing heart (Online Figure IIB). Therefore, labeling of the SV by Nfatc1-Dre as a lineage marker (Figure 2) can be explained by early embryonic Nfatc1 gene expression in the SV. Nfatc1 expression is known to be downregulated in VE at late time points after E11.5. To further examine Nfatc1 expression in coronary endothelium at later time points, we collected E15.5 Nfatc1-GFP heart sections and immunostained for GFP and the pan-endothelial marker CDH5 (VE-cadherin). We found that a subset of coronary ECs, in both subepicardial and intra-myocardial locations, was GFP positive (Online Figure IIC). Using NFATC1 antibody staining on E15.5 wild-type heart sections, we similarly found NFATC1-expressing coronary vascular ECs (Online Figure IID). These observations indicate continuing albeit perhaps sporadic Nfatc1 expression in coronary endothelium and are consistent with a previous report showing Nfatc1 in midgestation coronary vessels. To independently confirm Nfatc1 expression in coronary vasculature, we isolated E14.5 coronary ECs by fluorescence-activated cell sorting from Apln-CreER;Rosa26-mTmG mice that were treated with tamoxifen at E10.5. Apln is a specific marker for vascular ECs and is not expressed in endocardium. As a positive control, we isolated endocardial and vascular ECs as a single combined population from Tie2-GFP mouse hearts, and as a negative control, we isolated cardiomyocytes from Tnt-Cre;Rosa26-mTmG mice. Reverse transcription polymerase chain reaction analysis by 2 different sets of Nfatc1 primers showed expression of Nfatc1 in coronary vascular ECs (Online Figure IIE). Altogether, these data provide several candidate reasons why Nfatc1-Cre or Nfatc1-Dre labels coronary vessels—expression in the E8.5 SV, expression in the E9.5 to 10.5 VE, and expression in at least some coronary endothelium at E14.5 to E15.5. The previous interpretation that Nfatc1-Cre recombination is restricted to the endocardium, and therefore that VE contributes to the majority of coronary arteries, clearly requires further analysis by more restricted genetic lineage tools.

Identification and Characterization of Npr3 as a New Endocardial Marker

To find a marker that is more exclusively expressed in endocardium but not coronary ECs, we dissociated cells from E14.5 hearts, sorted cardiomyocytes, pan-ECs (including both endocardium and coronary endothelium) and smooth muscle cells by flow cytometry, and performed single-cell gene expression analysis using the Fluidigm platform. We could clearly distinguish these 3 main types of cardiovascular cells by gene expression signatures (Figure 3A). All ECs expressed the pan-endothelial markers platelet/endothelial cell adhesion molecule 1 (Pecam) and Cdih5. Importantly, we were able to divide the EC population into 2 subgroups based on expression of Fabp4, which is expressed in vascular ECs but not in endocardium. This analysis confirmed weak expression of Nfatc1 in both Fabp4+ and Fabp4− populations, consistent with our immunostaining and reverse transcription polymerase chain reaction data (Online Figure IIC–IIIIE) and supporting the interpretation that Nfatc1 is expressed in some coronary ECs at E14.5 to E15.5.

This analysis also revealed that a gene Npr3 was expressed in the EC population in a mutually exclusive manner with Fabp4 (Figure 3A) and thus might represent an endocardium-specific marker. Whole mount and section in situ hybridization showed that Npr3 was highly enriched in atrial endocardium and VE, but undetectable in SV (Figure 3B). To specifically co-stain Npr3+ cells with other lineage-specific markers, we generated the reporter mouse line Npr3-GFP, in which a GFP-Cre fusion cDNA was knocked into the 3′ untranslated region of the Npr3 gene by homologous recombination (Figure 3C). We stained Npr3-GFP heart sections with GFP antibody as a surrogate for Npr3 expression and found that Npr3 was expressed in atrial endocardium and VE but not in the SV at E10.5 hearts (Figure 3D). At E12.5 and E14.5, Npr3 is expressed in endocardium but not in coronary vascular ECs in the compact myocardium (Figure 3E and 3F). These data indicate that the newly identified endocardial gene Npr3 is not expressed in SV or coronary vessels.

For fate mapping approaches, it was important to confirm that Npr3 is co-expressed with Nfatc1 in endocardial cells in the early developing heart. Because Nfatc1 is reduced in expression after E9.5, we used Nfatc1-Dre;R26-RSR-tdTomato to mark Nfatc1+ cells and their derivatives. The rox-Stoprox reporter is a substrate only for Dre recombinase and is not recognized by Cre recombinase. By generating Npr3-CreER;Nfatc1-Dre;R26-RSR-tdTomato mice, we could take advantage of the Dre-Rox system to mark Nfatc1+ cells and used estrogen receptor (ESR) antibody to detect CreER as a surrogate for Npr3 expression. Immunostaining for ESR, tdTomato, and CDH5 on E9.5 and E10.5 embryonic heart sections showed that Npr3-CreER is expressed in almost all Nfatc1+ endocardial cells (Figure 4A and 4B). Altogether, the above data based on in situ hybridization, immunostaining, and single-cell quantitative polymerase chain reaction analysis prove Npr3 as a specific endocardial cell marker.

**Npr3-CreER Labels VE but Not SV**

Nfatc1-Cre or Nfatc1-Dre labels both VE and SV in the early heart, and Nfatc1 is expressed in a subset of coronary vascular ECs in E14.5 to E15.5 hearts, which compromise the utility of Nfatc1 as a lineage marker informative for the origins of coronary endothelium. We tested whether Npr3-CreER could label the endocardium in a more specific manner. By immunostaining for ESR and GFP on E8.5 Npr3-CreER and Npr3-GFP embryonic sections, respectively, we found Npr3 was expressed in atrium and VE but not SV (Online Figure IIIA and IIIB). We next collected Npr3-CreER embryos from E9.5 to E11.5; immunostaining against ESR showed that Npr3 was expressed in atrial endocardium and VE, but not in the SV at E9.5, E10.5, and E11.5 (Figure 5A–5C). In addition, Npr3 was also expressed in the ECs of the dorsal aorta at E9.5 and E10.5 (Figure 5A and 5B, arrowheads) and a subset of nonendothelial mesenchymal cells at E11.5 (Figure 5C, arrowheads). We also noticed that Npr3 was expressed in a subset of TBX18+ epicardial cells at E10.5, but not in TBX18+ proepicardial cells at E9.5 (Online Figure IVA and IVB). For technical controls, immunostaining for ESR on wild-type embryonic sections showed no detectable signals (Online Figure VA and VB). The point that Npr3 is expressed in VE but not SV is critical for this
Figure 3. Npr3 is expressed in endocardial cells. A, A heat map of cardiovascular lineage gene expression by fluorescence-activated cell sorter (FACS)-purified single cells from E14.5 heart. The 3 main cardiovascular cell types, cardiomyocyte (CM), endothelial cell (EC), and smooth muscle cell (SMC), are represented. The expression of genes in ECs (enlargement right) demonstrates a distinction between endocardial ECs and vascular ECs. B, Whole mount and section in situ of E9.5 to E13.5 hearts. C, Schematic diaphragm showing strategy for generation of Npr3-GFP knockin allele by homologous recombination. D–F, Immunostaining for GFP as surrogate for Npr3 with endothelial cell–specific markers CDH5 or PECAM on sections of E10.5 to E14.5 Npr3-GFP embryos. Yellow arrowheads indicate Npr3+ endocardial cells; white arrowheads indicate coronary vessels. Scale bars, 200 μm in B; 100 μm in D–F. A indicates atrium; H, heart; LA, left atrium; LV, left ventricle; Npr3, natriuretic peptide receptor 3; PECAM, platelet/endothelial cell adhesion molecule 1; RV, right ventricle; and V, ventricle.
study because it provides an opportunity to distinguish VE-derived coronary vessels from SV-derived coronary vessels. Based on this unique expression profile, we performed fate mapping analysis of Npr3-CreER;R26-tdTomato by tamoxifen injection at E8.5. We collected E10.5 and E11.5 embryos for examination of VE, SV, and the earliest coronary vessels in the atrioventricular groove. The E10.5 heart has no coronary ECs in the atrioventricular groove or in the compact myocardium, and 73.13±6.53% VE cells were labeled, whereas SV cells were not labeled (Figure 5D). The E11.5 heart shows the first coronary vessels appearing in the atrioventricular groove (Figure 5E). The 78.33±2.89% VE cells were labeled, whereas the SV and coronary ECs that are in the process of sprouting from the SV to the atrioventricular groove were not labeled (Figure 5E). These data show that Npr3 is expressed in VE but not in SV endothelium and that Npr3-CreER labels the VE but not SV in the E10.5 to E11.5 developing heart (Figure 5F).

**Npr3-VE Minimally Contributes to Coronary Vessels in Ventricular Free Walls**

We next collected hearts at later embryonic stage to quantitatively measure the contribution of Npr3-CreER-labeled VE
to coronary ECs. We labeled Npr3+ VE by tamoxifen induction at E8.5 and checked the coronary vessels in the compact myocardium of E12.5 to E15.5 hearts. Immunostaining for tdTomato and PECAM on E14.5 Npr3-CreER;R26-tdTomato embryos and detected tdTomato signal enriched in the core of the heart, with almost no signal in the ventricular free walls (Figure 6B). Immunostaining for tdTomato and PECAM on E14.5 heart sections showed that although most VE cells are labeled, only a few coronary ECs in the ventricular free walls are tdTomato positive (Figure 6C and 6D). Because of sectional orientation and the deep ingestion of some trabecular lacunae into the compact zone, some of the PECAM+ endocardium is ambiguous in morphology from coronary vascular ECs, possibly causing some VE cells to be mistakenly interpreted as coronary vessels. To avoid this possible caveat in coronary vessel quantification, we used the coronary vascular endothelial-specific marker FABP4. We co-stained FABP4 with tdTomato on E14.5 Npr3-CreER;R26-tdTomato embryos and found that tdTomato+ FABP4+ coronary vessels constitute the minority of

Figure 5. Expression and fate map of Npr3 in ventricular endocardium (VE) and sinus venosus (SV) of E9.5 to E11.5 hearts. A–C, Immunostaining images of CDHS and estrogen receptor (ESR) as surrogate for Npr3 detection on E9.5 to E11.5 Npr3-CreER embryonic sections. A1–C1 and A2–C2 are magnified images for ventricle and SV regions, respectively. Arrowheads in A and B indicate dorsal aortic endothelial cells, in C indicate nonendothelial mesenchymal cells. D and E, Immunostaining for tdTomato and PECAM on E10.5 and E11.5 Npr3-CreER;R26-tdTomato embryonic sections. Tamoxifen was injected at E8.5. White arrowheads indicate SV cells, yellow arrowheads indicate sprouting endothelial cells in atrioventricular groove (asterisk). F, Cartoon figure showing Npr3-CreER labels endocardium of ventricle (V) and atrium (A) but not SV. Scale bars, 100 μm. Npr3 indicates natriuretic peptide receptor 3; and PECAM, platelet/endothelial cell adhesion molecule 1.
FABP4+ vessels in ventricular free walls (Figure 6E and 6F). Immunostaining on serial sections across the heart showed that Npr3+ VE cells contribute to few coronary vessels in most parts of the ventricular free walls (Online Figure VII). Quantification of the number of labeled coronary vessels showed that although Npr3-CreER robustly labels ventricular...
endocardium, it labels significantly fewer coronary vessels in the right and left ventricle wall (Figure 6G). However, we did find that Npr3VE cells contribute to a substantial amount of coronary vessels in the ventricular septum and the most ventral part of ventricular free wall connecting to the ventricular septum (last row, Online Figure VIIIB). Immunostaining for tdTomato and FABP4 on E15.5 Npr3-CreER;R26-tdTomato hearts confirmed that VE cells minimally contribute to coronary vessels in the ventricular free wall, but amply to coronary vessels in the septum (88.75±4.84% in ventricular septum, Online Figure VIII). These results therefore suggested that ventricular endothelial cells minimally contribute to coronary vessels in the embryonic ventricular free wall.

**Nfatc1*Npr3 VE Minimally Contributes to Coronary Vessels in the Ventricular Free Wall**

Our data show that the VE is Nfatc1*Npr3, whereas the SV is Nfatc1*Npr3. We next aimed to perform lineage tracing directly of the Nfatc1 VE distinct from the Nfatc1 SV. By crossing of Nfatc1-Dre;Npr3-CreER mouse line with the dual recombination reporter line R26-Rox-Stop-Rox-Loxp-Stop-LoxptdTomato (Ai66),21 we established a strategy that labels the Nfatc1 VE only and dis-labels (subtracts labeling of) Nfatc1 SV. Dre and Cre are 2 types of recombinases that exclusively target their own recombination sites Rox and LoxP, respectively (Online Figure IXA). We validated that Cre does not recombine Rox sequences, and Dre does not recombine LoxP sites (Online Figure IXA). In the Ai66 reporter line, only cells expressing both Cre and Dre (and their descendants) are labeled by tdTomato (Online Figure IXB and IXC). In E10.5 and E11.5 Nfatc1-Dre;Npr3-CreER;Ai66 hearts after tamoxifen injection at E8.5, the Nfatc1*Npr3 VE was labeled, whereas the Nfatc1*Npr3 SV was not (Figure 7A and 7B). In a side-by-side comparison of Nfatc1-Dre alone with Nfatc1-Dre;Npr3-CreER embryos, we found that dis-labeling of the SV from the Nfatc1 domain dramatically reduced the labeling of both intramyocardial and subepicardial coronary vessels in E13.5 and E15.5 hearts (Figure 7C and 7D). Quantification of labeled coronary ECs in the ventricular free walls showed that Nfatc1 cells (VE+SV cells) contributed to 77.81±5.62% coronary vessels, whereas Nfatc1*Npr3VE cells contributed to 3.13±0.72% coronary vessels. These results are consistent with an SV origin of endocardium forming the coronary system.3 Our study reinterpret the contribution of Nfatc1 endothelial cells to coronary vessels in ventricular free walls and indicates that labeling of the SV rather than the VE by Nfatc1-Cre in the previous study2 could be a better explanation for the origins of the coronary endocardium in the embryonic ventricular free walls.

**Identification of Other Markers for Endocardium or Coronary Endothelium**

Endocardial cells and coronary ECs are 2 distinctive types of ECs with different molecular profiles: endocardial cells express relatively high level of Nfatc1, Npr3, whereas coronary ECs express high level of Apln and Fabp4.5,7,19 To provide the genome-wide information of their distinct gene expression profiles, we took advantage of Npr3-CreER and Fabp4-Cre lines to label enriched population of endocardium and coronary endothelium, respectively. We generated Npr3-CreER;Ai47 (GFP reporter line) and labeled endothelial cells by tamoxifen injection at E10.5. Immunostaining for GFP showed most GFP cells are restricted to endocardial cells in E14.5 heart (Figure 8A). We generated Fabp4-CreER;Ai47 mice, in which coronary ECs were labeled by GFP at E14.5 (Figure 8D). We isolated the GFP+ cells and GFP cells from Npr3-CreER;Ai47 or Fabp4-CreER;Ai47 ventricles by flow cytometry and collected RNA from purified cells for gene analysis (Figure 8B and 8E). Quantitative reverse transcription polymerase chain reaction of conventional pan-EC markers Pecam, Cd68, and Flk1 showed that these genes were highly enriched in GFP populations compared with GFP populations (Figure 8C and 8F), confirming the isolation of ECs in high purity. We next performed RNA sequencing on endocardium-enriched and coronary endothelium–enriched populations and compared the differentially gene expression between 2 GFP populations (Figure 8G; Online Table I). In addition to Npr3 and Fabp4 that were highly enriched in endocardium or coronary endothelium samples, respectively, we identified additional genes that are significantly higher in expression in either endocardium or coronary endothelium. For instance, the expression of Gpr126, Hapln1, Col23a1, and so on are significantly higher in endocardium samples, whereas the expression of Aoc3, Gpihbp1, Sema7a, and so on were significantly higher in coronary endothelium samples (Figure 8H). Altogether, the information on new molecular markers for endocardium or coronary endothelium would be valuable for the future study in cardiovascular research field.

**Discussion**

The main findings of this study are that (1) in addition to VE, Nfatc1-Cre or Dre labels both SV and subepicardial coronary vessels; (2) the newly identified marker Npr3 is co-expressed with Nfatc1 in VE, but is not expressed in the SV; (3) VE contributes to only a small minority of coronary vessels in ventricular free walls of embryonic hearts; (4) the Nfatc1*Npr3 SV is likely the major source of coronary vasculature in ventricular free walls; and (5) we provide information of new molecular markers specific for endocardium or coronary endothelium.

Determining the origin of coronary arteries is pivotal to further understanding the developmental program of coronary vessel formation and provides new insights to potential new treatments to coronary artery diseases. Many studies and reviews in the past years have made valuable contributions to the ongoing debate on the origins of coronary vessels in the developing heart.5,8,14,23-27 A consensus has emerged that there are 3 important sources for coronary vessels: proepicardium, VE, and SV.1,4,28 Each source represents a unique developmental origin that may determine different models of coronary vessels formation. However, there is controversy over whether VE or SV is the major source for most coronary vessels. Wu’s study concluded that ventricular endocardial cells invade the compact myocardium to form the majority of intramyocardial coronary vessels, whereas other previous studies suggested that subepicardial vessels originally from the SV invade the compact myocardium to form the majority of coronary vessels. The 2 different tissue origins thus lead to two inverse models of coronary vessel formation in the compact myocardium.
Figure 7. Intersectional genetic lineage tracing reveals requirement of SV labeling for tracing coronary vessels. A and B, Immunostaining for tdTomato and PECAM on E10.5 and E11.5 embryonic hearts. SV was not significantly labeled in Nfatc1-Dre;Npr3-CreER;Ai66 embryos. Arrowheads indicate SV endothelial cells sprouting into atrioventricular groove (*). C and D, Immunostaining for tdTomato and PECAM on E13.5 and E15.5 embryonic hearts. Coronary vessels (arrowheads) in the compact myocardium (Comp) of ventricular free walls were not significantly labeled in Nfatc1-Dre;Npr3-CreER;Ai66 embryos. Scale bars, 100 μm. E, Cartoon figure showing dis-labeling of SV results in the majority of coronary vasculature unlabeled in lineage tracing. A indicates atrium; Cu, cushion; L, liver; LV, left ventricle; Npr3, natriuretic peptide receptor 3; PECAM, platelet/endothelial cell adhesion molecule 1; SV, sinus venosus; Trab, trabecular myocardium; V, ventricle; and VS, ventricular septum.
By Nfatc1-Cre lineage tracing, Wu et al interpreted that the majority of coronary arteries in the ventricular free walls were from VE. However, this conclusion is not substantiated because Nfatc1-Cre also labels the SV extensively. We confirmed that Nfatc1 constitutive recombinase labeled both VE and SV efficiently. Our study showed that Nfatc1 was expressed in the SV of E8.0 to E8.5 embryos, which explains why the SV is labeled by Nfatc1-Cre or Nfatc1-Dre at later stages even when the Nfatc1 gene itself is no longer expressed. In addition, interpretation of Nfatc1-Cre lineage tracing hinges on an absence of Cre expression in coronary vascular ECs. We found that Nfatc1 was expressed in coronary vascular ECs, in agreement with previous report.13 Such that recombinase expression, even in a trace amount that is hard for detection, in coronary vascular ECs might genetically label vessels in situ. Although we could not infer from this expression data whether Nfatc1-Cre recombination events do take place in mature ECs, it is more cautious to perform inducible lineage tracing, such as with CreERT2, to label candidate precursor cells before coronary vessels start to form.

In this study, we also identified a new endocardial gene, Npr3, which is specifically expressed in VE but not SV. Taking advantage of this unique expression pattern, we performed lineage tracing based on Npr3-CreER tools. Npr3-CreER distinguishes VE from SV, thus eliminating the confounding labeling issue associated with Nfatc1-Cre. Npr3 is expressed in almost all Nfatc1+ ventricular endocardial cells, and the genetic labeling of VE by Npr3-CreER is efficient in our study for subsequent analysis. Our lineage tracing data showed that only a minority of coronary vessels is derived from Npr3-CreER-labeled VE cells. As an internal control, we did find that Npr3-CreER-labeled VE cells contribute to the majority of coronary vessels in the interventricular septum, suggesting that Npr3-CreER-labeled VE cells do contribute to coronary vessels (septal vessels), but in a specific compartment of developing heart.7 Although the VE alone minimally contributes to coronary vessels in ventricular free walls, the VE and SV together (ie, the Nfatc1+ lineage) contribute to the majority of coronary vessels (Figures 1 and 2). By using a dual recombinase-based intersectional lineage tracing strategy, we could subtract the
SV from the Nfatc1+ recombination domain and compare its fate with that of labeled Nfatc1+ SV. However, direct demonstration of coronary vessel formation from SV ECs requires usage of an inducible Cre line that specifically label SV but not endocardial cells, such as Apj-CreER. Interestingly, we also found that Npr3 is expressed in a minority of epicardial cells but not proepicardial cells. Proepicardium/epicardium has been reported to contribute to a substantial number of coronary vessels in the chicken model. However, Cre-based lineage tracing in mouse studies showed that at most a small fraction of coronary vascular ECs arise from (pro)epicardium, in this study, although a few epicardial cells were labeled by Npr3-CreER, Npr3+ cells (including Npr3+ epicardial lineage) minimally contributed to coronary vessels in theventricular free walls. Taken together, our data are consistent with previous work suggesting the SV as the major source for coronary vessels in the ventricular free walls.

In addition to concluding a VE source for coronary arterial endothelium, Wu et al also noted a requirement for vascular endothelial growth factor A (VEGF) signaling in coronary arteriogenesis (VEGFR2 deletion by Nfatc1-Cre) and interpreted this requirement as a specific program associated with coronary artery formation from the VE. Because our study suggests that the majority of the coronary endothelium in ventricular free walls is derived from the SV, the role of VEGF signaling in this process must be reconsidered. Coronary ECs from the SV migrate to the atrioventricular groove and form an immature subepicardial vascular plexus over the ventricular surface during the E11.5 to 13.5 period. A maturation process then results in the differentiation of this plexus into arteries and veins. Plexus maturation and arterial differentiation are both dependent on VEGF signaling, which explains the requirement for myocardial–endothelial VEGF signaling observed by Wu et al.

It remains intriguing why 2 different coronary developmental programs coexist, originating from 2 distinct sources with inverse distribution in the developing heart. Our data suggest that sprouting from the SV is the primary program that supports early coronary vasculogenesis. The SV is located on the dorsal side of the heart facing the atrioventricular groove, and from the atrioventricular groove, SV-derived ECs form a vascular plexus over the surface of the ventricle in a dorsal to ventral direction. This plexus then differentiates into the coronary arteries and veins in the ventricular walls. Because the ventricular septum facing the ventral side of the heart is the most distant to the origin of these coronary endothelium, this part might otherwise remain undervascularized. Compaction of trabecular myocardium into the ventricular septum and conversion of VE to coronary endothelium might therefore have arisen as an alternate way to initiate coronary perfusion through the septum.

A similar program of trabecular compaction associated with conversion of VE to coronary endothelium seems to also occur in the ventricular free wall at perinatal stage. Taken together, our study suggests that 2 different programs, each based on a different embryonic lineage origin, form the coronary vascular network in different compartments of the developing heart. Derivation from the SV is the major source of both arterial and venous coronary vascular ECs in the embryonic ventricular free walls.

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Disclosures

None.

References

Novelty and Significance

**What Is Known?**
- Coronary endothelial cells have multiple developmental origins.
- Sinus venous and ventricular endocardium are considered 2 principal sources.
- Ventricular endocardium is reported to contribute to the majority of coronary arteries in embryonic ventricular free walls.

**What New Information Does This Article Contribute?**
- **NFATC1** is expressed in sinus venous endothelial cells at early embryonic stages.
- **Ventricular** endocardium minimally contributes to coronary vessels in embryonic ventricular free walls.
- Nfatc1-Cre genetic tagging identifies sinus venous as a major source of coronary vessels in embryonic ventricular free walls.

Coronary artery disease causes myocardial infarction and heart failure, making it the leading cause of death worldwide.

Understanding the developmental origin of coronary artery formation could inspire novel regenerative treatments for cardiac diseases. However, the developmental origin(s) of coronary arteries has been debated for several decades. Previous studies have identified endocardium as a major source of coronary arteries in the embryonic heart and have shown separate origin of coronary arteries and veins in the mouse heart. The findings of the present study show that endocardium is not the major source for coronary vessels in the embryonic ventricular free walls. Instead, sinus venous contributes to the majority of coronary vessels (arteries and veins alike) in ventricular free walls. The findings could help to resolve the recent controversy over the developmental origin(s) of coronary arteries in embryonic ventricular free walls, providing the basis for studying coronary vessel formation and regeneration after injury.
Endocardium Minimally Contributes to Coronary Endothelium in the Embryonic Ventricular Free Walls
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Supplemental Materials

Endocardium Minimally Contributes to Coronary Endothelium in the Embryonic Ventricular Free Walls

A. Detailed Materials and Methods
B. Supplemental References
C. Supplemental Figures (Online Figure I – IX)

A. Detailed Materials and Methods

Animal Breeding and Genotyping

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Science. Tnt-Cre, Tie2-GFP, Rosa26-mTmG, R26-tdTomato, Ai66, Apln-GFP, Apln-LacZ, Apln-CreER, Fabp4-Cre, Ai47, Rosa26-loxp-LacZ, Rosa26-rox-LacZ, CAG-Dre and Nfatc1-Dre mouse lines were reported previously.1-6 The R26-RSR-tdTomato mouse line was generated by crossing Ai66 mouse with ACTB-Cre,7 which removed the transcriptional stop cassette flanked by two LoxP sites. Nfatc1-Cre knock-in mouse line was generated by targeting exactly the same genomic locus of Nfatc1 as described previously.8 Briefly, Nfatc1-Cre mouse line was generated by homologous recombination using Red/ET recombineering,9 and the knock-in strategy inserted IRES-Cre cassette into XhoI in the Nfatc1 3’ UTR. After G418 selection, over 180 clones were selected for retrieval of genomic DNA and screening of positive clones. To screen the correct targeted clones, we used long PCR assays with primer pairs spanning the targeting vector and flanking genomic DNA. After confirmation of correct targeting and karyotype, we expanded three positive ES clones and injected ES cells into blastocyst for mice generation. The obtained chimeric mouse lines were crossed to C57B/6 lines for germ-line transmission. Nfatc1-Dre and Nfatc1-GFP mice lines were generated by homologous recombination using CRISPR/Cas9 methods.6 For Nfatc1-Dre, Dre recombinase cDNA followed by a polyadenylation
sequence was inserted into the 9th coding exon of Nfatc1, and a 2A peptide sequence was used to link Nfatc1 coding region and Dre cDNA allowing expression of both Nfatc1 and Dre. For Nfatc1-GFP mouse line, the cDNAs encoding GFP and Cre fusion protein and 2A peptide were inserted into the same sites as Nfatc1-Dre. Npr3-CreER and Npr3-GFP were generated by conventional homologous recombination in ES cells and subsequent blastocyst injection of correctly targeted ES cell clones. For Npr3-CreER, a cDNA encoding Cre recombinase fused with a mutant form of the estrogen receptor hormone-binding domain (CreER\textsuperscript{12}) was inserted into frame with the translational start codon of the Npr3 gene.\textsuperscript{10} For Npr3-GFP, we generated this knock-in line by inserting a cDNA encoding GFP and Cre fusion protein into the last coding exon of Npr3. In this line, 2A peptide was used to link Npr3 coding region and GFP cDNA allowing expression of both endogenous Npr3 and inserted GFPCre cDNA. All mice lines were maintained on a C57BL6/ICR background. The Nfatc1-Cre, Nfatc1-Dre, Nfatc1-GFP, Npr3-CreER and Npr3-GFP mouse lines were generated by Shanghai Biomodel Organism Co., Ltd. Genomic DNA for genotyping was prepared from embryonic yolk sac or mouse tail. Tissues were lysed by incubation with Proteinase K overnight at 55\textdegree C. DNA was precipitated by adding isopropanol, and washed in 70% ethanol. Detailed information for genotyping primers for each mouse line could be provided upon request.

**Immunofluorescent Staining**

We performed immunostaining according to the previous protocols.\textsuperscript{11} Briefly, embryos or embryonic hearts from timed pregnant mice were collected and washed in PBS, and then fixed in 4% paraformaldehyde (PFA) for 15 minutes to 30 minutes based on the size of tissues. After three times washing in PBS, embryonic hearts with fluorescence reporters were observed and photographed using fluorescence microscopy (Zeiss AXIO Zoom. V16). The embryos or hearts were incubated in 30% sucrose in PBS and embedded in optimum cutting temperature, O.C.T. (Sakura). Cryosections, collected at 8-10 \textmu m thickness, were air dried for 1 hour at room temperature, and then were blocked with blocking buffer (5% donkey serum, 0.1% Triton X-100 in PBS) for 30 minutes at room temperature. Primary antibodies were incubated overnight at 4\textdegree C. The following primary antibodies were used: RFP/tdTomato (Rockland, 600-401-379, 1:1000), RFP/tdTomato
(ChromoTek, ABIN334653, 1:200), Pecam (BD, 553370, 1:500), GFP (Nacalai Tesque, 04404-84, 1:100), CDH5 (R&D, AF1002, 1:100), ESR (Abcam, ab27595, prediluted), FABP4 (Abcam, ab13979, 1:500), NKX2-5 (Santa Cruz Biotech, sc-8697, 1:100), TNNI3 (Abcam, ab56357, 1:100), CX40 (Alpha Diagnostic, CX40-A, 1:100), NFATC1 (Santa Cruz Biotech, sc-7294, 1:100), TBX18 (Santa Cruz Biotech, sc-17869, 1:100). Fluorescence-conjugated Alexa secondary antibodies (Invitrogen) were used at the dilution of 1:1000 in blocking buffer, and sections were incubated with secondary antibodies for 30 minutes at room temperature. For weak signals, we used HRP-conjugated second antibodies (Vector) with Tyramide Signal Amplification kit (TSA, PerkinElmer) to develop the signals. After washing in PBS three times, sections were counterstained with DAPI (Vector lab). Pictures were acquired by Olympus confocal microscope (FV1000) or Zeiss confocal microscope (LSM510).

**Whole Mount Immunostaining**

We performed whole mount immunostaining as described previously. Embryos were collected in PBS and fixed in 4% PFA at 4°C overnight. After washing in PBS, embryos were dehydrated through a methanol gradient (25, 50, 75 and 100% methanol in PBS) for 15 minutes each at room temperature. Then the embryos were rehydrated through 100, 75, 50, and 25% methanol in PBS for 15 minutes each at room temperature. Followed by washing in PBS for 10 minutes, embryos were blocked in blocking solution (5% donkey serum, 0.1% Triton X-100 in PBS) for 1 hour at 4°C. Primary antibodies diluted in blocking solution were incubated overnight at 4°C. Then the embryos were washed in PBT (0.1% Triton X-100 in PBS) for 4 hours at 4°C and left in PBT overnight with constant rotation. After incubation with second antibodies for 2 hours at room temperature, the embryos were washed in PBT for 4 hours. Primary antibodies were used as listed: NKX2-5 (Santa Cruz, sc-8697, 1:100), TNNI3 (Abcam, ab27595, 1:100), GFP (Invitrogen, A21311, 1:100). Donkey anti-goat 555 (Invitrogen, A21432, 1:250) and Donkey anti-rabbit 488 (Invitrogen, A21206, 1:250) were used as the second antibodies. Images were acquired by Leica stereomicroscope (M165FC).

**In Situ Hybridization**
In situ hybridization was performed as previously described. For whole mount in situ hybridization, dissected embryos or hearts were fixed in 4% PFA for 2 hours or overnight at 4°C. After washing in DEPC treated PBS, embryos or hearts were dehydrated through a methanol gradient (25, 50, 75, 100% methanol in DEPC treated PBS) for 5 minutes each on ice with rotation. Then the embryos or hearts were rehydrated through 100, 75, 50, and 25% methanol and rinsed in DEPC treated PBS. After bleached in 6% hydrogen peroxide that was diluted in DEPC treated PBS, embryos or hearts were treated with 20 µg/ml Proteinase K for 10 to 30 minutes at room temperature. Followed by washing three times in PBS for 5 minutes each time, embryos or hearts were re-fixed in 0.2% glutaraldehyde and 4% PFA for 20 minutes at room temperature. Then embryos or hearts were incubated in hybridization solution containing 1 µg/ml probes overnight at 70°C. After washing in different buffers to remove redundant probes, embryos or hearts were incubated with preabsorbed anti-digoxigenin antibodies (Roche) overnight at 4°C. BM purple (Roche, 11442074001) was used to develop the color. For in situ hybridization on cryosections, embryos or hearts were sectioned at 8-µm thickness. Slides were hybridized overnight with 1 µg/ml probes. After washing in SSC buffer and treated with ribonucleases at 37°C for 30 minutes, slides were incubated with alkaline phosphatase coupled anti-digoxigenin antibodies (Roche) overnight. BCIP/NBT (Promega, S3771) were used to develop the color in dark to the desired extent. Then slides were mounted with glycerol and images were acquired by Olympus microscope (BX53). The following primers were used to generate Npr3 probes: forward, 5’-GGAGGAGAGGAGACAAACACGAC-3’, reverse, 5’-GGACAGAGGACGACAGAAAAACG-3’.

**Fluorescence Activated Cell Sorting (FACS) and Reverse Transcription PCR (RT-PCR)**

We isolated cardiomyocytes from Tnt-Cre;Rosa26-mTmG mouse hearts; coronary vascular endothelial cells from Apln-CreER;Rosa26-mTmG mouse hearts; pan-endothelial cells from Tie2-GFP mice. Fluorescence labeled cells were sorted by FACS Aria Cell Sorter (BD) at core facility of the Institute for Nutritional Science according to
previous protocols. RNA was extracted with Trizol according to the manufacturer’s instruction (Invitrogen) and converted the RNA to cDNA using Prime Script RT kit (Takara). For controls, RNA was added into reverse transcription buffer without reverse transcriptase. Primers used were listed as following: Gapdh (Forward, 5’-GAAGGGCTCATGACCACAG-3’, reverse, 5’-GATGCAGGGATGATGTTCTG-3’), Nfatc1 set 1 (Forward, 5’- CTGGCCATAACTTTCTGCAA-3’, reverse, 5’-CTTCCATCTCCAGACGTG-3’), Nfatc1 set 2 (Forward, 5’-ACCTGTGCAAGCCAAATTC-3’, reverse, 5’-ACGCTGGTACTGGCTTCTCT-3’).

**Single Cell Preparation and Real-time PCR Analysis**

Single cell preparation and analysis were performed as described previously. Mouse embryos were harvested from pregnant CD1 female mice at 14.5 days post coitum and their hearts were treated with trypsin to dissociate into single cells. Each kind of target cells was isolated with FACS based on the expression level of CD31 (BD, 558737) and THY1 (eBioscience, 17-0902-81) (EDCs: CD31+; FB: THY1+; CM: CD31-/THY1-; SMC: aorta derived CD31- cells). Single cells were then manually pipetted into one well of 96-well plate at one cell per well in lysis buffer. For pre-amplification, each plate underwent reverse transcription and PCR amplification with the following conditions: 50°C for 15 min, 70°C for 2 min, 20 cycles of 95°C for 15s and 60°C for 4 min. After pre-amplification, the DNA products were diluted 5-fold with Tris-EDTA and used as templates for microfluidic PCR reactions (48x48 Fluidigm Dynamic Arrays) using lineage-specific gene primers.

**RNA-sequencing Preparation and Real-time qPCR Analysis**

For isolating endocardial cells, tamoxifen was administrated to Npr3-CreER;Ai47 at E10.5 and embryonic hearts were collected at E14.5. For isolating coronary endothelial cells, hearts were harvested from Fabp4-Cre;Ai47 at E14.5. Ventricles were then minced with fine scissors. After digestion in lysis buffer containing collagenase, trypsin and DNase I, supernatant was collected and filtered through a 70 µm disposable cell strainer. Then isolated cells were pelleted and re-suspended in PBS, which are ready for sorting by FACS (BD Aria IIu Cytometer). After GFP positive cells were isolated by flow cytometry,
RNA was extracted with Trizol according to the manufacturer’s instruction (Invitrogen). RNA-sequencing and analysis were carried out by Beijing Genomics Institute (BGI). We converted the RNA to cDNA using Prime Script RT kit (Takara). SYBR Green qPCR master mix (Applied Biosystems) was used and cDNA was amplified on a StepOnePlus™ real-time PCR system (Applied Biosystems). Detailed information for qRT-PCR primers could be provided upon request.

**Whole Mount X-gal Staining**

Whole mount x-gal staining was performed as protocols described previously. Briefly, embryos from timed pregnancies were fixed in 2% PFA and 0.2% glutaraldehyde in PBS for 30 minutes, and then fixed in LacZ fix solution (0.2% glutaraldehyde, 5mM EGTA, 100mM MgCl2 in PBS) for another 30 minutes. After washing in LacZ washing buffer (2mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40 in 100mM sodium phosphate buffer) for 15 minutes, embryos were incubated in LacZ staining buffer (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) in LacZ washing buffer) for 1 hour at 37°C. Images were acquired by Leica stereomicroscope (M165FC).

**Statistical Analysis**

All data were collected from at least 3 independent experiments as indicated. For analysis of contribution of endocardial cells to coronary endothelial cells, we collected four heart samples for each experiment. 10 tissue sections from each heart were collected, and 4 fields in each section were taken images for analysis. The labeling percentage was then calculated as tdTomato+PECAM/CDH5+ cells in total PECAM/CDH5+ endothelial cells. Quantification was performed by a lab member who was blinded to the sample identification. Data were presented as mean values ± s.e.m. Statistical comparisons between data sets were done by a two-side unpaired Student’s t test for comparing differences between two groups. P < 0.05 was considered to be statistically significant.

**B. Supplemental References**


Online Figure I. Nfatc1-Cre labels both endocardium, SV and coronary endothelium. A, Schematic showing two contrasting models for explanation of coronary artery formation in developing heart. LV, left ventricle; Epi, epicardium. B, Whole-mount and bright-field fluorescence views showing tdTomato labeling of E13.5 Nfatc1-Cre;R26-tdTomato heart. Magnification image shows labeled SV. C, Immunostaining for tdTomato, FABP4 and CDH5 on heart sections shows Nfatc1-Cre labels both endocardium (arrows) and coronary vessels (arrowheads). Scale bars, 500 μm.
Online Figure II. Expression of Nfatc1 in sinus venosus of E8.0 - E8.5 hearts and coronary vasculature in E15.5 hearts. A, Whole-mount view of E8.0-E8.5 Nfatc1-GFP embryos stained with GFP and cardiac markers NKX2-5 and TNNI3. B, Immunostaining for NKX2-5 or TNNI3 and GFP on Nfatc1-GFP embryonic sections shows Nfatc1 expression in right horn of SV. C, Immunostaining for GFP and CDH5 on E15.5 Nfatc1-GFP hearts shows GFP is expressed in a subset of coronary vessels (arrows). D, Immunostaining for NFATC1 and CDH5 on E15.5 wildtype mouse hearts shows NFATC1 is expressed in a subset of coronary vessels (arrows). E, RT-PCR of Nfatc1 expression in isolated coronary vascular endothelial cells (Coronary EC, samples 1 - 8), cardiomyocytes (CM, samples 9,10) or pan-endothelial cells (Pan EC, samples 11-14). Coronary EC was collected from Apin-CreER;Rosa26-mTmG mice (E14.5, tamoxifen was administered at E10.5); CM was collected from Tnt-Cre;Rosa26-mTmG mice; Pan EC was collected from Tie2-GFP mice. Scale bars, 100 μm; LV, left ventricle; SV, sinus venosus.
Online Figure III. Expression of Npr3 in early embryonic heart. A. Immunostaining for ESR and CDH5 on E8.5 Npr3-CreER sections shows ESR is expressed in atrium (a), ventricle (V) but not SV endocardium. B. Immunostaining for GFP and CDH5 on E8.5 Npr3-GFP sections shows GFP is expressed in atrium (a), ventricle (V) but not SV endocardium. Scale bars, 100 μm. Each image is representative of 3 individual samples.
Online Figure IV. Npr3 is expressed in a subset of epicardial cells but not in proepicardial cells. A,B. Immunostaining for ESR and TBX18 on E9.5 and E10.5 Npr3-CreER sections. No ESR+TBX18+ cells were detected in proepicardium (*) at E9.5 (A). A few epicardial cells express weak ESR at E10.5 (arrowheads, B). Scale bars, 100 μm.
Online Figure V. Immunostaining for ESR antibody on wild-type embryonic sections. A, B, Immunostaining for ESR and CDH5 on E9.5 and E10.5 wild-type embryonic sections. No signal was detected in ESR channel. Scale bars, 100 μm.
Online Figure VI. Coronary vascular endothelial cells are not present in atrium free wall at E12.5–E13.5. A, Immunostaining for GFP and PECAM on E12.5 Apln-GFP embryonic heart. Arrowheads indicate coronary vascular endothelial cells in the ventricle. B, Whole-mount X-gal staining of E12.5 Apln-LacZ heart. C, Immunostaining for ESR on E12.5 Apln-CreER heart. Arrowheads indicate coronary vascular endothelial cells in the ventricle. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; SV, sinus venosus. D, Immunostaining for tdTomato and FABP4 on E13.5 Npr3-CreER,R26-tdTomato embryonic section. Npr3-CreER labelled cells in right and left atrium are not FABP4+ coronary vessels. Scale bars, 100 µm. Each image is representative of 3 individual samples.
Online Figure VII. *Npr3-CreER* labeled cells in developing heart. A. Whole mount view of *Npr3-CreER;R26-tdTomato* heart and immunostaining of tdTomato and FABP4 on sections. Yellow arrowheads indicate tdTomato+ coronary VECs and white arrowheads indicate tdTomato+ epicardial cells. RV, right ventricle; VS, ventricular septum; LV, left ventricle; Epi, epicardium. B. Consecutive sections stained with tdTomato and FABP4 from dorsal side to ventral side. Scale bars, 100 μm.
Online Figure VIII. Contribution of Npr3+ endocardial cells to coronary vasculature. A, Whole-mount fluorescence and bright-field view of E15.5 Npr3-CreER;R26-tdTomato hearts. Dotted lines outline the epicardium. B, Immunostaining for tdTomato and FABP4 on E15.5 Npr3-CreER;R26-tdTomato heart sections. Boxed regions are magnified in C. C, Magnified regions of right ventricle (RV), ventricular septum (VS) and left ventricle (LV) showing Npr3-CreER labels most FABP4+ vessels (white arrowheads) in VS, but very few coronary vessels in RV or LV. Magenta arrowheads indicate a subset of epicardial cells labeled by Npr3-CreER. Comp., compact myocardium; Trab., trabecular myocardium; Epi, epicardium. Each image is representative of 3 individual samples. Scale bars, 500 μm in A and B; 100 μm in C.
Online Figure IX. Strategies for labeling and dis-labeling of SV from Nfatc1+ cell lineage tracing. A, Whole-mount X-gal staining of E9.5 embryos shows that Cre-loxp and Dre-rox are two exclusive recombination systems. ACTB-Cre and CAG-Dre were crossed with Rosa26-loxp-Stop-loxp-LacZ (Rosa26-loxp-LacZ) and Rosa26-rox-Stop-rox-LacZ (Rosa26-rox-LacZ). Scale bars, 0.5 mm. B, Nfatc1+ cell lineage tracing strategies with SV labeled or unlabeled. Ventricle endocardium constitutes Nfatc1+Npr3+ cells and SV constitutes Nfatc1+Npr3- cells. In Nfatc1-Dre;R26-RSR-tdTomato mice, SV is labeled by tdTomato; while in Nfatc1-Dre;Npr3-CreER;Ai66 mice, SV is unlabeled. C, Cartoon figure showing the labeling of SV by Nfatc1-Dre and dis-labeling of SV by Nfatc1-Dre;Npr3-CreER system.