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

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ABSTRACT

Rationale: There is persistent uncertainty regarding the developmental origins of coronary vessels, with two principal sources suggested as ventricular endocardium or sinus venosus (SV). These two 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 *Nfatc1-Cre* and *Nfatc1-Dre* lineage tracers for endocardium labeling. We found that Nfatc1 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.

Keywords:
Coronary development, coronary artery, coronary origin, lineage tracing, blood vessel, coronary.

Nonstandard Abbreviations and Acronyms:

VE	Ventricular endocardium
SV	Sinus venosus
CM	Cardiomyocyte
EC	Endothelial cell
SMC	Smooth muscle cell
UTR	Untranslated region
Nfatc1	Nuclear factor of activated T cells
Npr3	Natriuretic peptide receptor 3
CDH5	Cadherin 5
CX40	Gap junction protein, alpha 5
Nkx2-5	NK2 homeobox 5
TNNI3	Troponin I type 3
Pecam	Platelet/endothelial cell adhesion molecule 1
Fabp4	Fatty acid binding protein 4
ESR	Estrogen receptor
TBX18	T-Box 18
Gpr126	G protein-coupled receptor 126
Hapln1	Hyaluronan and proteoglycan link protein 1
Col23a1	Collagen type XXIII alpha 1
Aoc3	Amine oxidase, copper containing 3
Gpihbp1	GPI-anchored HDL-binding protein 1
Sema7a	Semaphorin 7A

INTRODUCTION

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 (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 prior to further maturation. Coronary veins remain in a subepicardial location on the heart surface. Studies of the origin of coronary arterial endothelium implicated two major candidate sources: ventricular endocardium (VE) and sinus venosus (SV), but there is ongoing controversy over their relative contribution.[2, 5-11] Resolving this issue is important, because these two proposed origins represent two completely different development mechanisms for coronary vessel formation. A 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, 8] 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, and which therefore have a developmental origin distinct from subepicardial veins (model 2, Online Figure IA).[7] There is consensus that both models are involved in coronary arterial formation; however, quantification by different groups reached vastly different estimates of the relative contribution of these two sources.[5, 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 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 Nfatc1-Cre lineage tracing to show that Nfatc1-derived cells constitute the majority of coronary arteries in the developing heart.[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.[13] 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.[5] 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,[8] 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.[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 IA), suggesting that the sinus venosus is the major source of coronary endothelium in the developing heart (model 1, Online Figure IA).

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 (IACUC) 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.[15] The *Nfatc1-Cre*, *Nfatc1-Dre*, *Nfatc1-GFP*, *Npr3-CreER* and *Npr3-GFP* mouse lines were generated by Shanghai Biomodel Organism Co., Ltd. All data were collected from at least 3 independent experiments as indicated, and 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.

RESULTS

Nfatc1 constitutive recombinases label both VE and SV.

In order to evaluate the lineage and fate of Nfatc1$^+$ cells, we first generated *Nfatc1-Cre* by knockin of IRES-Cre into the Nfatc1 3' UTR (Figure 1A), which targets exactly the same gene location as the mouse line reported previously.[7] We crossed *Nfatc1-Cre* with reporter line *R26-tdTomato* to permanently label Nfatc1-expressing cells and their descendants (Figure 1B). We collected E9.5 – E15.5 *Nfatc1-Cre;R26-tdTomato* embryos (Figure 1C) and performed immunostaining for genetic lineage marker tdTomato and endothelial cell marker VE-cadherin (CDH5). The tdTomato$^+$ cells, derived from the Nfatc1$^+$ precursors, are detected in most SV endothelial cells as well as VE at E9.5 and E11.5 (Figure 1D and 1E). By staining for coronary endothelial cell specific marker FABP4, we found that Nfatc1-derived cells (tdTomato$^+$) contribute to the majority of FABP4$^+$ subepicardial endothelial cells as well as intramyocardial coronary vessels in E13.5 and E15.5 embryonic heart (Figure 1F,G and Online Figure IB,C). These data demonstrated that *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, Nfatc1-Dre, for lineage tracing of Nfatc1$^+$ cells.[15] We crossed *Nfatc1-Dre* with its specific reporter line *Rosa26-Rox-Stop-Rox-tdTomato* (*R26-RSR-tdTomato*) to perform lineage tracing of Nfatc1$^+$ cells in the developing heart (Figure 2A). In E10.5 and E11.5 *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 endothelial cells at E12.5 (Figure 2E) and to the majority of intramyocardial coronary endothelial cells 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 two 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 (CX40) 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. Since both *Nfatc1-Cre* and *-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, since previous work implied that Nfatc1 mRNA and protein are not expressed in the SV.[7] It should be noted that this negative expression was based on observations of E9.5 and E10.5 embryos.[7] Since 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* knock-in 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 two horns of the SV at E8.0 – E8.5 (Online Figure IIA). In sections, it was evident that Nfatc1 (as visualized by GFP) 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 down regulated in VE at later time points after E11.5.[7, 16] 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 endothelial cells, in both subepicardial and intramyocardial 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 endothelial cells (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.[13] To independently confirm Nfatc1 expression in coronary vasculature, we isolated E14.5 coronary endothelial cells by fluorescence activated cell sorting from *Apln-CreER;Rosa26-mTmG* mice that were treated with tamoxifen at E10.5.[8] Apln is a specific marker for vascular endothelial cells, and is not expressed in endocardium.[5, 17] As a positive control, we isolated endocardial and vascular endothelial cells 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 PCR (RT-PCR) analysis by two different sets of *Nfatc1* primers showed expression of *Nfatc1* in coronary vascular endothelial cells (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 E9.5-10.5 VE, and expression in at least some coronary endothelium at E14.5 – E15.5. The previous interpretation that *Nfatc1-Cre* recombination is restricted to the endocardium, and therefore that ventricular endocardium 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 endothelial cells, we dissociated cells from E14.5 hearts, sorted cardiomyocytes (CM), pan-endothelial cells (ECs; including both endocardium and coronary endothelium) and smooth muscle cells (SMC) by flow cytometry, and performed single cell gene expression analysis using the Fluidigm platform. We could clearly distinguish these three main types of cardiovascular cells by gene expression signatures (Figure 3A). All ECs expressed the pan-endothelial markers *Pecam* and *Cdh5*. Importantly, we were able to divide the EC population into two subgroups based on expression of *Fabp4*, which is expressed in vascular ECs but not in endocardium.[18, 19] This analysis confirmed weak expression of *Nfatc1* in both Fabp4^{+} and Fabp4^{-} populations, consistent with our immunostaining and RT-PCR data (Online Figure IIC-E) and supporting the interpretation that *Nfatc1* is expressed in some coronary endothelial cells at E14.5 – E15.5.

This analysis also revealed that a gene *Natriuretic Peptide Receptor 3* (*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 and ventricular endocardium, but undetectable in sinus venosus (Figure 3B). To specifically co-stain Npr3^{+} cells with other lineage specific markers, we generated the reporter mouse line *Npr3-GFP*, in which a GFPCre fusion cDNA was knocked into the 3' UTR 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 and ventricular endocardium but not in the SV in E10.5 hearts (Figure 3D). At E12.5 and E14.5, Npr3 is expressed in endocardium but not in coronary

vascular endothelial cells 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. Since Nfatc1 is reduced in expression after E9.5, we used *Nfatc1-Dre;R26-RSR-tdTomato* to mark Nfatc1$^+$ cells and their derivatives. The rox-Stop-rox reporter is a substrate only for Dre recombinase and is not recognized by Cre recombinase.[20] 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 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,B). Altogether, the above data based on *in situ* hybridization, immunostaining, and single cell qPCR 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 endothelial cells in E14.5 – E15.5 hearts, which compromise the utility of Nfatc1 as a lineage marker informative for the origins of coronary endothelium. We tested if *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 ventricular endocardium but not SV (Online Figure IIIA,B). We next collected *Npr3-CreER* embryos from E9.5 to E11.5; immunostaining against ESR showed that Npr3 was expressed in atrial and ventricular endocardium, but not in the SV at E9.5, E10.5 and E11.5 (Figure 5A-C). In addition, Npr3 was also expressed in the endothelial cells of the dorsal aorta at E9.5 and E10.5 (Figure 5A-B, arrowheads), and a subset of non-endothelial 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,B). For technical controls, immunostaining for ESR on wild-type embryonic sections showed no detectable signals (Online Figure VA,B). The point that Npr3 is expressed in VE but not SV is critical for this study, as 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 endothelial cells in the atrioventricular groove or in the compact myocardium, and 73.13 ± 6.53 % VE cells were labeled while 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, while the SV and coronary endothelial cells 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 – 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 endothelial cells. We labeled Npr3$^+$ VE by tamoxifen induction at E8.5 and checked the coronary vessels in the compact myocardium of E12.5 – E15.5 hearts. Immunostaining for tdTomato and PECAM on E12.5 hearts showed that tdTomato$^+$PECAM$^+$ endothelial cells constitute a 4.56 ± 2.20% of subepicardial coronary vessels in the ventricular wall (Figure 6A). We also found that 80.21 ± 14.21% endocardial cells are labeled in the same hearts (Figure 6A). Npr3-CreER also labels atrial endocardial cells and there was almost no coronary vessels detected in the atrial free wall at this stage (Online Figure VI). We next collected E14.5 *Npr3-CreER;R26-tdTomato* hearts 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 while most VE cells are labeled, only a few coronary endothelial cells in the ventricular free walls are tdTomato positive (Figure 6C and 6D). Due to sectional orientation and the deep ingression of some trabecular lacunae into the compact zone, some of the PECAM$^+$ endocardium is ambiguous in morphology from coronary vascular endothelial cells, possibly causing some VE cells to be mistakenly interpreted as coronary vessels. To avoid this possible caveat in coronary vessel quantification, we employed the coronary vascular endothelial specific marker FABP4.[18, 19] 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 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 while Npr3-CreER robustly labels ventricle endocardium, it labels significantly fewer coronary vessels in the right and left ventricle wall (Figure 6G). However, we did find that Npr3$^+$ VE 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 VIIB). 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 endocardial 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$^+$ while 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-Loxp-tdTomato* (*Ai66*),[21] we established a strategy that labels the Nfatc1$^+$ VE only and dis-labels (subtracts labeling of) Nfatc1$^+$ SV.[22] Dre and Cre are two 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,C). In E10.5 and E11.5 *Nfatc1-Dre;Npr3-CreER;Ai66* hearts following tamoxifen injection at E8.5, the Nfatc1$^+$Npr3$^+$ VE was labeled, while 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 endothelial cells in the ventricular free walls showed that Nfatc1$^+$ cells (VE + SV cells) contributed to 77.81 ± 5.62% coronary vessels while Nfatc1$^+$Npr3$^+$ VE cells contributed to 3.13 ± 0.72% coronary vessels. These results are consistent with a sinus venosus origin of endothelium forming the coronary system.[5] Our study reinterprets the contribution of Nfatc1$^+$ endocardial 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 study[7] could be a better explanation for the origins of the coronary endothelium in the embryonic ventricular free walls.

Identification of other markers for endocardium or coronary endothelium.

Endocardial cells and coronary endothelial cells are two distinctive types of endothelial cells with different molecular profiles, as endocardial cells express relatively high level of Nfatc1, Npr3 while coronary endothelial cells 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 endocardial 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 endothelial cells were labeled by GFP at E14.5 (Figure 8D). We isolated the GFP^+ cells and GFP^- cells from *Npr3-CreER;Ai47* or *Fabp4-Cre;Ai47* ventricles by flow cytometry and collected RNA from purified cells for gene analysis (Figure 8B,E). qRT-PCR of conventional pan-endothelial cell markers *Pecam*, *Cdh5* and *Flk1* showed that these genes were highly enriched in GFP^+ populations compared with GFP^- populations (Figure 8C,F), confirming the isolation of endothelial cells in high purity. We next performed RNA-sequencing on endocardium-enriched and coronary endothelium-enriched populations, and compared the differentiated gene expression between two GFP^+ populations (Figure 8G and 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* et al. are significantly higher in endocardium samples while the expression of *Aoc3*, *Gpihbp1*, *Sema7a* et al. 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 ventricular endocardium, *Nfatc1-Cre* or *Dre* labels both sinus venosus and subepicardial coronary vessels. (2) The newly identified marker Npr3 is co-expressed with Nfatc1 in ventricular endocardium, but is not expressed in the sinus venosus. (3) Ventricular endocardium contributes to only a small minority of coronary vessels in ventricular free walls of embryonic hearts. (4) The $Nfatc1^+Nrp3^-$ sinus venosus is likely the major source of coronary vasculature in ventricular free walls. (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 three important sources for coronary vessels: proepicardium, ventricular endocardium and sinus venosus.[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 ventricular endocardium or sinus venosus 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, while other previous studies suggested that subepicardial vessels originally from the sinus venosus invade the compact myocardium to form the majority of coronary vessels. The two different tissue origins thus lead to two inverse models of coronary vessel formation in the compact myocardium.

By Nfatc1-Cre lineage tracing, Wu et al. interpreted the labeling of the majority of coronary vessels in the compact myocardium as evidence that their origin was the VE. However, this conclusion is not substantiated as 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 – 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 endothelial cells. We found that Nfatc1 was expressed in coronary vascular endothelial cells, in agreement with previous report.[13] Such that recombinase expression, even in a trace amount that is hard for detection, in coronary vascular endothelial cells might genetically label vessels in situ. Although we could not infer from this expression data if Nfatc1-

Cre recombination events do take place in mature endothelial cells, 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 [5]. While the VE alone minimally contributes to coronary vessels in ventricular free walls, the VE and SV together (i.e., the Nfatc1$^+$ lineage) contribute to the majority of coronary vessels (Figure 1 and Figure 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 endothelial cells requires usage of an inducible Cre line that specifically label SV but not endocardial cells, such as Apj-CreER.[10] 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 vascular endothelial cells in chicken model.[24] However, Cre-based lineage tracing in mouse studies showed that at most a small fraction of coronary vascular endothelial cells arise from (pro)epicardium.[6, 28-32] In this study, although a few epicardial cells were labeled by *Npr3-CreER*, Npr3$^+$ cells (including Npr3$^+$ epicardial cells) minimally contributed to coronary vessels in the ventricular 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.[5]

In addition to concluding a VE source for coronary arterial endothelium, Wu et al also noted a requirement for 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 endothelial cells from the SV migrate to the atrioventricular groove and form an immature subepicardial vascular plexus over the ventricular surface during the E11.5-13.5 period. A maturation process then results in the differentiation of this plexus into arteries and veins.[14] 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 two different coronary developmental programs co-exist, originating from two 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 endothelial cells 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, which is the most distant to the origin of these coronary endothelium, 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.[10] A similar program of trabecular compaction associated with conversion of VE to coronary endothelium appears to also occur in the ventricular free wall at perinatal stage.[9] Taken together, our study suggests that two 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 endothelial cells in the embryonic ventricular free walls.

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DISCLOSURES
None.

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FIGURE LEGENDS

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,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. A, atrium, V, ventricle; SV, sinus venosus. Scale bars, 100 μm in D,E; 500 μm in F,G.

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,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 four E15.5 hearts. **H**, Immunostaining for tdTomato, PECAM with arterial marker CX40 shows Nfatc1-derived cells contribute to arterial endothelial cells (arrowheads). A, atrium; V, ventricle; SV, sinus venosus; RV, right ventricle; LV, left ventricle. Scale bars, yellow 500 μm; white 100 μm.

Figure 3. Npr3 is expressed in endocardial cells. A, A heat map of cardiovascular lineage gene expression by FACS-purified single cells from E14.5 heart. The three 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* knock-in 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 - 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 C,E-G. H, heart; LV, left ventricle; LA, left atrium; RV, right ventricle; V, ventricle; A, atrium.

Figure 4. Npr3 is expressed in Nfatc1$^+$ ventricular endocardial cells. A,B, Immunostaining of ESR, tdTomato and CDH5 on sections of E9.5 and E10.5 embryos. ESR is a surrogate for Npr3, and Nfatc1$^+$ endocardial cells are shown as tdTomato$^+$ cells from *Nfatc1-Dre* lineage tracing. Arrowheads indicate Nfatc1$^+$Npr3$^+$CDH5$^+$ endocardial cells. Scale bars, 50 μm.

Figure 5. Expression and fate map of Npr3 in VE and SV of E9.5–E11.5 hearts. A-C, Immunostaining images of CDH5 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,B indicate dorsal aortic endothelial cells, in C indicate non-endothelial mesenchymal cells. **D,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 sinus venosus (SV). Scale bars, 100 μm.

Figure 6. Npr3-CreER labeled endocardial cells contribute to the minority of coronary vessels in the ventricular free walls. A, Immunostaining for tdTomato and PECAM on E12.5 *Npr3-CreER;R26-tdTomato* heart sections. Tamoxifen was administered at E8.5. Arrowheads indicate coronary endothelial cells. Right panel shows quantification of the percentage of *Npr3-CreER* labeled coronary vessels in the ventricular free walls of E12.5 hearts. **P* < 0.05; n = 4. **B**, Whole mount fluorescence view of E14.5 *Npr3-CreER;R26-tdTomato* embryonic heart. Tamoxifen was administered at E8.5. **C**, Immunostaining for tdTomato and PECAM on heart sections. Boxed regions are magnified in D. **D**, Magnified images of right ventricle (RV), ventricular septum (VS) and left ventricle (LV) show *Npr3-CreER* labeled endocardial cells contribute to few coronary vessels in ventricular free walls. White arrowheads indicate tdTomato+ endothelial cells, and magenta arrowheads indicate tdTomato$^+$ epicardial cells. **E,F**, Immunostaining of tdTomato and FABP4 on consecutive heart section shows few coronary vessels are tdTomato$^+$ cells (white arrowhead). A subset of epicardial cells are tdTomato$^+$ (magenta arrowhead). Boxed regions in E are magnified in F. **G**, Quantification of the percentage of *Npr3-CreER* labeled coronary vessels in the left and right ventricular free walls of E14.5 hearts. Student's *t*-test was used to analyze differences, and values are shown as means ± s.e.m.; **P* < 0.05; n = 4. Scale bars, 100 μm. Endo., endocardium; Epi, epicardium; Trab, trabecular myocardium; Comp, compact myocardium.

Figure 7. Intersectional genetic lineage tracing reveals requirement of SV labeling for tracing coronary vessels. A,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 (*). Cu, cushion; A, atrium; V, ventricle; L, liver; SV, sinus venosus. **C,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. LV, left ventricle; VS, ventricular septum; Trab., trabecular myocardium. Scale bars, 100μm. **E**, Cartoon figure showing dis-labeling of SV results in the majority of coronary vasculature unlabeled in lineage tracing.

Figure 8. Transcriptional profiles of endocardial and coronary vascular endothelial cells. A,**D**, Immunostaining for GFP and PECAM on heart sections from E14.5 Npr3-CreER;Ai47 mice (A) and Fabp4-Cre;Ai47 mice (D). **B,E**, Dissociation of GFP$^+$ and GFP- cardiac cells by FACS. **C,F,** *qRT-PCR* of pan-endothelial cell markers *Pecam*, *Cdh5* and *Flk1* from GFP$^+$ and GFP$^-$ cell populations. **G**, RNA-Sequencing data showed differentiated expressed genes (difference > 2 between two groups) from GFP$^+$ cells of *Npr3-CreER;Ai47* or *Fabp4-Cre;Ai47*. **H,** Relative gene expression detected by *qRT-PCR* in comparison to *Gapdh*. Data are shown as mean ± S.E.M., n = 4, **P* < 0.05.

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Novelty and Significance

What Is Known?

- Coronary endothelial cells have multiple developmental origins.
- Sinus venosus and ventricular endocardium are considered two 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 venosus endothelial cells at early embryonic stages.
- Ventricular endocardium minimally contributes to coronary vessels in embryonic ventricular free walls.
- Nfatc1-Cre genetic tagging identifies sinus venosus 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 venosus 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.

DOI: 10.1161/CIRCRESAHA.116.308749 15

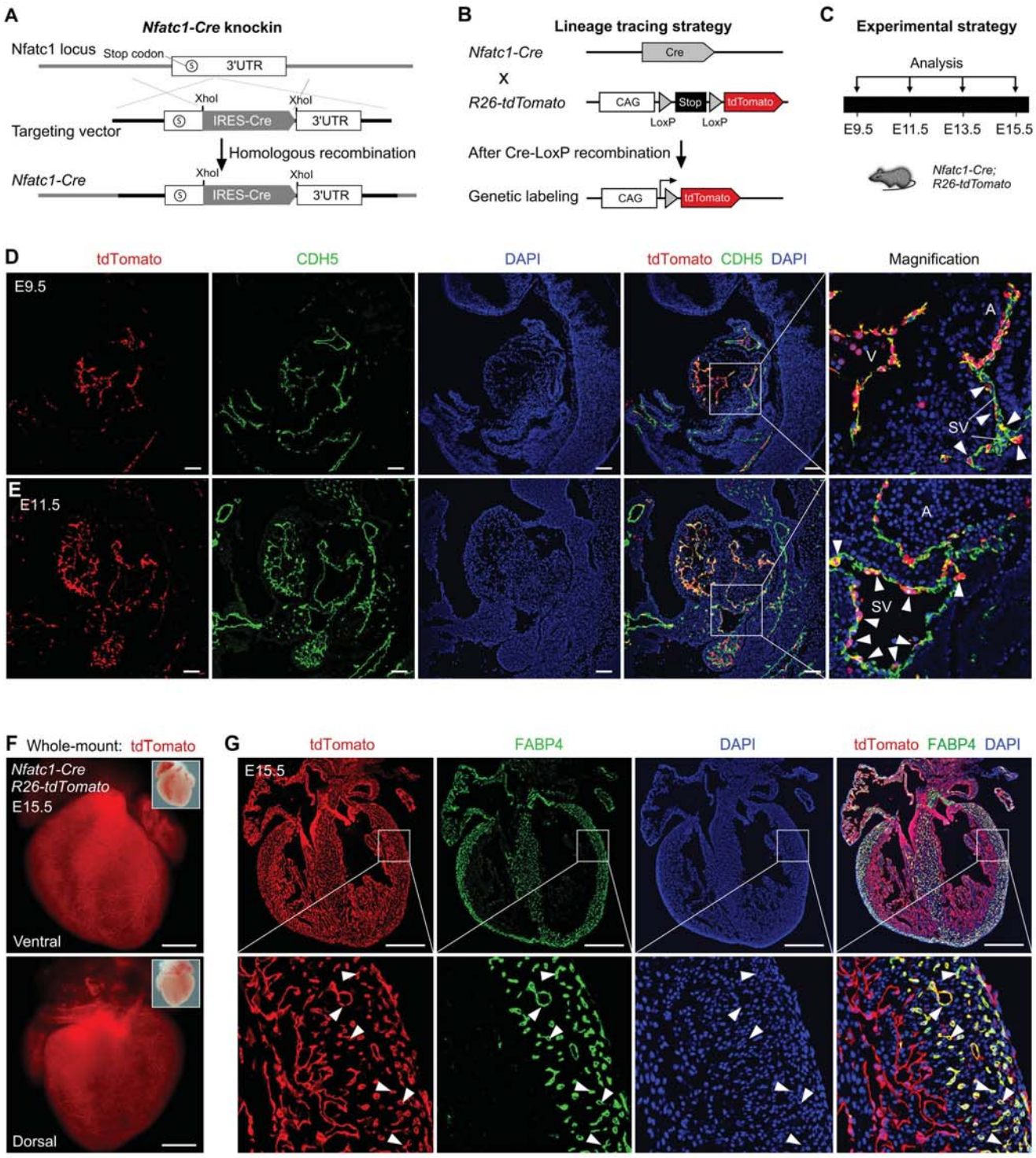
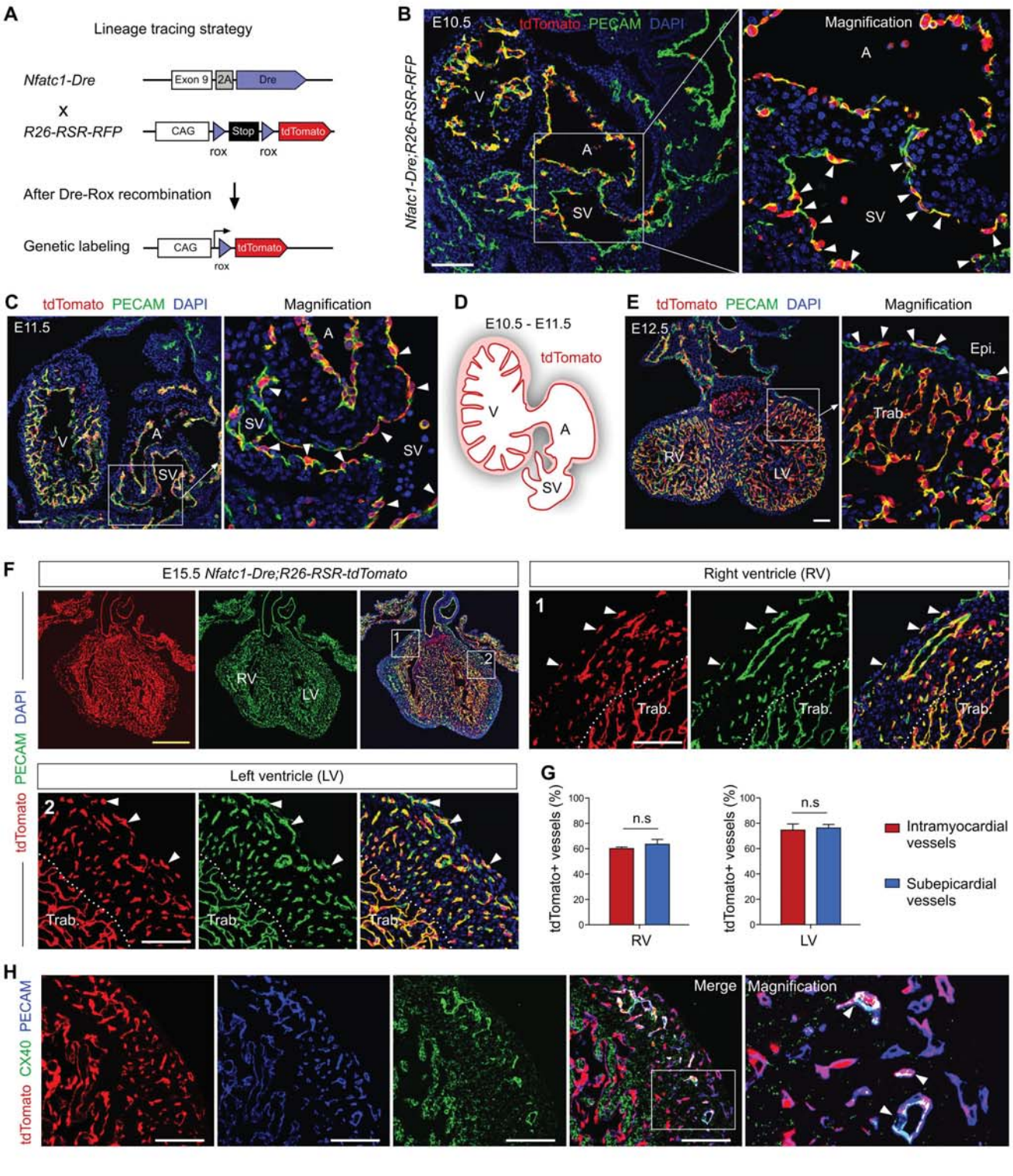


Figure 1



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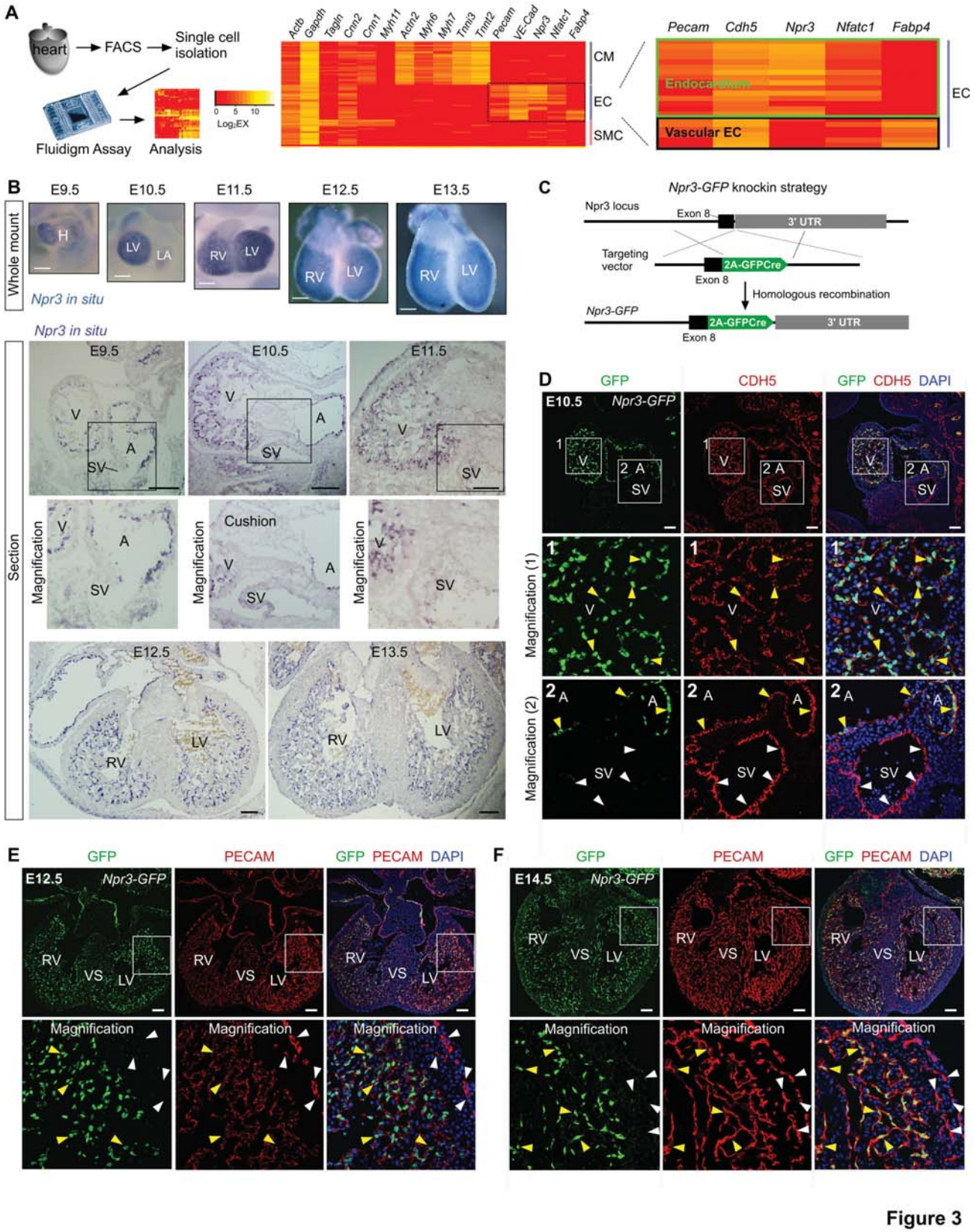

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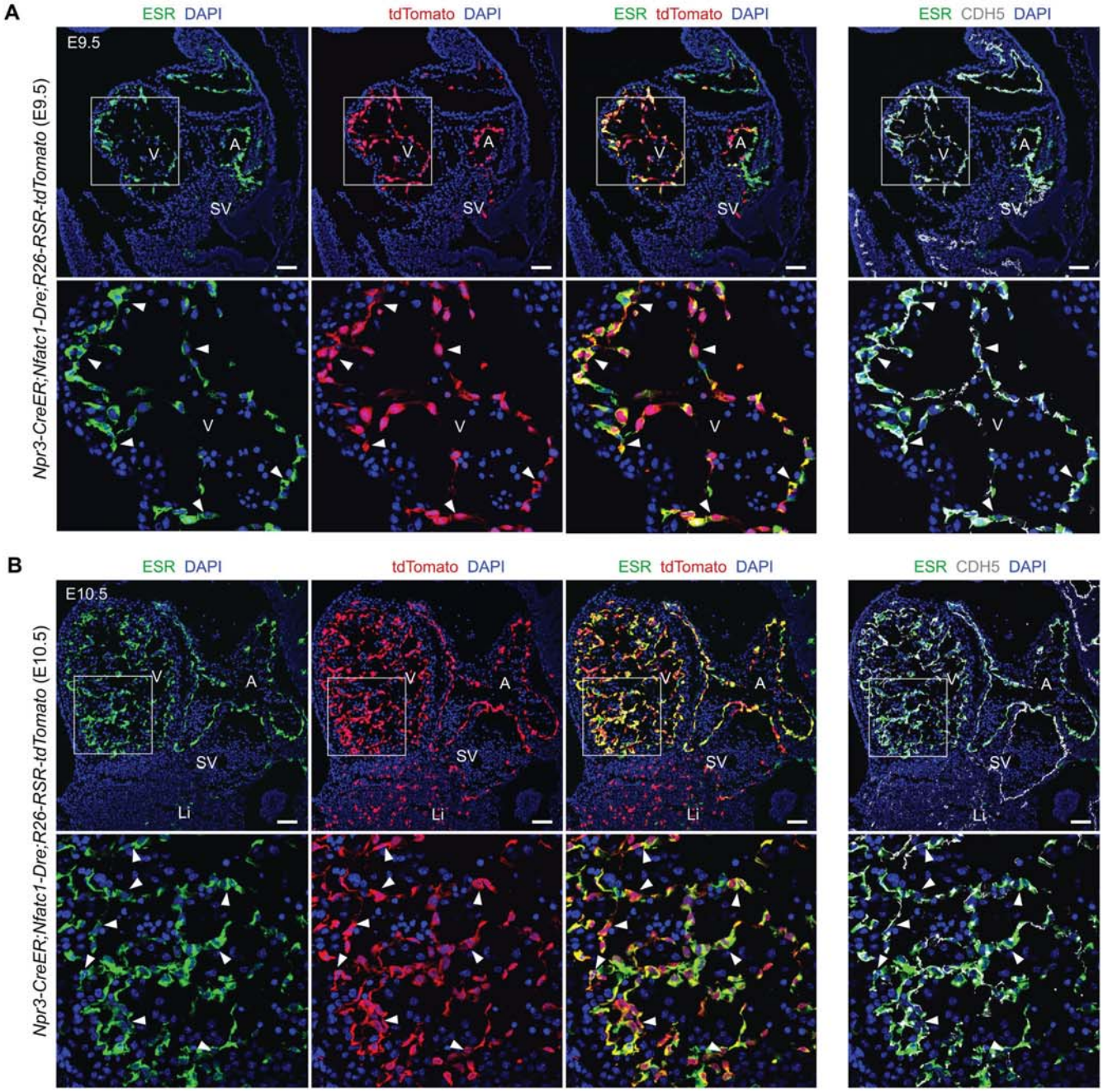

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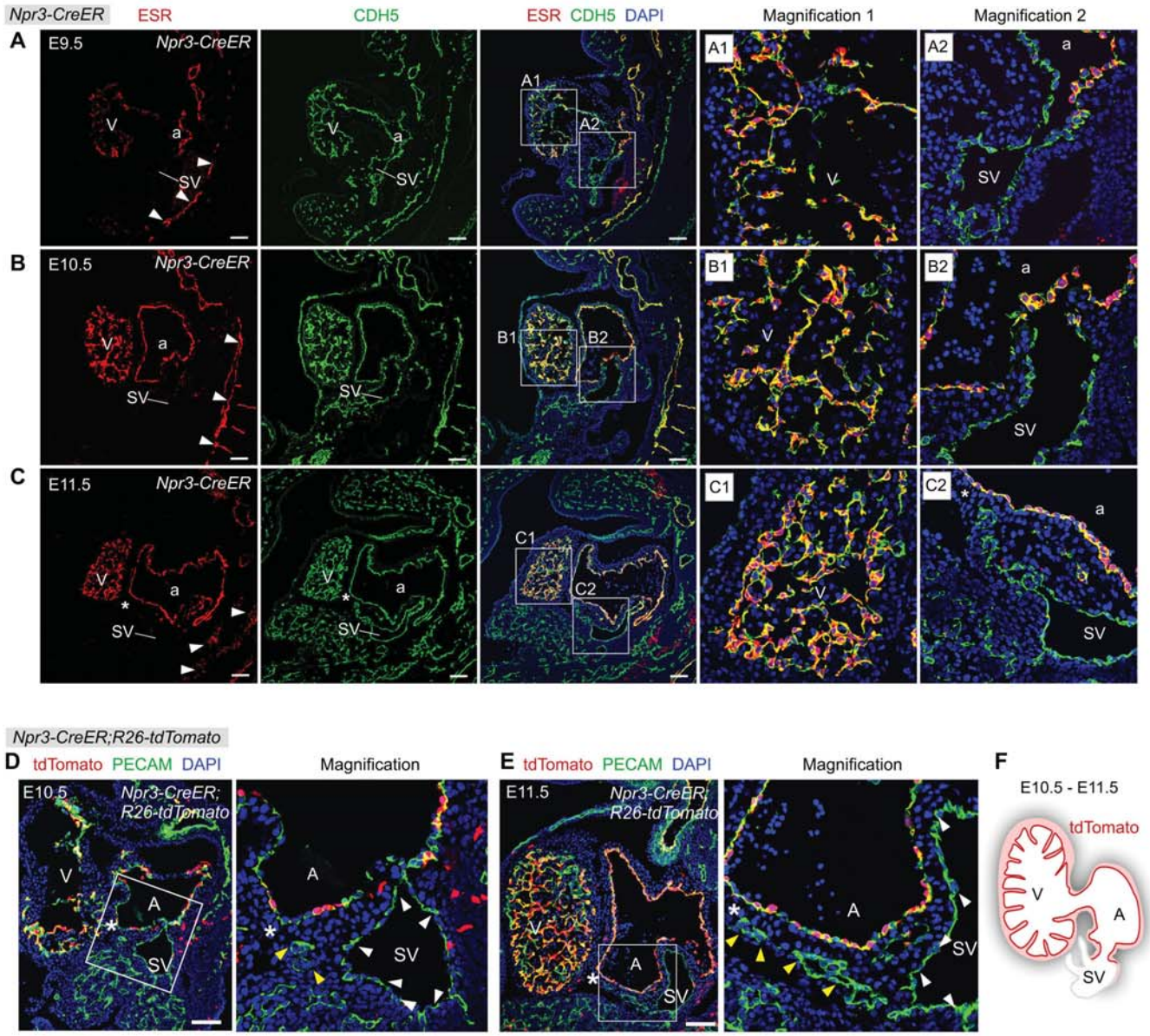

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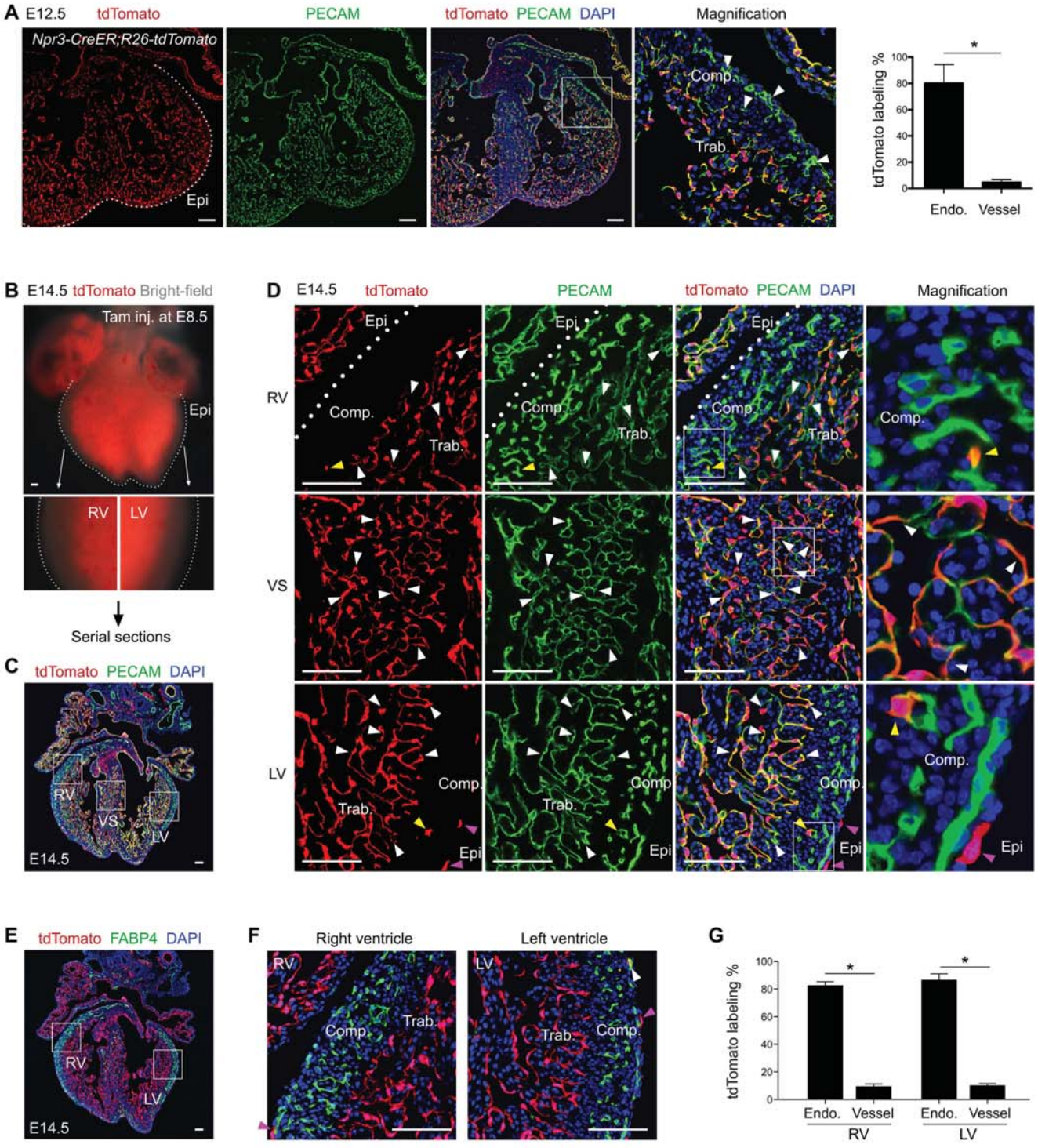

Figure 6

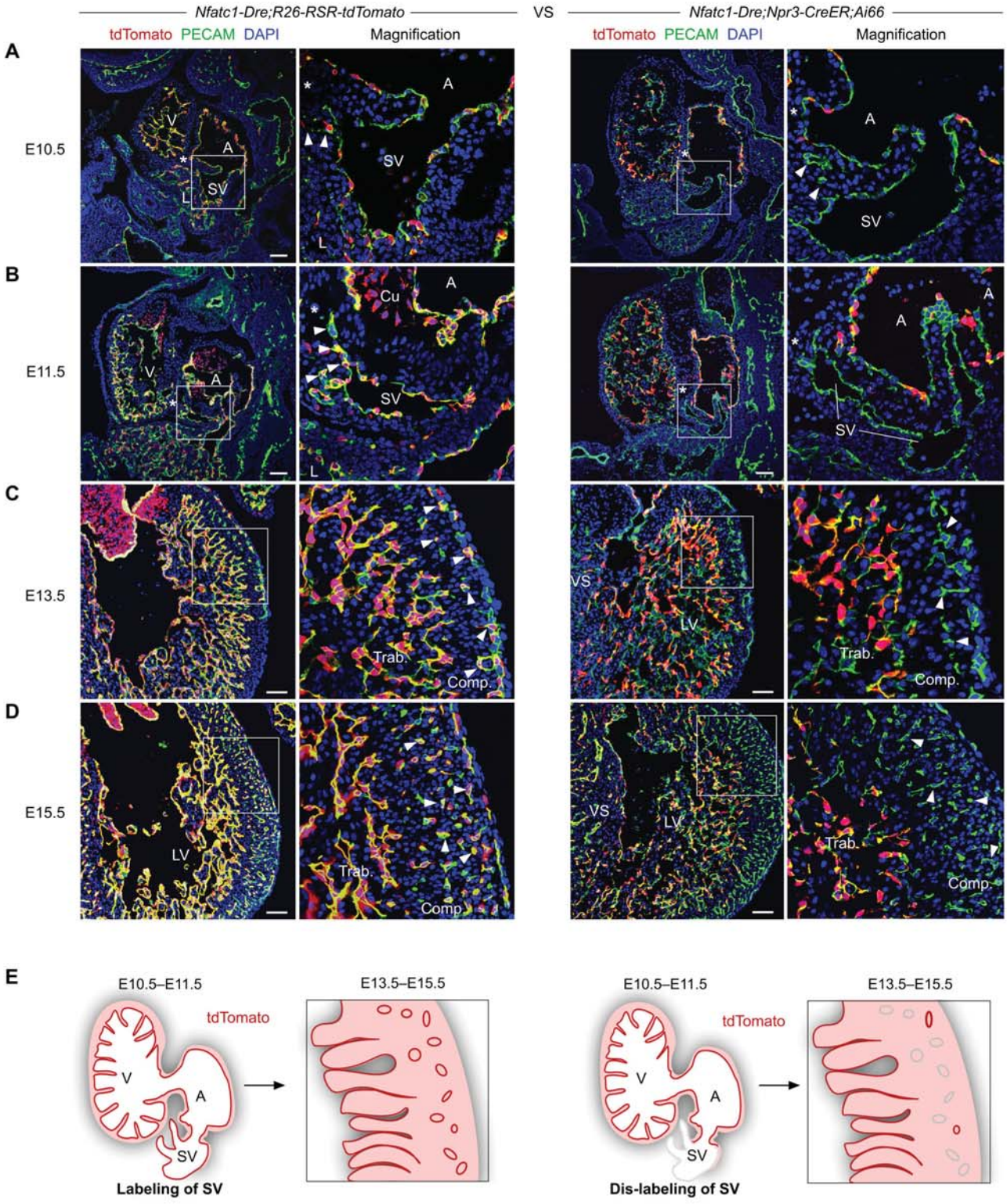

Figure 7

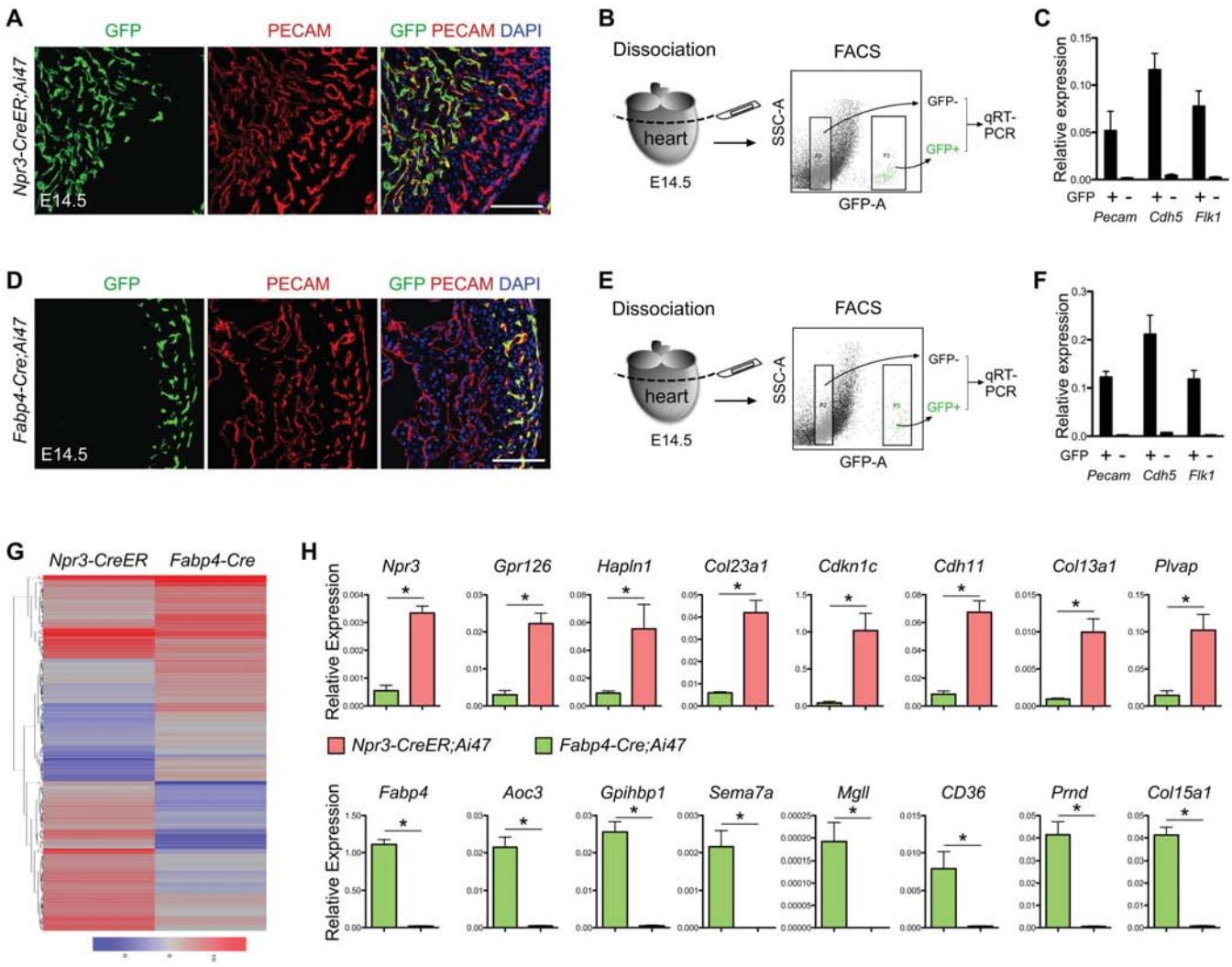

Figure 8

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

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 and Bin Zhou

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Supplemental Materials

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

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 ($CreER^{T2}$) was inserted into frame with the translational start codon of the Npr3 gene.[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°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.[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 μm 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°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.[12] 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.[13] 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'-GGACAGAGGACAGCAGAGAAAACG-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.[14] 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'-CTTCCATCTCCCAGACGTG-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.[15] 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.[16]

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 (BDAria 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^{TM} 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.[17] 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 $MgCl_2$ in PBS) for another 30 minutes. After washing in LacZ washing buffer (2mM $MgCl_2$, 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

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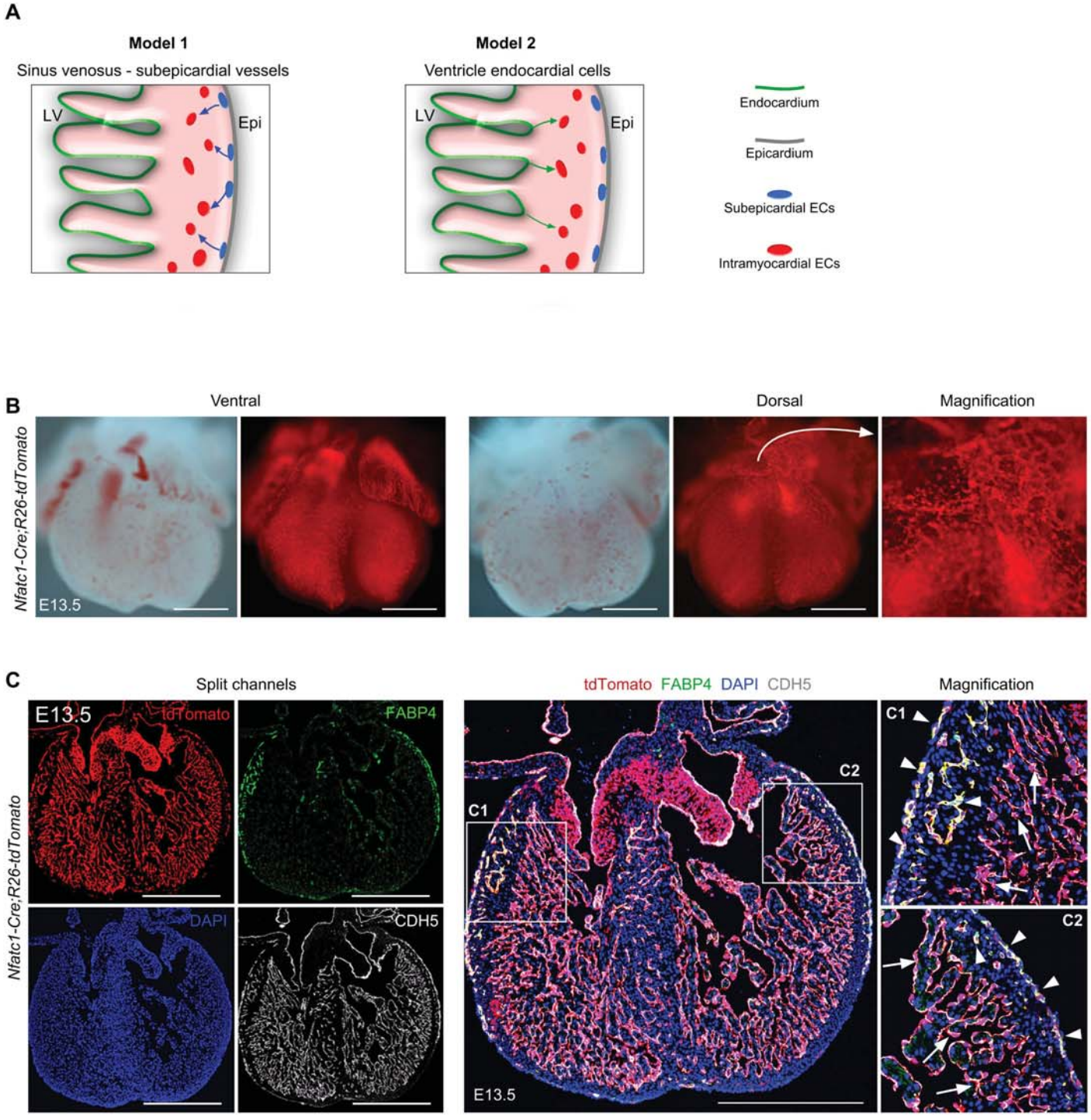
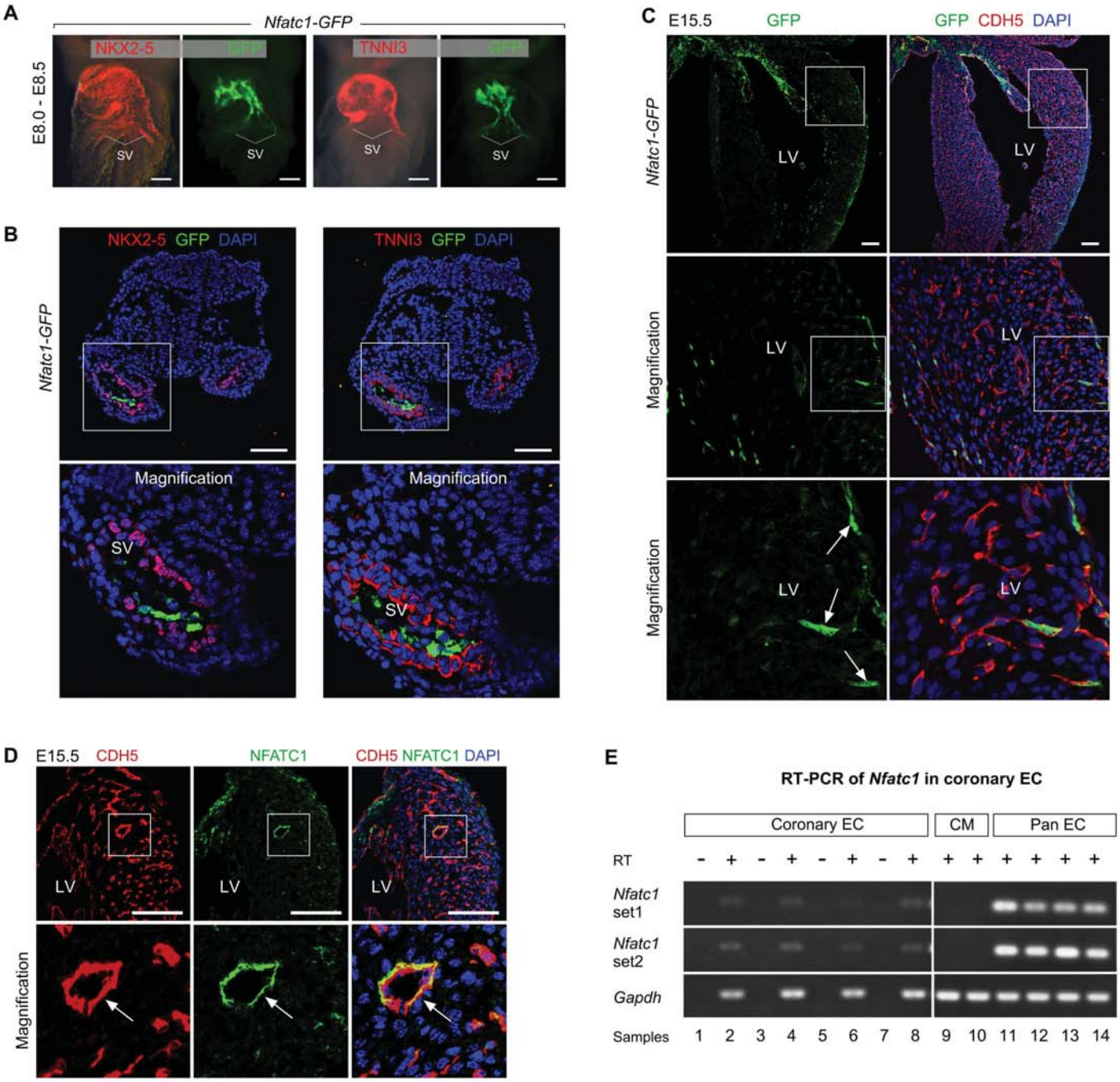
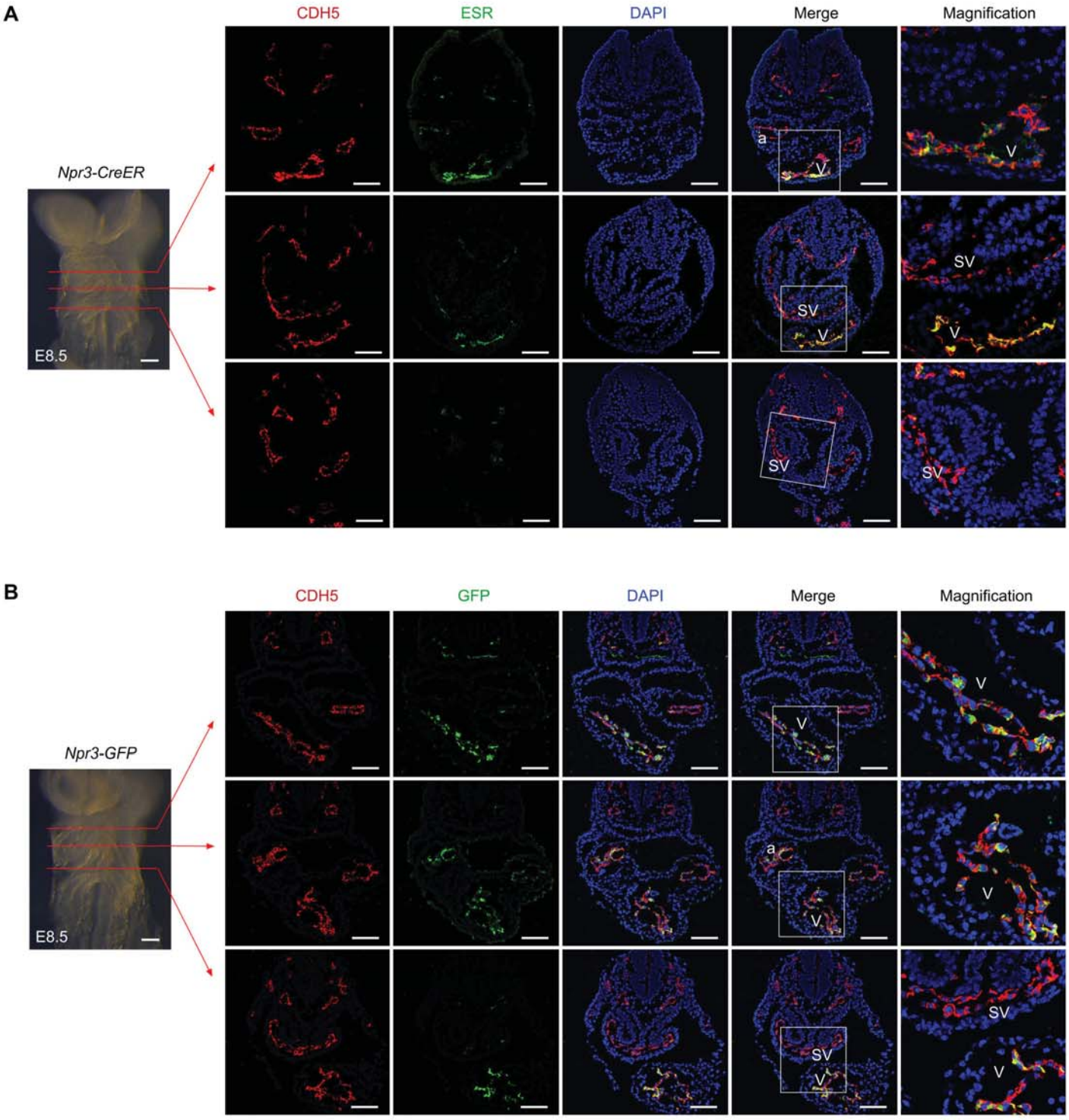


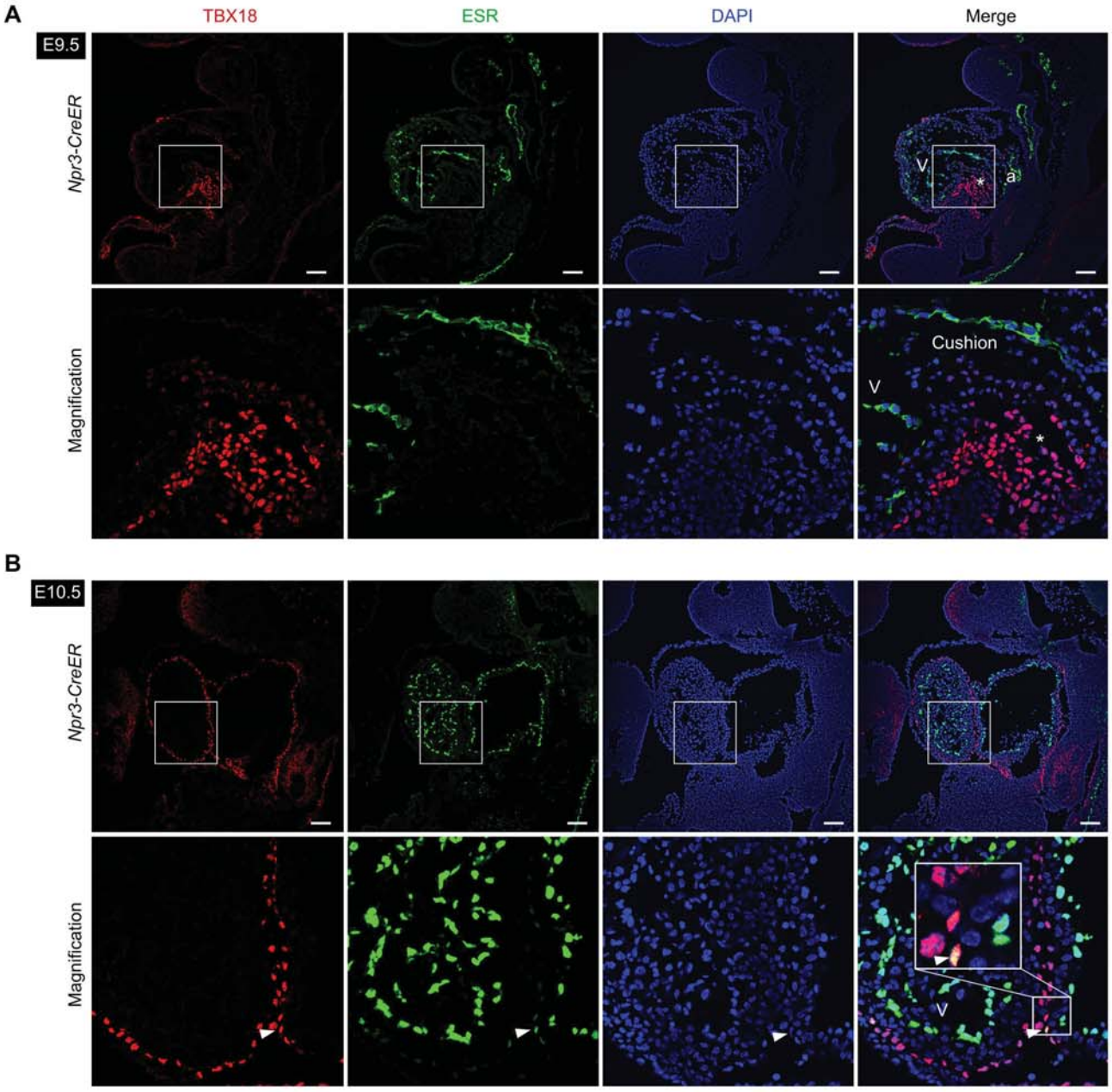
Online Figure I. Nfatc1-Cre labels both endocardium, SV and coronary endothelium. A, Schematic showing two contrasting models for explanation of coronary artery formation in developing heart. LV, left ventricle; Epi, epicardium. **B**, Whole-mount and bright-field fluorescence views showing tdTomato 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.



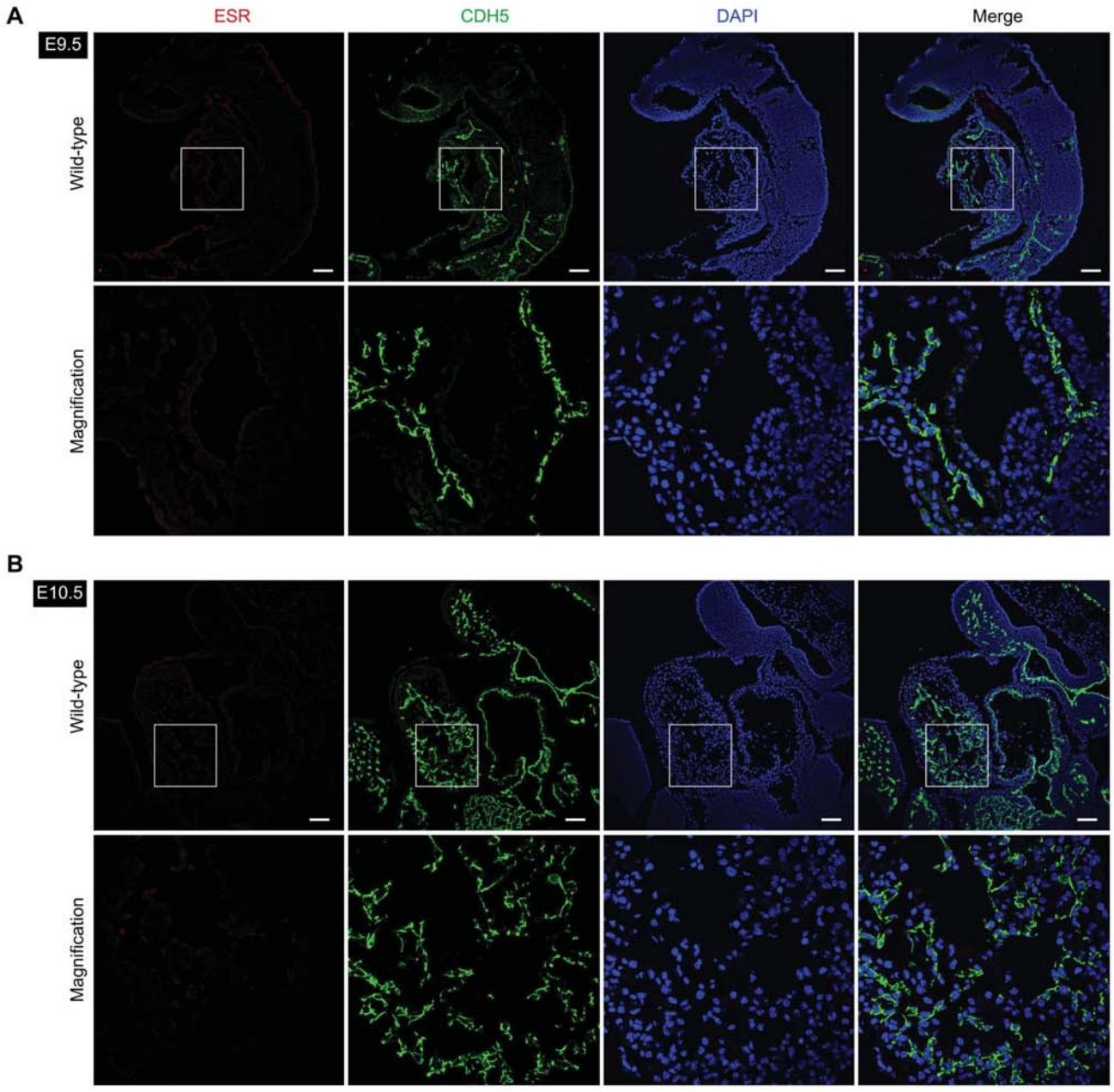
Online Figure II. Expression of Nfatc1 in sinus venosus of E8.0 - E8.5 hearts and coronary vasculature in E15.5 hearts. A, Whole-mount view of E8.0-E8.5 *Nfatc1-GFP* embryos stained with GFP and cardiac markers NKX2-5 and TNNI3. **B**, Immunostaining for NKX2-5 or TNNI3 and GFP on *Nfatc1-GFP* embryonic 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-endotheial cells (Pan EC, samples 11-14). Coronary EC was collected from *Apln-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.



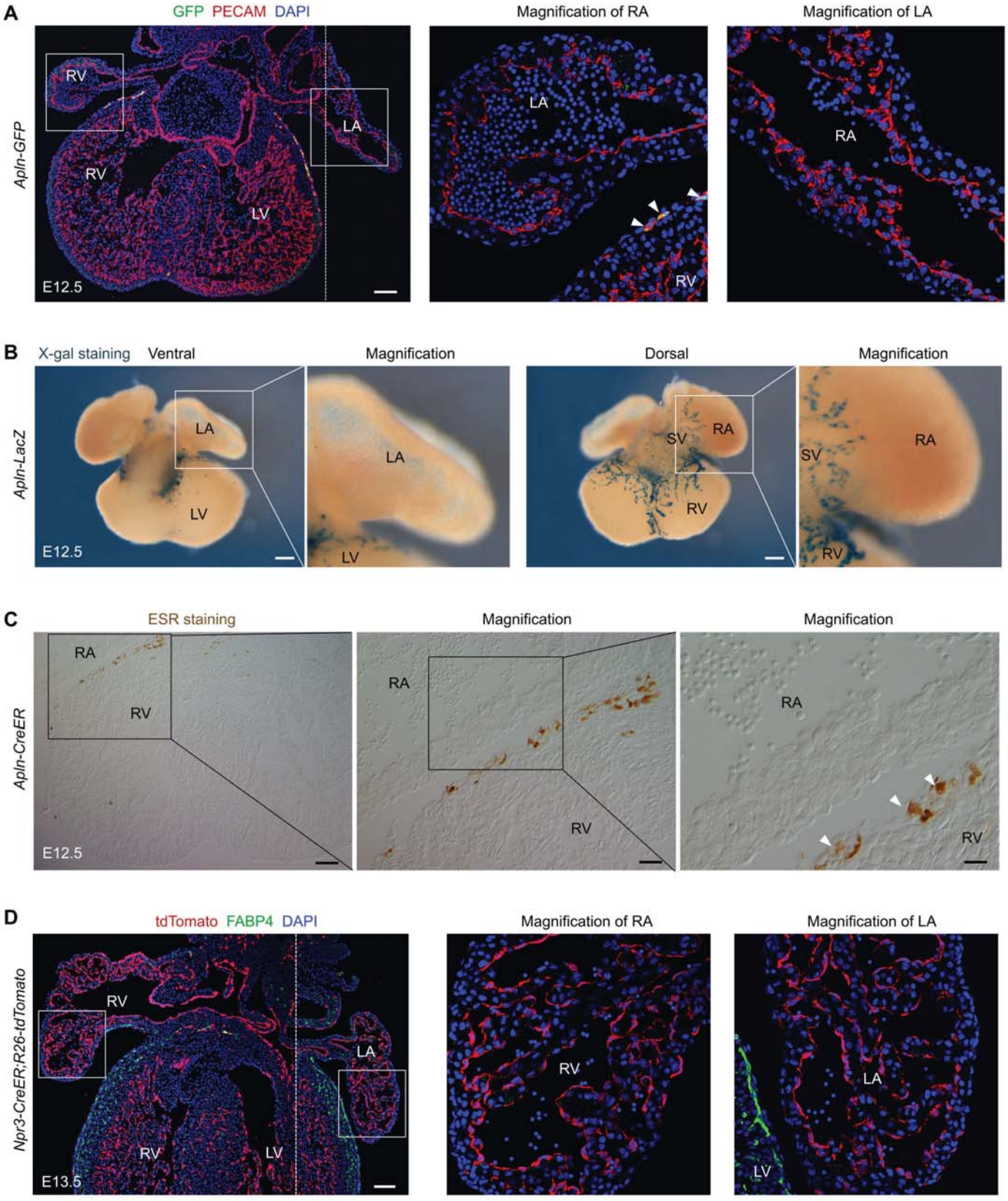
Online Figure III. Expression of Npr3 in early embryonic heart. A, Immunostaining for ESR and CDH5 on E8.5 *Npr3-CreER* sections shows ESR is expressed in atrium (a), ventricle (V) but not SV endocardium. **B**, Immunostaining for GFP and CDH5 on E8.5 *Npr3-GFP* sections shows GFP is expressed in atrium (a), ventricle (V) but not SV endocardium. Scale bars, 100 μm. Each image is representative of 3 individual samples.



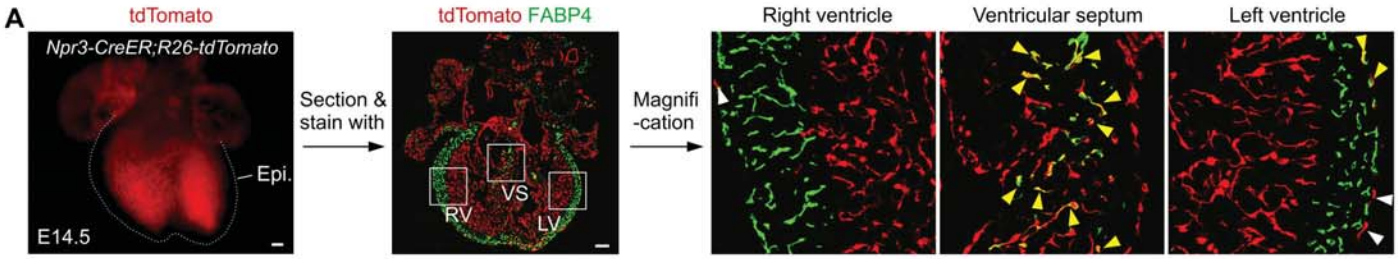
Online Figure IV. Npr3 is expressed in a subset of epicardial cells but not in proepicardial cells. A,B, Immunostaining for ESR and TBX18 on E9.5 and E10.5 *Npr3-CreER* sections. No ESR+TBX18+ cells were detected in proepicardium (*) at E9.5 (A). A few epicardial cells express weak ESR at E10.5 (arrowheads, B). Scale bars, 100 μm.

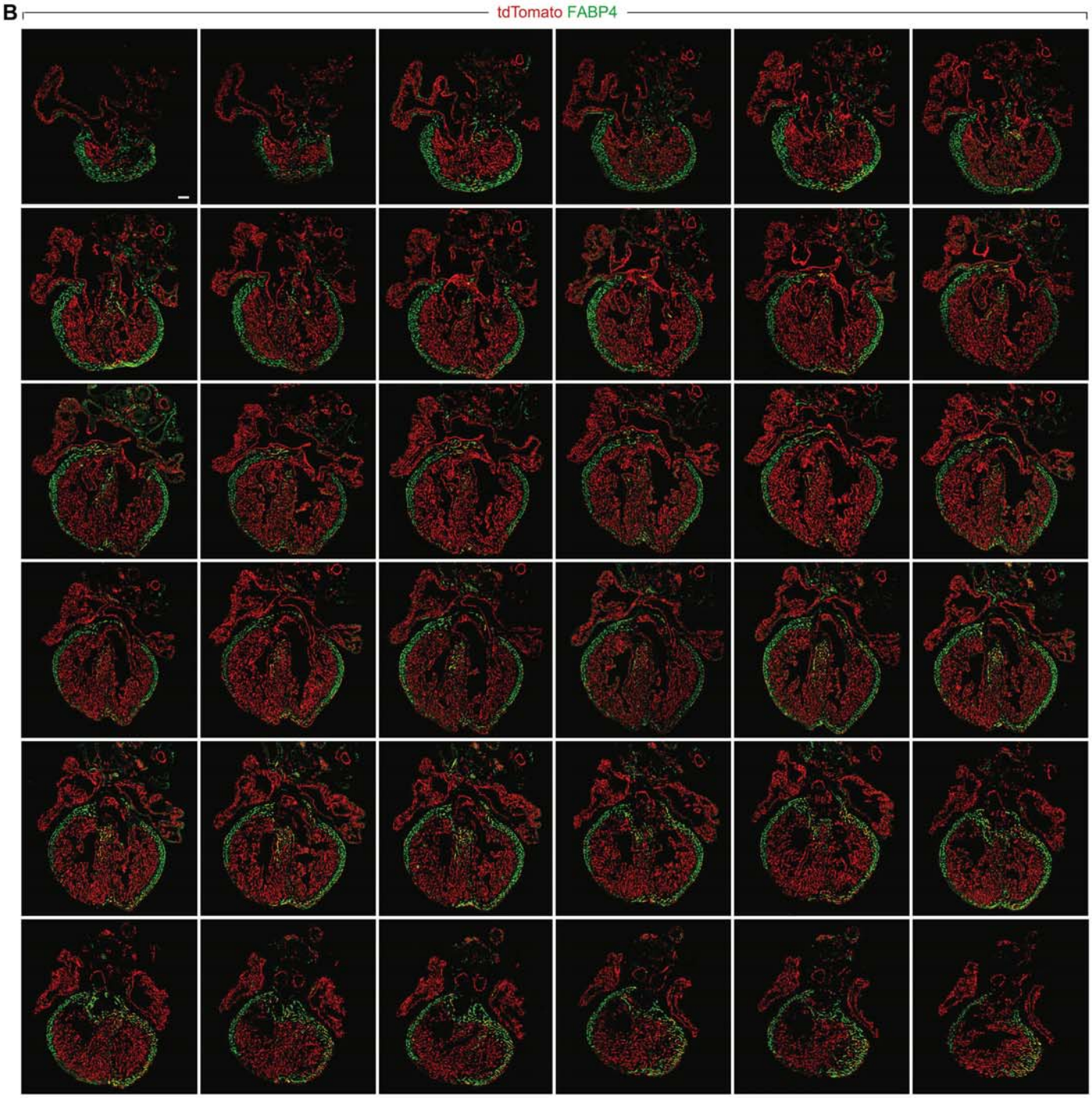


Online Figure V. Immunostaining for ESR antibody on wild-type embryonic sections. A,B, Immunostaining for ESR and CDH5 on E9.5 and E10.5 wild-type embryonic sections. No signal was detected in ESR channel. Scale bars, 100 μm.

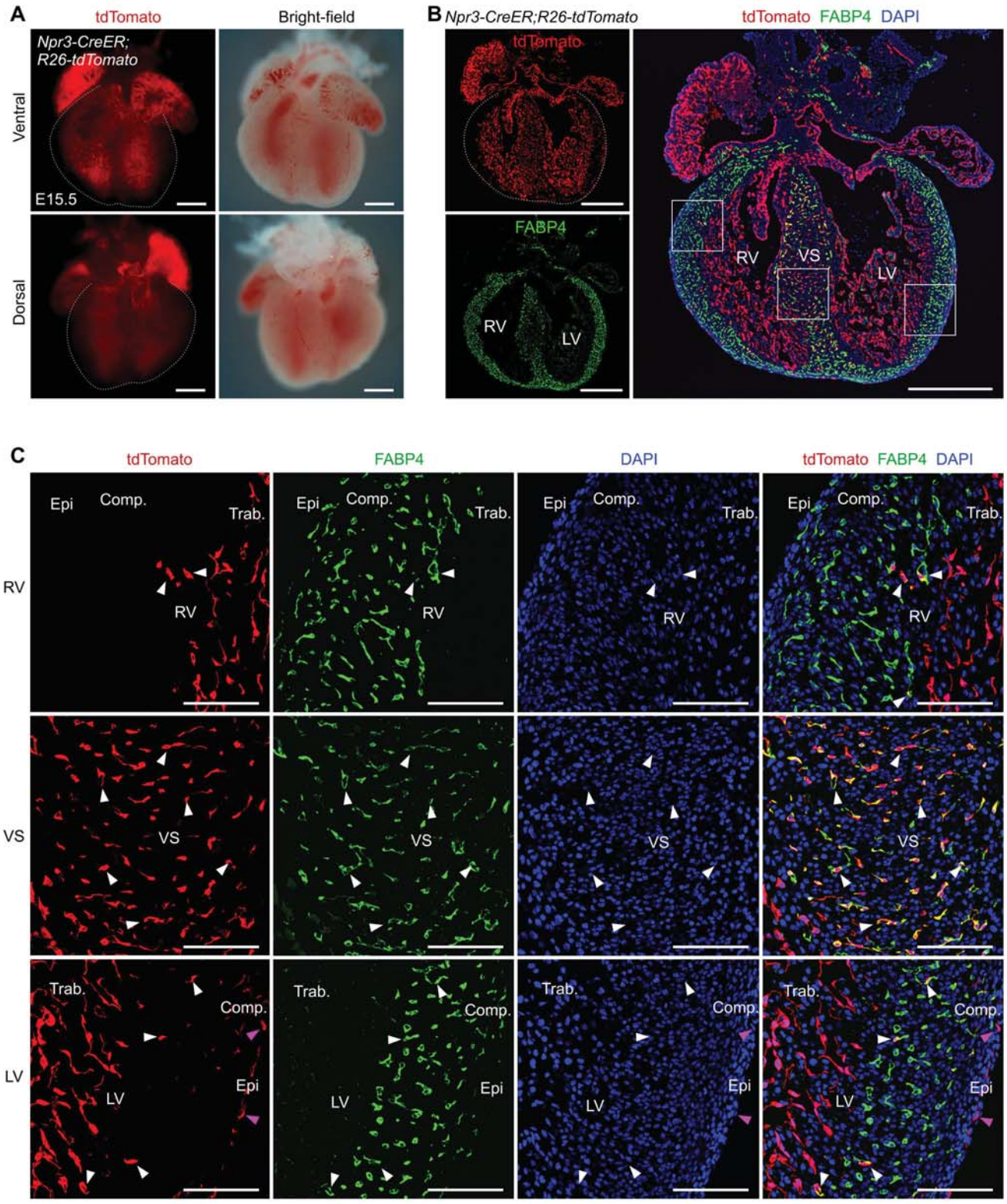


Online Figure VI. Coronary vascular endothelial cells are not present in atrium free wall at E12.5–E13.5. A, Immunostaining for GFP and PECAM on E12.5 *Apln-GFP* embryonic heart. Arrowheads indicate coronary vascular endothelial cells in the ventricle. **B**, Whole-mount X-gal staining of E12.5 *Apln-LacZ* 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* labeled cells in right and left atrium are not FABP4+ coronary vessels. Scale bars, 100 μm. Each image is representative of 3 individual samples.

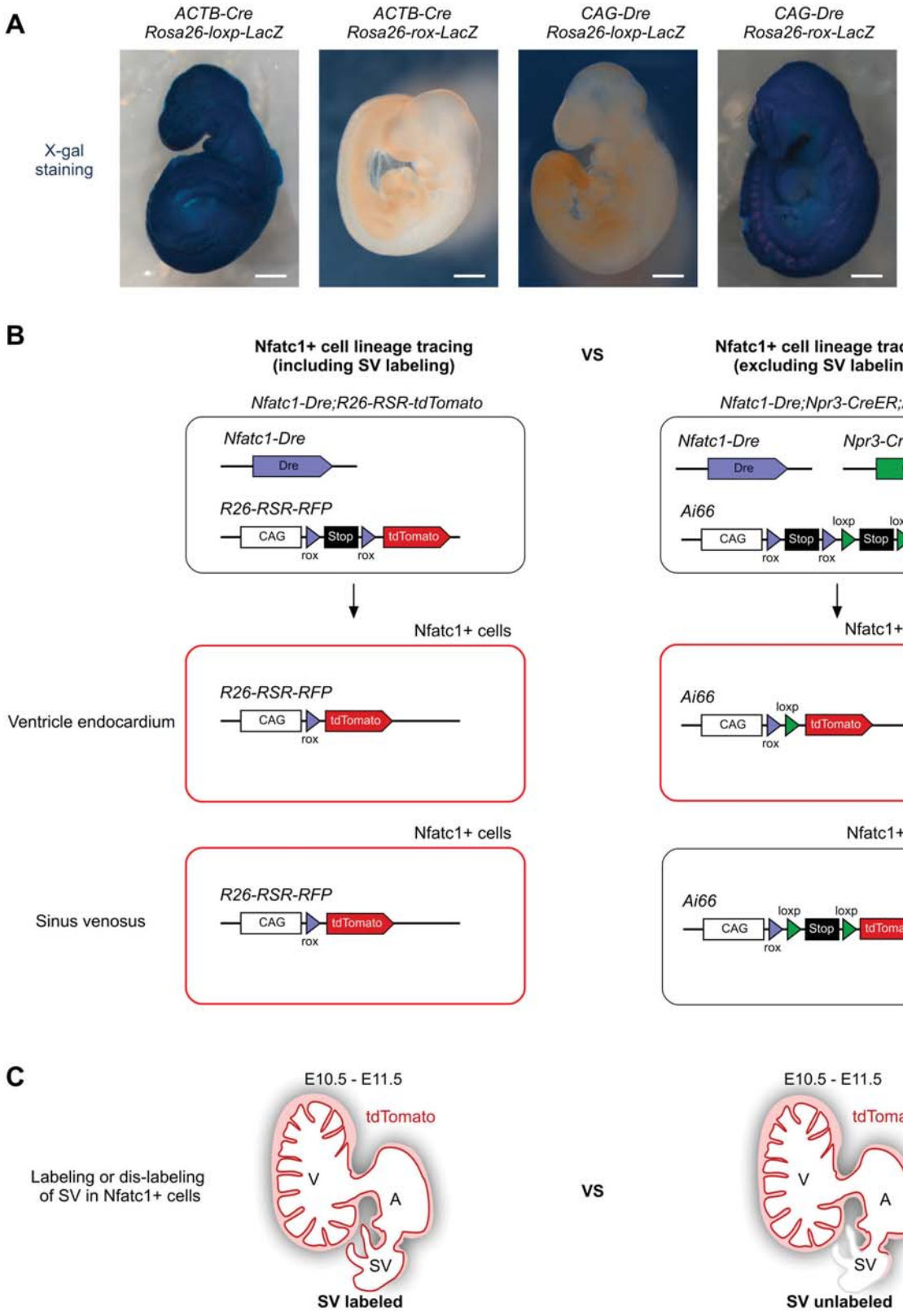




Online Figure VII. *Npr3-CreER* labeled cells in developing heart. A, Whole mount view of *Npr3-CreER;R26-tdTomato* heart and immunostaining of tdTomato and FABP4 on sections. Yellow arrowheads indicate tdTomato+ coronary VECs and white arrowheads indicate tdTomato+ epicardial cells. RV, right ventricle; 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, LV, left ventricle; RV, right ventricle; VS, ventricular septum. 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.