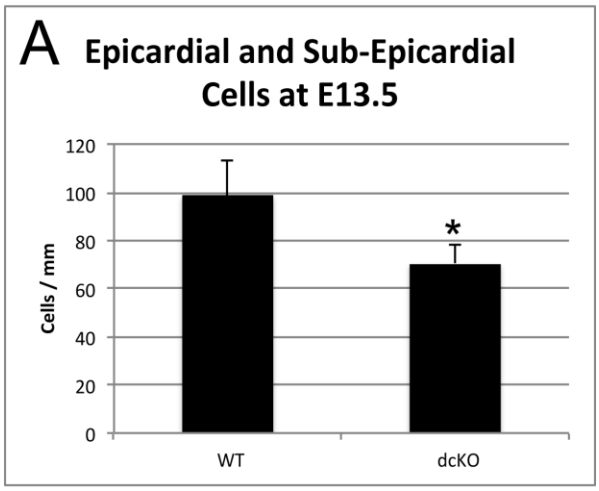
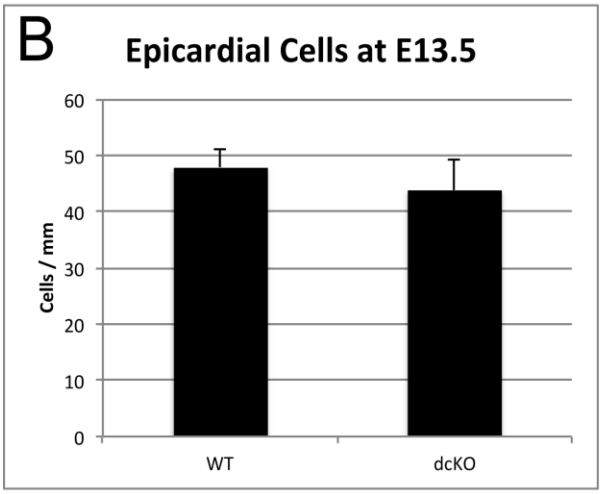
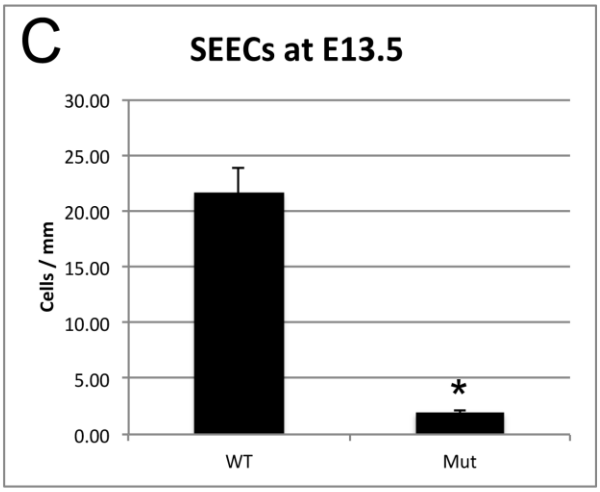

A
Epicardial and Sub-Epicardial Cells at E13.5
Cells / mm
120
100
80
60
40
20
0
*
WT
dcKO


B
Epicardial Cells at E13.5
Cells / mm
60
50
40
30
20
10
0
WT
dcKO


C
SEECs at E13.5
Cells / mm
30.00
25.00
20.00
15.00
10.00
5.00
0.00
*
WT
Mut

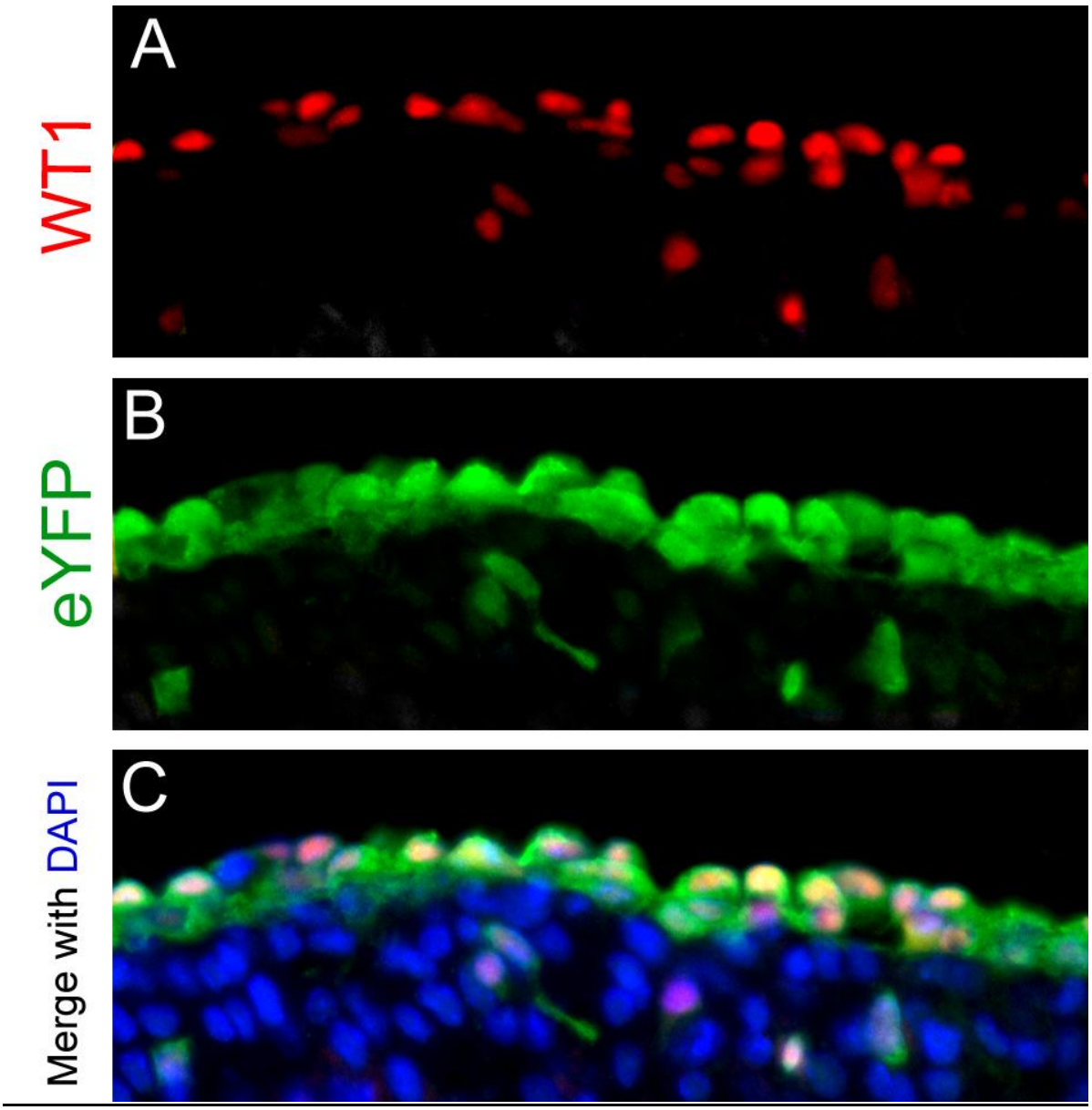
657

658

659

Supplemental Figure 1. ***WT1(RP23-8C14)-Cre*** **labels the epicardium**

Immunofluorescent staining of E12.5 hearts with antibodies against WT1 (A) and eYFP (B) controlled by *WT1(RP23-8C14)-Cre*. We found extensive co-staining in the epicardium (C). Nuclei were labeled with DAPI (Blue, C).

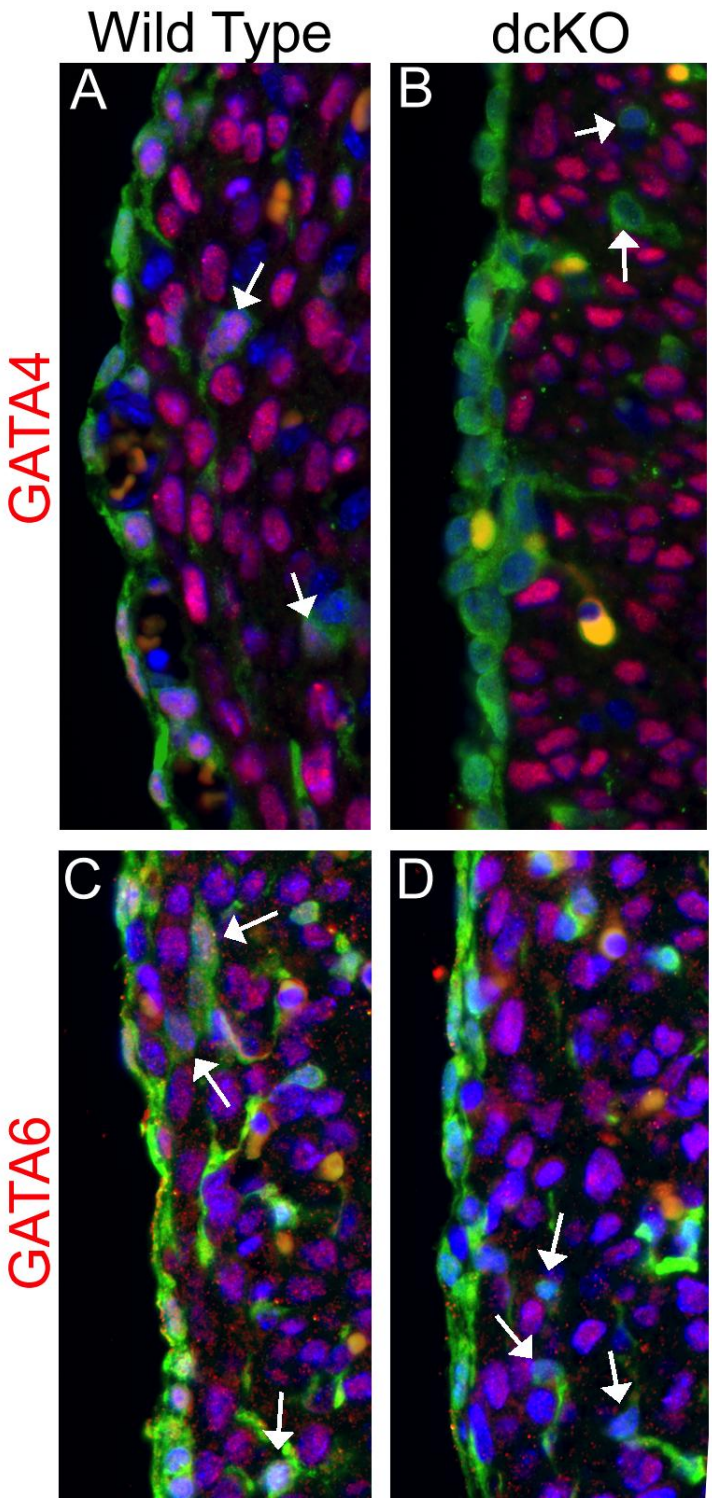


666

667

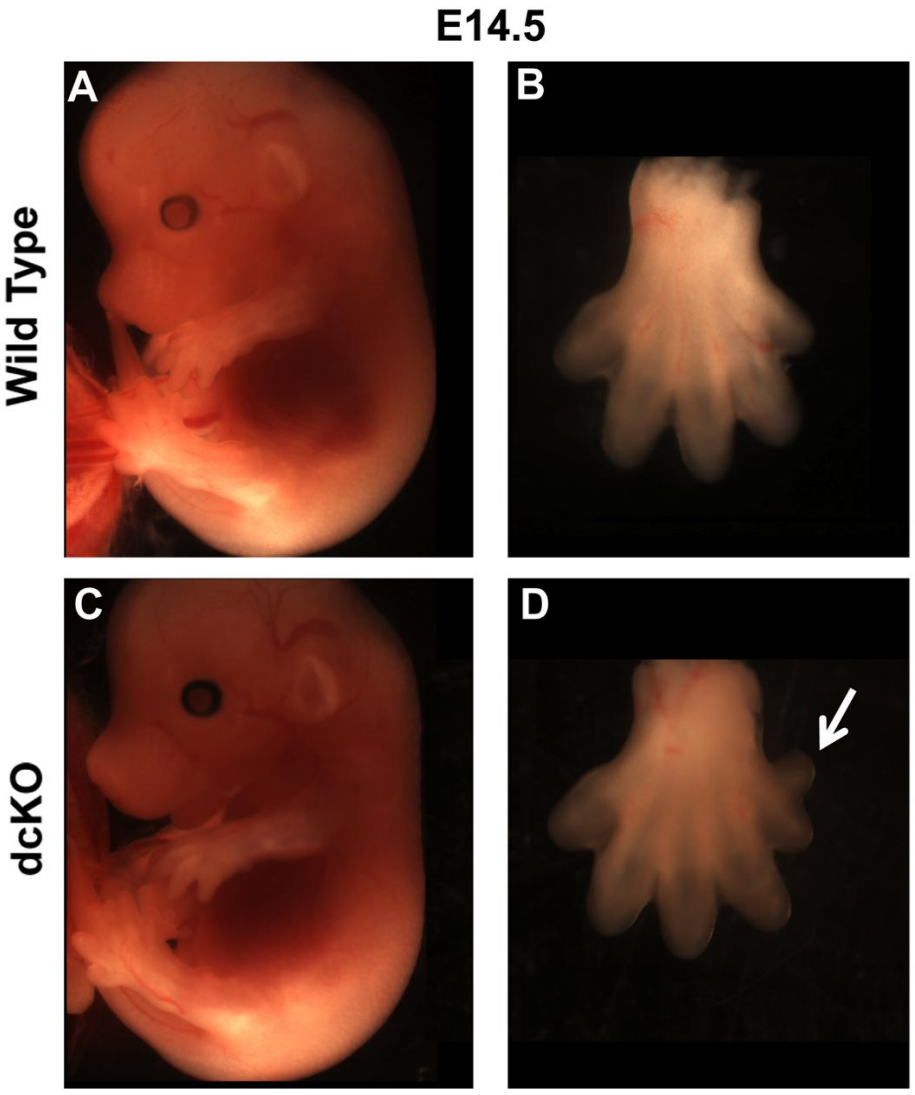
Supplemental Figure 2. *WT1(RP23-8C14)*-Cre disrupts expression of GATA6 and GATA4 in epicardial derived cells

The expression of GATA6 and GATA4 in epicardial derived cells at E14.5 was visualized with immunofluorescence for GATA6 (A-B) and GATA4 (C-D). GATA6 (A) and GATA4 (C) were found to be expressed in epicardial and epicardial derived cells (arrows) of wild type hearts. In dcKO hearts, there is an observable loss of GATA6 (B) and GATA4 (D) expression in epicardial derived cells (arrows) when compared to the wild type. Hearts were co-stained against eYFP (Green) and DAPI (Blue) to visualize nuclei.


Wild Type
dcKO
A
B
C
D
GATA4
GATA6

Supplemental Figure 3. dckO embryos exhibit polydactyly

dcKO embryos (C) at E14.5 appear grossly normal compared to wildtype (A). dcKO hind limbs demonstrate polydactyl (arrow, D). (A,C) and (B,D) images were taken at the same magnification respectively.

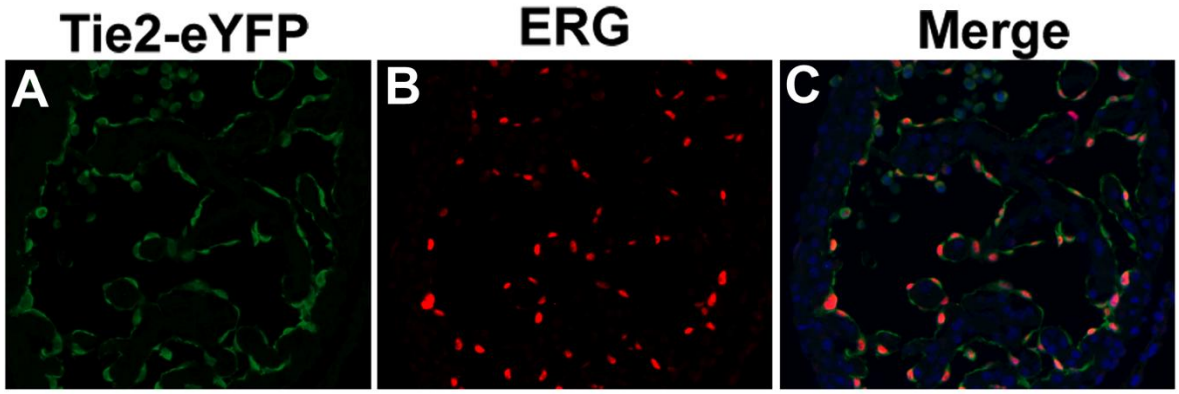


687

688

Supplemental Figure 4. ERG Labels Endothelial Cells

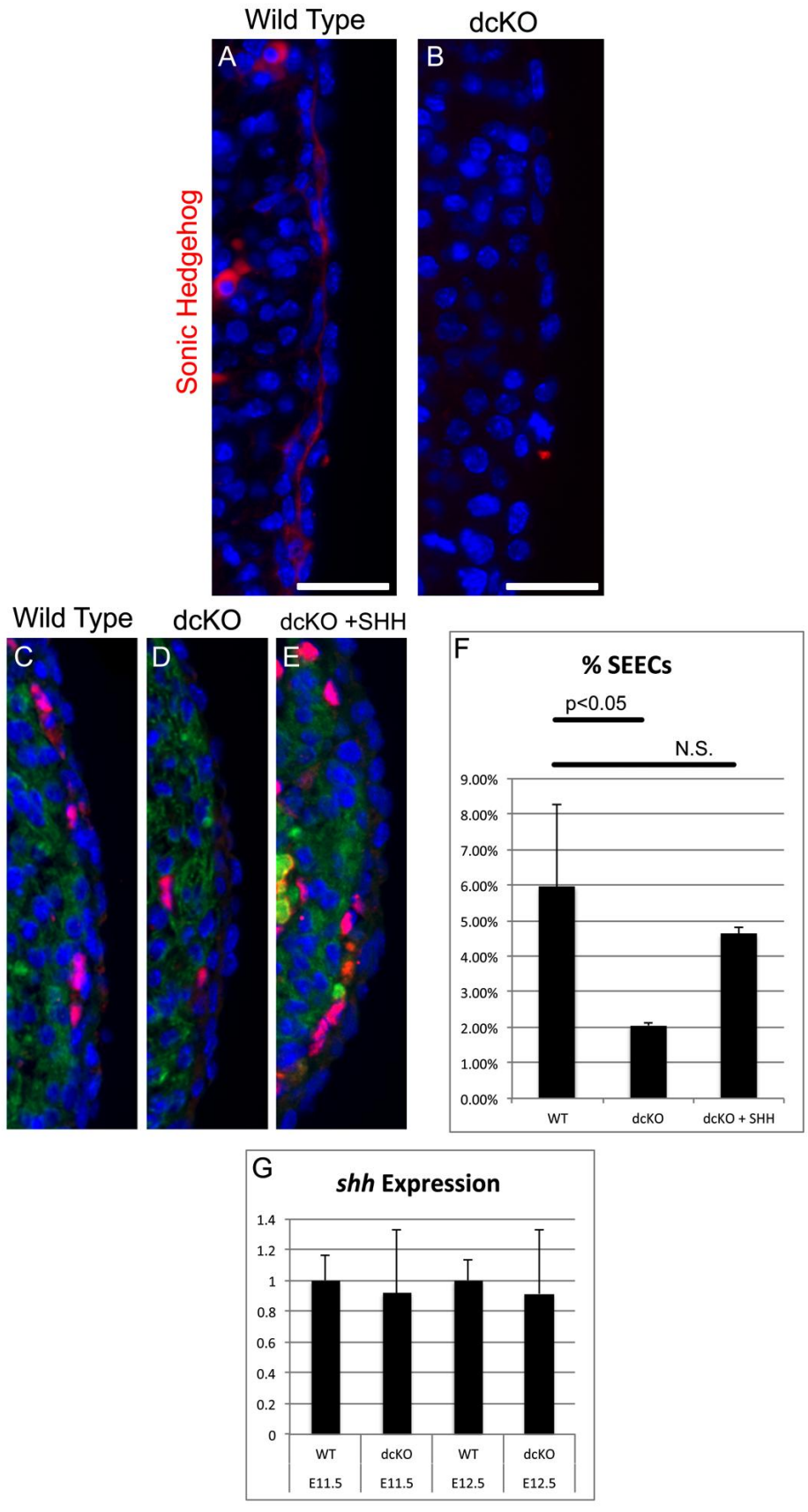
Immunofluorescent staining of an E10.5 *Tie2-Cre+/Rosa26R.EYFP* heart with antibodies against eYFP (A, Green) and ERG (B, Red) shows a high level of co-staining (C) between EYFP and ERG. DAPI (Blue) was used to label nuclei.



694
695

Supplemental Figure 5. Epicardial GATA regulate plexus formation via SHH

(A) E13.5 wild type heart immunofluorescently stained for SHH (red) showing expression in the sub-epicardium (Arrows). (B) E13.5 dcKO heart shows SHH expression is lost in the sub-epicardium. E11.5 whole hearts were isolated, cultured, and immunofluorescently stained to quantify the percentage of SEECs (C-D). The epicardium/sub-epicardium of WT hearts were found to contain 5.96% SEECs (C, F), while the dcKO hearts were found to contain 2.05% SEECs (D,F,*=$p<0.05$). Addition of SHH to the dcKO (E,F) increased the percentage of SEECs in the epicardium/sub-epicardium to 4.62% resulting in no significant (ns) difference between the WT and dcKO + SHH (F). Sections were counterstained with sarcomeric myosin (MF20, Green) to visualize the myocardium DAPI to label nuclei. Error bars represent standard deviation. (G) RT-PCR results showing no significant change in the relative expression of *shh* between the wild type and dcKO hearts at E11.5 and E12.5. Values are relative to the expression of *shh* in the wild type hearts at each age. Error bars represent standard deviation.


Wild Type
dcKO
A
B
Sonic Hedgehog
Wild Type
dcKO
dcKO +SHH
C
D
E
F
% SEECs
p<0.05
N.S.
9.00%
8.00%
7.00%
6.00%
5.00%
4.00%
3.00%
2.00%
1.00%
0.00%
WT
dcKO
dcKO + SHH
G
shh Expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WT E11.5
dcKO E11.5
WT E12.5
dcKO E12.5

Supplemental Methods:

Whole Heart Culture Assay

Similar to Lavine et al., E11.5 hearts were isolated in Hank's buffered salt solution. Hearts were placed in glass scintillation vials with 1 mL of media (DMEM / 10% FBS / 2 mg/L heparin / penicillin and streptomycin) (Lavine et al., 2006). For SHH rescue, media was supplemented with 1 mg/L SHH (R&D Systems). Hearts were incubated for 48hrs on a rocker at 37°C/5% CO_2. Following incubation, hearts were fixed, embedded, and stained. SEECs were quantified in the same manner as Figure 5.

RT-PCR

Embryonic hearts were isolated from time pregnant females and processed for RNA (Qiagen RNeasy Minikit) and cDNA template (SABiosciences RT^2 First Strand Kit) using published protocols. Samples were probed using SYBR Green with ROX reference (SABiosciences RT^2 Real-Time SYBR Green/Rox Master Mix PA-012) using 100nm primer oligos with standard protocols in a Stratagene Mx3005P Real-Time PCR System. Primers utilized are available upon request. Samples were normalized based on *Gapdh* expression.