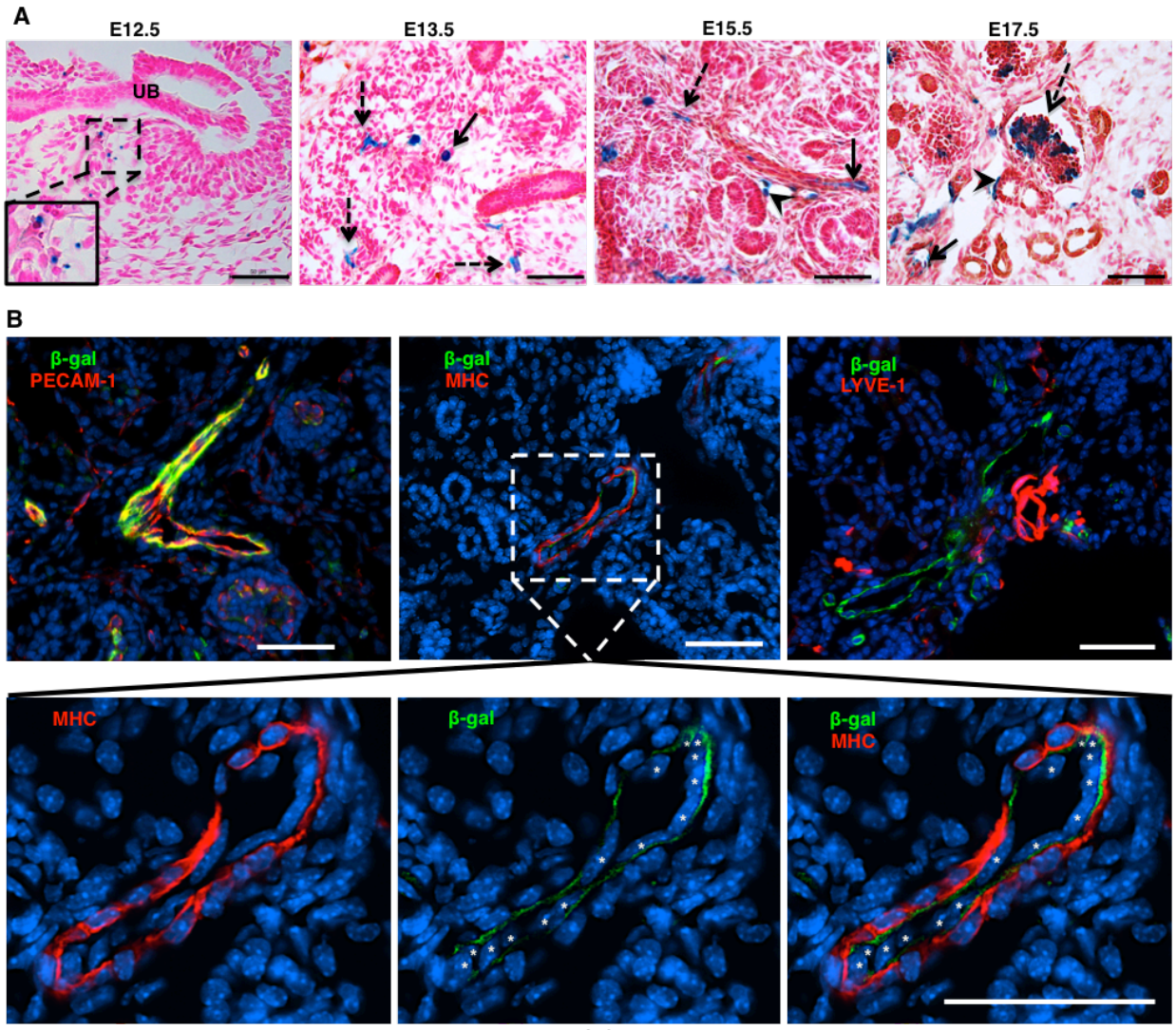
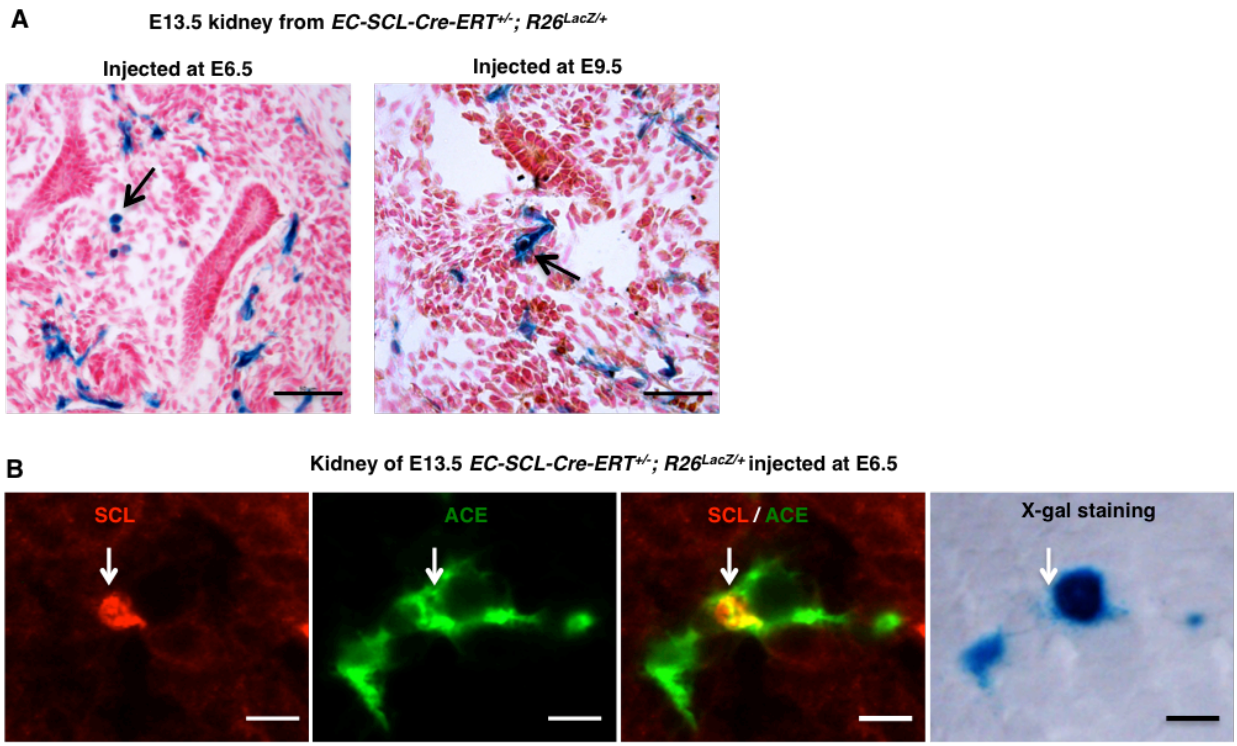
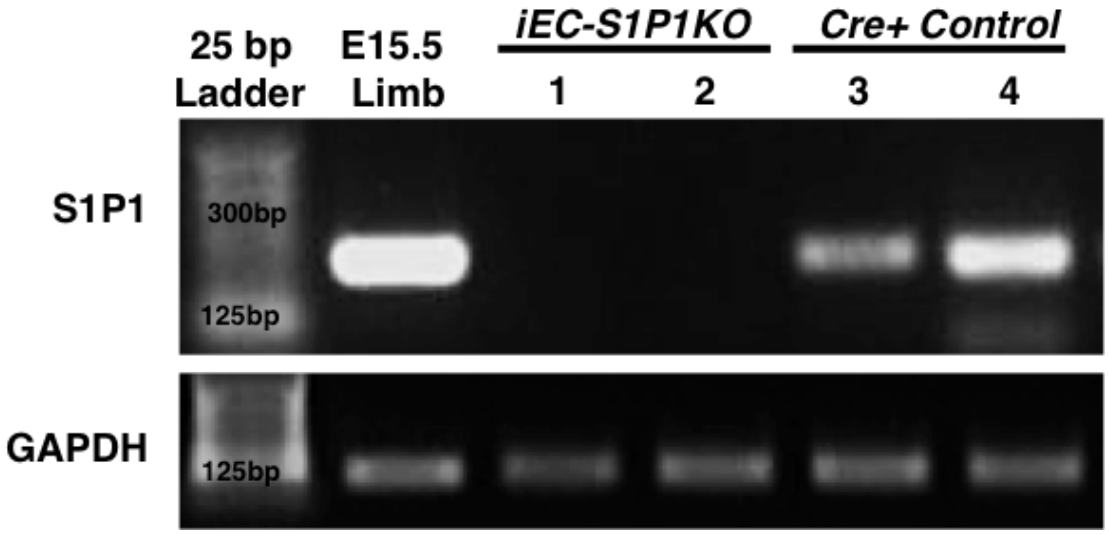
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



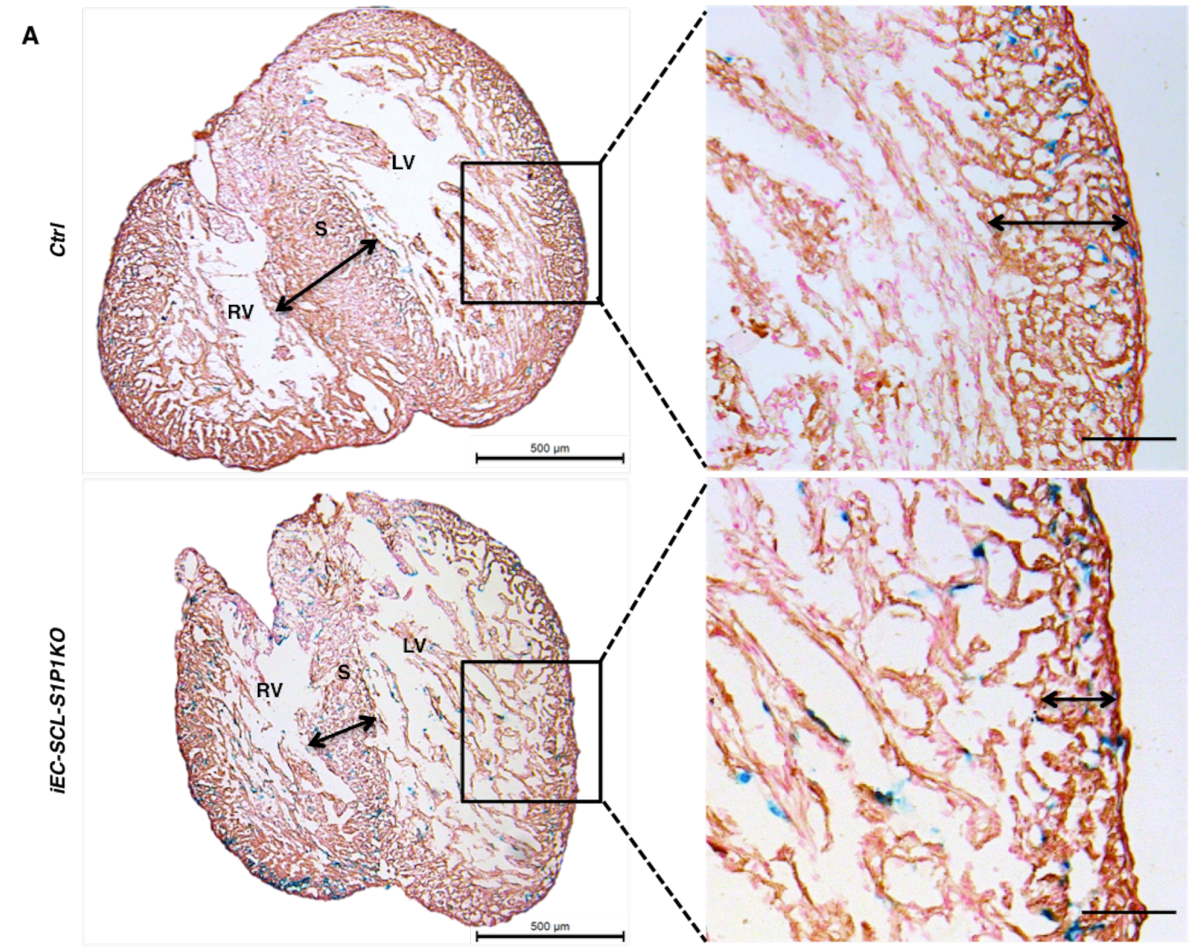
Supplemental Figure 1 related to Figure 1. *SCL*+ hematopoietic precursors give rise to renal endothelium but not mural cells in the embryonic kidney. (A) Kidneys of *HSC-SCl-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$ mice showing scattered HSC-SCL progenitor derived cells labeled by X-gal in blue (β-gal+) close to the ureteric bud (UB) at E12.5 are shown in the inset. β-gal+ cells adopted round (arrow) and spindle-shape (dashed arrows) configuration in E13.5 kidneys. A subset of β-gal+ ECs of renal arteries (arrows), veins (arrow heads) and peritubular capillaries (E15.5, dashed arrows) and glomerular capillaries (E17.5, dashed arrow) were found at E15.5 and E17.5. (B) β-gal+ (green) HSC-SCL lineage cells co-expressed the EC marker PECAM-1 (red), but not the mural marker myosin heavy chain (MHC, red) or the lymphatic EC marker LYVE-1 (red). The lower panels show β-gal and MHC staining at high magnification. Asterisks indicate nuclei of β-gal+ ECs. Nuclei were stained with hoechst in blue. Scale bars: 50μm

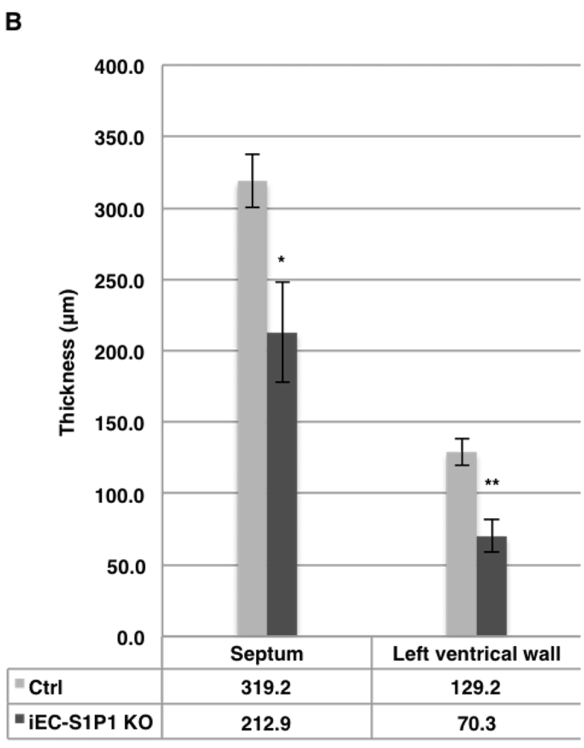


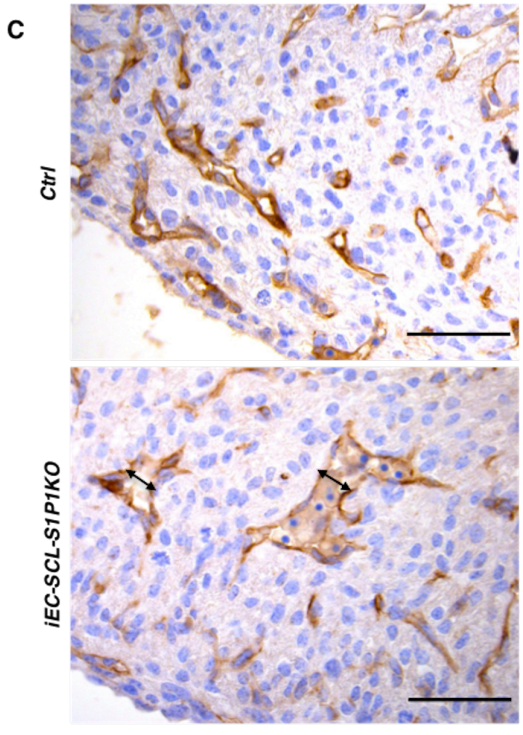
Supplemental Figure 2 related to Figure 2. The early embryonic kidney possesses hematopoietic precursors. (A) E13.5 kidneys showing blood like labeled cells, in blue, derived from endothelial precursors from *EC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$ mice labeled at E6.5 and from E9.5 to E13.5 (black arrows). (B) E13.5 embryonic kidneys from *EC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$ mice treated with tamoxifen at E6.5 showed (by double immunofluorescence) coincidence of β-gal+ cells derived from SCL+ precursors with SCL (red) and ACE (green). Scale bars: 50μm (A); 10μm (B).



Supplemental Figure 3 related to Figure 4. Deletion of *S1P1* in *SCL*+ ECs from *iEC-S1P1KO-mTmG* mice. RT-PCR for S1P1 and GAPDH from GFP+ cells sorted from kidneys of E15.5 *iEC-S1P1KO-mTmG* (*EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{\Delta/fl}$*; R26*$^{mTmG/+}$) mice (1 and 2) and GFP+ cells sorted from kidneys of Cre+ control (*EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{\Delta/+}$*; R26*$^{mTmG/+}$) siblings (3 and 4). The GFP+ cells from *iEC-S1P1KO-mTmG* mice show no expression of S1P1 whereas GFP+ cells from Cre+ control kidneys express S1P1. cDNA from E15.5 limb was used as a positive control.


A
Ctrl
LV
S
RV
500 µm
iEC-SCL-S1P1KO
LV
S
RV
500 µm


B
Thickness (µm)
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
*
**
Septum
Left ventrical wall
Ctrl
319.2
129.2
iEC-S1P1 KO
212.9
70.3


C
Ctrl
iEC-SCL-S1P1KO

Supplemental Figure 4 related to Figure 5. *iEC-SCL-S1P1KO* mice develop an abnormal heart. (A) X-gal staining and immunostaining for α-SMA in longitudinal heart sections of control (Ctrl) and *iEC-SCL-S1P1KO* mice at E15.5. The *iEC-SCL-S1P1KO* mice developed thinner inter-ventricular septum (S) and inter-ventricular myocardium compact layer, especially in the left ventricle (LV). RV, right ventricle. (B) Thickness of septum and left ventricle wall of E15.5 control (n=4) and *iEC-SCL-S1P1KO* (n=4) mice. Values are expressed as mean± SEM. * $p<0.05$, ** $p< 0.01$. (C) Immunostaining for PECAM-1 in heart sections of control and *iEC-SCL-S1P1KO* mice at E15.5 showing that the intra-myocardial vessels in the *iEC-SCL-S1P1KO* heart have larger diameter (double head arrows) than those in the control heart. Scale bars: 500μm (A); 100μm (A, inset); 50μm (C).

SUPPLEMENTAL TABLE

Supplemental Table 1. Genotyping results of *S1P1* conditional knockout embryos

	cKO	*Cre+ control*	*Cre- control*	*WT*	Total
Number of embryos	30	27	23	30	110
% of embryos	27.3%	25.6%	20.9%	27.3%	100%
Number of dead embryos	16	1	0	1	18
% of dead embryos	53.3%	3.7%	0	3.3%	16.4%

SUPPLEMENTAL MATERIAL AND METHODS

Mice

ER-GFP-Cre$^{+/-}$*;R26*$^{LacZ/+}$ embryos at 13.5 days of gestation (E13.5) (n=4) were used for tracing the fate of erythroblasts.

EC-SCL-Cre-ERT$^{+/-}$*;R26*$^{LacZ/+}$ and *HSC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$ embryos at E12.5 (EC n=22, HSC n=9), E13.5 (EC n=11, HSC n=11), E15.5 (EC n=3, HSC n=2) and E17.5 (EC n=5, HSC n=2) were used for fate tracing studies.

To generate *iEC-SCL-S1P1KO* mice, first we crossed *S1P1*$^{fl/fl}$ [1] mice to *EIIA-Cre*$^{+/-}$ mice [2] to obtain *EIIACre*$^{+/-}$*; S1P1*$^{\Delta/+}$ mice. Then this mouse line was crossed to wt Bl6 mice to remove the *EIIA-Cre*. The *S1P1*$^{\Delta/+}$ mice were born at the expected Mendelian ratio and were fertile. These mice were crossed with *EC-SCL-Cre-ERT* mice to generate *EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{\Delta/+}$ mice which next were crossed to the *S1P1*$^{fl/fl}$*; R26*$^{LacZ/LacZ}$ mice to generate the *EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{\Delta/fl}$*; R26*$^{LacZ/+}$ (*iEC-SCL-S1P1KO)* mice and their control siblings (*EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{fl/+}$*; R26*$^{LacZ/+}$).

To assess the deletion of S1P1 we crossed *EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{\Delta/+}$ to *S1P1*$^{fl/fl}$*; R26*$^{mTmG/mTmG}$ to generate *iEC-S1P1KO-mTmG* (*EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{\Delta/fl}$*; R26*$^{mTmG/+}$) mice, with GFP reporter expression in ECs with S1P1 deletion and heterozygous controls with GFP reporter expression in ECs with one intact S1P1 allele (*EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{\Delta/+}$*; R26*^{mTmG}).

EC-SCL-Cre-ERT$^{+/-}$*;R26*$^{LacZ/+}$ (n=9), *EC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$ (n=4), *HSC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$ (n=12), *HSC-SCL-Cre-ERT*$^{-/-}$*;R26*$^{LacZ/+}$ (n=4), *EC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{LacZ/+}$*;R26*$^{DTA/+}$ *(n=4)*, *EC-SCL-Cre-ERT*$^{-/-}$*;R26*$^{LacZ/+}$*;R26*$^{DTA/+}$ (n=5), *iEC-SCL-S1P1KO* (*EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{\Delta/fl}$*; R26*$^{LacZ/+}$) (n=5) and Cre+ control siblings (*EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{fl/+}$*; R26*$^{LacZ/+}$) (n=4) embryos at E12.5 were used for cross-transplantation experiments.

EC-SCL-Cre-ERT$^{+/-}$*;R26*$^{mTmG/+}$ (n=6) and *HSC-SCL-Cre-ERT*$^{+/-}$*;R26*$^{mTmG/+}$ (n=4) embryos at E12.5 were used for Colony-forming cell assays.

E12.5 embryos (n=14) from the cross between *Hoxb7-Cre* mice [3] and *R26*$^{LacZ/LacZ}$ mice were used for embryonic kidney culture studies,.

All procedures were performed in accordance with the Guiding Principles for Research Involving Animals and Human Beings by the American Physiological Society and were approved by the University of Virginia Animal Care Committee.

Genotyping of *iEC-SCL-S1P1KO* Mice

Genomic DNA from embryonic tissue was genotyped for *Cre*, wild type *S1P1* (*S1P1 wt*) and *S1P1* deletion (*S1P1Δ*) as previousely described. [4, 5] Four genotypes, including conditional *S1P1* knockout, *Cre*+ control, *S1P1* with one functional allele and wild type (wt) mice were obtained. Each genotype had equal opportunity based on Mendelian Ratio (25%). Conditional *S1P1* knockout (cKO) mice were *Cre* positive, *S1P1Δ* positive and *S1P1wt* negative; *Cre*+ control mice were *Cre* positive, *S1P1Δ* negative and *S1P1wt* positive; the *Cre*- control mice were *Cre* negative, *S1P1Δ* positive and *S1P1wt* positive; the wt mice were *Cre* negative, *S1P1Δ* negative and *S1P1wt* positive. The genotyping results are shown in Supplemental Table 1.

Tamoxifen Treatment of *iEC-SCL-S1P1KO* Mice

To minimize the toxicity and maximize the efficiency of Cre induction, 137 embryos from 21 litters were studied with different dose and number of injections. We found that mice with two injections (one at E10.5 and one at E12.5) (1mg/30g per injection) showed less toxicity and sufficient Cre recombination. 110 embryos of 15 litters at ages ranging from E14.5 to E16.5 (E14.5 n=28, E15.5 n=65, E16.5 n=17) were treated following this protocol. Based on genotyping results, 27.3% of the embryos (30 of 110) were conditional S1P1 knockout, which

matched the Mendelian ratio (25%). 53.3% of the conditional S1P1 knockout mice were dead at the time of harvesting (Supplemental Table 1).

Cell sorting and RT-PCR

Whole kidneys from E15.5 *iEC-S1P1KO-mTmG* (*EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{\Delta/fl}$*; R26*$^{mTmG/+}$) mice and their Cre+ control siblings (*EC-SCLCre-ERT*$^{+/-}$*; S1P1*$^{\Delta/+}$*; R26*$^{mTmG/+}$) (treated with tamoxifen at E10.5 and E12.5 by maternal injections) were micro-dissected and enzymatically dissociated to isolate single cells. GFP+ cells from *iEC-S1P1KO-mTmG* and control kidneys were sorted and collected using a Becton Dickinson Influx cell sorter.

Total RNA extraction and cDNA preparation from sorted cells were performed using a FastLane Cell cDNA kit from QIAGEN, according to the manufacturer's instructions. Quantitative PCR was performed in a CFXConnect system (Biorad, Hercules, CA) using a QuantiTect SYBR Green PCR kit from QIAGEN, following the manufacturer's instructions. The primers used for RT-PCRs were:

S1P1 forward 5'-AACTTTGCGAGTGAGCTGGT-3', *S1P1* reverse 5'-CTAGAGGGCGAGGTTGAGTG-3' (product size: 227bp), GAPDH forward 5'-TTGATGGCAACAATCTCCAC-3' and GAPDH reverse 5'-CGTCCCGTAGACAAAATGGT-3' (product size: 125bp). PCR conditions were: 95°C, 58°C, 72°C, 45 cycles.

Immunohistochemistry and X-gal reaction

7μm sections of 4% PFA fixed and cryo-embedded tissues were subjected to X-gal staining and/or immunostained following standard protocols. [6] The antibodies used were anti-β-gal (1:1000 dilution, Abcam, ab134435), anti-α-SMA (1:10000 dilution, Sigma, a2547), anti-Hb (1:500 dilution, DAKO, A0118), anti-Nanog (1:100 dilution, Abcam, ab84447), anti-SCL (1:100 dilution, Abcam, ab75739), anti-Runx1 (1:500 dilution, Abcam, ab23980), anti-ACE (1:200 dilution, Abcam, ab2092), anti-LYVE-1 (1:1000 dilution, Abcam, ab14917), anti-ppH3 (1:100

dilution, Cell Signaling #9701S), goat anti-PECAM-1 (1:200 dilution, Santa Cruz, sc1505, for Figure 4 and supplemental Figure 3) and rabbit anti-PECAM-1 (1:500 dilution, Santa Cruz, sc1506-R). Immunofluorescence was performed using Alexa Fluor 568 donkey anti-rabbit IgG (H+L) (SCL, pHH3), Alexa Fluor 488 goat anti-chicken IgG (H+L) (β-gal), Alexa Fluor 488 donkey anti-mouse IgG (H+L) (ACE), Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (PECAM-1) and Alexa Fluor 488 donkey anti-goat IgG (H+L)(PECAM-1) secondary antibodies (1:500 dilution; Life Technologies, Carlsbad, CA). Images were obtained using a Leica DFC310 FX digital camera connected to a Leica DFC 480 microscope.

Embryonic Kidney Cross-transplantation

The cross-transplantations of embryonic kidneys under the kidney capsule of adult hosts were performed as described previously.[7] The host mice were anesthetized with tribromoethanol (0.25 mg/g body weight). The host kidney capsule was incised with Vannas scissors, and the incision was extended with a 27-gauge needle. Embryonic kidneys were freshly micro-dissected and placed under the renal capsule of adult wild type or fluorescent reporter hosts via the capsular incision. After surgery, host mice were injected with analgesic drug buprenorphine (0.2mg/kg body weight) i.p. daily for 2 days and tamoxifen (1mg/30g body weight) i.p. daily for 7 days. The transplanted embryonic kidneys within the host kidneys were harvested and fixed in 4% PFA followed by X-gal staining and/or immunohistochemistry.

Measurement of Thickness of Septum and Left Ventricle Wall of E15.5 *iEC-SCL-S1P1KO* and control Mice

Longitudinal 5μm heart sections near the central conduction system from control (Ctrl) (*EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{fl/+}$*; R26*$^{LacZ/+}$) (n=4) and *iEC-SCL-S1P1KO* (*EC-SCL-Cre-ERT*$^{+/-}$*; S1P1*$^{\Delta/fl}$*; R26*$^{LacZ/+}$) (n=4) mice were used to measure the thickness of septum and left ventricle wall using Image J software. The thickest parts in the septum and left ventricle compact myocardium were selected for measuring wall thickness.

Statistical Analysis

Data are shown as mean ± SEM. Statistical analysis was carried out by student's t test and ANOVA. A $p<0.05$ was considered significant.

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