SUPPLEMENTAL MATERIALS

Single-cell Transcriptome Analysis Reveals Embryonic Endothelial Heterogeneity at Spatial-Temporal Level and Multi-functions of MicroRNA-126 in Mice

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Short title: Endothelium heterogeneity and miR-126 functions

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Expanded Materials & Methods

Generation of miR-126-ecKO mice and animal work

All animal experiments were performed in accordance with the institutional guidelines of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. For Tie2cre-miR126^{Flox} targeting construct, the first loxP was inserted into host gene Egfl7 intron 7-5' end, and the second loxP together with neomycin-resistant gene flanked by FRT sites was inserted into intron 7-3' end. Genetic transmission was confirmed by backcrossing the chimera with C57BL/6J mice and the Neo-cassette was removed using FLPeR mice. The floxed mice used in this study were maintained in C57BL/6J with at least four backcrosses. The Tie2-cre mice were obtained from Shanghai Model Organism. Briefly, the Tie2-Cre transgene was excised from the plasmid vector backbone. Microinjection into fertilized C57BL/6J x SJLF1 oocytes and other surgical procedures were performed as described[82]. We used male mice because this simplified the breeding strategy (as miR-126 is located on the X chromosome) and also to minimize estrogen-dependent fluctuations in response to angiogenesis and glucose metabolism[26, 27]. For the genotyping of Tie2-miR126 knockout allele, the primers used were forward primer (ATTTGCCTGCATTACCGGTC), and the reverse primer (ATCAACGTTTTCTTTTCGG).

Single cell suspension preparation

E14.5 mouse embryos were cut into pieces with very sharp knives, followed by dissociating in collagenase I solution(2mg/ml) for 45 minutes at 37°C. Before FACS, the Blood cells were depleted by Red Blood cells lysis buffer (BD Pharmingen). Cells were then labeled with PE rat anti-mouse CD31 (MEC 13.3, BD Pharmingen, 1:50), PE-cy7 rat anti-mouse CD144 (55-7H1, BD Pharmingen, 1:50), FITC rat anti-mouse CD45(30-F11, BD Pharmingen, 1:50) and CD31^{+}CD144^{+}CD45^{-} cells were sorted through a MoFlo FACS machine (MoFlo Astrios EQ, Beckman Coulter) directly into PBS containing 0.04% BSA.

Single-cell RNA-seq library preparation and sequencing

The single-cell RNA library was built via 10x Chromium platform, and 10000 counted FACS-enriched cells were loaded per channel approximately[81]. The miR-126-ecKO and Littermate control sample were loaded together with one chip. The libraries were built via the Chromium platform and Chromium Single Cell 3' v2 chemistry. Finally, the libraries were loaded on an illumine Hiseq 2500 Rapid flowcell with a PE150 sequencing strategy. At last, 70553 reads per cell and 56566 reads per cell were obtained from miR-126-ecKO and Littermate control ECs.

Single-cell RNA-seq data analysis

After aggregation of the sequencing data from miR-126-ecKO and Littermate control E14.5 embryos, R package Seurat 3.0 was used for cell filter, data normalization, variable gene selection, unsupervised clustering, and uniform manifold approximation and projection (UMAP) according to their recommended steps[80]. Briefly, Seurat objects were created from the aggregated library as matrix containing gene-by-cell expression data. Cells with less than 200 genes expression or a

percentage of more than 0.05 mitochondrial genes were filtered out. Then data were log-normalized and scaled for subsequent analysis. Variable genes were found and used for principal component analysis (PCA), which was performed for dimension reduction. ElbowPlot function was used for the determination of the numbers of principal components, followed by unsupervised clustering and UMAP. FindAllMarkers function was used to identify the genes exclusively expressed in each cluster. Visualization of total profiles of each cluster was generated with Seurat function DimPlot. Visualization of gene expression with feature plot, dot plot, and heatmap was generated with Seurat function FeaturePlot, DotPlot and DoHeatmap, respectively. Differentially expressed genes ($P<0.01$) between 2 identities were found with FindMarkers function. GO biological function analysis was performed with marker genes of each cluster found by FindMarkers function with average adjusted $p<0.05$ on toppgene website (https://toppgene.cchmc.org/) and then plotted with R package Goplot.

Immunofluorescence of whole-mount embryonic dorsal skin

E14.5 mouse embryos were fixed in 4% paraformaldehyde overnight at 4°C. Then the embryos were put into pre-cold methanol at -20 °C for at least 1hours. Embryos' dorsal skin tissues were dissected carefully with forceps, leaving the superficial vessel and lymphatic network layer within the dorsal skin intact. Samples were blocked in 10% heat inactivated donkey serum in PBS containing 0.2% Triton X-100 for 2 hours at room temperature. Samples were then incubated in blocking buffer containing primary antibodies overnight at 4°C, followed by Alexa-488 (Invitrogen) 1:500 2hours at room temperature for immunofluorescence detection. Whole-mount samples were mounted on glass slides before analyzed with a confocal microscope. The IF antibody against mouse were as follows: PECAM1(553370, BD). Secondary antibodies were Alexa Fluor 488 and 555 obtained from Molecular Probes, Inc. (Invitrogen).

Immunofluorescence of P7 pups' retina

The P7 nuts' retina were harvested by forceps[83]. Then, immediately put into methanol and rehydrated by 50% methanol. Finally, incubated with primary antibody overnight at 4°C, Appropriate Alexa 488 (Invitrogen), Cy5 (Jackson) conjugated secondary antibodies were used for staining for 2 hours at room temperature and analyzed with a confocal microscope (Car Zeiss 710).

Masson Staining (identify fibrosis in tissue sections)

Masson staining were carried out with a commercial kit (Solarbio, catalog: G1346). And the tissues were processed by 4% Paraformaldehyde fixation which was followed by Paraffin embedding and sectioning.

Alizarin Red S Staining (identify calcium in tissue sections)

The Alizarin Red S Staining was performed according to the kit (Solarbio, catalog: G3280), briefly, Washing the De-paraffinize slides to distilled water and Staining slides with the Alizarin Red Solution for 30 seconds to 5 minutes, then, observing the reaction microscopically, finally, Shaking off excess dye and blot sections. Dehydrating in acetone, 20 dips. Then in Acetone-Xylene (1:1)

solution, 20 dips. Clearing in xylene and mount in a synthetic mounting medium.

Intraperitoneal Pyruvate Tolerance Test (IPTT)

After 14 hours fasting, IPGTT were conducted by intraperitoneal injection of 2.5 g/kg of pyruvate and blood glucose levels were measured by a glucometer at 0, 15, 30, 30, 60, 90, 120 minutes after glucose administration. At least 6 miR126 KO and WT adult mice were used at one performance， and each time point was measured three times.

Intraperitoneal Glucose Tolerance Test (IGTT)

After 14 hours fasting, IPGTT were conducted by intraperitoneal injection of 1.5 g/kg of glucose and blood glucose levels were measured by a glucometer at 0, 15, 30, 30, 60, 90, 120 minutes after glucose administration. At least 6 miR126 KO and WT adult mice were used at one performance， and each time point was measured three times.

Adult hepatic endothelial cells separation and enrichment.

The adult hepatic endothelial cells were separated by collagenase perfusion and followed by Percoll-gradient separation. Briefly, after the mice were anesthetized by intraperitoneal injection of sodium pentobarbital, they were fixed on the operating plate, the liver was perfused through the inferior vena cava, and the in-situ digestion was performed. Firstly, transfer the digestion solution to a clean 50mL centrifuge tube and put it in a 37℃ constant temperature water bath for digestion for 10-15 minutes. Secondly, digested tissues were centrifuge at 100g for 5 minutes, and the supernatant is collected. Thirdly, Centrifuge the collected supernatants at 400g for 10 minutes and discard the supernatants. Finally, the precipitate was resuspended in 25% Percoll solution, mixed thoroughly, and carefully added to the surface of the 50% Percoll solution to form an obvious interface. Then, centrifuge at 900g for 20 minutes without brake. Collect the turbidity zone, which were mainly occupied with Hepatic ECs, between 25% and 50% Percoll, and dilute it with RPMI-1640 10 times, centrifuge at 2000r/min for 10 minutes, and discard the supernatant. Take 2ml of ECM and resuspend the pellet, spread it into a 6-well plate treated with 0.1% gelatin, and make up to 3ml. Next day, the adherent endothelial cells were collected and were used with following experiments such as Western Blotting and quantitative PCR.

***In vivo* administration of miR-126-3p agomir**

To investigate the effects of miR-126 on blood glucose metabolism, the chemically modified agomir were used to increase miRNA expression in vivo. The miRNA agomir is a chemically modified stable miRNA mimic, and it's *in vivo* delivery resulted in target silencing was similar to the effects induced by the overexpression of endogenous miRNA. The miR-126-3p agomir was synthesized by RiboBio (Guangzhou, China). Scrambled mimics that did not target any miRNA were injected as a NC. The mice received either agomir or their NCs (200 μl) via tail vein injection (100nM/kg body weight, n=6 per group) for three consecutive days. Then the Intraperitoneal Glucose Tolerance Test (IGTT) were carried out 7 days after the third tail vein injection.

Microscopy

Specimens were mounted with fluorescence mounting medium (Dako, Glostrup, Denmark) or Mowiol mounting medium, and confocal images (e.g., cells and sections) were generally captured using a Zeiss LSM-710 Falcon with 20X and 40X oil objectives. Images were processed with Car Zeiss software.

Statistical analysis

For experimental studies, all quantitative data were evaluated whether they followed the normal distribution by the Shapiro-Wilk test and equal variance by F-test. For data passed both tests, data are expressed as means ± SEM, Student's t-test was used for the comparison between 2 groups, and for comparison among multiple groups, the data were analyzed by one-way ANOVA with Tukey's post hoc test. For the data that were not normally distributed, nonparametric test (Mann-Whitney U test) was performed and presented as median ± SD. All P values are 2-sided, and $P<0.05$ was considered a statistically significant difference.

Supplemental Figures and legends

Figure S1

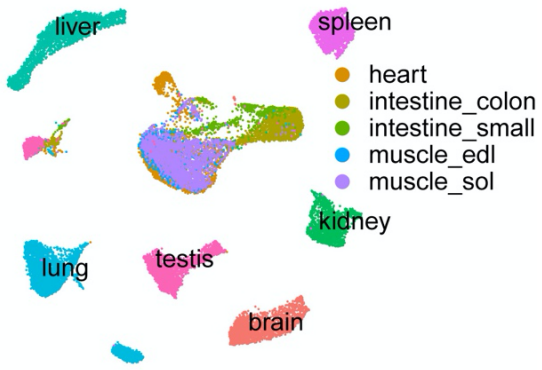
A

Organ/Tissue	Number
brain	3482
heart	3219
intestine_colon	2832
intestine_small	974
kidney	4001
liver	3481
lung	3972
muscle_edl	1157
muscle_sol	2457
spleen	3448
testis	3544
Total	**32567**

ECs →

Organ/Tissue	Number
brain	2175
heart	2524
intestine_colon	2271
intestine_small	722
kidney	2048
liver	2851
lung	3506
muscle_edl	1013
muscle_sol	2247
spleen	2287
testis	2730
Total	**24374**

B



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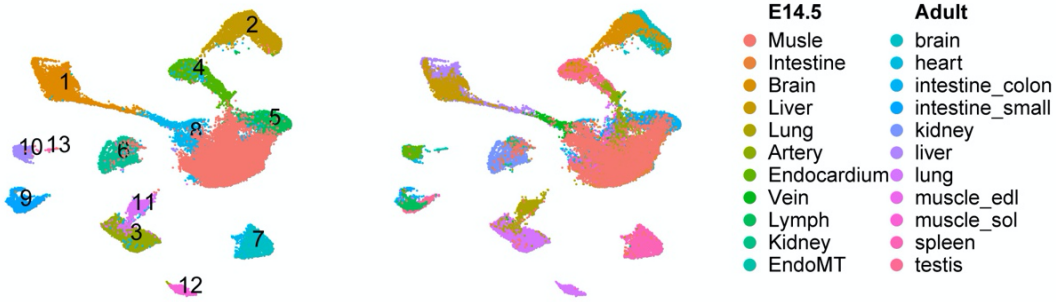
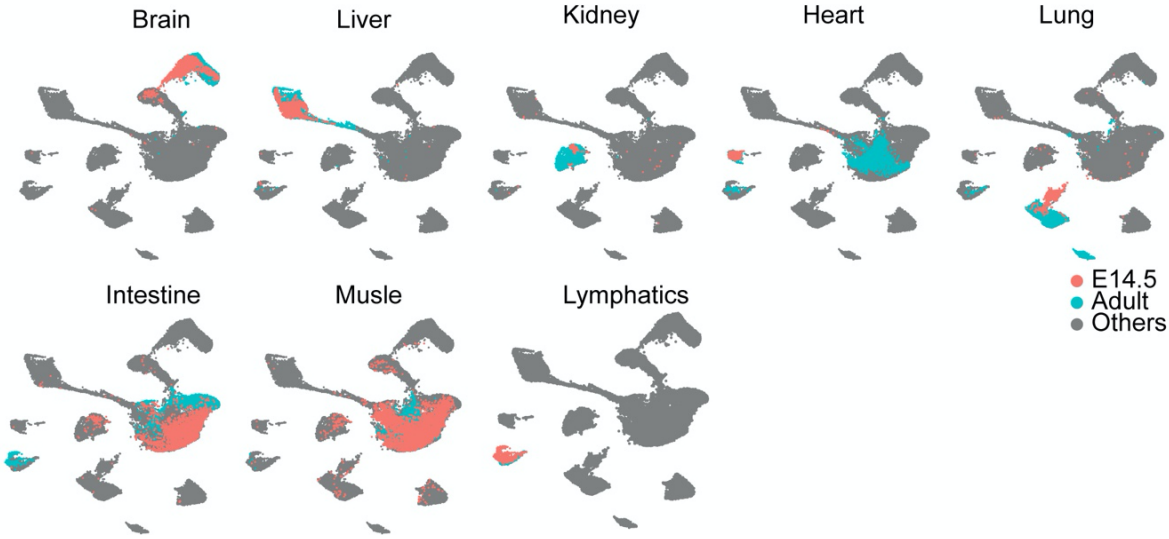


D



Supplemental Figure S1. Integration of eECs and aECs extracted from published dataset E-MTAB-8077 indicated the tissue sources of eECs.

A, aECs were extracted from published single-cell dataset E-MTAB-8077 and filtered by UMI number (Pecam1>0, Cdh5>0, Ptprc=0, Alas2<15). **B,** Uniform Manifold Approximation and Projection (UMAP) was performed to integrated aECs by Seurat. Colors denote different tissues. **C,** eECs and aECs extracted from E-MTAB-8077 were aggregated. Colors denote different clusters or tissues. **D,** eECs and aECs with the same sources were highlighted and the aECs were acted as anchors. eECs: embryonic endothelial cells; aECs: adult endothelial cells.

Figure S2

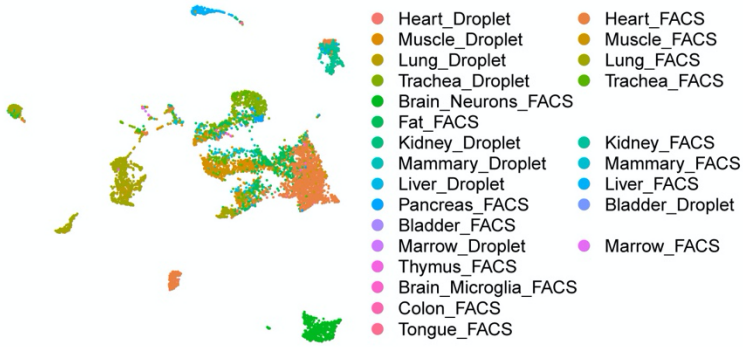
A

Organ/Tissue	FACS	Droplet
Heart	7115	654
Muscle	2102	4543
Lung	1923	1476710
Trachea	1391	1474560
Brain_Neurons	5799	0
Fat	5862	0
Kidney	865	2782
Mammary	2663	4481
Liver	981	1924
Pancreas	1961	0
Bladder	1638	2500
Marrow	5355	4112
Thymus	1580	1431
Brain_Microglia	4762	0
Colon	4149	0
Tongue	1432	7538
Skin	202	0
Spleen	1718	3458
Total	51498	2984693

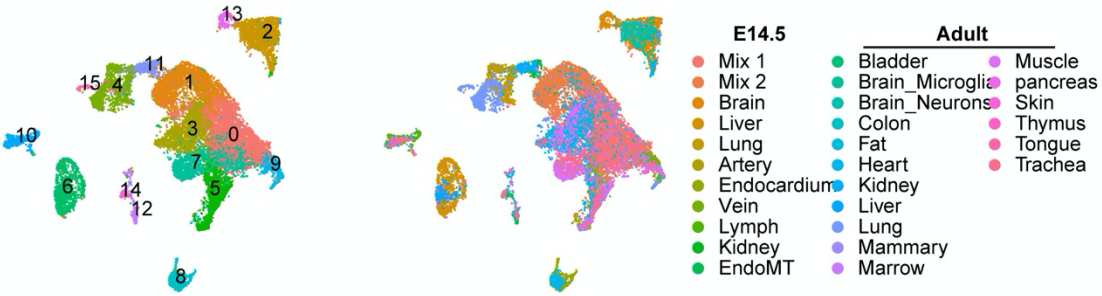
ECs →

Organ/Tissue	FACS	Droplet
Heart	1277	218
Muscle	181	1094
Lung	602	395
Trachea	110	739
Brain_Neurons	577	0
Fat	548	0
Kidney	83	309
Mammary	45	224
Liver	172	19
Pancreas	66	0
Bladder	6	34
Marrow	4	7
Thymus	9	0
Brain_Microglia	6	0
Colon	5	0
Tongue	5	0
Skin	0	0
Spleen	0	0
Total	3697	3039

B



C

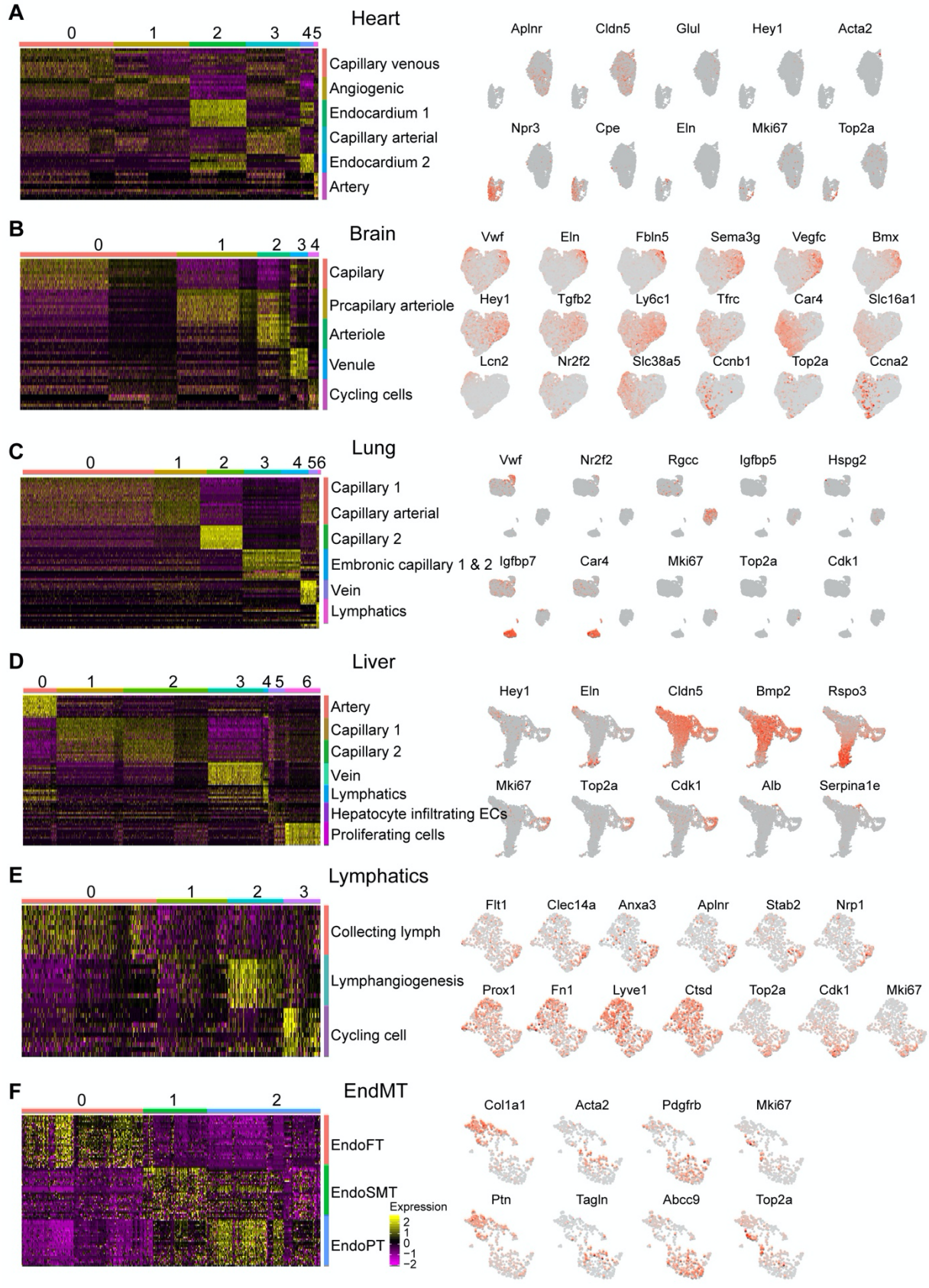


D

Supplemental Figure S2. Integration of eECs and aECs extracted from published dataset GSE109774 verified the eECs clustering.

A, ECs with different sequencing strategies (10X genomics and Droplet) were extracted from published single-cell dataset GSE109774 by UMI (Pecam1>0, Cdh5>0, Ptprc=0, Alas2<15). **B,** Uniform Manifold Approximation and Projection (UMAP) was performed to integrated aECs by Seurat. Colors denote different tissues with different sequencing methods. **C,** eECs and aECs extracted from GSE109774 were aggregated. Colors denote different clusters or tissues. **D,** eECs and aECs with the same sources were highlighted and the aECs were acted as anchors. eECs: embryonic endothelial cells; aECs: adult endothelial cells.

Figure S3

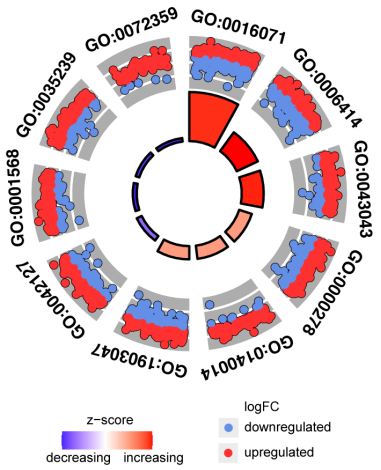

A
Heart
0 1 2 3 4 5
Capillary venous
Angiogenic
Endocardium 1
Capillary arterial
Endocardium 2
Artery
Aplnr Cldn5 Glul Hey1 Acta2
Npr3 Cpe Eln Mki67 Top2a
B
Brain
0 1 2 3 4
Capilary
Prcapilary arteriole
Arteriole
Venule
Cycling cells
Vwf Eln Fbln5 Sema3g Vegfc Bmx
Hey1 Tgfb2 Ly6c1 Tfrc Car4 Slc16a1
Lcn2 Nr2f2 Slc38a5 Ccnb1 Top2a Ccna2
C
Lung
0 1 2 3 4 5 6
Capillary 1
Capillary arterial
Capillary 2
Embronic capillary 1 & 2
Vein
Lymphatics
Vwf Nr2f2 Rgcc Igfbp5 Hspg2
Igfbp7 Car4 Mki67 Top2a Cdk1
D
Liver
0 1 2 3 4 5 6
Artery
Capillary 1
Capillary 2
Vein
Lymphatics
Hepatocyte infiltrating ECs
Proliferating cells
Hey1 Eln Cldn5 Bmp2 Rspo3
Mki67 Top2a Cdk1 Alb Serpina1e
E
Lymphatics
0 1 2 3
Collecting lymph
Lymphangiogenesis
Cycling cell
Flt1 Clec14a Anxa3 Aplnr Stab2 Nrp1
Prox1 Fn1 Lyve1 Ctsd Top2a Cdk1 Mki67
F
EndMT
0 1 2
EndoFT
EndoSMT
EndoPT
Expression
2
1
0
−1
−2
Col1a1 Acta2 Pdgfrb Mki67
Ptn Tagln Abcc9 Top2a

Supplemental Figure S3. The vascular bed heterogeneity still existed within eECs tissues, related to figure 3.

A, Heat map of the top 10 enriched genes in each subcluster of cardiac ECs and expression profiles of of marker genes in cycling cells (Mki67, Top2a, Cdk1), endocardium cells (Npr3, Cpe, Eln), arterial ECs (Glul, Hey1) and venous ECs (Aplnr, Cldn5). **B,** Heat map of the top 10 enriched genes in each subcluster of cerebral ECs and expression profiles of marker genes in in atrial ECs (Vwf, Eln, Fbln5, Sema3g, Vegfc, Bmx, Hey1, Tgfb2, Ly6c1), capillary ECs (Tfrc, Car4, Slc16a1), venous ECs (Lcn2, Nr2f2, Slc38a5), and proliferation (Ccna2, Top2a, Ccnb1). **C,** Heat map of the top 10 enriched genes in each subcluster of pulmonary ECs and expression profiles of of enriched genes in large vessel ECs (Vwf, Nr2f2), embryonic pulmonary ECs (Rgcc, Igfbp5, Hspg2, Igfbp7, Car4) and proliferating ECs (Mki67, Top2a, Cdk1). **D,** Heat map of the top 10 enriched genes in each subcluster of hepatic ECs and expression profiles of of marker genes in arterial ECs (Vwf,Eln), capillary ECs (Cldn5, Bmp2), venous ECs (Rspo3), proliferating ECs (Mki67, Top2a, Cdk1) and hepatocyte infiltrating ECs (Alb, Serpina1e). **E,** Heat map of the top 10 enriched genes in each subcluster of lymphatics ECs and expression profiles of marker genes in collecting lymphatic ECs (Ctsd, Fn1, Prox1 and Lyve1), ECs of lymphangiogenesis (Aplnr, Flt1, Anxa3, Stab2, Clec14a, and Nrp1), and proliferative cells (Top2a, Mki67, and Cdk1). **F,** Heatmap of the top 10 enriched genes in each subcluster of EndMT ECs and expression profiles of marker genes in fibroblast (Col1a1, Ptn), smooth muscle cell (Acta2, Tagln), pericyte (Pdgfrb, Abcc9) and proliferating ECs (Mki67, Top2a).

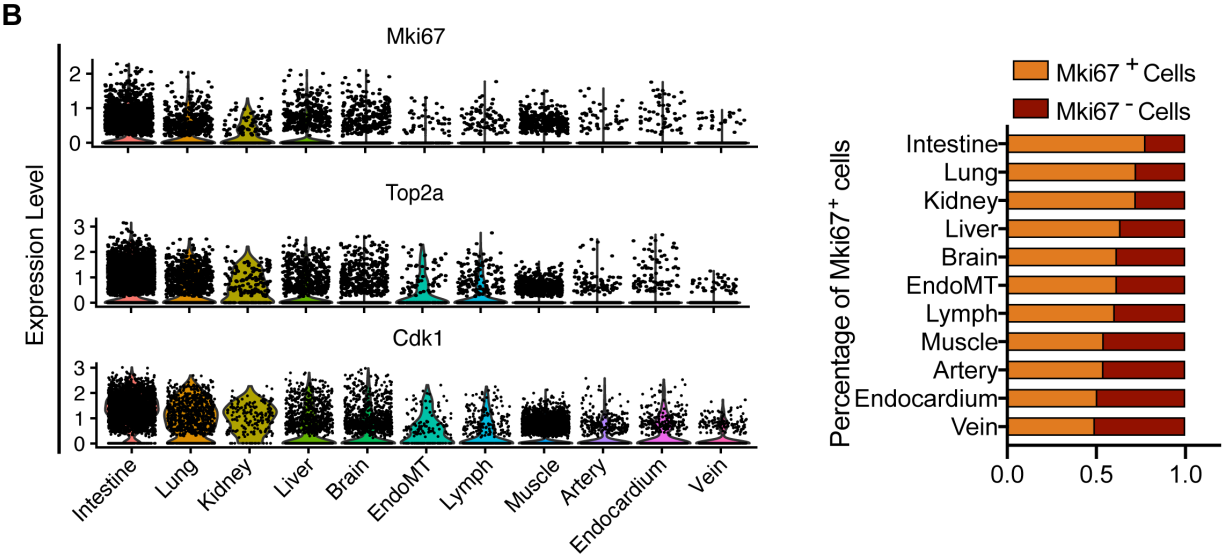
Figure S4

A



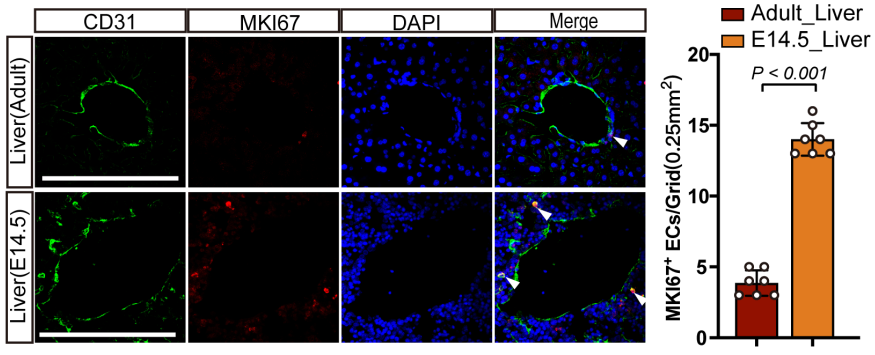
ID	Description
GO:0016071	mRNA metabolic process
GO:0006414	Translational elongation
GO:0043043	Peptide biosynthetic process
GO:0000278	Mitotic cell cycle
GO:0140014	Mitotic nuclear division
GO:1903047	Mitotic cell cycle process
GO:0042127	Regulation of cell population proliferation
GO:0001568	Blood vessel development
GO:0035239	Tube morphogenesis
GO:0072359	Circulatory system development

B



C

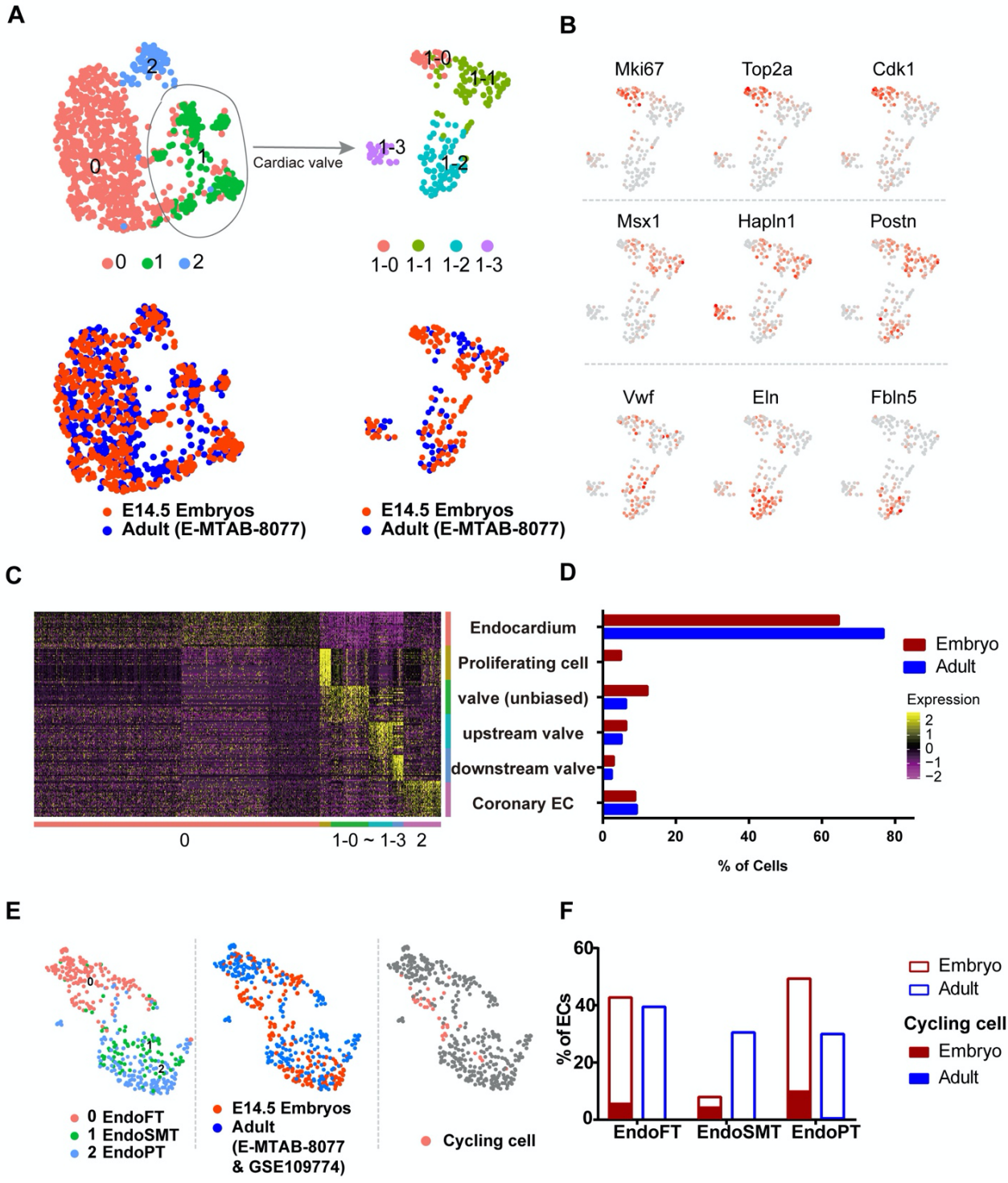
D



Supplemental Figure S4. Proliferation related genes were expressed more highly in embryonic ECs than in Adult's.

A, Gene Ontology analysis on different expressed genes (DEG) between embryonic ECs and adult ECs. The Biological Processes are mainly related to mitotic cell cycle, cell population proliferation and tube morphogenesis. **B,** Expression profile analysis on proliferation related genes across different organs or tissues by Vnplot. And quantification of Mki67+ cells proportion in each subcluster. **C,** Proliferation related genes' expression profile showed by FeaturePlot. **D,** double immunofluorescence staining of MKI67 and PECAM1 in embryonic and adult hepatic tissue frozen section. The white arrows indicate double-positive (MKI67+&PECAM1+) endothelial cells.

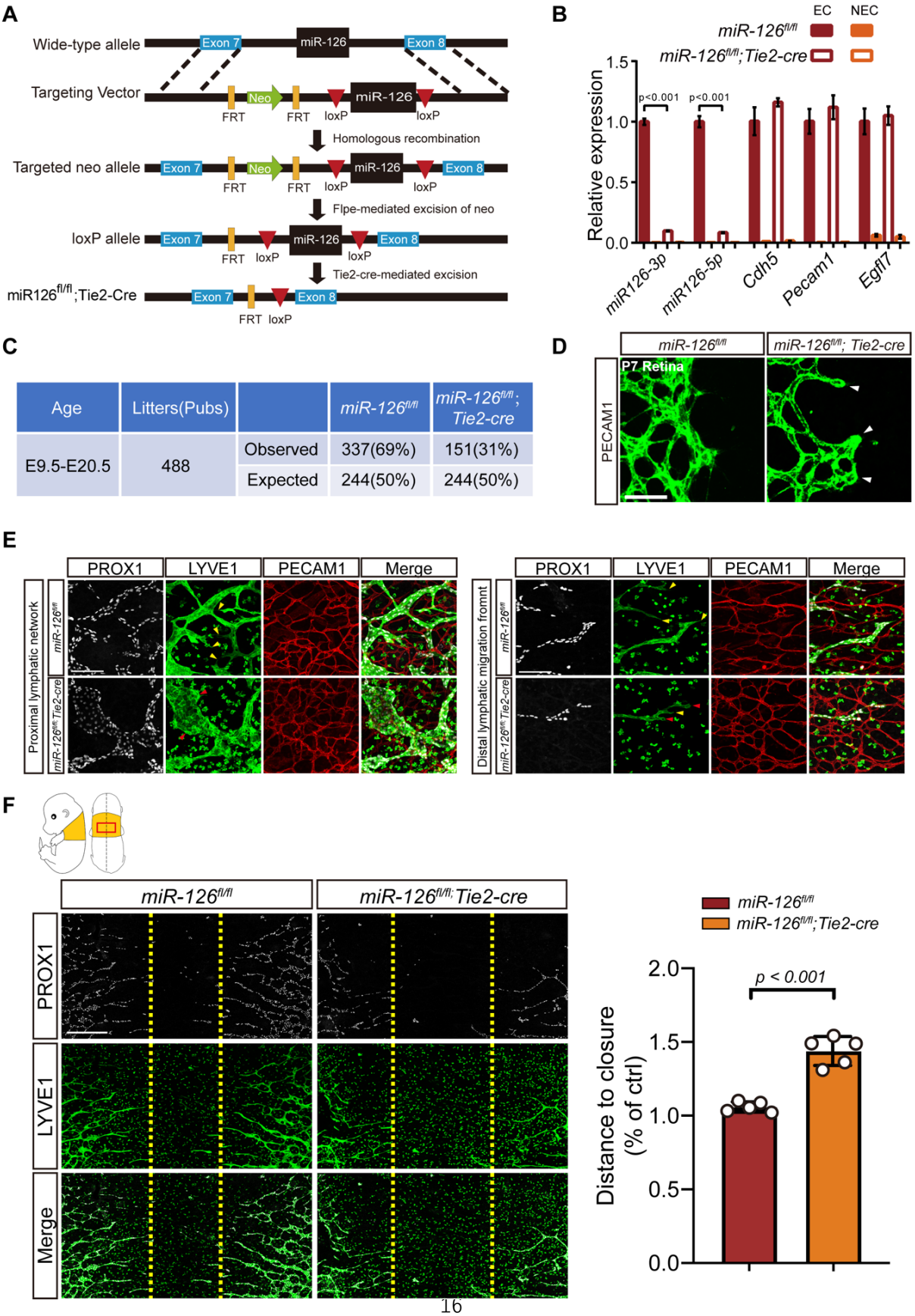
Figure S5


A
2
0
1
Cardiac valve
1-0
1-1
1-3
1-2
0 1 2
1-0 1-1 1-2 1-3
E14.5 Embryos
Adult (E-MTAB-8077)
E14.5 Embryos
Adult (E-MTAB-8077)
B
Mki67
Top2a
Cdk1
Msx1
Hapln1
Postn
Vwf
Eln
Fbln5
C
0
1-0 ~ 1-3 2
Endocardium
Proliferating cell
valve (unbiased)
upstream valve
downstream valve
Coronary EC
D
Embryo
Adult
Expression
2
1
0
−1
−2
0
20
40
60
80
% of Cells
E
0
1
2
0 EndoFT
1 EndoSMT
2 EndoPT
E14.5 Embryos
Adult
(E-MTAB-8077
& GSE109774)
Cycling cell
F
60
40
20
0
% of ECs
EndoFT
EndoSMT
EndoPT
Embryo
Adult
Cycling cell
Embryo
Adult

Supplemental Figure S5. Endocardium was potentially differentiated into multiple cells.

A, Endocardial aECs and eECs were classified into 3 subclusters. Colors denote different subclusters or mouse origin. **B,** Gene expression profiles of conventional endocardium (Npr3), cardiac valve (Postn, Hapln1, and Tbx20), and coronary ECs (Aplnr and Fabp4). **C,** Heat map of the top 10 enriched genes in each endocardial subcluster reveals the transcriptional diversity. **D,** Fractions of endocardial aECs and eECs in each subcluster. **E,** aECs and eECs transited to mesenchymal cells (cluster 12 and cluster 14 in Figure S2C) were classified into 3 subclusters and the atlas of cycling cells in EndoMT. **F,** Fractions of endocardial aECs and eECs in each subcluster. eECs: embryonic endothelial cells; aECs: adult endothelial cells.

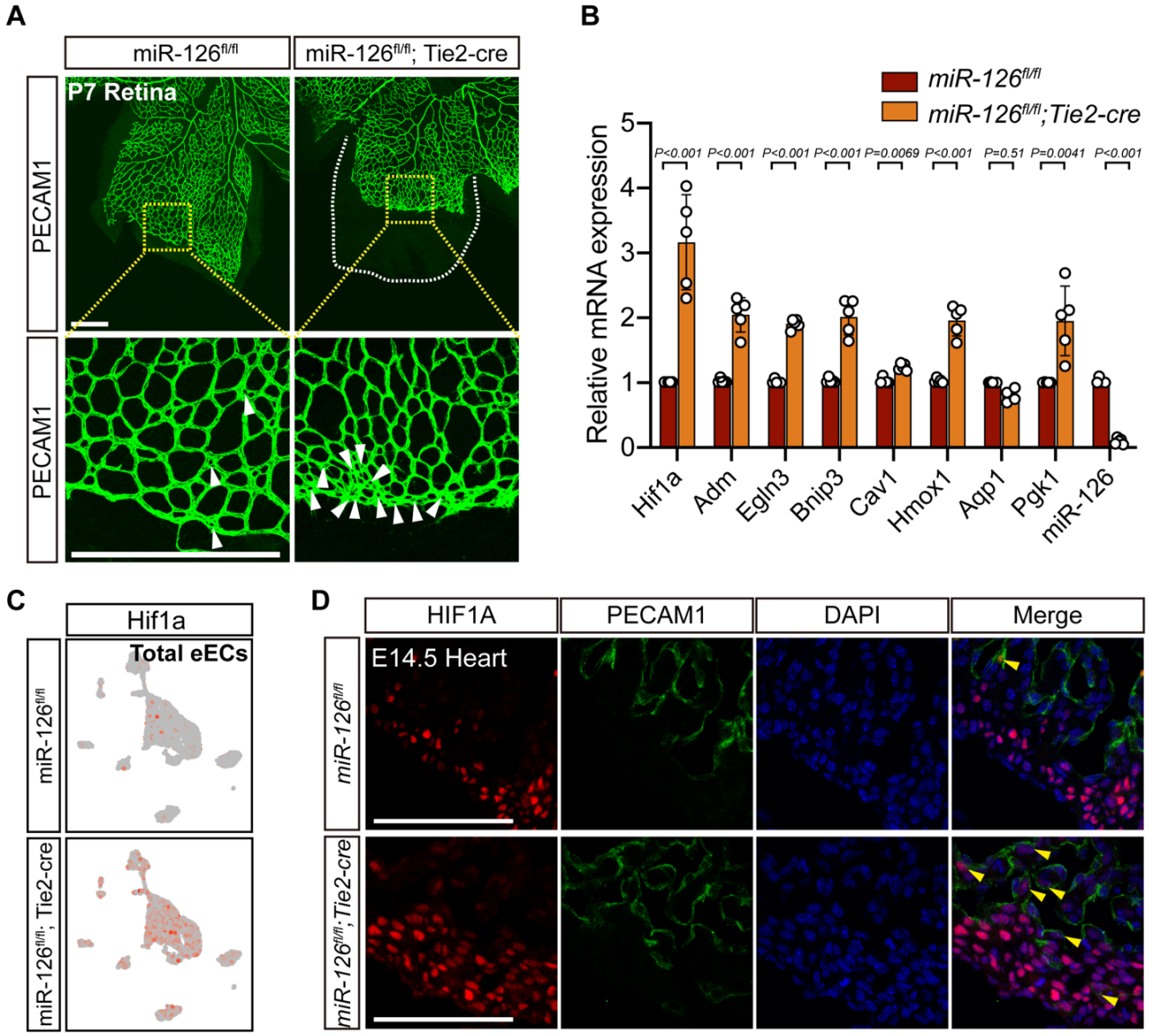
Figure S6


A
Wide-type allele
Exon 7
miR-126
Exon 8
Targeting Vector
Neo
FRT
FRT
loxP
miR-126
loxP
Homologous recombination
Targeted neo allele
Exon 7
Neo
FRT
FRT
miR-126
loxP
Exon 8
loxP
Flpe-mediated excision of neo
loxP allele
Exon 7
FRT
loxP
miR-126
loxP
Exon 8
Tie2-cre-mediated excision
miR126fl/fl;Tie2-Cre
Exon 7
FRT
loxP
Exon 8
B
EC
NEC
miR-126fl/fl
miR-126fl/fl;Tie2-cre
Relative expression
1.5
1.0
0.5
0.0
p<0.001
p<0.001
miR126-3p
miR126-5p
Cdh5
Pecam1
Egfl7
C
Age
Litters(Pubs)
miR-126fl/fl
miR-126fl/fl; Tie2-cre
E9.5-E20.5
488
Observed
337(69%)
151(31%)
Expected
244(50%)
244(50%)
D
miR-126fl/fl
miR-126fl/fl; Tie2-cre
PECAM1
P7 Retina
E
PROX1
LYVE1
PECAM1
Merge
Proximal lymphatic network
miR-126fl/fl
miR-126fl/fl;Tie2-cre
Distal lymphatic migration fromnt
F
miR-126fl/fl
miR-126fl/fl;Tie2-cre
PROX1
LYVE1
Merge
miR-126fl/fl
miR-126fl/fl;Tie2-cre
p < 0.001
Distance to closure
(% of ctrl)
2.0
1.5
1.0
0.5
0.0

Supplemental Figure S6. Sprouting angiogenesis was decreased in miR-126-ecKO embryos.

A, Strategy to generate miR-126 mutant mice by homologous recombination. Details were shown in methods. **B,** Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of miR-126 and ECs-related genes confirmed the specific knockout of miR-126 in ECs. **C,** The actual and expected number of mice for each genotype at E9.5~20.5 was shown. **D-E,** Vascular (**D**) and lymphatic (**E**) vessels sprouting were decreased in miR-126-ecKO embryos as indicated with blunted terminal, showed with white arrow heads. **F,** Lymphatic vessel migration rate was decreased in miR-126-ecKO embryos as indicated with marker genes PROX1 and LYVE1 by staining lymphatic vessels on dorsal skin at E14.5. Scale bar=500μm.

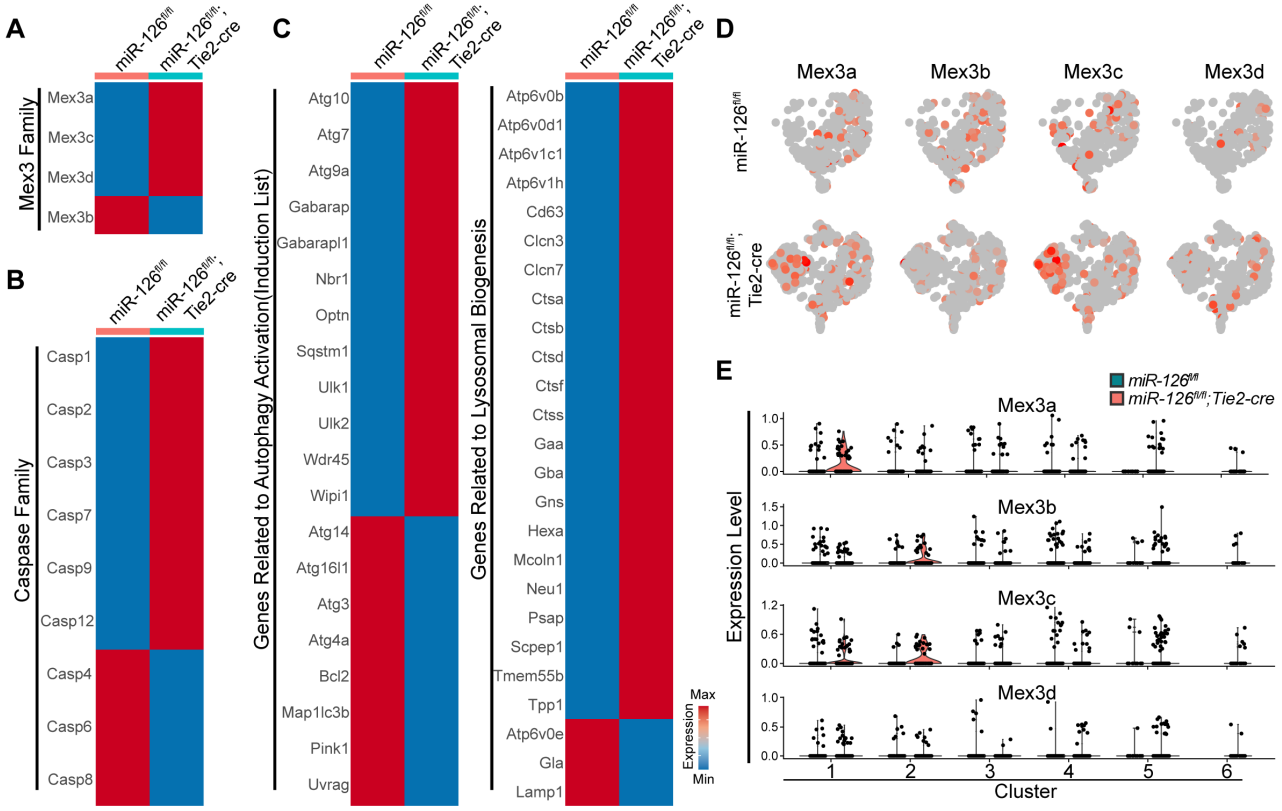
Figure S7

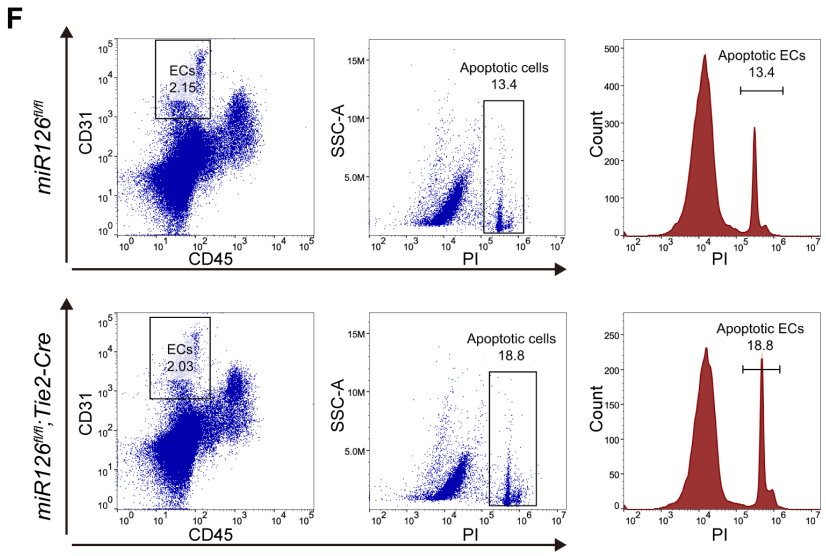


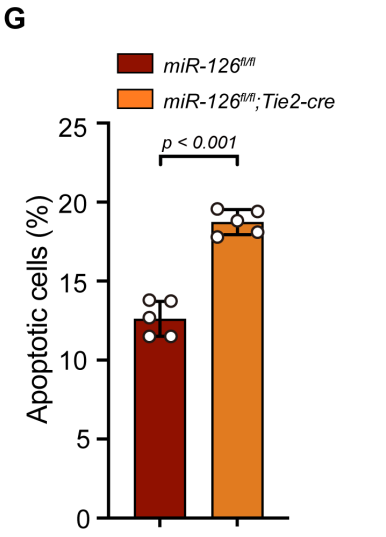
Supplemental Figure S7. Genes related to hypoxia response were preferentially upregulated in embryonic ECs of miR-126-ecKO mice.

A, immunofluorescence of ECs marker gene PECAM1 on miR-126-ecKO mice and littermate controls retina at postnatal Day 7. **B,** Genes related with hypoxia response and aberrantly up-regulated after miR-126 knocked out were confirmed by Realtime PCR analysis on embryonic endothelial cells which obtained by FACS from miR-126 deficient mice and littermate control at E14.5. **C,** Feature plot of Hif1a in total miR-126-deficient endothelial cells and littermate controls. **D,** Dual immunofluorescence of HIF1A and PECAM1, the yellow arrows indicate the endothelial cells which expressed with HIF1A.

Figure S8

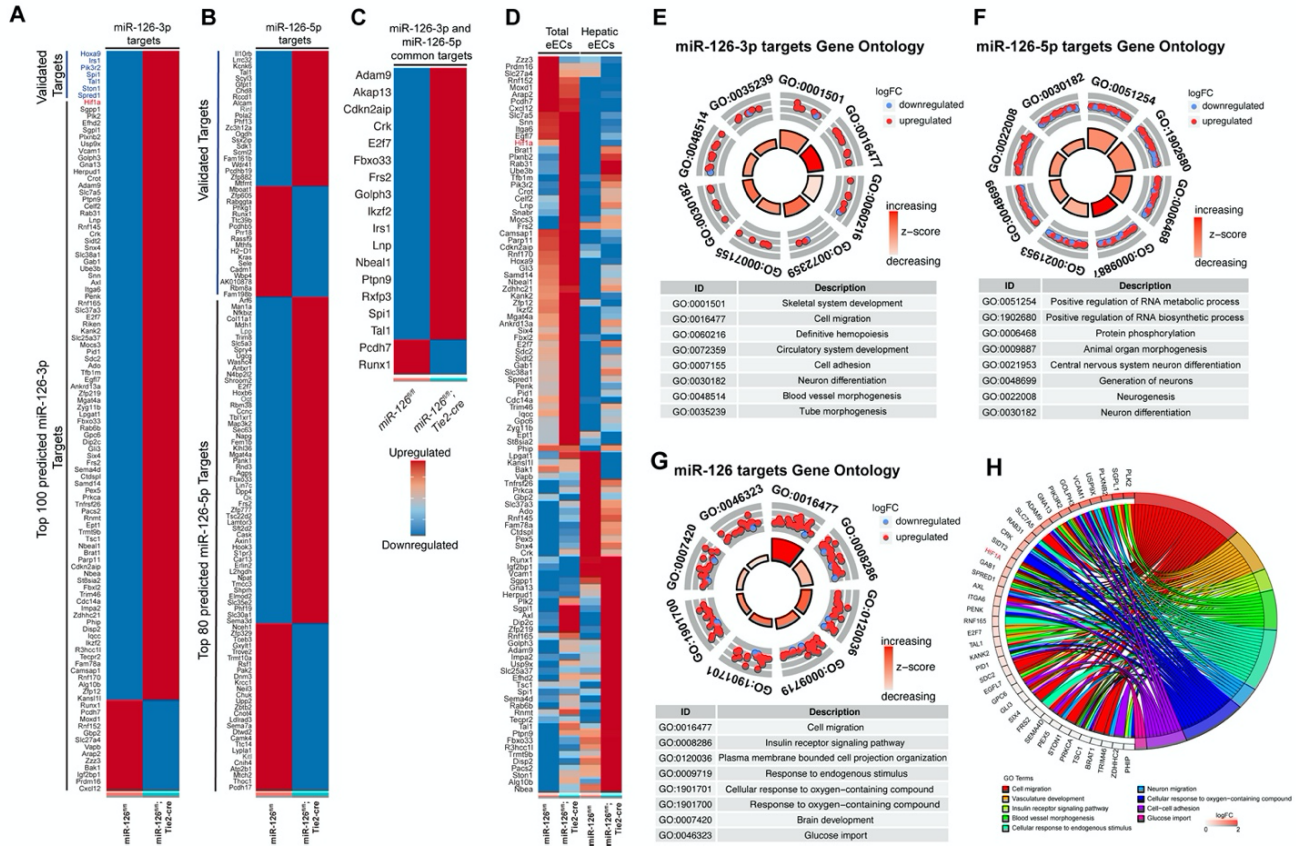

A
miR-126fl/fl
miR-126fl/fl;Tie2-cre
Mex3 Family
Mex3a
Mex3c
Mex3d
Mex3b
B
Caspase Family
Casp1
Casp2
Casp3
Casp7
Casp9
Casp12
Casp4
Casp6
Casp8
C
Genes Related to Autophagy Activation(Induction List)
Atg10
Atg7
Atg9a
Gabarap
Gabarapl1
Nbr1
Optn
Sqstm1
Ulk1
Ulk2
Wdr45
Wipi1
Atg14
Atg16l1
Atg3
Atg4a
Bcl2
Map1lc3b
Pink1
Uvrag
Genes Related to Lysosomal Biogenesis
Atp6v0b
Atp6v0d1
Atp6v1c1
Atp6v1h
Cd63
Clcn3
Clcn7
Ctsa
Ctsb
Ctsd
Ctsf
Ctss
Gaa
Gba
Gns
Hexa
Mcoln1
Neu1
Psap
Scpep1
Tmem55b
Tpp1
Atp6v0e
Gla
Lamp1
Max
Expression
Min
D
Mex3a
Mex3b
Mex3c
Mex3d
miR-126fl/fl
miR-126fl/fl;Tie2-cre
E
miR-126fl/fl
miR-126fl/fl;Tie2-cre
Mex3a
Mex3b
Mex3c
Mex3d
Expression Level
Cluster
1
2
3
4
5
6


F
miR126fl/fl
CD31
CD45
ECs
2.15
SSC-A
PI
Apoptotic cells
13.4
Count
Apoptotic ECs
13.4
miR126fl/fl;Tie2-Cre
ECs
2.03
Apoptotic cells
18.8
Apoptotic ECs
18.8


G
miR-126fl/fl
miR-126fl/fl;Tie2-cre
p < 0.001
Apoptotic cells (%)

Supplemental Figure S8. Genes related to apoptosis were upregulated in miR-126 deficient eECs. **A-C,** Heatmap analysis showing expression prefile of genes related to autophagy including Mex3 Family, Caspase Family, genes related to autophagy activation and genes related to lysosomal biogenesis. **D and E,** Feature plot and Vnplot analysis of genes composed of Mex3 family. **F,** Apoptosis analysis on embryonic ECs (eECs) at E14.5 by flow cytometry. **G,** Quantification of apoptosis rate changes after miR-126 being deficient in embryonic ECs. The $CD45^-$, $CD31^+$ and PI^+ cells are apoptotic ECs.

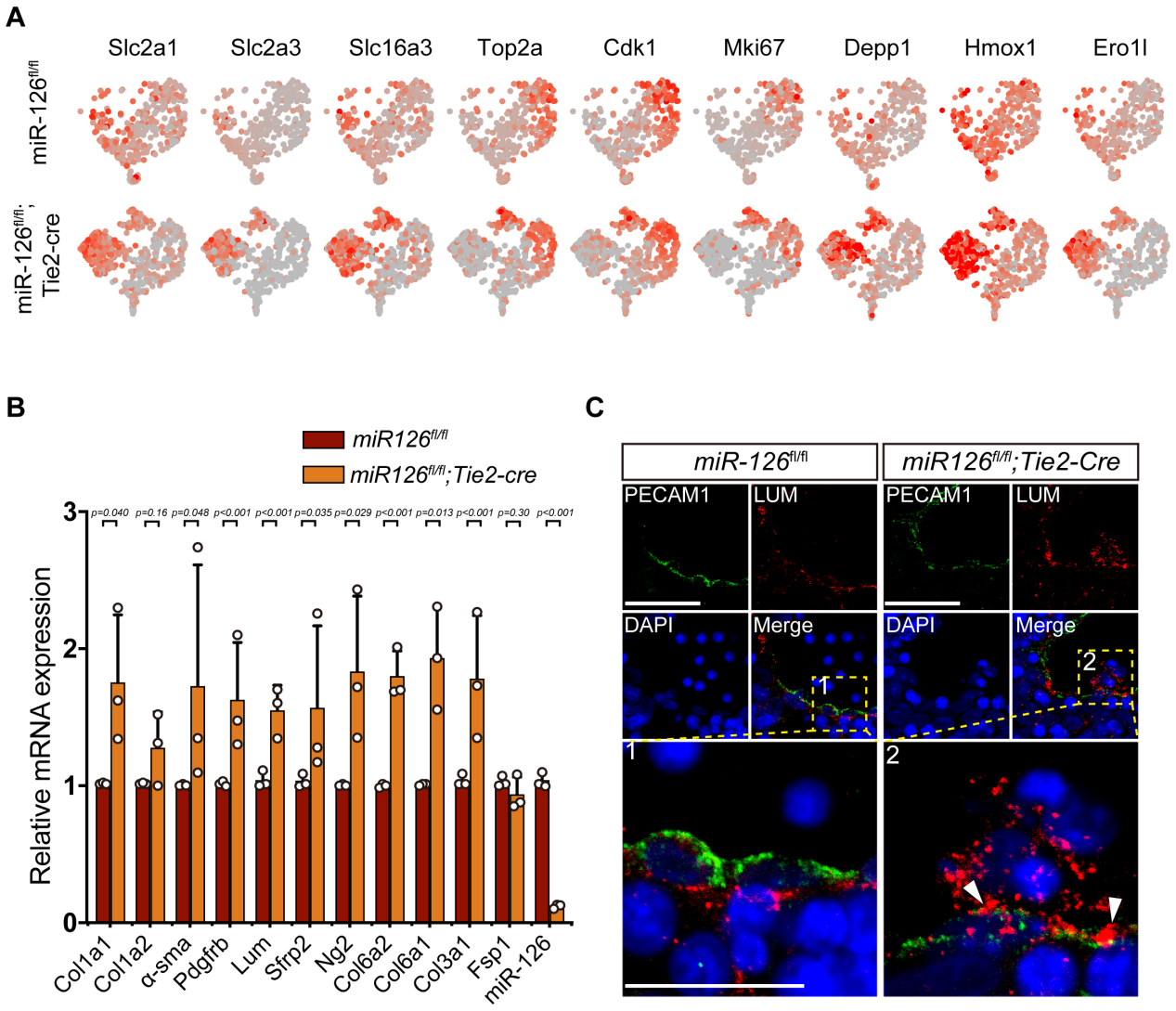
Figure S9



Supplemental Figure S9. MiR-126 targets were mainly upregulated in miR-126 deficient eECs and had tissue heterogeneity.

A-C, Heatmap analysis showing miR-126-3p targets, miR-126-5p targets and their common targets expression profile in miR-126 deficient mice and littermate controls eECs single-cell data. **D,** Heatmap shows miR-126 targets expression profile changes after miR-126 knocked out. **E & F**, Gene Ontology analysis on miR-126-3p and miR-126-5p targets by *GoPlot*. **G,** GO analysis on screened miR-126 targets via R. package *GOPlot*. **H,** Display of the relationship between representative genes and terms by *GOchord.*

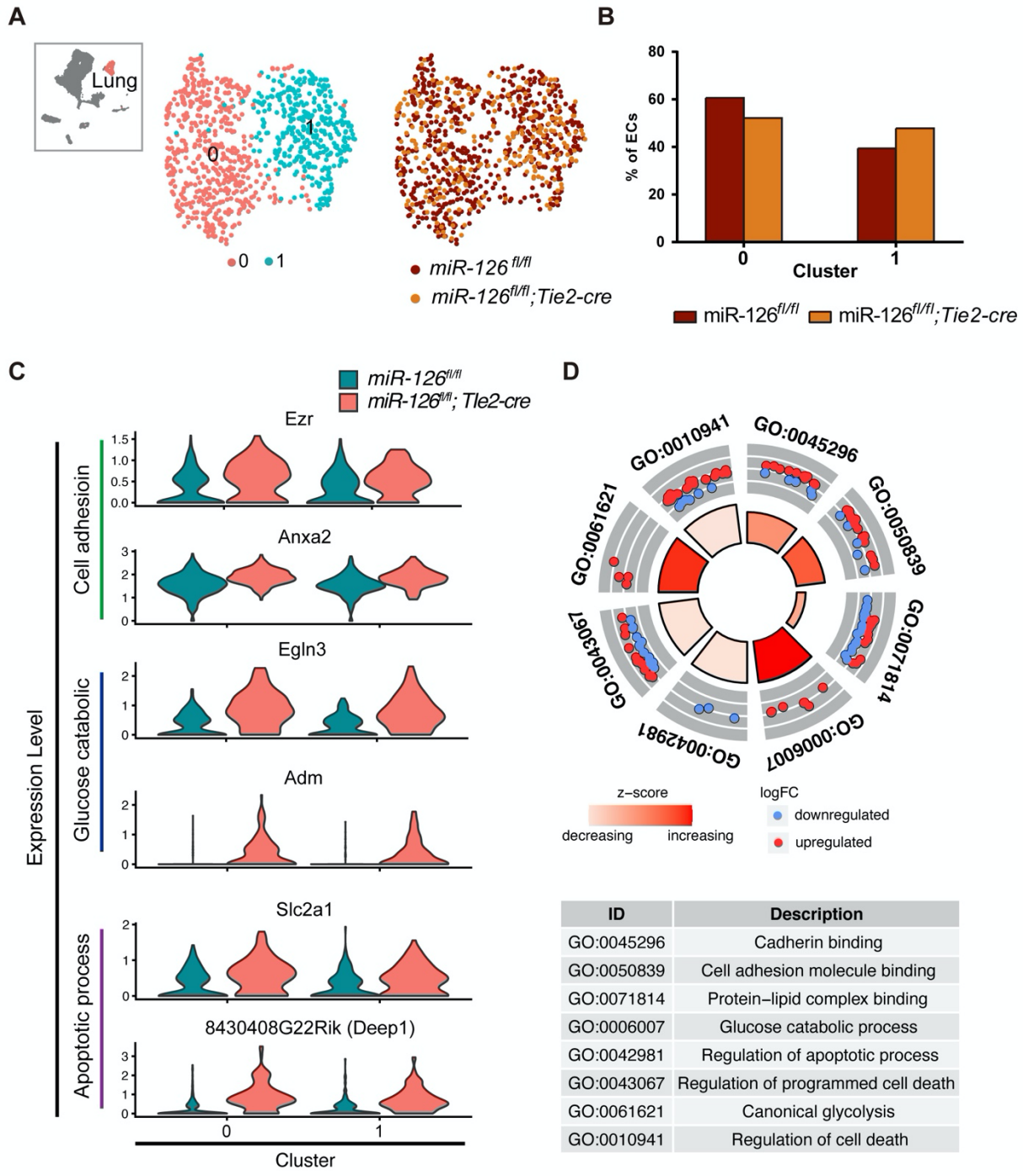
Figure S10



Supplemental Figure S10. Genes related to extracellular matrix (ECM) were upregulated in ECs of miR-126-ecKO embryos.

A, Expression file changes of genes related to Glucose transmembrane, cell proliferation and hypoxia after miR-126 knocked out in mice ECs. **B,** Quantitative PCR analysis on miR-126 deficient embryonic ECs and littermate controls. **C,** Dual immunofluorescence of LUM and PECAM1 on miR-126-deficient and littermate control hepatics tissues. Scale bar = 100um.

Figure S11



ID	Description
GO:0045296	Cadherin binding
GO:0050839	Cell adhesion molecule binding
GO:0071814	Protein–lipid complex binding
GO:0006007	Glucose catabolic process
GO:0042981	Regulation of apoptotic process
GO:0043067	Regulation of programmed cell death
GO:0061621	Canonical glycolysis
GO:0010941	Regulation of cell death

Supplemental Figure S11. Pulmonary ECs were decreased evenly, and cell adhesion related genes were upregulated in miR-126-ecKO embryos.

A, 2 subclusters were obtained from EndoMT (cluster 4 in Figure 6A). Colors denote different subclusters or mice line. **B,** Fractions of pulmonary ECs from miR-126-ecKO or littermate controls in each subcluster. **C and D,** Expression profile and Gene Ontology analysis of genes which are altered apparently after miR-126 knocked out in pulmonary embryonic ECs.

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mouse	Laboratory Animal Center, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences	C57BL/6J	Male	

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	Tie2-cre mice	TheJackson Laboratory	C57BL/6J		
Parent - Female	miR-126$^{flox/flox}$ mice	Chinese Academy of Sciences	C57BL/6J		

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Persistent ID / URL
PECAM1	BD pharmingen	553370	5ug/mL for IF	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/purified-rat-anti-mouse-cd31.553370
PORX1	R&D SYSTEMS	AF2727	10ug/mL for IF	https://www.rndsystems.com/cn/products/human-prox1-antibody_af2727
LYVE1	Abcam	ab14917	10ug/mL for IF	https://www.abcam.com/lyve1-antibody-bsa-and-azide-free-ab14917.html
CD31-PE	BD Biosciences	553373	4ug/mL	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/pe-rat-anti-mouse-cd31.553373
CD45-FITC	BD Biosciences	553080	4ug/mL	https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/single-color-antibodies-ruo/fitc-rat-anti-mouse-cd45.553080
CDH5-PE-Cy7	Biolegend	138006	4ug/mL	https://www.biolegend.com/en-us/search-results/alexa-fluor-647-anti-mouse-cd144-ve-cadherin-antibody-6569
HIF1A	Abcam	ab179483	10ug/mL for IF 1ug/mL for WB	https://www.abcam.com/hif-1-alpha-antibody-epr16897-ab179483.html
GLUT1	Abcam	ab15309	10ug/mL for IF 1ug/mL for WB	https://www.abcam.com/glucose-transporter-glut1-antibody-ab15309.html
GLUT2	Abcam	ab192599	1ug/mL for WB	https://www.abcam.com/glucose-transporter-glut2-antibody-epr16550-ab192599.html
GLUT3	Abcam	ab41525	1ug/mL for WB	https://www.abcam.com/glucose-transporter-glut3-antibody-ab41525.html
PIK3R2	KleanAB	P202564	1ug/mL for WB	http://www.kleanab.com/English/productDetail.jsp?id=20600
GAPDH	Proteintech	60004-1-Ig	0.5ug/mL for WB	https://www.ptgcn.com/products/GAPDH-Antibody-60004-1-Ig.htm

MKI67	Abcam	ab15580	10ug/mL for IF	https://www.abcam.com/ki67-antibody-ab15580.html	
COL1A1	Abcam	ab21286	10ug/mL for IF		https://www.abcam.com/collagen-i-antibody-ab21286.html
LUMICAN(LUM)	Abcam	ab168348	10ug/mL for IF		https://www.abcam.com/lumican-antibody-epr88982-ab168348.html

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL
NA			

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
NA			

Other

Description	Source / Repository	Persistent ID / URL
Chromium Single Cell 3' Library & Gel Bead Kit v2	10 X Genomics	https://www.10xgenomics.com/products/single-cell-gene-expression
Collagenase Type I	Sigma/flu/Ald	https://www.sigmaaldrich.com/HK/zh/product/sigma/c0130
Annexin V-FITC/PI Apoptosis Detection Kit	Vazyme	http://www.vazyme.com/product/26.html
miR-126 agomir	RiboBio	https://www.ribobio.com/product_detail/?sku=miR40000138-4-5

Oligos used in this study

Name	Sequence (5'-3')	Description
miR-126-3p-RT	TGGAGCGACCGTGTCGTGGAGTCGGCTAATGGTCGCTCCAGCATT	RT primer
miR-126-5p-RT	TGGAGCGACCGTGTCGTGGAGTCGGCTAATGGTCGCTCCACGCGTA	RT Primer
U6-RT	CGCTTCACGAATTTGCGTGTCAT	RT primer
qU6-F	GCTTCGGCAGCACATATACTAAAAT	qPCR primer
qU6-R	CGCTTCACGAATTTGCGTGTCAT	qPCR primer
qActb-F	GGCTGTATTCCCCTCCATCG	qPCR primer
qActb-R	CCAGTTGGTAACAATGCCATGT	qPCR primer
qmiR-126-3p-F	GACACTCCAGCAGCGTCGTACCGTGAGTAATA	qPCR primer
qmiR-126-3p-R	ATAGAGCGGTGTCGTGGAGTCGGCTAATGGTC	qPCR primer
qmiR-126-5p-F	GACACTCCAGCAGCG	qPCR primer
qmiR-126-5p-R	ATAGAGCGGTGTCGTGGAGTCGGCTAATGGTC	qPCR primer
qPecam1-F	CTGCCAGTCCGAAAATGGAAC	qPCR primer
qPecam1-R	CTTCATCCACCGGGGCTATC	qPCR primer
qCdh5-f	CACTGCTTTGGGAGCCTTC	qPCR primer
qCdh5-R	GGGGCAGCGATTCATTTTTCT	qPCR primer
qEgfl7-F	CTGCTTGTAGCATGGTTTCTAGT	qPCR primer
qEgfl7-R	TACGGCTGGGTCTGTAGACAT	qPCR primer
qHif1a-F	ACCTTCATCGGAAACTCCAAAG	qPCR primer
qHif1a-R	CTGTTAGGCTGGGAAAAGTTAGG	qPCR primer
qAdm-F	CCTACGGGACTCGCTGATGA	qPCR primer
qAdm-R	TGGGCTTCGCTCTGATTGC	qPCR primer

qEgln3-F	AGGCAATGGTGGCTTGCTATC	qPCR primer
qEgln3-R	GCGTCCCAATTCTTATTCAGGT	qPCR primer
qBnip3-F	TCCTGGGTAGAACTGCACTTC	qPCR primer
qBnip3-R	GCTGGGCATCCAACAGTATTT	qPCR primer
qCav1-F	ATGTCTGGGGGCAAATACGTG	qPCR primer
qCav1-R	CGCGTCATACACTTGCTTCT	qPCR primer
qHmox1-F	AAGCCGAGAATGCTGAGTTCA	qPCR primer
qHmox1-R	GCCGTGTAGATATGGTACAAGGA	qPCR primer
qAqp1-F	AGGCTTCAATTACCCACTGGA	qPCR primer
qAqp1-R	GTGAGCACCGCTGATGTGA	qPCR primer
qPgk1-F	ATGTCGCTTTCCAACAAGCTG	qPCR primer
qPgk1-R	GCTCCATTGTCCAAGCAGAAT	qPCR primer
qCol1a1-F	GCTCCTCTTAGGGGCCACT	qPCR primer
qCol1a1-R	CCACGTCTCACCATTGGGG	qPCR primer
qCol1a2-F	GTAACTTCGTGCCTAGCAACA	qPCR primer
qCol1a2-R	CCTTTGTCAGAATACTGAGCAGC	qPCR primer
qα-sma-F	GTCCCAGACATCAGGGAGTAA	qPCR primer
qα-sma-R	TCGGATACTTCAGCGTCAGGA	qPCR primer
qPdgfrb-F	TTCCAGGAGTGATACCAGCTT	qPCR primer
qPdgfrb-R	AGGGGGCGTGATGACTAGG	qPCR primer
qLum-F	CTCTTGCCTTGGCATTAGTCG	qPCR primer
qLum-R	GGGGGCAGTTACATTCTGGTG	qPCR primer
qSfrp2-F	CGTGGGCTCTTCCTCTTCG	qPCR primer
qSfrp2-R	ATGTTCTGGTACTCGATGCCG	qPCR primer
qNg2-F	GGGCTGTGCTGTCTGTTGA	qPCR primer
qNg2-R	TGATTCCCTTCAGGTAAGGCA	qPCR primer
qCol6a2-F	AAGGCCCCATTGGATTCCC	qPCR primer
qCol6a2-R	CTCCCTTCCGACCATCCGAT	qPCR primer
qCol6a1-F	CTGCTGCTACAAGCCTGCT	qPCR primer
qCol6a1-R	CCCCATAAGGTTTCAGCCTCA	qPCR primer
qCol3a1-F	CTGTAACATGGAAACTGGGGAAA	qPCR primer
qCol3a1-R	CCATAGCTGAACTGAAAACCACC	qPCR primer
qFsp1-F	CTGCCTACCGCAGTGCATT	qPCR primer
qFsp1-R	ACGCCATCATTTCTGCCCA	qPCR primer