Single-Cell Transcriptome Analysis Reveals Embryonic Endothelial Heterogeneity at Spatiotemporal Level and Multifunctions of MicroRNA-126 in Mice

Fang-Hao Guo (郭方浩)*, Ya-Na Guan (关亚娜)*, Jun-Jun Guo (郭君君), Lu-Jun Zhang (张陆军), Jing-Jing Qiu (邱京晶), Yong Ji (季勇), Alex F. Chen (陈丰原), Qing Jing (荆清)

BACKGROUND: Endothelial cells (ECs) play a critical role in angiogenesis and vascular remodeling. The heterogeneity of ECs has been reported at adult stages, yet it has not been fully investigated. This study aims to assess the transcriptional heterogeneity of developmental ECs at spatiotemporal level and to reveal the changes of embryonic ECs clustering when endothelium-enriched microRNA-126 (miR-126) was specifically knocked out.

METHODS: C57BL/6J mice embryos at day 14.5 were harvested and digested, followed by fluorescence-activated cell sorting to enrich ECs. Then, single-cell RNA sequencing was applied to enriched embryonic ECs. Tie2 (Tek receptor tyrosine kinase)-cre–mediated ECs-specific miR-126 knockout mice were constructed, and ECs from Tie2-cre–mediated ECs-specific miR-126 knockout embryos were subjected to single-cell RNA sequencing.

RESULTS: Embryonic ECs were clustered into 11 groups corresponding to anatomic characteristics. The vascular bed (arteries, capillaries, veins, lymphatics) exhibited transcriptomic similarity across the developmental stage. Embryonic ECs had higher proliferative potential than adult ECs. Integrating analysis showed that 3 ECs populations (hepatic, mesenchymal transition, and pulmonary ECs) were apparently disorganized after miR-126 being knocked out. Gene ontology analysis revealed that disrupted ECs were mainly related to hypoxia, glycometabolism, and vascular calcification. Additionally, in vivo experiment showed that Tie2-cre–mediated ECs-specific miR-126 knockout mice exhibited excessive intussusceptive angiogenesis; reductive glucose and pyruvate tolerance; and excessive accumulation of calcium. Agonist miR-126-3p agomir significantly rescued the phenotype of glucose metabolic dysfunction in Tie2-cre–mediated ECs-specific miR-126 knockout mice.

CONCLUSIONS: The heterogeneity of ECs is established as early as the embryonic stage. The deficiency of miR-126 disrupts the differentiation and diversification of embryonic ECs, suggesting that miR-126 plays an essential role in the maintenance of ECs heterogeneity.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: endothelial cells | gene ontology | mice | microRNA | transcriptome

Vascular endothelial cells (ECs) are the inner layer of blood vessels, which directly respond to physical and chemical signals related to vascular tension, cell adhesion, antithrombosis, smooth muscle cell proliferation, and vascular wall inflammation. ECs in different tissues have heterogeneous phenotypes,
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microRNA-126 (miR-126) regulates angiogenesis and as evidence for endothelial plasticity. Whether endothelial heterogeneity exists in embryos, which provided a powerful discovery tool and resource explored the endothelial diversity between and within tissues. With the development of single-cell RNA sequencing (scRNA-seq) technology, the heterogeneity of ECs was further characterized at the level of transcriptomics and epigenomics. Recently, EC atlases of 11 adult mouse tissues characterized at the level of transcriptomics and epigenomics. With common marker genes PECAM1 (platelet endothelial cell adhesion molecule-1) and CDH5 (Cadhernin 5), ECs can be enriched for further analysis and application. Whether endothelial heterogeneity exists in embryonic stage and how early it exists need further investigation.

The formation of vasculature starts with vasculogenesis and is followed by angiogenesis. Vasculogenesis initiates the vascular system during embryonic development by the differentiation and migration of mesoderm-derived angioblasts or ECs. Angiogenesis expands the vascular system by sprouting or intussusception. Sprouting angiogenesis is characterized by sprouts composed of ECs. Intussusceptive angiogenesis (IA) involves formation of blood vessels by splitting process in which elements of interstitial tissues invade existing vessels, forming transvascular tissue pillars that expand.

ECs exist in another special characteristic, namely, endothelial-to-mesenchymal transition (EndoMT). EndoMT, which parallels the epithelia-to-mesenchymal transition, describes a process in which ECs lose their endothelial specification gradually and gain features of mesenchymal cell. Mesenchyme transited ECs obtain characteristics of endothelial and mesenchymal cells simultaneously in both morphology and transcriptome. Although accounting for a minority in normal conditions, EndoMT is essential in embryonic development, such as the formation of cardiac valve. In addition, EndoMT participates in multiple pathology processes, including fibrosis. EndoMT also serves as evidence for endothelial plasticity. As ECs-exclusively enriched microRNA, microRNA-126 (miR-126) regulates angiogenesis and vascular integrity. Its dysfunction is related to various vascular diseases such as hypertension, atherosclerosis, and tumorogenesis.

To illuminate the transcriptional heterogeneity of embryonic ECs (eECs) and functions of miR-126 at single-cell resolution, ECs from wild-type and Tie2 (Tek receptor tyrosine kinase)-cre–mediated ECs-specific miR-126 knockout (miR-126-ecKO) mice were enriched by FACS at embryonic day 14.5 and were followed by scRNA-seq separately. With reported organ or tissue-specific genes, the whole-embryo hierarchical landscape of eECs was described. The spatio-temporal heterogeneity of ECs was explored by aggregating public single-cell datasets. In addition, more vascular abnormal phenotypes caused by deficiency of miR-126 were identified.

### MATERIALS AND METHODS

#### Data Availability

The authors declare that all data (including scRNA-seq data and R scripts for single-cell data analysis) and methods supporting the findings of this study are available in the Supplemental Methods or from the corresponding authors on reasonable request.

An expanded Methods section is available in the Supplemental Methods. All animal experiments were performed in accordance with the institutional guidelines of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. We used male mice because this minimized estrogen-dependent fluctuations in response to angiogenesis and glucose metabolism.

#### 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 that passed both tests, data are expressed as means±SEM. Student’s test was used for the comparison between 2 groups, and for comparison among multiple groups, the data were analyzed by 1-way ANOVA with

| Nonstandard Abbreviations and Acronyms |
|-----------------------------|
| aECs | adult ECs |
| ECs | endothelial cells |
| eECs | embryonic ECs |
| EndoMT | endothelial-to-mesenchymal transition |
| EndoSMT | endothelial-to-smooth muscle cells transition |
| FACS | fluorescence-activated cell sorting |
| IA | intussusceptive angiogenesis |
| miR-126-ecKO | Tie2-cre–mediated ECs-specific miR-126 knockout |
| Tie2 | Tek receptor tyrosine kinase |

**Highlights**

- The heterogeneity of endothelial cells (ECs) is established as early as the embryonic stage.
- Embryonic ECs (eECs) has spatiotemporal heterogeneity and higher proliferation potential than adult ECs.
- During development, regular embryonic ECs clustering can be disrupted in ECs-specific microRNA-126 knockout mice, and the emerged abnormal embryonic ECs clusters eventually lead to disordered glycometabolism, excessive intussusceptive angiogenesis, and increased endothelial-to-mesenchymal transition.
- MicroRNA-126-HIF1A (hypoxia-inducible factor 1-alpha)-GLUT1 (glucose transporter 1) pathway is involved in glycometabolism, and microRNA-126-3p agomir restored the abnormal blood glucose tolerance in microRNA-126-deficient mice.

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RESULTS

Embryonic ECs Can Be Divided Into 11 Hierarchical Populations

Four wild-type embryos at stage embryos at day 14.5 (E14.5) were harvested and dissociated into single-cell suspension by collagenase IV. FACS was used to purify live (DAPI−), nonmonocyte ECs (PECAM1 [CD31+], CDH5+, and CD45−), followed by scRNA-seq based on 10X Genomics strategy. False-positive cells including blood mononuclear cells and mesenchymal cells which may also be picked due to autofluorescence were excluded by unique molecular identifiers (Pecam1>0, Cdh5>0, Ptprc=0, Alas2<15). Finally, 5806 and 6278 eECs were obtained from 2 repetitions, respectively, and then integrated together. Eleven clusters were identified by clustering analysis, and cells isolated from the M1 (embryos from mouse 1) and M2 (embryos from mouse 2) were well integrated (Figure 1A).

Established endothelial markers in various organs or tissues were used to annotate each cluster (Figure 1B). ECs in the vasculature are composed of blood vascular ECs and lymphatic ECs. Genes preferentially expressed in lymphatic ECs include Lyve1, Prox1, and Ccl21a (cluster 8 in Figure 1B). Blood vascular ECs differ from the microvessels hierarchically organized, just as the anatomic structure of tissues were used to annotate each cluster (Figure 1B). ECs in the vasculature are composed of blood vascular ECs and lymphatic ECs. Genes preferentially expressed in lymphatic ECs include Lyve1, Prox1, and Ccl21a (cluster 8 in Figure 1B). Blood vascular ECs differ from the microvessels hierarchically organized, just as the anatomic structure of tissues were used to annotate each cluster (Figure 1B)

Embryonic ECs Are Heterogeneous in Tissues and Have High Proliferation Potential

To study the intratissue EC heterogeneity, we performed the graph-based subclustering of ECs (aECs and eECs) integrated dataset (published dataset E-MTAB-8077 for aECs and our scRNA-seq dataset for eECs) in Figure S1 in the Supplemental Material) for each tissue separately.

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Figure 1. Endothelial cells (ECs) at embryonic day 14.5 were divided into 11 populations. 
A, ECs were enriched for single-cell RNA sequencing analysis. Collagenase I was used to dissect embryos at day 14.5 (E14.5). Fluorescence-activated cell sorting (FACS) was used to isolate live (DAPI−), nonmonocyte (CD45−) ECs (PECAM1 [CD31+] and CDH5+). Seurat 3.0 was used for single-cell analysis. Colors denoted different clusters or different origins of mouse. B, Known endothelial mark genes in different tissues were used to define clusters of embryonic ECs (eECs). C, eECs were classified into 11 populations. Colors denoted different populations. DAPI indicates 4',6-diamidino-2-phenylindole; EndoMT, endothelial-to-mesenchymal transition; M1, embryos from mouse 1; M2, embryos from mouse 2; and PECAM1, platelet endothelial cell adhesion molecule-1.
Uniform manifold approximation and projection visualization analysis obtained comparable results (Figure 3). The expressions of the top 10 marker genes for each tissue-specific EC subclusters were shown by heatmap (Figure S3 in the Supplemental Material). According to established (canonical) marker genes of ECs subtypes,
Figure 3. The vascular heterogeneity still existed within embryonic endothelial cells (eECs) tissues which had higher proliferation potential than adult ECs (aECs). 

A, Five clusters subclustered from integrated heart subset and the fraction of each subcluster calculated separately from cardiac aECs and eECs. 

B, Five clusters subclustered from integrated brain subset and the fraction of each subcluster calculated separately from cerebral aECs and eECs. 

C, Seven clusters subclustered from integrated lung subset and the fraction of each subcluster calculated separately from pulmonary aECs and eECs. 

D, Seven clusters subclustered from integrated liver subset and the fraction of each subcluster calculated separately from hepatic aECs and eECs. 

E, Four clusters subclustered from integrated lymphatic subset and the fraction of each subcluster calculated separately from lymphatic aECs and eECs. E14.5 indicates embryos at day 14.5. E-MTAB-8077: published dataset E-MTAB-8077 from Kalucka et al. GSE109774: published dataset GSE109774 from Schaum et al.
the ECs subclusters were annotated. For instance, the large vessel markers, Vwf and Vcam1, which are related to the diameter blood vessel, were enriched in some arterial and venous EC clusters. Therefore, we classified them as artery/vein. Some reports showed that capillary ECs express both large vessel and capillary markers. We, therefore, classified them as capillary arterial and venous (Figure 3A and 3B). Some EC subclusters express unexpected genes, such as Serpina1e and Alb (subcluster 6 in Figure 3C), which are highly expressed in hepatocytes.46,47 We, therefore, define them as organogenic potential during development (Figure S5F in the Supplemental Material). The percentage of proliferating cells in eECs was extremely higher than in aECs (Figure S5D in the Supplemental Material), indicating that endocardial eECs have higher plasticity.

Classic mesenchymal cells include but are not limited to fibroblasts, mural cells (pericytes and smooth muscle cells), osteocytes, chondrocytes, and adipocytes. Wondering if EndoMT varies with the diverse mesenchymal cells, we extracted aECs and eECs transited to mesenchymal cells (cluster 12 and cluster 14 in Figure S2C in the Supplemental Material) and found EndoMT can be further classified into 3 subclusters (Figure S5E in the Supplemental Material). Then, the endothelial-to-pericyte transition, endothelial-to-smooth muscle cell transition (EndoSMT), and endothelial-to-fibroblast transition were classified with the expression profiles of gene markers in pericytes (Pdgfb,32 Abcc946), smooth muscle cells (Acta246 Tagfr45), and fibroblasts (Col1a1 and Pbn45,64), respectively (Figure S5E and S5G in the Supplemental Material). EndoMT and endothelial-to-pericyte transition can be named EndoMT. When considering the developmental stages, the proportion of endothelial-to-pericyte transition was significantly higher in embryos, whereas EndoSMT was in reverse (Figure S5F in the Supplemental Material). The potential reason may be that the shear stress in embryos is relatively low. Besides, the cycling cells of EndoMT were found distributed predominantly in the embryonic stage through exploring the ratio of cycling cells in each subcluster (Figure S5E in the Supplemental Material). Additionally, the ratio of cycling cells in each subcluster was different, and it is the highest in the EndoSMT. More than half of EndoSMT in embryos were cycling cells, which indicated its augmented potential during development (Figure S5F in the Supplemental Material).

Knockout of miR-126 Affects eECs Clustering and the Abnormality of eECs Is Mainly Related to Hypoxia and Apoptosis

MicroRNA-126 (miR-126) is an endothelium-enriched miR and functions in vascular integrity and angiogenesis.21,22,24-26 To reinforce the phenotype, EC-specific miR-126 knockout mice (miR-126-ecKO) were constructed (Figure S6A and S6B in the Supplemental Material). As expected, miR-126-ecKO embryos showed vascular leakage and subcutaneous embryonic edema (Figure 4A) and a higher lethality (Figure S6C in the Supplemental Material) compared with littermate control (miR-126fl/fl). Besides, impaired migration and sprouting of ECs in embryonic lymph and postnatal retinal was observed in miR-126-ecKO, as indicated by decreased vascular area (Figure 4B, Figure S6E and S6F in the
Figure 4. Knock out of microRNA-126 (miR-126) disturbed the embryonic endothelial cells clustering during development.

A. Embryos of Tie2 (Tek receptor tyrosine kinase)-cre–mediated ECs-specific miR-126 knockout (miR-126-ecKO) mouse demonstrated vascular leakage (white arrowheads) and hemorrhaging (yellow arrowheads), scale bar=2000 μm. B. The migration of ECs in the retina at p7 was reduced in miR-126-ecKO mouse. The retina vessels were stained by ECs marker gene PECAM1 (platelet endothelial cell adhesion molecule-1) in miR-126–deficient mice and wild types at P7. And the quantification of vascular area changes after miR-126 was knocked out in endothelial cells. The white dash lines indicate terminals of retina and the yellow dash lines indicate terminals of retina vessels. Scale bar, 1000 μm. C. The ECs of miR-126-ecKO embryos were aggregated with the littermate controls (miR-126fl/fl), and dashed lines demarcated the different groups. D. Quantitatively compare and analyze the proportion of each cell population in the 2 samples. E. Gene Ontology (GO) biological function analysis of differentially expressed gene in miR-126-ecKO and the littermate controls. F. Vascularization in the skin of miR-126-ecKO mouse was more complex than their littermate controls. Pillars were indicated with white arrowheads. Number of branches points was counted on the right (n=6), scale bar=200 μm. E14.5 indicates embryos at day 14.5, EndoMT, endothelial-to-mesenchymal transition; FC, fold change; and P7, posnatal day 7.
To elucidate the effect of miR-126 on the development of ECs from a single-cell perspective, 5203 ECs of miR-126-ecKO embryos were enriched and sequenced, followed by aggregation with eECs from the littermate control. Eleven populations were obtained and defined. And the fraction of most populations accounted comparable between miR-126-ecKO and littermate control embryos, except hepatic, pulmonary, and mesenchymal-transited ECs (Figure 4C and 4D), which will be further discussed below. GO analysis of genes enriched only in miR-126-ecKO eECs showed that 5 out of top 10 GO terms were related to hypoxia (Figure 4E). To confirm the analysis result, the eECs were enriched by FACS from both miR-126-eKO and littermate control mice, and the quantitative polymerase chain reaction results showed that genes related with hypoxia including Hif1a (hypoxia-inducible factor 1-alpha) were upregulated apparently after miR-126 was knocked out (Figure S7B in the Supplemental Material). In addition, feature plot analysis and coimmunofluorescence of HIF1A and PECAM1 indicated the expression profile was higher in miR-126–deficient cardiac ECs than in littermate control (Figure S7C and S7D in the Supplemental Material). Previous study reported that hypoxia stimulates excessive IA. Intriguingly, we did find excessive IA in the embryonic skin and the terminal region of postnatal retina in miR-126-eKO mouse by immunofluorescence staining of PECAM1 (white arrow in Figure 4F and Figure S7A in the Supplemental Material), which means that miR-126 is essential to homeostasis of vascular network at embryonic stage. Therefore, we preliminarily concluded that knockout of miR-126 in ECs would affect angiogenesis both in sprouting angiogenesis and IA, but in opposite directions. GO analysis of genes enriched only in miR-126–deficient eECs showed that 4 out of 5 top terms were related to cell apoptosis and cell death. Genes related to apoptosis (Mex3 family, caspase family, genes related to autophagy activation, and genes related to lysosomal biogenesis) had been identified in the affected ECs clusters. As the results showed, 3 members of Mex3 family were upregulated after miR-126 knocked out (Figure S8A, S8B, and S8E in the Supplemental Material). Considering that Mex3a could form a complex with miR-126-5p to facilitate antia apoptotic effect,58 the upregulation of Mex3a at transcripts level may be due to some compensatory mechanisms in miR-126–deficient eECs. Most members of Caspase family were upregulated in miR-126–deficient eECs (Figure S8B in the Supplemental Material). Moreover, a toolbox for deducing autophagy status which was built by Bordi et al.59 had been detected in our integrated single-cell data. Only sixty percentage of genes related to autophagy activation (Induction List) were upregulated after miR-126 knocked out. Although over 80% of genes related to lysosomal biogenesis were upregulated in miR-126–deficient eECs (Figure S8C in the Supplemental Material). This indicates that the activation of autophagy in miR-126–deficient eECs might be earlier than E14.5 days. Considering the higher embryonic mortality observed in miR-126–deficient mice, the apoptosis on eECs at E14.5 was analysis by flow cytometry. The result showed that the percentage of PI (propidium iodide)-positive cells were apparently increased after miR-126 was knocked out in eECs (Figure S8F and S8G in the Supplemental Material). The increased cell apoptosis contributed to the higher embryonic lethality observed in miR-126-ecKO mice (Figure S5C in the Supplemental Material).

Next, we analyzed the expression profiles of the miR-126 target genes (predicted targets by TargetScan7.2 and validated targets by published research papers) in the integrated scRNA-seq data. The results showed that most of target genes were upregulated after miR-126 was knocked out (Figure S9A through S9C in the Supplemental Material). Interestingly, many miR-126 target genes showed tissue heterogeneity; some targets were predominantly upregulated in hepatic eECs, whereas others were mainly upregulated in EndoMT eECs (Figure S9D in the Supplemental Material). Furthermore, GO analysis was conducted on the screened miR-126-3p targets, miR-126-5p targets, and total miR-126 targets, respectively, and the results showed that the upregulated biological progresses were mainly related to cell migration, response to oxygen-containing compound (Figure S9E through S9H in the Supplemental Material). Notably, among miR-126 targets, glucose transport–related genes denoted as glucose import were also upregulated. This indicated that it may act downstream of miR-126 targets.

**Knockout of miR-126 Disturbs Hepatic eECs Clustering to Affect Glucose Metabolism and Fibrosis**

To compare the hepatic eECs with or without miR-126 in detail, we performed reclustering analysis of eECs from the liver (cluster 3 in Figure 4C) and obtained5 subclusters. Of which clusters 5 and 6 varied most in miR-126-ecKO ECs (Figure 5A and B). Most DEG in clusters 5 and 6 were similar, except for proliferative genes (Figure S10A in the Supplemental Material). GO biological progress analysis of DEG in clusters 5 and 6 revealed multiple statistically significant GO terms associated with glucose regulation and hypoxia (Figure 5C). Some glucose transmembrane genes including Slc2a1, Slc2a3, and Slc16a3 were highly expressed in clusters 5 and 6 (Figure 5D and Figure S10A in the Supplemental Material). Glucose tolerance test and pyruvate tolerance test were conducted.
Knock out of microRNA-126 (miR-126) in endothelial cells (ECs) disturbed blood glucose metabolism and induced fibrosis in hepatic tissue.

A, 5 subclusters were obtained by further clustering of hepatic ECs (cluster 3 in Figure 6A). Colors denote different subclusters or mice line. B, Fractions of hepatic ECs from Tie2 (Tek receptor tyrosine kinase)-cre–mediated ECs-specific miR-126 knockout (miR-126-ecKO) or littermate controls in each subcluster. C, Gene Ontology biological function analysis of differentially expressed genes in cluster 2 and 3. D, Vpplot of genes whose expression profiles were significantly different between miR-126-ecKO and the littermate controls. E, Masson staining of embryonic liver at embryos at day 14.5 (E14.5), scale bar=200μm. F, Postprandial blood glucose was elevated in miR-126-ecKO mouse compared with wild types verified by Intraperitoneal glucose tolerance test (IGTT). Sample size: n=6. G, Gluconeogenesis was elevated in miR-126-ecKO mouse compared with miR-126fl/fl mouse verified by Intraperitoneal pyruvate tolerance test (IPTT). Sample size: n=6. AUC indicates area under curves.
Figure 6. Endothelial-to-mesenchymal transition (EndoMT) and vascular calcification were increased in Tie2 (Tek receptor tyrosine kinase)-cre–mediated ECs-specific microRNA-126 (miR-126) knockout (miR-126-ecKO) embryos. 
A. Five subclusters were obtained by further clustering of EndoMT (cluster 10 in Figure 6A). Colors denote different subclusters or mice line. 
B. Fractions of EndoMT from miR-126-ecKO or miR-126fl/fl in each subcluster. 
C. Gene Ontology biological function analysis of differentially expressed genes in cluster 3. 
D. Feather plot of representative genes in chondrogenesis and endothelial-to-mesenchymal which were enriched in cluster 3. 
E. Double immunofluorescent staining of COL1A1 (alpha-1 type I collagen) and PECAM1 (platelet endothelial cell adhesion molecule-1) proteins in embryonic hepatic tissue to identify the EndoMT (Endothelial-to-Mesenchymal Transition). 
F. Alizarin Red S Staining to identify the calcium in the adult’s brain and lung slides. Scale bar=100 μm. DAPI indicates 4’,6-diamidino-2-phenylindole.
to verify whether knocking out miR-126 in ECs affected glycose metabolism. The miR-126-ecKO mouse showed reduced tolerance to glucose and pyruvate (Figure 5F and 5G). In fact, previous studies have declared a negative correlation between miR-126 and blood glucose level, but the causality was unknown. The results suggested a potential function of miR-126 in glucose regulation. The pathways and metabolisms involved warrant further investigation. Considering that hepatic hypoxia may lead to fibrosis, Masson staining was performed and showed increased collagenous fibers in miR-126-ecKO mouse (Figure 5E). Collectively, the disorganized hepatic ECs caused by miR-126 deficiency contributes to liver fibrosis and abnormal glycose metabolism at adult stage.

Knockout of miR-126 in eECs Leads to Excessive EndoMT and Vascular Calcification

To determine the effect of miR-126 knockout on EndoMT, reclustering of mesenchymal-transited ECs (cluster 10 in Figure 4C) was performed and 5 subclusters were identified. Of which cluster 3 and cluster 4 varied most in miR-126-ecKO eECs (Figure 6A and 6B). GO analysis of DEG in cluster 3 revealed most terms related to extracellular matrix organization and skeletal system development (Figure 6C). Genes enriched in the extracellular matrix and cartilage were also highly expressed in cluster 3 (Figure 6D). Additionally, quantitative polymerase chain reaction analysis confirmed a higher expression of ECM (extracellular matrix) enriched genes in miR-126-deficient eECs (Figure S10B in the Supplemental Material). Dual immunofluorescence for an EC marker (PECAM1) and markers of extracellular matrix (COL1A1 [COL1A1 (alpha-1 type I collagen)] and LUM [lumican]) confirmed the excessive EndoMT in miR-126 knocked out eECs (Figure 6E and Figure S10C in the Supplemental Material). Previous reports demonstrated that the endochondral and intramembranous bone formation in vessels may lead to ectopic vascular calcification and eventually lead to various vascular diseases, such as atherosclerosis or hypertrophy. Multiple cells were the potential sources for calcification, including smooth muscle cells, pericytes, and ECs. Alizarin red staining demonstrated that vascular calcification in the brain and lung of miR-126-ecKO mouse increased (Figure 6F). Our analysis indicated knockout of miR-126 resulted in vascular calcification by endothelial-to-cartilage transition.

Knockout of miR-126 in eECs Leads to Upregulation of Cell Adhesion-Related Genes in Pulmonary eECs

To study the abnormality of pulmonary ECs, further clustering (cluster 4 in Figure 4C) identified 2 subclusters (Figure S11A in the Supplemental Material). The ratio of ECs in each group did not change much (Figure S11B in the Supplemental Material). Expression profiles and GO analysis showed that the different genes are mainly related to 3 molecular functions: cell adhesion, glycose catabolic, and cell apoptotic process (Figure S11C and S11D in the Supplemental Material). The biological process of glycose catabolic and programmed cell death has been observed in other tissues or whole body eECs as well. However, the abnormal cell adhesion process was found only in pulmonary subclusters (Figure S11D in the Supplemental Material). This indicated that there might be a defect with junctions between the ECs.

MiR-126-HIF1A-GLUT1 Pathway Is Involved in Glycometabolism, and Treatment With miR-126 Agomir Rescues Blood Glycose Imbalance in miR-126-ecKO Mice

The glucose transport proteins family (including GLUT1, GLUT2, GLUT3, and GLUT4) was reported to play critical roles in blood glycose metabolism. Heilig et al found that overexpression of GLUT1 (Slc2a1) in rat mesangial cells cultured in a normal glycose milieu mimicked the diabetic phenotype. Additionally, published articles claimed that overexpression of miR-126 directly inhibited Hif1a in ECs. This means that knocking out miR-126 in ECs may result in upregulation of HIF1A. We thus proposed that when miR-126 was knocked out in ECs, hepatic ECs partially upregulated the genes related to hypoxia. The upregulated hypoxia-related genes eventually induced the upregulation of Slc2a1 (GLUT1) and Slc2a3 (GLUT3). Finally, the upregulated GLUT1 led to a disorder in blood glycose metabolism.

To verify this speculation and confirm bioinformatic analysis results in Figure 5, dual immunofluorescence was performed on hepatic tissues of miR-126-deficient and littermate control. The results showed that HIF1A and GLUT1 were both expressed more highly in miR-126-deficient eECs (Figure 7A and 7B). To figure out whether the effects of miR-126 on hepatic eECs are conservative in embryonic and adult stage. The adult hepatic ECs were enriched by collagenase perfusion which was followed by Percoll-gradient separation. The results of Immunoblotting and quantification data showed that HIF1A and GLUT1 were both upregulated in hepatic ECs when miR-126 was knocked out (Figure 7C and 7D). Conversely, miR-126 knockout had little influence on GLUT2 (SLC2A2) and GLUT3 (SLC2A3) expression profile in hepatic ECs.

The blood glycose metabolism was found disrupted in miR-126 knocked out mice (Figure 5E). It is important to know whether restoring miR-126 recovers the defect. Hence, we treated miR-126-deficient mice with miR-126-3p agomir via tail vein injection. The intraperitoneal glycose tolerance test results revealed that miR-126-3p treatments displayed a significant restoration of impaired
**Figure 7.** HIF1A-GLUT1 pathway was upregulated in microRNA-126 (miR-126)–deficient endothelial cells (ECs), and miR-126-3p agomir restored the disrupted glycometabolism in miR-126 knocked out mice.  

A, Dual immunofluorescence of GLUT1 (glucose transporter 1) and PECAM1 (platelet endothelial cell adhesion molecule-1) in miR-126–deficient and littermate control hepatic tissues at embryos at day 14.5 (E14.5). Scale bar=100 μm. B, Dual immunofluorescence of HIF1A (hypoxia-inducible factor 1-alpha) and PECAM1 in miR-126–deficient and littermate control hepatic tissues at E14.5. Scale bar=100 μm. C, Western Blotting of glucose transport protein family (GLUT1, GLUT2 and GLUT3) and HIF1A in adult miR-126–deficient and wild-type hepatic ECs. D, Quantification of immunoblotting results in C. E, Intraperitoneal glucose tolerance test (IGTT) on wild types, miR-126–deficient mice treated with miR-126-3p agomir, agomir-negative control (NC) and no treats. Sample size: n=6. F, Area under curves (AUC) analysis IGTT results. Sample size: n=6. PIK3R2 indicates phosphoinositide-3-kinase regulatory subunit 2; and SLC2A, solute carrier family.
blood glucose tolerance in miR-126 knocked out mice (Figure 7E and 7F). The results indicated that miR-126-Hif1a-Glut1 pathway participated in murine blood glucose metabolism in vivo.

**DISCUSSION**

The present study provided a novel insight into the heterogeneity of ECs at embryonic stage and investigated the effect of miR-126 on ECs development at single-cell levels. The heterogeneous transcriptome of ECs in adult stage was investigated previously but rarely studied on developmental eECs. Single-cell analysis of the whole-embryo ECs revealed 11 clusters, in agreement with the hierarchical structure of vasculature in anatomy. Using public single-cell datasets (E-MTAB-8077 and GSE109774), the definition of each population was confirmed by aggregation of aECs and eECs. The cardiac, cerebral, pulmonary, hepatic, and lymphatic ECs were clustered in accordance with their anatomic structures. The heart ECs were further divided into endocardium, cycling cells, and cardiac valve ECs. In addition, the cardiac valves ECs were even subclustered into upstream valves, downstream valves, and coronary ECs. When considering the developmental stage (adult and embryo), eECs differed with aECs mostly by the high potential of proliferation and less shear stress. Thus, ECs are spatiotemporally characterized as heterogeneous.

Tie2 was first reported by Dumont et al and Tie2 transcripts were firstly detected in the extraembryonic on embryonic day 7 during development. Both Tie2-cre and Cdh5-cre have been used widely as constitutive active pan-endothelial Cre mouse models. Payne et al had given a comprehensive comparison among endothelial-specific Cre mouse models including Tie2-cre and Cdh5-cre. They reported that the earliest pan-endothelial cre activity in Tie2-cre model was around E7.5, whereas the earliest pan-endothelial cre activity in Cdh5-cre model is around E9.5. Hence, we applied Tie2-cre mouse model to investigate what happens to ECs when genes are knocked out in their initial development stage.

To clarify whether the heterogeneity in embryonic ECs is stable, we focused on a key regulator, miR-126, which is expressed predominantly in ECs and is critical for angiogenesis. In this study, we demonstrate that miR-126 is essential for regular eECs clustering. GO analysis identified that top 50 differently expressed genes are mainly related to hypoxia and cell apoptosis. And genes related to apoptosis, including Mex3 family, Caspase family, and a toolbox for deducing autophagy status which was built by Bordi et al, had also been analyzed in single-cell data. As expected, most apoptosis-related genes were upregulated in miR-126-deficient eECs. In addition, three eECs subgroups, including hepatic, pulmonary, and EndoMT ECs, showed significant changes in the clustering via integrated analysis. Unexpectedly, the upregulated genes are different among 3 subgroups. This indicated that the influence of miR-126 on the development of eECs may be heterogeneous across tissues.

The previous studies described that circulating miR-126 is closely associated with type 2 diabetes. However, the detailed mechanisms were unclear. Here, we characterized hepatic eECs clusters which were mostly affected when miR-126 was knocked out and found that genes related to glycometabolism, such as Slc2a1 and Slc2a3, were greatly upregulated. The experimental results verified this influence could last until adulthood, suggesting that loss of endothelial miR-126 is not only a feature of type 2 diabetes but also a cause. Previous works claimed that overexpression of miR-126 directly inhibits Hif1a in porcine endothelial cell, and hypoxia was reported to induce GLUT1 (Slc2a1) expression in ECs. Our results validated that miR-126 targets Hif1a which is a potent stimulus for induction of Slc2a1 mRNA in ECs. Tail vein administration miR-126 agomir apparently rescued impaired glucose tolerance in miR-126-ecKO mice. Hence, we demonstrate that miR-126-Hif1a-Slc2a1 pathway plays a protection role in murine glycometabolism in vivo. Additional investigation is required to identify downstream genes that mediate this effect.

EndoMT is a common cellular transdifferentiation during development. It also contributes to fibrosis under pathological condition. In the present work, we classify EndoMT subclusters in eECs. Specifically, EndoMT was divided into 3 subpopulations (endothelial-to-fibroblast transition, EndoSMT, and endothelial-to-pericyte transition) according to various mesenchymal cell types. In addition, comparative analysis of scRNA-seq data demonstrated that EndoMT can be disrupted when miR-126 is knocked out. Moreover, excessive extracellular matrix in ECs and fibrosis in hepatic tissue were observed in miR-126-ecKO mice, which confirmed the analysis results. Therefore, these findings suggest that miR-126 may function as a guidance cue to EndoMT.

Hypoxia is reported to induce excessive IA. Excessive IA phenotypes in miR-126-ecKO embryos indicated the potential role of miR-126 in the homeostasis of mouse embryonic vascular network. However, the mechanism of miR-126 on IA deserves further exploration. Some new abnormal phenotypes were observed from miR-126-ecKO mice including aberrant glycometabolism, fibrosis, and vascular calcification. In addition, we demonstrated that pathway including miR-126, hypoxia-induced factors (HIF1A), and GLUT1 may play critical roles in blood glucose metabolism. Although the comprehensive interactions and causal relationships between all these new
phenotypes were elusive, our findings may facilitate further study on miR-126.

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