

# Circ-0010928 Negatively Regulates Hypoxia-Induced Endothelial Cell Angiogenesis Through The miR-921/LSM14A axis.

Tian Rong Zhang

Guangxi Medical University

WeiQiang Huang (✉ [hwq2388@sina.com](mailto:hwq2388@sina.com))

Guangxi Medical University

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## Research

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# Abstract

## Background

Angiogenesis is an important factor in promoting vascular repair and a valuable process in the treatment of cardiovascular diseases. Circular RNAs (circRNAs) are widely expressed in eukaryotic cells and play an important role in the regulation of endothelial cells (ECs). In our study, bioinformatics analysis and real-time fluorescent PCR detection revealed that circRNA 0010928 (circ-0010928) is differentially expressed in human cardiac microvascular endothelial cells (HCMECs).

## Material & Methods

We evaluated the role of circ-0010928 in HCMECs. Then, we can verify the function of circ-0010928 in HCMECs by cell counting kit-8 (CCK8), scratch test, transwell experiment, tube forming experiment, flow cytometry. Use dual luciferase experiment to detect the binding relationship between circ-0010928, miR-921 and LSM14A.

## Results

Overexpression of circ-0010928 inhibited the proliferation, migration and tube formation of HCMECs under hypoxic conditions and promoted their apoptosis. In addition, dual luciferase reporter assays confirmed that circ-0010928 acted as a sponge of miR-921 and LSM14A as a downstream target gene of miR-921. Silencing miR-921 could also inhibit the proliferation, migration and tube formation of HCMECs and negatively regulate angiogenesis.

## Conclusion

CircRNA-0010928 may inhibit the function of miRNA-921 by combining with miRNA-921, and then miRNA-921 plays a role in regulating LSM14A, thereby regulating the state of angiogenesis.

## Background

Cardiovascular disease (CVD) is one of the diseases with the highest morbidity and mortality in the world and is the main cause of disability and death [1-3]. Although many drugs for the treatment of CVD have been developed in recent years, such as aspirin, statins, renin-angiotensin system inhibitors and thrombolytic therapy [4], CVD events are still in the high-risk category. Therefore, it is necessary to better understand the molecular mechanisms that contribute to the pathogenesis of CVDs to improve prevention and treatment. Angiogenesis is closely related to many CVDs. Vascular endothelial cells have an important barrier function in blood vessels, promoting the proliferation, migration and tube formation

of endothelial cells, which is beneficial to angiogenesis [5-7]. Therefore, the function of endothelial cells can be manipulated to further improve CVD treatments.

Circular RNAs (circRNAs) are endogenous non-coding RNAs (ncRNAs) that are circularized by connecting the 3' end of the RNA to the 5' end through back-splicing. CircRNAs exist in eukaryotic cells and are stable and highly expressed [8, 9]. In the past, circRNAs were generally considered to be by-products of incorrect splicing or mRNA processing [10]. With the development of high-throughput sequencing technology and bioinformatics, an increasing number of circRNAs have been discovered and have been shown to have important biological functions, such as sponging miRNAs and participating in protein translation [11]. In addition, circRNAs can be used as diagnostic and therapeutic markers and therapeutic targets [12-14]. Many studies have confirmed that circRNAs can participate in disease processes, such as hsa\_circ\_103809 acting as an miR-620 sponge to inhibit the proliferation and invasion of hepatocellular carcinoma [15]. Cdr1as promotes myocardial infarction by inhibiting the regulatory effect of miR-7a on the expression of its target genes [16]. Moreover, cZNF609 regulates vascular dysfunction via the cZNF609/miR-615-5p/MEFZA network [17].

Although many studies have reported that circRNAs act as sponges for miRNAs, there are few studies on angiogenesis. Salzman et al. [18] confirmed that circ-0010928 in HUVECs has obvious expression differences and statistical significance. However, the specific impact of circ-0010928 on HUVECs is still unknown. Here, we conducted a series of endothelial cell function tests to further explore the regulatory mechanism of circ-0010928 on vascular proliferation and migration.

## Materials And Methods

### Cell culture and hypoxia treatment

Human cardiac microvascular endothelial cells (HCMECs) were purchased from ScienCell Laboratory (Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd., Shanghai, China). This cell line is commonly used for angiogenesis research. HCMECs were cultured in endothelial cell medium (ECM) supplemented with 5% FBS, 1% endothelial cell growth supplement and 1% penicillin–streptomycin solution (ScienCell, USA). The cells were cultured in an incubator at 37°C with 5% CO<sub>2</sub>. Hypoxic treatment was performed when HCMECs grew to 60%-70%. HCMECs were cultured in RPMI 1640 medium, and the cells were placed in a three-gas incubator containing 95% N<sub>2</sub> and 5% CO<sub>2</sub> for continuous hypoxia for 24 hours.

### Oligonucleotide transfection

The circ-0010928 overexpression plasmid and negative control (NC) plasmid and miR-921-specific small interfering RNAs (siRNAs) and si-NC were constructed by Hanbio Biotechnology (Shanghai, China). According to the manufacturer's instructions, all oligonucleotides were transfected into HCMECs at a final concentration of 50 nM using Lipofectamine 2000.

### Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to extract total RNA from the cells. The PrimeScript™ RT reagent kit or Mir-X miR First-Strand Synthesis Kit (TaKaRa, Japan) was used to reverse transcribe total RNA into complementary DNA (cDNA). Then, PCR was performed with TB Green Premix Ex Taq II (TaKaRa, Japan) in an AB7500 thermocycler. GAPDH was used as the internal control for circ-0010928 and LSM14A. U6 was used as the internal control for miR-921, and the expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method. The primer sequences are shown in Table 1.

**Table 1:**

Gene name	Forward (5'>3')	Reverse (5'>3')
Circ-0010928	CTCATACCATGCCTGTGGTG	CTCGGACTTGTCCATTTCGAT
GAPDH	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT
MiR-921	GAGGGACAGAACCAGGATTCAA	TCCTCCTCTCCTTCCTTCTC
U6	GGAACGATACAGAGAAGATTAGC	TGGAACGCTTCACGAATTTGCG

## Luciferase reporter assay

The synthetic circ-0010928 sequence or LSM14A sequence containing the wild-type or mutant binding site of miR-921 was cloned into the dual luciferase reporter vector (Hanbio Biotechnology, Shanghai, China). The wild-type and mutant luciferase reporter gene plasmids and miR-921 mimics were co-transfected into 293T cells. After transfection, the cells were cultured for another 48 hours. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega).

## Cell Counting Kit-8 (CCK-8) assay

The cells were seeded in a 96-well plate at a density of  $3 \times 10^3$ /well and incubated at 37°C in a 5% CO<sub>2</sub> incubator for 24 hours. Ten microlitres of CCK-8 solution (Dojindo Laboratories, Japan) was added to each well and incubated for 2 hours. Subsequently, optical density (OD) values at 450 nm were obtained using a microplate reader (Thermo Fisher Scientific, USA).

## Tube formation assay

Matrigel (Corning, NY, USA) was placed into a 96-well plate at 100 µl per well, spread evenly on the bottom and placed at 37°C in a 5% CO<sub>2</sub> incubator for 30 min. The cells were seeded in each well at a density of  $3 \times 10^5$ /ml, and tube formation was observed under an optical microscope (Olympus, Japan) 8 hours later.

## Transwell assay

A 24-well plate with a Transwell chamber with an 8-µm pore size (BD, Franklin Lake, New Jersey, USA) was used to determine the cell migration ability. HCMECs ( $4 \times 10^4$  cells) were inoculated with 200 µl serum-free medium per well in the upper chamber, and 600 µl medium containing 10% FBS was added to

the lower chamber. After 24 hours of incubation, the migrating cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Solarbio, Beijing, China). Then, the number of stained migrated cells was observed with a microscope.

## Scratch assay

Lines were drawn parallel to the plate at the bottom of a six-well plate, and 3 marks were added for each well. The cells were inoculated in the six-well plate at  $3 \times 10^4$ /well and incubated at 37°C in a 5% CO<sub>2</sub> incubator until the density was close to 95%. A 200- $\mu$ l pipette tip was used perpendicular to the marked line to scratch the cell monolayer. After washing with PBS, medium containing 1% FBS was added and incubated again for 24 hours. The cell migration distance was observed under a microscope.

## Flow cytometry for apoptosis and cell cycle analysis

An Annexin V-APC/7-AAD Apoptosis Kit (Multisciences, Hangzhou, China) and a Cell Cycle and Apoptosis Analysis Kit (Meilunbio, Dalian, China) were used to perform flow cytometry to analyse apoptosis and the cell cycle, respectively. The cells were seeded into a six-well plate at  $3 \times 10^4$ /well and incubated to reach a cell density of over 95%, after which the cells were digested into a cell suspension. For apoptosis experiments, the cells were resuspended in 500  $\mu$ l buffer and incubated for 5 min in the dark with 10  $\mu$ l 7-AAD and 5  $\mu$ l APC. For the cell cycle experiments, the cells were fixed with 75% absolute ethanol. After 24 hours, 500  $\mu$ l staining solution containing PI was added to each sample and incubated for 30 min at 37°C in the dark.

## Statistical analyses

All data were analysed with SPSS 25.0 software (IBM, Somers, NY, USA) and are expressed as the mean  $\pm$  standard deviation (M $\pm$ SD). Student's t-test was used to compare two different groups, and one-way ANOVA was used to compare multiple sets of data. A P-value less than 0.05 was considered statistically significant.

## Results

### The role of circ\_0010928 in HCMECs under hypoxia

By searching the literature related to angiogenesis and conducting bioinformatics analyses, such as searching the databases circBase and circBank, we identified several circRNAs with high scores. qPCR experiments were performed to explore the expression of different circRNAs under hypoxic conditions, which confirmed that under hypoxic conditions, the expression of circ\_0010928 was significantly downregulated compared with that of other circRNAs (Fig. 1A). Therefore, we evaluated the biological function of circ\_0010928 in vascular endothelial cells. First, we performed qPCR experiments on cells overexpressing circ\_0010928, and the expression of circ\_0010928 was demonstrated to be significantly upregulated (Fig. 1B). Then, CCK-8, Transwell, scratch and tube formation assays showed that overexpression of circ\_0010928 inhibited the viability, migration and angiogenesis of HCMECs (Fig. 1C-

1F). Apoptosis analysis by flow cytometry showed that compared with the control treatment, circ\_0010928 overexpression enhanced the apoptosis of HCMECs (Fig. 1G). In addition, the cell cycle assay results indicated that overexpression of circ\_0010928 inhibited cell proliferation (Fig. 1H). The above results show that circ\_0010928 can inhibit cell proliferation, migration and tube formation and promote cell apoptosis. Overall, circ\_0010928 plays a negative regulatory role in angiogenesis.

## **Circ\_0010928 acts as an miR-921 sponge to regulate angiogenesis**

Many studies have reported that circRNAs have a large number of miRNA binding sites and regulate corresponding functions. Therefore, we further explored whether circ\_0010928 can bind to miRNAs to regulate the angiogenesis of HCMECs. The miRNAs bound to circ\_0010928 were predicted through websites such as TargetScan, starBase, RegRNA and miRDB. We identified eight miRNAs (miR-921, miR-591, miR-637, miR-124, miR-1322, miR-4237, miR-1283, miR-1204) with the highest correlation with circ\_0010928 and performed qPCR verification. The results showed that under hypoxic conditions, compared with that of other miRNAs, the expression of miR-921 was the most upregulated (Fig. 2A). Then, we performed qPCR analysis of miR-921 in cells overexpressing circ\_0010928, and the results showed that the expression of miR-921 was reduced. This result demonstrates that circ\_0010928 very likely acts as an miR-921 sponge (Fig. 2B). We designed a construct with a mutated binding site between circ\_0010928 and miR-921 (Fig. 2C). Measurement of the luciferase activity of the dual luciferase reporter construct showed that compared with that of the mutant group, the fluorescence intensity of the wild-type miR-921 group was significantly reduced (Fig. 2D). This finding further confirms that circ\_0010928 is a miR-921 sponge. Then, we conducted a functional test of the role of miR-921 in angiogenesis.

## **Silencing miR-921 inhibits the proliferation of HCMECs and promotes apoptosis**

To further verify the role of miR-921 in angiogenesis, we first performed qPCR experiments, and the results showed that the expression of miR-921 was significantly reduced in the miR-921 group compared with the control group (Fig. 3A). Subsequently, we carried out CCK-8, Transwell, scratch and tube formation experiments, which demonstrated that the viability, migration and tube formation ability of human umbilical vein endothelial cells (HUVECs) transfected with si-miR-921 were reduced (Fig. 3C-F). Flow cytometry detection of apoptosis and the cell cycle analysis showed that inhibiting miR-921 reduced the anti-apoptotic and proliferative abilities of HUVECs (Fig. 3G-H). In addition, qPCR detection of miR-921-silenced cells indicated that the expression of circ\_0010928 was significantly increased (Fig. 3B). The above experimental results show that miR-921 can promote HUVEC proliferation, migration, and tube formation and inhibit apoptosis. Moreover, miR-921 can inhibit the upstream regulator circ\_0010928. In addition, circ\_0010928 overexpression and miR-921 silencing had the same inhibitory effect. These findings further confirm that circ\_0010928 regulates angiogenesis by interacting with miR-921.

# Circ\_0010928 regulates hypoxia-induced HCMECs via the miR-921/LSM14A axis

To further study the specific molecular mechanism by which the circ\_0010928/miR-921 axis regulates angiogenesis, we investigated the miR-921 downstream target genes. We used websites such as TargetScan and starBase to predict the potential targets of miR-921. The qRT-PCR results showed that under hypoxic conditions, the changes in LSM14A expression were the most obvious (Fig. 4A), and overexpressing circ\_0010928 and silencing miR-921 in cells could promote the expression of LSM14A (Fig. 4B). Next, we designed a mutation of the binding site between LSM14A and miR-921 (Fig. 4C). The dual luciferase reporter assay also confirmed that LSM14A is a downstream target of miR-921 (Fig. 4D). The above results verify that HCMECs induced by hypoxia can regulate vascular regeneration by regulating the circ\_0010928/miR-921/LSM14A network (Fig. 4E).

## Discussion

CVDs, such as coronary atherosclerosis, acute myocardial infarction, diabetes and hypertension, are widespread diseases that have consistently shown high prevalence and mortality worldwide [19, 20]. Intravascular cells are an important barrier to blood vessels, and promoting vascular regeneration is of great significance for the prevention and treatment of CVDs [21, 22].

CircRNAs initially received little attention, and their functions and effects are poorly understood [23]. In recent years, in-depth research on the structure and function of circRNAs has demonstrated that many circRNAs can play an important role in CVDs as regulators of angiogenesis. For example, circRNAs can be used as sponges of miRNAs to regulate the biological functions of endothelial cells through the circRNAs/miRNAs/target gene axis [24, 25].

In this study, we established hypoxic conditions to simulate the ischaemic and hypoxic environment of coronary heart disease. Bioinformatics analysis and qRT-PCR experiments confirmed that the expression of circ\_0010928 was downregulated, and the overexpression of circ\_0010928 could inhibit the proliferation, migration and tube formation of HCMECs and promote apoptosis, indicating that circ\_0010928 is not conducive to the repair of HCMECs. Then, we further explored the mechanism by which circ\_0010928 regulates vascular regeneration and identified miR-921 through bioinformatics analysis and PCR experiments. Silencing miR-921 inhibited the proliferation, migration and tube formation of HCMECs, promoted apoptosis, and upregulated the expression of circ\_0010928. These results show that miR-921 has an inhibitory effect on circ\_0010928 and has a positive regulatory effect on HCMECs. Overall, circ\_0010928 acts as a sponge for miR-921 to interfere with the function of endothelial cells. In addition, we found that miR-921 binds to LSM14A. John et al. [26] reported that miR-378 is overexpressed in ovarian cancer cells, and LSM14A was predicted to be a potential downstream target of miR-378, exerting an anti-angiogenic effect. This effect is consistent with the anti-vascular effect of circ\_0010928, further confirming that LSM14A can act as a downstream target of miR-921 to regulate angiogenesis.

## Conclusion

Our research confirmed the molecular mechanism by which circ\_0010928 induces the biological functions of HCMECS and regulates angiogenesis through the circ\_0010928/miR-921/LSM14A axis under hypoxic conditions. In conclusion, the results and data from this study provide new insights into the effect of circRNAs, specifically circ\_0010928, on angiogenesis.

## Declarations

### Ethics approval and consent to participate

Not applicable

### Consent for publication

All participants in the experiment agreed to publish.

### Availability of data and materials

Data and materials are collected through the cell room of the cardiovascular research laboratory, and the experimental data is authentic and usable.

### Competing interests

The authors declare that they have no competing interests, and all authors can confirm the accuracy of this statement.

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### Authors' contributions

The author participated in the whole experiment, including cell culture, data collection and processing, and article writing.

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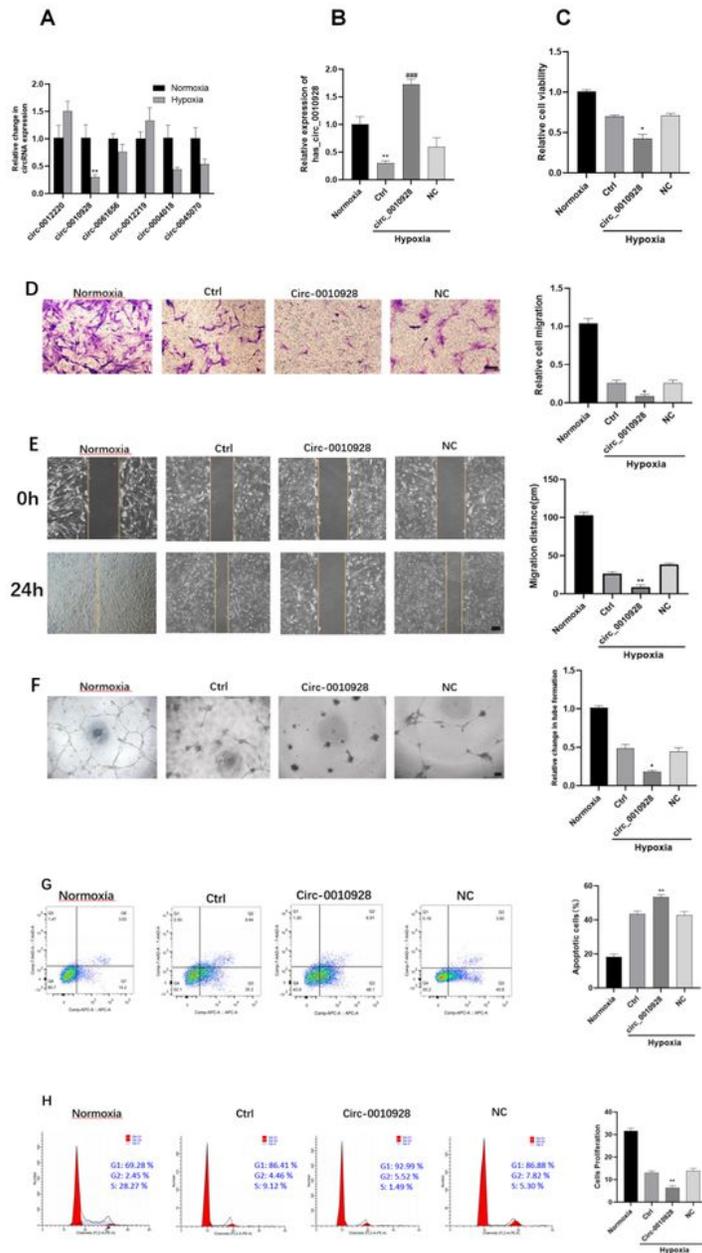
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## Figures



**Figure 1**

The effect of circ\_0010928 on HCMECs. (A) qRT-PCR experiment to measure the expression of circRNAs. (B) qRT-PCR analysis of changes in the expression of circ-0010928 in HCMECs treated with hypoxia and overexpressing circ-0010928 and NC (\*\*p<0.001 compared to normoxia and ###p<0.0001 compared to the ctrl). (C) The CCK-8 assay was used to analyse the viability of HCMECs overexpressing circ-0010928 and NC. (D-E) Transwell and scratch experiments were used to analyse the migration ability of HCMECs

overexpressing circ-0010928 and NC. Scale bar, 100  $\mu$ m. (F) A tube formation experiment was used to analyse the angiogenic ability of HCMECs overexpressing circ-0010928 and NC. Scale bar, 100  $\mu$ m. (G) Flow cytometry analysis of the apoptosis of HCMECs overexpressing circ-0010928 and NC. (H) Flow cytometry analysis of the proliferation ability of HCMECs overexpressing circ-0010928 and NC. Data are presented as the mean  $\pm$  SD; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001 compared to the ctrl.

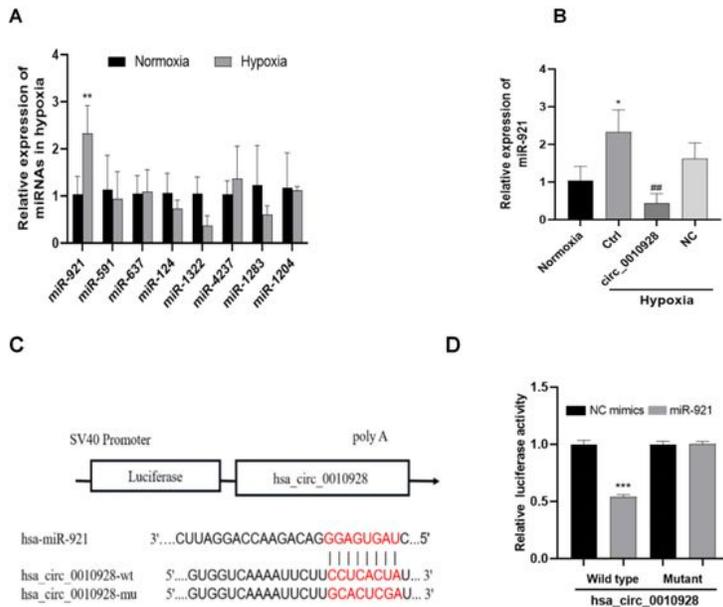


Figure 2

Circ-0010928 functions as a sponge of miR-921 in HCMECs. (A) qRT-PCR was performed to measure the expression of miRNAs. (B) qRT-PCR analysis of changes in miR-921 expression in HCMECs overexpressing circ-0010928 and NC (\* $p < 0.05$  compared to normoxia and ## $p < 0.001$  compared to the ctrl). (C) The predicted complementary binding sites of miR-921 in circ-0010928. (D) The luciferase reporter assay confirmed the binding of circ-0010928 and miR-921. Data are presented as the mean  $\pm$  SD; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  compared to the NC mimic of WT.

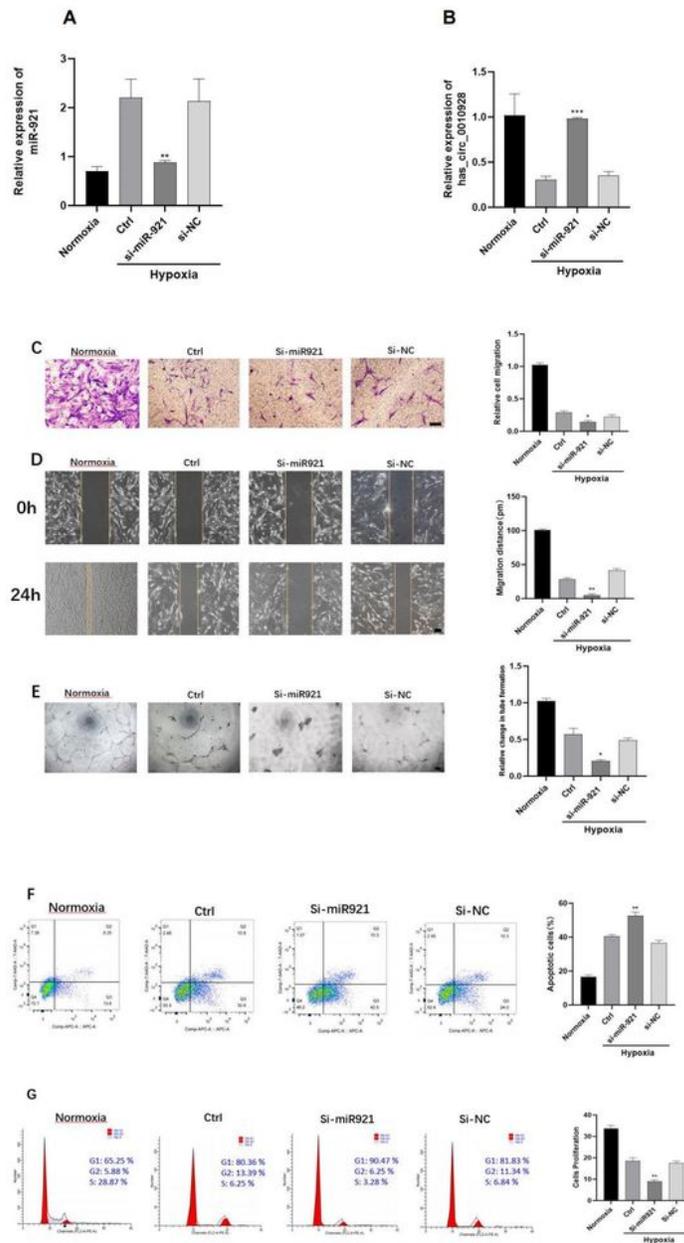
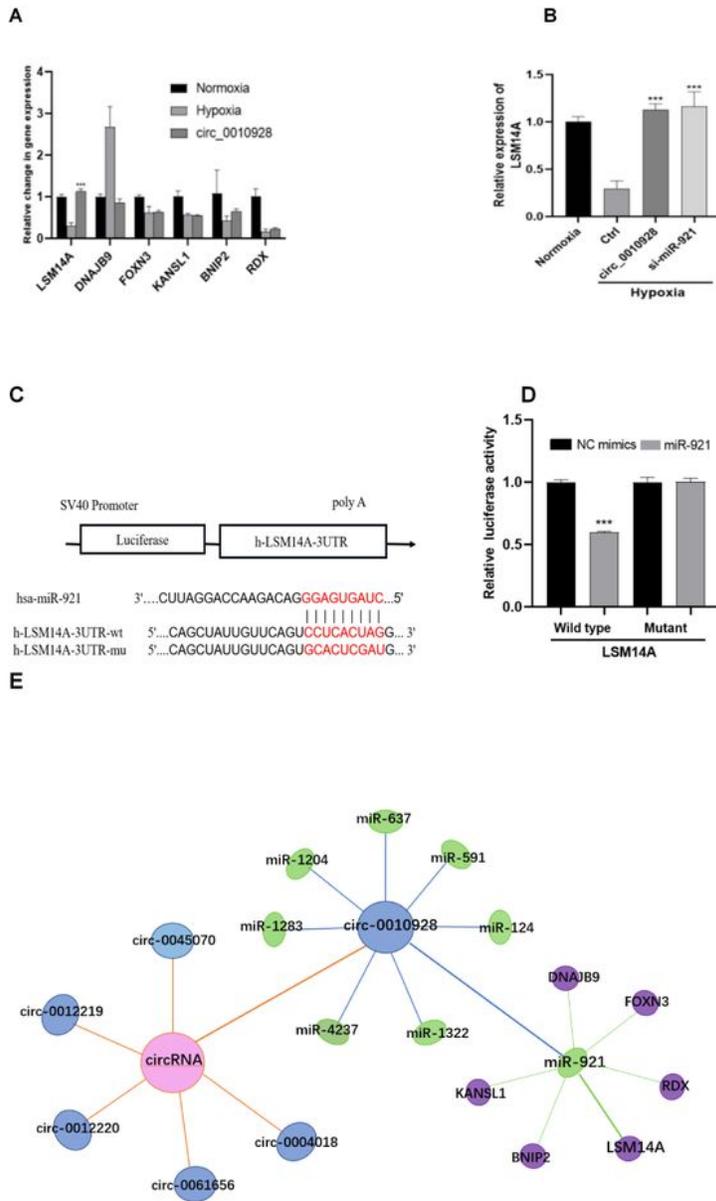


Figure 3

The downregulation of miR-921 inhibits the proliferation and promotes the apoptosis of HCMECs. (A) qRT-PCR analysis of the changes in miR-921 expression in HCMECs transfected with si-miR-921 and si-NC. (B) qRT-PCR analysis of changes in circ-0010928 expression in HCMECs transfected with si-miR-921 and si-NC. (C) A CCK-8 assay was used to analyse the viability of HCMECs transfected with si-miR-921 and si-NC. (D-E) Transwell and scratch experiments were used to analyse the migration ability of HCMECs transfected with si-miR-921 and si-NC. Scale bar, 100  $\mu$ m. (F) Tube formation experiment to analyse the angiogenesis ability of si-miR-921- and si-NC-transfected HCMECs. Scale bar, 100  $\mu$ m. (G) Flow cytometry analysis of the apoptosis of si-miR-921- and si-NC-transfected HCMECs. (H) Analysis of the proliferation ability of HCMECs transfected with si-miR-921 and si-NC by flow cytometry. Data are presented as the mean  $\pm$  SD; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001 compared to the ctrl.



**Figure 4**

Circ-0010928 regulates hypoxia-induced angiogenesis via the miR-921/LSM14A axis. (A) qRT-PCR experiment measuring the expression of downstream targets of miR-921 ( $***p < 0.0001$  compared to hypoxia). (B) qRT-PCR analysis of changes in LSM14A expression in HCMECs transfected with a circ-0010928 overexpression plasmid and si-miR-921 ( $***p < 0.001$  compared to the ctrl). (C) The predicted complementary binding sites of miR-921 in LSM14A. (D) The luciferase reporter assay confirmed the

binding of miR-921 and LSM14A. (E) Bioinformatics analysis predicts circRNAs, miRNAs and downstream targets and the established circ\_0010928/miR-921/LSM14A network. Data are presented as the mean  $\pm$  SD, \*\*\*p<0.001 compared to the NC mimic of WT.