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Circ-100338 induces angiogenesis after myocardial ischemia-reperfusion injury by sponging miR-200a-3p

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Abstract. – OBJECTIVE: To investigate the effect of circular RNA circ-100338 on angiogenesis of human umbilical vein endothelial cells (HUVEC) cells after hypoxia/reoxygenation (H/R) and its molecular mechanism.

MATERIALS AND METHODS: We evaluated the role of circ-100338 in coronary artery endothelial cells using human coronary endothelial cells (HCAEC). Then, we verified the function of circ-100338 in HUVEC cells through cell counting kit-8 (CCK8), scratch test, Tube forming experiment, 5-Ethynyl-2'-deoxyuridine (EdU) staining. Dual-Luciferase reporter gene experiment and RNA Pull-Down experiments were used to detect the binding effect of circ-100338 and miR-200a-3p, miR-200a-3p and FUS.

RESULTS: QRT-PCR results showed that the expression of circ-100338 decreased in HCAEC after H/R treatment. Overexpression of circ-100338 promotes angiogenesis. The Dual-Luciferase reporter gene assay and RNA pull-down assay consistently indicated the specific binding effect between circ-100338 and miR-200a-3p, miR-200a-3p and FUS, and circ-100338 promoted the angiogenesis phenotype in HUVEC cells.

CONCLUSIONS: CircRNA-100338 may inhibit the function of miRNA-200a-3p by combining with miRNA-200a-3p, and then miRNA-200a-3p plays a role in regulating FUS, thereby regulating the state of angiogenesis.

Key Words:
Circ-100338, MiR-200a-3p, Angiogenesis, Myocardial ischemia-reperfusion injury (I/RI).

Introduction

Myocardial ischemia-reperfusion injury (I/RI) refers to the myocardial recovery of blood flow perfusion within a short period of time after ischemia, which leads to aggravation of ischemic myocardial injury[1,2]. These injuries mainly include arrhythmia, myocardial contractile dysfunction, and irreversible myocardial reperfusion damage[3]. In recent years, with the widespread application of thrombolytic therapy, PCI, and surgery for cardiovascular system diseases, the mortality of patients with myocardial infarction has decreased markedly, but we cannot ignore the damage to the heart muscle caused by I/RI. The mechanisms of I/RI include excessive production of reactive oxygen species (ROS), overload of calcium ions, excessive apoptosis of cardiomyocytes, and inflammatory responses[4,5]. Reducing I/R myocardial cell injury is of great significance to the clinical prognosis of patients.

Angiogenesis (AG) refers to a biological process that, based on the original blood vessels, forms a new capillary network through changes in the proliferation and migration of vascular endothelial cells and adapts its functions to local needs, involving endothelial cell division, degradation of blood vessel basement membrane and extracellular matrix and migration of endothelial cells[6]. Therapeutic angiogenesis, that is to say, induces and promotes the angiogenesis of normal tissues surrounding ischemia or establishes collateral circulation[7]. It forms a new circulation bypass around the blocked or narrow arteries, completing the self-bypass of the ischemic site, which can reduce the necrosis of the ischemic tissue and thus perform a series of treatment methods.

In recent years, non-coding RNA (ncRNA) has received widespread attention, including small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), long non-coding RNA (long non coding RNA, lncRNA) and

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circular RNA (circulRNA, circRNA), they play an important regulatory role in the occurrence and development of various diseases[8]. Among them, circRNA was once considered as a useless by-product in the transcription process, and it has only gradually been paid attention in recent years[9]. Thanks to the rapid development of sequencing technology, a large number of circRNAs have been discovered. With the deepening of research, circRNAs have also been detected to have complex biological functions. Some circRNAs function as competing endogenous RNAs (ceRNAs), which are defined as miRNA sponges that bind miRNAs and thus prevent them from binding and suppressing their target mRNAs[10]. It has been shown that several abundant circRNAs can act as miRNA sponges. The mouse circSry contains 16 target sites for miR-138[11]. CircHIPK2 can be a sponge of miR124-2HG and can modulate astrocyte activation during autophagy and endoplasmic reticulum stress[12]. The study of myocardial I/RI and its prevention and protection is of great significance, but after decades of research, there are still many problems and unknown mechanisms that need to be solved and explored. CircRNA provides a new direction and angle for the study of myocardial I/RI. Although circRNA has received extensive attention and research, there are still few researches on circRNA in the cardiovascular field, and in particular on myocardial I/RI. Huang et al[13] reported that circRNA-100338 can induce angiogenesis and then promote the metastasis of hepatocellular carcinoma. We speculate whether circRNA-100338 can play a similar role in myocardial I/R. This study intends to perform functional verification from *in vitro*, combined with bioinformatics analysis and step-by-step verification to explore its possible molecular mechanism.

Materials and Methods

Cell Culture and Transfection

HUVEC cells (Cell Culture Center, Shanghai, China) were incubated in a carbon dioxide incubator (Jiangnan, Hangzhou, China), with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Rockville, MD, USA) and antibiotics (Gibco, Rockville, MD, USA). Replace the medium every 2-3 days. To simulate *in vitro* myocardial ischemia-reperfusion injury, DMEM medium was replaced with DMEM medium without glucose, and cells were continuously cultured under anaerobic conditions containing 95% N_2 and 5% CO_2 for 6 hours, and then replaced the medium containing sugar and placed it in the cell incubator for 4 hours. Circ-100338 overexpressed plasmid (circ-100338) and negative control (vector) were synthesized by GenePharma (Shanghai, China). Three days after transfection was selected as the best harvest time.

Cell Counting Kit-8 (CCK8) Assay

HUVEC cells were inoculated in 96-well plates, incubated for 24 hours, then 10 μL CCK-8 solution (JianCheng, Nanjing, China) was added to each well, and incubated for another 2 hours, with the absorbance at 450 nm of the enzyme marker.

RNA Pull-Down

The 3'-end biotin-labeled RNA production and RNA pull-down assay were carried out using a Pierce Magnetic RNA-Protein Pull-Down Kit according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). All of the RNA was purified by a Thermo GeneJET RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative Real Time-Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from myocardial tissue or HUVEC cells by TRIzol method (Thermo Fisher Scientific, Waltham, MA, USA), 1.0 g total RNA was taken, and complementary deoxyribose nucleic acid (cDNA) was reversed-transcribed by oligo (dT) and random primers to detect circ-100338 and miR-200a-3p levels. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference for the detection of circ-100338 and miR-200a-3p, and U6 was used as an internal reference for the detection of miR-200a-3p. PCR reactions and data analysis were performed in the vii A7 Quantitative PCR System (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta CT}$ method to calculate circ-10033, the relative expression level of microRNAs. Primers used were shown in Table I.

Western Blot

Radioimmunoprecipitation assay (RIPA) protein lysate (Beyotime, Shanghai, China) was added to HUVEC cells, which was cleaved on ice, centrifuged at 4°C 12000 r/min for 15 min, and the supernatant was taken for protein quanti-

Table I. Real Time-PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
MiR-200a-3p	AGCGTAACACTGTCTGGTAA	TCCTCCTCTCCTTCCTTCTC
CircRNA-100,338	AAAAGCAAGCAGTGCCCATA	GCTCGAATCAGGTCCACCA
U6	GCTTCGGCAGCACATATACTAAAAT	CGCTTCACGAATTTGCGTGTCAT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

fication and then separated. Then, we added 5×loading buffer, heated at 70°C for 10 min to denature the protein, and then conduct polyacrylamide gel electrophoresis. Polyvinylidene difluoride (PVDF) membrane (Roche, Basel, Switzerland) was used to transfer the protein, and 5% skim milk powder was sealed for 2 h, and the corresponding antibodies anti-FUS (1:1000, Abcam, Cambridge, MA, USA, Rabbit) and anti-VEGF (1:2000, Abcam, Cambridge, MA, USA, Rabbit) were incubated overnight at 4°C. The membrane was washed with Tris-Buffered Saline and Tween-20 (TBST), and the second antibody (1:2000) was added and incubated at room temperature for 2 h. Enhanced chemiluminescence (ECL) kit was developed. GAPDH (1:2000, Proteintech, Rosemont, IL, USA) as internal reference, the gray value was scanned and the relative protein expression content was analyzed.

Scratch Test

First, we used a Marker pen on the back of the 6-well plate to draw a horizontal line, about 0.5-1 cm wide, across the hole, and passed at least 5 lines per hole. Then, we added 50 μL of fibronectin (Corning, Corning, NY, USA) at a concentration of 10 μg/mL to each well in a 6-well plate, put it in a 4°C refrigerator overnight, and added the Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Corning, Corning, NY, USA) containing 10% FBS the next day. Cells in a logarithmic growth phase were inoculated at a concentration of about 1×10^6 / mL in a 6-well culture plate and cultured in an incubator. The cells were grown into a monolayer under an inverted microscope, and then treated with the test. After that, 10 μL of sterile micropipette was used. The liquid gun head was scratched along the ruler on the cell plate. The scraped off cells were removed with phosphate-buffered saline (PBS), and the serum-free medium was added to continue the culture. After the scratches, samples were taken regularly, observed under an inverted microscope and photographed. Image Pro-Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA) measures the width of scratches at any eight locations of cells in each group at the same time point, calculates cell migration distance and mobility, and then performs data statistics.

Tube Forming Experiment

A 48-well plate was used in the experiment, and 150-200 μL of Matrigel (Corning, Corning, NY, USA) was added to each well, so that the bottom of the plate was evenly covered and placed in an incubator for more than 2 hours to allow Matrigel to solidify. Then, about 2×10^4 cells were seeded per well, and the AG was observed after 4-6 hours.

5-Ethynyl-2′-Deoxyuridine (EdU) Staining

The cells were removed from the incubator and eluted with a PBS solution. It was then fixed with 4% paraformaldehyde (JianCheng, Nanjing, China). The excess paraformaldehyde was then eluted with PBS. The Triton X-100 solution (Kaiji, Nanjing, China) was further subjected to a film breaking treatment. After the film breaking was completed, a blocking solution was added and blocked for 1 hour. Primary antibody EdU (Ye Sen, Guangzhou, China) was then incubated overnight. The next day, add a secondary antibody and incubate for 1 hour in the dark, and then stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear staining solution (Ye Sen, Guangzhou, China). Finally, the condensing microscope was used to detect the image after sealing with glycerol.

Luciferase Reporter Analysis

Circ-100338 overexpression was co-transfected with reporter gene plasmids: 293T (Thermo Fisher Scientific, Waltham, MA, USA) culture was performed on 24-well plates. 48 h later, adenovirus (Genechem, Shanghai, China) contain-

ing circ-100338 was infected [4 x 10^5 cells with 20 μL virus (10^9 TU/mL) and polybrene (final concentration 5 mg/mL)]. FUS-WT/Mut was subcloned into pmirGLO Dual-Luciferase plasmid (Genechem, Shanghai, China) to co-transfected with miR-200a-3p mimics or NC into 293T cells. Luciferase activity was detected on day 5.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 25.0 statistical software (IBM, Armonk, NY, USA) was used for analysis. The measurement data were expressed as mean ± standard deviation. Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test, followed by Post-Hoc Test (Least Significant Difference). The Bonferroni corrected *t*-test was used for the pair-wise comparison between groups. $p<0.05$ was considered statistically significant.

Results

The Role of CircRNA-100338 in H/R Model

We evaluated the role of circ-100338 in coronary endothelial cells using human coronary endothelial cells (HCAEC). First, we performed qPCR verification of circRNA-100338. As a result, we found that the expression of circRNA-100338 was significantly downregulated in the I/R model (Figure 1A). Secondly, to find the function of circRNA-100338, we will immediately overexpress circRNA-100338 (Figure 1B), so as to preliminary explore whether circRNA-100338 exerts protective or damaging effects. First, we examined the effect of circRNA-100338 on HUVEC viability. Divided into two groups of control group and hypoxia / reoxygenation group (H/R), each group is divided into control group (Vector group) and overexpression group (Circ-100338). The cells were plated and treated accordingly, and the H/R model was constructed according to the method described above (6 hours of hypoxia and 2 hours of reoxygenation). Then, CCK-8 assay was used to detect the viability of myocardial cells in each group. We found that overexpression of circRNA-100338 under normoxic conditions did not cause upregulation of cardiomyocyte viability. After myocardial cells underwent hypoxia-reoxygenation injury, the cell viability of the over-expressed circRNA100338 group was significantly improved, suggesting that circRNA100338 has a protective effect on HUVEC (Figure 1C). We, then, performed cell scratch experiments and angiogenesis experiments and found that overexpression of circRNA-100338 can promote the migration and tube formation of HUVEC (Figure 1D and 1E). EdU staining showed that in the experimental group overexpressing circRNA-100338, the cell proliferation ability was markedly increased (Figure 1F). WB results also showed that the expression of VEGF increases when overexpressing circ-100338 (Figure 1G).

MiRNA-200a-3p Combined With CircRNA-100338 Regulates Angiogenesis

We first explored whether circRNA-100338 has the potential to bind miRNA. It is reported in the literature that the binding of circRNA and miRNA depends on AGO2 protein as a carrier or medium, so the binding of circRNA and AGO2 protein is the basis for its role as a miRNA sponge. The AGO2 pull down experiment was performed to test whether the circRNA-100338 was enriched on the AGO2 protein, and the possibility of circRNA-100338 binding to miRNA can be preliminarily judged. The results showed that compared with the vector group, the content of circRNA-100338 in the over-expressed AGO2 protein group was remarkably increased, that is, circRNA-100338 (Figure 2A) was enriched in the AGO2 protein, suggesting that circRNA-100338 has the potential of miRNA molecular sponge. Then, we used the website prediction method and combined with q-PCR to verify that in the over-expressing circRNA-100338 group, the expression of miRNA-200a-3p significantly decreased (Figure 2B). Next, we first designed point mutation primers for miRNA-200a-3p and circRNA-100338 binding sites, and constructed corresponding binding site mutation plasmids. Transfection of miRNA mimics with Luciferase and Luciferase transfection experiments revealed that compared with the non-mutated group, the fluorescence signal value of miRNA-200a-3p rose back after mutation at the binding site (Figure 2C), suggesting that miRNA-200a-3p and circRNA-100338 no longer bind after the binding site mutation. From this part of the results, we believe that there is a high possibility of binding circRNA-100338 to miRNA-200a-3p. Then we transfected HUVEC cells with miRNA-200a-3p mimics and NC respectively. We investigated whether miRNA-200a-3p can regulate the ability

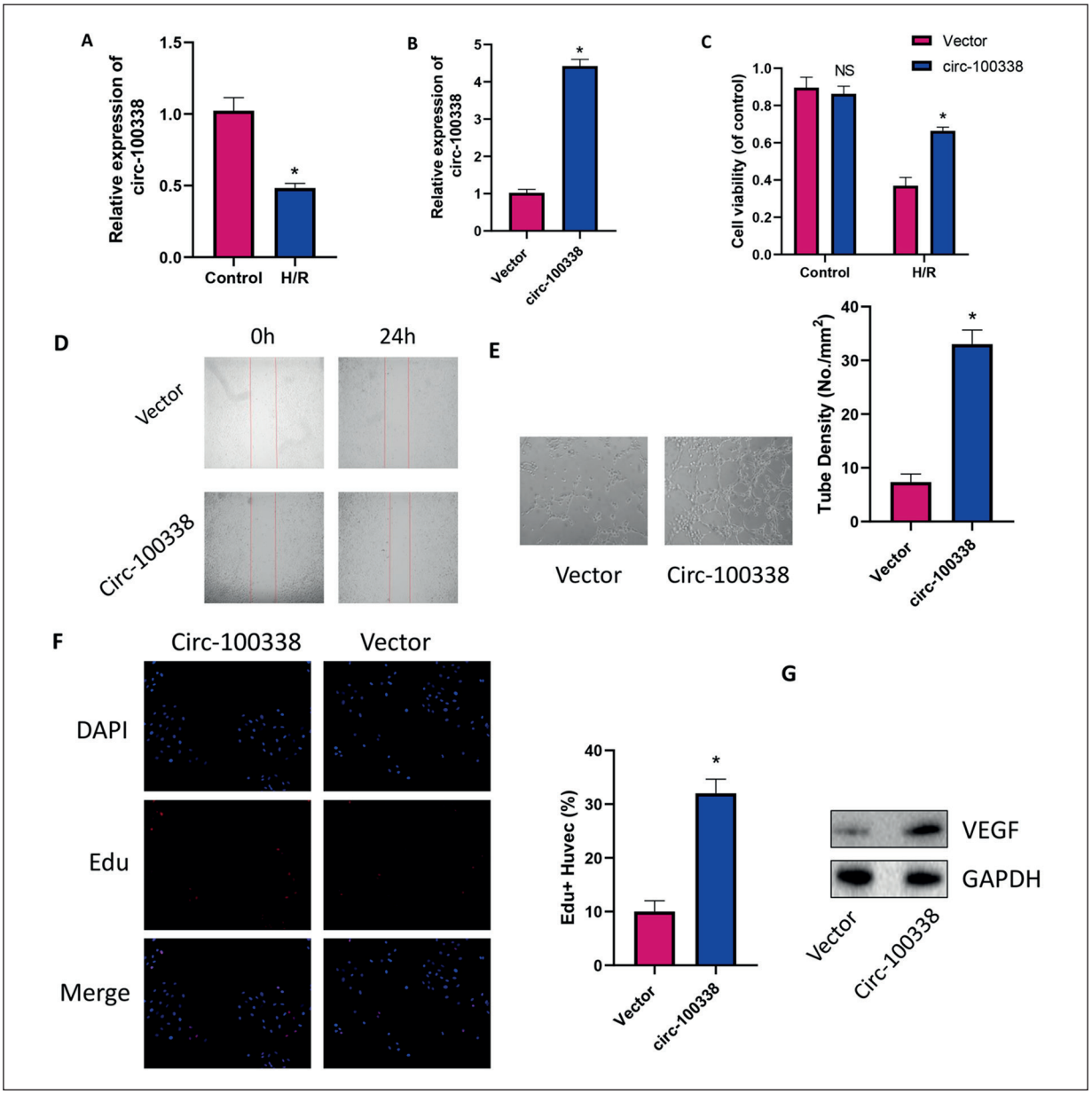

Figure 1. The role of circRNA-100338 in H/R model. **A,** The expression levels of circ-100338 in HCAEC of Control and H/R groups. ("*" indicates statistical difference from the Control group $p<0.05$) **B,** The expression levels of circ-100338 after transfection with circ-100338. ("*" indicates that compared with the Vector group $p<0.05$). **C,** CCK-8 showed the cell viability. ("*" indicates statistical difference from vector group $p<0.05$). **D,** Cell migration was examined by Scratch test (magnification: 10×). **E,** Tube forming experiment showed the tube density (magnification: 10×). ("*" indicates that compared with the Vector group $p<0.05$). **F,** Edu staining in vector or circ100338 overexpression plasmid-treated HUVEC cells. Quantification of Edu^+ cells presented as the % Edu-positive cells and DAPI-stained nuclei. (magnification: 200×). **G,** Western blot bands of VEGF ("*" indicates that compared with the Vector group $p<0.05$).

of angiogenesis through cell scratch experiments, tube formation experiments, EdU staining, and WB experiment. It was found that miRNA-200a-3p can inhibit cell migration, tube formation and proliferation, and inhibit angiogenesis (Figure 2D-2G).

CircRNA-100338 Regulates Angiogenesis by Binding to MiRNA-200a-3p

Simultaneously with H/R, we gave stimulation under different conditions, divided into four groups: vector + NC, circRNA-100338 + NC, vector + miRNA-200a-3p mimics and cir-

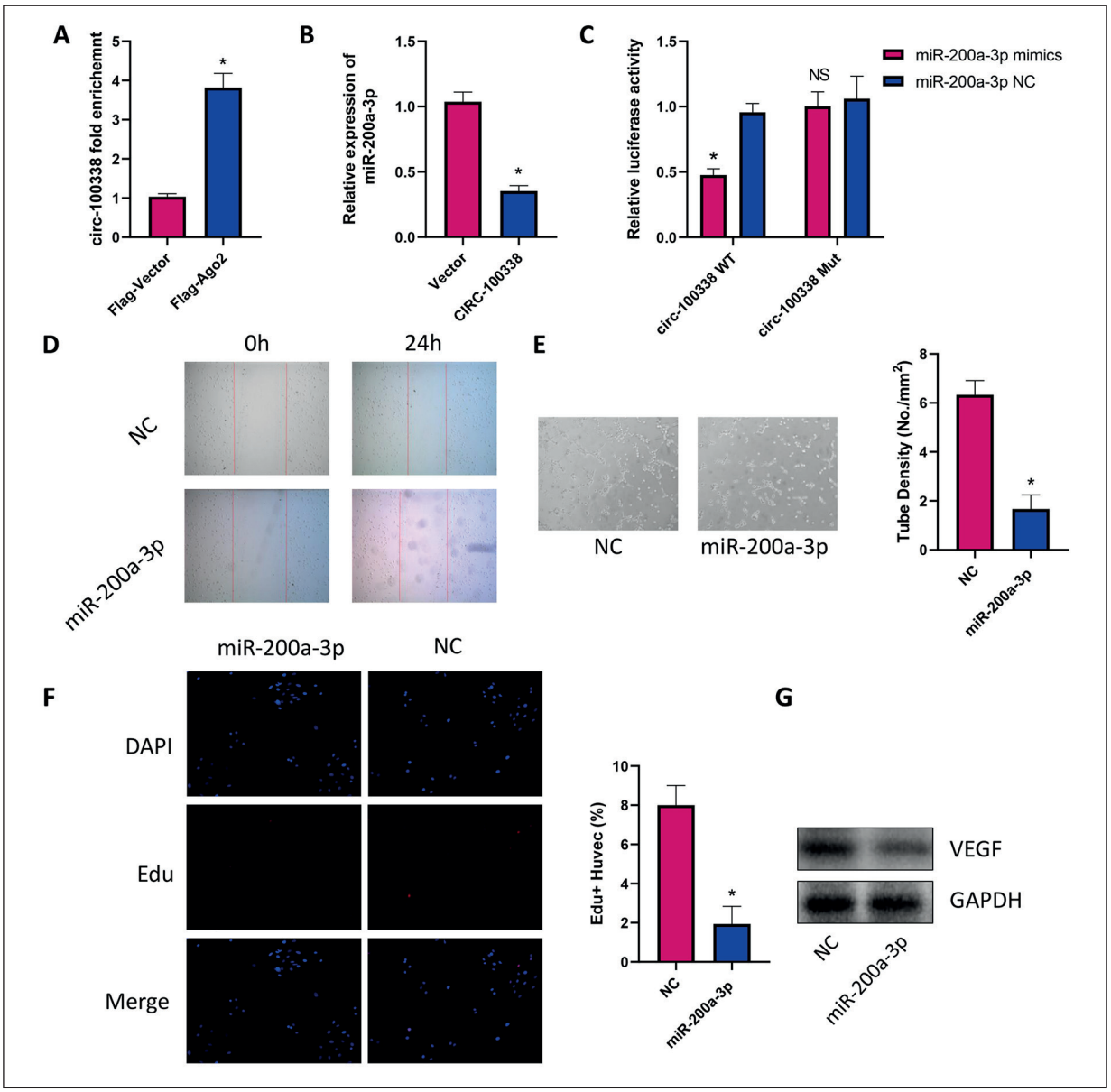

Figure 2. MiRNA-200a-3p combined with circRNA-100338 regulates angiogenesis. **A,** RT-qPCR detects the relative expression of circ-100338. ("*" indicates statistical difference from the Flag-vector group $p<0.05$). **B,** RT-qPCR detects the relative expression of miR-200a-3p ("*" indicates statistical difference from the Flag-vector group $p<0.05$). **C,** The Luciferase report assay. ("*" indicates statistical difference from the circ-100338 WT+miR-200a-3p NC group $p<0.05$). **D,** Cell migration was examined by Scratch test (magnification: 10×). **E,** Tube forming experiment showed the tube density (magnification: 10×). ("*" indicates that compared with the NC group $p<0.05$) **F,** Edu staining in NC or miR-200a-3p mimics treated HUVEC cells. Quantification of Edu^+ cells presented as the % Edu-positive cells and DAPI-stained nuclei. (magnification: 200×). **G,** Western blot bands of VEGF ("*" indicates that compared with the NC group $p<0.05$).

cRNA-100338 + miRNA-200a-3p mimics. Then, we observe its changes in the neovascular energy of HUVEC. We found that the angiogenesis ability of the circRNA-100338 + miRNA-200a-3p mimics group was better than the vector + miRNA-200a-3p mimics group, but it was worse than the circRNA-100338 + NC group (Figure 3A-3D), indicating 100338 can indeed regulate angiogenesis by binding to miRNA-200a-3p.

MiRNA-200a-3p Inhibits Angiogenesis by Regulating Fused in Sarcoma (FUS)

First, we predicted the possible downstream binding sites of miRNA-200a-3p through the

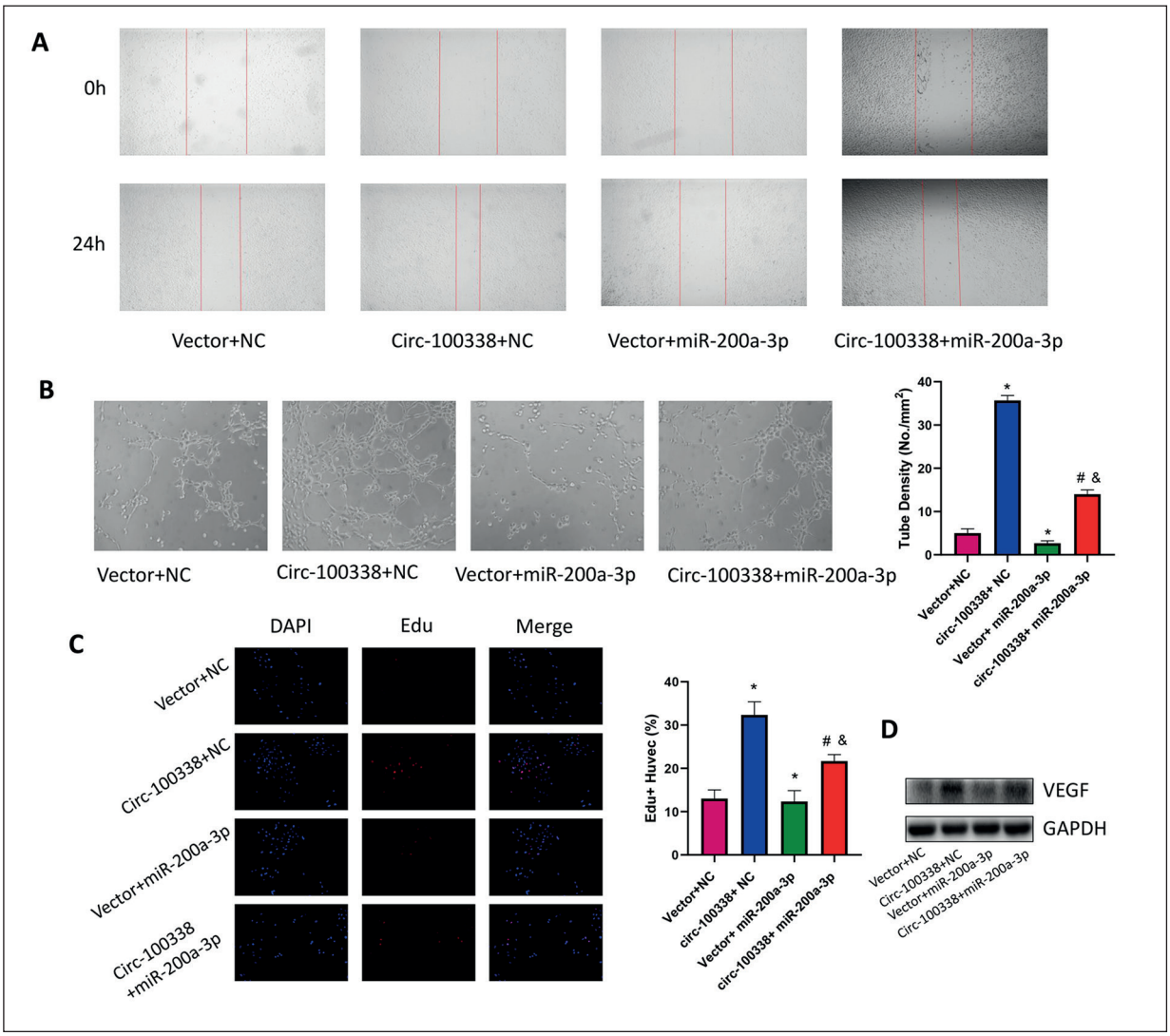

Figure 3. CircRNA-100338 regulates angiogenesis by binding to miRNA-200a-3p. **A,** Cell migration was examined by Scratch test (magnification: 10×). **B,** Tube forming experiment showed the tube density (magnification: 10×). ("*" indicates that compared with the Vector+NC group, "#" indicates that compared with the circ-100338+NC group, "&" indicates that compared with the Vector+miR-200a-3p group mimics $p<0.05$) (magnification: 20×). **C,** Edu staining in Vector+NC, circ-100338+NC group, Vector+miR-200a-3p mimics and circ-100338+miR-200a-3p mimics treated HUVEC cells. Quantification of Edu^+ cells presented as the % Edu-positive cells and DAPI-stained nuclei. (magnification: 200×). **D,** Western blot bands of VEGF ("*" indicates that compared with the Vector+NC group, "#" indicates that compared with the circ-100338+NC group, "&" indicates that compared with the Vector+miR-200a-3p mimics group $p<0.05$).

Targetscan website, and verified that FUS was the downstream binding site of miRNA-200a-3p (Figure 4A and 4B) through Luciferase reporter gene experiments and WB experiments. To study whether miRNA-200a-3p can inhibit AG through FUS, we knocked out or overexpressed FUS in HUVEC with or without miRNA-200a-3p to detect the ability of angiogenesis, respectively. The angiogenesis ability in the 200a-3p + OE-FUS group was higher than that of miRNA-200a-3p + siRNA FUS, indicating that there was an interaction between miRNA-200a-3p and FUS (Figure 4C and 4D).

Discussion

CircRNA has been discovered as early as the 1970s, but due to technical limitations, the detected circRNAs have low level and few spe-

cies, so they are considered to be mis-expression or splicing during pre-mRNA splicing. The "useless" by-products have not received further research and attention. Until several decades later, in the same journal of Nature in 2013, two circRNA research reports were published in succession[14,15]. Since then, circRNA related research has grown rapidly and gradually became a new star molecule in the field of non-coding RNA. CircRNA has been shown to be involved in the occurrence and development of many diseases through direct or indirect effects, but the most important thing is to focus on the field of oncology. CircRNA CDRlaS combines with miR-7 competitively to promote the expression of CCNEl and thereby promote the occurrence of liver cancer[16]. Inhibition of circRNA hsa circ 0000096 can dramatically affect the cell cycle and cell migration ability of gastric cancer cells[17]. In addition, circRNA has also been reported in many aspects in targeted therapy and clinical diagnosis of tumors[18]. However, there are still few researches and reports on circRNA in the cardiovascular system.

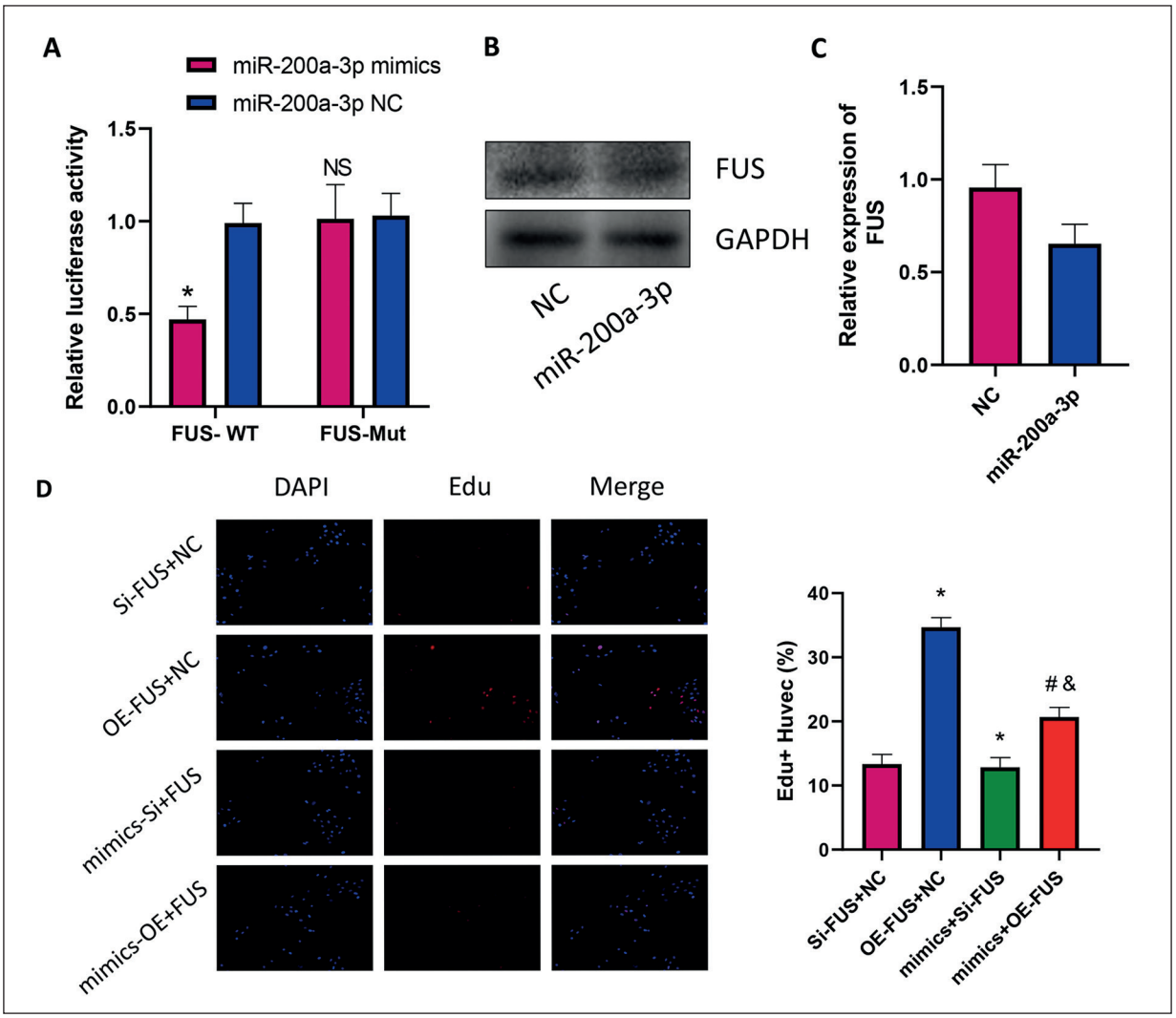

Figure 4. MiRNA-200a-3p inhibits angiogenesis by regulating fused in sarcoma (FUS). **A,** The Luciferase report assay. ("*" indicates statistical difference from the FUS-WT+miR-200a-3p NC group $p<0.05$). **B,** Western blot bands of FUS. **C,** Relative expression of FUS detected by RT-qPCR. ("*" indicates statistical difference from the NC group $p < 0.05$). **D,** Edu staining in si-FUS+NC, OE-FUS+NC, miR-200a-3p mimics+si-FUS, miR-200a-3p mimics+OE-FUS treated Huvec cells. Quantification of Edu^+ cells presented as the % Edu-positive cells and DAPI-stained nuclei (magnification: 200×) ("*" indicates that compared with the si-FUS+NC group, "#" indicates that compared with the OE-FUS+NC group, "&" indicates that compared with the miR-200a-3p mimics+si-FUS group $p<0.05$).

In this study, we found that circRNA-100338 was also downregulated in the rat I/R model. Secondly, in order to determine whether circRNA-100338 plays a protective or injury role in myocardial I/R. Our results show that circRNA-100338 plays an important role in coronary AG and circRNA-100338 can promote the proliferation, migration and tube formation of HCAEC. With regard to its mechanism exploration, circRNA-100338 has been shown to perform its function through sponge-related miRNAs[19], and bioinformatics analysis has also shown that circRNA-100338 enriches the seed sequence of miRNA-200a-3p. It was found through experiments that the circRNA-100338/miRNA-200a-3p pathway is involved in the proliferation of HUVEC cells, indicating that the mechanism of circRNA-100338 promoting endothelial cells depends on the sponge effect of miRNA. Later, we discovered that miRNA-200a-3p can bind to FUS. FUS has been shown to play important roles in a variety of cellular processes, including transcription, cell cycle progression, angiogenesis, and apoptosis[20,21]. Therefore, our research suggests that miRNA-200a-3p can regulate FUS expression.

Conclusions

We suggest that circRNA-100338 may inhibit the function of miRNA-200a-3p by combining with miRNA-200a-3p, and then miRNA-200a-3p plays a role in regulating FUS, thereby regulating the state of AG. It is the first time to indicate that circ-100338 can induce angiogenesis and then regulate the metastasis of myocardial I/R. As circRNAs are stable and have unique structural conformations, further studies are warranted to explore their potential as therapeutic agents and biomarkers.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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