MiR-423-5p promotes angiogenesis by targeting LHX6 in oxygen–glucose deprivation and reperfusion (OGD/R) induced Human Umbilical Vein Endothelial Cells (HUVEC)

Di Jin  
First Affiliated Hospital of Zhengzhou University

Wenjing Deng (✉ 13676965683@126.com)  
First Affiliated Hospital of Zhengzhou University

Junfang Teng  
First Affiliated Hospital of Zhengzhou University

Guoliang Xiang  
First Affiliated Hospital of Zhengzhou University

Yanan Zhao  
First Affiliated Hospital of Zhengzhou University

Research Article

Keywords: Ischemia/reperfusion injury, miR-423-5p, LHX6, angiogenesis

Posted Date: July 21st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3176697/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License
Abstract

Background

The prognosis of ischemic stroke is poor, moreover, ischemia-reperfusion (I/R) injury following revascularization therapy can give rise to more severe outcomes. Therefore, finding other effective and new methods for treating ischemic stroke is necessary. According to studies some microRNAs are involved in the process of angiogenesis which plays an increasingly vital role in I/R injury. In the present study, We selected miR-423-5p as our research object because of our previous clinical results.

Methods

To contrast the I/R injury model in vitro, we used oxygen–glucose deprivation and reperfusion (OGD/R) induced Human Umbilical Vein Endothelial Cells (HUVEC) as our study subjects. The level of miR-423-5p expression was detected by reverse transcription quantitative polymerase chain reaction(RT-qPCR). Transwell assay, scratch assay and tube formation assay were used to evaluate the proangiogenic activity with miR-423-5p mimic or inhibitor in vitro. We adopted western blot and RT-qPCR to test the expression of LIM homeobox 6(LHX6), and a luciferase reporter assay was carried out to confirm whether LHX6 is a direct target of miR-423-5p.

Results

We found miR-423-5p was significantly down-regulated in OGD/R induced HUVEC. The overexpression of miR-423-5p stimulated HUVEC proliferation and migration, instead, miR-423-5p inhibitor played the opposite role. In further research, we identified LHX6 as a downstream gene of miR-423-5p by the luciferase reporter assay. Western blot and RTqPCR analysis confirmed that LHX6 expression was negatively related to the expression of miR-423-5p.

Conclusions

In summary, this study suggests that miR-423-5p mediated the proangiogenic activity of HUVEC by relying on LHX6. It could be an underlying therapeutic target for I/R injury that warrants further studies.

Introduction

Stroke, a huge threat to human's health and lives throughout our country even the world, is the commonest type of cerebrovascular disease (Saini et al. 2021). It is normally classified as either ischemic or hemorrhagic stroke although the vast majority of cases belong to ischemic event. There have been no marked progress in the treatment for hemorrhagic stroke in recent years, and for ischemic stroke, the treatment is limited to two options: intravenous thrombolysis with alteplase and mechanical
thrombectomy. However, both above the methods are widely underutilized, because of their narrow time window. There is an urgent need for more effective therapies to significantly improve progression. According to the research, there are two possible causes of the damages following ischemic stroke, the primary and secondary insult. The primary insult is induced by its own ischemic process, while the second relate to I/Rc injury after revascularization therapy (Gauberti et al. 2018). During the course, a series of pathophysiologic processes such as oxidative stress, cell apoptosis, excitotoxicity, inflammatory response and angiogenesis occur (Lambertsen et al. 2019). We should therefore pay more attention to different post-ischemic stages in the matter of therapeutic approaches. Among the pathophysiologic processes angiogenesis is currently a hotspot of researches in ischemic stroke (Paro et al. 2022).

According to previous studies, promoting angiogenesis can ensure the nutrient and oxygen supply for hypoxic brain tissues and then promote the function recovery (Yang and Torbey 2020). In recent years, a growing body of research has demonstrated that microRNAs (miRNAs) are related to the regulation of angiogenesis as angiogenic stimulators or inhibitors (Heydari et al. 2020). In the present study, we collected blood plasma from ve normal subjects and ve patients with acute cerebral infarction ruling out serious heart disease, diabetes mellitus, cancers and so on. Six differentially expressed miRNAs were identified by bioinformatics analysis (Table 1). We selected miR-423-5p as our research object because of its highest expression abundance.

MiRNAs, consisting of about 20 to 22 nucleotides, are endogenously expressed RNA molecules that can be connected to the 3’ untranslated regions of mRNA to regulate its posttranscriptional regulation. They play a significant role in the pathophysiological processes of ischemic stroke. Furthermore, previous researchers have yielded some findings that they can regulate oxidative stress inflammatory response apoptosis and angiogenesis (Khoshnam et al. 2017). All of above processes are vital for the prognosis of ischemic stroke. For example, previous research found that miR-126 overexpression may inhibit oxidative stress and inflammatory response to weaken OGD/R injury (Li et al. 2021). And, miR-191 could inhibit angiogenesis by targeting VEZF1 in acute ischemic stroke (Du et al. 2019). MiR-423-5p is also reported that can exert its angiogenic function by targeting Sufu (Xu et al. 2019). What’s more, several studies have reported that it plays a vital role in cardiovascular disease and cancers. As a crucial pro-angiogenic factor, miR-423-5p overexpression in glioma cells can increase the quantity of microvessels and then promote tumorigenesis (Li et al. 2017). However, whether miR-423-5p can be utilized as a biomarker for ischemic stroke diagnosis and prognosis is rarely known. In this study, we focused to investigate its expression and role in acute ischemic stroke patients as well as OGD/R induced HUVECs.

LHX6, also known as LHX6.1 or hLHX6, belongs to the LIM Homobox gene family. They can encode a LIM homeodomain transcription factor that have to do with histological development and morphogenesis (Zhou et al. 2015). However, there’s increasing evidence that it may contribute to suppress tumor growth such as cervical cancer or lung cancer through some specific mechanism or signaling pathway. In non-small cell lung cancer, it was reported that down regulating of LHX6 expression may lead to drug resistance and increase cell migration ability via activation of the Wnt/β-catenin pathway which is involved in angiogenesis after ischemic stroke (Wang et al. 2020). So there’s reason to believe that LHX6 may play some role in angiogenesis after ischemic stroke. The bioinformatics study in our experiment
illustrated that miR-423-5p can target LHX6. Therefore, a deeper understanding of the relationship
between miR-423-5p and LHX6 in ischemic stroke may provide helpful insights and shed new light on
areas of research for identifying diagnostic markers and therapeutic approaches for treating
angiogenesis-related diseases such as cerebral infarction.

Materials and methods

Cell culture and OGD/R intervention

HUVECs were bought from American Type Culture Collection (ATCC; Manassas, VA). The cells were grown
in Endothelial Cell Medium (ECM; ScienCell) made of 5% fetal bovine serum (FBS; ScienCell) and 1%
endothelial cell growth supplement (ECGS, ScienCell) and 1% penicillin/streptomycin solution (P/S,
ScienCell) in a humidified incubator filled with 95% air and 5% CO 2 at 37°C. The 3th to 7th passage of
cells were used in this study. Culture media were exchanged according to the condition of cell growth,
normally every two days. To induce OGD/R injury, we replaced the medium with Earle's balanced salt
solution (Leagene Biotech Co. Beijing, China), then placed the cells into an anaerobic incubator suffused
with 94% N 2, 5% CO 2 and 1% O 2 for 6h at 37°C. Following OGD, cells were feded with complete ECM
medium and returned to the incubator under normoxia condition at 37°C in 5% CO2 and 95% air for 12
hours. HUVECs in normoxia group were cultured in a complete medium for six hours and then exchanged
fresh complete ECM medium in a humidified incubator filled with 95% air and 5% CO 2 at 37°C at the
same time for 12 hours. Before exposed to OGD, the density of cells was controlled between 40–60%
confluent.

Transfection

MiR-423-5p mimic (forward, 5'UGAGGGGCAGAGCGAGACUUU-3' and reverse, 5'-
AAAGUCUCGUCCUGCCCUCA-3'), mimic negative control (forward, 5'-
UCACAACCUCUAGAAAGUGAGA-3' and reverse, 5'-UCUACUCUUUCGGAGGUUGUGA-3'), miR-423-5p
inhibitor (reverse, 5'-AAAGUCUCGUCUCUGCCCUCA-3') and inhibitor negative control (reverse, 5'-
UCUACUCUUUCGGAGGUUGGA-3'), all of the above were synthesized by Hanbio Biotechnology
(Shanghai, China). Transfection of miR-423-5p mimic or its negative control at a final concentration of
20nM, while miR-423-5p inhibitor or its negative control at an ultimate concentration of 10nM were
carried out using RANFit (Hanbio Biotechnology, Shanghai, China), on the basis of manufacturer's
protocols. At 24 hours after transfection, transfection efficiency was examined by RT-qPCR. What calls for
special attention is that, some cells need to subjected to OGD/R injury after transfection.

RTqPCR

We used TRIzol reagent (Life Technologies, USA) to extract total RNA according to the manufacturer's
directions. Then RNA was reversely transcribed using miRNA The First Strand cDNA Synthesis Kit (by
stem-loop) (Sangon Biotech, Shanghai, China) for miRNA or PrimeScript™ RT Master Mix(Perfect Real
Time)(TAKARA, RR036Q, Japan) for mRNA.
Real-time PCR was performed using Applied Biosystems™ QuantStudio™ Real-Time PCR System with TB Green Premix Ex Taq™(Tli RNaseH Plus) (Code No.RR820A) following the manufacturer's protocol. All results were repeated for three times independently and the relative expression levels of genes were analyzed using the 2^-\Delta\Delta Ct method. U6 and β-actin were used as internal control respectively for miRNA and mRNA. All the primers used were designed and supplied by Sangon Biotech as followed: miR-423-5p, forward, 5'-GTGAGGGGCAGAGAGCGA-3', and reverse, 5'-AGTGCAGGGTCAGAGGTATT-3'; U6, forward, 5'-CTCCTTCGCAGCACA-3', and reverse, 5'-AACGCTTCACGAATTGGCT-3'; LHX6, forward, 5'-CGATATCTGCTAAGGTCAACA-3', and reverse, 5'-CTTGGCTCTTGGATGAGGTAGCT-3'; β-actin, forward, 5'-CCTGGCACCCAGCACAAT-3', and reverse, 5'-GGGCCGGACTCGTCATAC-3'.

**Cell migration assay**

We conducted the scratch assay and transwell assay to assess the motility of cells both in the horizontal direction and in the vertical direction. HUVECs were seeded at 2.5× 10^5 cells per well in 6-well plates and incubated with complete medium under normoxic condition at 37°C for the scratch assay. The second day, when the density of cells was achieved to 40–60% confluent, OGD/R injury was induced. In particular, cells should be transfected with mimic or inhibitor before induced to OGD/R injury for the transfection groups. In this case, the seeded concentration should be more thinner. Then the cells were scratched with 200µl pipette tips and washed with 2ml Phosphate buffer saline (PBS, meilunbio, Dalian, China) to remove nonadherent cells. The images of original wound area were taken with microscope (Olympus, 8H44507, JAPAN). After that HUVECs were cultured in ECM containing 2% FBS at 37°C. Pictures were taken from the same location of wound area with the same microscope after 6 hours and 12 hours. Image J software was used to analyze the images. Finally, migration rate was calculated as: % wound closure = [(Width of original wound—Width of wound after healing)/ Width of original wound] × 100%.

For the transwell assay, The chamber (8.0 µm pore size, Corning, NY, USA) was placed in 24- well plates, then 5x10^4 of cells in 200µl 1% FBS ECM were seeded into the upper chamber, while the lower chamber was filled with 500ul 5% FBS ECM. After 24h of incubation, the chambers were taken out and fixed in Ethanol absolute(SUN, Tianjin, China) for 15 minutes. We removed the nonmigrated cells with moist cotton swabs, then stained the cells moved to the bottom of the membrane with 0.1% Cryatal violet solution (Solarbio, Beijing, China) for 20 minutes. Subsequently, cells were washed with PBS for three times and dried in the air. The number of migrated cells was counted in ten randomly selected visual fields using an microscope(Olympus, 8H44507, JAPAN).

**Tube formation assay**

HUVECs were seeded into 6cm dish and exposed to 6 h of hypoxia followed by 12 h of re-oxygenation. Then, cells were digested, counted, and suspended in 1% FBS ECM, and the final cell density was 2*10^5/l. 50 µl of Matrigel Basement Membrane Matrix (Corning, USA) was plated in each well of a pre-cold 96-well plate and allowed gel solidification at 37°C in 5% CO2 for 40 minutes. Then, 50µl of HUVECs suspension was added to each well. Next, the plates of different groups were incubated at 37°C in the
normoxic condition. Tube formation was observed using an microscope (Olympus, 8H44507, Japan) after two hours, four hours and six hours respectively. Image J software was used to analyze the total branching length and the number of nodes in 6–10 randomly selected visual fields.

**Western blot**

Total protein was separated from HUVECs of different groups using RIPA buffer (high efficiency) (Solarbio LIFE SCIENCES, Beijing, China). What called for special attention was that PMSF should be added before use, protease inhibitor mixture and phosphatase inhibitors mixture can cooperate with RIPA buffer. Then the concentration of protein was detected using BCA protein assay kit (Solarbio LIFE SCIENCES, Beijing, China) based on the manufacturer's directions. Equal amounts of protein (30µg) was electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), next the protein was transferred to PVDF membranes. After blocking with 5% fat-free milk dissolving in TBST buffer, the membranes were incubated with anti-LHX6 (1:200, Proteintech Group, Chicago, USA) and anti-tubulin (1:2000, DINGGUO CHANGSHENG BIOTECHNOLOGY CO.LTD, Beijing, China), respectively, at 4°C overnight. The membranes were thereafter incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for one hour. Finally, proteins were visualized using ECL regent on Amersham Imager 680 (General Electric Company, USA).

**Luciferase assay**

TargetScan version 7.2 (http://www.targetscan.org/vert_72/) was used to predict the putative binding site between miR-423-5p and LHX6. We inserted the wild-type (WT) LHX6 3′-UTR fragments and the mutant-type (MUT) separately into the downstream of the luciferase gene in the pSI-Check2 plasmid. After the above step, both types of plasmid were transfected into HUVECs, accompanied by miR-423-5p mimic and negative control. After incubating for 48 hours, cells were collected and luciferase reaction reagent was added after a series of steps. Finally, the luciferase activities of renilla and firefly were measured by the Dual Luciferase Reporter System (Promega, WI, USA) using a Tecan Infinite M200 Pro instrument. Relative luciferase activity was presented in renilla luciferase/firefly luciferase form.

**Statistical analysis**

All of the data were analyzed and calculated using GraphPad Prism version9.3.1(471) (GraphPad Software, Inc.) and presented as the mean ± standard deviation. For two groups, the data were analyzed using Student’s t tests while one-way ANOVA followed by Tukey’s post hoc test was used to analyze data among multiple groups. A statistically significant difference was defined as P < 0.05.

**Results**
Table 1

Six differentially expressed miRNAs were identified by bioinformatics analysis. The first column is the name of detected differentially expressed miRNAs. And the second column is the change trend of every miRNA after ischemic stroke.

<table>
<thead>
<tr>
<th>Differentially expressed miRNA</th>
<th>tendency</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-1303</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-423-5p</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-4732-5p</td>
<td>down</td>
</tr>
<tr>
<td>hsa-miR-509-3-5p</td>
<td>up</td>
</tr>
<tr>
<td>hsa-miR-5189-3p</td>
<td>up</td>
</tr>
<tr>
<td>hsa-miR-618</td>
<td>up</td>
</tr>
</tbody>
</table>

miR-423-5p expression in HUVECs

The miRNA assay-based RT-PCR was used to validate the expression levels of miR-423-5p in the control group, OGD/R group and the transfection group. As shown in Fig. 1A, miR-423-5p expression of the OGD/R group was significantly lower than that of the control group. Based on the change in miR-423-5p expression and previous studies, it was speculated that modulating its expression may enhance the angiogenic capacity of HUVECs. To verify this assumption, we firstly transfected HUVECs with 10nM miR-423-5p inhibitor(siRNA) and 20nM miR-423-5p mimic to down and up-regulate the expression of miR-423-5p, respectively (Fig. 1B, 1D). Secondly, the transfected cells were induced to OGD/R injury which would further change the expression of miR-423-5p (Fig. 1C, 1E).

The migration ability was assessed by scratch assay and Transwell assay

We performed scratch assay and transwell assay with the purpose of investigating the function of miR-423-5p in HUVECs migration in different groups. The migration rate and migrated cells are two important indicators we observed. Experiments suggested that the migrated ability of HUVECs induced to OGD/R injury were increased compared with control group. What’s more, HUVECs transfected with miR-423-5p mimic before subjected to OGD/R (m + OGD/R) were found to facilitate the close up of scratch wounds (Fig. 2 Figure 4A) and accelerate cell migration(Fig. 3 Figure 4B) as compared with the OGD/R group and negative control group(mn + OGD/R), while inhibition of miR-423-5p(si + OGD/R) played the opposite role. It demonstrated that OGD/R facilitated the migration of HUVECs in vitro while miR-423-5p could strengthen this effect.
**Function of miR-423-5p in HUVECs tube-forming activity**

Experimental results showed that HUVECs which experienced OGD/R have enhanced tubular formation, as indicated by increased total branching length and number of nodes, as compared with control group under normoxia. In addition, HUVECs transfected with miR-423-5p mimic before subjected to OGD/R were found to have stronger tube forming ability when compared with the OGD/R group and negative control group, in contrast, inhibition of miR-423-5p played the opposite role (Fig. 5).

**LHX6 is a target gene of miR-423-5p**

We used the TargetScanHuman 7.2 (https://www.targetscan.org/vert_72/) and found that LHX6 was a target gene, whose 3’UTR region can be matched with miR-423-5p. Compared to the WT-LHX6-3’UTR + NC group, cotransfection of WT-LHX6-3’UTR with miR-423-5p mimic could reduce the relative activity of luciferase significantly (p < 0.05). However, there was no difference for luciferase activity between the MUT-LHX6-3’UTR + NC group and MUT-LHX6-3’UTR + mimic group (all P > 0.05) (Fig. 6). All of the above suggested that LHX6 was the target gene of miR-423-5p. We used qRT-PCR and western blot(Figure 7)to detect the expression content of LHX6 among the four groups which were consist of control group OGD/R group m + OGD/R group and mn + OGD/R group. Then we discovered that OGD/R led to a decreased expression level of LHX6, what’s more, the mRNA or protein expressions of LHX6 further declined when HUVECs transfected with miR-423-5p mimic before subjected to OGD/R(m + OGD/R group).

**Discussion**

MiRNAs are involved in a variety of pathophysiological processes after cerebral I/R injury (Todoran et al. 2023). During these processes, angiogenesis could play a crucial role. As important regulators of angiogenesis, the role of miRNAs has been confirmed by many previous studies. Such as, miR-191 could inhibit angiogenesis via targeting VEZF1 to promote ischemic brain injury (Du et al. 2019). Inhibiting the expression of miR-103 might induce angiogenesis of ischemic stroke and consequently reduce infarction volume by targeting VEGF (Shi et al. 2018). Here we successfully used OGD/R-cultured HUVECs to construct model in vitro. And this model has been used widely to mimic ischemic injury. This study evaluated the change of miR-423-5p in patients with ischemic stroke and HUVECs model of OGD and the interaction between miR-423-5p and LHX6. MiR-423-5p was downregulated in the plasma of ischemic stroke patients together with in OGD/R-exposed HUVECs. That is to say the two variation tendency are consisted with each other. Over-expression of miR-423-5p promoted migration and tube-forming in HUVECs subjected to OGD/R, while inhibition of miR-423-5p displayed opposite results. Mechanistically, miR-423-5p played the role in promoting angiogenesis by targeting LHX6 which is a tumor suppressor (Nathalia et al. 2018). Therefore, miR-423-5p might be a hot topic for the treatment of ischemic stroke.

Angiogenesis is a vital process in which new blood vessels rebuild from original vascular network through endothelial cells remodeling and proliferating (Hatakeyama et al. 2020). Fen Xu et al demonstrated that miR-423-5p played a vital role in promoting angiogenesis by targeting sufu but no
specific disease was involved (Xu et al. 2019). Though, several researches have reported that miR-423-5p has important roles in cancers and cardiovascular disease, the role it plays in ischemic stroke is rarely known. In our study, we screened out some differentially expressed miRNAs within the plasma of ischemic stroke patients and normal ones, and miR-423-5p was chose as the research object finally. Then we carried out a variety of assays related to angiogenesis consisted of scratch assay, transwell assay and tube formation assay and found that miR-423-5p was a promoter of angiogenesis in HUVECs. Enhancing the expression of miR-423-5p could promote proliferation, migration, and tube formation in HUVECs. Not only did overexpression of miR-423-5p promote HUVECs proliferation and migration, but it also could act as a tumor promoter through various mechanisms and promote tumorigenicity (Li et al. 2020). However, a recent study have demonstrated that suppressing the expression of miR-423-5p could improve ischemic stroke prognosis, specifically, it could reduce cerebral infarct volume and nerve tissue damage (Luo et al. 2022). These results seemed to be inconsistent with ours, however they didn't conducted assays in vitro and no angiogenesis related experiments were performed. It still needs further studies to explore the function of miR-423-5p in ischemic stroke.

As reported, LHX6 is remarkably conserved and has significant relationship with morphogenesis of multiple organs such as palate development (Cesario et al. 2015). LHX6 acts as a transcription factor which can be regulated by many other transcription factors such as fibroblast growth factors wingless-int(Wnt)/β-catenin and so on (Yang et al. 2017). In turn, these transcription factors can also be regulated by LHX6. What calls for special attention is that LHX6 could inhibit some tumor cells growth and invasion by suppressing the Wnt/β-catenin signaling pathway which plays a vital role in promoting angiogenesis after cerebral I/R injury (Chen et al. 2019; Khamchai et al. 2022). Therefore, it is reasonable that LHX6 down-regulation could induce angiogenesis through activating some special pathway such as Wnt/β-catenin signaling pathway. Our research suggested that LHX6 was a substrate of miR-423-5p at post-translational level. LHX6 was proved to be a target of miR-423-5p by Luciferase reporter assay. However, deeper studies are needed to explore the specific signaling pathways downstream of LHX6 after miR-423-5p interference.

In conclusion, this study indicates that miR-423-5p could promote angiogenesis refer to brain I/R damage in vitro by targeting LHX6. MiR-423-5p might be used as a effective therapeutic target for promoting angiogenesis after stroke.

**Abbreviations**

HUVEC, Human Umbilical Vein Endothelial Cell; OGD/R, oxygen–glucose deprivation and reperfusion; I/R, ischemia/ reperfusion; LHX6, Lim Homeobox 6.

**Declarations**

**Author Contributions**
Wenjing Deng and Junfang Teng designed the research, interpreted the data, and contributed to revising the manuscript. Di Jin performed the research, and analyzed the data, and wrote the manuscript. Guoliang Xiang and Yanan Zhao contributed to recruitment of patients and clinical diagnosis of disease.

Acknowledgments

We would like to acknowledge center for translational medicine of the first affiliated hospital of Zhengzhou university for their supply of instruments.

Conflicts of interest

The authors declare that they have no conflict of interest.

Funding

This work was financially supported by grants from the National Natural Science Foundation of China(8210051959) and the Natural Science Foundation of Henan Province(212300410262).

References


8. Li J, Yang C, Wang Y (2021) miR-126 overexpression attenuates oxygen-glucose deprivation/reperfusion injury by inhibiting oxidative stress and inflammatory response via the


Figures

Figure 1

Expression of miR-423-5p in HUVECs

**A** The expression of miR-423-5p in OGD/R group decreased significantly compared with control group. **B–D** MiR-423-5p relative expression in HUVECs that were transfected with miR-423-5p inhibitor(B) or miR-423-5p mimic(D) as assessed by RT-qPCR. **C–E** After transfection with inhibitor(C) or mimic(E), the HUVECs were induced to OGD for 6 hours and recovery for 12 hours compared to control. Means ± SEM. ### and #### or **** indicate significant differences at P 0.0002 and P 0.0001, respectively.
Figure 2

The effect of miR-423-5p on HUVECs migration was determined by scratch wound. Contrasting images taken with a microscope of HUVECs at 0h and 12h after scratching of six groups represented the control group, OGD/R group, m+OGD/R group transfected with miR-423-5p mimic following by OGD/R, mn+OGD/R group transfected with miR-423-5p mimic negative control following by OGD/R, si+OGD/R group transfected with miR-423-5p inhibitor following by OGD/R and sn+OGD/R group transfected with miR-423-5p inhibitor negative control following by OGD/R, respectively. White lines indicate the wound area.
Figure 3

Transwell assay was performed to prove the effect of miR-423-5p on HUVECs’ transfer ability. Pictures of HUVECs moved and adhered to the bottom membrane of a transwell chamber. Specific grouping was same to scratch wound healing assay.

![Transwell assay images](Image)

Figure 4

The migration rate in scratch assay and migrated cells in transwell assay within different groups

![Graph images](Image)
Means ± SEM. ### and #### or **** indicate significant differences at P 0.0002 and P 0.0001 respectively.

Figure 5

The tube formation ability of HUVECs was evaluated by the the vessel-like structure formation assay. The migration number of nodes and total branching length are two important indicators we observed. Means ± SEM. # and ### indicate significant differences at P 0.05 and P 0.0002 respectively. ** indicates significant differences at p <0.002.
Figure 6

LHX6 was proved to be the target gene of miR-423-5p by luciferase reporter gene assay

A Possible binding sites between miR-423-5p and the coding sequence region of LHX6 3’UTR B Detection of relative luciferase activity after cotransfection of MUT/WT-LHX6-3’UTR plasmids with miR-423-5p mimic or NC; ****, \( P < 0.0001 \) compared with NC group.

### A

<table>
<thead>
<tr>
<th>hsa-miR-423-5p</th>
<th>3’....UUUCAGAGCGAGAGACGGGGAGU...5’</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt-LHX6-3’UTR</td>
<td>5’....GGGCGCCCCCAACAGCUGCCCCUCUCA... 3’</td>
</tr>
<tr>
<td>mut-LHX6-3’UTR</td>
<td>5’....GGGCGCCCCCAACAGCUCACAUAA... 3’</td>
</tr>
</tbody>
</table>
Figure 7

A RT-qPCR was used to discover the expression difference of LHX6 mRNA between different groups. B Intuitive images show the expression of LHX6 protein in every group by western blot. C Expression of LHX6 protein of each group in the form of bar charts. *, $P<0.05$ compared with the normal control group; #, $P<0.05$ compared with the OGD group.