

miR-152-3p aggravates vascular endothelial cell dysfunction by targeting DEAD-box helicase 6 (DDX6) under hypoxia

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ABSTRACT

Stroke is a main cause of disability and death worldwide, and ischemic stroke accounts for most stroke cases. Recently, microRNAs (miRNAs) have been verified to play critical roles in the development of stroke. Herein, we explored effects of miR-152-3p on vascular endothelial cell functions under hypoxia. Human umbilical vein endothelial cells (HUVECs) were treated with hypoxia to mimic cell injury *in vitro*. Reverse transcription quantitative polymerase chain reaction revealed that miR-152-3p exhibited high expression in HUVECs treated with hypoxia. The inhibition of miR-152-3p reversed hypoxia-induced decrease in cell viability and the increase in angiogenesis, according to the results of cell counting kit-8 assays and tube formation assays. miR-152-3p inhibition reversed the increase in endothelial cell permeability mediated by hypoxia, as shown by endothelial cell permeability *in vitro* assays. In addition, the increase in protein levels of angiogenic markers and the decrease in levels of tight junction proteins induced by hypoxia were reversed by miR-152-3p inhibition. Mechanistically, miR-152-3p directly targets 3'-untranslated region of DEAD-box helicase 6 (DDX6), which was confirmed by luciferase reporter assays. DDX6 is lowly expressed in HUVECs under hypoxic condition, and mRNA expression and protein level of DDX6 were upregulated in HUVECs due to miR-152-3p inhibition. Rescue assays showed that DDX6 knockdown reversed effects of miR-152-3p on cell viability, angiogenesis and endothelial permeability. The results demonstrated that miR-152-3p aggravates vascular endothelial cell dysfunction by targeting DDX6 under hypoxia.

ARTICLE HISTORY

Received 12 May 2021

Revised 19 July 2021

Accepted 20 July 2021

KEYWORDS

Mir-152-3p; ddx6; hypoxia; angiogenesis; endothelial cell permeability

Introduction

Stroke is the second cause of death and the primary cause of disability in adults worldwide [1]. Stroke is characterized by the failure of blood flow to complete normal cerebral circulation and thus results in oxidative stress and inflammatory injury to brain tissues [2]. Stroke can be classified into ischemic stroke and hemorrhagic stroke [3]. Ischemic stroke, as the most common type of stroke, is responsible for 80% of all stroke cases [4], which is resulted from the lack of blood flow that contains oxygen and nutrients [5].

The decrease in blood supply after stroke can activate angiogenesis, which is a compensation for the lack of oxygen and is necessary for recovering neurovascular injuries [6]. Previous studies have verified that angiogenesis exerts critical functions

in improving neurological functional recovery after stroke [7,8].

Endothelial function is essential to maintain normal brain function, and the endothelium of brain vasculature serves as blood-brain barrier (BBB). The failure of BBB can be characterized by levels of tight junction proteins such as zonula occludens-1 (ZO-1) and occludin are downregulated due to ischemia [9–11]. Therefore, the decrease in ZO-1 and occludin protein levels can be deemed as a biomarker of endothelial dysfunction [12].

Mature microRNAs (miRNAs) are transcripts containing approximately 18–22 nucleotides in length [13]. miRNAs can regulate the expression of downstream messenger RNAs (mRNAs) post-transcriptionally, which are involved in the

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 Supplemental data for this article can be accessed [here](#)

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development of individuals or diseases [13,14]. Specifically, miRNAs bind with 3'-untranslated regions (3'-UTR) of mRNAs to accelerate the degradation of mRNAs [15]. Emerging evidence has revealed that miRNAs are significant regulators of endothelial cell functions [16,17]. For example, miR-124-3p overexpression was discovered to suppress the proliferation and migration of HUVECs as well as angiogenesis *in vitro* [18]. miR-182 promotes BBB failure by exacerbating endothelial cell apoptosis via the mTOR/forkhead box O1 (FOXO1) pathway during cerebral ischemia [19]. miR-874-3p overexpression inhibits C-X-C motif chemokine ligand 12 (CXCL12) expression to promote angiogenesis in HUVECs [20]. Herein, we intended to explore effects of miR-152-3p on functions of vascular endothelial cells under hypoxia. According to previous investigation, miR-152-3p overexpression promotes the proliferation of mammary epithelial cells [21]. In addition, miR-152-3p has been verified to show low expression in the brain tissues of mice treated with ischemic/reperfusion [22] and in patients with acute ischemic stroke [23].

We herein aimed to explore the effects of miR-152-3p on vascular endothelial cell viability and permeability as well as angiogenesis *in vitro*. In addition, the miRNA-target network in HUVECs was also under investigation. We hypothesized that miR-152-3p may affect vascular endothelial cell dysfunction by targeting certain downstream mRNA. The study may provide novel insight into the role of miRNAs in vascular endothelial cells.

Materials and methods

Bioinformatics analysis

The mRNAs containing binding site with miR-152-3p were predicted using the starBase (<http://starbase.sysu.edu.cn/>) [24]. Six mRNAs (DDX6, PNPLA6, ATP2A2, QKI, SLC25A44, GADD45A) were identified under the condition of Cross Linking and Immunoprecipitation-sequence (CLIP) Data: strict stringency (≥ 5); Degradome Data: high stringency (≥ 3); Pan-Cancer: 2 cancer types; Program Number: 1 program; and Predicted Program: microT, miRanda, miRmap, PITA, PicTar and TargetScan (supplementary Table 1).

Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in endothelial cell growth medium (ECGM; Thermo Fisher, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher) in a humidified atmosphere containing 21% O₂, 5% CO₂ at 37°C. For hypoxia treatment, cells were incubated in a hypoxic incubator containing 1% O₂, 5% CO₂ and 94% N₂ at 37°C for 6 or 24 hours [25,26].

Cell transfection

The plasmid miR-152-3p inhibitor was employed to suppress miR-152-3p expression, and vectors containing short hairpin RNAs against DDX6 (sh-DDX6) were utilized to knockdown DDX6 expression in cells. The above plasmids and vectors as well as their correspondent negative controls (NC inhibitor and sh-NC) were purchased from GenePharma (Shanghai, China). For cell transfection, Lipofectamine 2000 was utilized based on manufacturer's recommendations [27]. Transfection efficiency was determined utilizing RT-qPCR after 2 days.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA in HUVECs was extracted utilizing TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Reverse Transcription Kits (Promega, Madison, WI, USA) were used for the reverse transcription of RNA into cDNAs. Afterward, a SYBR-Green qPCR Master Mix Kit (Takara, Tokyo, Japan) was applied to perform the quantitative PCR on ABI biosystems (Foster City, CA, USA). RNA levels were calculated by the $2^{-\Delta\Delta C_t}$ method [28]. GAPDH was an internal control for candidate mRNAs while U6 snRNA was a control for miR-152-3p. Sequences of primers are listed in Table 1.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 (Dojindo, Kumamoto, Japan) was employed to measure the viability of HUVECs treated with hypoxia. Approximately 5×10^3 cells

Table 1. Sequences of primers used for RT-qPCR.

Gene	Sequence (5'→3')
miR-152-3p forward	TCAGTGCATGACAGAACTTGG
miR-152-3p reverse	CTCTACAGCTATATTGCCAGCCA
DDX6 forward	TTGCTAGCCAAGAAGATTCTC
DDX6 reverse	ACGATTTTCGATGTTCTCTGC
ATP2A2 forward	GTATGGCAGGAAAGAAATGCT
ATP2A2 reverse	CTGTCGATACACTTTGCC
QKI forward	TTTCTGTGGACGCCTAGAG
QKI reverse	TTCCGTACTCTGCTAATTCTG
SLC25A44 forward	TTCTATGCAGGTTGAGGGC
SLC25A44 reverse	AGATGATTCTGGCCGAGAG
GADD45A forward	AACGACATCAACATCCTGC
GADD45A reverse	AATGTGGATTCTGCACCAG
GAPDH forward	CCTCCTGTTTCGACAGTCAG
GAPDH reverse	CATACGACTGCAAAGACCC
U6 forward	CTTGGCAGCACATATACCA
U6 reverse	CTCATTGAGGGCCATGCT

were seeded in 96-well plate. After transfection, culture medium was replaced by 100 μ L ECGM containing 10 μ L CCK-8 reagent for 2 hours of incubation at 37°C. Finally, cells were observed at 570 nm wavelength on a Microplate Reader (Bio-Rad, Hercules, CA, USA) [29].

Tube formation assay

HUVECs (3×10^4 cells/well) were added with endothelial cell matrix gel (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's recommendations. Next, cells were added in triplicate to 96-well sterile plates at 37°C for 16 hours. The number of meshes and the length of branches were analyzed using light microscopy [25].

Western blot analysis

Western blot analysis was conducted according to the previous report [30]. HUVECs were homogenized in RIPA lysis buffer (Beyotime, Shanghai, China) to extract total proteins. The protein concentration was determined by BCA Protein Assay Kits. After the protein contents (50 μ g) were separated by 10% SDS-PAGE, they were transferred to PVDF membranes (Millipore, Billerica, MA, USA) for 1 hour of blocking with 5% skim milk at room temperature. Then, the membranes were incubated with primary antibodies (Abcam, Cambridge, UK) at 4°C overnight. Afterward, the membranes were washed and then incubated with secondary antibodies (Abcam) for 1 hour. The protein bands were visualized by

enhanced chemiluminescent detection system (Thermo Fisher). GAPDH was set as an internal control. The primary antibodies were against vascular endothelial growth factor A (VEGFA; ab46154; 1:1000), angiopoietin 2 (ANGII; ab155106; 1:5000), ZO-1 (ab276131; 1:1000), occludin (ab242202; 1:1000) and GAPDH (ab9485; 1:2500).

Endothelial cell permeability *in vitro* assay

The assay was conducted according to the previous study [9]. The transwell method was employed to assess endothelial cell permeability. HUVECs were seeded in the upper chamber under hypoxia. The fluorescein isothiocyanate (FITC)-dextran molecules migrating to the lower chamber from the upper chamber through a 3 μ m pore size filter were collected. At last, 96-well plate fluorescence reader was employed to measure the fluorescence intensity of these molecules at the wavelength of 495 nm.

Luciferase reporter assay

The binding site between miR-152-3p and DDX6 was predicted from the starBase. Wild-type (WT) and mutated (MUT) fragments of DDX6 3'UTR containing potential binding site with miR-152-3p were synthesized and respectively subcloned into pmirGLO vectors (Promega) to construct pmirGLO-DDX6-WT/MUT reporters. Phusion Site-Directed Mutagenesis Kits (Thermo Fisher) were applied to mutate the predicted binding site. The above reporters were cotransfected with miR-152-3p inhibitor or NC inhibitor into HUVECs using Lipofectamine 3000 reagent (Invitrogen). The luciferase activities of DDX6-WT/MUT were determined utilizing Dual-Glo[®] Luciferase Assay System Kits (Promega) after 48 hours of cotransfection [31].

Statistical analysis

Statistical Product and Service Solutions (SPSS) software (Chicago, IL, USA) was utilized for statistical analysis [32]. All assays were performed three times and data were shown as the mean \pm standard deviation. Student's *t* test or one-way analysis of variance followed by Tukey's *post hoc*

test were employed to analyze differences among two or more groups. Statistically significant was defined as $p < 0.05$.

Results

miR-152-3p was reported to be low-expressed in the hippocampus of ischemia/reperfusion mice according to the previous study. However, the function and mechanism of miR-152-3p have not been explored yet. The study aimed to investigate the effects of miR-152-3p on endothelial cell viability, permeability and angiogenesis. We hypothesized that miR-152-3p might affect endothelial functions of HUVECs by targeting a downstream mRNA. The results indicated that miR-152-3p aggravates endothelial cell permeability and angiogenesis by targeting DDX6 under hypoxia.

Inhibition of miR-152-3p mitigates hypoxia-induced angiogenesis in HUVECs

The expression of miR-152-3p in HUVECs under normoxia and hypoxia, and its effects on cell viability, tube formation and endothelial cell permeability were investigated. RT-qPCR exhibited that miR-152-3p expression was significantly ($***p < 0.001$) increased in HUVECs under hypoxia (Figure 1a). Next, we suppressed miR-152-3p expression by transfection of miR-152-3p inhibitor into HUVECs under hypoxia to conduct loss-of-function assays. As shown by RT-qPCR, miR-152-3p expression was successfully ($***p < 0.001$) decreased in cells treated with hypoxia (Figure 1b). CCK-8 assays illustrated that miR-152-3p inhibition partially ($**p < 0.01$) reversed the decrease ($***p < 0.001$) in cell viability mediated by hypoxia (Figure 1c). Tube formation assays were performed to evaluate angiogenesis *in vitro*. As a result, miR-152-3p inhibition significantly ($**p < 0.01$) reversed hypoxia-induced increase ($***p < 0.001$) in tube formation ability of HUVECs (Figure 1d). According to western blot analysis, miR-152-3p inhibition markedly ($***p < 0.001$) reversed hypoxia-mediated upregulation ($***p < 0.001$) of vascular endothelial growth factor A (VEGFA) and angiopoietin-2 (ANGII) protein levels in cells (Figure 1e).

Overall, miR-152-3p was high-expressed in HUVECs under hypoxia. Inhibition of miR-152-3p reverses hypoxia-induced decrease in cell viability and hypoxia-activated tube formation of HUVECs.

Inhibition of miR-152-3p alleviates hypoxia-induced endothelial permeability of HUVECs

Western blot analysis and endothelial cell permeability *in vitro* assays were conducted to measure endothelial cell permeability. As shown by western blot analysis, miR-152-3p inhibitor partially ($*p < 0.05$) reversed hypoxia-mediated decrease ($***p < 0.001$) in ZO-1 and occludin protein levels in cells (Figure 2a). Endothelial cell permeability *in vitro* assays revealed that miR-152-3p inhibitor partially ($**p < 0.01$) reversed the promotion ($***p < 0.001$) of endothelial permeability induced by hypoxia (Figure 2b). The results suggested that inhibition of miR-152-3p alleviates endothelial permeability promoted by hypoxia.

DDX6 is targeted by miR-152-3p

Subsequently, we hypothesized that miR-152-3p might contribute to endothelial cell dysfunction due to the miRNA-target mechanism. To verify the hypothesis, bioinformatics analysis was performed to seek for possible mRNAs possessing binding capacity for miR-152-3p. Six mRNAs (DDX6, NPLA6, ATP2A2, QKI, SLC25A44 and GADD45A) binding to miR-152-3p were predicted utilizing the starBase (supplementary Table 1). RT-qPCR was employed to assess expression levels of these candidate genes in HUVECs with transfection of miR-152-3p inhibitor or NC inhibitor. Compared with the expression of other genes, DDX6 expression was significantly ($***p < 0.001$) upregulated by miR-152-3p inhibitor in HUVEC (Figure 3a). Next, we probed the effects of silencing miR-152-3p on DDX6 protein level in cells. As suggested by western blot analysis, miR-152-3p inhibitor markedly ($***p < 0.001$) upregulated the protein level of DDX6 in HUVECs (Figure 3b). Afterward, we explored the interaction between miR-152-3p and DDX6. The potential binding site between miR-152-3p and

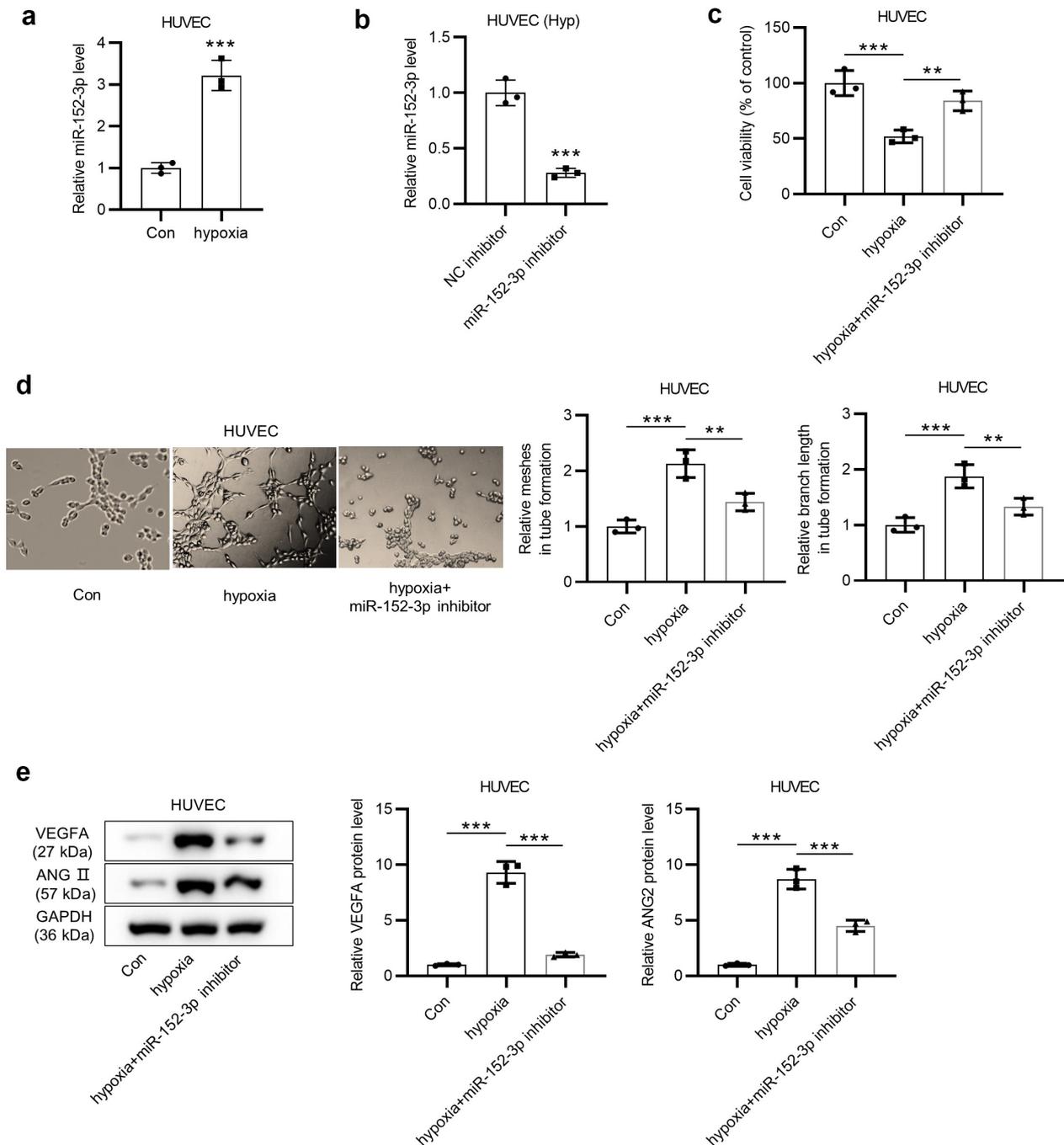


Figure 1. Inhibition of miR-152-3p mitigates hypoxia-induced angiogenesis in HUVECs. (a) miR-152-3p expression in HUVECs under hypoxia was examined by RT-qPCR in thrice. (b) The efficiency of miR-152-3p downregulation was measured by RT-qPCR in triplicate. (c) CCK-8 assays were conducted to detect the viability of HUVECs transfected with miR-152-3p inhibitor under hypoxia. each experiment was repeated three times. (d) Tube formation assays were applied to determine effects of miR-152-3p inhibitor on angiogenesis. the experiment was conducted in triplicate. (e) Western blot analyses were conducted three times to examine levels of angiogenesis-associated proteins (VEGFA and ANGII). ** $p < 0.01$, *** $p < 0.001$.

DDX6 was predicted with the starBase, and the sequence of DDX6 was mutated. Luciferase reporter assays revealed that the luciferase activity of DDX6-WT was markedly (** $p < 0.01$) increased by

miR-152-3p inhibitor while no significant changes were examined in that of DDX6-MUT (Figure 3c). Additionally, RT-qPCR analysis suggested that DDX6 expression was significantly (***) $p < 0.001$

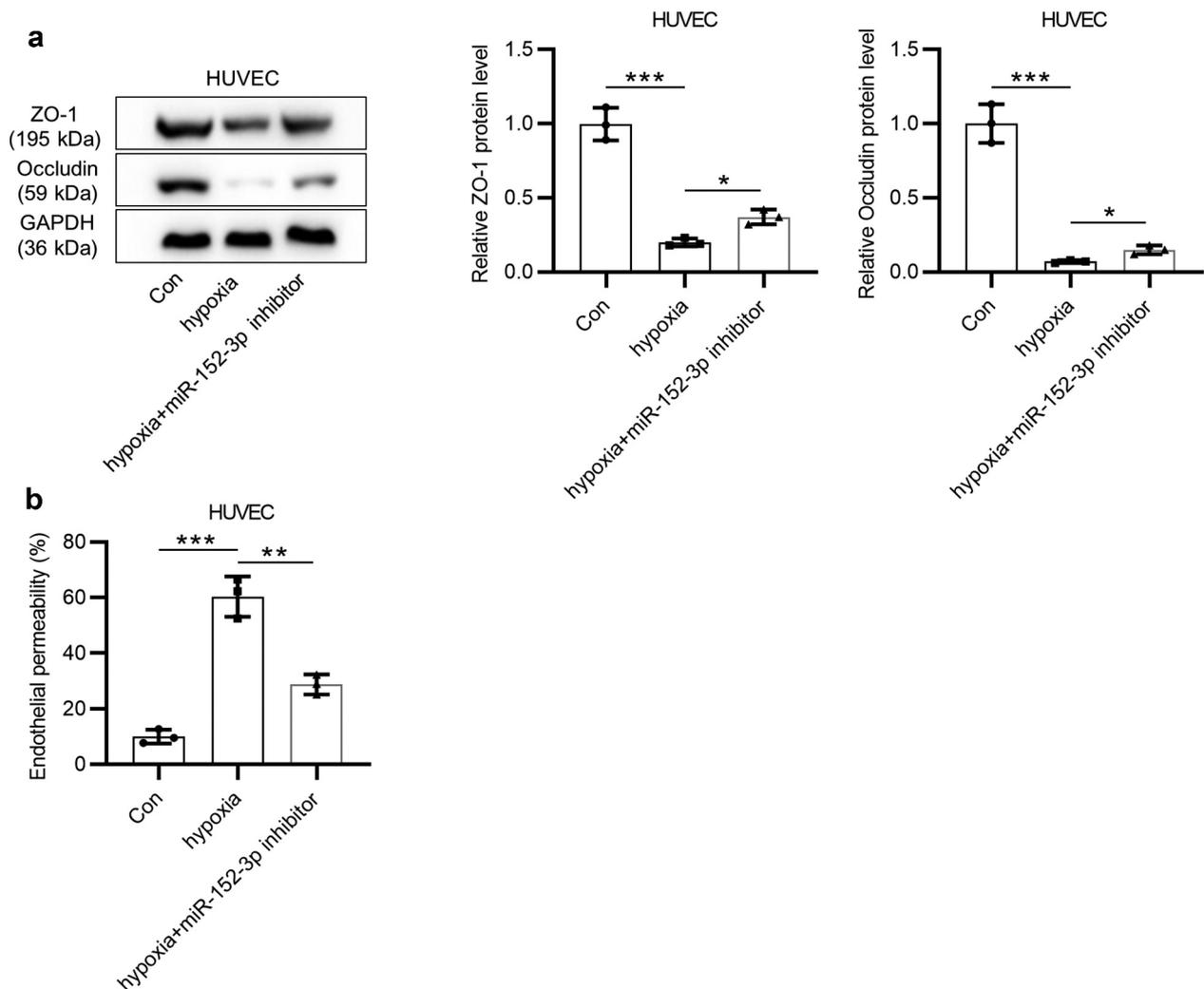


Figure 2. Inhibition of miR-152-3p alleviates hypoxia-induced endothelial permeability of HUVECs. (a) Western blot analysis was employed to examine levels of tight junction proteins (ZO-1 and occludin) in HUVECs under hypoxia. Each experiment was performed in triplicate. (b) Endothelial permeability of indicated cells was detected utilizing endothelial cell permeability *in vitro* assays. The assay was performed in thrice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

downregulated in HUVECs treated with hypoxia (Figure 3d). In conclusion, miR-152-3p targets DDX6 and negatively modulates DDX6 expression in HUVECs.

miR-152-3p promotes tube formation of HUVECs by targeting DDX6

To validate whether miR-152-3p suppresses cell viability while accelerates tube formation under hypoxia by targeting DDX6, rescues assays were carried out in HUVECs transfected with miR-152-3p inhibitor, NC inhibitor or co-transfected with sh-DDX6 and miR-152-3p inhibitor under hypoxia. First, the knockdown efficiency of

DDX6 expression was detected utilizing RT-qPCR analysis, which suggested a significant (** $p < 0.001$) decrease in DDX6 expression in cells (Figure 4a). CCK-8 assays revealed that DDX6 knockdown partially (** $p < 0.01$) rescued the decrease (** $p < 0.001$) in cell viability induced by miR-152-3p inhibition (Figure 4b). As shown in tube formation assays, DDX6 silencing partially (** $p < 0.01$) rescued the suppressive effect (** $p < 0.001$) of miR-152-3p inhibitor on tube formation in cells (Figure 4c). The protein levels of VEGFA and ANGII were greatly (** $p < 0.001$) downregulated by miR-152-3p inhibition and then partially (* $p < 0.05$) reversed by DDX6 knockdown in cells (Figure 4d). The above results verified that

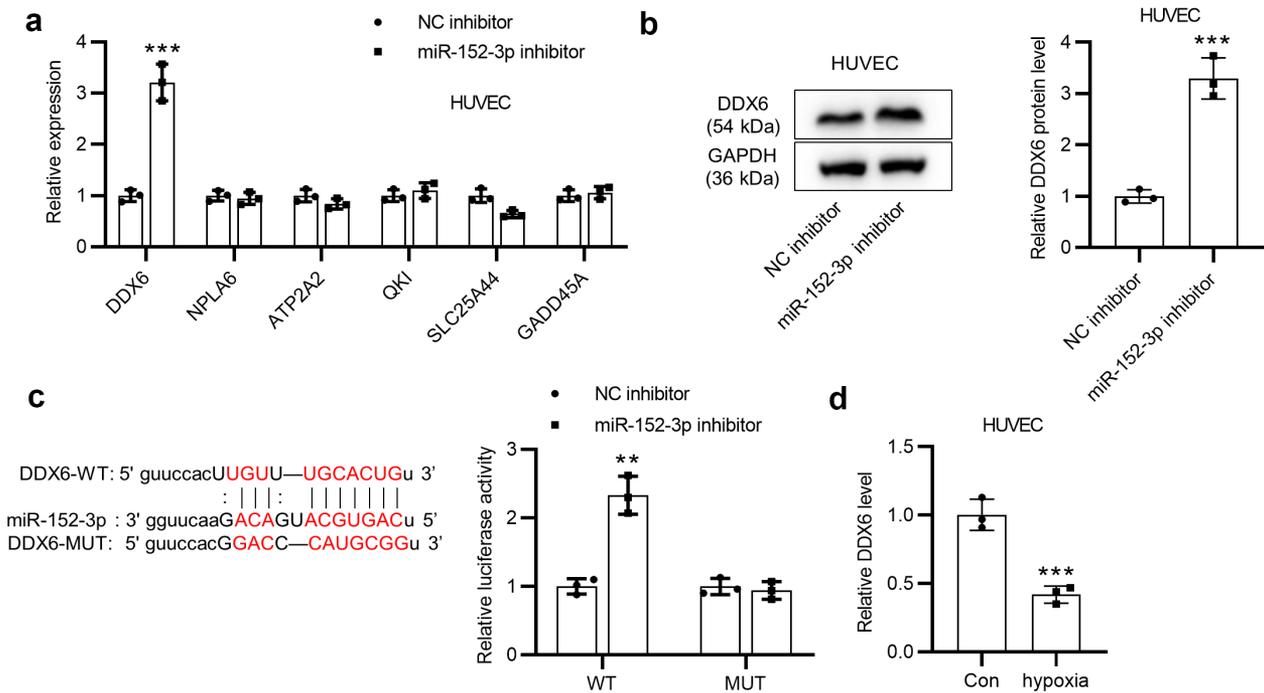


Figure 3. DDX6 is targeted by miR-152-3p. (a) Expression levels of candidate mRNAs in HUVECs transfected with miR-152-3p inhibitor or NC inhibitor were examined utilizing RT-qPCR in thrice. (b) Western blot analyses were conducted three times to measure effects of miR-152-3p inhibitor on the protein level of DDX6 in cells. (c) The possible binding site between miR-152-3p and DDX6 was predicted from the starBase, and luciferase reporter assays were performed in thrice to explore the interaction between miR-152-3p and DDX6. (d) DDX6 expression in HUVECs under hypoxia was examined utilizing RT-qPCR analysis in triplicate. ** $p < 0.01$, *** $p < 0.001$.

miR-152-3p inhibits cell viability while promotes angiogenesis under hypoxia by targeting DDX6.

miR-152-3p facilitates endothelial permeability of HUVECs by downregulating DDX6 expression

Western blot analyses and endothelial cell permeability *in vitro* assays were performed to verify whether miR-152-3p accelerates endothelial cell permeability by targeting DDX6. Protein levels of tight junction markers (ZO-1 and occludin) in HUVECs under hypoxia were examined by western blot. DDX6 depletion partially ($*p < 0.05$) reversed the upregulation ($***p < 0.001$) of ZO-1 and occludin protein levels mediated by miR-152-3p inhibition in cells (Figure 5a). According to endothelial cell permeability *in vitro* assays, DDX6 knockdown partially ($***p < 0.001$) rescued the inhibitory effect ($***p < 0.001$) of miR-152-3p inhibition on endothelial cell permeability (Figure 5b). Overall, miR-152-3p facilitates endothelial permeability of HUVECs by downregulating DDX6 expression.

Discussion

Stroke is a deadly disease that is threatening lives worldwide [33]. Endothelial permeability, inflammation and oxidative stress are factors influencing the outcome of stroke, and angiogenesis is critical to recover blood supply after stroke [34,35]. MicroRNAs (miRNAs) are deemed as diagnostic and therapeutic biomarkers for stroke [36], and its regulation of endothelial cell functions was extensively reported [37–39]. Previously, miR-152-3p was found to be low-expressed in the hippocampus of ischemia/reperfusion and postconditioning mice [22]. In addition, decreased miR-152-3p was detected in patients who suffered acute ischemic stroke with National Institute of Health stroke scale (NIHSS) score ≥ 7 , suggesting that decreased miR-152-3p is related to the severity of endothelial injury [23]. Moreover, miR-152-3p is associated with detrimental microvascular functions in oxygen-induced retinopathy [40].

Herein, we discovered that miR-152-3p exhibited high expression in HUVECs under hypoxia. Loss-of-function assays revealed that miR-152-3p

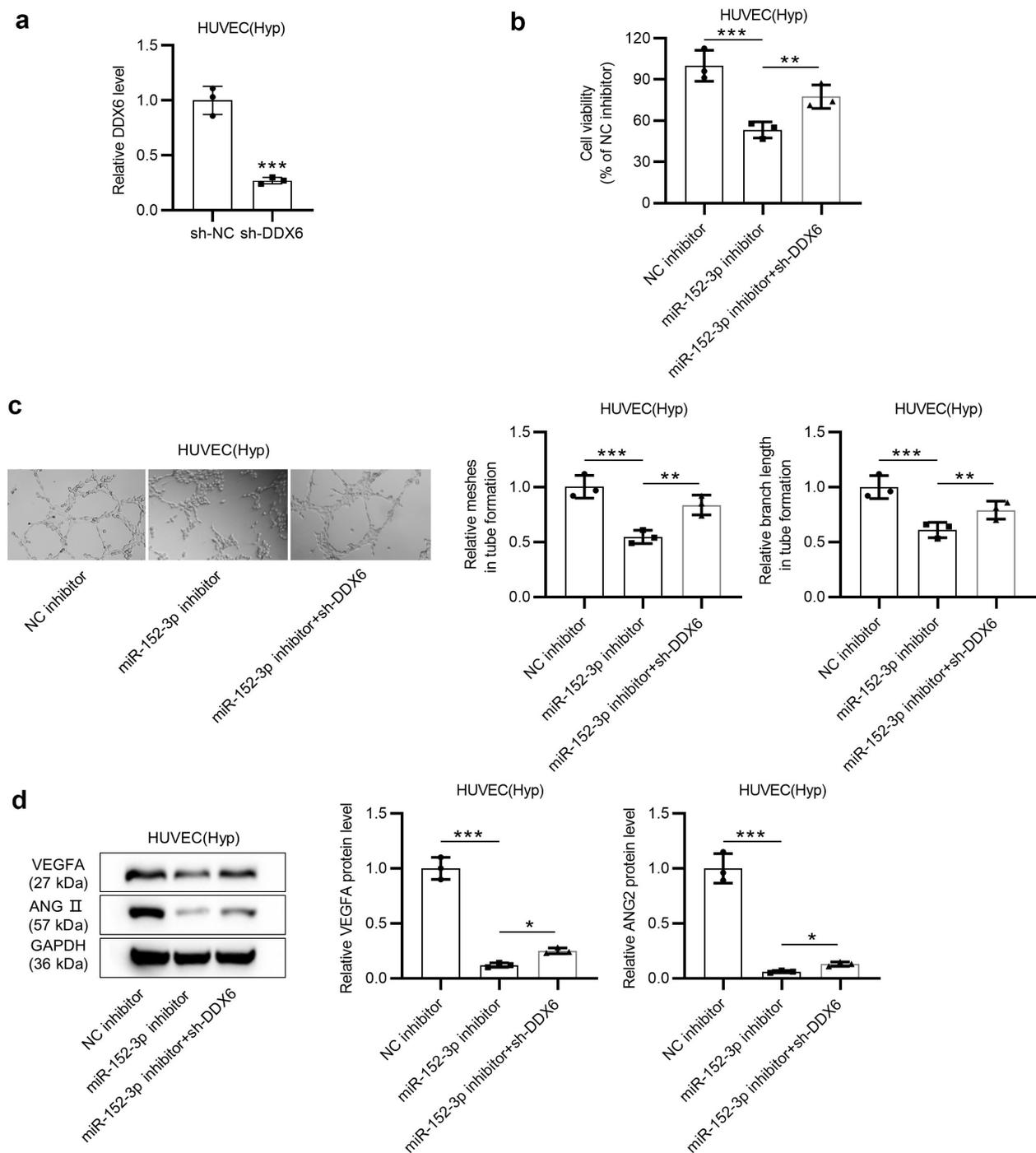


Figure 4. miR-152-3p promotes tube formation of HUVECs by targeting DDX6. (a) The knockdown efficiency of DDX6 in HUVECs under hypoxia was detected by RT-qPCR in thrice. (b) CCK-8 assays were carried out three times to probe effects of miR-152-3p inhibition and DDX6 silencing on cell viability. (c) The angiogenesis in cells with different transfection was measured by tube formation assays. Each experiment was repeated in triplicate. (d) Western blot analyses were employed to probe protein levels of angiogenesis-associated factors in cells with transfection of miR-152-3p inhibitor and sh-DDX6. Each analysis was performed three times. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

inhibition reversed hypoxia-mediated increase in endothelial cell permeability and angiogenesis.

Angiogenesis is a compensatory response to the reduction of oxygen, which contributes to the

restoration of blood flow and thereby promotes the recovery of ischemic stroke [7]. Vascular endothelial growth factor A (VEGFA) and angiotensin 2 (ANGII) are essential factors related to

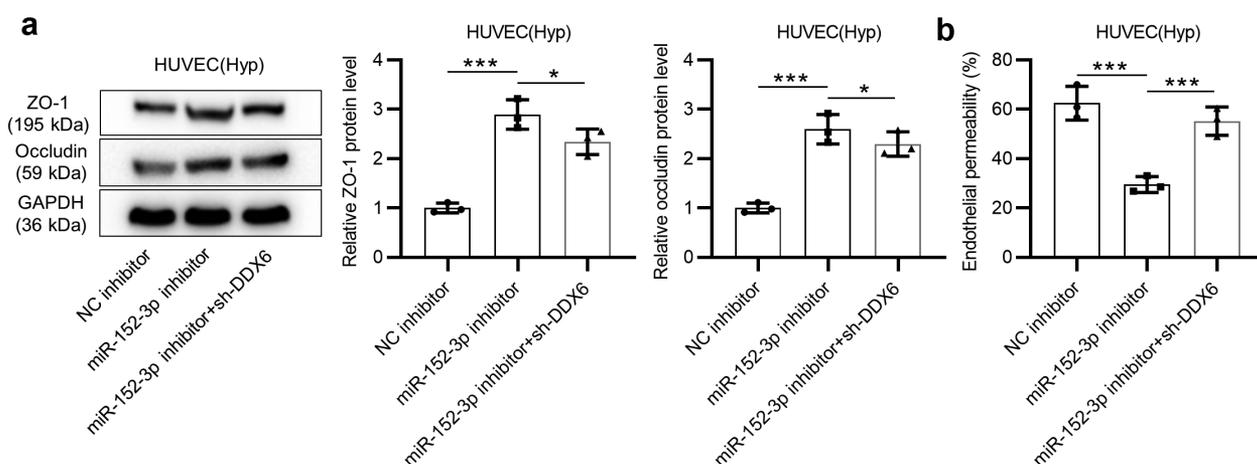


Figure 5. miR-152-3p facilitates endothelial permeability of HUVECs by downregulating DDX6 expression. (a) Protein levels of ZO-1 and occludin in cells with transfection of NC inhibitor, miR-152-3p inhibitor or miR-152-3p inhibitor + sh-DDX6 were examined by western blot analyses. each analysis was performed in thrice. (b) The rescue effect of DDX6 on the decrease in cell permeability induced by miR-152-3p inhibition was probed utilizing endothelial cell permeability *in vitro* assays. Each experiment was performed in thrice. * $p < 0.05$, *** $p < 0.001$.

angiogenesis [41–43]. In our study, VEGFA and ANGII protein levels in cells and tube formation ability of HUVECs were significantly upregulated by hypoxia while miR-152-3p inhibition reversed the increase, suggesting that miR-152-3p promotes angiogenesis under hypoxia.

Neurological functions can be damaged by the increase in endothelial permeability [35]. Tight junctions existing between cerebral endothelial cells form a diffusion barrier that prevents most blood-borne substances from entering the brain [44]. The barrier is known as blood-brain-barrier (BBB). As a selectively permeable cellular monolayer, the BBB affects the homeostasis within the brain [34,45]. Tight junction proteins such as occludin and ZO-1 are strongly related with BBB stability [46]. Herein, we found that miR-152-3p inhibition rescued hypoxia-induced decrease in ZO-1 and occludin expression in HUVECs. Moreover, endothelial cell permeability *in vitro* assays suggested that inhibiting miR-152-3p expression reversed the promotion of endothelial permeability mediated by hypoxia. The results suggest that miR-152-3p promotes endothelial cell permeability under hypoxia.

Mechanistically, miRNAs regulate mRNA expression post-transcriptionally by binding to 3'UTR of mRNAs [47]. In previous studies, miR-152-3p was discovered to modulate cell

proliferation and invasion by targeting forkhead box F1 (FOXF1) in keloid fibroblasts [48]. The miR-152-3p/Gadd45 correlation is also investigated in PC12-Derived nerve cells [49]. To explore the downstream molecule of miR-152-3p, mRNAs binding with miR-152-3p were predicted using the starBase, and DDX6 was finally selected for further study due to its increased expression mediated by miR-152-3p inhibition in cells. DDX6, a member of the DDX RNA helicase family, is an RNA-binding protein that correlates with different aspects of gene expression regulation [50]. DDX6 was demonstrated to increase the activity of miRNA let-7a in neural stem cells [51]. DDX6 was found to increase the activity of miR-21 and miR-124 by interacting with Tau protein in tauopathies [52]. Functions of DDX6 in vascular endothelial cells still require more investigation. Herein, DDX6 expression was downregulated in HUVECs under hypoxia. MiR-152-3p inhibition upregulated mRNA expression and protein levels of DDX6 in cells. According to luciferase reporter assays, miR-152-3p directly targeted the 3'UTR of DDX6 in cells. In addition, rescue assays showed that DDX6 knockdown reversed the alleviation of angiogenesis and endothelial permeability mediated by miR-152-3p inhibition. The results indicated that miR-152-3p promotes angiogenesis and endothelial permeability by downregulating DDX6 expression.

Furthermore, DDX6 was reported to have a strong binding capacity for vascular endothelial growth factor (VEGF) 5'-UTR [25]. VEGF contributes to the formation of blood vessels [53]. Under hypoxic conditions, VEGF expression is mediated by the increase in mRNA synthesis, stability and translation. Elevated VEGF mRNA synthesis under hypoxia is induced by hypoxia-inducible factor 1 (HIF-1) which is a transcription factor composing of a constitutive β -subunit and an O₂-labile α -subunit (HIF-1 α) [25]. HIF-1 α is essential for the maintenance of oxygen homeostasis [54]. Another study reveals that HIF-1 α mRNA is released from the P-bodies or does not enter into P-bodies through decreased DDX6 [54]. Herein, we did not further explore how DDX6 affects angiogenesis, endothelial cell permeability and viability under hypoxia. We hypothesized that the relationship between DDX6 and HIF-1 α might be the answer, which will be further explored in future studies.

In conclusion, miR-152-3p aggravates angiogenesis in vascular endothelial cells and promotes endothelial cell permeability by targeting DDX6 under hypoxia. The study may provide novel insight into miRNA investigation in ischemic stroke. However, how hypoxia treatment affects miR-152-3p and DDX6 expression in HUVECs are not investigated and *in vivo* animal experiments have not been designed in the study, which will be included in our future studies. In addition, the upstream gene that may regulate miR-152-3p expression and the possible signaling pathway mediated by DDX6 in HUVECs will be investigated in our future studies.

Conclusion

miR-152-3p is highly expressed in HUVECs under hypoxia. miR-152-3p inhibition elevates the decrease in cell viability induced by hypoxia. miR-152-3p inhibition mitigates the activation of angiogenesis and endothelial cell permeability under hypoxia. Mechanistically, DDX6 is targeted by miR-152-3p in HUVECs. DDX6 deficiency reverses the effects of miR-152-3p inhibition on angiogenesis, endothelial cell viability and permeability under hypoxia. Overall, miR-152-3p

aggravates vascular endothelial cell dysfunction by targeting DDX6 under hypoxia.

HIGHLIGHTS

- (1) miR-152-3p is high-expressed in HUVECs under hypoxia.
- (2) Inhibition of miR-152-3p mitigates hypoxia-activated angiogenesis.
- (3) Inhibition of miR-152-3p alleviates hypoxia-induced endothelial cell permeability.
- (4) DDX6 is targeted by miR-152-3p.
- (5) DDX6 depletion reverses effects of miR-152-3p inhibition on cell dysfunction.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Nature Science Foundation of China [Grant No. 81860229, for Shixiong Huang].

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References

- [1] Feigin VL, Forouzanfar MH, Krishnamurthi R, et al. Global and regional burden of stroke during 1990-2010: findings from the global burden of disease study 2010. *Lancet*. 2014 Jan 18;383(9913):245-255.
- [2] Zhong C, Yin C, Niu G, et al. MicroRNA miR-497 is closely associated with poor prognosis in patients with cerebral ischemic stroke. *Bioengineered*. 2021 Dec;12(1):2851-2862.
- [3] Chauhan G, Debette S. Genetic risk factors for ischemic and hemorrhagic stroke. *Curr Cardiol Rep*. 2016 Dec;18(12):124.
- [4] GBD 2015 Mortality and Causes of Death Collaborators. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. 2016 Oct 8;388(10053):1459-1544.
- [5] El Amki M, Wegener S. Improving cerebral blood flow after arterial recanalization: a novel therapeutic strategy in stroke. *Int J Mol Sci*. 2017 Dec 9;18:12.

- [6] Kong Q, Hao Y, Li X, et al. HDAC4 in ischemic stroke: mechanisms and therapeutic potential. *Clin Epigenetics*. 2018 Sep 12;10(1):117.
- [7] Hatakeyama M, Ninomiya I, Kanazawa M. Angiogenesis and neuronal remodeling after ischemic stroke. *Neural Regen Res*. 2020 Jan;15(1):16–19.
- [8] Krupinski J, Kaluza J, Kumar P, et al. Role of angiogenesis in patients with cerebral ischemic stroke. *Stroke*. 1994 Sep;25(9):1794–1798.
- [9] Zhang B, Li J. Phoenixin-14 protects human brain vascular endothelial cells against oxygen-glucose deprivation/reoxygenation (OGD/R)-induced inflammation and permeability. *Arch Biochem Biophys*. 2020 Mar 30;682:108275.
- [10] Greene C, Campbell M. Tight junction modulation of the blood brain barrier: CNS delivery of small molecules. *Tissue Barriers*. 2016 Jan-Mar;4(1):e1138017.
- [11] Liu W, Wang P, Shang C, et al. Endophilin-1 regulates blood-brain barrier permeability by controlling ZO-1 and occludin expression via the EGFR-ERK1/2 pathway. *Brain Res*. 2014 Jul 21;1573:17–26.
- [12] Yuan S, Liu KJ, Qi Z. Occludin regulation of blood-brain barrier and potential therapeutic target in ischemic stroke. *Brain Circ*. 2020 Jul-Sep;6(3):152–162.
- [13] Mellis D, Caporali A. MicroRNA-based therapeutics in cardiovascular disease: screening and delivery to the target. *Biochem Soc Trans*. 2018 Feb 19;46(1):11–21.
- [14] Nong A, Li Q, Huang Z, et al. MicroRNA miR-126 attenuates brain injury in septic rats via NF- κ B signaling pathway. *Bioengineered*. 2021 Dec 12;12(1):2639–2648.
- [15] Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*. 2010;79(1):351–379.
- [16] Gong M, Yu B, Wang J, et al. Mesenchymal stem cells release exosomes that transfer miRNAs to endothelial cells and promote angiogenesis. *Oncotarget*. 2017 Jul 11;8(28):45200–45212.
- [17] Arderiu G, Peña E, Aledo R, et al. MicroRNA-145 regulates the differentiation of adipose stem cells toward microvascular endothelial cells and promotes angiogenesis. *Circ Res*. 2019 Jun 21;125(1):74–89.
- [18] Shi Y, Xu X, Luan P, et al. miR-124-3p regulates angiogenesis in peripheral arterial disease by targeting STAT3. *Mol Med Rep*. 2020 Dec;22(6):4890–4898.
- [19] Zhang T, Tian C, Wu J, et al. MicroRNA-182 exacerbates blood-brain barrier (BBB) disruption by down-regulating the mTOR/FOXO1 pathway in cerebral ischemia. *Faseb J*. 2020 Oct;34(10):13762–13775.
- [20] Xie K, Cai Y, Yang P, et al. Upregulating microRNA-874-3p inhibits CXCL12 expression to promote angiogenesis and suppress inflammatory response in ischemic stroke. *Am J Physiol Cell Physiol*. 2020 Sep 1;319(3):C579–c588.
- [21] Yang Y, Fang X, Yang R, et al. MiR-152 regulates apoptosis and triglyceride production in MECs via Targeting ACAA2 and HSD17B12 Genes. *Sci Rep*. 2018 Jan 11;8(1):417.
- [22] Miao W, Bao TH, Han JH, et al. Neuroprotection induced by post-conditioning following ischemia/reperfusion in mice is associated with altered microRNA expression. *Mol Med Rep*. 2016 Sep;14(3):2582–2588.
- [23] Song P, Sun H, Chen H, et al. Decreased serum exosomal miR-152-3p contributes to the progression of acute ischemic stroke. *Clin Lab*. 2020 Aug 1;66(8/2020):8.
- [24] Li JH, Liu S, Zhou H, et al. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res*. 2014 Jan;42(D1):D92–7.
- [25] De Vries S, Naarmann-de Vries IS, Urlaub H, et al. Identification of DEAD-box RNA helicase 6 (DDX6) as a cellular modulator of vascular endothelial growth factor expression under hypoxia. *J Biol Chem*. 2013 Feb 22;288(8):5815–5827.
- [26] Lee YC, Chang YC, Wu CC, et al. Hypoxia-preconditioned human umbilical vein endothelial cells protect against neurovascular damage after hypoxic ischemia in neonatal brain. *Mol Neurobiol*. 2018 Oct;55(10):7743–7757.
- [27] Dalby B, Cates S, Harris A, et al. Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods*. 2004 Jun;33(2):95–103.
- [28] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{(-Delta Delta C(T))} Method. *Methods*. 2001 Dec;25(4):402–408.
- [29] Hu C, Bai X, Liu C, et al. Long noncoding RNA XIST participates hypoxia-induced angiogenesis in human brain microvascular endothelial cells through regulating miR-485/SOX7 axis. *Am J Transl Res*. 2019;11(10):6487–6497.
- [30] Duan MX, Zhou H, Wu QQ, et al. Andrographolide protects against HG-induced inflammation, apoptosis, migration, and impairment of angiogenesis via PI3K/AKT-eNOS signalling in HUVECs. *Mediators Inflamm*. 2019;2019:6168340.
- [31] Zheng J, Zhuo YY, Zhang C, et al. LncRNA TTTY15 regulates hypoxia-induced vascular endothelial cell injury via targeting miR-186-5p in cardiovascular disease. *Eur Rev Med Pharmacol Sci*. 2020 Mar;24(6):3293–3301.
- [32] Bezzaouha A, Bouamra A, Ammimer A, et al. Non-parametric tests on SPSS to compare two or more means on matched samples. *Tunis Med*. 2020 Dec;98(12):932–941.
- [33] Kuriakose D, Pathophysiology XZ. Treatment of stroke: present status and future perspectives. *Int J Mol Sci*. 2020 Oct 15;21(20):20.
- [34] Yang C, Hawkins KE, Doré S, et al. Neuroinflammatory mechanisms of blood-brain

- barrier damage in ischemic stroke. *Am J Physiol Cell Physiol.* **2019** Feb 1;316(2):C135–c153.
- [35] Sandoval KE, Witt KA. Blood-brain barrier tight junction permeability and ischemic stroke. *Neurobiol Dis.* **2008** Nov;32(2):200–219.
- [36] Mirzaei H, Momeni F, Saadatpour L, et al. MicroRNA: relevance to stroke diagnosis, prognosis, and therapy. *J Cell Physiol.* **2018** Feb;233(2):856–865.
- [37] Venkat P, Cui C, Chopp M, et al. MiR-126 mediates brain endothelial cell exosome treatment-induced neurorestorative effects after stroke in type 2 diabetes mellitus mice. *Stroke.* **2019** Oct;50(10):2865–2874.
- [38] Wang J, Chen S, Zhang W, et al. Exosomes from miRNA-126-modified endothelial progenitor cells alleviate brain injury and promote functional recovery after stroke. *CNS Neurosci Ther.* **2020** Dec;26(12):1255–1265.
- [39] Bernstein DL, Zuluaga-Ramirez V, Gajghate S, et al. miR-98 reduces endothelial dysfunction by protecting blood-brain barrier (BBB) and improves neurological outcomes in mouse ischemia/reperfusion stroke model. *J Cereb Blood Flow Metab.* **2020** Oct;40(10):1953–1965.
- [40] Desjarlais M, Rivera JC, Lahaie I, et al. MicroRNA expression profile in retina and choroid in oxygen-induced retinopathy model. *PLoS One.* **2019**;14(6):e0218282.
- [41] Roslavtceva VV, Salmina AB, Prokopenko SV, et al. [The role of vascular endothelial growth factor in the regulation of development and functioning of the brain: new target molecules for pharmacotherapy]. *Biomed Khim.* **2016** Mar-Apr;62(2):124–133.
- [42] Scholz A, Plate KH, Reiss Y. Angiopoietin-2: a multifaceted cytokine that functions in both angiogenesis and inflammation. *Ann N Y Acad Sci.* **2015** Jul;1347(1):45–51.
- [43] Sun B, Liu Y, Huang W, et al. Functional identification of a rare vascular endothelial growth factor a (VEGFA) variant associating with the nonsyndromic cleft lip with/without cleft palate. *Bioengineered.* **2021** Dec;12(1):1471–1483.
- [44] Ballabh P, Braun A, Nedergaard M. The blood-brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiol Dis.* **2004** Jun;16(1):1–13.
- [45] Nian K, Harding IC, Herman IM, et al. Blood-brain barrier damage in ischemic stroke and its regulation by endothelial mechanotransduction. *Front Physiol.* **2020**;11:605398.
- [46] Reinhold AK, Rittner HL. Barrier function in the peripheral and central nervous system - a review. *Pflugers Arch.* **2017** Jan;469(1):123–134.
- [47] Friedman RC, Farh KK, Burge CB, et al. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* **2009** Jan;19(1):92–105.
- [48] Wang R, Bai Z, Wen X, et al. MiR-152-3p regulates cell proliferation, invasion and extracellular matrix expression through by targeting FOXF1 in keloid fibroblasts. *Life Sci.* **2019** Oct 1;234:116779.
- [49] Łuczowska K, Rogińska D, Ulańczyk Z, et al. Effect of bortezomib on global gene expression in PC12-derived nerve cells. *Int J Mol Sci.* **2020** Jan 23;21(3):3.
- [50] Marcon BH, Rebelatto CK, Cofré AR, et al. DDX6 helicase behavior and protein partners in human adipose tissue-derived stem cells during early adipogenesis and osteogenesis. *Int J Mol Sci.* **2020** Apr 9;21(7):7.
- [51] Nicklas S, Okawa S, Hillje AL, et al. The RNA helicase DDX6 regulates cell-fate specification in neural stem cells via miRNAs. *Nucleic Acids Res.* **2015** Mar 11;43(5):2865–2874.
- [52] Chauderlier A, Gilles M, Spolcova A, et al. Tau/DDX6 interaction increases microRNA activity. *Biochim Biophys Acta Gene Regul Mech.* **2018** Aug;1861(8):762–772.
- [53] Karaman S, Leppänen VM, Alitalo K. Vascular endothelial growth factor signaling in development and disease. *Development.* **2018** Jul 20;145(14):14.
- [54] Saito K, Kondo E, MicroRNA MM. 130 family regulates the hypoxia response signal through the P-body protein DDX6. *Nucleic Acids Res.* **2011** Aug;39(14):6086–6099.