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MiR-145 protected the cell viability of human cerebral cortical neurons after oxygen-glucose deprivation by downregulating EPHA4

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ABSTRACT

Our previous study indicated that microRNA 145 (miR-145) and its predicated target, erythropoietin-producing hepatoma (EPH) receptor A4 (EPHA4), was closely associated with ischemic stroke. In this study, we aimed to further explore their function in a model of oxygen-glucose deprivation (OGD). The expression of miR-145 in the blood of 44 patients with ischemic stroke and 37 normal controls was detected by qRT-PCR. After transfection with either the wild- or mutant-type pGL3-promoter EPHA4 3′UTR into the miR-145 mimic and miR-145 inhibitor, a dual-luciferase reporter assay was performed to explore the interaction between miR-145 and EPHA4. qRT-PCR and Western blot were performed to further explore the effects of miR-145 on EPHA4 expression after an miR-145 mimic, an miR-145 inhibitor or LV-sh-EPHA4 was transfected into cerebral cortical neurons. The expression of miR-145 was significantly upregulated in the blood of patients with ischemic stroke compared to that of normal controls. Dual-luciferase reporter assay, qRT-PCR and Western blot results indicated that miR-145 indeed targets EPHA4 through its 3′-UTR and regulates the expression level of EPHA4 at both the mRNA and protein levels. Moreover, the OGD model was successfully constructed, and miR-145 exerted a protective effects in cell viability in the OGD model by downregulating EPHA4. The expression of LOC105376244 could be regulated by the miR-145-EPHA4 interaction. MiR-145 exerted a protective effects in cell viability in the OGD model by downregulating EPHA4, which suggested their potential roles in ischemic stroke and requires further research.

1. Introduction

Ischemic stroke is a neurological disease that serves as one of the main causes of long-term morbidity and mortality for both developed and developing countries [1]. Thrombolytic therapy is the only effective clinical treatment, but the narrow therapeutic window and the risk of subsequent intracerebral haemorrhage has limited its use [2]. Therefore, how to effectively protect the cranial nerves and promote the recovery of damaged nerve cells has always been a research hotspot.

The underlying mechanisms of neuroprotection against ischemic brain injury are still not fully understood. MiR-145 has been reported to be associated with the pathologies of ischemic stroke by regulating angiogenesis, hyperlipidaemia, nerve injury and inflammatory reactions ([4,8,13,19–21]. Our previous results show that miR-145 was upregulated in the blood of patients after ischemic stroke and was involved in the process of post-ischemic neuronal damage [11]. The erythropoietin-producing hepatoma (EPH) family of receptor tyrosine kinase and its receptor ligands play key roles in the nervous system [17]. EPH receptor A4 (EPHA4) is mainly expressed in the central nervous system [15] and interacts with all ephrin ligands [14]. Lemmens et al. reported that reduction of EphA4 improves motor function after experimental stroke and demonstrate that ROCK inhibition is a promising therapeutic strategy to enhance recovery after ischemic stroke [16]. EphA4-mediated ephrin-A3 reverse signalling to be a crucial mechanism for astrocytes to control glial glutamate transporters and protect hippocampal neurons from glutamate excitotoxicity under ischemic conditions [22]. Jia et al. suggested that miR-145 upregulated in acute ischemic stroke might be a new biomarker for acute ischemia stroke evaluation [13]. Wei et al. found that an rs4705342 T > C polymorphism in the promoter of miR-145 is associated with a

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decreased risk of ischemic stroke [21]. To further research the role of miR-145 in ischemic stroke, our RNA-sequencing analysis indicated that EPHA4 was a predicated target of miR-145 and was dysregulated in the blood of patients after ischemic stroke with three co-expressed long non-coding RNAs: LOC105370660, LINC01128 and LOC105376244 [12]. Moreover, EPHA4 has been reported to regulate the damage of hippocampal neuronal and glutamate transport in astrocytes in transient cerebral ischaemia and in motor function after ischemic stroke by interacting with downstream target genes [6,16,22]. We hypothesized that the miR-145-EPHA4 interaction may play an important role in ischemic stroke.

In this study, we established a model of oxygen-glucose deprivation (OGD) to mimic the post-ischemic environment. The miR-145-EPHA4-LOC105370660/LINC01128/LOC105376244 interaction was further researched in the OGD model, which may provide clues for the molecular mechanism of neuroprotection against ischemic brain injury and, accordingly, provide a theoretical basis for exploring new drug targets for stroke and treatment strategies for ischemic brain injury.

2. Materials and methods

2.1. Patients

A total of 44 patients with ischemic stroke and 37 matched normal controls were recruited from the First Affiliated Hospital of Shantou University Medical College. The aetiology of stroke was classified according to the TOAST classification criteria. Patients with ischemic stroke were included in this study. Patients with neurological diseases, cardiac embolism, transient ischemic attack, haemorrhagic infarction, occult cerebrovascular malformation, or traumatic cerebrovascular disease were excluded. Age- and sex-matched normal controls without a history of stroke, head trauma or operation, heart surgery or neurological diseases were included in this study. This study was approved by the institutional review board of the First Affiliated Hospital of Shantou University Medical College. Moreover, signed informed consent was obtained from the study subjects.

2.2. Expression of miR-145 in patients with ischemic stroke

Blood samples were collected from 44 ischemic stroke patients and 37 normal controls at day 1 and week 1. Total RNA from blood samples was isolated by using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. By using miRcute miRNA first-strand cDNA synthesis kits (TIAN- GEN), miRNA reverse transcription was performed according to the manufacturer's instructions. Then, we performed qRT-PCR reactions with SuperReal PreMix Plus (Invitrogen, USA) in an ABI 7500 real-time PCR detection system. Relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. Human U6 were used as endogenous controls for miRNA expression in analysis. Primer sequences of miR-145 is as follows: 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′.

2.3. Culture of human cerebral cortical neurons and identification by immunofluorescence

Human cerebral cortical neurons primary cells (ScienCell Research Laboratories, San Diego, CA) were cultured at 37 °C and 5% CO_2 in an incubator with a primary neuronal cell culture system (iCell Bioscience Inc., Shanghai). Human cerebral cortical neurons were divided into two groups, i.e., the case group and the negative control group. In the case group, cells were incubated with the primary antibody, NF-M rabbit anti-human polyclonal antibody and the secondary antibody, conjugated goat anti-rabbit IgG (H + L). In the negative control group, cells were incubated with 5% BSA instead of the primary antibody and then incubated with conjugated goat anti-rabbit IgG (H + L) (Alexa Fluor® 594). Bright-field images of human cerebral cortical neurons were obtained by fluorescence microscopy. Fluorescent images of cells in both groups were obtained at 300 ms exposure time by fluorescence microscopy.

2.4. Transfection of cells

In the present study, we aimed to explore miRNA-145 and its predicated target, EPHA4, in ischemic stroke. RNA interference was performed through a vector-based shRNA. shRNAs are stem-loop RNA structures that can be used to silence gene expression via RNA interference after processing by DICER. Three siRNA sequences within the EPHA4 gene (si-EPHA4-1, si-EPHA4-2, si-EPHA4-3), negative control siRNA and FAM-labelled control siRNA were synthesized chemically. MiR-145 mimetics (sequence: 5′-GTCCAGTTTTCCCAGGAATCCCT-3′), miR-145 inhibitors (sequence: 5′-ACGGAUUCCUGGGAAAACUG GAC-3′) and their corresponding negative controls (sequence: 5′-UUC UCCGAACGUGUCACGUTT-3′) were synthesized chemically. The transfection of these sequences into cerebral cortical neurons was conducted by combined use of Lipofectamine LTX and FuGENE HD. The effects of these three siRNA sequences (si-EPHA4-1 (sequence: 5′-GCA GCACCATCATCCATTG-3′), si-EPHA4-2 (sequence: 5′-TCCGAACCTAC CAAGTGTG-3′), si-EPHA4-3 (sequence: 5′-TCATGAAGCTGAACAC CGA-3′)), miR-145 mimetics and miR-145 inhibitors on the expression of EPHA4 were assessed by using qRT-PCR and Western blot 48 h after transfection. Moreover, the siRNA sequence with the best knockdown efficiency was inserted into lentiviral vectors.

2.5. The interaction between miR-145 and EPHA4 was detected by dual-luciferase reporter assay

Our previous study found that three lncRNAs (LOC105370660, LINC01128 and LOC105376244) were co-expressed with EPHA4 in patients with ischemic stroke. The interaction between EPHA4 and these three lncRNAs was further explored in the OGD model.

Interactions between miRNA-145 and EPHA4 were further explored. First, the binding site of miRNA-145-5p and the 3′UTR region of transcription products encoded by EPHA4 were predicted with miRDB (http://www.mirdb.org/). Second, we constructed a luciferase reporter vector as follows: a total of approximately 500 bp window up- and downstream of the binding site between miRNA-145-5p and the 3′UTR region of the transcription products encoded by wild-type EPHA4 was synthesized chemically. Third, this sequence was ligated to the pGL3-promoter vector (wild-type pGL3-promoter EPHA4 3′UTR). In addition, the 500 bp mutant sequence was synthesized by point mutation with the wild-type 3′UTR region as a template and was ligated to the pGL3-promoter vector (mutant-type pGL3-promoter EPHA4 3′UTR). Fourth, transfection was conducted by the combined use of Lipofectamine LTX and FuGENE HD. Wild-type pGL3-promoter EPHA4 3′UTRs were transfected into cerebral cortical neurons with miR-145 mimetics, inhibitors and negative controls. Mutant-type pGL3-promoter EPHA4 3′UTRs were transfected into cerebral cortical neurons with miR-145 mimetics, inhibitors and negative controls. The pRL-TK vector was transfected into cerebral cortical neurons that served as an internal control reporter. Finally, a luciferase reporter assay kit was used to measure the luciferase activity according to the manufacturer's protocol. After washing cells with PBS, cells were lysed with a passive lysis buffer, cultured for 15 min on a rocking platform at room temperature and subsequently assayed for luciferase activity in cell lysate. Each 10 μL lysate was mixed with 10 μL of luciferase assay reagent and placed in a luminometer (Berthold). Firefly luciferase activity was measured first, followed by the addition of 10 μL Stop&Glo substrate to measure Renilla luciferase.

2.6. Establishment the model of oxygen-glucose deprivation (OGD)

Cerebral cortical neurons were cultured under hypoxic conditions to mimic the hypoxic process in ischemic stroke. The specific steps were as

follows: after digestion and resuspension, cells were counted, and 3×10^5 cells were inoculated into a 35 mm petri dish with DMEM medium (glucose concentration 4.5 g/L) so that the degree of cell confluence reached approximately 70% for the next day. After renewing the culture medium on the next day, the cells were cultured in glucose-free Earle's medium and placed in a three-gas incubator to conduct experiments of normoxia and hypoxia. In the normoxia group, cells were cultured under 21% O_2, 5% CO_2 and 78% N_2; in the hypoxia group, cells were cultured under 1% O_2, 5% CO_2 and 94% N_2. Cells in both groups were harvested after 12 h, 24 h, and 48 h, respectively.

2.7. CCK-8 assay

The CCK-8 assay was used to measure the effects of miR-145 and EPHA4 on cell viability in the OGD model. After the medium in the primary neuronal cell culture system was replaced with medium containing 10% CCK-8, cerebral cortical neurons were incubated at 37 °C for 1 h. After incubation, the supernatant was transferred to a 96-well plate, and the absorbance was measured at 450 nm using an ELISA reader.

2.8. qRT-PCR

Total RNA from cerebral cortical neurons cells was isolated by using E.Z.N. A FFPE RNA isolation kit (OMEGA) was used according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using random hexamer oligos (Fermentas) and SuperScriptIII reverse transcriptase (Invitrogen). The qRT-PCR reaction was conducted in an ABI 7500 real-time PCR system using SuperReal PreMix Plus (Invitrogen). The fold change for the relative gene expression was determined by using the $2^{-\Delta\Delta Ct}$ method. In our analysis, we regard human 18srRNA as an endogenous control for mRNA expression.

2.9. Western blot

Two days after transfection, cells were lysed in RIPA lysis buffer (Beyotime) with PMSF (Beyotime). The concentration of protein was quantified with the BCA protein kit (Thermo). Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and proteins were transferred to polyvinylidene difluoride membranes (Millipore). After blocking, the membrane was incubated with the primary antibodies, rabbit polyclonal antibody against EPHA4 (1:500; 21,875–1-AP, 120 kDa, Proteintech) and mouse monoclonal antibody against β-actin (1:5000, mouse, 43 kDa, Sigma) at 4 °C overnight. After washing three times with TBST, the membrane was incubated with horseradish peroxidase-conjugated secondary goat anti-mouse and anti-rabbit antibodies (1:2000, Cell Signalling) for 2 h at room temperature. After washing three times with TBST, an ECL system (Thermo Fisher) was used to visualize the protein bands.

2.10. Statistical analysis

Values are expressed as the mean ± standard deviation. The number of replicates in each group is illustrated in the figure. Data analysis was performed using Prism GraphPad 7.0. A Student's paired *t*-test was used for comparing level of miR-145 in ischemic stroke and control normal. ANOVA were conducted to compare groups sampled at different time points. When more than two groups were compared, one-way ANOVA was used. A value of $p < 0.05$ was considered statistically significant.

Table 1
Baseline clinical characteristics of the IS patients and normal controls.

Characteristics	IS(n = 44)	Control (n = 37)	*P* value
Male/female	30/14	24/13	0.9371
Age	63 ± 11.26	62 ± 10.69	0.7928
GLU (mmol/L)	7.70 ± 3.52	6.06 ± 2.40	0.0158
TG (mmol/L)	2.26 ± 1.91	1.59 ± 0.96	0.2205
HDL-C (mmol/L)	1.08 ± 0.31	1.16 ± 0.29	0.2401
LDL-C (mmol/L)	3.25 ± 0.97	3.05 ± 0.96	0.3481
Hypertension, n (%)	40(90.9)	19(51.3)	0.0002
Diabetes, n (%)	22(50.0)	11(29.7)	0.1047
Hyperlipidemia, n (%)	20(45.4)	14(37.8)	0.2014
Smoker, n (%)	19(43.1)	11(29.7)	0.3087
Drinking n (%)	8(18.1)	3(8.1)	0.3208

Abbreviations: GLU, glucose; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglyceride.
Data were represented as mean ± standard deviation or n (%). *P* value was calculated by *t*-test or χ^2 test.

3. Results

3.1. Expression of miR-145 in patients with ischemic stroke

According to our previous analysis, miR-145 was upregulated in the blood of patients after ischemic stroke. To further research the role of miR-145 in ischemic stroke, we detected the expression of miR-145 in patients with ischemic stroke. Baseline clinical characteristics of 44 patients with ischemic stroke and 37 matched normal controls were displayed in Table 1. Here, the qRT-PCR was performed to detect the expression of miR-145 in ischemic stroke patients. The total expression of miR-145 was significantly upregulated in the blood of patients with ischemic stroke compared to that of normal controls (Fig. 1a). Moreover, miR-145 was upregulated in the blood of patients with ischemic stroke at 1 day and 1 week after ischemic stroke compared to levels in normal controls (Fig. 1b).

3.2. Identification of human cerebral cortical neurons by immunofluorescence

To detect purity of human cerebral cortical neurons primary cells, we used the immunofluorescence to identify the human cerebral cortical neurons. Cells were imaged using bright-field microscopy (Fig. 2a). Fluorescence microscopy images of nuclei that were non-specifically stained with DAPI, specifically the neurofilament protein NF-M and the merged image, are shown in Fig. 2b. These images indicated that cells were in good condition with uniform morphology and that the purity of cells was > 95%.

3.3. Interaction between miR-145 and EPHA4 confirmed by dual-luciferase reporter assay

Our RNA-sequencing analysis indicated that EPHA4 was a predicated target of miR-145 and was dysregulated in the blood of patients after ischemic stroke. Here, the dual-luciferase reporter assay was performed to detect interaction between miR-145 and EPHA4. Dual-luciferase reporter assay detects the transcriptional regulation of promoters or regulatory elements by detecting the amount of firefly luciferase. The binding site of miRNA-145 and the 3′UTR region of the transcription products encoded by wild- and mutant-type EPHA4 are displayed in Fig. 3a. Sequencing results indicated that the wild- and mutant-type pGL3-promoter EPHA4 3′UTRs were successfully constructed (Fig. 3b). Luciferase activity in cells transferred with wild-type pGL3-promoter EPHA4 3′UTR and miR-145 mimic was significantly lower than that in the wild-type pGL3-promoter EPHA4 3′UTR alone and in negative controls ($p < 0.05$, Fig. 3c). No significant difference in luciferase activity was observed between mutant-type pGL3-

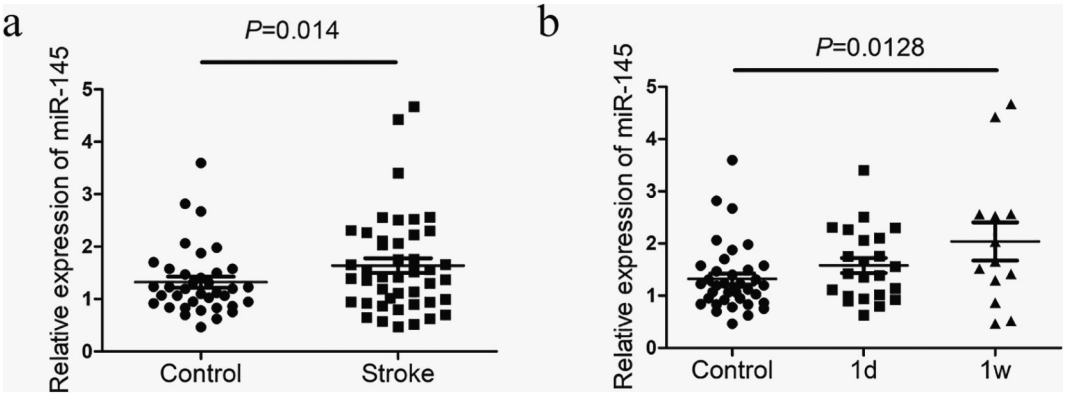

Fig. 1. The expression of miR-145 in blood between patients with ischemic stroke and normal controls.
a). The total expression of miR-145 in blood between patients with ischemic stroke and normal controls. The x-axis indicated control and ischemic stroke group and y-axis indicated relative expression of miR-145. b). The total expression of miR-145 in blood between patients with ischemic stroke and normal controls. The x-axis indicated control, ischemic stroke group at 1 day and 1 week after ischemic stroke, y-axis indicated relative expression of miR-145. The assays were performed three times independently (ischemic stroke $n = 44$, control $n = 37$). Scale bar is 100 μm. * indicated p-value < 0.05, ** indicated p-value < 0.01.

promoter EPHA4 3′UTRs with the miR-145 mimic and those with the negative control ($p < 0.01$, Fig. 3d). Luciferase activity in cells transferred with the wild-type pGL3-promoter EPHA4 3′UTR and an miR-145 inhibitor was significantly higher than that in the cells with the wild-type pGL3-promoter EPHA4 3′UTR and that in negative controls. No significant difference in luciferase activity was observed in the mutant-type pGL3-promoter EPHA4 3′UTR with an miR-145 inhibitor or in negative controls. These results indicate that miR-145 can bind to EPHA4 using the binding site predicted by miRDB and can down-regulate the expression of EPHA4.

3.4. The effect of miR-145 on EPHA4 expression was detected by qRT-PCR and Western blot

To further research the effect of miR-145 on EPHA4 expression in ischemic stroke, the qRT-PCR and Western blot were used to confirm the effect of miR-145 on EPHA4 expression. Based on the qRT-PCR results (Fig. 4a), the expression of EPHA4 was significantly lower in cells with the miR-145 mimic than in negative controls ($p < 0.01$); the expression of EPHA4 was significantly higher in cells with the miR-145 inhibitor than that in negative controls ($p < 0.05$). Based on Western blot results (Fig. 4b–c), the expression of EPHA4 was significantly lower in cells with the miR-145 mimic than that in negative controls ($p < 0.05$); the expression of EPHA4 was significantly higher in cells with the miR-145 inhibitor than that in negative controls ($p < 0.05$). These results indicated that the miR-145 mimic could downregulate the expression of EPHA4, while the miR-145 inhibitor could upregulate the expression of EPHA4 at both the mRNA and protein levels, which was consistent with the results of our dual-luciferase reporter assay.

3.5. Construction of sh-EPHA4 lentivirus vectors

Based on qRT-PCR (Fig. 5a) and Western blot results (Fig. 5b), all three siRNAs targeting EPHA4 (i.e., si-EPHA4-1, si-EPHA4-2 and si-EPHA4-3) significantly reduced the expression of EPHA4 at both the mRNA and protein levels. Moreover, si-EPHA4-1 had the best knockdown efficiency. Finally, the si-EPHA4-1 sequence was inserted into lentiviral vectors (LV-sh-EPHA4). LV-sh-EPHA4 significantly downregulated the expression of EPHA4 at both the mRNA ($p < 0.05$, Fig. 5c) and protein ($p < 0.05$, Fig. 5d–e) levels.

3.6. Construction of OGD models

Based on the CCK-8 assay (Fig. 6), the cell survival rate in the OGD

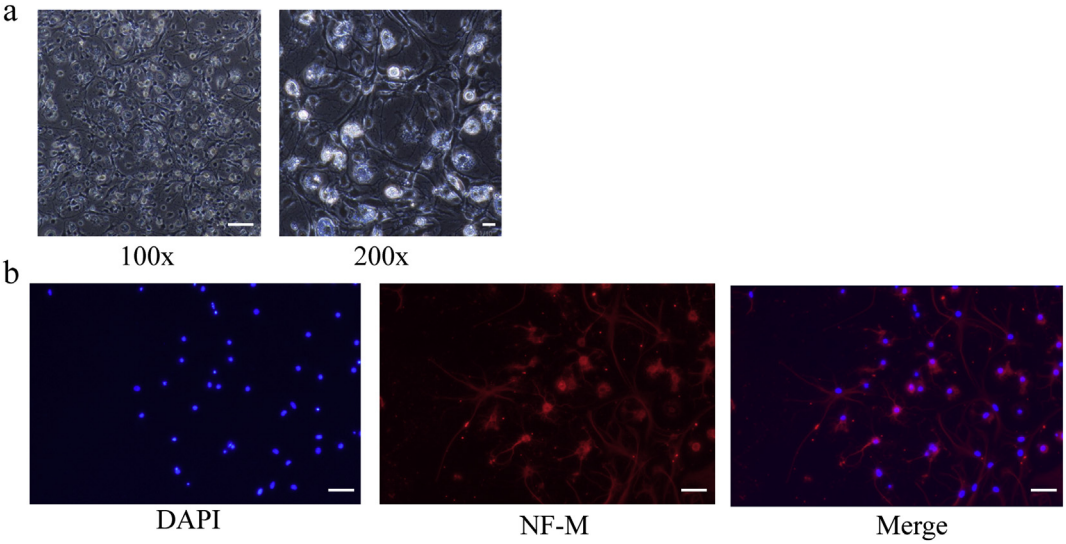

Fig. 2. Identification of human cerebral cortical neurons.
a). Brightfield images (100× and 200×). b). Non-specifically stained nuclei of DAPI, specifically stained neurofilament protein NF-M and their merged images.

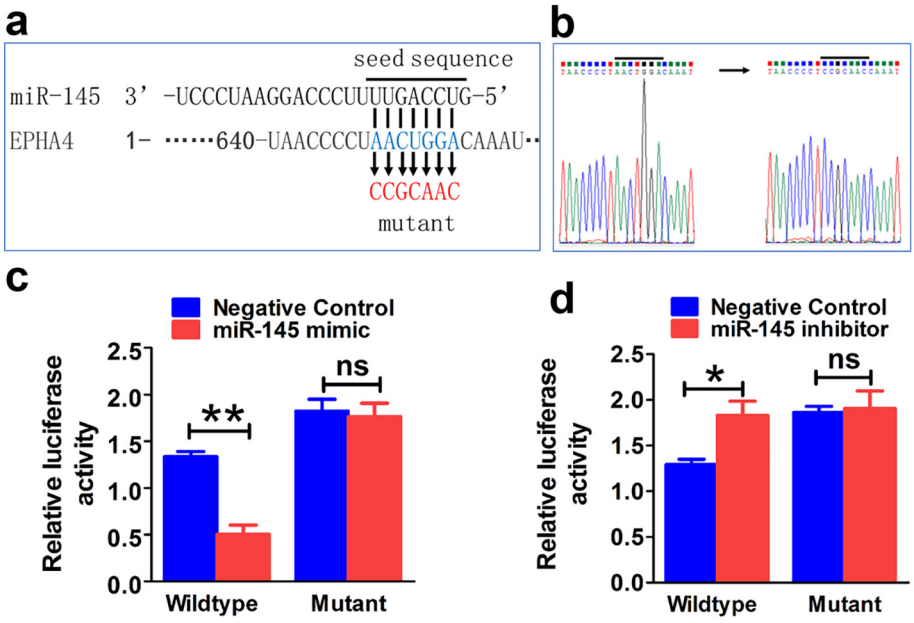

Fig. 3. Interaction between miR-145 and EPHA4 confirmed by dual-luciferase reporter assay.
a). The binding site of miRNA-145 and the 3′UTR region of transcription products encoded by wild- and mutant-type EPHA4. b). Sequencing results of wide- and mutant-type pGL3-promotor-EPHA4-3′UTR. c). Luciferase activity in cells transferred with Wide/Mutant-type pGL3-promotor-EPHA4-3′UTR and miR-145 mimic. c). Luciferase activity in cells transferred with Wide/Mutant-type pGL3-promotor-EPHA4-3′UTR and miR-145 inhibitor. The data are the mean ± S.D. of three independent assays ($n = 3$). * indicated p-value < 0.05, * *indicated p-value < 0.01. ns, not significant.

model was significantly lower than that in the control group when cultured for 12 h ($p < 0.05$), 24 h ($p < 0.01$), and 48 h ($p < 0.001$). The results indicated that cell viability in the OGD group was significantly decreased, and the OGD model was successfully constructed.

3.7. The effect of miR-145 on EPHA4 expression was detected by qRT-PCR and Western blot in the OGD model

In the OGD model, the miR-145 mimic and miR-145 inhibitor increased and decreased the cell survival rate, respectively (Fig. 7a). LV-sh-EPHA4 increased the cell survival rate in the OGD model (Fig. 7a). These results indicated that both miR-145 and the knockdown of EPHA4 exerted protective effects on cell viability in the OGD model. Fig. 7b indicates that the miR-145 inhibitor significantly down-regulated cell viability in the OGD model, while this downregulation was counteracted by the knockdown of EPHA4 (LV-sh-EPHA4). Fig. 7c indicates that the miR-145 mimic significantly up-regulated cell viability in the OGD model, while this upregulation was counteracted by the knockdown of EPHA4 (LV-sh-EPHA4). Taken together, miR-145 exerted a protective role in cell viability in the OGD model by downregulating EPHA4.

3.8. Effect of the miR-145 mimic/inhibitor and knockdown of EPHA4 on the expression of lncRNAs in the OGD model

Our RNA-sequencing analysis indicated that EPHA4 was a predicated target of miR-145 and was dysregulated in the blood of patients after ischemic stroke with three co-expressed long non-coding RNAs: LOC105370660, LINC01128 and LOC105376244. Both LOC105370660 and LOC105376244 were significantly upregulated in the OGD model compared to the control groups (Fig. 8a and g). Moreover, the miR-145 inhibitor significantly up-regulated LOC105376244 expression in the OGD model (Fig. 8g and h), while this upregulation was counteracted by the knockdown of EPHA4 (Fig. 8h). The miR-145 mimic significantly down-regulated LOC105376244 expression in the OGD model (Fig. 8g and i), while this downregulation was counteracted by the knockdown of EPHA4 (Fig. 8i). Taken together, these results show that the expression of LOC105376244 in the OGD model can be regulated by the miR-145–EPHA4 interaction.

4. Discussion

Ischemic stroke often results in irreversible neuronal damage and neuron death and leads to severe neurological deficits [5]. At present, effective treatment for damage to the central nervous system (CNS) after ischemic stroke is limited. There is an urgent need to explore the

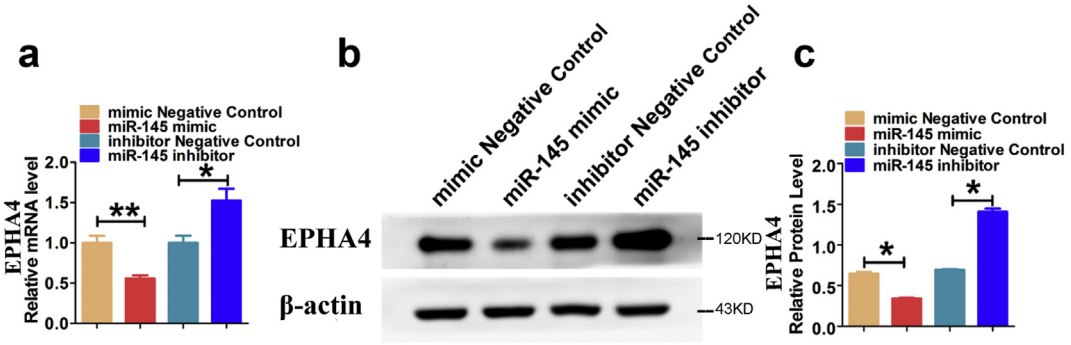

Fig. 4. The effect of miR-145 on EPHA4 expression was detected by qRT-PCR and Western blot.
a). mRNA expression of EPHA4 in human cerebral cortical neurons transfected with miR-145 mimic and miR-145 inhibitor. b and c shown the protein expression of EPHA4 in human cerebral cortical neurons transfected with miR-145 mimic and miR-145 inhibitor. The data are the mean ± S.D. of three independent assays (n = 3). * indicated p-value < 0.05, **indicated p-value < 0.01.

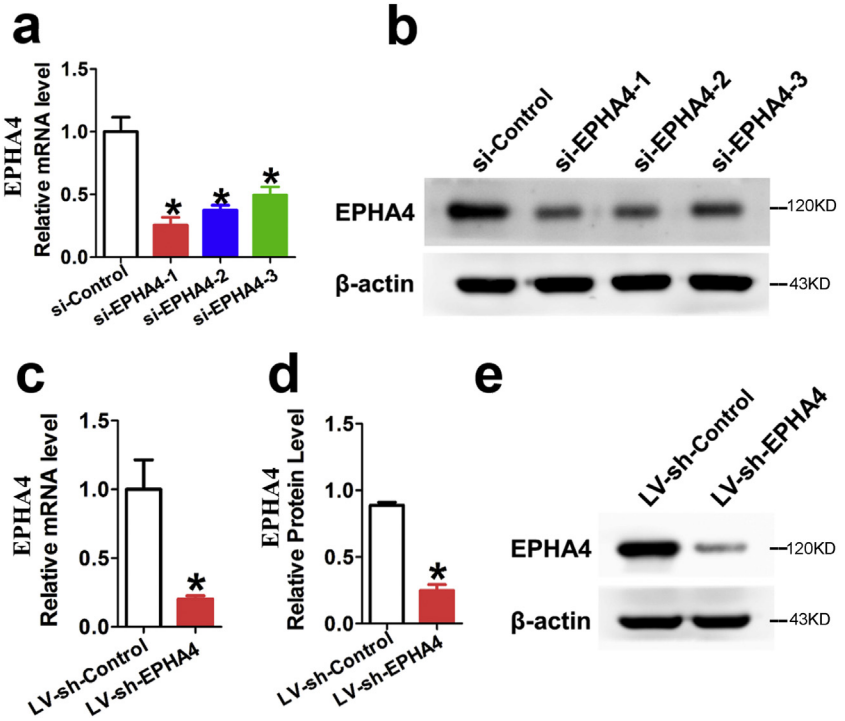

Fig. 5. Knockdown of EPHA4.
a). mRNA expression of EPHA4 in human cerebral cortical neurons transfected with three siRNAs targeting EPHA4 (si-EPHA4-1, si-EPHA4-2 and si-EPHA4-3). b). protein expression of EPHA4 in human cerebral cortical neurons transfected with three siRNAs targeting EPHA4 (si-EPHA4-1, si-EPHA4-2 and si-EPHA4-3). c). mRNA expression of EPHA4 in LV-sh-EPHA4. d and e shown the protein expression of EPHA4 in LV-sh-EPHA4. The data are the mean ± S.D. of three independent assays ($n = 3$). * indicated p-value < 0.05.

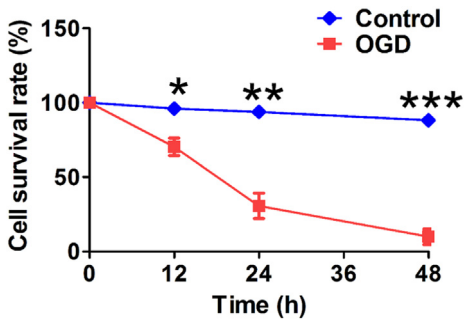

Fig. 6. The OGD model.
Cell survival rate between OGD model and control group cultured for 12 h, 24 h, and 48 h. The assays were performed three times independently (n = 3). * indicated p-value < 0.05, **indicated p-value < 0.01, ***indicated p-value < 0.001.

molecular mechanism of damage repair after ischemic stroke.

Ischemic stroke is an ongoing process involving various pathophysiological neuronal survival stages [18]. Limited blood and oxygen supply can induce ionic imbalance, oxidative stress, excitotoxicity, and inflammation, which can induce the subsequent worsening of clinical outcomes [18]. In this study, OGD was utilized to explore the mechanism of ischemic stroke.

Previous studies have indicated that miR-145 is closely associated with ischemic stroke. The over-stimulation of the glutamate receptor is a major mechanism for neuronal cell death during stroke, and miR-145 serves as one of the superoxide dismutase targets involved in glutamate toxicity [3]. Moreover, miR-145 is the most abundant miRNA in normal arteries and is mainly localized to vascular smooth muscle cells (VSMCs); thus, miR-145 could play a role in reperfusion injury induced by ischemic stroke via regulating the proliferation of VSMCs [7,10]. Additionally, miR-145 could be involved in ischemic stroke by regulating the inflammatory response [10]. Both our previous study [11] and the present study found that miR-145 was significantly upregulated

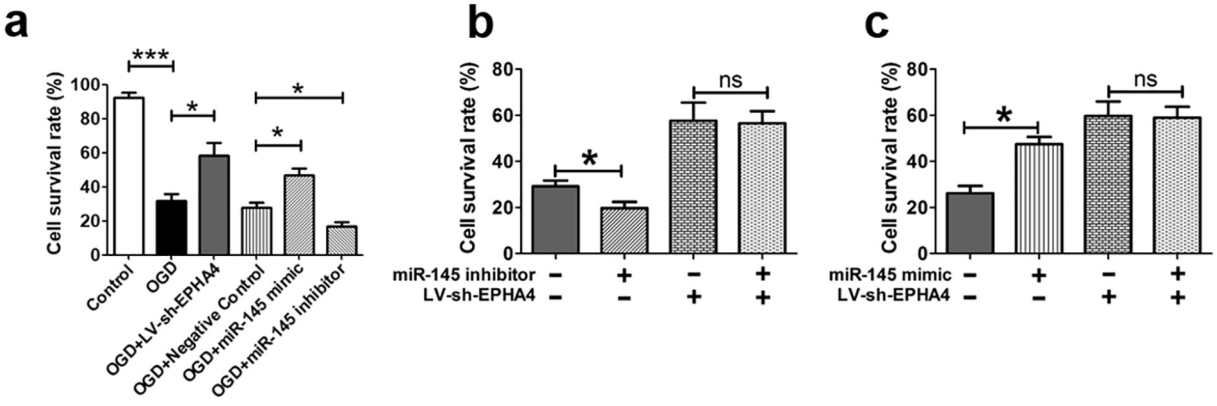

Fig. 7. Interaction between miR-145 and EPHA4 in OGD model.
a). Cell survival rate in OGD model and OGD model transfected with LV-sh-EPHA4, miR-145 mimic and miR-145 inhibitor. b). Effect of LV-sh-EPHA4 on cell survival rate in OGD model transfected with miR-145 inhibitor. c). Effect of LV-sh-EPHA4 on cell survival rate in OGD model transfected with miR-145 mimic. The assays were performed three times independently (n = 3). * indicated p-value < 0.05, ** indicated p-value < 0.01, *** indicated p-value < 0.001. ns, not significant.

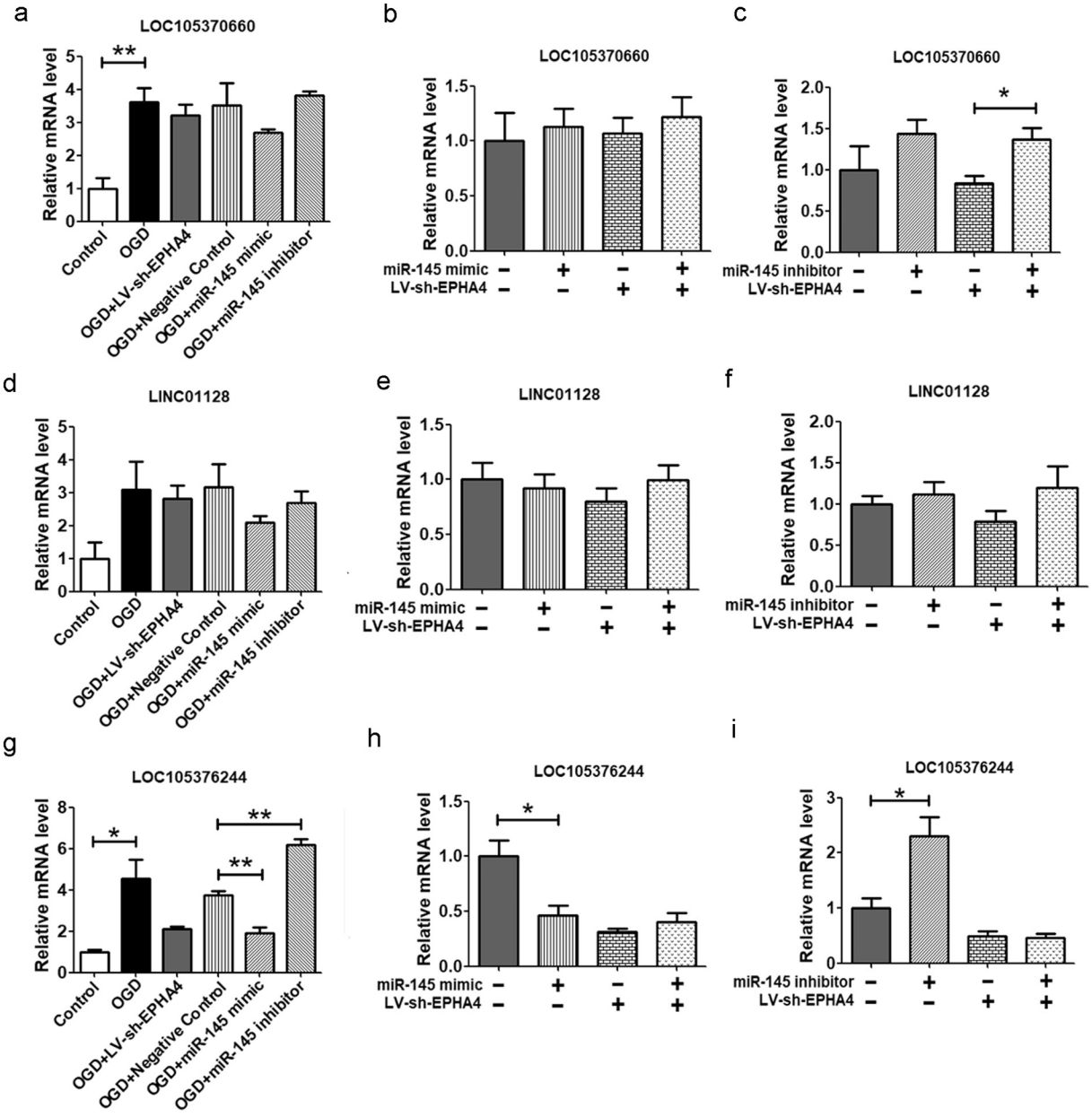

Fig. 8. Effect of miR-145 mimic/inhibitor and knockdown of EPHA4 on the expression of lncRNAs in OGD model.
a). Effect of miR-145 mimic/inhibitor and knockdown of EPHA4 on the expression of LOC105370660 in OGD model. b). Effect of LV-sh-EPHA4 on the expression of LOC105370660 in OGD model transfected with miR-145 mimic c). Effect of LV-sh-EPHA4 on the expression of LOC105370660 in OGD model transfected with miR-145 inhibitor. d). Effect of miR-145 mimic/inhibitor and knockdown of EPHA4 on the expression of LINC01128 in OGD model. e). Effect of LV-sh-EPHA4 on the expression of LINC01128 in OGD model transfected with miR-145 mimic f). Effect of LV-sh-EPHA4 on the expression of LINC01128 in OGD model transfected with miR-145 inhibitor. g). Effect of miR-145 mimic/inhibitor and knockdown of EPHA4 on the expression of LOC105376244 in OGD model. h). Effect of LV-sh-EPHA4 on the expression of LOC105376244 in OGD model transfected with miR-145 mimic i). Effect of LV-sh-EPHA4 on the expression of LOC105376244 in OGD model transfected with miR-145 inhibitor. The data are the mean ± S.D. of three independent assays (n = 3). * indicated p-value < 0.05, ** indicated p-value < 0.01.

in the blood of patients with ischemic stroke compared to that of normal controls. Moreover, miR-145 mimetics improved the cell survival rate in the OGD model, while the miR-145 inhibitor decreased the cell survival rate, which suggested the protective effects of miR-145 for cell viability after ischemic stroke. These findings highlight the importance of miR-145 in ischemic stroke. We speculated that miR-145 is involved in ischemic stroke by regulating its downstream targets.

EPHA4 was indicated as a predicated target of miR-145 in our previous study. In this study, miR-145 mimetics significantly reduced luciferase activity in cells transfected with the wild-type pGL3-promoter EPHA4 3′UTR, but not the mutant-type pGL3-promoter EPHA4 3′UTR; miR-145 inhibitors significantly increased luciferase activity in cells transfected with the wild-type pGL3-promoter EPHA4 3′UTR but not the mutant-type pGL3-promoter EPHA4 3′UTR. Moreover, our qRT-PCR and Western blot results showed that miR-145 mimetics and inhibitors down- and upregulated the expression of EPHA4, respectively. These findings first indicated that miR-145 indeed targets EPHA4 through its 3′-UTR and regulates the expression levels of EPHA4 at both the mRNA and protein levels.

EPHA4 is a member of the ephrin family, and the ephrin family has been demonstrated to play a role in neurological functional recovery after ischemic stroke by regulating various processes such as angiogenesis, neurogenesis, axonal reorganization and synaptic plasticity [16]. EPHA4 is the most abundant ephrin receptor in the CNS, which is

distributed on pyramidal neurons of the hippocampus and is able to interact with all ephrin ligands [9,14,15]). The upregulated protein expression of EPHA4 and its ephrin-A3 ligand was found in the hippocampus during the early stages of ischaemia [9]. The application of clustered EPHA4 downregulated the expression of astrocytic glutamate transporters and aggravated neuronal loss and hippocampus-dependent spatial memory impairment by activating ephrin-A3 reverse signalling [22]. Additionally, Robin et al. indicated that motor recovery after photothrombotic stroke was markedly enhanced in transgenic mice with reduced levels of EPHA4 [16]. In the present study, the knockdown of EPHA4 by siRNA was found to improve the cell survival rate in the OGD model, which suggested that the knockdown of EPHA4 protected cell viability in the OGD model. Furthermore, this protection of EPHA4 was mediated by miR-145, which highlighted that the miR-145-EPHA4 interaction might play a role in the process of ischemic stroke.

To further research the underlying mechanism of the miR-145-EPHA4 interaction in ischemic stroke, three lncRNAs (LOC105370660, LINC01128 and LOC105376244) that co-expressed EPHA4 in the blood of patients with ischemic stroke and whose functions were not known were obtained in our previous RNA-sequencing study [12]. In the present study, the upregulation of LOC105370660 and LOC105376244 was observed in the OGD model, which suggested that these two lncRNAs might be involved in the process of ischemic stroke. By regulating EPHA4, the miR-145 inhibitor and miR-145 mimic upregulated or downregulated the expression of LOC105376244, respectively, in the OGD model. Hence, we hypothesized that LOC105376244 might be involved in ischemic stroke regulated by the miR-145-EPHA4 interaction.

In conclusion, this study first demonstrated that miR-145 indeed targets EPHA4 and regulates the expression of EPHA4 at both the mRNA and protein levels. Under oxygen-glucose deprivation, miR-145 exerted a protective role against cell viability in the OGD model by downregulating EPHA4, which suggests their potential roles in ischemic stroke. Moreover, LOC105376244, an aberrantly expressed lncRNA in patients with ischemic stroke but not normal controls, was regulated by the miR-145–EPHA4 interaction, which might also be involved in ischemic stroke. Further research using animal models is needed to confirm our findings and explore the mechanism of the miR-145-EPHA4-LOC105376244 interaction in ischemic stroke.

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Conflict of interest statement

The authors declare that they have no conflict of interest

Authors' contributions

Wenzhen He, De Cai and Duncan Wei made substantial contributions to conception and design; Siqia Chen and Xianguang Chen performed the experiment. Shunxian Li and Wenjie Chen collected and analyzed the data; De Cai and Duncan Wei interpreted the data; All authors were involved in drafting and revising the manuscript and gave final approval of the manuscript.

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