

Long Non-Coding RNA SNHG7 Alleviates Oxygen and Glucose Deprivation/Reoxygenation-Induced Neuronal Injury by Modulating miR-9/SIRT1 Axis in PC12 Cells: Potential Role in Ischemic Stroke

This article was published in the following Dove Press journal:
Neuropsychiatric Disease and Treatment

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Objective: The roles of long non-coding RNA (lncRNAs) in ischemic stroke (IS) have been widely illustrated. Here, we focused on the function and mechanism of lncRNA *SNHG7* in IS.

Methods: Middle cerebral artery occlusion (MCAO) was used for inducing *mice* to establish IS models in vivo. Oxygen and glucose deprivation/reoxygenation (OGD/R) was used for treating PC12 cells to establish IS models in vitro. Relative expression of *SNHG7* and *miR-9* was determined by qRT-PCR. The neuronal injury was assessed by measuring relative activity of ROS, malondialdehyde (MDA) level and cell viability. Cell viability was determined by MTT assay. Dual-luciferase reporter (DLR) assay was employed to test the target of *SNHG7* or *miR-9*. Western blot was used to determine the protein expression of *SIRT1*. Apoptosis rate was measured by flow cytometry.

Results: *SNHG7* was down-regulated and *miR-9* was up-regulated by MCAO treatment in brain tissues of *mice* and by OGD/R treatment in PC12 cells. Overexpression of *SNHG7* or suppression of *miR-9* decreased the relative activity of ROS and the MDA level as well as enhancing cell viability, and *SNHG7* reduced apoptosis rate in OGD/R-induced PC12 cells (IS cells). *miR-9* was targeted by *SNHG7* and *SIRT1* was targeted by *miR-9*. The protein expression of *SIRT1* was reduced by OGD/R treatment in PC12 cells. The suppressive effects of *SNHG7* on the relative activity of ROS, the MDA level and apoptosis rate as well as the promotion effect of *SNHG7* on cell viability were reversed by *miR-9* mimics or sh-*SIRT1* in IS cells.

Conclusion: lncRNA *SNHG7* alleviated OGD/R-induced neuronal injury by mediating *miR-9/SIRT1* axis in vitro.

Keywords: ischemic stroke, oxygen and glucose deprivation/reoxygenation, small nucleolar RNA host gene 7, miR-9, silent information regulator factor 2-related enzyme 1

Introduction

Ischemic stroke (IS) is considered as a cerebrovascular disease with high disability and mortality, and it has high prevalence in the elderly.¹ The major symptoms of IS include facial numbness, impair of memory, trouble speaking and partial paralysis.^{2,3} In the past few years, there is an increasing incidence rate of IS in people, which may result from multitudinous factors, such as hypercholesterolemia, hypertension, obesity and diabetes.⁴ Although traditional clinical treatments for IS⁵ including thrombolytic therapy, percutaneous intravascular interventions and

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medication have achieved effectiveness, the unsatisfactory result of IS therapy urges us seeking for new strategies to relieve IS.

Long non-coding RNAs (lncRNAs), an abundant component of the mammalian transcriptome, are a subtype of RNA with a length of more than 200 nucleotides and lack protein-coding ability.⁶ Previous studies have reported the potential roles of lncRNAs in IS.⁷ Chen et al have reported that silencing of lncRNA *ROR* can alleviate injury of PC12 cells in IS cell models induced by hypoxia/reoxygenation (H/R).⁸ Lv et al have revealed that lncRNA *SNHG1* alleviates apoptosis and inflammation in IS cell model.⁹ Xiang et al have indicated that inhibition of lncRNA *MEG3* markedly enhances cell viability and reduces cell apoptosis in IS cell model induced by oxygen-glucose deprivation and reoxygenation (OGD/R).¹⁰ Specifically, lncRNA small nucleolar RNA host gene 7 (*SNHG7*), located on chromosome 9q34.3, has been reported to make imperative impacts on diverse diseases.^{11–13} *SNHG7* suppresses cell viability of cardiac fibroblasts in cardiac fibrosis.¹¹ *SNHG7* can inhibit the high glucose-induced proliferation and migration of human retinal endothelial cells in diabetic retinopathy (DR).¹² *SNHG7* can promote viability of cartilage cells and suppress apoptosis of cartilage cells in osteoarthritis.¹³ However, the role and mechanism of *SNHG7* in IS have not been elucidated till now.

MicroRNAs (miRNAs), a class of small intracellular molecules with 18–23 nucleotides in length, can regulate gene expression at the post-transcriptional level.¹⁴ Many miRNAs are emerging as important molecular mediators of IS, such as *miR-143*,¹⁵ *miR-27b*¹⁶ and *miR-191*.¹⁷ As one of the most widely explored miRNAs, *miR-9* has also been revealed to exert vital roles in IS.^{18–20} The suppression of *miR-9* can reduce inflammation and neuronal apoptosis to alleviate neuronal injury in IS cell models induced by OGD/R.¹⁸ Overexpression of *miR-9* promotes post-ischemic cell viability of the neuronal cells in IS cell models induced by OGD.¹⁹ Overexpression of *miR-9* inhibits the apoptosis of neurons in IS cell models induced by OGD.²⁰ Nonetheless, the regulatory effect of *SNHG7* on *miR-9* in IS has not been clarified.

Sirtuins (*SIRT*s) are a family of nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylases.²¹ According to the report, seven closely-related *SIRT* family members have been identified, including silent information regulator factor 2-related enzyme 1 (*SIRT1*), *SIRT2*, *SIRT3*, *SIRT4*, *SIRT5*, *SIRT6* and *SIRT7*.^{22,23} Abundant

studies have revealed the roles of lncRNAs and miRNAs in these sirtuin genes, such as the *miR-140-5p/SIRT2* axis in myocardial oxidative stress,²⁴ the *miR-421/SIRT3* axis in H/R-induced oxidative stress,²⁵ the lncRNA *HMMR-AS1/miR-138/SIRT6* axis in lung adenocarcinoma²⁶ and the *miR-20b/SIRT7* axis in high glucose-induced podocyte apoptosis.²⁷ *SIRT1* is a NAD⁺-dependent deacetylase.²⁸ Given that the expression of *SIRT1* reduces with aging and *SIRT1* slows down cellular senescence, researchers have focused on the involvement of *SIRT1* in IS.^{29,30} Teertam et al have reported that *miR-149-5p* plays a regulatory role in neuronal cell death through *SIRT1/p53* axis during IS.³¹ Rao et al have revealed that repression of *miR-217* protects neurons against OGD/R-induced injury by targeting *SIRT1*.³² However, the regulatory mechanism between *SIRT1* and *miR-9* has not been explored in IS. In the current study, the expression and role of *SNHG7* and *miR-9* were probed in IS cell models. Furthermore, to further understand the molecular mechanism of *SNHG7* in IS, the regulatory interrelation among *SNHG7*, *miR-9* and *SIRT1* was explored in IS cell models. Our finding might provide a novel target for the prevention and treatment of IS.

Materials and Methods

The Establishment of the Middle Cerebral Artery Occlusion (MCAO) Model

This study was performed after obtaining local ethical committee approval of Zibo First Hospital (No. 2,018,013), and all experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Male C57BL/6 mice (6–8 weeks) were bought from Shanghai Animal Laboratory Center (Shanghai, China). All mice were housed under conditions of constant temperature and humidity. Food and water were available ad libitum. Mice were divided into two groups (the sham group and the MCAO group), with five mice in each group. Mice in the MCAO group were anesthetized with 1.5–3% isoflurane. A 6–0 surgical nylon filament, coated with 1% poly-L-lysine and blunted at the tip, was inserted 10 mm into the internal carotid to occlude the origin of the middle cerebral artery (MCA) in mice. Cerebral blood flow (CBF) was determined in the area of MCA by laser Doppler flowmetry (Moor Instruments, Devon, UK). Only mice with >80% decrease of CBF in the MCA territory were included in the present study. After MCA obstruction for 1 h, the sutures were removed to recover blood flow and mice were allowed

to recover for 24 h. In the sham group, all procedures were identical except for inserting an intraluminal filament. Finally, *mice* were anesthetized with pentobarbital sodium (50 mg/kg) and sacrificed by cervical dislocation. Brain tissues of *mice* were collected for following experiments.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from PC12 cells or brain tissues of *mice* by using TRIzol[®] reagent (Takara, Dalian, China) and converted to complementary DNAs (cDNAs) with a PrimeScript[™] RT reagent kit with gDNA Eraser (Takara). The qRT-PCR was performed by using SYBR Premix Ex Taq II (Takara) and the reaction procedure was shown as follows: initial denaturation at 95°C for 30 s, 40 cycles of 95°C for 10 s, 72°C for 45 s and 60°C for 15 s. The primers used in the current study were shown in Table 1. Finally, the relative expression of *SNHG7* and *miR-9* was calculated by the $2^{-\Delta\Delta C_t}$ method, and *GAPDH* was chosen as an internal reference.

Cell Culture

A *rat* adrenal pheochromocytoma cell line PC12 with specific characteristic of neural cells was used in accordance with previous studies.^{33–36} PC12 cells were bought from American Type Culture Collection (ATCC, USA). PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and incubated at 37°C under a humidified atmosphere with 5% CO₂.

Establishment of OGD/R Model

The OGD/R model in PC12 cells was established as previously reported.³⁷ Briefly, PC12 cells cultured in glucose-free DMEM medium were incubated at 37°C in a hypoxia condition with 5% CO₂ and 95% N₂. After hypoxia treatment for 4 h, PC12 cells were transferred back to normal DMEM medium containing 10% FBS and 4.5 g/L glucose, and cells were incubated at 37°C under an atmosphere of 95% air and 5% CO₂ to recover. Then PC12 cells were

harvested after recovery for 4 h, 8 h, 12 h and 24 h for following experiments. PC12 cells in the sham group did not receive the treatment of OGD/R.

Cell Transfection

The pcDNA3.1 (His tag in-frame with the 3'-end of the cDNA; CMV promoter; neomycin resistance), pcDNA-*SNHG7* (containing full-length *rat SNHG7*), short hairpin (sh)-negative control (NC), sh-*SNHG7*, miR-NC, *miR-9* mimics, mimics NC, *miR-9* inhibitor, inhibitor NC and sh-*SIRT1* were bought from RiboBio Company (Beijing, China). Additionally, we have listed the sequences of shRNAs or miRs as follows: sh-*SNHG7* (5'-GCCTGGGTGTTGCTGTGTATT-3'), sh-*SIRT1* (5'-CCATTCTTCAAGTTTGCAA-3'), *miR-9* mimics (5'-UCAUACAGCUAGAUAAACCAAAGA-3'), mimics NC (5'-UCACAGUGAACCGGUCUCUUU-3'), *miR-9* inhibitor (5'-GCTAGATAACCAAAG-3') and inhibitor NC (5'-ACGTC TATACGCCA-3'). When cells grew 80–90% confluence, the above transcripts were transfected into PC12 cells with Lipofectamine3000 (Invitrogen, Carlsbad, CA, USA) for 48 h.

Detection of Reactive Oxygen Species (ROS) and Malondialdehyde (MDA)

A lipid peroxidation product MDA assay kit (ab118970, Abcam, MA, USA) was used for measuring the MDA level under the guidance of the manufacturer's instructions. A cellular ROS assay kit (ab113851, Abcam) was employed to determine relative activity of ROS.

3-(4, 5-Dimethyl-2-Thiazolyl)-2, 5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

PC12 cells were plated in 96-well plates at a density of 5×10^3 /well and then incubated with 20 μ L MTT solution (Beyotime, Shanghai, China). After incubation for 4 h, dimethyl sulfoxide (DMSO; 150 μ L) was added to dissolve the remaining formazan crystals. Cell viability was evaluated by measuring the optical density (OD) at 450

Table 1 Primers for Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in Current Study

Gene	Forward	Reverse
<i>SNHG7</i>	5'-GTGACTTCGCCTGTGATGGA-3'	5'-GGCCTCTATCTGTACCTTTATTCC-3'
<i>miR-9</i>	5'-GCCCGCTCTTTGGTTATCTAG-3'	5'-CCAGTGCGAGGGTCCGAGGT-3'
<i>SIRT1</i>	5'-ATGAAGCACCAACCGTATC-3'	5'-CTGAATTGACCTTGACTGATG-3'
<i>GAPDH</i>	5'-ATTCCACCCATGGCAAATTC-3'	5'-AGCATCGCCCCACTTGATT-3'

nm with a microplate reader (BMG LABTECH, Durham, NC, USA).

Dual-Luciferase Reporter (DLR) Assay

The 3'-UTR fragment of wild type (WT) *SNHG7* or WT *SIRT1* containing the complementary sequence of *miR-9* was synthesized and introduced into a pGL3 Basic Vector (Promega, Madison, WI) to generate *SNHG7* WT vector or *SIRT1* WT vector. In a similar way, the 3'-UTR fragment of mutant type (mut) *SNHG7* or mut *SIRT1* including the mutant sequence of *miR-9* was synthesized and inserted into a pGL3 Basic Vector (Promega) to form *SNHG7* mut vector or *SIRT1* mut vector. Then PC12 cells were co-transfected with a WT vector/mut vector and *miR-NC*/*miR-9* mimics for 48 h. Then relative luciferase activity was measured by Dual-Luciferase Reporter Assay System (Promega).

Western Blot

Total proteins were extracted from PC12 cells and brain tissues of mice by RIPA buffer (Beyotime). Equal proteins of each sample were separated by 10% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE). Next, separated proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA) and blocked with 5% non-fat milk for 1 h. Then protein samples were incubated with the following primary antibodies overnight at 4°C: anti-*SIRT1* (1:1000, ab189494, Abcam) and anti- β -actin (1:6000, ab115777, Abcam). After the membranes were washed with tris buffered saline Tween (TBST), the horseradish-conjugated secondary antibody (1:10,000, ab205718, Abcam) was added to incubate at 37°C for 2 h. The immune-reactivity was visualized with a Chemiluminescence Detection Kit (Thermo Fisher Scientific, Shanghai, China) and the relative protein expression of *SIRT1* over the β -actin was quantified by Gel-pro Analyzer software (Media Cybernetics, Maryland, USA).

Flow Cytometry

Apoptosis rate was analyzed by an Annexin V-FITC/PI kit (Beyotime) on flow cytometry. Apoptotic cells (PC12 cells) were labeled with FITC-AnnexinV and PI for 30 min in the dark. The fluorescence was determined on cytoFLEX LX flow cytometer (Beckman-Coulter Electronics, Jiangsu, China) using CytExpert software. Quadrants were positioned on Annexin V/PI plots to distinguish apoptotic cells

(Annexin V + /PI−, Annexin V + /PI+). Apoptotic rate = apoptotic cells/total cells × 100%.

Bioinformatic Analysis

The targets of *SNHG7* were predicted by starbase (<http://starbase.sysu.edu.cn/agoClipRNA.php?>), and a total of 82 targets for *SNHG7* were predicted. Among these targets, *miR-9* plays an important role in IS, and the regulatory effect of *SNHG7* on *miR-9* in IS has not been investigated. Thus, we chose *miR-9* as a target in this study.

The targets of *miR-9* were predicted by starbase (<http://starbase.sysu.edu.cn/agoClipRNA.php?>), and a total of 5,413 targets for *miR-9* were predicted. Among these targets, *SIRT1* is important in IS, and the regulatory mechanism between *miR-9* and *SIRT1* has not been explored in IS. Additionally, we have verified the targeting relationship between *SIRT1* and *miR-9* by miRDB. Therefore, we chose *SIRT1* as a target in this study.

Statistical Analysis

Experiments of the present study were implemented in triplicate. The SPSS Statistics 22.0 was utilized for the statistical analysis of data. All data were shown as the mean ± standard deviation. Student's *t*-test was employed to assess the differences between two groups. The One-way ANOVA method was used for multiple comparisons, followed by a Tukey's multiple comparisons test for pairwise comparisons. The *P* value less than 0.05 was regarded as statistically significant.

Results

SNHG7 Was Down-Regulated in Brain Tissues of MCAO-Induced Mice and OGD/R-Induced PC12 Cells, and It Mitigated OGD/R-Induced Neuronal Injury in PC12 Cells

In order to explore the function of *SNHG7* in IS, we firstly determined the relative expression of *SNHG7* in brain tissues of MCAO mice and OGD/R-induced PC12 cells by qRT-PCR. The result showed that *SNHG7* was markedly decreased in brain tissues of MCAO mice compared with brain tissues of mice without MCAO treatment ($P < 0.001$, Figure 1A). *SNHG7* was also remarkably decreased by OGD/R treatment at 4 h, 8 h and 12 h in PC12 cells ($P < 0.001$, Figure 1B).

Then we silenced and overexpressed *SNHG7*. As shown in Figure 1C, *SNHG7* was up-regulated by transfection of pcDNA-*SNHG7* and down-regulated by transfection of sh-*SNHG7* in PC12 cells (all $P < 0.001$). The results of functional assays demonstrated that the relative activity of ROS and the MDA level of PC12 cells in the OGD/R group were increased compared with the NC group, while cell viability of PC12 cells in the OGD/R group was notably decreased in comparison with the NC group (all $P < 0.001$, Figure 1D–F). Of note, the relative activity of ROS and the MDA level were reduced by overexpression of *SNHG7* in OGD/R-induced PC12 cells, and cell viability was enhanced by overexpression of *SNHG7* in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 1D–F). In comparison to overexpression of *SNHG7*, knock-down of *SNHG7* had opposite effects on cell viability,

relative activity of ROS and the MDA level in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 1D–F).

SNHG7 Could Serve as a Competing Endogenous RNA (ceRNA) for miR-9

To further explore the mechanism of *SNHG7* in IS, we predicted the target genes of *SNHG7* by starbase2.0. We observed that *SNHG7* had potential binding sites for *miR-9* (Figure 2A). Then the interaction between *SNHG7* and *miR-9* was tested by DLR assay. The result showed that relative luciferase activity of PC12 cells was significantly reduced by co-transfection of *SNHG7* WT vector and *miR-9* mimics ($P < 0.001$, Figure 2B), whereas relative luciferase activity of PC12 cells showed no statistical change after co-transfection of *SNHG7* mut vector and *miR-9* mimics (Figure 2B). Relative expression of *miR-9* was increased in brain tissues

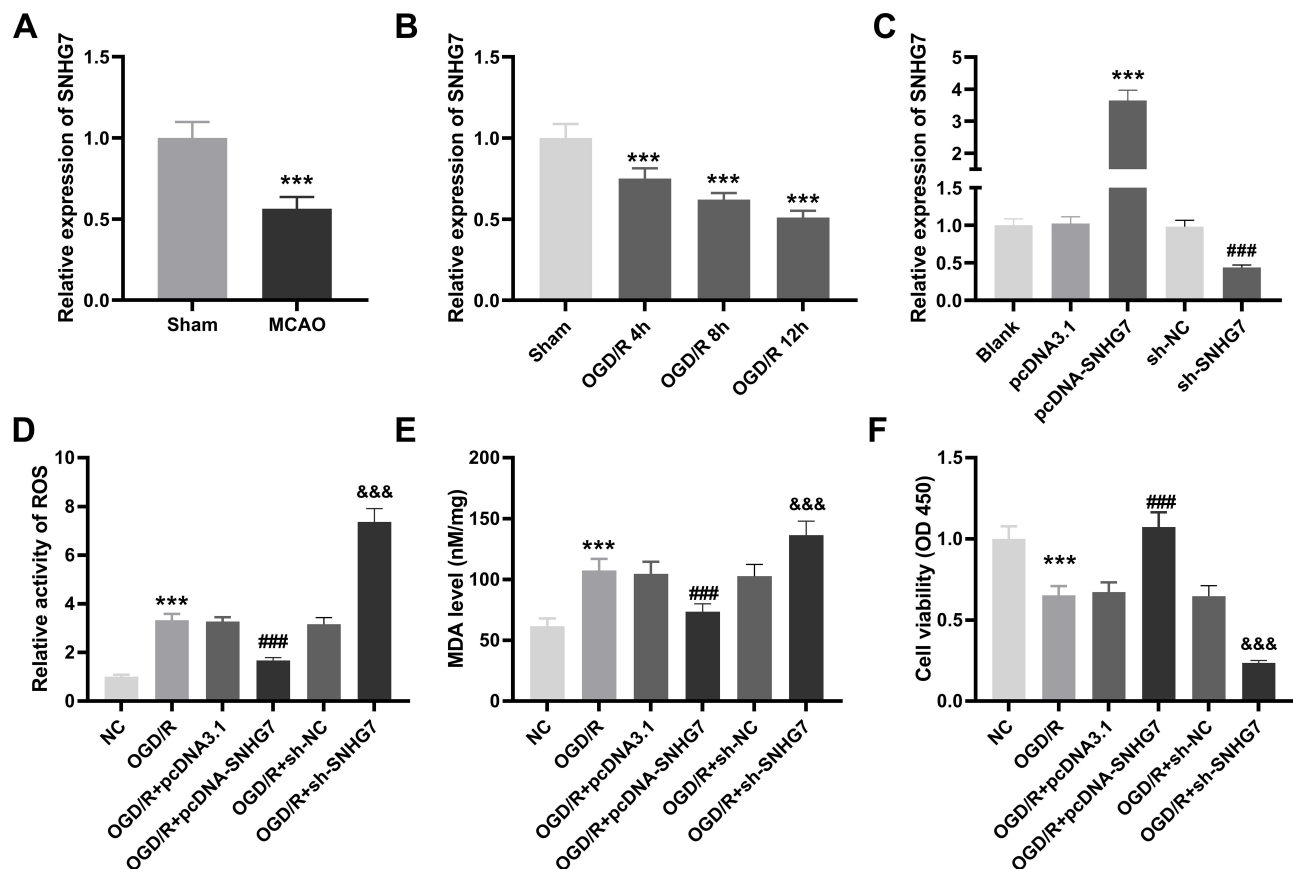


Figure 1 *SNHG7* is down-regulated in brain tissues of middle cerebral artery occlusion (MCAO)-induced mice and oxygen and glucose deprivation/reoxygenation (OGD/R)-induced PC12 cells, and it mitigates OGD/R-induced neuronal injury in PC12 cells. **(A)** Relative expression of *SNHG7* was detected by quantitative real-time polymerase chain reaction (qRT-PCR) in brains tissues of MCAO mice. *** $P < 0.001$, vs Sham. $n = 5$. **(B)** Relative expression of *SNHG7* was determined by qRT-PCR in OGD/R-induced PC12 cells. *** $P < 0.001$, vs Sham. There are three replicates in each experimental group. **(C)** Relative expression of *SNHG7* was determined by qRT-PCR in PC12 cells after transfection of pcDNA-*SNHG7* or sh-*SNHG7*. *** $P < 0.001$, vs pcDNA3.1. **** $P < 0.001$, vs sh-NC. There are three replicates in each experimental group. **(D)** Relative activity of ROS was determined in OGD/R-induced PC12 cells. *** $P < 0.001$, vs NC. **** $P < 0.001$, vs OGD/R + pcDNA3.1. &&& $P < 0.001$, vs OGD/R + sh-NC. **(E)** The MDA level was determined in OGD/R-induced PC12 cells. *** $P < 0.001$, vs NC. **** $P < 0.001$, vs OGD/R + pcDNA3.1. &&& $P < 0.001$, vs OGD/R + sh-NC. **(F)** The cell viability was determined by MTT assay in OGD/R-induced PC12 cells. *** $P < 0.001$, vs NC. **** $P < 0.001$, vs OGD/R + pcDNA3.1. &&& $P < 0.001$, vs OGD/R + sh-NC. The experiments in Figure 1D–F were implemented at 12 h after OGD/R treatment, and there are three replicates in each experimental group.

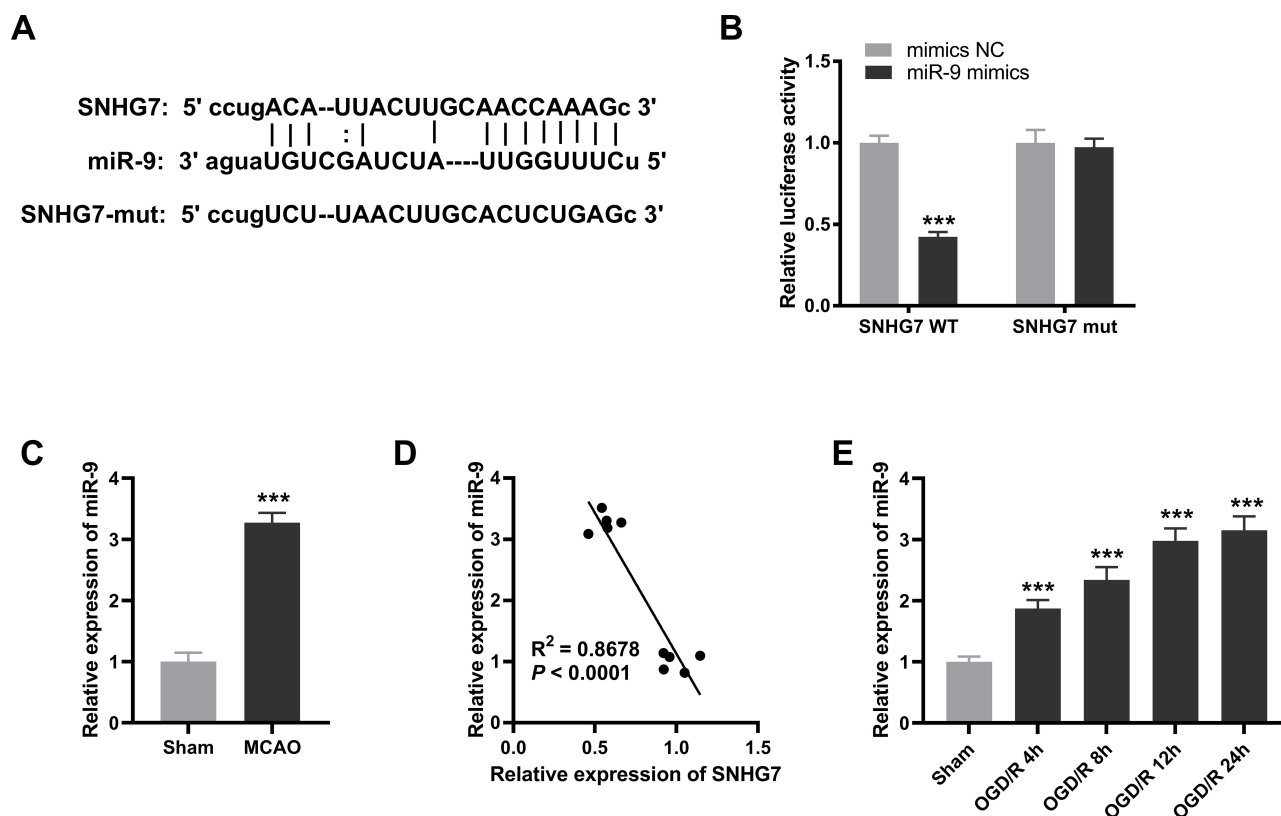


Figure 2 SNHG7 can serve as a competing endogenous RNAs (ceRNA) for miR-9. **(A)** The binding sequences between SNHG7 and miR-9 were predicted by starbase2.0. **(B)** The interaction between SNHG7 and miR-9 in PC12 cells was validated by dual-luciferase reporter (DLR) assay. *** $P < 0.001$, vs miR-NC. There are three replicates in each experimental group. **(C)** Relative expression of miR-9 was detected by quantitative real-time polymerase chain reaction (qRT-PCR) in brain tissues of middle cerebral artery occlusion (MCAO) mice. *** $P < 0.001$, vs Sham. $n = 5$. **(D)** The relationship between SNHG7 and miR-9 in brain tissues of MCAO mice was analyzed by Pearson's correlation analysis. $P < 0.0001$. $n = 5$. **(E)** Relative expression of miR-9 in oxygen and glucose deprivation/reoxygenation (OGD/R)-induced PC12 cells was detected by qRT-PCR. *** $P < 0.001$, vs Sham. There are three replicates in each experimental group.

of MCAO mice in relative to that in brain tissues of mice without MCAO treatment ($P < 0.001$, Figure 2C). The result of Pearson's correlation analysis showed a negative correlation between SNHG7 and miR-9 in brain tissues of MCAO mice ($P < 0.0001$, Figure 2D). In addition, relative expression of miR-9 was elevated by OGD/R treatment at 4 h, 8 h, 12 h and 24 h in PC12 cells ($P < 0.001$, Figure 2E).

Inhibition of miR-9 Alleviated OGD/R-Induced Neuronal Injury in PC12 Cells

Afterwards, we explored the expression and function of miR-9 in PC12 cells. We observed that the expression of miR-9 was obviously elevated by OGD/R treatment in PC12 cells ($P < 0.001$, Figure 3A). MiR-9 was evidently increased by transfection of miR-9 mimics and decreased by transfection of miR-9 inhibitor in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 3A). The relative activity of ROS and the MDA level of PC12 cells in the OGD/R group were increased in comparison with the NC group, whereas the viability of PC12 cells in the OGD/R group was significantly

decreased in comparison with the NC group (all $P < 0.001$, Figure 3B–D). Importantly, the relative activity of ROS and the MDA level were reduced by inhibition of miR-9 in OGD/R-induced PC12 cells, while cell viability was enhanced by inhibition of miR-9 in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 3B–D). In contrast to inhibition of miR-9, up-regulation of miR-9 had opposite effects on cell viability, the relative activity of ROS and the MDA level in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 3B–D).

SIRT1 Was Targeted by miR-9

Subsequently, we predicted the target genes of miR-9 with starbase2.0. It was observed that SIRT1 had a conserved binding region of miR-9 (Figure 4A). The result of DLR revealed that relative luciferase activity of PC12 cells was reduced by co-transfection of SIRT1 WT vector and miR-9 mimics ($P < 0.001$, Figure 4B), whereas relative luciferase activity of PC12 cells showed no significant change after co-transfection of SIRT1 mut vector and miR-9 mimics (Figure 4B). The result of Western blot showed that

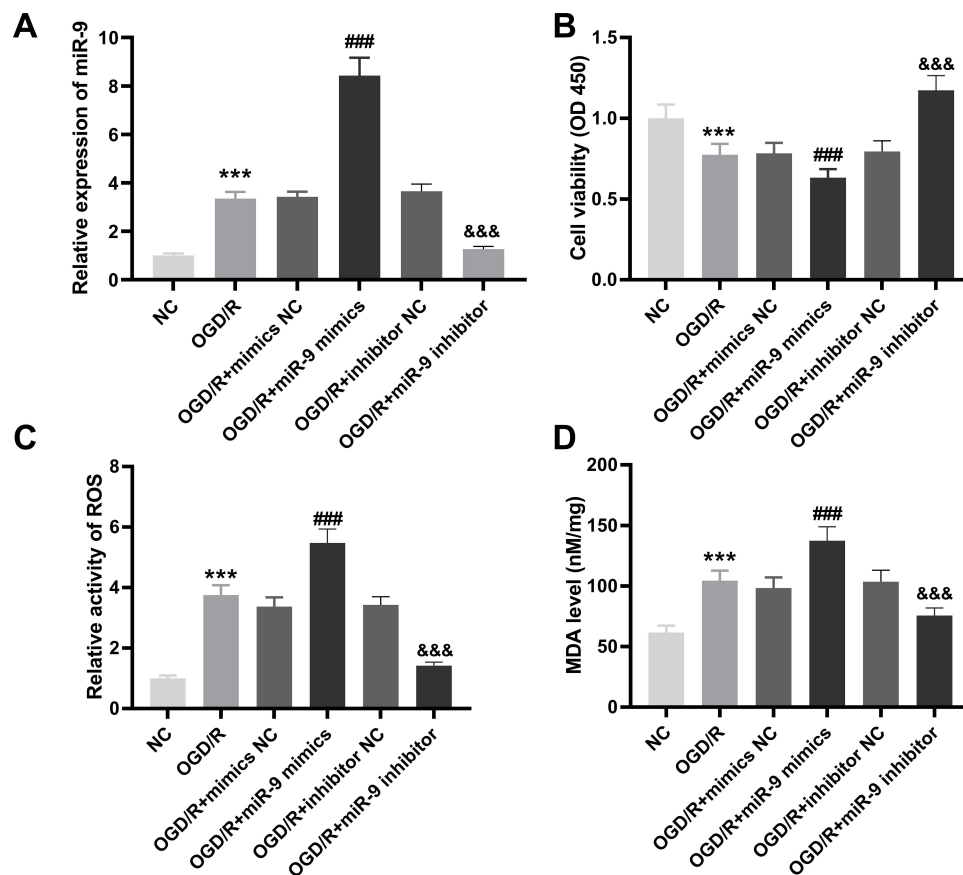


Figure 3 Inhibition of *miR-9* alleviates oxygen and glucose deprivation/reoxygenation (OGD/R)-induced neuronal injury in PC12 cells. **(A)** Relative expression of *miR-9* was determined by real-time polymerase chain reaction (qRT-PCR) in OGD/R-induced PC12 cells. *** $P < 0.001$, vs. NC. #### $P < 0.001$, vs OGD/R + mimics NC. &&& $P < 0.001$, vs OGD/R + inhibitor NC. **(B)** The cell viability was determined by MTT assay in OGD/R-induced PC12 cells. *** $P < 0.001$, vs NC. #### $P < 0.001$, vs OGD/R + mimics NC. &&& $P < 0.001$, vs OGD/R + inhibitor NC. **(C)** Relative activity of ROS was determined in OGD/R-induced PC12 cells. *** $P < 0.001$, vs NC. #### $P < 0.001$, vs OGD/R + mimics NC. &&& $P < 0.001$, vs OGD/R + inhibitor NC. **(D)** The MDA level was determined in OGD/R-induced PC12 cells. *** $P < 0.001$, vs NC. #### $P < 0.001$, vs OGD/R + mimics NC. &&& $P < 0.001$, vs OGD/R + inhibitor NC. The experiments in Figure 3 were implemented at 12 h after OGD/R treatment, and there are three replicates in each experimental group.

SIRT1 was down-regulated in brain tissues of MCAO mice in relative to that in brain tissues of mice without MCAO treatment ($P < 0.001$, Figure 4C). There was a negative correlation between *SIRT1* and *miR-9* in brain tissues of MCAO mice ($P < 0.0001$, Figure 4D). Furthermore, *SIRT1* was decreased by OGD/R treatment at 4 h, 8 h, 12 h and 24 h in PC12 cells ($P < 0.001$, Figure 4E).

LncRNA SNHG7 Mitigated OGD/R-Induced Neuronal Injury via Regulating *miR-9/SIRT1* Axis in PC12 Cells

Based on above results, we hypothesized that SNHG7 might repress the injury of PC12 cells through mediating *miR-9/SIRT1* axis. To verify this, we firstly explored the interrelation among SNHG7, *miR-9* and *SIRT1*. The result of Western blot demonstrated that *SIRT1* was up-regulated by overexpression of SNHG7, and the promotion effect of SNHG7 on

SIRT1 was reversed by *miR-9* mimics or sh-*SIRT1* in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 5A). Then rescue assays were performed. The results indicated that cell viability was promoted by overexpression of SNHG7, and the promotion effect of SNHG7 on cell viability was reversed by overexpression of *miR-9* or knockdown of *SIRT1* in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 5B). The relative activity of ROS and the MDA level were reduced by overexpression of SNHG7, and the suppression effects of SNHG7 on the relative activity of ROS and the MDA level were reversed by overexpression of *miR-9* or knockdown of *SIRT1* in PC12 cells induced by OGD/R (all $P < 0.001$, Figure 5C and D). Moreover, apoptosis rate of OGD/R-induced PC12 cells was reduced by up-regulation of SNHG7, while the reduction effect of SNHG7 on apoptosis rate was reversed by up-regulation of *miR-9* or knockdown of *SIRT1* in OGD/R-induced PC12 cells (all $P < 0.001$, Figure 5E).

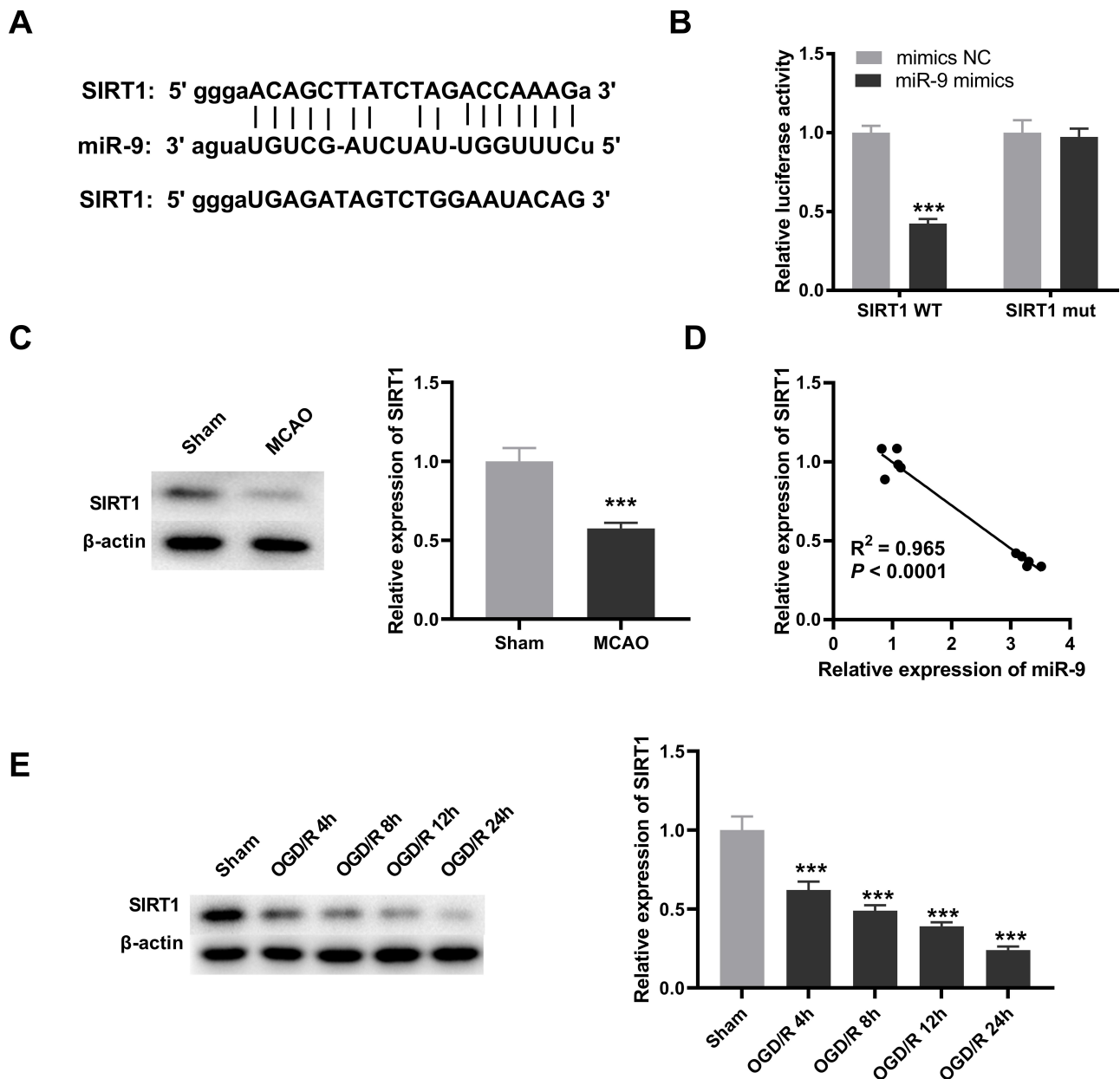


Figure 4 *SIRT1* is targeted by *miR-9*. **(A)** The binding sequence between *SIRT1* and *miR-9* was predicted by starbase2.0. **(B)** The interaction between *SIRT1* and *miR-9* in PC12 cells was validated by dual-luciferase reporter (DLR) assay. *** $P < 0.001$, vs *miR-NC*. There are three replicates in each experimental group. **(C)** Relative expression of *SIRT1* was detected by Western blot in brain tissues of middle cerebral artery occlusion (MCAO) mice. *** $P < 0.001$, vs Sham. $n = 5$. **(D)** The relationship between *SIRT1* and *miR-9* in brain tissues of MCAO mice was analyzed by Pearson's correlation analysis. $P < 0.0001$. $n = 5$. **(E)** Relative expression of *SIRT1* in oxygen and glucose deprivation/reoxygenation (OGD/R)-induced PC12 cells was detected by Western blot. *** $P < 0.001$, vs Sham. There are three replicates in each experimental group.

Discussion

IS is a severe neurological disease with serious impairment of the neurological function.³⁸ Growing literature has shown that down-regulation of lncRNAs is implicated in the pathological process of IS, such as lncRNA *Oprm1*,³⁹ lncRNA *ZFAS1*⁴⁰ and lncRNA *ANRIL*.³⁴ Coincident with expression trend of previous studies, we also observed that *SNHG7* was down-regulated by MCAO treatment in brain tissues of mice and by OGD/R treatment in PC12 cells, implying that

SNHG7 might involve in the pathologies of IS. Besides, numerous studies have revealed the key role of *SNHG7* in many diseases.^{11–13} *SNHG7* can inhibit the high glucose-induced proliferation and migration of human retinal endothelial cells in DR.¹² *SNHG7* promotes cell viability and represses cell apoptosis in osteoarthritis.¹³ *SNHG7* suppresses cell viability of cardiac fibroblasts in cardiac fibrosis.¹¹ In the present study, we found that the relative activity of ROS, apoptosis rate and the MDA level were

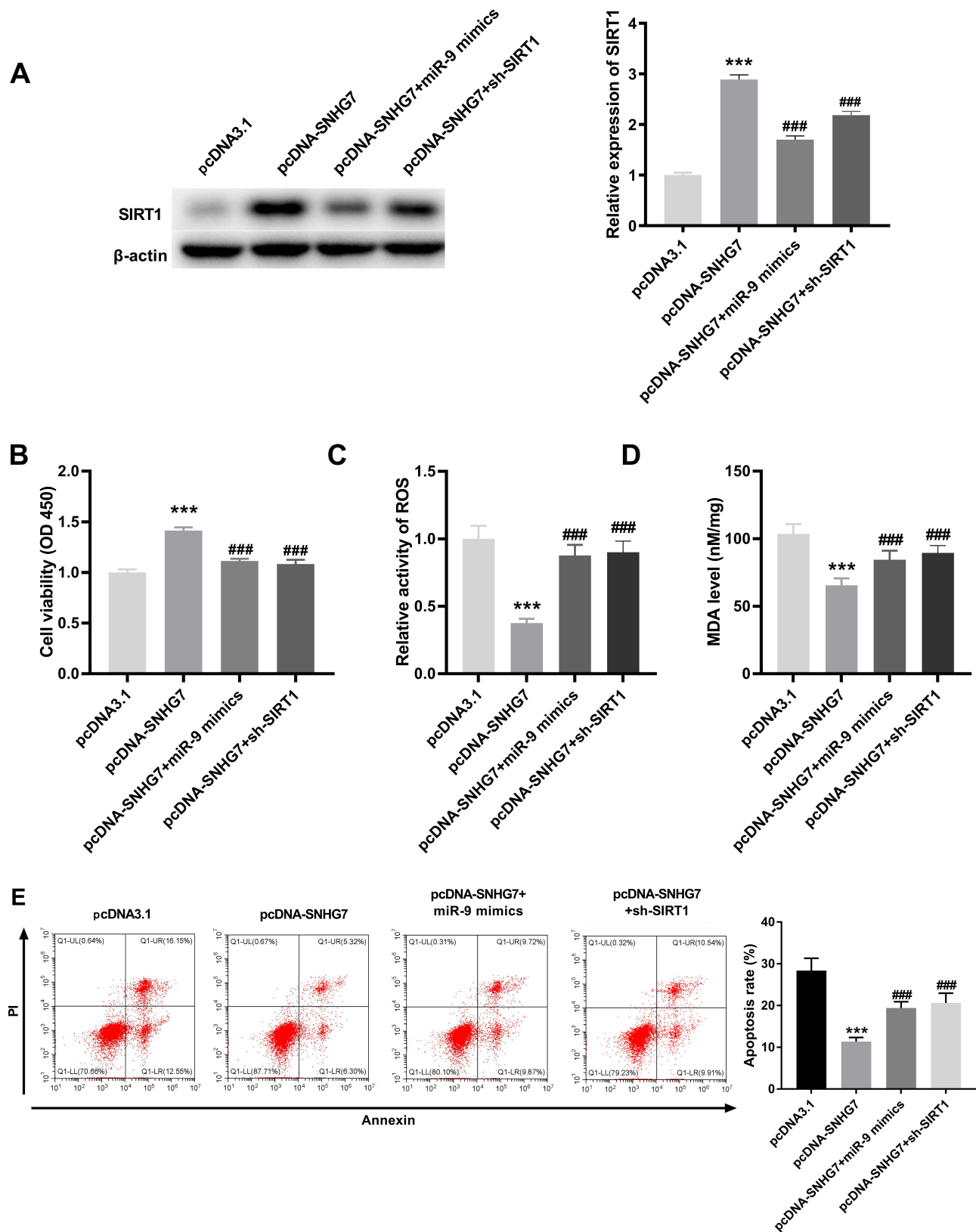


Figure 5 LncRNA *SNHG7* lightens oxygen and glucose deprivation/reoxygenation (OGD/R)-induced neuronal injury via regulating *miR-9/SIRT1* axis in PC12 cell. **(A)** Relative expression of *SIRT1* was determined by Western blot in OGD/R-induced PC12 cells. *** $P < 0.001$, vs pcDNA3.1. #### $P < 0.001$, vs pcDNA-SNHG7. **(B)** The cell viability was determined by MTT assay in OGD/R-induced PC12 cells. *** $P < 0.001$, vs pcDNA3.1. #### $P < 0.001$, vs pcDNA-SNHG7. **(C)** Relative activity of ROS was determined in OGD/R-induced PC12 cells. *** $P < 0.001$, vs pcDNA3.1. #### $P < 0.001$, vs pcDNA-SNHG7. **(D)** The MDA level was determined in OGD/R-induced PC12 cells. *** $P < 0.001$, vs pcDNA3.1. #### $P < 0.001$, vs pcDNA-SNHG7. **(E)** Apoptosis rate was determined by flow cytometry in OGD/R-induced PC12 cells. *** $P < 0.001$, vs pcDNA3.1. #### $P < 0.001$, vs pcDNA-SNHG7. The experiments in figure 5 were implemented at 12 h after OGD/R treatment, and there are three replicates in each experimental group.

reduced by *SNHG7*, while the viability was stimulated by *SNHG7* in PC12 cells induced by OGD/R, suggesting that *SNHG7* mitigated OGD/R-induced neuronal injury in PC12 cells. Based on these outcomes, we inferred that *SNHG7* might be a promising target for IS therapy.

In recent years, the expression and function of *miR-9* in IS have drawn wide attention.^{18,19,41} Xue et al have revealed that *miR-9* is up-regulated by OGD treatment in neurons, and the suppression of *miR-9* remarkably alleviates neuronal injury in IS cell models by decreasing OGD-induced neuronal apoptosis.¹⁸ Ji et al have revealed that the level of serum *miR-9* is markedly higher in acute IS patients than that in non-stroke volunteers.⁴¹ Nampoothiri et al have reported that inhibition of *miR-9* enhances viability of neuronal cells in IS cell models.¹⁹ Consistently, we also observed that *miR-9* was increased by MCAO treatment in brain tissues of mice and by OGD/R induction in PC12 cells. Moreover, we observed that down-regulation of *miR-9* reduced the relative activity of ROS and the MDA level in PC12 cells induced by OGD/R, while promoted viability of PC12 cells induced by OGD/R. These results of functional assays revealed that inhibition of *miR-9* alleviated neuronal injury in IS cell models induced by OGD/R, and the role of *miR-9* in our study was similar to previous studies. Besides, *miR-9* has been revealed to interact with *SNHG7* in skeletal fracture⁴² and in malignant melanoma.⁴³ Similarly, we also found that *miR-9* was targeted by *SNHG7* and negatively correlated with *SNHG7*. Meanwhile, we discovered that over-expression of *miR-9* reversed the suppression effects of *SNHG7* on the relative activity of ROS and the MDA level as well as the promotion effect of *SNHG7* on cell viability in OGD/R-induced PC12 cells. Collectively, *SNHG7* might alleviate neuronal injury by sponging *miR-9* in IS cell models.

Previously, many studies have reported the expression and role of *SIRT1* in IS.^{29,30} Yan et al have revealed that *SIRT1* is down-regulated in the stroke group compared with the normal group, and rosuvastatin ameliorates cerebral infarction condition of rats with cerebral IS by *SIRT1*/NF- κ B pathway.⁴⁴ Teertam et al have indicated that the expression of *SIRT1* is down-regulated in the MCAO group in comparison with the sham group, and overexpression of *SIRT1* decreases the loss of pyramidal neuron in brains of IS mice.³¹ In line with previous literature, we found that *SIRT1* was down-regulated by MCAO treatment in brain tissues of mice and by OGD/R treatment in PC12 cells, suggesting that

SIRT1 might alleviate IS. Furthermore, it has been reported that *SIRT1* can act as the downstream target of *miR-199a* in cerebral ischemia⁴⁵ and *miR-149-5p* in acute IS.³¹ Here, we observed that *SIRT1* was targeted by *miR-9* and negatively correlated with *miR-9*. Based on above results, we speculated that inhibition of *miR-9* alleviated neuronal injury by targeting *SIRT1* in IS cell models. Meantime, we discovered that the knockdown of *SIRT1* reversed the inhibition effects of *SNHG7* on the relative activity of ROS, the MDA level and apoptosis rate as well as the promotion effect of *SNHG7* on cell viability in OGD/R-induced PC12 cells. At length, we deduced that *SNHG7* might attenuate OGD/R-induced neuronal injury by mediating *miR-9*/*SIRT1* axis in vitro.

Conclusions

To sum up, *SNHG7* was strikingly down-regulated by MCAO treatment in brain tissues of mice and by OGD/R treatment in PC12 cells. DLR assay verified that *SNHG7* could act as the sponge of *miR-9* and *SIRT1* was the downstream target of *miR-9* in brain tissues of mice. Overall, our results indicated that *SNHG7* mitigated neuronal injury through competition with *miR-9* to regulate the expression of *SIRT1* in IS cell models induced by OGD/R. *SNHG7* was a promising therapeutic target for attenuating IS. However, the functional experiments of *SNHG7* in vivo were not performed in our study, and further study is needed to verify our findings.

Abbreviations

Long non-coding RNA, lncRNAs; ischemic stroke, IS; Middle cerebral artery occlusion, MCAO; Oxygen and glucose deprivation/reoxygenation, OGD/R; Malondialdehyde, MDA; Dual-luciferase reporter, DLR.

Ethics Approval

This study was conducted after obtaining local ethical committee approval of Zibo First Hospital (No. 2018013) and We confirm that all experiments were executed in accordance with the Guide for the Care and Use of Laboratory Animals.

Funding

There is no funding to report.

Disclosure

The authors report no conflicts of interest for this work.

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