

Upregulation of miR-20b Protects Against Cerebral Ischemic Stroke by Targeting Thioredoxin Interacting Protein (TXNIP)

Dejiang Yang¹, Yu Tan¹, Huanhuan Li¹, Xiaowei Zhang¹, Xinming Li¹ and Feng Zhou^{2*}

¹Department of Neurology, the Third Affiliated Hospital of Nanchang University, Nanchang 330008,

²Department of Neurology, the Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai 519000, PR. China

Dysregulation of microRNAs (miRNAs) is involved in abnormal development and pathophysiology in the brain. Although miR-20b plays essential roles in various human diseases, its function in cerebral ischemic stroke remains unclear. A cell model of oxygen glucose deprivation/reoxygenation (OGD/R) and a rat model of middle cerebral artery occlusion/reperfusion (MCAO/R) were constructed. qRT-PCR and western blot were used to evaluate the expression of miR-20b and TXNIP. Cell viability was detected by MTT assay, and cell apoptosis was evaluated by flow cytometry. Targetscan and Starbase were used to predict the potential targets of miR-20b. Luciferase reporter assay was applied to determine the interaction between miR-20b and TXNIP. Rescue experiments were conducted to confirm the functions of miR-20b/TXNIP axis in cerebral ischemic stroke. miR-20b was significantly downregulated after I/R both *in vitro* and *in vivo*. Upregulation of miR-20b inhibited OGD/R-induced neurons apoptosis and attenuated ischemic brain injury in rat model. Bioinformatic prediction suggested that TXNIP might be a target of miR-20b, and luciferase reporter assay revealed that miR-20b negatively regulated TXNIP expression by directly binding to the 3'-UTR of TXNIP. Downregulation of TXNIP inhibited OGD/R-induced neurons apoptosis *in vitro* and ischemic brain injury *in vivo*. Rescue experiments indicated that downregulation of TXNIP effectively reversed the effect of miR-20b inhibitor in neurons apoptosis after OGD/R-treatment and ischemic brain injury in a mouse model after MCAO/R-treatment. Our study demonstrated that upregulation of miR-20b protected the brain from ischemic brain injury by targeting TXNIP, extending our understanding of miRNAs in cerebral ischemic stroke.

Key words: Cerebral ischemic stroke, miR-20b, TXNIP, Apoptosis, Ischemic brain injury

INTRODUCTION

Stroke has becoming one of the leading causes of death in the world with a higher mortality and also a major cause of long-term disability [1]. Approximately 85% of all reported strokes are because of cerebral ischemia that always occurs when an embolus or thrombus blocks the major cerebral artery and then leads to the cell death [2]. Until now, despite many clinical trials for stroke therapies have been completed, the only effective treatment to date

is thrombolysis such as tissue plasminogen activator [3]. However, due to the narrow therapeutic window (less than 4.5 h), the strict indications for therapy and the certain risk of secondary hemorrhage, only a few stroke patients may benefit from it [4]. Therefore, the well understanding of critical mediators involved in the progression of cerebral ischemic stroke may eventually lead us to discover efficient diagnosed or treated targets for ischemic stroke.

MicroRNAs (miRNAs) are emerging as a novel class of non-coding RNAs approximately with 20~25 nucleotides in length, and can regulate a series of gene through directly binding to the 3'-UTR of their targeted mRNAs at the post-transcriptional level [5]. Accumulative evidence have found that cerebral ischemia significantly changes the expression profiles of miRNAs, which play essential functions as critical mediators of ischemia [6]. For example, miR-26a is upregulated after cerebral infarction injury *in vitro* and

Submitted September 11, 2020, Revised February 26, 2021,
Accepted March 16, 2021

*To whom correspondence should be addressed.
TEL: 86-0791-88862226, FAX: 86-0791-88862226
e-mail: FengZhouZhuhai@163.com

in vivo, and promotes angiogenesis in a rat model of cerebral ischemic by modulating PI3K/AKT and MAPK/ERK pathways [7]. MiR-19a-3p expression is significantly elevated in OGD/R and I/R models, and inhibition of miR-19a-3p protects neurons against ischemic stroke by regulating glucose metabolism and neuronal apoptosis [8]. MiR-20b has been identified to participate in various biological processes of many human diseases. MiR-20b inhibits mycobacterium tuberculosis-induced inflammation by targeting NLRP3/caspase-1/IL-1 β pathway in the lung of mouse model [9]. LncRNA CHAR is found to function as a competitive endogenous RNA (ceRNA) of miR-20b to govern cardiac hypertrophy [10]. In addition, one previous study found that miR-20b was significantly upregulated in Type 2 diabetes mellitus patients with non-alcoholic fatty liver disease, suggesting that miR-20b may be a potential biomarker for the early diagnosis [11]. Although miR-20b has been identified to play crucial roles in various human diseases, its function in ischemic stroke remains unclear.

Increasing evidences demonstrate that there is a rapid increase of reactive oxygen species (ROS) production immediately after acute ischemic stroke, resulting in tissue damage [12]. Thioredoxin interacting protein (TXNIP) is an endogenous inhibitor of the thioredoxin system, a major cellular thiol-reducing and antioxidant system [13]. Linking oxidative stress to inflammation in ischemic stroke, the inhibition of TXNIP was shown to decrease the activation of inflammasome-dependent pathways to attenuate ischemic brain injury [14]. Therefore, targeting TXNIP might be a potential therapeutic strategy for ischemic stroke. Recently, one study revealed that downregulation of TXNIP attenuated oxidative stress injury of neurons in ischemic stroke [15]. Conversely, the activation of TXNIP exacerbates neuronal apoptosis and brain infarct area in ischemic stroke [16]. These studies suggest that TXNIP is an activator of ischemic injury in cerebral ischemic stroke.

In this study, we explored the role of miR-20b in cerebral ischemic stroke, and found that the expression of miR-20b was significantly downregulated in OGD/R-treated neurons and brain tissues of MCAO/R-treated rats. Further, our results demonstrated that upregulation of miR-20b could effectively inhibited apoptosis of OGD/R-treated neurons *in vitro* and attenuated ischemic brain injury of MCAO/R-treated rats *in vivo* by directly targeting TXNIP (graphical abstract), providing a novel regulatory mechanism of miR-20b in cerebral ischemic stroke and suggesting that miR-20b might be a therapeutic target for treatment of ischemic stroke.

MATERIALS AND METHODS

Animal model

Adult male SD rats (8~10 weeks, approximately 270 \pm 17 g) and 17-day-old SD rats were all obtained from the Experimental Animal Center of the Chinese Academy of Medical Sciences, People's Republic of China. All rats were maintained under a pathogen-free facility. All procedures were approved by Institutional Animal Care and Use Committee of the Fifth Affiliated Hospital of Sun Yat-sen University. The rat middle cerebral artery occlusion/reperfusion (MCAO/R) model of ischemic stroke was constructed as previously described with minor modulations [17]. In brief, when rats were anesthetized with 40 mg/kg sodium pentobarbital, the left common artery and the left external carotid artery were exposed. A piece of 6-0 monofilament nylon suture with its cusp slightly rounded by heat was plugged via the right internal carotid artery to the base of the middle cerebral artery. After 2 h of MCAO, rats were permitted to recover for 24 h. For the sham group, rats underwent similar operations to expose the carotid arteries without the occlusion of the middle cerebral artery. Finally, rats were sacrificed by cervical dislocation and brains were removed for the subsequent experiments. Of which, the lesion area was used to determine the mRNA and protein expression, as well as the immunohistochemistry staining. Six rats in each group.

Cell culture and OGD/R model

Neurons were isolated from the cerebral cortex of approximately 17-day-old SD rats as previously described [18]. Neurons were cultured in DMEM medium (Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) at 37 $^{\circ}$ C with 5% CO₂. To mimic the ischemic conditions *in vitro*, neurons were treated with oxygen glucose deprivation (OGD) by replacing the DMEM medium with deoxygenated glucose-free Hanks' Balanced Salt Solution (Invitrogen) in a hypoxic chamber containing 5% CO₂, 95% N₂ and 1% O₂ at 37 $^{\circ}$ C for 2 h. Subsequently, the cultures were exposed to reoxygenation by exchanging the medium to glucose-containing DMEM medium supplementing with 10% FBS and kept under normoxic conditions for 24 h. Control neuronal cells were cultured with glucose-containing DMEM and not subjected to OGD/R, simultaneously.

Cell transfection

Neurons were transfected with 20 nM miR-20b mimic, miR-20b inhibitor, or sh-TXNIP or corresponding negative controls (miR-NC, inhibitor NC and sh-NC) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection for 48 h, neurons were subjected to OGD/R,

R treatment and used for the subsequent experiments. MiR-20b mimic, miR-20b inhibitor, sh-TXNIP and negative controls were all purchased from Ambion (Austin, TX, USA). The sequences were listed as follows: sh-TXNIP sense: 5'-GATC-CGCCAGCCAACTCAAGAGGCAAAGAAATTCAAGAGATTTCTTTGCCTCTTGAGTTGGCTGGTTTTTTG-3'; anti-sense: 5'-AATTCAAAAAACAGCCAACTCAAGAG-GCAAAGAAATCTCTTGAATTTCTTTGCCTCTT-GAGTTGGCTGGCG-3'; sh-NC sense: 5'-GATCCGCCA-CAACAACGGAGAAACGCGAAATTCAGAGATTTTC-GCGTTTCTCCAGTTGTTGTGGTTTTTTG-3'; anti-sense: 5'-AATTCAAAAAACACAACAACGGAGAAACGC-GAAATCTCTTGAATTTTCGCGTTTCTCCAGTTGTTGTG-GCG-3'.

Cortical injection of siRNAs

Cortical injections of siRNAs into rats were administered as reported previously [19]. Briefly, rats were deeply anesthetized with sodium pentobarbital, and fixed in a stereotaxic apparatus (anteroposterior, 0.8 mm; mediolateral, 1.5 mm; depth, 3.5 mm). Lentiviral sh-TXNIP (109 TU/ml) or its control (109 TU/ml) was mixed homogeneously with the cationic lipid polybrene (4 µg/µl, GenePharma) and incubated for 15 minutes, and 7 µl mixture was administered through cortical injection. 100 µM miR-20b mimics, miR-20b inhibitor, or negative controls were mixed with the siRNA-Mate (GenePharma) and incubated for 20 minutes, then cortically injected into rats similarly. After injection, rats were exposed to MCAO/R treatment and used for the subsequent analysis.

Measurement of infarct volume

Brains were removed from rats and cut into 1.0 mm-thick coronal sections. The brain sections were incubated with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich; Merck KGaA) at 37°C for 15 min. Subsequently, the brain sections were fixed with 4% paraformaldehyde overnight. Finally, the slices were photographed and the infarct size was quantified using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Evaluation of neurological deficits

Neurological status was evaluated based on a neurologic deficit score following ischemia at 24 h reperfusion using the scales as described by Longa et al. [20]. 0: no observable neurological deficits; 1: failure to extend left forepaw; 2: circling to the left; 3: falling to the left; 4: cannot walk spontaneously.

Evaluation of brain water content

Brain water contents were determined 24 h after reperfusion.

Infarct brain hemispheres were quantified with an electronic scale (wet weight), dried overnight at 105°C in a desiccating oven, and weighed (dry weight). The total brain water was calculated as the following formula: [(wet weight-dry weight)/wet weight]×100%.

Immunohistochemistry staining

The brain tissues were fixed with 4% formaldehyde and cut into 5 mm-thick serial sections, and immunohistochemistry staining was performed according to the immunohistochemical kit (Boster Biological Technology, Wuhan, China) by using the specific cleaved caspase-3 antibody (1:300; ab2302, Abcam). A blinded investigator used a microscope to take images and chose images randomly for each section. The expression of cleaved caspase-3 was analyzed by the Image-Pro Plus software.

qRT-PCR analysis

Total RNA of brain tissues or cultured cells was extracted by using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was obtained using a Prime Script™ RT reagent kit (Takara, Shiga, Japan). qRT-PCR analysis was performed on a 7900HT Fast Real-Time PCR machine (Applied Biosystems) based on a standard SYBR Green PCR kit (Toyobo, Osaka, Japan). GAPDH and U6 were considered as the internal references. The relative expression levels of targets were calculated by using the $2^{-\Delta\Delta C_t}$ method. The primers used in this study were listed as follows: miR-20b forward: 5'-TGCAG-TAGTTTTGGCATGA-3', reverse: 5'-TCAACAAGAGATTTGT-TATCCAAGAG-3'; TXNIP forward: 5'-AGTTACCCGAGT-CAAAGCCG-3', reverse: 5'-TCTCGTTCTCACCTGTAGGC-3'; GAPDH forward: 5'-TGTTGCCATCAATGACCCCTT-3', reverse: 5'-CTCCACGACGTACTIONCAGCG-3'; U6 forward: 5'-GCTTCGGCAGCACATATACT-3', reverse: 5'-AAC-GCTTCACGAATTTGCGT-3'.

Western blot

Total protein of brain tissues or cells was extracted by using RIPA lysis buffer (Sigma-Aldrich) on ice. Approximately equal amounts of protein samples were separated by 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk, the membrane was incubated with primary antibody against TXNIP (1:500, ab188865, Abcam) and GAPDH (1:1,000, ab9485, Abcam) at 4°C overnight. The following day, the membranes was then incubated with the secondary antibody labeled with horseradish peroxidase (HRP) (1: 5,000, ab6721, Abcam) for 1 h at room temperature. Finally, the proteins of interest were detected by using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA) and band

intensity was quantified by Image-Pro Plus 6.0 software (Media Cybernetic).

Luciferase reporter assay

Targetscan database (http://www.targetscan.org/vert_72/, v7.2) and starBase (<http://starbase.sysu.edu.cn/>, v2.0) were used to predict the potential targets of miR-20b. To determine the interaction between miR-20b and TXNIP, the wild type (WT) and mutant type (MUT) 3'-UTR of TXNIP containing putative binding sites of miR-20b were amplified by PCR and cloned into pmirGLO dual luciferase reporter vector (Promega, Madison, WI, USA). The recombinant luciferase reporter vectors were co-transfected with miR-20b mimics or miR-NC into neurons by using Lipofectamine 2000. 48 h after transfection, cells were lysed and relative luciferase activities were detected by using the dual-luciferase assay system (Promega).

MTT assay

Cell viability was evaluated by using the MTT Cell Viability Assay Kit (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, after transfection and OGD/R treatment, 10 μ l of MTT stock solution was added and incubated for 4 h at 37°C. Then, 100 μ l of dissolution reagent was added for another 4 h. Finally, the absorbance at 490 nm was measured with a microplate absorbance reader (Bio-Rad, Sunnyvale, CA, USA).

Flow cytometry analysis

The apoptotic rate of neurons was evaluated by using the Annexin V-FITC Apoptosis kit (Beyotime) according to the manufacturer's instructions. In brief, after washing with cold PBS, cells were stained with Annexin V conjugated with Propidium iodide (PI) for 15 min at 25°C. The apoptotic cells were quantified by a FACSAria II flow cytometer (BD Biosciences, USA).

Statistical analysis

All experiments were performed in triplicate. Data were presented as mean \pm SD. Difference between the two groups was analyzed by the unpaired Student's *t* test, and difference among multiple groups was determined by using one-way ANOVA. *p* value<0.05 was considered as the significant threshold.

RESULTS

miR-20b was significantly downregulated after I/R both *in vitro* and *in vivo*

To explore the role of miR-20b in cerebral ischemic stroke, experimental model of stroke in cells and rats were applied. *In vitro*,

cell viability of neurons subjected to OGD/R was significantly decreased compared with control cells without OGD/R treatment ($p<0.01$) (*t* test, $t_{(4)}=8.696$, $p=0.001$) (Fig. 1A). Flow cytometry assay showed that OGD/R treatment significantly promoted neurons apoptosis compared with control group ($p<0.01$) (*t* test, $t_{(4)}=-9.815$, $p=0.001$) (Fig. 1B). The expression of miR-20b in OGD/R-treated neurons was evaluated by qRT-PCR and the results indicated that miR-20b was markedly downregulated in OGD/R-treated neurons compared with control group ($p<0.01$) (*t* test, $t_{(4)}=11.076$, $p=0.000$) (Fig. 1C). *In vivo*, rats subjected to MCAO/R treatment (I/R group) showed a severe neurological deficit compared with sham group ($p<0.01$) (*t* test, $t_{(10)}=-23.000$, $p=0.000$) (Fig. 1D). The percentage of infarct volume was obviously increased in I/R rats compared with sham group ($p<0.01$) (*t* test, $t_{(10)}=-14.312$, $p=0.000$) (Fig. 1E). Similarly, the expression of miR-20b in brain tissues of I/R rats was significantly downregulated compared with that in sham group ($p<0.01$) (*t* test, $t_{(10)}=9.008$, $p=0.001$) (Fig. 1F). These results suggested that miR-20b was downregulated after I/R both *in vitro* and *in vivo*, and might play an essential role in cerebral ischemic stroke.

Upregulation of miR-20b protected neurons against OGD/R-induced injury *in vitro*

To determine the function of miR-20b, neurons were transfected with miR-20b mimics to overexpress miR-20b, or transfected with miR-20b inhibitor to knockdown miR-20b. qRT-PCR assay showed that miR-20b mimics significantly increased the expression of miR-20b compared with miR-NC in neurons ($p<0.01$) (ANOVA, $F_{(4,10)}=113.428$, $p=0.000$; LSD test, $t=-15.120$, $p=0.000$); miR-20b inhibitor decreased miR-20b expression compared with inhibitor NC ($p<0.01$) (ANOVA, $F_{(5,12)}=113.428$, $p=0.000$; LSD test, $t=4.203$, $p=0.002$) (Fig. 2A). MTT assay indicated that OGD/R treatment obviously decreased the viability of neurons ($p<0.01$) (ANOVA, $F_{(5,12)}=33.817$, $p=0.000$; LSD test, $t=8.539$, $p=0.000$), miR-20b mimics significantly enhanced cell viability of OGD/R-treated neurons compared with miR-NC ($p<0.05$) (ANOVA, $F_{(5,12)}=33.817$, $p=0.000$; LSD test, $t=-4.567$, $p=0.010$), while miR-20b inhibitor decreased cell viability of OGD/R-treated neurons compared with inhibitor NC ($p<0.05$) (ANOVA, $F_{(5,12)}=33.817$, $p=0.000$; LSD test, $t=3.442$, $p=0.026$) (Fig. 2B). Meanwhile, cell apoptosis was evaluated by flow cytometry and the results revealed that OGD/R treatment promoted neurons apoptosis ($p<0.01$) (ANOVA, $F_{(5,12)}=37.578$, $p=0.000$; LSD test, $t=-8.105$, $p=0.000$), miR-20b mimics significantly inhibited apoptosis of OGD/R-treated neurons compared with miR-NC ($p<0.01$) (ANOVA, $F_{(5,12)}=37.578$, $p=0.000$; LSD test, $t=4.731$, $p=0.000$), while miR-20b inhibitor inversely promoted apoptosis of OGD/R-treated neurons

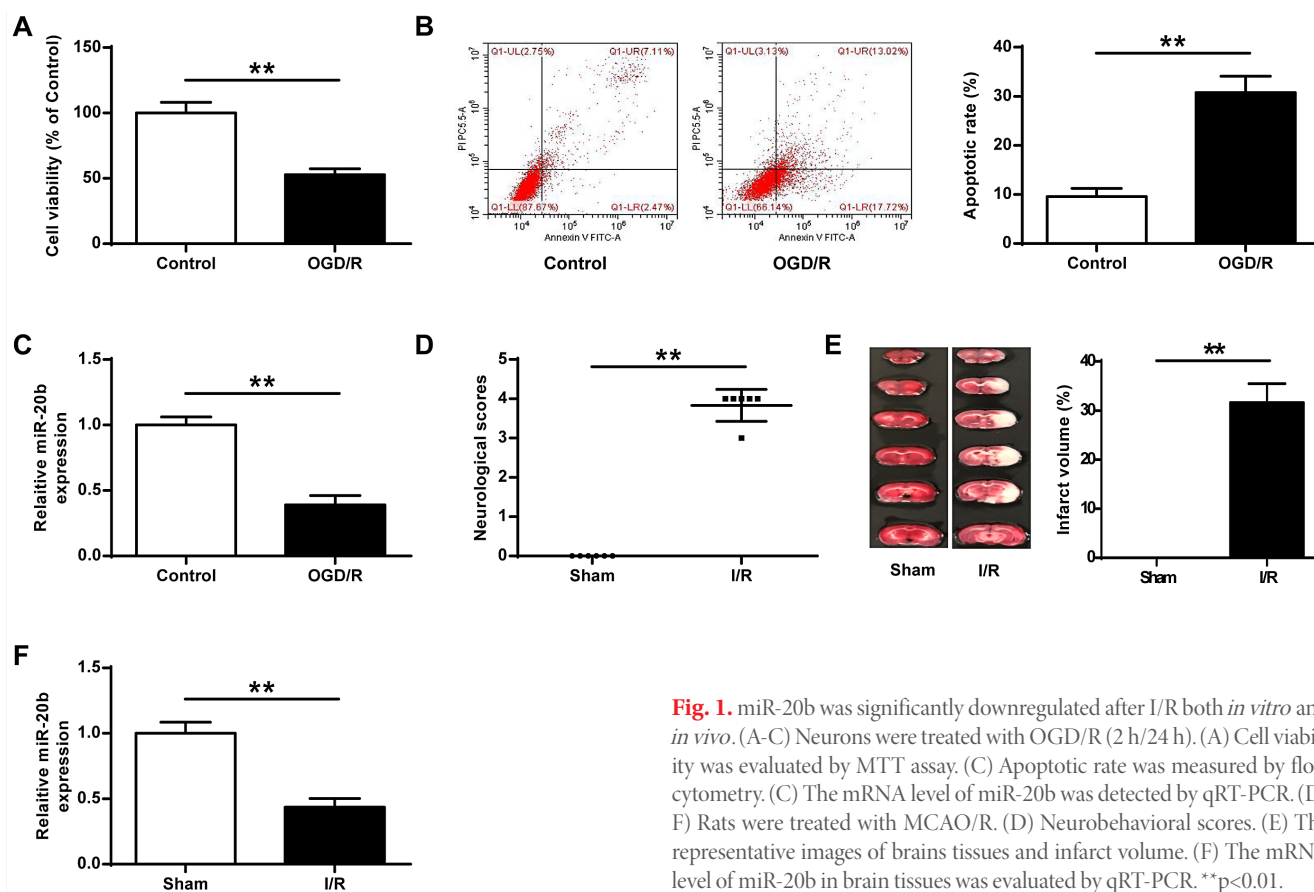


Fig. 1. miR-20b was significantly downregulated after I/R both *in vitro* and *in vivo*. (A-C) Neurons were treated with OGD/R (2 h/24 h). (A) Cell viability was evaluated by MTT assay. (C) Apoptotic rate was measured by flow cytometry. (C) The mRNA level of miR-20b was detected by qRT-PCR. (D-F) Rats were treated with MCAO/R. (D) Neurobehavioral scores. (E) The representative images of brains tissues and infarct volume. (F) The mRNA level of miR-20b in brain tissues was evaluated by qRT-PCR. ** $p < 0.01$.

compared with inhibitor NC ($p < 0.05$) (ANOVA, $F_{(5,12)} = 37.578$, $p = 0.000$; LSD test, $t = -3.829$, $p = 0.019$) (Fig. 2C). These data indicated that upregulation of miR-20b protected neurons against OGD/R-induced injury *in vitro*.

Upregulation of miR-20b attenuated ischemic brain injury *in vivo*

In order to determine the role of miR-20b in cerebral ischemic stroke, miR-20b mimic, miR-20b inhibitor and negative controls were injected into the cerebral cortex of rats. The expression of miR-20b in brains tissues was evaluated by qRT-PCR, and the results showed that MCAO/R treatment (I/R group) obviously decreased the expression of miR-20b compared with sham group ($p < 0.01$) (ANOVA, $F_{(5,30)} = 57.817$, $p = 0.000$; LSD test, $t = 10.353$, $p = 0.000$), miR-20b mimics significantly increased the expression of miR-20b compared with miR-NC in I/R rats ($p < 0.01$) (ANOVA, $F_{(5,30)} = 57.817$, $p = 0.000$; LSD test, $t = -7.959$, $p = 0.000$), and miR-20b inhibitor decreased miR-20b expression compared with inhibitor NC in I/R rats ($p < 0.01$) (ANOVA, $F_{(5,30)} = 57.817$, $p = 0.000$; LSD test, $t = 5.059$, $p = 0.000$) (Fig. 3A). Moreover, neurological scores of I/R rats were higher than that of sham group ($p < 0.01$)

(ANOVA, $F_{(3,20)} = 118.698$, $p = 0.000$; LSD test, $t = -15.872$, $p = 0.000$), and miR-20b mimics significantly decreased the neurological scores compared with miR-NC in I/R rats ($p < 0.01$) (ANOVA, $F_{(3,20)} = 118.698$, $p = 0.000$; LSD test, $t = 9.661$, $p = 0.000$) (Fig. 3B), indicating that overexpression of miR-20b could effectively reduce the neurological scores. Meanwhile, MCAO/R treatment significantly increased infarct volume in rat brains compared with sham operation ($p < 0.01$) (ANOVA, $F_{(5,30)} = 42.464$, $p = 0.000$; LSD test, $t = -10.305$, $p = 0.000$), miR-20b mimics obviously attenuated infarct volume compared with miR-NC in I/R rats ($p < 0.01$) (ANOVA, $F_{(5,30)} = 42.464$, $p = 0.000$; LSD test, $t = 4.374$, $p = 0.001$), while miR-20b inhibitor increased infarct volume compared with inhibitor NC in I/R rats ($p < 0.05$) (ANOVA, $F_{(5,30)} = 42.464$, $p = 0.000$; LSD test, $t = -3.396$, $p = 0.005$) (Fig. 3C). The effect of miR-20b on brain edema was also evaluated and the results showed that I/R treatment significantly increased brain water content of rats compared with sham group ($p < 0.01$) (ANOVA, $F_{(5,30)} = 19.385$, $p = 0.000$; LSD test, $t = -6.102$, $p = 0.000$), while miR-20b mimics efficiently reduced brain water content compared with miR-NC in rats ($p < 0.05$) (ANOVA, $F_{(5,30)} = 19.385$, $p = 0.000$; LSD test, $t = 4.293$, $p = 0.013$) (Fig. 3D). In addition, the neuron apoptosis was evaluated by using

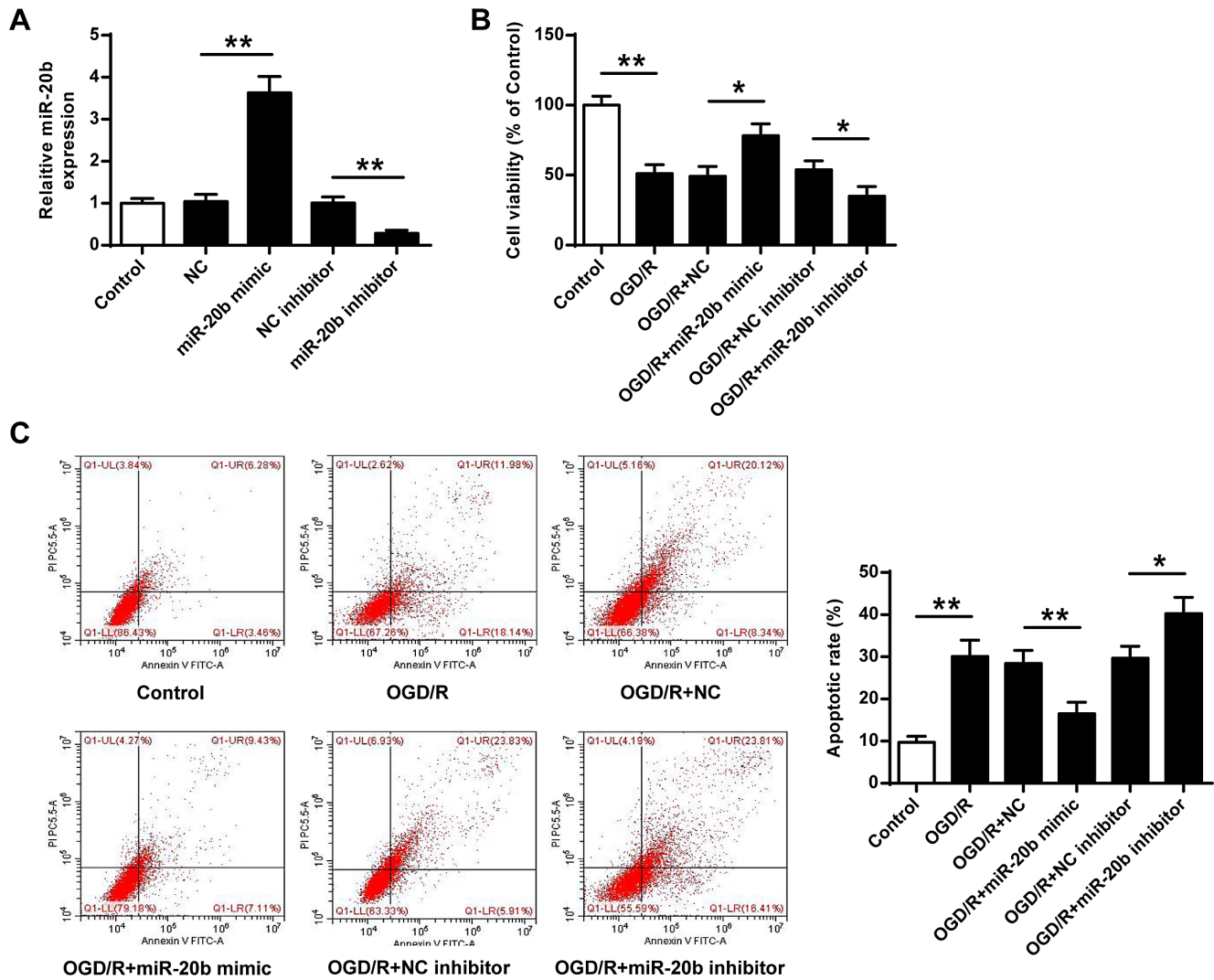


Fig. 2. Upregulation of miR-20b protected neurons against OGD/R-induced injury *in vitro*. Neurons were transfected with miR-20b mimics, miR-NC, miR-20b inhibitor, or inhibitor NC, and then then treated with OGD/R. (A) The mRNA level of miR-20b was detected by qRT-PCR. (B) Cell viability was evaluated by MTT assay. (C) Apoptotic rate was detected by flow cytometry. * $p < 0.05$, ** $p < 0.01$.

immunohistochemical staining with specific cleaved caspase-3 antibody and the results indicated that I/R treatment significantly increased the number of c-caspase 3 positive cells compared with sham group ($p < 0.01$) (ANOVA, $F_{(5,30)} = 63.814$, $p = 0.000$; LSD test, $t = -10.328$, $p = 0.000$), while miR-20b mimics obviously reduced c-caspase 3 positive cells compared with miR-NC in rats ($p < 0.01$) (ANOVA, $F_{(5,30)} = 63.814$, $p = 0.000$; LSD test, $t = 9.051$, $p = 0.000$) (Fig. 3E and F). These results suggested that upregulation of miR-20b could attenuate ischemic brain injury *in vivo*.

TXNIP was a target of miR-20b

To further explore the mechanism of miR-20b in ischemic stroke, TargetsCan and Starbase database were used to predict the potential targets of miR-20b. The prediction results showed that there

were two putative binding sites between miR-20b and 3'-UTR of TXNIP (Fig. 4A), indicating that TXNIP might be a target of miR-20b. Then luciferase reporter assay was performed to determine the interaction and the results revealed that miR-20b mimics significantly decreased the relative luciferase activity of WT 3'-UTR of TXNIP in both site 1 ($p < 0.01$) (ANOVA, $F_{(3,8)} = 39.876$, $p = 0.000$; LSD test, $t = 9.141$, $p = 0.000$) and site 2 ($p < 0.01$) (ANOVA, $F_{(3,8)} = 39.800$, $p = 0.000$; LSD test, $t = 8.627$, $p = 0.000$) compared with miR-NC, and exhibited no obvious effect in MUT 3'-UTR of TXNIP (Fig. 4B). In addition, OGD/R treatment significantly up-regulated the expression of TXNIP both at mRNA level ($p < 0.01$) (ANOVA, $F_{(5,30)} = 44.698$, $p = 0.000$; LSD test, $t = -7.167$, $p = 0.000$) (Fig. 4C) and protein level ($p < 0.01$) (ANOVA, $F_{(5,30)} = 38.130$, $p = 0.000$; LSD test, $t = -5.652$, $p = 0.000$) (Fig. 4D) compared with

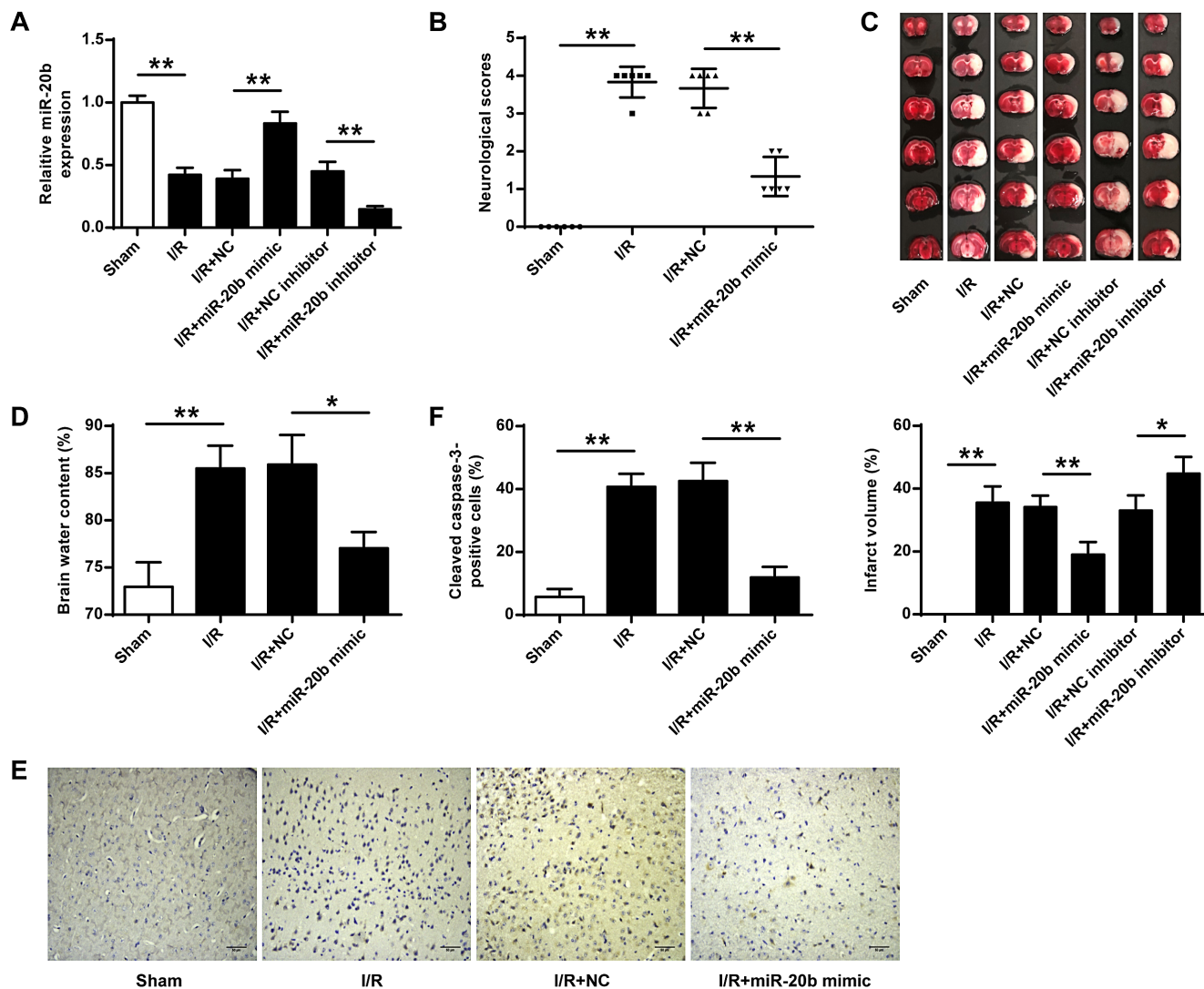


Fig. 3. Upregulation of miR-20b attenuated ischemic brain injury *in vivo*. MiR-20b mimic, miR-20b inhibitor and negative controls were injected into the cerebral cortex of rats, and rats were then treated with MCAO/R. (A) The mRNA level of miR-20b in brain tissues of rats was evaluated by qRT-PCR. (B) Neurobehavioral scores. (C) The representative images of brains tissues and infarct volume. (D) The effect of miR-20b on brain edema after cerebral ischemia. (E) Immunohistochemical staining of cleaved caspase-3 positive cells in cortical neuron ($\times 200$ magnification, scale bar=100 μ m). (F) Quantitative analysis of immunohistochemical staining. * $p < 0.05$, ** $p < 0.01$.

control group in neurons; miR-20b mimics obviously decreased the expression of TXNIP both at mRNA level ($p < 0.01$) (ANOVA, $F_{(5,30)} = 44.698$, $p = 0.000$; LSD test, $t = 6.164$, $p = 0.000$) (Fig. 4C) and protein level ($p < 0.01$) (ANOVA, $F_{(5,30)} = 38.130$, $p = 0.000$; LSD test, $t = 4.942$, $p = 0.000$) (Fig. 4D) compared with miR-NC in OGD/R-treated neurons; while miR-20b inhibitor increased the expression of TXNIP both at mRNA level ($p < 0.01$) (ANOVA, $F_{(5,30)} = 44.698$, $p = 0.000$; LSD test, $t = -5.961$, $p = 0.000$) (Fig. 4C) and protein level ($p < 0.01$) (ANOVA, $F_{(5,30)} = 38.130$, $p = 0.000$; LSD test, $t = -5.941$, $p = 0.000$) (Fig. 4D) compared with inhibitor NC in OGD/R-treated neurons. These data indicated that the effect of miR-20b in OGD/R-induced injury was mediated by TXNIP.

Downregulation of TXNIP inhibited OGD/R-induced neurons apoptosis *in vitro* and ischemic brain injury *in vivo*

To explore the effect of TXNIP in cerebral ischemic stroke, silencing of TXNIP was conducted in neuronal cells and rat brains. *In vitro*, sh-TXNIP (knockdown of TXNIP) significantly decreased the expression of TXNIP both at mRNA level ($p < 0.01$) (ANOVA, $F_{(2,6)} = 96.870$, $p = 0.000$; LSD test, $t = 12.341$, $p = 0.000$) (Fig. 5A) and protein level ($p < 0.01$) (ANOVA, $F_{(2,6)} = 121.571$, $p = 0.000$; LSD test, $t = 13.292$, $p = 0.000$) (Fig. 5B) in neurons compared with sh-NC control. MTT assay showed that OGD/R-treatment obviously decreased neurons viability compared with control ($p < 0.01$)

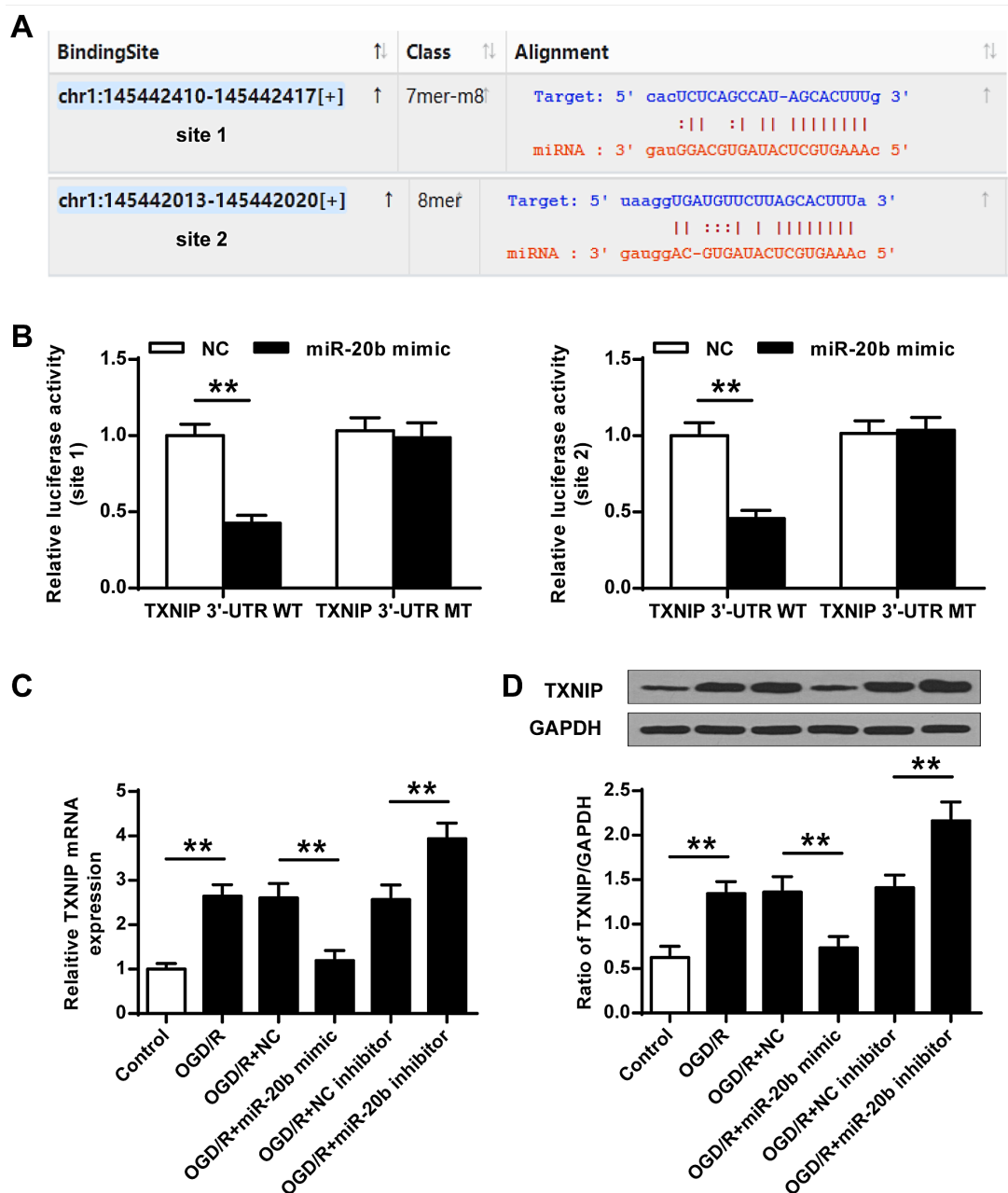


Fig. 4. TXNIP was a target of miR-20b. (A) The putative binding sites between miR-20b and TXNIP were predicted by TargetsScan and Starbase databases. (B) The WT or MUT 3'-UTR of TXNIP both in site 1 and site 2 was cloned into pmirGLO reporter vector, and co-transfected with miR-20b mimics or miR-NC into neurons. The relative luciferase activity was detected by dual luciferase reporter system. (C, D) Neurons were transfected with miR-20b, miR-NC, miR-20b inhibitor, or inhibitor NC, and treated with OGD/R. The expression of TXNIP was evaluated by qRT-PCR (C) and western blot (D). **p<0.01.

(ANOVA, $F_{(3,8)}=21.086$, $p=0.000$; LSD test, $t=6.520$, $p=0.000$), and sh-TXNIP significantly enhanced the viability of OGD/R-treated neurons compared with sh-NC ($p<0.05$) (ANOVA, $F_{(3,8)}=21.086$, $p=0.000$; LSD test, $t=-3.604$, $p=0.023$) (Fig. 5C). Meanwhile, the apoptotic rate of neurons in OGD/R-treated neurons was significantly increased compared with control without OGD/R

treatment ($p<0.01$) (ANOVA, $F_{(3,8)}=33.497$, $p=0.000$; LSD test, $t=-8.483$, $p=0.000$), and sh-TXNIP effectively inhibited cell apoptosis of OGD/R-treated neurons compared with sh-NC ($p<0.05$) (ANOVA, $F_{(3,8)}=33.497$, $p=0.000$; LSD test, $t=4.594$, $p=0.010$) (Fig. 5D). *In vivo*, the expression of TXNIP in ischemic brain of I/R rats was significantly upregulated both at mRNA level ($p<0.01$)

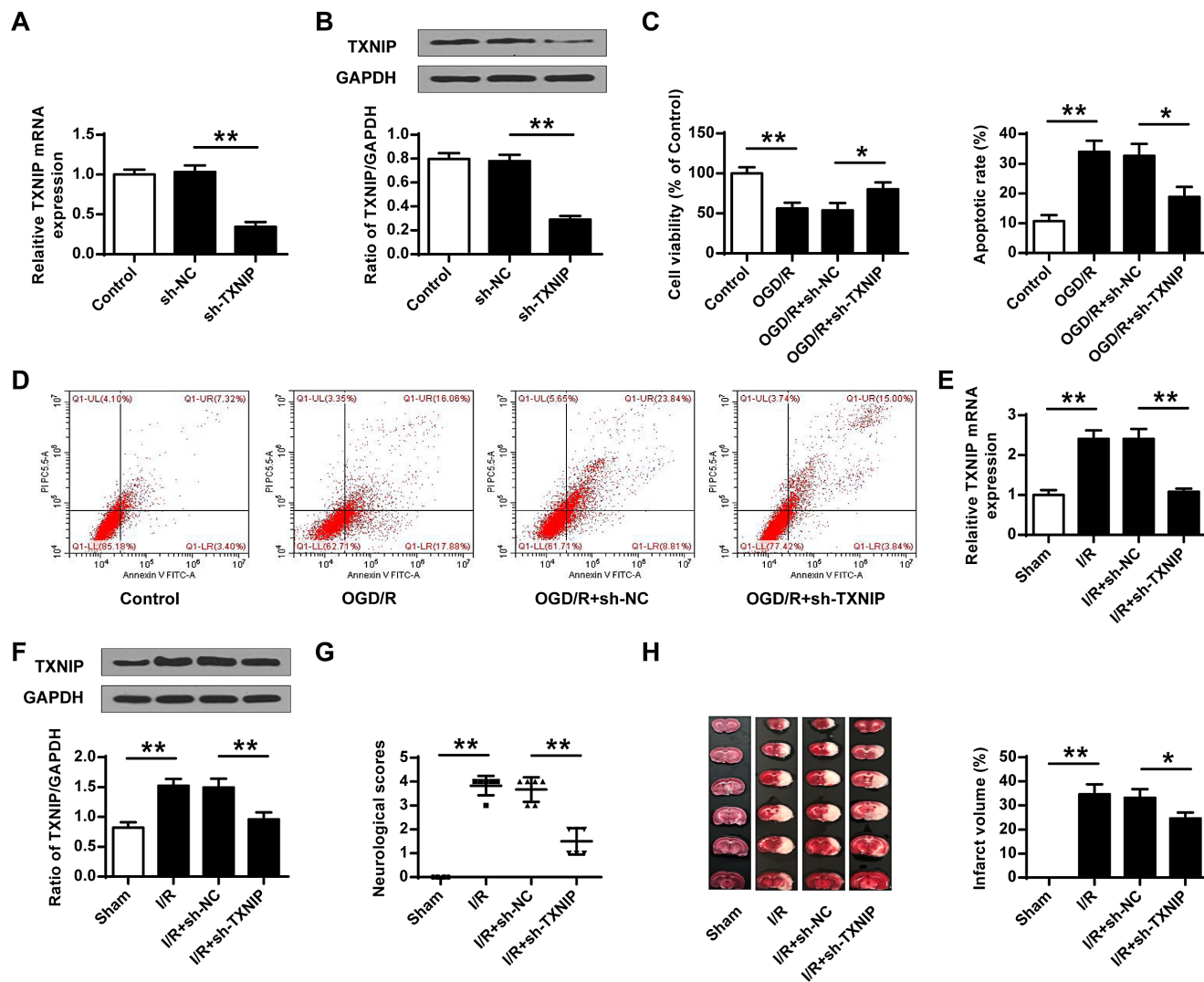


Fig. 5. Downregulation of TXNIP inhibited OGD/R-induced neurons apoptosis *in vitro* and ischemic brain injury *in vivo*. (A and B) Neurons were transfected with sh-TXNIP or sh-NC. The expression of TXNIP was detected by qRT-PCR (A) and western blot (B). (C, D) Neurons were transfected with sh-TXNIP or sh-NC, and then treated with OGD/R. (C) Cell viability was evaluated by MTT assay. (D) Apoptotic rate was detected by flow cytometry. * $p < 0.05$, ** $p < 0.01$. (E-H) sh-TXNIP or sh-NC was injected into the cerebral cortex of rats, and rats were then treated with MCAO/R. The expression of TXNIP in ischemic brain was evaluated by qRT-PCR (E) and western blot (F). (G) Neurobehavioral scores. (H) The representative images of brains tissues and infarct volume. * $p < 0.05$, ** $p < 0.01$.

(ANOVA, $F_{(3,20)}=58.051$, $p=0.000$; LSD test, $t=-9.608$, $p=0.000$) (Fig. 5E) and protein level ($p < 0.01$) (ANOVA, $F_{(3,20)}=27.335$, $p=0.000$; LSD test, $t=-7.174$, $p=0.000$) (Fig. 5F) compared with that of sham rats; and sh-TXNIP obviously decreased the expression of TXNIP both at mRNA level ($p < 0.01$) (ANOVA, $F_{(3,20)}=58.051$, $p=0.000$; LSD test, $t=9.040$, $p=0.000$) (Fig. 5E) and protein level ($p < 0.01$) (ANOVA, $F_{(3,20)}=37.335$, $p=0.000$; LSD test, $t=5.466$, $p=0.001$) (Fig. 5F) in ischemic brain of I/R rats compared with sh-NC. Moreover, the neurobehavioral scores in I/R rats were higher than that in sham rats ($p < 0.01$) (ANOVA, $F_{(3,20)}=110.606$, $p=0.000$; LSD test, $t=-15.506$, $p=0.000$), and sh-TXNIP significantly decreased neu-

robehavioral scores of I/R rats compared with sh-NC ($p < 0.01$) (ANOVA, $F_{(3,20)}=110.606$, $p=0.000$; LSD test, $t=8.764$, $p=0.000$) (Fig. 5G). Meanwhile, MCAO/R treatment exacerbated cerebral infarction volume of rats compared with sham operation ($p < 0.01$) (ANOVA, $F_{(3,20)}=86.766$, $p=0.000$; LSD test, $t=-14.256$, $p=0.000$), while sh-TXNIP attenuated infarction volume of rats compared with sh-NC ($p < 0.05$) (ANOVA, $F_{(3,20)}=86.766$, $p=0.000$; LSD test, $t=3.405$, $p=0.027$) (Fig. 5H). All these data revealed that downregulation of TXNIP could effectively attenuate OGD/R-induced neurons apoptosis *in vitro* and ischemic brain injury *in vivo*.

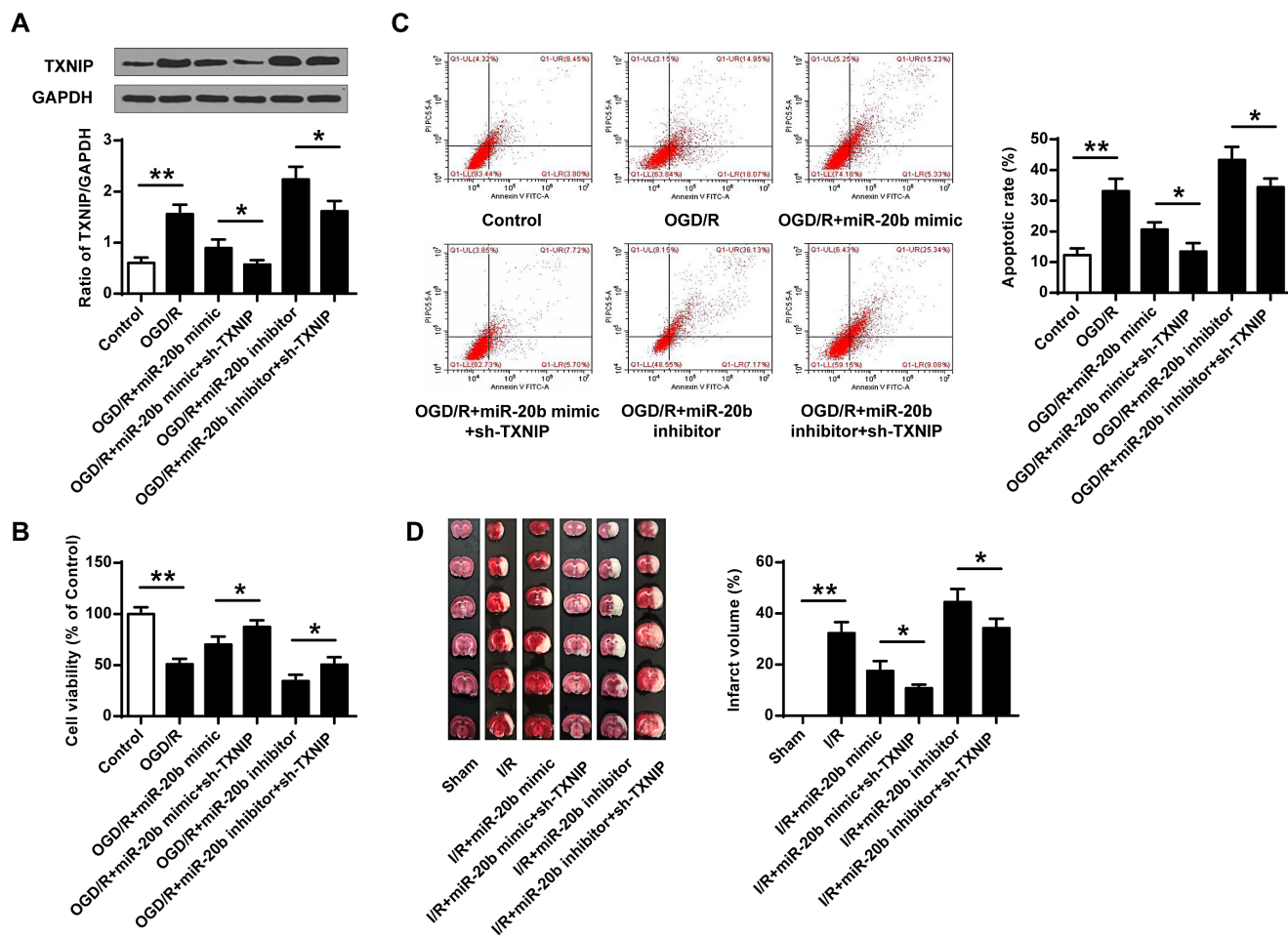


Fig. 6. Upregulation of miR-20b attenuated OGD/R-induced neurons apoptosis *in vitro* and ischemic brain injury through targeting TXNIP (A-C) Neurons were transfected with miR-20b mimics, miR-20b inhibitor, or co-transfected with miR-20b mimics and sh-TXNIP, or miR-20b inhibitor and sh-TXNIP. Then cells were treated with OGD/R. (A) The expression of TXNIP was evaluated by western blot. (B) Cell viability was detected by MTT assay. (C) Apoptotic rate was measured by flow cytometry. * $p < 0.05$, ** $p < 0.01$. (D) Rats were cortical injected with miR-20b mimics, miR-20b inhibitor, or co-injection with miR-20b mimics and sh-TXNIP, or miR-20b inhibitor and sh-TXNIP. Then rats were treated with MCAO/R. The representative images of brains tissues and infarct volume. * $p < 0.05$, ** $p < 0.01$.

Upregulation of miR-20b decreased OGD/R-induced neurons apoptosis in vitro and ischemic brain injury through targeting TXNIP

To further explore whether the effect of miR-20b in ischemic brain injury was mediated by TXNIP, the rescue experiments both *in vitro* and *in vivo* were performed. *In vitro*, co-transfection of miR-20b mimics and sh-TXNIP further decreased the expression of TXNIP in OGD/R-treated neurons compared with miR-20b mimics group ($p < 0.05$) (ANOVA, $F_{(5,12)}=43.149$, $p=0.000$; LSD test, $t=2.258$, $p=0.043$), while additional sh-TXNIP obviously attenuated miR-20b inhibitor-induced expression of TXNIP in OGD/R-treated neurons ($p < 0.05$) (ANOVA, $F_{(5,12)}=43.149$, $p=0.000$; LSD test, $t=3.351$, $p=0.029$) (Fig. 6A). MTT assay indicated that co-transfection of miR-20b mimics and sh-TXNIP further enhanced

the viability of OGD/R-treated neurons compared with miR-20b mimics group ($p < 0.05$) (ANOVA, $F_{(5,12)}=41.566$, $p=0.000$; LSD test, $t=-2.930$, $p=0.043$), while additional sh-TXNIP significantly rescued the inhibitory effect of miR-20b inhibitor in the viability of OGD/R-treated neurons ($p < 0.05$) (ANOVA, $F_{(5,12)}=41.566$, $p=0.000$; LSD test, $t=-2.946$, $p=0.012$) (Fig. 6B). Meanwhile, co-transfection of miR-20b mimics and sh-TXNIP significantly inhibited apoptotic rate of OGD/R-treated neurons compared with miR-20b mimics group ($p < 0.05$) (ANOVA, $F_{(5,12)}=47.544$, $p=0.000$; LSD test, $t=2.752$, $p=0.018$), while additional sh-TXNIP obviously attenuated miR-20b inhibitor-induced apoptosis of OGD/R-treated neurons ($p < 0.05$) (ANOVA, $F_{(5,12)}=47.544$, $p=0.000$; LSD test, $t=3.012$, $p=0.039$) (Fig. 6C). Moreover, co-injection of miR-20b mimics and sh-TXNIP further decreased infarct volume of I/R rat

brains compared with miR-20b mimics group ($p < 0.05$) (ANOVA, $F_{(5,30)} = 66.522$, $p = 0.000$; LSD test, $t = 2.327$, $p = 0.038$), while additional sh-TXNIP effectively attenuated miR-20b inhibitor-exacerbated infarct volume ($p < 0.05$) (ANOVA, $F_{(5,30)} = 66.522$, $p = 0.000$; LSD test, $t = 2.830$, $p = 0.047$) (Fig. 6D). These results revealed that the protective effect of miR-20b upregulation in ischemic brain injury was mediated by TXNIP.

DISCUSSION

In the present study, we found for the first time that the expression of miR-20b was significantly upregulated in neurons and brains tissues of mouse model after I/R treatment. Overexpression of miR-20b inhibited OGD/R-treated neurons apoptosis and attenuated I/R-induced nervous disorder, as well as infarct volume. Luciferase reporter assay determined that miR-20b could directly targeting 3'-UTR of TXNIP. Silencing of TXNIP inhibited neurons apoptosis and attenuated infarct volume of I/R rats. In addition, sh-TXNIP significantly attenuated miR-20b inhibitor-caused growth defect and apoptosis in OGD/R-treated neurons, as well as infarct volume of I/R rats. In a word, our results revealed that upregulation of miR-20b could effectively protect against cerebral ischemic stroke both *in vitro* and *in vivo* by targeting TXNIP and inhibiting its expression, suggesting that miR-20b might be a novel therapeutic target for cerebral ischemic stroke.

In the past decades, more and more miRNAs have been identified to be closely involved in the development and progression of cerebral ischemic stroke through various manners. For instance, inhibition of miRNA-27b promotes angiogenesis in mouse ischemic stroke model by activating adenosine monophosphate-activated protein kinase (AMPK), which is positively related to the tube formation and migration [21]. MiRNA-126 facilitates vascular remodeling and decreases fibrosis and has been identified to act as an essential regulatory factor during the pathogenesis of cardiovascular diseases and cerebral stroke [22]. Upregulation of miR-3473b contributes to the pathogenesis of ischemic stroke by promoting post-stroke neuroinflammation injury through directly targeting SOCS3 [23]. MiRNA-335 promotes stress granule formation to suppress neurons apoptosis via downregulating ROCK2 in acute ischemic stroke [24]. MiRNA-210 induces the apoptosis of neuronal cells of mouse model with cerebral ischemia by activating the HIF-1 α -VEGF pathway [25]. Upregulation of miRNA-199b-3p effectively suppresses the apoptosis of cerebral microvascular endothelial cells in ischemic stroke by modulating the MAPK/ERK/EGR1 axis [26]. These evidences all demonstrates that miRNAs are closely involved in the development of ischemic stroke. Although miR-20b has been found to play important roles

in a series of different types of human cancers such as metastatic colorectal cancer [27], prostate cancer [28], breast cancer [29], esophageal cancer [30], and so on, its function in cerebral ischemic stroke has not been reported. Our study revealed that OGD/R treatment in neurons and MCAO/R-treatment in mouse model both decreased the expression of miR-20b, and overexpression of miR-20b significantly attenuated ischemic injury. Therefore, miR-20b was a crucial miRNA closely associated with the progression of cerebral ischemic stroke, and might be a potential biomarker for the diagnosis and treatment.

ROS plays an important role in normal physiological processes and is also involved in a number of disease processes, which can mediate the damage in cell structures, including lipids, membranes, proteins, and DNA [31]. Increasing reports have revealed that ischemic stroke is caused by a blockage of cerebral blood flow, leading to neuronal and glial hypoxia and resulting in inflammatory and ROS-mediated cell death [32]. Hence, inhibition of ROS production is an effective approach to attenuate the ischemic injury and several agents have been identified to protect against ischemic stroke by inhibiting ROS-mediated neurons deaths including 3H-1,2-Dithiole-3-thione [33], 3-n-butylphthalide [34], leonurine (SCM-198) [35] and isoquercetin [36]. In addition, there are some endogenous molecules also can affect the balance between antioxidant and oxidant [37]. TXNIP, an endogenous inhibitor and regulator of thioredoxin, and a major cellular thiol-reducing and antioxidant system, which can prevent against ROS production [13]. Previous studies demonstrated that downregulation TXNIP could trigger the assembly and oligomerization of the inflammasome caused by ROS [38]. Previous studies demonstrated that inhibiting the expression of TXNIP could effectively inhibit the activation of inflammasome during ischemic stroke [39, 40]. TXNIP has also been demonstrated that functions as a direct of miRNAs to interfere the neuronal apoptosis after neonatal hypoxic-ischemic injury. For example, TXNIP mediates the protective roles of miR-17 in in rats and PC12 cells after hypoxic-ischemic injury [16]. TXNIP is identified to function as the direct target of miR-21-5p, which participates the inhibitory effect of gastrodin combined with rhynchophylline in cerebral ischaemia-induced inflammasome activation [41]. These studies suggest that miRNAs may participate in the development of ischemic stroke by interacting with TXNIP. Interestingly, our study identified that TXNIP was a target of miR-20b, and downregulation of TXNIP inhibited OGD/R-induced neurons apoptosis *in vitro* and ischemic brain injury *in vivo*. Moreover, rescue experiments revealed that downregulation of TXNIP significantly attenuated the effect of miR-20b inhibitor in neurons apoptosis and infarct volume. Taken together, we provided a new regulatory axis of miR-20b and TXNIP in cerebral

ischemic stroke, which might contribute to understand the pathogenesis of ischemic stroke.

Previous studies have demonstrated that tissue plasminogen activator (tPA) was a first therapeutic drug for stroke patients which was approved by Food and Drug Administration (FDA) [42]. Meanwhile, tPA has been also reported to be involved in post stroke complications like increasing inflammation [43]. However, it is interesting to explore the effect of miR-20b on tPA.

In summary, our results demonstrated that miR-20b was significantly downregulated in OGD/R-treated neurons and the brains of MCAO/R-treated rats, and overexpression of miR-20b could effectively attenuate OGD/R-induced neurons apoptosis *in vitro* and MCAO/R-induced ischemic brain injury *in vivo* by directly targeting TXNIP (graphical abstract), suggesting that miR-20b might a potential therapeutic target for cerebral ischemic stroke.

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