

Down-Regulation of miRNA-30a Alleviates Cerebral Ischemic Injury Through Enhancing Beclin 1-Mediated Autophagy

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Abstract The understanding of molecular mechanism underlying ischemia/reperfusion-induced neuronal death and neurological dysfunction may provide therapeutic targets for ischemic stroke. The up-regulated miRNA-30a among our previous identified 19 MicroRNAs (miRNAs) in mouse brain after 6 h middle cerebral artery occlusion (MCAO) could negatively regulate Beclin 1 messenger RNA (mRNA) resulting in decreased autophagic activity in tumor cells and cardiomyocytes, but its role in ischemic stroke is unclear. In this study, the effects of miRNA-30a on ischemic injury in N2A cells and cultured cortical neurons after oxygen glucose deprivation (OGD), and mouse brain with MCAO-induced ischemic stroke were evaluated. The results showed that miRNA-30a expression levels were up regulated in the brain of mice after 6 h MCAO without reperfusion, but significantly down regulated in the peri-infarct region of mice with 1 h MCAO/24 h reperfusion and in N2A cells after 1 h OGD/6–48 h reoxygenation. Both the conversion ratio of microtubule-associated protein 1 light chain 3 (LC3)-II/LC3-I and Beclin 1 protein level increased in N2A cells and cultured

cortical neurons following 1 h OGD/24 h reoxygenation. The down-regulated miRNA-30a could attenuate 1 h OGD/24 h reoxygenation-induced ischemic injury in N2A cells and cultured cortical neurons through enhancing Beclin 1-mediated autophagy, as miRNA-30a recognized the 3'-untranslated region of *beclin 1* mRNA to negatively regulate Beclin 1-protein level via promoting *beclin 1* messenger RNA (mRNA) degradation, and Beclin 1 siRNA abolished anti-miR-30a-induced neuroprotection in 1 h OGD/24 h reoxygenation treated N2A cells. In addition, anti-miR-30a attenuated the neural cell loss and improved behavioral outcome of mice with ischemic stroke. These results suggested that down-regulation of miRNA-30a alleviates ischemic injury through enhancing beclin 1-mediated autophagy, providing a potential therapeutic target for ischemic stroke.

Keywords miRNA-30a · Beclin-1 · Microtubule-associated protein 1 light chain 3 (LC3) · Autophagy · Oxygen glucose deprivation (OGD) · Ischemic stroke

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Introduction

Stroke, a serious damage to human health and life safety in the world refractory diseases, has high incidence, high morbidity and high mortality characteristics. Recent clinical trials have demonstrated that there are numerous opportunities to improve stroke prevention strategies, effectively intervene in and treat acute stroke [1]. However, the molecular mechanism of stroke-induced neuronal death and neurological dysfunction are not fully known.

MicroRNAs (miRNAs) are important small noncoding endogenous RNAs of 21–23 nucleotides that negatively modulate gene expression by binding to the 3'-untranslated

region (3'-UTR) of mRNA [2, 3]. miRNAs are highly conserved, and able to regulate a wide range of biological processes including cell proliferation and differentiation, metabolism and apoptotic cell death [4, 5]. In addition, several miRNAs have also been reported to be involved in autophagy modulation by regulating the expression of autophagy-related genes [6–9]. Beclin 1, the mammalian homologue of yeast Atg6, was first described as a Bcl-2-interacting protein [10], and its mediated autophagy plays an important role in the regulation of cell survival and death [11, 12].

Recently, the accumulating evidence has linked the changes in cerebral miRNAs expression to the occurrence and development of ischemic stroke [13–16]. By using large-scale miRNA microarrays, we have identified 19 differentially expressed miRNAs in the brain of mice with hypoxic preconditioning (HPC) and 6 h middle cerebral artery occlusion (MCAO) without reperfusion-induced ischemic stroke. It should be noted that among these 19 miRNAs, the up-regulated miRNA-30a in peri-infarct region of 6 h MCAO mice could be inhibited by HPC pretreatment [17]. miRNA-30a could negatively regulate Beclin 1 mRNA resulting in decreased autophagic activity in tumor cells [18–20] and cardiomyocytes [21, 22]. However, the role of miRNA-30a in cerebral ischemic injury and whether it can influence autophagy by regulating Beclin 1 remain unclear. In this study, we found that the miRNA-30a expression was up regulated in peri-infarct region of mice following 6 h MCAO without reperfusion, but down regulated in the brain of mice with 1 h MCAO/24 h reperfusion and 1 h oxygen-glucose deprivation (OGD)/6–48 h reoxygenation-treated N2A cells. The down-regulated miRNA-30a could alleviate neural cell ischemic injuries by enhancing autophagy in vitro and in vivo through targeting 3'-UTR of Beclin 1 mRNA.

Experimental Procedure

Except the individually indicated agents and antibodies in the text, the chemicals were purchased from Sigma-Aldrich (St. Louise, MO 63103, USA). Adult male C57BL/6 J mice (weighing 22–25 g) were maintained in temperature-controlled rooms (12-h light–dark cycle) with access to food and water ad libitum. Experimental procedures were performed according to the guidelines set by the Animal Care and Use Committee of Capital Medical University and were consistent with the NIH Guide for the care and use of laboratory animals.

MCAO-Induced Ischemic Stroke Mouse Model

The MCAO-induced ischemic stroke mouse model was prepared as described before [23–25]. In brief, the left

common and left external carotid arteries were exposed and ligated through a ventral midline neck incision under the condition of anesthesia with pentobarbital sodium (60 mg/kg i.p.). A 5-0 surgical nylon monofilament (0.23 mm in diameter) was gently inserted through external and internal carotid arteries to occlude the middle cerebral artery (a point approximately 12 mm distal to the carotid bifurcation). According to the experimental requirements, two ischemic stroke mouse models were produced through 6 h MCAO without reperfusion and 1 h MCAO followed by 24 h reperfusion. At these time points, the infarct volume is still unstable, but the ischemic penumbra or peri-infarct region exits more apparently. Sham-operated mice received the same procedure, without inserting the nylon monofilament. The mouse brains were removed after transcardial perfusion first with ice cold phosphate buffered saline (PBS) then 4 % paraformaldehyde in PBS for Nissl staining; and the cortexes from peri-infarct region were dissected according to the previous reports after 6 h MCAO or 1 h MCAO/24 h reperfusion for RT-PCR or Western blot analysis [23–25].

Measurement of Neurological Deficit

At 24 h after 1 h MCAO, mice were tested for neurological deficits according to neurological disability status scale reported by Rodriguez et al. [26]. Briefly, the six major steps indicate: 0, no neurological dysfunction; 2, slight decrease in mobility and the presence of passivity; 4, moderate neurological dysfunction and including additional alterations, such as moderate hypomobility, flattened posture, lateralized posture, hunched back, ataxic gait, decreased body tone and muscular strength and slight motor incoordination; 6, corresponding to more handicapped animals but still able to walk, with more marked hypomobility, circling, tremor, jerks and/or convulsions, forelimb flexion and moderate motor incoordination; 8, corresponding to respiratory distress, and total incapacity to move/coordinate. Status 10 refers to death due to 1 h MCAO/24 h reperfusion. In all cases, if the criteria for the precise grade were not met, the nearest appropriate number was utilized: 1, 3, 5, 7 and 9.

Nissl Staining

Mouse brains were removed after transcardial perfusion with 4 % paraformaldehyde in PBS. Brains were post-fixed in 4 % paraformaldehyde. After dehydration by successive immersion in 20 and 30 % sucrose solution, brains were cut into 20 μ m-thickness sections and then stained with 0.04 % cresyl violet (Sigma-Aldrich) dissolved in acetate buffer for 1 h. Six sections per brain were used for cell counting. Staining cells in the injured side of the cerebral

cortex were calculated in three views under the light microscope (Nikon, 50i, Japan). The final average number of the six sections from each sample was used for analysis.

Stereotaxic Administration of Lentiviral Vectors

Cortical injection of lentiviral vectors was carried out 5 days before treatment using a stereotaxic instrument according to the measure reported by Zhou et al. [27]. Briefly, mice were anesthetized with pentobarbital sodium and fixed in a stereotaxic apparatus. 0.7 μ l of lentivirus suspension containing 2×10^9 TU/ml (GeneChem, Shanghai, RP China) was injected into each point (0.3 mm anterior, 0.8 and 1.9 mm posterior to the bregma with 3 mm lateral and 2 mm deep for point 1, 2 and 3, respectively) by using a cannula (28-gauge, inner diameter 0.18 mm, outer diameter 0.36 mm) at a rate of 0.2 μ l/min. All the target points were in the left hemisphere (i.e., ipsilateral to the MCAO). The needle was withdrawn over a course of 10 min. The mice were subjected to MCAO at 5 days after injection of lentiviral vector.

N2A Cell Culture and Treatment

Mouse N2A neuroblastoma cells were generous gift from the lab of Dr Yun Wang (Peking University) and were grown to 60 % confluence in growth culture medium of Dulbecco's modified Eagle's medium (DMEM; Gibco Inc., Grand island, NY, USA) supplemented with 10 % fetal bovine serum. Then, cells were transfected with pri-miR-30a, anti-miR-30a plasmids or their controls (GeneChem, Shanghai, RP China), and Beclin 1 siRNA (GCTGCCGT TATACTGTTCT) and negative control siRNAs (GTTCTC CGAACG TGTCACGT, GenePharma, Shanghai, PR China) at a final concentration of 20 μ M by using Lipofectamine 2000 (Invitrogen Tec., Carlsbad, CA, USA) according to the manufacturer's instruction. The medium was replaced with the growth culture medium after 6 h transfection, and then N2A cells were subjected to 1 h OGD and 24 h reoxygenation post 48 h transfection.

To mimic ischemic-like conditions in vitro, 1 h OGD/0-48 h reoxygenation treatment was performed on N2A cells. Cells were transferred into a 37 °C anaerobic chamber (Thermo Electron LED GmbH, Langensfeld, Germany) in hypoxic condition (1 % O₂/5 % CO₂/94 % N₂). The culture medium was replaced with glucose-free DMEM (Gibco Inc., Grand island, NY14072, USA) and cells were maintained in the hypoxic chamber for 1 h. After 1 h OGD exposure, cells were maintained in growth culture medium under normoxic condition (21 % O₂/5 % CO₂/74 % N₂) for 0–48 h reoxygenation. Control group was kept in growth culture medium under normoxic condition.

Primary Cortical Neurons Culture and Lentiviral Transduction

Primary cortical neurons were obtained from postnatal 24 h C57BL/6 J mice. Cortical neurons were dissociated and seeded onto plates at a density of 5×10^5 cells per cm². Cortical neurons were cultured in neurobasal medium (Gibco Inc), with 2 % B27 supplement (Gibco Inc). Half of the culture medium was replaced by fresh medium every 3 days. We transduced cells with lentiviral vectors containing pri-miR-30a, anti-miR-30a or their controls (GeneChem, Shanghai, RP China) at a multiplicity of infection of 20 after 6 days according to the manufacturer's instructions. The efficiency of transducing the lentiviral vector of containing pri-miR-30a, anti-miR-30a into primary culture of neurons is about 70 %. After 48 h, cortical neurons subjected to 1 h OGD and 24 h reoxygenation.

For cell viability assessment, the extent of N2A cell and cultured cortical neuron death were determined by using thiazolyl blue tetrazolium bromide (MTT, 0.5 mg/mL; AppliChem Inc., Omaha, NE, USA) and the CytoTox 96[®] Non-Radioactive Cytotoxicity Assay (LDH; Promega Cor, Madison, WI, USA) following the manufacturer's instructions.

Reverse Transcription Quantitative Real-time Polymerase Chain Reaction (RT-qPCR) for mRNA and miRNA Quantification

Total RNA was isolated from cerebral tissues, N2A cells and cultured cortical neurons with the NucleoSpin[®] miRNA kit (Macherey–Nagel, Germany) according to the manufacturer's instruction. First strand cDNA synthesis and amplification were performed by using ProtoScript[®] M-MuLV First Strand cDNA Synthesis Kit (New England Biolabs Inc. MA 01915, USA). The Brilliant II SYBR[®] Green QPCR Master Mix (Agilent Tech., CA 95051, USA) were used for PCR amplifications according to the manufacturer's protocol. The primers were used as follows: beclin 1 (Forward: 5'-GACCGAGTGACCATTTCAGGAC-3'; Reverse: 5'-GGTTCT CCATGGTGCCACCATCAG-3') and β -actin (Forward: 5'-ATATCGCTGCGCTG GTCGTC-3'; Reverse: 5'-AGGATGGCGTGAGGGAGAG C-3'). The RT-PCR amplification was performed with an Mx3000P[™] (Agilent Tech., CA 95051, USA). All quantifications were normalized to an endogenous β -actin control. miRNA-30a expression levels were validated by using the miRCURY LNATM Universal RT microRNA PCR (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's instruction. U6 was used as an internal control. Relative expression level between treatments was then calculated using the following equation: relative gene expression = $2^{-(\Delta\text{Ct sample} - \Delta\text{Ct control})}$.

Western-Blot Analysis

Antibodies in this study were rabbit anti-beclin 1 monoclonal antibody (1:1,000; Cell Signaling Technology, catalogue number #3495, USA), anti-microtubule-associated protein 1 light chain 3 (LC3) polyclonal antibody (1:1,000; Cell Signaling Technology, catalogue number #2775, USA), anti-caspase-3 polyclonal antibody (1:1,000; Cell Signaling Technology, #9662, USA), mouse anti- β -actin monoclonal antibody (1:3,000; Sigma-Aldrich Corp. St. Louis, MO 63103, USA), and the horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG as secondary antibody (1:5,000; Stressgen Biotechnologies Corporation, Victoria BC, Canada). Total protein was extracted from the brain, N2A cells or cultured cortical neurons according to the previous report [28, 29]. 30 μ g of total protein were loaded for SDS-PAGE (12 % SDS gel). Proteins were then electrophoresed and transferred onto polyvinylidene difluoride membrane (GE Healthcare, UK) and blocked with 10 % non-fat milk in Tween/Tris-buffered salt solution (TTBS, 20 mM Tris-Cl, pH 7.5, 0.15 M NaCl and 0.05 % Tween-20) for 1 h. Following incubation with the primary and secondary antibodies, the Enhanced Chemiluminescence kit (GE Healthcare, UK) was used to detect the signals. The amount of proteins were quantified by densitometry and normalized to β -actin, an internal standard.

Luciferase Assays

A 567 bp fragment from the 3'-UTR of Beclin 1 mRNA containing the predicted miR-30a binding sequences was amplified by PCR from 3T3 cell genomic DNA, and then cloned into the pmiR-RB-REPORTTM luciferase reporter vector (RIBOBIO, Guangzhou, China). The primer sets were used as follows: 3'-UTR of Beclin1 mRNA (Forward: 5'-CCGCTCGAGCTTGCTCCTTAG GGGATGTTT G-3'; Reverse: 5'-GAATGCGGCCGCAATAGACCATAGCAA ATCCTTTATTAC-3') and 3'-UTR mutant of Beclin1 mRNA (Forward: 5'-CCACAACGTGTAAATACCAAAA TCCACAAAAG-3'; Reverse: 5'-TTTGGTATTTACACG TTGTGGTTTAATATTACC-3'). Both wild type and mutant were confirmed by sequencing.

Mouse N2A cells were plated at 0.5×10^5 cells per well in 24-well plates. The following day, cells were co-transfected with pmiR-RB-REPORTTM luciferase reporter vector, including the 3'-UTR of beclin 1 mRNA either wild type or mutant miRNA-30a binding sites, and pri-miR-30a or pri-miR-30a control plasmid by using Lipofectamine 2000. Luciferase assays were performed with a GloMax 20 Luminometer 48 h after transfection using Dual-Luciferase[®] Reporter Assay System (Promega Cor, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

The GelDoc-2000 Imagine System was used to perform quantitative analysis of Western blot. For Beclin-1 expression and conversion of LC3-I to LC3-II, the ratio (band density of Beclin-1/band density of β -actin or band density of LC3-II/band density of LC3-I) was expressed as 100 % in the Control group, and then the other group was expressed as percentage of that from Control group. Statistical analysis was conducted by one-way analysis of variance (ANOVA) followed by all pair wise multiple comparison procedures using Bonferroni test. The values were presented as mean \pm SEM, and the significance was regarded as at least $p < 0.05$.

Results

Changes of miRNA-30a, Beclin 1 and LC3-I/II Expression Levels in Ischemic Cortex of MCAO Mice and OGD-Treated N2A Cells

To determine miRNA-30a expression status in cerebral cortex of mice after ischemic stroke, we found that miRNA-30a expression level was up-regulated in the peri-infarct region of mice with 6 h MCAO without reperfusion, but significantly decreased after 1 h MCAO/24 h reperfusion when compared with that of the sham group (Fig. 1a). To verify the result in vitro, the miRNA-30a expression level was also tested in N2A cells after 1 h OGD/0–48 h reoxygenation. As shown in Fig. 1b, the level of miRNA-30a expression was not changed after 1 h OGD only, and then was significantly down-regulated in mouse N2A cells during 6–48 h reoxygenation post 1 h OGD. Similarly, the N2A cell survival rate decreased significantly at 6–48 h reoxygenation and reached the lowest level of 45 % at 24 h reoxygenation post 1 h OGD (Fig. 1c).

To confirm the existence of autophagy in 1 h OGD/0–48 h reoxygenation treated N2A cells, the Beclin 1 and LC3 conversion levels were determined by using Western blot (Fig. 1d). The quantitative analysis demonstrated that both levels of conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II and Beclin 1 increased gradually and reached the highest level in N2A cells following 24 h reoxygenation post 1 h OGD (Fig. 1e, f), suggesting an important role of autophagy in ischemic injury.

Effect of miRNA-30a on OGD-Induced Autophagy and Ischemic Injury in Vitro

To explore the role of miRNA-30a in OGD-induced autophagy and cell death, the N2A cells were transfected

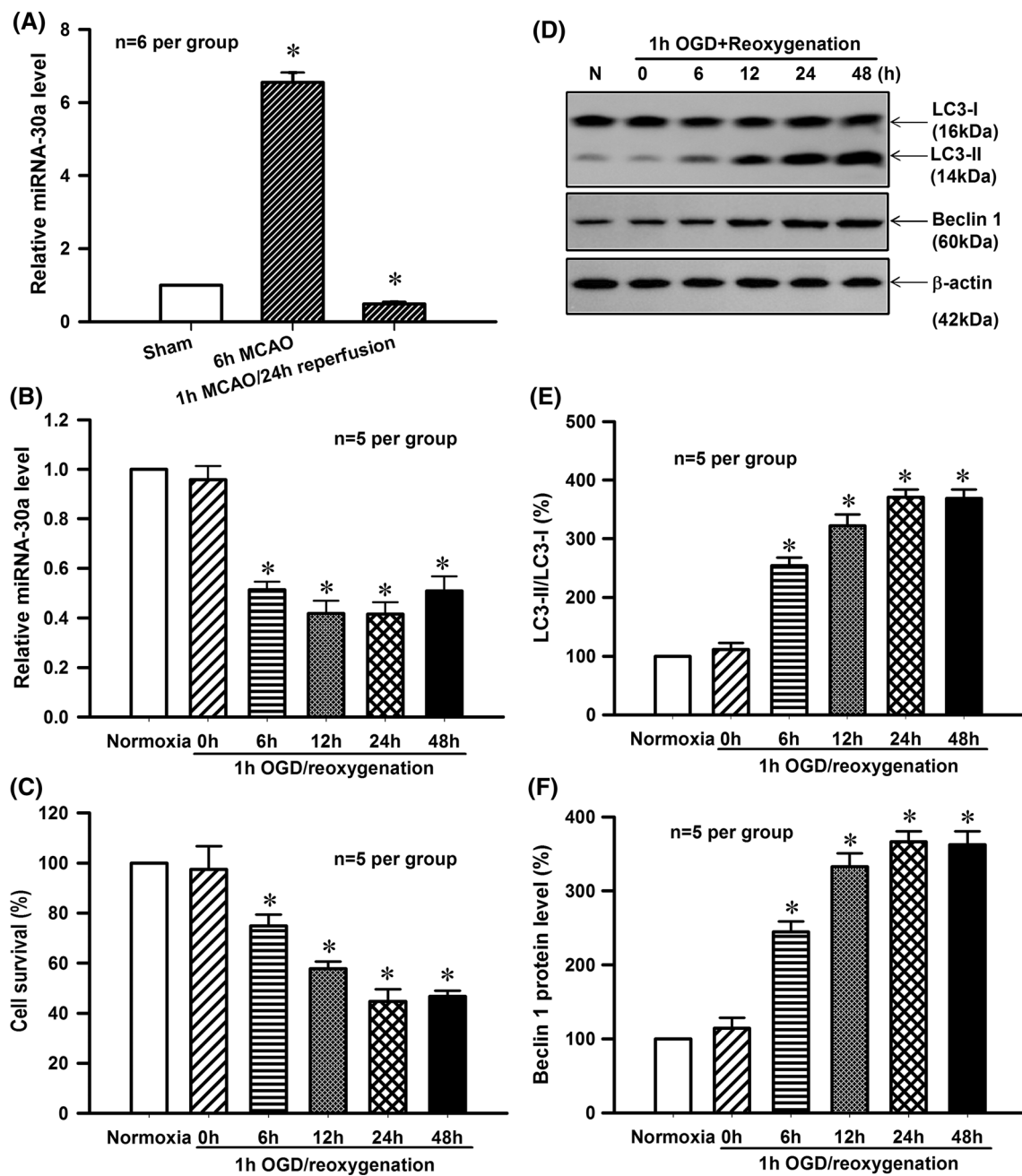


Fig. 1 Changes of miRNA-30a, Beclin 1 and LC3-I/II expression levels in ischemic cortex of MCAO mice with ischemic stroked and N2A cells after OGD-induced ischemic injury. **a** The RT-PCR results showed that the up- and down-regulated miRNA-30a expression could be observed in ischemic cortex of mice following 6 h MCAO and 1 h MCAO/24 h reperfusion, respectively (n = 6 per group). **b** The miRNA-30a expression levels were significantly down-regulated in N2A cells during 6–48 h reoxygenation post 1 h OGD (n = 5 per group). **c** The MTT assays results demonstrated a

significant decrease in cell survival rate of N2A cells during 6–48 h reoxygenation post 1 h OGD (n = 5 per group). **d** Typical results of Western blot showed changes of Beclin-1 and LC3-I/II protein levels in N2A cells during 0–48 h reoxygenation post 1 h OGD. The quantitative analysis demonstrated that the ratio of LC3-II/LC3-I (**e**) and Beclin 1 protein levels (**f**) increased significantly in N2A cells 6–48 h reoxygenation post 1 h OGD (n = 5 per group). **P* < 0.05 versus Sham or Normoxia

with pri-miR-30a or anti-miR-30a plasmids and the cultured cortical neurons were transfected with lentivirus vectors. As shown in Fig. 2a, miRNA-30a expression levels were up- and down-regulated in N2A cells after 48 h

transfection of pri-miR-30a and anti-miR-30a, respectively. Accordingly, the ratio of LC3-II to LC3-I and Beclin 1 expression level were further down and up regulated in 1 h OGD/24 h reoxygenation-treated N2A cells after 48 h

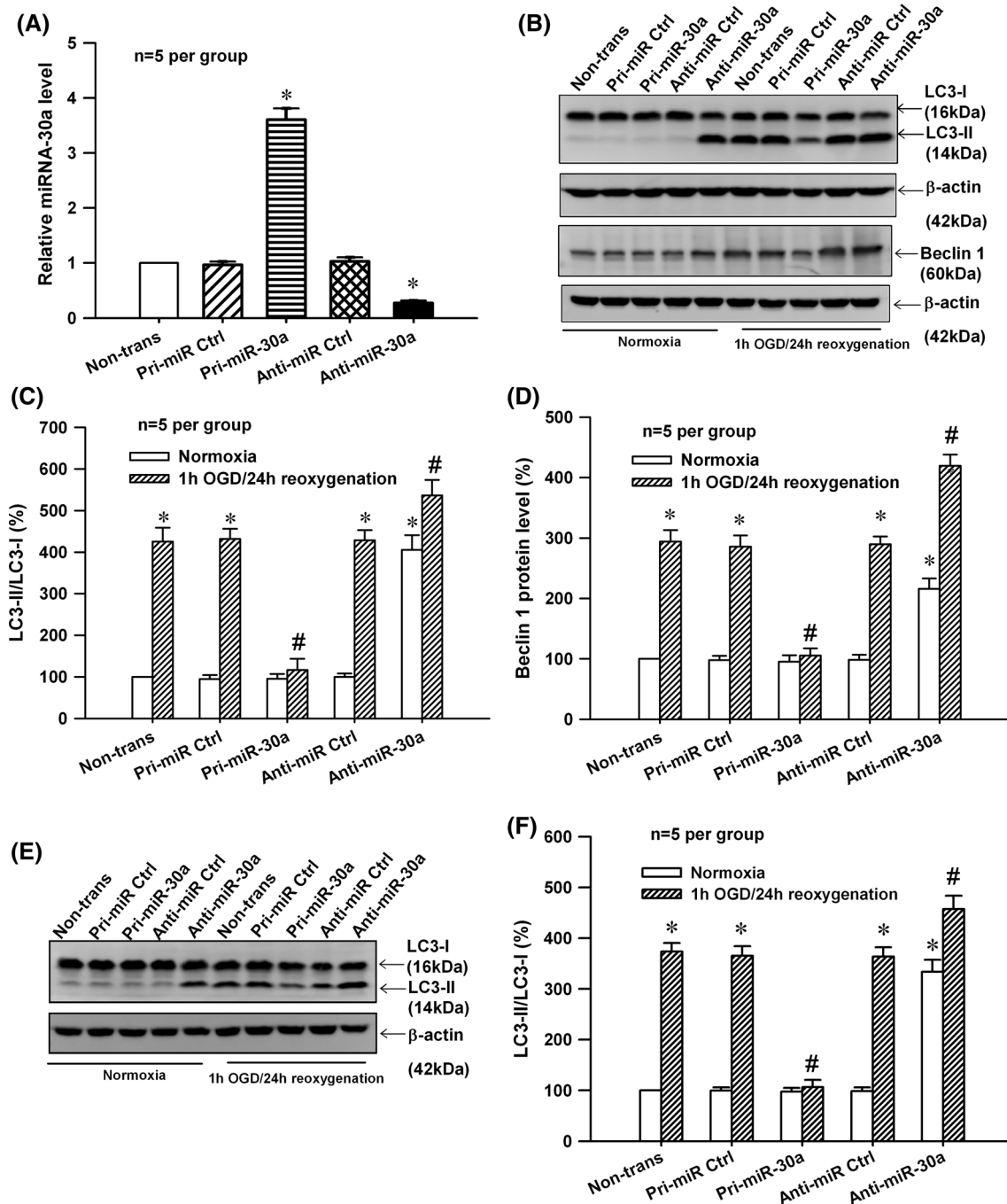


Fig. 2 Effect of miRNA-30a on the conversion of LC3-I to LC3-II and Beclin 1 protein levels in OGD-treated N2A cells and cortical neurons. **a** The RT-PCR results showed that the miRNA-30a levels could be up- and down-regulated in pri-miR-30a and anti-miR-30a transfected N2A cells after 1 h OGD/24 h reoxygenation, respectively (n = 5 per group). **b** Typical results of Western blot showed the effects of miRNA-30a on LC3-I/II and Beclin-1 protein levels in N2A cells after 1 h OGD/24 h reoxygenation. The quantitative analysis

demonstrated that the ratio of LC3-II/LC3-I (**c**) and Beclin 1 protein levels (**d**) could be down- and up-regulated significantly in pri-miR-30a and anti-miR-30a transfected N2A cells after 1 h OGD/24 h reoxygenation (n = 5 per group). **e**, **f** Western blot analysis of LC3-I conversion in cultured cortical neurons after 1 h OGD/24 h reoxygenation (n = 5 per group). **P* < 0.05 versus Non-trans in normoxic condition, #*P* < 0.05 versus Non-trans in condition of 1 h OGD/24 h reoxygenation

transfection of pri-miR-30a and anti-miR-30a (Fig. 2b–d). As shown in Fig. 2e, f, the same result of LC3-II/LC3-I was observed in cultured cortical neurons. In addition, we

found that pri-miR-30a increased 1 h OGD/24 h reoxygenation-induced cell death, whereas the anti-miR-30a effectively reduced 1 h OGD/24 h reoxygenation-induced

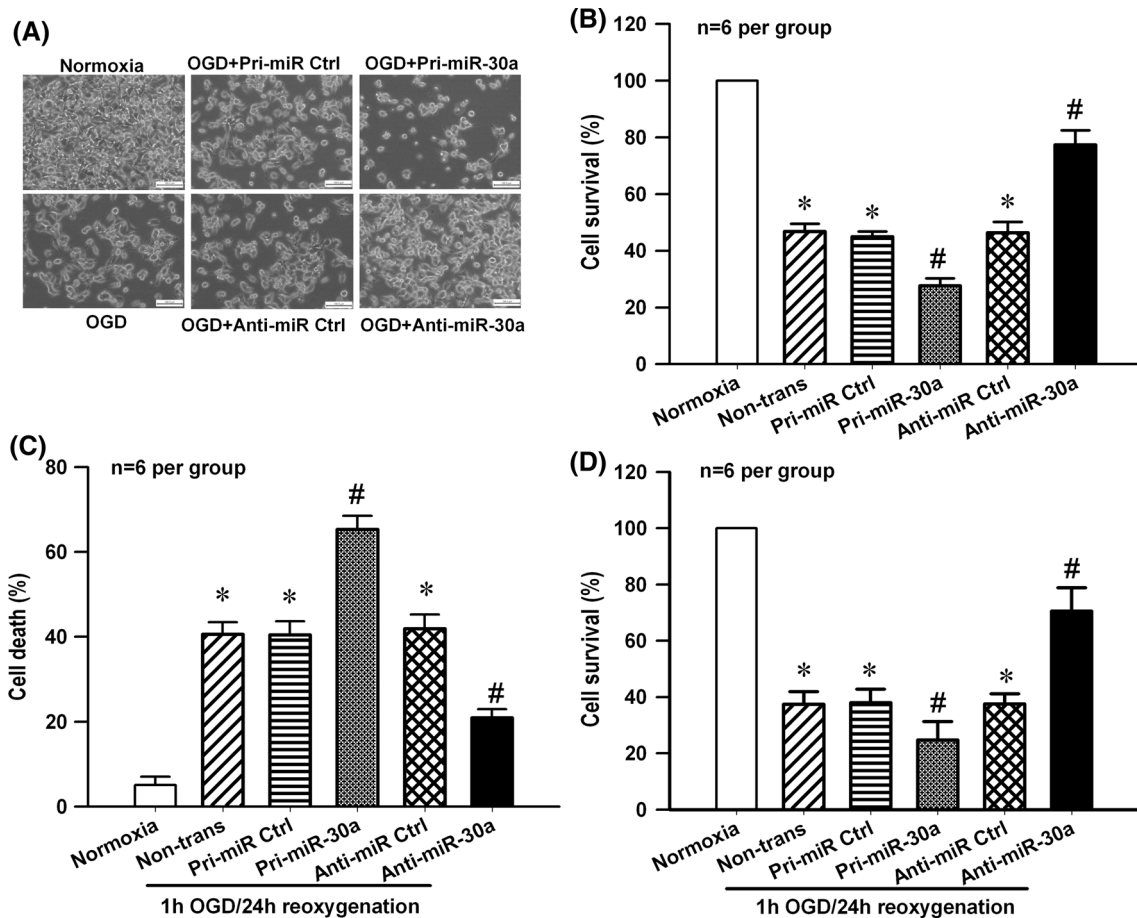


Fig. 3 Effect of miRNA-30a on OGD-induced ischemic injury in N2A cells and cultured cortical neurons. **a** Representative results of microscopic images showed the effects of pri-miR-30a and anti-miR-30a on OGD-induced N2A cell injury (scale bar = 100 μm). The results of MTT (**b**) and LDH (**c**) assays indicated that up-regulation of miRNA-30a by transfection with pri-miR30a significantly decreased N2A cell survival rate (**b**) and increased N2A cell death rate (**c**), but

down-regulation of miRNA-30a by anti-miR30a transfection could attenuate 1 h OGD/24 h reoxygenation-induced cell death when compared with that of normoxia and their control groups (n = 6 per group). **d** The results of MTT assays in cultured cortical neurons (n = 6 per group). *P < 0.05 versus Non-trans in normoxic condition, #P < 0.05 versus Non-trans in condition of 1 h OGD/24 h reoxygenation

cell death when compared with that of normoxia and their control groups (Fig. 3a–d). These results support the conclusion that down-regulation of miRNA-30a attenuates OGD-induced ischemic injury in vitro through enhancing Beclin 1-mediated autophagy.

Down-Regulation of miRNA-30a Ameliorated OGD-Induced Ischemic Injury Via Targeting Beclin 1

Using bioinformatics analysis, the miRNA-30a binding site was predicted at the 3'-UTR of *beclin 1* mRNA with high possibility ranking. To test whether miRNA-30a directly recognizes the 3'-UTR of *beclin 1* mRNA to repress its translation or promote its degradation, we constructed a luciferase reporter vector in which T7 driven-luciferase cDNA was followed by a fragment of the 3'-UTR from *beclin 1* mRNA containing the predicted miRNA-30a

binding sequences or its mutant 3'-UTR (Fig. 4a). The luciferase activity assay indicated that pri-miR-30a not its mutant significantly decreased luciferase activity of the reporter vector containing miRNA-30a binding sequences of *beclin 1* mRNA 3'-UTR (Fig. 4b). In addition, pri-miR-30a and anti-miR-30a could down- or up-regulate both *beclin 1* mRNA (Fig. 4c) and protein expression levels in OGD-treated N2A cells (Fig. 2d). These results suggested that miRNA-30a could directly recognize the 3'-UTR of *beclin 1* mRNA to negatively regulate Beclin 1-protein levels through promoting degradation of its mRNA.

To demonstrate the contribution of Beclin 1 to the biological effects of miRNA-30a in OGD-induced ischemic injury, we co-transfected Beclin 1 siRNA and anti-miR-30a in N2A cells, and then detected LC3-I conversion (Fig. 5a, b), Beclin 1 protein expression (Fig. 5a, c), and cell survival rate (Fig. 5d) in N2A cells after 1 h OGD/24 h reoxygenation. The results indicated that inhibition of

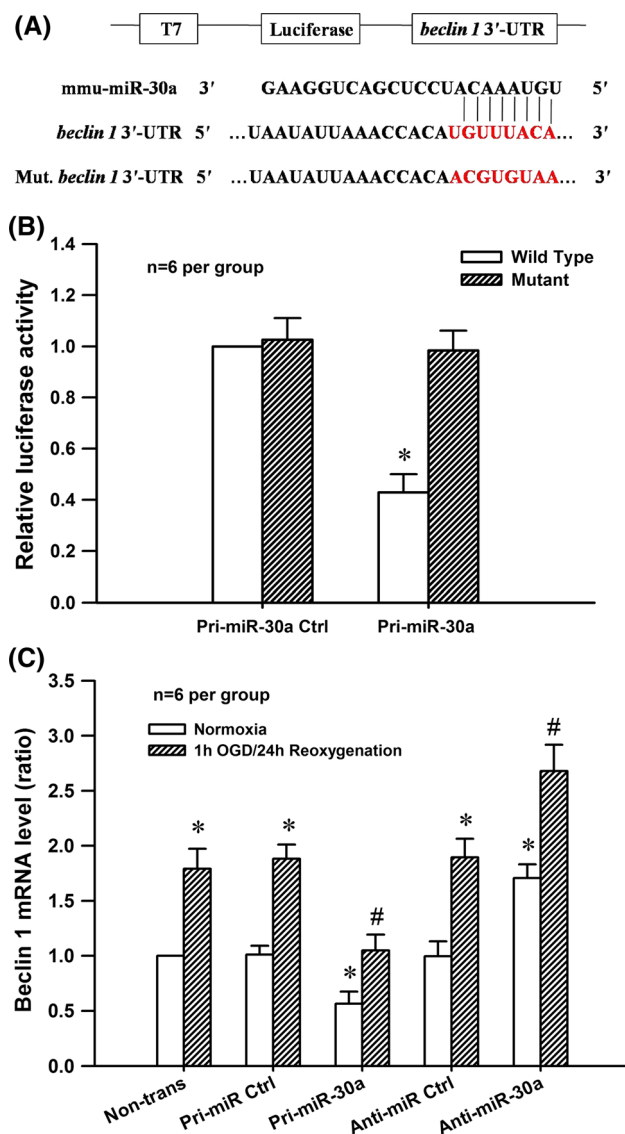


Fig. 4 miRNA-30a directly recognizes the 3'-UTR of Beclin1 mRNA and regulates its mRNA expression levels in N2A cells. **a** Showed the design of a miRNA-30a luciferase reporter vector containing a T7-driven-luciferase cDNA fused to a 3'-UTR of Beclin 1 mRNA or mutated Beclin 1 mRNA. The sequence of miRNA-30a and the putative binding position in the 3'-UTR of Beclin 1 mRNA was also shown. **b** Luciferase reporter assay was performed by co-transfection of luciferase reporter containing 3'-UTR (wild type or mutant) of Beclin 1 mRNA with pri-miR-30a or its control into N2A cells. The results of luciferase activity demonstrated that co-transfection of pri-miR-30a with wild type (not mutant) vector resulted in a significant decrease of luciferase activity at 48 h after transfection (n = 6 per group). **c** The RT-PCR results showed that pri-miR-30a and anti-miR-30a could significantly down- or up-regulate Beclin 1 mRNA levels in N2A cells under both conditions of normoxia and OGD (n = 6 per group). **P* < 0.05 versus Pri-miR-30a Ctrl (**b**) or Non-trans in normoxic condition (**c**), #*P* < 0.05 versus Non-trans in condition of 1 h OGD/24 h reoxygenation

Beclin 1 expression by using siRNA could abolish anti-miR-30a-induced neuroprotective effect in 1 h OGD/24 h reoxygenation treated N2A cells.

Effect of miRNA-30a on MCAO-Induced Autophagy and Ischemic Injury in Vivo

To further evaluate the role of miRNA-30a in cerebral ischemic injury in vivo, we used stereotaxic injection of lentivirus-based pri-miR-30a, anti-miR-30a and their controls into cerebral cortex of mice. The up- and down-regulated miRNA-30a levels in ischemic cortex of MCAO mice were confirmed by using quantitative RT-PCR (Fig. 6a). As shown in Fig. 6b–d, pri-miR-30a could decrease the conversion of LC3-I to LC3-II and Beclin 1 expression, while anti-miR-30a increased the ratio of LC3-II/LC3-I and Beclin 1 protein levels in ischemic cortex of mice after 1 h MCAO/24 h reperfusion. Consequently, pri-miR-30a enhanced neural cell loss and neurological deficit, whereas anti-miR-30a could effectively attenuate ischemic injury by reducing neural cell loss in ischemic cortex and neurological scores of mice after 1 h MCAO/24 h reperfusion (Fig. 7a–c). These data suggested that down-regulation of miRNA-30a can provide neuroprotection against ischemic injuries in vivo through enhancing Beclin 1-mediated autophagy.

Discussion

In this study, we reported three main findings as follows. The first is that miRNA-30a expression was down regulated in N2A cells during 6–48 h reoxygenation post 1 h OGD-induced ischemic injury in vitro and in the perinfarct region of mice after 1 h MCAO/24 h reperfusion-induced ischemic stroke in vivo. However, the expression level of miRNA-30a was up regulated in the brain of mice after 6 h MCAO without reperfusion, which is consistent with our previous results of large-scale miRNA microarray [17]. Secondly, we confirmed that the differentially expressed miRNA-30a in brain of mice with ischemic stroke could directly recognize the 3'-UTR of *beclin 1* mRNA to negatively regulate Beclin 1-protein levels through promoting degradation of its mRNA. The third is that down-regulation of miRNA-30a can provide neuroprotection against ischemic injuries in vitro and in vivo through enhancing Beclin 1-mediated autophagy. The difference of miRNA-30a expression in brain of mice after 6 h MCAO without reperfusion and 1 h MCAO/24 h reperfusion might be due to the duration of MCAO and reperfusion. Further studies are needed to explore whether the high expression of miRNA-30a can serve as biomarker for clinical diagnosis in acute cerebral ischemia.

A subset of miRNAs are abundantly expressed in the human brain [30], and play important roles in numerous brain diseases [31–33]. In our previous study, miRNAs profiling techniques were performed to identify the

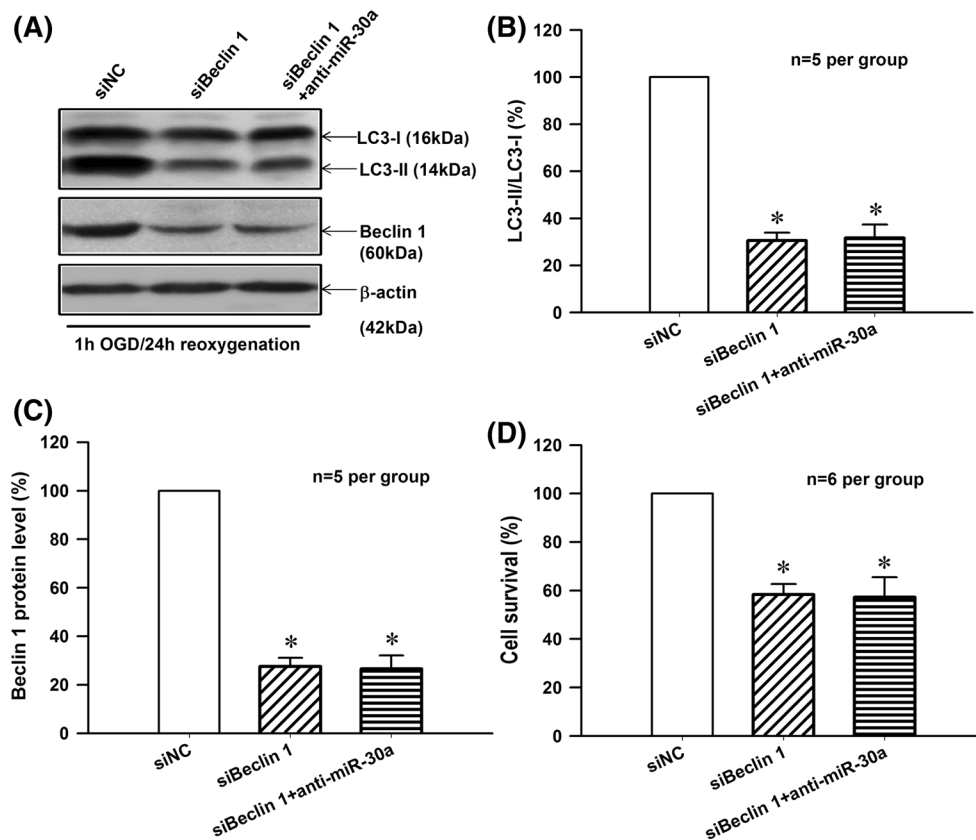


Fig. 5 Beclin 1 siRNA blocks the neuroprotective effect of anti-miR-30a in OGD-induced ischemic injury of N2A cells. **a** Representative results of Western blot showed the effect of Beclin 1 siRNA on LC3-I, LC3-II and Beclin 1 protein expression levels in N2A cells after 1 h OGD/24 h reoxygenation; The quantitative analysis results demonstrated that the ratio of LC3-II/I (**b**) and Beclin 1 protein levels

(**c**) decreased significantly in OGD treated N2A cells after Beclin 1 siRNA transfection or co-transfection with anti-miR-30a (n = 5 per group). **d** MTT assay results indicated that Beclin 1 siRNA could block the anti-miR-30a-induced neuroprotective effect in 1 h OGD/24 h reoxygenation treated N2A cells (n = 6 per group). **P* < 0.05 versus siNC, siNC: Beclin 1 siRNA control group

significantly changed miRNAs in the brain of mice with ischemic stroke. miRNA-30a, one of the 19 differentially expressed miRNAs, was up-regulated in both cerebral cortex of HPC and the peri-infarct region of 6 h MCAO treated mice [17]. There were several studies about miRNA-30a target genes and their involvements in pathophysiological process. For examples, miRNA-30a is significantly down-regulated in highly metastatic colorectal cancer (CRC) cell lines and metastatic tissues, and may be a potential therapeutic target to block CRC metastasis [34]; the down-regulated miRNA-30a in non-small-cell lung cancer inhibits invasion and metastasis by targeting Snail [35]; as a potential prognostic marker, miRNA-30a could inhibit breast tumor growth, metastasis and invasion by targeting metadherin and vimentin [36]. Interestingly, recent reports also revealed that miRNA-30a is enriched in layer III pyramidal neurons, and negatively regulates brain derived neurotrophic factor expression in prefrontal cortex [37]; decreased miRNA-30a expression possibly contributes to the neuroprotective effect of neuropeptide Y in rat

cortical neurons exposed to A β [38]; and the circulating miRNA-30a may serve as a potential biomarker for acute myocardial infarction [39].

Autophagy plays key cellular functions such as degradation of long-lived proteins, organelle turnover, and adaptation to nutrient depletion, cellular development, and anti-aging. However, the role of autophagy in brain after ischemia/reperfusion injury is controversial. Some studies show that excessive autophagy plays a death-promoting role in neuronal death after stroke [40–42] and others support that autophagy is neuroprotective [43–45]. These contradictory conclusions might be due to both the extent and time point of autophagy induction in determining the result after cerebral ischemia/reperfusion injury. During autophagy, LC3 is processed from LC3-I (16 kDa) to LC3-II (14-kDa), which is recruited to autophagosomes, and the increase in the LC3-II/I ratio is an indicator of up-regulated autophagy [46]. As expected, we found that after ischemia/reperfusion injury, the ratios of LC3-II to LC3-I were increased in cerebral cortex and in N2A cells. The results

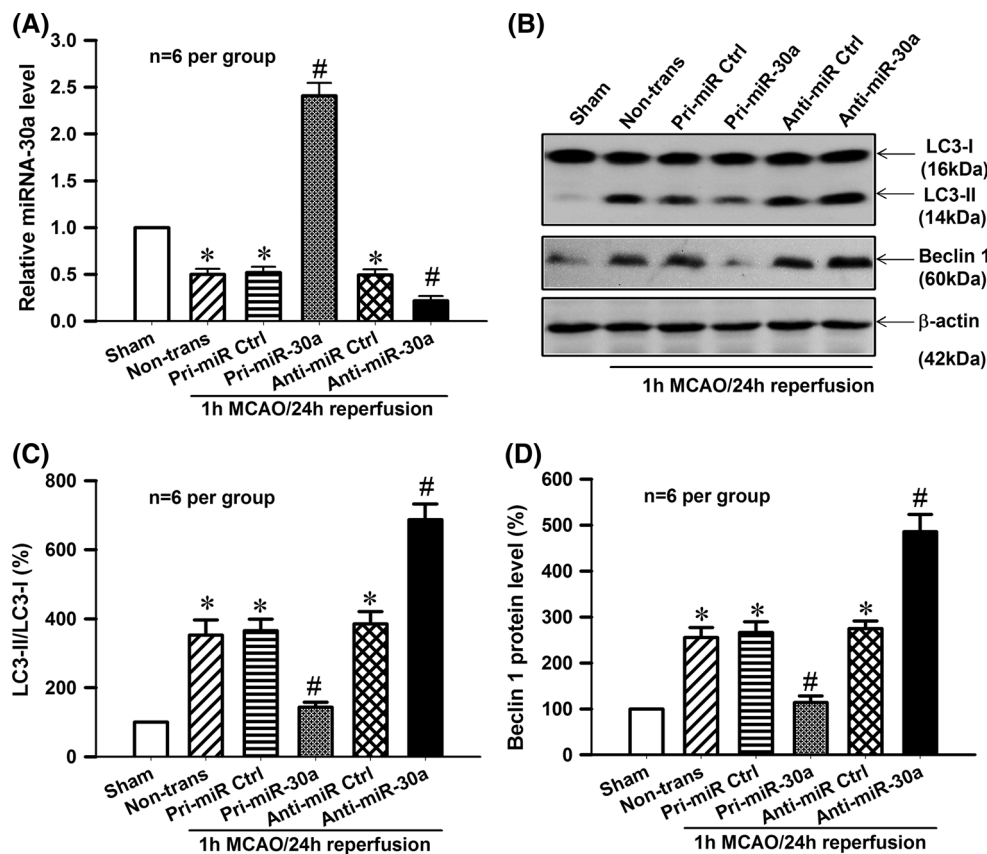


Fig. 6 Effect of miRNA-30a on the ratio of LC3-II/LC3-I and Beclin 1 protein levels in ischemic cortex of MCAO mice with ischemic stroke. **a** Stereotaxic injection of lentivirus-based pri-miR-30a and anti-miR-30a could effectively up- and down-regulated miRNA-30a expression level in ischemic cortex of 1 h MCAO/24 h reperfusion treated mice with ischemic stroke. **b** Typical result of Western blot showed the effect of miRNA-30 on the ratio of LC3-II/LC3-I and Beclin 1 protein levels. **c, d** The quantitative analysis results

demonstrated that up-regulation of miRNA-30a by pri-miR-30a could inhibit the ratio of LC3-II/I and reduced Beclin 1 protein expression level. In contrast, down-regulation of miRNA-30a by anti-miR-30a injection could effectively elevate the ratio of LC3-II/I and increased Beclin 1 protein expression level in ischemic cortex of 1 h MCAO/24 h reperfusion treated mice with ischemic stroke. * $P < 0.05$ versus Sham, # $P < 0.05$ versus Non-trans in condition of 1 h MCAO/24 h reperfusion, $n = 6$ per group

of our study also indicate that upon ischemia/reperfusion conditions, activation of autophagy was enhanced in anti-miR-30a treated group, whereas autophagy was inhibited in pri-miR-30a treated group. These demonstrated that protective autophagy was induced in our models because targeting miRNA-30a-mediated autophagy exacerbated neural cell death after ischemia/reperfusion injury, suggesting that after a period of reperfusion, autophagy may rescue cells by eliminating damaged organelles and protein aggregates.

According to the prediction of miRNA targets in human (<http://www.targetscan.org>) and in vertebrates (<http://pic tar.mdc-berlin.de/>), miRNA-30a may regulate the expression of beclin 1 at the post-transcriptional level by pairing with partially complementary sites in the 3'-UTR of *beclin 1* mRNA. In additional experiments, we also demonstrated that miRNA-30a could directly bind with the 3'-UTR of *beclin 1* mRNA and promote its mRNA degradation. Pri-miR-30a decreased the expression of beclin 1, while anti-miR-30a increased its level in mouse brain after MCAO-

ischemic stroke in vivo and in N2A cells after OGD-induced ischemic injury in vitro. Furthermore, beclin 1 siRNA could block anti-miR-30a-mediated autophagy and neuroprotection in OGD-treated N2A cells. Taken together, these results provide strong evidence that miRNA-30a mediates the autophagy through negatively regulating autophagy-related gene *beclin 1* mRNA level in cerebral ischemic injury. However, there's also a report that phosphoinositide 3-kinase (PI3 K) catalytic subunit delta is a direct target of miR-30a as miR-30a binds directly to the 3'-UTR of PI3 K catalytic subunit delta mRNA [34]. The PI3 K/protein kinase B (PKB also Akt)/the mammalian target of Rapamycin (mTOR) signalling pathway negatively regulate autophagy under certain conditions [47, 48]. The role of PI3 K/Akt/mTOR signaling pathway in miR-30a-mediated autophagy after ischemic stroke should be observed in the future experiments.

In addition, we should notice that down-regulation of miRNA-30a in cultured neuronal cells with OGD/

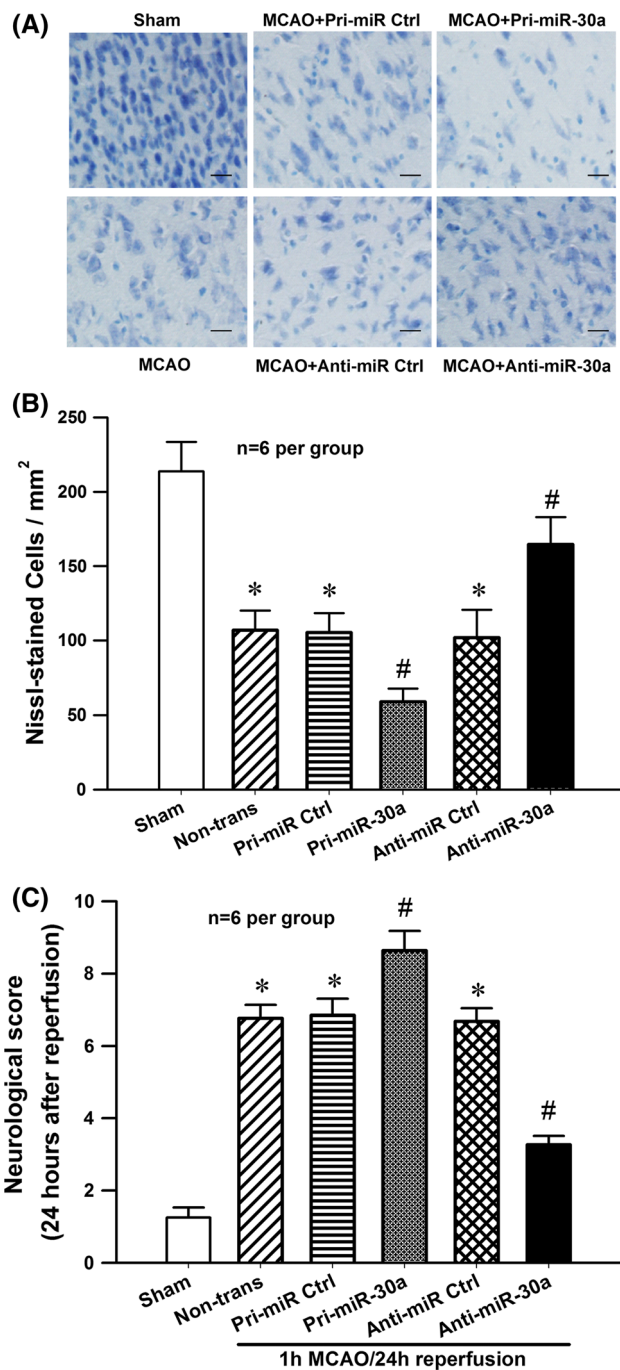


Fig. 7 Effect of miRNA-30a on neural cell loss in ischemic cortex and neurological scores of MCAO mice with ischemic stroke. **a** Typical images of Nissl staining showed the effect of miRNA-30a on neural cell loss in ischemic cortex of mice after 1 h MCAO/24 h reperfusion (scale bar = 100 μm). **b** Quantitative analysis demonstrated that up- and down-regulation of miRNA-30a through injection of lentivirus-based pri-miR-30a and anti-miR-30a could significantly enhance and attenuate the neural cell loss in ischemic cortex of mice after 1 h MCAO/24 h reperfusion, respectively (n = 6 per group). **c** Results of the neurological score indicated that pri-miR-30a and anti-miR-30a could separately aggravate and improve the neurological deficit of MCAO mice with ischemic stroke (n = 6 per group). *P < 0.05 versus Sham, #P < 0.05 versus Non-trans in condition of 1 h MCAO/24 h reperfusion

reoxygenation and brain of mice with MCAO/reperfusion provides neuroprotective effects through beclin 1-mediated autophagy during ischemia. Although the endogenous miRNA-30a levels were down regulated in mouse brain after MCAO/reperfusion and N2A cells after OGD/reoxygenation, the cell survival rate was still decreased. This may due to the protective and harmful factors were induced at the same time in cultured neuronal cells with 1 h OGD/6–48 h reoxygenation and brain of mice with 1 h MCAO/24 h reperfusion. The down-regulation of miRNA-30a that

reduced cell loss by increasing beclin 1-mediated autophagy might not be enough to provide protective effects at these conditions.

In summary, we have provided evidence that the down-regulation of miRNA-30a in brain of mice with ischemic stroke and in OGD-treated neurons could alleviate ischemic injury through enhancing beclin 1-mediated autophagy. The findings point out a novel mechanism for the regulation of ischemic neural cell death through miRNA-30a, and suggest that regulation of miRNA-30a and/or beclin1 in the brain could be potential therapeutic targets for ischemic stroke. Further work is needed to explore the stroke-related function of this particular miRNA in more time points of brain ischemia and reperfusion injury.

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Conflict of interest The authors confirm that there are no conflicts.

References

1. Marsh JD, Keyrouz SG (2010) Stroke prevention and treatment. *J Am Coll Cardiol* 56:683–691
2. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136:215–233
3. Fasanaro P, Greco S, Ivan M, Capogrossi MC, Martelli F (2010) microRNA: emerging therapeutic targets in acute ischemic diseases. *Pharmacol Ther* 125:92–104
4. Huang Y, Shen XJ, Zou Q, Zhao QL (2010) Biological functions of microRNAs. *Bioorg Khim* 36:747–752
5. Zhao Y, Srivastava D (2007) A developmental view of microRNA function. *Trends Biochem Sci* 32:189–197
6. Frankel LB, Wen J, Lees M, Hoyer-Hansen M, Farkas T, Krogh A, Jaattela M, Lund AH (2011) microRNA-101 is a potent inhibitor of autophagy. *EMBO J* 30:4628–4641
7. Chang Y, Yan W, He X, Zhang L, Li C, Huang H, Nace G, Geller DA, Lin J, Tsung A (2012) miR-375 inhibits autophagy and reduces viability of hepatocellular carcinoma cells under hypoxic conditions. *Gastroenterology* 143:177–187
8. Korkmaz G, Le SC, Tekirdag KA, Agami R, Gozuacik D (2012) miR-376b controls starvation and mTOR inhibition-related

- autophagy by targeting ATG4C and BECN1. *Autophagy* 8:165–176
9. Kovaleva V, Mora R, Park YJ, Plass C, Chiramel AI, Bartenschlager R, Dohner H, Stilgenbauer S, Pscherer A, Lichter P, Seiffert M (2012) miRNA-130a targets ATG2B and DICER1 to inhibit autophagy and trigger killing of chronic lymphocytic leukemia cells. *Cancer Res* 72:1763–1772
 10. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J* 19:5720–5728
 11. Cao Y, Klionsky DJ (2007) Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. *Cell Res* 17:839–849
 12. Wang J (2008) Beclin 1 bridges autophagy, apoptosis and differentiation. *Autophagy* 4:947–948
 13. Rink C, Khanna S (2011) MicroRNA in ischemic stroke etiology and pathology. *Physiol Genomics* 43:521–528
 14. Kosik KS (2006) The neuronal microRNA system. *Nat Rev Neurosci* 7:911–920
 15. Dharap A, Bowen K, Place R, Li LC, Vemuganti R (2009) Transient focal ischemia induces extensive temporal changes in rat cerebral microRNAome. *J Cereb Blood Flow Metab* 29:675–687
 16. Tan JR, Koo YX, Kaur P, Liu F, Armugam A, Wong PT, Jeyaseelan K (2011) microRNAs in stroke pathogenesis. *Curr Mol Med* 11:76–92
 17. Liu C, Peng Z, Zhang N, Yu L, Han S, Li D, Li J (2012) Identification of differentially expressed microRNAs and their PKC-isoform specific gene network prediction during hypoxic preconditioning and focal cerebral ischemia of mice. *J Neurochem* 120:830–841
 18. Zhu H, Wu H, Liu X, Li B, Chen Y, Ren X, Liu CG, Yang JM (2009) Regulation of autophagy by a beclin 1-targeted microRNA, miR-30a, in cancer cells. *Autophagy* 5:816–823
 19. Zou Z, Wu L, Ding H, Wang Y, Zhang Y, Chen X, Chen X, Zhang CY, Zhang Q, Zen K (2012) MicroRNA-30a sensitizes tumor cells to cis-platinum via suppressing beclin 1-mediated autophagy. *J Biol Chem* 287:4148–4156
 20. Yu Y, Yang L, Zhao M, Zhu S, Kang R, Vernon P, Tang D, Cao L (2012) Targeting microRNA-30a-mediated autophagy enhances imatinib activity against human chronic myeloid leukemia cells. *Leukemia* 26:1752–1760
 21. Pan W, Zhong Y, Cheng C, Liu B, Wang L, Li A, Xiong L, Liu S (2013) MiR-30-regulated autophagy mediates angiotensin II-induced myocardial hypertrophy. *PLoS ONE* 8:e53950
 22. Yin X, Peng C, Ning W, Li C, Ren Z, Zhang J, Gao H, Zhao K (2013) miR-30a downregulation aggravates pressure overload-induced cardiomyocyte hypertrophy. *Mol Cell Biochem* 379:1–6
 23. Bu X, Zhang N, Yang X, Liu Y, Du J, Liang J, Xu Q, Li J (2011) Proteomic analysis of cPKCbetaII-interacting proteins involved in HPC-induced neuroprotection against cerebral ischemia of mice. *J Neurochem* 117:346–356
 24. Feng S, Li D, Li Y, Yang X, Han S, Li J (2013) Insight into hypoxic preconditioning and ischemic injury through determination of nPKCepsilon-interacting proteins in mouse brain. *Neurochem Int* 63:69–79
 25. Zhang N, Yin Y, Han S, Jiang J, Yang W, Bu X, Li J (2011) Hypoxic preconditioning induced neuroprotection against cerebral ischemic injuries and its cPKCgamma-mediated molecular mechanism. *Neurochem Int* 58:684–692
 26. Rodriguez R, Santiago-Mejia J, Gomez C, San-Juan ER (2005) A simplified procedure for the quantitative measurement of neurological deficits after forebrain ischemia in mice. *J Neurosci Methods* 147:22–28
 27. Zhou L, Li F, Xu HB, Luo CX, Wu HY, Zhu MM, Lu W, Ji X, Zhou QG, Zhu DY (2010) Treatment of cerebral ischemia by disrupting ischemia-induced interaction of nNOS with PSD-95. *Nat Med* 16:1439–1443
 28. Li J, Qu Y, Zu P, Han S, Gao G, Xu Q, Fang L (2006) Increased isoform-specific membrane translocation of conventional and novel protein kinase C in human neuroblastoma SH-SY5Y cells following prolonged hypoxia. *Brain Res* 1093:25–32
 29. Li J, Niu C, Han S, Zu P, Li H, Xu Q, Fang L (2005) Identification of protein kinase C isoforms involved in cerebral hypoxic preconditioning of mice. *Brain Res* 1060:62–72
 30. Bak M, Silahatoglu A, Moller M, Christensen M, Rath MF, Skryabin B, Tommerup N, Kauppinen S (2008) MicroRNA expression in the adult mouse central nervous system. *RNA* 14:432–444
 31. Hebert SS, De SB (2009) Alterations of the microRNA network cause neurodegenerative disease. *Trends Neurosci* 32:199–206
 32. Liu DZ, Tian Y, Ander BP, Xu H, Stamova BS, Zhan X, Turner RJ, Jickling G, Sharp FR (2010) Brain and blood microRNA expression profiling of ischemic stroke, intracerebral hemorrhage, and kainate seizures. *J Cereb Blood Flow Metab* 30:92–101
 33. Teplyuk NM, Mollenhauer B, Gabriely G, Giese A, Kim E, Smolsky M, Kim RY, Saria MG, Pastorino S, Kesari S, Krichevsky AM (2012) MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity. *Neuro Oncol* 14:689–700
 34. Zhong M, Bian Z, Wu Z (2013) miR-30a suppresses cell migration and invasion through downregulation of PIK3CD in colorectal carcinoma. *Cell Physiol Biochem* 31:209–218
 35. Kumarswamy R, Mudduluru G, Ceppi P, Muppala S, Kozlowski M, Niklinski J, Papotti M, Allgayer H (2012) MicroRNA-30a inhibits epithelial-to-mesenchymal transition by targeting Snai1 and is downregulated in non-small cell lung cancer. *Int J Cancer* 130:2044–2053
 36. Cheng CW, Wang HW, Chang CW, Chu HW, Chen CY, Yu JC, Chao JI, Liu HF, Ding SL, Shen CY (2012) MicroRNA-30a inhibits cell migration and invasion by downregulating vimentin expression and is a potential prognostic marker in breast cancer. *Breast Cancer Res Treat* 134:1081–1093
 37. Mellios N, Huang HS, Grigorenko A, Rogaev E, Akbarian S (2008) A set of differentially expressed miRNAs, including miR-30a-5p, act as post-transcriptional inhibitors of BDNF in prefrontal cortex. *Hum Mol Genet* 17:3030–3042
 38. Croce N, Gelfo F, Ciotti MT, Federici G, Caltagirone C, Bernardini S, Angelucci F (2013) NPY modulates miR-30a-5p and BDNF in opposite direction in an in vitro model of Alzheimer disease: a possible role in neuroprotection? *Mol Cell Biochem* 376:189–195
 39. Long G, Wang F, Duan Q, Yang S, Chen F, Gong W, Yang X, Wang Y, Chen C, Wang DW (2012) Circulating miR-30a, miR-195 and let-7b associated with acute myocardial infarction. *PLoS ONE* 7:e50926
 40. Zheng YQ, Liu JX, Li XZ, Xu L, Xu YG (2009) RNA interference-mediated downregulation of Beclin1 attenuates cerebral ischemic injury in rats. *Acta Pharmacol Sin* 30:919–927
 41. Shi R, Weng J, Zhao L, Li XM, Gao TM, Kong J (2012) Excessive autophagy contributes to neuron death in cerebral ischemia. *CNS Neurosci Ther* 18:250–260
 42. Wen YD, Sheng R, Zhang LS, Han R, Zhang X, Zhang XD, Han F, Fukunaga K, Qin ZH (2008) Neuronal injury in rat model of permanent focal cerebral ischemia is associated with activation of autophagic and lysosomal pathways. *Autophagy* 4:762–769
 43. Papadakis M, Hadley G, Xilouri M, Hoyte LC, Nagel S, McMenamin MM, Tsaknakis G, Watt SM, Drakesmith CW, Chen R, Wood MJ, Zhao Z, Kessler B, Vekrellis K, Buchan AM

- (2013) Tsc1 (hamartin) confers neuroprotection against ischemia by inducing autophagy. *Nat Med* 19:351–357
44. Carloni S, Buonocore G, Balduini W (2008) Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury. *Neurobiol Dis* 32:329–339
45. Wang P, Guan YF, Du H, Zhai QW, Su DF, Miao CY (2012) Induction of autophagy contributes to the neuroprotection of nicotinamide phosphoribosyltransferase in cerebral ischemia. *Autophagy* 8:77–87
46. Mizushima N, Yoshimori T, Levine B (2010) Methods in mammalian autophagy research. *Cell* 140:313–326
47. Tanida I (2011) Autophagosome formation and molecular mechanism of autophagy. *Antioxid Redox Signal* 14:2201–2214
48. Ge W, Ren J (2012) mTOR-STAT3-notch signalling contributes to ALDH2-induced protection against cardiac contractile dysfunction and autophagy under alcoholism. *J Cell Mol Med* 16:616–626