Downregulation of microRNA-196a attenuates ischemic brain injury in rats by directly targeting HMGA1

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Abstract. – OBJECTIVE: Dysfunction of the microRNA (miRNA) network is a major regulator in neurological diseases. However, little is known about the functional significance of miRs in ischemic brain injury. This study was designed to investigate the functional behaviors and regulatory mechanisms of miR-196a in ischemic brain injury.

MATERIALS AND METHODS: Cerebral ischemia was induced by middle cerebral artery occlusion (MCAO) in rats. The expression levels of miR-196a and HMGA1 were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), Western blotting, and/or immunocytochemistry. The role of miR-196a in cerebral infarction and brain cell apoptosis was determined by infarct volume estimation and Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay, respectively. Bioinformatics software and Luciferase analysis were used to predict and confirm the targets of miR-196a.

RESULTS: The results showed that the miR-196a expression was significantly increased in rat brain after MCAO and in cultured neonatal rat cortical neurons after oxygen-glucose deprivation (OGD). Pretreatment with antago-miR-196a by intracerebroventricular markedly reduced the miR-196a mRNA and HMGA1 protein of the brain in rats subjected to MCAO. Notably, the knockdown of miR-196a could protect MCAO rats against cerebral infarction and brain cell apoptosis. In the OGD model, apoptosis and miR-196a expressions were inhibited, while High-Mobility-Group-A1 (HMGA1) levels were increased in cortical neurons in a time-dependent manner. Moreover, HMGA1 was the target gene of miR-196a. MiR-196a overexpression promoted OGD-induced cortical neurons apoptosis possibly through negative regulation of HMGA1.

CONCLUSIONS: These findings indicated a crucial role of miR-196a in regulating infarct volume and neuronal cell death under cerebral ischemia, thus offering a new target for the development of therapeutic agents against ischemic brain injury.

Introduction

Ischemic brain injury is characterized by oxidative stress, hypoxia, inflammation, and glutamate excitotoxicity, which is caused by insufficient blood flow to the brain, eventually leading to cell apoptosis and death[1]. Ischemic stroke is a leading cause of death and long-term disability in the world[2]. Neonatal hypoxic-ischemic brain injury or neonatal stroke is a major cause of hypoxic-ischemic encephalopathy and cerebral palsy[3]. The advances in suitable therapy for the purpose of decreasing ischemic brain injury have been limited because the pathophysiological mechanisms remain unknown. Therefore, it is of great importance to understand the pathological mechanism of ischemia-related cell death for developing effective therapies for ischemic brain injury. MicroRNAs (miRs) are an abundant family of small RNAs (-22 nucleotides) that fine-tune the expression of the genes implicated in biological processes such as tumor cells differentiation, proliferation and apoptosis[4,5]. Kosik[6] has indicated that miRNAs are abundantly expressed in the nervous system and identified as crucial molecular mediators in the regulation of neuronal cell survival. For example, miR-497 was the first found to promote ischemic neuronal death by negatively regulating anti-apoptotic proteins, bcl-2 and bcl-w[7]. MiR-210, miR-29b, miR-124 were demonstrated to participate in the biological processes in nervous system. These findings suggest miRs could be potential candidates for possible biomarkers or therapeutic targets in stroke. MiR-

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196a was originally identified as an oncogene which is associated with apoptosis, invasion, and proliferation. Increasing evidence has suggested that the aberrant expression of miR-196a is a frequent event in various cancers, including head and neck squamous cell carcinomas, laryngeal cancer, pancreatic cancer and gastric cancer[8-11]. In addition, miR-196a is a putative diagnostic biomarker or therapeutic target for various human diseases including adrenomyeloneuropathy, chronic hepatitis and Huntington's Diseases[12-14]. However, little is known about the correlation between miR-196a and ischemic brain injury. In the present work, we found that the expression of miR-196a was significantly increased during OGD *in vitro* and MCAO *in vivo*. In addition, down-regulated miR-196a attenuated the ischemic brain injury which could be also be accomplished by High-Mobility-Group-A1 (HMAG1) overexpression. Furthermore, Luciferase assay verified that miR-196a directly targeted HMAG1 and inhibited its expression.

Materials and Methods

Rat Cortical Neurons Isolation and Culture

Primary cortical neurons were obtained from neonatal Sprague-Dawley (SD) rats (<24 h). All implementation procedures of animals were approved by the Institutional Animal Care and Use Committee of the Shanghai Jiao Tong University. The neonatal brain was removed and fragmented in D-hanks solution (Gibco, Grand Island, NY, USA). The cortex was sliced into 1 mm^3 fragments. Subsequently, the cortical pieces were dissociated by 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) contained with 100 ng/ml DNase (Roche, Basel, Switzerland) at 37°C for 35 min. The digestion was terminated with Dulbecco's Modified Eagle Medium (DMEM; Hyclone, South Logan, USA) with 10% FBS (Gibco, Grand Island, NY, USA). Cells were plated in 6-well dishes at a density of 1×10^6 cells/well in Neurobasal-A medium supplemented with 2% B27, 1% glutamine (Gibco, Grand Island, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. In this process, cells were incubated in a humidified incubator with 5% CO_2 at 37°C, with the medium changed every 3 days. After 7 days in culture, the cells were used for subsequent oxygen-glucose derivation (OGD) model analysis.

Establishment of Male Cerebral Artery Occlusion (MCAO) Models

The experiments were carried out on SD adult male rats (289 ± 10 g). Animals were randomly divided into four groups: sham, MACO, antagomiR control, and antagomiR-196a. Either the antagomiR or antagomir-196a was intracerebroventricularly administrated 30 min before the operation. MCAO model was performed as described by Engel et al[15] with slight changes. Briefly, rats were anaesthetized using 1% sodium pentobarbital (40 mg/kg), and a midline neck incision was made to expose the right common carotid artery (CCA), external carotid artery (ECA) and internal carotid artery (ICA). Subsequently, a piece of 4/0 monofilament nylon suture with its tip slight rounded by heat was inserted through the ECA and entered into the right ICA to the base of the middle cerebral artery, thus occluding blood flow to the cortex and striatum. The wound was closed with a small suture clip and subjected to 90 min of MCAO. Then, the filament was withdrawn to allowed reperfusion for 24 h. The laser-Doppler flow meter (moorVMS-LDF1) was used to verify successful occlusion and reperfusion during MCAO. Only the rats that showed a reduction greater than 70% in cerebral blood flow were considered as ischemic and chosen for the following research. Sham group animals were subjected to similar operations to expose the carotid arteries without occlusion of the middle cerebral artery.

Infarct Volume Analysis

The rats were sacrificed and the brain tissue was removed, sliced into 1.0-mm-thick coronal sections and frozen for 30 min at -20°C. The sections were placed in a 1% solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at 37°C and fixed in 4% formalin for 24 h. The infarct region lacks staining and appears white, whereas the normal non-infarct tissue appears red. Both sides of each stained coronal slice were photographed using a digital camera, and infarction was measured with digital image analysis software Image J (Bethesda, MD, USA). The infarct volume was expressed as a percentage: the total infarct volume of ipsilateral structure/total volume of contralateral structure×100%.

Transferase-Mediated Deoxyuridine Triphosphate-Biotin Nick End Labeling (TUNEL) Assay

After the establishment of MCAO in adult rats, coronal brain tissues were fixed in 10% parafor-

maldehyde for 24 h, dehydrated in alcohol, and embedded in paraffin. The specimens were then cut into slices of 4 μm. Transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay was performed to detect MCAO and OGD-mediated apoptosis in rat brains and cortical neurons using TUNEL analysis kit (Roche, Basel, Switzerland). The cell nucleus was presented as blue color and the positive nucleus was indicated as green color. The number of positive nuclei was determined by manually counting (magnification× 400) the positively labeled nuclei in five randomly selected fields under a microscope. Apoptosis rate was considered as the percentage of TUNEL-positive cells from the total cell number.

Immunocytochemistry

The 4 μm sections were deparaffinized and rehydrated through graded alcohols followed by exposure to the antigen retrieval system (10 mM sodium citrate, 0.05% Tween 20, pH = 6.0) 15 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 15 minutes at room temperature. Then, the slides were rinsed in 0.01 mol/L phosphate-buffered saline solution (PBS; Gibco, Grand island, NY, USA) 3 times and subsequently incubated with the primary mouse polyclonal antibody (1:200, Abcam, Cambridge, MA, USA) for 1 hour at room temperature. After washing the slides with PBS, the slides were incubated with horseradish peroxidase (HRP) labeled goat anti-mouse antibody (Beyotime, Shanghai, China). Then, the slides were stained with DAB peroxidase substrate kit (Beyotime, Shanghai, China). Positive nuclei were indicated as brown color. Slides were then observed under a microscope, positive cells were counted and photographed.

Quantitative Real Time-Polymerase Chain Reaction

Total RNA was extracted with TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. For complementary DNA (cDNA) samples, 1 μg of total RNA was reverse-transcribed into cDNA with TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in a 15 μL total volume. The expression levels of miR-196a and HMGA1 were quantified by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) using human TaqMan MicroRNA Assay Kits (Applied Biosystems, Foster City, CA, USA) on an IQ5 Real Time-PCR system (Bio-Rad, Hercules, CA, USA). The U6RNA and GAPDH were used as internal control for miR-196a and HMGA1 expression normalization. The primers were used as follows: miR-196a (forward: 5′-CGTCAGAAGGAATGATGCACAG-3′ and reverse: 5′-ACCTGCGTAGGTAGTTTCATGT-3′), HMGA1 (forward: 5′-GGCCCAAATCGACCATAAAGG-3′ and reverse: 5′-GGACAAATCATGGCTACCCCT-3′), GAPDH (forward: 5′-AGAGGCAGGGATGATGTTCTG-3′ and reverse: 5′-GACTCATGACCACAGTCCATGC-3′) and U6 (forward: 5′-CTCGCTTCGGCAGCACA-3′ and reverse: 5′-AACGCTTCACGAATTTGCGT-3′). The quantification was performed by the comparative $2^{-\Delta\Delta CT}$ method.

Cell Transfection

Approximately 2×10^5 cortical neurons were plated in 6-well plates and cultured for 24 h. For transfection, cells were transfected with miR-196a mimics, miR Control, miR-196a inhibitor, HMGA1, or miR-196a+HMGA1 (final concentration of 30 mM) using DharmaFECT 1 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions. The miRIDIAN mimics (miR-196a), miR-196a miRIDIAN Hairpin Inhibitor (miR-196a inhibitor, IH-300529-06), and miR Control were purchased from GenePharma (Shanghai, China), while the HMGA1 expressing vector pIRES-puro 3 was obtained from Life Technologies (#13466, Gaithersburg, MD, USA). Cortical neurons were harvested 48 h post-transfection for the following Western blotting, RT-qPCR, or TUNEL assay.

OGD Analysis

For OGD, the transfected (HMGA1, miR-196a, and miR-196a+HMGA1) and non-transfected control newborn cortical neurons were cultured with glucose-free Earl's balanced salt solution (EBSS) medium (in mg/l: 6800 NaCl, 400 KCl, 264 CaCl$_2$ * 2H$_2$O, 200 MgCl$_2$ * 7H$_2$O 2200 NaHCO$_3$, 140 NaH$_2$PO4 * H$_2$O; pH = 7.2), which was purged with N$_2$/CO$_2$ (95%/5%) for 20 min as a result of oxygen concentration of 1%. Cortical neuronal cell cultures were washed three times with glucose-free EBSS medium and incubated for 0 h, 12 h and 24 h in oxygen-free N$_2$/CO$_2$ (95%/5%) gas[16]. The cells of the control group were grown in EBSS supplemented with 10 mM glucose in a standard incubator.

Luciferase Assays

For the construction of Luciferase reporter vectors, a region of the 3'UTR of HMGA1 containing

the putative target site (WT) or their mutant forms (MUT) was designed using TargetScan software (http://www.target scan.org) and synthesized by Shanghai Sangon Biological Engineering Technology and Service (Shanghai, China). Before being inserted into the PGL3-Basic Luciferase vector (Promega, Madison, WI, USA), the oligonucleotide was hotted for 5 min at 90°C and cooled to 37°C for 10 min to form a duplex. Cortical neurons cells were plated at a density of 4×10^3 cells/well in 96-well plates and cultured for 24 h. Cells were co-transfected with 40 ng pGL3-HMGA1-3'UTR-WT or pGL3-HMGA1-3'UTR-MUT vectors and miR-196a mimics or scrambled oligonucleotides (RiboBio, Guangzhou, China) at a final concentration of 45 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Luciferase assays were performed with a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA) 48 h after transfection. Renilla Luciferase activity was normalized to that of Firefly Luciferase.

Western Blot Analysis

Total proteins were extracted with radioimmunoprecipitation assay lysis buffer (RIPA; Beyotime Biotechnology, Shanghai, China). 30 μg of protein was separated by 10% Tris-glycine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA) with the electrophoretic transfer system (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat dry milk in Tris-HCl buffered saline (TBS, pH 7.4) containing 0.1% Tris-Buffered Saline and Tween 20 (TBST; Sigma-Aldrich, St. Louis, MO, USA) for 1 h at room temperature followed by incubated with primary antibody against HMGA1 (1:1000, Abcam, Cambridge, MA, USA), caspase-3 (1:1000, ProteinTech, Chicago, IL, USA) and GAPDH (1:1000, Abcam, Cambridge, MA, USA) overnight at 4°C. Subsequently, incubate the membranes with appropriate horseradish peroxidase-conjugated secondary antibodies for 2 hours followed by detection with a Super Signal Enhanced Chemiluminescence kit (Pierce, Waltham, MA, USA). Quantification of bands was made by scanned densitometric analysis and Image J analysis system (Bethesda, MD, USA).

Statistical Analysis

All data are expressed as mean ± standard deviation (SD) from at least three separate experiments. The differences between the groups were analyzed using Student's *t*-test. The difference was deemed statistically significant at $p < 0.05$.

Results

Antagomir-196a Treatment Reduced Infarct Volume and Attenuated Cortical Neurons Apoptosis in MCAO Rats

To explore the impact of miR-196a on ischemic brain injury, antagomir-196a and antagomir control were injected intracerebroventricularly into adult rats, respectively. AntagomiR-196a administration blocked the infarct volume upregulation induced by MCAO, displaying smaller infarct volume than the control (Figure 1A and B). Notably, it now appears that nerve cell apoptosis may occur following either focal or global cerebral ischemia[17]. To determine whether miR-196a affected cell apoptosis in rat brains, TUNEL staining assay was carried out. As a result, the proportion of apoptotic cells was much lower in miR-196a antagomiR injected rat brains than in antagomiR control (Figure 1C and D). When non-injected rats were subjected to MCAO, the miR-196a mRNA increased remarkably in comparison with the sham group. Meanwhile, pretreatment with antagomiR-196a decreased the post-MCAO miR-196a mRNA expressions compared with the group of antagomiR (Figure 1E). Considered the abnormal expression of HMGA1 in the previous research *in vitro*, we evaluated the expression of HMGA1 using immunocytochemistry in the rat brain subjected to MCAO. As shown in Figure 1F and G, the expression of HMGA1 was down-regulated in the MCAO group compared with that in control. Antagomir-196a injection enhanced the HMGA1 expression in comparison with antagomir control. Consistently, pretreatment with antagomiR-196a markedly decreased the expression of caspase-3 in MCAO rats (Figure 1H). These results indicate that the intracerebroventricularly injection of antagomiR-196a could protect against cerebral infarction and cerebral cells apoptosis in MCAO rats, which was accompanied with elevated HMGA1.

The Expression of MiR-196a and HMGA1 were Altered in Cortical Neurons Subjected to OGD

TargetScan prediction tool was performed to predict the target gene of miR-196a. We then focused on HMGA1, a molecular that has been found to be associated with apoptosis. The OGD
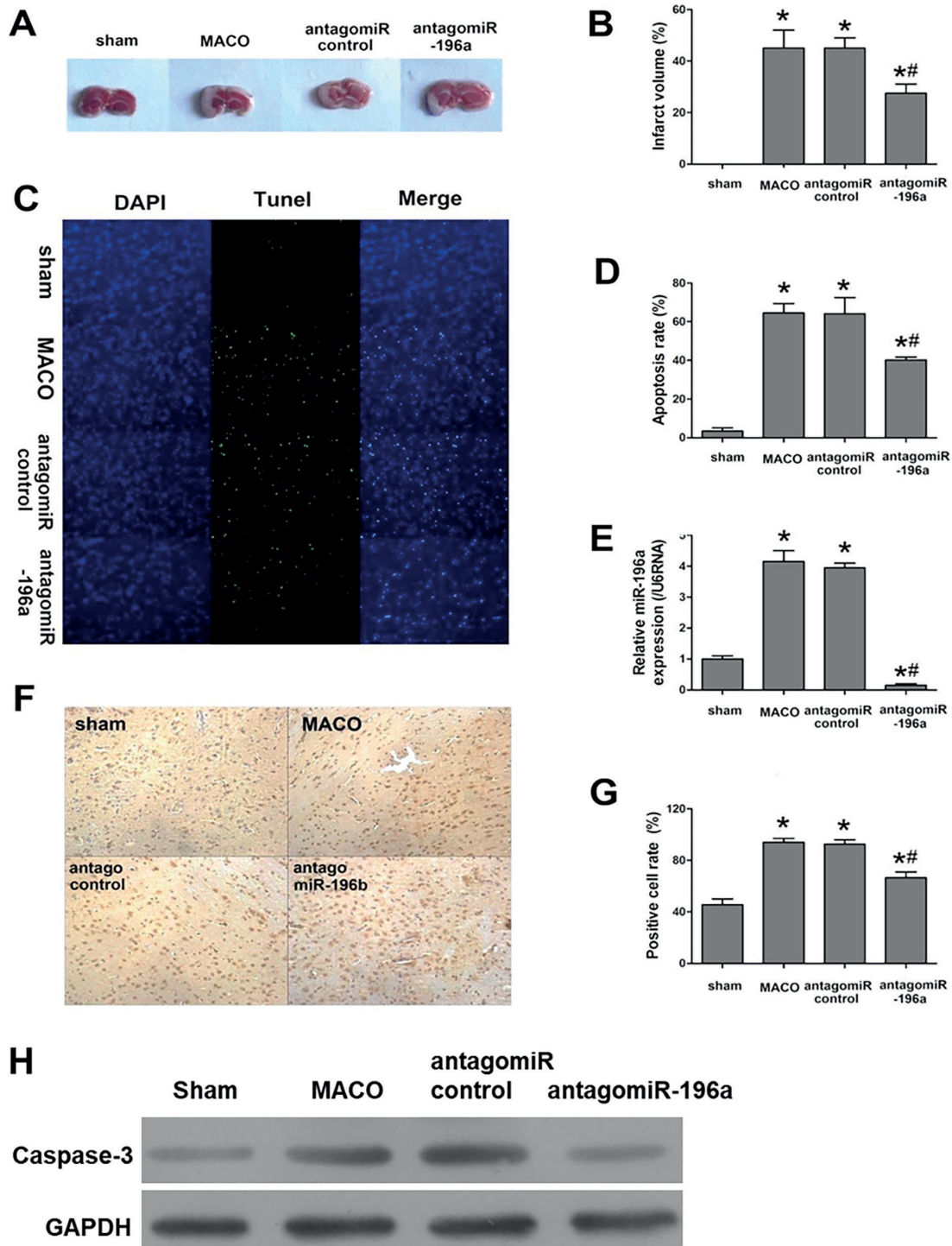
Figure 1. AntagomiR-196a reduced neuronal apoptosis and infarction volume after MCAO *in vivo*. *A,* Rats un-pretreated or pretreated with antagomir were subjected to 1.5 h MCAO followed by 24 h of reperfusion, 1% TTC-stained coronal sections were shown under different treatments. *B,* The infarction volume was analyzed with software Image J and expressed as a proportion of the ipsilateral hemispheric volume. *C-D,* Apoptosis of brain tissue after MCAO was evaluated by TUNEL assay. *E,* MiR-196a expression of brain tissue was detected by qRT-PCR. *F-G,* HMGA1 protein expression was investigated by immunocytochemistry in brain tissue. Positive nuclei were indicated as brown color; Magnification (200×); *H,* The expression of caspase-3 was determined using Western blotting in brain tissue after MCAO. Data are expressed as mean ± SD; n = 8 *$p <$ 0.05 *vs*. control, #$p <$ 0.05 *vs*. OGD group.

models were constructed from cortical neurons of neonatal SD rats. Expectedly, the apoptosis rate was time-dependently promoted under OGD conditions (Figure 2A and B). Furthermore, a significant upward trend was observed in the expression of miR-196a mRNA in the OGD model (Figure 2C). In contrast, 12 h OGD had an inhibitory effect on the HMGA1 mRNA and protein expressions in cultured neonatal rat cortical neurons, and the suppressions were strengthened at 24 h after OGD (Figure 2D and E). These observations showed that OGD induced time-dependent alters of miR-196a and HMGA1 expression levels.

MiR-196a Directly Targeted to the 3'UTR of HMGA1 mRNA

To reveal the relation between miR-196a and HMGA1, HMGA1 WT or MUT 3'UTR vector with scramble oligonucleotides or miR-196a mimics were co-transfected into 293T cells. The co-transfection of HMGA1 3'UTR with miR-196a mimics resulted in reduced activities of Luciferase (Figure 3A and B). A mutation in ACUACCUC motif in the 3'UTR of HMGA1 mRNA completely abrogated the suppression of Luciferase by miR-196a mimics (Figure 3A and B). The results demonstrated that HMGA1 is a direct miR-196a target. Western blot was conducted to determine whether HMGA1

expression was regulated post-transcriptionally by miR-196a. As shown in Figure 3C, miR-196a mimics transfection down-regulated HMGA1 protein expression in the neonatal cortical neurons that exposed to OGD, compared to the control groups, whereas cortical neurons transfected with miR-196a inhibitor, subjected to OGD, showed a partial recovery of HMGA1 protein levels. These data proposed that miR-196a may act as a repressor to negatively regulate HMGA1 post-transcriptionally.

MiR-196a and HMGA1 Modulated OGD-Induced Neonatal Cortical Neurons Apoptosis

To test whether miR-196a and HMGA1 effect on cortical neurons apoptosis was induced by OGD, the cells transfected with miR-196a mimics, HMGA1, or miR-196a+HMGA1 were subjected to OGD. As shown in Figure 4 A and B, the apoptotic rate was elevated significantly with miR-196a mimics-treatment but decreased treated with HMGA1. It is worth noting that the apoptosis rate of the miR-196a+ HMGA1 group was statistically higher than that of the miR-196a group, but lower than the HMGA1 group. These results indicate that miR-196a mimics enhanced OGD-cortical neurons apoptosis possibly through negative modulation of HMGA1.
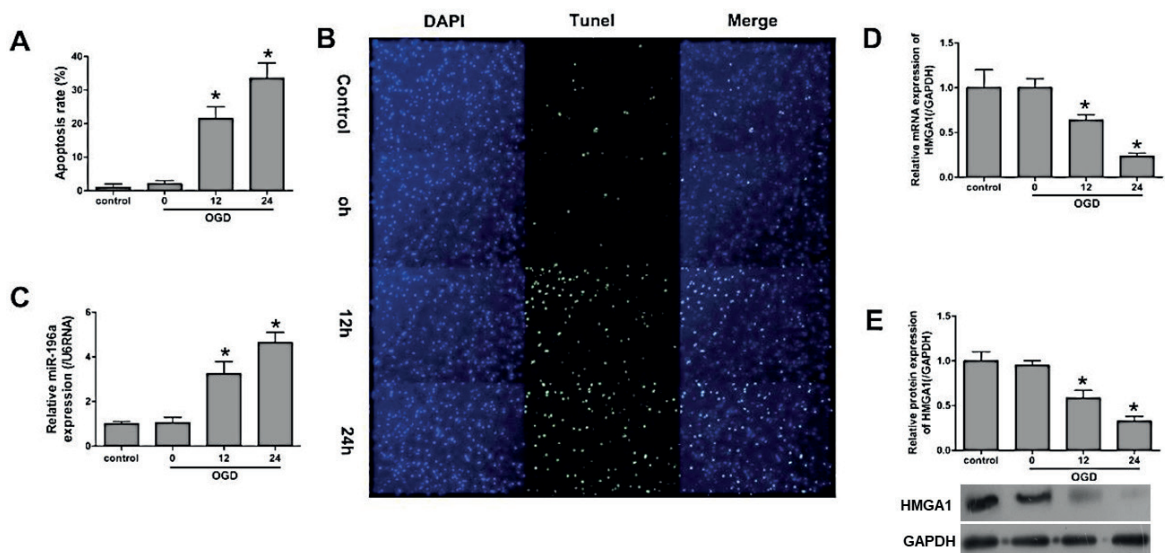
Figure 2. The expression of miR-196a and HMGA1, as well as the apoptosis rate, were changed in cortical neurons subjected to OGD. *A-B,* TUNEL assay was used to evaluate the apoptosis of cortical neurons after OGD. *C-D,* QRT-PCR was used to assess the mRNA expression of miR-196a and HMGA1. *E,* Western blot was carried out to investigate the protein expression of HMGA1. Magnification (200×); Data are expressed as mean ± SD; n = 8 *$p < 0.05$ *vs.* control.
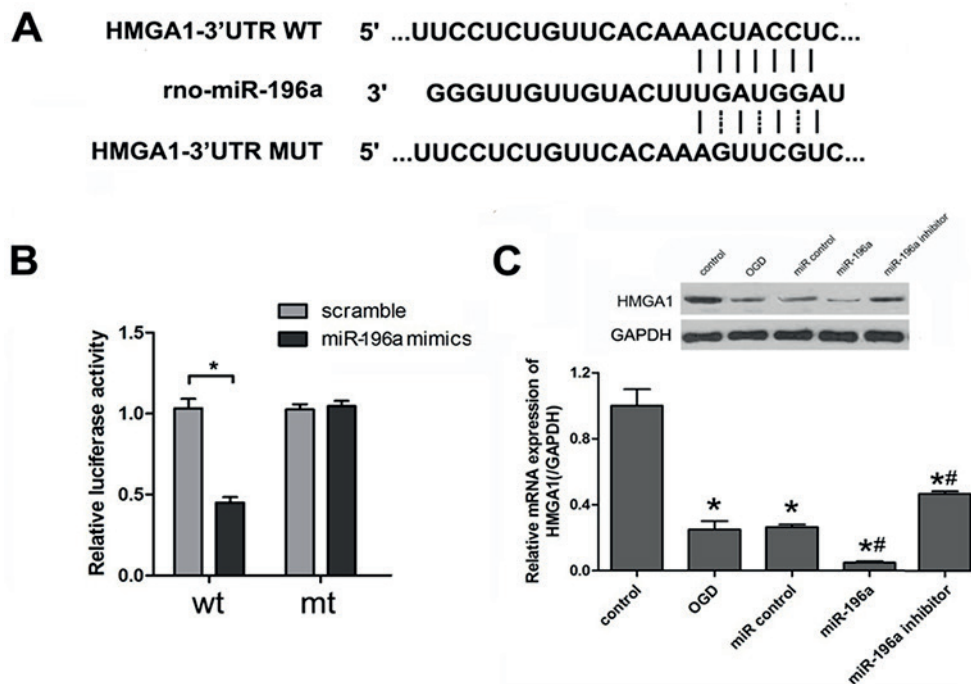
Figure 3. HMGA1 is directly targeted and negatively regulated by miR-196a. *A-B,* At 48 h post-co-transfection of pGL3-HMGA1-3'-UTR-WT or pGL3-HMGA1-3'-UTR-MUT with scramble oligonucleotides or miR-196a mimics, Luciferase activity was measured using the Dual-Luciferase Reporter Assay System. *C,* HMGA1 protein expressions of cortical neurons under different treatment were evaluated by Western blot. Data are expressed as mean ± SD; n = 8 *$p < 0.05$ *vs.* control, #$p < 0.05$ *vs.* OGD group.

Discussion

Accumulating evidence suggests that a subset of miRNAs are abundantly expressed in the human brain[18], and play important roles in the pathophysiology of brain seizures, ischemia, and trauma[19,20]. In the present work, we first demonstrated that miR-196a expression was enhanced notably during OGD *in vitro* and MCAO *in vivo*. The downregulation of miR-196a expression reduced brain infarct size and protected against ischemic induced brain cell apoptosis. Moreover, HMGA1 was a direct transcriptional target of miR-196a. Cerebral ischemia generates widespread molecular and biochemical changes, inflammatory responses, oxidative stress, free radical production and neuronal apoptosis[21-23]. After a stroke, the irreversible ischemia neuronal damage usually occurs in the areas of dense ischemia[24]. The necrotic core regions are surrounded by "ischemic penumbra" in which are less affected tissue, where cerebral blood flow is evidently reduced to disturb cell membrane potentials and ionic homeostasis[25-27]. Infarction would happen if the penumbra area or ischemia go on for a long time[28]. Recent studies[29,30] showed that several miRNAs

can serve as potential biomarkers for diagnosis of acute cerebral infarction, such as miR-124, miR-21 and miR-24. Concretely, plasma miR-124 concentration is markedly up-regulated in the rat MCAO model; miR-21 and miR-24 levels of mouse N2A neuroblastoma cells were increased after OGD and reoxygenation. Here, miR-196a expression profiles could be induced by MCAO in cerebral cells and by OGD in cortical neurons, indicating that miR-196a might serve as a new cerebral infarction biomarker. HMGA1 protein is a non-histone protein that regulates chromatin structure and gene expression during embryogenesis, tumorigenesis and immune responses, which is considered as a key hub for several oncogenic pathways, such as the Wnt/ß-catenin, Notch pathways and Ras/ERK signaling[31,32]. Neuronal apoptosis is the prominent cause of cell death in the secondary brain damage, which greatly affects the ischemic injury functional outcome[33]. Previous studies[34] have indicated that HMGA1 represses p53 apoptotic activity by promoting the cytoplasmic relocalization of the p53 pro-apoptotic activator HIPK2. Moreover, the anti-apoptotic effect of HMGA1 occurs by deregulation of Bcl-2[35]. In this work, after being treated with
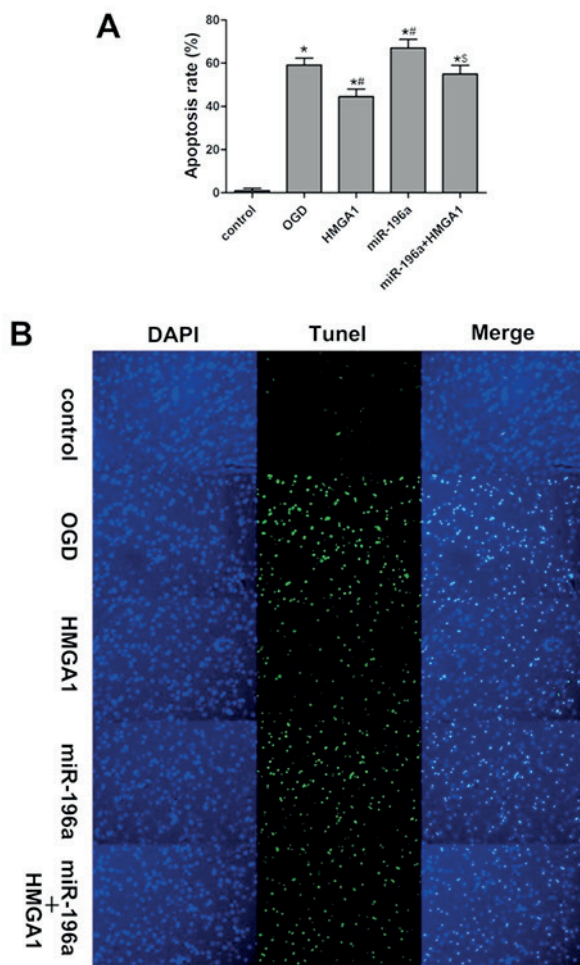
Figure 4. HMGA1 alleviated apoptosis of cortical neurons that is promoted by miR-196a under OGD. *A, B,* Apoptosis of cortical neurons under different treatments were evaluated by TUNEL assay. Data are expressed as mean ± SD; n = 8 *$p < 0.05$ *vs.* control, #$p < 0.05$ *vs.* OGD group, $$p < 0.05$ *vs.* miR-196a group.

OGD, cortical neurons apoptosis was attenuated by overexpression of HMGA1 and heightened by high expression of miR-196, while HMGA1 and miR-196a co-transfection created an intermediate level of apoptosis rate. Additionally, TargetScan and Luciferase activity assay demonstrated that HMGA1 as a direct target for miR-196a. These studies suggest that miR-196a promotes cortical neurons apoptosis by targeting HMGA1 through binding to "seedless" 3'UTR miRNA interaction sites. Thus, miR-196a-mediated inhibition of survival of cerebral cells contributed to infarction under cerebral ischemia. However, the molecular mechanisms underlying apoptosis needs further exploration. Whether HMGA1 regulate p53 or

Bcl-2 to realize its biological function is on the following working list.

Conclusions

We demonstrated that miR-196a is induced by ischemia and contributes to the pathogenesis of ischemic brain injury by directly targeting HMGA1. Accordingly, the inhibition of miR-196a may become a potential therapeutic option for ischemia-related brain damage. However, further studies using neuron-specific miR-196a are warranted.

Conflict of Interest
The Authors declare that they have no conflict of interest.

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