ARTICLE
Journal of Cellular Biochemistry 116:233–241 (2015)

Journal of Cellular Biochemistry

Neuroprotective Effects of Viral Overexpression of microRNA-22 in Rat and Cell Models of Cerebral Ischemia-Reperfusion Injury

Houyou Yu,[1] Mingchun Wu,[2] Peng Zhao,[1] Yang Huang,[1] Wei Wang,[1] and Wen Yin[1]*

[1]*Department of Emergency Medicine, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China*
[2]*Department of Anaesthesiology, Wuhan General Hospital of Guangzhou Command, Wuhan 430070, China*

ABSTRACT

Several studies have reported that microRNA (MIR) is involved in the pathogenesis and progression of ischemic diseases, including cerebral ischemia, and that MIR-22 may inhibit the inflammatory response and cell apoptosis, which contribute to ischemia/reperfusion (I/R) injury. However, the specific function of MIR-22 in cerebral I/R injury remains far from clear. This study aimed to examine the potential protective effect of MIR-22 against cerebral I/R injury and its mechanism. As predicted, adenovirus-mediated MIR-22 overexpression markedly reduced the neurological score and infarct size ($P < 0.05$). We demonstrated that MIR-22 overexpression resulted in a reduction in inflammatory cytokines TNF-α, IL-6, COX-2, and iNOS, whereas the level of IL-10 was enhanced. MIR-22 overexpression significantly inhibited NF-κB activity by decreasing NF-κB coactivator NCOA1 expression. Furthermore, we found that MIR-22 could reduce the apoptotic rate of cortical neurons. Caspase-3 activity was inhibited by MIR-22, and the expression of the anti-apoptosis gene Bcl-2 in neurons was increased and that of the pro-apoptosis gene Bax decreased following MIR-22 overexpression. Our results suggest that MIR-22 could be used to treat cerebral I/R injury and that its neuroprotective effect may be attributed to a reduction in inflammation and apoptosis. J. Cell. Biochem. 116: 233–241, 2015.

KEY WORDS: CEREBRAL ISCHEMIA/REPERFUSION INJURY; microRNA-22; INFLAMMATORY; APOPTOSIS

Ischemic brain injury threatens human health and life, causing high mortality and long-term disability [Hackett et al., 2000; Rosamond et al., 2008]. Cerebral ischemia-reperfusion (I/R) injury is increasingly recognized as a complication of restoring blood flow to ischemic brain tissue [Hallenbeck and Dutka, 1990]. Ischemia-reperfusion injury is the main cause of the aggravation of cerebral injury and functional impairment, and also leads to revascularization and cerebral infarction after thrombolysis [Schaller and Graf, 2004]. Accumulating evidence has demonstrated that excess calcium inside neurocytes, the excessive formation of free radicals, the cytotoxic effect of excitatory amino acids and cascade inflammatory reactions are the main factors contributing to cerebral I/R injury [Cao et al., 2007].

Evidence suggests that cerebral ischemia elicits an inflammatory response that is augmented by reperfusion [Soriano et al., 1999]. Inflammatory cascade reactions, as the main cause of aggravation of cerebral injury, have been the focus of attention [Huang et al., 2006]. Some evidence indicates that cerebral I/R injury may induce the up-regulation of cytokine expression, inflammatory cell aggregation and increased expression of intercellular adhesion molecules [Cao et al., 2007]. Moreover, anti-inflammatory agents displayed a neuroprotective effect in an animal model [Khan et al., 2004]. Additionally, accumulating evidence suggests that cerebral I/R injury also leads to neuronal death, especially in the hippocampal formation [Nitatori et al., 1995]. Moreover, extensive research has shown that anti-apoptotic reagents could attenuate cerebral I/R [Cao et al., 2011; Tao et al., 2013]. MicroRNA has been found to play an important role in the inflammatory response and neuronal death induced by ischemia-reperfusion injury, although the mechanism remains unclear.

Houyou Yu and Mingchun Wu have contributed equally to this study.
Conflict of interest: none.

Grant sponsor: National Natural Science Foundation of China (NSFC); Grant number: 81101367; Grant sponsor: Science and technology research and development projects of Shaanxi Province; Grant number: 2011K12–54.

*Correspondence to: Wen Yin, Department of Emergency Medicine, Xijing Hospital, Fourth Military Medical University, 17 Changle West Road, Xi'an 710032, China. E-mail: wenyinxian029@163.com

Manuscript Received: 20 April 2014; Manuscript Accepted: 29 August 2014
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 4 September 2014
DOI 10.1002/jcb.24960 • © 2014 Wiley Periodicals, Inc.

MicroRNAs (MIRs) are non-coding 19–24 nucleotide RNA molecules that act as negative regulators of post-transcriptional gene expression by binding to target messenger RNAs [Ambros, 2004; Wang et al., 2013]. Several reports have demonstrated that MIRs are involved in the pathogenesis and progression of many ischemic diseases, including cerebral ischemia; moreover, MIRs also hold great therapeutic potential. There is evidence that MIRs are involved in gene expression and function; however, little is known of their function in I/R injury [Chang et al., 2012]. Recent studies suggested that the expression of specific MIRs could affect neurogenesis, neuronal differentiation, neurodevelopmental function, and neural cell specification [Kosik, 2006; Schratt et al., 2006]. There is some evidence that MIR-22 protects against myocardial I/R injury through anti-apoptosis in the rat, and that MIR-22 could inhibit the inflammatory response by suppressing the activity of NF-κB [Takata et al., 2011; Yang et al., 2014]. Recent studies conducted by Ana Jovicic and colleagues showed that MIR-22 overexpression is neuroprotective via general anti-apoptotic effects and may also target specific Huntington's disease-related mechanisms [Jovicic et al., 2013]. However, limited information is available on the exact role of MIR-22 in cerebral I/R injury in vitro.

Herein, using a focal cerebral I/R injury rat model and an oxygen-glucose-deprivation/RP model of cultured neonatal rat cortical neurons as our experimental model, we found that MIR-22 overexpression attenuates cerebral I/R injury. Our findings also demonstrate that MIR-22 can prevent cerebral I/R injury through the attenuation of inflammation and neuron apoptosis. Understanding the mechanism by which MIR-22 promotes protection against ischemic injury is integral to the identification and development of novel brain ischemic injury therapeutics.

MATERIALS AND METHODS

ANIMALS

Male Sprague–Dawley rats (n = 41), weighing 260–320 g, were randomly divided into four groups: (1) sham-operation (n = 5); (2) MCAO group (n = 12); (3) MCAO + Scramble group (n = 12); (4) MCAO + MIR-22 group (n = 12). All rats received a standard diet and free water. All experimental protocols used in this study were carried out in accordance with the Guide for Animal Experimentation of the Fourth Military Medical University and were approved by the Ethics Committee for Animal Experimentation of Xijing Hospital, the Fourth Military Medical University, Shaanxi Province, China.

MIR-22 EXPRESSION VECTOR CONSTRUCT

Adenovirus expressing MIR-22 (Ad-MIR-22) was constructed as described previously [Yang et al., 2014]. A DNA segment encompassing the MIR-22 was synthesized by Genechem (Shanghai, China). The obtained sequences were fully sequenced (Sangon, Shanghai, China). Ad-MIR-22 or control Ad-Scramble was generated using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA) according to the operating protocols. The obtained adenoviruses were further packaged and amplified in HEK293 cells, and then purified using CsCl banding.

IN VIVO GENE TRANSFER AND ANIMAL MODEL OF FOCAL CEREBRAL ISCHEMIA AND REPERFUSION

The animals were subject to adenovirus-mediated gene transfer, and a subsequent focal brain ischemia-reperfusion surgical procedure as described previously with minor modifications [Li et al., 2012]. In brief, rats were anesthetized and then a burr hole was drilled 2.5 mm lateral from the sagittal suture and parallel to the bregma. A 20-μl solution of Ad-MIR-22 (1×10^9 PFU), Ad-Scramble (1×10^9 PFU) or saline was injected into two sites in the brain. Following injection, the wound was closed and the rat was allowed to recover. Cerebral ischemia and reperfusion treatment was performed 3 days later. Focal brain ischemia was induced via intraluminal occlusion of the right middle cerebral artery (MCAO) as described previously [Chang et al., 2007]. Briefly, rats were re-anesthetized, and the rectal temperature was maintained between 36.5 and 37.5°C throughout the surgical procedure and up to 1.5 h after reperfusion. In both the control and MIR overexpression groups, the filament remained in the lumen for 1.5 h, and was then withdrawn to allow reperfusion for 24 h.

NEUROLOGICAL EXAMINATION

Threats were neurologically assessed by an investigator who was unaware of animal grouping 24 h after reperfusion. An 25-point neurological scoring system with modifications was carried out in each group of rats for neurological assessment [Garcia et al., 1995].

DETERMINATION OF INFARCT SIZE

At 24 h after reperfusion, the rats (n = 12 for each group) were anesthetized. The brain was rapidly removed and sliced into six sequential sections (±5 mm, ± 3 mm, and ± 1 mm from the bregma). Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma–Aldrich, St Louis, MO) for 15 min at 37°C and then fixed in 4% formaldehyde solution. The infarct area in each brain slice was photographed with a digital camera (Kodak DC240, East-man Kodak Co.). Infarct volume was calculated according to the formula as follows: lesion area of each section = (contralateral hemisphere area/ipsilateral hemisphere area) x ipsilateral lesion area [Leach et al., 1993]. The volume of the lesions was estimated by the summation of the lesion areas of all sections multiplied by the slice thickness.

PRIMARY CORTICAL NEURON CULTURE

Rat primary cortical neurons were obtained from newborn Sprague–Dawley rats as described previously [Gong et al., 2007]. Briefly, cortices taken from Sprague-Dawley rats were collected and dissected. The cerebral tissues were minced and then incubated in 0.125% trypsin for 30 min. The reaction was terminated using DMEM/F12 medium with fetal bovine serum. The cell suspension was filtered and centrifuged (3,000 *g*, 10 min), then the sediment was re-suspended in DMEM/F12 medium. Cells were adjusted to 1×10^6/mL and plated on 96-well plates coated with 10 mg/L poly-L-lysine. After 72 h arabinosylcytosine (5 μg/mL) was added to the cell cultures to prevent the growth of non-neuronal cells. After 24 h, the normal medium was changed and then refreshed every 72 h.

SIMULATION OF ISCHEMIA AND REPERFUSION IN VITRO AND ADENOVIRUS INFECTION

Oxygen-glucose deprivation/reperfusion (OGD/RP) was performed as described previously [Gong et al., 2007]. Briefly, cortical neurons were exposed to glucose-free Earl's solution (5.4 mmol/L KCl, 116.4 mmol/L NaCl, 0.8 mmol/L $MgSO_4$, 1.8 mmol/L $CaCl_2$, 26.2 mmol/L $NaHCO_3$, 2.6 mmol/L NaH_2PO_4, and 20.1 mmol/L HEPES [pH 7.4]) and cultured in an incubator in an atmosphere of 5% CO_2 and 95% N_2 (OGD) for 2 h at 37°C. Then, glucose-free Earl's solution supplemented with 5.6 mmol/L glucose was added to terminate the OGD reaction, and the cells were cultured in an atmosphere of 5% CO_2 and 95% O_2 for another 12 h. Cortical neurons were infected with control adenovirus or MIR-22-expressing adenovirus for 6 h and then replaced with standard culture medium. At 72 h after adenovirus treatment, cells were subjected to OGD/RP.

TOTAL RNA EXTRACTION AND QUANTITATIVE REAL TIME PCR

The transcription of MIR-22 was detected according to the RT–PCR method described previously [Yang et al., 2014]. Total RNA of primary cultured neurons was extracted using Trizol reagent (Biostar, Shanghai, China) according to the manufacturer's instructions. MIR-22 levels were determined using the mirVana^{TM}qRT-PCR miRNA detection kit (Ambion). The ABI Prism 7,500 system (PE Applied Biosystems) was used to amplify and detect specific products. U6 was used as an internal control and the expression levels of the relative genes were calculated using the $2^{-\Delta\Delta CT}$ method [Livak and Schmittgen, 2001].

WESTERN BLOT

Total protein of OGD/RP-treated cortical neurons was isolated using RIPA lysis buffer (Beyotime, Nantong, China) according to the manufacturer's instructions. Protein (40 μg/lane) was subjected to 12% SDS–PAGE and electroblotted onto nitrocellulose (Amersham Pharmacia, Germany). Immunodetection of p65, Bcl-2, Bax, COX-2, iNOS and β-actin was performed using p65 antibody (Abcam, Cambridge, MA), Bcl-2 antibody (Abcam, Cambridge, MA), Bax antibody (Abcam, Cambridge, MA), COX-2 antibody (Abcam, Cambridge, MA), iNOS antibody (Abcam, Cambridge, MA), and β-actin antibody (Abcam, Cambridge, MA). Goat anti- Rabbit IgG (Abcam, Cambridge, MA) was used as the secondary antibody. The bound antibodies were visualized using ECL reagent (Boehringer Mannheim, Mannheim, Germany). All experiments were repeated three times.

ELISA

The expression of pro-inflammatory cytokines IL-6, TNF-α, and IL-10 in cortical neurons was detected using a commercially available ELISA kit (Aviscera Bioscience, Santa Clara, CA) according to the manufacturer's instructions.

shRNA

NCOA1-shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cells were transduced with lentiviral particles and then selected using puromycin.

DETECTION OF APOPTOTIC CELLS BY FLOW CYTOMETRY

The apoptosis of cortical neurons was measured by flow cytometry (Beckman-Coulter, Brea, CA) using an Annexin V-FITC/PI kit (BD PharMingen, San Diego, CA). Briefly, 1×10^6 cells/sample were collected after OGD/RP treatment and plated in 24-well plates. Cells were incubated with Annexin V/FITC for 15 min at room temperature, the cells were washed, and propidium iodide (PI) was then added. After 30 min the cells were ready for analysis by flow cytometry and the cell apoptotic ratio was assayed.

CASPASE ACTIVITY ASSAY

Caspase-3 activity was measured using the Caspase-Glo®3/7 Assay (Promega) according to the manufacturer's instructions [Jovicic et al., 2013]. In brief, cortical neurons (50 μL/well) were cultured in 96-well plates and then cells were co-cultured with 50 μL of Caspase-Glo reagent for 30 min. Luminescence was measured on a TECAN GenioPro plate reader in the linear range of the instrument.

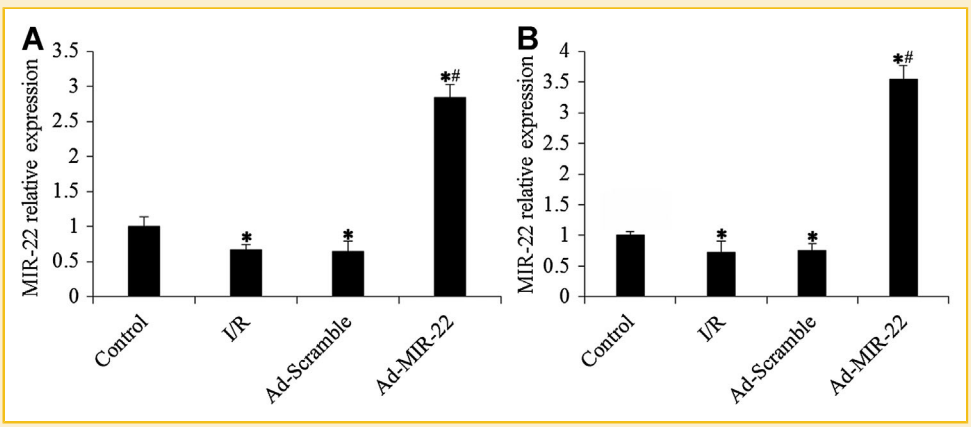

Fig. 1. Regulation of MIR-22 after cerebral I/R. MIR-22 levels was determined using the mirVana^{TM}qRT-PCR miRNA detection kit. (A) MIR-22 was down-regulated in the I/R rat model, while adenovirus-mediated MIR-22 gene transfer in vivo up-regulated its expression. (B) MIR-22 was down-regulated in the OGD treated cortex cell, while adenovirus-mediated MIR-22 gene transfers in vitro up-regulated its expression. All experiments were repeated at least three times. Values are presented as mean ± SEM. *$P < 0.05$ vs. control, #$P < 0.05$ vs. IR.

Caspase activity was measured at 24 h for neurons treated with OGD/RP treatment.

STATISTICAL ANALYSIS

All results are reported as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's *t*-test subsequent to ANOVA. A value of $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

RESULTS

CEREBRAL I/R INDUCED DOWN-REGULATION OF MIR-22 EXPRESSION

To overexpress MIR-22 in cerebral tissue and primary cortical neurons, we created an adenovirus vector that encodes MIR-22 in normal conditions. As shown in Figure 1A, after 24 h of reperfusion, MIR-22 expression was significantly down-regulated relative to the control group without injury. Three days after transfection of MIR-22 into the brain, MIR-22 expression was markedly increased compared with the control group and I/R group ($P < 0.05$). The expression of MIR-22 in cortical neurons was also determined. Figure 1B shows that MIR-22 expression was significantly up-regulated in neurons transduced with Ad- MIR-22 ($P < 0.05$). Moreover, Ad-Scramble had no apparent effect on the expression level of MIR-22 compared with the I/R group.

EFFECTS OF MIR-22 ON NEUROLOGICAL SCORES AND INFARCT VOLUME

All animals survived until 24 h after reperfusion. Many studies have suggested that I/R injury leads to a deterioration in neurological

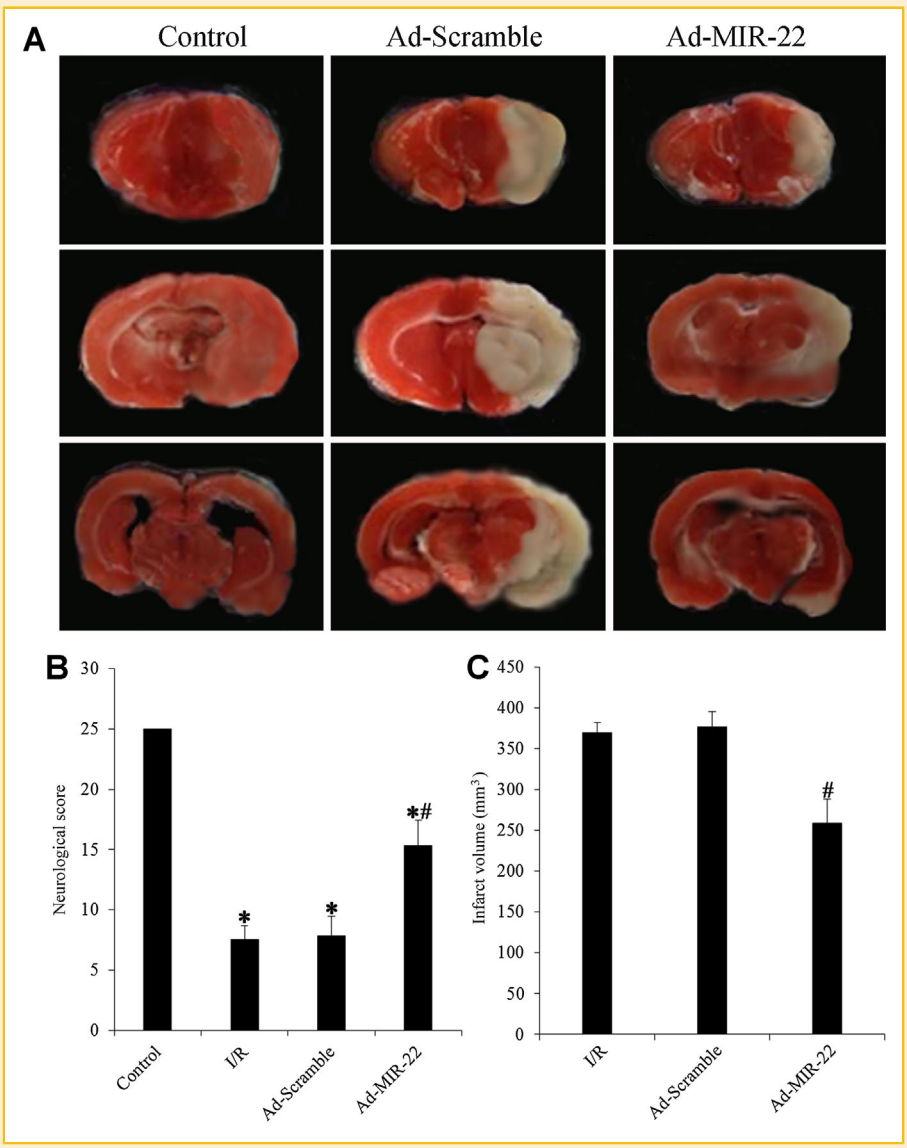

Fig. 2. The effect of MIR-22 overexpression on neurological score and infarct volume. (A) Representative TTC staining in cerebral sections from control, Scramble and MIR-22 overexpression groups. (B) Quantitative analysis of neurological score 24 h after reperfusion. (C) Quantitative analysis of infarct volume 24 h after reperfusion. Values are presented as mean ± SEM. *$P < 0.05$ vs. control, #$P < 0.05$ vs. IR.

function, and we first detected the deterioration of neurological function (Fig. 2A). We then determined the neurological scores, and found that I/R injury significantly reduced the neurological scores compared with the control group ($P < 0.05$). Moreover, the neurological score was significantly higher in the Ad-MIR-22-treated group (neurological score: 15.31 ± 2.17, $P < 0.05$), compared with the I/R group (neurological score: 7.85 ± 1.63) (Fig. 2B). At 24 h after reperfusion, the infarct volumes in all groups were measured. As shown in Figure 2C, the infarct volumes in the Ad-MIR-22-treated group were also lower than in the vehicle group ($P < 0.05$) (Fig. 2C).

MIR-22 SUPPRESSED INFLAMMATORY CYTOKINE EXPRESSION IN OGD-TREATED PRIMARY CORTICAL NEURONS

There is ample evidence that inflammatory mediator genes such as TNF-α, IL-6, iNOS, and COX-2, play a chief role in inflammatory cerebral IR injury [Yi et al., 2007]. In this study, ELISA and Western blot were performed to measure the content of IL-6, TNF-α, iNOS, COX-2, and IL-10. After OGD treatment primary cortical neurons showed a significant increase in TNF-α, IL-6, iNOS, and COX-2 levels compared with the control group (Fig. 3A–D). Overexpression of MIR-22 in cortical neurons significantly attenuated the increases in IL-6, TNF-α, iNOS, and COX-2 levels compared with the OGD-treated group (Fig. 3A–D). In contrast to the levels of IL-6,TNF-α, iNOS, and COX-2, OGD treatment caused a significant reduction in IL-10 expression compared with the control group; this reduction was significantly reversed by MIR-22 overexpression (Fig. 3E).

THE EFFECTS OF MIR-22 ON NF-κB ACTIVITY

Existing evidence suggests that NF-κB is activated in cerebral ischemia, and that the inflammatory response plays an important role in the ischemic brain damage associated with elevated NF-κB [Liu et al., 2009]. Thus, we next analyzed the effects of MIR-22 on NF-κB activity. In the present study, we observed a marked p65 subunit translocation in primary cortical neurons after OGD treatment, which was attenuated by MIR-22 overexpression (Fig. 4A and B). These results suggest that the anti-inflammatory effects of MIR-22 may be associated with inhibition of the NF-κB signaling pathway. Previous reports suggested that MIR-22 could target the NF-κB coactivator NCOA1 [Takata et al., 2011], and thus further studies were performed to demonstrate that MIR-22 inhibits the activity of NF-κB by inhibiting the expression of NCOA1. Western blot results showed that cortical neurons infected with MIR-22-overexpressing adenovirus exhibited decreased expression of NCOA1 (Fig. 4C), suggesting that NCOA1 expression is regulated

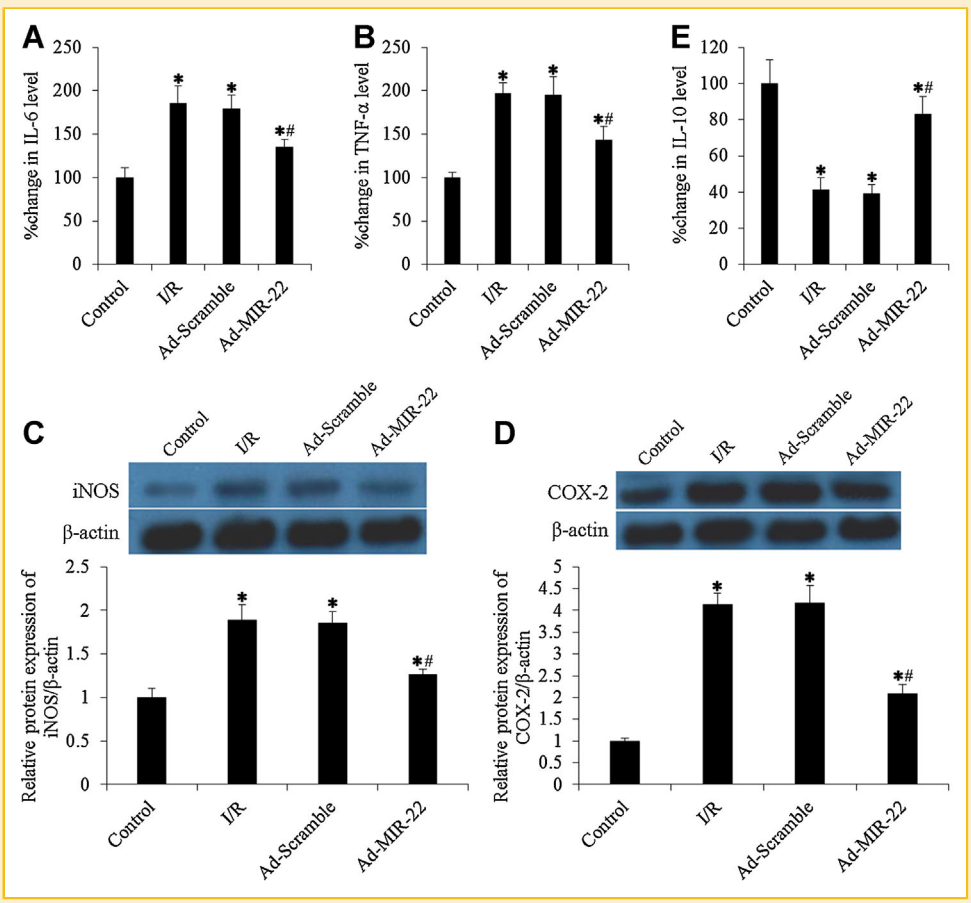

Fig. 3. Effect of MIR-22 overexpression on cytokine (TNF-α, IL-6, IL-10, COX-2, and iNOS) levels in cerebral cortical neurons after OGD treatment. Effect of MIR-22 overexpression on IL-6 (A), TNF-α (B), and IL-10 (E) expression detected by ELISA. Western blot analysis of the expression of iNOS (C) and COX-2 (D). Values are presented as mean ± SEM. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. IR.

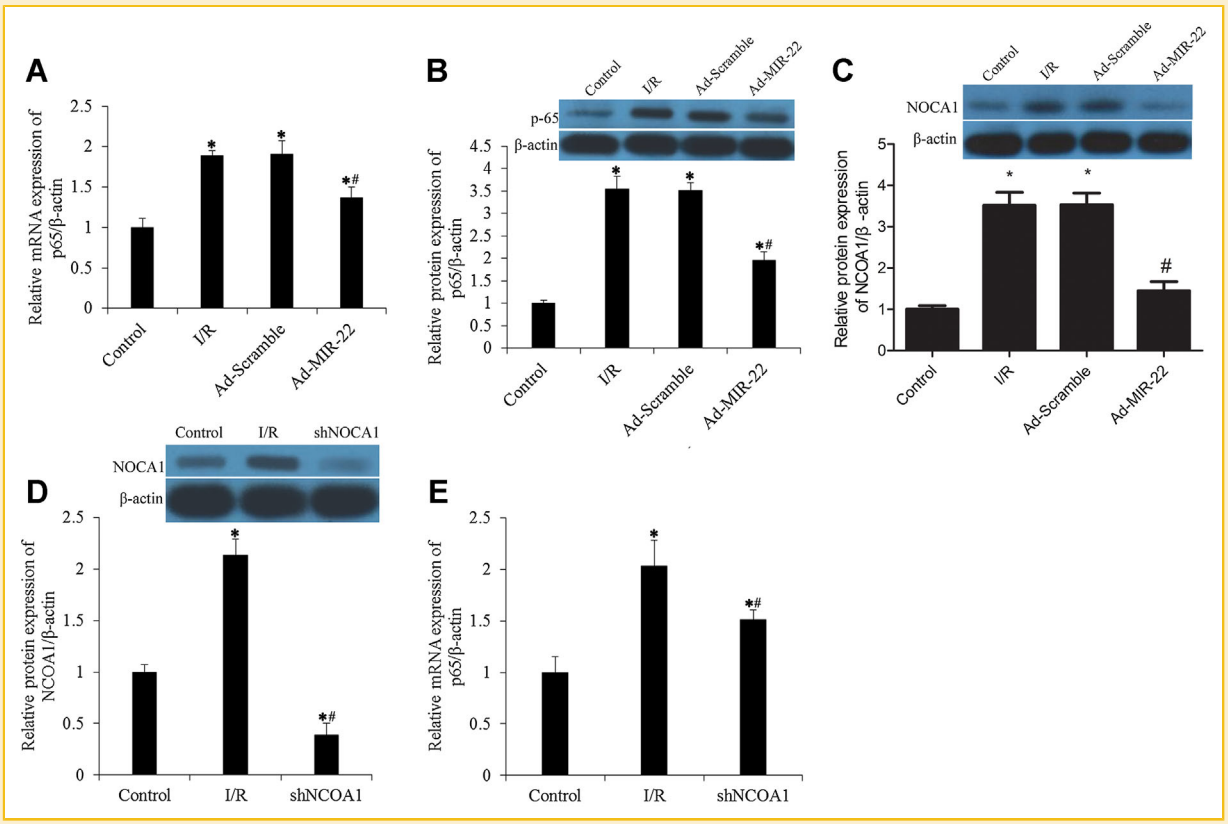

Fig. 4. Overexpression of MIR-22 suppresses NF-κB activity by reducing NCOA1 expression. (A) The mRNA expression of p65 was detected by RT-PCR. (B) The expression of p65 was detected by Western blot. (C) NCOA1 expression was measured by Western blot. Cortical neurons were infected with MIR-22-expressing adenovirus. (D) Western blot analysis of the level of NCOA1 in cortical neurons infected with NCOA1 shRNA-expressing lentiviral particles. (E) Knockdown of NCOA1 reduced NF-κB activation The mRNA expression of p65 was detected by RT-PCR. Values are presented as mean ± SEM. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. IR.

by MIR-22. In addition, NF-κB activity was inhibited by the knockdown of NCOA1 (Fig. 4D and E). These results suggest that MIR-22 regulates NF-κB activity by modulating the expression of the NF-κB coactivator NCOA1.

THE EFFECTS OF MIR-22 ON NEURONAL APOPTOSIS

In addition to the inflammatory response, neuron apoptosis also contributed to cerebral ischemia injury [Villa et al., 2003]. We used flow cytometry to detect the apoptotic rate of neurons in all groups, and found that primary cortical neurons showed a significant increase in cell apoptotic rate compared with the control group after OGD treatment (Fig. 5A and B). Overexpression of MIR-22 in cortical neurons significantly decreased the apoptotic rate, whereas Ad-Scramble had no apparent effect on the apoptotic rate of neurons compared with the OGD treated group.

THE EFFECTS OF MIR-22 ON THE EXPRESSION OF APOPTOSIS-RELATED GENES

As the above results indicated that MIR-22 overexpression could inhibit the apoptosis of OGD-treated cortical neurons, we next sought to evaluate whether MIR-22 could inhibit the upstream manifestations of apoptosis. We tested this hypothesis by measuring the effect of MIR-22 overexpression on the activity of effector caspase-3 and the expression of Bcl-2 and Bax. As expected, we observed significant activation of caspase-3 in the OGD-treated group, and the expression of Bcl-2 and Bax was both increased, moreover, the Bax expression was markedly increased compared with the change of Bcl-2 level (Fig. 6A and B). Compared with the I/R group, the Western blot analysis showed higher Bcl-2 expression (6.5-fold compared with 3.9-fold) and lower Bax expression (2.9-fold compared with 4.6-fold) in the MIR-22 overexpression group at 24 h after ischemia ($P < 0.05$ vs. I/R, Fig. 6B). Taken together, the above results show the therapeutic potential of MIR-22 mediated by both general anti-apoptotic and anti-inflammatory effects.

DISCUSSION

The major findings of this study demonstrate that: (1) MIR-22 overexpression can improve neuronal behavioral scores and reduce the infarct volume after cerebral I/R injury in rats; (2) MIR-22 overexpression can inhibit the expression of inflammatory cytokines induced by OGD treatment in primary cortical neurons; (3) the anti-inflammatory effect of MIR-22 is apparently involved in regulating the activity of NF-κB by modulating the expression of the NF-κB coactivator NCOA1; (4) MIR-22 overexpression can also attenuate

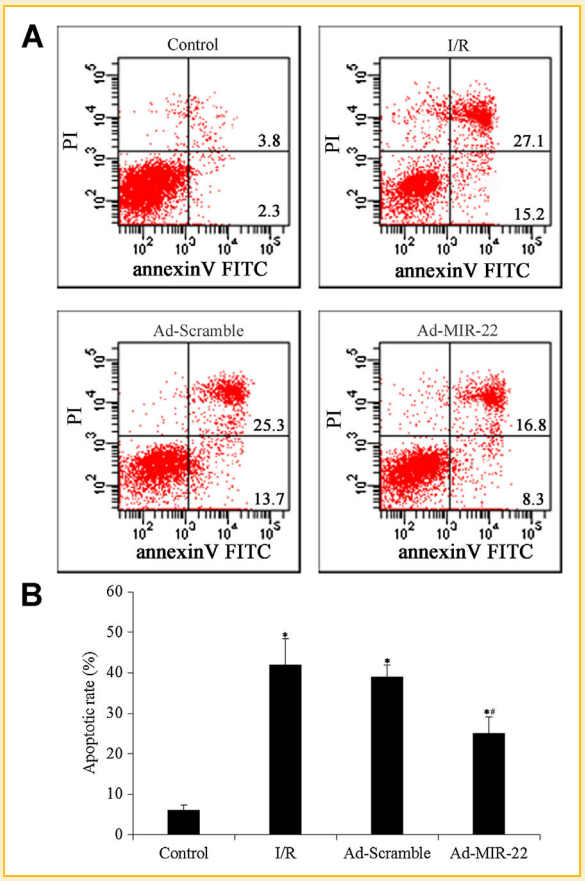

Fig. 5. The apoptosis of cortical neurons in the control, OGD, Scramble and MIR-22 overexpression group, as measured by flow cytometry. (A) Flow cytometry analysis the apoptosis of neurons in the control, OGD, Scramble and MIR-22 overexpression group respectively. (B) Quantitative analysis of apoptotic neurons of different groups. Values are presented as mean ± SEM. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. IR.

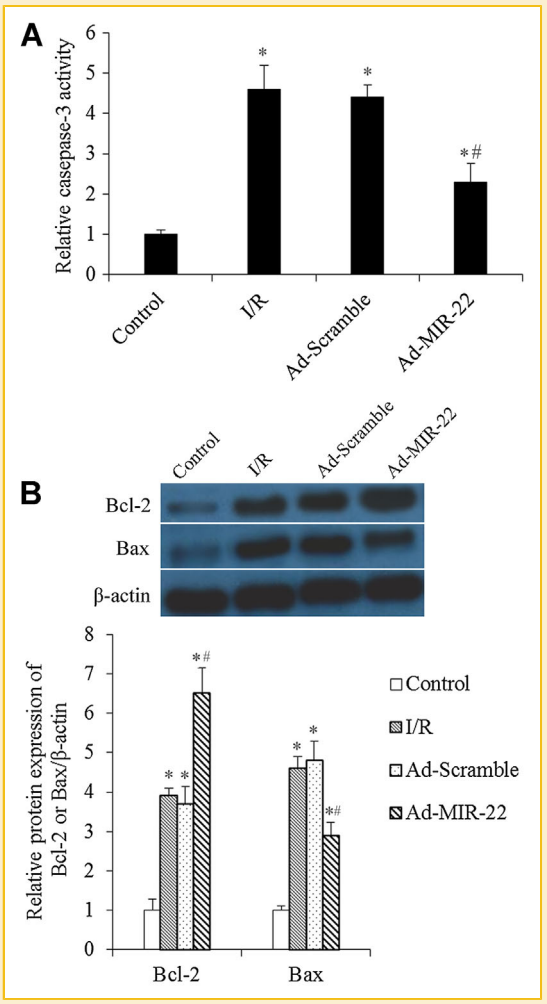

Fig. 6. MIR-22 overexpression modulates caspase-3 activation and apoptotic gene expression. (A) MIR-22 overexpression inhibits caspase-3 activation. Caspase-3 activation was determined by Caspase-Glo® 3/7 Assay. (B) NaB modulates the expression of apoptotic genes in cortical neurons. The expression of Bax and Bcl-2 was measured by Western blot analysis. Values are presented as mean ± SEM. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. IR.

the apoptosis of cortical neurons induced by OGD; and (5) the anti-apoptotic effect of MIR-22 is apparently involved in regulating the expression of apoptosis-related genes, caspase-3, Bcl-2 and Bax. These data demonstrate for the first time that MIR-22 is neuroprotective in vivo and in in vitro models of cerebral I/R injury, which may be explained by the anti-inflammatory and anti-apoptotic effects of MIR-22 in I/R injury.

In this study, we use the MCAO and OGD method to build cerebral ischemia animal and cell models. RT-PCR and Western blot results showed that cerebral I/R induced the down-regulation of MIR-22 expression in vivo and in vitro. We also observed that MIR-22 overexpression can improve neuronal behavioral scores and reduce the infarct volume after cerebral I/R injury in rats. It is well established that cerebral I/R injury is a complex pathophysiological process in which inflammation and apoptosis play a crucial role [Villa et al., 2003; Yi et al., 2007]. In the current study, we found that Ad- MIR-22 exerted a potent protective effect against I/R-induced inflammation and apoptosis in cerebral cortical neurons.

A number of MIRs such as MIR-21 [Deng et al., 2013], MIR-181 [Ouyang et al., 2012], MIR-375 [Wang et al., 2014] and MIR-424 [Zhao et al., 2013] were found to be involved in cerebral I/R injury by altering the expression of key genes associated with inflammation and cell apoptosis. In the current study, we found that MIR-22 could protect against cerebral I/R injury via anti-apoptotic and anti-inflammatory effects. MIR-22 is considered to be a tumor-suppressing microRNA in many cancers [Ling et al., 2012]. Moreover, evidence has also demonstrated that MIR-22 has an apparent neuroprotective effect by targeting the related genes and inhibiting neuron apoptosis [Jovicic et al., 2013]. Recently, MIR-22 has been reported as a critical regulator of myocardial I/R injury. However, the role of MIR-22 in cerebral I/R injury remains unclear at this point.

In this study, we found that MIR-22 was significantly down-regulated in ischemic cerebral tissue subjected to occlusion followed by 24 h reperfusion in vivo and in neurons treated with OGD. Overexpression of MIR-22 reduced the level of TNF-α, IL-6, iNOS,

and COX-2, but increased the level of IL-10. Moreover, we found that the expression of NF-κB p65 was significantly higher in I/R-treated cells than those without I/R treatment, and in comparison with I/R-treated cells without transfection, no significant differences in the above mentioned gene and protein expression were found in the blank plasmid transfected cells, while the expression levels in the MIR-22 overexpression group were significantly lower. Additionally, we found that cortical neurons infected with MIR-22-overexpressing adenovirus exhibited lower levels of expression of NF-κB coactivator NCOA1. In order to determine the effects of MIR-22, NF-κB activity was detected by the knockdown of NCOA1. These results demonstrated that MIR-22 regulates NF-κB activity by modulating the expression of the NF-κB coactivator NCOA1. Additionally, our study also provides evidence that MIR-22 overexpression could reduce the apoptotic rate of cerebral cortical neurons induced by OGD. Moreover, the molecular mechanisms involved in the anti-apoptotic effect of MIR-22 were also determined. We found that MIR-22 overexpression could reduce the activity of effector caspase-3, whereas the expression of Bcl-2 was significantly increased and the level of Bax expression decreased.

CONCLUSION

In summary, our study reveals that dys-regulation of MIR-22 expression contributes to cerebral I/R injury. Overexpression of MIR-22 provides protection against I/R-induced inflammation and cerebral cortical neuron apoptosis. Furthermore, MIR-22 could inhibit the activity of NF-κB by modulating the expression of the NF-κB coactivator NCOA1. The activity of caspase-3 and the expression of Bcl-2 and Bax were also regulated by MIR-22, thus modulating the apoptosis of cortical neurons. These findings support the notion that increasing MIR-22 expression might be a desirable therapeutic approach for the treatment of cerebral I/R injury.

ACKNOWLEDGMENTS

Financial support was provided by National Natural Science Foundation of China (NSFC) (NO. 81101367) and Science and technology research and development projects of Shaanxi Province (NO. 2011K12-54 HSP72).

REFERENCES

Ambros V. 2004. The functions of animal microRNAs. Nature 431:350–355.

Cao Y, Mao X, Sun C, Zheng P, Gao J, Wang X, Min D, Sun H, Xie N, Cai J. 2011. Baicalin attenuates global cerebral ischemia/reperfusion injury in gerbils via anti-oxidative and anti-apoptotic pathways. Brain Res Bull 85:396–402.

Cao C-X, Yang Q-W, Lv F-l, Cui J, Fu H-B, Wang J-Z. 2007. Reduced cerebral ischemia-reperfusion injury in Toll-like receptor 4 deficient mice. Biochem Biophys Res Commun 353:509–514.

Chang Y, Hsiao G, Chen SH, Chen YC, Lin JH, Lin KH, Chou DS, Sheu JR. 2007. Tetramethylpyrazine suppresses HIF-1alpha, TNF-alpha, and activated caspase-3 expression in middle cerebral artery occlusion-induced brain ischemia in rats. Acta Pharmacol Sin 28:327–333.

Chang K-P, Lee H-C, Huang S-H, Lee S-S, Lai C-S, Lin S-D. 2012. MicroRNA signatures in ischemia-reperfusion injury. Ann Plast Surg 69:668–671.

Deng X, Zhong Y, Gu L, Shen W, Guo J. 2013. MiR-21 involve in ERK-mediated upregulation of MMP9 in the rat hippocampus following cerebral ischemia. Brain Res Bull 94:56–62.

Garcia JH, Liu K-F, Ho K-L. 1995. Neuronal necrosis after middle cerebral artery occlusion in Wistar rats progresses at different time intervals in the caudoputamen and the cortex. Stroke 26:636–643.

Gong Q-H, Wang Q, Shi J-S, Huang X-N, Liu Q, Ma H. 2007. Inhibition of caspases and intracellular free Ca 2+ concentrations are involved in resveratrol protection against apoptosis in rat primary neuron cultures. Acta Pharmacol Sin 28:1724–1730.

Hackett ML, Duncan JR, Anderson CS, Broad JB, Bonita R. 2000. Health-related quality of life among long-term survivors of stroke results from the Auckland stroke study, 1991–1992. Stroke 31:440–447.

Hallenbeck JM, Dutka AJ. 1990. Background review and current concepts of reperfusion injury. Arch Neurol 47:1245.

Huang J, Upadhyay UM, Tamargo RJ. 2006. Inflammation in stroke and focal cerebral ischemia. Surg Neurol 66:232–245.

Jovicic A, Jolissaint JFZ, Moser R, Santos MdFS, Luthi-Carter R. 2013. MicroRNA-22 (miR-22) overexpression is neuroprotective via general anti-apoptotic effects and may also target specific Huntington's disease-related mechanisms. PLoS One 8:e4222.

Khan M, Sekhon B, Jatana M, Giri S, Gilg AG, Sekhon C, Singh I, Singh AK. 2004. Administration of N-acetylcysteine after focal cerebral ischemia protects brain and reduces inflammation in a rat model of experimental stroke. J Neurosci Res 76:519–527.

Kosik KS. 2006. The neuronal microRNA system. Nat Rev Neurosci 7:911–920.

Leach M, Swan J, Eisenthal D, Dopson M, Nobbs M. 1993. BW619C89, a glutamate release inhibitor, protects against focal cerebral ischemic damage. Stroke 24:1063–1067.

Li M, Qu YZ, Zhao ZW, Wu SX, Liu YY, Wei XY, Gao L, Gao GD. 2012. Astragaloside IV protects against focal cerebral ischemia/reperfusion injury correlating to suppression of neutrophils adhesion-related molecules. Neurochem Int 60:458–465.

Ling B, Wang G-X, Long G, Qiu J-H, Hu Z-L. 2012. Tumor suppressor miR-22 suppresses lung cancer cell progression through post-transcriptional regulation of ErbB3. J Cancer Res Clin Oncol 138:1355–1361.

Liu Y, Zhang X-J, Yang C-H, Fan H-G. 2009. Oxymatrine protects rat brains against permanent focal ischemia and downregulates NF-κB expression. Brain Res 1268:174–180.

Livak KJ, Schmittgen TD. 2001. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2ΔΔCT Method. Methods 25:402–408.

Nitatori T, Sato N, Waguri S, Karasawa Y, Araki H, Shibanai K, Kominami E, Uchiyama Y. 1995. Delayed neuronal death in the CA1 pyramidal cell layer of the gerbil hippocampus following transient ischemia is apoptosis. J Neurosci 15:1001–1011.

Ouyang Y-B, Lu Y, Yue S, Xu L-J, Xiong X-X, White RE, Sun X, Giffard RG. 2012. MiR-181 regulates GRP78 and influences outcome from cerebral ischemia *in vitro* and *in vivo*. Neurobiol Dis 45:555–563.

Rosamond W, Flegal K, Furie K, Go A, Greenlund K, Haase N, Hailpern SM, Ho M, Howard V, Kissela B. 2008. Heart disease and stroke statistics–2008 update: A report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 117:e25.

Schaller B, Graf R. 2004. Cerebral ischemia and reperfusion: the pathophysiologic concept as a basis for clinical therapy. J Cereb Blood Flow Metab 24:351–371.

Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. 2006. A brain-specific microRNA regulates dendritic spine development. Nature 439:283–289.

Soriano SG, Coxon A, Wang YF, Frosch MP, Lipton SA, Hickey PR, Mayadas TN. 1999. Mice deficient in Mac-1 (CD11b/CD18) are less susceptible to cerebral ischemia/reperfusion injury. Stroke 30:134–139.

Takata A, Otsuka M, Kojima K, Yoshikawa T, Kishikawa T, Yoshida H, Koike K. 2011. MicroRNA-22 and microRNA-140 suppress NF-κB activity by regulating the expression of NF-κB coactivators. Biochem Biophys Res Commun 411:826–831.

Tao T, Liu Y, Zhang J, Xu Y, Li W, Zhao M. 2013. Therapeutic hypercapnia improves functional recovery and attenuates injury via antiapoptotic mechanisms in a rat focal cerebral ischemia/reperfusion model. Brain Res 1533:52–62.

Villa P, Bigini P, Mennini T, Agnello D, Laragione T, Cagnotto A, Viviani B, Marinovich M, Cerami A, Coleman TR. 2003. Erythropoietin selectively attenuates cytokine production and inflammation in cerebral ischemia by targeting neuronal apoptosis. J Exp Med 198:971–975.

Wang Y, Dong X, Li Z, Wang W, Tian J, Chen J. 2014. Downregulated RASD1 and Upregulated miR-375 are involved in protective effects of Calycosin on cerebral ischemia/reperfusion rats. J Neurol Sci 339:144–148.

Wang X, Ha T, Liu L, Zou J, Zhang X, Kalbfleisch J, Gao X, Williams D, Li C. 2013. Increased expression of microRNA-146a decreases myocardial ischaemia/reperfusion injury. Cardiovasc Res 97:432–442.

Yang J, Chen L, Yang J, Ding J, Li S, Wu H, Zhang J, Fan Z, Dong W, Li X. 2014. MicroRNA-22 targeting CBP protects against myocardial ischemia–reperfusion injury through anti-apoptosis in rats. Mol Biol Rep 41:555–561.

Yi J-H, Park S-W, Kapadia R, Vemuganti R. 2007. Role of transcription factors in mediating post-ischemic cerebral inflammation and brain damage. Neurochem Int 50:1014–1027.

Zhao H, Wang J, Gao L, Wang R, Liu X, Gao Z, Tao Z, Xu C, Song J, Ji X. 2013. MiRNA-424 protects against permanent focal cerebral ischemia injury in mice involving suppressing microglia activation. Stroke 44:1706–1713.