MicroRNA-29c Correlates with Neuroprotection Induced by FNS by Targeting Both Birc2 and Bak1 in Rat Brain after Stroke

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Keywords

Apoptosis; Cerebral ischemia; Electrical fastigial nucleus stimulation; MicroRNA.

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Received 3 November 2014; revision 23

December 2014; accepted 24 December 2014

SUMMARY

Aims: Studies showed fastigial nucleus stimulation (FNS) reduced brain damage, but the mechanisms of neuroprotection induced by FNS were not entirely understood; MicroRNAs are noncoding RNA molecules that regulate gene expression in a posttranscriptional manner, but their functional consequence in response to ischemia-reperfusion (IR) remains unknown. We investigated the role of microRNA-29c in the neuroprotection induced by FNS in rat. **Methods:** The IR rat models were conducted 1 day after FNS. Besides, miR-29c antagomir (or agomir or control) was infused to the left intracerebroventricular 1 day before IR models were conducted. We detected differential expression of Birc2 mRNA (also Bak1 mRNA and miR-29c) level among different groups by RT-qPCR. The differential expression of Birc2 protein (also Bak1 protein) level among different groups was surveyed via Western blot. The neuroprotective effects were assessed by infarct volume, neurological deficit, and apoptosis. **Results:** MiR-29c was decreased after FNS. Moreover, miR-29c directly bound to the predicted 3'-UTR target sites of Birc2 and Bak1 genes. Furthermore, over-expression of miR-29c effectively reduced Birc2 (also Bak1) mRNA and protein levels, increased infarct volume and apoptosis, and deteriorated neurological outcomes, whereas down-regulation played a neuroprotective role. **Conclusions:** MiR-29c correlates with the neuroprotection induced by FNS by negatively regulating Birc2 and Bak1.

doi: 10.1111/cns.12383

Introduction

Although the descending tendency in overall stroke mortality, stroke is still a leading cause of death or disability [1]. Ischemic strokes, which occupy about 80–88% of all strokes [2], have caused many stroke-related complications. Thus, improvement in the poor prognosis of stroke or therapy of recurrent stroke now depends much on improving our understanding of the complex molecular mechanisms.

The fastigial nucleus (FN), which is located at the top of the fourth ventricle, contains adrenergic intrinsic neurons and nerve fibers. Fibers can be excited by electrical stimulation, leading to many reactions including reflexive vascular expansion, elevated blood pressure, and increased cerebral blood flow [3]. The neuroprotective effects of FNS in cerebral ischemia are involved in multiple mechanisms including the inhibition of electrical activity, excitotoxic damage, and inflammatory response, as well as apoptosis. Furthermore, FNS can promote nerve tissue repair and improve stroke-related complications [4]. Nevertheless, the molecular mechanisms are still not completely understood.

MicroRNAs (miRNAs) regulate gene expression by binding to the 3'untranslated region (3'UTR) of target genes [5]. So far, there are more than 2000 annotated human mature miRNAs [6]. Evidence indicates that miRNAs have specific expression profiles in many human disorders, including cancer and vascular disease, and play a role in the pathogenesis of these diseases. For example, miR-29c, which is down-regulated in ischemic brain, has been shown to regulate the ischemic brain damage [7]. Thus, miRNAs might act as significant prognostic markers and constitute a new therapeutic target for ischemic stroke.

We had previously performed the global profile of miRNAs after a 1-h electrical FNS using miRNA microarray. Our results show that many miRNAs, including miR-29c, exhibited significant differential expression (data not shown). Using our previously published three miR target prediction software [8] and a luciferase reporter assay *in vitro*, we identified apoptotic proteins Birc2 and Bak1 as real targets of miR-29c. In this study, over-expression of miR-29c with agomir administration *in vivo* resulted in increased sensitivity to IR injury, whereas down-regulation of endogenous miR-29c with antagomir administration

in vivo was neuroprotective. Taken together, our findings implicate miR-29c may be involved in molecular processes of neuroprotection induced by FNS.

Materials and Methods

Animals

Male Sprague Dawley rats (280–300 g) were purchased from the Animal Experiment Center of Guangxi Medical University. Animals were handled according to the guidelines of the Council for International Organization of Medical Sciences on Animal Experimentation (World Health Organization, Geneva, Switzerland). The Guangxi Medical University Animal Care and Use Committee approved the animal protocols. During the surgery, rats were intraperitoneally anesthetized with 3.5% chloral hydrate (1.0 mL/100 g; Sigma, Missouri, USA), and all efforts were made to minimize suffering. After the surgery, the animals were allowed to recover from anesthesia and returned to the cage with *ad libitum* access to food and water.

Electrical Stimulation of the Fastigial Nucleus and Transient Focal Cerebral Ischemia

The fastigial nucleus was accurately targeted using a stereotaxic atlas of the rat brain. The posterior border of bregma was set as the zero point, and a hole (1.1 mm lateral, 11.1 mm posterior, 5.6 mm deep according to Paxinos and Watson [9]) was made for electrode attachment. A YC-2 programmed electrical stimulation instrument (Chengdu Instrument Factory, Chengdu, China) was used to apply a 70- μ A direct-current square-wave pulse (50 Hz). Each electrical stimulation lasted 1 h. An established focal cerebral IR model was employed 24 h later. Focal cerebral ischemia was produced by 2 h of middle cerebral artery occlusion (MCAO), as described previously [10]. Rats in the sham-operated group underwent only vascular separation without filament insertion. Minimum number of animals ($n = 6$) were used for each category.

Plasmid Construction

Birc2 and Bak1 3'UTR sequences were, respectively, amplified from rat genomic DNA and then cloned into the XhoI/NotI site of pmiR-RB-REPORTTM vector (RiboBio, Guangzhou, China). The following primer sets were used to generate specific fragments: Birc2-3'UTR-F: 5'CCGCTCGAGATGAAGGAAGCTGTCTGAACAA 3' and Birc2-3'UTR-R: 5'GAATGCGGCCGCAACATTTTAAAGATT ATTATGTACACAGT 3'; Bak1-3'UTR-F: 5'CCGCTCGAGCTGCCCC GGGAGTCTCAGCC 3' and Bak1-3'UTR-R: 5'GAATGCGGCCGCGG GGTGTTGGGGCATTGCACC 3'.

Birc2 and Bak1 3'UTR mutant plasmids were, respectively, performed by creating a point mutation in the miR-29c binding site using the QuickChange[®] XL site-directed mutagenesis kit (Stratagene, California, USA) as per manufacturer's instructions. The miR-29c binding site UGGUGCU is, respectively, mutated into UGCUCGU and UCGUCGU. Sequences of the wild-type and mutant vectors were confirmed with restriction digestion and automated DNA sequencing.

Luciferase Assays

HEK293T cells (4×10^3 cells/well) were, respectively, transfected with pmiR-Birc2 and Bak1 3'UTR plasmid (with either wild-type or mutant-type miR-29c binding sites) together with 50 nM miR mimics or control (RiboBio) using Lipofectamine 2000 (Invitrogen, California, USA) as per manufacturer's instructions. 48 h after transfection, cells were lysed and subjected to a double luciferase reporter assay system (Promega, Wisconsin, USA). Renilla luciferase activity was normalized to that of firefly luciferase.

Intracerebroventricular Infusion of MiR-29c Antagomir or Agomir

MiR-29c antagomir, agomir, and respective N-controls (RiboBio) were dissolved in artificial CSF to obtain a concentration of 20 nmol/mL [11]. The following are the structure and sequence of miR-29c antagomir, agomir, and respective N-controls.

miR-29c antagomir: 5'-mUmAmAmCmCmGmAmUmUmCmAmAmUmGmGmUmGmCmUmA-/3chol/-3'; antagomir Ncontrol: 5'-mCmAmGmUmAmCmUmUmUmUmGmUmGmUmAmGmUmAmCmAmAmA-/3chol/-3'; miR-29c agomir: 5'-mUmAmGmCmAmCmCmAmUmUmUmGmAmAmAmUmCmGmUmUmA-/3chol/-3'; agomir Ncontrol: 5'-mUmUmUmGmUmAmCmUmAmCmAmCmAmAmAmAmGmUmAmCmUmG-/3chol/-3'.

Rats were anesthetized as described above and fixed in a stereotaxic apparatus. The microsyringe (Gaoge, Shanghai, China) was stereotactically implanted into the left lateral ventricle of the brain (bregma: 0.8 mm posterior, -4.8 mm dorsoventral, -1.5 mm lateral; based on the rat brain atlas of Paxinos and Watson [9]) and secured to the skull. A total of 120 rats were randomly divided into five groups. The miR-29c antagomir, agomir, respective N-controls (0.2 nmol/ μ L, total 5 μ L, at a rate of 1 μ L/10 min), and NS were infused into rat brain left lateral ventricles. One day after intracerebroventricular (ICV) infusion, rats were subjected to 2-h transient MCAO and were killed at 1 day of reperfusion for RT-qPCR, Western blotting, the TUNEL assay, and TTC staining; six rats from each group were used for each method, and all rats were evaluated for neurological deficits after waked.

RT-qPCR for MiR-29c Quantitation

RNA was reverse-transcribed into cDNA, and then, real-time quantitative PCR was performed according to our previously published SYBR quantitative reverse transcription-PCR technique [8]. In brief, RNA was reverse-transcribed into cDNA using the SYBR[®] PrimeScriptTMmiRNA RT-PCR Kit (Code No. RR716) according to the manufacturer's instructions. In this study, U6 expression was used as an internal control. Real-time quantitative PCR with SYBR[®] Premix Ex Taq TM II (Perfect Real Time; Takara, Dalian, China) was performed in total volume of 20 μ L containing 2 μ L reverse-transcribed reaction product, 10 μ L SYBR[®] Premix Ex Taq II ($2 \times$), 0.8 μ L miR-29c or U6b (Takara) qPCR primer mix, and 7.2 μ L RNase Free dH₂O using the running conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 20 s, and finally 65°C for 15 s. All

reactions were conducted in triplicate, and controls were performed with no template or no reverse transcription for each gene. The expression was normalized to U6 by the $2^{-\Delta\Delta C_t}$ method, where $\Delta C_t = C_t \text{ miR-29c} - C_t \text{ U6}$.

RT-qPCR for mRNA Quantitation

RNA was reverse-transcribed into cDNA, and then, real-time quantitative PCR was performed according to our previously published SYBR quantitative reverse transcription-PCR technique [8]. In brief, RNA was reverse-transcribed into cDNA using the PrimeScript[®] RT reagent kit with gDNA eraser (Perfect Real Time) according to the manufacturer's instructions. In this study, GAPDH expression was used as an internal control. Real-time quantitative PCR with the SYBR[®] Premix Ex Taq[™] II (Tli RNase H Plus) was performed in total volume of 20 μL containing 2 μL reverse-transcribed reaction product, 10 μL SYBR[®] Premix Ex Taq II (2 $\times$), 1.6 μL specific gene or GAPDH (Takara) qPCR primer mix, and 6.4 μL RNase Free dH₂O using the running conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 20 s, and finally 65°C for 15 s. All reactions were conducted in triplicate and controls were performed with no template or no reverse transcription for each gene. The expressions were normalized to GAPDH by the

$2^{-\Delta\Delta C_t}$ method, where $\Delta C_t = C_t \text{ specific gene} - C_t \text{ GAPDH}$. Primer sequences (Takara) were as follows: Birc2: forward 5'-CAGCTTTGTGCAGACTTTGCTTTC-3', reverse 5'-CCTTGTTCCAGAGGTAGCGAGTG-3'; Bak1: forward 5'-CTTCGGATCTTTGTCTTCAAAC-3', reverse 5'-ACCTGGTCCTGTCCGGATG-3' and GAPDH: forward 5'-TATGACTCTACCCACGGCAAGT-3', reverse 5'-ATACTCAGCACCAGCATCACC-3'.

Western Blotting Analysis

Protein was extracted from brain tissue with RIPA lysis buffer (Beyotime, Haimen, China). Protein concentration of each specimen was detected by the Bradford method to maintain the same loads. Isolated protein was heat denatured at 95°C for 5 min, electrophoretically separated on 10% SDS-PAGE, and transferred to polyvinylidene fluoride membranes (PVDF; Millipore Corp, Massachusetts, USA). The membranes were blocked with a buffer containing 5% skim milk in TBS with Tween for 1 h, incubated on ice overnight with antibody against Birc2 (1:200, ab2399; Abcam, Cambridge, UK) and GAPDH (1:5000; AG019, Beyotime), and then washed and incubated with secondary 1:10,000 anti-rabbit antibody (042-06-18-06; KPL, Washington, USA) at room temperature for 2 h. There also are Bak1 (1:500, TA302647;

Figure 1 Reciprocal expression of miR-29c and Birc2 and Bak1. (A) The expression of miR-29c in three groups. (B) and (C) The expression of Birc2 mRNA and Bak1 mRNA in rat brains. (D) and (E) The expression of Birc2 protein and Bak1 protein in three groups. * $P < 0.05$ vs. sham controls; # $P < 0.05$ vs. MCAO group.

Origene, MD, USA) and its secondary anti-goat antibody (1:10,000, CW0105; CWBIO, Beijing, China). The bands were scanned with the LICOR Odyssey infrared imaging system (LICOR Bioscience, Nebraska, USA), according to the manufacturer's instructions, and the data were analyzed using LICOR Odyssey software V3.0. GAPDH (1:5000) was used as an internal control.

Measurement of Infarct Volume and Evaluation of Neurological Deficit

Rat brains were sliced into six 2-mm-thick coronal sections on a rat brain matrix. The slices were stained with 2% TTC (2,3,5-triphenyltetrazolium chloride) (Sangon Biotech, Shanghai, China) for 15 min at 37°C. Infarct volume was computed using the Swanson formula: $100\% \times (\text{contralateral hemisphere volume} - \text{non-infarct ipsilateral hemisphere volume}) / \text{contralateral hemisphere volume}$ [12]. When rats began to wake, they were evaluated scores according to a 5-point scale [8].

TdT-Mediated dUTP-Biotin Nick End Labeling (TUNEL) Assay

As described previously [8], the 3- μm brain slices were subjected to TUNEL assay according to the manufacturer's specifications (Roche Applied Science, Mannheim, Germany). Reaction was visualized under a fluorescence microscope (Germany). All specimens were performed in triplicate, and at least five fields from each independent experiment were imaged using microscopy (Germany). Positively labeled nuclei (brown color) were identified from negatively unstained nuclei (blue color). The number of positive nuclei was determined by counting (magnification $\times 400$) and all the positively labeled nuclei present in five random visual fields under a microscope. The percentage of TUNEL-positive nuclei to all nuclei counted was used as apoptotic index (AI). The apoptotic cells had the following characteristics: single cells, no inflammation, curling of cell membrane, brown particulate, or fragmented nuclei.

Figure 2 The effect of FNS in rat brains in vivo IR. **(A)** Infarct volume was decreased after FNS. **(B)** Number of TUNEL-positive nuclei was reduced after FNS. **(C)** Neurological scores were reduced after FNS. * $P < 0.05$.

Statistical Analysis

All data represent at least three independent experiments, and the numbers of animals are indicated in the figure legends. Data are reported as the means $\pm$ standard deviation (SD). Statistical significance was determined using t-tests to compare two groups or analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test for experiments with more than two groups. Values of $P < 0.05$ were considered significant.

Results

Expression of MiR-29c after FNS

Our previous results had showed that many miRNAs exhibited significant differential expression after a 1-h electrical FNS using miRNA microarray (data not shown). In the present study, we focused our measurements on miR-29c levels by real-time PCR *in vivo* IR brain after FNS. Compared to sham group and only MCAO-treated group, levels of miR-29c were significantly decreased after FNS (Figure 1A). These findings indicated that miR-29c level was obviously down-regulated in IR rat brains after FNS.

The Neuroprotective Role of FNS after IR Injury

Rats were subjected to 2 h of MCAO and 24-h reperfusion after FNS, and then, the expressions of Birc2 and Bak1 mRNAs and protein levels were up-regulated (Figure 1B–E). The infarct volume, apoptosis, and neurological scores were significantly reduced in rat brains *in vivo* IR after FNS (Figure 2).

MiR-29c Predicted to Target 3'UTR of Birc2 and Bak1

Using TargetScan, PITA, and miRanda, we analyzed the targets of rat miR-29c. TargetScan predicted 797 targets, and PITA predicted 3363 targets, while miRanda predicted 1306 targets, and 165 of those are conserved targets predicted by three algorithms. Birc2 and Bak1 are two of the predicted miR-29c targets identified by the three prediction tools with a very high score and a low mean free energy, and they are two apoptosis-related genes. The minimum free energy of binding was -71.7 and -74.5 kcal/mol, respectively, which was calculated using RNAhybrid. The respective miR-29c seed sequences of Birc2 and Bak1 were shown in Figure 3A and D.

MiR-29c Directly Targets 3'UTRs of Birc2 and Bak1

To conclusively validate the miR–target relationship, we, respectively, cotransfected wild-type Birc2 and Bak1 3'UTR vector and mutant Birc2 and Bak1 3'UTR vector (miR-29c binding site UGGUGCU is, respectively, mutated into UGCUCGU and UCGUCGU) with miR-29c mimics or Ncontrol in HEK 293T cells (Figure 3B and E; the underlined nucleotides). Renilla luciferase activity was normalized to that of firefly luciferase. The miR-29c mimics prevented wild-type Birc2 and Bak1 3'UTR expression. Mutation of the miR-29c binding site completely eliminated the inhibition of luciferase activity

Figure 3 Luciferase reporter assays showed Birc2 and Bak1 were authentic targets of miR-29c; (A) and (D) Predicted binding sites of miR-29c in the 3'UTR of Birc2 and Bak1. (B) and (E) Design of a miR-29c luciferase reporter vector with HSV-driven expression of a luciferase vector fused to a wild Birc2 and Bak1 3'UTR or mutated Birc2 and Bak1 3'UTR. The underlined nucleotides were subsequently mutated for the 3' UTR-miRNA binding studies. (C) and (F) Rno-miR-29c mimics decreased expression of luciferase containing a wild-type miR-29c binding site (left two columns) but not a mutant binding site (right two columns). * $P < 0.05$ vs. Ncontrol.

by miR-29c mimics. There was also no significant change in luciferase reporter activity when Ncontrol was cotransfected with wild-type Birc2 and Bak1 3'UTR vector (Figure 3C and F). Overall, these data showed that Birc2 and Bak1 were potential targets of miR-29c.

MiR-29c Down-regulates Expression of Birc2 and Bak1

Theoretically predicted by TargetScan, PITA and miRanda, miR-29c has hundreds of potential targets which could be

Figure 4 The consequences of *in vivo* experiment. (A) The expression of miR-29c in five groups. (B) and (C) The expression of Birc2 mRNA and Bak1 mRNA in rat brains. (D) and (E) The expression of Birc2 protein and Bak1 protein in five groups. MiR-29c antagomir treatment decreased the infarct volume (F), apoptosis (G) and improved neurological scores (H), while miR-29c agomir treatment increased the infarct volume (F), apoptosis (G) and aggravated neurological scores (H). * $P < 0.05$ vs. antagomir control; # $P < 0.05$ vs. agomir control.

divided into two subgroups: proapoptotic and antiapoptotic proteins. Considering the significance of these candidates in ischemic stroke, we selected the following two predicted targets (antiapoptotic: Birc2; proapoptotic: Bak1). As expected, the levels of two proteins were significantly increased in antagomir-injected group and were significantly decreased in agomir-injected group (Figure 4D and E). These findings show that miR-29c inhibitor effectively increases, whereas miR-29c mimic decreases the expression of Birc2 and Bak1 (*in vivo*; Figure 4B–E). Taken together, these data indicated miR-29c efficiently repressed Birc2 and Bak1 expression *in vivo*.

Anti-MiR-29c Reduces IR-Induced Injury

We shifted miR-29c levels by injecting antagomir, agomir, or Ncontrol, respectively, by intracerebroventricular (ICV) infusion (Figure 4A). Down-regulation of miR-29c alleviated ischemic damage and decreased infarct volume (Figure 4F). Furthermore, neurological scores were effectively reduced related to antagomir control group (Figure 4H). Additionally, the number of TUNEL-positive cells was significantly reduced in antagomir-injected group (Figure 4G), whereas over-expression of miR-29c enhanced ischemic damage and increased infarct volume (Fig-

ure 4F). Meanwhile, neurological scores were effectively increased related to agomir control group (Figure 4H). Additionally, the number of TUNEL-positive cells was significantly increased in agomir-injected rats (Figure 4G). These results demonstrated that down-regulation of miR-29c protected against IR-induced cellular damage *in vivo*.

Discussions

Extensive researches have demonstrated that FNS can reduce ischemia damage [4,13–16]. In our previous study, 1 h of electrical FNS reduced the infarct volume by ~40%. Moreover, apoptotic cells in the ischemic area and penumbra area were decreased [17]. The identities of the neuronal elements that mediate this neuroprotection are not known. Neuroprotection is partly mediated by the suppression of apoptosis.

MiRNAs regulate gene expression via RNA-induced silencing complexes and contribute to the regulation of many cellular functions, including proliferation, differentiation, and apoptosis [18,19]. Studies demonstrated ischemic stroke changes miRNA expression profiles in rodents as well as human beings [20,21]. However, the role of a specific miRNA in ischemic stroke is just emerging. We had previously performed miRNA arrays to detect the expression pattern of miRNAs after FNS. Surprisingly, we observed that miR-29c expression was distinctly decreased after FNS (data not shown). Thus, we now focus on elucidating the functional consequences of miR-29c *in vivo* using gain-of-function and loss-of-function approaches. We found continuous delivery of antagomir effectively reduced brain infarction and apoptosis and improved neurological outcomes, whereas elevation of miR-29c showed the opposite effect. Overall, we demonstrated decreasing levels of miR-29c rendered neuroprotection against IR injury. Collectively, these data suggested miR-29c may be involved in molecular processes of neuroprotection induced by FNS.

Human miR-29 family contains three mature members, miR-29a, miR-29b, and miR-29c. Mature miR-29s are well conserved in human, mouse, and rat, and they share identical sequences at nucleotide positions 2–7, the seed region that plays a crucial role in determining which protein-coding genes a microRNA would target [22]. Hence, predicted target genes by bioinformatics for the miR-29 family members extensively overlap. Several target genes of miR-29c have been discovered such as RCC2, collagen genes, MMPs, and the ADAM family of metalloprotease-disintegrins [23,24].

Bioinformatics analysis showed Birc2 and Bak1 are putative targets of miR-29c. To further validate this computational finding that miR-29c may negatively regulate Birc2 and Bak1, we generated a

luciferase construct harboring the 3'-UTR fragment of Birc2 and Bak1 containing a broadly conserved binding site of miR-29c (Luc-Birc2, Luc-Bak1, Figure 3B,E) and a mutant luciferase construct with deletion of the binding site (Luc-Birc2 -mu, Luc-Bak1 -mu, Figure 3B,E). Luciferase assay showed that miR-29c significantly repressed the luciferase activity in the HEK293T cell line transiently transfected with Luc-Birc2 and Luc-Bak1, compared with cells transfected with Luc-Birc2-mu and Luc-Bak1-mu, suggesting that Birc2 and Bak1 are genuine target of miR-29c.

Birc2 (also known as cIap1) belongs to the family of antiapoptotic regulators regarded as inhibitors of apoptosis (IAP) proteins. Expression of Birc2 is induced in conditions of endoplasmic reticulum (ER) stress via the phosphatidylinositol 3-kinase (PI3K)–Akt signaling pathway and contributes importantly to cellular adaptation to stress due to repressing the ER stress-induced apoptotic process [25,26].

While Bak1 (BCL2-antagonist/killer 1) is constitutively localized to the outer mitochondrial membrane and is strongly associated with apoptosis [27]. Thus, it seems to be a contradiction that miR-29c may function as either antiapoptotic (Birc2) or proapoptotic (Bak1) in our study. Nevertheless, the PI3K/Akt inhibitor can lead to activation of proapoptotic proteins Bax and Bak and release of Bim and Bak from Mcl-1. It also induced ER stress- and Bax/Bak-mediated apoptosis of cancer cells [28,29]. Therefore, Birc2 and Bak1 may be involved in the PI3K–Akt signaling pathway. It was plausible that miR-29c might work depending on the individual contribution of these targets to the specific pathway.

In this study, we investigated the association between miR-29c expression and neuroprotective effects by FNS in stroke. Consequently, miR-29c attenuates ischemic neuronal apoptosis by negatively regulating apoptotic proteins Birc2 and Bak1. Therefore, miR-29c may be involved in apoptosis processes of neuroprotection induced by FNS in stroke. The present observations have shown that it may partly be associated with apoptosis protein pathways such as the PI3K–Akt pathway. However, further experiments should focus on Bak1 and its signaling pathway.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81160168 to L.J.L.).

Conflict of Interest

The authors declare no conflict of interest.

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