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miR-181c regulates ischemia/reperfusion injury-induced neuronal cell death by regulating c-Fos signaling

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MicroRNA-181c (miR-181c) has been reported to be highly expressed in the brain, but downregulated in acute ischemic stroke patients. However, the underlying mechanism of miR-181c in regulating cerebral ischemic injury (I/R) remains poorly understood. The aim of this study was to explore the molecular basis of miR-181c in regulating cerebral I/R. It was found that the overexpression of miR-181c mediated by recombinant adeno-associated virus (AAV) vector infection significantly promoted neuron death induced by oxygen-glucose deprivation (OGD)/reoxygenation in hippocampal neuron, whereas the inhibition of miR-181c provided protective effects against OGD/reoxygenation-induced cell death. In addition, c-Fos expression was decreased and increased though overexpression or inhibition of miR-181c. c-Fos was further determined to a putative target of miR-181c by dual-luciferase reporter assay. miR-181c overexpression also inhibited the expression of the downstream gene of c-Fos, including AP-1 and NFATc1, whereas the inhibition of miR-181c upregulated the expression of AP-1 and NFATc1 in neurons after OGD/reoxygenation. Interestingly, c-Fos siRNA apparently abolished the protective effect of anti-miR-181c on OGD/reoxygenation-induced cell death. These observations determine that downregulated miR-181c ameliorates I/R *via* increasing the expression of c-Fos and its downstream genes, which will provide a new and promising therapeutic target for cerebral I/R.

1. Introduction

Ischemic stroke is among the death-related brain diseases with high morbidity and high mortality in adults, which is due to the insufficient blood supply to the brain tissues (Yao et al. 2016). The cells are in anoxic and low-nutrient state in the cerebral ischemia tissues, which inevitably causes damage to the cells. When the injury is light, the cells survive through a series of adaptive reactions, and in severe cases, apoptosis or necrosis occurs (Arumugam et al. 2009). Although there are some treatments for acute stroke, few patients receive proper therapies at the right time window (Nguyen et al. 2011). Therefore, the precise mechanism of ischemia-induced neuronal death needs urgently to be understood for developing effective therapies for acute stroke.

MicroRNAs (miRNAs) are a class of endogenous highly conserved small non-coding RNAs of about 19 to 23 nucleotides in length. miRNAs cleave or inhibit transcription of a target mRNA by binding to the 3'-untranslated region (3'-UTR) of their target mRNA (Raychaudhuri et al. 2017). MiRNAs play important roles in various cellular processes, including cell proliferation, apoptosis, and differentiation, and provide novel insights in disease research, treatment, diagnosis, and prognosis (Huang 2017; Moridikia et al. 2018). Study results have revealed that a variety of miRNAs were involved and functional in cerebral ischemia reperfusion injury. miR-181c has been suggested to be highly expressed in the brain, and downregulated in miRNA expression profiles of acute ischemic stroke patients (Ma et al. 2016). Thereby, miR-181c may be extensively implicated in ischemia reperfusion-induced brain injury. However, the molecular mechanism remains to be elucidated.

c-Fos has been revealed in the neuronal responses to ischemia and it is an inducible and widely applicable marker for neural systems activated by a variety of extracellular stimuli (Schlossberg et al. 1996). While c-Fos has been shown to be induced in parvocellular neurosecretory corticotropin-releasing factor neurons in response to stress (Verbalis et al. 2010). The activation of c-Fos has been demonstrated to be associated with neuronal survival (Efimova et al. 2007). Sublethal ischemic preconditioning has been demonstrated to induce c-Fos expression exhibiting ischemic tolerance against subsequent severe ischemic injury (Sommer et al. 1995; Kaur et al. 2013). These studies implied that c-Fos is a promising molecular target for the prevention and treatment of cerebral ischemia reperfusion injury.

In this study, we investigated the role of miR-181c in regulating neuron death using oxygen-glucose deprivation (OGD) model of

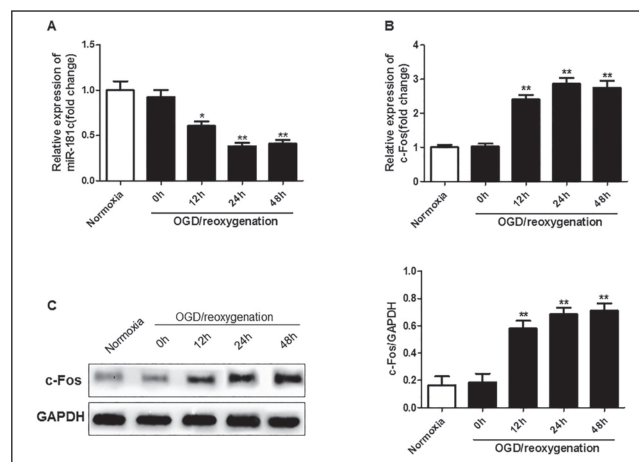

Fig.1: Changes of miR-181c and c-Fos expression levels in hippocampal neurons after OGD-induced ischemic injury. (A) RT-qPCR analysis of miR-181c expression levels in neurons during 12–48 h of reoxygenation post-3 h OGD. RT-qPCR (B) and (C) western blot analysis were detected to the mRNA and protein expression levels of c-Fos in neurons during 12–48 h of reoxygenation post-3 h OGD. *p<0.05 and **p<0.01 vs. Normoxia.

cell ischemia and explored its underlining mechanism. The results showed that miR-181c played an important role in OGD-induced neuron death using the overexpression and knockdown of miR-181c. Furthermore, c-Fos was predicted and identified as a target gene of miR-181c.miR-181c silence could increase the expression of c-Fos and activate the expression of downstream genes that might protect neurons from ischemia-induced cell death.

2. Investigations and results

2.1. *MiR-181c was downregulated in OGD/reoxygenation-treated neurons*

As shown in Fig. 1A, the expression of miR-181c was significantly decreased in OGD/reoxygenation-treated hippocampal neurons by RT-qPCR and continued to be downregulated during 12–48 h reoxygenation post-OGD when compared with the normoxic group. In addition, the mRNA (Fig. 1B) and protein expression (Fig. 1C) exhibited that c-Fos expression was significantly increased following 12–48 h of reoxygenation post-OGD. These results suggested a critical role of miR-181c and c-Fos in ischemic injury.

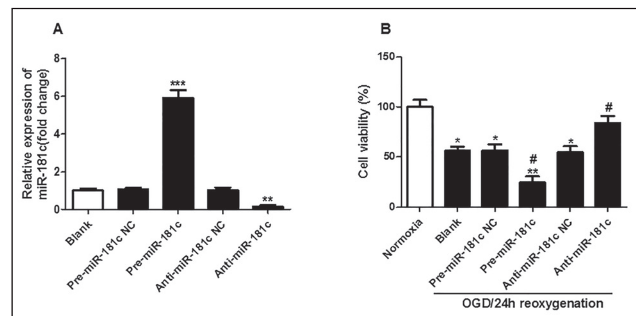

Fig.2: Effect of miR-181c on OGD-induced cell death. (A) RT-qPCR analysis of miR-181c expression level in different treated groups: Blank cells without infection, Pre-miR NC cells infected with AAV-NC pre-miRNAs; Pre-miR-181c cells infected with AAV-pre-miR-181c; Anti-miR NC cells infected with AAV-NC anti-miRNAs; Anti-miR-181c cells infected with AAV-anti-miR-181c. After transfection for 48 h, cells were harvested for analysis. (B) Cell viability was detected using MTT assays after 24 h of reoxygenation post-OGD. *p<0.05 and **p<0.01 vs. Normoxia; #p<0.05 vs. Pre-miR-181c NC or Anti-miR-181c NC.

2.2. *MiR-181c is involved in regulating OGD-induced cell death*

As shown in Fig. 2A, miR-181c expression levels were significantly upregulated or downregulated in hippocampal neurons after 48-h infection of pre-miR-181c or anti-miR-181c, respectively. In addition, miR-181c overexpression contributed to the OGD/reoxygenation-induced cell death, where the knockdown of miR-181c effectively inhibited OGD/reoxygenation-induced cell death in comparison with the control or normoxia groups (Fig. 2B).

2.3. *MiR-181c directly regulates c-Fos expression*

The biological effect of miR-181c on c-Fos expression in neurons during OGD/reoxygenation was detected to explore the potential mechanism of miR-181c in regulating OGD-induced cell death. The results showed that mRNA (Fig. 3A) and protein (Fig. 3B) expression levels of c-Fos were significantly decreased by miR-181c overexpression during OGD/reoxygenation, whereas the knockdown of miR-181c markedly increased c-Fos expression level. Moreover, a dual luciferase reporter assay was performed to determine whether miR-181c regulates c-Fos expression directly by targeting the 3′-UTR of c-Fos mRNA. Bioinformatics analysis demonstrated that c-Fos was a putative target gene of miR-181c (Fig. 4A). Co-transfection of pGL3-c-Fos-3′-UTR with pre-miR-181c in HEK293 cells significantly downregulated the relative luciferase activity in comparison with the control group, whereas cells that co-transfected miR-181c with of pGL3-c-Fos-Mut-3′-UTR containing mutations in the predicted consensus sequences

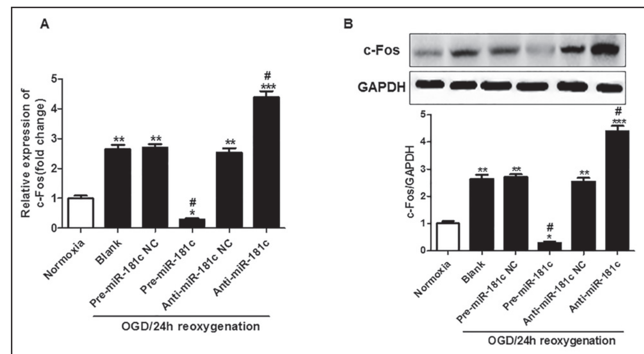

Fig.3: Effect of miR-181c on c-Fos expression. (A) RT-qPCR analysis of c-Fos mRNA level in different groups. (B) Western blot analysis of c-Fos protein in different groups. Different treated cells subjected to 24 h of reoxygenation post-OGD were harvested for analysis. *p<0.05 and **p<0.01 vs. Normoxia; #p<0.05 vs. Pre-miR-181c NC or Anti-miR-181c NC.

for miR-181c had no apparent effect on luciferase activity (Fig. 4B). These findings suggested that c-Fos represents a direct target gene of miR-181c.

2.4. *MiR-181c is involved in regulating the expression of AP-1 and NFATc1*

To further validate the regulatory effect of miR-181c on c-Fos, we detected the s protein expression of its downstream genes (AP-1 and NFATc1) by western blot analysis (Fig. 5). The results showed that miR-181c overexpression significantly decreased the protein expression of AP-1 and NFATc1 in comparison with control groups in neurons during OGD/reoxygenation. In contrast, knockdown of miR-181c significantly increased the expression levels of AP-1 and NFATc1 in comparison with control groups.

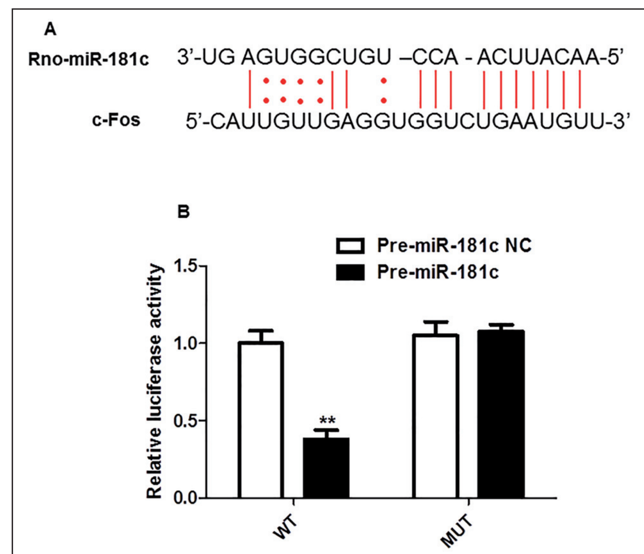

Fig. 4: c-Fos is a direct target gene of miR-181c. (A)The putative binding sequences of 3′-UTR of c-Fos mRNA with miR-181c. (B) Luciferase activity assay was performed to detect the interaction between miR-181c and 3′-UTR of c-Fos mRNA. The wild-type (WT) or mutant (MUT) 3′-UTR of c-Fos mRNA was co-transfected with pre-miR-181c or pre-miR-181c NC for 48 h before being harvested for analysis. **p<0.01 vs. pre-miR-181c NC.

2.5. *Knockdown of c-Fos abrogates the neuroprotective effect of si-miR-181c in OGD-induced ischemic injury*

In order to further confirm the contribution of c-Fos to the biological role of miR-181c in OGD-induced cell injury, the cell viability improved by anti-miR-181c was markedly inhibited by c-Fos knockdown (Fig. 6A) in neurons OGD/24 h reoxygenation. In addition, c-Fos siRNA and anti-miR-181c were co-transfected in

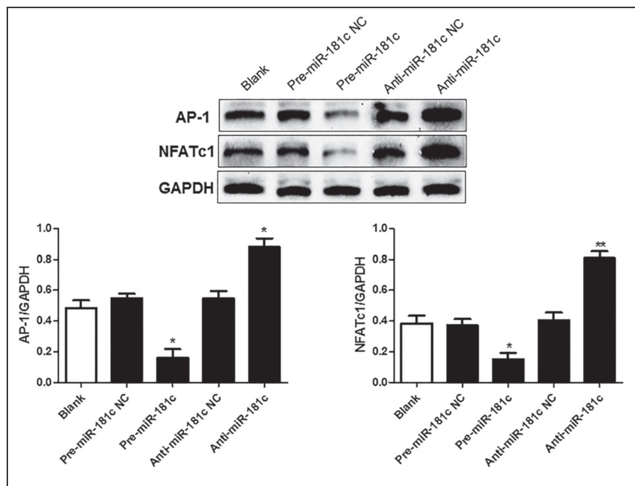

Fig. 5: Effect of miR-181c on the expression of AP-1 and NFATc1. Western blot analysis of the protein expression levels of AP-1 and NFATc1 in different treated groups after 24 h of reoxygenation post-OGD. *p<0.05 and **p<0.01 vs. blank.

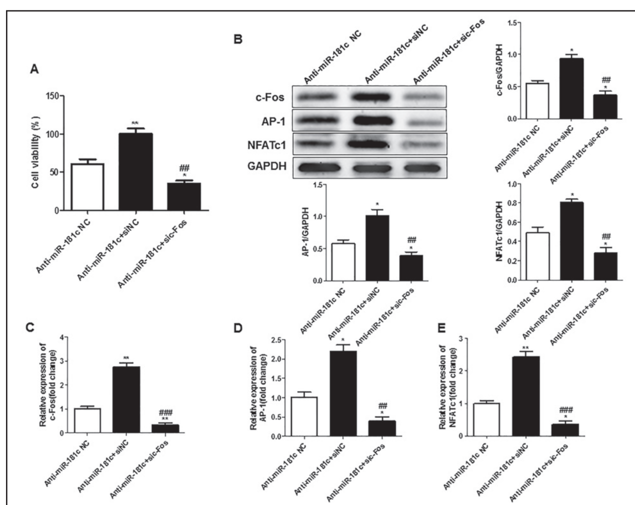

Fig. 6: Effect of c-Fos siRNA on anti-miR-181c-induced neuroprotective effect in OGD/reoxygenation-treated neurons. (A) Detection the effect of cotransfection of anti-miR-181c and c-Fos siRNA on cell viability using MTT assays after 24 h of reoxygenation post-OGD. (B) Western blot analysis of c-Fos, AP-1 and NFATc1 protein expression levels in different groups. Anti-NC cells treated with NC anti-mRNAs; Anti-miR-181c+siNC cells treated with anti-miR-181c and nonspecific siRNA; Anti-miR-181c+ sic-Fos cells treated with anti-miR-181c and c-Fos siRNA. (B) RT-qPCR analysis of c-Fos (C), AP-1 (D) and NFATc1 (E) mRNA level levels in different groups. *p<0.05 and **p<0.01 vs. anti-miR-181c NC; #p<0.05 and ##p<0.01 vs. siNC.

neurons. Western blot results showed that the stimulation effects of anti-miR-181c on the expression levels of c-Fos, AP-1, and NFATc1 were apparently blocked by c-Fos siRNA (Fig. 6B). Also, RT-qPCR was shown the same results (Fig. 6C-E). These results indicated that inhibition of c-Fos by siRNA abrogated anti-miR-181c-induced neuroprotective effect in OGD/reoxygenation-treated neurons.

3. Discussion

It has been reported that miR-181c is decreased in the brain tissues of mice subjected to middle cerebral artery occlusion. Most recently, it has been demonstrated that miR-181c controls microglia-mediated neuronal apoptosis by suppressing tumor necrosis factor after ischemia/hypoxia and microglia-mediated neuronal injury (Zhang et al. 2012). Consistently, we found that miR-181c was downregulated in primary hippocampal neurons subjected to OGD/reoxygenation *in vitro* in the present study. Also, overexpression of miR-181c accelerated cell death induced by OGD/reoxygenation, whereas knockdown of miR-181c provided protective effects against ischemic injury. Our study further confirmed the importance of miR-181c in regulating cerebral ischemic injury that might contribute to the development of intervention therapy for cerebral ischemic stroke.

Currently, an increasing number of studies have demonstrated that miRNAs play a critical role in regulating neuronal cell death, which is the key factor of stroke pathophysiology. miR-497 and miR-302b regulate ethanol-induced neuronal cell death through BCL2 protein and Cyclin D2 (Sanjay et al. 2011). Boris et al. (2014) have reported that downregulation of miR-23a and miR-27a following experimental traumatic brain injury induces neuronal cell death through activation of proapoptotic Bcl-2 proteins. MiR-21 has been demonstrated to represses FasL in microglia and protect against microglia-mediated neuronal cell death following hypoxia/ischemia (Li et al. 2012). In addition, Yao et al. (2016) have reported that miR-455 inhibits neuronal cell death by targeting TRAF3 in cerebral ischemic stroke. In the present study, we identified and characterized that miR-181c directly targeted the 3′-UTR of c-Fos mRNA and that the inhibition of miR-181c enhanced the expression of c-Fos. Interestingly, Li et al. (2015) reported that miR-181c negatively regulates the inflammatory response in OGD microglia by targeting TLR4. These findings suggested that miR-181c regulates ischemic injury, not only through a single target gene but also through the involvement of different target genes. Recently, it has been reported that miR-181c is firstly found in cardiomyocytes from the nuclear genome, but it can be translocated to mitochondria and affect the synthesis of proteins encoded by the mitochondrial genome. Overexpression of mi R-181c can lead to the remodeling of complex IV. Mitochondrial dysfunction and increased production of reactive oxygen species (Das et al. 2014). It was also found that miR-181c ameliorated cognitive impairment induced by chronic cerebral hypoperfusion in rats (Fang et al. 2017). MiR-181c has been highly expressed in the brain, and downregulated in miRNA expression profiles of acute ischemic stroke patients (Ma et al. 2016). miR-181c may mediate the cognitive impairment induced by chronic cerebral hypoperfusion associated with upregulation of TRIM2 (Fang et al. 2017). Therefore, upregulation of miR-181c may be responsible for the pathologies of diseases. The downregulation of miR-181c during ischemia may be a protection mechanism initiated by biology itself to avoid cell injury. Therefore, inhibition of miR-181c provided protective effects against ischemia.

It has been reported that c-Fos exerts critical roles in various diseases including cerebral ischemic models (Kovács 1998). The activation of c-Fos has been demonstrated to be associated with neuronal survival. Sublethal ischemic preconditioning has been demonstrated to induce c-Fos expression, exhibiting ischemic tolerance against subsequent severe ischemic injury (Yang et al. 2019). The activation of c-Fos could upregulate the downstream genes, including AP-1 and NFATc1. Here, we demonstrated that overexpression of miR-181c inhibited c-Fos expression, whereas inhibition of miR-181c significantly upregulated the protein levels of c-Fos, as well as downstream genes including AP-1 and NFATc1. Furthermore, knockdown of c-Fos significantly abrogated the protective effect of anti-miR-181c on OGD-induced cell death. In summary, these data imply that c-Fos plays an important role in ischemic injury regulated by miR-181c.

Taken together, our study suggested that miR-181c was a promising target for the prevention of cerebral ischemic injury. However, miRNA-based therapy for the treatment of the cerebral ischemia/reperfusion injury still has limitations. Recombinant AAV for gene delivery has been reported to be capable of passing through the blood brain barrier, which has been implied as a promising vehicle for gene therapy. In the present study, we have obtained the recombinant AAV carrying anti-miR-181c. However, we only investigated their effect in hippocampal neurons subjected to OGD *in vitro*. Thereby, the further effects and safety of AAV-anti-miR-181c in treatment of cerebral ischemic injury need to be assessed and investigated using animal models in vivo.

4. Experimental

4.1. Cell culture

Human embryonic kidney 293 (HEK293) cell was obtained from the cell bank of the Chinese Academy of Science (Shanghai, China) and grown in 1640 culture medium containing 10% fetal bovine serum (FBS, Gibco), 100 IU/ml penicillin, and 100 IU/ml streptomycin in 5% CO_2 incubator at 37 °C and 95% humidity. Primary hippocampal neuron cultures were prepared from neonatal SD rats, as previously described with modifications (Weidong et al. 2015). Briefly, the hippocampus tissues were dissected and dissociated in trypsin-EDTA (0.25%) and primary hippocampal neurons were maintained in neurobasal media (Life Technologies, Carlsbad, CA, USA) in supplement with GlutaMAX and B27 plus glucose (4.5 g/l) for 7 days. Then, cells were cultured in a medium containing 5 % horse serum (Sigma) and 5 % FBS (Sigma) supplemented with 15 mM glucose for 14 days. All subsequent procedures described were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (2011, 8th Edition).

4.2. Recombinant adeno-associated virus vector construction

Recombinant adeno-associated virus (AAV) vectors were constructed by Biowit Technologies (Biowit, Shenzhen, China). Briefly, the expression sequences encoding premiR-181c or anti-181c were subcloned into AAV-shuffer plasmid pSSHG which were then co-transfected with two helper packaging plasmid pAAV/Ad and adenoviral plasmid PFG140 to HEK293 cells via the calcium phosphate precipitate method. The recombinant AAV were harvested and concentrated by CsCl gradient centrifugation. The titers were determined by dot blot analysis before AAV vectors were used.

4.3. Oxygen-glucose deprivation of hippocampal neurons

The culture medium were replaced with glucose-free DMEM, and cells were cultured in hypoxic conditions (1% O_2/94% N_2/5% CO_2) at 37 °C for 3 h. Thereafter, the media were discarded and normal DMEM containing glucose was added and continued to culture for 0–48 h of reoxygenation under normoxic condition (95% air/5% CO_2). Cells cultured in growth culture medium under normoxic condition were used as control.

4.4. RT-qPCR

Total RNA from the human tissues and culture cells was extracted using Trizol reagent following the protocols. Extracted RNA (6 µl) was reverse transcribed using the TIANScript RT Kit (TiangenBiotech, Beijing, China) according to the provider's protocol. Quantitative PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR systems (Applied Biosystems). GAPDH and U6 SnRNA were used as internal control of mRNA or miRNA, respectively. Relative gene expression was quantified by $2^{-\Delta\Delta Ct}$ method.

4.5. Western blot analysis

After homogenized, the cells were lysed with RIPA buffer (Beyotime, Shanghai, China) and an BCA Protein Concentration Assay Kit (Beyotime, Shanghai, China) was used to the quantify the protein concentration. An equal amount of protein (approximately 50 µg) was loaded with 10 % SDS-PAGE (Mini-Protean-3, Bio-Rad, Hercules, CA, USA) and transferred onto a PVDF transfer membrane (Millipore, Massachusetts, USA). After blocking the membrane, they were incubated with the appropriate concentration of specific antibodies overnight at 4 °C followed by further incubation with the secondary antibody for 2 h at room temperature. Visualization of protein bands were detected with ECL chemoluminescence staining detection kit (Bio-Rad) using densitometry of Bandscan 5.0 software that was used for quantifying the density of each protein band. With GAPDH as control, the expression of total protein was normalized.

4.6. MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to detect the proliferation of cells according to the manufacturer's instructions. The absorbance was measured at a wavelength of 490 nm using a microplate reader (BioTek ELx808, BioTek Instruments, Winooski, VT, USA).

4.7. Dual-luciferase reporter assay

The fragment from the 3′-UTR of c-Fos mRNA containing the predicted miR-181c-binding sequences was amplified and subcloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The pGL3 vector containing of c-Fos mRNA or mutated forms was co-transfected with AAV-pre-miR-181c or controls into HEK293 cells and incubated for 48 h. Then, cells were harvested and lysed, and the luciferase activity was detected using the dual-luciferase reporter assay kit (Promega, Madison, WI, USA) as per standard protocols. The relative luciferase activities were normalized to that of the control cells.

4.8. Cell transfection

Lipofectamine 3000 was used to perform the transfection. The cells were infected with AAV-anti-miR-181c vectors, and c-Fos small interfering RNA (siRNA) and nonspecific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for12 h for further study.

4.9. Statistical analysis

Values are expressed as mean+standard deviation (SD). Significant differences between means were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparisons, and differences were considered significant at $P < 0.05$.

Conflicts of interest: The authors declare that they have no conflicts of interest.

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