

## Original Article

# Knockdown of miR-29a-3p protects the injury of PC-12 cells induced by H<sub>2</sub>O<sub>2</sub>

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**Abstract:** This study aimed to investigate the effects of miR-29a-3p on the injury of PC-12 cells induced by H<sub>2</sub>O<sub>2</sub>. PC-12 cells were collected to examine the mRNA levels of miR-29a-3p by real-time quantitative PCR (qRT-PCR). siRNA was used to down-regulate miR-29a-3p expression in PC-12 cells and to observe the changes in cell proliferation and apoptosis. Cell Counting Kit-8 assay was used to assess cell proliferation. The number of apoptotic cells was assessed using annexin V-FITC/PI apoptosis detection kit. Flow cytometry analysis was used to assess total reactive oxygen species (ROS). The mRNA and protein levels of P13K, p-P13K, AKT, p-AKT were determined by qRT-PCR and Western blot. The results showed that H<sub>2</sub>O<sub>2</sub> inhibited the proliferation and promoted apoptosis of PC-12 cells, which induced injury of PC-12 cells. H<sub>2</sub>O<sub>2</sub> promoted the expression of miR-29a-3p in PC-12 cells. The down-regulated miR-29a-3p reduced cell damage induced by H<sub>2</sub>O<sub>2</sub> and alleviated the reduction of IGF1 expression. Moreover, the down-regulation of miR-29a-3p enhanced the expression levels of p-P13K/P13K, p-AKT/AKT, indicating that the down-regulation of miR-29a-3p activated PI3K/AKT pathway. In conclusion, Knockdown miR-29a-3p protected the injury of PC-12 cell induced by H<sub>2</sub>O<sub>2</sub> via negatively regulating the expression of IGF1 and the activation of PI3K/AKT pathway.

**Keywords:** miR-29a-3p, PC-12 cells, H<sub>2</sub>O<sub>2</sub> injury, IGF1, PI3K/AKT pathway

## Introduction

Spinal cord injury (SCI) is a common trauma with symptoms of motor dysfunction or paralysis, which is caused by excessive stretching, spinal lateral bending, and excessive bending [1]. It shows a variety of neurological deficit, social, and financial burdens depending on the different level of injuries [2]. SCI not only brings the physical and psychological harm to patients, but also causes huge economic burdens to the whole society. SCI can cause localized oxidative damage, so the molecular mechanism of oxidative damage is critical for the treatment of SCI. A large amount of evidences show that the distinctive characteristic in many neurodegenerative diseases is an excess of reactive oxygen species (ROS). ROS does damage to deoxyribonucleic acid, proteins and lipid membranes, which will result in the injury or death of neuronal cells [3-5]. H<sub>2</sub>O<sub>2</sub> can result in oxidative stress and induce the apoptosis in cells. More and more evidences discovered that the neuro-

nal cell damage in rat pheochromocytoma line 12 (PC-12) cells has been commonly used as an ideal model in vitro [5-7]. Therefore, H<sub>2</sub>O<sub>2</sub> is used in this study to induce oxidative damage model of PC-12 cells.

MicroRNAs (miRNAs) are a class of small endogenous, non-coding, single-stranded RNAs with the length of 20-22nt [8, 9], which suppress protein expression by inhibiting the translation of messenger RNAs. Mounting evidence suggests that miRNAs play a critical role in a variety of biological processes such as apoptosis, differentiation and migration [10], and the dysfunction of miRNAs is involved in many diseases including cancers [11, 12]. Plenty of miRNAs have association with patient survival or tumor types, which might be used as prognostic or diagnostic markers. miR-29 family is a conserved family of miRNAs, which regulates the expression of lots of oncogenes [13, 14]. miR-29a-3p is a member of miR-29 family, which has been reported to have tumor suppressor

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functions [15], and is down-regulated in many tumor cells [16]. In the present study, the effect of miR-29a-3p on cell viability and apoptosis in H<sub>2</sub>O<sub>2</sub>-treated PC-12 cells were investigated. And the expression of IGF1, P13K, p-P13K, AKT, p-AKT, and the ROS were evaluated.

### Materials and methods

#### *Cell culture and H<sub>2</sub>O<sub>2</sub> treatment*

The PC-12 cells were purchased from Institute of Zoology, Chinese Academy of Sciences (Beijing, China) and used to analysis throughout the study. Dulbecco modified Eagle medium (DMEM) containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin was used to culture PC-12 cells (final density: 1 × 10<sup>4</sup> cells/ml). The cells were cultured in a humidified incubator containing 95% air and 5% CO<sub>2</sub> at 37°C. Culture medium was changed every other day. For the H<sub>2</sub>O<sub>2</sub> treatment, the cells were plated in CELLSTAR cell culture multi-well plates (Greiner, Germany) at a density of 5 × 10<sup>4</sup> cells/ml for 24 h. Then the medium was removed and the cells were treated with fresh medium with different concentrations of H<sub>2</sub>O<sub>2</sub> (0-500 µM) for 24 h to construct the injury model. The control group was treated with the same medium without H<sub>2</sub>O<sub>2</sub>.

#### *Cell counting Kit-8 assay*

Cells were seeded in 96-well plate with 5000 cells/well. Then cells were transfected with various synthetic miRNA mimics and corresponding control (50 Nmol), and 100-Nmol inhibitor and corresponding control, using Lipofectamine-2000 (Invitrogen) according to the manufacturer's instructions. Cellular growth was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Briefly, after stimulation, the CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 hour at 37°C in humidified 95% air and 5% CO<sub>2</sub>. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

#### *Apoptosis assay*

The apoptotic cells was identified and quantified by flow cytometry analysis using Annexin V-FITC/PI apoptosis detection kit ((BD Biosciences). The PC-12 cells (100,000 cells/well)

were seeded in 6-wellplate. Treated cells were washed twice with cold PBS and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and analyzed by using flow cytometer (Becton Dickinson, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

#### *Total reactive oxygen species (ROS) assay*

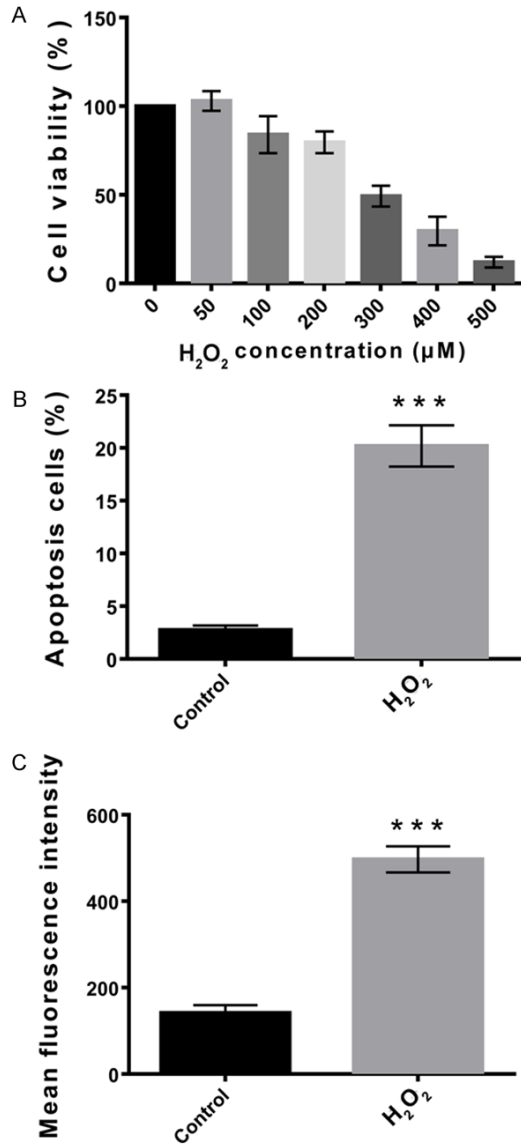
Flow cytometry was used to measure ROS by using 2, 7-dichlorofluorescein diacetate (DCFH-DA) (Nanjing Jiancheng, Nanjing, China) [17]. The cells were seeded into a 6-well plate. The treated cells were washed twice with PBS and resuspended in buffer. And then cells were co-cultured with serum-free culture medium containing 10 µM DCFH-DA for 20 min at 37°C in darkness. Then the cells were washed with PBS, and samples were collected by using trypsin digestion method. All samples were centrifuged and the supernatants were removed. The cells were resuspended to 500 µl PBS and the fluorescent intensities were measured using a flow cytometer (488 nm excitation, 521 nm emission).

#### *qRT-PCR*

Total RNA was extracted from PC-12 cells and tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa Biotechnology Co., Ltd., Dalian, China). The PCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II with the TaqMan MicroRNA Assay of miR-29a-3p and U6 (Applied Biosystems, Foster City, CA, USA) were used for testing the expression levels of miR-29a-3p in cells. The 2<sup>-ΔΔCT</sup> method was used in the analysis of the relative expression ratio of miRNA [18].

#### *miRNAs transfection*

miR-29a-3p mimic, si-miR-29a-3p and the NC controls were synthesized by GenePharma Co. (Shanghai, China). Cell transfections were conducted using Lipofectamine 3000 reagent

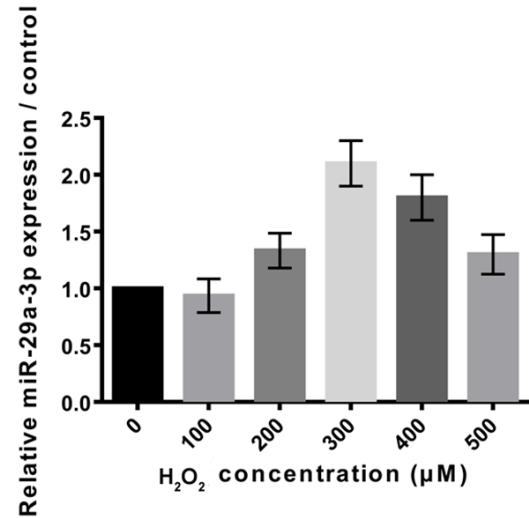


**Figure 1.** The effect of H<sub>2</sub>O<sub>2</sub> on PC-12 cells. A. H<sub>2</sub>O<sub>2</sub> induced the proliferation of PC-12 cells. B. H<sub>2</sub>O<sub>2</sub> induced the apoptosis of PC-12 cells. C. H<sub>2</sub>O<sub>2</sub> induced the increase of ROS in PC-12 cells. \*\*\**P* values <0.001.

(Invitrogen) following the manufacturer's instructions.

#### Western blot

The protein used for western blot was extracted using RIA lysis buffer (Thermo Scientific, Worcester, MA) containing protease inhibitors (Sukahon, Shandong, China). Homogenates were centrifuged at 13,000×*g* for 30 minutes at 4°C. The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI,

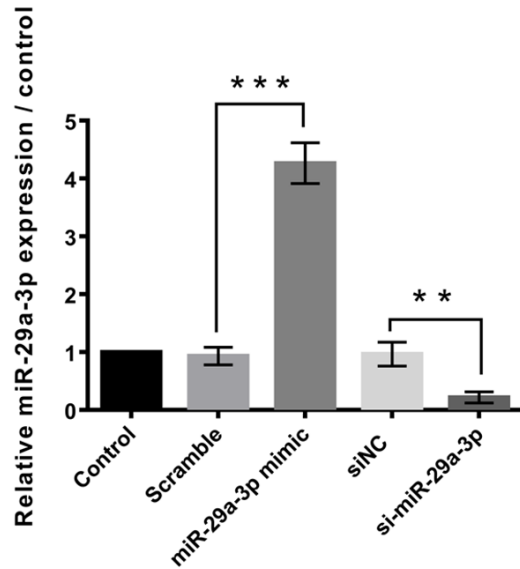


**Figure 2.** H<sub>2</sub>O<sub>2</sub> induced the up-regulation of miR-29a-3p in PC-12 cells. The real-time quantitative PCR method was used to analyze the effect of H<sub>2</sub>O<sub>2</sub> on expression of miR-29a-3p in PC-12 cells.

USA). The western blot system was established using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. GAPDH antibody was purchased from Sigma. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1,000. Primary antibody was incubated with the membrane overnight at 4°C, then washed and incubated with secondary antibody marked with horseradish peroxidase for 1 hour at room temperature. After rinsing, the polyvinylidenedifluoride (PVDF) membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using a Genomic and Proteomic Gel Documentation (Gel Doc) Systems from Syngene (Frederick, MD).

#### Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. The *P*-values were calculated using a one-way analysis of variance (ANOVA). A *P*-value of <0.05 was considered to indicate a statistically significant result.



**Figure 3.** Up-regulation and down-regulation of miR-29a-3p in PC-12 cells. \*\**P* values <0.01. \*\*\**P* values <0.001.

## Results

### *H<sub>2</sub>O<sub>2</sub> induced PC-12 cell model*

$H_2O_2$  can induce oxidative stress of cells and further result in apoptosis [19], so it is usually used in experimental models of ROS. The cells were cultured in CELLSTAR cell culture multi-well plates for 24 h, and then were administrated with  $H_2O_2$  (0-500  $\mu$ M) for 24 h. As shown in **Figure 1A**, the cell viability was reduced with the increase of  $H_2O_2$  concentration. At the concentration of 300  $\mu$ M, the cell viability was more than 50%. So in the study 300  $\mu$ M was used as the suitable concentration of  $H_2O_2$  for PC-12 cell model.

The effect of  $H_2O_2$  on apoptotic PC-12 cells was observed by flow cytometry analysis. The results showed that the treatment with 300  $\mu$ M  $H_2O_2$  for 24 h significantly promoting the apoptosis of PC-12 cells (**Figure 1B**). Similarly, the results of flow cytometry analysis showed that  $H_2O_2$  obviously increased intracellular ROS compared with control group (**Figure 1C**).

### *H<sub>2</sub>O<sub>2</sub> promoted the expression of miR-29a-3p in PC-12 cells*

The real-time quantitative PCR method was used to analyze the effect of  $H_2O_2$  on expres-

sion of miR-29a-3p in PC-12 cells. The results in **Figure 2** showed that  $H_2O_2$  promoted the expression of miR-29a-3p in PC-12 cells, and the promoting effect was enhanced with the increase amount of  $H_2O_2$ . The expression of miR-29a-3p was highest when the  $H_2O_2$  concentration was 300  $\mu$ M. The results indicated that increased expression of miR-29a-3p might play a certain role in PC-12 cells.

### *Up-regulation and down-regulation of miR-29a-3p in PC-12 cells*

To investigate the function of miR-29a-3p in PC-12 cells, we transfected PC-12 cells with a miR-29a-3p-specific siRNAs. The overexpression of miR-29a-3p up-regulated mRNA expression of miR-29a-3p in PC-12 cells, while the knockdown of miR-29a-3p down-regulated mRNA expression of miR-29a-3p respectively compared to control cells (**Figure 3**).

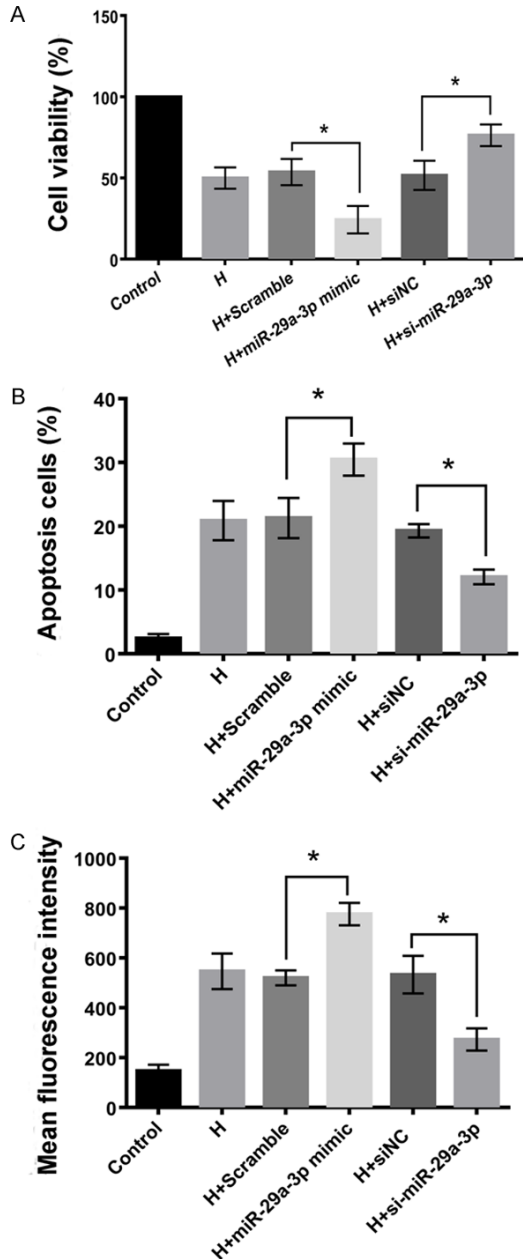
### *The knockdown of miR-29a-3p reduced cell damage induced by H<sub>2</sub>O<sub>2</sub>*

The expression levels of miR-29a-3p had correlation with the degree of differentiation which partially lied on cell proliferation. CCK-8 assay was used to evaluate the effect of miR-29a-3p on cell proliferation. As shown in **Figure 4A**, miR-29a-3p inhibited cell proliferation compared to the control group, while miR-29a-3p mimics further inhibited cell proliferation. However, the knockdown of miR-29a-3p alleviated the inhibition effect.

Flow cytometry analysis was used to evaluate the effect of miR-29a-3p on apoptosis and ROS. The results showed that miR-29a-3p mimics significantly promoted apoptosis, while the knockdown of miR-29a-3p alleviated apoptosis (**Figure 4B**). Similarly, miR-29a-3p mimics obviously increased intracellular ROS compared with control group, while the knockdown of miR-29a-3p alleviated the increase of ROS (**Figure 4C**).

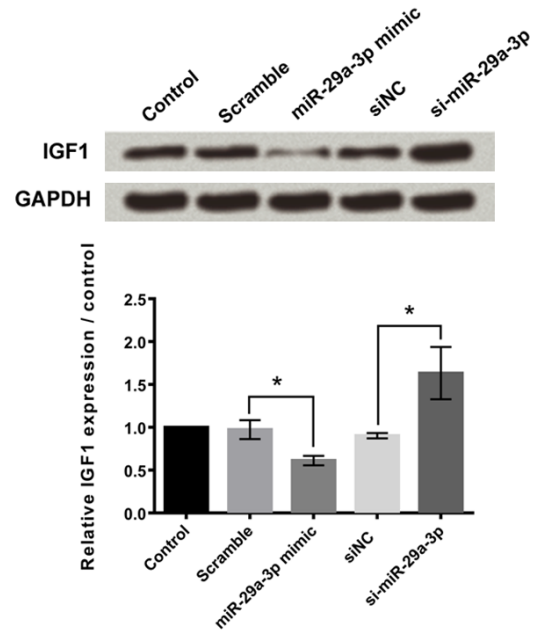
### *miR-29a-3p negatively regulated the expression of IGF1*

In order to further explore the effect of miR-29a-3p on PC-12 cells, Western blot was used to analyze the insulin-like growth factors 1 (IGF1), which had the function of promoting cell differentiation or wound repair. As shown in



**Figure 4.** The effects of up-regulation of miR-29a-3p on proliferation, apoptosis and ROS generate in PC-12 cells. A. The up-regulation of miR-29a-3p further inhibited the proliferation of PC-12 cells. The down-regulation of miR-29a-3p alleviated the inhibition effect of H<sub>2</sub>O<sub>2</sub> on proliferation of PC-12 cells. B. The up-regulation of miR-29a-3p further promoted the apoptosis of PC-12 cells. The down-regulation of miR-29a-3p decreased the apoptosis rate. C. The up-regulation of miR-29a-3p increased the ROS in PC-12 cells and the down-regulation of miR-29a-3p decreased the ROS in PC-12 cells. ROS: reactive oxygen species. \*P values <0.05.

**Figure 5,** miR-29a-3p mimics obviously reduced the expression of IGF1, while the knockdown of



**Figure 5.** miR-29a-3p negatively regulated the expression of IGF1. Western blot was used to analyze IGF1. IGF1: insulin-like growth factors 1. \*P values <0.05.

miR-29a-3p alleviated the reduction of IGF1 expression, indicating that miR-29a-3p negatively regulated the expression of IGF1.

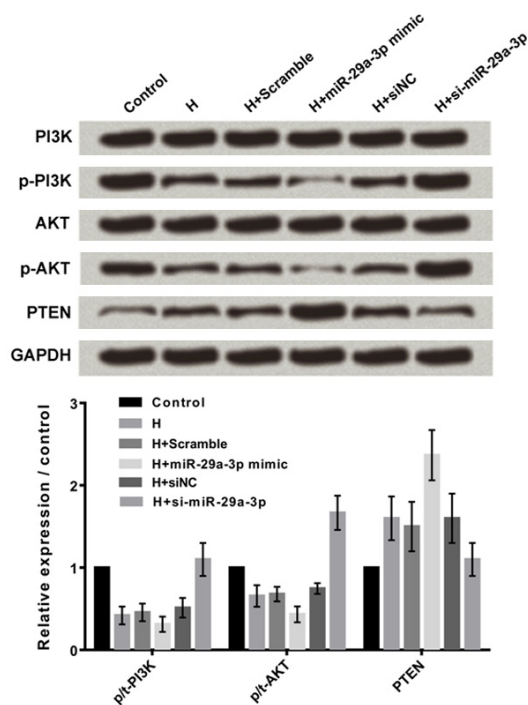
#### miR-29a-3p inhibited the activation of PI3K/ AKT pathway

In order to further explore the effect of miR-29a-3p on PC-12 cells, we examined the expression of P13K, p-P13K, AKT, p-AKT, PTEN by qRT-PCR and Western blot. The results showed that up-regulation of miR-29a-3p inhibited the levels of p-P13K/P13K, p-AKT/AKT, which indicated that overexpression of miR-29a-3p suppressed PI3K/AKT pathway. On the contrary, the down-regulation of miR-29a-3p enhanced the expression levels of p-P13K/P13K, p-AKT/AKT to some extent, indicating that the down-regulation of miR-29a-3p activated PI3K/AKT pathway (Figure 6).

#### Discussion

miRNAs regulates gene expression and participates in many cellular processes such as cell differentiation, proliferation, apoptosis, cell-cycle and metabolism [8]. It has been reported that miRNAs regulate their related target genes during the biological process of cancer [20]. Growing evidences have indicated that the

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**Figure 6.** The expression levels of P13K, p-P13K, AKT, p-AKT had negative relationship with the expression level of miR-29a-3p. GAPDH was used as the loading control.

abnormal expression of miR-29s can easily be found in multiple cancers, which indicates the important role of miR-29s in cancer progression [21-23]. This study for the first explored the effect of miR-29a-3p on H<sub>2</sub>O<sub>2</sub>-induced oxidative damage of PC-12 cells. The results showed up-regulated expression of miR-29a-3p in PC-12 cells, indicating that miR-29a-3p might play a regulatory role in the oxidative damage of PC-12 cells. Some studies have discovered the up-regulation of miR-29a in human B cell chronic lymphocytic leukemia (B-CLL) and acute myeloid leukemia (AML) [24, 25]. However, some studies have reported the down-regulation of the miR-29 family in acute myeloid leukemia [26], lung cancer [27], brain tumors and chronic lymphocytic leukemia [24]. The seemingly contradictory conclusions indicated that the dysregulation of miR-29a in different cancers might have correlation with the microenvironment of cells. In order to explore the effect of miR-29a-3p on cell damage induced by H<sub>2</sub>O<sub>2</sub>, we detected the expression levels of miR-29a-3p transcript in PC-12 cells. The results showed that the expression level of miR-29a-3p was closely associated with cell proliferation and apopto-

sis. The knockdown of miR-29a-3p promoted cell proliferation and inhibited apoptosis.

Insulin-like growth factor-1 (IGF-1) is known to modulate a large amount of intracellular pathways [28]. IGF-1 receptor (IGF-1R) acts as crucial oncogene in the development of cancers, and is regulated by various miRNAs in cancers [29]. It reported that miR-494 suppressed tumor growth of epithelial ovarian carcinoma by targeting IGF1R [30]. In the present study, we found that miR-29a-3p negatively regulated the expression of IGF1, indicating that IGF1 played important roles in suppressing tumor cells. The PI3K/AKT pathway plays an important role in many biological responses, including cellular proliferation and survival. In order to further explore whether the effect of miR-29a-3p on cell damage induced by H<sub>2</sub>O<sub>2</sub> through the PI3K/AKT pathway, the expressions of P13K, p-P13K, AKT, p-AKT, PTEN were analyzed. The results showed that the levels of P13K, p-P13K, AKT, p-AKT had negative relationship with the expression level of miR-29a-3p, indicating that miR-29a-3p inhibited the activation of PI3K/AKT pathway. From the above results, we found that the knockdown of miR-29a-3p inhibited apoptosis and promoted cell proliferation by activating PI3K/AKT pathway.

In conclusion, the present study revealed that miR-29a-3p was up-regulated in PC-12 cells treated with H<sub>2</sub>O<sub>2</sub>. The down-regulation of miR-29a-3p reduced oxidative damage and apoptosis in PC-12 cells via the activation of PI3K/AKT pathway. These findings provided evidence for the values of miR-29a-3p in the clinical treatment of spinal cord injury.

### Disclosure of conflict of interest

None.

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