Mechanism underlying circularRNA_014301-mediated regulation of neuronal cell inflammation and apoptosis

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Abstract. Spinal cord injury (SCI) causes damage to the spinal cord owing to trauma or disease and myelinated fiber tracts that transmit sensation and motor signals to and from the brain. Circular RNAs (circRNAs) are a recently discovered class of regulatory molecules, and their roles in SCI are still unknown. circRNA_014301 was indicated to be differentially expressed in the spinal cord at the site of SCI in a rat model. To analyze the role of circRNA_014301 in SCI, we exposed rat adrenal pheochromocytoma PC12 cells were exposed to increasing concentrations of lipopolysaccharide (LPS) and to construct a PC12 cell inflammatory model. Cell Counting Kit-8 assay was used to analyze cell viability. Reverse transcription-quantitative PCR and ELISA were used to detect the expression of inflammatory factors (IL-1β, IL-6 and TNF-α). Annexin V-FITC/PI double staining was employed to detect cell apoptosis, and western blotting was performed to detect the expression of apoptotic proteins (Bax/Bcl-2/cleaved caspase-3) and NF-κB. The results demonstrated that LPS induced inflammation in PC12 cells as evidenced by the reduced cell proliferation and enhanced expression of inflammatory and apoptotic factors under increasing LPS concentrations. Western blotting analyses indicated that circRNA_014301 induced the expression of p-NF-κB/NF-κB, Bax and cleaved caspase-3, and decreased the expression of Bcl-2 following LPS-induced inflammation, and this apoptosis-promoting effect was relieved by small interfering-RNA-mediated knockdown of circRNA_014301. Thus, circRNA_014301 silencing alleviated apoptosis and inflammation in PC12 cells. SCI is invariably associated with spinal cord inflammation, and LPS was used to stimulate apoptosis and inflammatory injury in PC12 cells, and create a cell model of SCI. By promoting PC12 cell apoptosis under inflammatory conditions, it was indicated that circRNA_014301 may suppress SCI. Therefore, circRNA_014301 may represent a potential target for SCI diagnosis and therapy.

Introduction

Spinal cord injury (SCI) is a serious neurological disorder that involves damage to the spinal cord caused by trauma or disease and its most typical manifestation is paralysis at the injured site (1). SCI not only causes physical and psychological trauma for patients, but is also associated with an important economic burden for the entire society (2,3). The annual SCI incidence worldwide is 15-40 cases per million people (2), and the treatment of patients with SCI is currently limited (4). Several factors can cause SCI, including oxygen-free radicals, inflammation, ion disturbances, neurotransmitter accumulation, excitatory amino acid accumulation, axon loss, glial scar formation and apoptosis (5-10). Therefore, there is an urgent requirement for novel effective treatment methods for SCI.

Initial stage SCI involves a series of vascular, biochemical and cellular events, and it is the most complicated and the least understood stage of SCI (11,12). The vascular alterations that occur during this stage lead to serious complications, including oxidative stress, blood flow reduction, edema, tissue disorders, depolarization, metabolic dysfunction, loss of cell function, tissue dissolution and neuronal cell death (13,14). Subsequent cellular changes include increases in the numbers of macrophages and neutrophils, apoptosis, Wallerian degeneration and severe inflammatory response (15,16). This secondary SCI is a delayed progressive tissue injury after the initial injury (17,18), during which inflammatory cells such as macrophages, microglia, T cells and neutrophils cross the blood-brain barrier and enter the injured area. These cells expand and release inflammatory factors, such as TNF-α and IL-1β, whose levels peak at 6-12 h and continue to increase within 4 days after injury.

Biochemical and cellular alterations in the local environment that occur after SCI gradually affect the neurons, oligodendrocytes and astrocytes (19). For example, the center and areas distal to the site of injury contain oligodendrocytes undergoing apoptosis. Apoptosis leads to demyelination of the axons of the preserved oligodendrocytes. Moreover, the

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phagocytic inflammatory cells attracted to the injured area release reactive oxygen species, thereby causing a number of reactions, such as DNA oxidative damage, protein oxidation and lipid peroxidation (19).

More than three quarters of the human genome are transcribed, but <2% of the RNA is translated into proteins (20). RNAs that are not translated into proteins but exhibit other cellular functions are called non-coding RNAs (ncRNAs) (21). In the past two decades, different types of ncRNAs have been discovered alongside rRNAs and tRNAs, including microRNAs (miRNAs/miRs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs) (22). Whereas miRNAs and lncRNAs are linear, circRNAs are characterized by a covalently closed continuous loop without the 5' cap and 3' poly(A) tail (22). In contrast to lncRNAs, circRNAs are usually derived from protein-coding genes and complete exons (23). Various types of circRNAs exist according to their biogenesis, as follows: circRNAs formed by reverse splicing and exon circularization, circovirus RNA genomes, circRNA intermediates, intronic circRNAs and exonic circRNAs (24). Due to the lack of free ends, circRNAs are resistant to exonucleases; moreover, they exhibit the potential for rolling circle amplification (25).

Research on the role of circRNAs in nerve damage has rapidly evolved. Numerous studies have revealed the expression pattern of circRNAs in traumatic brain injury and neuropathic pain models through DNA microarrays and RNA-sequencing (26,27). For instance, circRNA_0006928 may regulate neuronal apoptosis by binding to miR-184 (26). In chronic inflammatory pain, circRNA-Filip1l, which is negatively regulated by miR-1224 through binding and splicing, increases chronic inflammatory pain and can regulate nociception by targeting ubiquitin protein ligase E3 component n-recognin 5 (28). circRNA expression increased in the rat spinal cord after traumatic SCI, which indicated that there was a relationship between circRNA and SCI (29). In addition, differentially expressed circRNAs were identified in the rat sciatic nerve compression model, and downregulated circRNAs were revealed compared with control rats. Among them, circRNA_2837 was demonstrated to regulate neuronal autophagy by acting as a binding sponge for the miR-34 family (30). Furthermore, silencing circRNA_2837 can induce autophagy in primary spinal cord neurons by targeting miR-34a (30). Although these findings suggested that circRNAs exhibit regulatory functions in nerve injury, their precise role is still unknown.

A previous study using a rat SCI model revealed that a total of 150 circRNAs were significantly differentially expressed in the rat spinal cord after SCI (fold-change ≥2; P≤0.05). Of these, 99 circRNAs were upregulated, and 51 were downregulated (31). Among them, circRNA_014301 was highly expressed at the injury site and is therefore of interest (29,31). Although these studies revealed that circRNA_014301 was significantly induced following SCI in the rat model, whether it exhibits a specific regulatory function in SCI remains unknown (32). The present study aimed to analyze the effect of circRNA_014301 on the inflammation and apoptosis of PC12 cells to assess the possible regulatory role of circRNA_014301 in a cellular model of SCI and identify a potential therapeutic target for SCI.

Materials and methods

Cell culture and treatments. The rat adrenal pheochromocytoma cell line PC12 was obtained from The World Cell Factory (CyberKang (Shanghai) Biotechnology Co., Ltd.). The cells were maintained in a complete medium containing RPMI-1640 (Sigma-Aldrich; Merck KGaA) plus 2 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES and 1 mM sodium pyruvate, supplemented with 10% heat-inactivated horse serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 5% FBS (Biological Industries), in a 5% CO_2 incubator at 37°C with no antibiotics. When cell confluence reached >90%, the cells were passaged at a ratio of 1:2 or 1:3 before use.

Different concentrations of lipopolysaccharide (LPS; 0, 1, 2.5, 5 and 10 μg/ml; Sigma-Aldrich; Merck KGaA) were used to treat PC12 cells for 24 h and to construct a PC12 cell inflammatory model (33-35).

The small interfering (si)RNA for circRNA_014301 (si-circRNA_014301; 5'-CAGACAGGAGCTACTCGGATA TGAT-3') and the si-negative control (si-NC; 5'-CATCTCCCA GCAGTGACACTGACTT-3') were purchased from Shanghai GenePharma Co., Ltd. Cell transfection was performed using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), as per the manufacturer's instructions. PC12 cells were seeded into 6-well plates at $2x10^4$ cells/well. Cells were transfected with 100 nM of siRNA. The culture plate was placed in a CO_2 incubator at 37°C for 12 h. Cells were subsequently stimulated with 5 μg/ml LPS for 24 h to construct an SCI inflammation model. After the intervention is over, cells are collected and tested. After transfection, RNA was extracted, and the silencing efficiency was determined via reverse transcription-quantitative (RT-q) PCR.

The experimental protocol was divided into four treatments: Control (normally cultured cells without LPS), LPS (5 μg/ml) treatment, si-circRNA_014301 + LPS treatment and si-NC + LPS treatment. Following transfection with si-circRNA_014301 or si-NC for 24 h, LPS treatment was performed for 24 h to establish the inflammatory model.

Total RNA extraction and purification. Total RNA was extracted from PC12 cells using the RNeasy Protect Mini kit (Qiagen, Inc.) following the manufacturer's manual. RNA concentration was quantified using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.), and its integrity was evaluated on an agarose gel. The RNA was then purified with RNase-Free DNase Set (Qiagen, Inc.) and digested for 30 min at 37°C with RNase R (20 mg/ml; cat. no. RNR07250; Epicenter Biotechnologies; Lucigen Corporation) to remove linear RNA according to the manufacturer's instructions.

RNase R is known to degrade linear RNAs leaving circRNAs intact, and is used to validate the depletion of linear RNAs and the resistance of circRNAs to RNase R treatment (36). miRDB database (http://mirdb.org/) was utilized to predict miRNA-mRNA interactions.

Isolation of nuclear and cytoplasmic fractions. The nuclear and cytoplasmic fractions were isolated using a PARIS™ kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The expression of circRNA_014301 in the
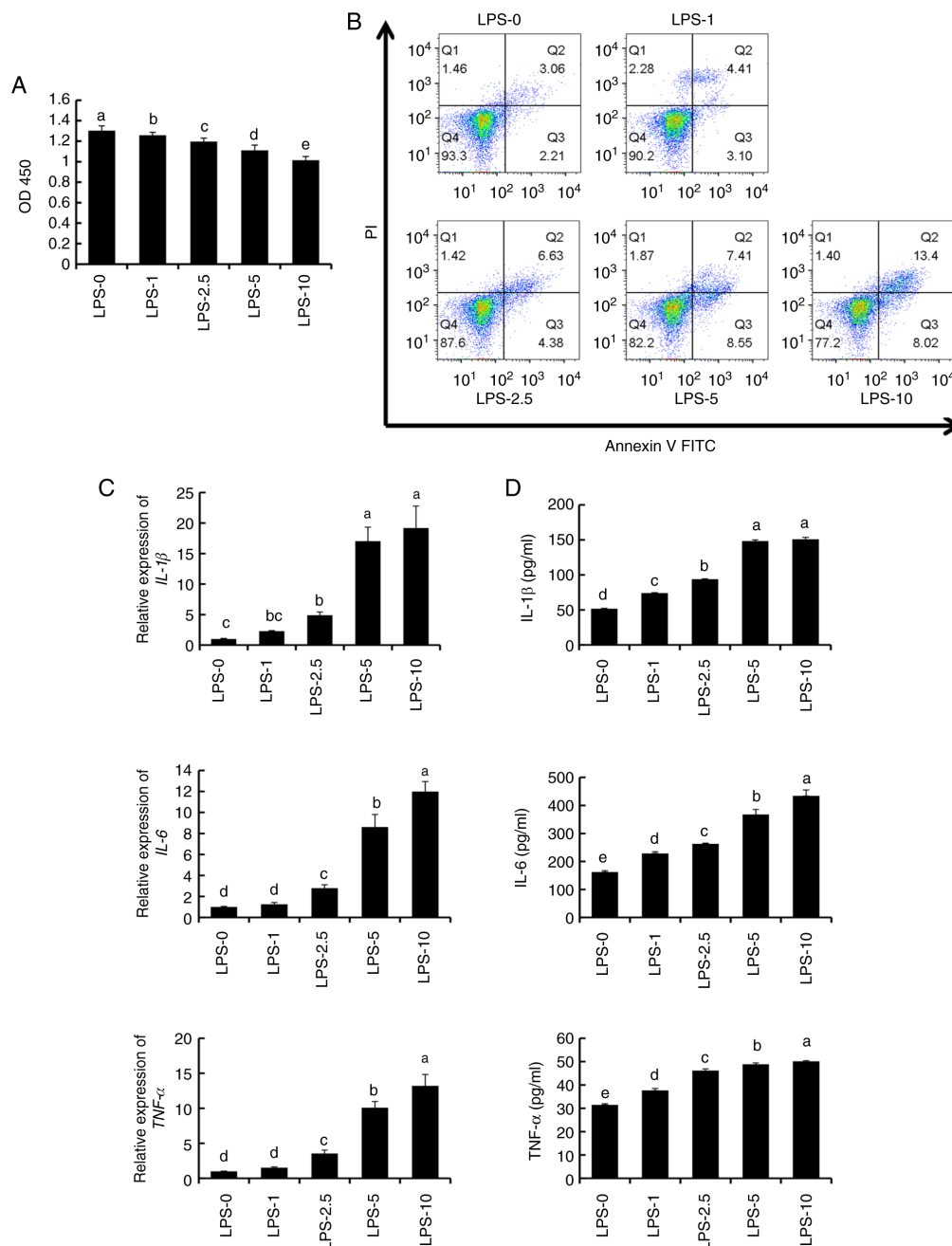
Figure 1. LPS promotes inflammation in PC12 cells. (A) PC12 cell viability decreased with increasing LPS concentration. (B) Apoptosis of PC12 cells detected via flow cytometry. The proportion of PC12 cells undergoing early and late apoptosis was the highest with 5 and 10 μg/ml LPS. Increasing LPS levels (C) promote the relative expression of inflammatory factors or (D) increase their concentration in PC12 cells. The data are presented as the mean ± SD of three independent experiments. Groups labelled with the same lower-case letter are not significantly different to one another; groups with different letters are significantly different (P<0.05). LPS, lipopolysaccharide; OD, optical density; PI, propidium iodide.

nuclear and cytoplasmic was tested via qPCR. U6 snRNA and 18S rRNA were employed as positive controls for nuclear and cytoplasmic fractions, respectively.

RT-qPCR. The purified, RNase R-digested RNA was reverse-transcribed to cDNA using PrimeScript RT reagent Kit (Takara Biotechnology Co., Ltd.) for 15 min at 37°C. cDNA amplification was performed using SYBR® Green Realtime PCR Master Mix (Toyobo Life Science). The settings of the LightCycler 96 Real-Time PCR system (Roche Diagnostics) were as follows: 94°C for 30 sec; followed by 40 cycles of 94°C for 5 sec and 61°C for 35 sec; followed by 97°C

for 10 sec, 65°C for 1 min, and 97°C for 1 sec. Each reaction included three replicates. GAPDH (forward, 5'-GCTCTC TGCTCCTCCCTGTTCTA-3'; and reverse, 5'-TGGTAACCA GGCGTCCGATA-3') and U6 (forward, 5'-AAAGCAAAT CATCGGACGACC-3'; and reverse, 5'-GTACAACACATT GTTTCCTCGGA-3') were used as the internal reference genes (37,38). The primer sequences of 18S RNA (forward, 5'-TGTGCCGCTAGAGGTGAAATT-3', and reverse 5'-TGG CAAATGCTTTCGCTTT-3') were based on Tao *et al* (39). The primer sequences of circRNA_014301 (forward, 5'-GCT GCTCTAGTGGTGACTCATG-3'; and reverse 5'-TTCTCC ATTCATCCAATCAACTTCG-3'), IL-1β (forward, 5'-GAC

CTTCCAGGATGAGGACA-3'; and reverse 5'-AGCTCA TATGGGTCCGACAG-3'), IL-6 (forward, 5'-AGTTGCCTT CTTGGGACTGA-3'; and reverse 5'-CAGAATTGCCAT TGCACAAC-3') and TNF-α (forward, 5'-ACGGCATGGATC TCAAAGAC-3'; and reverse 5'-GTGGGTGAGGAGCAC GTAGT-3') were based on Gonzales *et al* (40). The primer sequences of MTY1L (forward, 5'-GTCATGGTGTGAGGG GTCCC-3', and reverse 5'-CACACTCTGTGATTCTTCAG-3') were based on Bruno *et al* (41). Gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (31,42).

Cell viability assay. The viability of PC12 cells was measured using Cell Counting Kit-8 (CCK-8) assay (Beyotime). Briefly, LPS treatment after transfection was performed as aforementioned, and then the cells were seeded onto 96-well plates at $2x10^4$ cells/well and incubated in a humidified incubator at 37°C for 6 h. Subsequently, 10 μl of CCK-8 solution was added to each well, and the plate was incubated for 1 h at 37°C. The absorbance of each well at 450 nm was recorded using a microplate reader (BioTek Instruments, Inc.). The higher the OD 450 value, the faster the cell proliferation.

ELISA. The contents of IL-1β (cat. no. RLB00), IL-6 (cat. no. R6000B) and TNF-α (cat. no. RTA00) in the PC12 cells were measured using corresponding Quantikine ELISA kits (R&D Systems, Inc.) according to the manufacturer's instructions. Each experiment was performed in three biological replicates.

Flow cytometry analysis of apoptosis. PC12 cells in the various groups were harvested and centrifuged at room temperature at 10,000 x g for 5 min, followed by treatment with Annexin V-FITC binding buffer and 1 mg/ml of PI solution (cat. no. G003-1-2; Nanjing Jiancheng Bioengineering Institute) at room temperature for 15 min. Apoptotic cells were detected using flow cytometry (CytoFLEX; Becton, Dickinson and Company) and analyzed using CytExpert software 2.0 (Becton, Dickinson and Company).

Western blotting. Total protein from the PC12 cell line was acquired using RIPA buffer (cat. no. P0013B; Beyotime Institute of Biotechnology) and centrifuged at 4°C for 15 min, protein concentrations were determined using BCA assays (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd), and samples (20 μg) were separated via 10% SDS-PAGE and transferred to PVDF membranes. The membranes were incubated at room temperature for 2 h in 5% nonfat dried milk. The target protein antibodies (1:1,000) and anti-GAPDH (1:3,000) were incubated with the membranes at 4°C overnight. The primary antibodies used were as follows: Bax (cat. no. ab32503; Abcam), Bcl-2 (cat. no. 3498; Abcam), cleaved caspase-3 (cat. no. 9661; Abcam), phosphorylated (p)-NF-κB p65 (cat. no. 3033; Abcam), NF-κB p65 (cat. no. 8242; Abcam), with and GAPDH (cat. no. M1000110; Beijing Solarbio Science & Technology Co., Ltd.) as the reference antibody. The secondary antibodies [goat anti- rabbit IgG-HRP (1:5,000; cat. no. SE134; Beijing Solarbio Science & Technology Co., Ltd.) and goat anti-mouse IgG-HRP (cat. no. GB23301; Wuhan Servicebio Technology Co., Ltd.)] were subsequently incubated with membranes at room temperature for 1 h. ECL
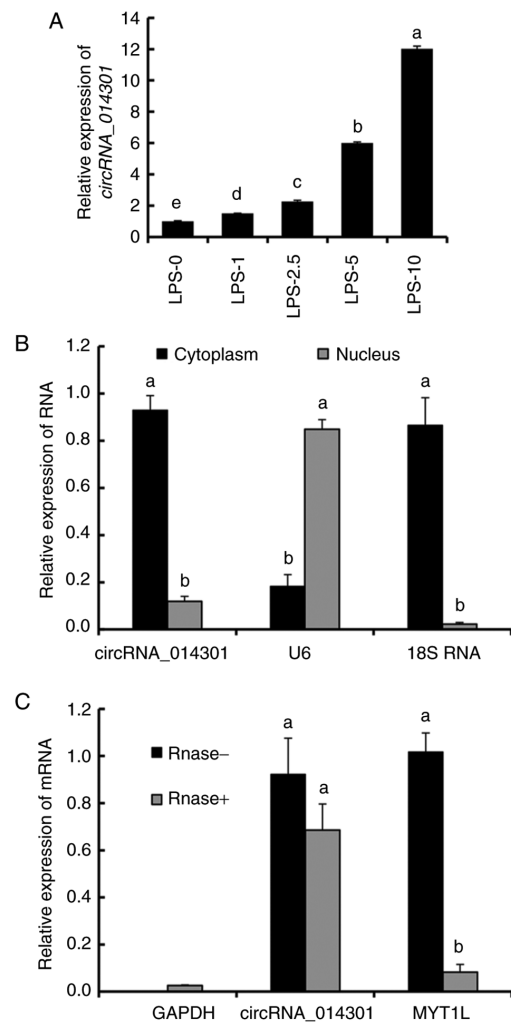

Figure 2. Spatial expression of circRNA_014301 and *MYT1L* in PC12 cells. (A) The relative expression of circRNA_014301 was enhanced with increasing concentrations of LPS as detected via RT-qPCR. (B) Cytoplasmic expression of circRNA_014301 determined using RT-qPCR. (C) RT-qPCR detection of the relative expression of circRNA_014301 and *MYT1L* before and after RNase digestion. The data are presented as the mean ± SD of three independent experiments. Groups labelled with the same lower-case letter are not significantly different to one another; groups with different letters are significantly different (P<0.05). LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR; circRNA, circular RNA.

color developing solution (cat. no. PE0010; Beijing Solarbio Science & Technology Co., Ltd.) was added to the PVDF film for exposure and photographing. Quantity One software 4.0 (Bio-Rad Laboratories, Inc.) was used to perform grayscale analysis of protein bands.

Statistical analysis. The results of three biological replicates are presented as the mean ± standard deviation. SPSS 21.0 software (IBM Corp.) was used for statistical analyses, and the data were analyzed using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was used to indicate a statistically significant difference.

Results

LPS treatment promotes inflammatory response in PC12 cells. As presented in Fig. 1A, PC12 cell viability was reduced
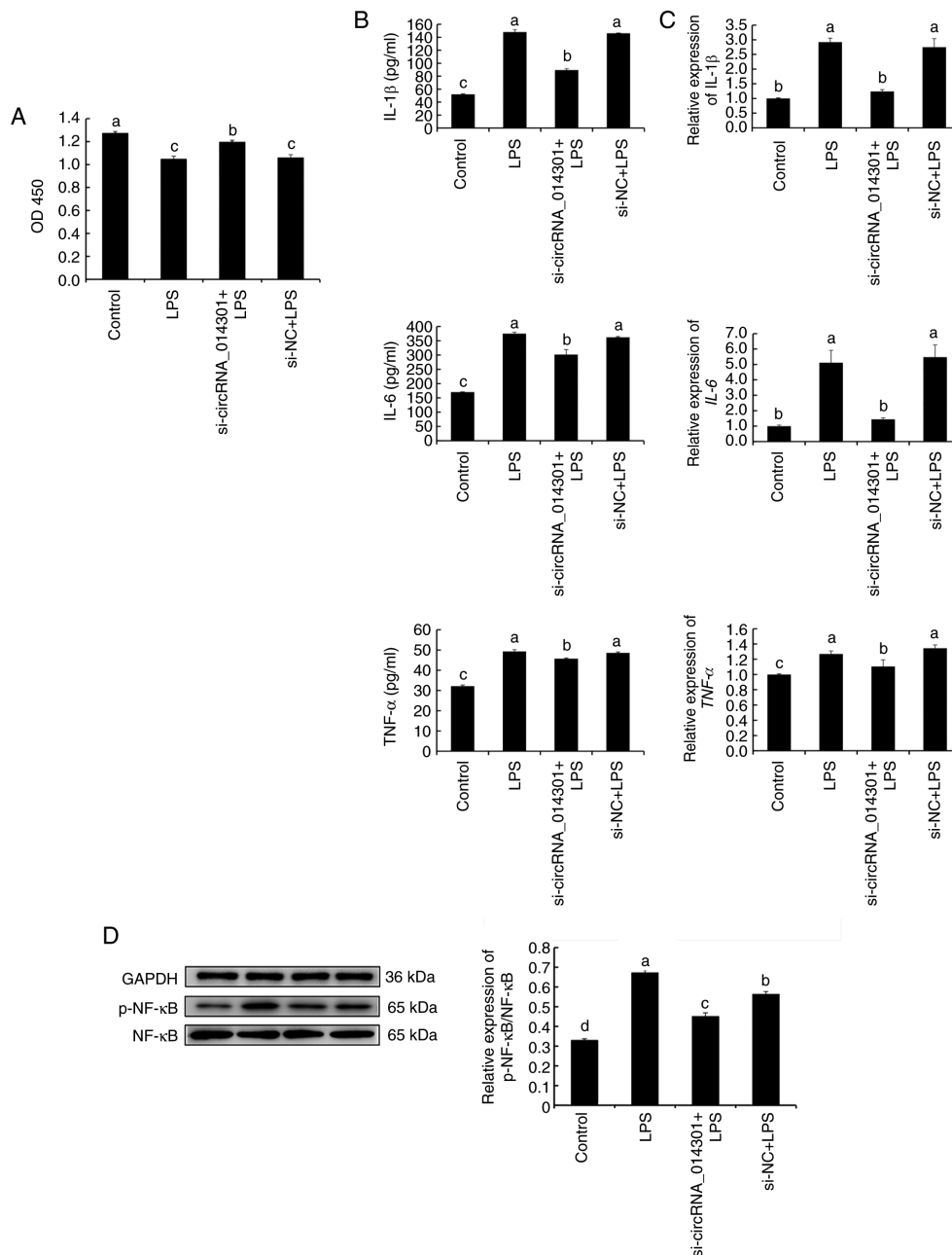
Figure 3. circRNA_014301 promotes inflammation in PC12 cells. (A) PC12 cell viability was decreased with LPS treatment compared with the control, but it was increased in LPC-treated cells after siRNA silencing of circRNA_014301. (B) Compared with control treatment, the concentration of inflammatory factors (IL-1β, IL-6 and TNF-α) was increased after LPS treatment, but it was reduced after siRNA silencing of circRNA_014301. (C) LPS promoted the expression of inflammatory factors (IL-1β, IL-6 and TNF-α) compared with control treatment, which was suppressed by the siRNA-mediated circRNA_014301 knockdown. (D) LPS increased the ratio of p-NF-κB/NF-κB compared with control treatment, whereas siRNA-mediated silencing of circRNA_014301 suppressed it. Groups labelled with the same lower-case letter are not significantly different to one another; groups with different letters are significantly different (P<0.05). LPS, lipopolysaccharide; circRNA, circular RNA; OD, optical density; p, phosphorylated; si, small interfering; NC, negative control.

with increasing LPS concentration. Cell viability was low with 5 µg/ml LPS treatment, but this was significantly higher than that of the 10 µg/ml LPS treatment.

As demonstrated in Fig. 1B, while 93.3% of the cells were unaffected after the control treatment (LPS-0), cells underwent apoptosis under increasing LPS concentrations. Moreover, higher LPS concentrations enhanced the apoptotic effect. The proportion of early cell apoptosis was 4.41, 6.63, 7.41 and 13.4% with LPS-1, LPS-2.5, LPS-5 and LPS-10 treatment, respectively, whereas the proportion of late cell apoptosis was 3.10, 4.38, 8.55 and 8.02% with LPS-1, LPS-2.5,

LPS-5 and LPS-10 treatment, respectively. Furthermore, apoptosis was most pronounced with LPS-5 and LPS-10 treatments (Fig. 1B). Thus, 5 µg/ml LPS evoked a sufficiently high inflammatory response while mildly affecting cell viability.

The expression and concentration of inflammatory factors (IL-1β, IL-6 and TNF-α) both increased with increasing LPS concentration (Fig. 1C and D). The expression levels of IL-1β, IL-6 and TNF-α increased slightly with 1-2.5 µg/ml LPS, increased notably with 5 µg/ml LPS, and peaked with 10 µg/ml LPS. However, there was no significant difference in the IL-1β concentration in the 5-10 µg/ml LPS range.
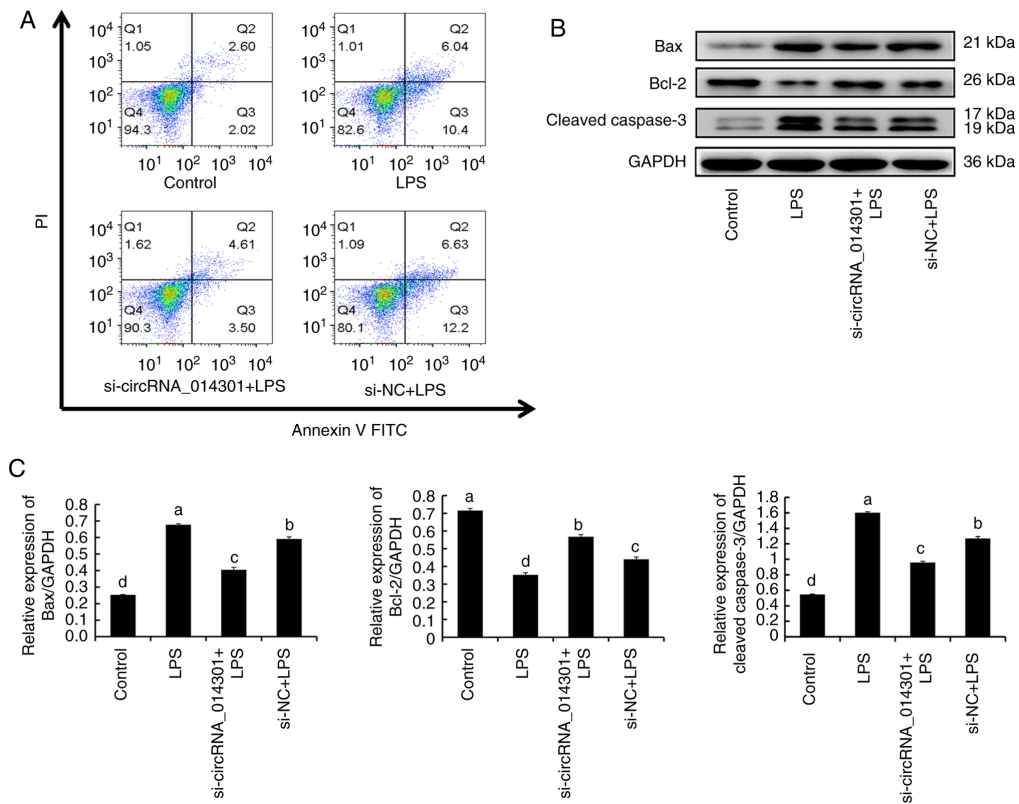
Figure 4. circRNA_014301 inhibits the apoptosis of PC12 cells. (A) Apoptotic PC12 cells detected via flow cytometry. The proportion of PC12 cells undergoing early and late apoptosis was lower in the si-circRNA_014304 + LPS group compared with the LPS or the si-NC + LPS treatments. (B) LPS promoted the protein expressions of Bax and caspase-3, whereas these effects were suppressed by the siRNA-mediated silencing of circRNA_014301; Bcl-2 expression presented the opposite tendency compared with Bax and caspase-3. (C) Quantification of western blot analysis. Groups labelled with the same lower-case letter are not significantly different to one another; groups with different letters are significantly different (P<0.05). LPS, lipopolysaccharide; circRNA, circular RNA; PI, propidium iodide; si, small interfering; NC, negative control.

LPS treatment promotes circRNA_014301 expression in PC12 cells. The relative expression of circRNA_014301 increased with increasing LPS concentration (Fig. 2A). It was slightly upregulated with 1-2.5 μg/ml LPS but was 6 and 12 times higher than that of the control with 5 μg/ml and 10 μg/ml LPS treatments, respectively. These results suggested that LPS induced circRNA_014301 expression in PC12 cells.

Referring to the miRDB database, it was therefore predicted that the following miRNAs could bind to circRNA_014301: miR-200a-3p, miR-141-3p, miR-3120, miR-16-5p, miR-15a-5p, miR-15b-5p, miR-195-5p, miR-497-5p, miR-322-5p, miR-344b-1-3p, miR-410-3p, miR-331-5p and miR-501-5p (Table SI).

Whereas circRNA_014301 and 18S RNA were highly expressed in the cytoplasm, U6 showed high enrichment in the nucleus (Fig. 2B). There were no significant changes in the expression of circRNA_014301 with RNase⁻ and RNase⁺ treatments. However, the expression of *MYT1L* was significantly lower with the RNase⁺ treatment compared with the RNase⁻ treatment, implying that *MYT1L* was cleaved by the RNase (Fig. 2C). These results suggested that circRNA_014301 was predominantly expressed in the cytoplasm.

circRNA_014301 silencing inhibits inflammation in PC12 cells. As indicated in Fig. S1 siRNA eliminated circRNA_014301 expression in PC12 cells, thus confirming the efficiency of the siRNA-mediated knockdown. Compared

with the control, PC12 cell viability was reduced with LPS, si-circRNA_014301 + LPS and si-NC + LPS treatments. It was also more enhanced with si-circRNA_014301 + LPS treatment than with LPS and si-NC + LPS treatments (Fig. 3A).

As demonstrated in Fig. 3B and C, the expression levels and concentration of inflammatory factors (IL-1β, IL-6 and TNF-α) with LPS and si-NC + LPS treatments were significantly higher than those of the control and si-circRNA_014301 + LPS treatments, as analyzed using ELISA and RT-qPCR. The expression level and concentration of TNF-α with si-circRNA_014301 + LPS treatment were significantly higher than those of the control treatment. The concentrations of IL-1β and IL-6 with si-circRNA_014301 + LPS treatment were significantly higher than those of the control treatment. However, there were no significant changes in the expression levels of IL-1β and IL-6 between the control and si-circRNA_014301 + LPS treatment.

The ratio of p-NF-κB/NF-κB was higher with LPS treatment compared with that of the control, followed by the si-NC + LPS and si-circRNA_014301 + LPS treatments (Fig. 3D).

circRNA_014301 silencing inhibits apoptosis in PC12 cells. As demonstrated in Fig. 4A, 2.60 and 2.02% of PC12 cells underwent early and late apoptosis, respectively, with the control treatment. Compared with the control, cell apoptosis increased with all other treatments. With LPS treatment,

6.04 and 10.4% of cells underwent early and late apoptosis, respectively. With si-circRNA_014301 + LPS treatment, the apoptotic effect was milder, with 4.61 and 3.50% of cells undergoing early and late apoptosis, respectively. Remarkably, apoptosis was the highest with si-NC + LPS treatment, where 6.63 and 12.2% of cells underwent early and late apoptosis, respectively. These findings suggested that the siRNA-mediated knockdown of circRNA_014301 expression alleviated apoptosis in PC12 cells.

As presented in Fig. 4B, the protein expression of apoptotic markers (Bax, Bcl-2 and cleaved caspase-3) differed with the various treatments. The relative protein expression of Bax and cleaved caspase-3 was similar, being the highest with LPS treatment, followed by si-NC + LPS, si-circRNA_014301 + LPS and control treatments (Fig. 4C). Conversely, the protein expression levels of Bcl2 were the highest on the control treatment, followed by si-circRNA_014301 + LPS, si-NC + LPS and LPS treatments (Fig. 4C).

Discussion

SCI is invariably associated with spinal cord inflammation that adversely affects the outcome of SCI (43). The PC12 cell line was derived from a pheochromocytoma of rat adrenal medulla. Tischler (44) discovered that these cells differentiate into neurons under treatment with nerve growth factor. He also indicated that they can synthesize and store several neurotransmitters, such as dopamine. PC12 cells can be used as an in vitro cell model of neuronal cells for high-throughput experiments (45). Compared with primary cultured nerve cells, PC12 cell uniformity allows experimental consistency, long-enough cell survival time for observation and maintained nerve cell characteristics (46). The PC12 cell line has been widely used to study nervous system diseases and pathological and physiological characteristics of neurons and to create SCI cellular models (47). In the preset study, increasing concentration of LPS were used to stimulate apoptosis and inflammatory injury in PC12 cells and create a cell model of SCI (34,48). Jiang and Wang (33) reported that 5 μg/ml LPS treatment could induce inflammatory injury in PC12 cells. Indeed, 5 μg/ml LPS treatment evoked a strong inflammatory response while mildly reducing the cell viability. Therefore, 5 μg/mL LPS treatment was deemed suitable for generating a PC12 cell inflammatory model.

Ashwal-Fluss et al (49) demonstrated that circRNAs are produced co-transcriptionally and compete with mRNAs for regular splicing. Therefore, the biogenesis of circRNAs results in reduced synthesis of mRNAs from the same locus. Thus, the production of circRNA acts as an RNA trap for mRNA production (50). circRNA_014301 (51,52) and *MYT1L* are both located in the cytoplasm. Whereas *MYT1L* was digested under the presence of RNase, circRNA_01430 was unaffected by the addition of the enzyme. These results suggested that circRNA_014301 was indeed located in the cytoplasm in accordance with Capel et al (51) and Patop et al (52), who revealed that this circRNA_014301 was predominantly cytoplasmic.

A representative function of circRNA is that it can act as a miRNA sponge to regulate the stability or translational efficiency of other RNAs (53). circRNAs and miRNAs can also act as transcriptional regulators or protein-bound RNAs and can even directly be translated into proteins (54). As this type of circRNAs regulates target gene expression, they are called competitive endogenous RNAs (ceRNAs) (53). Further investigation of circRNA and miRNA regulatory pathways will be the focus of follow-up research.

Inflammation serves a crucial role in the pathogenesis of SCI (55). The induced inflammation may result in a further decrease in functional recovery due to the development of scar tissue and the necrosis or apoptosis of neurons and oligodendrocytes (56). Nonetheless, potentially beneficial effects of the inflammatory process have also been reported, illustrating the dual nature of inflammation after SCI trauma (57). Apoptosis is essential for the clearance of potentially injurious inflammatory cells and the subsequent efficient resolution of inflammation (58). The expression of inflammatory genes (IL-1 and IL-1β) and the regulation of inflammatory agents (nitric oxide synthase and cyclooxygenase 2) that serve a potential role in the inflammatory pathways mediating damage of the central nervous system are regulated by the NF-κB family of transcription factors (59). It has been revealed that inhibiting the activation of NF-κB regulates the secondary damage in SCI to a large extent (60). The present study demonstrated that NF-κB activation was suppressed following siRNA-mediated knockdown of circRNA_014301, indicating that circRNA_014301 is involved in the NF-κB pathway, and this hypothesis requires further experimental verification.

Large circRNAs are associated with the biological activities of endothelial cells (61). Li et al (35) indicated that circRNA hsa_circ_0003575 silencing promoted cell proliferation and angiogenesis in oxidized low-density lipoprotein-induced endothelial cells. Furthermore, Dang et al (62) demonstrated that the knockdown of circRNA hsa_circ_001079 suppressed proliferation and promoted apoptosis in hypoxia-induced endothelial cells. circRNA_014301 silencing suppressed PC12 cell inflammation in the present study. The protective regulatory effect of circRNA_014301 silencing may inhibit the development of SCI. Subsequent in vivo experiments should be performed for in-depth verification of circRNA_014301 function. Based on the aforementioned findings, it is hypothesized that circRNA_014301 may constitute a potential biomarker for SCI detection.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XX performed the experiments, analyzed the results and wrote the manuscript. YX and KX contributed to data analysis and

manuscript revision. All authors have read and approved the final manuscript. XX and YX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Park S, Park K, Lee Y, Chang KT and Hong Y: New prophylactic and therapeutic strategies for spinal cord injury. J Lifestyle Med 3: 34-40, 2013.
2. Sekhon LHS and Fehlings MG: Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine (Phila Pa 1976) 26 (Suppl 24): S2-S12, 2001.
3. Bellon K, Kolakowsky-Hayner SA, Chen D, McDowell S, Bitterman B and Klaas SJ: Evidence-based practice in primary prevention of spinal cord injury. Top Spinal Cord Inj Rehabil 19: 25-30, 2013.
4. Wouda EMN and Stienstra Y: vander WTS, Kerstjens H, deLange WCM, Coppes M, Kuijlen J, Marga T, and Akkerman OW: Neurological and functional recovery in tuberculosis patients with spinal cord injury in The Netherlands. Neuro Rehab 40: 439-445, 2017.
5. Tator CH: Review of experimental spinal cord injury with emphasis on the local and systemic circulatory effects. Neurochirurgie 37: 291-302, 1991.
6. Torres-Espín A, Forero J, Fenrich KK, Lucas-Osma AM, Krajacic A, Schmidt E, Vavrek R, Raposo P, Bennett DJ, Popovich PG, *et al*: Eliciting inflammation enables successful rehabilitative training in chronic spinal cord injury. Brain 141: 1946-1962, 2018.
7. Agrawal SK and Fehlings MG: Mechanisms of secondary injury to spinal cord axons in vitro: Role of Na+, Na(+)-K(+)-ATPase, the Na(+)-H+ exchanger, and the Na(+)-Ca2+ exchanger. J Neurosci 16: 545-552, 1996.
8. Kiyatkin EA and Sharma HS: Not just the brain: Methamphetamine disrupts blood-spinal cord barrier and induces acute glial activation and structural damage of spinal cord cells. CNS Neurol Disord Drug Targets 14: 282-294, 2015.
9. Mazzone GL, Veeraraghavan P, Gonzalez-Inchauspe C, Nistri A and Uchitel OD: ASIC channel inhibition enhances excitotoxic neuronal death in an in vitro model of spinal cord injury. Neuroscience 343: 398-410, 2017.
10. Casha S, Yu WR, and Fehlings MG: Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat. Neuroscience 103: 203-218, 2001.
11. Darian-Smith C: Synaptic plasticity, neurogenesis, and functional recovery after spinal cord injury. Neuroscientist 15: 149-165, 2009.
12. Tator CH and Fehlings MG: Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75: 15-26, 1991.
13. Liu D, Xu GY, Pan E and McAdoo DJ: Neurotoxicity of glutamate at the concentration released upon spinal cord injury. Neuroscience 93: 1383-1389, 1999.
14. Goodman JH, Bingham WG Jr and Hunt WE: Platelet aggregation in experimental spinal cord injury. Ultrastructural observations. Arch Neurol 36: 197-201, 1979.
15. Beattie MS, Farooqui AA and Bresnahan JC: Review of current evidence for apoptosis after spinal cord injury. J Neurotrauma 17: 915-925, 2000.
16. Silva NA, Sousa N, Reis RL and Salgado AJ: From basics to clinical: A comprehensive review on spinal cord injury. Prog Neurobiol 114: 25-57, 2014.

17. Yip PK and Malaspina A: Spinal cord trauma and the molecular point of no return. Mol Neurodegener 7: 6, 2012.
18. Norenberg MD, Smith J and Marcillo A: The pathology of human spinal cord injury: Defining the problems. J Neurotrauma 21: 429-440, 2004.
19. Jain NB, Ayers GD, Peterson EN, Harris MB, Morse L, O'Connor KC and Garshick E: Traumatic spinal cord injury in the United States, 1993-2012. JAMA 313: 2236-2243, 2015.
20. Fatica A and Bozzoni I: Long non-coding RNAs: New players in cell differentiation and development. Nat Rev Genet 15: 7-21, 2014.
21. Cech TR and Steitz JA: The noncoding RNA revolution-trashing old rules to forge new ones. Cell 157: 77-94, 2014.
22. Qu S, Yang X, Li X, Wang J, Gao Y, Shang R, Sun W, Dou K and Li H: Circular RNA: A new star of noncoding RNAs. Cancer Let 365: 141-148, 2015.
23. Pamudurti NR, Bartok O, Jens M, Ashwal-Fluss R, Stottmeister C, Ruhe L, Hanan M, Wyler E, Perez-Hernandez D, Ramberger E, *et al*: Translation of CircRNAs. Mol Cell 66: 9-21.e7, 2017.
24. Chen LL: The biogenesis and emerging roles of circular RNAs. Nat Rev Mol Cell Biol 17: 205-211, 2016.
25. Lasda E and Parker R: Circular RNAs: Diversity of form and function. RNA 20: 1829-1842, 2014.
26. Zhou J, Xiong Q, Chen H, Yang C and Fan Y: Identification of the spinal expression profile of non-coding RNAs involved in neuropathic pain following spared nerve injury by sequence analysis. Front Mol Neurosci 10: 91, 2017.
27. Zhao RT, Zhou J, Dong XL, Bi CW, Jiang RC, Dong JF, Tian Y, Yuan HJ and Zhang JN: Circular ribonucleic acid expression alteration in exosomes from the brain extracellular space after traumatic brain injury in mice. J Neurotrauma 35: 2056-2066, 2018.
28. Pan Z, Li GF, Sun ML, Xie L, Liu D, Zhang Q, Yang XX, Xia S, Liu X, Zhou H, *et al*: MicroRNA-1224 splicing circularRNA-Filip1l in an Ago2- dependent manner regulates chronic inflammatory pain via targeting Ubr5. J Neurosci 39: 2125-2143, 2019.
29. Zhou ZB, Du D, Chen KZ, Deng FL, Niu YL and Zhu L: Differential expression profiles and functional predication of circRNA in traumatic spinal cord injury of rats. J Neurother 36: 2287-2297, 2019.
30. Zhou ZB, Niu YL, Huang GX, Lu JJ, Chen A and Zhu L: Silencing of circRNA.2837 Plays a Protective Role in Sciatic Nerve Injury by Sponging the miR-34 Family via Regulating Neuronal Autophagy. Mol Ther Nucleic Acids 12: 718-729, 2018.
31. Liu Y, Liu J and Liu B: Identification of circular RNA expression profiles and their implication in spinal cord injury rats at the immediate phase. J Mol Neurosci 70: 1894-1905, 2020.
32. Qin C, Liu CB, Yang DG, Gao F, Zhang X, Zhang C, Du LJ, Yang ML and Li JJ: Circular RNA expression alteration and bioinformatics analysis in rats after traumatic spinal cord injury. Front Mol Neurosci 11: 497, 2019.
33. Jiang J and Wang G: Matrine protects PC12 cells from lipopolysaccharide-evoked inflammatory injury via upregulation of miR-9. Pharm Biol 58: 314-320, 2020.
34. Li R, Yin F, Guo Y, Ruan Q and Zhu Q: *Angelica polysaccharide* protects PC-12 cells from lipopolysaccharide-induced injury via down-regulating microRNA-223. Biomed Pharmacother 108: 1320-1327, 2018.
35. Li CY, Ma L and Yu B: Circular RNA hsa_circ_0003575 regulates oxLDL induced vascular endothelial cells proliferation and angiogenesis. Biomed Pharmacother 95: 1514-1519, 2017.
36. Pandey PR, Rout PK, Das A, Gorospe M and Panda AC: RPAD (RNase R treatment, polyadenylation, and poly(A)+ RNA depletion) method to isolate highly pure circular RNA. Methods 155: 41-48, 2019.
37. Wang C, Wang Q, Lou Y, Xu J, Feng Z, Chen Y, Tang Q, Zheng G, Zhang Z, Wu Y, *et al*: Salidroside attenuates neuroinflammation and improves functional recovery after spinal cord injury through microglia polarization regulation. J Cell Mol Med 22: 1148-1166, 2018.
38. Yanbin Z and Jing Z: CircSAMD4A accelerates cell proliferation of osteosarcoma by sponging miR-1244 and regulating MDM2 mRNA expression. Biochem Biophys Res Commun 516: 102-111, 2019.
39. Tao L, Fan X, Sun J and Zhang Z: Metformin prevented high glucose-induced endothelial reactive oxygen species via OGG1 in an AMPKα-Lin-28 dependent pathway. Life Sci 268: 119015, 2021.

40. Gonzales AM and Orlando RA: Curcumin and resveratrol inhibit nuclear factor-kappaB-mediated cytokine expression in adipocytes. Nutr Metab (Lond) 5: 17, 2008.

41. Bruno IG, Karam R, Huang L, Bhardwaj A, Lou CH, Shum EY, Song HW, Corbett MA, Gifford WD, Gecz J, et al: Identification of a microRNA that activates gene expression by repressing nonsense-mediated RNA decay. Mol Cell 42: 500-510, 2011.

42. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

43. Anwar MA, Al Shehabi TS and Eid AH: Inflammogenesis of secondary spinal cord injury. Front Cell Neurosci 10: 98, 2016.

44. Greene LA and Tischler AS: Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc Natl Acad Sci USA 73: 2424-2428, 1976.

45. Dichter MA, Tischler AS and Greene LA: Nerve growth factor-induced increase in electrical excitability and acetylcholine sensitivity of a rat pheochromocytoma cell line. Nature 268: 501-504, 1977.

46. Hillion JA, Takahashi K, Maric D, Ruetzler C, Barker JL and Hallenbeck JM: Development of an ischemic tolerance model in a PC12 cell line. J Cereb Blood Flow Metab 25: 154-162, 2005.

47. Zhang G, Liu Y, Xu L, Sha CH, Zhang HB and Xu WB: Resveratrol alleviates lipopolysaccharide-induced inflammation in PC12 cells and in rat model. BMC Biotechnol 19: 10, 2019.

48. Xie Y, Zhang H, Zhang Y, Wang C, Duan D and Wang Z: Chinese Angelica polysaccharide (CAP) alleviates LPS-induced inflammation and apoptosis by down-regulating COX-1 in PC12 cells. Cell Physiol Biochem 49: 1380-1388, 2018.

49. Ashwal-Fluss R, Meyer M, Pamudurti NR, Ivanov A, Bartok O, Hanan M, Evantal N, Memczak S, Rajewsky N and Kadener S: circRNA biogenesis competes with pre-mRNA splicing. Mol Cell 56: 55-66, 2014.

50. Jens M and Rajewsky N: Competition between target sites of regulators shapes post-transcriptional gene regulation. Nat Rev Genet 16: 113-126, 2015.

51. Capel B, Swain A, Nicolis S, Hacker A, Walter M, Koopman P, Goodfellow P and Lovell-Badge R: Circular transcripts of the testis-determining gene Sry in adult mouse testis. Cell 73: 1019-1030, 1993.

52. Patop IL, Wüst S and Kadener S: Past, present, and future of circRNAs. EMBO J 38: e100836, 2019.

53. Bak RO and Mikkelsen JG: miRNA sponges: Soaking up miRNAs for regulation of gene expression. Wiley Interdiscip Rev RNA 5: 317-333, 2014.

54. Liu J, Li Z, Teng W and Ye X: Identification of downregulated circRNAs from tissue and plasma of patients with gastric cancer and construction of a circRNA-miRNA-mRNA network. J Cell Biochem 121: 4590-4600, 2020.

55. Beattie MS: Inflammation and apoptosis: Linked therapeutic targets in spinal cord injury. Trends Mol Med 10: 580-583, 2004.

56. Acarin L, González B and Castellano B: Neuronal, astroglial and microglial cytokine expression after an excitotoxic lesion in the immature rat brain. Eur J Neurosci 12: 3505-3520, 2000.

57. Hausmann ON: Post-traumatic inflammation following spinal cord injury. Spinal Cord 41: 369-378, 2003.

58. Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, Caldicott A, Martinez-Losa M, Walker TR, Duffin R, et al: Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med 12: 1056-1064, 2006. Erratum in: Nat Med 12: 1434, 2006.

59. Hu X, Nesic-Taylor O, Qiu J, Rea HC, Fabian R, Rassin DK and Perez-Polo JR: Activation of nuclear factor-kappaB signaling pathway by interleukin-1 after hypoxia/ischemia in neonatal rat hippocampus and cortex. J Neurochem 93: 26-37, 2005.

60. Brambilla R, Bracchi-Ricard V, Hu WH, Frydel B, Bramwell A, Karmally S, Green EJ and Bethea JR: Inhibition of astroglial nuclear factor kappaB reduces inflammation and improves functional recovery after spinal cord injury. J Exp Med 202: 145-156, 2005.

61. Qin M, Wang W, Zhou H, Wang X, Wang F and Wang H: Circular RNA circ_0003645 silencing alleviates inflammation and apoptosis via the NF-κB pathway in endothelial cells induced by oxLDL. Gene 755: 144900, 2020.

62. Dang RY, Liu FL and Li Y: Circular RNA hsa_circ_0010729 regulates vascular endothelial cell proliferation and apoptosis by targeting the miR-186/HIF-1α axis. Biochem Biophys Res Commun 490: 104-110, 2017.