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MiR-103 alleviates autophagy and apoptosis by regulating SOX2 in LPS-injured PC12 cells and SCI rats

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ABSTRACT

Objective(s): Recent studies revealed that microRNAs (miRNAs) may play crucial roles in the responses and pathologic processes of spinal cord injury (SCI). This study aimed to investigate the effect and the molecular basis of miR-103 on LPS-induced injuries in PC12 cells *in vitro* and SCI rats *in vivo*.

Materials and Methods: PC12 cells were exposed to LPS to induce cell injuries to mimic the *in vitro* model of SCI. The expression of miR-103 and SOX2 in PC12 cells were altered by transient transfections. Cell viability and apoptotic cell rate were measured by CCK-8 assay and flow cytometry assay. Furthermore, Western blot analysis was performed to detect the expression levels of apoptosis- and autophagy- related proteins, MAPK/ERK pathway- and JAK/STAT pathway-related proteins. In addition, we also assessed the effect of miR-103 agomir on SCI rats.

Results: LPS exposure induced cell injuries in PC12 cells. miR-103 overexpression significantly increased cell viability, reduced cell apoptosis and autophagy, and opposite results were observed in miR-103 inhibition. miR-103 attenuated LPS-induced injuries by indirect upregulation of SOX2. SOX2 overexpression protected PC12 cells against LPS-induced injuries, while SOX2 inhibition expedited LPS-induced cell injuries. Furthermore, miR-103 overexpression inhibited MAPK/ERK pathway and JAK/STAT pathway through upregulation of SOX2. We also found that miR-103 agomir inhibited cell apoptosis and autophagy in SCI rats.

Conclusion: This study demonstrates that miR-103 may represent a protective effect against cell apoptosis and autophagy in LPS-injured PC12 cells and SCI rats by upregulation of SOX2 expression.

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Introduction

Spinal cord injury (SCI) is a dyscapacitating pathology with a high incidence and high morbidity and has a significant impact on the human health, life quality and life expectancy (1). It has been reported that there exist two different mechanisms for the pathogenesis of SCI: an initial primary mechanical injury and a secondary injury which comprises several interrelated damage processes that include vascular alterations, biochemical disturbances and cellular responses (2, 3). The second phase of injury are induced by multiple biological processes, including excitotoxicity, apoptosis, inflammation, and demyelination (4-6). It's imperative for us to understand the progress of SCI and explore the underlying molecular mechanism. MicroRNAs (miRNAs), a class of small non-coding RNAs with a length of 18–25 nucleotides, are endogenous and function as negative regulators of gene expression at the post-transcriptional level through directly cleaving target messenger RNA (mRNA) or translational repression (7). Emerging evidences have manifested that miRNAs play pivotal roles in a wide variety of biological processes such as cell proliferation, invasion, differentiation, migration and metastasis in various cancers (8). And recent studies reported that miRNAs are estimated to regulate 60% of all genes in the human genome and may widely influence the signaling networks leading to pathological responses after SCI (9, 10).

MiR-103, first sequenced by Mourelatos *et al.* (11), was found to be central players in regulating intracellular CoA (12). Meanwhile, it was reported that miR-103 could target the Dicer gene, and it may impact the expression level of multiple miRNAs (13). MiR-103 could regulate insulin sensitivity through targeting caveolin-1 (14). In addition, miR-103 is also identified to be involved in the process of cell metabolism. For example, miR-103 is up-regulated when pre-adipocyte differentiates into mature adipocyte and overexpression of miR-103 accelerated adipogenesis (15-17). Moreover, recent study shown that miR-103 could affect neuronal migration by modulating CDK5R1 expression (18). However, the role of miR-103 in SCI and the potential signaling

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pathway that miR-103 acts through in SCI still remains unclear.

PC12 cells were derived from a transplantable rat pheochromocytoma and shared many similarities in the structures and functions with neurons. Furthermore, PC12 cells had general characteristics of neuroendocrine cells, and were easy to subculture compared with primary neurons. Thus, it was frequently used to the model *in vivo* for neurophysiological and neuropharmacological research and was used as the research for spinal cord injury (19-21).

The aim of the present study is to explore the function of miR-103 in LPS-induced injuries in PC12 cells and in SCI rats, and further to investigate the underlying signaling cascade it acts through. We found that miR-103 overexpression attenuated LPS-induced injuries in PC12 cells, while inhibition of miR-103 functioned as an opposite effect. We also found that SOX2 was positively regulated by miR-103 and the cross-regulation between miR-103 and SOX2 played a pivotal role in LPS-induced injuries in PC12 cells. In addition, we found that the expression of miR-103 and SOX2 were both down-regulated in SCI rats. Moreover, miR-103 agomir up-regulated the expression of SOX2, and inhibited cell apoptosis and autophagy in SCI rats. These data will shed a light on further study of miRNAs on central nervous system (CNS) disease.

Materials and Methods

Cell culture and LPS treatment

The PC12 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA) and cultured in Dulbecco's modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA), containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, Life Technologies, Grand Island, NY) in an humidified atmosphere with 5% CO_2 at 37 ˚C. When cells reach about 80% of confluence in appropriate culture dishes, cells were pre-starved using DMEM supplemented with 0.1% FBS for 1 hr and then were treated with LPS in a series of concentration for 12 hr.

MiRNAs transfection

MiR-103 mimic, miR-103 inhibitor or the corresponding negative control (NC) constructs (mimic control, and inhibitor control) were synthesized by GenePharma (Shanghai, China). PC12 cells were seeded onto a 6-well plate before transfection, and were transfected with miR-103 mimic, miR-103 inhibitor and the NC controls when they reached 50% confluence. Cell transfections were conducted using lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After transfection for 48 hr, the cells were processed for further analysis. The sequences of miR-103 inhibitor/mimic or the corresponding negative controls were shown as in Table 1.

Table 1. The sequences of miR-103 inhibitor/mimic/negative control

Name	Sequences
miR-103 mimic	sense: AGCAGCAUUGUACAGGGCUAUGA antisense: AUAGCCCUGUACAAUGCUGCUUU
mimic control	sense: AGCAGCAGUUUAGGGCACUAUGA antisense: AUAGUGCCCUAAACUGCUGCUUU
miR-103 inhibitor	UCAUAGCCCUGUACAAUGCUGCU
inhibitor control	UCAUAGGCGUGUACAAUGCUGCU

Transfection of SOX2 expressing vector and shRNA

For the analysis of SOX2 functions, the SOX2 full-length sequences and short-hairpin RNA (shRNA) directed against SOX2 was conducted in pEX-2 vector (pEX-SOX2) and pPU6/GFP/Neo vector (sh-SOX2) (GenePharma). The lipofectamine 3000 reagent (Invitrogen) was used for the cell transfection according to the manufacturer's instructions. A non-targeting sequence of plasmid and an empty plasmid were respectively used as NC of pEX-SOX2 and sh-SOX2, and referred as to pEX and shNC. The stably transfected cells were selected by the culture medium containing 0.5 mg/ml G418 (Invitrogen). After approximately 4 weeks, G418-resistant cell clones were established.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and extracted from transfected cells and tissues by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed to cDNA by using a Reverse Transcription Kit (Takara, Dalian, China). The mRNA expression of SOX2 was determined by qRT-PCR using the SYBR Green Master Mix (Takara). The expression of miR-103 was measured by using Taqman MicroRNA Assay (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instruction. GAPDH and U6 were used for the normalization of mRNA and miRNA. The results were presented as fold changes relative to U6 or GAPDH and were calculated using the $2^{-\Delta\Delta CT}$ method. The sequences of primers for PCR were shown as in Table 2.

CCK-8 assay

Cell viability was detected by CCK-8 Assay Kit (Dojindo, Kumamoto, Japan) according to the protocol. Briefly, cells were seeded at 96-well plates with a concentration of 1×10^4 cells/well for 24 hr. After treatment with a series of concentration of LPS (1, 5, and 10 μg/ml) for 12 hr, cells were incubated with 10 μl CCK-8 solution for additional 1 hr in the dark at 37 ˚C.

Table 2. The primer sequences of SOX2/miR-103 for PCR

Genes	Primer sequences (5'-3')
SOX2	Forward: CGCGGATCCATGTACAACATGATGGAGACGGAGC Reverse: CCGGAATTCGATTTATCGCGTCGACTCACATG
miR-103	Forward: GGGAGCAGCATTGTACAGGG Reverse: CAGTGCGTGTCGTGGAGT

Absorbance was measured at 450 nm wavelength on a microplate reader (Bio-Rad Laboratories, Orlando, FL, USA).

For the analysis of miR-103 and SOX2 effect on cell viability, PC12 cells were transfected with miR-103 inhibitor, miR-103 mimic or their corresponding controls, or shPSEN1, pcPSEN1 or their corresponding controls. Next, cells or transfected cells were exposed to 5 μg/ml LPS for 12 hr, and then cell viability was evaluated as mentioned earlier.

Apoptosis assay

Cell apoptosis was measured by using an Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Biotechnology, Shanghai, China) according to the instruction. Briefly, cells were collected and washed with phosphate buffered saline (PBS) for three times. Then, cells were resuspended with 500 μl 1× binding buffer and were mixed with 5 μl Annexin V-FITC and 5 μl PI for 30 min in the dark at 37 ˚C. Cell apoptosis was detected by a FACS can (Beckman Coulter, Fullerton, CA, USA) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Western blot

The protein of PC12 cells and tissues were extracted using RIPA lysis buffer (Beyotime,) containing protease inhibitors (Roche, Basel, Switzerland). BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) was used to quantify the concentration of proteins. Equivalent Denatured proteins (30 μg) were loaded and detached by using a Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Subsequently, the membrane was blocked with 5% BSA (Roche) in TBST at room temperature for 1 hr and rinsed with TBST for three times. Then the membranes were incubated with respectively primary antibodies (a dilution of 1:1000 in 5% BSA) at 4 ˚C overnight. Primary antibodies: anti-Bcl-2 (#4223), anti-Bax (#5023), anti-caspase-3 (#9662), anti-caspase-9 (#9502), anti-GAPDH (#2118), anti-LC (#12741), anti-Beclin-1(#3495), anti-p62 (#8025), anti-SOX2 (#14962), anti-p-ERK (#9101), anti-ERK (#9102), anti-p-MAPK (#9211), anti-MAPK (#9212), anti-p-JAK1(#74129), anti-JAK1(#3344), anti-p-STAT1 (#7649), anti-STAT1 (#14994), anti-p-STAT3 (#9145), anti-STAT3 (#4904) were purchased from Cell Signaling Technology (Beverly, MA, USA). Thereafter, the membranes were washed with TBST and further incubated with secondary antibody marked by horseradish peroxidase (Sigma-Aldrich) at a 1:5000 dilution for 2 hr at room temperature. The blots were developed with ECL solution (Pierce) and visualized by using Image Lab™ Software (Bio-Rad, Hercules, CA, USA).

Animals and experiments group

A total of 54 Sprague Dawley (SD) male rats were used (weight, 180–220 g; Sun Yat-Sen University, Guangdong Province, China) and were age- and weight-matched between groups within an experiment. All animal procedures were approved by the Laboratory Animal Users Committee of Yat-Sen University.

Among the 54 SD rats, 32 SD rats of them were used to explore the expression of miR-103 and SOX2 in Sham and SCI model groups. Those 32 rats were randomly divided into 4 groups (n=8 for each group): (1) sham; (2) SCI for 1 day; (3) SCI for 3 days; (4) SCI for 7 days.

Another 24 rats were used to explore the effect of overexpression of miR-103 on the SOX2 expression, apoptosis-related and autophagy-related factors levels post-SCI injury. Those 24 rats post-SCI for 3 days were randomly divided into 3 groups (n=8 for each group): (1) saline; (2) agomir negative control (NC); (3) miR-103 agomir.

Establishment of SCI rat model

After anesthetized by intraperitoneal injection of 0.3 mg/g body weight chloral hydrate (Kermel, Tianjing, China), the experimental rats were shaved and cleaned their backs and then fixed in the prone position. A skin incision was made at the T9/T10 along the spinous process with a sterile ophthalmic scissor, spinal cord contusions were induced using a modified Allen's weight drop apparatus (8 g weight at a vertical height of 40 mm, 8 g×40 mm). The SCI model was successfully induced based on the following symptoms: spinal cord edema and hemorrhage; tail with spastic swing; retraction-like flutter and flaccid paralysis of bilateral hindlimbs (22).

Intraperitoneal injection of miR-103 agomir

A partial laminectomy at T12/T13 was performed for the placement of an intrathecal catheter. miR-103 agomir and miR-103 agomir NC were obtained from RiboBio (RiboBio Co., Ltd, Guangzhou, China). Briefly, the miR-103 agomir or miR-103 agomir NC was dissolved in 0.9% saline and was continuously delivered into (1 μl/h) the intrathecal space of SCI rats post-injury for 3 days, as previously described via subcutaneously implanted osmotic mini-pumps (Alzet 1030D, CA, USA) connected to a subdural-implanted catheter (23). Each pump was primed overnight at 37 ˚C to confirm immediate delivery after implantation. After the implantation, muscles were sutured in layers, the skin incision was closed with 3-0 silk threads, and 5 ml of lactated Ringer's solution was administered intraperitoneally. All surgeries were performed in a warm environment to maintain body temperature. After the surgery, rats were placed on fresh dry cages and given free access to food and water. Penicillin G (Beyotime.) was administered daily for 3 d to prevent rats from infection. Bladders were manually expressed twice daily until full voluntary or autonomic voiding was obtained.

Preparation of SCI samples

The animals were sacrificed at various time points 1 d, 3 d and 7 d after SCI by injection of high-dose (200

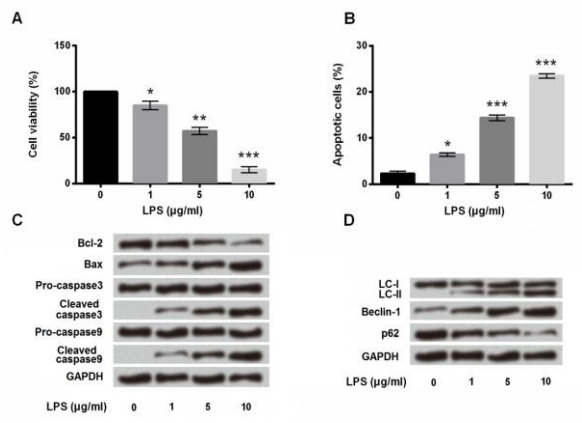

Figure 1. LPS exposure induced cell apoptosis and autophagy in PC12 cells. PC12 cells were treated with LPS in a series of concentration (1, 5, and 10 μg/ml) for 12 hr. (A) Cell viability was measured by CCK-8 assay analysis. (B) Apoptotic cell rate was detected by flow cytometric analysis of Annexin V-FITC/PI staining. (C) The expression of apoptosis-related protein was measured by Western blot. (D) The protein levels of autophagy-related factors were analyzed by Western blot. * P<0.05, ** P<0.01, *** P<0.001

mg/kg) pentobarbital (Nembutal, Oak Pharmaceuticals, Lake Forest, IL, USA). Spinal cord tissues were collected from the operated area from all the groups. The collected spinal cord tissues were initially flash frozen in liquid nitrogen tank and stored at -80°C for qRT-PCR or Western blot assay.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad Software, San Diego, CA, USA). 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.

Results

LPS exposure induced cell apoptosis and autophagy in PC12 cells

As shown in Figure 1A, LPS treatment significantly decreased cell viability in PC12 cells with increasing dosage (P<0.05, P<0.01, or P<0.001). LPS exposure also induced cell apoptosis in PC12 cells in a dose dependent manner, as a significantly increase of apoptotic cell rate (P<0.05, or P<0.001) (Figure 1B), an increased protein expression of pro-apoptosis factor (Bax, Cleaved caspase-3 and Cleaved caspase-9) and a decreased expression of Bcl-2 (Figure 1C). In addition, cell autophagy also induced by LPS treatment in PC12 cells, evidenced by down-regulation of p62, and upregulation of LC-II and Beclin-1 (Figure 1D). Considering that 5 μg/ml LPS resulted in a significantly decrease of cell viability and apoptotic cell rate, 5 μg/ml was selected as a LPS-stimulating condition for use in the following experiments. These results indicated that LPS treatment induced cell injuries in PC12 cells.

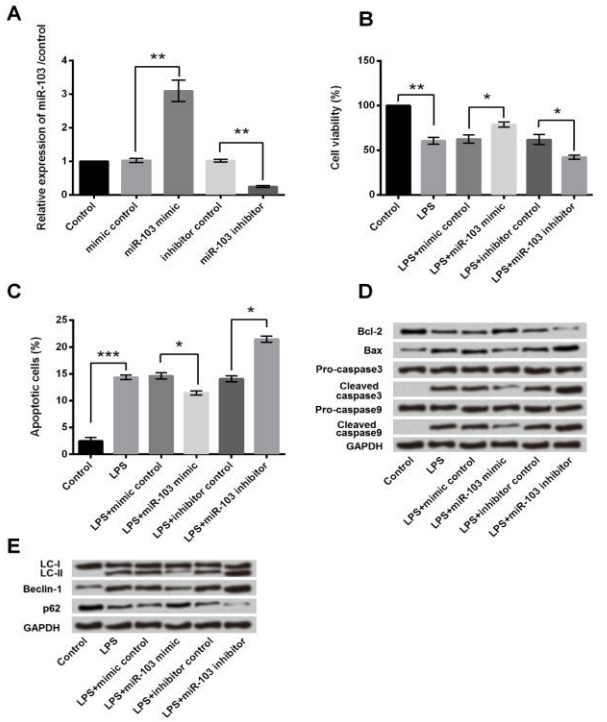

Figure 2. MiR-103 attenuated LPS-induced cell injuries in PC12 cells. PC12 cells were transfected with miR-103 inhibitor, miR-103 mimic or their corresponding controls, *i.e.*, inhibitor control and mimic control. Then cells were incubated with 5 μg/ml LPS for 12 hr. (A) The efficiency of transfection was verified by qRT-PCR (B) Cell viability, (C) apoptotic cell rate, and (D) the expressions of apoptosis-related proteins were respectively assessed by CCK-8 assay, flow cytometry, and Western blot. (E) The protein expressions of autophagy-related factors were measured by Western blot. * P<0.05, ** P<0.01, *** P< 0.001

MiR-103 attenuated LPS-induced cell injuries in PC12 cells

To evaluate the effect of miR-103 on LPS-induced injuries in PC12 cells, PC12 cells were transfected either with miR-103 mimic or inhibitor and the efficiency of transfection was performed by qRT-PCR analysis. As expected, the expression of miR-103 was remarkable elevated after transfection with miR-133a mimic and was declined with miR-103 inhibitor (P<0.01) (Figure 2A). As shown in Figure 2B, miR-103 mimic significantly increased cell viability in PC12 cells after treatment with LPS, while miR-103 inhibitor reduced cell viability (P<0.05, or P<0.01). We further investigated functional significance of miR-103 on cell apoptosis and autophagy in LPS-injured PC12 cells. We found that miR-103 mimic significantly reduced apoptotic cell rate and miR-103 inhibitor increased apoptotic rate (P<0.05, or P< 0.001) (Figure 2C). Consistently, miR-103 mimic up-regulated the expression of anti-apoptosis protein and down-regulated pro-apoptosis factors (Figure 2D). As shown in Figure 2E, miR-103 overexpression reduced the expression of LC-II and Beclin-1, and increased the levels of p62. However, miR-103 inhibition exerted an opposite effect on these factors. These results suggested that miR-103 exerted a protective effect on LPS-induced injuries in PC12 cells.

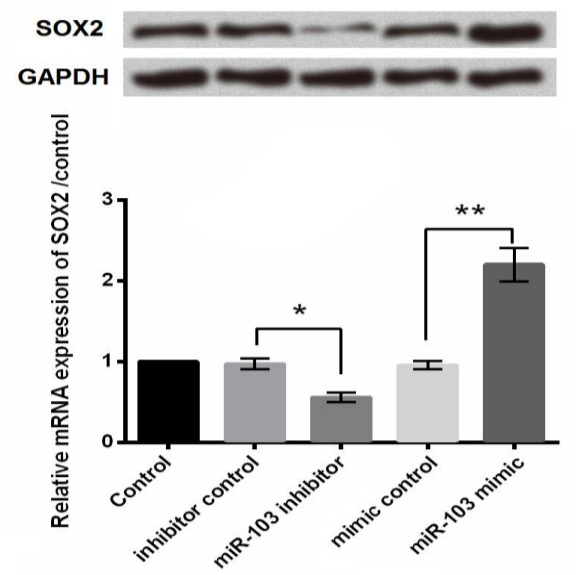

Figure 3. MiR-103 up-regulated SOX2 expressions. PC12 cells were transfected with miR-103 inhibitor, miR-103 mimic or their corresponding controls, *i.e.*, inhibitor control and mimic control. Then the protein and mRNA expressions of SOX2 in PC12 cells were assessed by Western blot and qRT-PCR. * P<0.05, ** P<0.01

MiR-103 up-regulated SOX2 expressions

As shown in Figure 3, the mRNA and protein expression of SOX2 was down-regulated in miR-103 inhibitor transfected PC12 cells (P<0.01). However, inverse upregulations were found in cells which were transfection with the miR-103 mimic (P<0.01), which indicating SOX2 was positively correlated to miR-103.

MiR-103 inhibitor accelerated LPS-induced injures in PC12 cells through down-regulating of SOX2

To investigate whether miR-103 regulated cell apoptosis and autophagy through regulating SOX2, PC12 cells were transfected with vector and shRNA specific targeted SOX2. As shown in Figure 4A, the mRNA and protein expression of SOX2 were significantly augmented after transfected with pEX-SOX2 (P<0.01), while were restrained in cells which were transfection with sh-SOX2 (P<0.01). We found that the effect of miR-103 inhibitor on the cell viability and apoptotic cell rate was reversed by the overexpression of SOX2 through transfection with pEX-SOX2 (P<0.01) (Figure 4B and 4C). Similar results were observed in the regulation of autophagy-related factors, as SOX2 overexpression reduced the expressions of LC-II and Beclin-1, while increased the levels of p62 (Figure 4D). Overall, these results revealed that miR-103 inhibition may expedite LPS-induced injures in PC12 cells through down-regulation of SOX2.

SOX2 was involved in LPS-induced cell injuries in PC12 cells

To investigate the role of SOX2 in LPS-induced cell injuries in PC12 cells, PC12 cells were transfected with pEX-SOX2 and sh-SOX2. And then cell viability, cell

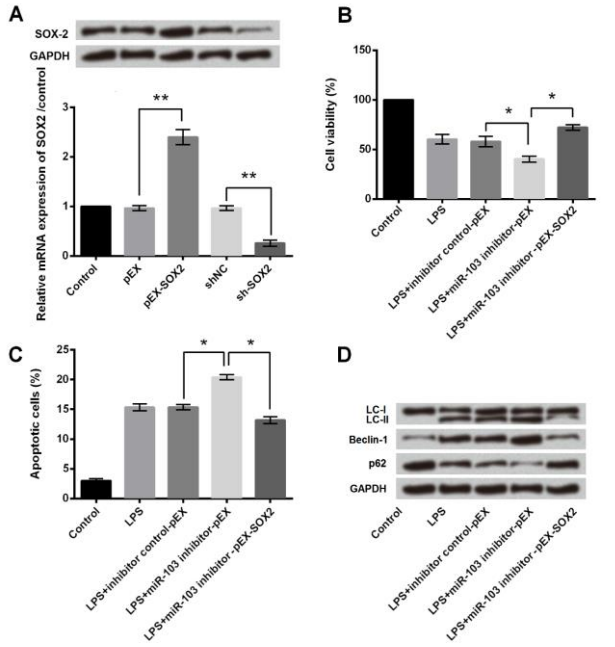

Figure 4. MiR-103 inhibitor accelerated LPS-induced injures in PC12 cells through down-regulation of SOX2. PC12 cells were transfected with pEX-SOX2, sh-SOX2 or their corresponding controls, *i.e*, pEX and shNC, or co-transfected with miR-103 inhibitor and pEX-SOX2. Then cells were treated with 5 μg/ml LPS for 12 hr. (A) The efficiency of transfection was verified by qRT-PCR and Western blot. (B) Cell viability and (C) apoptotic cell rate were respectively assessed by CCK-8 assay and flow cytometry. (D) The protein expressions of autophagy-related factors were measured by Western blot. * P<0.05, ** P<0.01

and autophagy was investigated. Results revealed that SOX2 inhibition enhanced LPS-induced cell injuries, as decreased cell viability (P<0.05) (Figure 5A), induced

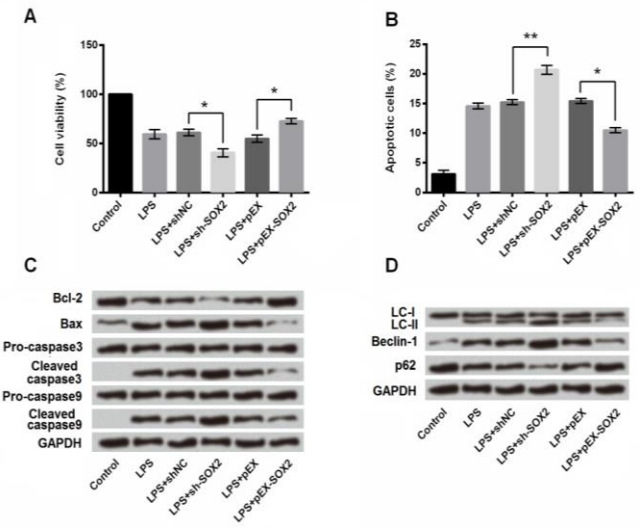

Figure 5. SOX2 was involved in LPS-induced cell injuries in PC12 cells. PC12 cells were transfected with pEX-SOX2, sh-SOX2 or their corresponding controls, *i.e.*, pEX and shNC. Then, 5 μg/ml LPS were exposed to PC12 cells for 12 hr to induce injury. (A) Cell viability, (B) apoptotic cell rate, and (C) the expressions of apoptosis-related proteins were respectively assessed by CCK-8 assay, flow cytometry, and Western blot. (D) The protein expressions of autophagy-related factors were measured by Western blot. * P<0.05, ** P<0.01

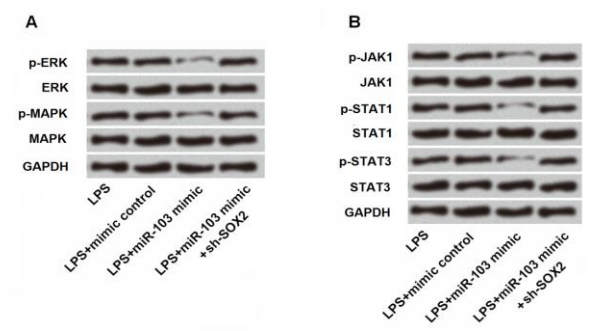

Figure 6. MiR-103 inhibited MAPK/ERK pathway and JAK/STAT pathway through upregulation of SOX2. PC12 cells were transfected with miR-103 mimic and mimic control, or co-transfected with sh-SOX2. And cells were incubated with 5 μg/ml LPS for 12 hr. The protein expressions of core factors related with (A) MAPK/ERK signaling pathway and (B) JAK/STAT signaling pathways were measured by Western blot

apoptosis (P<0.05, or P<0.01) (Figure 5B and 5C) and autophagy (Figure 5D). Of contrast, inverse regulations were found in cells which were transfection with pEX-SOX2 (P<0.05, or P<0.01) (Figure 5A-5D). These results suggested that SOX2 was participated in the regulation of LPS-induced injuries in PC12 cells.

MiR-103 inhibited MAPK/ERK pathway and JAK/STAT pathway through upregulation of SOX2

Here, we investigated the regulation of miR-103 on MAPK/ERK pathway and JAK/STAT pathway in LPS-injured PC12 cells. As shown in Figure 6A, miR-103 mimic reduced the expression of p-ERK and p-MAPK, and the regulatory effects were reversed by SOX2 inhibition. We also found miR-103 overexpression reduced the phosphorylation of JAK1, STAT1 and STAT3. Interestingly, the regulation of miR-103 on the JAK/STAT signaling pathway was blocked by SOX inhibition (Figure 6B). These results implied that overexpression of miR-103 inhibited MAPK/ERK pathway and JAK/STAT pathway through upregulation of SOX2.

MiR-103 agomir inhibited cell apoptosis and autophagy in SCI rats

To further confirm the effect of miR-103 and SOX2 on SCI rats, we first assessed the expression of miR-103 and SOX2 in SCI rats by qRT-PCR. As show in Figure 7A, the expression of miR-103 was significantly down-regulated in SCI model rats at 1, 3 and 7 days post injury (P<0.05, or P<0.01). Similar results were observed in the expression of SOX2 that SOX2 levels were dramatically reduced in the SCI model rats at 1, 3 and 7 days post injury (P<0.01, or P<0.001) (Figure 7B). Then, rats were treated with miR-103 agomir to overexpress the miR-103. The efficiency of miR-103 agomir was verified by the qRT-PCR. The expression of miR-103 was significantly up-regulated by miR-103 agomir injection (P<0.01) (Figure 7C). Further, we investigated the effect of miR-103 agomir on SOX2 expression in rats. Results in Figure 7D and 7E showed that miR-103 agomir

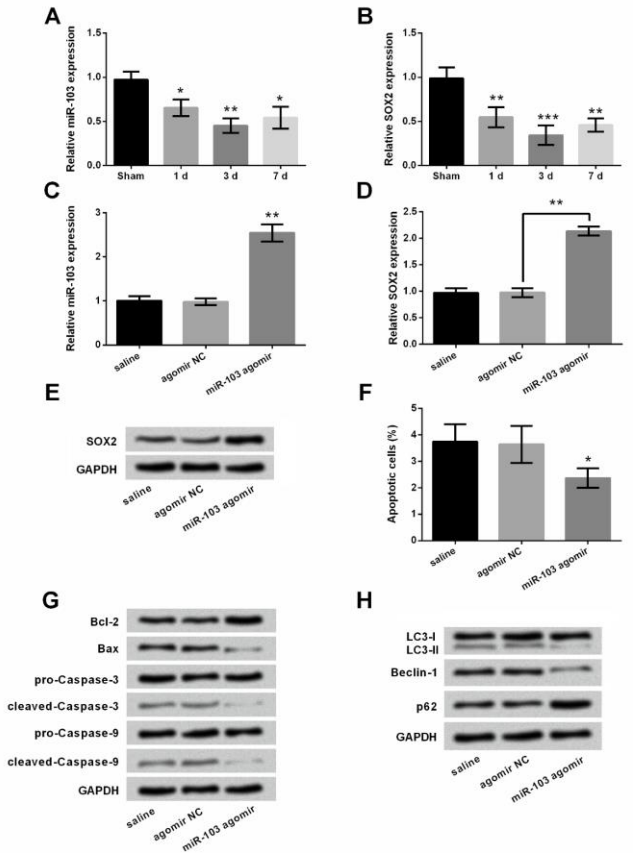

Figure 7. MiR-103 agomir inhibited cell apoptosis and autophagy in SCI rats. (A) The expression of miR-103 on SCI rats after injury at 1, 3 and 7 days by Western blot. (B) The expression of SOX2 on SCI rats after injury at 1, 3 and 7 days by Western blot. (C) Qrt-PCR was performed to assess the effect of miR-103 agomir on miR-103 expression in SCI rats. (D)The mRNA and (E) protein expression of SOX2 in SCI rats after miR-103 agomir treatment was measured by qRT-PCR and Western blot, respectively. (F) Flow cytometry analysis was used to detect the apoptotic cell rats in SCI rats after miR-103 agomir injection. The expression of (G) apoptosis-related and (H) autophagy-related factors were measured by Western blot

significantly elevated the mRNA and protein expression of SOX2 in rats (P<0.01). In addition, we also explored the effect of miR-103 agomir on the apoptosis and autophagy. As shown in Figure 7F and 7G, miR-103 agomir notably reduced the apoptotic cell rates and regulated the protein expression of apoptosis-related factors (P<0.05). MiR-103 agomir also inhibited the expression of LC-II and Beclin-1 but enhanced the p62 expression (Figure 7H). Overall, these results indicated that miR-103 overexpression also reduced cell apoptosis and autophagy in SCI rats.

Discussion

Increasing evidence suggests that the aberrant expression of miRNAs contributes to changes in gene expression following neural injury by regulating the expression of their target genes and downstream signaling pathway (24-26). Recent studies revealed that miRNAs may play critical roles in the responses and pathologic processes

of SCI and the expression of miRNAs up-regulated or down-regulated after SCI (27-29). Therefore, miRNAs provide an especially promising therapeutic means for functional recovery following SCI. Our study presented that overexpression of miR-103 dramatically increased cell viability, reduced cell apoptosis and autophagy in LPS-injured PC12 cells, and inhibition of miR-103 accelerated the LPS-induced injuries. Moreover, we found that miR-103 attenuated cell injuries through upregulation of SOX2. In addition, the *in vivo* data demonstrated that the expression of miR-103 and SOX2 were both down-regulated in SCI rats. Moreover, miR-103 agomir up-regulated the expression of SOX2, and inhibited cell apoptosis and autophagy in SCI rats. These data suggest that miR-103 may play a crucial role in LPS-injured PC12 cells and SCI rats.

MiR-103 usually up-regulated in cancer cells and tissues and has been identified as an oncogene in multiple cancers as its function for anti-apoptosis and pro-metastasis (30, 31). Previous studies revealed that miR-103 was up-regulated at 4 hr post-SCI and subsequently down-regulated at 1 and 7 days post-SCI (3, 26). In addition, it has been reported that miR-103 was able to directly interact with the CDK5R1 3'-UTR and reduced the migration ability of SK-N-BE neuroblastoma cells through down-regulating the expression of CDK5R1 (18). Furthermore, miR-103/107 coordinately suppressed macropinocytosis and preserved end-stage autophagy, thereby contributing to maintenance of a stem cell–enriched epithelium (32, 33). Our results demonstrated that miR-103 reduced apoptotic cell rate, inhibited the protein expression of pro-apoptotic factors and autophagy-related factors in LPS-injured PC12 cells. What'more, the expression of miR-103 was down-regulated in SCI rats, and miR-103 agomir reduced cell apoptosis and autophagy in SCI rats. It may better define the function of miR-103 in SCI and its anti-apoptosis role.

SOX2, a "founder member" of the SOX gene family, was first identified to be related to the sex-determining gene Sry by the possession of an HMG-box DNA-binding domain and as a regulator of the Fgf-4 gene, wihch is essential for survival of the early mouse embryo (34). It has been previously reported that SOX2 was required for neuronal differentiation in brain (35) and contributed to the self-renew of neural stem cells (36). Conditional neural-specific SOX2 deletion could cause impairment of embryonic brain development and neurogenesis and even was lethal at birth (37). In addition, SOX2 has been identifies as an oncogenic gene in various cancers and it may promote cell proliferation and migration (38). Our present study showed that SOX2 was involved in the LPS-induced injuries of PC12 cells. We found that miR-103 up-regulated the expression of SOX2 and overexpression of SOX2 may increase cell viability, reduce cell apoptosis and autophagy. Taken together, miR-103 may alleviate LPS-induced injuries in PC12 cells through upregulation of SOX2.

It has been reported that the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling pathway is one of the most important transducing signals from the cell surface to the nucleus in response to cytokines (39). The activation of JAK/STAT signalling pathway may also be involved in the neuronal or glial differentiation of precursor cells in the development of brain (40). Previous study has shown that the JAK/STAT signaling pathway was activated by IL-6 in neurons of the SCI and the phosphorylation of JAK, STAT1 and STAT3 was increased after SCI (41, 42) Meanwhile, inhibition of the JAK/STAT pathway activation could induce cell apoptosis in pancreatic cancer cells (43). The activation of mitogen-activated protein kinase (MAPK) signaling pathways also has been identified as an important role in SCI. It was reported that enhanced activation of MAPK/ERK signaling pathway was observed in the injured spinal cord after traumatic SCI (44), and PD98059, a specific inhibitor of the activation of MAPK, alleviated the development of inflammation and tissue injury associated with spinal cord trauma (45). In addition, it has been reported that SOX2 overexpression could inhibit the activation of p38 MAPK pathway in osteoblast cell lines (46). Furthermore, SOX2 has been reported to regulate apoptosis through MAP4K4-survivin signaling pathway in human lung cancer cells (47). Moreover, it has been reported that MAP4K4 is a novel MAPK/ERK pathway regulator required for lung adenocarcinoma maintenance (48). Meanwhile, MAP4K4 has also been reported to activate a number of other proteins, including kinases within the JAK/STAT pathway (49). Thus, we hypothesized that miR-103 might be involved in the regulation of MAPK/ERK and JAK/STAT signaling pathway through SOX2. In our present study, we showed that miR-103 overexpression inhibited the expression of p-JAK, p-STAT1 and p-STAT3, which was blocked by SOX2 down-regulation. And similar results were observed in the MAPK/ERK signaling pathway. These results suggested that miR-103 may inhibit MAPK/ERK and JAK/STAT pathway through upregulation of SOX2 and it might be correlated with the protective effect of miR-103 on SCI.

Conclusion

The present study demonstrated that miR-103 attenuated LPS-induced cell apoptosis and autophagy through upregulation of SOX2 in PC12 cells. The possible pathway of miR-103 effect on apoptosis and autophagy in LPS-induced PC-12 cells and SCI rats has been displayed in Fig 8. Our study might further help to understand the pathophysiological mechanisms of SCI and develop targeted therapies for SCI. It may provide a new insight that miR-103 may act as a critical role in SCI development and clinical therapy. We will further explore the potential mechanism of the effect of miR-103 and SOX2 on the MAPK/ERK and JAK/STAT signaling cascade in SCI for our future research.

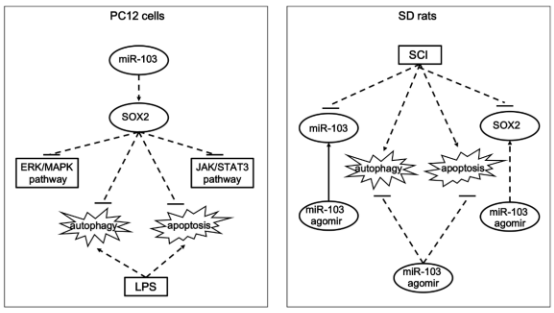

Figure 8. Proposed mechanisms of miR-103 effect on apoptosis and autophagy in LPS-injured PC12 cells and SCI rats. In pc12 cells, miR-103 enhances the expression of SOX2, and SOX2 further reduced LPS-induced cell apoptosis and autophagy. In addition, SOX2 inhibits the activation of ERK/MAPK and JAK/STAT3 pathway. In SCI rats, SCI induces the down-regulation of miR-103 and SOX2. miR-103 agomir promotes the expression of SOX2, and suppresses cell apoptosis and autophagy in SCI rats. * $P<0.05$, ** $P<0.01$, *** $P<0.001$

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