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Overexpression of miR-381-3p promotes the recovery of spinal cord injury

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Abstract. – OBJECTIVE: To study the effects of miR-381-3p on spinal cord injury and its underlying mechanism.

MATERIALS AND METHODS: After the spinal cord injury rat model of was established, Sprague Dawley (SD) rats were randomly divided into the control group and the acute spinal cord injury (ASCI) group. Microglial BV2 cells were used as experimental cells, and the cells were divided into the control group and the lipopolysaccharide (LPS) group. The mRNA and protein expression level of miR-381-3p, IKKβ, inflammatory factors, and p-p65 were detected by quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) and Western blot, respectively. Dual-luciferase reporter gene assay and Western blot were used to detect the regulatory effect of IKKβ on miR-381-3p. Changes in grip ability and rotary performance of rats in the ASCI group were evaluated after miR-381-3p overexpression *in vivo*.

RESULTS: The expression of miR-381-3p was downregulated in rats of the ASCI group, while the expression of IKKβ and p-p65 were upregulated. In vitro experiments demonstrated that LPS could inhibit the expression of miR-381-3p and promote the upregulation of IKKβ and p-p65. Overexpression of miR-381-3p could inhibit the mRNA and protein expression of IKKβ. The upregulated expression of IKKβ, p-p65, tumor necrosis factor-alpha (TNF-α), and interleukins-1β (IL-1β) induced by LPS in BV2 cells were reversed by miR-381-3p mimic transfection. Besides, upregulated TNF-α and IL-1β induced by miR-381-3p inhibitor in BV2 cells were reversed by IKKβ inhibitor (BMS-345541). Results of animal experiments indicated that miR-381-3p was overexpressed in rats of the ASCI group. The protein levels of IKKβ and p-p65, and the mRNA expression levels of inflammatory cytokines TNF-α and IL-1β were remarkably decreased in the ASCI group than those of the control group. The grip ability, coordination, and anti-fatigue performance of rats in the ASCI group recovered quicker than those of the control group.

CONCLUSIONS: MiR-381-3p was downregulated in ASCI rats. The overexpression of miR-381-3p could recover the motor ability of rats in the ASCI group earlier and might inhibit injury aggravation by inhibiting inflammatory responses via the IKKβ-NF-κB pathway.

Key Words:
ASCI, MiR-381-3p, IKKβ-NF-κB, p-p65, TNF-α, IL-1β.

Introduction

Spinal cord injury (SCI) is one of the serious injuries, accompanied by sudden loss of sensory, motor, and autonomic nerves. SCI exhibits great physical and mental impact on affected patients[1,2]. The annual incidence rate of SCI is around 3.6-195.4/1,000,000[3]. However, the current treatment of SCI cannot effectively recover the neurological function of SCI patients. Most SCI patients are accompanied by substantial neurological function disorders and lifelong disabilities, which also brings heavy economic burden to the patient. According to the degree of injury and paralysis, SCI costs about 111-472 million dollars in the whole life of each SCI patient[4]. Therefore, it is urgent to thoroughly investigate the potential mechanism of SCI.

SCI is a pathophysiological process that involves a series of cytological and molecular reactions. It's known to all that SCI can be divided into primary and secondary injury[5,6]. Primary injury is an irreversible death of neuronal cells caused by external forces and cannot be regenerated[7]. Secondary injury refers to further neuronal damage and the extension of injury area within minutes to hours upon the occurrence of primary injury. The inhibition of secondary injury as early as possible can reduce the enlargement of injury area and the damage of anatomic structures, so as to recover neurological functions[8].

Inflammatory response is the major pathophysiology of acute injury, and it still persists in sec-

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ondary injury[9]. SCI can activate the inflammatory response, release a large number of pro-inflammatory mediators and neurotoxins, eventually leading to cell death. The nuclear factor-kappa B (NF-κB) is a major factor that regulates the expression of several pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukins-1β (IL-1β). It can also regulate the expression level of various cytokines, including chemokines and adhesion molecules[10-15]. Direct inhibition of the NF-κB pathway can decrease the expression of inflammation-related factors, thereby reducing the secondary damage caused by inflammation and improving the recovery of neurological function[16-18]. NF-κB is mainly regulated by NF-κB inhibitor IKKs, among which IKKβ subunits play a major role[19-21]. In this study, we aimed to primarily investigate the mechanism of the *IKKβ-NF*-κB pathway in regulating the inflammation of SCI.

Materials and Methods

Establishment of the SCI Model

The experiment was approved by the Ethic Committee of the Second Affiliated Hospital of Nanchang University, China. Totally 12 adult female SD rats were randomly divided into two groups: the control group and the acute spinal cord injury (ASCI) group, with 6 rats in each group. Rats were fed in a sterile environment with controlled temperature and alternating illumination between light and dark for 12 h. Before operation, the rat were fasted for 12 h, and were refrained from drinking water for 4 h. 10% chloral hydrate (3 mL/kg) was intraperitoneally injected for general anesthesia[22]. Then the rats were fixed at the pronation position, and laminectomy was performed at the spinal cord of T9-11. After exposure, the spinal cord at T10 level was clamped with forceps for 20 s. Finally, paralysis of the lower limbs indicated the successful establishment of the ASCI model. Rats in the control group underwent the same procedure without SCI treatment.

Separation of Microglia

The rats were sacrificed, and the spinal cord was isolated and placed in phosphate-buffered saline (PBS) buffer containing 0.2% glucose. The meninges were removed, and the spinal cords were smashed and homogenized. Each portion of the tissue samples was dissolved in 5 mL of PBS and filtered through a 40-μm filter, followed by resuspension and centrifugation with the Percoll stratified solution. The purified microglia were located at the Percoll gradient interface. Microglial cells were then cultured in Dulbecco's Modified Eagle Medium (DMEM) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA).

Separation of Primary Microglia

Methods for the isolation of microglial cells from the central nervous system were described in a previous report[23]. 1-3-month old SD rats were sacrificed in CO_2. The cerebral cortex of the parietal lobe was isolated, digested with enzymes, and placed in PBS containing 0.25% trypsin. After culture for 15 min, DMEM medium containing 5% fetal calf serum, 10% calf serum and 1% penicillin and streptomycin were added for terminate digestion. Cells were re-suspended, seeded in a culture flask, and maintained in a 37°C, 5% CO_2 incubator for 7-9 days. The microglia-enriched medium was then collected and cultured for 24-48 h. Then, the cells were digested with trypsin, followed by the treatment with interferon for 1 h. Subsequently, primary microglia were treated with lipopolysaccharide (LPS) for 24 h, and the solution was changed every day.

Cell Culture and Transfection

BV2 cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and the cells were maintained in a 37°C, 5% CO_2 incubator. MiR-381-3p mimic, miR-381-3p inhibitor or corresponding controls were transfected according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Finally, the cells were harvested 48 h after transfection for subsequent experiments.

Dual Luciferase Reporter Assay

The 3'UTR region of IKKβ was cloned into the psiCHECK-2 vector to construct the psiCHECK-IKKβ-3'UTR luciferase reporter gene. BV2 cells were co-transfected with miR-381-3p mimic, miR-381-3p inhibitor or corresponding controls, respectively. The luciferase activity was measured 48 h after transfection.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Assay

QRT-PCR was used to determine the mRNA expression of miR-381-3p, IKKβ, TNF-α, and IL-1β. Total RNA was extracted according to

the instructions of the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, complementary deoxyribose nucleic acid (cDNA) was synthesized by using a reverse transcription kit. Subsequently, the mRNA expression of relevant genes was detected by qRT-PCR. The reaction conditions were: pre-denaturation at 95°C for 30 s, 95°C for 5 s, and 60°C for 31 s, for a total of 40 cycles. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references. Primers used in this study were as follows: MiR-381-3p: CGTGAATGATAGTGAGGAAC, R: GTGAACGATTTGCCACACACA; *IKKβ*: F: CCTTGTCCTATAGAAGCACAAC, R: GTCATTTCCACAGCCCTGTGA; *TNF-α*: F: CCACCACGCTCTTCTGTCTACTG, R: GGGCTACGGGCTTGTCACT; *IL-1β*: F: CCCTGCAGCTGGAGAGTGTGG, R: TGTGCTCTGCTTGAGAGGTGCT; U6: F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; *GAPDH*: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Western Blotting

Cells were lysed with the radio-immunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) and sonicated on ice. Total protein was then extracted by centrifugation, and the protein concentration was determined according to the bicinchoninic acid (BCA) method (Abcam, Cambridge, MA, USA). Proteins were separated by gel electrophoresis, and were transferred to polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland) by the wet transfer method. The membranes were then blocked with 5% nonfat milk for 1 h, followed by incubation with corresponding primary antibodies at 4°C overnight. After that, the membranes were washed with Tris-buffered saline-Tween (TBST) (Beyotime, Shanghai, China) for three times, and corresponding secondary antibodies was added for incubation at room temperature for 1 h. The membranes were washed with TBST for three times. Finally, the images were visualized by using the electrochemiluminescence (ECL) method (Thermo Fisher Scientific, Waltham, MA, USA).

Injection of miRNAs in SCI Rats

SCI rats were randomly divided into two groups: the control group (miR-381-3p negative control) and the miR-381-3p overexpression group (miR-381-3p mimic), with 6 rats in each group. The rats were anesthetized and injected intrathecally with 100 nmol miR-381-3p mimic or miR-381-3p negative control once a week for a total of six weeks.

Determination of Grip Ability, Coordination and Anti-Fatigue Performance

We measured the grip ability, coordination and anti-fatigue performance of ASCI rats 1 day before the ASCI procedure and on the 3rd, 7th, 14th, 21st, 28th, 35th, and 42th day after the operation, respectively. Specifically, rat's tail was gently lifted to the crossbar to grip the crossbar actively with the lower limbs. The rats were pulled at the crossbar in the horizontal direction. The maximum gripping force of bilateral forelimbs exhibited on the grip strength meter was recorded. The grip ability of each rat was measured for three times, and the maximum grip strength was recorded for statistical analysis. We then used a roller to measure the coordination and anti-fatigue performance of the rats, and the maximum rotational speed of the rats to maintain their balance was recorded. The rotarod test contained 18 stainless steel rods, the speed of which was increased by 1.5 rpm per second[24].

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 13.0 Software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. The measured data were expressed as mean ± standard deviation (mean ± SD). The unpaired *t*-test was used for comparing the difference between groups. $p<0.05$ was considered statistically significant.

Results

Expression of miR-381-3p and Relative Genes in the IKKÐ-NF-κB Signaling Pathway in SCI Rats

Totally 12 adult SD rats were randomly divided into two groups: the control group (n=6) and the ASCI group (n=6). MiR-381-3p was downregulated in the ASCI group (Figure 1A). The mRNA and protein level of IKKβ and p-p65 were both upregulated in the ASCI group (Figure 1B-1C). The LPS treatment of primary gliocytes could downregulate the expression of miR-381-3p (Figure 1D). Meanwhile, the mRNA and protein level of IKKβ and p-p65 were also upregulated after LPS treatment (Figure 1E-1F).

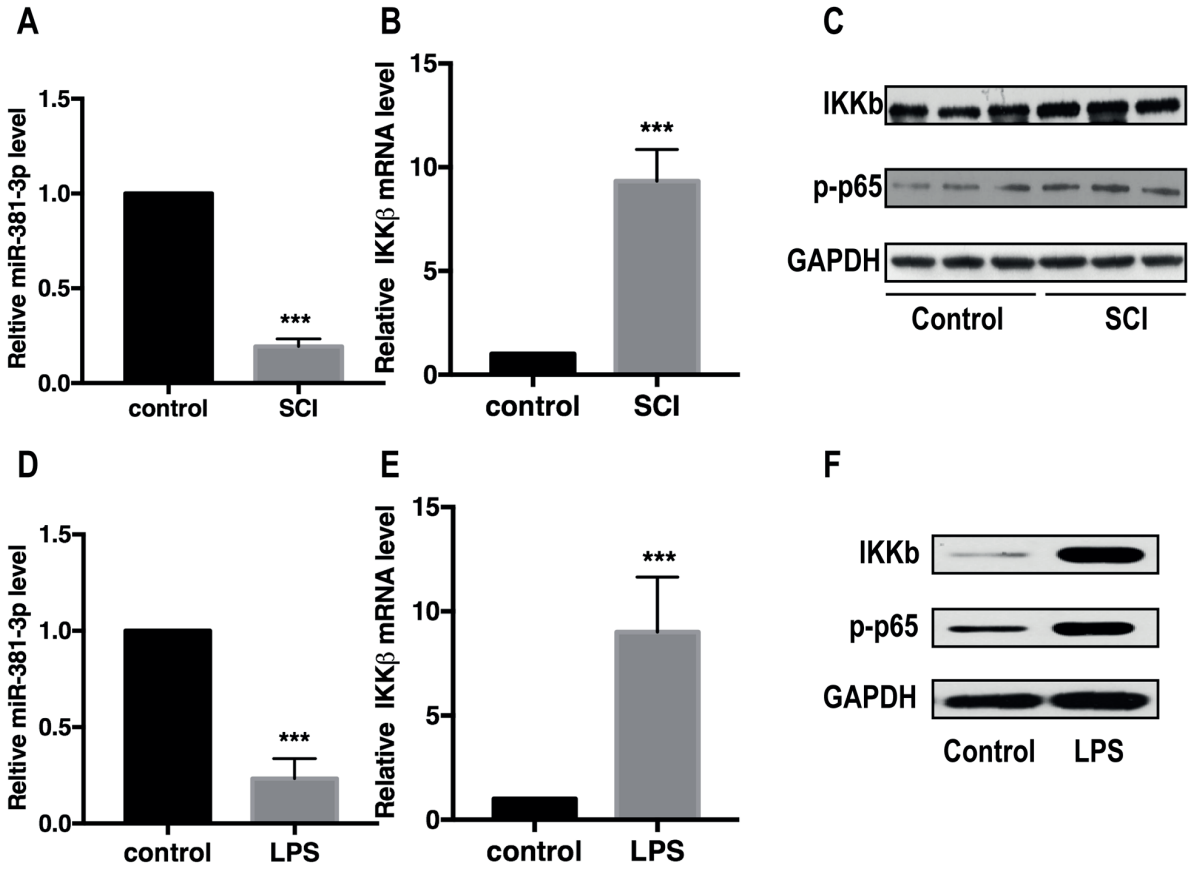

Figure 1. Expression and regulation of miR-381-3p and the IKKβ-NF-κB signaling pathway in rats with SCI. A total of 12 adult SD rats were randomly divided into 2 groups: the control group (n=6) and the ASCI group (n=6). ***A,*** MiR-381-3p was downregulated in the SCI group. ***B,*** and ***C,*** The mRNA and protein expression of IKKβ and p-p65 were increased in the ASCI group. ***D,*** The level of miR-381-3p was downregulated in the LPS treated group. ***E,*** and ***F,*** The mRNA and protein expression of IKKβ and p-p65 were increased in the LPS treated group.

Relationship Between miR-381-3p and IKKβ

In SCI rats, miR-381-3p and IKKβ were both abnormally expressed. Specifically, miR-381-3p was downregulated, whereas IKKβ was upregulated. Studies have shown that miRNAs are involved in post-transcriptional regulation, which may induce degradation or inhibit the translation process of target mRNAs[25]. To investigate whether there was a regulatory relationship between miR-381-3p and IKKβ, we detected the mRNA expression, protein expression, and gene activity of IKKβ by transfecting miR-381-3p plasmids in BV2 cells (Figure 2A). We found that overexpression of miR-381-3p mimic could inhibit the mRNA expression of IKKβ, and vice versa (Figure 2B). Dual luciferase reporter gene assay confirmed that miR-381-3p could directly bind to IKKβ (Figure 2C), which was further verified by Western blot (Figure 2D).

Effect of miR-381-3p and IKKβ on Inflammatory Response

The IKKβ-NF-κB signaling pathway is an important pathway that regulates inflammation, and miR-381-3p has been proven to regulate this pathway. We further verified whether miR-381-3p regulated inflammatory response *via* the IKKβ-NF-κB pathway. BMS-345541 is a novel selective inhibitor of IKKβ that can inhibit IKKβ activity[26]. BV2 cells were treated with LPS or simultaneously with miR-381-3p mimic. The mRNA and protein level of IKKβ, p-p65, IL-1β and TNF-α in each group were detected by qRT-PCR and Western blot, respectively. The results suggested that overexpression of miR-381-3p partially reversed the upregulated effect of LPS on IKKβ (Figure 3A-3B) and p-p65 (Figure 3B). Moreover, overexpression of miR-381-3p could also partially reverse the upregulated effect of LPS on TNF-α and IL-1β (Figure 3C-3D). Next, we detected the

mRNA level of IL-1β and TNF-α in BV2 cells co-transfected with miR-381-3p inhibitor and BMS-345541. The results indicated that BMS-345541 could neutralize the upregulated effect of miR-318-3p inhibitor on the expression level of TNF-α and IL-1β. All these results demonstrated that miR-381-3p could regulate the expression of IKKβ, thus regulating the expression of inflammatory factors.

Effect of miR-381-3p on ASCI Rats

In vitro experiments confirmed that miR-381-3p was involved in the ASCI inflammatory response. We further explored whether miR-318-3p participated in the regulation of inflammation in the animal model. Rats in the control group and the experimental group were treated with miR-381-3p negative control and miR-381-3p mimic, respectively. The grip ability and rod rotating ability of each rat were detected. Six weeks later, all the rats were sacrificed. The protein levels of IKKβ and p-p65 were detected by Western blot, and the mRNA levels of TNF-α and IL-1β were detected by qRT-PCR. The results showed that miR-381-3p was overexpressed, whereas IKKβ and p-p65 were downregulated in ASCI rats (Fig-

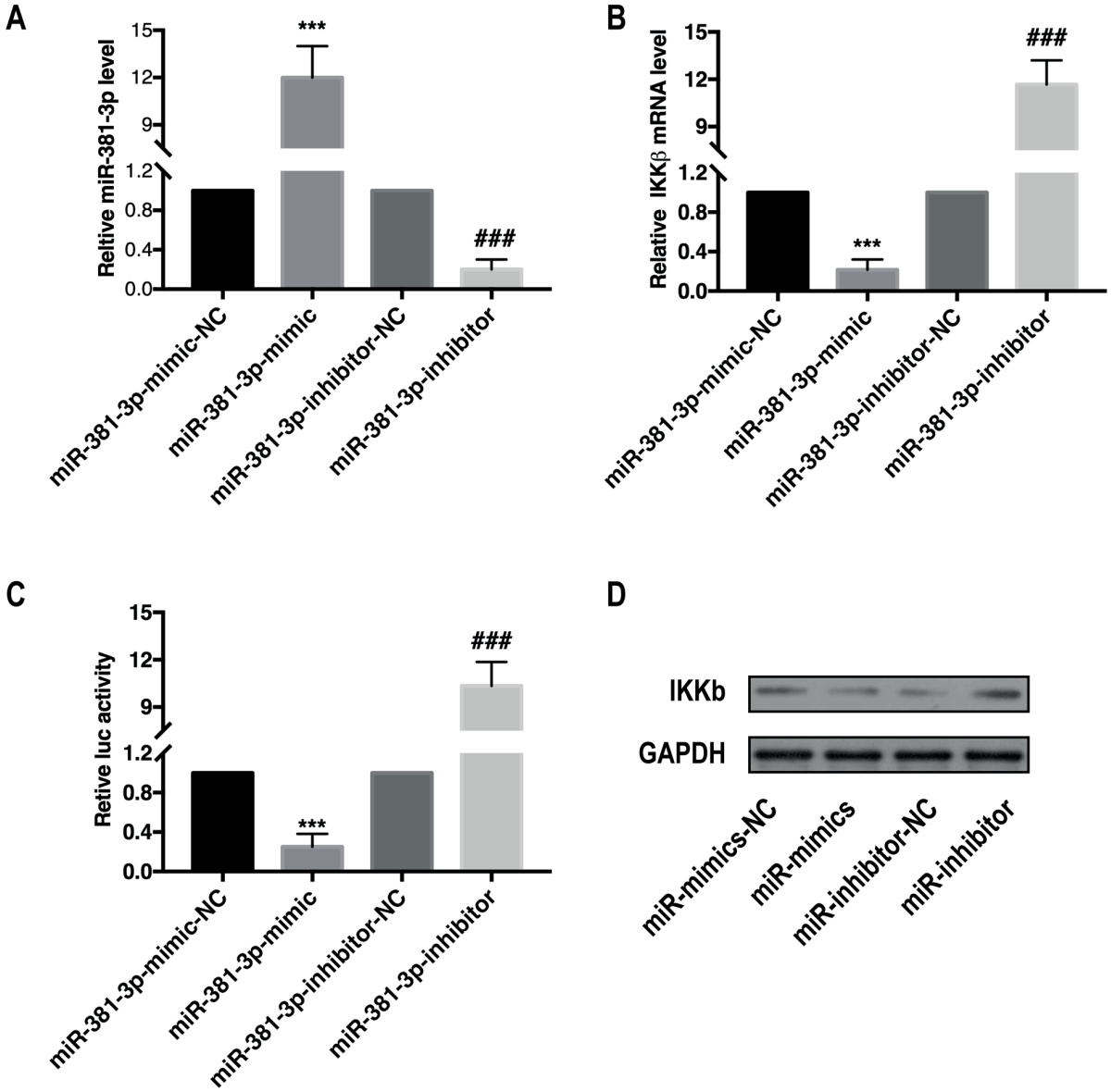

Figure 2. Identification of the relationship between miR-381-3p and IKKβ. ***A-D,*** Downregulated mRNA level of IKKβ. ***A and B,*** Luciferase activity and protein level of IKKβ; ***C,*** IKKβ induced by the overexpression of miR-381-3p was significantly reversed.

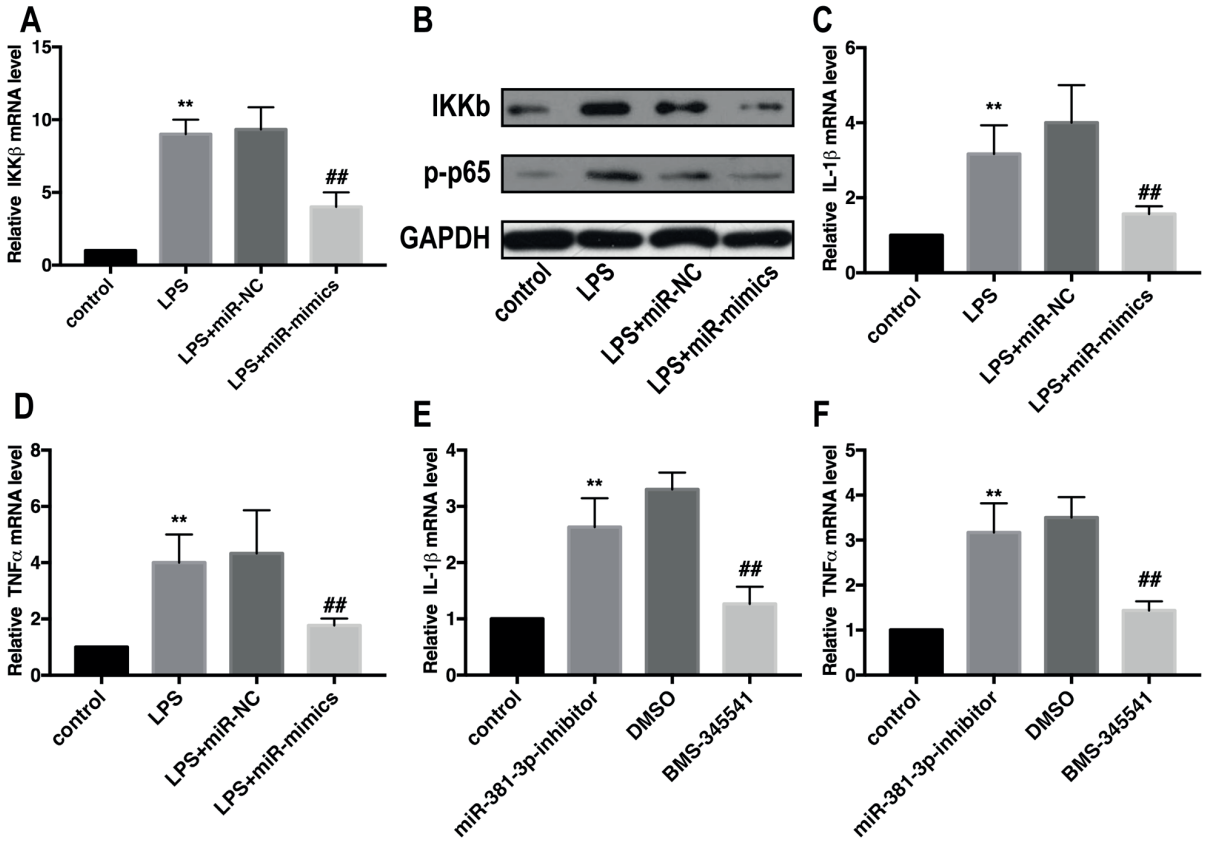

Figure 3. The effect of miR-381-3p and IKKβ on inflammatory response. ***A,*** and ***B,*** MiR-381-3p overexpression could reverse the upregulation of LPS on IKKβ and p-p65 expression in BV2 cells. ***C,*** and ***D,*** MiR-381-3p overexpression could reverse the LPS-induced upregulation of TNF-α and IL-1β in BV2 cells. ***E,*** and ***F,*** BMS-345541 could reverse the upregulated effect of miR-381-3p-inhibitor on the expression of TNF-α and IL-1β in BV2 cells.

ure 4A). The expression levels of inflammatory cytokines TNF-α and IL-1β were also decreased (Figure 4B). Behavioral tests suggested that the grip ability (Figure 4C) and rod rotating ability (Figure 4D) of ASCI rats treated with miR-381-3p mimic recovered more quickly than those of negative controls.

Discussion

The specific pathogenesis of SCI has not been fully elucidated. Inflammatory response induced by SCI is believed to play an important role in the prognosis of neurological function[27]. Inflammatory response is the major component of secondary SCI[9]. In particular, the IKKβ-NF-κB signaling pathway is the major transcriptional regulatory factor that mediates the expression of inflammatory genes[10,11]. In mammalian cells, NF-κB is mainly presented as heterodimers of p65 and p50[28]. SCI stimulates the abnormal activation of *NF*-κB in neuronal cells and gliacytes[9,16,29]. A large number of pro-inflammatory factors and chemotatic factors are overexpressed after SCI, such as TNF-α and IL-1β[12-15]. Subsequently, the infiltration of inflammatory cells in SCI tissues may further stimulate secondary injury, which is manifested as neuronal cell apoptosis and astrocyte activation, scar tissues formation, and can inhibit the proliferation of axons[15,16,30-32].

Numerous studies[33-35] have shown that miRNAs play an important role in the central nervous system, and participates in the occurrence, growth and development of neurocytes, the formation of synaptic connections, myelination, and others. MiRNAs are also involved in the development of spinal cord. Differentially expressed miRNAs suggest that miRNAs are associated with the development of spinal cord. Microarray analysis, RT-PCR and *in situ* hybridization have found that totally 44 miRNAs are differentially expressed in

mouse central nervous system[36]. Liu et al[37] have analyzed the expression changes of miRNAs after SCI, and have found that 60 of 350 miRNAs are remarkably changed by the miRBase platform. Studies have suggested that these changes are related to immune responses, such as immune cell invasion and the regulation of inflammatory signaling pathways. It can be concluded that miRNAs play an important role in the regulation of inflammatory signaling pathways and pathological immune responses[38].

In this study, we found that miR-381-3p was significantly downregulated in the rat SCI model, suggesting that miR-381-3p was greatly involved in SCI. Inflammatory reaction is the central link of secondary SCI, and IKKβ-NF-κB has been proven to regulate inflammation. We also found that the protein expression of IKKβ and p-p65 were significantly upregulated, further confirming that SCI was resulted from inflammation. To explore the relationship between miR-381-3p, the IKKβ-NF-κB signaling pathway and inflammation, our *in vivo* and *in vitro* experiments both confirmed that miR-381-3p could negatively regulate the IKKβ-NF-κB signaling pathway. In the animal model, we examined the effect of overexpressed miR-381-3p on motor ability. The results found that the motor ability of ASCI rats with miR-381-3p overexpression recovered much earlier than those of negative controls.

Conclusions

We showed that miR-381-3p was downregulated in SCI rats. The overexpression of miR-381-3p can promote the recovery of motor ability in SCI rats much earlier. Moreover, miR-381-3p inhibits the inflammatory response of SCI *via* the IKKβ-NF-κB signaling pathway.

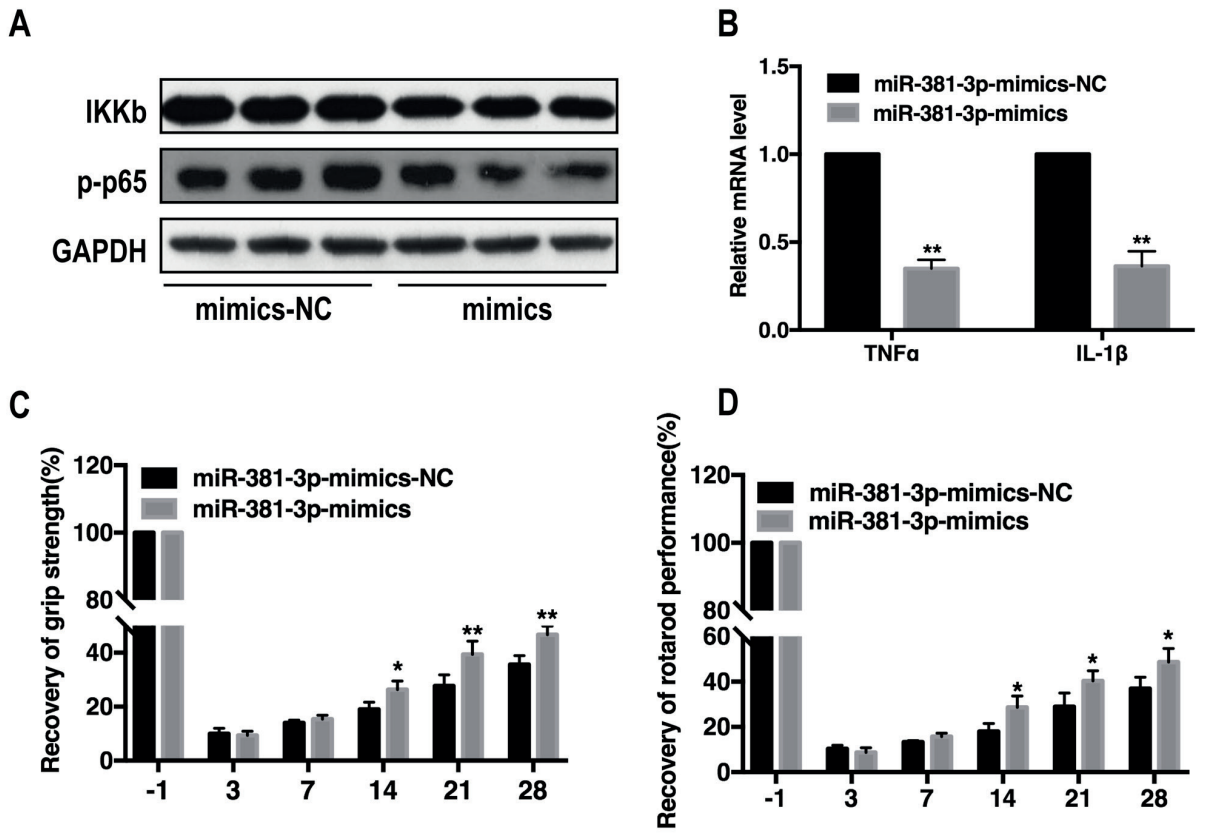

Figure 4. The effect of miR-381-3p overexpression on the development of ASCI rats. ***A,*** IKKβ and p-p65 protein expressions were detected by Western blot. ***B,*** The mRNA level of TNF-α and IL-1β was detected by qRT-PCR. ***C,*** Functional recovery of forelimb strength. ***D,*** Rotarod performance in rats were measured.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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