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MiR-219-5p promotes spinal cord injury recovery by inhibiting NEUROD2-regulated inflammation and oxidative stress

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Abstract. – OBJECTIVE: The aim of this study was to explore the specific role of miR-219-5p in spinal cord injury (SCI), and to investigate its underlying mechanism.

MATERIALS AND METHODS: The SCI model was first constructed in mice, and the motor function of each mouse was evaluated by the Basso Beattie Bresnahan (BBB) method. The protein and mRNA expression levels of miR-219-5p and NEUROD2 in SCI mice were detected by Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), respectively. Subsequently, all mice were assigned into 3 groups, including the sham operation group, the SCI group and the SCI+miR-219-5p group. The levels of inflammatory factors (TNF-α, IL-1β and IL-6) were detected by Western blot and qRT-PCR. Meanwhile, reactive oxygen species (ROS) were detected by flow cytometry. Target genes of miR-219-5p were predicted by TargetScan and verified by Luciferase reporter gene assay. For in vitro experiments, the possible molecule mechanism of miR-219-5p in regulating NEUROD2 was detected by Western blot.

RESULTS: MiR-219-5p was significantly downregulated after SCI. The expression level of miR-219-5p was decreased in the SCI group than that of the sham operation group in a time-dependent manner, which reached the lowest level on the 7th day. Besides, the mRNA and protein levels of NEUROD2 in the SCI group were both remarkably increased in a time-dependent manner, which reached a peak on the 7th day. The levels of inflammatory factors (TNF-α, IL-1β and IL-6) and ROS were significantly higher in the SCI group, which could be reversed by miR-219-5p mimics transfection in SCI mice. Meanwhile, the BBB score in the SCI group was remarkably lower than that of the SCI + miR-219-5p group from the 4th day after SCI. TargetScan predicted that NEUROD2 was the target gene of miRNA-219-5p. In addition, Western blot results indicated that miR-219-5p could regulate NEUROD2, eventually promoting the recovery of SCI.

CONCLUSIONS: Overexpressed miR-219-5p promotes SCI recovery and motor function elevation via alleviating NEUROD2-regulated inflammation and oxidative stress.

Key Words:

Spinal cord injury, MicroRNA-219-5p (MiR-219-5p), NEUROD2, Inflammation, Oxidative stress.

Introduction

Acute spinal cord injury (SCI) is a common type of motor system trauma with poor prognosis. SCI frequently occurres in traffic accidents, sports injuries, high altitude falls, and violent injuries. It is estimated that the number of newly-increased SCI cases is about 11,000 in the United States[1]. However, the incidence of SCI per year is 19.5-56.1/1,000,000 in Asia[2]. Sensorimotor disorders to the corresponding segment, especially cervical spinal cord injury, may affect the function of upper limbs. This eventually leads to the loss of labor ability and brings a heavy burden on SCI patients and their families[3].

As a complicated and dynamic pathological process, SCI involves functional changes of multiple systems. Currently, researchers have demonstrated that SCI consists of two stages, namely primary injury and secondary injury[4]. Primary injury is an instantaneous mechanical injury caused by external forces, mainly due to the displacement of the spinal column[5]. However, secondary injury is the pathophysiological process after primary injury. It's known to all that secondary injury is accompanied by alterations of cellular metabolism and gene expression, including excitatory amino acid release, calcium influx, free

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radical damage, inflammation and apoptosis. Secondary injury immediately starts several minutes after primary injury, which can last for several weeks or even months[6]. Moreover, differentially expressed genes also exert an essential role in the pathophysiology of acute SCI[7].

MicroRNAs (MiRNAs) are a kind of non-coding, small molecules with 19-22 nucleotides in length. It's reported that miRNAs are evolutionarily highly conserved. Bioinformatics predicts that at least 30% of encoding proteins in the human genome are regulated by miRNAs[8]. Hence, miRNAs may provide a novel idea for studying the pathogenesis and treatment strategies of human diseases. Recent studies[9,10] have shown that multiple miRNAs are specifically expressed in the mammalian spinal cord and other nervous systems. Functionally, miRNAs exert a crucial role in the central nervous system, which participates in the development of neurons, formation of synaptic connections and myelination[11-13]. Meanwhile, various disorders in the central nervous system are associated with miRNA dysregulation[14]. Several animal experiments have confirmed that immune dysfunction and inflammatory response after SCI are closely involved in SCI and its recovery[15-17]. Therefore, the aim of this study was to investigate the exact role of miR-219-5p in SCI and to investigate the possible underlying mechanism.

Materials and Methods

Construction of the Spinal Cord Injury Model in Mice

Adult C57BL/6 mice weighing 20-25 g were randomly assigned into 3 groups, including the sham operation group, the SCI group and the SCI + miR-219-5p group. For constructing the SCI model, all mice were cut open alongside the neck after anesthesia. After dissecting the skin and fascia, the C5 spinous process was removed under a microscope. Subsequently, the incision was sutured layer by layer. Mice in the sham operation group were cut open without removal of the C5 spinous process. Meanwhile, mice in the SCI + miR-219-5p group were additionally administrated with miR-219-5p mimics. The Basso Beattie Bresnahan (BBB) method was used to evaluate the recovery of motor function in each mouse. mouse. This study was approved by the Animal Ethics Committee of Soochow University Animal Center.

RNA Extraction and Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Total RNA in spinal cord tissues (4 mm within the SCI area) was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into complementary deoxyribonucleic acid (cDNA) in accordance with PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The specific qRT-PCR reaction conditions were: denaturation at 95°C for 30 s, followed by annealing at 95°C for 5 s and extension at 60°C for 31 s, for a total of 40 cycles. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references. Each sample was repeated for 3 times. Primers used in this study were as follows: MiR-219-5p, F: CTTTTTGCGGTCTGGGCTTG, R: AACGCTTCACGAATTTGCGT; U6, F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; NEUROD2, F: CGGCTAGCCGCATGCTGACCCGCCTGTT, R: CGGGATCCCGGGCCGGACAAAGGCAAAG; TNF-α (tumor necrosis factor-α), F: CCACCACGCTCTTCTGTCTACTG, R: GGGCTACGGGCTTGTCACT; IL-1β (interleukin-1β), F: CCCTGCAGCTGGAGAGTGTGG, R: TGTGCTCTGCTTGAGAGGTGCT; IL-6 (interleukin-6), F: ACAACCACGGCCTTCCCTACT, R: CTCATTTCCACGATTTCCCAGA; GAPDH, F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Western Blot

Total protein of SCI tissues was extracted by radio-immunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The concentration of extracted protein was detected by the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Subsequently, extracted protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk, the membranes were incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) at 4°C overnight. After washing with Tris-Buffered Saline with Tween 20 (TBST), the membranes were incubated with corresponding secondary antibody. Finally, immunoreactive bands were exposed by the enhanced chemiluminescence method (ECL) (Thermo Fisher Scientific, Waltham, MA, USA).

Cell Culture

HEK-293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Hyclone, South Logan, UT, USA), and maintained in a 37°C, 5% CO_2 incubator.

Dual Luciferase Reporter Gene Assay

Target genes of miR-219-5p were predicted by TargetScan, and NEUROD2 was screened out. Corresponding reporter plasmids of NEUROD2 were constructed by Ribobio (Guangzhou, China). Mutant-type (NEUROD2-1 mut) or wild-type of NEUROD2 (NEUROD2 wt) and miR-219-5p mimics or inhibitor were co-transfected into HEK-293 cells, respectively. After culturing for 48 h, relative Luciferase activity was detected based on the instructions of the Dual-Glo®Luciferase Assay System (Promega, Madison, WI, USA).

Statistical Analysis

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

Results

MiR-219-5p was Downregulated in SCI Mice

After the construction of the SCI model in mice, we evaluated the recovery of motor function by the BBB method. Results showed that compared with the sham operation group, the BBB score in the SCI group decreased remarkably in a time-dependent manner (Figure 1A). Liu et al[18] have found that 97 miRNAs are differentially expressed after SCI, among which miR-219-5p is remarkably downregulated (Figure 1B). In this study, we detected the expression of miR-219-5p at the different time points after SCI. Results demonstrated that compared with the sham operation group, miR-219-5p expression in the SCI group decreased remarkably in a time-dependent manner and reached the lowest level on the 7th day (Figure 1C).

NEUROD2 was Overexpressed in SCI Mice

TargetScan predicted that NEUROD2 was the target gene of miR-219-5p. Previous studies[19,20] have confirmed that NEUROD2 exerts an essential role in the differentiation and migration of neurons. Our findings indicated that both mRNA (Figure 2A) and protein (Figure 2B) levels of NEUROD2 were significantly elevated in a time-dependent manner, thereby playing a crucial role in SCI recovery.

MiR-219-5p Decreased the Levels of Inflammation and Oxidative Stress

Studies[15-17] have shown that the inflammatory response is closely related to secondary SCI. Multiple pathophysiological responses further exaggerate SCI, including oxidative stress, excessive release of excitatory amino acids and inflammatory response[21]. Oxidative stress stimulates the production of ROS and reactive nitrogen species (RNS) in the body[22]. In this paper, we detected the expression levels of inflammatory factors (TNF-α, IL-1β, and IL-6) and ROS in SCI mice. Results demonstrated that compared with the sham operation group, the expression levels of TNF-α (Figure 3A, F), IL-1β (Figure 3B, F), IL-6 (Figure 3C, 3F), and ROS (Figure 3D, 3F) were significantly elevated in the SCI group. However, the overexpression of miR-219-5p resulted in downregulated inflammation and oxidative stress. Furthermore, we determined motor function recovery by the BBB method and found that mice in the SCI group presented the worst motor function and the lowest recovery. However, mice in the SCI + miR-219-5p group exhibited a stronger motor function on the 4th day after SCI (Figure 3E).

MiR-219-5p Inhibited SCI via Regulating NEUROD2

Binding sites of miR-219-5p and NEUROD2 were first predicted by TargetScan (Figure 4A) and then confirmed by Luciferase reporter gene assay. The lowest Luciferase activity was found in HEK-293 cells co-transfected with miR-219-5p inhibitor and NEUROD2 mut plasmid (Figure 4B). This suggested that NEUROD2 was the target gene regulated by miR-219-5p. Moreover, miR-219-5p mimics transfection remarkably downregulated NEUROD2 in HEK-293 cells, indicating that miR-219-5p could inhibit NEUROD2 expression (Figure 4C).

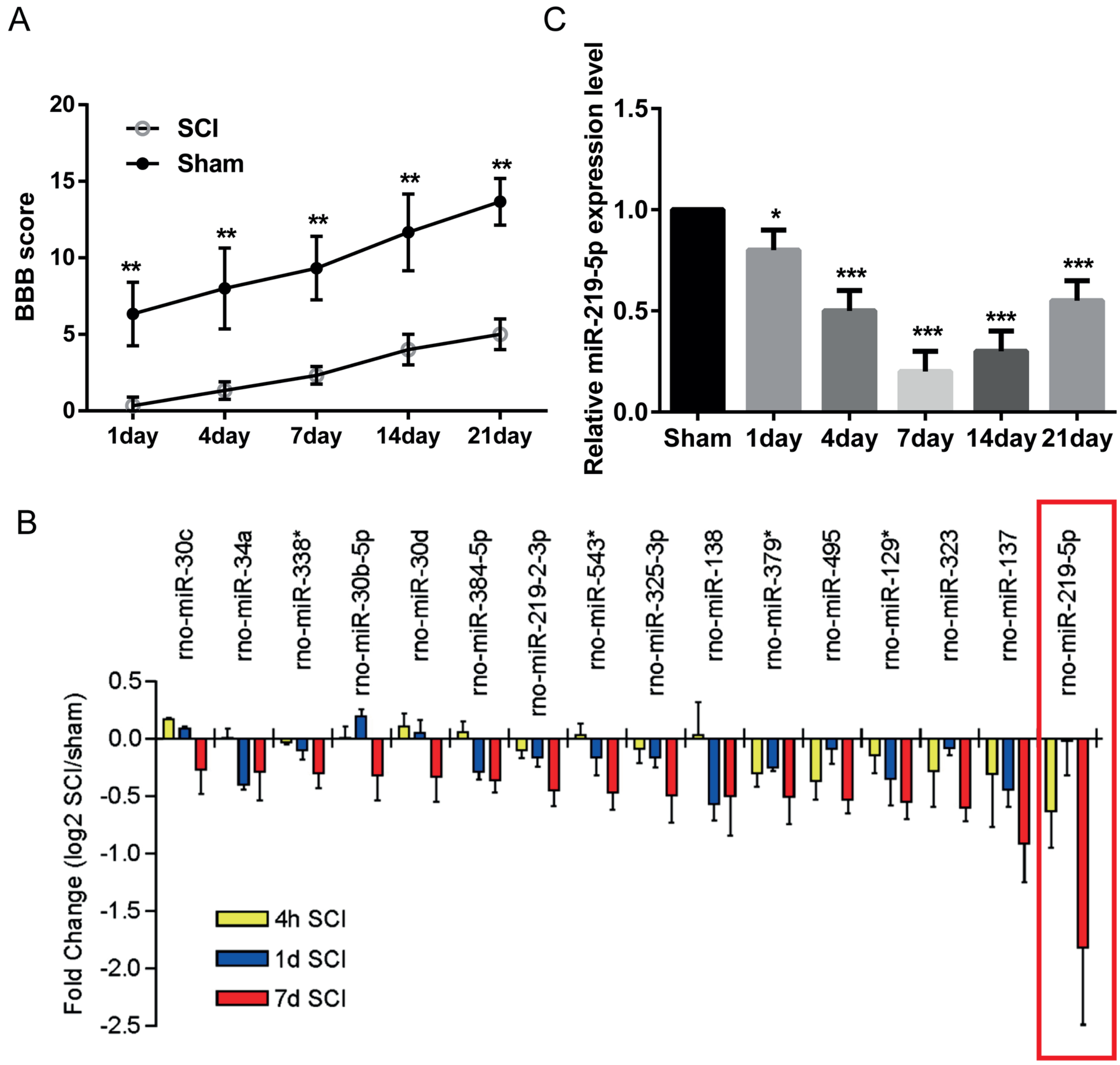

Figure 1. MiR-219-5p was downregulated in SCI mice. ***A,*** Compared with the sham operation group, BBB score in the SCI group decreased remarkably in a time-dependent manner. ***B,*** MiR-219-5p was remarkably downregulated after SCI. ***C,*** Compared with the sham operation group, miR-219-5p expression in the SCI group was significantly decreased in a time-dependent manner and reached the lowest level on the 7th day.

Discussion

SCI is a kind of acute traumatic disease that involves multiple pathophysiological changes. It may eventually lead to spinal cord interstitial edema and hemorrhage. Free radical response is considered to be one of the major factors of secondary SCI[23,24]. When SCI occurs, ischemia, hypoxia as well as spinal cord hemorrhage stimulates the release of a large number of oxygen free radicals[25]. Destroyed permeability and integrity of neuronal membranes result in neuronal death. Meanwhile, the inflammatory response is also involved in the process of secondary SCI. Recent studies have proved that accumulating secretion of pro-inflammatory and chemokines (including TNF-α, IFN-γ, TGF-β, and ILs) leads to demyelination of residual nerve fibers. As a result, it may cause the degeneration or even the death of a large number of neurons[26]. These inflammation-related genes, however, can gradually return to normal level in the late stage of SCI[27]. NEUROD2, a neurogenic differentiation factor, is a member of the bHLH family. Functionally, NEUROD2 participates in the differentiation and maturation of central and peripheral neurons

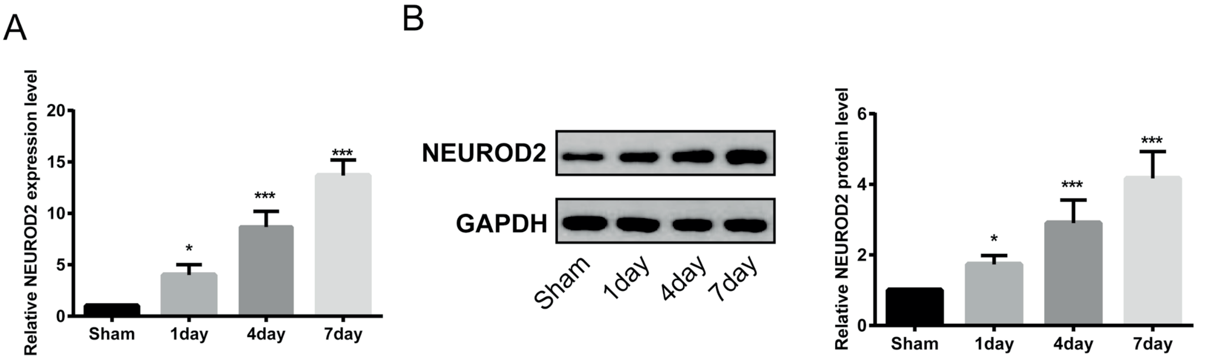

Figure 2. NEUROD2 was overexpressed in SCI mice. ***A-B,*** The mRNA (*A*) and protein (*B*) levels of NEUROD2 were elevated in a time-dependent manner.

in the late stage of spinal cord development[28]. In this study, we found that NEUROD2 was gradually upregulated in SCI mice with the prolongation of injury time, indicating that NEUROD2 was involved in the process of SCI.

In recent years, the effects of miRNAs on spinal cord diseases have been well elucidated. For example, miRNAs can regulate the expression of multiple factors related to spinal nerve differentiation and spinal cord development[29-31]. Meanwhile, miRNAs are also considered to regulate inflammation and immune function in SCI[32-34]. Previous studies have reported that some miRNAs participate in the regulation of calcium signals, oxidative stress and astrocyte activation after SCI. Besides, miRNAs are correlated with cell apoptosis after SCI[32].

MiR-219-5p is the mature form of human miR-219 family with multiple functions[35]. It has been reported that miR-219-5p is involved in

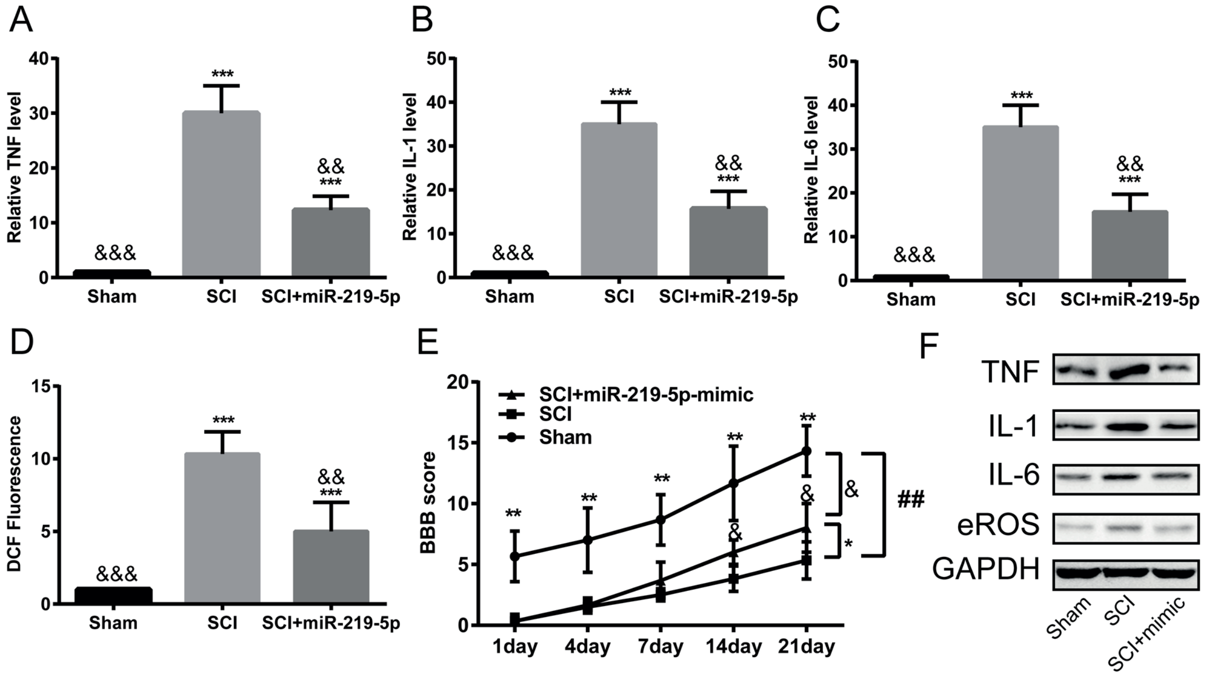

Figure 3. MiR-219-5p decreased the levels of inflammation and oxidative stress ***A-C,*** Elevated levels of TNF-α (*A*), IL-1β (*B*) and IL-6 (*C*) were found in the SCI group than those of the sham operation group. ***D,*** The ROS level was significantly increased in the SCI group than that of the sham operation group. ***E,*** Mice in the SCI group presented the worst motor function and lowest recovery, whereas mice in the SCI + miR-219-5p group exhibited a stronger motor function on the 4th day after SCI. ***F,*** The levels of inflammatory factors and ROS were remarkably decreased in the SCI + miR-219-5p group than those of the SCI group.

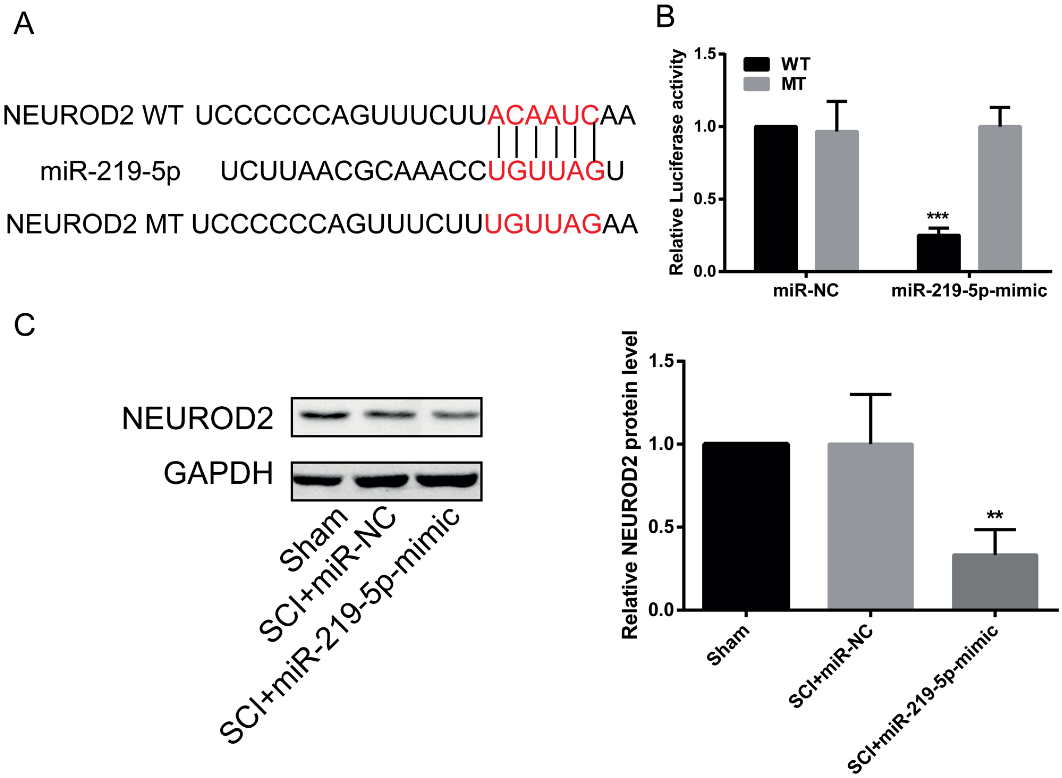

Figure 4. MiR-219-5p inhibited SCI *via* regulating NEUROD2. ***A,*** Binding sites of miR-219-5p and NEUROD2 were predicted by TargetScan. ***B,*** Lowest Luciferase activity was found in HEK-293 cells co-transfected with miR-219-5p inhibitor and NEUROD2 mut plasmid. ***C,*** Downregulated NEUROD2 was found in HEK-293 cells transfected with miR-219-5p mimics.

the growth and development of neurons, suggesting that it may serve as a tumor suppressor gene in gliomas[36,37]. In 2009, Liu et al[18] first discovered that miR-219-5p was significantly downregulated in SCI mice. In the present study, we verified that miR-219-5p was remarkably downregulated after SCI. NEUROD2 was predicted as the target gene of miR-219-5p by TargetScan. *In vitro* experiments further demonstrated that NEUROD2 was regulated by miR-219-5p. Moreover, significant downregulated inflammatory factors and oxidative stress were observed in SCI mice administrated with miR-219-5p mimics. More importantly, motor function recovery in SCI mice was remarkably elevated after miR-219-5p overexpression.

Conclusions

We found that MiR-219-5p was downregulated after SCI. In addition, overexpressed miR-219-5p promoted SCI recovery and motor function elevation *via* alleviating NEUROD2-regulated inflammation and oxidative stress.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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