

MiR-92b-5p inhibitor suppresses IL-18 mediated inflammatory amplification after spinal cord injury via IL-18BP up-regulation

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Abstract. – OBJECTIVE: The aim of this study was to investigate the effects of miR-92b-5p inhibitor and interleukin-18-binding protein (IL-18BP) on interleukin-18 (IL-18)-mediated inflammatory response after spinal cord injury (SCI).

MATERIALS AND METHODS: In this work, microglia was isolated from the newborn C57/B6J mice spinal cord to *in vitro* culture. The expression of IL-18BP and IL-18 was measured by the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) after transfection of miR-92b-5p into activated microglia. The expression of IL-18BP and IL-18 was determined following miR-92b-5p inhibitor treatment. In addition, the spinal cord injury model was established in mice. The expressions of miR-92b-5p, IL-18BP, and IL-18 were measured by qRT-PCR, and the expressions of inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF- α) and interleukin-1 β (IL-1 β) were determined by Western blot. After intrathecal injection of miR-92b-5p inhibitor, the mRNA expression of miR-92b-5p, IL-18BP, and IL-18 and the expression of iNOS, TNF- α , and IL-1 β in the injured area of the spinal cord of mice were measured. Basso Mouse Scale (BMS) was used to determine the recovery of locomotor function after spinal cord injury and miR-92b-5p inhibition.

RESULTS: After miR-92b-5p transfection, the expression of IL-18BP was significantly decreased compared with that of untransfected microglia cells, whereas the level of IL-18 mRNA was significantly increased. However, the level of IL-18BP elevated significantly and the level of IL-18 reduced markedly after treatment with corresponding inhibitors. In addition, compared with the sham operation group (Sham), the RNA level of miR-92b-5p in the SCI group was significantly higher than that in the Sham, but the expression of IL-18BP was evidently declined and the expression of IL-18 was significantly increased in the SCI group. Meanwhile, the expression of miR-

92b-5p in miR-92b-5p inhibitor intrathecal injection mice was remarkably lower than that in SCI group, the expression level of IL-18BP was significantly increased, and the RNA expression of IL-18 was weakened accordingly. Moreover, the protein expression of iNOS, TNF- α , and IL-1 β in miR-92b-5p inhibitor-treated mice was significantly lower than that in the SCI group. The locomotor evaluation of miR-92b-5p inhibitor group was dramatically higher than that of the SCI group.

CONCLUSIONS: Suppressing the expression of miR-92b-5p after SCI can effectively intensify the level of IL-18BP, reduce the expanded inflammatory effect of IL-18, decline the release of iNOS, TNF- α , and IL-1 β , thus alleviate the neuronal injury and improve the locomotor function after SCI.

Key Words:

MiR-92b-5p, Interleukin-18-binding protein, Spinal cord injury, BMS.

Introduction

Spinal cord injury (SCI) is the most serious complication after spinal trauma, which often leads to the collapse of spinal neural network^{1,2}, thereby causing severe long-term neurological dysfunction and sensory and motor dysfunction of limbs below the injured segment³. The poor prognosis of SCI brings unbearable pain to patients and also places a huge burden on the family. The microglia-mediated secondary inflammatory injury is crucial, which, due to the massive release of pro-inflammatory factors, the effect of proteases and the production of reactive oxygen species, aggravates the SCI⁴⁻⁶.

Interleukin-18 (IL-18) is regarded as a member of the IL-1 family (IL-1F) due to the similar molecular structure, processing mechanism, complex receptor tissue and signal transduction pathway to the parent cytokine IL-1 β ^{7,8}. IL-18, similar to IL-1 β , lacks a classical secretory signal peptide and exists as a precursor protein, which can be secreted and activated only by secondary cleavage^{9,10}. IL-18 widely exists in macrophages, monocytes, microglia and Kupffer cells, which, as a typical inflammatory amplifier, plays a positive role in resisting infection and regulating immunity¹¹. However, the unrestricted expression of IL-18 in chronic inflammation will lead to damage and even death of tissue cells¹². IL-18 will activate immune cells, promote the release of classical inflammatory factors IL-1 β and TNF- α , and induce the synthesis of nitric oxide and formation of chemokines¹³. In the central nervous system (CNS), IL-18 may also participate in and induce the development of such diseases like neurodegeneration, acute brain injury and schizophrenia^{14,15}. IL-18 binding protein (IL-18 BP), a natural and specific inhibitor of IL-18, is a kind of soluble protein (40 kDa) which can selectively bind to IL-18 in a mature state (instead of the IL-18 precursor) and has high affinity, so that it cannot bind to the IL-18 receptor to exert effects¹⁶⁻¹⁸. IL-18 BP α is secreted the most with the highest affinity¹⁹. The inflammation is reduced in transgenic mice with IL-18 BP overexpression, proving the importance of reducing the IL-18 activity in improving autoimmune and inflammatory diseases, such as osteoarthritis, Alzheimer's disease and even traumatic brain injury²⁰.

Micro-ribonucleic acid (miRNA) is a kind of endogenous non-coding RNA molecule with 22-24 nucleotides in length, which is able to maintain the gene expression and transcriptional regulation in the pathophysiological activities²¹. A lot of miRNAs have been found in the CNS of mammals, including the brain and spinal cord, which play key roles in keeping the normal neurological function and may serve as important intervention targets in neurological diseases²². After SCI, the changes in the expression levels of various miRNAs may suggest that they are involved in the pathological process²³. In SCI of rats, miR-126 promotes angiogenesis and effectively controls the inflammatory response²⁴. MiR-155 facilitates the recovery of motor function after SCI by inhibiting the differentiation of Th17 cells²⁵. The administration of miR-133b *via* lentivirus improves the motor function of SCI mice²⁶.

However, the correlation between the expression of miR-92b-5p and SCI has not been reported recently, and there have been no studies confirming the role of miR-92b-5p in the prognosis of SCI. In this work, it was found that inhibiting miR-92b-5p could increase the IL-18 BP level, thus inhibiting the IL-18-mediated inflammatory amplification and promoting the recovery of motor function in mice after SCI.

Materials and Methods

Primary Culture of Microglia

The whole spinal cord tissues were extracted from neonatal male C57/B6J 5 days old mice obtained from Soochow University, crushed into individual cells and inoculated into a 75 cm² culture flask containing poly-L-lysine. Then, the Dulbecco's Modified Eagle Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin was added for culture in an incubator at 37°C for 2 weeks. The mixture was shaken slightly and filtered through nylon mesh to obtain the microglia. The purified microglia was inoculated into a 6-well plate containing poly-L-lysine, activated by lipopolysaccharide (LPS, 100 ng/mL) for 24 h and cultured for subsequent experiments. This study was approved by the Animal Ethics Committee of Soochow University Animal Center.

Animals

A total of 18 male C57/B6J mice obtained from Soochow University weighing about 20-25 g aged 6-8 weeks were normally fed in a capacious place under 12/12 h light/dark cycle every day, and randomly divided into 3 experimental groups with 6 mice in each group: 1) sham-operation group (only treated with laminectomy), 2) SCI group (the moderate spinal contusion model was established, and an equal volume of normal saline to that in inhibitor group was injected), and 3) miR-92b-5p inhibitor group (intrathecal injection of exogenous miR-92b-5p inhibitor at the injury site).

SCI Model of Mice

To establish the SCI model of mice, the moderate spinal contusion was caused through impact against the thoracic spinal cord. The operation is as follows: after the mice were anesthetized *via* intraperitoneal injection of 5% chloral hydrate, the hair at the surgical site was shaved off in a

prone position. After the skin was disinfected with iodophor, it was cut and the fascia and dorsal muscle were separated to completely expose the vertebral plate. The T9-T11 vertebral plates were removed *via* laminectomy, and the same segment of the spinal cord was exposed. The spinal cord exposed was impacted using the impactor (5 g×4 cm). The spinal congestion, straight swinging of both lower limbs, tail flick reflex and delayed paralysis indicated the successful modeling. Then, after hemostasis, the muscle and dorsal skin were sutured. SCI mice were fed at a suitable temperature (2 mice/cage) and had a normal diet.

Quantitative Real Time-polymerase Chain Reaction (qRT-PCR) Detection

The microglia cultured and fresh spinal cord tissues extracted after euthanasia (3 mm above and below the center of injury, taken from the corresponding spinal cord in the sham-operation group) were ground in a mortar filled with pre-cooled TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and added with chloroform, isopropanol and ethanol to extract RNA. The RNA concentration was determined using the nano-drop method. Then the RNA extracted was synthesized into the complementary deoxyribonucleic acid (cDNA) using the reverse transcription kit, followed by qPCR with SYBR mix. The relative expression levels of RNA in miR-92b-5p, IL-18 BP and IL-18 were calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are as follows: miR-92b-5p (forward): 5'-AGGGACGGGACGCGGTGCA-GTG-3'; miR-92b-5p (reverse): 5'-GCGAGCA-CAGAATTAATACGAC-3'. IL-18BP (forward): 5'-CAGGTACGCAGCTGTGCAA-3'; IL-18BP (reverse): 5'-ACGACGTGACGCTGGACAA-3'. IL-18 (forward): 5'-GCCTCTATTTGAAGATA-TGACTGA-3'; IL-18 (reverse): 5'-GAGATAGTA-CAGCCATACCTCTA-3'. GAPDH (forward): 5'-ACCACAGTCCATGCCATCAC-3'; GAPDH (reverse): 5'-TCCACCACCCTGTTGCTGTA-3'.

Western Blotting

The microglia cultured and fresh spinal cord tissues extracted were lysed using the whole cell lysis buffer containing the protease inhibitor, and the total protein was extracted on ice. The concentration of total protein was analyzed using the bicinchoninic acid (BCA; Pierce, Rockford, IL, USA). After the protein to be detected was separated *via* 10% sodium dodecyl sulfate-polyacrylamide gel, it was transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA,

USA), and sealed with 5% skim milk at room temperature for 1 h. After the membrane was washed with Tris-Buffered Saline with Tween-20 (TBST) 3 times (5 min/time), the protein was incubated by anti-iNOS antibody of rabbit (1:250), anti-TNF- α antibody of rabbit (1:500), anti-IL-1 β antibody of rabbit (1:200) and rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) at 4°C overnight. On the next day, the membrane was washed again with TBST 3 times (10 min/time), the protein was incubated by goat anti-rabbit antibody (1:10000) at room temperature for 1 h, and the membrane was washed 3 times, followed by observation and analysis of protein to be detected using enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA).

Behavioral Analysis

The motor function of all mice in experimental groups was evaluated *via* BMS at 1 d, 3 d, 5 d, 7 d, and 14 d after injury, and the activity and coordination of ankle in hind limbs, paw posture, body stability and tail posture were mainly observed and scored. The motor behavior of mice was scored [0 point (no ankle movement)-9 points (mostly normal movement)] in an open field for 4 min by two researchers.

Statistical Analysis

Statistical Product and Service Solutions (SPSS 18.0) software (SPSS Inc., Chicago, IL, USA) was used for the analysis of experimental data obtained. The data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). The *t*-test was used for the difference between the two groups. $p < 0.05$ suggested that the difference was statistically significant.

Results

The Level of IL-18BP in Microglia Transfected with MiR-92b-5p Decreased yet the Level of IL-18 Increased In vitro

As a typical inflammatory amplification factor, IL-18 can aggravate the inflammatory effect. However, IL-18BP can bind to IL-18, antagonize its pro-inflammatory effect, and alleviate the release of inflammatory cytokines. MiR-92b-5p may be a possible regulatory target after spinal cord injury, and may participate in the process of secondary inflammation. The mRNA levels of IL-18 BP and IL-18 were detected by qRT-PCR after transfection of MiR-92b-5p into microglia cells. It was found that the expression of IL-18BP was

downregulated and the expression of IL-18 was upregulated (Figure 1A, 1B). These results suggest that the expression of miR-92b-5p can increase the inflammatory effect of IL-18 by inhibiting the expression of IL-18BP.

MiR-92b-5p Inhibitors Can Upregulate the Expression of IL-18BP and Inhibit IL-18 In vitro

Then, we examined the effect of miR-92b-5p inhibitor intervention on the expression of IL-18BP and IL-18. After the treatment of miR-92b-5p transfected microglia with inhibitor, it was found that the mRNA expression of IL-18BP was significantly increased, yet the expression of IL-18 was significantly decreased compared with only transfected miR-92b-5p cells (Figure 2). The results showed that the involvement of miR-92b-5p inhibitor could effectively increase the effect of IL-18BP and decrease the expression of IL-18.

Accumulation of Inflammatory Factors Following SCI is Associated with Upregulation of MiR-92b-5p Levels

Compared with the Sham group, the production of iNOS TNF- α and IL-1 β increased significantly 3 days after spinal cord injury in mice (Figure 3A, 3B). We found that the expression of miR-92b-5p was also increased (Figure 3C). Meanwhile, the expression of IL-18 BP was also inhibited, whereas the expression of IL-18 was significantly increased (Figure 3D). This suggests that the inflammatory effect after SCI may have the upregulating effect of miR-92b-5p and interfere with the involvement of IL-18BP in inhibiting the pathway of IL-18 bioefficacy. We

speculate that inhibiting the level of miR-92b-5p may reduce the re-injury of inflammatory spinal cord tissue after SCI, thus promoting the recovery of motor function in mice after SCI.

MiR-92b-5p Inhibitor Effectively Inhibits the Inflammatory Response of Injured Tissue After SCI by Increasing the Expression of IL-18BP

In the spinal cord of SCI mice injected with miR-92b-5p inhibitor, we found that the level of miR-92b-5p was significantly lower than that of the SCI group 3 days after injury (Figure 4A). However, the expression of IL-18BP was significantly elevated and the expression of IL-18 was inhibited (Figure 4B). More notably, the protein levels of TNF- α and IL-1 β were significantly downregulated (Figure 4C, 4D). These data suggest that miR-92b-5p inhibitors can effectively inhibit the inflammatory response of IL-18 by increasing the expression of IL-18BP.

Application of MiR-92b-5p Inhibitor Promotes the Recovery of Motor Function in SCI Mice

The locomotor function of the mice in the Sham group was evaluated by BMS at 1 d, 3 d, 5 d, 7 d and 14 d after injury. The results showed that the locomotor function of the Sham group was good (7.83 ± 0.36 , 8.32 ± 0.45 , 8.62 ± 0.27 , 8.71 ± 0.63 , 8.52 ± 0.56). The recovery of locomotor function in the SCI group was not satisfactory (0.56 ± 0.12 , 0.78 ± 0.22 , 0.97 ± 0.14 , 1.25 ± 0.26 , 2.21 ± 0.42), but the locomotor function in miR-92b-5p inhibitor group was significantly improved compared with the SCI group (0.76 ± 0.18 , 1.63 ± 0.23 , 2.27 ± 0.28 , 3.54 ± 0.41 , 4.69 ± 0.53) (Figure

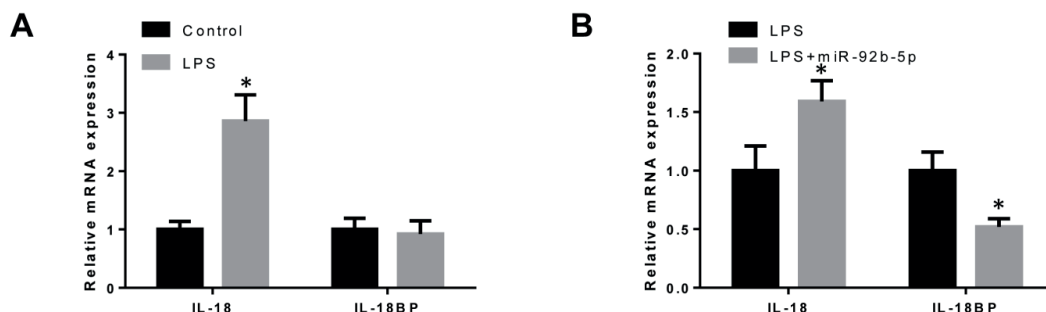


Figure 1. The level of IL-18BP in microglia transfected with miR-92b-5p decreased yet the level of IL-18 increased *in vitro*. **A**, The expression of IL-18 in microglial cells activated by LPS was significantly elevated, but the expression of IL-18BP was hardly changed. **B**, The level of IL-18 was significantly upregulated in activated cells after transfection of miR-92b-5p, but the level of IL-18BP was suppressed.

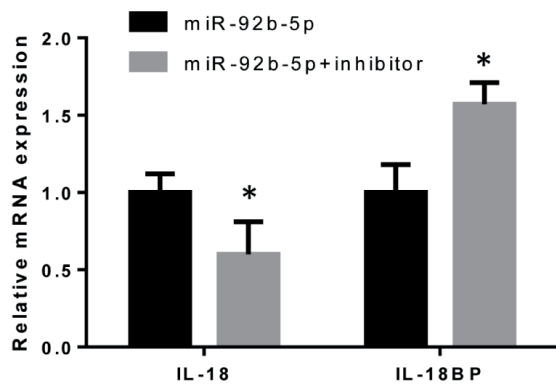


Figure 2. MiR-92b-5p inhibitors can upregulate the expression of IL-18BP and inhibit IL-18 *in vitro*. After MiR-92b-5p inhibitors administration, the expression of IL-18 was remarkably inhibited and the level of IL-18BP was evidently elevated.

5). The results showed that the locomotor function of the mice after SCI was significantly improved after treatment with miR-92b-5p inhibitor.

Discussion

With the deepening of research on miRNA, the intervention in miRNA has gradually become a potential therapeutic target in the development of the disease²⁷. In particular, there are changes in expressions of multiple miRNAs after SCI, affecting the development of disease. Therefore, many studies have demonstrated that the regulation on miRNA can improve the adverse consequences of SCI to a great extent, thus better recovering the neurological function²⁸. After knockout of miR-21 gene, the neurological recovery is effectively promoted after acute SCI²⁹. The upregulation of miR-486 after SCI can inhibit the expression of NeuroD6, 3 which is not conducive to neurological recovery and may mediate the production of reactive oxygen species, indicating that miR-486 is a new therapeutic target for SCI³⁰. MiR-133b also plays an important role in the functional reconstruction of SCI in adult zebrafish³¹. However, the role of

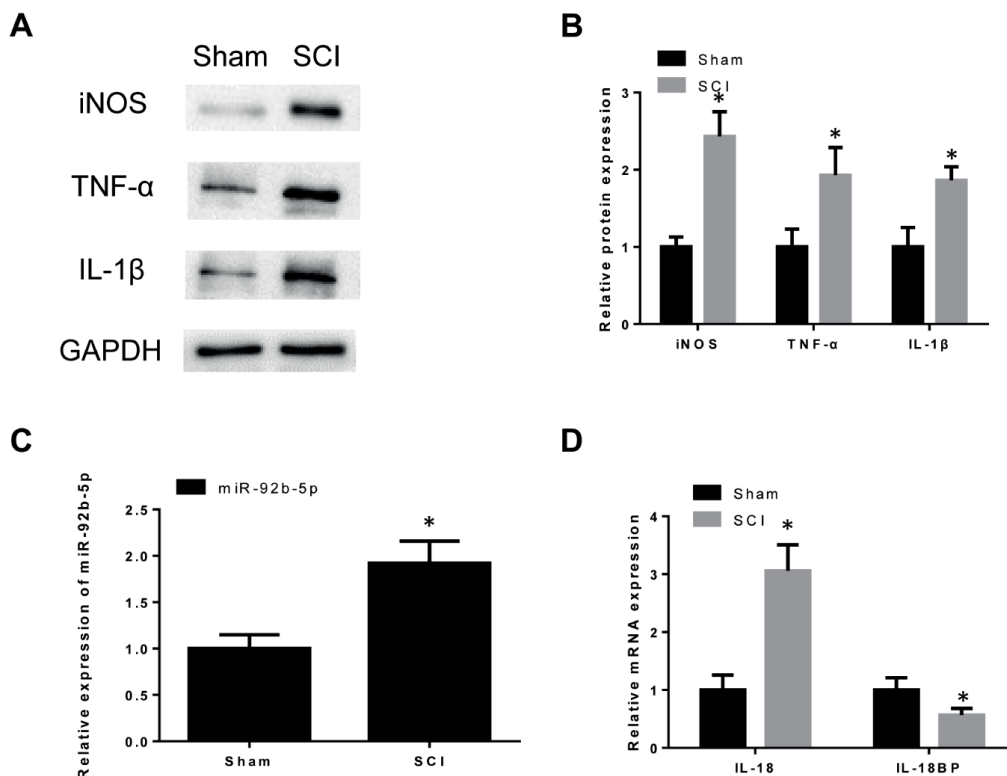


Figure 3. Accumulation of inflammatory factors following SCI is associated with upregulation of miR-92b-5p levels. **A**, After SCI, the protein levels of a variety of classic inflammatory factors increased sharply compared with Sham group at 3 day. **B**, The analysis showed that the increase of various inflammatory factors is statistically significant. **C**, The expression of miR-92b-5p was remarkably risen after SCI. **D**, Meanwhile, the level of IL-18 was significantly elevated and IL-18BP was significantly downregulated.

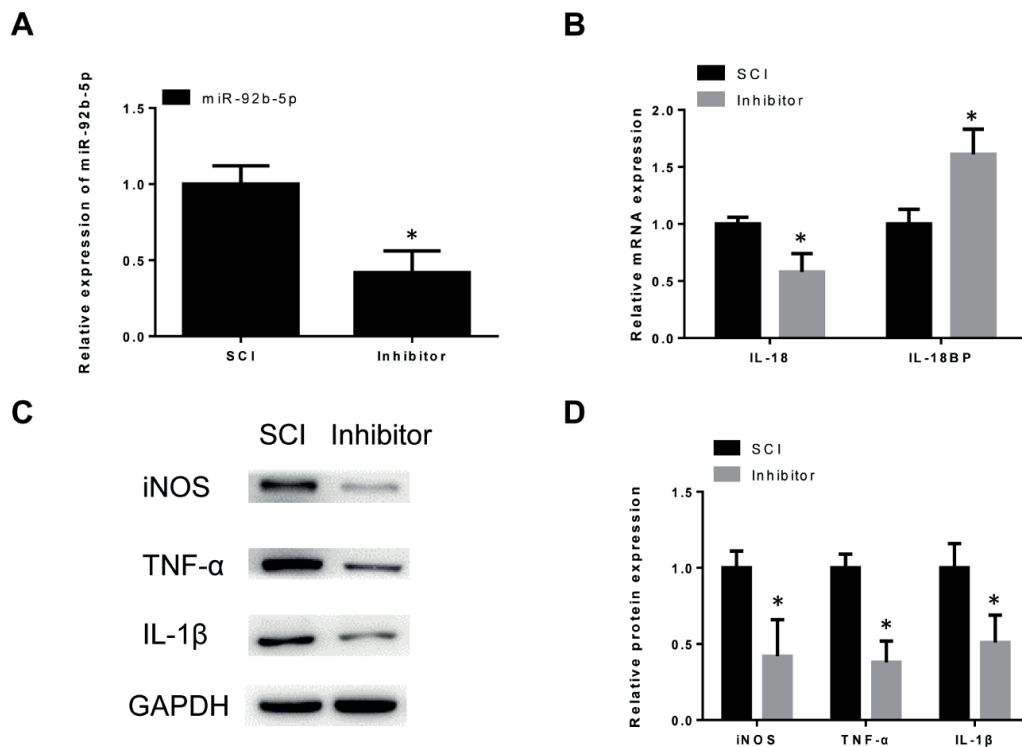


Figure 4. MiR-92b-5p inhibitor effectively inhibits the inflammatory response of injured tissue after SCI by increasing the expression of IL-18BP. **A**, RNA level of MiR-92b-5p was significantly suppressed after injection of MiR-92b-5p inhibitor at 3 day. **B**, Compared with the SCI group, the level of IL-18 decreased significantly and the level of IL-18bp increased significantly after inhibitor therapy. **C**, The expression of various inflammatory factors was significantly downregulated by MiR-92b-5p inhibitor treatment. **D**, It was found that the change of protein level of inflammatory factors was statistically significant.

changes in miR-92b-5p level in SCI has not been reported. In this study, it was found that after microglia was transfected with miR-92b-5p, the pro-inflammatory effect of IL-18 was enhanced by inhibiting IL-18 BP activity. However, inhibiting the miR-92b-5p level can effectively inhibit the biological activity of IL-18 by increasing the expression of IL-18 BP. At the same time, the increase in miR-92b-5p level and IL-18, and the decrease in IL-18 BP were detected after SCI; the release of the classical inflammatory factors iNOS, TNF- α and IL-1 β was also increased. It was found in SCI mice injected with the miR-92b-5p inhibitor that the miR-92b-5p level was downregulated, and IL-18 relieved the inflammatory response through the effective action of IL-18 BP. Moreover, the motor function of mice was also improved. All of these results suggest that miR-92b-5p is involved in the inflammatory response after SCI and enhances the inflammatory amplification of IL-18 *via* inhibiting the

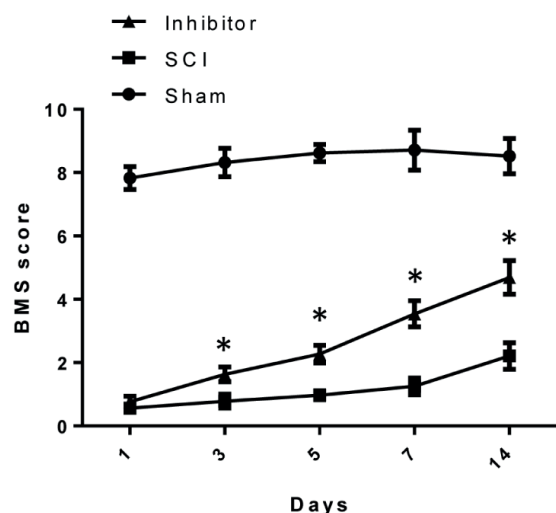


Figure 5. Application of miR-92b-5p inhibitor promotes the recovery of motor function in SCI mice. The BMS score of mice in the inhibitor group was significantly higher than in the SCI group according to the BMS evaluation after observation.

biological activity of IL-18 BP. The application of miR-92b-5p inhibitor can effectively alleviate the secondary inflammatory response after SCI, thus increasing the number of surviving neurons.

In summary, miR-92b-5p is a potential therapeutic target for improving the neurological recovery after SCI, and interference in its expression can alleviate the inflammation, thus exerting a neuroprotective effect. Therefore, the application of miR-92b-5p inhibitor can promote the recovery of motor function after SCI.

Conclusions

We revealed that miR-92b-5p inhibitor can effectively inhibit the effect of IL-18, alleviate the inflammatory effect, save and protect the neurons by reducing the interference in IL-18 BP after SCI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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