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MicroRNA-125b promotes the regeneration and repair of spinal cord injury through regulation of JAK/STAT pathway

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Abstract. – OBJECTIVE: Spinal cord injury (SCI) is a severe trauma to the central nervous system. Long non-coding RNAs have been reported to play essential roles in spinal cord injury. This study mainly explored the role of micro-125 in the regulation of spinal cord injury by regulating STAT3.

MATERIALS AND METHODS: The stable mouse model of cervical spinal cord contusion was established by Infinite Horizon spinal cord striker, and the model mice' motor function was analyzed. Bioinformatics databases were used to screen the target mRNAs of micro-125b. qRT-PCR was performed to detect the expression of micro-125b and its target genes in injury area of mice' spinal cord. Western Blot and ELISA were introduced to detect the expression of inflammation and apoptosis-related proteins in each group. The recovery status of spinal cord after SCI was assessed by motor function scores and axon counts of mice in each group.

RESULTS: Micro-125b appeared to be significantly down-regulated over-time after SCI. JAK1 and STAT1, two important neuregulin proteins, were predicted to be the target genes of micro-125b, and overexpression of micro-125b induced the decrease of phosphorylated JAK1 and STAT1. Enhanced micro-125b expression also allowed axons from the injury area of spinal cord to extend into the outer periphery of the damaged area, thus improving the motor function of the injured rats. Besides, overexpression of micro-125b demonstrated significant neuronal protective effects by reducing apoptosis and inflammatory responses in neurons.

CONCLUSIONS: Our data revealed that micro-125b was down-regulated in injured spinal cord, and overexpression of micro-125b promoted the repair and regeneration following spinal cord injury through the regulation of the JAK/STAT pathway.

Key Words:
microRNA-125b, Spinal cord injury, Axon regeneration.

Introduction

Spinal cord injury (SCI) is a kind of central nervous system disease with high incidence, mortality, morbidity and cost[1-3]. With the rapid development of society, economy and traffic construction, the incidence of SCI was increasing year by year. About 10,000 new cases of SCI patients were reported in the United States each year[4]. The nature of the violence at the time of the injury determined the extent of primary injury, and followed by secondary damage including free radical attack, apoptosis, inflammatory response and so on. However, the specific molecular mechanisms underlying this process and how to mitigate and reverse secondary damage are still not clear. Tissue repair and functional reconstruction after SCI are very difficult not only because of the weak regenerative capacity of the neurons, but also because they are associated with the scars formed after SCI[5].

With the development of molecular biology and the completion of the Human Genome Project, more and more attention has been paid to the research of microRNAs. Accumulated evidence indicates that abnormal microRNA expression occurred after SCI[5-8]. Previous study has showed that microRNAs were involved in the regulation of gene expression, which is closely related to pathological processes such as ischemic edema of spinal cord, inflammatory reaction and neu-

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ronal necrosis[8]. In addition, microRNAs were also reported to play important roles in the development and plasticity of spinal cord, some of which might be effective therapeutic targets to SCI[9-11]. Therefore, elucidating the function of microRNAs in the process of SCI not only can further clarify the pathogenesis of secondary SCI, but also provide new therapeutic targets and intervention strategies for the treatment and rehabilitation of SCI. There are three members of microNA-125 family: hsa-micro-125a, hsa-micro-125b-1 and has-micro-125b-2. Among them, hsa-micro-125b-1 and has-micro-125b-2 are located on chromosomes 11q23 and 21q21, respectively[12]. Micro-125b is a non-coding, multi-functional small RNA molecule, which plays a vital role in many processes such as cell proliferation, differentiation and apoptosis by down-regulating target mRNAs' levels or inhibiting their expression at the transcriptional level[13]. It has been reported that the micro-125 family participated in cellular physiology through the regulation of different transcription factors, matrix metalloproteinases and growth factors[14,15]. Ferretti et al[16] discovered that micro-125b inhibited tumor cell proliferation and promoted normal cell differentiation through Hedgehog signaling pathway by targeting the Smo gene. Yu et al[17] also found that micro-125b directly bound to the 3'UTR region of p53 and thus downregulated the expression level of its protein. At present, researches about micro-125b mainly focused on various types of tumors, such as thyroid cancer and breast cancer[18,19]. However, no research to date has emerged about the dysregulation of micro-125b in the SCI. In this study, the mouse SCI model was established to explore the role of micro-125b in SCI.

Materials and Methods

C5 Spinal Cord Contusion Model

A total of 30 mice were divided into two groups: injured group (15) and sham group (15). Mice in the injury group were subjected to C5 spinal blunt contusion, i.e. SCI model was established by 80 Kdyn dose acute spinal cord injury by Infinite Horizon spinal impact instrument. Mice in the sham group underwent simple laminectomy. Three rats in each group were selected for behavioral analysis, and motor function of mice was assessed before injury, on 1 day and 3 days after spinal cord injury, and 1-8 weeks weekly after operation. Spinal cord samples were collected from the mice sacrificed at 1 day, 3 days, 7 days and 14 days after SCI to analyze the expression of micro-125b. This study was approved by the Animal Ethics Committee of the Animal Center of Huai'an First People's Hospital.

Griping Strength Meter (GSM)

This GSM method was improved based on Khaing's and Anderson's studies[20,21]. Measurement of the two forelimbs grip force, left and right single forelimb grip force were repeated for 4 times. To ensure the accuracy of measurement results, adequate rest should be given to mice when they were tired during the measurement.

Real-time Quantitative PCR (qRT-PCR)

Total RNA of the spinal cord samples was extracted by using TRIzol (purchased from Invitrogen, Carlsbad, CA, USA) in accordance with the experimental instructions. After reverse transcription, polymerase chain reaction was performed to amplify the cDNA. The sequences of the primers are as follows: micro-125b (Forward) 5'-AGUGUUCAAUCCCAGAGUCCCU-3', micro-125b (Reverse) 5'-GGGGACTCTGGGATTGAACACT-3'; GAPDH (Forward) 5'-AGGAGCGAGATCCCGCCAACA-3', GAPDH (Reverse) 5'-CGGCCGTCACGCCACATCTT-3'.

Western Blot Analysis

Total protein was extracted from the spinal cord tissue by radioimmunoprecipitation assay (RIPA) lysis solution. Polyacrylamide gradient gels (10%) were used to separate the different proteins which were then transferred to 0.22 um polyvinylidene difluoride (PVDF) membrane. All the membranes were incubated in blocking buffer (5% fat-free milk) prior to incubation with primary antibodies at 4°C overnight. After incubation with the corresponding secondary antibody, these protein bands were imaged according to the instructions. Anti-JAK, anti-STAT and anti-caspase primary antibodies were purchased from Cell Signaling Technology (Dilution 1:1000, Danvers, MA, USA). GAPDH was used as an internal reference.

Axon Counting

The axons number of each layer was denoted as N upper 1, N upper 2, N upper 3…N under 1, N under 2, N under 3, etc. Based on the average number of biotinylated dextran amines (BDA) labeled axons in each slice in the upper spinal cord injury zone, we calculated the relative value of

axon counts in each segment. The specific calculation methods are as follows: N upper = (N upper 1 + N upper 2 + N upper 3)/3; Relative count of any level: n = N/N upper.

ELISA

Spinal cord samples were collected and stained by using of ELISA kits according to the instructions. The absorbance value at 450 nm was read by microplate reader.

Statistical Analysis

All data were analyzed by statistical product and service solutions (SPSS) 22.0 (IBM, Armonk, NY, USA) and expressed as the means ± SD of at least three independent experiments. All the statistic charts were made by GraphPad Prism 5.0 (Version X; La Jolla, CA, USA). Independent-samples *t*-tests between two groups were performed for statistical test. $p < 0.05$ was considered significant.

Results

Decreased Mouse Grip and Micro-125b Expression in Mouse SCI Model

In the Sham group, the grip strength of mice decreased temporarily in the early postoperative period, and recovered to the preoperative level in 1 week after surgery. In the injured group (SCI group), mice's motor function was severely impaired and unable to grasp within 1 week after surgery; 7 d-14 d after surgery, the grip value of SCI mice was improved, but still in a very low level. At 21 d-56 d, the grip strength of both forepaw and claw was improved slightly with time. Besides, the grip strength of mice in the injury group was significantly lower than that of the sham group at each time point after operation (Figure 1A-C).

In the Sham group, the expression of micro-125b in the injured spinal cord was maintained at a stable level ($p > 0.05$). The expression

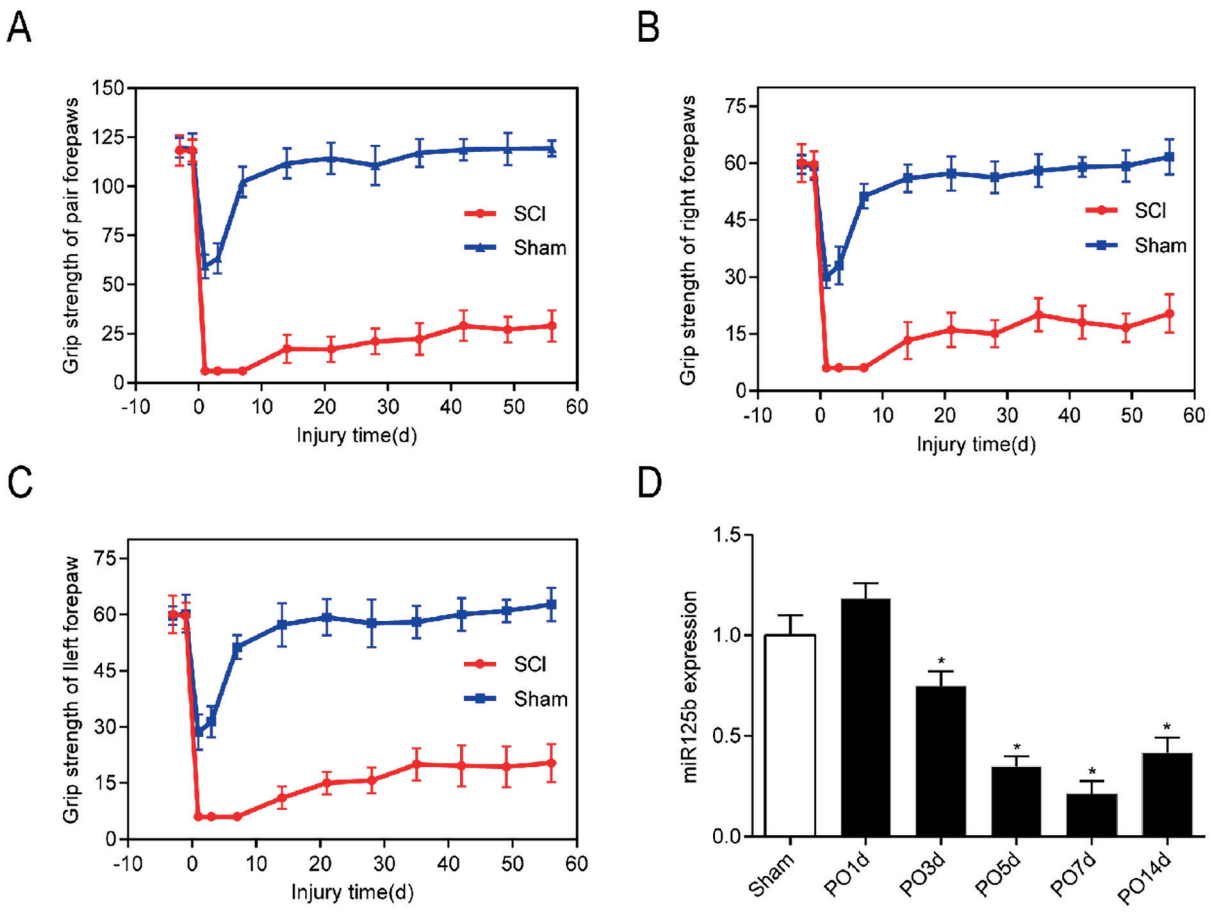

Figure 1. Decreased mouse grip and micro-125b expression in mouse SCI model. ***A, B,*** and ***C,*** Grip measurements of mice in each group. ***D,*** The expression of micro-125b in each group was detected by qRT-PCR.

of micro-125b in SCI group slightly increased on the 1st day after injury, but no significant difference was observed when compared to sham group ($p > 0.05$). However, the expression of micro-125b in the spinal cord of SCI mice started to show a marked decrease on the 3rd day after injury and reached the lowest value on the 7th day after injury (Figure 1C).

Overexpression of Micro-125b Reduced the Expression of JAK1/STAT1

After spinal cord injury, the expression level of micro-125b in the injured area of mice's spinal cord reduced significantly in each group. The expression of micro-125b in the spinal cord of micro-125b-overexpressed mice was significantly higher than that in SCI and Ng groups ($p < 0.05$), and reached the peak on the 3rd day after operation (Figure 2A).

JAK1 and STAT1 were predicted to be the target genes for micro-125b by using bioinformatics databases. Our results showed that the levels of P-JAK1 and P-STAT1 mRNA were significantly higher in micro-125b group than those in SCI group. However, there was no difference between the SCI group and Ng group in the content of P-JAK1 and P-STAT1 mRNA (Figure 2B-C). Consistent with mRNA expression, the protein levels of P-JAK1 and P-STAT1 in micro-125b group gradually increased over time and reached the highest point at 7th day after surgery, while the total protein level of JAK1 and STAT1 changed little. No difference was observed between the SCI group and Ng group in the content of P-JAK1 and P-STAT1 protein (Figure 2D-E).

Micro-125b Promotes the Repair and Regeneration of Injured Spinal Cord in Mice

In the micro-125b group, the function of forepaws of some mice began to recover from the 7th day after injury, and the grip strength was also

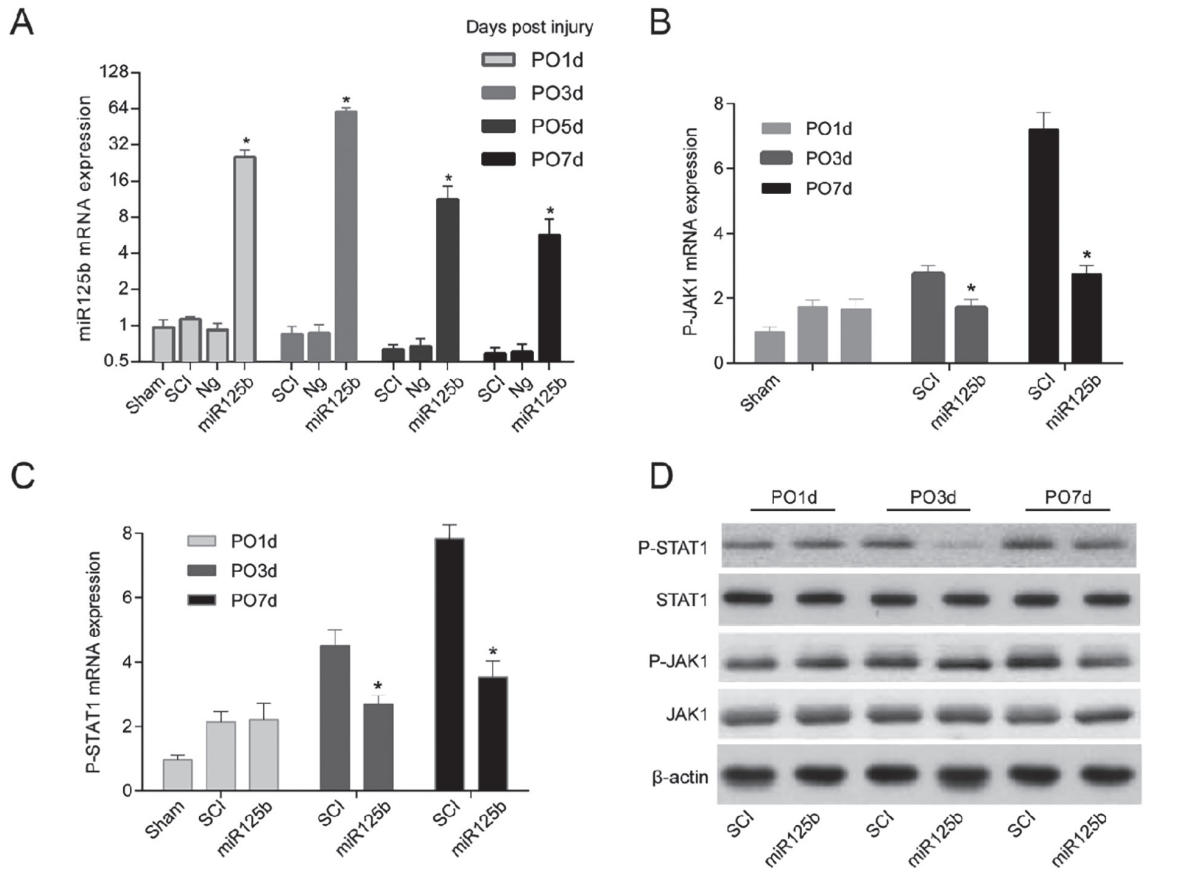

Figure 2. Overexpression of micro-125b reduced the expression of JAK1/STAT1. ***A,*** The expression level of micro-125b in the injury area of mice's spinal cord in each group. ***B,*** and ***C,*** Effect of overexpression of micro-125b on the mRNA expression of JAK1/STAT1 in injured area of spinal cord in mice. ***D,*** Effect of micro-125b overexpression on the protein expression of JAK1/STAT1 in injured area of spinal cord in mice.

improved with time. At 28 d-56 d after injury, the grip strength of mice in micro-125b group was markedly improved when compared with mice in SCI group but still significantly lower than mice in the simple injury group (Figure 3A-C). Besides, the relative counts of axons in the lower segment of each lesion in the SCI group had no difference with that of Ng group, while the axons counts of micro-125b group were significantly higher than that of simple injury group (Figure 3D).

Micro-125b Inhibited the Inflammatory Response and Apoptosis After Spinal Cord Injury in Rats

ELISA assay results showed that compared with Sham group, the MPO concentration in SCI group increased significantly, and the MPO concentration in micro-125b group was also markedly lower than that in SCI group (Figure 4A). In addition, protein levels of MCP-1 and Caspase 3 in SCI group were significantly higher than those in Sham group. However, overexpression of micro-125b decreased the expression of MCP-1 and Caspase 3 (Figure 4B-C).

Discussion

Patients with spinal cord injury may develop severe nerve damage and dysfunction[22,23]. Pathological manifestations of acute mechanical injury in spinal cord include acute spinal cord ischemia and secondary injury, which involve ischemia, calcium and sodium ion-mediated cell injury, cell death, inflammatory response and cell death[24,25]. As an evolutionarily protective pathway of eukaryotes, JAK/STAT signaling pathway is closely associated with cell growth, survival, development and differentiation[26].

JAK/STAT signal pathway is a common pathway for many kinds of cytokines and growth factors to transmit signals in cells and mediates various biological reactions including cell prolif-

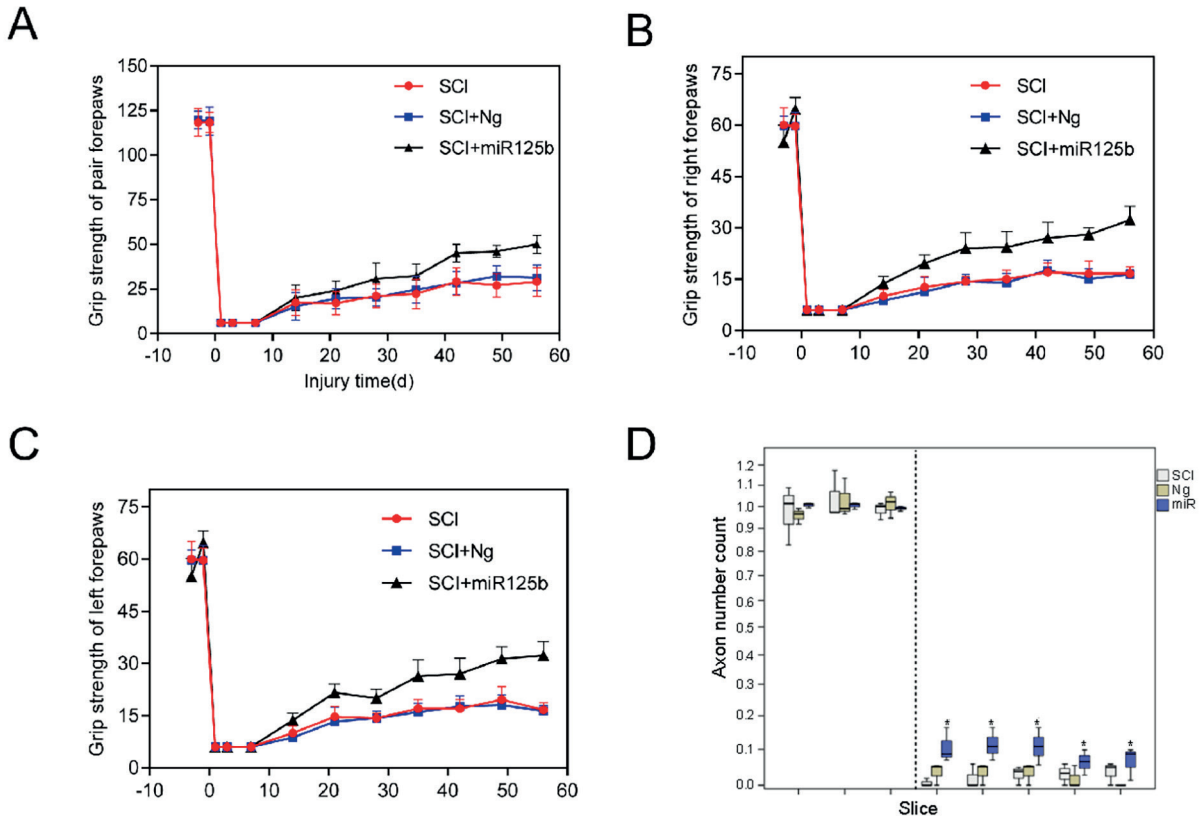

Figure 3. Micro-125b promotes the repair and regeneration of injured spinal cord in mice. ***A,*** The changes of the grip power of the forelimbs in mice at different time points after spinal cord injury in each group. ***B,*** Changes in the right forelimb grip strength of mice at different time points after spinal cord injury in each group. ***C,*** Changes in the left forelimb grip strength of mice at different time points after spinal cord injury in each group. ***D,*** Effect of micro-125b overexpression on axonal count in the inferior segment of spinal cord injured mice.

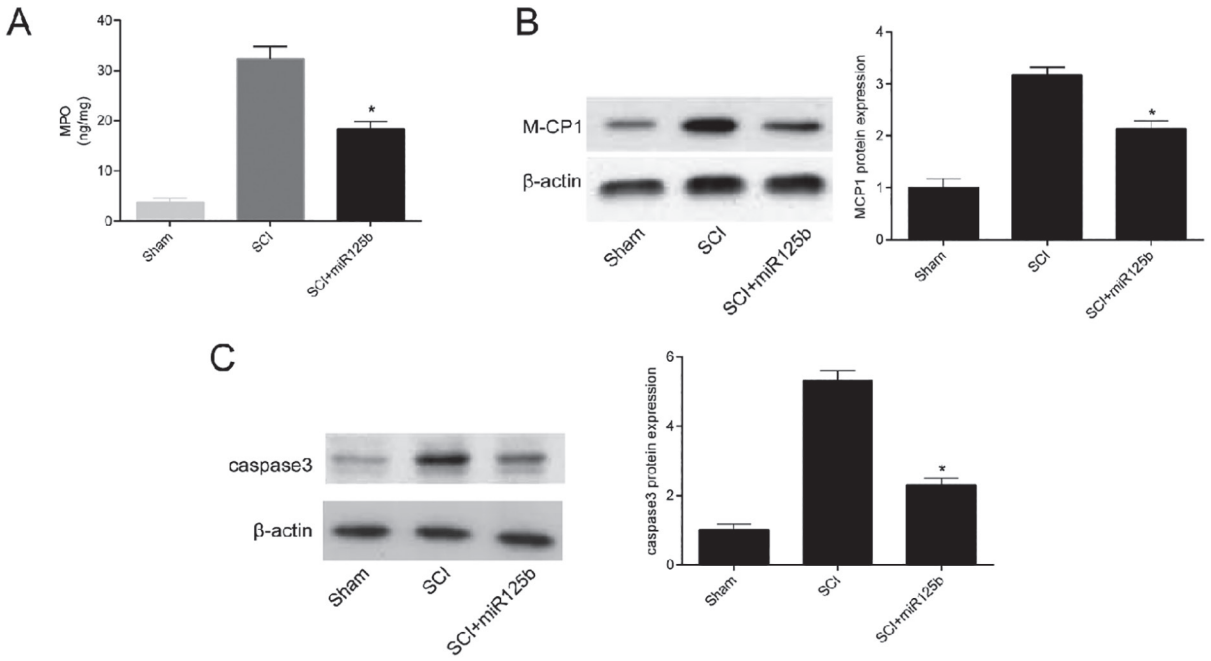

Figure 4. Micro-125b inhibited the inflammatory response and apoptosis after spinal cord injury in rats. ***A,*** Effect of micro-125b overexpression on inflammatory reaction (MPO) in spinal cord injury mice. ***B,*** Effect of micro-125b overexpression on inflammatory response related protein (MCP-1) in spinal cord injury mice. ***C,*** Effects of micro-125b overexpression on apoptosis related protein (Caspase-3) in spinal cord injury mice.

eration, differentiation, migration, apoptosis and immune regulation[27]. JAK/STAT signaling pathway is directly related to the neuronal growth and glial scar formation in the injury area of spinal cord. The activation and overexpression of STAT3 are important for neuronal protection after axon injury. Peptides like growth factors, hormones and cytokines can cause the activation of JAK by interacting with cell membrane receptors[28,29]. Studies also showed that STAT3 was activated in reactive astrocytes in the injured area[30,31].

Microarray studies have identified a number of microRNAs that are highly differentially expressed in the mouse spinal cord[11,32,33]. A large number of studies on the dysregulation of microRNAs after spinal cord injury have confirmed that microRNAs were involved in the regulation of various signaling pathways such as inflammatory response, apoptosis and inhibition of axon regeneration after spinal cord injury[5,13,33-38]. Micro-125b has been reported to play an important role in the maintenance of autonomic repair of damaged spinal cord in adult zebrafish. Therefore, the expression changes of micro-125b in spinal cord injury of mammals may be related to the inhibition of axonal regeneration. Many models have been developed for regenerative studies of spinal cord injury repair. A modified C5 spinal cord blunt contusion model was used in our study. By using the Infinite Horizon Spinal Strike, we can precisely control the impact dose to maximize the nature of clinical spinal cord injury[39]. Two evaluation systems were also introduced to this study: Forelimb Motor Function Score (FLS) and Forelimb Grip Measurement (GSM). FLS was used by assessing the posture of the mouse forelimb during exercise and walking[40-42]. In this study, micro-125b was significantly down-regulated over time after spinal cord injury. Exogenous micro-125b mimic can be efficiently taken up by damaged tissues by local injection and enters the metabolic chain to generate micro-125b. Bioinformatics databases were used to screen micro-125b's target genes, which were related to nerve growth and apoptosis including JAK1 and STAT1. QRT-PCR and Western Blot were performed to detect the expression of related target genes in each group; results showed that overexpression of micro-125b could reduce the phosphorylation levels of JAK1 and STAT1, two important nerve growth regulatory proteins. All above results suggested that micro-125b may be involved in the process of regeneration and

repair of spinal cord through the regulation of JAK1/STAT1 pathway after spinal cord injury. In addition, enhanced micro-125b expression could cause axons from injury area to penetrate into the periphery of the damaged area, reduce the apoptosis of neurons and the formation of inflammatory reaction, and eventually improve the motor function of spinal cord injured mice.

Conclusions

Our data revealed that micro-125b plays an important regulatory role in axonal regeneration and repair of spinal cord by directly targeting JAK1/STAT1 signaling pathway, which makes micro-125b a new potential therapeutic target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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