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LncRNA ZNF667-AS1 inhibits inflammatory response and promotes recovery of spinal cord injury via suppressing JAK-STAT pathway

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Abstract. – OBJECTIVE: The aim of this study was to explore the role of lncRNA ZNF667-AS1 in the recovery of spinal cord injury (SCI), and to investigate its underlying mechanism.

MATERIALS AND METHODS: Mice were randomly assigned to the SCI group, the sham group and the lncRNA ZNF667-AS1 group, with 10 mice in each group. With Infinite Horizon device at a dose of 80 Kdyn, mice in the SCI group and the lncRNA ZNF667-AS1 group experienced SCI by an acute hit on the C5 spinous process. Before animal procedures, mice in the lncRNA ZNF667-AS1 group were additionally injected with overexpression lentivirus of lncRNA ZNF667-AS1. On the contrary, mice in the sham group only received laminectomy. After successful construction of the SCI model in mice, grip strength was accessed. LncRNA ZNF667-AS1 expression in spinal cord tissues before and after SCI was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), respectively. Meanwhile, the protein expression levels of relative genes in Janus Kinase-signal transducer and activator of transcription (JAK-STAT) pathway were detected by Western blot.

RESULTS: Grip strength of forelimb in the SCI group recovered significantly slower than that of the sham group. With the prolongation of SCI, the expression of lncRNA ZNF667-AS1 was gradually decreased. However, the expression levels of JAK2, STAT3 and iNOS were upregulated in a time-dependent manner. In addition, mice in the lncRNA ZNF667-AS1 group presented remarkable grip strength recovery of forelimb after SCI.

CONCLUSIONS: LncRNA ZNF667-AS expression is gradually downregulated after SCI. Meanwhile, it inhibits the inflammatory response and promotes SCI recovery via suppressing the JAK-STAT pathway.

Key Words:
SCI, Inflammation, LncRNA ZNF667-AS1, JAK-STAT.

Introduction

Spinal cord injury (SCI) is a common disease resulted from various pathogenic factors[1]. SCI leads to sensory dyskinesia in the corresponding segment, and its prognosis is relatively poor. Particularly, cervical SCI accounts for 54% of all SCI cases, which is more severe and may eventually affect upper limb function. Therefore, SCI poses great physical and economic burdens on affected people and their families.

Studies have shown that primary damage at the early stage of SCI leads to a large number of neuron loss. Subsequently, secondary damage causes neuronal apoptosis and irreversible loss of nerve cells. Moreover, the formation of nerve scars resulted from keratinocyte regeneration also hinders the growth of nerve fibers[2,3]. In recent years, relative studies have indicated that endogenous factors may play a greater role in nerve repair after SCI[4].

LncRNA is a type of non-coding RNA with more than 200 nt in length[5]. It has been shown that lncRNA regulates gene expression at transcriptional and post-transcriptional level, thereby participating in multiple biological functions[6]. Evidence has proved that differentially expressed lncRNAs are involved in many diseases, such as tumors and neurodegenerative diseases[7,8]. Currently, studies have indicated that lncRNA ZNF667-AS1 can inhibit the proliferation of cervical cancer cells, which can serve as a prognostic hallmark for cervical cancer[9]. In the present study, we detected the expression level of lncRNA ZNF667-AS1 in mice with SCI. Furthermore, we assessed the regulatory effect of lncRNA ZNF667-AS1 on SCI recovery. Our study provides an experimental

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basis for improving SCI recovery. Meanwhile, lncRNA ZNF667-AS1 may be a potential therapeutic target.

Materials and Methods

Construction of the SCI Model in Mice

30 female C57BL/6 mice with 8 to 10 weeks old were obtained from the Model Animal Research Center of Qingdao University. This study was approved by the Animal Ethics Committee of Qingdao University Animal Center. Mice were randomly assigned into the SCI group, the sham group and the lncRNA ZNF667-AS1 group, with 10 mice in each group. Before animal procedures, mice in the lncRNA ZNF667-AS1 group received a subdural injection of overexpression lentivirus of lncRNA ZNF667-AS1 (1×10^7 TU). Mice in the SCI group and the lncRNA ZNF667-AS1 group underwent SCI, whereas those in the sham group only received laminectomy. Briefly, mice were cut open alongside the neck after anesthesia. After dissecting the skin and fascia, the C5 spinous process was hit by using Infinite Horizon device at a dose of 80 Kdyn and removed under a microscope. Subsequently, the incision was sutured layer by layer. Mice were anesthetized, and lesioned spinal cord tissues were harvested after animal procedures. Collected tissue samples were preserved at -80°C for the following experiments. Lentiviruses used in this study were constructed by Gene Pharma (Shanghai, China).

Griping Strength Meter (GSM)

Mice were gently held to make their tails brought to the bar of GSM. When their paws were grabbed in the bar, mice were pulled back quickly in the horizontal direction. Grip strength of the forelimb in mice was recorded when the grip was released. Meanwhile, grip strengths of the left or right forelimb were recorded, respectively. Totally four successful records were taken, and the average grip strength was calculated. The grip strength that mice could not grab in the bar was recorded as 0.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in impaired spinal cord tissues was extracted according to the instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was detected using a spectrometer and those samples with A260/A280 ratio of 1.8-2.0 were selected for the following qRT-PCR reaction. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), with 3 replicates in each group. QRT-PCR reaction parameters were as follows: denaturalization at 95°C for 60 s, extension at 95°C for 30 s, and annealing at 60°C for 40 s, for a total of 40 cycles. Relative gene expression was calculated by the $2^{-\Delta\Delta CT}$ method. U6 was used as the internal reference. Primer sequences used in this study were as follows: ZNF667-AS1, F: 5'-CAGGAACCTCCTTACTC-3', R: 5'-CTAGGGAGTCCGAAGGA-3'; JAK2, F: 5'-TCACCCACACCGTCAGCCGATTT-3', R: 5'- -CACCCATTCCCTTCACAGAGCAA-3'; STAT3, F: 5'-ACCCAACAGCCGCCGTAG-3', R: 5'-CAGACTGGTTGTTTCCATTCAGAT-3'. U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3',R:5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot

Total protein 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, 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). Then the membranes were routinely incubated with primary antibodies at 4° C overnight (diluted in 1: 500). After washing with Tris-buffered with Saline-Tween 20 (TBST), the membranes were incubated with the corresponding secondary antibody (diluted in l: 1000) at room temperature for 1 h. Immuno-reactive bands were exposed by the enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 software was used for all statistical analysis (IBM, Armonk, NY, USA). Continuous variables were expressed as mean ± standard deviation. Independent sample *t*-test was used

to compare the differences between two groups. $p<0.05$ was considered statistically significant.

Results

Recovery Obstacle of Forelimb Grip Strength after SCI

The expression level of lncRNA ZNF667-AS1 in mouse spinal cord tissues of the SCI group was detected on the postoperative 1st, 3rd, 5th, 7th, and 10th day by qRT-PCR, respectively. Results showed that with the prolongation of SCI, the expression level of lncRNA ZNF667-AS1 was gradually downregulated (Figure 1A). Grip strengths of forelimbs in mice were detected on the preoperative 3rd, 2nd, and 1st day, as well as the postoperative 1st, 3rd, 7th, 14th, 21st, 28th, 35th, and 42nd day, respectively. The behavioral test elucidated the recovery of forelimb grip strength in both the SCI group and the sham group after animal procedures. However, the recovery of grip strength in the SCI group was remarkably slower than that of the sham group (Figure 1B-1D).

JAK-STAT Pathway was Activated after SCI

We detected the expression levels of relative genes in the JAK-STAT signaling pathway on the postoperative 1st, 3rd, 5th, 7th, and 10th day, respectively. QRT-PCR results demonstrated that within the first 7 days after animal procedures, the mRNA levels of JAK2 and STAT3 were significantly upregulated in a time-dependent manner (Figure 2A and 2B). Western blot results also found the protein expression levels of JAK2, STAT3 and iNOS were remarkably upregulated after SCI (Figure 2C and 2D).

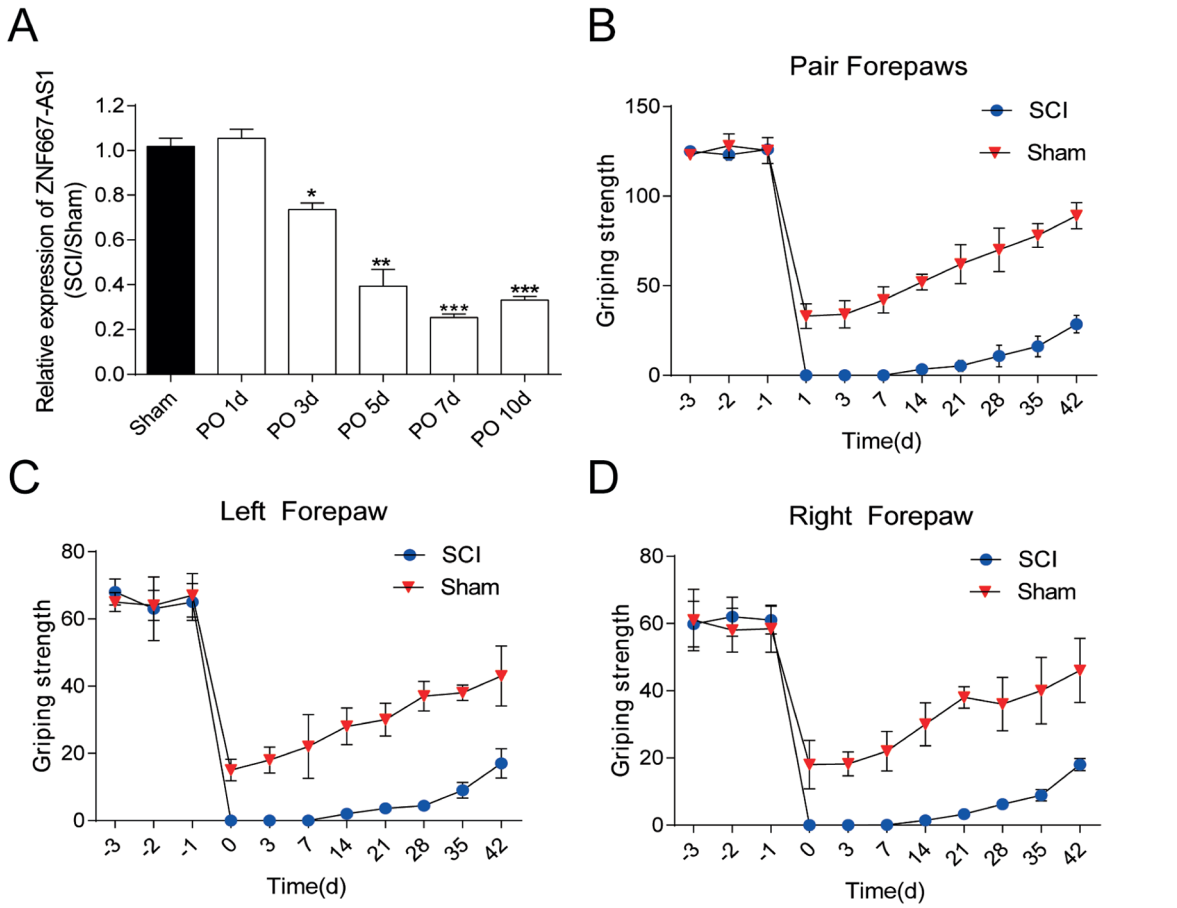

Figure 1. Recovery obstacle of forelimb grip strength after SCI. ***A,*** LncRNA ZNF667-AS1 expression was downregulated with the prolongation of SCI. ***B,*** Forelimb grip strength in the SCI group was lower than that of the sham group. ***C,*** Grip strength recovery of left forelimb in the SCI group was worse than that of the sham group. ***D,*** Grip strength recovery of right forelimb in the SCI group was worse than that of the sham group.

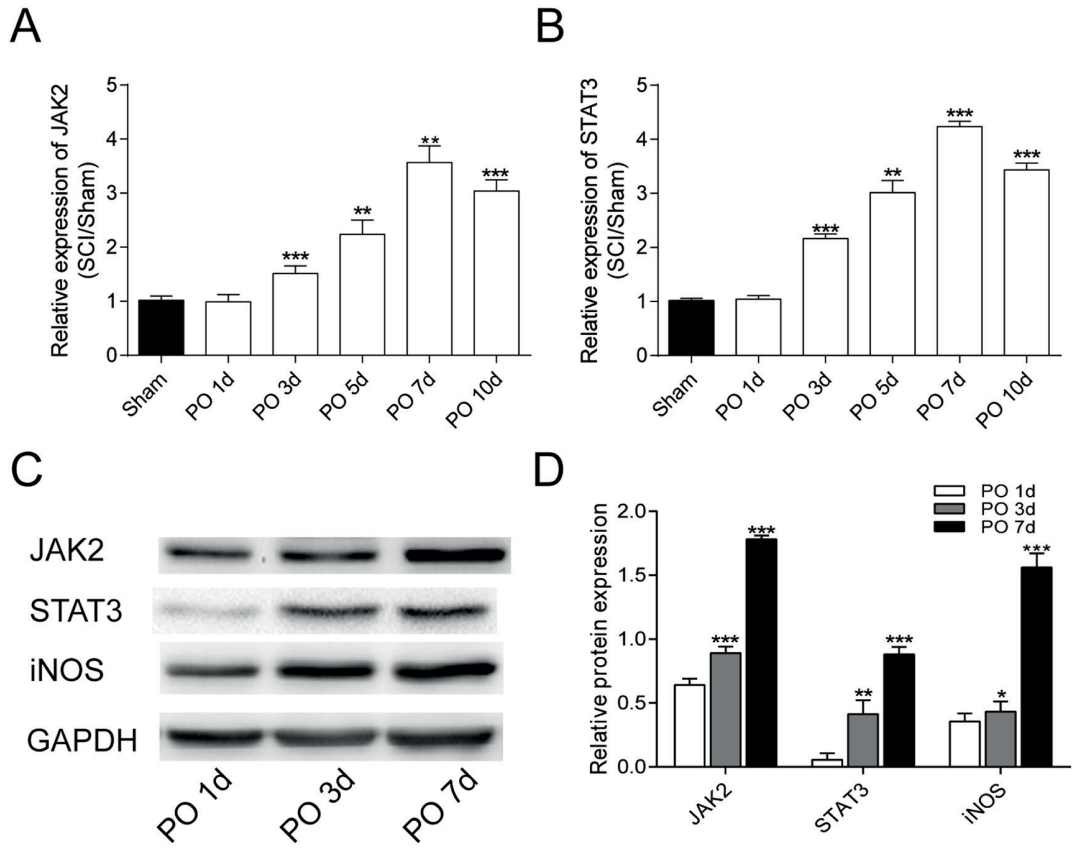

Figure 2. JAK-STAT pathway was activated after SCI. ***A,*** JAK expression in spinal cord tissues was remarkably upregulated with the prolongation of SCI. ***B,*** STAT3 expression in spinal cord tissues was upregulated with the prolongation of SCI. ***C*** and ***D,*** Protein expression levels of JAK2, STAT3 and iNOS in spinal cord tissues were significantly upregulated with the prolongation of SCI.

LncRNA ZNF667-AS1 Inhibited Inflammatory Response via JAK-STAT Pathway

Subsequently, we detected the expression level of lncRNA ZNF667-AS1 in the spinal cord tissues of the SCI group, the sham group and the lncRNA ZNF667-AS1 group by qRT-PCR. Results indicated that lncRNA ZNF667-AS1 expression was remarkably upregulated in the lncRNA ZNF667-AS1 group than that of the sham group and the SCI group on the postoperative 1st, 3rd, 5th, and 7th day (Figure 3A). Subsequent experiments also found that the postoperative mRNA levels of JAK2 and STAT3 in the lncRNA ZNF667-AS1 group were significantly lower than those of the SCI group (Figure 3B and 3C). Similarly, lower protein expression levels of JAK2, STAT3 and iNOS were observed in the lncRNA ZNF667-AS1 group when compared with those of the SCI group within the 7 days after SCI (Figure 3D).

LncRNA ZNF667-AS1 Promoted SCI Recovery

The behavioral test showed remarkable alleviation of the forelimb grip strength in the lncRNA ZNF667-AS1 group than that of the sham group and the SCI group (Figure 4A-4C). These findings suggested that lncRNA ZNF667-AS1 overexpression promoted SCI recovery.

Discussion

SCI is a complex and dynamic pathological process, involving multiple functional changes in the central nervous system. SCI gradually develops from primary injury to secondary injury, which may last for several months[10]. Traumatic SCI results from mechanical injuries, such as external force induced spinal cord compression, pulling, tearing or cutting[11]. Secondary injury

is a series of pathological processes secondary to primary injury[12,13]. During the progression of chronic injury, neuronal apoptosis and glial cells further deteriorate SCI[14]. Several months and even years after SCI, neuron death, scar formation, gliosis, and cavity formation all affect SCI recovery[15]. So far, no effective measures have been developed to cure SCI completely.

Recently, SCI mouse model has been frequently applied for investigating the potential mechanism of SCI. Compared with the neuron injury cell model, SCI mouse model is capable of simulating the microenvironment of the spinal cord. Meanwhile, the international standardized animal spinal cord impact device has been developed for *in vivo* animal models[16]. The device exerts the advantages of high repeatability and small trauma. In the present study, we first established the SCI model in mice. In addition, the behavioral test was performed to access neuronal injury recovery after SCI.

LncRNAs do not encode proteins themselves, but have specific secondary structures that are temporal and spatial in regulating gene expression[17]. Some studies have shown that lncRNA can regulate gene expression at epigenetic, transcriptional and post-transcriptional level. Meanwhile, lncRNAs participate in the process of species evolution, embryo development, material metabolism and disease development[18]. Researches have pointed out the crucial role of lncRNAs in the normal development of the central nervous system, especially in neuronal differentiation, brain development and synaptic plasticity[19]. Unlike other damaged tissues, nervous system damage cannot be regenerated after injury.

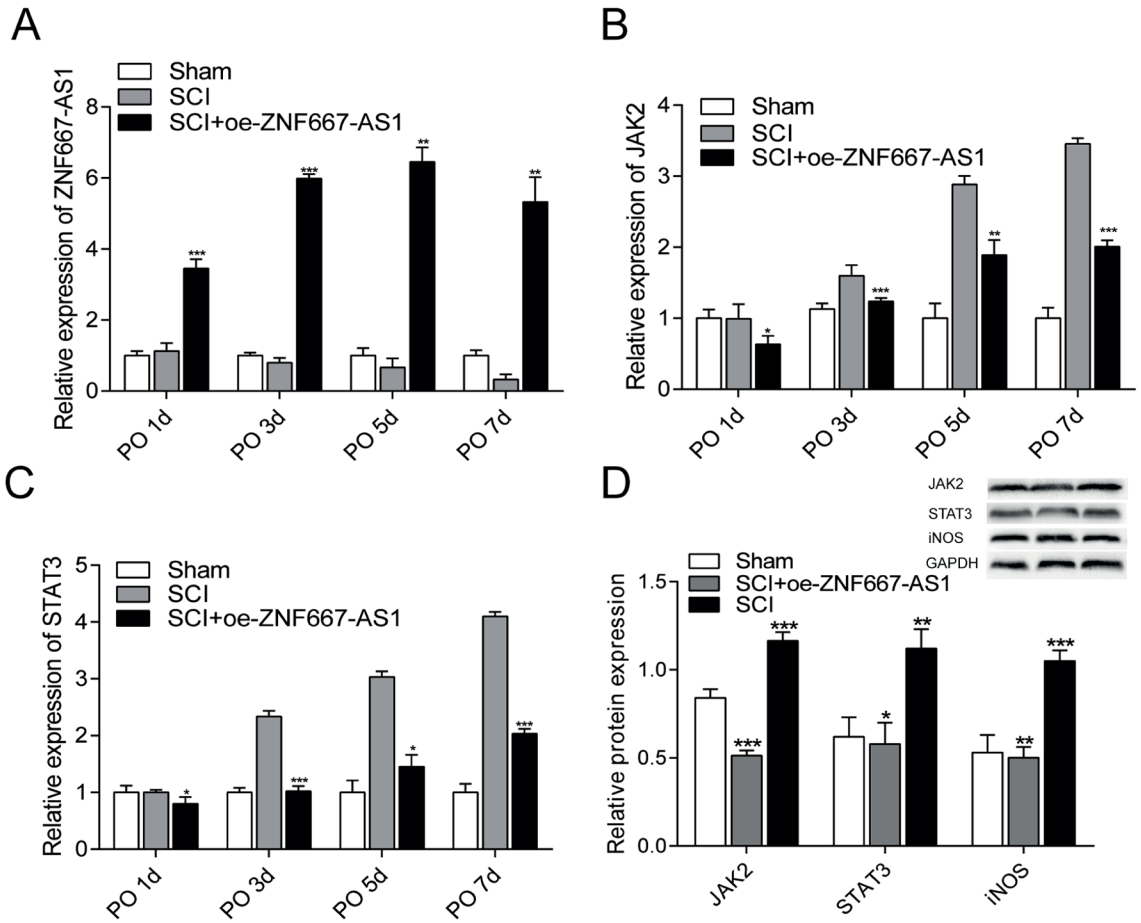

Figure 3. LncRNA ZNF667-AS1 inhibited inflammatory response via JAK-STAT pathway. ***A,*** LncRNA ZNF667-AS1 expression in mouse spinal cord tissues. ***B,*** The mRNA level of JAK2 in the lncRNA ZNF667-AS1 group. ***C,*** The mRNA level of STAT3 in the lncRNA ZNF667-AS1 group. ***D,*** Protein expression levels of JAK2, STAT3 and iNOS in mouse spinal cord tissues of the lncRNA ZNF667-AS1 group.

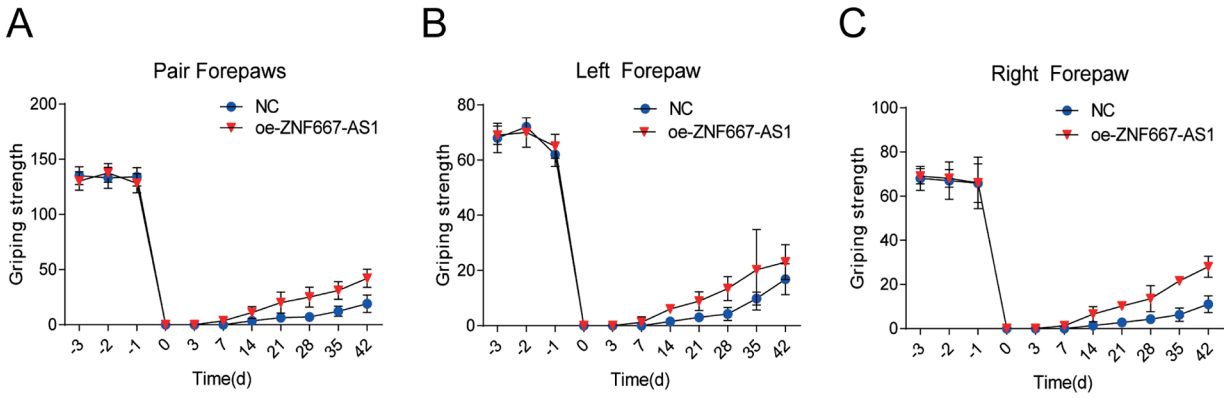

Figure 4. LncRNA ZNF667-AS1 promoted SCI recovery. ***A-C***, Grip strength of forelimbs (A), left forelimb (B) and right forelimb (C) of mice in the lncRNA ZNF667-AS1 group.

It has initially been believed that exogenous factors are the major reasons for the non-regeneration of the axon, such as inhibitory factors and glial scar in the damaged area. Subsequent studies have also proved that only a small amount of axons are regenerated after eliminating the exogenous factors, indicating their crucial role of in SCI[20]. The JAK-STAT signaling pathway is involved in multiple important biological processes, including cell proliferation, differentiation, apoptosis and immune regulation. Some investigations[21,22] have shown that the JAK-STAT pathway is closely related to spinal cord neuron death, spinal cord ischemia and others. Multiple studies have found that the JAK-STAT signaling pathway is activated after SCI, serving its biological function mainly through affecting the phosphorylation levels of JAK and STAT[22]. Meanwhile, the JAK-STAT pathway can also be activated during astrocyte proliferation and glial scar formation after SCI. In the acute phase of SCI, phosphorylated STAT in neurons plays a protective role in nerves. With the progression of injury, STAT is phosphorylated in microglial and astrocytes in the chronic phase of SCI, eventually protecting impaired nerves[23].

In the present study, we first observed that the expression level of lncRNA ZNF667-AS1 was gradually decreased during SCI recovery, which might involve the JAK-STAT pathway. By constructing the SCI mouse model, mice injected with overexpression lentivirus of lncRNA ZNF667-AS1 presented significantly better recovery after SCI. Our findings suggested that lncRNA ZNF667-AS1 might serve as a therapeutic target for the improvement of SCI recovery.

Conclusions

We demonstrated that lncRNA ZNF667-AS expression is gradually downregulated after SCI, which inhibits inflammatory response and promotes SCI recovery *via* suppressing JAK-STAT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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