

MicroRNA-409 promotes recovery of spinal cord injury by regulating ZNF366

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Abstract. – OBJECTIVE: To explore the role of microRNA-409 in spinal cord injury (SCI) recovery and its underlying mechanism.

MATERIALS AND METHODS: The mouse SCI model was first established, and the difference in recovery of grip strength was detected. MicroRNA-409 expressions in mice tissues from sham operation group and SCI group were detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). We further increased microRNA-409 level in mice spinal cord by plasmids transfection. Luciferase activity was detected to verify the direct binding of microRNA-409 and ZNF366.

RESULTS: The grip strength in both groups was temporarily decreased after surgery, while the grip strength in SCI group was always remarkably lower than that of the sham operation group since the first week after the surgery, suggesting the successful construction of mouse SCI model. MicroRNA-409 expression in the SCI group was gradually decreased from the postoperative 3rd day, which was remarkably lower than that of the sham group. Additionally, ZNF366 expression in the SCI group began to increase from the first day after the surgery, which was markedly higher than that of the sham group. After injection of exogenous microRNA-409, ZNF366 expression in the SCI group showed a remarkable decrease compared to that of the sham operation group. We also confirmed that ZNF366 was the target gene of microRNA-409 by bioinformatics analysis and luciferase activity assay.

CONCLUSIONS: MicroRNA-409 is downregulated after spinal cord injury. Overexpression of microRNA-409 directly targets ZNF366 and promotes the recovery of spinal cord injury.

Key Words:

Spinal cord injury, MicroRNA-409, ZNF366.

Introduction

Spinal cord injury (SCI) is characterized by high morbidity and poor performance, which se-

riously leads to permanent disability in some patients. Recently, the incidence of SCI in China has been increasing annually with over 60,000 newly reported cases each year. Although researches have been made on SCI, there is no effective treatment for SCI till now¹. In 1997, Li et al² used olfactory ensheathing cell transplantation to treat spinal cord injury in rats and obtained hindlimb exercise recovery. In 2000, Nogo, a myelin inhibitory protein found by Chen et al³ was considered to be able to cure SCI. However, no substantial breakthrough has been achieved so far in the treatment of SCI. Therefore, it is of great significance to explore the mechanism of SCI from various perspectives.

MicroRNAs (miRNAs) are a new class of non-coding single-stranded small RNAs. MiRNAs negatively regulate gene expressions at the post-transcriptional level by binding to the 3'UTR of the mRNAs, thus resulting in translational inhibition or mRNA degradation⁴. MiRNA is overexpressed in the central nervous system, especially in spinal cord tissue, which exerts a crucial role in nerve regeneration, neural development, and cellular response in synaptic plasticity⁵. Based on the powerful post-transcriptional regulation of miRNAs, they are thought to be important regulators of secondary injury in spinal cord⁶.

Accumulating studies^{7,8} have revealed the spatiotemporal expression changes of various unknown miRNAs after SCI. The target genes regulated by miRNAs play vital roles in the pathogenesis of apoptosis and regeneration of neurons, glial scar formation, and microglial activation. Thus, we suggested that differentially expressed miRNAs after SCI were closely related to the pathological processes of SCI. Further in-depth studies of miRNAs are necessary for investigating therapeutic intervention after SCI.

Materials and Methods

Construction of Mouse Spinal Cord Injury Model

Adult C57BL/6 mice weighing 20–25 g were randomly assigned to SCI group and control group. Grip strengths of both forelimbs were recorded 3, 2, and 1 days before SCI, as well as 0, 3, 7, 14, 21, 28, 35, and 42 days after SCI, respectively. Spinal cord samples were collected after SCI for 1, 3, 7, and 14 days, respectively.

For the construction of mouse SCI model, mice were cut open alongside the neck after anesthesia. After the skin and fascia were dissected, the C5 spinous process was removed under a microscope. After the surgical procedure, the incision was then sutured by layers. This study was approved by the Animal Ethics Committee of Affiliated Hospital of Weifang Medical University Animal Center.

Griping Strength Meter (GSM)

Mice were gently held so that their tails were brought to the bar of GSM. Mice were then pulled back quickly in the horizontal direction when their paws grabbed in the bar. Forelimb griping strength was recorded when the grip was released. Grip strengths of left or right forelimb were recorded, respectively. 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.

SCI Sample Collection

After mice were sacrificed, muscles and skins were cut open in layers, followed by unilateral laminectomy at C5. Spinal cord tissues were collected, extending 4 mm to the SCI area. Tissues were then placed in the 1.5 ml tube and preserved at -80°C for subsequent experiments.

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

50–100 g SCI tissues were selected for extraction of total RNA according to the instruc-

tions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was then performed based on the instructions of TaqMan MicroRNA kit (TaKaRa, Otsu, Shiga, Japan). The relative concentration was calculated by the $2^{-\Delta\Delta CT}$ method using U6 as the loading control. Primers used in this study were shown in Table I.

Dual Luciferase Reporter Gene

The 3'-UTR WT (wild-type) and MUT (mutant) plasmids of PGL3-ZNF366 were constructed based on a bioinformatics prediction website and were transfected according to the instructions of Lipofectamine 2000 (Thermo Fisher Scientific, Inc. Waltham, MA, USA). Cells were transfected with microRNA-409 overexpression + PGL3-Basic vector, microRNA-409 overexpression + 3'-UTR WT, or microRNA-409 overexpression + 3'-UTR MUT, respectively. Luciferase activities were detected according to the instructions of dual luciferase reporter gene kit.

Western Blot

The total protein was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Protein samples were then separated by 10% sodium dodecyl sulphate (SDS) protein electrophoresis after the concentration of each sample was adjusted to the same level. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane and routinely immunostained at 4°C overnight (diluted in 1:500) (Millipore, Billerica, MA, USA). Membranes were then incubated with the secondary antibody (1:1000) at room temperature for 1 h. All membranes were exposed by enhanced chemiluminescence method.

Statistical Analysis

Statistic package for social science (SPSS16.0, Chicago, IL, USA) software was used for statistical analysis. Continuous variables were shown as mean $\pm$ standard deviation. The independent sample *t*-test was used to compare the data between two groups. $p < 0.05$ indicated the difference was statistically significant.

Table I. qRT-PCR primer pairs.

Name	Forward	Reverse
MicroRNA409	5'-GAATGTTGCTCGGTGA-3'	5'-GTGCAGGGTCCGAGGT-3'
U6	5'-CTCGCTTCGGCAGCAC-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GTTGGAGGTCGGAGTCAACGG-3'	5'-GAGGGATCTCGCTCCTGGAGGA-3'
ZNF366	5'-CCCCATCCAGTACAACCTGCT-3'	5'-CTTCACGTCAGAGTGGACGA-3'

Results

Effect of Spinal Cord Injury on the Grip Strength of Mice

Due to the surgical wound, mice in the sham group were unable to complete a full-scale pull during the early measurement of grip strength, and a temporary drop in grip strength was observed. The grip strength in the sham group began to recover one week later. Motor function of forelimbs in the SCI group was severely impaired. Of note, no movement could be performed using the forelimbs of mice in the SCI group within the first week after the surgery. From the 7th day after the surgery, mice in the SCI group tried to grasp the measuring rod. Grip strength was gradually improved over time. However, the measured data were extremely low, indicating severe motor dysfunction in the SCI mice. In addition, the grip strength of mice in the SCI group was always remarkably lower than those of the sham group ($p < 0.05$) at different postoperative time points. Grip strength changes of both forelimbs were similar (Figure 1A-1C).

Spinal Cord Injury Led to Altered Expressions of MicroRNA-409 and ZNF366

QRT-PCR was performed to detect mRNA levels of microRNA-409 and ZNF366 in mouse spinal cord after SCI for 1 day, 3 days, 1 week, and 2 weeks, respectively. We found that microRNA-409 expression in the SCI group was gradually decreased from the 3rd day after SCI and was remarkably lower than that of the sham

group ($p < 0.05$, Figure 2A). Meanwhile, higher ZNF366 expression was found in the SCI group from the first postoperative day than that of the sham group ($p < 0.05$, Figure 2B).

Subsequently, we validated protein expression of ZNF366 in SCI mice by Western blot. From the 3rd postoperative day, protein expression of ZNF366 showed a time-dependent increase with a statistically significant difference ($p < 0.05$, Figure 2C).

MicroRNA-409 Promoted the Repair of Spinal Cord Injury Through ZNF366

To investigate the role of microRNA-409 in SCI, we injected exogenous microRNA-409 in the subdural area 5 min after the injury. The microRNA-409 level in the spinal cord was measured at the postoperative 1st, 3rd, and 7th day, respectively. The results showed that expression level of microRNA-409 in the SCI group was remarkably increased from the first day after surgery compared with that of sham operation group (Figure 3A). Meanwhile, ZNF366 expression was markedly decreased in mice injected with exogenous microRNA-409 and the difference was statistically significant ($p < 0.05$, Figure 3B).

We then examined the effect of exogenous injection of microRNA-409 on grip strength in mice. Serious limb injury was found both in the SCI group and microRNA-409 treatment group. Grip functions in mice of the microRNA-409 treatment group showed an initial recovery from the 3rd day after the injury, and the grip strength was gradually improved over time. A remarkable improvement of grip strength was found in the

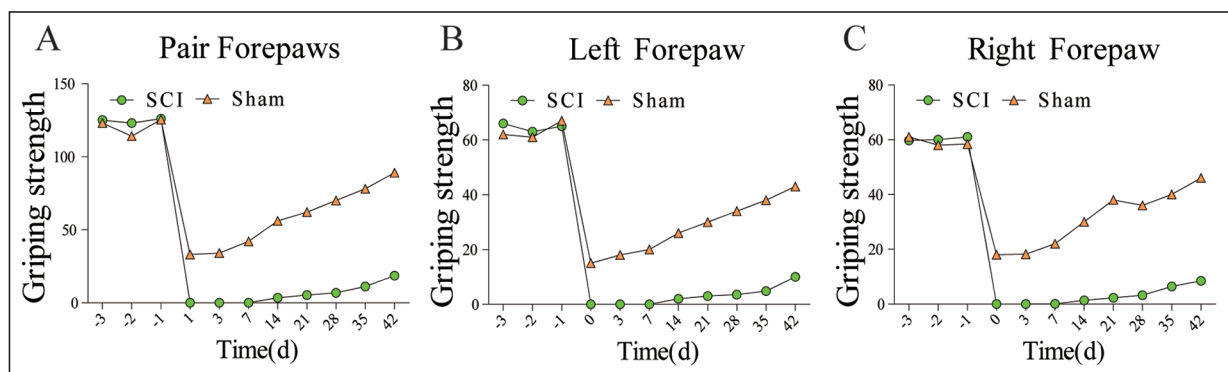


Figure 1. The recovery of forelimb grip strength in the SCI group is significantly more difficult than that in the sham operation group. **A**, The recovery of grip strength of the two forelimbs in the SCI group was significantly slower than that of the sham operation group. **B**, The recovery of grip strength of the left forelimb in the SCI group was significantly slower than that of the sham operation group. **C**, The recovery of grip strength of the right forelimb in the SCI group was significantly slower than that of the sham operation group.

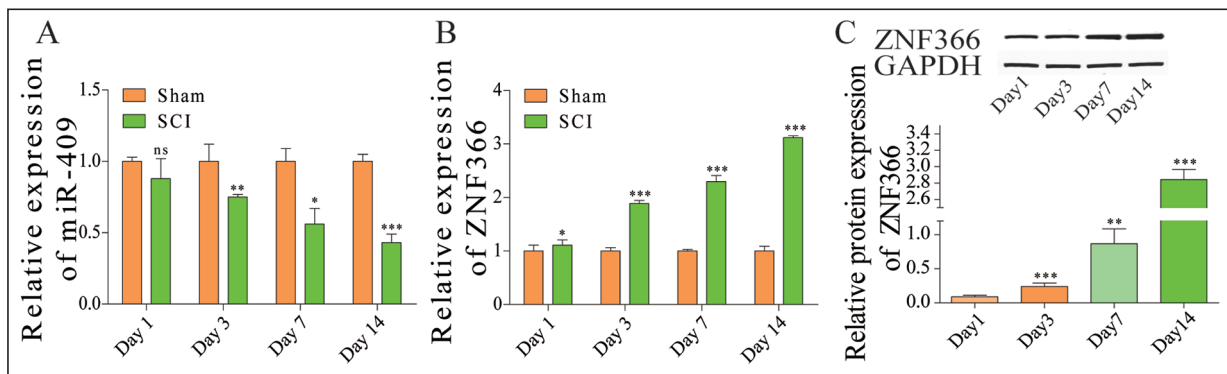


Figure 2. Spinal cord injury led to altered expression of microRNA-409 and ZNF366. **A**, The expression level of microRNA-409 decreased significantly in a time-dependent manner after spinal cord injury. **B-C**, The expression level of ZNF366 increased significantly in a time-dependent manner after spinal cord injury.

SCI group compared with that of microRNA-409 treatment group in the same period ($p < 0.05$, Figure 3C), indicating that microRNA-409 played a promoting role in the repair and regeneration of SCI in mice.

MicroRNA-409 Directly Targeted to ZNF366

To further investigate the role of microRNA-409 in repairing SCI, target genes of microRNA-409 were predicted using bioin-

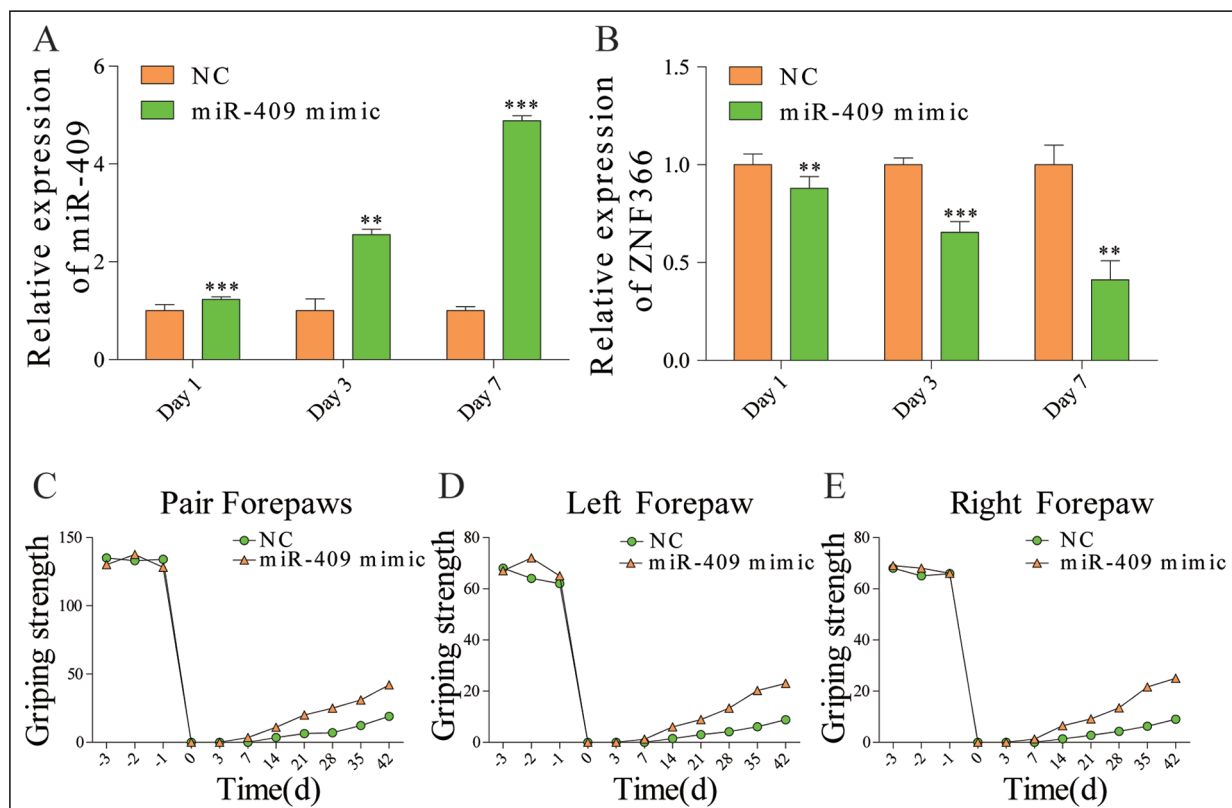


Figure 3. MicroRNA-409 promoted the repair of spinal cord injury through ZNF366. **A**, Expression levels of microRNA-409 in the injured spinal cord of mice in each group. **B**, Effects of overexpressed microRNA-409 in spinal cord injury areas of mice on the mRNA expression of ZNF366. **C-E**, Overexpression of microRNA-409 significantly improved the grip strength recovery of both forelimbs, left forelimb and right forelimb of SCI mice

formatics websites TargetScan, mirBase, and mirDB, respectively. The results showed that ZNF366 was predicted to be a candidate target gene for microRNA-409 (Figure 4A). We then constructed plasmids containing the ZNF366-WT or ZNF366-MUT sequence to verify the binding of microRNA-409 to ZNF366 (Figure 4B). The luciferase activity assay showed a lower luciferase activity in the ZNF366-WT group than that of the ZNF366-MUT group (Figure 4C), suggesting that microRNA-409 could directly bind to ZNF366. More importantly, up-regulated microRNA-409 resulted

in a significantly decrease in protein expression of ZNF366, which further validated the target bind of microRNA-409 to ZNF366 (Figure 4D).

Discussion

The spinal cord is a vulnerable site in the central nervous system. Spinal cord injuries would seriously lead to the loss of labor and self-care abilities, as well as many severe complications. Due to the non-renewable nature of nerves, es-

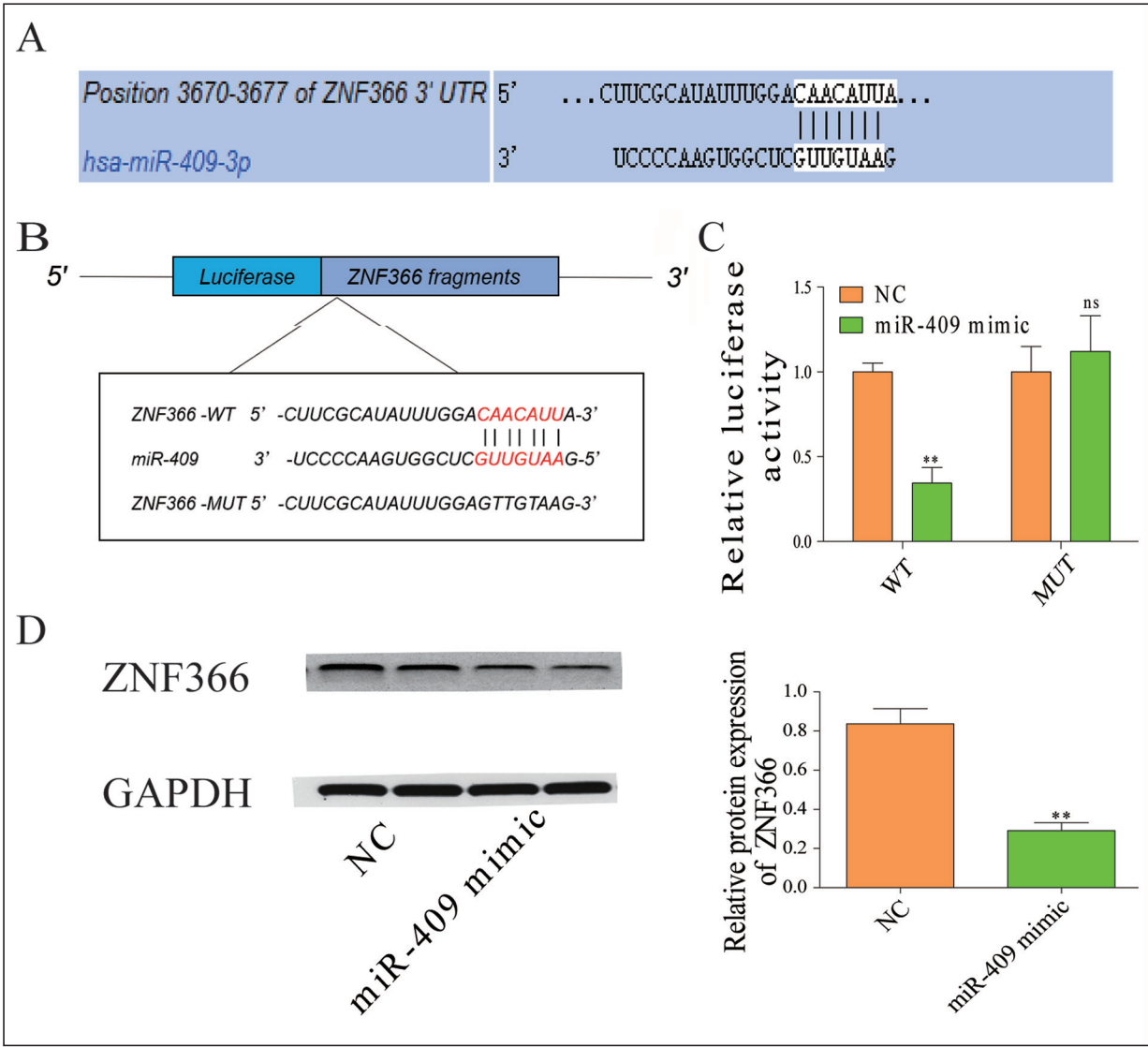


Figure 4. MicroRNA-409 directly targeted ZNF366. **A**, Binding sites were predicted for ZNF366 and microRNA-409 by bioinformatics website. **B**, Construction of sequences for ZNF366-WT and ZNF366-MUT. **C**, The luciferase activity was decreased in the ZNF366-WT group, but no significant difference in the ZNF366-MUT group was observed. **D**, Overexpression of microRNA-409 significantly decreased the protein expression of ZNF366.

pecially the central nervous system, there is currently no effective treatment for SCI. The high morbidity of SCI has plagued the majority of patients and has also brought a heavy psychological and economic burden. Therefore, the rehabilitation and treatment after SCI is an urgent problem for both doctors and patients.

MiRNA is a non-coding RNA with regulatory functions and tissue specificity, which plays a crucial role in gene expressions^{9,10}. It is reported^{11,12} that miRNAs participate in a series of important physiological activities such as cell cycle, cell differentiation, and spinal cord development of neurons. In this work, microRNA-409 was abnormally expressed in spinal cord tissues after SCI, suggesting that microRNA-409 might be involved in the repair process of SCI.

MicroRNA-409 is located on 14q32.31, and was first reported to be able of inhibiting invasion and metastasis of gastric cancer cells, as well as inducing apoptosis of gastric cancer cells^{13,14}. In fibrosarcoma, microRNA-409 could inhibit cell proliferation, angiogenesis and the growth of transplanted tumors¹⁵. However, the role of microRNA-409 in SCI remains unclear. Our results showed that microRNA-409 was downregulated after SCI in mice. Injection of exogenous microRNA-409 improved the grip strength of mice, confirming that microRNA-409 promoted SCI recovery.

ZNF366 that encodes a Kruppel zinc finger protein is highly conserved in evolution, which is located on chromosome 5. Previous studies¹⁶ have shown that ZNF366 could act as a co-repressor of the estrogen receptor and participate in regulating expressions of estrogen-responsive genes. However, there has been no report of ZNF366 in SCI so far. Our results showed that ZNF366 expression was gradually increased over time after injury. Moreover, microRNA-409 promoted the recovery of the grip function of mice after SCI by downregulating ZNF366, suggesting that microRNA-409 might promote SCI recovery through ZNF366. Luciferase activity assay further revealed that ZNF366 was a direct target of microRNA-409.

Conclusions

We found that microRNA-409 was downregulated in mice with spinal cord injury, while overexpressed microRNA-409 improved the grip strength of SCI mice to a certain ex-

tent by directly targeting ZNF366. Our data indicated that microRNA-409 participates in SCI repair, which provides new ideas for the treatment of SCI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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