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Downregulation of miRNA-127-5p aggravates spinal cord injury through activating MAPK1

C. ZHANG[1,2,3], M.-M. WANG[4], Y. ZHANG[3], L. YANG[3], M.-S. ZHU[3], Q.-R. DONG[1]

[1]Department of Orthopedics, The Second Affiliated Hospital of Soochow University, Suzhou, China
[2]Department of Trauma and Reconstructive Surgery, University Medicine Greifswald, Greifswald, Germany
[3]Department of Orthopedics, General Hospital of Pingmeishenma Medical Group, Pingdingshan, China
[4]Department of Health Care Management, University Medicine Greifswald, Greifswald, Germany

Chong Zhang and *Meimei Wang* contributed equally to this work

Abstract. – OBJECTIVE: To uncover the role of microRNA-127-5p (miRNA-127-5p) in aggravating motor dysfunction following spinal cord injury (SCI) by regulating the mitogen-activated protein kinase 1 (MAPK1) level.

MATERIALS AND METHODS: *In vivo* SCI model in mice was established by constructing spinal cord hitting injury. Mice were classified into sham group, SCI group, SCI+miRNA-127-5p mimics group, and SCI+miRNA-127-5p mimics+MAPK1 group, respectively. Grip strengths of mouse pair forepaws, right forepaw, and left forepaw at different time points were determined. Expression levels of miRNA-127-5p and MAPK1 in mice of each group at post-SCI were detected. Potential binding sites in promoter regions of miRNA-127-5p and MAPK1 were predicted by bioinformatics and further confirmed by Dual-Luciferase reporter gene assay and Western blot.

RESULTS: Grip strengths of SCI mice were much lower than those in sham group at different time points after SCI procedures. MiRNA-127-5p was markedly downregulated on the postoperative 3rd day in SCI group, and its level time-dependently decreased since after. *In vivo* overexpression of miRNA-127-5p in SCI mice improved their grip strengths from the postoperative 7th day. MAPK1 was the direct target of miRNA-127-5p. Transfection of miRNA-127-5p mimics downregulated protein level of MAPK1 in 293T cells. Overexpression of MAPK1 abolished the protective effect of miRNA-127-5p on motor function recovery following SCI.

CONCLUSIONS: Downregulation of miRNA-127-5p aggravates SCI-induced motor dysfunction through negatively regulating MAPK1 level.

Key Words:
SCI, MiRNA-127-5p, MAPK1.

Introduction

Spinal cord injury (SCI) is a severe trauma for the central nervous system (CNS) with high mortality, disability, and medical expense[1]. Due to the rapid development of economics and infrastructure, the increasing incidence of SCI poses a great burden on affected people and their families[2,3]. Effective treatment for SCI and the secondary damage is well concerned.

In the past 20 years, molecular biology and the Human Genome Project have been astonishingly advanced. A new type of 24-nucleotide non-coding RNAs transcribed by endogenous genes has been discovered, that is, microRNAs (miRNAs). MiRNAs participate in post-transcriptional regulations on gene expressions[4]. Relevant studies[5] have demonstrated the vital functions of miRNAs in the disease condition during and following SCI, showing a promising application in intervention and therapy for SCI. MiRNA-127-5p is previously reported to suppress the growth of hepatoma cells[6]. Its function in SCI, however, remains unclear.

Mitogen-activated protein kinase 1 (MAPK1) is a natural negative regulator of MAPKs and exerts an important role in MAPKs dephosphorylation[7]. A conserved threonine or tyrosine group in the MAPK regulatory site can be activated by phosphorylation of a dual-specificity protein kinase (DSPK). DSPK is able to dephosphorylate threonine or tyrosine groups at the same site, thus inactivating MAPKs mainly by dephosphorylating protein phosphatase[8]. In this paper, we established SCI mouse model and clarified the

Corresponding Author: Qirong Dong, MD, e-mail: sdfeydqr@163.com

function of miRNA-127-5p/MAPK1 regulatory loop in influencing motor function recovery at post-SCI.

Materials and Methods

Establishment of SCI Model in Mice

This study was approved by the Animal Ethics Committee of Soochow University Animal Center. A total of 12 female and 12 male adult mice, weighing 18-23 g, were housed in the Specific Pathogen Free (SPF)-level experimental animal center (room temperature of 25±3°C, humidity of 55±5% and light/dark cycle of 12 h/12 h). Mice were given free accesses to food and water. After anesthesia by intraperitoneal injection of 10% chloral hydrate (0.33 mL/kg), the mouse was fixed on the surgical table, and skin disinfection on the surgical area was performed. A 2-cm longitudinal incision on the back was made for exposure of T9-T11 spinous processes. Muscles attaching on the processes were separated, and T9-T10 laminas were removed. The mouse was subsequently fixed on the stereo positioner. SCI was established by hitting (25 mm × 10 g) from a height of 3 cm. Retracted hind legs and swaying tail of the mouse indicated the successful establishment of SCI. The incision was sutured layer by layer. Mice in sham group only underwent removal of laminas and spinous processes.

SCI mice were further assigned into sham group, SCI group, SCI+miRNA-127-5p mimics group, and SCI+miRNA-127-5p mimics+MAPK1 group. Subdural injection of the corresponding lentivirus in the lesioned area was conducted at 5 min after spinal hitting.

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

Cells were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA), and the latter was applied for PCR using the SYBR Green method (TaKaRa, Otsu, Shiga, Japan). U6 was considered as the internal reference. The primer sequences synthesized by Invitrogen Co., Ltd, Shanghai, China were listed as follows: miRNA-127-5p: forward: 5'-CTCTTCAAGCTCCAAACCAAAC-3' and reverse: 5'-GTATCCACCAGAACCACCAGG-3'; U6: forward: 5'-GCTTCGGCACATATACTAAAAT-3' and reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'; MAPK1: forward: 5'-TTTCCTCTGGATCAGCGTGT-3' and reverse: 5'-TGAGATGTCGGGGCTTCTTT-3'.

Grip Strength Determination

Mice were gently held so that their tails were brought to the bar of GSM (grip strength meter), and they were induced for grabbing the bar using their paws. Mice were pulled back quickly and gently in horizontal direction, and forepaw grip strength was recorded at the time of grip release. Grip strength for pair, left and right forepaws were recorded four times. Grip strength that mice could not grab in the bar was recorded as 0.

Western Blot

The total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). The protein sample was separated by electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, the membranes were incubated with primary antibodies overnight at 4°C, followed by the incubation of secondary antibody at room temperature for 1 h. The protein blot on the membrane was exposed by enhanced chemiluminescence (ECL).

Transfection

Cells were cultured in antibiotics-free medium overnight and transfected at 80% confluence using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). At 24 h, fresh medium was replaced.

Dual-Luciferase Reporter Gene Assay

Potential targets of miRNA-127-5p were predicted in TargetScan (http://www.targetscan.org/). MAPK1 WT and MAPK1 MUT luciferase vectors were constructed based on the binding sites in promoter regions of miRNA-127-5p and MAPK1. 293T cells were co-transfected with miRNA-127-5p mimics/NC and MAPK1 WT/MAPK1 MUT, respectively. After transfection of 48 h, cells were lysed for determining relative luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 22.0 statistical software was used for data analysis (IBM Corp., Armonk, NY, USA). All

data were expressed as mean ± SEM. The *t*-test was used for comparing differences between two groups. $p<0.05$ was considered to be statistically significant.

Results

Establishment of SCI Model in Mice

Grip strengths of mice were detected in sham group and SCI group. In mice of sham group, grip strengths of pair forepaws, right and left forepaws were remarkably reduced because of postoperative pain. Motor function recovery of mice in sham group emerged on the postoperative 3rd day. However, the motor function of SCI mice was severely damaged, and they were unable to perform any movement using the forepaws. A week later, SCI mice could complete the test of grip strengths. At any time point following SCI, grip strengths in SCI group were remarkably lower than those in sham group (Figure 1).

Protective Effect of MiRNA-127-5p on SCI Mice

MiRNA-127-5p was markedly downregulated on the postoperative 3rd day in SCI group, and its level time-dependently decreased since after (Figure 2A). After injection of miRNA-127-5p mimics in SCI mice, *in vivo* level of miRNA-127-5p was time-dependently upregulated (Figure 2B). From the 7th day following SCI, mouse grip strengths were remarkably recovered in those administrated with miRNA-127-5p mimics (Figure 2C-2E). The above data demonstrated a protective effect of miRNA-127-5p on SCI-induced motor dysfunction.

MAPK1 Was the Target Gene of MiRNA-127-5p

The binding sites in the promoter regions of miRNA-127-5p and MAPK1 were predicted by bioinformatics (Figure 3A). MAPK1 was upregulated in SCI mice, showing a time-dependent trend (Figure 3B). Moreover, MAPK1 level in SCI mice overexpressing miRNA-127-5p was dynamically detected, which was gradually reduced with the prolongation of SCI (Figure 3C). In 293T cells transfected with miRNA-127-5p mimics, the protein level of MAPK1 was downregulated (Figure 3D). Furthermore, the Dual-Luciferase reporter gene assay supported our speculation that MAPK1 was the target gene binding to miRNA-127-5p (Figure 3E).

MiRNA-127-5p Affected Motor Function at Post-SCI Through Negatively Regulating MAPK1

In 293T cells transfected with pcDNA-MAPK1, MAPK1 level was markedly upregulated, indicating a pronounced transfection efficacy (Figure 4A). Compared with SCI mice co-overexpressing miRNA-127-5p and MAPK1, those overexpressing miRNA-127-5p showed a better recovery of grip strengths (Figure 4B-4D). Therefore, we believed that MAPK1 was responsible for motor function recovery at post-SCI regulated by miRNA-127-5p.

Discussion

SCI causes necrosis and cytolysis of primary spinal cord tissues in the first phase, and secondary injury in the later phase, exerting a serious impact on the CNS and life quality of patients. Clinical manifestations of SCI include

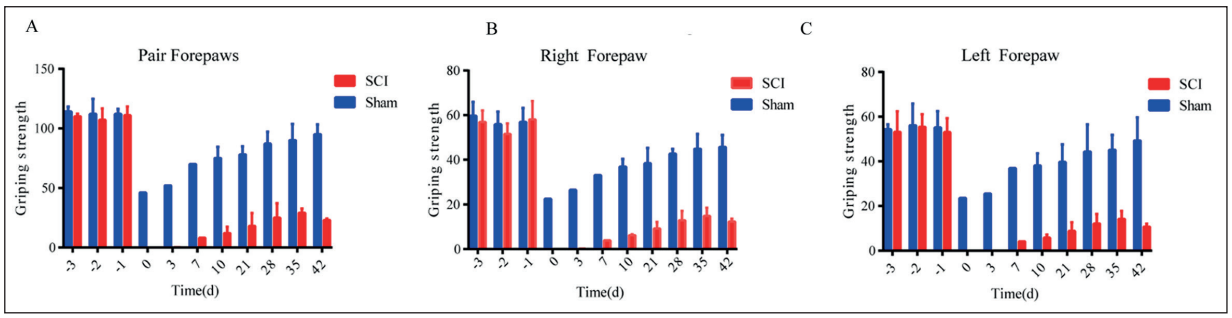

Figure 1. Establishment of SCI model in mice. Grip strengths of pair forepaws **(A),** right forepaw **(B),** and left forepaw **(C),** in mice of SCI group and sham group at different time points before and after SCI.

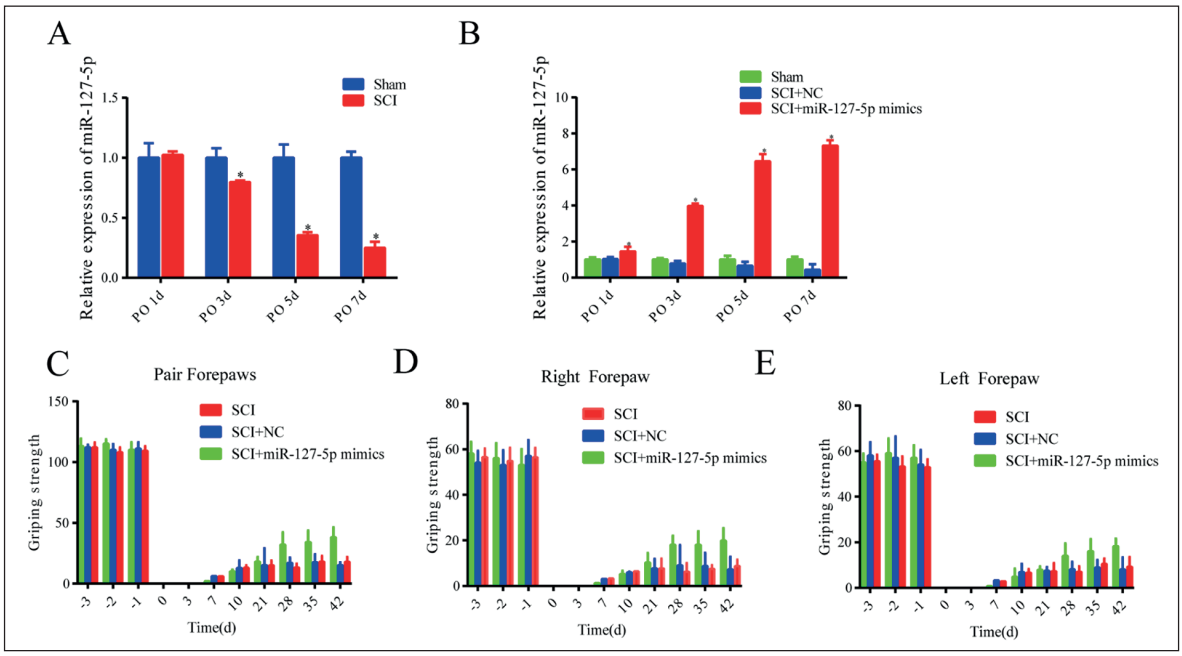

Figure 2. Protective effect of miRNA-127-5p on SCI mice. **A,** MiRNA-127-5p levels in mice of sham group and SCI group at different time points following SCI. **B,** MiRNA-127-5p levels in mice of sham group, SCI+NC group and SCI+miRNA-127-5p mimics group at different time points following SCI. Grip strengths of pair forepaws **C,** right forepaw **D,** and left forepaw **E,** in mice of sham group, SCI+NC group and SCI+miRNA-127-5p mimics group at different time points before and after SCI.

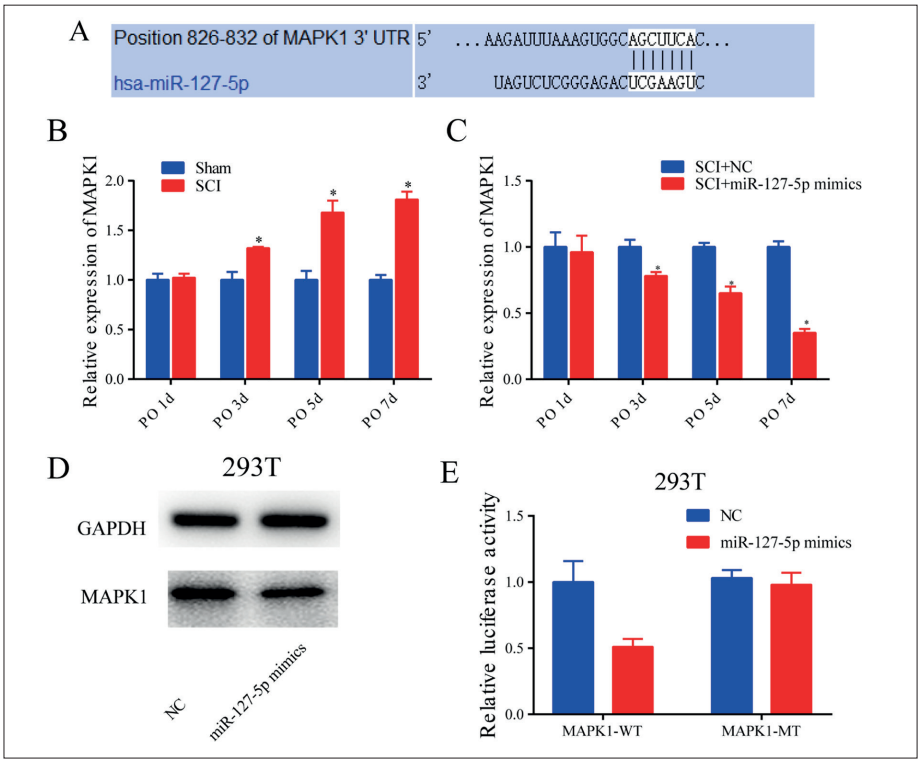

Figure 3. MAPK1 was the target gene of miRNA-127-5p. **A,** Binding sites between miRNA-127-5p and MAPK1. **B,** MiRNA-127-5p levels in mice of sham group and SCI group at different time points following SCI. **C,** MiRNA-127-5p levels in mice of SCI+NC group and SCI+miRNA-127-5p mimics group at different time points following SCI. **D,** Protein level of MAPK1 in 293T cells transfected with NC or miRNA-127-5p mimics. **E,** Luciferase activity in 293T cells co-transfected with miRNA-127-5p mimics/NC and MAPK1 WT/MAPK1 MUT.

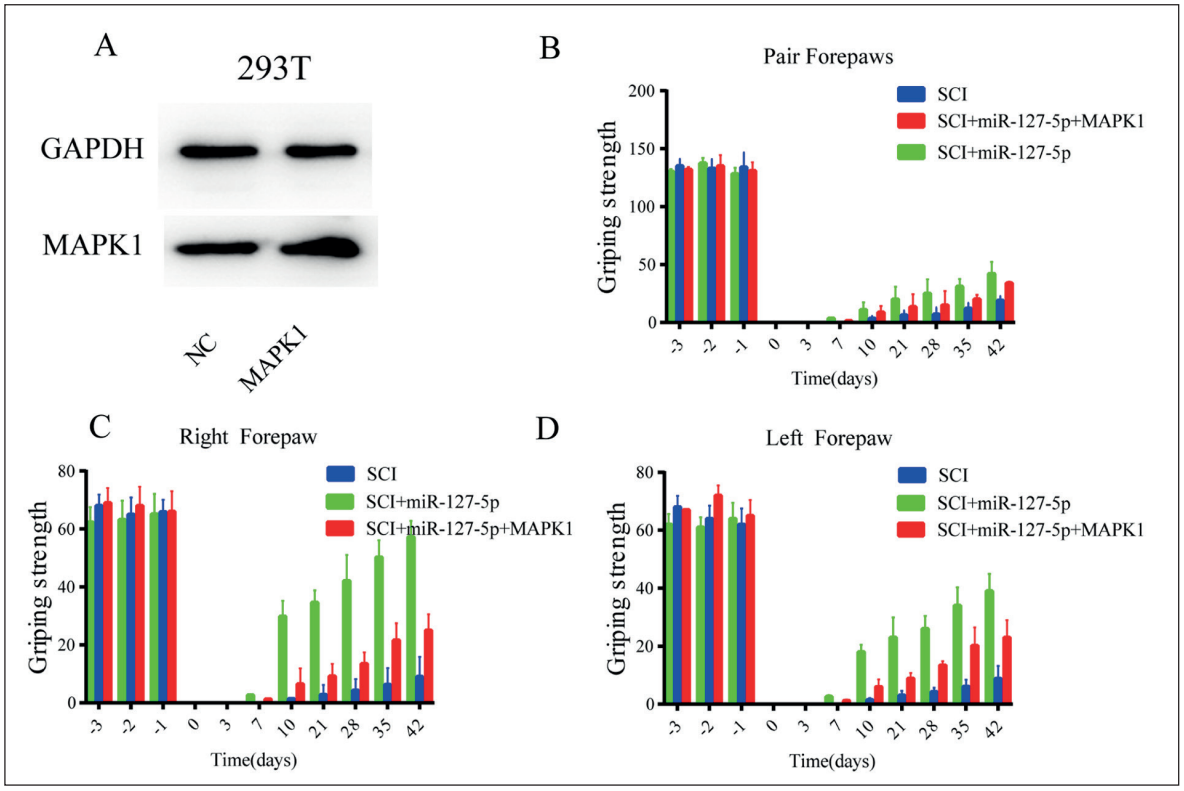

Figure 4. MiRNA-127-5p affected motor function at post-SCI through negatively regulating MAPK1. **A,** Transfection efficacy of pcDNA-MAPK1 in 293T cells. Grip strengths of pair forepaws **(B),** right forepaw **(C),** and left forepaw **(D),** in mice of SCI group, SCI+miRNA-127-5p mimics group and SCI+miRNA-127-5p mimics+MAPK1 group at different time points before and after SCI.

motor sensation dysfunction below the injury level and complete or incomplete loss of sphincter function[9]. After the occurrence of SCI, primary and secondary pathophysiological processes are complex and variable, including tissue ischemic edema, local immune-inflammatory response, and extensive neuronal necrosis. In clinical treatment, surgery and drugs are applied for alleviating secondary injury, improving nerve recovery and regeneration following SCI[10]. Unfortunately, effective therapeutic methods for SCI are still lacking[11,12].

Nowadays, the biological functions of miRNAs have been well concerned. About 17,000 miRNAs have been discovered in microorganisms, animals, and plants, including more than 1,000 miRNAs in humans[13]. These miRNAs may be utilized for diagnosis, monitoring, and treatment of human diseases. Under physiological circumstances, miRNAs contribute to maintain normal immunity. Nevertheless, dysregulated miRNAs under the stimuli of stress or diseases result in imbalanced immune tolerance, thus damaging normal cells, tissues, and organs[14]. Moreover, miRNAs are involved in cellular behaviors, organ development, immune response, etc[15-17]. Several miRNAs are abnormally expressed at post-SCI, exerting a potential function in regulating SCI-induced inflammatory response[18]. Our findings uncovered that miRNA-127-5p was downregulated in SCI mice. Notably, *in vivo* overexpression of miRNA-127-5p improved grip strengths of SCI mice, suggesting that overexpression of miRNA-127-5p improved motor function at post-SCI.

Liu et al[19] pointed out that some inflammation factors, including TNF-α, IL-1β, and ICAM-1, are potential downstream genes of differentially expressed miRNAs at post-SCI. These factors are downregulated following SCI, indicating the occurrence of the inflammatory response as the secondary damage resulted from SCI. These differentially miRNAs and target genes play important roles in the inflammatory response, oxidative stress and apoptosis. Maldonado-Bouchard et al[20] illustrated an evident upregulation of TNF-α, IL-1β, IL-6, and arachidonic acid metabolites within 6 h following SCI. Izumi et al[21] reported that miR-223 is upregulated in the lesioned spi-

nal cord in the early phase of secondary injury following SCI, accompanied by elevations of inflammatory factor abundances.

In this paper, MAPK1 was found to be the direct target of miRNA-127-5p. Transfection of miRNA-127-5p mimics downregulated protein level of MAPK1 in 293T cells. Of note, overexpression of MAPK1 abolished the protective effect of miRNA-127-5p on SCI. Hence, miRNA-127-5p/MAPK1 regulatory loop may be utilized for therapeutic targets of SCI.

Conclusions

We first showed that downregulation of miRNA-127-5p aggravates motor function at post-SCI through negatively regulating MAPK1 level.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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