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MicroRNA-490-3p inhibits inflammation and apoptosis induced by spinal cord injury via targeting MK2

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Abstract. – OBJECTIVE: To investigate the regulatory role of microRNA-490-3p in the recovery process of spinal cord injury (SCI) and its underlying mechanism.

PATIENTS AND METHODS: Expression levels of microRNA-490-3p and MK2 in peripheral blood of SCI patients and healthy controls were detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Glial cells C8-D1A and C8-B4 were induced by H_2O_2 for constructing in vitro SCI model, followed by detection of microRNA-490-3p and MK2 levels. After glial cells were transfected with microRNA-490-3p mimic or inhibitor, respectively, cell apoptosis and levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were detected. Bioinformatics was used to predict the binding site between microRNA-490-3p and MK2, which was further verified by dual-luciferase reporter gene assay. After construction of MK2 overexpression plasmid, rescue experiments were carried out to confirm the regulatory effect of microRNA-490-3p on MK2 in mediating SCI recovery.

RESULTS: MicroRNA-490-3p expression was remarkably lower, whereas MK2 expression was higher in peripheral blood of SCI patients than those of healthy controls. Downregulated microRNA-490-3p and upregulated MK2 were observed in glial cells after H_2O_2 induction in C8-D1A and C8-B4 cells. Overexpression of microRNA-490-3p remarkably decreased levels of TNF-α and IL-6, as well as alleviated apoptosis in glial cells. Both protein and mRNA levels of MK2 were negatively regulated by microRNA-490-3p. Dual-luciferase reporter gene assay confirmed that MK2 was the target gene of microRNA-490-3p. Finally, rescue experiments verified that the regulatory effects of microRNA-490-3p on alleviating inflammation and apoptosis after SCI were reversed by MK2 overexpression.

CONCLUSIONS: MicroRNA-490-3p is lowly expressed in glial cells after SCI. Overexpression of microRNA-490-3p alleviates inflammation and apoptosis via targeting MK2, thereafter promoting SCI recovery.

Key Words:
Spinal cord injury, MicroRNA-490-3p, MK2, Inflammation, Apoptosis.

Introduction

Spinal cord injury (SCI) is a common spinal trauma that can cause irreversible sensorimotor impairment. SCI poses great physical and psychological trauma, as well as the financial burden on affected patients and their families. SCI stimulates the secondary injuries, including free radical damage, apoptosis, and inflammatory reaction. The specific molecular mechanisms of SCI and its secondary injuries remain unclear, which are required for in-depth researches[1-3].

MicroRNAs (miRNAs) are an important class of non-coding small RNAs that regulate gene expression at the post-transcriptional level. MiRNA expression is tissue-specific and spacial-specific, which is greatly involved in the development and differentiation of tissues and cells[4,5]. MiRNAs are not only found in tissues and cells, but also in the circulatory system. Studies[6-8] have shown that circulating miRNAs are differentially expressed in tumors and are capable of regulating cell proliferation and apoptosis. Based on the regulatory effect of miRNAs at the post-transcriptional level, they are considered to be potent mediators in the progression of SCI[9,10]. MicroRNA-490-3p has been confirmed to participate in the pathogenesis of cancers, such as ovarian cancer, gastric cancer,

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lung cancer, bladder cancer, and liver cancer[11-15]. However, whether microRNA-490-3p exerts a regulatory effect on the development of SCI is not yet clear.

Differentially expressed miRNAs in the pathological progression of SCI may serve as potential therapeutic targets. To better determine the role of microRNA-490-3p in the development of SCI, we first detected microRNA-490-3p expression in peripheral blood of SCI patients and H_2O_2-induced glial cells. The regulatory effect of microRNA-490-3p on the inflammation and apoptosis of glial cells were further explored.

Patients and Methods

Subject Enrollment and Sample Collection

40 SCI patients and 40 healthy controls were enrolled. 5 mL of venous blood sample was extracted from each subject and preserved at -80°C. The Liaocheng People's Hospital Ethics Committee approved this study.

Cell Culture

Mouse glial cell lines C8-D1A and C8-B4 were incubated in DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS (fetal bovine serum), 100 U/mL penicillin and 100 μg/mL streptomycin (Hyclone, South Logan, UT, USA). Cells were incubated in a 5% CO_2 incubator at 37°C. Cell passage was performed until 85% of cell density.

In Vitro SCI Model Construction

Third-passage glial cells were incubated with gradient concentrations of H_2O_2 for 4 h. The experimental concentration was selected based on the cell counting kit-8 (CCK-8) results (Dojindo, Kumamoto, Japan) as the highest concentration that did not alter cell viability. Glial cells were then treated with the certain concentration of H_2O_2 for establishing the *in vitro* SCI model.

Cell Transfection

One day prior to cell transfection, cells in good growth condition were seeded into 6-well plates with serum-free medium. Transfection was performed when the confluence was up to 60% following the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Culture medium was replaced 6 hours later.

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

Total RNA in treated cells was extracted using the TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was detected using the spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). Primers used in the study were as follows: U6, F: 5'-CTCGCTTCGGCAGCACA-3', R: 5'-AACGCTTCACGAATTTGCGT-3'; GAPDH, F: 5'-GTTGGAGGTCGGAGTCAACGG-3', R: 5'-GAGGGATCTCGCTCCTGGAGGA-3'; MicroRNA-490-3, F: 5'-AACATGCCATGGGGGCCGGAGCGGAGT-3', R: 5'-CCAGAATTCAAAGCAGGAAGAGTAAGACTTCC-3'.

Cell Apoptosis Detection

Glial cells were seeded in the 6-well plate at a density of 1×10^5/ml. After 48 h cell culture, cells were washed with pre-cooled PBS (phosphate-buffered-saline) twice, incubated 10 min with FITC-Annexin V and PI (propidium iodide) in the dark. Glial cells were washed with PBS twice, followed by flow cytometry detection.

Dual-Luciferase Reporter Gene Assay

Wild-type (MK2-WT) and mutant-type MK2 (MK2-MUT) sequences were first constructed. Glial cells were seeded in the 96-well plates. After overnight incubation, cells were co-transfected with 50 pmol/L microRNA-490-3p mimics or negative control and 80 ng MK2-WT or MK2-MUT, respectively. 48 hours later, luciferase activity was detected using the dual-luciferase reporter gene assay kit (Promega, Madison, WI, USA).

Western Blot

Glial cells and peripheral blood samples were lysed for protein extraction. The concentration of each protein sample was determined by a BCA (bicinchoninic acid) kit (Abcam, Cambridge, MA, USA). The protein sample was separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Roche, Basel, Switzerland). After incubation with primary and secondary antibodies (Cell Signaling Technology, Danvers, MA, USA), immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA)

was used for data analysis. Data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Measurement data were compared using the *t*-test. $p < 0.05$ considered the difference was statistically significant.

Results

SCI Altered Expressions of MicroRNA-490-3p and MK2

We detected expression levels of microRNA-490-3p and MK2 in peripheral blood of SCI patients and healthy controls by qRT-PCR. The data showed that microRNA-490-3p expression was remarkably lower, whereas MK2 expression was higher in peripheral blood of SCI patients than those of healthy controls (Figure 1A and 1B). Western blot results also indicated that the protein level of MK2 was higher in peripheral blood of SCI patients (Figure 1D). For *in vitro* SCI model establishment, glial cells C8-D1A and C8-B4 were induced by H_2O_2. Similar results were obtained in glial cells as downregulated microRNA-490-3p and upregulated MK2 were observed after H_2O_2 induction (Figure 1C, 1E and 1F).

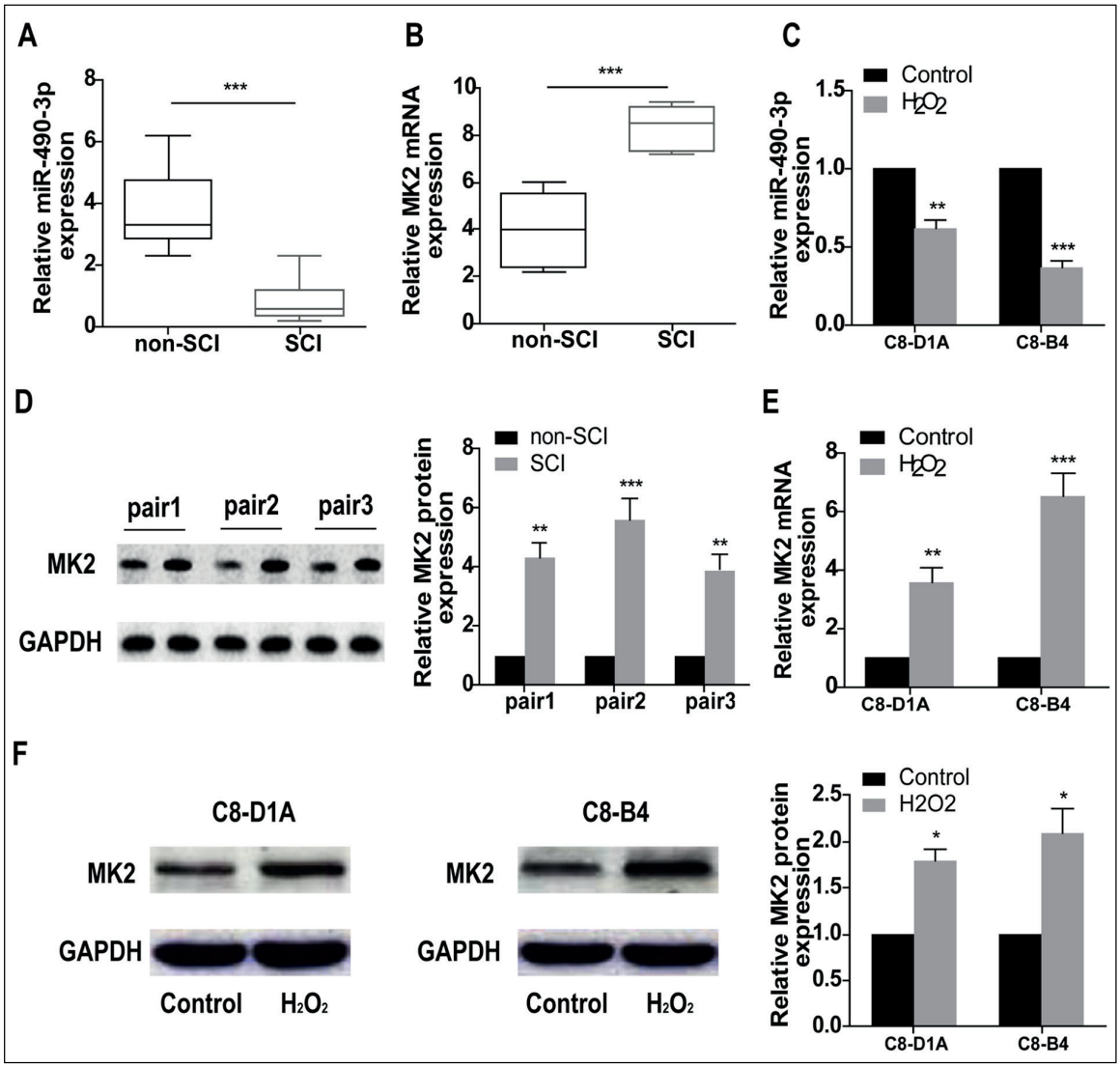

Figure 1. SCI altered expressions of microRNA-490-3p and MK2. ***A, B,*** MicroRNA-490-3p expression was remarkably lower, whereas MK2 expression was higher in peripheral blood of SCI patients than those of healthy controls detected by qRT-PCR. ***C,*** H_2O_2 induction downregulated microRNA-490-3p in C8-D1A and C8-B4 cells. ***D,*** Protein level of MK2 was upregulated in SCI patients. ***E,*** The mRNA level of MK2 was upregulated in C8-D1A and C8-B4 cells induced by H_2O_2. ***F,*** Protein level of MK2 was upregulated in C8-D1A and C8-B4 cells induced by H_2O_2.

MicroRNA-490-3p Inhibited Cell Apoptosis and Downregulated Levels of Inflammatory Factors

To explore the biological function of microRNA-490-3p in SCI, we first constructed microRNA-490-3p mimic and microRNA-490-3p inhibitor. Transfection efficacies of microRNA-490-3p mimic and microRNA-490-3p inhibitor in C8-D1A and C8-B4 cells were verified (Figure 2A). Subsequently, we found that the microRNA-490-3p overexpression remarkably downregulated the mRNA levels of IL-6 and TNF-α in glial cells. On the contrary, microRNA-490-3p knockdown upregulated the mRNA levels of IL-6 and TNF-α (Figure 2B). Western blot showed the similar trends in protein levels of IL-6 and TNF-α after transfection of microRNA-490-3p mimic or microRNA-490-3p inhibitor, respectively (Figure 2C). Additionally, microRNA-490-3p overexpression markedly reduced the apoptosis of C8-D1A and C8-B4 cells (Figure 2D). The above results suggested that microRNA-490-3p may alleviate inflammation and apoptosis of glial cells after SCI.

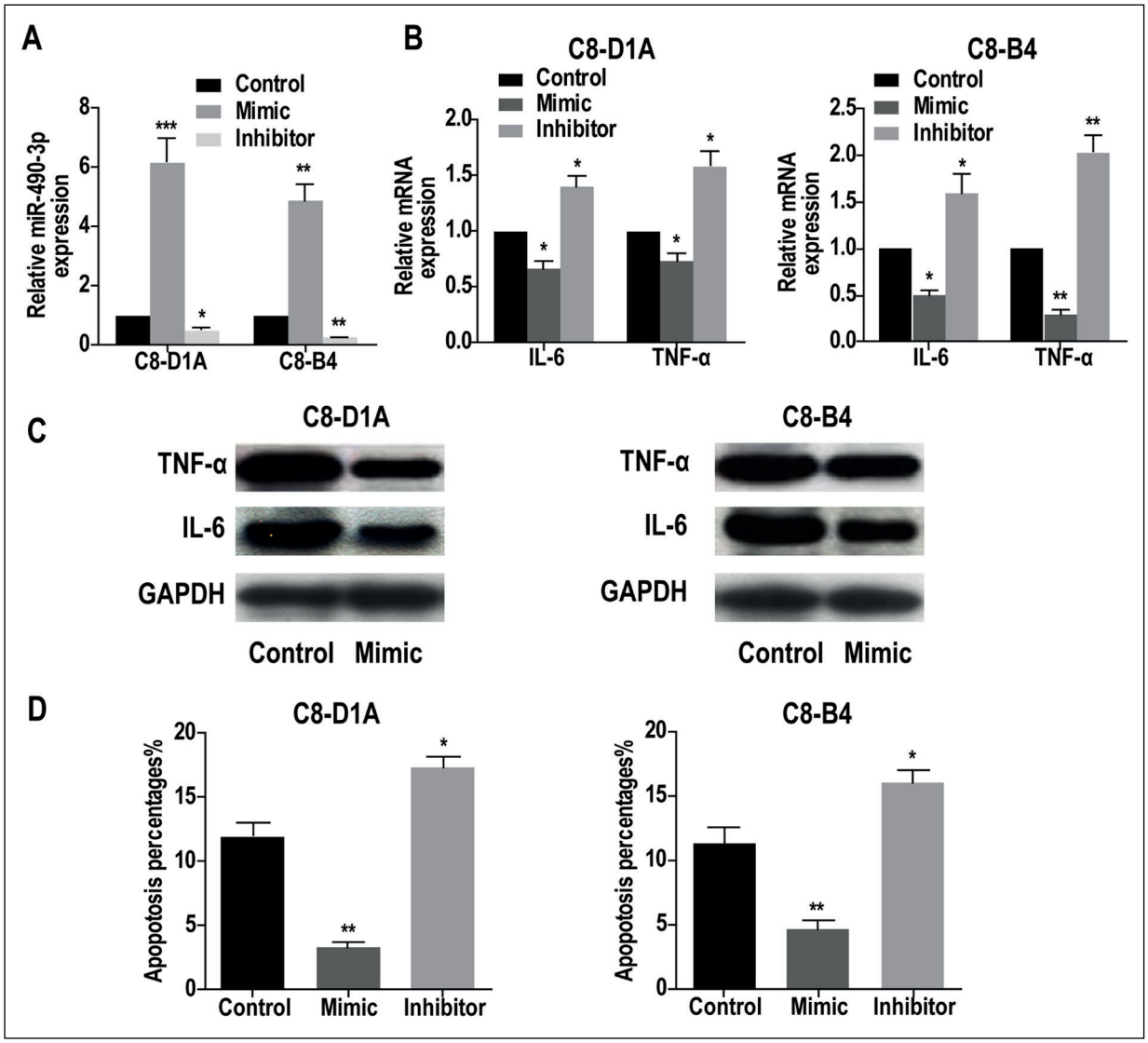

Figure 2. MicroRNA-490-3p inhibited cell apoptosis and downregulated inflammatory factors. ***A,*** Transfection efficacies of microRNA-490-3p mimic and microRNA-490-3p inhibitor in C8-D1A and C8-B4 cells. ***B,*** MicroRNA-490-3p overexpression remarkably downregulated mRNA levels of IL-6 and TNF-α. On the contrary, microRNA-490-3p knockdown upregulated mRNA levels of IL-6 and TNF-α. ***C,*** Protein levels of IL-6 and TNF-α were downregulated after microRNA-490-3p overexpression in C8-D1A and C8-B4 cells. ***D,*** MicroRNA-490-3p overexpression markedly reduced apoptosis of C8-D1A and C8-B4 cells.

MicroRNA-490-3p Downregulated MK2 Expression

QRT-PCR results showed that the mRNA level of MK2 was negatively regulated by microRNA-490-3p in C8-D1A and C8-B4 cells (Figure 3A). The protein expression of MK2 was also negatively regulated by microRNA-490-3p verified by Western blot (Figure 3B). Dual-luciferase reporter gene assay was then performed to verify the binding condition of microRNA-490-3p and MK2. Sequences of MK2-WT and MK2-MUT were first constructed (Figure 3C). Luciferase activity was remarkably decreased in glial cells co-transfected with microRNA-490-3p mimics and MK2-WT. However, no significant difference in luciferase activity was found after co-transfection with microRNA-490-3p mimics and MK2-MUT (Figure 3D). It is suggested that microRNA-490-3p binds to MK2 with a target interaction.

MK2 Reversed the Regulatory Effects of MicroRNA-490-3p on Alleviating Cell Apoptosis and Inflammatory Response

Rescue investigations were conducted to verify the regulatory effect of microRNA-490-3p on MK2. The downregulated levels of inflammatory factors induced by microRNA-490-3p overexpression were reversed by MK2 overexpression (Figure 4A). The reduced apoptosis of C8-D1A and C8-B4 cells after transfection of microRNA-490-3p mimic was also reversed by overexpression of MK2 (Figure 4B).

Discussion

The spinal cord is vulnerable to trauma in the central nervous system. Currently, there are no effective treatments for SCI. SCI patients may lose their labor and self-care abilities and are ac-

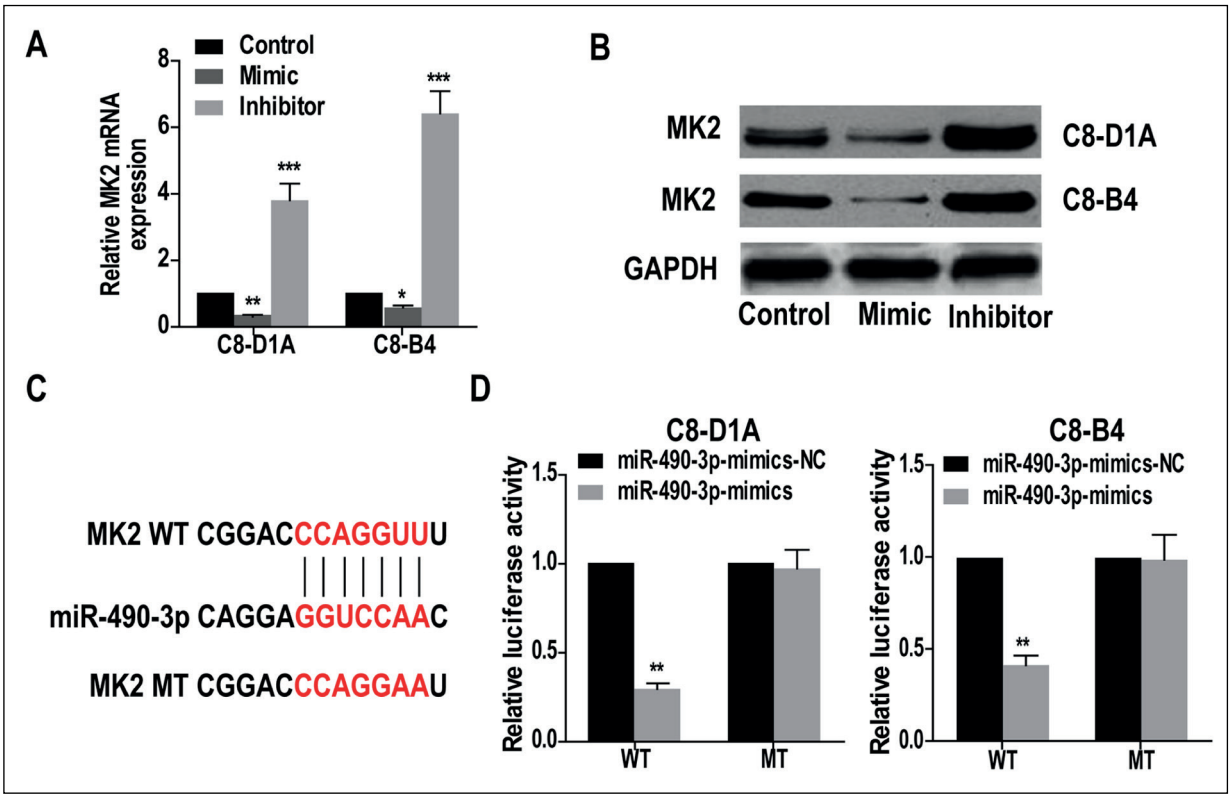

Figure 3. MicroRNA-490-3p downregulated MK2 expression. ***A,*** QRT-PCR results showed the mRNA level of MK2 was negatively regulated by microRNA-490-3p in C8-D1A and C8-B4 cells. ***B,*** Protein expression of MK2 was negatively regulated by miR-490-3. ***C,*** Construction of MK2-WT and MK2-MUT sequences. ***D,*** Luciferase activity was remarkably decreased in glial cells co-transfected with microRNA-490-3p mimics and MK2-WT. However, no significant difference in luciferase activity was found after co-transfection with microRNA-490-3p mimics and MK2-MUT.

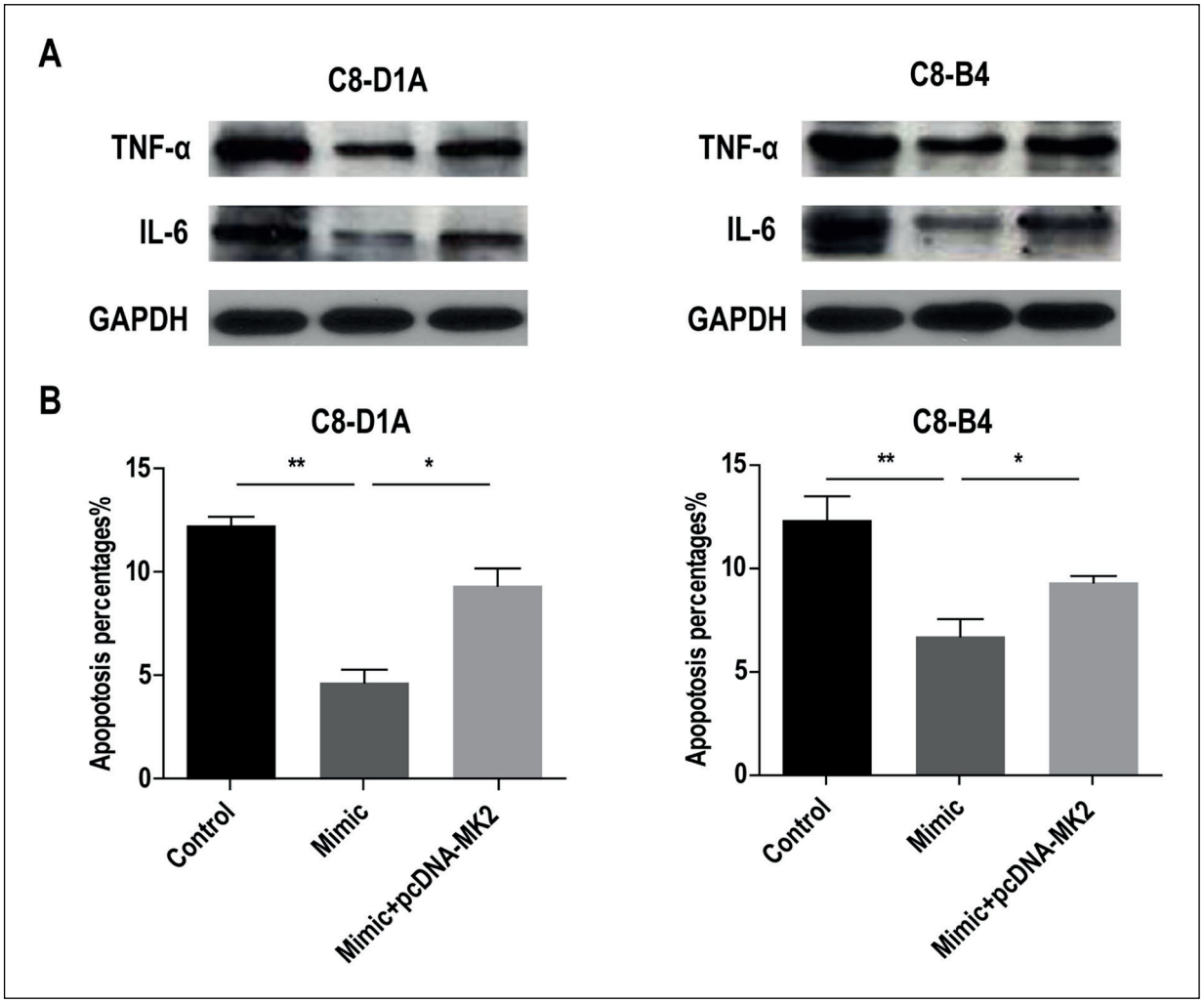

Figure 4. MK2 reversed the regulatory effects of microRNA-490-3p on alleviating cell apoptosis and inflammatory response. ***A,*** The downregulated levels of inflammatory factors induced by microRNA-490-3p overexpression were reversed by MK2 overexpression. ***B,*** The reduced apoptosis of C8-D1A and C8-B4 cells after transfection of microRNA-490-3p mimic were reversed by overexpression of MK2.

companied by serious complications. Therefore, rehabilitation and treatment after SCI are urgently needed to be improved[16-18]. MiRNAs have a certain regulatory role in the disease development. Some studies have identified multiple differentially expressed miRNAs in the central nervous system. It is reported that miRNAs participate in regulating vital transcriptional factors in the neuronal differentiation and cell type maintenance of spinal cord. The crucial role of miRNAs in SCI has been gradually confirmed[19-21].

In this study, we first detected microRNA-490-3p expression in peripheral blood of SCI patients and healthy controls. The results showed that microRNA-490-3p was remarkably downregulated in peripheral blood of SCI patients. Subsequently, we established *in vitro* SCI model by H_2O_2 induction to simulate the oxidative stress process after SCI. Similarly, microRNA-490-3p expression was downregulated in glial cells after H_2O_2 induction. Overexpression of microRNA-490-3p remarkably alleviated inflammation and apoptosis after SCI, manifesting as decreased levels of TNF-α and IL-6 in glial cells. MicroRNA-490-3p knockdown obtained the opposite results in regulating cell inflammation and apoptosis.

MK2 was the first discovered MAPK-activated protein kinase and purified in 1992. It is mainly activated by phosphorylation of p38. The biological function of MK2 in inflammation has been well studied, while its role in SCI has not

been fully elucidated[22-24]. In this study, MK2 was highly expressed in the peripheral blood of SCI patients. MK2 expression was also upregulated after H_2O_2 induction in glial cells. The overexpression of microRNA-490-3p downregulated the MK2 expression. Subsequent bioinformatics analysis and dual-luciferase reporter gene assay further revealed that MK2 is the target gene for microRNA-490-3p. The overexpression of MK2 could reverse the effects of microRNA-490-3p on the SCI-induced inflammatory response and apoptosis in glial cells.

Taken together, microRNA-490-3p regulates inflammation and apoptosis of glial cells after SCI *via* inhibiting MK2 expression. MicroRNA-490-3p exerts a protective role in the disease progression of SCI.

Conclusions

We found that microRNA-490-3p is lowly expressed in glial cells after SCI. Overexpression of microRNA-490-3p alleviates inflammation and apoptosis after SCI *via* targeting MK2, thereafter promoting the recovery of SCI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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