MicroRNA-34c promotes neuronal recovery in rats with spinal cord injury through the C-X-C motif ligand 14/Janus kinase 2/signal transducer and activator of transcription-3 axis

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Abstract

Background: Developing effective spinal cord repair strategies for spinal cord injury (SCI) is of great importance. Emerging evidence suggests that microRNAs (miRNAs) are closely linked to SCI recovery. This study aimed to investigate the function of *miR-34c* in the neuronal recovery in rats with SCI.

Methods: A rat model with SCI was established. Differentially expressed miRNAs were identified by a microarray analysis. *MiR-34c* expression in rats was measured by reverse transcription quantitative polymerase chain reaction. Altered expression of *miR-34c* or C-X-C motif ligand 14 (*CXCL14*) was introduced in SCI rats to measure their roles in neuronal recovery. Western blot analysis was performed to determine the phosphorylation of Janus kinase 2 (*JAK2*) and signal transducer and activator of transcription-3 (*STAT3*). Neuronal apoptosis in rat spinal cord tissues was detected. The concentrations of SCI recovery-related proteins thyrotropin releasing hormone (*TRH*), prostacyclin (*PGI2*), and ganglioside (*GM*) were evaluated by enzyme-linked immunosorbent assay. Data were analyzed using a *t*-test with a one-way or two-way analysis of variance.

Results: Rats with SCI presented decreased grip strength (112.03 ± 10.64 *vs.* 17.32 ± 1.49 g, $P < 0.01$), decreased *miR-34c* expression (7 days: 3.78 ± 0.44 *vs.* 0.95 ± 0.10, $P < 0.05$), and increased *CXCL14* expression (7 days: 0.61 ± 0.06 *vs.* 2.91 ± 0.27, $P < 0.01$). *MiR-34c* was found to directly bind to *CXCL14*. Overexpression of *miR-34c* increased grip strength (11.23 ± 1.08 *vs.* 31.26 ± 2.99 g, $P < 0.01$) and reduced neuronal apoptosis in spinal cord tissues ($53.61\% \pm 6.07\%$ *vs.* $24.59\% \pm 3.32\%$, $P < 0.01$), and silencing of *CXCL14* also increased the grip strength (12.76 ± 1.13 *vs.* 29.77 ± 2.75 g, $P < 0.01$) and reduced apoptosis in spinal cord tissues ($55.74\% \pm 6.24\%$ *vs.* $26.75\% \pm 2.84\%$, $P < 0.01$). In addition, *miR-34c* upregulation or *CXCL14* downregulation increased the concentrations of *TRH*, *PGI2*, and *GM*, and reduced phosphorylation of *JAK2* and *STAT3* in rats with SCI (all $P < 0.01$).

Conclusion: The study provided evidence that *miR-34c* could promote neuronal recovery in rats with SCI through inhibiting *CXCL14* expression and inactivating the *JAK2/STAT3* pathway. This study may offer new insights into SCI treatment.

Keywords: MIRN34C; CXCL14; JAK2/STAT3; Spinal cord injury; Apoptosis

Introduction

The traumatic spinal cord injury (SCI) is a worldwide medical concern bringing extensive physical, mental, and economic consequences to individuals, families, and even society as a whole.[1,2] Over 70% of SCI patients are afflicted by multiple injuries accompanied with spinal cord trauma, leading to high incidences of complications in acute and long-term care.[3] SCI can lead to several serious complications including neurogenic bladder or bowel impairment, autonomic hyperreflexia, respiratory and gastrointestinal troubles, sexual issues, urinary tract infections, psychological dysfunction, and so forth.[4,5] Currently, surgery, drug treatments, and cell transplantation therapies are hopeful treatments for SCI, but there is limited clinically available regenerative treatment to date that meets the stratification of patients.[6] Neurotoxicity, vascular dysfunction, glial scarring, neuroinflammation, and neuronal apoptosis are main causes of secondary degeneration that bring greater challenge in SCI treatment.[7] Therapeutic strategies that concern the neural regeneration and rehabilitation may be applied at varied stages of the post-injury response.

MicroRNAs (miRNAs) are a well-known class of endogenous small RNAs (18–24 nucleotides in length)

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without coding functions that regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of their complementary target messenger RNAs (mRNAs) and inducing translational repression and mRNA decay.[8-10] It is estimated that over half of all genes in the human genome are regulated by miRNAs, and emerging evidence suggests that a great number of miRNAs are widely expressed in the central nervous system (CNS).[11] Multiple miRNAs are aberrantly expressed following SCI, indicating their important roles in the development and pathologic processes of SCI.[9] *miR-34c* has been documented to alleviate neuropathic pain in mice with chronic constriction injury.[12] However, the specific role of *miR-34c* in SCI progression remains unknown. The computer-based online prediction suggested C-X-C motif ligand 14 (*CXCL14*) as a target gene of *miR-34c*. *CXCL14*, also known as breast and kidney-expressed chemokine, is a member of the chemokine family that mainly regulates the migration of immune cells and executes antimicrobial immunity.[13] However, there is little information concerning the role of *CXCL14* in the neuronal inflammation and the neuronal recovery from SCI. The Janus activated kinase (*JAK*)/signal transducer and activator of transcription (*STAT*) signaling pathway is an important regulatory mechanism involved in basic biological functioning and disease progression in the CNS.[14] Repressed activation of the *JAK2/STAT3* signaling pathway has been documented to promote nerve regeneration and repair.[15] Here, we hypothesized that *miR-34c* could interact with *CXCL14* and the *JAK2/STAT3* pathway to exert a protective role in SCI recovery, using a rat model with SCI established to test the hypothesis.

Methods

Ethical approval

The study was approved by the Clinical Ethical Committee of School of Medicine of Yan'an University (No. 201803-011). All experimental procedures were conducted in line with the ethical guidelines for the study of experimental pain in conscious animals.

Animal model establishment

A total of 120 Wistar rats (8–9 weeks old, 230–250 g) were purchased from the Experimental Animal Center of Xinjiang Medical University. A rat model with SCI was established by the modified Allen method. In brief, the rats were fixed on a stereotaxic apparatus in a prone position with the T10 spinal segment exposed. A round thin copper pad (diameter = 3 mm, size = 7 mm^2, weight = 0.1 g) was put on the surface of the exposed T10 spinal segment, and then a 10-g load was dropped on the copper pad at a height of 5 cm to induce the SCI model. Rats in the sham group only had the T10 spinal segment exposed and then had the skin sewed back up. The rats were numbered by weight. The numbers were divided by ten, and the rats with the same number remainder were allocated into the same group. Then the rats were randomly allocated in this manner into the sham group, SCI group, NC-LV group (negative control [NC] for lentiviral vector [LV]), *miR-34c*-LV group, short hairpin (sh)-NC group, and sh-*CXCL14* group, with 20 rats in each group. Rats in the sham group were injected with 100 μL sterile saline through the caudal vein. Rats in the SCI group were injected with 100 μL sterile saline outside the dura mater 1 day after surgery, while rats in the NC-LV, *miR-34c*-LV, sh-NC, and sh-*CXCL14* groups were injected with the corresponding vectors (all purchased from Vigene Bioscience Co., Ltd., Shandong, China).

Dual luciferase reporter gene assay

The target mRNAs of *miR-34c* were predicted using StarBase (http://starbase.sysu.edu.cn/). The DNA segment containing the binding site between the 3′-UTR of *CXCL14* and *miR-34c* was inserted into the luciferase reporter gene vector to construct the *CXCL14*-wild type (WT) vector, and the corresponding mutant type (MUT) vector *CXCL14*-MUT was constructed as well. Well-constructed *CXCL14*-WT and *CXCL14*-MUT vectors were co-transfected with either *miR-34c* mimic or the mimic NC into 293T cells. Then the luciferase activity of cells was measured.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

The spinal cord tissues of each group of rats were extracted at different time points after model establishment. Total RNA from the tissues was extracted using an RNAiso Plus kit (Takara Biotechnology, Dalian, Liaoning, China). RT was performed using a PrimeScript RT kit (Takara), and then the real-time qPCR was performed on a Stepone Plus PCR Kit (Applied Biosystems Inc., Carlsbad, CA, USA). The 10 μL PCR reaction volume contained 1.6 μL complementary DNA, 5 μL 2 × SYBR Green Taq PCR mixture (Takara), 0.2 μL forward primer (10 mmol/L), 0.2 μL reverse primer (10 mmol/L), and 3 μL double distilled water. The PCR conditions were as follows: pre-denaturation at 95°C for 5 min, followed by 60 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s and extension for 10 s, and a final extension at 72°C for 10 min. U6 was set as an internal reference for *miR-34c* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and the relative expression of *miR-34c* and *CXCL14* was determined by the $2^{-\Delta\Delta Ct}$ method. The primers are shown in Table 1.

Table 1: Primer sequences for RT-qPCR.

Gene	Primer sequence (5′-3′)
miR-34c	F: AGGCAGTGTAGTTAGCTGATTGC
	R: GTGCAGGGTCCGAGGT
CXCL14	F: TGGTTATCGTGACCACCAAG
	R: TCTCTCAACTGGCCTGGAGT
U6	F: GCTTCGGCAGCACATTATCTAAAAT
	R: CGCTTCACGAATTTGCGTGTCAT
GAPDH	F: GTTGGAGGTCGGAGTCAACGG
	R: GAGGGATCTCGCTCCTGGAGGA

RT-qPCR: Reverse transcription quantitative polymerase chain reaction; miR: microRNA; *CXCL14*: C-X-C motif ligand 14; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

Western blot analysis

The spinal cord tissues of each group of rats were extracted at different time points after model establishment. Then the tissue homogenate was prepared using Radio-Immunoprecipitation assay (RIPA) cell lysis buffer (Gibco Company, Grand Island, NY, USA) and a protease inhibitor (Sigma-Aldrich Chemical Company, St Louis, MO, USA). Then the tissue homogenate was centrifuged at 12,000 × *g* at 4°C for 10 min to collect the supernatant as protein samples. The concentration of the collected proteins was detected using a bicinchoninic acid kit (P0012, Beyotime Biotechnology Co., Ltd., Shanghai, China). Then the samples were preserved at –80°C and run on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. The membranes were sealed in skimmed milk for 2 h and then incubated with the primary antibodies *CXCL14* (ab36622), *JAK2* (ab108598), p-*JAK2* (ab32101), *STAT3* (ab68153), p-*STAT3* (ab76315), and *GAPDH* (ab181602) (All provided by Abcam Inc., Cambridge, MA, USA) at room temperature for 2 h. Then the membranes were washed in tris-buffered saline tween 3 times, 10 min each time, and then treated via dropper with a developer (A38555, ThermoFisher, St. Louis, USA), and photographed under a protein developer (Wes, ProteinSimple, Santa Clara, CA, USA). The signal intensity of the protein bands was determined on an Image Quant 350 (GE Healthcare, Fairfield, CT, USA). *GAPDH* was set as an internal reference, and the relative protein expression was evaluated based on the gray value ratio of the target protein relative to *GAPDH*.

Grip strength measurement (GSM)

The grip strength of rats was measured using a grip strength meter (TSE Systems, Thuringia, Germany). The rats were set on the meter with a forepaw on one side grasping the medal bar on the meter. Then the tip of the rat's tail was lifted quickly until the paws were released to evaluate the grip strength of the paws. The grip strength (g) of both right and left forepaws of each rat was calculated with four repeated experiments. The strength was 0 if the rat could not grasp the medal bar. Adequate intervals are provided between every two experiments to ensure the accuracy of the measurement outcomes. The grip strength of both left and right forepaws was measured at 1, 3, and 5 days before surgery and 3, 5, 7, 10, 20, 30, 40, and 50 days after model establishment, respectively.

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)

Spinal sections (3 μm) were prepared for TUNEL assay. An apoptosis detection kit (Roche Ltd., Basel, Switzerland) was utilized to measure apoptosis. The sections were baked at 60°C for 1 h, dewaxed, and reacted with 0.01 mol/L citrate buffer (pH 6.0) in a boiling water bath for 10 min and then cooled down at room temperature. Then the samples were washed with phosphate buffer saline (PBS) and labeled, and the remaining procedures were conducted as per the kit's instructions to measure cell apoptosis. The nuclei and nuclear membrane of apoptotic cells were labeled in dark brown. The cross-section of the spinal cord of each rat was chosen at random and observed. The apoptotic cells were observed under an optical microscope (Olympus, CH30, Japan) at a 200× magnification with five fields randomly selected.

Enzyme-linked immunosorbent assay (ELISA)

The spinal cord tissues of rats were collected, washed 3 times with PBS, and treated with RIPA and protease inhibitor to prepare the tissue homogenate. The homogenate was then centrifuged at 12,000 × *g* at 4°C for 10 min to collect the supernatant, and then the concentrations of the SCI recovery-related proteins thyrotropin releasing hormone (*TRH*), prostacyclin (*PGI2*), and ganglioside (*GM*) were measured using the corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The SPSS 21.0 (IBM Corp. Armonk, NY, USA) was used for data analysis. The Kolmogorov-Smirnov test checked whether the data were normally distributed. Measurement data were presented as mean ± standard deviation. Differences between every two groups were evaluated using a *t*-test, while differences among multiple groups were compared using one-way or two-way analysis of variance (ANOVA). Tukey multiple comparisons test was used for the pairwise comparisons after the ANOVA analysis. The *P* value was obtained from a two-tailed test, and a $P < 0.05$ was considered statistically significant.

Results

MiR-34c expression is decreased in SCI rats

Differentially expressed miRNAs between rats in the sham group and SCI group were analyzed using the GeneChip^{TM} miRNA 4.1 Array Strip (Thermo Fisher). The raw data was homogenized and then analyzed using a Limma Rstudio Package. A total of 189 differentially expressed miRNAs were screened out with $|\text{Log}_2\text{Fold Change}| > 2$ and $P < 0.05$ as the criteria, among which 104 miRNAs were up-regulated and 85 were down-regulated. A heatmap for the top 30 differentially expressed miRNAs is presented in Figure 1A, suggesting that *miR-34c* presents the highest differential expression. In addition, it has been reported before that *miR-34c* expression may be decreased following SCI.[16] Then, a rat model with SCI was established, and the grip strength of rat forepaws was determined. The GSM results showed that the grip strength of both right and left forepaws was considerably decreased at different time points before and after SCI model establishment [Figure 1B–D] [Supplementary Tables 1–3, http://links.lww.com/CM9/A281]. The RT-qPCR results suggested that *miR-34c* expression was decreased in SCI rats at different time points after model establishment compared to the sham-operated ones (1 day: 3.76 ± 0.41 *vs*. 1.21 ± 0.14, $t = 10.14$, $P < 0.05$; 3 days: 3.94 ± 0.43 *vs*. 1.16 ± 0.13, $t = 11.05$, $P < 0.05$; 5 days: 4.31 ± 0.39 *vs*. 1.08 ± 0.12, $t = 12.84$, $P < 0.05$; 7 days: 3.78 ± 0.44 *vs*. 0.95 ± 0.10, $t = 11.25$, $P < 0.05$) [Figure 1E].

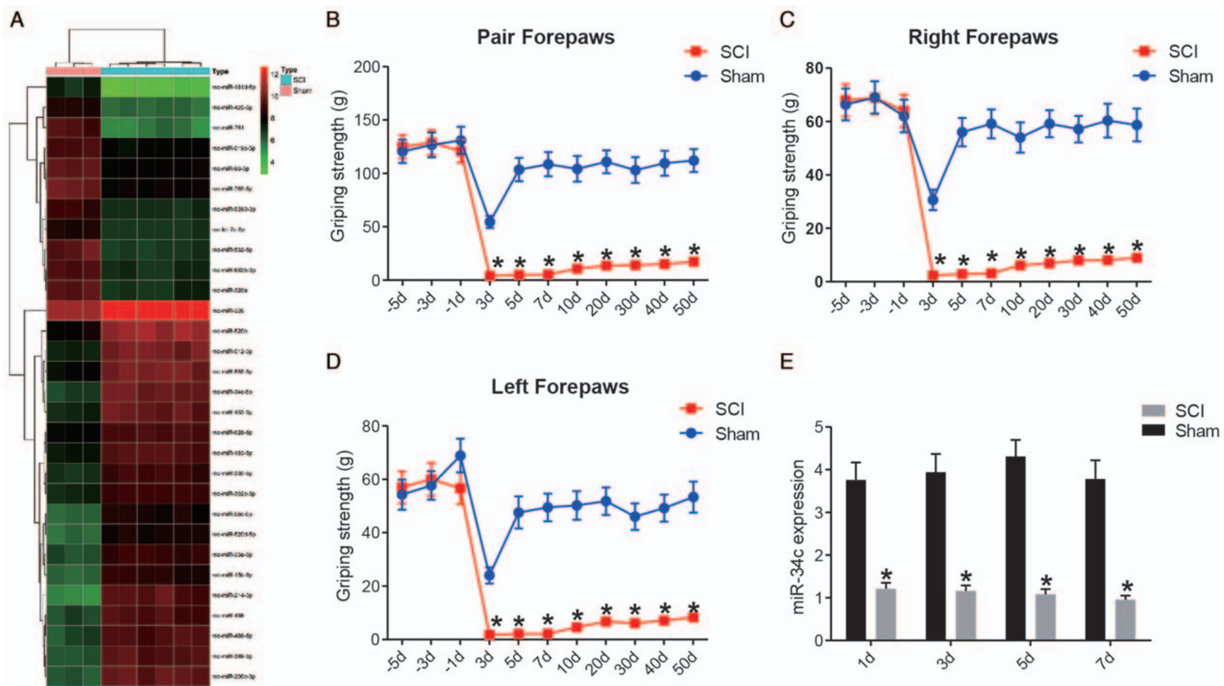

Figure 1: *miR-34c* expression is decreased in SCI rats. (A) differentially expressed miRNAs between rats in the sham group ($n = 20$) and the SCI group ($n = 20$) analyzed by microarray; the grip strength of rat pair (B), right (C), and left (D) forepaws at different time points evaluated by GSM; (E) *miR-34c* expression in rat spinal cord tissues determined by RT-qPCR. *: compared to the sham group, $P < 0.05$; measurement data were presented as mean ± SD; Repetition = 3. GSM: Grip strength measurement; SD: standard deviation; *miR-34c*: microRNA-34c; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; SCI: Spinal cord injury.

Overexpression of miR-34c promotes the neuronal recovery of SCI rats and inhibits the JAK2/STAT3 signaling pathway

To further investigate whether *miR-34c* expression affects the rats' recovery from SCI, artificial up-regulation of *miR-34c* was introduced in rats by transfecting *miR-34c*-LV. To validate the transfection efficiency, RT-qPCR was performed, and the results suggested that *miR-34c* expression was up-regulated in SCI rats after *miR-34c*-LV transfection compared to NC-LV transfection (1 day: 0.89 ± 0.09 *vs.* 2.97 ± 0.32, $t = 11.05$, $P < 0.01$; 3 days: 0.76 ± 0.08 *vs.* 2.74 ± 0.36, $t = 10.52$, $P < 0.01$; 5 days: 0.72 ± 0.08 *vs.* 2.68 ± 0.28, $t = 10.41$, $P < 0.01$; 7 days: 0.68 ± 0.10 *vs.* 2.61 ± 0.29, $t = 10.25$, $P < 0.01$) [Figure 2A]. The GSM results presented that *miR-34c*-LV transfection led to increased grip strength of rat forepaws [Figure 2B–D] [Supplementary Tables 4–6, http://links.lww.com/CM9/A281]. Moreover, the TUNEL assay suggested that the apoptosis of neurons in rat spinal cord tissues was significantly reduced when *miR-34c* was up-regulated (53.61 ± 6.07 *vs.* 2.67 ± 3.08, $t = 7.36$, $P < 0.01$) [Figure 2E and 2F]. To further identify the neuronal recovery, the levels of *TRH*, *PGI2*, and *GM* in rat spinal cord tissues were measured using ELISA, and the results showed that the levels of the above factors were increased (*TRH*, 6.94 ± 0.75 *vs.* 19.07 ± 1.86, $t = 6.60$, $P < 0.01$; *PGI2*, 12.34 ± 1.09 *vs.* 37.49 ± 3.52, $t = 13.68$, $P < 0.01$; *GM*, 8.69 ± 0.83 *vs.* 32.61 ± 3.48, $t = 13.02$, $P < 0.01$) following *miR-34c*-LV transfection [Figure 2G]. Moreover, the *JAK2/STAT3* signaling pathway has been recognized to be closely linked with SCI.[17] Herein, we measured the expression and phosphorylation of *JAK2* and *STAT3* in rat spinal cord tissues using a western blot analysis. The results suggested that after *miR-34c*-LV transfection, the expression of *JAK2* and *STAT3* showed no significant changes (*JAK2*, 1.67 ± 0.18 *vs.* 1.74 ± 0.16, $t = 0.63$, $P = 0.95$; *STAT3*, 1.46 ± 0.16 *vs.* 1.49 ± 0.15, $t = 7.75$, $P = 0.99$), but the phosphorylation of *JAK2* and *STAT3* reduced significantly (p-*JAK2*, 1.32 ± 0.14 *vs.* 0.46 ± 0.06, $t = 0.27$, $P < 0.01$; p-*STAT3*, 1.19 ± 0.13 *vs.* 0.38 ± 0.04, $t = 7.30$, $P < 0.01$) [Figure 2H and 2I]. These results identified that overexpression of *miR-34c* could promote rat recovery from SCI and inactivate the *JAK2/STAT3* signaling pathway.

miR-34cMiR-34c directly binds to CXCL14

To further identify the exact mechanism involved in *miR-34c*-mediated SCI recovery, we measured the aberrantly expressed mRNAs in the GSE102964 microarray using the Limma Rstudio package with $|Log_2Fold\ Change| > 1.5$ and $P < 0.05$ as the screening criteria. A total of 96 mRNAs were identified with differential expression, among which six mRNAs were down-regulated while 90 mRNAs were up-regulated, and the heatmap for the representative differentially expressed mRNAs is shown in Figure 3A. Moreover, the target mRNAs of *miR-34c* were predicted on miRSearch, and the outcomes were compared with the differentially expressed genes acquired by GSE102964 microarray, with *CXCL14* found intersected [Figure 3B]. RT-qPCR and western blot analysis were applied to analyze the *CXCL14* expression in rats. The results presented that the mRNA and protein expression was increased in rats with SCI compared to the sham-operated ones (mRNA: 1 day: 1.06 ± 0.12 *vs.* 3.24 ± 0.35, $t = 10.68$, $P < 0.01$; 3 days: 1.03 ± 0.11 *vs.* 3.41 ± 0.36,

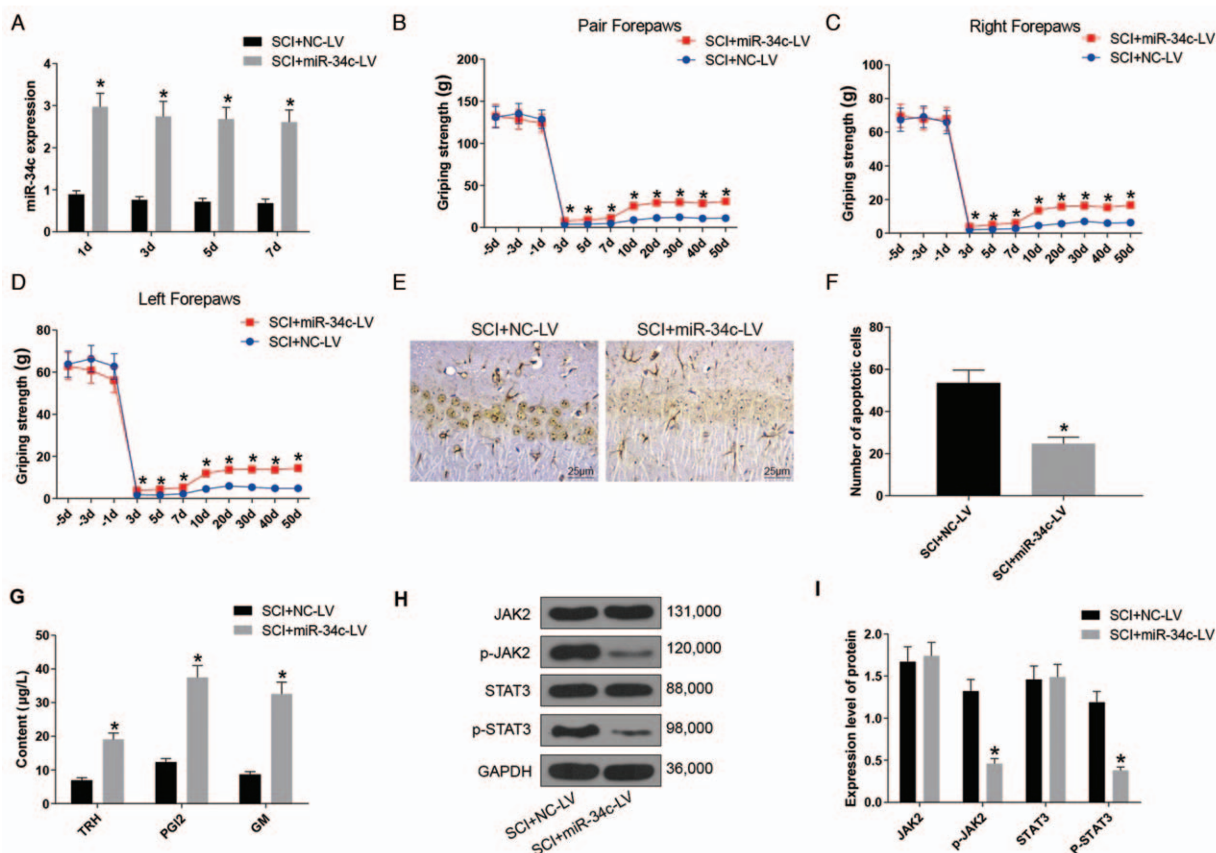

Figure 2: *miR-34c* promotes the neuronal recovery of SCI rats and inhibits the *JAK2*/*STAT3* signaling pathway. (A) *miR-34c* expression in rat spinal cord tissue detected using RT-qPCR; (B–D), the grip strength of rat pair (B), right (C), and left (D) forepaws evaluated by GSM; (E) figure for the TUNEL assay (original magnification ×400); (F) statistical chart for the number of apoptotic cells in rat spinal cord tissues; (G) protein levels of *TRH*, *PGI2*, and *GM* measured using ELISA kits; (H and I) levels and phosphorylation of *JAK2* and *STAT3* measured by western blot analysis. *: compared to the SCI + NC-LV group, $P < 0.05$. ELISA: Enzyme-linked immunosorbent assay; *GM*: Ganglioside; GSM: Grip strength measurement; *JAK2*/*STAT3*: Janus activated kinase2/signal transducer and activator of transcription3; *miR-34c*, microRNA-34c; NC-LV: negative control-lentiviral vector; *PGI2*: Prostacyclin; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; SCI: Spinal cord injury; *TRH*: Thyrotropin releasing hormone; TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling.

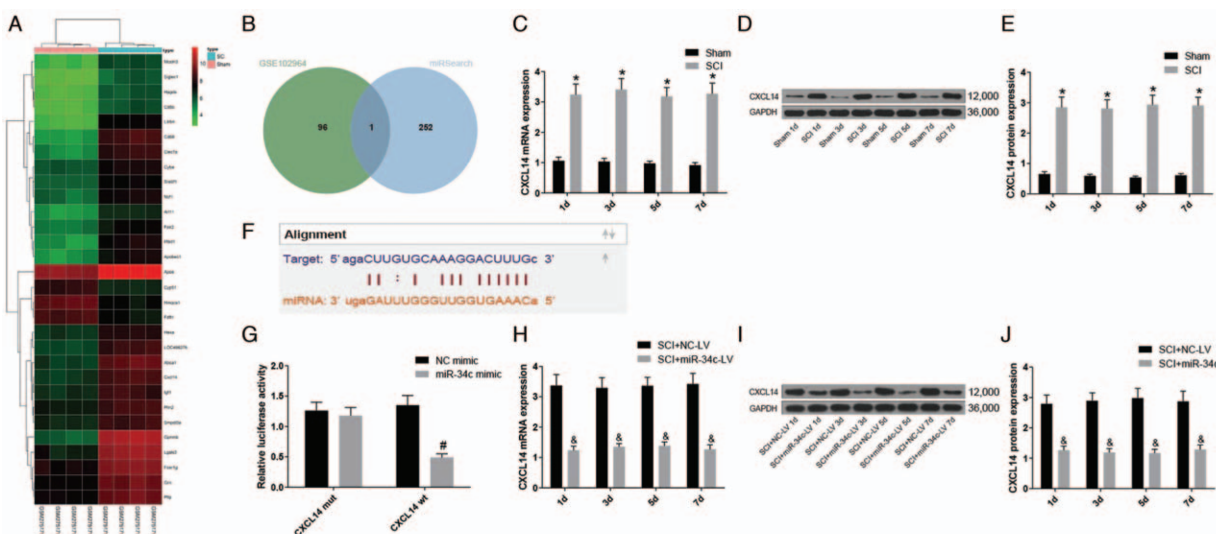

Figure 3: *miR-34c* directly binds to *CXCL14*. (A) differentially expressed mRNAs in SCI analyzed using the GSE102964 microarray; (B) intersection of the predicted *miR-34c* target genes on miRSearch and differentially expressed genes by GSE102964; (C) mRNA expression of *CXCL14* detected using RT-qPCR; (D and E) protein level of *CXCL14* in rat spinal cord tissues determined by western blot analysis; (F) binding site between *miR-34c* and *CXCL14* predicted on StarBase (http://starbase.sysu.edu.cn/); (G) binding relationship between *miR-34c* and *CXCL14* validated through a luciferase assay; (H–J), mRNA (H) and protein (I and J) expression of *CXCL14* in rat spinal cord tissues measured by RT-qPCR and western blot analysis, respectively. *: compared to the sham group, $P < 0.05$; †: compared to the NC mimic group, $P < 0.05$; ‡: compared to the SCI + NC-LV group, $P < 0.05$; measurement data were presented as mean ± SD; data were analyzed using two-way ANOVA; Repetition = 3. ANOVA: Analysis of variance; *CXCL14*: C-X-C motif ligand 14; *miR-34c*: microRNA-34c; NC-LV: Negative control-lentiviral vector; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; SCI: Spinal cord injury; SD: Standard deviation.

$t = 11.66$, $P < 0.01$; 5 days: 0.97 ± 0.08 *vs*. 3.18 ± 0.29, $t = 10.83$, $P < 0.01$; 7 days: 0.91 ± 0.09 *vs*. 3.27 ± 0.35, $t = 11.57$, $P < 0.01$; protein: 1 day: 0.65 ± 0.08 *vs*. 2.85 ± 0.34, $t = 12.32$, $P < 0.01$; 3 days: 0.59 ± 0.05 *vs*. 2.81 ± 0.29, $t = 12.43$, $P < 0.01$; 5 days: 0.54 ± 0.04 *vs*. 2.94 ± 0.31, $t = 13.44$, $P < 0.01$; 7 days: 0.61 ± 0.06 *vs*. 2.91 ± 0.27, $t = 12.88$, $P < 0.01$) [Figure 3C–E]. Then the binding site between *miR-34c* and *CXCL14* was predicted on a bio-information system (http://starbase.sysu.edu.cn/) and validated through a dual luciferase reporter gene assay [Figure 3F and 3G], which showed that overexpression of *miR-34c* inhibited the luciferase activity of the *CXCL14* reporter vector (1.36 ± 0.16 *vs*. 0.49 ± 0.06, $t = 0.76$, $P < 0.01$). To further confirm the target relationship between *miR-34c* and *CXCL14*, we measured the mRNA and protein expression of *CXCL14* after *miR-34c* up-regulation. The results suggested that over-expression of *miR-34c* led to decreased *CXCL14* expression in the spinal cord tissues of rats with SCI (mRNA: 1 day: 3.37 ± 0.36 *vs*. 1.24 ± 0.13, $t = 10.31$, $P < 0.01$; 3 days: 3.29 ± 0.34 *vs*. 1.35 ± 0.11, $t = 9.39$, $P < 0.01$; 5 days: 3.36 ± 0.28 *vs*. 1.39 ± 0.12, $t = 9.54$, $P < 0.01$; 7 days: 3.42 ± 0.35 *vs*. 1.27 ± 0.15, $t = 10.41$, $P < 0.01$; protein: 1 day: 2.79 ± 0.29 *vs*. 1.26 ± 0.14, $t = 7.96$, $P < 0.01$; 3 days: 2.89 ± 0.26 *vs*. 1.19 ± 0.13, $t = 8.85$, $P < 0.01$; 5 days: 2.98 ± 0.32 *vs*. 1.17 ± 0.12, $t = 9.42$, $P < 0.01$; 7 days: 2.87 ± 0.34 *vs*. 1.29 ± 0.15, $t = 8.22$, $P < 0.01$) [Figure 3H–J]. The above findings indicated that *miR-34c* could directly bind to *CXCL14*.

Silencing of CXCL14 promotes neuronal recovery of rats with SCI and inhibits the activation of the JAK2/STAT3 signaling pathway

To further identify the role of *CXCL14* in SCI, artificial silencing of *CXCL14* was introduced in SCI rats through transfection with a sh-*CXCL14* vector. RT-qPCR and western blot analysis results suggested that the mRNA and protein expression of *CXCL14* was significantly decreased after sh-*CXCL14* vector transfection (mRNA, 1 day: 3.45 ± 0.37 *vs*. 1.18 ± 0.11, $t = 10.72$, $P < 0.01$; 3 days: 3.37 ± 0.34 *vs*. 1.09 ± 0.10, $t = 10.77$, $P < 0.01$; 5 days: 3.49 ± 0.38 *vs*. 1.16 ± 0.12, $t = 11.01$, $P < 0.01$; 7 days: 3.37 ± 0.31 *vs*. 1.14 ± 0.09, $t = 10.54$, $P < 0.01$; protein: 1 day: 2.89 ± 0.29 *vs*. 0.89 ± 0.09, $t = 11.68$, $P < 0.01$; 3 days: 2.81 ± 0.27 *vs*. 0.94 ± 0.09, $t = 10.92$, $P < 0.01$; 5 days: 2.79 ± 0.29 *vs*. 0.98 ± 0.08, $t = 10.57$, $P < 0.01$; 7 days: 2.86 ± 0.28 *vs*. 0.86 ± 0.10, $t = 11.68$, $P < 0.01$) [Figure 4A–C]. The GSM results suggested that silencing of *CXCL14* led to decreased grip strength of rat forepaws [Figure 4D–F] [Supplementary Table 7–9, http://links.lww.com/CM9/A281]. TUNEL assay also found that silencing of *CXCL14* resulted in decreased number of apoptotic neurons in the spinal cord tissues of SCI rats (55.74 ± 6.24 *vs*. 26.75 ± 2.84, $t = 7.32$, $P < 0.01$) [Figure 4G and 4H]. Moreover, it was found that the concentrations of *TRH*, *PGI2*, and *GM* in rat spinal cord tissues were increased when *CXCL14* was silenced (*TRH*, 7.34 ± 0.81 *vs*. 18.67 ± 1.98, $t = 5.57$, $P < 0.01$; *PGI2*, 11.89 ± 1.14 *vs*.

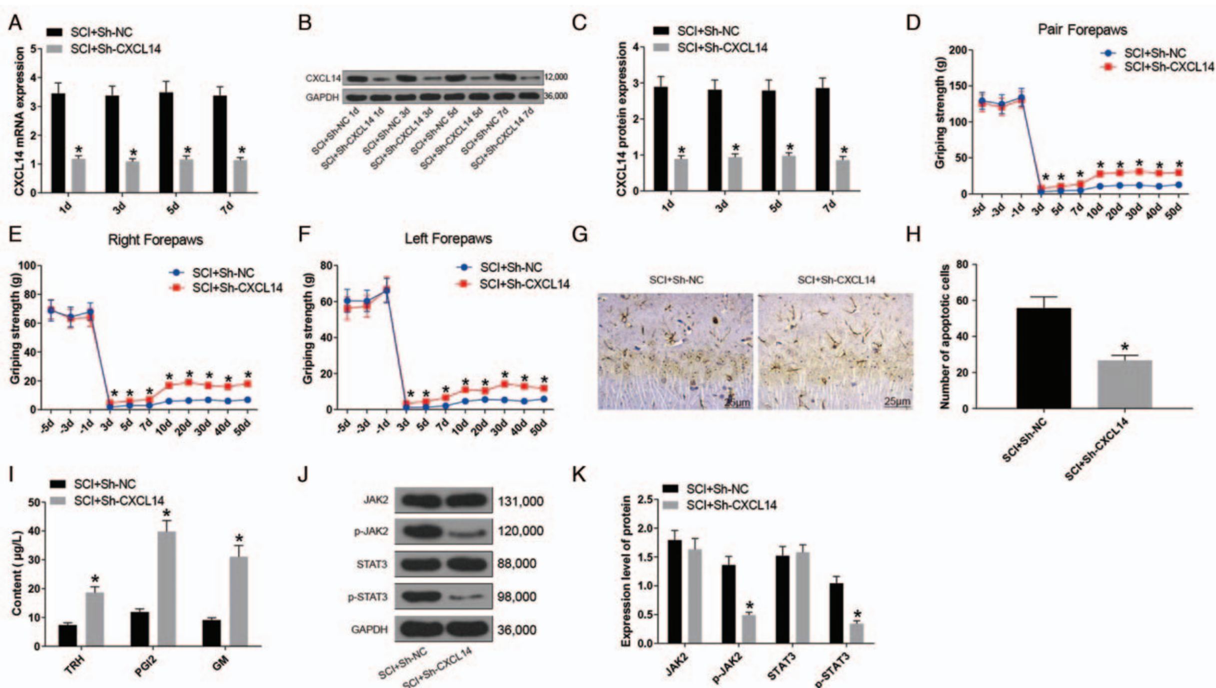

Figure 4: Silencing of *CXCL14* promotes neuronal recovery of rats with SCI and inhibits the activation of the *JAK2/STAT3* signaling pathway. (A) mRNA expression of *CXCL14* in rat spinal cord tissues detected using RT-qPCR; (B and C) protein level of *CXCL14* in rat spinal cord tissues determined by western blot analysis; (D–F) the grip strength of rat pair, right and left forepaws evaluated by GSM; (G and H) cell apoptosis in spinal cord tissues measured by TUNEL assay; (I) contents of neuronal recovery-related factors *TRH*, *PGI2*, and *GM* in rats measured using ELISA assay; (J and K) expression and phosphorylation of *JAK2* and *STAT3* determined by western blot analysis. *: compared to the SCI + sh-NC group, $P < 0.05$; measurement data were presented as mean ± SD (Repetition = 3). *CXCL14*: C-X-C motif ligand 14; ELISA: Enzyme-linked immunosorbent assay; *GM*: Ganglioside; GSM: Grip strength measurement; *JAK2/STAT3*: Janus activated kinase2/signal transducer and activator of transcription3; *PGI2*: Prostacyclin; SCI: Spinal cord injury; SD: Standard deviation; sh-NC: Short hairpin-negative control; *TRH*: Thyrotropin releasing hormone; TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling.

39.76 ± 3.88, $t = 13.70$, $P < 0.01$; *GM*, 9.08 ± 0.86 *vs.* 31.08 ± 3.95, $t = 10.81$, $P < 0.01$) [Figure 4I]. Western blot analysis also found that silencing of *CXCL14* led to decreased phosphorylation of *JAK2* and *STAT3* in SCI rats (*JAK2*, 1.79 ± 0.17 *vs.* 1.63 ± 0.19, $t = 1.43$, $P > 0.99$; *STAT3*, 1.52 ± 0.16 *vs.* 1.58 ± 0.13, $t = 0.54$, $P > 0.99$, p-*JAK2*, 1.36 ± 0.16 *vs.* 0.49 ± 0.05, $t = 7.80$, $P < 0.01$; p-*STAT3*, 1.04 ± 0.12 *vs.* 0.34 ± 0.05, $t = 7.30$, $P < 0.01$) [Figure 4J and 4K]. These results suggested that the silencing of *CXCL14* could promote the SCI recovery in rats and inhibit the *JAK2/STAT3* signaling pathway.

Discussion

SCI leads to cytolysis and necrosis of spinal cord tissues in the first phase and secondary complications in the later phase, seriously influencing the CNS and leading to poor quality of life for patients.[18] miRNAs are important gene mediators that also play crucial roles in almost every aspect of SCI with neural growth, neurogenesis, glial differentiation, and other processes frequently involved.[19] Here, our study identified *miR-34c* as a promoter of neural regeneration in SCI using a rat model, which might alleviate SCI through down-regulating CXCR14 expression and inactivating the *JAK2/STAT3* signaling pathway.

Initially, a microarray analysis was performed to screen out the mostly aberrantly expressed miRNAs in SCI with *miR-34c* identified. Decreased *miR-34c* expression in rats following SCI was further quantified via RT-qPCR. A large number of miRNAs have been identified to be abnormally expressed and exert key functions in SCI, such as miR-362-3p,[20] miR-133b,[21] and miR-486.[22] Likewise, *miR-34c* has been suggested to be down-regulated in rats with SCI[23] and in rats with spinal nerve ligation-induced lesions.[16] However, the specific role of *miR-34c* in SCI progression, to the best of our knowledge, has not been elucidated before. Herein, based on the above finding, we further introduced over-expression of *miR-34c* in SCI rats. It was found that over-expression of *miR-34c* promoted the neural recovery and function in SCI rats, presenting as increased grip strength of rat forepaws and increased concentrations of SCI recovery-related proteins *TRH*, *PGI2*, and *GM* in rat spinal tissues. *TRH* is well known for its broad-spectrum neuropharmacological action and it is crucial in CNS recovery following brain and spinal injury.[24] *PGI2* was reported to reduce impaired mobility following SCI through inhibiting tumor necrosis factor production and the neutrophil accumulation that follows.[25] Likewise, the involvement of *GM* on nerve function recovery has been identified previously.[26] Taken together, these results suggested that *miR-34c* could promote SCI recovery in rats.

Following these findings, we next explored the possible mechanisms involved in the *miR-34c*-mediated neuroprotective events. Importantly, our study found that overexpression of *miR-34c* led to decreased phosphorylation of *JAK2* and *STAT3*, namely, it inactivated the *JAK2/STAT3* signaling pathway. *JAK2/STAT3* is not phosphorylated under normal physiological conditions, but it is phosphorylated immediately when the body is under stress.[27] The *JAK2/STAT3* signaling pathway has been documented to mediate immune reactions and tumor pathogenesis.[28] It has been reported that JAK–STAT is a main downstream pathway of interleukin (IL-6), which is implicated in the neurological injury and impaired neurological repair in several CNS disorders.[29] Activation of *JAK2/STAT3* has also been found in rats with SCI accompanied with increased cell apoptosis- and inflammation- related cytokines.[27] Then we inferred that *miR-34c* might exert the neuroprotective functions through *JAK2/STAT3* inhibition. Next, we further intersected the target genes of *miR-34c* and the differentially expressed genes in SCI rats, with *CXCL14* subsequently identified. The luciferase assay validated *CXCL14* as a target gene of *miR-*

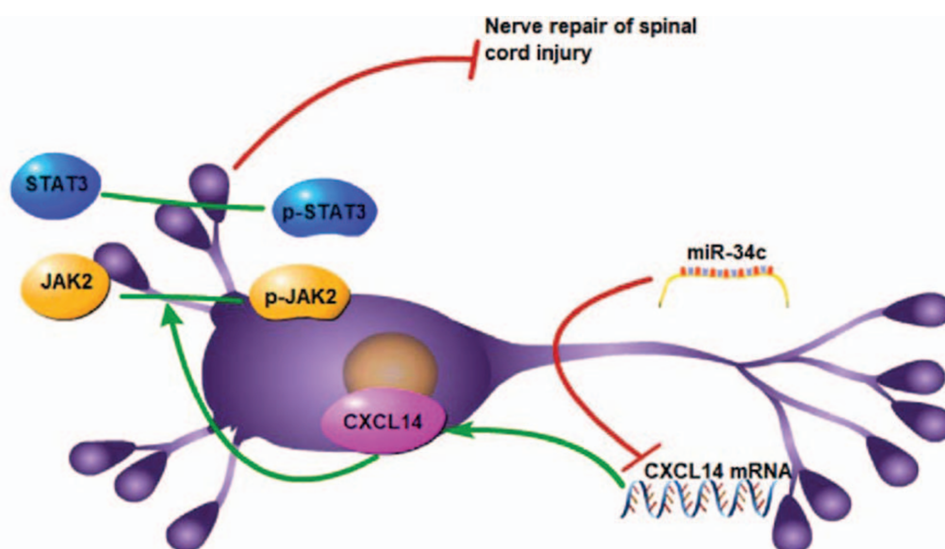

Figure 5: A diagram for the molecular mechanism. *CXCL14* inhibits neuronal recovery in the injured spinal cords by activating the *JAK2/STAT3* signaling pathway. *MiR-34c* negatively regulates *CXCL14* expression, thus promoting neuronal recovery in rats with SCI. *CXCL14*: C-X-C motif ligand 14; *JAK2/STAT3STAT3*: Janus activated kinase2/signal transducer and activator of transcription-3; *miR-34c*: microRNA-34c; SCI: Spinal cord injury.

34c. *CXCL14* has been identified as an emerging immune and inflammatory regulator.[13] However, its role in neural regeneration has been poorly studied. Our study found that the silencing of *CXCL14* stimulated neuronal recovery in SCI rats, presenting as increased grip strength of rat forepaws and *TRH*, *PGI2*, and *GM* concentrations. Importantly, artificial silencing of *CXCL14* was found to decrease the phosphorylation of *JAK2/STAT3* in rats. The direct links between *CXCL14* and *JAK2/STAT3* has not been investigated before, though, it has been found that the *JAK2/STAT3* was activated in chicken IL 26-induced inflammation, during which *CXCL14* was up-regulated as well.[30] Here, we proposed that *CXCL14* could activate the *JAK2/STAT3* signaling pathway, that and inhibited *CXCL14* expression and the following *JAK2/STAT3* inactivation might be responsible for *miR-34c*-mediated neuroprotection in SCI rats.

To conclude, our current study provided evidence that could promote the neuronal repair and functions in SCI rats by down-regulating *CXCL14* expression and activating the *JAK2/STAT3* signaling pathway [Figure 5]. However, the mechanisms involved in SCI is too complicated to be fully explored and explained. Here our study only focused on this *miR-34c/CXCL14/JAK2/STAT3* axis with the grip strength and neuronal apoptosis as the major markers in nerve repair evaluation. Though there are limitations, the findings of the paper might provide novel insights into SCI treatment. We hope more studies in this field will be carried out in the near future to validate our findings, as well as explore other underlying molecular mechanisms involved in the progression of SCI.

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Conflicts of interest

None.

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