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MicroRNA-21a-5p promotes fibrosis in spinal fibroblasts after mechanical trauma

Wenzhao Wang[a,1], Shi Tang[b,1], Hongfei Li[a], Ronghan Liu[a], Yanlin Su[a], Lin Shen[a], Mingjie Sun[a], Bin Ning[a,*]

[a] Department of Spinal Surgery, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong, China
[b] Department of Radiology, West China Hospital of Sichuan University, Chengdu, Sichuan, China

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ABSTRACT

Traumatic spinal cord injury (SCI) causes permanent disability to at least 180,000 people per year worldwide. Early regulation of spinal fibroblast proliferation may inhibit fibrotic scar formation, allowing the creation of a favorable environment for neuronal regeneration and thereby enhancing recovery from traumatic SCIs. In this study, we aimed to identify the role of microRNA-21a–5p (miR-21a-5p) in regulating spinal fibroblasts after mechanical trauma and to investigate the dysregulation of miR-21a-5p in the pathological process of spinal SCI. We investigated the differential expression of microRNAs in primary spinal fibroblasts after mechanical trauma and found that the expression of miR-21a-5p was higher in spinal fibroblasts after scratch damage (SD). In addition, mouse spinal fibroblasts were transfected with miR-21a-5p mimics/inhibitor, and the role of miR-21a-5p in spinal fibrogenic activation was analyzed. These experiments demonstrated that miR-21a-5p overexpression promoted fibrogenic activity in spinal fibroblasts after mechanical trauma, as well as enhancing proliferation and attenuating apoptosis in spinal fibroblasts. Finally, the potential role of miR-21a-5p in regulating the Smad signaling pathway was examined. MiR-21a-5p activated the Smad signaling pathway by enhancing Smad2/3 phosphorylation. These results suggest that miR-21a-5p promotes spinal fibrosis after mechanical trauma. Based on these findings, we propose a close relationship between miR-21a-5p and spinal fibrosis, providing a new potential therapeutic target for SCI.

1. Introduction

Traumatic spinal cord injury (SCI) is a severe pathological state associated with loss of motor function, sensation, and automatic function [1] that causes permanent disability to at least 180,000 people per year worldwide [2]. While SCI causes long-term physical impairments, current treatments are mostly limited to supportive measures. Thus, studies are needed to elucidate the pathophysiology of SCI and identify promising therapies.

The regeneration of spinal axons in the epicenter of SCI lesions has been demonstrated to be the key element to recovery from an SCI [3]; however, there are many impediments to regrowth, including fibrosis in particular [4]. Fibrosis is defined as the overgrowth, hardening, or scarring of tissues and is ascribed to the excess deposition of extracellular matrix (ECM) components [5,6]. The fibrotic response is perhaps more deleterious in the central nervous system (CNS) than in other tissues, as the CNS ECM normally contains relatively small amounts of fibrous proteins and adhesive glycoproteins [7]. After SCI, fibroblasts that detach from the meninges and perivascular cells invade and reside in the lesions, multiplying and expressing ECM components including fibronectin (FN), type I collagen (Col I), and type IV collagen (Col IV) to form a fibrotic scar with accessory glia limitans [8]. The invasion of spinal fibroblasts into the lesion epicenter results in the formation of a fibrotic scar that impedes axonal regrowth by constituting a physical as well as a biochemical barrier [9]. Thus, early regulation of spinal fibroblast proliferation may inhibit fibrotic scar formation, allowing the creation of a favorable environment for neuronal regeneration and thereby enhancing recovery from traumatic

Abbreviations: SCI, spinal cord injury; miRNA, microRNA; SD, scratch damage; ECM, extracellular matrix; CNS, central nervous system; FN, fibronectin; Col1a1, type I a1collagen; Col1a2, type I a2 collagen; Col IV, type IV collagen; qRT-PCR, quantitative real-time PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen; PBS, phosphate-buffered saline; DAPI, 4',6-diamidino-2-phenylindole; TGF-β, transforming growth factor-β; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3K, phosphatidylinositol 3 kinase; CST, corticospinal tract

* Correspondence to: Jinan Central Hospital Affiliated to Shandong University, No. 105, Jiefang Road, Jinan, Shandong 250013, China.
E-mail address: ningbin@sdu.edu.cn (B. Ning).
[1] These authors contributed equally to this work and should be considered co-first authors.

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SCIs.

Fibrosis is regulated by numerous signaling molecules that target different sources of fibroblasts [10]. MicroRNAs (miRNAs) are non-coding RNAs of about 22 nucleotides each that regulate gene expression by binding to the 3′ untranslated regions of messenger RNAs (mRNAs), resulting in translational repression or mRNA degradation [11]. In particular, microRNA-21–5p (miR-21a-5p), a well-known pro-fibrotic miRNA, has been shown to promote fibrosis in several organs, causing various diseases [12]. Likewise, the Smad signaling pathway plays a crucial role in the progression of fibrosis by enhancing the proliferation of fibroblasts and production of ECM components [13]. Moreover, the role of miR-21a-5p in fibrosis after SCI has not yet been investigated. In a prior study, we found that miR-21a-5p was significantly upregulated in the lesion core of spinal tissues after SCI [14]. Thus, we inferred that miR-21a-5p might play a key role in the Smad signaling pathway and its relationship to fibrosis after traumatic SCI. However, the role of miRNAs in the regulation of traumatic spinal fibroblasts has not been well studied.

While many studies have investigated the pathological process of fibrosis in SCI, its underlying mechanisms remain unclear. In this regard, research that probes the mechanism associated with the process of fibrosis in SCI is needed. Here, in an effort to identify the roles of miRNAs in regulating spinal fibroblasts after traumatic SCI, we aimed to establish a spinal fibroblast scratch damage (SD) model, which was assessed using microarray analysis. To extend the study, miR-21a-5p mimics/inhibitor were used for transfection, and we further investigated the role of the Smad signaling pathway in the progression of miR-21a-5p-related spinal fibrosis. Finally, we demonstrated that miR-21a-5p promotes spinal fibrosis, whereas inhibition of miR-21a-5p attenuates this activity. Overall, our study evaluated the fibrotic effects of miR-21a-5p in spinal fibroblasts after traumatic SCI and provides novel insight into the mechanism of fibrotic scar formation and neuron regeneration after SCI.

2. Materials and methods

2.1. Cells culture and mechanical trauma model

Primary spinal fibroblasts (PriCells, Wuhan, China) isolated from mouse spinal cord tissues were placed in modified Eagle's medium (Gibco, Shanghai, China) containing 8% fetal bovine serum (Gibco, Brisbane, Australia) and 100 IU/mL penicillin–streptomycin (Solarbio, Beijing, China) and were cultured at 37 °C in a humidified atmosphere of 5% CO_2. Cells at passages 2–4 were used for the subsequent experiments. To investigate the fibrogenic effect of mechanical trauma, primary spinal fibroblasts in 35-mm dishes at 70–80% confluence were scratched longitudinally and latitudinally with a 200-μL micropipettor pipette tip every 0.5 cm at right angles to each other and were observed under an inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan) for various lengths of time (0, 12, 24, and 48 h post-scratch). Fibroblasts were harvested at 48 h post-scratch.

2.2. Oligonucleotide transfection

Transfection of miR-21a-5p mimics/inhibitor/scrambled controls (RiboBio, Guangzhou, China) was initiated using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). MiR-21a-5p mimics were designed to mimic endogenous miR-21a-5p, thereby upregulating miR-21a-5p activity and enabling functional analysis of miR-21a-5p [15]. MiR-21a-5p inhibitor was also designed to specifically inhibit endogenous miR-21a-5p molecules, thus downregulating miR-21a-5p activity and leading to the loss of miR-21a-5p function [16]. Transfection procedures were carried out according to manufacturer suggestions when the cells were 30–50% confluent. After 48 h, cells were scratched, and they were harvested at 48 h.

2.3. RNA isolation and quantitative real-time PCR

Total RNA was isolated from fibroblasts using RNAiso™ Plus (Takara, Dalian, China). RNA concentration and quality were determined by ultraviolet spectrophotometry (absorbance at 260 nm/280 nm, Invitrogen) The expression levels of miR-21a-5p were measured using quantitative real-time PCR (qRT-PCR) performed with the Bulge-Loop™ miRNA Reverse Transcription Kit (RiboBio), the Bulge-Loop™ miRNA qRT-PCR Starter Kit (RiboBio), and miR-21 primers (RiboBio) and were normalized using small nuclear RNA (U6 snRNA, RiboBio) as the internal control.

Next, mRNAs were reverse-transcribed with the PrimeScript RT Reagent Kit (Takara). The expression levels of type I a1 collagen (Col1a1), type I a2 collagen (Col1a2), type IV collagen (Col IV), and fibronectin (FN), which are commonly used as markers of fibrotic scars, were measured by qRT-PCR with SYBR Premix Ex Taq™ (Takara) using a 7500 RT-PCR system (Applied Biosystems, Wilmington, DE, USA). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. The $2^{-\Delta\Delta CT}$ value was used for comparative quantitation. All PCR reactions were performed in triplicate. The primer pairs used included mmu-miR-21a-5p (forward: 5′-UAGCUUAUCAGACUGAUGUUGA-3′, reverse: 5′-UCA ACAUCAGUCUGAUAAGCUA-3'), mouse Col1a1 (forward: 5′-GACATG TTCAGCTTTGTGGACCTC-3′, reverse: 5′-GGGACCCTTAGGCCATTGT GTA-3′), mouse Col1a2 (forward: 5′-TGCTTGCAGTAACTTCGTGC CTA-3′, reverse: 5′-CATGGGACCATCAACACCATC-3′), mouse Col IV (forward: 5′-CTTCGCCTCCAGGAACGACTA-3′, reverse: 5′-ATGGCCGG TGCTTCACAA-3′), mouse FN (forward: 5′-GCTTTGGCAGTGGTCATTT CAG-3′, reverse: 5′-ATTCCCGAGGCATGTGCAG-3′), and mouse GAPDH (forward: 5′-TGTGTCCGTCGTGGATCTGA-3′, reverse: 5′-TTGCTGTTG AAGTCGCAGGAG-3′).

2.4. Protein isolation and western blot analysis

Total protein was harvested from each experimental group and then centrifuged at 12,000 × *g* for 10 min at 4 °C. Protein concentrations in the supernatant were detected using the enhanced BCA Protein Assay Kit (Beyotime, Shanghai, China). Then, the supernatant samples containing 1 mg protein/500 μL were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Solarbio) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After blocking with 2% bovine serum albumin (Thermo Fisher Scientific, Waltham, MA, USA), the blots were incubated at 4 °C overnight with the following primary antibodies: anti-FN antibody (1:5000; Abcam, Cambridge, UK), anti-Smad7 antibody (1:500; Cell Signaling Technology, Danvers, USA), anti-p-Smad2 antibody (1:500; Cell Signaling Technology), anti-p-Smad3 antibody (1:500; Cell Signaling Technology), anti-Smad2/3 antibody (1:500; Cell Signaling Technology), anti-B-cell lymphoma 2 (Bcl-2; 1:1000; Cell Signaling Technology), anti-Bax (1:1000; Cell Signaling Technology), anti-proliferating cell nuclear antigen (PCNA; 1:1000; Cell Signaling Technology), anti-β-actin monoclonal antibody (1:1000; Cell Signaling Technology), and anti-GAPDH antibody (1:5000; Abcam). After washing, the membranes were probed with goat anti-rabbit and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Proteintech, Wuhan, China) for 45 min at room temperature (22–25 °C) and visualized using West Pico ECL Substrate (Solarbio). All experiments were performed in triplicate.

2.5. Immunofluorescence staining

Cultured mouse spinal fibroblasts on sterile glass cover slips were washed briefly with phosphate-buffered saline (PBS) three times and fixed with 4% paraformaldehyde for 15 min. Then, cells were treated with 0.3% Triton X-100 for 30 min and blocked with normal goat serum for 30 min at room temperature. Subsequently, cells were incubated