MicroRNA-21 regulates cell proliferation and apoptosis in H_2O_2-stimulated rat spinal cord neurons

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Abstract. Oxidative stress can alter the expression level of microRNAs (miRNAs) and has a role in oxidative damage generated by reactive oxygen species (ROS). While previous studies have demonstrated that miR-146a, miR-21 and miR-150 are essential for ROS production in heart disease, the role of these miRNAs in spinal cord injuries has not yet been examined. The present study focused on examining the role of miR-146a, miR-21 and miR-150 during H_2O_2 stimulation in rat neuronal spinal cord (RN-sc) cells. RN-sc cells were treated with H_2O_2, and cells were harvested for reverse transcription quantitative polymerase chain reaction (RT-qPCR) to detect the expression levels of miR-146a, miR-21 and miR-150. The results demonstrated that miR-146a, miR-21 and miR-150 expression was upregulated during H_2O_2 treatment. T-cell death and apoptosis were investigated using an MTT assay and flow cytometric analysis, respectively. Following miR-21 silencing, H_2O_2-induced cell death and apoptosis were reduced in RN-sc cells, while miR-150 silencing had no effect. Furthermore, Smad7 was identified as a direct target of miR-21 using a Luciferase reporter assay, RT-qPCR and western blot analysis. In addition, while H_2O_2 downregulated Smad7 protein expression, this was reversed by inhibiting miR-21 expression. Based on previous studies, it was predicted that miR-21 has a role in ROS production through regulating Smad7 in rat spinal cord neurons.

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Introduction

Reactive oxygen species (ROS) are oxygen-derived radicals and include members such as the highly reactive superoxide (O_2^-), hydroxyl (OH^-) and peroxyl (RO^{2-}), as well as non-radicals, including hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). In healthy individuals, ROS and antioxidants remain in balance; however, an ROS overabundance results in oxidative stress. Previous studies have demonstrated that oxidative stress can alter microRNA (miRNA) expression. miRNAs are non-coding RNAs, ~22 nucleotide (nt) in length, that are evolutionarily conserved and function as sequence-specific regulators of gene expression through translational repression and/or transcriptional cleavage (1-6). In addition, miRNAs have a role in cellular oxidative damage caused by ROS (7,8).

The association between miRNAs and ROS has been investigated in various diseases, including cancer, vascular diseases and cardiometabolic diseases (9-11). UV, H_2O_2, ionizing radiation and anticancer drugs that produce ROS are known to modulate miRNA expression (12-14). Numerous studies have focused on miRNA profiling following oxidative stress exposure in various tissues and have demonstrated the importance of miRNA modulation in the cellular response to a redox imbalance (10). The interaction between ROS and miRNAs remains to be elucidated, with certain studies suggesting that miRNA expression levels could be regulated by ROS, including miR-17-92 (15), while others suggest that miRNAs, including miR-34a and miR-23b, are able to modulate ROS production (8,16). Additionally, miR-23b can either inhibit or promote ROS during transcriptional regulatory processes, thus causing an anti- or pro-oxidant effect (16).

Spinal cord injuries (SCI) are one of the most debilitating pathologies and lead to huge rehabilitation challenges (17,18). SCI is a comprehensive consequence of a primary mechanical insult followed by a sequence of progressive secondary pathophysiological events, with experimental evidence indicating that ROS are important mediators of secondary damage (19-22). Furthermore, increased ROS levels can cause oxidative damage leading to neuronal death and neurological dysfunction (23-25) in uninjured rat spinal cords. Therefore, finding a regulator to reduce ROS damage of the central nervous system may reduce secondary SCI.

The present study aimed to investigate the expression of miR-146a, miR-21 and miR-150 in H_2O_2-stimulated rat spinal cord neurons (RN-sc). In addition, the present study assessed whether inhibition of miR-21 and miR-150 affects cell proliferation and apoptosis.

Materials and methods

Cell culture. RN-sc cells (ScienCell Research Laboratories, San Diego, CA, USA) isolated from the E14 rat spinal cord were and cultured in neuronal medium (ScienCell Research Laboratories; cat. no. 1521).

H_2O_2 treatment, miRNA mimics synthesis and transfection. RN-scs were seeded in 6-well plates, treated 24 h post-seeding with 100 mM H_2O_2 for 6 h and harvested for quantitative polymerase chain reaction (qPCR). Cells were plated to 50% confluency and transfected with 200 nM miR-150, miR-21 mimic or negative control (NC; Guangzhou RiboBio Co., Ltd., Guangzhou, China) using HiPerFect HTS Reagent (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Cells were exposed to 50 mM H_2O_2 24 h post-transfection for varying lengths of time and harvested for further experimentation. For the MTT assay, RN-sc cells were pre-treated with anti-sense-miR-21 or anti-sense-miR-150 mimics for 24 h prior to exposure to 50 μM H_2O_2 for 0, 2, 4 or 6 days. For flow cytometric analysis, RN-sc cells were pre-treated with anti-sense-miR-21 or anti-sense-miR-150 mimics for 24 h, stimulated with a high concentration of H_2O_2 (200 μM) for 12 h and harvested for flow cytometric analysis

Reverse transcription (RT)-qPCR. Total RNA was extracted using TRIzol reagent and reverse transcribed to cDNA using stem loop RT primers specific to miR-146a, miR-150 or miR-21 (Guangzhou RiboBio Co., Ltd., Guangzhou, China). The following PCR primer sequences were used: Smad, forward 5'-TTTTGAGGTGTGGTGGGT-3' and reverse 5'-GAGGCAGTAAGACAGGGATGA-3'. All reactions were performed using SYBR Green mix (Takara Bio Inc., Shiga, Japan) under the following PCR conditions: 94˚C for 5 min, 40 cycles of 94˚C for 30 sec, 55˚C for 30 sec and a final incubation at 72˚C for 20 sec, with fluorescence detected following each cycle and continuously traced using an Applied Biosystems 7500 system (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA). Relative expression levels were calculated as ratios normalized to β-actin. All experimentation was performed in triplicate with the results expressed as the mean ± standard deviation.

Cell proliferation assay. Cell proliferation was monitored using the MTT assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Rat spinal neurons cells were seeded at $1x10^3$ per well in 96-well plates 24 h post-transfection, with the cellular proliferation assay performed on days 0, 2, 4 and 6. To perform the assay, 10 μl MTT reagent was added to each well and the plate was incubated for 4 h at 37˚C. Prior to the end of the incubation period, the absorbance was measured at 570 nm using a Vmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA), with each sample assayed in triplicate.

Cell apoptosis assay. Rat spinal neuron cells were harvested 48 h post-transfection, with $1x10^6$ cells (500 μl) added into FACS tubes, mixed with 25 ng/ml Annexin V-fluorescein isothiocyanate and 10 mg/ml propidium iodide and incubated for 15 min at room temperature in the dark. The cells were then immediately analyzed by flow cytometry on a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. RN-sc cells ($2x10^6$) were collected and washed twice with ice-cold phosphate-buffered saline. The cell pellets were suspended in RIPA lysis buffer, incubated on ice for 40 min and the lysates were centrifuged at 12,000 x g for 15 min at 4˚C. Proteins were isolated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. Membranes were blocked for 1 h at 37˚C with 5% non-fat milk and incubated with rabbit anti-human Smad7 monoclonal antibody (1:1,000; cat no. ab124890; Abcam, Cambridge, MA, USA) or rabbit anti-human β-actin monoclonal antibody (1:2,000; cat no. ab119716; Abcam). Following washing with Tris-buffered saline with 0.5% Tween 20 (TBST), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (H+L) secondary antibody (1:10,000, cat no. ab97080; Abcam) at room temperature for 40 min, washed again with TBST and imaged with enhanced chemiluminescence captured on X-ray films.

Plasmid construction and luciferase reporter assay. To construct a luciferase reporter vector, the wild-type 3' untranslated region (UTR) and mutant 3'UTR of Smad7 containing putative binding sites for miR-21 were subcloned into the psi-CHECK-2 vector. For the luciferase reporter assay, 293T cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were plated at $5x10^4$ cells per well in 24 well plates. On the following day, psiCHECK-2 luciferase vectors containing the 3'UTR of Smad7 and the miR-21 mimic or the negative control oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The luciferase assay was performed 48 h post-transfection using the dual luciferase reporter assay system (Promega Corporation) according to the manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All numerical data were analyzed using Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of H_2O_2 on miR-146a, miR-21 and miR-150 expression in RN-sc cells. To investigate the possibility of miRNAs modulating reactive oxygen injury, the expression levels of miR-146a, miR-21 and miR-150 were monitored during H_2O_2 exposure. The results demonstrated that the expression of miR-146a, miR-150 and miR-21 was upregulated 4.1-fold, 8.4-fold and 9.2-fold, respectively, during H_2O_2 treatment relative to the control samples (Fig. 1). Based on these findings, miR-21 and miR-150 were selected for further evaluation.

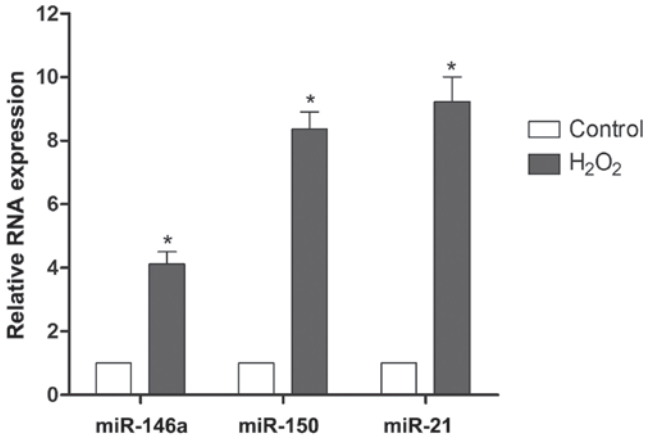

Figure 1. Effect of H_2O_2 on miR-146a, miR-21 and miR-150 expression in rat neuronal spinal cord cells. Cells were treated with the vehicle or H_2O_2 (100 μM) for 6 h. miRNA levels were determined by quantitative polymerase chain reaction, with data presented as the mean ± standard deviation. *P<0.05 compared with control group. miR, microRNA.

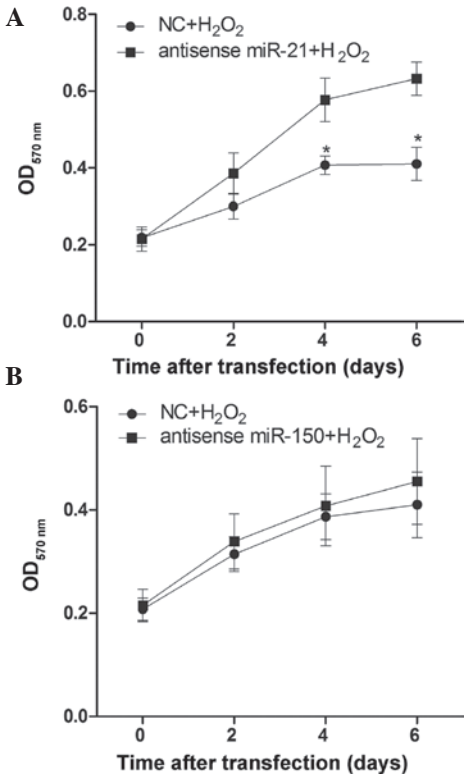

Figure 2. Effect of miR-21 and miR-150 on cellular proliferation during H_2O_2 treatment. RN-sc cells pre-treated with anti-sense-miR-21 or anti-sense-miR-150 mimics for 24 h prior to exposure with 50 μM H_2O_2 for 6 days. The cells were harvested for MTT assays, with the OD determined to assess cellular proliferation at days 0, 2, 4 and 6. Effect of (A) miR-21 and (B) miR-150 on cellular proliferation. Values are expressed as the mean ± standard deviation. *P<0.05 vs. NC + H_2O_2 group. miR, microRNA; OD, optical density; NC, negative control.

Effect of miR-21 and miR-150 on cell proliferation during H_2O_2 treatment. To verify whether decreasing miR-21 and miR-150 has an effect on cellular proliferation following H_2O_2 treatment, RN-sc cells were pre-treated with anti-sense-miR-21 or anti-sense-miR-150 mimics for 24 h, stimulated with 50 μM H_2O_2 for 6 days and harvested for MTT assays at different

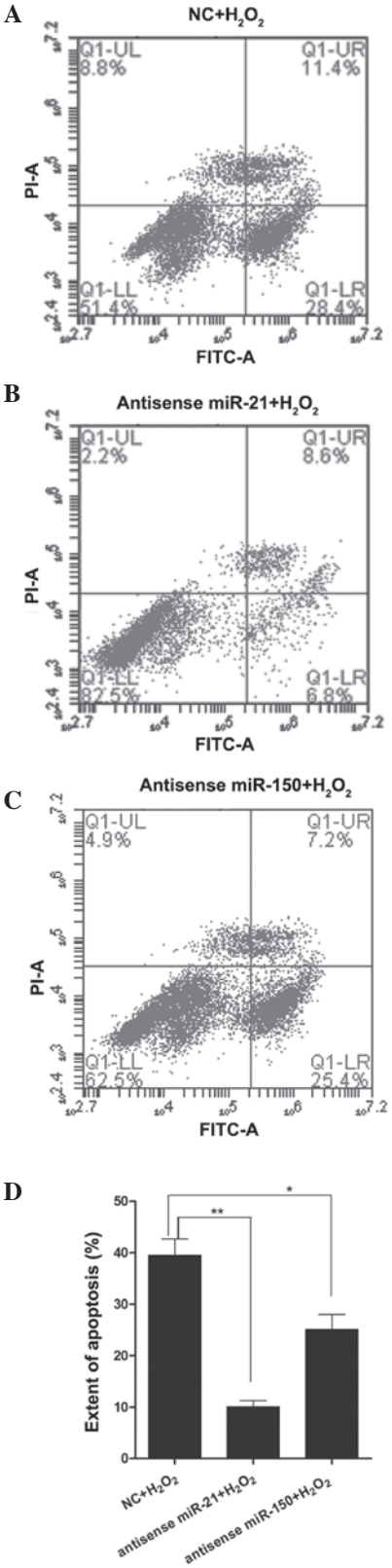

Figure 3. Effect of miR-21 and miR-150 on cellular apoptosis during H_2O_2 treatment. rat neuronal spinal cord cells were pre-treated with anti-sense-miR-21 or anti-sense-miR-150 mimics for 24 h, stimulated with a high concentration of H_2O_2 (200 μM) for 12 h and harvested for flow cytometric analysis with Annexin FITC/PI staining. Images depict (A) the effect of the NC on cell apoptosis; (B) the effect of miR-21 on cell apoptosis; (C) the effect of miR-150 on cell apoptosis and (D) the extent of apoptosis among different experimental groups. The apoptotic ratio combined the early apoptosis percentage plus late apoptosis percentage, with values expressed as the mean ± standard deviation. *P<0.05 vs. NC + H_2O_2 group. miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; NC, negative control.

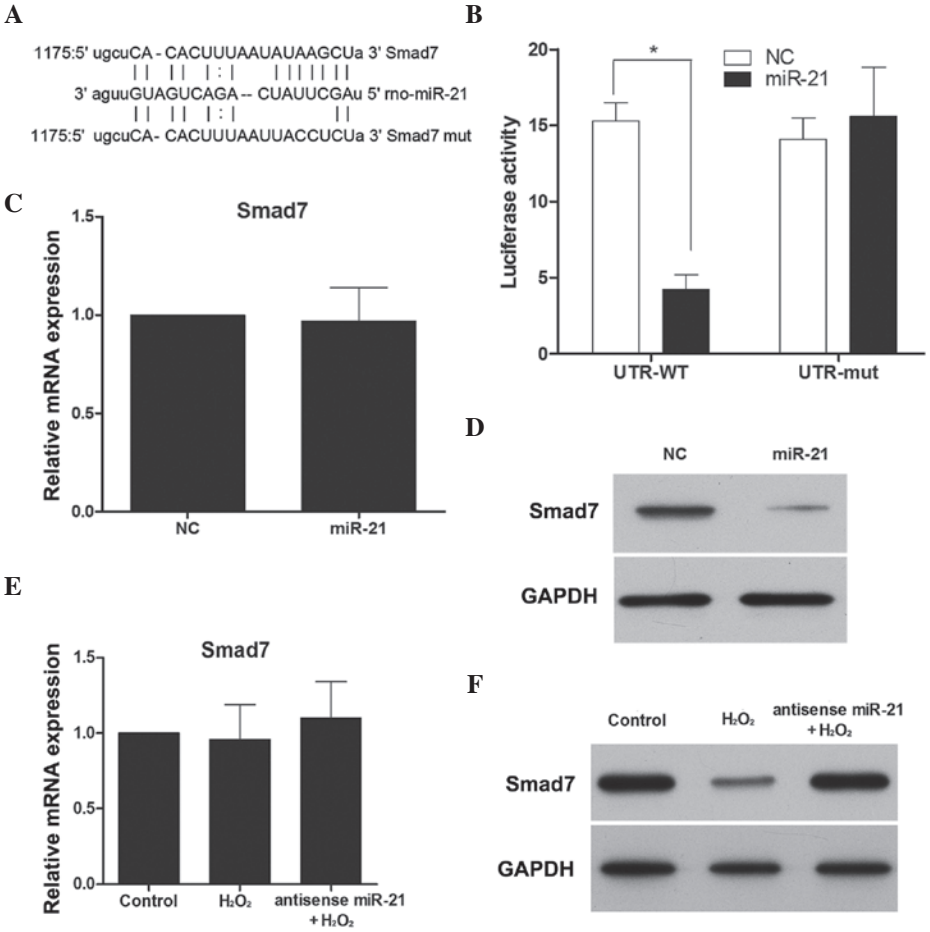

Figure 4. Smad7 is a direct target of miR-21. (A) Predicted duplex formation between the Smad7 3'UTR and miR-21. (B) Luciferase activity of the wild-type (UTR-WT) or mutant (UTR-mut.) SMAD7 3'UTR reporter gene in 293T cells infected with miR-21 or negative control mimics. (C) qPCR of Smad7 in RN-sc cells infected with miR-21 or the negative control mimic. Data were normalized to the level of GAPDH mRNA. (D) Western blot analysis of Smad7 in RN-sc cells infected with miR-21 or the negative control mimics. (E) qPCR of Smad7 in RN-sc cells treated with H_2O_2 with or without an anti-sense miR-21 mimic. Data were normalized to the level of GAPDH mRNA. (F) Western blot analysis of Smad7 in RN-sc cells treated with H_2O_2 in the presence or absence of an anti-sense miR-21 mimic. *P<0.05. miR, microRNA; 3'UTR, 3' untranslated region; WT, wild type; qPCR, quantitative polymerase chain reaction; RN-sc, rat neuronal spinal cord.

time points. The results demonstrated that pre-treatment with the anti-sense-miR-21 mimic could reduce H_2O_2-induced inhibition of cellular proliferation (Fig. 2A), while the anti-sense-miR-150 mimic pre-treatment had no pronounced effect (Fig. 2B).

Effect of miR-21 and miR-150 on cell apoptosis during H_2O_2 treatment. To verify whether a decrease in miR-21 and miR-150 affects apoptosis following H_2O_2 treatment, RN-sc cells were pre-treated with anti-sense-miR-21 or anti-sense-miR-150 mimics for 24 h and stimulated with a high concentration of H_2O_2 (200 μM) for 12 h. Subsequently, the cells were harvested for flow cytometric analysis. The results demonstrated that anti-sense-miR-21 mimic pre-treatment markedly reduced H_2O_2-induced cellular apoptosis relative to the control samples (Fig. 3A, B and D), while anti-sense-miR-150 mimic pre-treatment reduced apoptosis to a lesser extent than anti-sense-miR-21 pre-treatment (Fig. 3A, C and D).

Smad7 is a direct target of miR-21 under H_2O_2 treatment. The above results demonstrated that miR-21, but not miR-150, could regulate proliferation and apoptosis during H_2O_2 treatment in RN-sc cells. To understand the mechanisms by which miR-21 affects cellular apoptosis and proliferation, several computational methods on MIRANDA (http://www.microrna.org) were employed to identify miR-21 targets in rats. Among the predicted targets, Smad7 was of particular interest (Fig. 4A). To assess whether Smad7 was a direct target of miR-21, a luciferase reporter assay was performed. Luciferase inhibition was observed following transfection with the wild-type Smad7 3'-UTR, while no inhibition was noted in the presence of the mut-type Smad7 3'UTR (Fig. 4B). Subsequently, the effect of miR-21 over-expression on Smad7 mRNA and protein expression was examined. Although miR-21 overexpression did not cause degradation of Smad7 mRNA (Fig. 4C), it did reduce the activity of the luciferase reporter gene fused to the wild-type Smad7 3'-UTR, thus indicating that miR-21 targets Smad7 through translational inhibition. In support of these results, a clear reduction in the levels of endogenous Smad7 protein in miR-21-overexpressing RN-sc cells was observed (Fig. 4D).

To further establish interactions between miR-21 and Smad7 during H_2O_2 treatment, Smad7 mRNA and protein expression was monitored in the presence and absence of an anti-sense miR-21 mimic and H_2O_2. While H_2O_2 had no effect on Smad7 mRNA expression, Smad7 protein expression was

downregulated (Fig. 4E and F), and this effect was reversed when miR-21 expression was inhibited (Fig. 4F).

Discussion

In the present study, it was found that H_2O_2 could clearly alter the expression level of miR-21 and miR-150 in RN-sc cells. Previous studies examining cardiac myocytes found that miR-150 and miR-21 expression was upregulated following H_2O_2 treatment and that miR-150 and miR-21 silencing could decrease H_2O_2-induced cardiac cell death and apoptosis (26,27). Another study found that ROS promote gastric carcinogenesis via upregulating miR-21 expression, which in turn downregulates the expression of programmed cell death protein 4 (PDCD4) (28). In a previous study examining cardiac myocytes, H_2O_2-mediated upregulation of miR-21 was confirmed by qPCR, with H_2O_2-induced cardiac cell death and apoptosis increased by a miR-21 inhibitor and decreased by pre-miR-21 (27). Based on these results, it was proposed that miR-21 and miR-150 may have a role in oxidative stress damage in rat spinal cord neurons.

To assess the potential role of miR-21 and miR-150 in H_2O_2-mediated SCI in rat neurons, miR-21 and miR-150 expression was modulated via targeted inhibition. Notably, downregulation of miR-21 expression inhibited H_2O_2-mediated apoptosis and proliferative inhibition in RN-sc cells, while downregulation of miR-150 had no effect. These results suggest that miR-21 downregulation has a protective effect in H_2O_2-mediated apoptosis and proliferative inhibition.

It is clear that miRNAs downregulate the expression of target genes by either inducing mRNA degradation or inhibiting mRNA translation. In lung squamous carcinomas, miR-21 simultaneously regulates multiple pathways that enhance cell proliferation, apoptosis and tumor invasiveness by targeting phosphatase and tensin homolog, reversion-inducing cysteine-rich protein with Kazal motifs and B-cell lymphoma-2 (29). In addition, PDCD4 is an important target gene of miR-21 and miR-21-PDCD4 signaling has been demonstrated to be involved in the regulation of ROS-induced physiological processes (30). However, the exact mechanisms of a given miRNA can remain elusive due to their numerous target genes and potential differences in the role of each target gene. In the present study, Smad7 was identified as a new target of miR-21 in rat spinal cord neurons. Furthermore, Smad7 protein level could be inhibited by H_2O_2, with this effect reversed following the suppression of miR-21 expression. Another study also noted inhibition of Smad7 expression following H_2O_2 exposure and demonstrated that Smad7 inhibited ROS production in angiotensin II-induced cardiac fibroblasts (31). Therefore, it was predicted that miR-21 may be important in ROS production through the regulation of Smad7 in rat spinal cord neurons.

In conclusion, the present study demonstrated that H_2O_2 could upregulate the expression of miR-21 and downregulate the expression of Smad7. Additionally, Smad7 is a target gene of miR-21 and it was predicted that miR-21 may be important in ROS production through Smad7 regulation in rat spinal cord neurons. Future studies are required to further examine the association between SCI, ROS, miR-21 and Smad7 *in vivo*.

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