MicroRNA-218 aggravates H_2O_2-induced damage in PC12 cells via spred2-mediated autophagy

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Abstract. The current study aimed to investigate the effects and underlying mechanism of miR-218 in H_2O_2-induced neuronal injury. The impacts of miR-218 knockdown on cell viability, apoptosis and autophagy-associated proteins were detected by Cell Counting Kit-8 assay, flow cytometry and western blotting in H_2O_2-injured PC12 cells, respectively. Reverse transcription-quantitative PCR (RT-qPCR) and western blotting was executed to explore the expression level of miR-218 and sprouty-related EVH1 domainprotein2 (spred2) in H_2O_2-stimulated cells. Besides, the regulatory association between miR-218 and spred2 was explored through bioinformatics and luciferase reporter assay. Following knockdown of miR-218 and spred2, the functions of miR-218 and spred2 in H_2O_2-injured cells were further studied. High expression level of miR-218 was observed in H_2O_2-disposed PC12 cells, while spred2 expression level was downregulated. Knockdown of miR-218 expression alleviated H_2O_2-induced PC12 cell injury by increasing cell proliferation, and decreasing apoptosis and autophagy. Furthermore, spred2 was identified as a direct target of miR-218 and was negatively regulated by miR-218. Moreover, suppression of spred2 abrogated the protective effects of miR-218 inhibition on H_2O_2-injured PC12 cells. Depletion of miR-218 protected PC12 cells against H_2O_2-induced cell injury via the upregulation of spred2, which provided a promising therapeutic strategy for spinal cord injury.

Introduction

Spinal cord injury (SCI) is a devastating neurological disorder, leading to the motor, sensory and autonomic dysfunction below the injured segment, which not only seriously impacts the quality of life of the patients and represents economic burdens to the society (1). It is well documented that the number of deaths, paraplegia and quadriplegia caused by SCI is gradually increasing. Although numerous patients with SCI have a response to surgery, cell transplantation and gene therapy and drug therapy, highly disabling disease is common, and relapse represents the major cause of treatment failure (2,3). It has become increasingly clear that the primary mechanical injury and secondary oxidative stress, neuronal inflammation and apoptosis are involved in the pathogenesis of SCI (4,5).

miRNAs are small single-stranded RNAs of 18-25 nucleotides in length that are transcribed from DNA (6). miRNA is partially or completely complementary to the 3'UTR region of the target gene by base-pairing interactions to inhibit mRNA translation or induce its degradation, thereby functioning to control the expression of the target gene (7). Emerging research suggest that several miRNAs play pivotal roles in regulating cell differentiation, proliferation and survival (8,9). A variety of miRNAs are differentially expressed in the onset and development of SCI, and are involved in the regulation of multiple physiological processes such as the proliferation, apoptosis and autophagy, as well as oxidative damage of neuronal cells (10,11). There is emerging evidence that the excess production of reactive oxygen species, such as H_2O_2, has been implicated in the secondary damage after SCI (12). Further clarification of the key miRNA molecules regulating H_2O_2-caused cell injury exploits new ideas for the diagnosis and treatment of SCI. It was recently reported that miR-218-5p was significantly upregulated in oxygen-glucose deprivation/reoxygenation (OGD/R)-treated PC12 cells, and downregulation of miR-218-5p protected against OGD/R-induced cell injury through attenuating oxidative stress, inflammation and apoptosis (13). A large quantity of evidence has revealed abnormal expression of miR-218 in cancer (14), cardiovascular diseases (15) and neurological disorders (16). However, little was known about the roles of miR-218 in SCI to the best of our knowledge.

The present study was designed to investigate the effect of miR-218 on H_2O_2-induced apoptosis and autophagy of rat neuronal PC12 cells, and to further examine the underlying mechanism.

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Materials and methods

Cell culture and treatment. Rat neuronal PC12 cell line, obtained from BioVector NTCC, were cultured in DMEM medium containing 10% fetal bovine serum and penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere with 5% CO_2. Culture medium was changed every other day. For the H_2O_2 treatment, PC12 cells were seeded into cell culture multi-well plates and incubation was continued for 24 h. To induce the cell injury model, these cells were then exposed to fresh medium containing 200 μM H_2O_2 for 24 h as previously described (17,18). The cells treated with the same medium without H_2O_2 were used as controls.

Cell transfection. The mirVana® miRNA mimic rno-miR-218 (Assay ID: MC20167), mirVana™ miRNA Mimic Negative Control, mirVana® miRNA inhibitor anti-rno-miR-218 (Assay ID: MH20167), mirVana™ miRNA Inhibitor Negative Control, predesigned Silencer® Select siRNA spred2 (Assay ID: s156883) and Silencer® Select siRNA negative control were all were purchased from Thermo Fisher Scientific, Inc.. The PC12 cells were transfected with the vectors after confluence reached 70% using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were collected after 48 h transfection for the following experiments. The sequences of the transfection agents are shown in Table I.

Table I. Sequences of transfection vectors.

Vector	Sequence, 5'-3'
miR-218 mimics	UUGUGCUUGAUCUAACCAUGU
miR-NC	UCGCUUGGUGCAGGUCGGGAA
miR-218-inhibitor	ACATGGTTAGATCAAGCACAA
NC-inhibitor	UUCUCCGAACGUGUCACGUTT
si-spred2	GGAAGAUGAUGAAGAGAUAGU
si-NC	CAGUACUUUUGUGUAGUACAA

miR, microRNA; NC, negative control; si-, small interfering RNA; spred2, sprouty-related EVH1 domainprotein2.

Luciferase reporter assay. The coding sequence of spred2 containing the predicted binding site of miR-218 was amplified, which was inserted into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation) to establish the vectors of spred2-wild-type (spred2-wt). The spred2-mutated-type (spred2-mut) reporter vector carrying the mutated binding site of miR-218 in the spred2 was constructed as negative controls. Luciferase assays were performed in PC12 cells after co-transfection with the wild/mutated types of spred2 promoter reporters and miR-218 mimics or miR-NC using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h of transfection, the luciferase activities were tested using the Dual-Luciferase Reporter Assay system (Promega Corporation).

Cell viability assay. Transfected PC12 cells were re-seeded into 96-well plates at a density of $2x10^3$ cells per well, and 10 μl Cell Counting Kit-8 (CCK-8) reagent (Beyotime Institute of Biotechnology) per well was added at 24 h. After incubation for 2 h, the absorbance was analyzed at 450 nm.

Apoptosis detection. PC12 cells ($1x10^5$ cells/well) were cultured in a 96-well plate and labeled with Annexin V/PI fluorescent double staining (BD Biosciences) for 30 min as previously described (19). The apoptotic cells were analyzed with flow cytometry (FACScan; BD Biosciences) using FlowJo software (version X; FlowJo LLC).

RNA isolation, reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using TRIzol® reagents (Thermo Fisher Scientific, Inc.) and reverse-transcribed to cDNA using the TaqMan™ MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) and the Prime Script RT reagent kit (Takara Bio, Inc.) following the manufacturer's instructions, respectively. The relative expression levels of miR-218 and spred2 were normalized to U6 and β-actin as an internal control using the Primer-Script™ RT-PCR kit (Takara), respectively. The specific primers were as follows: miR-218 forward, 5'-AAG ACACCCTGGACGAAGCC-3' and reverse, 5'-ACAACC AGAGTCCACCGGCG-3'; U6 forward, 5'-ATTGGAACG ATACAGAGAAGATT-3' and reverse, 5'-GGAACGCTT CACGAATTTG-3'; spred2 forward, 5'-TATATTGTGCGT GTCAAGGCTG-3' and reverse, 5'-GGGGTGCATGAC CTTACAGA-3'; β-actin forward, 5'-CATGTACGTTGC TATCCAGGC-3 and reverse, 5'-CGCTCGGTGAGGATC TTCATG-3'.

Western blotting. Protein was extracted through radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology) and protease inhibitors (Roche Diagnostics). Total protein was measured using a bicinchoninic acid (BCA) assay. The protein (30 μg per lane) was separated by 10% polyacrylamide gel electrophoresis and electro-blotted to polyvinylidene fluoride membranes. After blocking with 5% nonfat milk in TBST for 2 h at room temperature, the membranes were subsequently incubated overnight at 4˚C with the indicated antibodies (1:1,000 dilution), followed by incubation with secondary antibodies (1:2,000 dilution) for 2 h at room temperature. The primary antibodies included rabbit polyclonal to spred2 antibody (cat. no. ab153700; Abcam), monoclonal rabbit p62 antibody (cat. no. 8025; Cell Signaling Technology, Inc.), monoclonal rabbit LC3-I antibody (cat. no. 4599; Cell Signaling Technology, Inc.), monoclonal rabbit LC3-II antibody (cat. no. 3868; Cell Signaling Technology, Inc.), monoclonal rabbit β-actin antibody (cat. no. 4970; Cell Signaling Technology, Inc.). The secondary antibodies were HRP-conjugated goat anti-rabbit IgG antibodies (cat. no. 7074; Cell Signaling Technology, Inc.). The gray values were measured using ImageJ software (National Institutes of Health).

Statistical analysis. All data were statistically analyzed by SPSS software (version 19.0; IBM Corp.) and expressed as

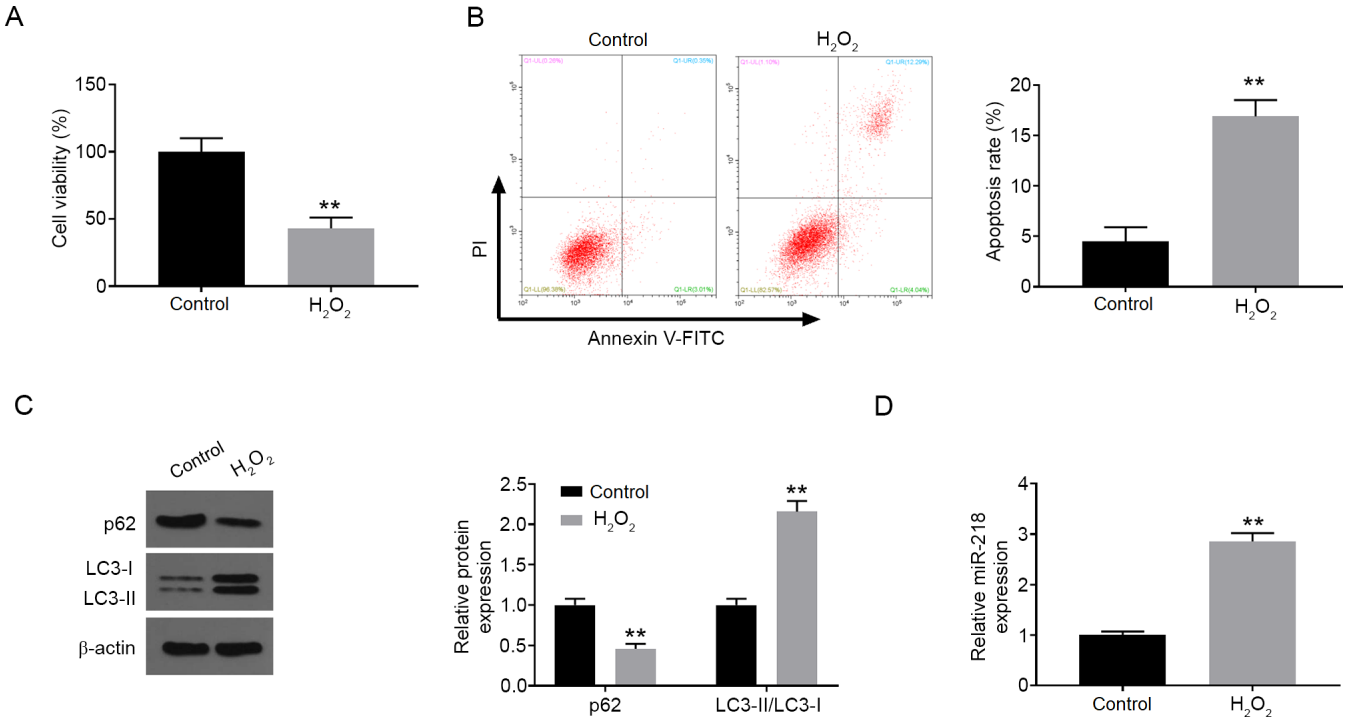

Figure 1. H_2O_2 induces PC12 cell injury and miR-218 upregulation. After treatment with 200 μM H_2O_2 for 24 h in PC12 cells, cell viability was detected by Cell Counting Kit-8 assay (A); cell apoptosis was tested via flow cytometry (B); the protein levels of autophagy-associated proteins were determined by western blotting (C); and the expression of miR-218 was determined by reverse transcription-quantitative PCR (D). All experiments were repeated three times. Data are expressed as mean ± SD. **P<0.01. miR, microRNA.

mean ± standard deviation (SD). Unpaired Student's t-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used to assess the difference between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

H_2O_2 induces PC12 cell injury and miR-218 upregulation. In order to construct the *in vitro* neuronal cell injury model, PC12 cells were treated with 200 μM H_2O_2 for 24 h. Compared with the control group, H_2O_2 treatment markedly suppressed cell viability detected by CCK-8 assay (Fig. 1A). Furthermore, the percentage of cell apoptosis was significantly increased after H_2O_2 treatment compared with the control group (Fig. 1B). Moreover, the expression level of p62 was dramatically downregulated, while the ratio of LC3-II/I was observably increased after H_2O_2 treatment in comparison with the control group (Fig. 1C). In addition, H_2O_2 stimulation also resulted in increased expression of miR-218 in PC12 cells (Fig. 1D).

Inhibition of miR-218 increases proliferation and decreases apoptosis and autophagy in H_2O_2-treated PC12 cells. To confirm whether miR-218 is involved in the regulation of H_2O_2-disposed neuronal cell injury, the expression of miR-218 was thus silenced in PC12 cells by transfection with miR-218 inhibitor (Fig. 2A). In the subsequent experiments, the knockdown of miR-218 markedly inhibited H_2O_2-induced decrease in cell viability (Fig. 2B) and alleviated H_2O_2-induced cell apoptosis (Fig. 2C and D), as well as ameliorating H_2O_2-induced cell autophagy by reversing the changes in the expression levels of autophagy-associated proteins (Fig. 2E).

Spred2 is a direct target of miR-218 in PC12 cells. Using the online database starBase, spred2 was predicted as the potential target of miR-218 (Fig. 3A). The results of luciferase assay in PC12 cells further verified that miR-218 mimics significantly suppressed the luciferase report activity of spred2-wt, but not spred2-mut (Fig. 3B). The overexpression of miR-218 was achieved in PC12 cells by transfection with miR-218 mimic, which was further confirmed by RT-qPCR (Fig. S1). Additionally, the mRNA and protein levels of spred2 were memorably decreased after miR-218 overexpression, but were elevated by miR-218 silencing (Fig. 3C and D). The expression levels of spred2 in PC12 cells were detected after H_2O_2 treatment. The present study data showed that the expression of spred2 at mRNA and protein levels was markedly decreased following treatment with H_2O_2 compared with the control group (Fig. 3E and F).

miR-218 enhances H_2O_2-induced PC12 cell injury by targeting spred2. Subsequently, it was investigated whether miR-218 regulated H_2O_2-induced injury via decreasing spred2 expression. Spred2 was knocked down in PC12 cells by transfection with si-spred2 (Fig. 4A). As shown in Fig. 4B, spred2 inhibition significantly reversed miR-218 downregulation-induced promotion of cell proliferation in H_2O_2-exposed PC12 cells (Fig. 4B). Moreover, the inhibitory effects of miR-218 inhibition on cell apoptosis (Fig. 4C and D) and autophagy (Fig. 4E)

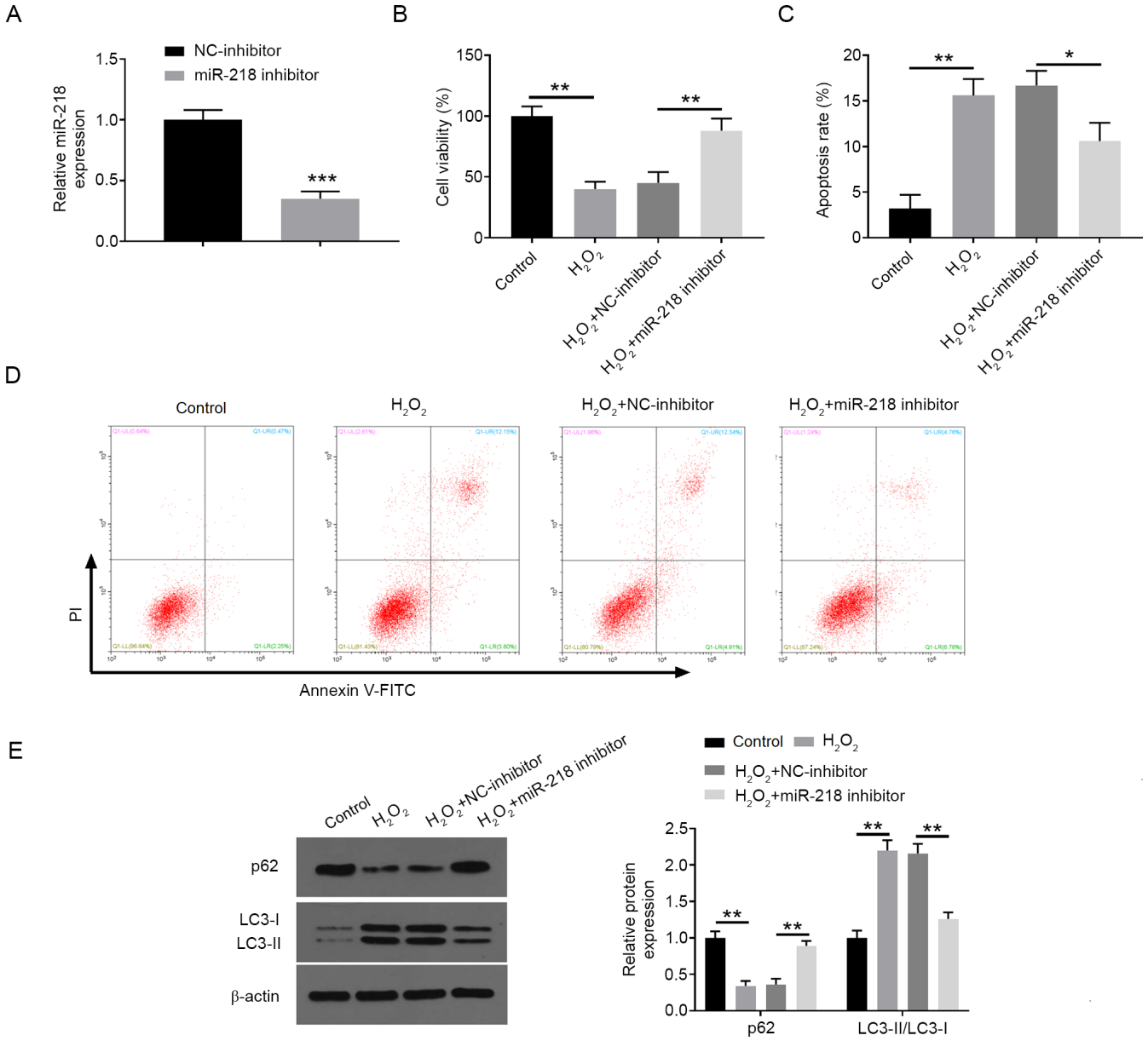

Figure 2. Inhibition of miR-218 increases proliferation and decreases apoptosis and autophagy in H_2O_2-treated PC12 cells. The expression of miR-218 was determined after transfection with miR-218 inhibitor or NC inhibitor in PC12 cells (A). Cell viability (B), cell apoptosis (C and D), and the protein levels of autophagy-associated proteins (E) was determined in PC12 cells transfected with miR-218 inhibitor or NC inhibitor, following treatment with 200 μM H_2O_2 for 24 h. All experiments were repeated three times. Data are expressed as mean ± SD. *P<0.05, **P<0.01, ***P<0.001. miR, microRNA; NC, negative control.

were also reversed by spred2 knockdown in H_2O_2-treated PC12 cells.

Discussion

In the current study, a cell injury model induced by H_2O_2 was constructed to investigate the protective role of miR-218 knockdown in H_2O_2-injured PC12 cells. The study initially indicated that miR-218 expression was significantly increased in PC12 cells after H_2O_2 treatment. Moreover, suppression of miR-218 alleviated H_2O_2-induced PC12 cell injury by enhancing cell proliferation and inhibiting apoptosis and autophagy. Furthermore, it was identified that spred2 was a direct target of miR-218 and was negatively regulated by miR-218. Further investigation demonstrated that miR-218 mediated H_2O_2-induced PC12 cell injury through regulating spred2.

SCI is a severe neurological disease with a high disability rate (20). To date, emerging effort has been made to identify critical regulators, which are involved in the pathogenesis of SCI, and the pharmacological therapies targeting these regulators have been developed for clinical trials to prevent neurological dysfunction (21,22). Recently, there is emerging evidence that a larger number of miRNAs are aberrantly expressed following SCI and might play a key role in the development of SCI (23). There are abundant cancer-associated research regarding the anti-tumor roles of miR-218 (24,25). A previous study by Zhu *et al* (13) demonstrated that downregulation of miR-218 protected against OGD/R-induced injuries of PC12 cells through lowering inflammation, oxidative stress,

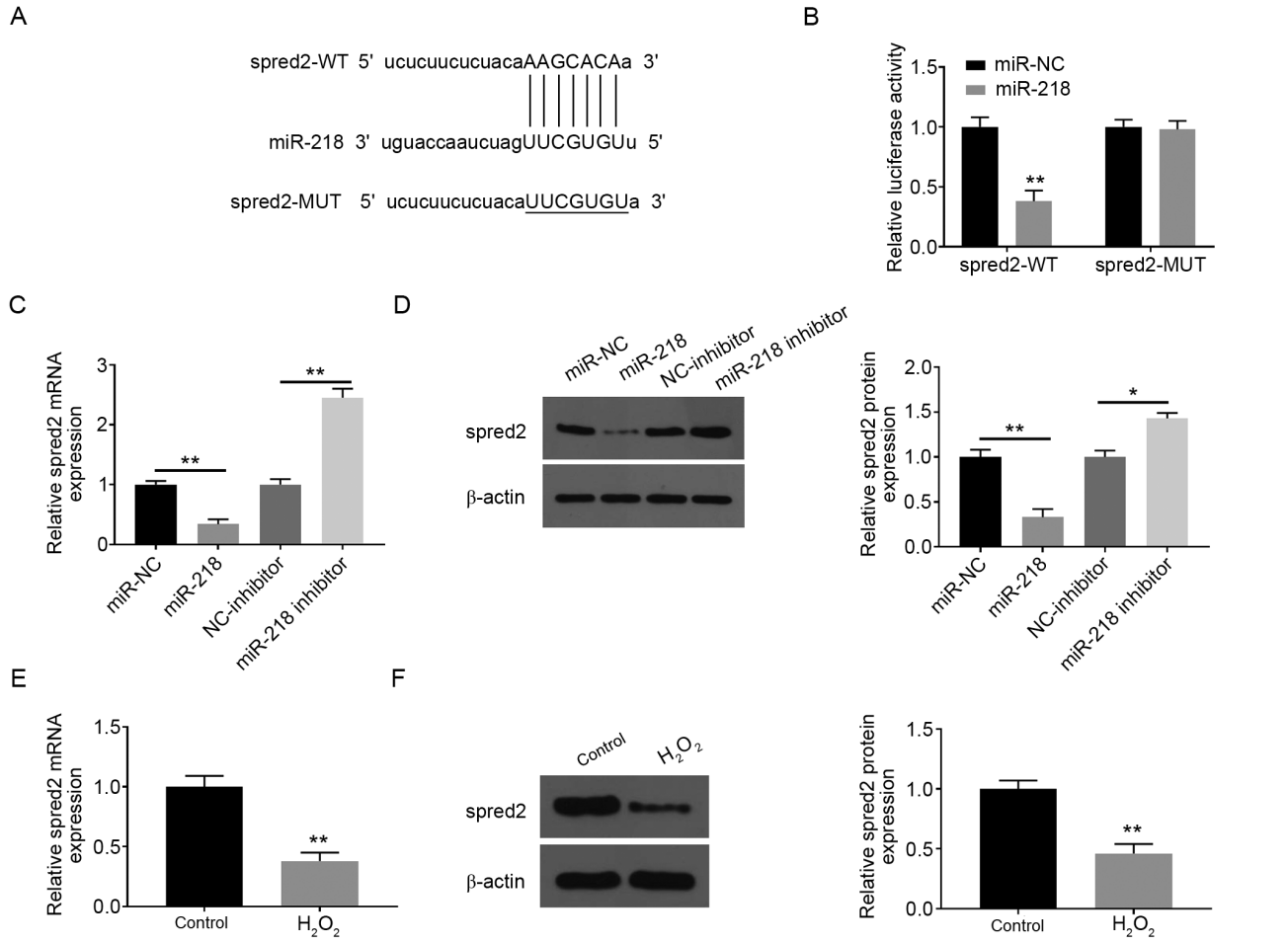

Figure 3. Spred2 is a direct target of miR-218 in PC12 cells. (A) Predicted sequence between miR-218 and spred2 using starBase online database. (B) The luciferase report activity of spred2-WT/-MUT in PC12 cells. (C and D) mRNA and protein levels of spred2 in PC12 cells after transfection with miR-218 mimics, miR-218 inhibitor and their controls. (E and F) The mRNA and protein levels of spred2 in PC12 cells after H_2O_2 treatment. All experiments were repeated three times. Data are expressed as mean ± SD. *P<0.05, **P<0.01. Spred2, sprouty-related EVH1 domainprotein2; miR, microRNA; WT, wild type; MUT, mutant, NC, negative control.

apoptosis and maintenance of endovascular homeostasis; nevertheless, the involvement of miR-218 in SCI remains unclear.

In recent years, several studies have proposed that oxidative stress caused by free radicals is implicated in the pathophysiological processes of secondary injury following SCI. The suppression of oxidative injury is therefore considered effective therapeutic strategy to improve SCI. Exposure of neurons to H_2O_2 results in oxidative stress and the activation of a cascade of intracellular toxic events, ultimately resulting in cell death (26). Autophagy is a metabolic process degrading long-lived proteins and organelles, which is not only a highly-conserved defense mechanism, but also functions as a mechanism of programmed cell death involving the degradation of damaged organelles and misfolded proteins in eukaryotes, which is associated with numerous pathological or physiological processes (27,28), including the pathogenesis of SCI (29). In the present study, H_2O_2 exposure at a concentration of 200 μM induced proliferation inhibition and increase in apoptosis and autophagy in PC12 cells, indicating that H_2O_2 treatment successfully induced cell injury in PC12 cells. It was demonstrated that miR-218 expression was significantly augmented in H_2O_2-stimulated PC12 cells. Employing H_2O_2-induced PC12 cells, it was also discovered that miR-218 inhibition ameliorated H_2O_2-induced decrease in cell viability and increase in apoptosis and autophagy.

Furthermore, the present study findings also confirmed that spred2 might be a target gene of miR-218, by means of bioinformatics prediction and luciferase reporter assays. Spred2 is a negative regulator of the ERK-MAPK pathway and is essential for cell survival, inflammation and angiogenesis (30,31). Moreover, spred2 is recently identified as a vital regulator of autophagy and cell death by interacting with LC3 and p62 (32,33). Studies showed that spred2 interaction with NBR1 downregulated fibroblast growth factor (FGF) signaling in the regulation of receptor trafficking (34). Chen *et al* (35) demonstrated that miR-210 directly inhibited spred2 expression, and spred2 decreased oxLDL-mediated migration of vascular cells via the ERK/c-Fos/MMPs pathway. Spred2 was reported as a target gene of miR-221-3p, and was involved in the development of LPS-induced lung inflammation by negatively regulating the ERK1/2 pathway (36,37). Spred2 was also identified as the downstream target of miR-1246, wherein downregulated spred2 further reversed the inhibition of the MAPK pathway (38). The expression of spred2 in PC12

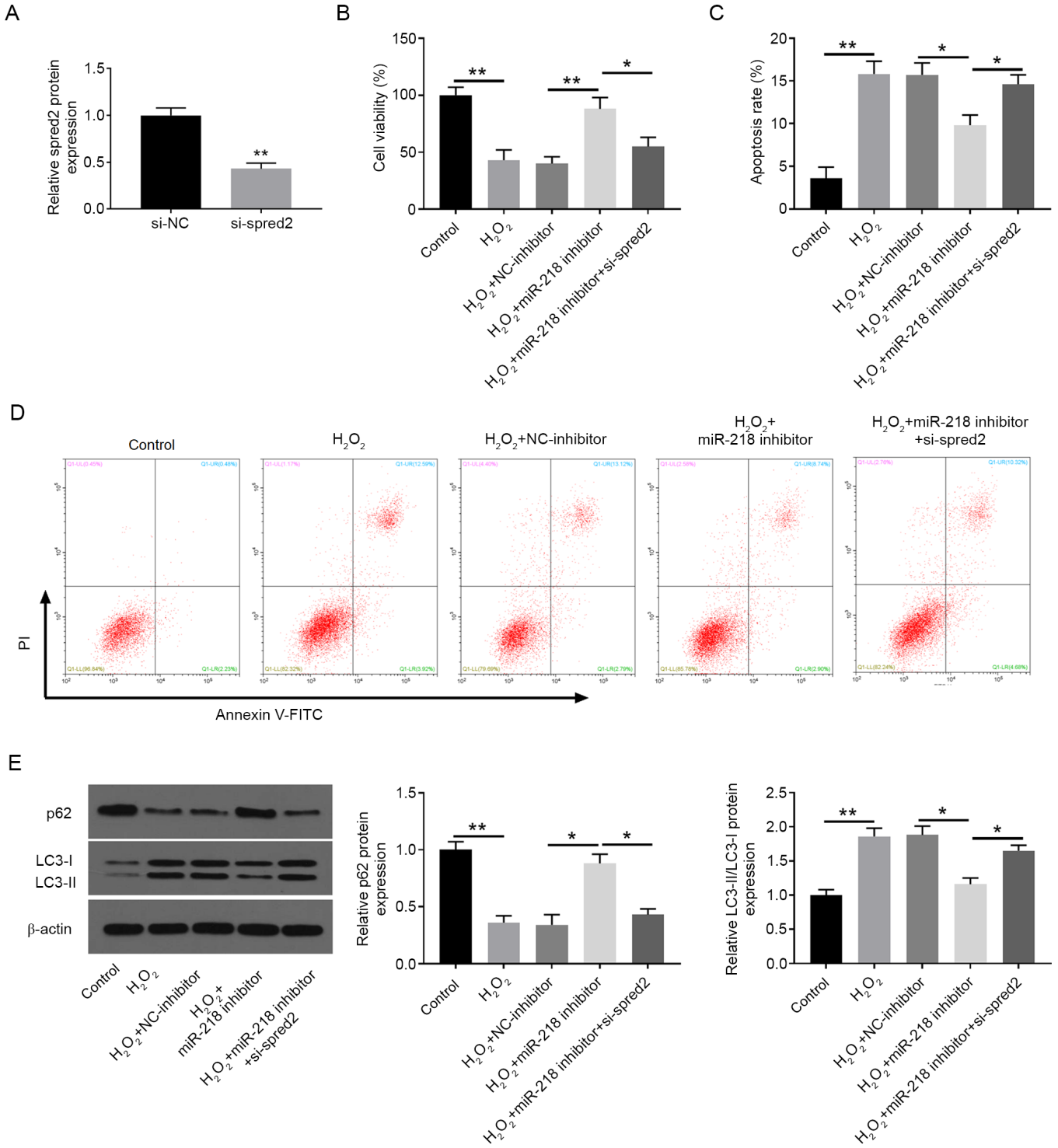

Figure 4. miR-218 enhances H_2O_2-induced PC12 cell injury by targeting spred2. The protein levels of spred2 were determined in PC12 cells after transfection with si-spred2 or si-NC (A). Cell viability (B), cell apoptosis (C and D) and the protein levels of autophagy-associated proteins (E) were detected in PC12 cells co-transfected with miR-218 inhibitor and si-spred2, followed by treatment with 200 μM H_2O_2 for 24 h. All experiments were repeated three times. Data are expressed as mean ± SD. *P<0.05, **P<0.01. miR, microRNA; spred2, sprouty-related EVH1 domainprotein2; si-, small interfering RNA-; NC, negative control.

cells was downregulated after H_2O_2 stimulation in the present study. Loss- and gain-of-function analyses further revealed that spred2 knockdown overturned the anti-apoptotic and anti-autophagy effects of miR-218 suppression in H_2O_2-stimulated PC12 cells. Following results, it was shown that miR-218 deficiency impaired autophagy and apoptosis by targeting spred2 in PC12 cells under H_2O_2 stimulation. According to the previous studies, differentiated PC12 cells appeared to be significantly more resistant to H_2O_2 than naive PC12 cells (39). Further research is necessary to investigate the detected mechanisms in differentiated PC12 cells.

Overall, despite the lack of clinical data and *in vivo* experiments, as well as the use of only one cell line, the present study findings provided a novel insight into the effects

and mechanisms of miR-218 in H_2O_2-induced PC12 cell injury. It was preliminarily confirmed that the downregulation of miR-218 may protect PC12 cells from H_2O_2-disposed injury via targeting spred2. The present study is the first to demonstrate that the miR-218-spred2 axis may be a promising therapeutic strategy for SCI.

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Availability of data and materials

The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

DC and CL were involved in the conception and design, acquisition of data, and drafting the article, and performed the analysis and interpretation of data, and revised the manuscript critically for important intellectual content. RL revised the manuscript, analyzed the data and gave approval of the version to be published and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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