Original Article

MiR-495 regulates cell proliferation and apoptosis in H_2O_2 stimulated rat spinal cord neurons through targeting signal transducer and activator of transcription 3 (STAT3)

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Background: MicroRNA-495 (miR-495) is a post-translational modulator that performs several functions, and it is involved in several disease states. On the other hand, the physiological functions of miR-495 in H_2O_2 stimulated mouse spinal cord neuronal dysfunction have not yet been fully understood.

Methods: In this study, we speculated that miR-495 may regulate the expression of STAT3 in the processes of neuronal proliferation and apoptosis following spinal cord injury (SCI). Cell viability was assessed with methyl thiazolyl tetrazolium (MTT) assay. Caspase-3 activity was assayed with ELISA. Cellular apoptotic changes were measured with TUNEL assay. Intracellular ROS production was determined by measuring uptake of dichlorodihydrofluorescein diacetate (DCFH-DA; PCR was used to assay the mRNA expression of STAT3 gene bearing predicted targeting positions for miR-495, while qRT-PCR was used to measure miR-495 mRNA.

Results: The results demonstrated that treatment of SCNs with H_2O_2 led to a significant decrease in cell survival, while it enhanced apoptosis. The H_2O_2 treatment induced cell membrane dysfunction, and increased ROS levels and DNA damage. Interestingly, the expression of miR-495 was markedly suppressed when SCNs were exposed to H_2O_2. However, miR-495 overexpression reversed H_2O_2-induced cytotoxicity and apoptosis in SCNs. Moreover, H_2O_2 exposure elevated protein and mRNA concentrations of STAT3 in SCNs. Bioinformatics analysis showed likely binding domains of miR-495 in the 3'-untranslated regions of STAT3 in SCNs. MiR-495 loss-of-function and gain-of-function significantly up-regulated and down-regulated both STAT3 mRNA and protein expressions, respectively, in SCNs.

Conclusions: miR-495 overexpression inhibited H_2O_2-induced SCN dysfunction. This mechanism was mediated through the down-regulation of STAT3 expression.

Keywords: MicroRNA-495 (miR-495); spinal cord injury (SCI); STAT3; neural differentiation

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Introduction

Spinal cord injury (SCI) is a debilitating condition that contributes to post-traumatic tissue functional deficits (1). Initial damage to the spinal cord may simultaneously disintegrate cell membranes, injure myelin and axons, and cause damage to micro-vessels, thereby activating worse secondary injuries (2). The secondary injury cascades are functional biological processes which include local inflammation, free radical production, and increased oxidative stress, all of which may induce neuronal apoptosis, glial cell death and axonal destruction, ultimately leading to permanent neurological disability (3,4). Previous studies have demonstrated that hypoxia (5), ischemia (6), lipid peroxidation (7), and ROS (7) are implicated in the underlying pathophysiology of SCI-induced neuronal apoptosis. Interestingly, hydrogen peroxide (H_2O_2) induces neuronal death in the spinal cord through intracellular ROS generation and the modulation of genes related to apoptosis (8). Moreover, the microRNA-21 (miR-21) has been shown to regulate cell proliferation and apoptosis in H_2O_2-stimulated neurons of the spinal cord, and it controlled ROS generation by its effect on Smad7 in mouse SCNs (9). Although the internal and external signals modulating the apoptosis of neurons have been much explored, the mechanisms that underlie the effect of miRNAs on neuronal apoptosis following SCI are still unclear.

MicroRNAs, a category of endogenous molecules (18-25 nucleotides) which do not code for amino acids, exert regulatory influence on transcription via interaction with the 3'-UTRs of mRNA targets (10). MiRNAs play important roles in cell proliferation, differentiation, and apoptosis in various cell types (11,12). Several studies have found that they are associated with several pathological tissue lesions, as well as uncommon miRNA expressions, although various cell signaling pathways regulate the development of neurons (13,14). MiR-93, by targeting Eph-A4, has been shown to increase neurite formation from neurons of the spinal cord (15). Furthermore, the up-regulation of miR-26a promoted neurite growth, and also protected the spinal cord against local analgesia-induced nerve damage (16). MiR-9 has been shown to modulate the apoptosis of neurons by targeting monocyte chemotactic protein-induced protein-1 expression in a mouse model of SCI (17). MiR-124 is highly expressed in neurons and has important functions in embryonic neurogenesis and postnatal neuronal differentiation. However, miR-124 expression in neurons was down-regulated after SCI in a mouse model (18). These results indicate that miRNAs are the upstream molecular regulators of neural differentiation, and may be potential therapeutic targets for SCI. Importantly, many miRNAs have been shown to be markedly up-regulated or down-regulated in H_2O_2-stimulated rat SCNs (9). However, the molecular mechanisms involved in the effect of miR-459 on SCI, and the involvement of STAT3 remain unknown.

In the present study, H_2O_2 was added to cell cultures so as to induce oxidative stress, a phenomenon related to secondary SCI, and we hypothesized that microRNA-495 (miR-495) may regulate STAT3 expression and its involvement in responses to H_2O_2-stimulated rat SCNs. We present the following article in accordance with the MDAR reporting checklist (http://dx.doi.org/10.21037/atm-21-102).

Methods

Cultural conditions

Primary neurons of the spinal cord were harvested from embryonic day 14 Sprague-Dawley rats as previously described (19). The cells were cultured at 37 ℃ in high-glucose DMEM containing 10% heat-inactivated FBS and 5% heat-inactivated horse serum (Gibco Lab) in a humidified atmosphere containing 5 % CO_2 and 95% O_2.

MTT assay

To assess cell viability, the MTT assay was performed on SCN outgrowths using an assay kit (Shanghai Jining Industrial Co., LTD., China) in line with the instructions on the kit manual.

Measurement of caspase 3 activity

SCNs were lysed and then stained using anti-caspase-3 (Shanghai Enzyme-linked Biotechnology Co., Ltd. China). Immunocomplexes were formed after 2 hours, and the absorbance of the p-nitroaniline liberated was read at 405 nm in an ELISA instrument.

TUNEL assay

Apoptosis was measured using terminal dUTP nick-end labeling (TUNEL) assay. Following trypsinization, SCNs were exposed to 4% paraformaldehyde, and subjected to permeabilization with Triton-X-100 in 0.1% Na citrate. After washing, cells were put in an incubator along with

the reaction mixture for 60 minutes at 37 ℃, after which they were immediately analyzed using FACScan (Becton Dickinson) and the Cellquest program.

Lactate dehydrogenase (LDH) activity assay

SCNs were seeded in 96-well plates, and then treated with H_2O_2. After 24 hours, cells were centrifuged to obtain the supernatants which were subjected to LDH assay using assay kits (Keygen, Nanjing, China) in line with manual protocol. Data were normalized with the concentration of podocyte protein lysates.

ROS measurements

Intracellular ROS generation was determined by measuring the uptake of DCFH-DA. The SCNs were seeded in well plates and treated with the dye for 24 hours. Then, the cells were analyzed using flow cytometry (Becton Dickinson).

Comet assay

Frozen slides were fully precoated with 0.8% agarose in PBS of pH approximately 7.4, and also blanketed with 22 mm × 22 mm glass coverslips, and kept for 20 min at laboratory temperature. Next, a 3:7 volume ratio of cell culture and 1% low MP agarose was evenly spread over every corner of the precoated slides, blanketed, and imaged with an Olympus fluorescence microscope bearing a CCD camera. Comet tail lengths were calculated in every group with Image proR plus appliance. Olive tail moment (OTM) denoted DNA damage, and it was quantified viz:

$$OTM\left(\%\right)=\frac{\left(Head\ mean\right)\times Tail\ \%\ DNA}{100} \quad [1]$$

Luciferase reporter assay

The 3'-untranslated regions of the STAT3 gene bearing the predicted target points for miR-495 were got via polymerase chain reaction amplification. The construct was placed into several cloning points in pMIR-REPORT luciferase miRNA reporter vector. SCNs were subjected to co-transfection with Lipofectamine 2000 using 0.1 μg of luciferase reporters bearing miR-495 mimics and STAT3 3'-UTR. Cell lysates were obtained 48 hours after transfection, and luciferase was measured using the dual luciferase reporter assay kits.

Transfection of miR-495 inhibitors and mimics

FAM-derivatized 2'-OMe-oligonucleotides were produced by GenePharma, Shanghai, China. The sequence of 2'-OMe miR-495 mimic which comprised RNA duplexes was: 5'-AAACAAACAUGGUGCACUUCUU-3, while 2'-OMe miR-495 inhibitor and 2'-OMe scramble oligonucleotides had sequences of 5'-AAGAAGUGCACCAUGUUUGUU U-3' and 5'-ACUUGCGUUAUUGGUGACCUAC-3', respectively. Transfection was done with Lipofectamine 2000, with change of medium after 24 hours and cell analysis after 48 hours.

RT-Polymerase chain reaction

Total RNA was extracted with TRIzol. Then, 4 μg of RNA was reverse-transcribed to cDNA using MMLV reverse transcriptase in addition to oligo(dT) primers (Fermentas) in line with the kit protocols. MiR-495 was quantified using mirVana qRT-PCR miRNA detection kit (Ambion, Austin, USA) with SYBR Green. The following primers were used:

STAT3: 5'-GGGTGGAGAAGGACATCAGCGGTA A-3' (sense); 5'-GCCGACAATACTTTCCGAATCC-3' (anti-sense);

GAPDH: 5'-GCACCGTCAAGCTGAGAAC-3' (sense); 5'-TGGTGAAGACGCCAGTGGA-3' (antisense).

The relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Immunoblot assay

SCNs were lysed using Nonidet P-40 Lysis buffer, boiled for e few minutes, and then centrifuged to obtain the supernatants. Solutions with 50 μg protein were subjected to 10% SDS-polyacrylamide gel electrophoresis, followed by transfer onto NC membranes which were then blocked with 5% (w/v) fat-free milk powder prior to incubation for 12 hours at 4 ℃ with 10 antibodies for Bcl-2, Bax, caspase 3, and STAT3. Following rinsing with TBST, incubation with horse radish peroxidase-conjugated-antibody was carried out at room temperature for 2 hours, followed by visualization with ECL.

Statistical analysis

Data are shown as mean ± SEM, and were analyzed using analysis of variance and Tukey's test. GraphPad Prism version 6 was employed analysis. Statistical significance was

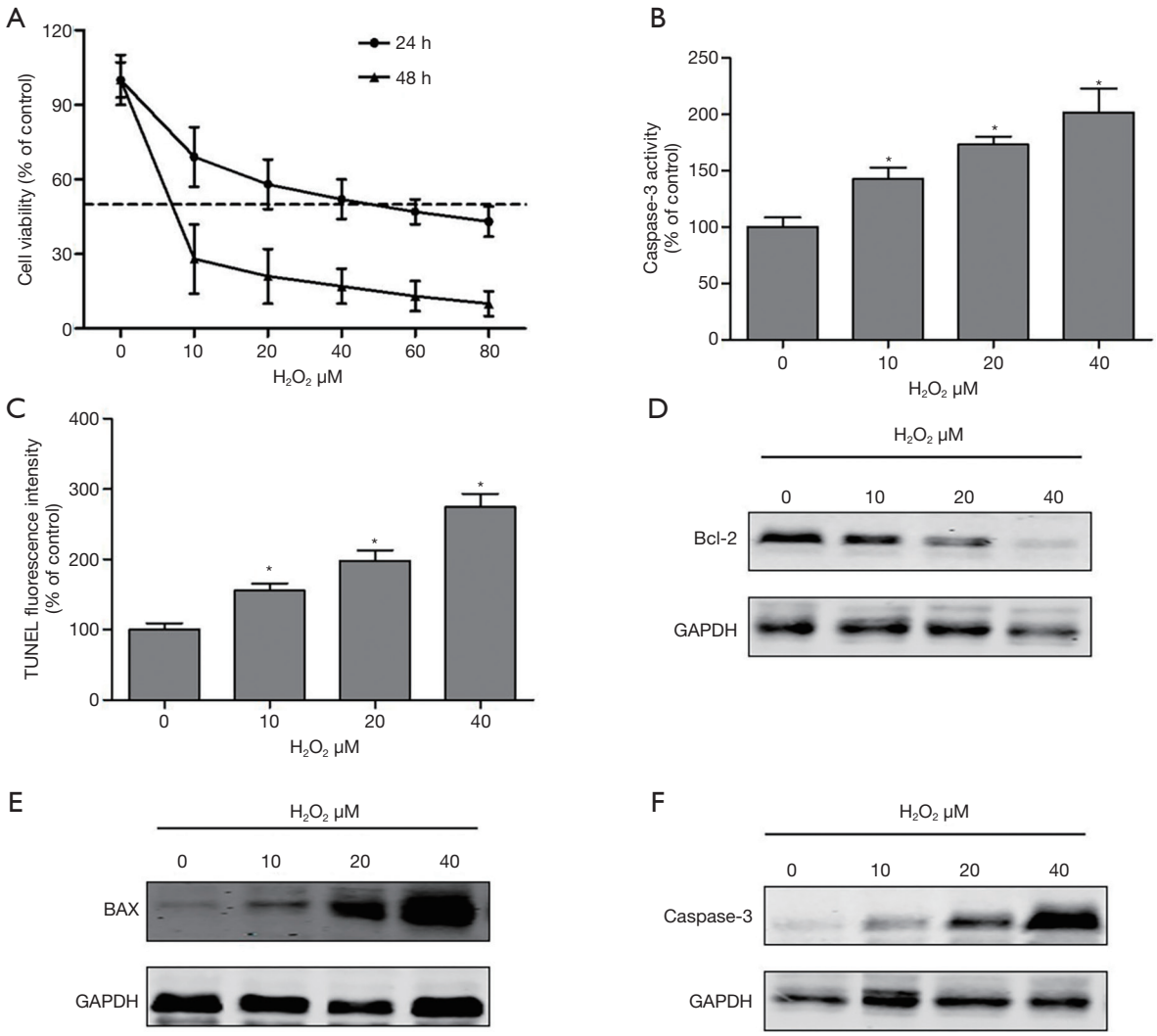

Figure 1 Effects of different concentrations of H_2O_2 on cytotoxicity and apoptosis of SCNs. (A) Spinal cord neurons (SCNs) were incubated with varying levels of H_2O_2 for 24–48 hours. Cell viability was examined using the MTT assay. (B) Caspase-3 levels in SCNs. (C) Results of TUNEL assay. (D) Protein concentrations of Bcl-2, (E) Bax, and (F) caspase-3, as measured using immunoblotting. *P<0.05, *vs.* untreated SCNs.

assumed at P<0.05.

Results

H_2O_2-induced cytotoxicity and apoptosis in SCNs

The cytotoxicity and apoptosis of SCNs were assessed after exposure to different concentrations of H_2O_2. The H_2O_2 exposure decreased the viability of SCNs in a dose- and time-dependent manner (*Figure 1A*). The mean lethal concentration of H_2O_2 in SCNs after 24 hours incubation was approximately 40 μM (*Figure 1A*). Moreover, we examined H_2O_2-induced apoptosis in SCNs using caspase-3 levels and TUNEL staining 24 hours after H_2O_2 incubation. As shown in *Figures 1B,C*, H_2O_2 exposure induced cell apoptosis and increased the levels of caspase 3 in SCN lysates in a dose-reliant fashion. Furthermore, protein expressions of markers relating to apoptosis were measured using western blot assay. H_2O_2 treatment resulted in down-regulated expression of anti-apoptotic Bcl-2 (*Figure 1D*), and up-regulated expressions Bax (*Figure 1E*) and caspase-3 (*Figure 1F*) in SCNs.

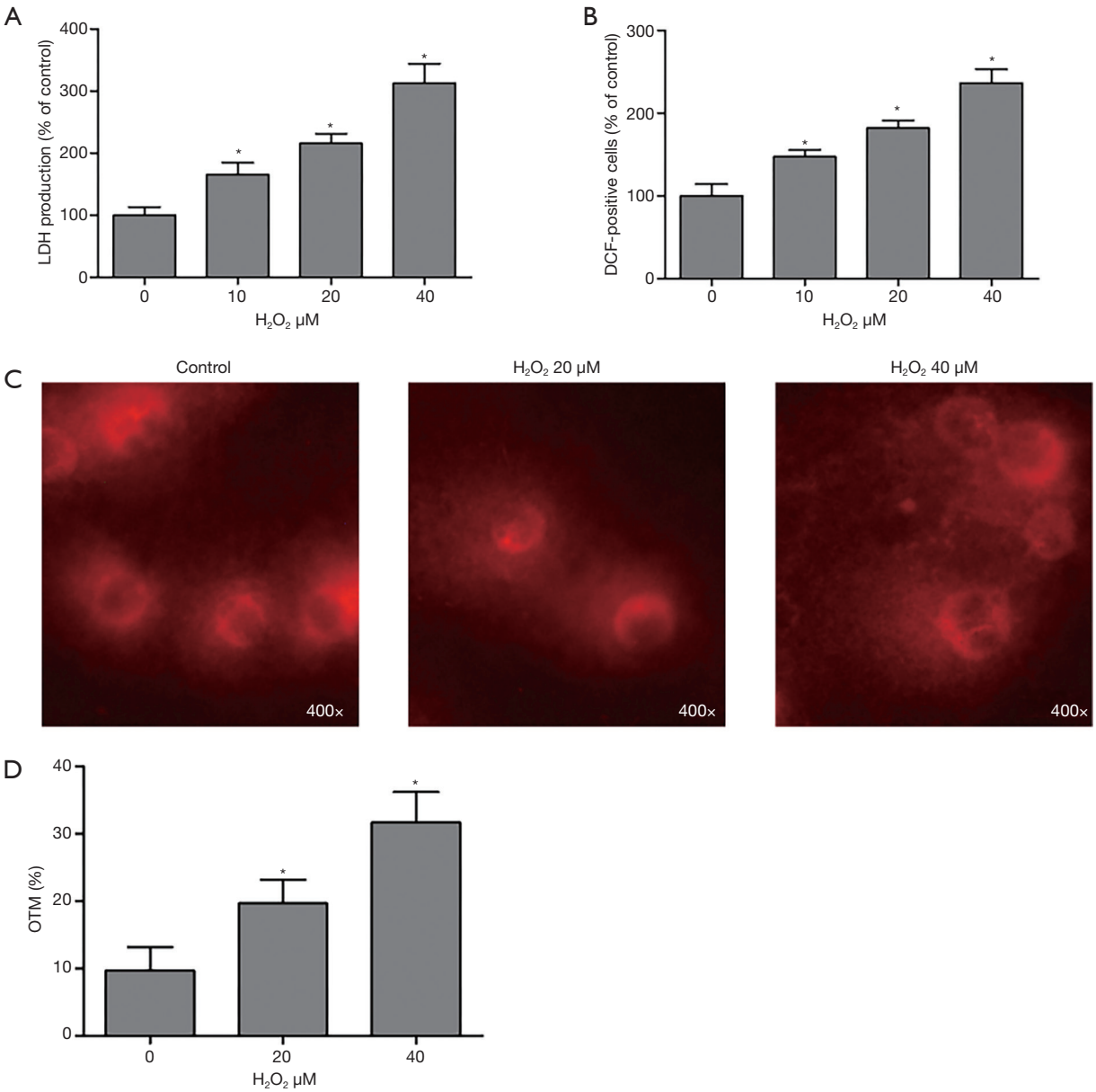

Figure 2 H_2O_2-induced cell membrane dysfunction, ROS, and DNA damage. (A) Lactate dehydrogenase (LDH) levels were measured after spinal cord neurons (SCNs) were exposed to H_2O_2 for 24 hours. (B) Intracellular reactive oxygen species levels after 12 hours treatment with H_2O_2. (C) Cell DNA damage (Comet assay, 400×) and (D) tail lengths of the comet calculated in each group using Image proR plus software. Data are mean ± SD, n=3 per group. *P<0.05 *vs.* non-treated SCNs.

H_2O_2-induced cell membrane dysfunction, ROS production, and DNA damage

A concentration-dependent increase in extracellular LDH activity was observed in SCNs exposed to H_2O_2 at concentrations greater than 10 μM (*Figure 2A*). Moreover, the results indicated that H_2O_2 exposure led to marked rise in ROS levels in a concentration-dependent manner (*Figure 2B*). The comet assay was performed to assess the genotoxicity of H_2O_2 in SCNs. As shown in *Figures 2C,D*, when the SCNs were treated with H_2O_2, olive tail moment was markedly higher than that in the control group.

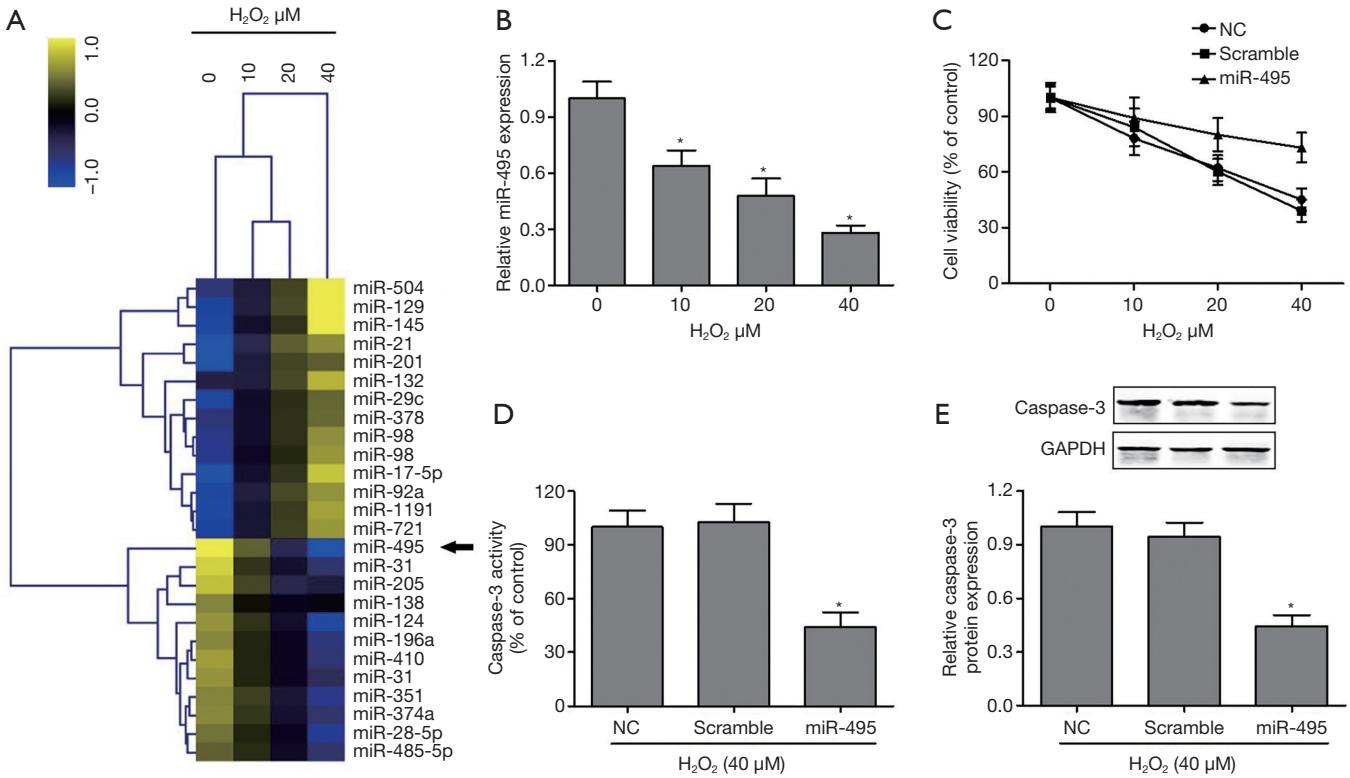

Figure 3 Overexpressed miR-495 inhibited H_2O_2-induced SCN apoptosis. (A) Unsupervised hierarchical cluster of differentially-expressed miRNAs in SCNs treated with H_2O_2 for 24 hours. (B) MiR-495 levels, in the non-treated and H_2O_2-treated group after exposure to different concentrations for 24 hours, as assayed using real-time PCR. (C) Viabilities of spinal cord neurons (SCNs) transfected with miR-495 mimics or scramble. (D) Caspase 3 activity in SCNs lysates, and (E) Caspase-3 protein concentration, as assayed using ELISA and immunoblot, respectively. Values are mean ± SD. *P<0.05 *vs.* un-treated or NC SCNs.

Overexpressed miR-495 inhibited H_2O_2-induced SCN apoptosis

To identify the differentially expressed miRNAs in cultured SCNs in response to H_2O_2 exposure, we performed a microarray assay with miRNA libraries generated using total RNA extracted from H_2O_2-stimulated (0, 10, 20, or 40 μM) SCNs for 24 hours. We found that miR-495 was significantly lowly expressed in H_2O_2-treated SCNs, relative to control (*Figure 3A*). Therefore, we further investigated the function of miR-495 in SCNs when they were exposed to H_2O_2. The dose-dependent experiments showed that H_2O_2 markedly suppressed miR-495 expression in SCNs (*Figure 3B*). However, miR-495 gain-of-function reversed H_2O_2-induced cytotoxicity (*Figure 3C*) and apoptosis (*Figure 3D,E*) in SCNs. Moreover, we found that H_2O_2-treated SCNs had up-regulated mRNA (*Figure 4A*) and protein (*Figure 4B*) expressions of STAT3.

Based on the miRBase database (http://www.mirbase.org), we found the hypothetical interaction domain of miR-495 in 3'-UTR of STAT3 in mice (*Figure 4C*). To verify whether STAT3 was a direct target of miR-495, the 3'-UTR of the wild-type and mutant STAT3 genes were cloned and co-transfected along with miR-495 or NC oligonucleotides into SCNs. Luciferase assays were performed 24 hours post-transfection. As shown in *Figure 4D*, there was reduced activity of the enzyme in miR-495-transfected SCNs, when compared to NC cells. In contrast, after co-transfection of miR-495 into STAT3 mutant 3'-UTR cells, the activity of luciferase was not significantly different from that of the NC. Next, we observed that transfection of SCNs with miR-495 mimic oligonucleotides or miR-495 inhibitors significantly down-regulated or up-regulated both STAT3 mRNA (*Figure 4E*) and protein (*Figure 4F*) expression levels, respectively. Thus, miR-495 exerted a mitigating influence against H_2O_2-induced SCNs by suppressing the expression of STAT3.

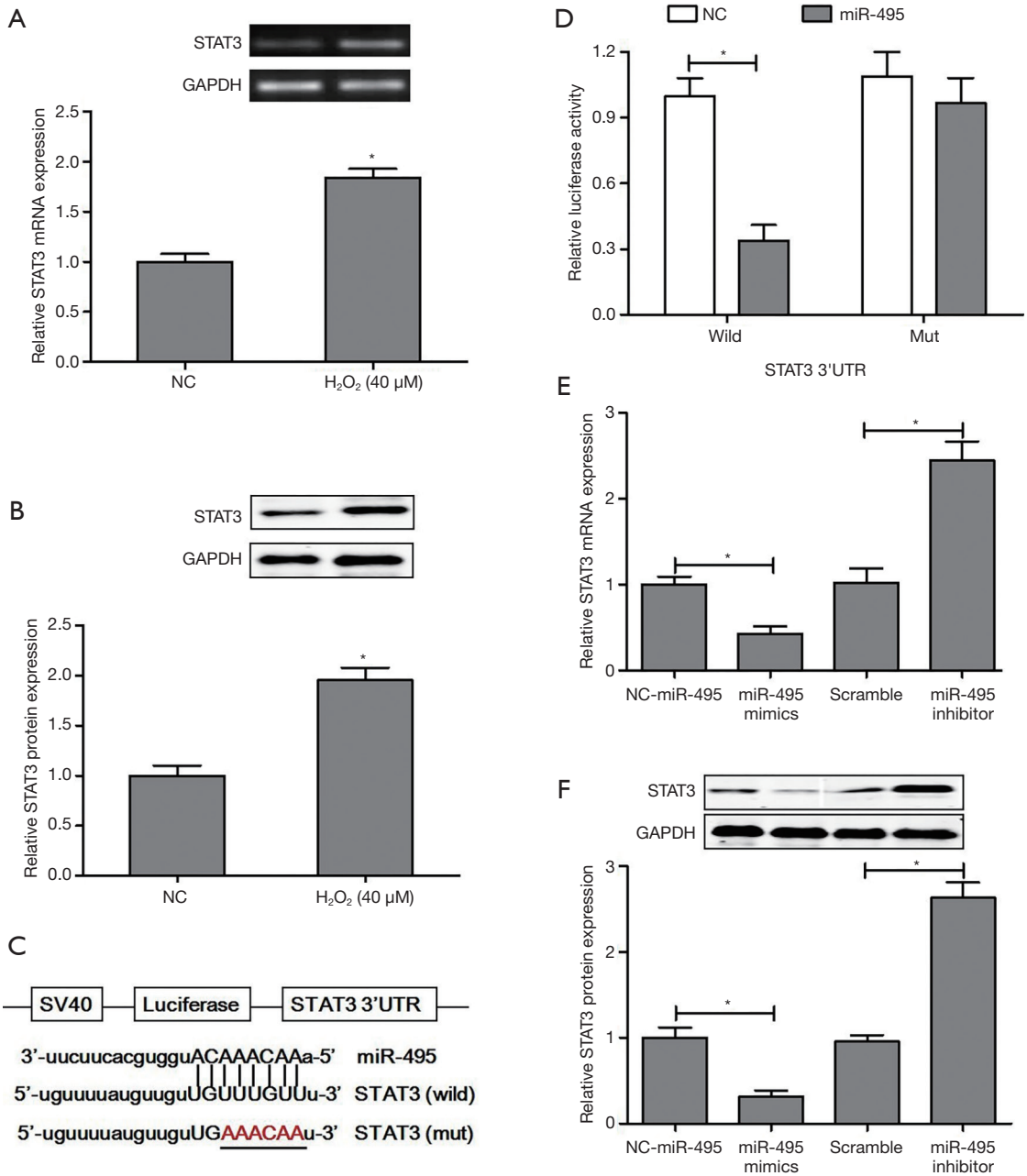

Figure 4 Spinal cord neurons (SCNs) were incubated with H_2O_2 (40 μM) for 24 hours. (A) STAT3 mRNA and (B) STAT3 protein levels. (C) Potential interaction sites of miR-495 in 3'UTR of STAT3, as determined by Targetscan, and (D) luciferase activity assay. (E) STAT3 mRNA expression, and (F) STAT3 protein expressions. *P<0.05 *vs.* NC.

Discussion

Our data suggest that miR-495 was involved in H_2O_2-induced SCN dysfunction. Moreover, H_2O_2 induced apoptosis, ROS, and DNA damage in SCNs. Simultaneously, H_2O_2 exposure down-regulated miR-495 levels and up-regulated STAT3 levels in SCNs. Interestingly, overexpressed miR-495 reversed H_2O_2-induced cytotoxicity and apoptosis in SCNs, and inhibited the mRNA and protein expression of STAT3. Therefore, we conclude that miR-495 may exert a protective effect against H_2O_2-induced SCN dysfunction by suppressing the expression of STAT3.

MiRNAs regulate translation by interacting with the 3'-UTR of target mRNAs, thereby suppressing protein translation in cells (20,21). In this study, we found potential miR-495 binding domains within 3'-UTR of STAT3 in rats. In addition, miR-495 regulated luciferase expression by interacting with the 3'-UTR of STAT3 in SCNs. Moreover, our results indicated that STAT3 expression was suppressed by miR-495 overexpression at the transcriptional and translational levels. MiR-495 is involved in the pathogenesis of several types of cancers (22,23). However, to our knowledge, there are no extant studies on the neuroprotective effect of miR-495 against SCI. In response to H_2O_2-stimulated SCN dysfunction in rats, miR-495 had the lowest expression in the presence of H_2O_2. However, overexpressed miR-495 reversed H_2O_2-induced cytotoxicity and apoptosis in SCNs. Importantly, we addressed the post-translational phenomenon of miR-495 and the possible targets of miR-495 during neuronal apoptosis following SCI. Bioinformatics analysis showed the potential miR-495 binding domains in 3'-UTR of STAT3 in rat SCNs.

Previous studies have indicated that STAT3 is a key regulator of astrocytes (which are reactive in nature) during the repair process after SCI. Therefore, it is an important target for the treatment of CNS injury (24,25). It has been reported that activation of STAT3 route may be implicated in the development of neuropathic pain in rats (26). Moreover, the activation of STAT3 is involved in IL-17-induced spinal cord neuroinflammation after SCI in rats (27). In contrast, sustained activation of STAT3 has been shown to enhance corticospinal remodeling and functional recovery after SCI (28). This investigation has shown that H_2O_2 treatment up-regulated mRNA and protein expressions of STAT3 in SCNs. STAT3, a widely studied transcription factor which is known to be involved in the protection of neurons and nerve regeneration, exists in cytosol in a sedentary state. However, peripheral damage of the nerve increases the transcription STAT3 (29). In a mouse model of hypoglossal nerve damage, miR-124 was shown to be implicated in the regulation of the expression of STAT3, which was crucial for proper nerve regeneration (30). In the present study, we cloned the 3'-UTR of the wild-type or mutant-type STAT3 gene and co-transfected it along with miR-495 or scramble sequences into SCNs, which resulted in reduced luciferase activity in miR-495-transfected cells. These results showed that miR-495 was capable of suppressing endogenous STAT3 expression via targeting its 3'-UTRs.

In summary, this study has indicated that miR-495 suppresses STAT3 in SCNs. Overexpression of miR-495 might play a protective role against H_2O_2-induced SCN dysfunction by suppressing the expression of STAT3.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at http://dx.doi.org/10.21037/atm-21-102

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-21-102

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-21-102). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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