

# miR-34a alleviates spinal cord injury via TLR4 signaling by inhibiting HMGB-1

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**Abstract.** The aim of the present study was to investigate the effect of microRNA (miR)-34a on spinal cord injury (SCI)-induced inflammation and the possible underlying mechanisms. The results indicated that miR-34a expression was downregulated in a rat model of SCI compared with the control group. Furthermore, miR-34a knockdown was demonstrated to aggravate inflammation, inhibit cell proliferation and enhance apoptosis in an *in vitro* model of SCI. MiR-34a inhibition was demonstrated to upregulate the expression of inducible nitric oxide synthase and nitric oxide, as well as inducing the expression of toll-like receptor 4 (TLR4) and high mobility group box-1 (HMGB-1) in an *in vitro* model of SCI. TLR4 inhibitor reduced the effects of miR-34a down-regulation on inflammation and cell growth in SCI. Together, these results suggest that miR-34a is able to alleviate SCI via inhibiting HMGB-1 expression in TLR4 signaling.

## Introduction

Spinal cord injury (SCI) is one of the most common injuries that requires spinal surgery (1). SCI is often caused by traffic accidents, falls, construction accidents and sports injuries (1). The incidence of SCI has increased with urban and transportation development (1). Recent epidemiological studies have suggested that 11,000 new cases of SCI are reported each year in the USA (1,2). SCI is often accompanied by serious complications, including respiratory dysfunction and failure, pneumonia, pulmonary edema and embolism (1).

High mobility group box-1 (HMGB-1) is a non-histone protein that is located in eukaryotic nuclei and comprises 215 amino acid residues (1,3). It has a highly conserved structure and is primarily synthesized in damaged cells or peripheral mononuclear macrophages (1,4). HMGB-1 activates the

downstream inflammatory signaling pathway. As such, it serves a role in a variety of tumors, inflammatory reactions and organ damage (1,4). HMGB-1 receptors include toll-like receptor (TLR)2, TLR4 and the receptor for advanced glycation end products, which is also a member of the TLR family (5). The HMGB-1/TLR4 pathway has previously been demonstrated to serve a vital role in the pathogenesis of SCI caused by burn, trauma and shock (6). Furthermore, HMGB-1 is reported to be involved in a number of central nervous system injuries (6). A recent study indicated that HMGB-1 mediates SCI-related localized neuronal apoptosis (6).

Number of microRNAs (miRs or miRNAs) are associated with and serve important roles in the progression from SCI to ASCI (7). A microarray study of ASCI in rats suggested that many miRNAs are differentially expressed following ASCI (7). Bioinformatics analysis revealed that changes in miRNA expression serve a role in the pathogenesis of ASCI in rats (7). Furthermore, changes in miRNA expression are crucial for cell apoptosis, oxidative stress, angiogenesis, inflammatory response and other mechanisms (7). However, the specific functions and underlying mechanisms of differentially expressed miRNAs are widely unknown. Yuan *et al* (8) demonstrated that miR-34a modulates endothelial inflammation after fetal cardiac bypass in the goat placenta. The aim of the present study was to investigate the effect of miR-34a on SCI-induced inflammation and the possible underlying mechanism.

## Materials and methods

**Animals and spinal cord surgery.** A total of 12 Male Wistar rats weighing 220-250 g and aged 10-12 weeks old were purchased from The Animal Centre of Nanchang University (Nanchang, China) and housed in our laboratory at 22-23°C in 55-60% humidity, with a 12 h light/dark cycle, and free access to food and water. The rats underwent urinary bladder massage at least twice per day until the recovery of spontaneous micturition (9). The present study was approved by the Research Council and Animal Care and Use Committee of Shangrao People's Hospital (Shangrao, China), and performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (9). Rats were randomly divided into two groups: Control (n=6) and SCI model (n=6). Rats in the SCI model group were anesthetized using 35 mg/kg pentobarbital sodium (intravenous injection; Sigma-Aldrich; Merck

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KGaA, Darmstadt, Germany), following which an incision was made on the back posterior to the lower thoracic region. Back muscles were separated and the dorsal surface of the spinal cord was exposed at T10. The lower thoracic cord was transected using fine scissors and the surgical wound was closed in two layers. Rats in the control group were anaesthetized using 35 mg/kg pentobarbital sodium and did not undergo surgery. At 12 h following spinal surgery, rats in all groups were anaesthetized using 35 mg/kg pentobarbital sodium and sacrificed by decollation. The back muscles were then separated at T10 and the spinal cord was harvested. Spinal cord tissues was collected from spinal surgery and washed with PBS. Tissue samples were then fixed with 4% paraformaldehyde for 24 h at room temperature.

**MicroRNA quantification.** Total RNA was extracted from the spinal cord using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). First-strand cDNA was synthesized using a Takara RNA PCR kit (Takara Bio, Inc., Otsu, Japan) at 37°C for 30 min and 84°C for 10 sec. miR-34a expression was measured using SYBR Select Master Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a CFX 96™ Connect Real-Time system (Bio-Rad Laboratories, Inc.). The PCR conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Primers used were as follows: miR-34a forward, 5'-TCTGTCTCTCTTGGCAGTGTCTT-3' and reverse, 5'-CTCGCTTCGGCAGCA CA-3'; U6 forward, 5'-GCTTCGGCAGCACATATACTAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The thermocycling conditions were as follows: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. Relative mRNA expression was quantified using the  $2^{-\Delta\Delta C_q}$  method (10).

**Cell culture and transfection.** PC12 cells were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO<sub>2</sub>. MiR-34a mimics (miR-34a overexpression), anti-miR-34a (miR-34a knockdown) and negative control miRNA (control) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Cells were transfected with 100 ng of miR-34a mimics (miR-34a overexpression), anti-miR-34a (miR-34a knockdown) and negative control miRNAs (control) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). At 4 h post-transfection, PC12 cells were treated with lipopolysaccharide (50 ng/ml; Invitrogen; Thermo Fisher Scientific, Inc.) and TAK-242 (1 nM; MedChemExpress, Shanghai, China) for 24, 48 and 72 h at 37°C for cell proliferation assays and for 48 h for all other assays.

**Cell proliferation assay.** MTT (10 ml; 5 mg/ml; Beyotime Institute of Biotechnology, Haimen, China) was added to cells after transfecting the cells for 24, 48 or 72 h; MTT was incubated with the cells at 37°C for 4 h in the dark. Dimethyl sulfoxide was added for 20 min at 37°C after the culture medium was removed. The absorbance was measured using a microplate reader (FluoDia T70; Photon Technology

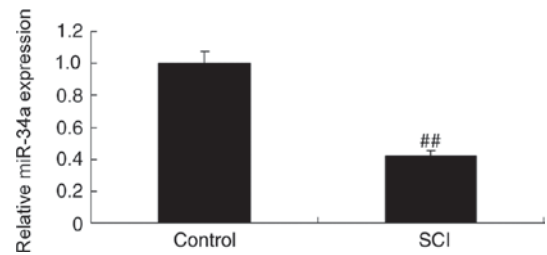


Figure 1. miR-34a expression in a rat model of SCI. <sup>##</sup>P<0.01 vs. control. miR, microRNA; SCI, spinal cord injury.

International, Lawrenceville, NJ, USA) at a wavelength of 490 nm.

**Flow cytometry.** At 48 h post-transfection, cells were washed three times with PBS and resuspended with 5  $\mu$ l annexin V-fluorescein isothiocyanate and 5  $\mu$ l propidium iodide (BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 15 min. Apoptosis was measured using a CyAn™ ADP cytometer (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA).

**Western blotting.** Proteins were extracted from cells at 48 h after transfection using radioimmunoprecipitation assay buffer (Kaiji, Shanghai, China) and quantified using a BCA assay. Proteins (50  $\mu$ g/lane) were separated by 8-10% SDS-PAGE and blotted onto polyvinylidene fluoride membranes. Membranes were subsequently blocked using 5% non-fat dry milk in 0.1% TBS/Tween at 37°C for 1 h, following which they were incubated with antibodies against TLR4 (sc-8694; 1:1,000), HMGB-1 (sc-26351; 1:1,000) and GAPDH (sc-293335; 1:2,000; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. The membranes were next washed with 0.1% TBS/Tween and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (sc-2004; 1:2,000; Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. Proteins were visualized using an enhanced chemiluminescent detection reagent (Pierce; Thermo Fisher Scientific, Inc.). Protein bands were measured using Image Lab 3.0 software (Bio-Rad Laboratories, Inc.).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance was used for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**miR-34a expression in an in vitro model of SCI.** In order to investigate the mechanism of miR-34a in SCI, miR-34a expression was measured in SCI model rats and control rats. The results indicate that miR-34a was downregulated in the SCI model group compared with the control group (Fig. 1). This suggests that miR-34a expression may be associated with the pathogenesis of SCI.

**Effects of miR-34a on inflammation in an in vitro model of SCI.** MiR-34a mimics and anti-miR-34a mimics were used to

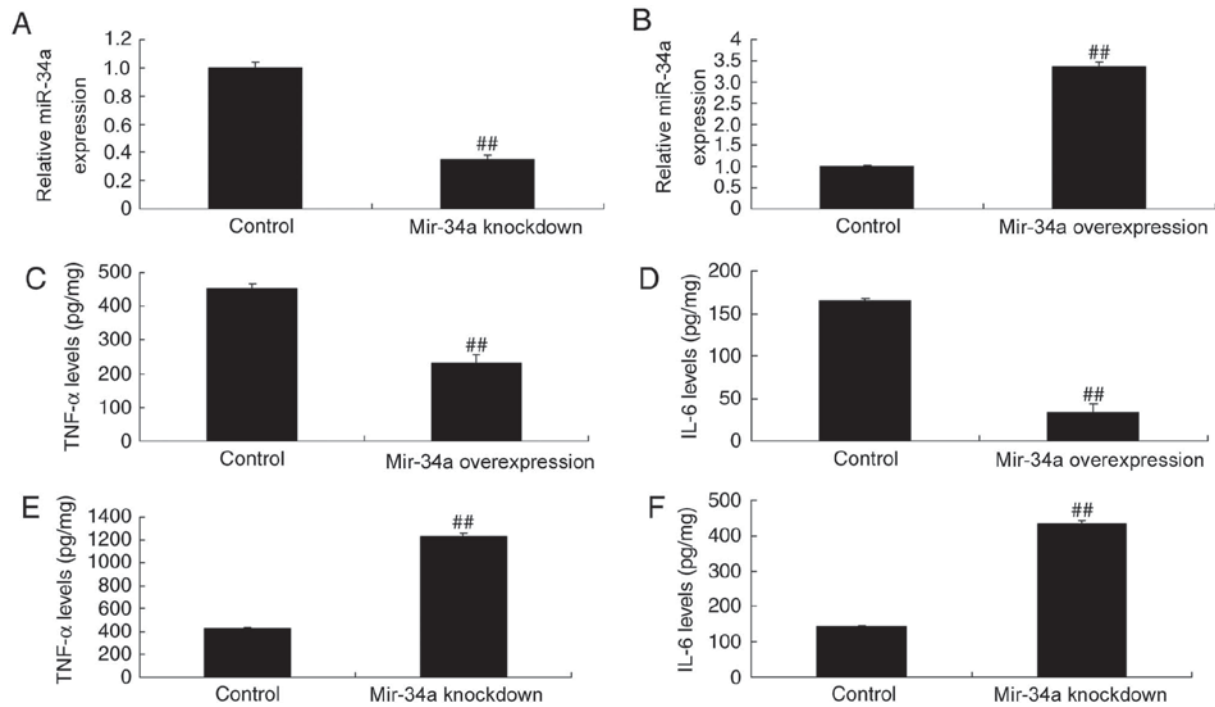


Figure 2. Effects of miR-34a on inflammation in an *in vitro* model of spinal cord injury. miR-34a expression in (A) miR-34A knockdown or (B) miR-34a overexpression cells. TNF-α expression in (C) miR-34A knockdown or (D) miR-34a overexpression cells. IL-6 expression in (E) miR-34A knockdown or (F) miR-34a overexpression cells. ##P<0.01 vs. control. miR, microRNA; TNF, tumor necrosis factor; IL, interleukin.

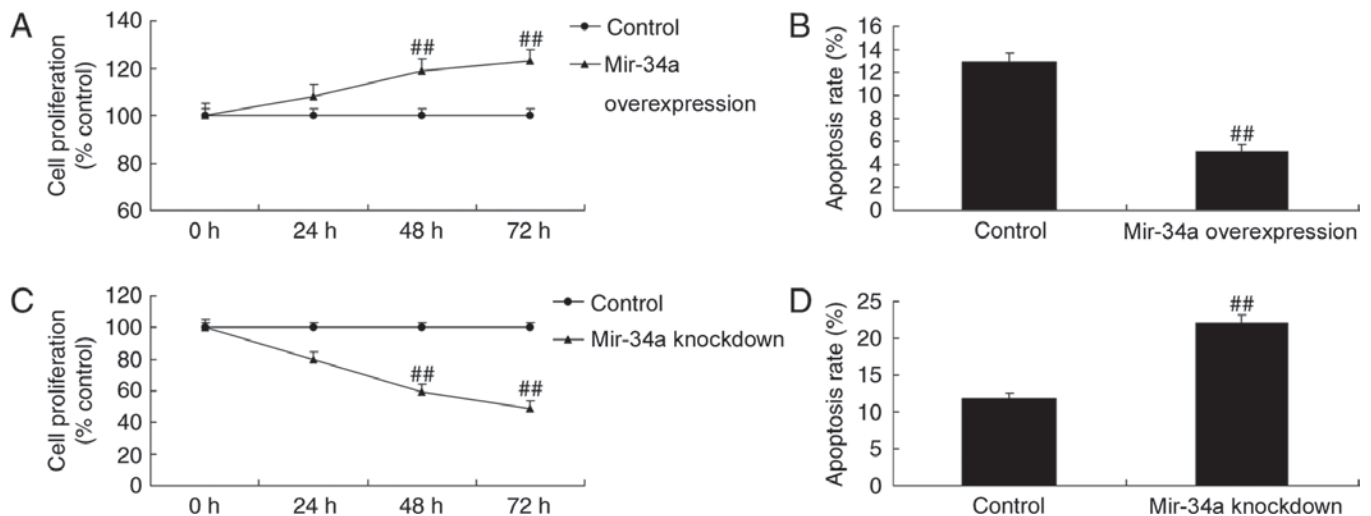


Figure 3. Effects of miR-34a on cell growth in an *in vitro* model of spinal cord injury. (A) Cell proliferation and (B) apoptosis rate in miR-34a overexpression cells. (C) Cell proliferation and (D) apoptosis rate in miR-34A knockdown cells. ##P<0.01 vs. control. miR, microRNA.

induce miR-34a overexpression and knockdown, respectively, *in vitro* (Fig. 2A and B). MiR-34a overexpression downregulated TNF-α and IL-6 in SCI expression compared with control cells (Fig. 2C and D), while miR-34a knockdown upregulated TNF-α and IL-6 (Fig. 2E and F).

**Effects of miR-34a on cell growth in an *in vitro* model of SCI.** MiR-34a overexpression increased cell proliferation and reduced apoptosis in an *in vitro* SCI model compared with control cells (Fig. 3A-B). In cell proliferation was inhibited and apoptosis was increased in miR-34a knockdown cells compared with the control (Fig. 3C-D).

**Effects of miR-34a on COX-2 and NF-κB protein expression in an *in vitro* model of SCI.** COX-2 and NF-κB were downregulated in miR-34a overexpression cells compared with the control, whereas they were upregulated in miR-34a knockdown cells (Fig. 4).

**Effects of miR-34a on TLR4 and HMGB-1 protein expression in an *in vitro* model of SCI.** TLR4 and HMGB-1 expression was assessed using western blotting. The results revealed that TLR4 and HMGB-1 were downregulated in miR-34a overexpression cells compared with the control; however, they were upregulated in miR-34a knockdown cells (Fig. 5).

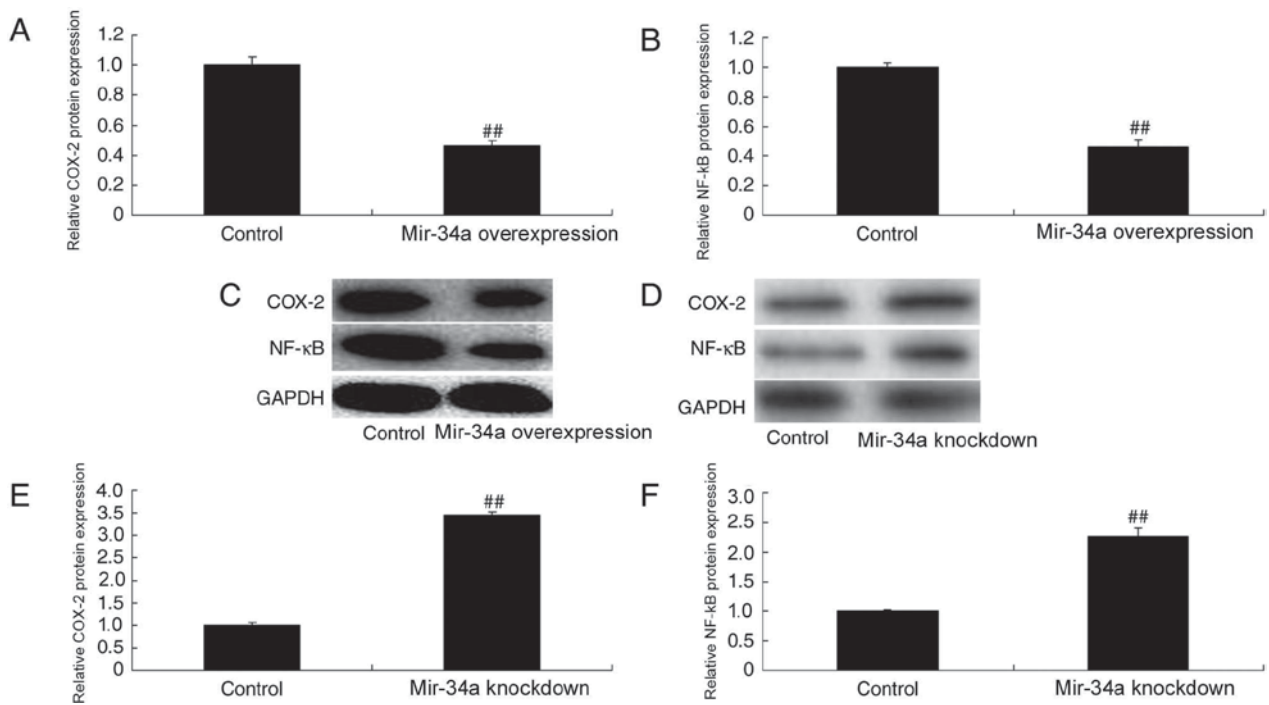


Figure 4. Effects of miR-34a expression on COX-2 and NF-κB in an *in vitro* model of spinal cord injury. (A) COX-2 and (B) NF-κB expression in miR-34a overexpression cells. Representative western blots of COX-2 and NF-κB expression in (C) miR-34a overexpression and (D) miR-34a knockdown cells. (E) COX-2 and (F) NF-κB expression in miR-34a knockdown cells. <sup>##</sup> $P < 0.01$  vs. control. miR, microRNA; COX-2, cyclooxygenase-2; NF, nuclear factor.

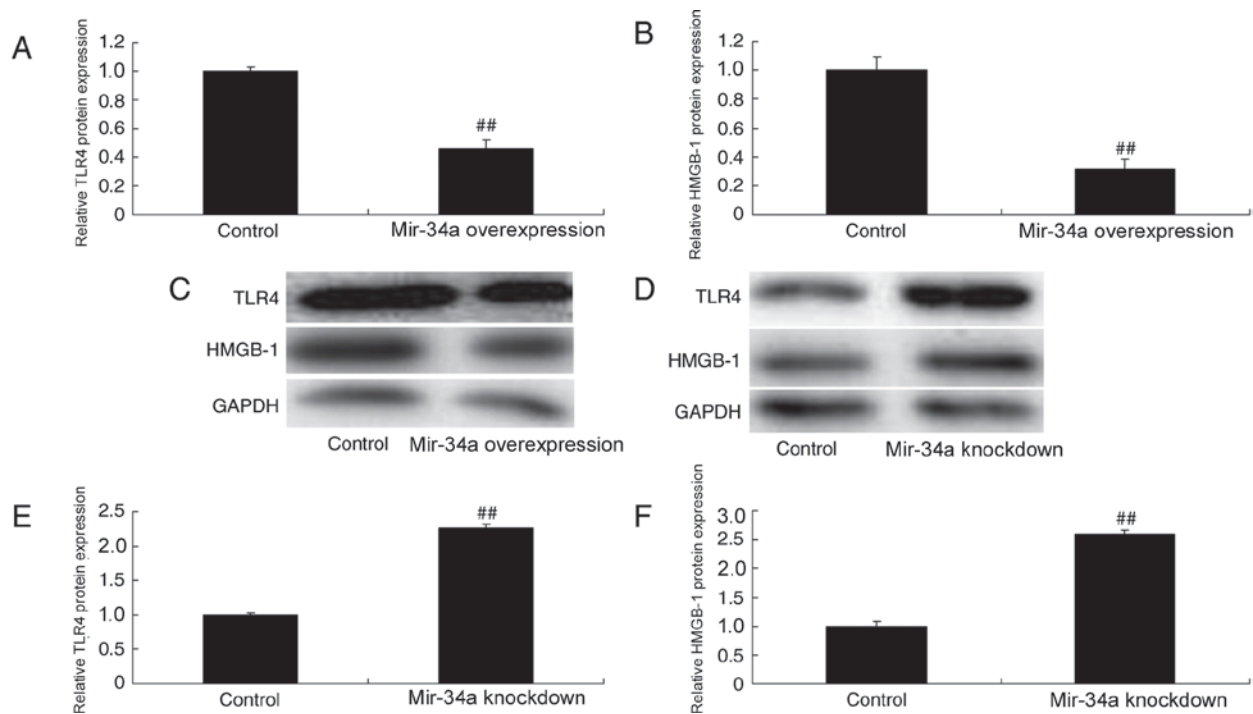


Figure 5. Effects of miR-34a on TLR4 and HMGB-1 protein expression in an *in vitro* model of spinal cord injury. (A) TLR4 and (B) HMGB-1 expression in miR-34a overexpression cells. Representative western blots of TLR4 and HMGB-1 expression in (C) miR-34a overexpression and (D) miR-34a knockdown cells. (E) TLR4 and (F) HMGB-1 expression in miR-34a knockdown cells. <sup>##</sup> $P < 0.01$  vs. control. miR, microRNA; TLR4, toll-like receptor 4; HMGB-1, high mobility group box-1.

*TLR4 inhibitor attenuates the effect of miR-34a downregulation and reduces TLR4 and HMGB-1 protein expression in SCI.* The role of TLR4 in miR-34a downregulation in SCI was further assessed using a TLR4 inhibitor. The results revealed that TLR4 inhibitor ameliorated the miR-34a

knockdown-induced overexpression of TLR4 and HMGB-1 in an *in vitro* model of SCI (Fig. 6).

*TLR4 inhibitor decreases the effect of miR-34a downregulation on inflammation and cell growth in SCI.* Treatment



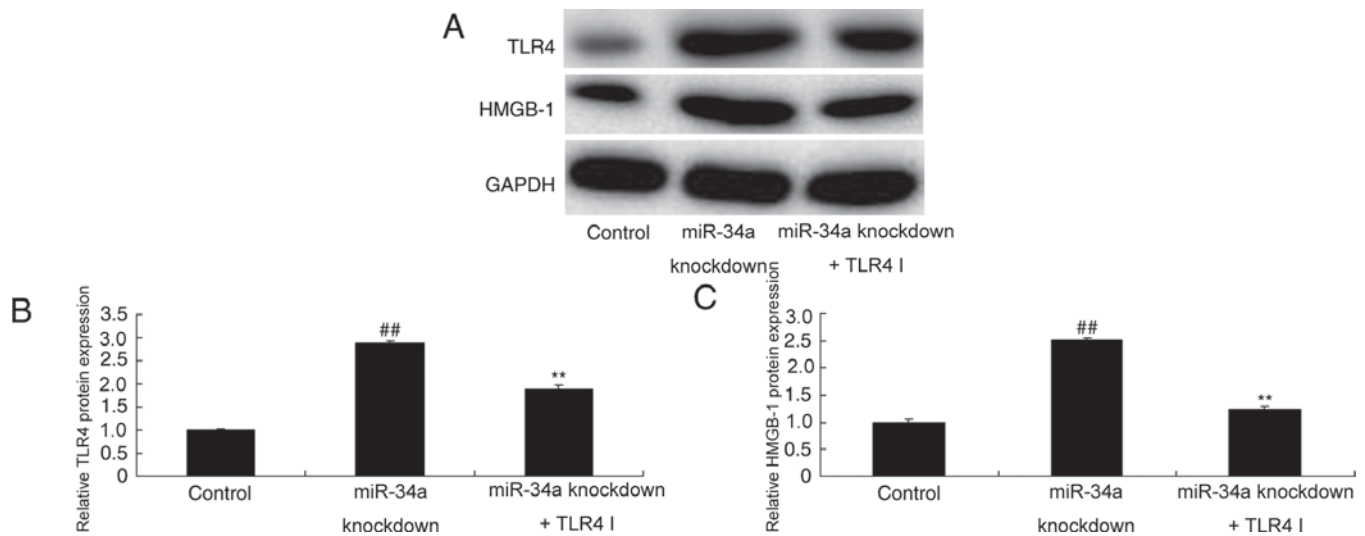


Figure 6. TLR4 inhibitor decreases the effects of miR-34a downregulation on TLR4 and HMGB-1 protein expression in spinal cord injury. (A) Representative western blot of TLR4 and HMGB-1 protein expression in miR-34a knockdown and overexpression cells. Quantified western blotting data for (B) TLR4 and (C) HMGB-1 expression. <sup>##</sup>P<0.01 vs. control and <sup>\*\*</sup>P<0.01 vs. miR-34a knockdown. TLR4, toll-like receptor 4; miR, microRNA; HMGB-1, high mobility group box-1.

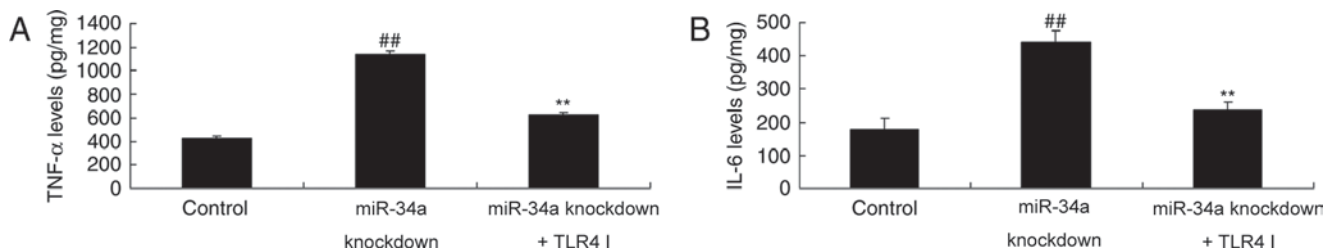


Figure 7. TLR4 inhibitor decreases the effects of miR-34a downregulation on inflammation and cell growth in spinal cord injury. (A) TNF-α and (B) IL-6 levels expression in miR-34a knockdown and overexpression cells. <sup>##</sup>P<0.01 vs. control and <sup>\*\*</sup>P<0.01 vs. miR-34a knockdown. TLR4, toll-like receptor 4; miR, microRNA; TNF, tumor necrosis factor; IL, interleukin.

with the TLR4 inhibitor was demonstrated to ameliorate the miR-34a knockdown-induced overexpression of TNF-α and IL-6 levels and reverse the effects of miR-34a knockdown on cell proliferation and apoptosis (Fig. 7).

*TLR4 inhibitor decreases the effect of miR-34a downregulation on COX-2 and NF-κB protein expression in SCI.* TLR4 inhibitor was demonstrated to suppress the expression of COX-2 and NF-κB proteins in an *in vivo* model of SCI model following miR-34a knockdown, compared with untreated miR-34a knockdown cells (Fig. 8).

## Discussion

SCI can be classified as primary or secondary according to its mechanism (1,2). Primary SCI occurs as a result of direct or indirect external force on the spinal cord, while secondary SCI results from destructive lesions in the integrated tissues that occur due to primary SCI through a series of physiological and biochemical mechanisms (11). These mechanisms include oxidative stress, increased inflammatory response and overexpression of excitatory amino acids (1). As such, secondary injury may further aggravate SCI and expand the scope of injury. The inflammatory response following SCI is complicated, involving the nervous system, immune system

and various other dynamic factors (12). A number of studies have reported that the SCI-induced inflammatory response has a dual-effect of nerve injury and neuroprotection (1). In the present study, miR-34a expression was demonstrated to be reduced in a rat model of SCI compared with the control group.

The oxidative activity of serum inflammatory cells, including neutrophils, has been reported to be increased in SCI patients (13). Furthermore, levels of free radicals are elevated, NF-κB is upregulated and myeloperoxidase activity is increased (13). Damaged nerve cells have been demonstrated to generate and release certain inflammatory factors and other stimulating proteins during the pathogenesis of SCI (14). They enter the circulation through the injured blood-brain barrier, thus mediating the systemic inflammatory response and causing lung injury (14). The results of the present study suggest that suppressing miR-34a expression aggravates inflammation, inhibits cell proliferation, enhances apoptosis and upregulates iNOS protein expression and NO levels in an *in vitro* model of SCI. Yuan *et al* (8), demonstrated that miR-34a is able to modulate endothelial inflammation after fetal cardiac bypass in the goat placenta, which is consistent with the present study.

Necrotic nerve cells release a variety of inflammatory proteins during the progression of SCI (15). They are able to mediate SCI and may participate in systemic organ damage (15). HMGB-1 has been demonstrated to be closely associated with SCI (15),

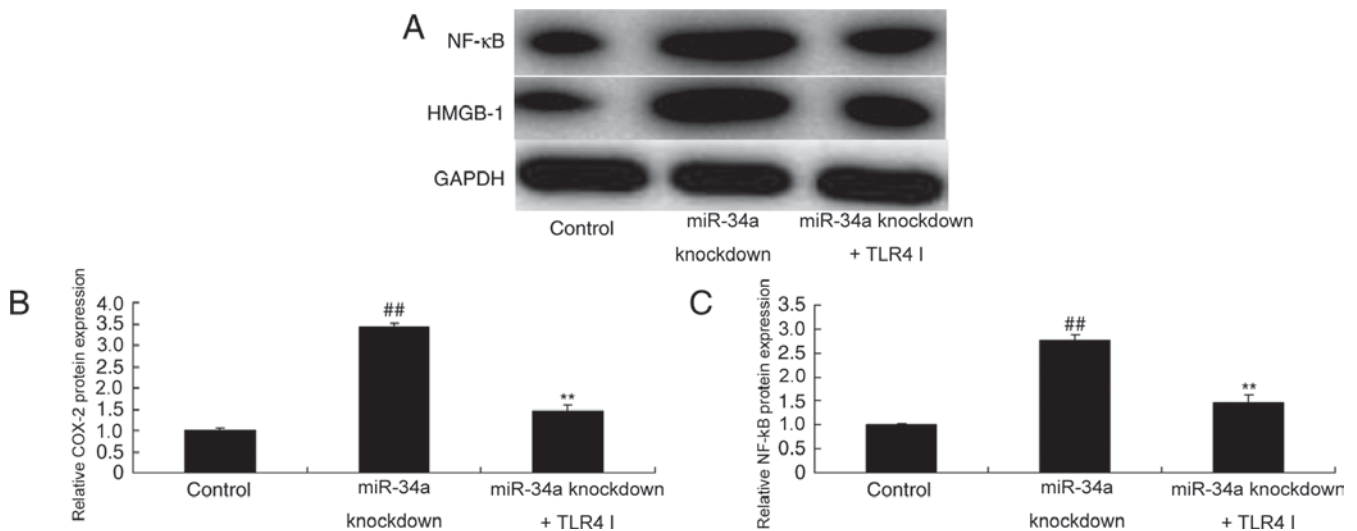


Figure 8. TLR4 inhibitor decreases the effects of miR-34a downregulation on COX-2 and NF-κB protein expression in spinal cord injury. (A) Representative western blot of COX-2 and NF-κB expression in miR-34a knockdown and overexpression cells. Quantified western blotting data for (B) COX-2 and (C) NF-κB expression. <sup>##</sup>P<0.01 vs. control and <sup>\*\*</sup>P<0.01 vs. miR-34a knockdown. TLR4, toll-like receptor 4; miR, microRNA; COX-2, cyclooxygenase-2; NF, nuclear factor.

while TLR4 can mediate multiple inflammation and injury processes (1,16). Injured nerve cells release HMGB-1 during early SCI and is highly expressed in spinal cord tissues (1,17). In the present study, miR-34a knockdown induced TLR4 and HMGB-1 protein expression in an *in vitro* model of SCI.

HMGB-1 released by necrotic nerve cells is able to bind with TLRs receptor as an endogenous ligand to activate the downstream inflammatory pathway and mediate secondary SCI (18). Importantly, HMGB-1 has been reported to be of great significance during the occurrence of lung injury (19). Consequently, it is able to initiate the corresponding signaling pathway to activate NF-κB and other transcription factors through the MyD88-dependent or MyD88-independent pathway (17). This in turn leads to the transcription of inflammatory factors and other immunomodulatory molecules and contributes to SCI (17). TLR4 inhibitor was revealed to ameliorate the effects of miR-34a downregulation on inflammation and cell growth in SCI. Jiang *et al* (20), suggested that the miR-34a/TLR4 axis serves an important role in the development of hepatocellular carcinoma (1), which was consistent with the present study.

In conclusion, the results of the present study suggest that miR-34a expression is associated with SCI. However, the significance of these findings needs to be confirmed in studies with a larger sample size.

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#### Availability of data and materials

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

#### Authors' contributions

JZ designed the experiment, analyzed the data and wrote the manuscript. OS, JL, ZC, CW and WW performed the experiments.

#### Ethics approval and consent to participate

The present study was approved by the Research Council and Animal Care and Use Committee of Shangrao People's Hospital (Shangrao, China), and performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (9).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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