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miR-141 alleviates LPS-induced inflammation injury in WI-38 fibroblasts by up-regulation of NOX2

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ABSTRACT

Aims: The roles of miR-141 in various types of cancers and inflammatory bowel diseases are researched, whereas, little information about its function in lung inflammation is available. This study was designed to explore the effect of miR-141 on inflammation injury in WI-38 cells, possibly providing basis for targeted therapeutic strategy for treatment of infantile pneumonia.
Main methods: WI-38 cells were treated with LPS to construct cell model with inflammation injury. Expressions of miR-141 and NOX2 were altered by transfection assay and expressions of them were detected by qRT-PCR or Western blot. Cell viability and apoptosis were evaluated by CCK-8 assay and flow cytometry, respectively. The tested pro-inflammatory factors were analyzed by qRT-PCR and Western blot; and their productions were quantified by ELISA. Main proteins participating in regulation of apoptosis, p38 MAPK pathway and NF-κB pathway were analyzed by Western blot.
Key findings: miR-141 was down-regulated in LPS-treated cells and elevating miR-141 level reduced inflammation extent of WI-38 cells by promoting viability, inhibiting apoptosis, and inhibiting production of tested pro-inflammatory cytokines. NOX2 was up-regulated by miR-141 overexpression. NOX2 silence impaired the cell-protective effect of miR-141. miR-141 inhibited LPS-induced activations of p38 MAPK and NF-κB pathways, which was also mediated by NOX2.
Significance: miR-141 alleviated LPS-induced inflammation injury in WI-38 fibroblasts by up-regulating NOX2 and further inhibiting p38 MAPK and NF-κB pathways.

1. Introduction

Infantile pneumonia is a common severe respiratory disease occurred in young children, which contributes to high mortality due to children's under-developed immunity and respiratory [1]. Its main clinical manifestations are fever, cough, dyspnea, and even breathing disorder, heart failure, and septicemia. Nowadays, pneumonia remains the leading cause of death in children outside the neonatal period, in spite of advances in prevention and management [2,3].

Increasing evidences apply targeted therapy for treating many types of inflammatory disease and cancer. MicroRNAs (miRNAs), an abundant class of small non-coding RNAs (≈ 22 nt), can regulate mRNA expression both at transcriptional and post-transcriptional levels, and thereby further influence cellular activities in disease progression [4,5]. Some miRNAs have been detected in the pathogenesis of pneumonia and found to be related with the development of pneumonia. Abd-El-Fattah et al indicated that serum levels of miR-21, miR-155, and miR-197 were significantly increased in pneumonia patients compared with controls [6]. In addition, some miRNAs including miR-374a, miR-875-5p, miR-342-3p, miR-150, miR-15b, miR-376a/c, and miR-214 were up-regulated and also some miRNAs including miR-29c, miR-1247, and miR-1233 were down-regulated in patients suffered with pneumonia [7].

miR-141 belonging to miR-200 family, is one of the known cancer-related miRNAs, such as breast cancer [8], gastric cancer [9], prostate cancer [10], lung cancer [11], and so on. For example, miR-141 was significantly elevated in sera of lung cancer patients and was one of the important signatures able to distinguish lung cancer patients with non-cancer subjects [11]. miR-141 has been reported to participate in pathogenesis of inflammatory bowel disease. Expression of miR-141 was decreased in Crohn's disease and ulcerative colitis [12]. Up-regulation or down-regulation of miR-141 alleviated or aggravated trinitrobenzene sulfonic acid-induced experimental colitis, respectively, by targeting CXCL12β [13]. However, no recent reports focused on the

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role of miR-141 in pneumonia.

It is well-known that miRNAs function by suppressing targeted mRNA expression directly [14]. On the contrary, miRNA-mediated promotion of mRNA expression is also reported [15]. This study revealed that NADPH oxidase 2 (NOX2) translation was activated by miR-141, indicating NOX2 might be involved in inflammation response-mediating effect of miR-141. The participation of NOX2 in inflammatory diseases has been widely studied, such as arthritis, neuroinflammatory, and lung inflammation [16]. In the present study, WI-38 fibroblasts were administrated with LPS to induce inflammation and then the effects of altered miR-141 and NOX2 expression on inflammation response of LPS-treated cells as well as the underlying mechanism were studied.

2. Materials and methods

2.1. Cell culture, treatment and transfection

Human WI-38 fibroblasts purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. To induce inflammation, WI-38 cells were treated by 1, 2, 5, or 10 μg/mL of LPS for 12 h. DMEM, FBS, antibiotics, and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA).

miR-141 mimic, miR-141 inhibitor and their negative control (NC) synthesized by GenePharma Co. (Shanghai, China) were used to regulate miR-141 expression in WI-38 cells. The designed siRNA targeting NOX2 (si-NOX2) synthesized by GenePharma Co. was used to down-regulate NOX2. Cell transfection assay was conducted using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

2.2. Cell viability assay

Cell viability was determined using Cell Counting Kit-8 (CCK-8) solution (Beyotime, Shanghai, China). Cells were cultured in 96-well plate (5000 cells/well) at 37 °C in a humidified atmosphere with 5% CO_2 for 24 h. After incubation, 10 μL of CCK-8 solution was added to each well. Cells were further incubated for 2 h. Following that, changes in absorbance at 450 nm were measured by microplate reader (Bio-Tek, Winooski, VT, USA).

2.3. Cell apoptosis assay

Cell apoptosis was determined using Annexin V-FITC/PI double staining method combined with flow cytometry analysis. Cells were seeded in 6-well plate (100,000 cells/well) at 37 °C in a humidified atmosphere with 5% CO_2. After incubation of 24 h, cells were treated by Annexin V-FITC/PI apoptosis detection kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction. Apoptotic cells were determined by flow cytometry (Becton-Dickinson, Franklin Lakes, NJ, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

After different treatments on WI-38 cells, cell culture supernatant was collected from 24-well plates and inflammatory cytokines were quantified by using specific ELISA kits for tests of MCP-1 (ab100586), IL-6 (ab46042), and IL-8 (ab46032, Abcam, Cambridge, MA, USA). Three independent experiments were performed and data was normalized and calculated on the basis of linear calibration curves obtained by standard solutions.

2.5. Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and then purified using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) and RNeasy Mini kit (Qiagen, Duesseldorf, Germany), respectively. For determination analysis of miR-141, RNA samples were reversed by TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA), and qRT-PCR was performed using TaqMan MicroRNA Assay supplemented with the TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA). For determination analyses of MCP-1, IL-6, IL-8, and NOX2, RNA samples were reversed by Multiscribe™ Reverse transcription Kit (Applied Biosystems, CA, USA) supplemented with random hexamers or oligo (dT) and qRT-PCR was performed using Fast START Universal SYBR Green Master (ROX) (Roche, Heiman, Germany). Data were analyzed through $2^{-\triangle\triangle Ct}$ method. miR-141 expression and tested mRNA expressions were normalized to U6 and GAPDH, respectively.

2.6. Western blot

Collected cells with different administration were washed twice in 1 × PBS, then lysed in ice-cold lysis buffer for 1 h. Obtained cell lysates were centrifuged for 10 min at 12,000 rpm at 4 °C. The protein samples were resolved on SDS-PAGE followed by transfer to PVDF membranes (Bio-Rad, Hercules, CA, USA). The blots were blocked with 5% non-fat milk for 1 h at room temperature, incubated overnight with the appropriate primary antibody at 4 °C, washed with Tris Buffered Saline (TBS), and incubated with peroxidase-conjugated secondary antibody for 1 h at room temperature. The primary antibodies used at a dilution of 1:1000 were listed as follows: Bcl-2 (ab32124), Bax (ab32503), pro-caspase-3 (ab44976), cleaved-caspase-3 (ab32042), pro-caspase-9 (ab32539), cleaved-caspase-9 (ab32539), MCP-1 (ab151538), IL-6 (ab6672), IL-8 (ab7747), NOX2 (ab80508), p38 (ab170099), p-p38 (ab47363), p65 (ab16502), p-p65 (ab86299), IκBα (ab32518), and p-IκBα (ab32518, Abcam). Bands were monitored with the aid of enhanced chemiluminescence reagent. GAPDH (ab9485, Abcam) was used as the internal control for ensuring equal protein loading. The intensity of the bands was quantified using ImageJ 1.49 (National Institute of Health, Bethesda, MD).

2.7. Statistical analysis

All experiments were repeated three times in triplicates. Results are presented as mean ± SD and statistical analyses were performed using GraphPad Prism 6.0 software. The P values were calculated using a one-way analysis of variance (ANOVA) or student t-test. P values of < 0.05, 0.01, or 0.001 were considered to indicate a statistically significant result, which are marked as *, **, or *** in figures.

3. Results

3.1. Inflammatory injury occurs in WI-38 cells after LPS treatment

WI-38 cells were treated by LPS with 1, 2, 5, and 10 μg/mL concentrations. Cell viability was inhibited (Fig. 1A); cell apoptosis was increased (Fig. 1B); Bcl-2 expression was suppressed (Fig. 1C and D); and Bax and cleaved-caspase-3/9 expressions were enhanced (Fig. 1C and D). All of these were changed in a dose dependent manner. WI-38 cells were then treated with 5 μg/mL LPS. According to qRT-PCR analysis, mRNA levels of tested pro-inflammatory cytokines MCP-1, IL-6, and IL-8 were elevated ($P < 0.001$, $P < 0.05$, and $P < 0.001$, Fig. 1E). Western blot analysis showed excessive protein expressions of MCP-1, IL-6, and IL-8 after 5 μg/mL LPS treatment (all $P < 0.001$, Fig. 1F and G). ELISA further quantified productions of MCP-1, IL-6, and IL-8 and they all significantly increased (all $P < 0.001$, Fig. 1H). Results indicate that LPS induced inflammatory injury in WI-38 cells.

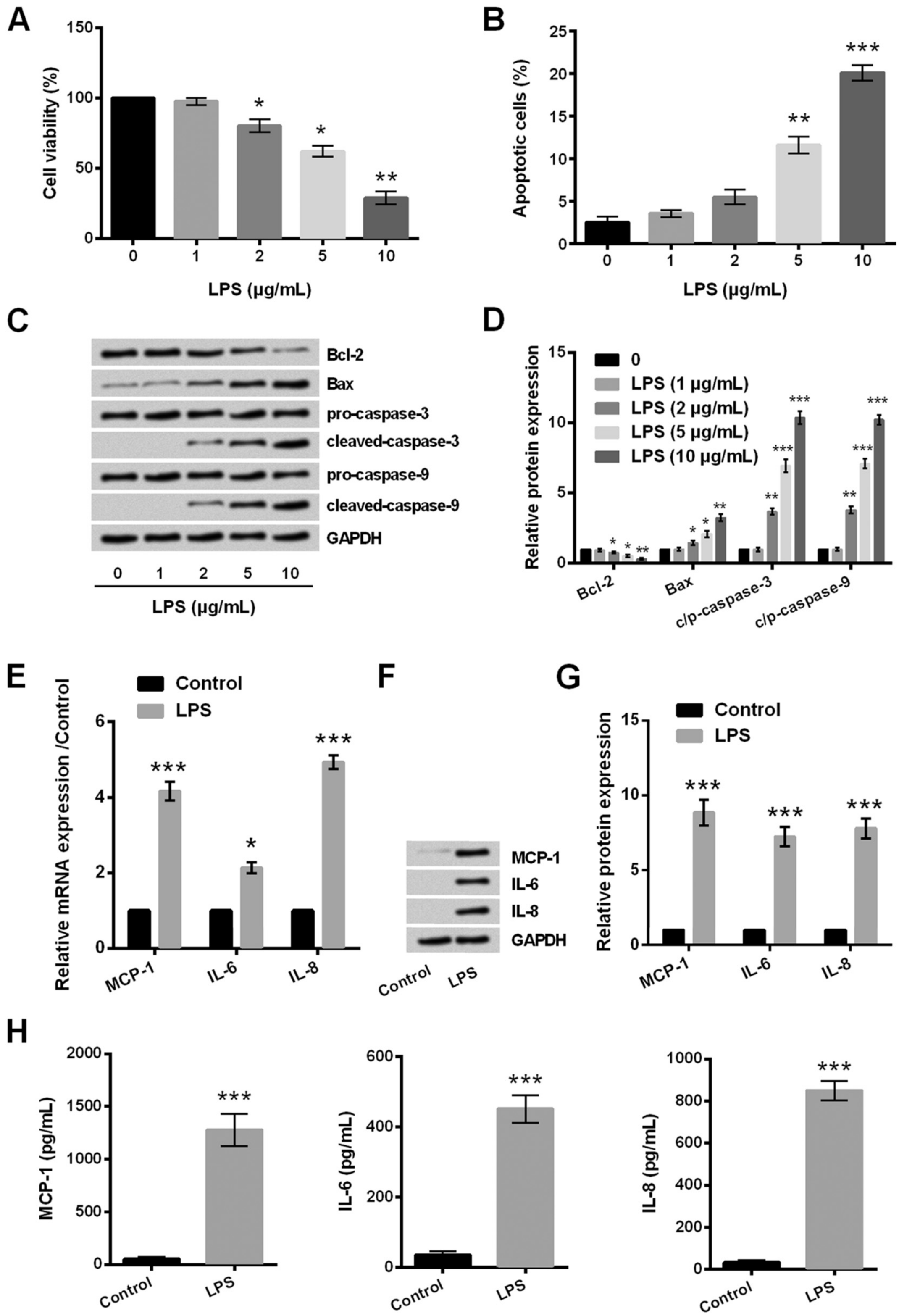

Fig. 1. LPS induced inflammation in WI-38 cells. (A) LPS induced inhibition of cell viability (higher than 2 μg/mL), (B) promotion of apoptosis (higher than 5 μg/mL), and (C and D) abnormal expression of apoptosis-related proteins (higher than 2 μg/mL). LPS at 5 μg/mL induced excessive (E) mRNA expressions and (F, G and H) protein expressions of tested pro-inflammatory factors.

3.2. miR-141 protects WI-38 cells from inflammatory injury

miR-141 was down-regulated in LPS-injured WI-38 cell ($P < 0.001$, Fig. 2A), indicating miR-141 might be related with the inflammation of WI-38 cells. We conducted transfection assay to alter miR-141 expression, which was significantly elevated after transfection with the specific mimic ($P < 0.001$) and significantly inhibited after transfection with the specific inhibitor ($P < 0.01$, Fig. 2B). WI-38 cells treated with miR-141 mimic exhibited higher viability and less apoptosis in comparison with cells treated with NC; however, WI-38 cells treated

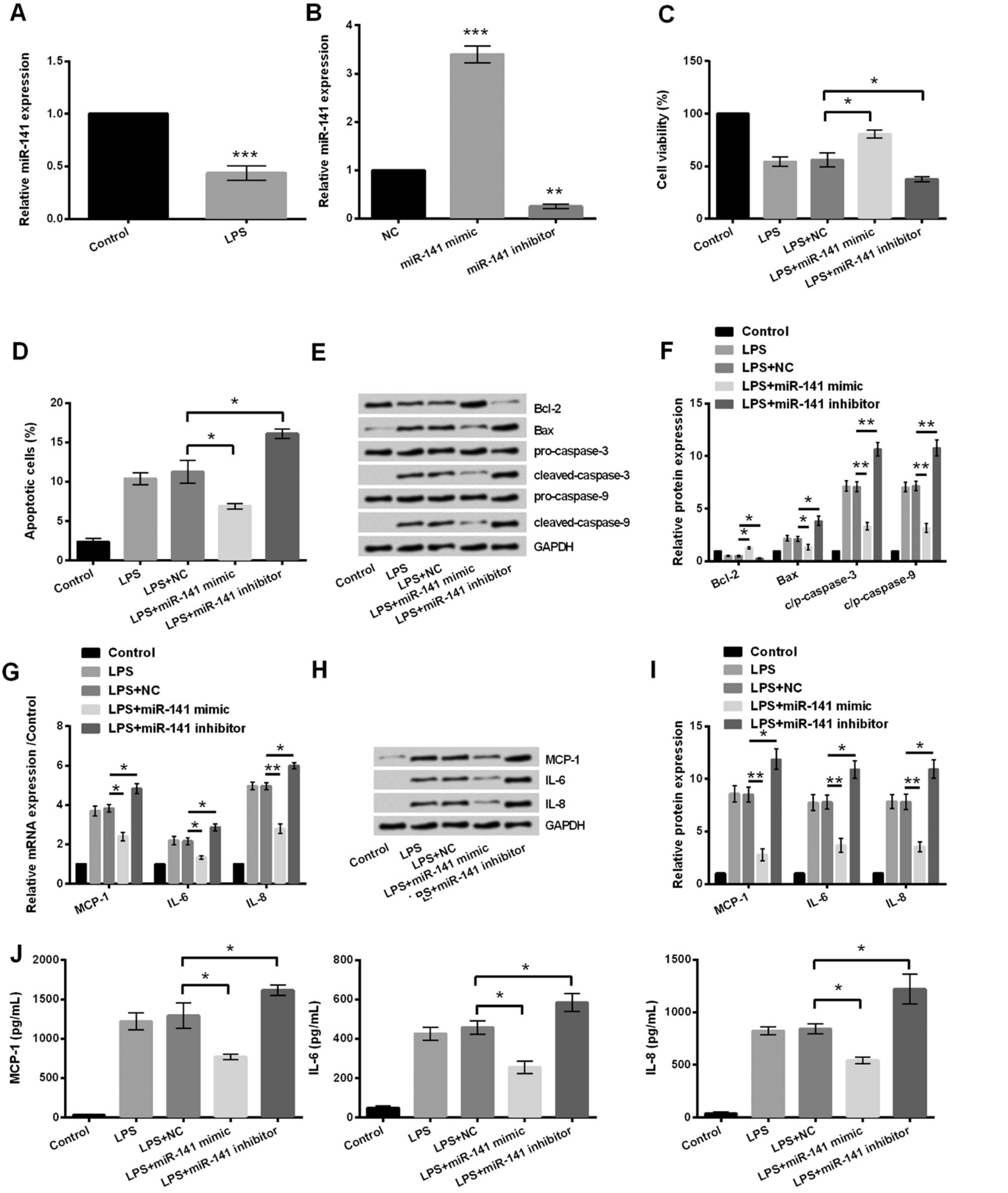

Fig. 2. miR-141 affected extent of inflammation in WI-38 cells with overexpression inhibiting it and silence promoting it. (A) Down-regulation of miR-141 in WI-38 cells was induced by LPS. (B) Expression of miR-141 was altered though transfection assay. (C) Viability was promoted or inhibited after transfected with miR-141 mimic or inhibitor. (D) Apoptosis was repressed or enhanced after transfected with miR-141 mimic or inhibitor. (E and F) Apoptosis-related proteins exhibited opposite changes after transfected with miR-141 mimic and inhibitor. Tested pro-inflammatory factors exhibited opposite changes both in (G) mRNA level and (H, I and J) protein level after transfected with miR-141 mimic and inhibitor.

with miR-141 inhibitor exhibited the contrary results (all $P < 0.05$, Fig. 2C and D). The anti-apoptosis protein Bcl-2 was up-regulated after miR-141 overexpression but down-regulated after miR-141 silence. The pro-apoptosis proteins Bax and cleaved-caspase-3/9 were down-regulated after miR-141 overexpression but up-regulated after miR-141 silence (Fig. 2E and F). The mRNA levels of tested pro-inflammatory cytokines MCP-1, IL-6, and IL-8 were significantly decreased in miR-141-overexpressed cells ($P < 0.05$, $P < 0.05$, and $P < 0.01$) but significantly increased in miR-141-knocked out cells (all $P < 0.05$, Fig. 2G). Low protein expressions of MCP-1, IL-6, and IL-8 were found

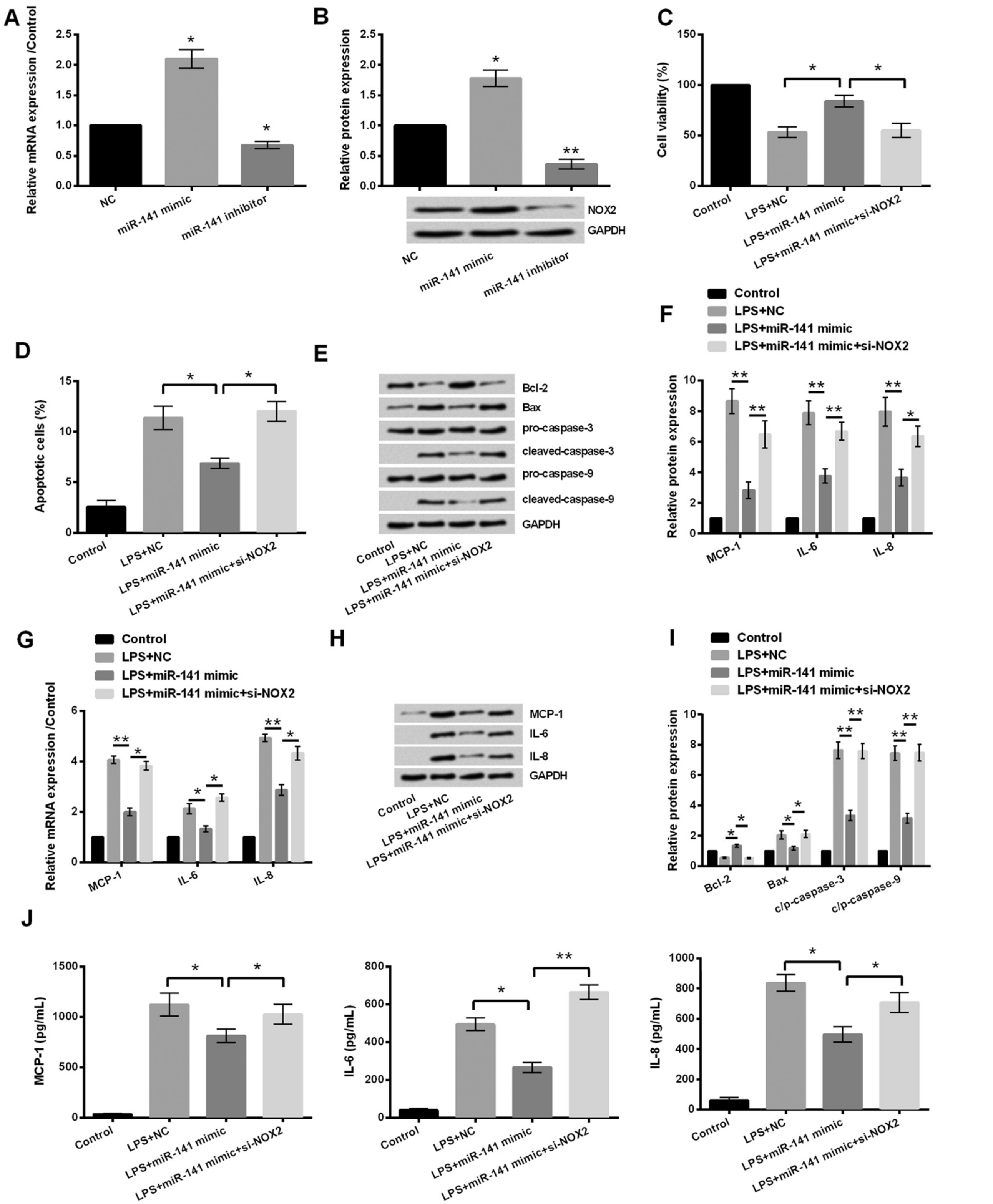

Fig. 3. miR-141 reduced extent of inflammation in WI-38 cells via increasing expression of NOX2. (A and B) NOX2 expression was positively related with miR-141 expression. Down-regulating NOX2 (C) inhibited cell viability, (D) promoted apoptosis, (E and F) affected expression of apoptosis-related proteins, accelerated production of tested pro-inflammatory factors both in (G) mRNA level and (H, I and J) protein level.

in miR-141-overexpressed cells. However, excessive protein expressions of MCP-1, IL-6, and IL-8 were detected in miR-141-knocked out cells (Fig. 2H, I and J).

3.3. NOX2 regulated by miR-141 protects WI-38 cells against inflammatory injury

miR-141 mimic up-regulated NOX2 both in mRNA level and protein level; in contrast, miR-141 inhibitor down-regulated them (Fig. 3A and

B). Since miR-141 was capable of promoting NOX2, we next determined the role of NOX2 in LPS-treated WI-38 cells. Compared with miR-141 mimic group, viability was significantly suppressed ($P < 0.05$, Fig. 3C), and apoptosis was significantly increased in miR-141 mimic + si-NOX2 group ($P < 0.05$, Fig. 3D). miR-141 mimic + si-NOX2 treatment showed the contrary effect on Bcl-2, Bax, and cleaved-caspase-3/9 in comparison with miR-141 mimic treatment (Fig. 3E and F). The mRNA expression levels of MCP-1, IL-6, and IL-8 were all increased after miR-141 mimic + si-NOX2 transfection compared with miR-141 mimic (all $P < 0.05$, Fig. 3G). The protein expression levels of them exhibited the similar changes (Fig. 3H and I). The increased contents of these cytokines in miR-141 mimic + si-NOX2-transfected cells were detected compared with miR-141 mimic-transfected cells ($P < 0.05$, $P < 0.01$, and $P < 0.05$, Fig. 3J). All data imply that miR-141 protected WI-38 cells via up-regulating NOX2, which may also explain partly why NOX2 suppression impaired the protective function of miR-141.

3.4. *miR-141 inhibits p38 MAPK and NF-κB pathways*

To explore the underlying mechanism of cell-protective effect of miR-141, Western blot was conducted to detect protein expressions of some key elements in MAPK signaling pathway and NF-κB signaling pathway. LPS treatment induced overexpression of phosphorylated p38 ($P < 0.01$, Fig. 4A and B) as well as overexpression of phosphorylated p65 and suppression of phosphorylated IκBα (both $P < 0.05$, Fig. 4C and D), while all of them were reversed after miR-141 mimic transfection. However, the alterations of these three proteins induced by LPS were further enhanced after miR-141 mimic + si-NOX2 transfection (all $P < 0.01$). These results suggest that miR-141 protected WI-38 cells from LPS-induced injury possibly via up-regulating NOX2, thereby inhibiting activations of p38 MAPK and NF-κB signaling pathways.

4. Discussion

In pneumonia, pathogens modulate expressions of miRNAs and mRNAs by lung neutrophils, which likely form inflammatory and immune responses against them and subsequent repair in the lungs [17]. Identifying miRNAs and mRNAs changes caused by organisms may provide an opportunity to map the signaling pathways that are crucial to the host immune response to infection. In this study, decreased miR-141 expression was identified after WI-38 cells were administrated with LPS and positive correlation between NOX2 expression and miR-141 expression were detected, making us to speculate that miR-141-NOX2 might participate in the regulation of inflammation response in WI-38 cells. Thus, we explored the role of miR-141-NOX2 in LPS-injured WI-38 cells.

Actually, a large amount of evidences reveal that miR-141 is a tumor regulator [11,18] and also related with several autoimmune diseases, such as lupus and psoriasis [19,20]; however, little information is available on the expression pattern of miR-141 in pulmonary inflammation, and whether the aberrant expression of miR-141 in pulmonary inflammation is associated with inflammation degree remains unknown. Several previous studies reported the role of miR-141 in intestinal inflammation. Wu et al showed slight reduction of miR-141 in ulcerative colitis patients [21]. Additionally, another study exhibited the expression profiles of the miR-200 family in inflammatory bowel diseases, ulcerative colitis and Crohn's disease. miR-141 was down-regulated in both diseases; and expression repression of miR-200 family induced epithelial-mesenchymal transition (EMT) leading to fibrosis, an

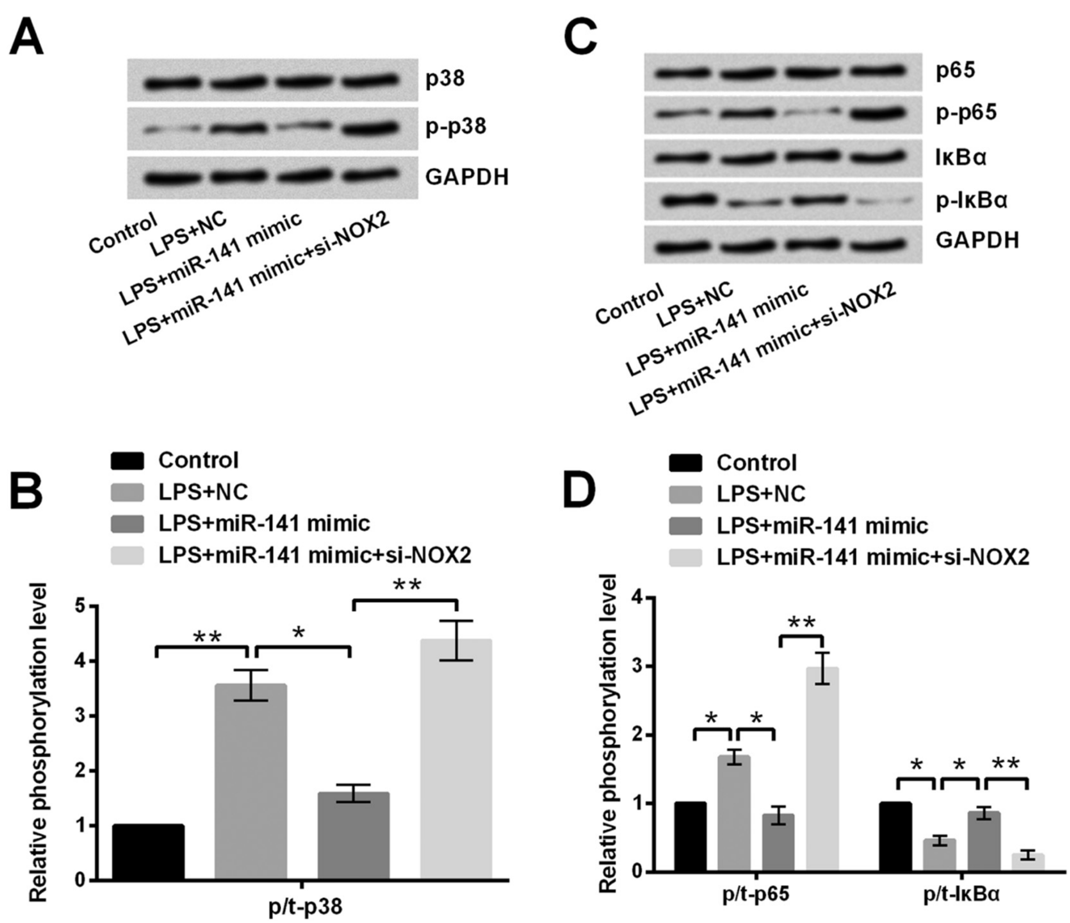

Fig. 4. miR-141 inhibited activations of (A and B) p38 MAPK and (C and D) NF-κB signaling pathways by up-regulating NOX2.

important feature of inflammatory bowel diseases [12]. Fibrosis not only occurs in inflammatory bowel diseases but also on lung inflammation. The similar results were observed in one previous study, which also mentioned that stimulation of some pro-inflammatory cytokines during inflammation process contributed to the down-regulation of miR-141 [13]. There are evidences that miR-141 was reduced in TNF-α-stimulated cells [21,22]. Pre-miR-141 on mice treatment could ameliorate colitis in vivo [13], indicating the anti-inflammation effect of miR-141, which was also proved in our study. miR-141 overexpression protected WI-38 cells from LPS-induced inflammation through promoting viability, inhibiting apoptosis, decreasing production of MCP-1, IL-6, and IL-8.

NOX complexes, a family of transmembrane electron-carrying enzymes, are responsible for reactive oxygen species (ROS) production using NADPH and oxygen as an electron donor and acceptor, respectively, which comprise seven currently known members, including NOX2 [23]. NOX-derived superoxide had a bacterial-killing role, which protected human from inflammatory lung injury, including pneumonia [24,25]. According to our data, NOX2 expression was positively related with miR-141, implying the involvement of NOX2 in process of miR-141-mediated inflammation response in WI-38 cells. miR-141 protected WI-38 cells likely through up-regulating ROX2. A previous study revealed the role of NOX2 in resolution of systemic inflammatory responses and specifically in limiting pulmonary inflammation [26]. NOX2 absence in WT mice augmented systemic progression of acute inflammatory phase [26] and NOX2 deficiency enhanced allergen-induced airway inflammation by producing inflammatory cytokines [27], implying the pro-inflammation of NOX2 silence, which are consistent with our results. Also, NOX2-activating drugs were considered as a potentially strategy for controlling inflammatory diseases [23].

In acute and chronic inflammatory diseases, released inflammatory mediators activate multiple intracellular signaling cascades, including MAPK and NF-κB signal transduction pathway [28,29]. LPS treatment could induce activations of MAPK and NF-κB signaling pathways, promoting inflammation response [30,31]. In this study, LPS likewise introduced activations of p38 MAPK and NF-κB signaling pathways, which were inhibited after miR-141 was overexpressed; however, they were activated after NOX2 expression was repressed. Results imply that miR-141 protected WI-38 cells via mediating NOX2 expression and then inhibited NOX2-regulated p38 MAPK and NF-κB pathways.

5. Conclusions

Together, our study revealed that augmenting miR-141 expression would inhibit the WI-38 cell injury induced by LPS. Regulative relations of miR-141-NOX2-p38 MAPK/NF-κB might provide a better understanding of the mechanism by which miR-141 alleviated effects of LPS on WI-38 cells. In the future, miR-141 might be a novel target for treating infantile pneumonia.

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Authorship

Conceives, designed the experiments and wrote the paper: Ruirui Xue. Performed the experiments and analyzed the data: Beibei Quan and Huating Zhang.

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None.

Conflict of interest

Authors declare that there is no conflict of interests.

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