FOXA2 attenuates lipopolysaccharide-induced pneumonia by inhibiting the inflammatory response, oxidative stress and apoptosis through blocking of p38/STAT3 signaling

ZHIBIN XUE[1], YINGLIN LI[1], SHIJI XIAO[1], HANQING ZHANG[1] and JIANZHANG XU[2]

[1]Department of Pediatrics, The Affiliated Hospital of Putian University; [2]Department of Pediatrics, Putian Children's Hospital, Putian, Fujian 351100, P.R. China

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Abstract. Pneumonia is a severe inflammatory disease of the lung. Forkhead box protein A2 (FOXA2) has been demonstrated to serve an important regulatory role in various pulmonary diseases; however, the role of FOXA2 in pneumonia remains to be elucidated. The present study aimed to explore the functional effects and regulatory mechanism of FOXA2 in pneumonia. An *in vitro* pneumonia model was induced using lipopolysaccharide (LPS) in WI-38 cells. The mRNA and protein expression levels of FOXA2 were determined by reverse transcription-quantitative PCR and western blotting, respectively. Cell viability was assessed using a Cell Counting Kit-8 assay. Inflammatory cytokines were evaluated using ELISA kits and oxidative stress markers were assessed using a malondialdehyde assay kit, superoxide dismutase assay kit and CATalase assay kit. Cell apoptosis was evaluated using flow cytometry and the caspase3 activity was determined. Western blotting was performed to examine the protein expression levels of endoplasmic reticulum stress (ERS)-associated factors. For a rescue assay, a p38 MAPK activator, U46619, was used to investigate the regulatory mechanism of FOXA2 involving p38/STAT3 signaling. FOXA2 was downregulated in LPS-induced WI-38 cells. FOXA2 overexpression alleviated LPS-induced inflammation, oxidative stress, apoptosis and ERS in WI-38 cells. Furthermore, the inhibitory effects of FOXA2 on inflammation, oxidative stress and apoptosis, as well as ERS in LPS-induced WI-38 cells were partly weakened by additional treatment with U46619. In conclusion, FOXA2 served a protective role against LPS-induced pneumonia by regulating p38/STAT3 signaling, providing a novel idea for the development of targeted therapeutic strategies for pneumonia.

Correspondence to: Dr Jianzhang Xu, Department of Pediatrics, Putian Children's Hospital, 181 Meiyuan East Road, Licheng, Putian, Fujian 351100, P.R. China
E-mail: xujianzhang920123@163.com

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Introduction

Pneumonia, a common inflammatory disease of the lung, is a huge burden on both society and individuals, and is associated with high incidence and mortality rates, with ~120 million new cases and 1.3 million deaths every year (1), especially in children and seniors >65 years old. It has been reported that ~921 000 children under the age of 5 years died from pneumonia in 2015 globally (2). Pneumonia, the typical clinical symptoms of which include cough, dyspnea, fever and chest pain, may develop into respiratory distress syndrome, accompanied by multiple complications, such as heart failure and parapneumonic pleurisy (3-5). Pneumonia is caused by various types of infection, with viruses such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, bacteria such as respiratory syncytial virus parainfluenza virus, fungi and other pathogens (6,7). In the last few years, superinfection with difficult-to-treat fungi such as *Fusarium* and the resistance of germs to common antibiotics has made treating and curing pneumonia difficult, despite the clinical advantages of the latest generation antibiotics such as cefiderocol (8-11). Furthermore, the novel coronavirus (severe acute respiratory syndrome coronavirus 2) causing the COVID-19 disease pandemic, which can induce respiratory damage and progress to pneumonia or damage to the whole body, has infected millions of individuals, leading to >8,000,000 cases and >400,000 deaths (12-15). Thus, pneumonia is a severe clinical problem and an improved understanding of the pathological mechanism of pneumonia is urgently required for the exploration of effective therapeutic modalities.

Forkhead box protein A2 (FOXA2; also referred to as hepatocyte nuclear factor 3β), belongs to the forkhead transcription factor family, which contains a winged-helix DNA-binding domain (16). FOXA2 was initially identified in liver tissues by Jackson *et al* (17), who found that FOXA2 could control the expression of liver-specific genes. At present, accumulating evidence has revealed that FOXA2 is widely distributed in multiple tissues, and abnormal FOXA2 expression is associated with various diseases. For instance, high expression levels of FOXA2 are observed in various types of cancer, including esophageal squamous cell carcinoma, prostate cancer and lung cancer, and are associated with tumorigenesis

or tumor metastasis (18-20). In addition, FOXA2 is depleted by microbial infection and proinflammatory responses in diseased airways, causing the dysregulation of mucin biosynthesis and goblet cell development, and ultimately leading to acute deterioration of pulmonary functions (21). FOXA2 is also downregulated in chronic obstructive pulmonary disease, and FOXA2 overexpression serves a protective role against cigarette smoke-induced cellular senescence and lung inflammation (22). Although the aforementioned evidence suggests the critical regulatory role of FOXA2 in multiple pulmonary diseases, the functional effects and molecular mechanism of FOXA2 in pneumonia remain to be determined.

Therefore, the present study aimed to explore the expression and regulatory role of FOXA2 in pneumonia, and attempted to elucidate the regulatory mechanism of FOXA2, in order to provide novel strategies for the treatment of pneumonia.

Materials and methods

Cell culture and treatment. WI-38 human embryonic fibroblast cells were obtained from Procell Life Science & Technology Co., Ltd., and cultured in minimum essential medium (Procell Life Science & Technology Co., Ltd.) supplemented with 10% FBS (Procell Life Science & Technology Co., Ltd.) and 1% penicillin/streptomycin mixture in a humified incubator with 5% CO_2 at 37˚C. WI-38 cells were treated with increasing doses (5, 10 and 15 μg/ml) of lipopolysaccharide (LPS; cat. no. L2880; MilliporeSigma) for 24 h at 37˚C to establish an *in vitro* infantile pneumonia cell model as described previously (23,24). In addition, to investigate the molecular mechanism, WI-38 cells were pretreated with U46619 (2 mM; cat. no. sc-201242; Santa Cruz Biotechnology, Inc.), an activator of p38 signaling (25), for 4 h at 37˚C prior to LPS exposure. Normally cultured WI-38 cells were used as the control group.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using the Eastep® Super Total RNA Extraction Kit (Promega Corporation) according to the manufacturer's instructions. Following detection of the concentration and purity of the extracted RNA using a NanoDrop 3000 Spectrophotometer (Thermo Fisher Scientific, Inc.), total RNA was reverse transcribed into cDNA using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd.). The conditions for RT were as follows: 37˚C for 10 min, followed by 85˚C for 5 sec and holding at 4˚C. Subsequently, qPCR was performed using ChamQ SYBR Master Mix (Vazyme Biotech Co., Ltd.) and a LightCycler 96 (Roche Applied Science). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 10 min; followed by 40 cycles of 95˚C for 10 sec, 60˚C for 60 sec and 95˚C for 15 sec. The sequences of the primers used in the present study were as follows: FOXA2 forward, 5'-CGACTGGAGCAGCTACTATGC-3' and reverse, 5'-ATGTACGTGTTCATGCCGTTC-3'; and GAPDH forward, 5'-CAGGAGGCATTGCTGATGAT-3' and reverse, 5'-GAAGGCTGGGGCTCATTT-3'. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method (26) and normalized to GAPDH.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). Following detection of the protein concentration using a BCA kit (Thermo Fisher Scientific, Inc.), equal amounts of protein (30 μg per lane) were separated by SDS-PAGE on 12% gels and then transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked with 5% skimmed milk for 2 h at room temperature, followed by probing with primary antibodies against FOXA2 (cat. no. ab23630; 1:1,000; Abcam), cyclooxygenase-2 (Cox2; cat. no. ab179800; 1:1,000; Abcam), inducible nitric oxide synthase (iNOS; cat. no. ab178945; 1:1,000; Abcam), NADPH oxidase (Nox)2 (cat. no. ab129068; 1:5,000; Abcam), Nox4 (cat. no. ab154244; 1:1,000; Abcam), Bcl-2 (cat. no. ab32124; 1:1,000; Abcam), Bax (cat. no. ab32503; 1:1,000; Abcam), Cleaved-poly(ADP-ribose) polymerase 1 (PARP1; cat. no. ab32064; 1:1,000; Abcam), PARP1 (cat. no. ab227244; 1:1,000; Abcam), phosphorylated (p)-p38 (cat. no. 9215; 1:1,000; Cell Signaling Technology, Inc.), p38 (cat. no. 9212; 1:1,000; Cell Signaling Technology, Inc.), p-STAT3 (cat. no. 94994; 1:1,000; Cell Signaling Technology, Inc.), STAT3 (cat. no. ab109085; 1:1,000; Abcam), glucose-regulated protein 78 (GRP78; cat. no. ab21685; 1:1,000; Abcam), CHOP (cat. no. 5554; 1:1,000; Cell Signaling Technology, Inc.), X-box binding protein 1 (XBP1; cat. no. ab37152; 1:1,000; Abcam), activating transcription factor 6 (ATF6; cat. no. orb381900; 1:1,000; Biorbyt, Ltd.), p-eukaryotic translation initiation factor 2 subunit α (eIF2α; cat. no. orb15000; 1:1,000; Biorbyt, Ltd.), eIF2α (cat. no. orb480065; 1:1,000; Biorbyt, Ltd.) and GAPDH (cat. no. ab9485; 1:2,500; Abcam) at 4˚C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat-anti-rabbit IgG secondary antibody (cat. no. ab6721; 1:2,000; Abcam) for 1 h at room temperature. The blot signals were developed using Immobilon ECL Ultra Western HRP Substrate (MilliporeSigma), and were analyzed using ImageJ 1.52a software (National Institutes of Health).

Cell viability assay. Cell viability was examined using a Cell Counting Kit-8 (CCK-8) assay. In brief, WI-38 cells were seeded into 96-well plates at the density of $4x10^4$ cells/well and incubated with different concentrations of LPS for 24 h at 37˚C. Subsequently, 10 μl CCK-8 reagent (Beijing Solarbio Science & Technology Co., Ltd.) was added to each well and the cells were incubated at 37˚C for 2 h. Finally, the absorbance of each well at 450 nm was measured using a full wavelength microplate reader (Thermo Fisher Scientific, Inc.). The cell viability was calculated as follows: Cell viability (%)=experimental group OD/control group OD x100.

Cell transfection. FOXA2 overexpression vector (oe-FOXA2) was established by inserting the FOXA2 gene into the pcDNA3.1 vector (Shanghai GenePharma Co., Ltd.), referring to the empty vector as the negative control (overexpression-negative control; oe-NC) were obtained from Shanghai GenePharma Co., Ltd. After indicated treatment, WI-38 cells seeded in 6-well culture plates ($2x10^5$ cells/well) were transfected with 5 μg/ml oe-NC or oe-FOXA2 using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. Cells were harvested for the subsequent assays 48 h after transfection.

Measurement of cytokines. In brief, the cell medium was collected and the supernatant was collected after being

centrifuged at 2,000 x g for 10 min at 4˚C. The concentrations of inflammatory cytokines, including TNF-α, IL-6 and IL-1β, in the supernatants from WI-38 cells were assessed using ELISA kits (cat. nos. STA00D, S6050 and SLB50, respectively; R&D Systems, Inc.) according to the manufacturer's protocols.

Measurement of reactive oxygen species (ROS) and oxidative stress markers. The cellular ROS levels were evaluated using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) probe. In brief, WI-38 cells at a density of $5x10^5$ cells/ml were treated with 10 μM DCFH-DA (Cayman Chemical Company) for 45 min in the dark at 37˚C. Afterwards, the images were captured under a fluorescence microscope (Olympus Corporation).

In brief, the cell medium was collected and the supernatant was collected after being centrifuged at 2,000 x g for 10 min at 4˚C. The levels of malondialdehyde (MDA), and the activities of superoxide dismutase (SOD) and catalase (CAT) in the supernatants from WI-38 cells were assessed using their corresponding kits from Nanjing Jiancheng Bioengineering Institute (cat. nos. A003-1, A001-3 and A007-1, respectively) according to the manufacturer's protocols.

Flow cytometry. WI-38 cells were digested with trypsin, washed with ice-cold PBS and resuspended with 1X binding buffer (Beyotime Institute of Biotechnology). Subsequently, Annexin V-FITC (Beyotime Institute of Biotechnology) and PI (Beyotime Institute of Biotechnology) were added to stain cells for 30 min in the dark at 4˚C. The apoptotic cells were detected by flow cytometry (FACSCalibur; BD Biosciences) and FlowJo™ VX10 software (FlowJo LLC). The apoptosis rate was calculated as follows: Apoptosis rate %=(number of early apoptotic cells + number of late apoptotic cells)/total number of cells x100.

Caspase3 activity assay. Total protein was extracted from WI-38 cells using RIPA lysis buffer (Thermo Fisher Scientific, Inc.) and its concentration was determined using a BCA kit (Thermo Fisher Scientific, Inc.). Subsequently, the caspase3 activity was assessed using a Caspase-3 Activity Assay Kit (cat. no. BC3830; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol. The absorbance at 405 nm was measured using a full wavelength microplate reader (Thermo Fisher Scientific, Inc.). According to absorbance at 405 nm of standard sample in each concentration, the standard curve was drawn. The caspase-3 activity in each sample was calculated according to the standard curve, and the average was taken.

Statistical analysis. All data were analyzed utilizing GraphPad Prism version 8.0 (Dotmatics) and presented as the mean ± standard deviation of three independent experiments. Comparisons among groups were performed using one-way ANOVA with Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FOXA2 expression is downregulated in LPS-induced WI-38 cells. To study the role of FOXA2 in pneumonia, WI-38 cells were induced by LPS to simulate the inflammatory injury in pneumonia *in vitro*, and the expression levels of FOXA2 were examined. As shown in Fig. 1A and B, both the protein and mRNA expression levels of FOXA2 were gradually decreased with increasing concentrations of LPS, suggesting the downregulation of FOXA2 in an LPS-induced *in vitro* model of pneumonia in WI-38 cells. Additionally, the cell viability was decreased by LPS in a concentration-dependent manner (Fig. 1C). In particular, 15 μg/ml LPS led to a significant decrease in WI-38 cell viability and 5 μg/ml LPS led to a slight decrease in WI-38 cell viability, while 10 μg/ml LPS induced ~50% cell viability, thus 10 μg/ml LPS was used in subsequent experiments. Subsequently, oe-FOXA2 was transfected into WI-38 cells. The results in Fig. 1D and E demonstrated the transfection efficacy of the FOXA2 overexpression plasmids. Significant elevation of FOXA2 mRNA and protein expression was observed in the oe-FOXA2 group compared with the oe-NC group. Subsequently, LPS-induced WI-38 cells were also transfected with oe-FOXA2, and the data indicated that the decreased FOXA2 expression following LPS exposure was partly restored by transfection with oe-FOXA2 (Fig. 1F and G).

FOXA2 overexpression alleviates LPS-induced inflammation and oxidative stress in WI-38 cells. To explore the regulatory role of FOXA2 in pneumonia, inflammation and oxidative stress levels were examined. According to the ELISA, the levels of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, were increased in LPS-exposed WI-38 cells, whereas FOXA2 overexpression suppressed this increase (Fig. 2A). In addition, high ROS activity in the LPS group was observed based on the fluorescence images in Fig. 2B, and this was partly abolished by FOXA2 overexpression. Furthermore, LPS also upregulated MDA levels, and downregulated SOD and CAT activities in WI-38 cells, which was reversed by FOXA2 overexpression (Fig. 2C), suggesting that FOXA2 overexpression could inhibit LPS-induced oxidative stress in WI-38 cells. Furthermore, western blotting revealed that the expression levels of inflammation- and oxidative stress-related proteins (Cox2, iNOS, Nox2 and Nox4) were significantly increased following LPS treatment, which were then depleted again by FOXA2 overexpression (Fig. 2D).

FOXA2 overexpression alleviates LPS-induced apoptosis in WI-38 cells. The effect of FOXA2 on apoptosis was assessed in LPS-induced WI-38 cells. As shown in Fig. 3A and B, LPS treatment led to increased apoptosis in WI-38 cells, which was partially reversed by FOXA2 overexpression, suggesting an anti-apoptotic activity of FOXA2, which was then verified by the decreased Caspase3 activity following FOXA2 overexpression in LPS-induced WI-38 cells since Caspase3 is considered a primary executioner of apoptosis (Fig. 3C). Furthermore, the protein expression levels of the anti-apoptotic protein Bcl-2 were downregulated and the protein expression levels of the pro-apoptotic proteins Bax and Cleaved-PARP1/PARP1 were significantly upregulated following LPS exposure in WI-38 cells, and these were then partly reversed by FOXA2 overexpression (Fig. 3D and E).

FOXA2 restricts endoplasmic reticulum stress (ERS) by blocking p38/STAT3 signaling. Through western blotting, it

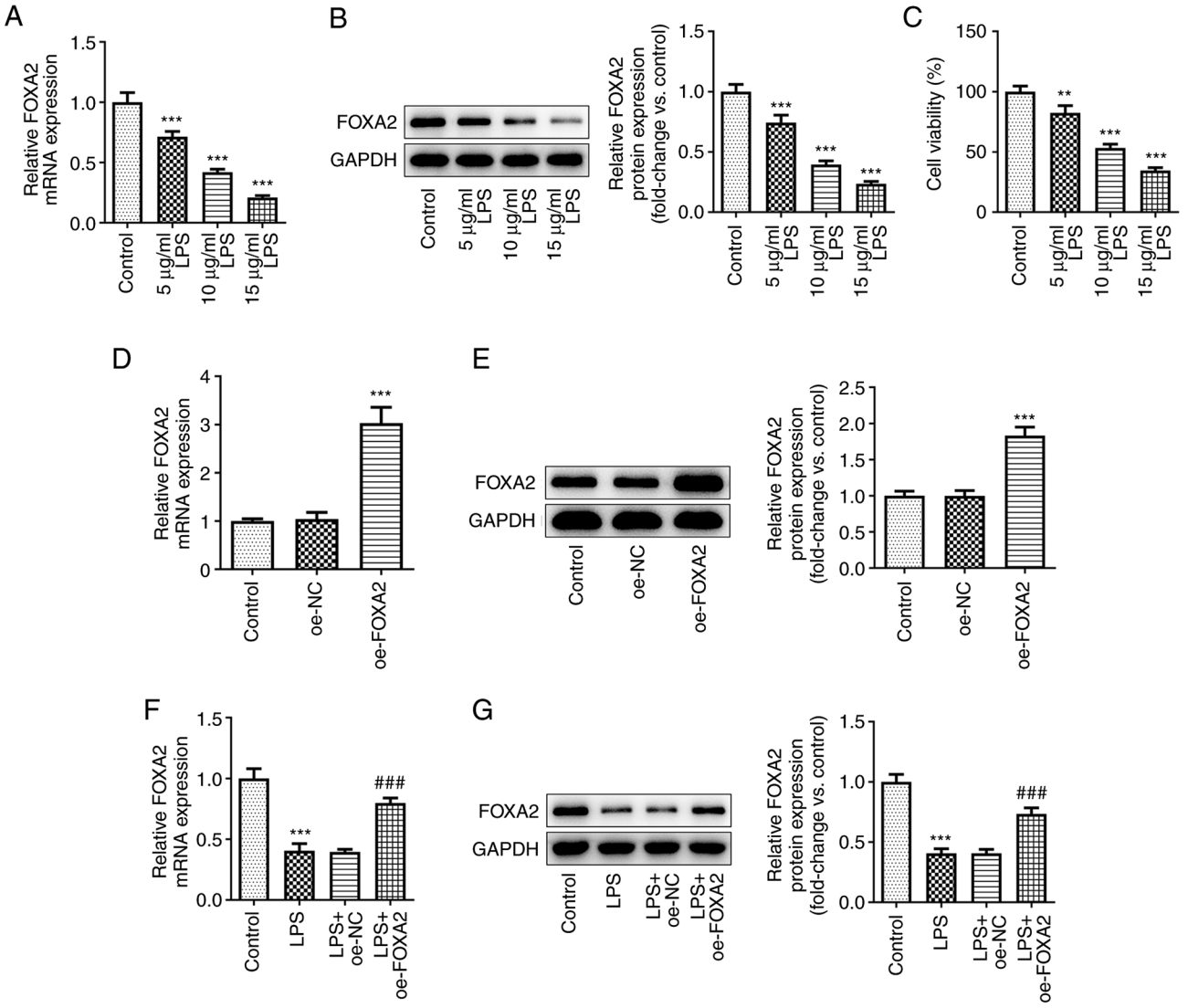

Figure 1. FOXA2 is downregulated in LPS-induced WI-38 cells. WI-38 cells were induced by different concentrations (5, 10 and 15 μg/ml) of LPS to simulate the inflammatory injury in pneumonia *in vitro*. (A) mRNA and (B) protein expression levels of FOXA2 were examined using RT-qPCR and western blotting, respectively. ***P<0.001 vs. Control. (C) Cell viability was detected using a Cell Counting Kit-8 assay. **P<0.01 and ***P<0.001 vs. Control. WI-38 cells were transfected with oe-FOXA2 or oe-NC, and the (D) mRNA and (E) protein expression levels of FOXA2 were examined using RT-qPCR and western blotting, respectively. ***P<0.001 vs. oe-NC. The untransfected or transfected WI-38 cells were induced by LPS (10 μg/ml), and the (F) mRNA and (G) protein expression levels of FOXA2 were examined using RT-qPCR and western blotting, respectively. ***P<0.001 vs. Control; $^{###}$P<0.001 vs. LPS + oe-NC. FOXA2, forkhead box protein A2; LPS, lipopolysaccharide; oe-FOXA2, FOXA2 overexpression vector; oe-NC, overexpression negative control; RT-qPCR, reverse transcription-quantitative PCR.

was revealed that the increased p-/total-(t)-p38 and p-/t-STAT3 levels in LPS-treated WI-38 cells were both decreased after FOXA2 was overexpressed (Fig. 4A), implying that p38/STAT3 signaling was activated by LPS stimulation in WI-38 cells, but was inactivated by FOXA2 overexpression. p38 MAPK and STAT3 signaling are important signal transduction pathways mediating ERS (27,28), thus whether FOXA2 influenced ERS via p38/STAT3 signaling was then assessed. As shown in Fig. 4B, LPS treatment triggered ERS in WI-38 cells, as evidenced by the upregulated protein expression levels of GRP78, CHOP, XBP1, ATF6 and p/t-eIF2α, which were reduced by FOXA2 overexpression. However, the inhibitory effect of FOXA2 on GRP78, CHOP, XBP1 and p/t-eIF2α protein expression levels in LPS-induced WI-38 cells was partly reversed by additional treatment with U46619, an activator of p38 signaling, indicating that FOXA2 may inhibit LPS-triggered ERS in WI-38 cells partly through inactivating p38 signaling.

FOXA2 protects WI-38 cells against LPS-induced oxidative stress, inflammation and apoptosis by inactivating p38/STAT3 signaling. Finally, to clarify whether FOXA2 attenuated LPS-induced cell injury by inactivating p38/STAT3 signaling, oxidative stress, inflammation and apoptosis were examined in FOXA2-overexpressing WI-38 cells with or without U46619 treatment. An ELISA revealed that the downregulated production of TNF-α, IL-6 and IL-1β following FOXA2 overexpression in LPS-induced WI-38 cells was partly elevated by U46619 treatment (Fig. 5A). Furthermore, FOXA2 overexpression was observed to reduce ROS and MDA levels, but increased SOD and CAT activities in LPS-induced WI-38 cells, whereas the impact of FOXA2 overexpression on ROS,

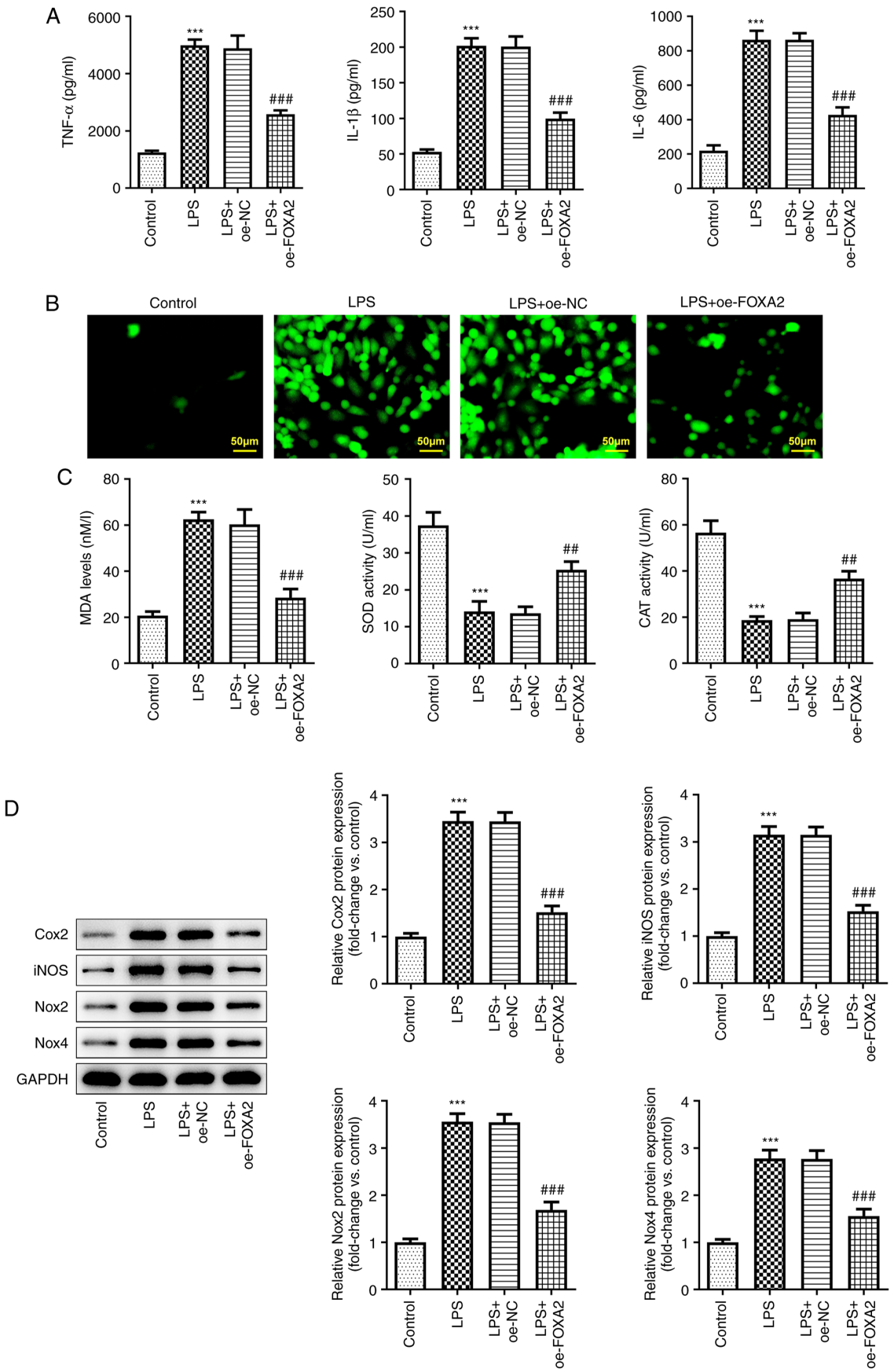

Figure 2. FOXA2 overexpression alleviates LPS-induced inflammation and oxidative stress in WI-38 cells. (A) Production of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, was examined by ELISA. (B) Cellular reactive oxygen species levels were detected using the 2',7'-dichlorofluorescein diacetate method. Magnification, x200. Scale bar, 50 μM. (C) MDA levels, SOD activity and CAT activity were assessed using their corresponding kits. (D) Protein expression levels of Cox2, iNOS, Nox2 and Nox4 were examined using western blotting. ***P<0.001 vs. Control; $^{##}$P<0.01 and $^{###}$P<0.001 vs. LPS + oe-NC. CAT, catalase; Cox2, cyclooxygenase-2; FOXA2, forkhead box protein A2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MDA, malondialdehyde; Nox, NADPH oxidase; oe-FOXA2, FOXA2 overexpression vector; oe-NC, overexpression negative control; SOD, superoxide dismutase.

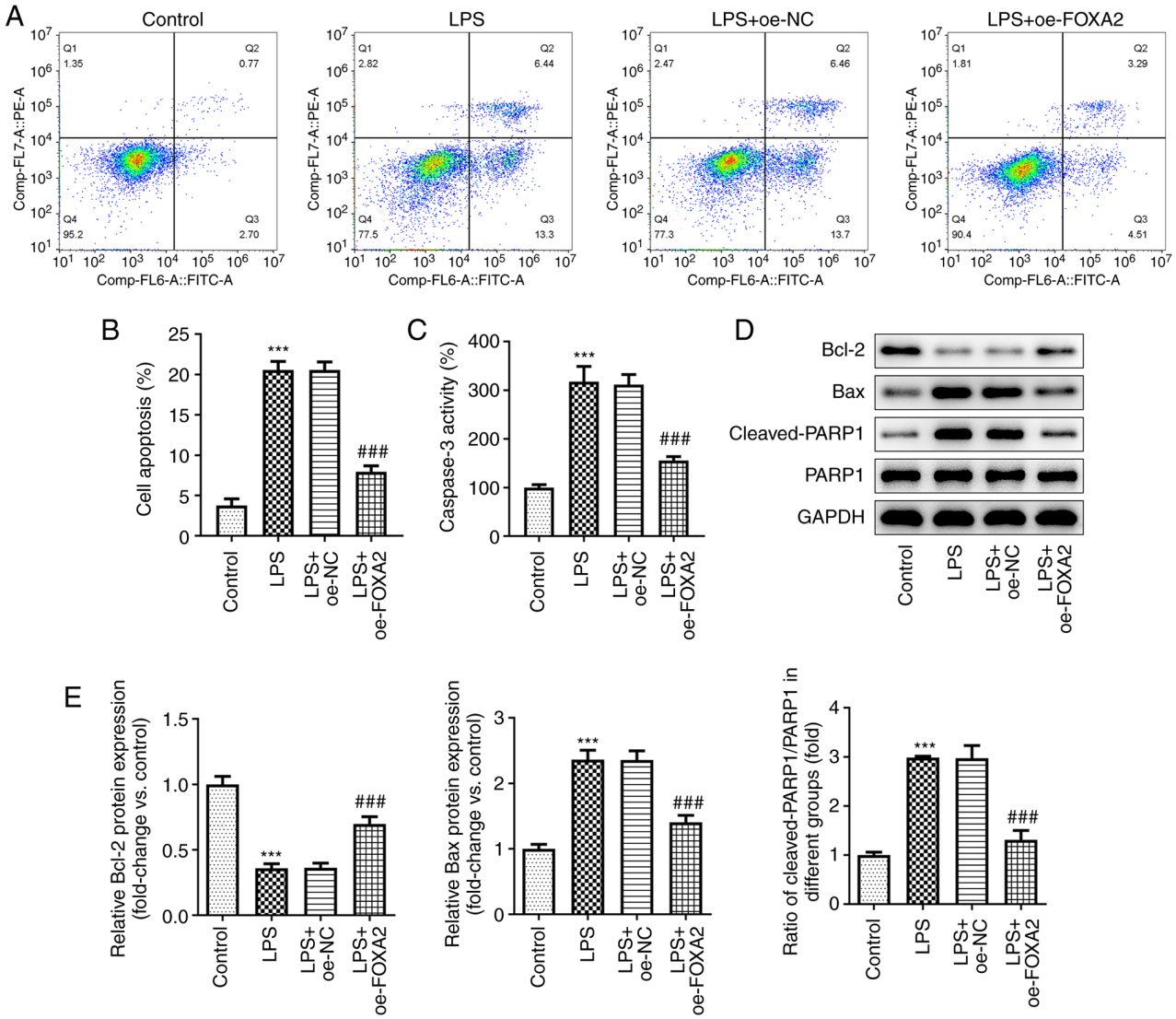

Figure 3. FOXA2 overexpression alleviates LPS-induced apoptosis in WI-38 cells. (A) Flow cytometry was performed to assess the cell apoptosis rate in each group and (B) quantified. (C) Caspase3 activity was determined using the related kit. (D) Expression levels of apoptosis-related proteins were examined using western blotting and (E) quantified. ***P<0.001 vs. Control; ###P<0.001 vs. LPS + oe-NC. FOXA2, forkhead box protein A2; LPS, lipopolysaccharide; oe-FOXA2, FOXA2 overexpression vector; oe-NC, overexpression negative control; PARP1, poly(ADP-ribose) polymerase 1.

MDA and CAT levels were partly reversed by U46619 treatment (Fig. 5B and C). The upregulated protein expression levels of Cox2, iNOS, Nox2 and Nox4 in the U46619 + LPS + oe-FOXA2 group compared with the LPS + oe-FOXA2 group further demonstrated that FOXA2 may protect WI-38 cells against LPS-triggered oxidative stress and inflammation partly through inactivating p38/STAT3 signaling (Fig. 5D). In addition, the anti-apoptotic activity of FOXA2 in LPS-induced WI-38 cells was weakened by U46619 treatment, as demonstrated by the elevated apoptosis rate and Caspase3 activity, accompanied by elevated protein expression levels of Bax and Cleaved-PARP1/PARP1, and reduced Bcl-2 protein expression levels in the U46619 + LPS + oe-FOXA2 group compared with the LPS + oe-FOXA2 group (Fig. 6).

Discussion

Pneumonia is a common, complicated and serious inflammatory disease of the lung, affecting numerous individuals worldwide, and is associated with a threat to life quality and an economic burden (29). LPS, a gram-negative bacterial endotoxin, serves a critical role in the initiation of pneumonia, and LPS-induced acute lung injury contributes to the development of pneumonia (30). Therefore, alleviating LPS-induced lung injury has been widely recognized as an effective strategy for the treatment of pneumonia (23,31,32). An *in vitro* pneumonia model was induced by LPS in WI-38 cells in the present study. Following LPS exposure, the expression levels of FOXA2 were reduced. Thereafter, gain-of-function experiments were conducted, which confirmed that FOXA2 overexpression exerted inhibitory effects on the LPS-triggered inflammatory response, oxidative stress, apoptosis and ERS in WI-38 cells, suggesting FOXA2 as a positive regulator for alleviating pneumonia. In terms of the mechanism, the protective role of FOXA2 against pneumonia was partially abolished by a p38 activator, indicating that FOXA2 blocked the progression of pneumonia partially through blocking of the p38/STAT3 signaling pathway. Therefore, we hypothesized

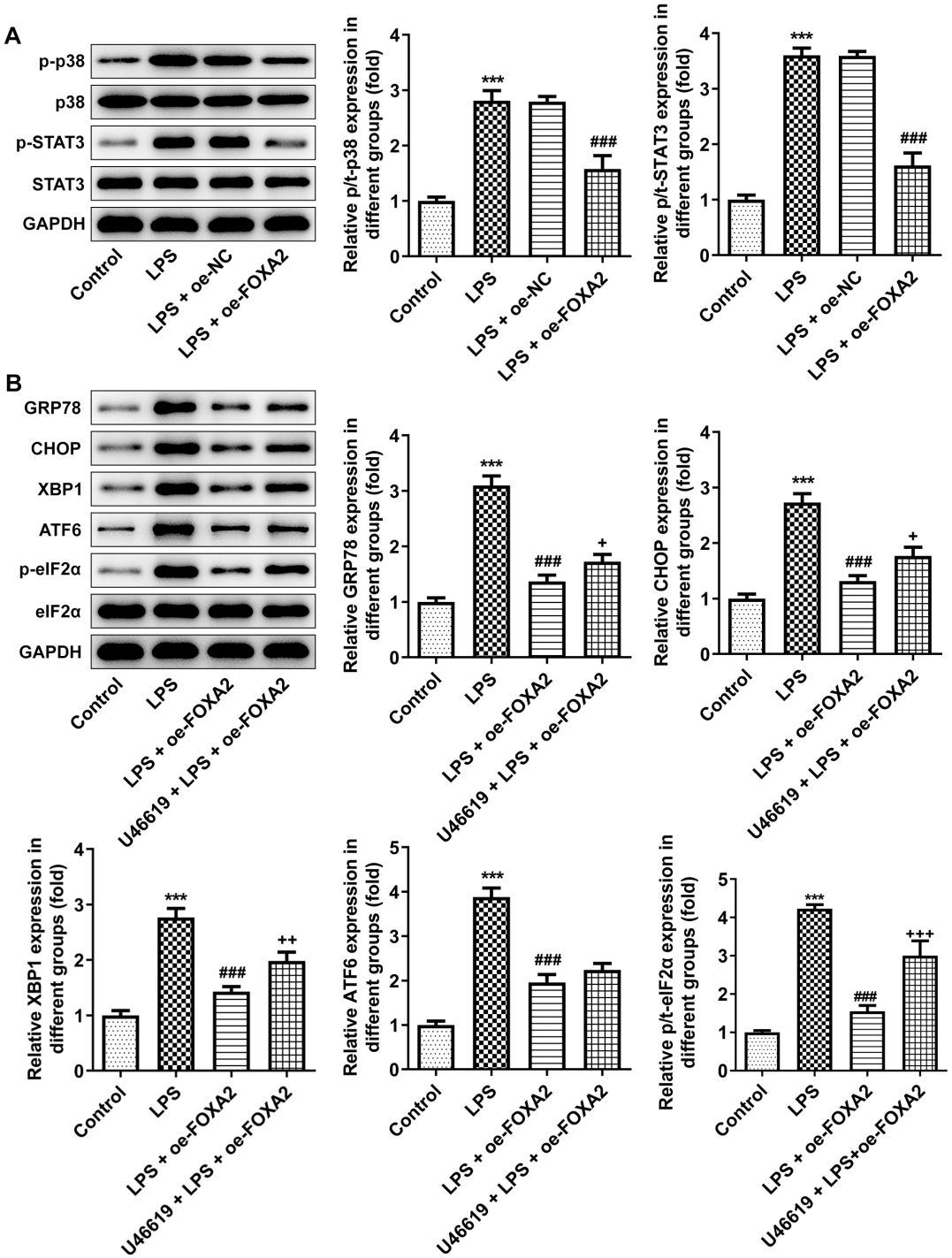

Figure 4. FOXA2 decreases ERS by blocking p38/STAT3 signaling. (A) Expression levels of p38/STAT3 signaling-related proteins were examined using western blotting. ***P<0.001 vs. Control; ###P<0.001 vs. LPS + oe-NC. (B) FOXA2-overexpressing WI-38 cells were pretreated with U46619, an activator of p38 signaling, and then induced by LPS. The expression levels of ERS-related proteins were detected using western blotting. ***P<0.001 vs. Control; ###P<0.001 vs. LPS; +P<0.05, ++P<0.01 and +++P<0.001 vs. LPS + oe-FOXA2. ATF6, activating transcription factor 6; eIF2α, eukaryotic translation initiation factor 2 subunit α; ERS, endoplasmic reticulum stress; FOXA2, forkhead box protein A2; GRP78, glucose-regulated protein 78; LPS, lipopolysaccharide; oe-FOXA2, FOXA2 overexpression vector; oe-NC, overexpression negative control; p-, phosphorylated; t-, total; XBP1, X-box binding protein 1.

that FOXA2 could serve as a promising target for the treatment of pneumonia.

Pneumonia can result in the release of pro-inflammatory cytokines, neutrophil infiltration and lung tissue destruction, and the activated neutrophils may further aggravate the inflammatory response by generating ROS, which in turn triggers oxidative stress, ultimately leading to apoptosis (33-36). Therefore, potential drugs or targets that possess anti-inflammatory, antioxidant and anti-apoptotic properties may be effective for the treatment of pneumonia. Increasing evidence has verified FOXA2 as a promising molecular target in pulmonary diseases through reducing inflammation,

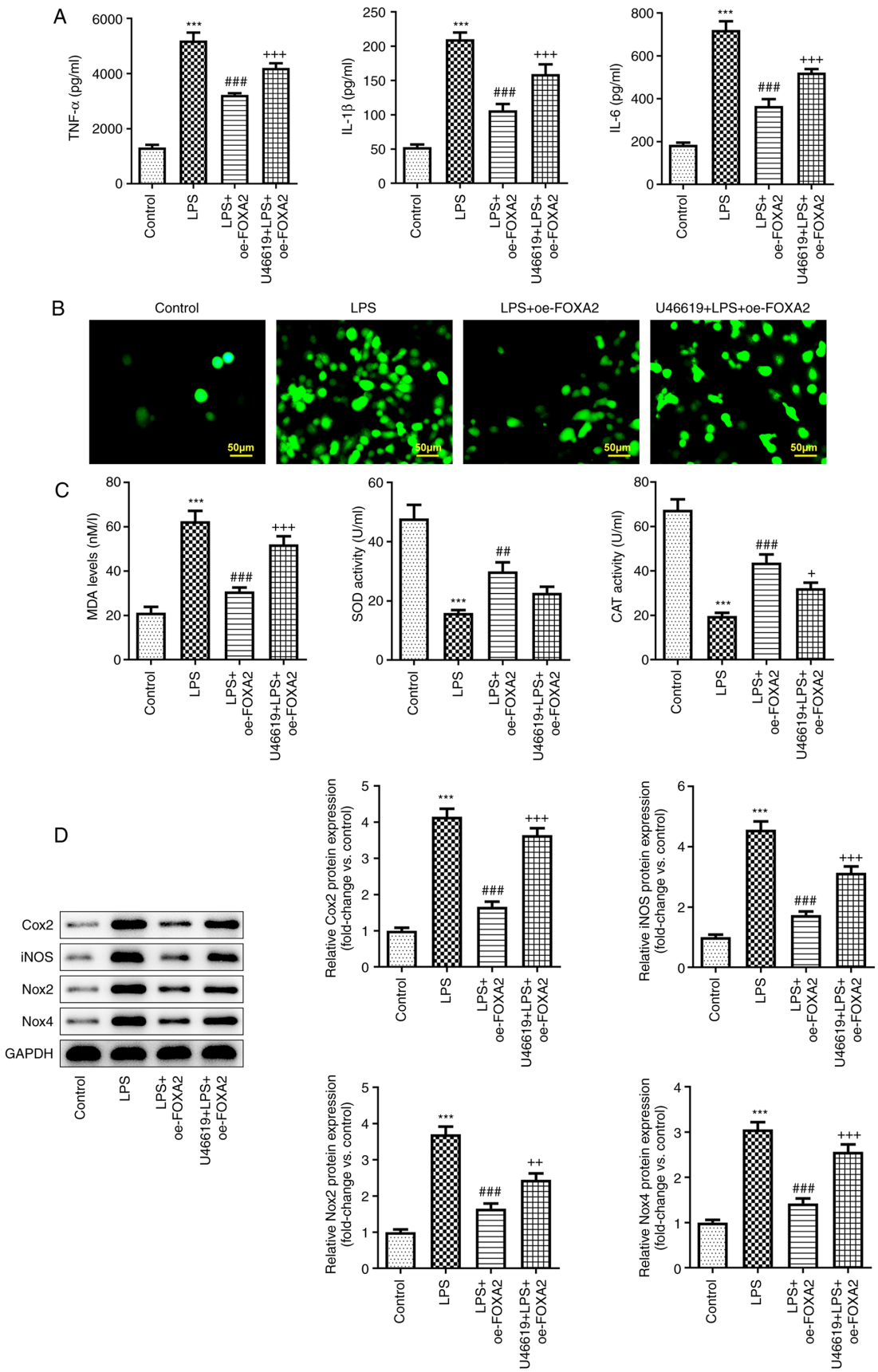

Figure 5. FOXA2 protects WI-38 cells against LPS-induced oxidative stress and inflammation via inactivation of p38/STAT3 signaling. (A) Production of pro-inflammatory cytokines, including TNF-α, IL-6 and IL-1β, was measured using ELISA. (B) Cellular reactive oxygen species levels were detected using the 2',7'-dichlorofluorescein diacetate method. Magnification, x200. Scale bar, 50 μM. (C) MDA levels, SOD activity and CAT activity were assessed using their corresponding kits. (D) Protein expression levels of Cox2, iNOS, Nox2 and Nox4 were examined using western blotting. ***P<0.001 vs. Control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS; $^{+}$P<0.05, $^{++}$P<0.01 and $^{+++}$P<0.001 vs. LPS + oe-FOXA2. CAT, catalase; Cox2, cyclooxygenase-2; FOXA2, forkhead box protein A2; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MDA, malondialdehyde; Nox, NADPH oxidase; oe-FOXA2, FOXA2 overexpression vector; SOD, superoxide dismutase.

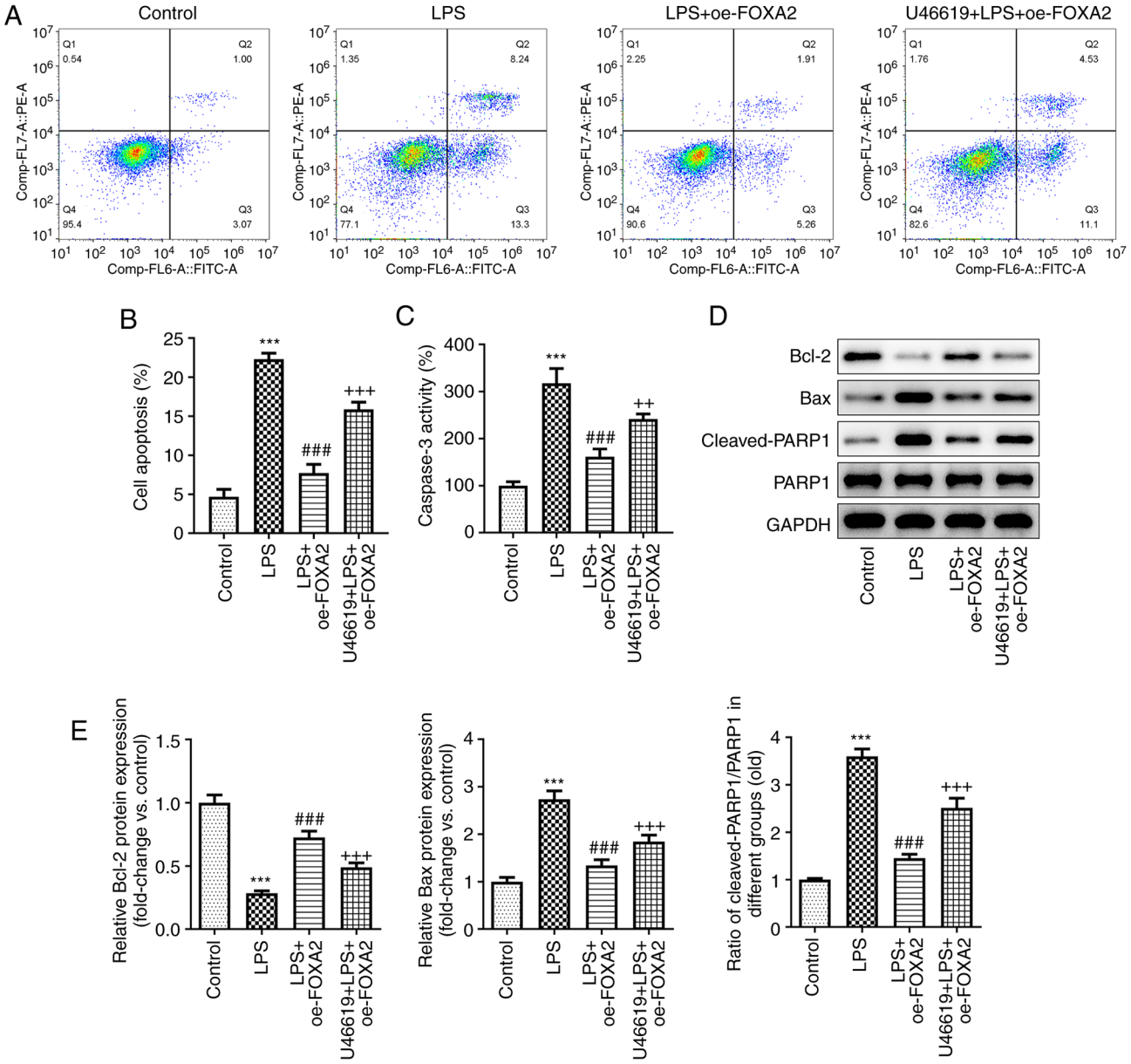

Figure 6. FOXA2 protects WI-38 cells against LPS-induced apoptosis via inactivation of p38/STAT3 signaling. (A) Flow cytometry was performed to assess the cell apoptosis rate in each group and (B) quantified. (C) Caspase3 activity was determined using the related kit. (D) Expression levels of apoptosis-related proteins were examined using western blotting and (E) quantified. ***P<0.001 vs. Control; $^{###}$P<0.001 vs. LPS; $^{++}$P<0.01 and $^{+++}$P<0.001 vs. LPS + oe-FOXA2. FOXA2, forkhead box protein A2; LPS, lipopolysaccharide; oe-FOXA2, FOXA2 overexpression vector; PARP1, poly(ADP-ribose) polymerase 1.

oxidative stress and apoptosis. For example, Yánez *et al* (37) revealed that FOXA2 deletion in T cells aggravated the T helper 2 inflammatory response in allergic airway inflammation. Increase in FOXA2 can also weaken cigarette smoke extract-induced cell inflammation to delay the progression of chronic obstructive pulmonary disease (22). Long non-coding RNA-NEF has been shown to inhibit hyperoxia-induced oxidative stress, inflammation and apoptosis in lung epithelial tissues by upregulating FOXA2 (38). Consistentlyin the present study, FOXA2 was found to be aberrantly decreased following stimulation with increasing concentrations of LPS, suggesting that FOXA2 mRNA and protein expression was decreased in pneumonia. Subsequently, FOXA2 overexpression was observed to attenuate the LPS-induced inflammatory response, oxidative stress and apoptosis in WI-38 cells, demonstrating that FOXA2 may exert anti-inflammatory, anti-oxidative stress and anti-apoptotic activities to protect against pneumonia. In addition, previous evidence has confirmed the crosstalk between ERS and LPS-induced lung inflammation (39). Furthermore, FOXA2 has been demonstrated to serve a vital role in oxidative stress and ERS, and to exert anti-apoptotic activity by regulating the cellular inhibitor of apoptosis protein 1 signaling pathway (40). In the present study, ERS was observed to be triggered by LPS in WI-38 cells, whereas FOXA2 overexpression inhibited the activation of ERS, thus indicating that FOXA2-induced suppression of inflammation, oxidative stress and apoptosis in pneumonia may be associated with inactivation of ERS.

The p38 MAPK signaling pathway is a crucial signaling pathway for the induction of the inflammatory response and

cell apoptosis (41,42). STAT3 is a member of the STAT family of transcription factors involved in cellular responses to various cytokines (43). It has been reported that STAT3 is a substrate of p38 MAPK and inhibition of p38 suppresses the activation of STAT3 (44). p38/STAT3 signaling has been recognized as a critical pathway participating in inflammation-related diseases. For instance, nilotinib has been shown to modulate LPS-induced neuroinflammatory responses by regulating p38/STAT3 signaling (45). Thymic stromal lymphopoietin may promote asthmatic airway remodeling by activating p38/STAT3 signaling (44). In agreement with the aforementioned studies, p38/STAT3 signaling was demonstrated to be activated by LPS stimulation in WI-38 cells in the present study, suggesting that p38/STAT3 signaling was activated in pneumonia. However, this effect was partly weakened by FOXA2 overexpression, suggesting that FOXA2 may exert an inhibitory effect on p38/STAT3 signaling, which was consistent with the previous evidence that FOXA2 overexpression could suppress p38 MAPK signaling to attenuate cigarette smoke-induced lung inflammation (46). In addition, to verify whether FOXA2 also functioned in LPS-induced *in vitro* model of pneumonia via inactivation of the p38/STAT3 pathway, a p38 activator U46619 was introduced in the present study. Treatment with U46619 partly weakened the inhibitory effects of FOXA2 on the LPS-triggered inflammatory response, oxidative stress and apoptosis, as well as ERS in WI-38 cells, indicating that FOXA2 may serve a protective role against pneumonia partly via regulation of the p38/STAT3 signaling pathway.

The present study had some limitations. The expression profile of FOXA2 was not validated in clinical patients with pneumonia. The regulatory role of FOXA2 in an animal model of pneumonia also needs to be verified. In addition, the present study only used a single embryonic fibroblast cell line and only addressed a small part of the potential regulatory mechanism of FOXA2, thus more cell lines should be used for in-depth research of the molecular mechanism of FOXA2 in pneumonia. All these limitations need to be addressed in future studies.

In conclusion, to the best of our knowledge, the present study was the first to reveal that FOXA2 exerted a protective effect against pneumonia by inhibiting the inflammatory response, oxidative stress and apoptosis, which may be partially achieved via modulation of the p38/STAT3 signaling pathway and ERS. These findings may provide a novel idea for the development of targeted therapeutic strategies for pneumonia.

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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

JX designed the study. ZX, YL, SX and HZ conducted the experiments to collect data. ZX, YL and SX analyzed and interpreted the data. ZX drafted the manuscript and JX revised the manuscript. ZX and JX confirm the authenticity of all the raw data. 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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