microRNA-3941 targets IGF2 to control LPS-induced acute pneumonia in A549 cells

SHINUAN FEI[1], LICHUN CAO[2] and LIANGZHI PAN[3]

[1]Department of Pediatrics, Edong Healthcare Group, Huangshi Maternity and Children's Health Hospital; Departments of [2]Pediatrics and [3]Medical Records, Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, Huangshi, Hubei 435000, P.R. China

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Abstract. The present study aimed to investigate the potential roles and regulatory mechanism of microRNA (miR)-3941 in lipopolysaccharides (LPS)-induced acute pneumonia. The expression of miR-3941 in child patients with acute pneumonia was detected and A549 cells were treated with LPS to establish the cellular model of acute pneumonia. The effects of miR-3941 in LPS-induced cell injury were investigated by assessing cell viability, apoptosis and inflammation. In addition, the regulatory relationship between miR-3941 and insulin-like growth factor 2 (IGF2) was explored, as well as the association between miR-3941 and the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) pathway. miR-3941 was significantly down-regulated in patients with acute pneumonia ($P<0.01$). In the cell model of acute pneumonia, LPS treatment significantly induced cell injury via inhibiting cell viability ($P<0.05$ or $P<0.01$), inducing cell apoptosis ($P<0.01$) and enhancing the production of cytokines [interleukin (IL)-6, IL-8 and tumor necrosis factor-α; $P<0.01$ or $P<0.001$]. LPS treatment also resulted in a significantly decreased expression of miR-3941 in A549 cells ($P<0.01$) and the overexpression of miR-3941 significantly alleviated LPS-induced cell injury ($P<0.05$). In addition, IGF2 was confirmed as a direct target gene of miR-3941. Knockdown of IGF2 significantly alleviated LPS-induced cell injury ($P<0.05$, $P<0.01$ or $P<0.001$), which was significantly reversed by suppression of miR-3941 ($P<0.05$, $P<0.01$ or $P<0.001$). Furthermore, inhibition of miR-3941 was demonstrated to activate the PI3K/AKT pathway, which was inhibited following knockdown of IGF2. The present study indicates that miR-3941 is downregulated in child patients with acute pneumonia and that downregulation of miR-3941 may promote LPS-induced cell injury in A549 cells via targeting IGF2 to regulate the activation of the PI3K/AKT pathway. Therefore, miR-3941 may be a potential therapeutic target for the treatment of acute pneumonia in child patients.

Correspondence to: Professor Lichun Cao, Department of Pediatrics, Huangshi Central Hospital, Affiliated Hospital of Hubei Polytechnic University, 293 Yiyuan Street, Huangshi, Hubei 435000, P.R. China
E-mail: lichuncao37@126.com

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Introduction

Pneumonia is a respiratory condition in which lung inflammation is caused by pathogens or other factors (1). In children aged <5 years, pneumonia is a major cause of mortality and morbidity (2), and acute pneumonia poses a serious risk to the life or health of children (3,4). It has been estimated that ~800,000 deaths caused by pneumonia occur each year among children <5 years of age (5). A better understanding of the mechanisms underlying acute pneumonia in children may facilitate the development of effective therapeutic strategy, thus reducing the burden of this disease.

microRNAs (miRNAs or miRs) are small, noncoding RNAs that have important functions in posttranscriptional regulation of gene expression (6,7). miRNAs have roles in a number of biological processes, such as cell proliferation, apoptosis, and innate immunity (8-10). miRNAs have also been identified as key regulators in the pathological processes of a number of diseases, including cancers (11), neurological diseases (12) and inflammatory disease (13,14). In the present study miR-3941 was investigated, which was recently determined to be associated with the malignant progression of lung cancer via regulating immunoglobulin (CD79a) binding protein 1 (15). miR-3941 has also been implicated in the tumorigenesis of colorectal cancer via interaction with ATP binding cassette subfamily A member 6 (16). miR-3941 is also associated with acute lymphoblastic leukemia in Chinese children via controlling insulin-like growth factor (IGF)1 (17). However, to the best of our knowledge the potential roles of miR-3941 in acute pneumonia in child patients have not yet been the subject of study.

In the present study, the expression of miR-3941 in child patients with acute pneumonia was detected. In previous studies, A549 cells treated with lipopolysaccharides (LPS) have been used as a cellular model for exploring the key mechanisms associated with respiratory diseases including acute lung injury and pneumonia (18-20). Therefore, to

investigate the potential roles of miR-3941 in regulating the development of acute pneumonia, the effects of miR-3941 in LPS-induced A549 cell injury were investigated by assessing cell viability, apoptosis and inflammation. In addition, the regulatory relationship between miR-3941 and IGF2 was explored, as well as the association between miR-3941 and the phosphatidylinositol-4,5-bisphosphate 3-kinase/protein kinase B (PI3K/AKT) pathway. It was hypothesized that down-regulation of miR-3941 may inhibit cell viability, induce cell apoptosis and enhance the production of cytokines via targeting IGF2 and activating the PI3K/AKT pathway, thus aggravating LPS-induced cell injury in A549 cells. The findings of the present study may provide new insights for the diagnosis and treatment of acute pneumonia in child patients.

Materials and methods

Subjects. Between May 2015 and April 2016, a total of 20 child patients with acute pneumonia (female:male, 8:12; age range 0.5-12 years; mean age 6.26±1.46 years) admitted to the Department of Pediatrics (Huangshi Central Hospital, Huangshi, China) were enrolled in the present study. Patients with nervous system diseases, endocrine system disease, diseases of the circulatory system, or failure of the heart, liver, kidney or other organs were excluded. In addition, a total of 20 health controls (female:male, 7:13; age range 0.4-12.3 years; mean age 6.38±1.29 years) who underwent a physical examination between April 2015 and March 2016 were also recruited. Variables including age, gender, height, weight and body mass index (BMI) between patients with acute pneumonia and healthy controls were comparable. The present study was approved by the Ethics Committee of the Huangshi Central Hospital (Hubei, China) and written informed consent was obtained from the parent(s) of each patient.

Collection of blood samples. A total of 3 ml fasting peripheral venous blood was harvested from each patient and control subject. Serum was obtained following centrifugation of blood samples at 4,000 x g at 4˚C for 10 min and stored at -80˚C.

Cell culture and treatment. The human pulmonary epithelial cell line A549 (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% glutamine (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2 for 24-48 h. Cells were treated with 1 μg/ml LPS (0.5 ng; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) for 24, 48 and 72 h. DMSO was used as the control and its concentration in the medium was kept at 0.1% to avoid toxicity.

Cell transfection. Cells ($1x10^4$ cells/well) were seeded in a 6-well plate. miR-3941 mimics (50 nM), normal control (NC) mimics (50 nM), miR-3941 inhibitors (150 nM), NC inhibitors (150 nM), IGF2 specific small interfering RNA (siRNA) (100 nM) or si-NC (100 nM) were all obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China) and then transfected into A549 cells using HiPerFect transfection reagent (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Primer sequences were as follows: miR-3941 mimics sense, 5'-UUACACACAACUGAGGAUCAUA-3' and mimic antisense 5'-UGAUCCUCAGUUGUGUGUAAUU-3'; miR-3941 inhibitors 5'-UAUGAUCCUCAGUUGUGUGUAA-3'; si-IGF2 5'-UCGUUGAGGAGUGCUGUUUdTdT3' and si-NC 5'-GGCAUAGAUGUAGCUGUAAdTdT3'. A total of 48 h following transfection, the cells were collected for further experiments.

MTT assay. Cell viability was determined via an MTT assay. Briefly, A549 cells were plated in a 96-well plate ($1x10^4$ cells/well; 37˚C) containing serum-free Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) overnight. Following treatment with LPS or transfection, cells were cultured with DMEM containing 10% FBS and 0.5 mg/ml MTT at 37˚C for a further 4 h. To dissolve the formazan crystals, DMSO was added. Cell viability was finally determined by measuring the absorbance at 550 nm with a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Flow cytometry. Cell apoptosis was assessed using flow cytometry. Briefly, A549 cells were cultured in a 96-well plate ($1x10^4$ cells/well) at 37˚C for 24-48 h in DMEM. Following treatment with LPS or transfection, cells were suspended in HEPES buffer containing annexin V-FITC and propidium iodide (PI) (all, BD Biosciences, Franklin Lakes, NJ, USA) for 15 min at room temperature. Following double staining for 1 h in the dark at 4˚C, cell apoptosis was assessed by flow cytometry using a FACScan flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Apoptotic cells were analyzed using BD CellQuest 3.0 software (BD Biosciences, Franklin Lakes, NJ, USA).

ELISA assay. To determine levels of interleukin (IL)-6, IL-8 and tumor necrosis factor (TNF)-α, an ELISA assay was conducted. ELISA kits for IL-6 (cat. no. D6050), IL8 (cat. no. D8000C) and TNF-α (cat. no. DTA00C) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Briefly, samples from A549 cells were incubated with diluted antibody solution for 1 h at room temperature, horseradish peroxidase (HRP) solution for another 1 h and 3,3',5,5'-tetramethylbenzidine solution for 15 min in the dark. Within 5 min following termination, the optical density at 450 nm was determined using a microplate reader (BioTek Instruments, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from A549 cells from different treatment groups using the microRNA Extraction and Purification kit (Shanghai Novland Co., Ltd., Shanghai, China). The relative expression levels of miR-3941 and IGF2 were detected using qPCR on an iQ5 Real-Time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a Two-Step Stemaim-it miR qRT-PCR Quantitation kit (SYBR Green; Shanghai Novland Co., Ltd.). The following thermocycling conditions were used for the PCR: Initial denaturation for 2 min at 95˚C, 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 2 min, and a final extension at 72˚C for 5 min. The following primer

sequences were used for the PCR: miR-3941 5'-TTACAC ACAACTGAGGATCATA-3'; U6 forward, 5'-GCTTCGGCA GCACATATACTAAAAT-3', reverse, 5'-CGCTTCACGAAT TTGCGTGTCAT-3'; IGF2 forward: 5'-ATGTCACCCATG TCACCAAG-3', reverse: 5'-GGCTTGTGCCAATTAGGT TCT-3'. β-actin forward, 5'-GGGCACAGTGTGGGTGAC-3', reverse 5'-CTGGCACCACACCTTCTAC-3'. U6 and β-actin were used as internal controls for quantitative normalization of miRNA and mRNA, respectively. The relative expression levels of miR-3941 and IGF2 were determined using the $2^{-\Delta\Delta Cq}$ method (21).

Bioinformatics analysis. TargetScan Human 6.2 software (http://www.targetscan.org) was used to predict biological targets of miRNAs.

Luciferase reporter assay. To determine whether IGF2 is a direct target of miR-3941, complementary oligonucleotide containing the miR-3941 target site from the 3'-untranslated region (3'-UTR) downstream of wild-type IGF2 (IGF2 3'UTR-WT) was synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). Following transfection, the DNA samples from the A549 cells were extracted. The miR-3941-predicted target sequence was then cloned in the p-MIR-REPORT luciferase plasmid (Sangon Biotech Co., Ltd., Shanghai, China). A mutated miR-3941 target sequence with identical flanking nucleotides of IGF2 (IGF2 3'UTR-MUT) Invitrogen (Thermo Fisher Scientific, Inc.) was used as the control. Cell transfection was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following 48 h of transfection, the lysate was analyzed using a Dual-Glo luciferase assay system (cat. no. E1960; Promega Corporation, Madison, WI, USA) and the luciferase activity was detected via a luminometer (Berthold Technologies GmbH & Co., KG, Wildbad, Germany). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Western blot analysis. Total protein from the A549 cells was extracted using RIPA lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.), and the concentration of protein extracts was determined using Bradford reagent (Bio-Rad Laboratories, Inc.). Following separation by (20 μg/lane) 10% SDS-PAGE, protein bands were transferred onto polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked in 1% TBS-Tween-20 (TBST) containing 5% skimmed milk for 1 h at room temperature and washed with PBS three times. Thereafter, the blots in membranes were incubated with primary antibodies against rabbit polyclone IGF2 (cat. no. sc-5622), mouse monoclonal phosphorylated (p)-PI3K (cat. no. sc-293172), mouse monoclonal PI3K (cat. no. sc-365290), rabbit polyclonal AKT (cat. no. sc-7985-R), rabbit polyclonal p-AKT (cat. no. sc-8312), mouse monoclonal B cell lymphoma 2 (Bcl-2) (cat. no. sc-7382), mouse monoclonal Bcl-2-associated X protein (Bax) (cat. no. sc-7480), mouse monoclonal pro-caspase-3 (cat. no. sc-7272), rabbit polyclone caspase-3 (cat. no. sc-7148), mouse monoclonal pro-caspase-9 (cat. no. sc-17784), mouse monoclonal caspase-9 (cat. no. sc-56073) and mouse monoclonal GAPDH (cat. no. sc-47724) at 4˚C overnight (all 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and subsequently probed with anti rabbit/mouse horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos. 5571 and 7074, respectively; both Cell Signaling Technology, Inc., Danvers, MA, USA). The protein bands were finally detected using an enhanced chemiluminescence detection kit (EMD Millipore).

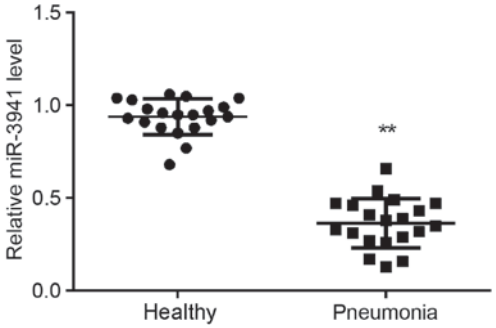

Figure 1. miR-3941 was significantly down-regulated in the serum of child patients with acute pneumonia compared with that in the serum of healthy controls. A total of 20 children patients with acute pneumonia and 20 healthy children were enrolled in the present study. Data are presented as the mean ± standard deviation (n=3). **P<0.01 vs. control. miR, microRNA.

Statistical analysis. All experiments were repeated three times and data obtained from multiple experiments were presented as the mean ± or + standard deviation. The normal distribution of data was assessed via one-sample Kologrov-Smirnov test. Statistical analysis of data was performed using one-way analysis of variance followed by a Tukey-Kramer's post hoc test or Student's t-test using SPSS 19.0 (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

miR-3941 is downregulated in the serum of child patients with acute pneumonia. A total of 20 child patients with acute pneumonia and 20 healthy controls were enrolled in the present study. As demonstrated in Fig. 1, miR-3941 expression was significantly decreased in the serum of children with acute pneumonia compared with that in the serum of controls (P<0.01).

LPS induces A549 cell injury and decreases miR-3941 levels. A549 cells were treated with 1 μg/ml LPS to establish a cellular model of acute pneumonia. During the 72 h experimental period, LPS treatment significantly inhibited cell viability at all time points (P<0.05; Fig. 2A). In addition, compared with the control group, treatment with 1 μg/ml LPS for 48 h significantly induced cell apoptosis (P<0.01; Fig. 2B) and markedly altered the expression of apoptosis-relation proteins: Bcl-2, pro-caspase-3 and pro-caspase-9 were down-regulated, whereas Bax, cleaved-caspase-3 and cleaved-caspase-9 were markedly upregulated (Fig. 2C). Furthermore, compared with the control group, LPS treatment was demonstrated to significantly increase the production of IL-6, IL-8 and TNF-α (P<0.01; Fig. 2D) in A549 cells, which indicated that the cellular model of acute pneumonia was successfully established. Therefore, the expression of miR-3941 was explored.

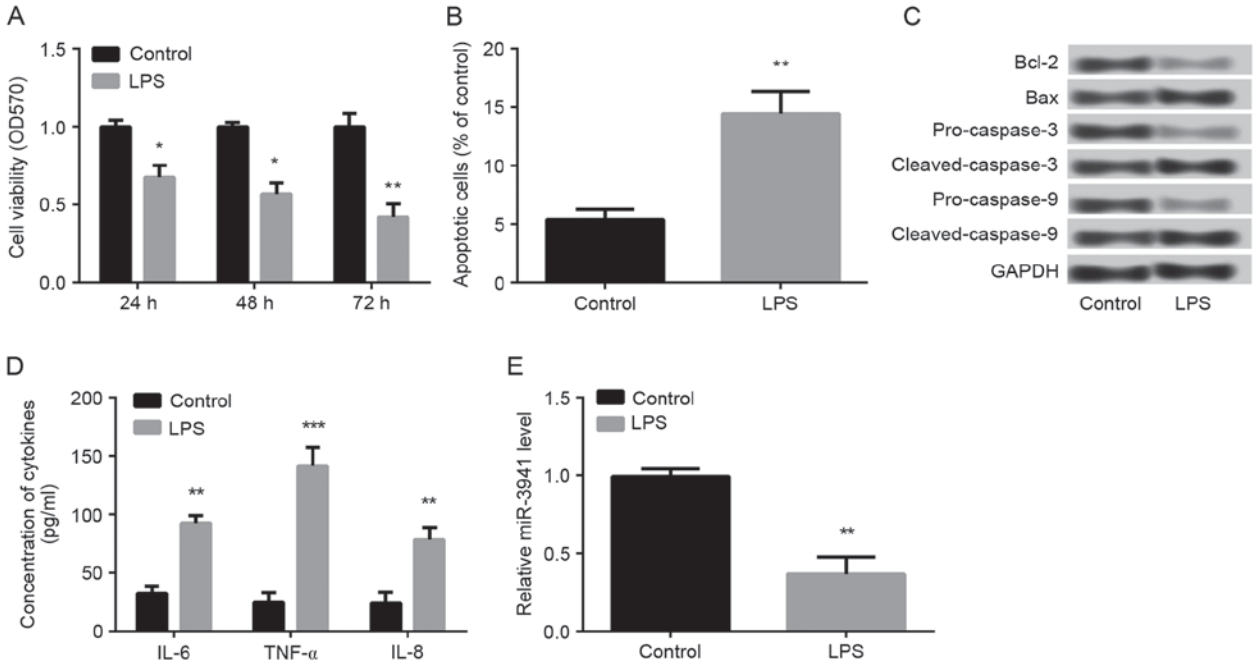

Figure 2. LPS induced A549 cell injury and decreased miR-3941 levels. Cells were treated with 1 μg/ml LPS or dimethyl sulfoxide (control). (A) MTT assay demonstrated that cell viability was significantly inhibited following treatment of LPS for 24, 48 and 72 h. (B) Flow cytometry revealed that cell apoptosis was significantly increased following treatment with 1 μg/ml LPS for 48 h. (C) Western blot analysis demonstrated the expression changes of the apoptosis-related proteins Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, pro-caspase-9 and cleaved-caspase-9 following treatment with 1 μg/ml LPS for 48 h. (D) ELISA analysis determined the enhanced production of IL-6, IL-8 and TNF-α in A549 cells following treatment with 1 μg/ml LPS for 48 h. (E) Reverse transcription-quantitative polymerase chain reaction showed that the expression of miR-3941 was significantly decreased following treatment with 1 μg/ml LPS for 48 h. Data are presented as the mean + standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control. LPS, lipopolysaccharides; miR, microRNA; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; IL, interleukin; TNF, tumor necrosis factor; OD, optical density.

The results demonstrated that LPS treatment resulted in the significantly decreased expression of miR-3941 in A549 cells (P<0.01; Fig. 2E), which suggested that miR-3941 may have a key role in the development of pneumonia.

Overexpression of miR-3941 alleviated LPS-induced cell injury. To further investigate the effects of miR-3941, miR-3941 was overexpressed in A549 cells by transfection with miR-3941 mimic. As expected, it was demonstrated that miR-3941 expression was significantly increased in cells transfected with miR-3941 mimic compared with cells transfected with mimic NC and control cells (P<0.01; Fig. 3A). Furthermore, overexpression of miR-3941 ameliorated the LPS-induced cell injury in LPS-treated cells via significantly promoting cell viability (P<0.01; Fig. 3B) and inhibiting cell apoptosis (P<0.01; Fig. 3C), markedly reversing the LPS-induced expression changes of apoptosis-related proteins (Fig. 3D), and significantly suppressing the production of IL-6, IL-8 and TNF-α (P<0.01; Fig. 3E).

IGF2 is a direct target gene of miR-3941. By means of TargetScanHuman, the predicted binding sequences of IGF2 and miR-3941 were obtained (Fig. 4A). The results of luciferase reporter analysis demonstrated that the relative luciferase activity containing the IGF2 3'UTR-WT was significantly decreased in miR-3941 mimic-transfected cells compared with controls (P<0.05; Fig. 4B). However, the relative luciferase activity containing the IGF2 3'UTR-MUT was not significantly different between miR-3941 mimic-transfected cells and control cells (Fig. 4B). Furthermore, IGF2 expression was significantly downregulated in the miR-3941 mimic group compared with mimic NC group, and upregulated in the miR-3941 inhibitor group (P<0.05; Fig. 4C). This was supported by similar, marked differences in IGF2 protein expression (Fig. 4D). LPS treatment was also demonstrated to significantly promote the mRNA expression (P<0.05; Fig. 4E) and markedly increase the protein expression (Fig. 4F) of IGF2 in A549 cells. These findings indicated that IGF2 was the direct target of miR-3941.

Effects of IGF2 suppression on cell viability, apoptosis, and expression of cytokines. The effects of IGF2 on the development of pneumonia were subsequently investigated. As demonstrated in Fig. 5A, the IGF2 protein expression was markedly decreased and mRNA expression was significantly decreased in the si-IGF2 group compared with the si-NC group (P<0.05), which indicated that IGF2 was successfully knocked down in A549 cells. In addition, compared with the LPS + si-NC + inhibitor NC group, significantly increased cell viability (P<0.05; Fig. 5B), significantly inhibited cell apoptosis (P<0.01; Fig. 5C), and significantly decreased production of IL-6, IL-8 and TNF-α (P<0.01; Fig. 5E) were observed in the LPS+si-IGF2 group, which demonstrated that knockdown of IGF2 significantly alleviated LPS-induced cell injury. In addition, suppression of miR-3941 by transfection with miR-3941 inhibitor significantly (P<0.05; Fig. 5B, C and E) and markedly (Fig. 5D) ameliorated the effects of IGF2 knockdown on LPS-induced cell injury.

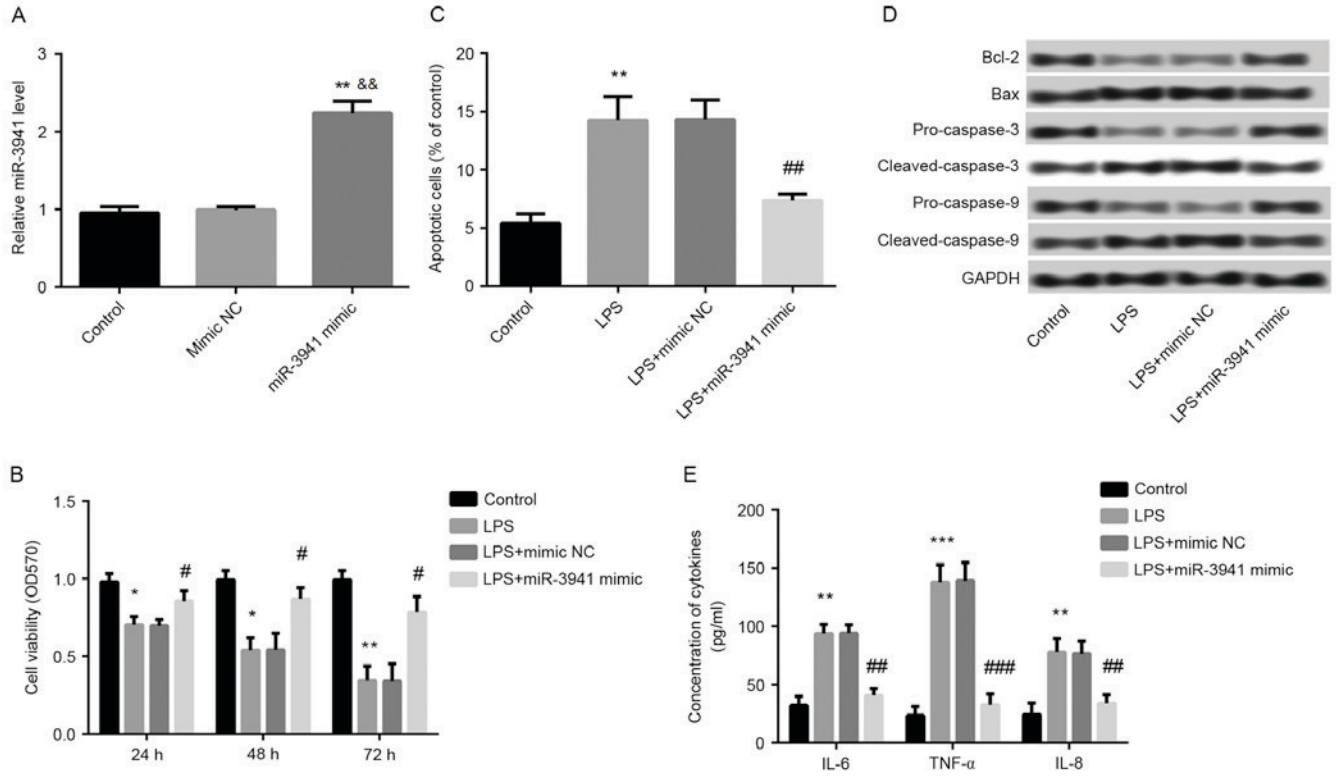

Figure 3. Overexpression of miR-3941 alleviated LPS-induced cell injury. Cells were treated with 1 μg/ml LPS or dimethyl sulfoxide (control). A part of LPS-induced cells were transfected with miR-3941 mimic or mimic NC. (A) Reverse transcription-quantitative polymerase chain reaction revealed the expression of miR-3941 in different treated groups. Compared with controls, miR-3941 expression was significantly increased in miR-3941 mimic transfected cells. (B) MTT assay showed cell viability in different treated groups. The LPS-induced decreased cell ability was significantly ameliorated by miR-3941 mimic. (C) Flow cytometry demonstrated cell apoptosis in different treated groups. The LPS-induced cell apoptosis was significantly reversed by miR-3941 mimic. (D) Western blotting revealed the expression changes of the apoptosis-related proteins Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, pro-caspase-9 and cleaved-caspase-9 in different treated groups. The LPS-induced decreased cell ability was significantly reversed by miR-3941 mimic. (E) ELISA indicated the production of cytokines (IL-6, IL-8 and TNF-α). The LPS-induced production of cytokines was reduced by miR-3941 mimic. Data are presented as the mean + standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\&\&}$P<0.01 vs mimic NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. LPS group. miR, microRNA; LPS, lipopolysaccharides; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; IL, interleukin; TNF, tumor necrosis factor; NC, normal control; OD, optical density.

Association between miR-3941 and the PI3K/AKT pathway. To further explore the regulatory mechanism of miR-3941, the association between miR-3941 and the PI3K/AKT pathway was investigated. As shown in Fig. 5F, LPS treatment led to the markedly increased phosphorylation level of p-PI3K and p-AKT compared with controls. Compared with the LPS group, the expression of p-PI3K and p-AKT were decreased in the LPS+si-NC+miR-3941 inhibitor group and increased in the LPS+si-IGF2 group. In addition, the expression of p-PI3K and p-AKT was markedly decreased in the LPS+si-IGF2+miR-3941 inhibitor group. These findings indicated that inhibition of miR-3941 may be able to activate the PI3K/AKT pathway, whereas knockdown of IGF2 may inhibit the activation of the PI3K/AKT pathway.

Discussion

miRNAs have been identified as regulators in the pathological process of a number of inflammatory diseases (22). In the present study, miR-3941 was demonstrated to be downregulated in children with acute pneumonia. In the cellular model of acute pneumonia, LPS treatment significantly induced cell injury via inhibiting cell viability, inducing cell apoptosis and enhancing the production of cytokines. LPS treatment also resulted in a significantly decreased expression of miR-3941 in A549 cells and overexpression of miR-3941 alleviated LPS-induced cell injury. In addition, IGF2 was confirmed as a direct target gene of miR-3941. Knockdown of IGF2 significantly alleviated LPS-induced cell injury, which was reversed by suppression of miR-3941. Furthermore, inhibition of miR-3941 was demonstrated to activate the PI3K/AKT pathway, whereas knockdown of IGF2 inhibited activation of the PI3K/AKT pathway, which suggested the regulatory functions of miR-3941 and IGF2 with the PI3K/AKT pathway. These findings merit further discussion.

miRNAs typically have crucial roles in several biological processes via regulating their target genes (23). In the present study, IGF2 was identified as a direct target gene of miR-3941. IGF2 is one of the most intricately regulated of all growth factors that has a crucial role in epigenetic regulation (24). In a previous study, the IGF axis consisting of two IGFs and six IGF-binding proteins was demonstrated to have important roles in children with inflammatory bowel disease (25). Furthermore, IGF2 has been shown to be associated with the development of lung adenocarcinoma (26,27). In addition, it has previously been reported that serum IGF2 levels have important prognostic values to predict the treatment outcome in children with bronchopneumonia (28,29). In the present study,

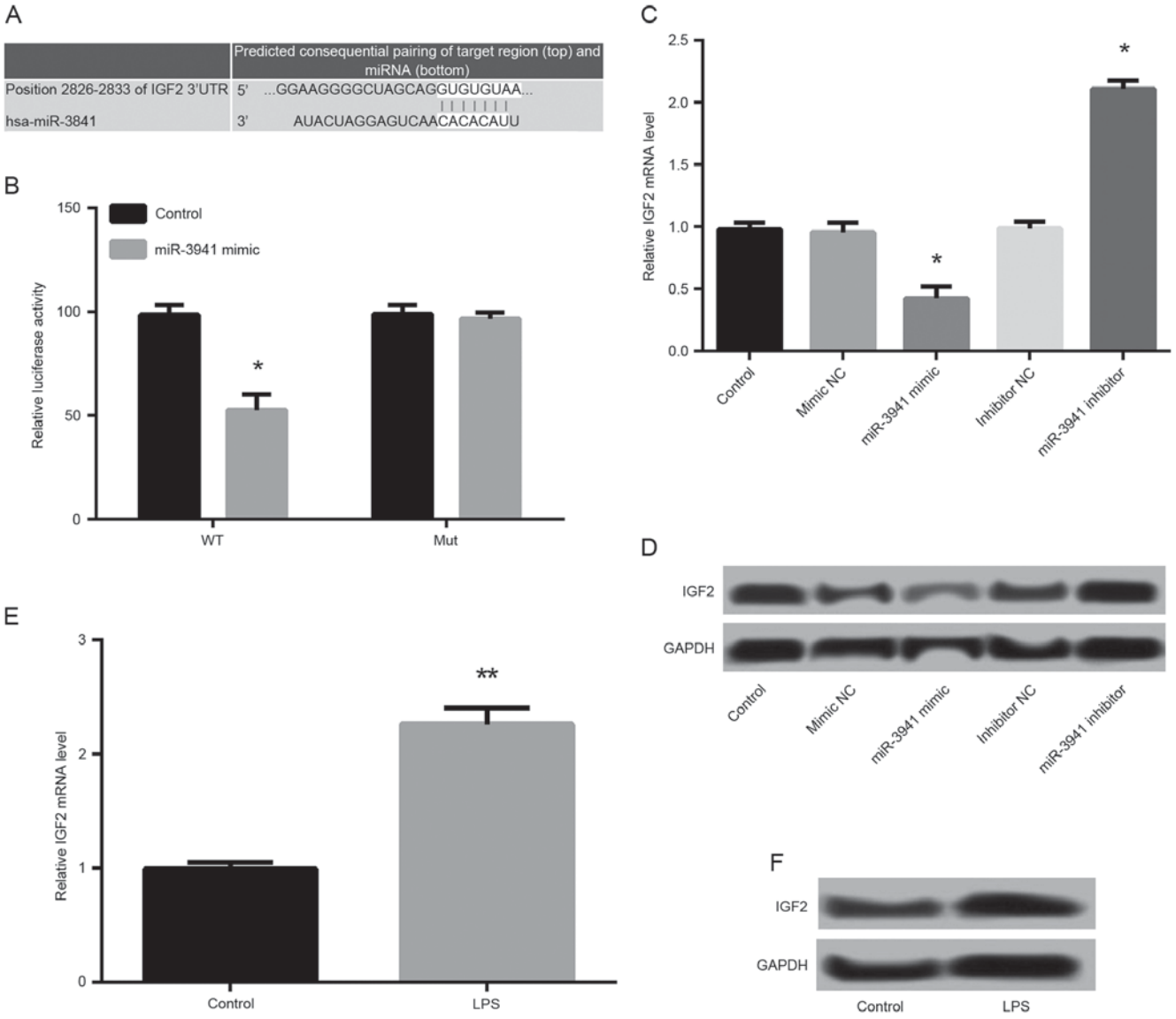

Figure 4. IGF2 was a direct target gene of miR-3941. (A) The predicted binding sequences of IGF2 and miR-3941. (B) Luciferase reporter analysis confirmed that the relative luciferase activity containing WT were significantly decreased in miR-3941 mimic-transfected cells compared with control. *P<0.05 vs. the control group. (C and D) Cells were transfected with miR-3941 mimic, mimic NC, miR-3941 inhibitor, or inhibitor NC. The mRNA and protein expression of IGF2 in different transfected groups was measured. IGF2 was downregulated in the miR-3941 mimic group compared with mimic NC group and upregulated in the miR-3941 inhibitor group. *P<0.05 vs. the mimic NC group. (E and F) The mRNA and protein expression of IGF2 were increased following LPS treatment. Data are presented as the mean + standard deviation (n=3). **P<0.01 vs. the control group. IGF2, insulin-like growth factor 2; miR, microRNA; 3'UTR, 3'-untranslated region; WT, miR-3941 target site from the 3'-UTR downstream of wild-type IGF2; NC, normal control; LPS, lipopolysaccharides; MUT, mutated miR-3941 target sequence with identical flanking nucleotides of IGF2.

knockdown of IGF2 significantly alleviated LPS-induced cell injury by increasing cell viability, inhibiting cell apoptosis, and decreasing production of cytokines, which was reversed by suppression of miR-3941. Furthermore, overexpression of miR-3941 alleviated LPS-induced cell injury. Taken together, these findings suggest that miR-3941 may serve a crucial role in LPS-induced acute pneumonia via regulating IGF2. However, IGF2 is known as a potent mitogen (30), and the possible mechanisms of IGF2 in regulating cell viability and apoptosis remain to be elucidated. Further study is required to investigate the functional regulatory mechanism of IGF2 and miR-3941.

Furthermore, the association between miR-3941 and the PI3K/AKT pathway was investigated to explore the regulatory mechanism of miR-3941. Pneumonia results from bacteria in the alveoli and type I alveolar epithelial cells are able to activate innate immune responses to defend pneumonia (31). The PI3K/AKT pathway has been demonstrated to have a role in innate immune cells (32,33). In addition, the PI3K/AKT pathway has been previously identified to be associated with epithelial-to-mesenchymal transition in human lung cancer A549 cells (34). The PI3K/AKT signaling pathway has also been confirmed to regulate the matrix metalloproteinase-2-induced VEGF-mediated angiogenesis in A549 lung cancer cells (35). In the present study, the activation of the PI3K/AKT pathway was increased following the inhibition of miR-3941, and was markedly inhibited following knockdown of IGF2, which suggests that IGF2 is able to inhibit

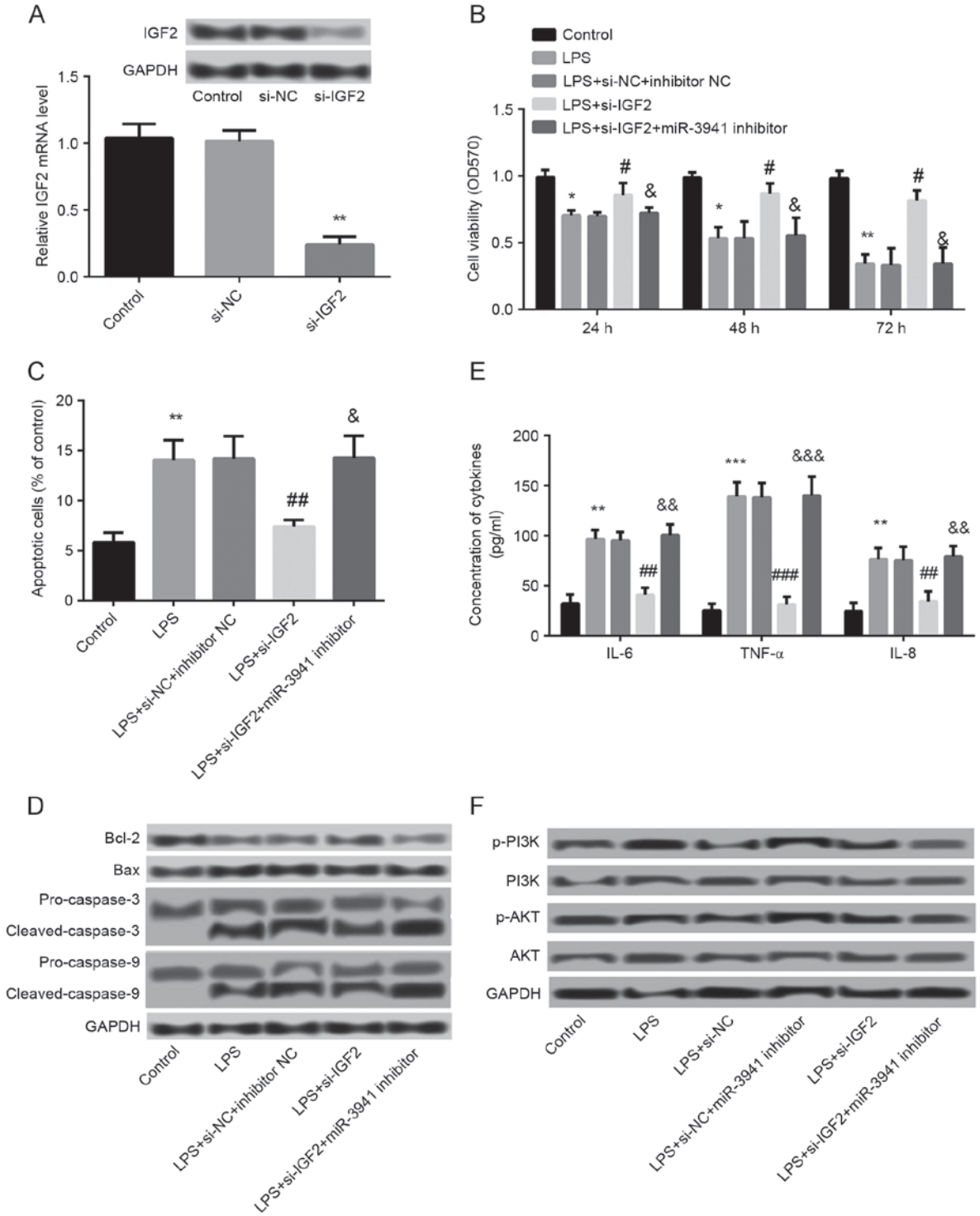

Figure 5. Effects of IGF2 suppression on cell viability, apoptosis, and expression of cytokines. (A) Cells were transfected with si-IGF2 and si-NC. The mRNA and protein expression of IGF2 was decreased in the si-IGF2 group compared with the si-NC and control groups. **P<0.01 vs. the si-NC group. (B) MTT assay showed cell viability in different groups which were treated with LPS and then transfected with si-IGF2, si-NC, miR-3941 inhibitor or inhibitor NC. (C) Flow cytometry revealed the cell apoptosis in different treated groups. (D) Western blotting demonstrated the expression changes of the apoptosis-related proteins Bcl-2, Bax, pro-caspase-3, cleaved-caspase-3, pro-caspase-9 and cleaved-caspase-9 in different treated groups. (E) ELISA indicated the production of cytokines IL-6, IL-8 and TNF-α in different treated groups. (F) Western blotting revealed the expression of PI3K, p-PI3K, AKT and p-AKT in different treated groups. Data are presented as the mean + standard deviation (n=3). *P<0.05, **P<0.01, ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. LPS + si-NC + inhibitor NC group; $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. LPS+si-IGF2 group. IGF2, insulin-like growth factor 2; siRNA, small interfering RNA; NC, normal control; LPS, lipopolysaccharides; miR, microRNA; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; IL, interleukin; TNF, tumor necrosis factor; NC, normal control; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; p, phosphorylated; AKT, protein kinase B; OD, optical density, si-NC, small interfering-normal control.

the miR-3941-induced activation of the PI3K/AKT pathway. Therefore, it was speculated that miR-3941 may target IGF2 to regulate the activation of the PI3K/AKT pathway to control the development of LPS-induced acute pneumonia.

In conclusion, the present study indicates that miR-3941 is downregulated in child patients with acute pneumonia and that the downregulation of miR-3941 may promote LPS-induced cell injury in A549 cells via targeting IGF2 to regulate the activation of the PI3K/AKT pathway. These findings provide a potential therapeutic strategy and target for the treatment of acute pneumonia in children.

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