Silence of MEG3 intensifies lipopolysaccharide-stimulated damage of human lung cells through modulating miR-4262

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ABSTRACT
Acute lung injury (ALI) is a serious threat for health and lives worldwide. Recently, IncRNA MEG3 has been well studied to participate in lung cancer, making us speculate that it may be essential for ALI. ALI was simulated by treatment of LPS in human lung fibroblast WI-38 cells and human PMVECs. Cell viability, migration, apoptosis and MEG3 level were assessed by flow cytometry/ Western blot and qRT-PCR assays, respectively. Utilizing bioinformatics methods, luciferase activity assay and assessments of cell viability, migration and apoptosis, the possible interacted microRNA were explored. We found that, LPS induced decreases of cell viability, migration and apoptosis, the possible interacted miRNA were down-regulation of miR-4262; KLF4

Introduction
Acute lung injury (ALI) is a clinical severe respiratory disorder and often leads to acute respiratory distress syndrome, resulting in remarkable annual morbidity, mortality and healthcare expenditures [1,2]. ALI is a multifactorial illness, which may be induced by diverse aetiologies, such as direct injury to the lung including pneumonia, toxic inhalation, near-drowning and aspiration, and indirect causes including burns, massive blood transfusion, sepsis, etc. [3]. Even though extensive literatures have focused on the relevant pathogenesis of ALI, the treatment of ALI remains to be a task with tremendous hardship [4]. Thus, innovative therapeutic strategies are of greatly unmet needs.

Lipopolysaccharide (LPS) is an endotoxin expressed on the outer membrane of Gram-negative bacteria [5]. Currently, LPS is accepted to induce release of pro-inflammatory cytokines, resulting in acute tissue injury [3,6]. Intratracheal administration or intranasal instillation of LPS has been widely used for the construction of animal model with ALI [7,8]. LPS-induced lung injury in the mice is a robust experimental model for the studies of ALI [9]. A previous study has revealed the possible roles of hedgehog signaling pathway in the LPS-induced cell injury of pulmonary microvascular endothelial cells (PMVECs) [10]. Thus, cell model with LPS-induced cell injury is of great importance for the deep mechanism research of ALI.

Long non-coding RNAs (LncRNAs) are a loosely classified group of long RNA transcripts (longer than 200 nucleotides) which were involved in diverse biological processes, including proliferation, migration and apoptosis [11,12]. With the developments of epigenomic technologies and bioinformatics, the number of novel LncRNAs is climbing [13]. Currently, several LncRNAs are reported to be involved in lung diseases, such as NANCI [14] and GAS5 [15]. LncRNA maternally expressed gene 3 (MEG3), transcribed from chromosome 14q32, is aberrantly expressed in multiple cancers [16] and its down-regulation has been reported to augment cisplatin resistance of lung cancer [17]. However, very little is known about the functional roles of MEG3 in lung injury.

Migration and proliferation of lung fibroblasts were observed at the early stage of ALI [18]. Additionally, lung fibroblasts are described as an essential player of lung repair during ALI [19]. Herein, in our study, human lung normal fibroblast WI-38 cells were treated with LPS to simulate ALI. Then, the expression and the roles of MEG3 in the LPS-treated WI-38 cells were explored. In the meantime, the effects of MEG3 on LPS-treated human PMVECs were also studied to support the results from WI-38 cells. Moreover, the molecular mechanism and possibly involved signaling pathways in the MEG3-associated modulation were investigated.

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Materials and methods

Cell culture and treatment

WI-38 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle medium (DMEM; GibCO, Grand Island, NY) containing 10% foetal bovine serum (FBS; Invitrogen, Carlsbad, CA), antibiotic/antimycotic solution (100 units) and gentamycin (50 μg/mL) with Na pyruvate (1 mM). Human PMVECs (Biofavor Biotech Service Co., Ltd., Wuhan, China) were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS, 1% penicillin/streptomycin and 0.5% fungizone (Invitrogen, Carlsbad, CA). Cells were grown at 37 °C in a humidified atmosphere (5% CO₂ in air). To construct cell model with cell injury, cells were stimulated with E. coli-derived LPS (10 μg/mL; Sigma-Aldrich, St. Louis, MO) for 5 h [20].

Cell counting kit-8 (CCK-8) assay

Cell viability was measured by a CCK-8 assay. Briefly, cells were seeded into a 96-well plate at a density of 5 × 10³ cells/well. After treatments, 10 μL CCK-8 solution (Dojindo Molecular Technologies, Gaithersburg, MD) was added to the culture medium of each well, followed by 1 h of incubation at 37 °C in a humidified incubator containing 5% CO₂ in air. Then, a Microplate Reader (Bio-Rad, Hercules, CA) was used for measurement of absorbance at 450 nm. Cell viability was presented as the absorbance in relation to the control group (set as 100%).

Migration assay

Cell migration was determined using Transwell chambers with 8-μm sized pores (BD Biosciences, Bedford, MA). Briefly, treated-cells were resuspended in FBS-free medium and seeded onto the upper compartments with a volume of 200 μL. Then, 600 μL complete medium was added into the lower compartments. Following 24 h of incubation at 37 °C, cells were fixed by methanol. Afterwards, the non-traversed cells staying on the upper surface of the filter were removed carefully with a cotton swab, whereas the cells that traversed to the lower side of the filter were stained with crystal violet and were counted under an inverted microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Apoptosis assay

Cell apoptosis was estimated by propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated Annexin V staining. In brief, treated-cells were washed by phosphate-buffered saline (PBS), fixed in 70% ethanol and washed again by PBS. Then, cells were collected and resuspended in binding buffer from an Annexin V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). Cells were subsequently stained by Annexin V-FITC and PI following the information of the manufacture. The apoptotic cells were differentiated by using a flow cytometer (Beckman Coulter, Fullerton, CA). Percentage of apoptotic cells was analysed by FlowJo software (Tree Star, San Carlos, CA).

Quantitative reverse transcription PCR (qRT-PCR)

For the measurement of MEG3 and mRNA, total RNA was extracted from treated-cells using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s recommendation. Then, quantitation of MEG3 was performed with One Step SYBR® PrimeScript™ PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Dalian, China). Quantitation of mRNAs was performed with MultiScribe™ Reverse Transcriptase kit (Applied Biosystems, Foster City, CA) and SYBR™ Green PCR Master Mix (Applied Biosystems, Foster City, CA) for reverse transcription and real-time PCR, respectively, according to the protocols provided by the suppliers. For the measurement of miR-4262, small RNA was isolated from treated-cells using RNAiso for Small RNA (TaKaRa Biotechnology, Dalian, China) following the protocol of supplier. Then, the Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II (both from Applied Biosystems, Foster City, CA) were used for reverse transcription and real-time PCR, respectively, on the basis of the information of supplier. Primers were all designed and synthesized by Sangon Co., Ltd. (Shanghai, China). Relative expression was calculated according to the 2⁻ΔΔCt method described previously [21], normalizing to U6 (for miR-4262) or GAPDH (for MEG3 and mRNAs).

Generation of stably transfected cells and miRNA transfection

Short-hairpin RNA directed against human MEG3 or Krüppel-like factor (KLF) 4 was sub-cloned into the pGPU6/Neo plasmid (GenePharma, Shanghai, China) to generate sh-MEG3 or sh-KLF4. The plasmid carrying a non-targeting sequence was used as a negative control (NC) of sh-MEG3 or sh-KLF4, termed sh-NC. For overexpression of KLF4, the full-length KLF4 sequences were ligated into pEX-2 plasmid (GenePharma, Shanghai, China) to generate pEX-KLF4, control of which was the empty pEX-2 plasmid, termed pEX. The Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) was utilized for cell transfection in line with the manufacturer’s instructions. G418 (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO) was added to the culture medium for the selection of stably transfected cells. G418-resistant cell clones were established after approximately 4 weeks. MiR-4262 mimic, scramble miRNAs, miR-4262 inhibitor and its NC were purchased from Life Technologies Corporation (Gaithersburg, MD). Cell transfection with miRNAs was also performed with Lipofectamine 3000 reagent, and cells were harvested at 72 h post-transfection in the subsequent experiments.

Construction of reporter vectors and luciferase reporter assay

The fragment from human MEG3 containing the putative miR-4262 binding site was sub-cloned into a pmirGLO
plasmid (Promega, Madison, WI) to form the reporter vector MEG3-wild-type (MEG3-Wt). To mutate the putative binding site of miR-4262 in the MEG3, the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was utilized following the manufacturer’s suggestions to generate MEG3-mutated-type (MEG3-Mt). After sequencing, 293T cells were co-transfected with miR-4262 mimics (scramble miRNAs) and MEG3-Wt (MEG3-Mt), followed by the determination of luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

Western blot analysis

The proteins of treated-cells were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Applygen Technologies Inc., Beijing, China). After quantification with the BCA™ Protein Assay Kit (Pierce, Appleton, WI), equivalent proteins were separated by SDS-PAGE. Then, proteins in the gels were blotted to polyvinylidene difluoride (PVDF) membranes, followed by blockage with 5% bovine serum albumin (BSA). PVDF membranes were then incubated at 4°C overnight with primary antibodies against B cell lymphoma-2 (Bcl-2, ab32124), Bcl-2-associated X protein (Bax, ab182733), procaspase-3 (ab90437), cleaved caspase-3 (ab2302), procaspase-9 (ab32539), cleaved caspase-9 (ab2324), KLF4 (ab72543), phosphatidylinositol-3-kinase (PI3K, ab86714), phospho-PI3K (p-PI3K, ab182651), Janus-activated kinase-1 (JAK1, ab133666), phospho-JAK1 (p-JAK1, ab138005), signal transducer and activator of transcription-1 (STAT1, ab3987), phospho-STAT1 (p-STAT1, ab109461), STAT3 (ab68153), phospho-STAT3 (p-STAT3, ab76315), GAPDH (ab128915) (all from Abcam, Cambridge, UK), phospho-AKT (p-AKT, #4060) and AKT (#4685) (both from Cell Signaling Technology, Beverly, MA). After rinsing and incubation with secondary antibodies marked by horseradish peroxidase for 1 h at room temperature, membranes were transferred into the Bio-Rad ChemiDoc™ XRS system for the visualization of the bands. Antibodies against B cell lymphoma-2 (Bcl-2, ab32124), Bcl-2-associated X protein (Bax, ab182733), procaspase-3 (ab90437), cleaved caspase-3 (ab2302), procaspase-9 (ab32539), cleaved caspase-9 (ab2324), KLF4 (ab72543), phosphatidylinositol-3-kinase (PI3K, ab86714), phospho-PI3K (p-PI3K, ab182651), Janus-activated kinase-1 (JAK1, ab133666), phospho-JAK1 (p-JAK1, ab138005), signal transducer and activator of transcription-1 (STAT1, ab3987), phospho-STAT1 (p-STAT1, ab109461), STAT3 (ab68153), phospho-STAT3 (p-STAT3, ab76315), GAPDH (ab128915) (all from Abcam, Cambridge, UK), phospho-AKT (p-AKT, #4060) and AKT (#4685) (both from Cell Signaling Technology, Beverly, MA). After rinsing and incubation with secondary antibodies marked by horseradish peroxidase for 1 h at room temperature, membranes were transferred into the Bio-Rad ChemiDoc™ XRS system for the visualization of the bands.

Statistical analysis

All experiments were repeated three times. The results were presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA). The p values were calculated using the one-way analysis of variance (ANOVA). A p < .05 was considered as a significant difference.

Results

LPS induced WI-38 cell injury

LPS-induced cell injury was evaluated by the alterations of cell viability, migration, apoptosis as well as apoptosis-associated proteins. Compared with control group, LPS significantly reduced cell viability (p < .05, Figure 1(A)), migration (p < .01, Figure 1(B)) and markedly enhanced the percentage of apoptotic cells (p < .001, Figure 1(C)). Figure 1(D) shows protein expression of Bcl-2 was markedly down-regulated (p < .01) but expression of Bax, cleaved/pro caspase-3 and cleaved/pro caspase-9 was remarkably up-regulated (p < .01 or p < .001) after stimulation of LPS. All the results demonstrated that cell model with cell injury was successfully constructed after stimulation of LPS for 5 h.

MEG3 was down-regulated by LPS in WI-38 cells and PMVECs

The expression of MEG3 was assessed by qRT-PCR after treatment of LPS. Compared with the control group, MEG3 was significantly down-regulated by LPS in both WI-38 cells (p < .01, Figure 2(A)) and PMVECs (p < .01, Figure 2(B)), indicating the possible involvements of MEG3 in the LPS-induced injury of lung cells.

Knockdown of MEG3 aggravated LPS-induced injury of lung cells

To explore the effects of MEG3 on LPS-induced cell injury, MEG3 was significantly down-regulated in WI-38 cells and PMVECs by transfection with sh-MEG3 compared to the sh-NC group (both p < .001, Figure 3(A)). When compared to the LPS + sh-NC group, LPS-induced decreases of cell viability and migration were markedly decreased by MEG3 knockdown (all p < .05, Figure 3(B,C)), whereas LPS-induced increase of apoptotic cells was significantly increased by MEG3 knockdown (both p < .05, Figure 3(D)) in WI-38 cells and PMVECs. Meanwhile, LPS-induced alterations of Bax, Bcl-2, cleaved/pro caspase-3 and cleaved/pro caspase-9 were further augmented by MEG3 knockdown compared with the LPS + sh-NC group (p < .05 or p < .01, Figure 3(E)). Results above suggested that LPS-induced injury of lung cells could be aggravated by MEG3 knockdown.

MiR-4262 was negatively correlated with MEG3 expression

Owing to the development of bioinformatics, MEG3 was predicted to bind to miR-4262. In Figure 4(A), MEG3 knockdown dramatically up-regulated level of miR-4262 compared with the sh-NC group (p < .01), indicating that miR-4262 was negatively regulated by MEG3. In addition, the luciferase activity was markedly reduced by co-transfection with MEG3-Wt and miR-4262 mimic compared with co-transfection with MEG3-Wt and scramble miRNAs (p < .05), whereas the difference of luciferase between co-transfections with MEG3-Mt was non-significant, suggesting the direct binding of MEG3 to miR-4262 (Figure 4(B)). Thus, we concluded that miR-4262 was negatively correlated with MEG3 expression.
Knockdown of MEG3 exacerbated LPS-induced WI-38 cell injury through up-regulating miR-4262

To explore the effects of miR-4262 on LPS-induced cell injury, WI-38 cells were transfected with miR-4262 mimic, miR-4262 inhibitor or their respective controls. In Figure 5(A), miR-4262 was significantly up-regulated by transfection with miR-4262 mimic compared with the scramble group (*p < .001) but was markedly down-regulated by transfection with miR-4262 inhibitor compared with the NC group (**p < .01). Subsequent experiments showed MEG3 knockdown-induced alterations of cell viability, migration and apoptosis in LPS-treated WI-38 cells were observably reversed by miR-4262 inhibition compared with the LPS + sh-MEG3 + NC group (**p < .01, cell viability in Figure 5(B); ***p < .001, migration in Figure 5(C); p < .001, apoptosis in Figure 5(D,E)). Collectively, MEG3 knockdown might affect LPS-induced WI-38 cell injury through up-regulation of miR-4262.

Figure 1. LPS induced WI-38 cell injury. (A) Cell viability by CCK-8 assay. (B) Cell migration by Transwell assay. (C) Cell apoptosis by flow cytometry. (D) Expression of apoptosis-associated proteins by Western blot analysis. Data presented are the mean ± SEM of at least three independent experiments. *p < .05; **p < .01; ***p < .001. C/P-: cleaved/pro; MW: molecular weight.

Figure 2. Long non-coding RNA MEG3 was down-regulated by LPS treatment. Expression of MEG3 in WI-38 cells (A) and human PMVECs (B) was assessed by qRT-PCR. Data presented are the mean ± SEM of at least three independent experiments. **p < .01.
KLF4 was negatively modulated by miR-4262 expression

Utilizing bioinformatics methods, the possible interacted genes of miR-4262 were screened. Among those genes, KLF4 was selected to be fully investigated. In Figure 6(A,B), mRNA and protein expression of KLF4 was down-regulated by miR-4262 overexpression ($p < .05$ or $p < .01$) while were up-regulated by miR-4262 inhibition (both $p < .01$), indicating the negative correlation between miR-4262 and KLF4.

Aberrantly expressed KLF4 affected LPS-induced WI-38 cell injury

Next, different plasmids were respectively transfected into WI-38 cells and the stable transfected cells were selected. In Figure 7(A), mRNA and protein levels of KLF4 were markedly increased by pEX-KLF4 (both $p < .01$) but were remarkably decreased by sh-KLF4 (both $p < .01$). Following experiments showed LPS-induced alterations of cell viability (Figure 7(B)), migration (Figure 7(C)) and apoptosis.

Figure 3. Knockdown of long non-coding RNA MEG3 aggravated LPS-induced injury of lung cells. (A) Expression of MEG3 by qRT-PCR. (B) Cell viability by CCK-8 assay. (C) Cell migration by Transwell assay. (D) Cell apoptosis by flow cytometry. (E) Expression of apoptosis-associated proteins by Western blot analysis. Data presented are the mean ± SEM of at least three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$. P: pro; C: cleaved; C/P: cleaved/pro; MW: molecular weight.
Figure 4. MiR-4262 was negatively correlated with long non-coding RNA MEG3 expression. (A) Expression of MEG3 by qRT-PCR. (B) Luciferase activity by luciferase reporter assay. Data presented are the mean ± SEM of at least three independent experiments. *p < .05; **p < .01.

Figure 5. Effects of long non-coding RNA MEG3 silence on LPS-induced WI-38 cell injury were alleviated by miR-4262 inhibition. (A) Expression of miR-4262 by qRT-PCR. (B) Cell viability by CCK-8 assay. (C) Cell migration by Transwell assay. (D) Cell apoptosis by flow cytometry. (E) Expression of apoptosis-associated proteins by Western blot analysis. Data presented are the mean ± SEM of at least three independent experiments. *p < .05; **p < .01; ***p < .001. P-: pro; C-: cleaved; C/P: cleaved/pro; MW: molecular weight; NC: negative control of miR-4262 inhibitor.
Figure 7(D,E) were significantly exacerbated by KLF4 silence (p < .05 or p < .01) while were markedly ameliorated by KLF4 overexpression (p < .05, p < .01 or p < .001). Results suggested that aberrantly expressed KLF4 affected LPS-induced WI-38 cell injury.

**KLF4 alleviated the LPS-induced inhibition of the PI3K/AKT and JAK/STAT pathways**

To reveal the underlying mechanisms of MEG3-associated regulations, the phosphorylated levels of key kinases in the PI3K/AKT and JAK/STAT pathways were assessed by Western blot analysis. Results in Figure 8(A,B) showed phosphorylated levels of PI3K, AKT, JAK1, STAT1 and STAT3 were all reduced by LPS (p < .05 or p < .01). Moreover, the LPS-induced down-regulations of phosphorylated kinases were further down-regulated by KLF4 silence (all p < .05) but were reversed by KLF4 overexpression (p < .05 or p < .01). Collectively, the LPS-induced inhibition of the PI3K/AKT and JAK/STAT pathways could be reversed by KLF4.

**Discussion**

With the high incidence and mortality, ALI is a critical illness that threatens health and lives worldwide [22]. In our study, MEG3 was identified to be down-regulated in LPS-treated WI-38 cells and human PMVECs. LPS-induced cell injury, presenting as the decreases of cell viability and migration and the increase of cell apoptosis, was aggravated by MEG3 knockdown by up-regulating miR-4262. KLF4, negatively regulated by miR-4262, could ameliorate LPS-induced WI-38 cell injury through activating the PI3K/AKT and JAK/STAT signaling pathways.

During ALI, large amount of neutrophils, inflammatory mediators, proteases and oxidants were accumulated, resulting in vascular endothelia and alveolar epithelial cell damage or death. Then, the blood-alveolar barrier is disrupted, leading to intrapulmonary pulmonary oedema and impaired gas exchange [23]. In our study, the LPS-induced cell injury was evaluated according to alterations of cell viability, migration and apoptosis. After stimulation with LPS, the cell viability and migration were reduced while cell apoptosis was elevated. Additionally, the pro-apoptotic Bax was up-regulated but anti-apoptotic Bcl-2 was down-regulated after LPS treatments, elucidating that LPS could induce cell apoptosis through the mitochondrial-related pathway. In the meantime, the active caspase-9 and active caspase-3 were both increased by LPS treatments, which implied that LPS activated the caspase pathway. These two activated pathways induced by LPS might provide an explanation for the changed cell viability and apoptosis.

MEG3 has been widely explored in diverse cancer types including lung cancer [24], thus we hypothesized that MEG3 might be of importance in ALI. First of all, the down-regulation of MEG3 in LPS-treated WI-38 cells and PMVECs powerfully suggested the possible association of MEG3 in LPS-induced lung cell injury. Then, MEG3 was stably knocked down, followed by assessments of cell viability, migration and apoptosis. Results presented that LPS-induced injury of WI-38 and PMVECs was aggravated by MEG3 knockdown. The inhibitory effect on cell proliferation as well as inducible effect on cell apoptosis of MEG3 knockdown in LPS-treated WI-38 cells differs from that in lung cancer reported in a previous literature [25]. However, MEG3 knockdown has been stated to inhibit epithelial–mesenchymal transition in lung cancer [26], which was consistent with the results in our study. The similar but not identical effects of MEG3 might be associated with the alterations from normal to cancer cells.

A previous study once stated that MEG3 repressed proliferation of hepatocellular carcinoma cells via negative modulation of miR-664 [27]. To explore the molecular mechanism of the MEG3-associated modulations in LPS-treated lung cells, bioinformatics software was applied to analyse the possible relevant miRNAs of MEG3. In our study, miR-4262 was negatively correlated with MEG3 expression, and the subsequent luciferase assay also proved the direct binding between MEG3 and miR-4262. In addition, the effects of MEG3 knockdown on LPS-induced WI-38 cell injury were ameliorated by miR-4262 inhibition, illustrating that MEG3 knockdown functioned through up-regulating miR-4262.
MiRs are small non-coding RNAs that participate in physiology and disease through down-regulating target mRNAs [28]. The roles of miR-4262 in cell proliferation, migration and apoptosis are controversial due to the different target mRNAs [29,30]. Thus, we next screened the possible interacted genes of miR-4262 via bioinformatics methods. KLF4 is a conserved transcriptional factor that participates in multiple cellular processes, such as cell proliferation, migration and apoptosis [31]. KLF4 acts as either a tumor suppressor or an oncogene in diverse cancers, and the suppressive role of KLF4 in lung cancer has been proven [32]. In another literature, ALI could be ameliorated by miR-7 deficiency through up-regulating KLF4 [33]. Hence, in our study, we focused on the interaction between miR-4262 and KLF4. Results implied KLF4 was negatively correlated with miR-4262 level and KLF4 overexpression could alleviate LPS-induced WI-38 cell injury. The protective role of KLF4 overexpression in LPS-treated WI-38 cells was consistent with the literature described above [33]. On the basis of the above results, it can be suggested that MEG3 knockdown might aggravate LPS-induced WI-38 cell injury through miR-4262-mediated down-regulation of KLF4.

Activation of the PI3K/AKT pathway has been proven to protect A549 cells from LPS-induced cell apoptosis [34]. Insulin has been proven to alleviate LPS-induced ALI through activation of the PI3K/AKT pathway [35]. Accumulating evidence has proven the JAK/STAT signaling pathway plays crucial roles in ALI [36,37]. In our study, these two signaling pathways were both inhibited by LPS, and were further inhibited by KLF4 knockdown. However, KLF4 overexpression could ameliorate the LPS-induced inhibition, indicating the possible involvements of the PI3K/AKT and JAK/STAT signaling pathways in the LPS-induced WI-38 cell injury. More details about the interactions between KLF4 and these two pathways should be investigated in the future.

To summarize, MEG3 was down-regulated by LPS and its down-regulation could exacerbate LPS-induced injury of lung cells through miR-4262-mediated silence of KLF4, involving the PI3K/AKT and JAK/STAT signaling pathways. This study

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**Figure 7.** LPS-induced WI-38 cell injury was ameliorated by KLF4 overexpression but was aggravated by KLF4 knockdown. (A) Expression of KLF4 by qRT-PCR and Western blot analysis. (B) Cell viability by CCK-8 assay. (C) Cell migration by Transwell assay. (D) Cell apoptosis by flow cytometry. (E) Expression of apoptosis-associated proteins by Western blot analysis. Data presented are the mean ± SEM of at least three independent experiments. *p < .05; **p < .01; ***p < .001. P-: pro; C-: cleaved; C/P: cleaved/pro; MW: molecular weight.
suggested that MEG3 might be a potential volunteer for the treatment of ALI. However, this application needs more validations performed in vivo.

**Disclosure statement**
The authors declare that there is no conflict of interest.

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