

## Original Article

# MicroRNA-124 alleviates hyperoxia-induced inflammatory response in pulmonary epithelial cell by inhibiting TLR4/NF- $\kappa$ B/CCL2

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**Abstract:** Background: Lung epithelial cell dysfunction induced by hyperoxia-associated oxidative stress is a prominent feature involved in the development of acute lung injury (ALI). How the underlying molecular mechanisms contributed to this process are poorly defined. In the present study, we sought to identify the role of miR-124 in hyperoxia-induced cell apoptosis and excessive inflammatory response in pulmonary epithelial cell. Methods: The miR-124 levels in pulmonary epithelial cell were assayed by qRT-PCR. MiR-124 mimics and inhibitors were transfected to gain or loss of miR-124 function. Cell proliferation was analyzed by CCK8 assay. Cell apoptosis was analyzed by flow cytometry. The targeted genes were predicted by a bioinformatics algorithm and confirmed by a dual luciferase reporter assay. The protein levels were assayed by western blotting. Results: The results showed that miR-124 was significantly down-regulated in Beas2B cells and primary LECs upon hyperoxia exposure conditions. However, over-expression of miR-124 dramatically attenuated hyperoxia-provoked TLR4, NF- $\kappa$ B and pro-inflammatory cytokines production. *In vitro*, the cell viability and apoptosis was significantly reversed following transfection with miR-124 mimics in the presence of hyperoxia. Furthermore, the 3'-untranslated region (3'-UTR) of CCL2 was bound by miR-124. Conclusion: It was concluded that miR-124 inhibited hyperoxia-induced apoptosis and excessive inflammatory response in Beas2B cells and primary LECs, at least partially, through the inhibition of TLR4/NF- $\kappa$ B/CCL2 signaling cascades.

**Keywords:** MiR-124, TLR4, NF- $\kappa$ B, CCL2, pulmonary epithelial cell

## Introduction

Hyperoxia therapy is routinely administered in patients with acute and chronic pulmonary diseases, including acute lung injury, acute respiratory distress syndrome (ARDS) and severe pneumonia [1]. However, accumulating evidence suggests that prolonged exposure to hyperoxia can also cause oxygen toxicity and accelerate lung injury [2, 3]. Moreover, supplemental oxygen for a prolonged period frequently results in neonatal bronchopulmonary dysplasia (BPD), which can lead to arrest of alveolar and vascular development [4]. Importantly, hyperoxia can affect the permeability of the alveolar epithelium and ultimately lead to epithelial barrier dysfunction and cell death, this process is closely related to inflammation-mediated signaling pathways [5]. Nu-

merous studies have reported that inflammatory response is involved in hyperoxia-induced lung injury [6-9]. Both chemokines and pro-inflammatory cytokines are critical in the pathogenesis of hyperoxia-induced lung injury [7, 10, 11]. For example, CXCR4 antagonism decreases lung inflammation and improves alveolar and vascular structure in neonatal rats with experimental BPD [3]. Pro-inflammatory cytokines, TNF- $\alpha$  and IL-1 $\beta$ , have been implicated in mediating neutrophil influx into hyperoxic lung in patients with ARDS [10, 11]. However, the underlying molecular mechanisms of pro-inflammatory chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein 1 (MCP1), in hyperoxia-induced inflammatory response in alveolar epithelial cell are not clearly defined.

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CCL2 is a member of the CC chemokines sub-family and a potent chemoattractant for monocytes/macrophages to inflammatory sites [12]. CCL2 and chemokine (C-C motif) receptor 2 (CCR2) have been well documented to induce inflammatory cytokines production [13, 14]. There is overwhelming evidence showing that CCL2 is elevated in various pulmonary diseases [15]. In hyperoxia-induced acute lung injury (ALI) mice, CCL2 level is dramatically up-regulated [16]. In contrast to that CCL2/CCR2 signalling pathway protects against hyperoxia-induced lung injury by suppressing induction of iNOS and ROS [17]. These findings suggest that the precise mechanism of CCL2 in hyperoxia-induced lung injury is complicated and remains controversial, and CCL2 may possess a dual effect on the pathological progression of lung injury. Therefore, it is necessary to further investigation to verify the exact mechanism of CCL2 in hyperoxia-induced lung injury.

MicroRNA (miR) is endogenous single-stranded noncoding RNAs and is consisted of 18-25 nucleotides in length, which plays an important role in regulating genes expression at transcription level or translation level by binding to the 3'-untranslated regions (3'-UTR) of target gene [18]. Recent studies have indicated that a large amount of miRs are differentially expressed in the pathological process of lung injury [19, 20]. For example, miR-185 promotes hyperoxia-induced lung epithelial cell death [21]. MiR-29 is prominently increased in the lungs of hyperoxic mice [22]. However, the entire miRNA expression profile involving in the pathological progression of hyperoxia-induced lung injury is unknown. In the present study, we found that miR-124 was significantly inhibited in hyperoxia-treated alveolar epithelial cell. The post-translational mechanisms of miR-124 were also investigated, and bioinformatics prediction showed that CCL2 was a target gene of miR-124. Therefore, we further examined whether overexpression of miR-124 could alleviates hyperoxia-induced alveolar epithelial cell dysfunction by targeting inflammation-related signaling pathways.

### Materials and methods

#### *Cell culture*

Beas2B cells were obtained from American Type Culture Collection (ATCC, Manassas, VA);

primary mouse lung epithelial cells (LECs) were isolated as previously described [23]. Beas2B cells and primary LECs were incubated in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% FBS, 100 µg/mL streptomycin and 100 IU/mL penicillin (all of them from Sigma-Aldrich). All cells were cultured at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and 95% air (Normoxia). For hyperoxia treatment, cells were exposed to 95% oxygen and 5% CO<sub>2</sub>. For N-acetyl-L-cysteine (NAC) and Mito-TEMPO were purchased from Sigma-Aldrich.

#### *CCK-8 proliferation assay*

The CCK-8 proliferation assay was determined as previously described [24]. Cell (1×10<sup>4</sup>) proliferation was measured using CCK-8 Cell Proliferation/Viability Assay Kit (Dojindo Japan).

#### *Enzyme-linked immunosorbent assay (ELISA)*

The levels of inflammatory cytokines, tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin 6 (IL-6) and pro-inflammatory cytokine chemokine (C-C motif) ligand 2 (CCL2) were measured by ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, China) with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA), according to the manufacturer's instructions.

#### *Flow cytometry for the detection of apoptosis*

Beas2B cells and primary LECs were collected after digestion and were washed twice with PBS and centrifuged at 1,000 rpm for 5 min. The supernatant was discarded, and the cells were then resuspended and fixed in ice-cold 75% ethanol and stored at 4°C. The cell apoptosis assay was determined as previously described [24]. Annexin V-FITC apoptosis detection kit was purchased from Invitrogen (Carlsbad, Calif, USA). The samples were analyzed using flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The data were processed by Cell Quest Software (version 5.1, BD Biosciences, USA).

#### *Transfection with miR-124 mimics, miR-124 inhibitors and miR-Con*

The sequences of the miR-124 mimics (5'-UAGGACGCGGUGAAUGCC-3'), miR-124 inhibito-

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rs (5'-GGCAUUCACCGCGUGCCUUA-3') and miR-Con (5'-UCCAAACAUCGGUGAAUGCC-3') were synthesized by RiboBio (Guangzhou, China). The cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 nM. At 48 h post-transfection, the cells were harvested for analysis.

### *Luciferase reporter gene activity assay*

The 3'-UTR of the CCL2 gene containing the predicated target sites for miR-124 was obtained by online predict software miRanda-mirSVR (<http://www.microrna.org>) and Target-Scan (<http://www.targetscan.org>), and synthesized by GenePharma, (Shanghai, China). The fragment was inserted into multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion; Thermo Fisher Scientific, Inc.). The cells were co-transfected with luciferase reporters containing the 3'-UTR of wild-type (WT) or mutant-type (MUT) CCL2 and miR-124 mimics or miR-Con using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The luciferase activity was measured using a dual luciferase reporter assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol.

### *RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. cDNA was synthesized by reverse transcription reactions with 2 µg total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). miR-124 levels were determined using the TaqMan qRT-PCR kit (Thermo Fisher Scientific, Inc.) on an ABI 7500 Real-Time PCR System (Applied Biosystems, San Mateo County, CA, USA). The PCR primers were used as following: forward, 5'-CGTGTTCACAGCGGACCTTG-3' and reverse, 5'-TGGTGTCTGGAGTCG-3' for miR-124; forward, 5'-CGCTTCGGCAGCACATATACTAA-3' and reverse, 5'-TATGGAACGCTTCACGAATTTGC-3' for U6. U6 levels were utilized to normalize the expression of miR-124. The relative expression levels of genes were calculated using the  $2^{-\Delta\Delta Ct}$  method [25].

### *Western blotting*

Protein was extracted using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). The concentration was determined using the Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich; Merck KGaA). Samples containing 50 µg of protein were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primary antibodies CCL2 (cat.no: sc-1784; dilution: 1:1,000), TLR4 (cat.no: sc-293072; dilution: 1:1,000) and NF-κB (cat.no: sc-514451; dilution: 1:1,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (cat.no: sc-516102; dilution: 1:10,000; Santa Cruz Biotechnology), following visualized using chemiluminescence (Thermo Fisher Scientific, Inc.). β-actin (cat.no: sc-130301; dilution: 1:2,000; Santa Cruz Biotechnology) signals were used to correct for unequal loading. Signals were analyzed with Quantity One® software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA).

### *Statistical analysis*

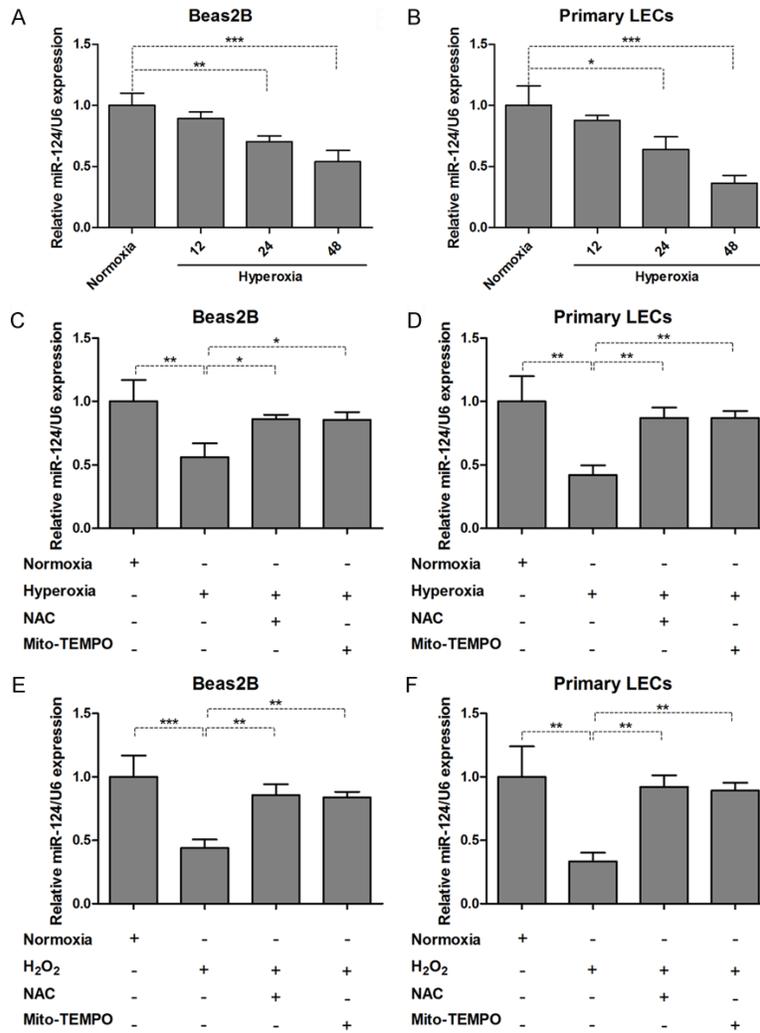
The data from these experiments were reported as mean ± standard deviation (SD) for each group. All statistical analyses were performed by using PRISM version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student *t*-test was used to analyze two-group differences. Inter-group differences were analyzed by one-way analysis of variance, followed by a post hoc Tukey test for multiple comparisons. *P*<0.05 was considered to indicate a statistically significant difference.

## **Results**

### *Hyperoxia-associated oxidative stress inhibits miR-124 expression in lung epithelial cells*

Initially, we found that hyperoxia (95% oxygen) significantly suppressed the expression of miR-124 compared with normoxia group in human lung epithelial Beas2B cells and mouse primary LECs in a time-dependent manner (**Figure 1A** and **1B**). To investigate whether hyperoxia inhibited miR-124 expression via regulating reactive oxygen species (ROS), a ROS inhibitor NAC (5

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**Figure 1.** Hyperoxia or oxidative stress induces miR-124 expression in lung epithelial cells. A and B: Time course of hyperoxia-induced expression of miR-124 in Beas2B cells and primary LECs was detected using real-time PCR. C and D: Beas2B cells and primary LECs were exposed to normoxia or hyperoxia for 24 h in the presence or absence of NAC (5 mM) or Mito-TEMPO (100  $\mu$ M), real-time PCR was performed to determine the level of miR-124. E and F: Beas2B cells and primary LECs were incubated with H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) for 6 h in the presence of NAC (5 mM) or Mito-TEMPO (100  $\mu$ M), real-time PCR was performed to determine the level of miR-124. n = 3 in each group. \* $P$ <0.05, \*\* $P$ <0.01 and \*\*\* $P$ <0.001.

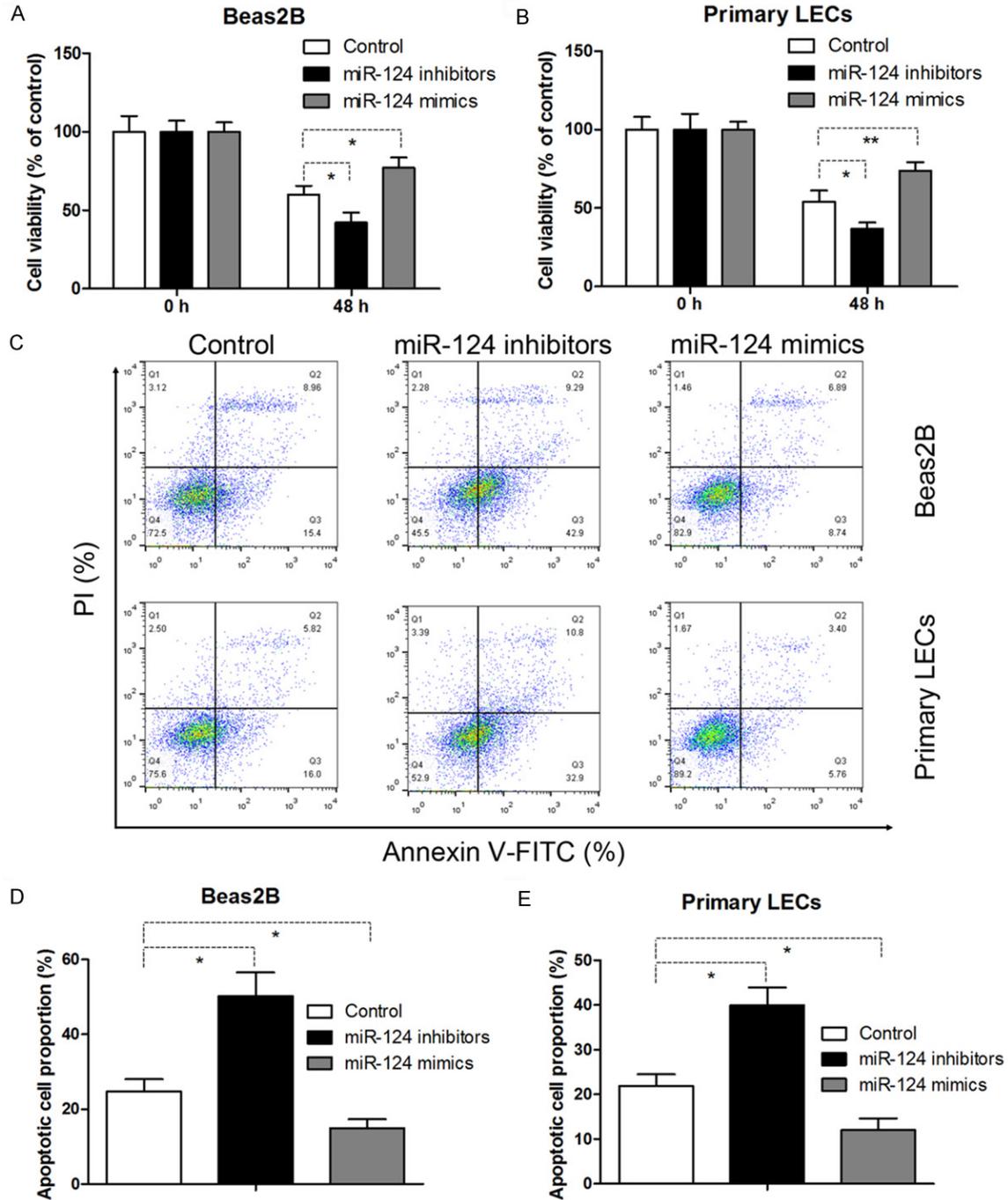
mM) or a mitochondrial antioxidant Mito-TEMPO (100  $\mu$ M) [21] was added into the Beas2B cells or primary LECs in the presence of hyperoxia, and the results demonstrated that both NAC and Mito-TEMPO markedly reversed hyperoxia-inhibited miR-124 in Beas2B cells (**Figure 1C**). These observations were confirmed in mouse primary LECs (**Figure 1D**). To further determine whether hyperoxia can inhibit miR-124 via ROS, we treated Beas2B cells and primary LECs with H<sub>2</sub>O<sub>2</sub>, a known ROS donor [23]. Multiple previ-

ous studies have confirmed that H<sub>2</sub>O<sub>2</sub> can induce ROS production in lung epithelial cells [26, 27]. Beas2B cells and primary LECs exposure to H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M) dramatically inhibited the expression level of miR-124, and these responses were also attenuated by NAC or Mito-TEMPO treatment (**Figure 1E** and **1F**).

### *miR-124 inhibits hyperoxia-induced lung epithelial cell death*

First, we analyzed the cell viability of Beas2B cells and primary LECs using CCK-8 assay after transfected with miR-124 mimics or inhibitors followed by exposure of hyperoxia. The results revealed that the cell viability of Beas2B cells and primary LECs was dramatically suppressed after exposure to hyperoxia. However, overexpression of miR-124 significantly improved the survival of Beas2B cells and primary LECs after hyperoxia exposure, in contrast to that suppression of miR-124 by inhibitors suppressed the survival of Beas2B cells and primary LECs after hyperoxia exposure (**Figure 2A** and **2B**). Next, we performed flow cytometry analysis to further evaluate whether miR-124 regulates Beas2B and primary LECs viability by inducing cell apoptosis. As shown in **Figure 2C-E**, we found that miR-124 inhibitors promoted apoptosis in Beas2B cells and primary LECs in the presence of hyperoxia. However, miR-124 mimics significantly inhibited apoptosis in Beas2B cells and primary LECs in the presence of hyperoxia. Based on above findings, we demonstrated that overexpression of miR-124 protected against hyperoxia-induced apoptosis in Beas2B cells and primary LECs.

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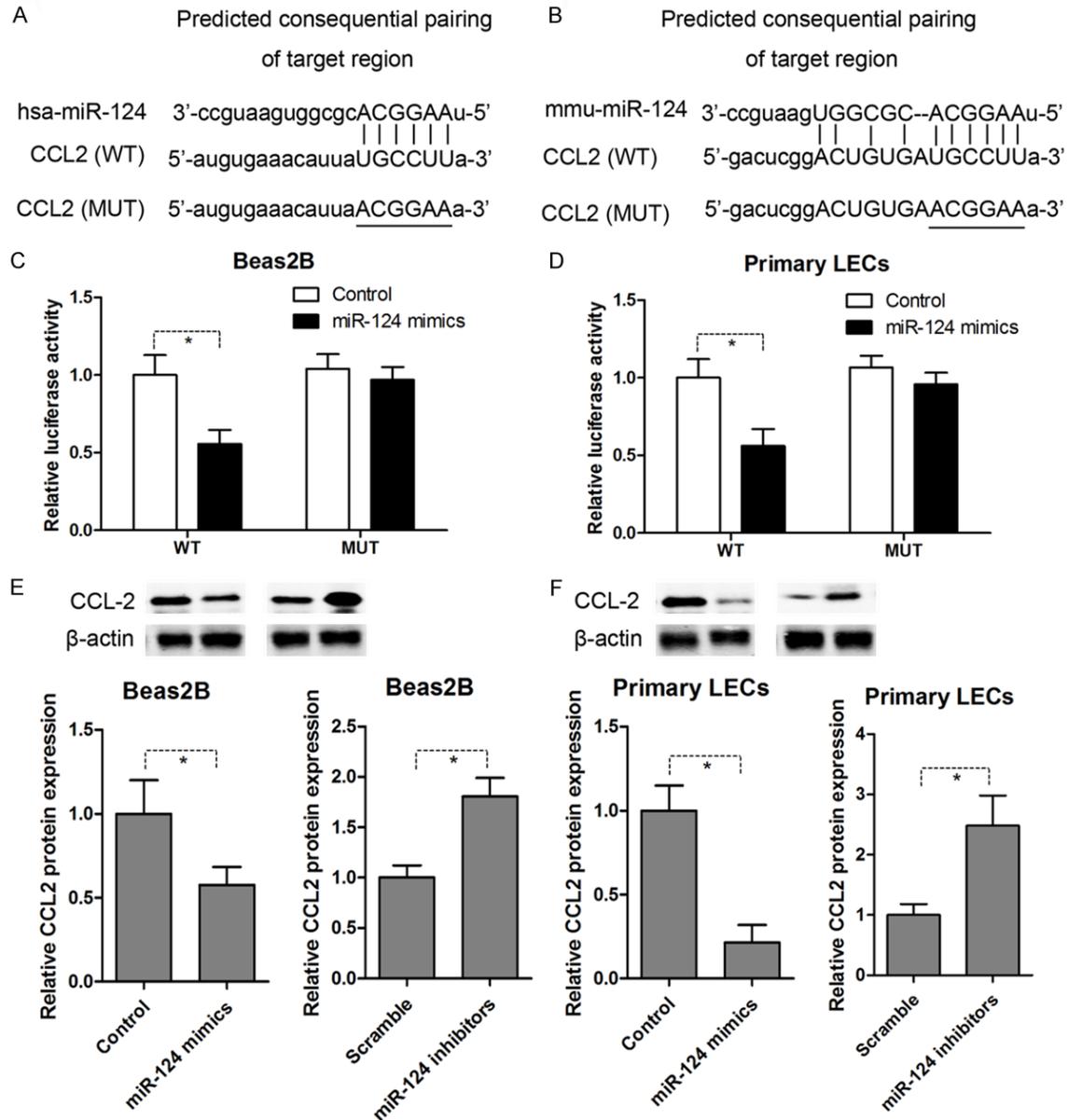
**Figure 2.** miR-124 inhibits hyperoxia-induced lung epithelial cell death. A and B: The effect of miR-124 on cell viability was detected using CCK8 after transfection of control, miR-185 inhibitors or mimics into Beas2B cells and primary LECs at 0 h (Normoxia) or 48 h (hyperoxia). C-E: After exposure to hyperoxia for 48 h, cell apoptosis was measured by flow cytometry after transfection of control, miR-185 inhibitors or mimics into Beas2B cells and primary LECs. n = 3 in each group. \* $P < 0.05$  and \*\* $P < 0.01$ .

*CCL2* is a target gene of miR-124

To find the target genes of miR-124, two algorithms (miRanda-mirSVR: <http://www.microrna.org>

and TargetScan: <http://www.targetscan.org>) were used for the prediction of the target genes. We found that *CCL2* was a highly specific target gene of miR-124 and could be iden-

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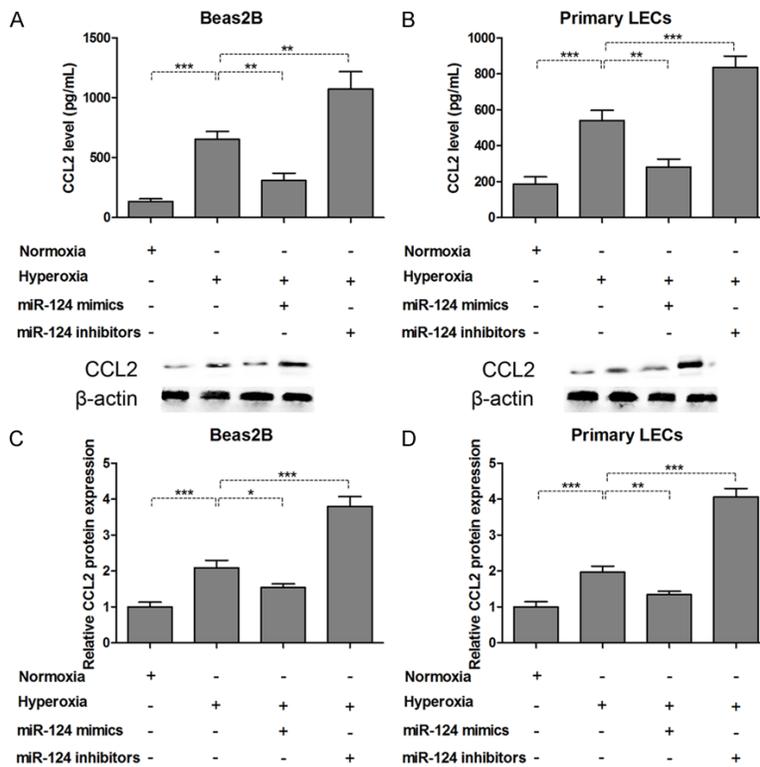


**Figure 3.** CCL2 is a target gene of miR-124. A and B: Schematic representation of the putative miR-124 binding site in the 3'-UTR of CCL2 was predicted by the online database. C and D: The Beas2B cells and primary LECs were co-transfected with the WT and MUT of 3'-UTR of CCL2 and miR-124 mimics or miR-Control, the luciferase activity assay was performed. E and F: After transfected with miR-124 mimics or inhibitors into Beas2B cells and primary LECs, the protein expression of CCL2 was detected by western blotting.  $n = 3$  in each group.  $*P < 0.05$ .

tified by both two algorithms in human and mouse species. According to the TargetScan or miRanda-mirSVR algorithm, sequence alignment of the miR-124 complementary sites in the 3'-untranslated region (3'-UTR) of CCL2 were predicted in human and mouse species (Figure 3A and 3B). To further determine whether miR-124 targeted CCL2 directly by its 3'-UTR, we cloned the fragments of the human or

mouse CCL2 3'-UTR containing the wild-type (WT) or mutant type (MUT) miRNA-binding sites into the luciferase reporter vectors and transfected them into cells combined with miR-124 mimics or control. As anticipated, transfection with miR-124 mimics significantly diminished luciferase activity elicited by the luciferase vector carrying the complementary sequence with WT 3'-UTR of CCL2. However, the luciferase

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**Figure 4.** miR-124 inhibits hyperoxia-induced the up-regulation of CCL2. A and B: Beas2B cells and primary LECs were exposed to normoxia or hyperoxia for 24 h with miR-124 mimics or inhibitors transfection, CCL2 levels in the supernatant were measured by ELISA analysis. C and D: Beas2B cells and primary LECs were exposed to normoxia or hyperoxia for 24 h with miR-124 mimics or inhibitors transfection, CCL2 protein expression was detected by western blotting. n = 3 in each group. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

enzyme activity had no significant difference between control group and miR-124 mimics group in the reporter vector containing MUT 3'-UTR of CCL2 (Figure 3C and 3D). Collectively, CCL2 is a target gene of miR-124 by binding with its 3'-UTR. To confirm the association between CCL2 and miR-124, western blot analyses were performed to investigate the expression levels of CCL2 in the Beas2B cells and primary LECs following transfection with miR-124 mimics or miR-124 inhibitor. As shown in Figure 3E and 3F, the protein expression levels of CCL2 were significantly decreased following transfection with the miR-124 mimics and increased following transfection with the miR-124 inhibitors in the Beas2B cells and primary LECs.

### miR-124 regulates TLR4/NF- $\kappa$ B/CCL2 signaling in hyperoxia-treated lung epithelial cell

To clarify the role of miR-124 in hyperoxia-induced proinflammatory response, we had

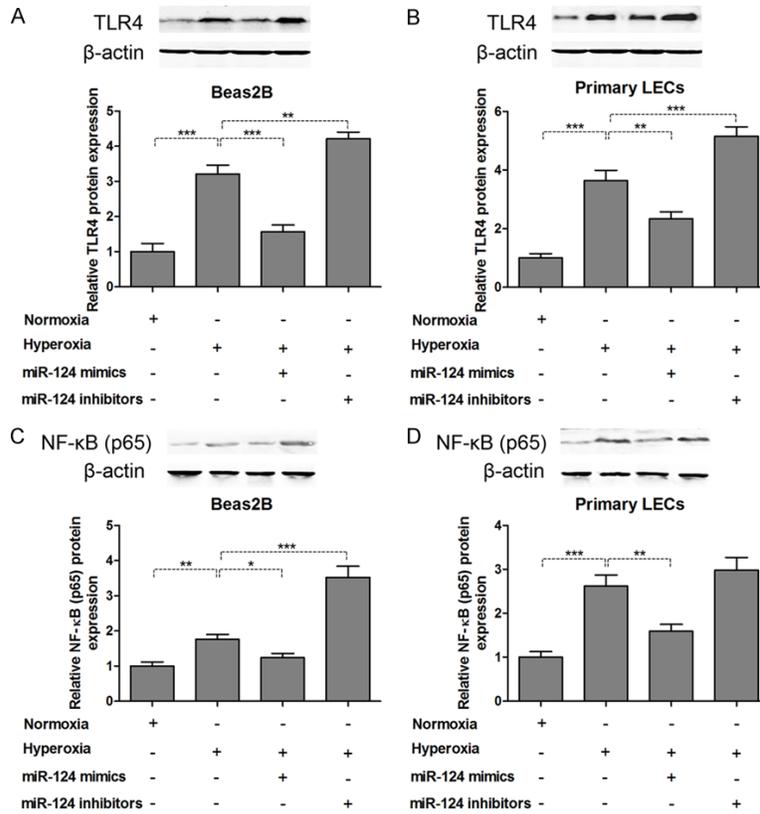
proposed a hypothesis that miR-124 regulated hyperoxia-induced proinflammatory response by CCL2 signaling. Previous studies indicate that CCL2 signaling is involved in hyperoxia-induced lung injury [17, 28]. We treated Beas2B cells and primary LECs with hyperoxia and found that exposure of Beas2B cells and primary LECs with hyperoxia caused a significant increase in MCP-1 secretion (Figure 4A and 4B). However, miR-124 mimics inhibited hyperoxia-induced overexpression of MCP-1 in the supernatant. In contrast to that miR-124 inhibitors accelerated MCP-1 secretion in Beas2B cells and primary LECs after exposure to hyperoxia. These observations were also confirmed by western blotting analysis in Beas2B cells and primary LECs (Figure 4C and 4D). A growing body of scientific evidence has suggested TLR4/NF- $\kappa$ B signaling is a proinflammatory pathway [29, 30], which can induce CCL2

expression in the inflammatory cascade with potential physiopathological implications [31]. To determine whether miR-124 regulated TLR4/NF- $\kappa$ B in hyperoxia-treated lung epithelial cell, western blot analysis was performed to examine the expression of TLR4 and NF- $\kappa$ B (p65) after transfected with miR-124 mimics or inhibitors. The results showed that the expression levels of TLR4 and NF- $\kappa$ B (p65) were significantly upregulated in hyperoxia-treated Beas2B cells and primary LECs compared with control group (Figure 5A-D). Compared with hyperoxia-treated group, the protein expression levels of TLR4 and NF- $\kappa$ B (p65) were significantly inhibited after transfected with miR-124 mimics (Figure 5A-D).

### miR-124 inhibits hyperoxia-induced pro-inflammatory cytokines in lung epithelial cell

Excessive proinflammatory response is the striking characteristic of hyperoxia-induced ALI [9]. However, little is known about the role of

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**Figure 5.** miR-124 inhibits hyperoxia-induced the up-regulation of TLR4 and NF-κB. A and B: Beas2B cells and primary LECs were exposed to normoxia or hyperoxia for 24 h with miR-124 mimics or inhibitors transfection, TLR4 protein expression was detected by western blotting. C and D: Beas2B cells and primary LECs were exposed to normoxia or hyperoxia for 24 h with miR-124 mimics or inhibitors transfection, NF-κB protein expression was detected by western blotting. n = 3 in each group. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ .

miR-124 in hyperoxia-induced proinflammatory response in Beas2B cells and primary LECs. Thus, we next studied the consequences of overexpression or inhibition of miR-124 on the inflammatory cytokines in cultured Beas2B cells and primary LECs. The concentrations of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were significantly increased in hyperoxia treatment group compared with control group, while administration of NAC or Mito-TEMPO in Beas2B cells and primary LECs significantly attenuated the effects of hyperoxia-induced pro-inflammatory cytokines (Figure 6A-F). Importantly, we found that overexpression of miR-124 inhibited the increase of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  levels in the supernatant of Beas2B cells and primary LECs exposure to hyperoxia. Furthermore, suppression of miR-124 in Beas2B cells and primary LECs using miR-124 inhibitors markedly promoted the up-regulation of TNF- $\alpha$ , IL-6 and

IL-1 $\beta$  levels in the supernatant of Beas2B cells and primary LECs in the presence of hyperoxia (Figure 6G-I).

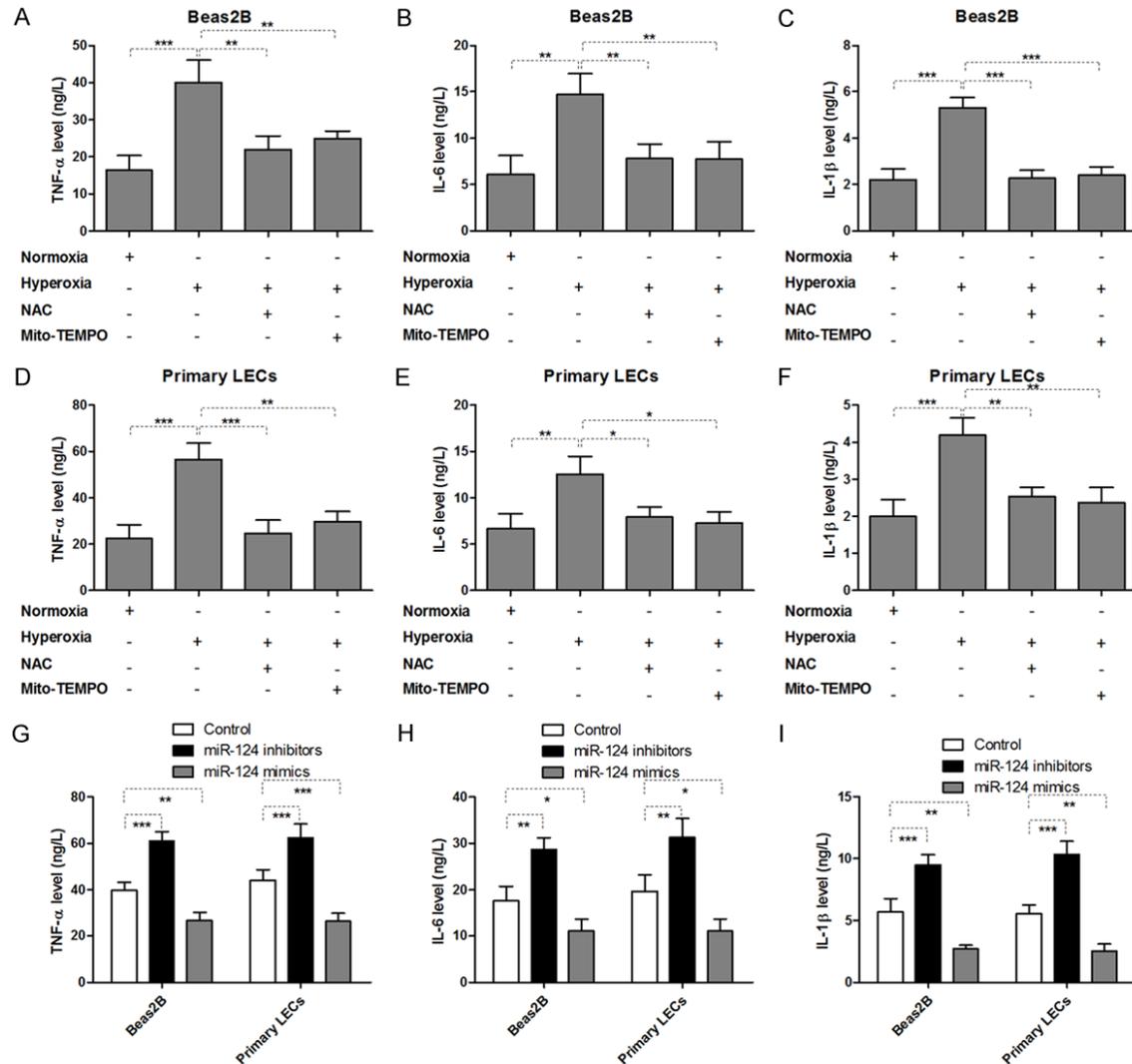
### Discussion

Numerous studies have been reported that miR-124 as tumor suppressor gene involves in the development and progression of a variety of tumors [32, 33]. These observations have been repeatedly reported in cancer research. However, the roles of miR-124 in the pathophysiological function of normal lung epithelial cells in response to hyperoxia remain unclear.

In recent years, a few studies have been done to focus on the expression and function of miR-124 in pulmonary epithelial cell [34, 35]. For example, the expression of miR-124 is a dynamic process during fetal lung development, and overexpression of miR-124 inhibits pulmonary epithelium maturation through the inhibition of nuclear factor I/B, a critical protein in fetal lung maturation [35].

Moreover, miR-124 directly targets TLR signaling components, including TLR6, myeloid differentiation factor 88 (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6) and TNF- $\alpha$  mediates attenuation of the *Mycobacterium bovis* Bacillus Calmette-Guerin-induced inflammatory response in A549 alveolar epithelial cells [34]. Our report is the first to explore the expression and inflammation-related regulatory mechanisms of miR-124 in normal lung epithelial cells in response to hyperoxia. miR-124 was significantly down-regulated in Beas2B cells and primary LECs exposure to hyperoxia conditions. However, overexpression of miR-124 dramatically attenuated hyperoxia-provoked TLR4, NF-κB and pro-inflammatory cytokines production. These findings are consistent with the results of other studies [34, 35].

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**Figure 6.** miR-124 inhibits hyperoxia-induced excessive inflammatory response. A-C: Beas2B cells were exposed to normoxia or hyperoxia for 24 h in the presence or absence of NAC (5 mM) or Mito-TEMPO (100 μM), TNF-α, IL-6 and IL-1β levels in the supernatant were measured by ELISA analysis. D-F: Primary LECs were exposed to normoxia or hyperoxia for 24 h in the presence or absence of NAC (5 mM) or Mito-TEMPO (100 μM), TNF-α, IL-6 and IL-1β levels in the supernatant were measured by ELISA analysis. G-I: Beas2B cells and primary LECs were exposed to hyperoxia for 24 h with miR-124 mimics or inhibitors transfection, TNF-α, IL-6 and IL-1β levels in the supernatant were measured by ELISA analysis. n = 3 in each group. \**P*<0.05, \*\**P*<0.01 and \*\*\**P*<0.001.

Hyperoxia-associated oxidative stress can rapidly regulate miRs expression, such as miR-15a, miR-16 and miR-185, in lung epithelial cells, deregulated miRs are closely related to the cell apoptosis and proliferation [21, 23]. Our results showed that miR-124 loss of function promoted Beas2B cells and primary LECs apoptosis in response to hyperoxic stress, which is a harmful factor involved in the development of ALI [2]. We had also found that miR-124 gain of function protected against hyperoxia-induced apoptosis in normal lung epithelial cells and per-

formed an opposite function in the cancer cells [36, 37].

TLR4/NF-κB is a crucial signaling of the inflammatory response-modulated lung injury and is activated by a wide array of physiologic and pathologic conditions, including lipopolysaccharide (LPS) [38], acute cigarette smoke [39], ischemia-reperfusion [40] and oxidative stress [41]. However, the majority of the current knowledge linking TLR4/NF-κB signaling to lung injury is focused on LPS. In the current study, we

investigated the mechanism of TLR4/NF- $\kappa$ B in hyperoxia-induced inflammatory response *in vitro*, and the results showed that TLR4/NF- $\kappa$ B signaling was activated in hyperoxia-cultured Beas2B cells and primary LECs, accompanied with the increasing of NF- $\alpha$ , IL-6 and IL-1 $\beta$  production. These findings were also confirmed by Chen et al in hyperoxia-induced lung injury in neonatal rats [41]. Subsequently, we found that miR-124 was able to attenuate a pro-inflammatory response in Beas2B cells and primary LECs, at least partially, through the inhibition of TLR4/NF- $\kappa$ B signal cascades. miR-124 possesses a post-transcriptionally regulatory capacity of targeting multiple components of the TLR signaling cascade, including transcription factors and pro-inflammatory factors [34]. In agreement with this notion, we found miR-124 could directly target to CCL2 in Beas2B cells and primary LECs. Overexpression miR-124 significantly inhibited CCL2 levels in Beas2B cells and primary LECs upon hyperoxia exposure. This observation demonstrated a crucial cross-talk between CCL2 and miR-124 in hyperoxia-induced inflammatory response, suggesting that CCL2 is a critical mediator through which miR-124 exerts its biological functions in alveolar epithelial cells in response to hyperoxia.

CCL2 is secreted by monocytes/macrophages or dendritic cells in response to infection or inflammation [42], whose activation can be regulated by TLR4/NF- $\kappa$ B signal cascades [31]. The activation of TLR4/NF- $\kappa$ B pathway accelerates CCL2 secretion in LPS-stimulated mouse macrophage RAW264.7 cells [43]. Moreover, TLR4-mediated NF- $\kappa$ B pathway involves in excessive inflammatory response, including release of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and CCL2, in LPS-treated human renal proximal tubular epithelial cells [44]. Numerous studies have identified that TLR4/NF- $\kappa$ B signal inflammatory response-induced lung pathological injury [38, 45]. In this study, our data revealed the activation of TLR4/NF- $\kappa$ B pathway and the up-regulation of a variety of inflammatory cytokines in Beas2B cells and primary LECs upon hyperoxia exposure. These findings suggest that TLR4/NF- $\kappa$ B-mediated release of inflammatory cytokines may involve in the pathological process of hyperoxia-treated pulmonary epithelial cell. Furthermore, our functional studies using miR-124 mimics or inhibitors showed that TLR4/NF- $\kappa$ B-mediated release of inflammatory cytokines in hyperoxia-treated pulmonary epithelial

cell could be inhibited by overexpression of miR-124.

In conclusion, our data provide new insights into the role miR-124 in protecting the pulmonary epithelial cell against hyperoxia-associated oxidative stress. TLR4/NF- $\kappa$ B signaling, CCL2 and other inflammatory cytokines are involved in pulmonary epithelial cell dysfunction *in vitro*. However, experiments using *in vivo* models of hyperoxia-induced lung injury through miR-124/TLR4/NF- $\kappa$ B/CCL2 need to be further expounded. More detailed analysis based on our results will help to elucidate the molecular and functional role of miR-124/TLR4/NF- $\kappa$ B/CCL2 in the pathogenesis of hyperoxia-induced lung injury and to evaluate their potential as novel therapeutic targets.

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### Disclosure of conflict of interest

None.

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