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Original Article

MicroRNA-21-5p antagonizes oxidant-mediated apoptosis in alveolar epithelial type II cells by targeting PDCD4

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Abstract: Acute respiratory distress syndrome (ARDS) is a condition characterized by acute inflammation in the lungs. Apoptosis of alveolar epithelial type II (ATII) cells contributes to the initiation and progression of the disease. miRNAs are tightly regulated and their dysregulation plays an important role in human diseases. One such miRNA, miR-21 is shown to be involved in several different diseases. However, its role in ARDS is still not known. Here, we hypothesize that miR-21-5p inhibits apoptosis in ATII cells and protects against ARDS. In the present study, 50 μM H_2O_2 was used to induce ATII cell damage to simulate ARDS *in vitro*. CCK-8 assay was performed to detect cell proliferation and flow cytometry was used to evaluate cell apoptosis. A dual-luciferase assay was performed to confirm whether miR-21 directly targeted the programmed cell death 4 (PDCD4) mRNA. Here, we found that ATII cell apoptosis increased after treatment with 0.5 mM H_2O_2. Overexpression of miR-21 or knockdown of PDCD4 promoted ATII cell proliferation and inhibited ATII cell apoptosis after treatment with H_2O_2. We further confirmed that miR-21 regulates PDCD4 expression by targeting its three prime untranslated region (3'-UTR). Our results suggest that miR-21 potentially antagonizes oxidant-mediated apoptosis in alveolar epithelial type II cells. These findings provide new insights in understanding the process of ARDS and also provide a potential target for the treatment of ARDS.

Keywords: ARDS, ATII cells, miR-21-5p, PDCD4, apoptosis

Introduction

The acute respiratory distress syndrome (ARDS) is a life-threatening clinical condition associated with arterial hypoxemia, bilateral radiographic pulmonary opacities, and is often the primary cause of cardiac failure [1]. It is characterized by lung endothelial injury and reduced lung compliance leading to increased vascular permeability and protein-rich alveolar filling [2]. Despite improvements in adult intensive care units (ICUs) over the last fifteen years, ARDS is still linked to high morbidity and mortality (40%) rates [3-5]. Previous studies found that the development and outcome of ARDS is associated with the activation of multiple inflammatory cells and the release of inflammatory mediators [2]. However, traditional anti-inflammatory drugs or monoclonal antibodies against a certain inflammatory factor have little curative effect on the treatment of ARDS [6, 7]. In spite of intense research over the past few decades, the pathogenesis of this disease remains poorly defined. Type II alveolar epithelial cells (ATII) are important in the defense and repair of lung injury [8, 9]. Previous studies found that apoptosis of ATII cells contributes to the initiation and progression of ARDS [10, 11]. Therefore, understanding the molecular mechanism of apoptosis of ATII cells may provide a new strategy for the prevention and treatment of ARDS.

MicroRNAs (miRNAs) are a class of endogenous, short, non-coding RNA molecules that are approximately 22-25 nucleotides in length and play important roles in diverse cellular pathways [12]. miRNAs regulate gene expression of target messenger RNAs (mRNAs) by binding to its 3'-untranslated region (3'-UTR), and result in either mRNA degradation or prevent mRNA from being translated to protein [13, 14]. Several studies have shown that miR-

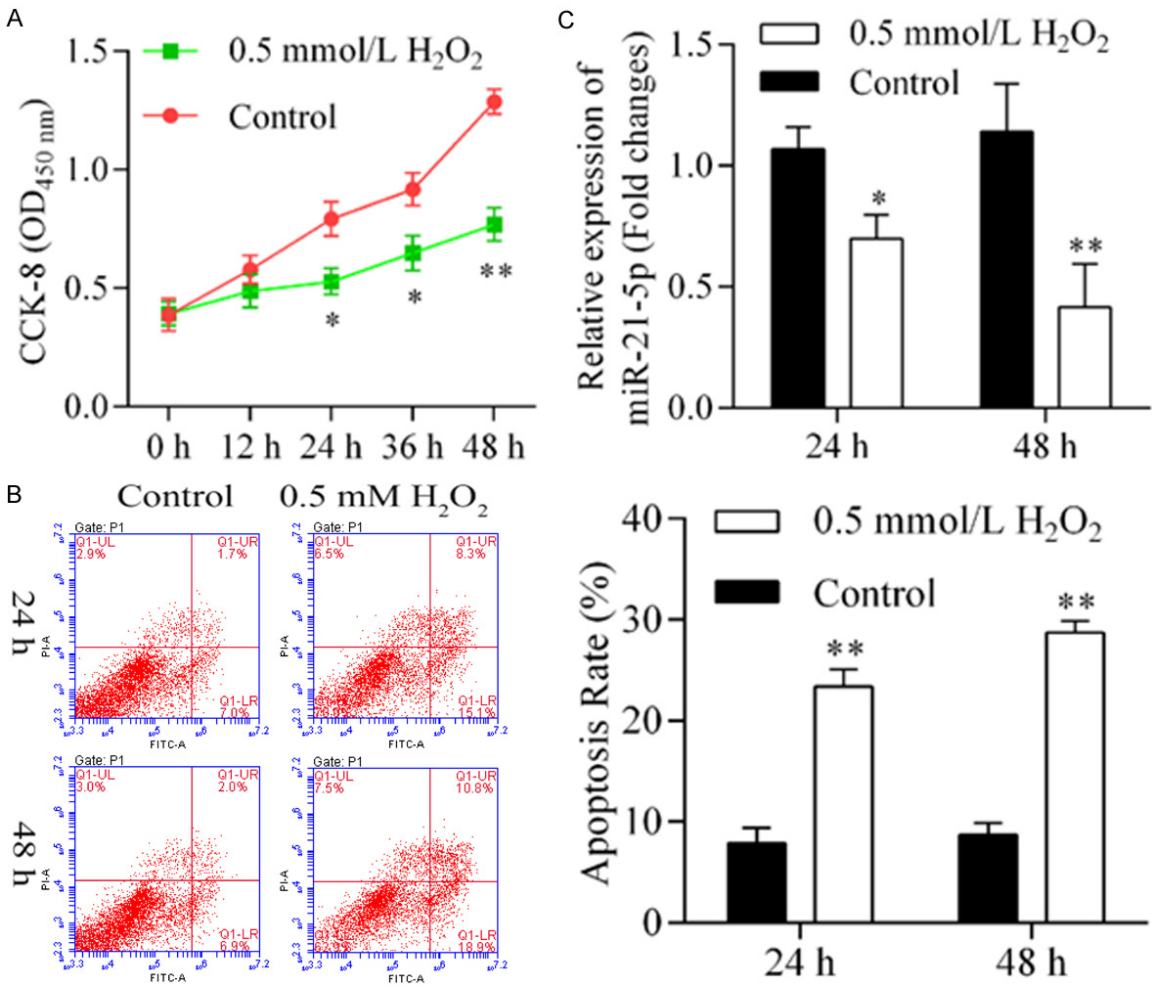

Figure 1. Oxygen suppressed ATII cell proliferation, induced ATII cell apoptosis and inhibited miR-21-5p expression. A. CCK-8 assay shows that ATII cell proliferation was suppressed after treatment with 50 μM H_2O_2, $^{*}P<0.05$, $^{**}P<0.01$. B. Flow cytometry shows that ATII cell apoptosis was induced after treatment with 50 μM H_2O_2, $^{**}P<0.01$. C. The expression of miR-21 was reduced in ATII cells after treatment with 50 μM H_2O_2, $^{*}P<0.05$, $^{**}P<0.01$.

NAs are involved in different cellular processes involving cell growth, differentiation, apoptosis and stress response [15, 16]. In addition, miRNAs have been reported to have both tumor suppressor and oncogenic activities [17]. As the core molecules of cell apoptosis regulators, miRNAs are involved in the cell's response to signals of death or survival. miR-221 and miR-222 have been shown to inhibit CDK (cyclin-dependent kinase) inhibitory proteins p27Kip1, p57 and c-Kit receptor, resulting in a decrease in cell growth and survival and repressing cell differentiation [18-20]. Overexpression of the miR-106b-25 cluster interferes with the expression of p21Waf1/Cip1 and Bim to control both cell cycle and apoptosis [21]. miR-122 expression decreased in human hepatocellular carcinoma, and miR-122 overexpression suppressed cell viability and activated apoptosis [22, 23]. A previous study found that miR-21 appears to function as an anti-apoptotic factor [24-26]. This suggests that miR-21 may be a potential anti-apoptotic factor in other diseases. However, the role of miR-21-5p in ATII apoptosis is still not known.

In our earlier work, we found that miR-21-5p represses apoptosis in type II alveolar epithelial cells and reduces acute lung injury in rats (data not published). Hence, we hypothesized that miR-21-5p inhibits ATII cell apoptosis to protect ARDS and may develop into a potential new treatment strategy. Since ATII cells are typically used in acute lung injury cell models, we used ATII cells in our present study. Specifically, we treated ATII cells with H_2O_2 to simulate an

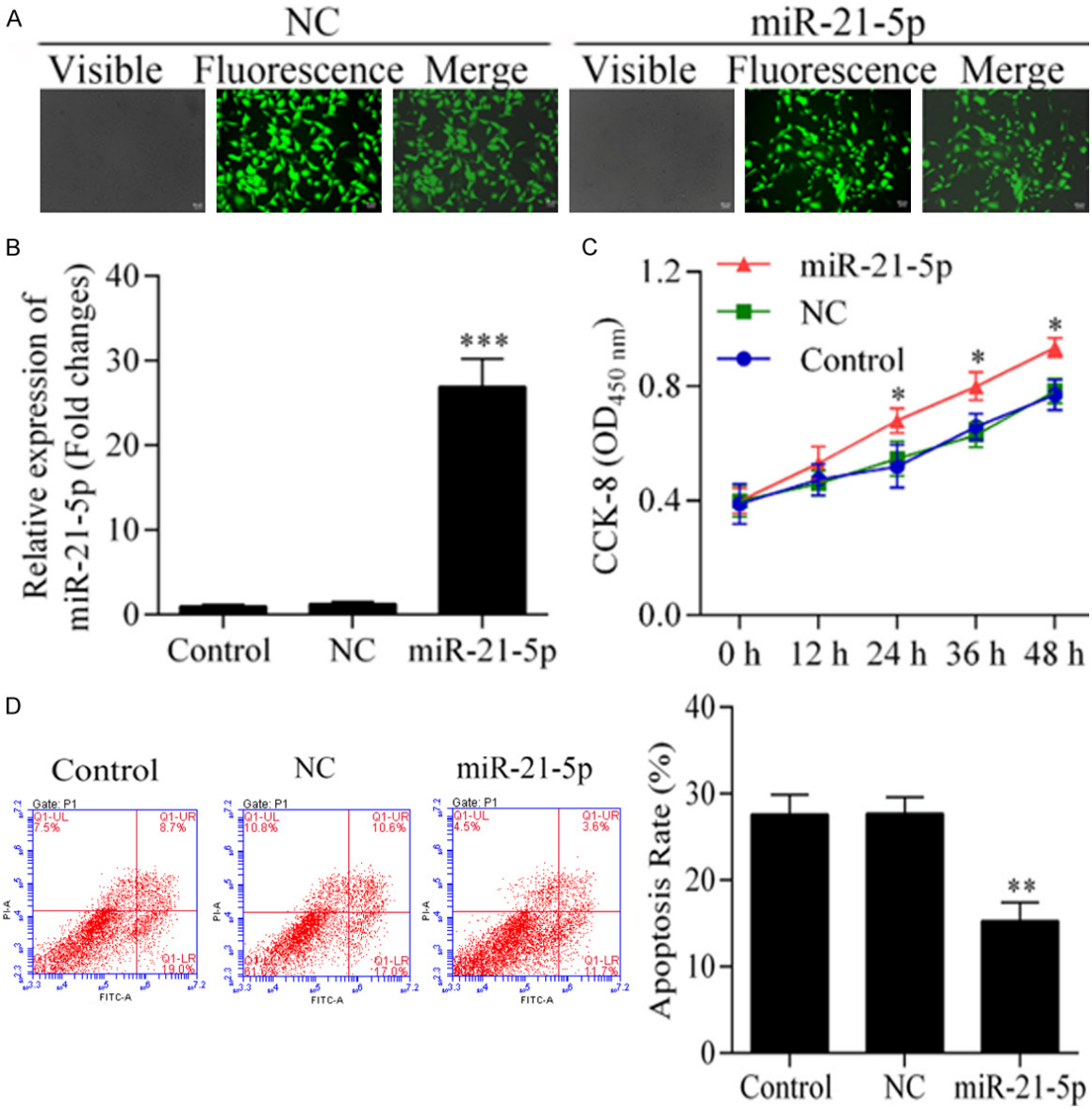

Figure 2. Overexpression of miR-21-5p suppresses oxygen-induced cell apoptosis and inhibits cell proliferation. A. Stable transfection was achieved with the lentivirus method, MOI = 200. B. miR-21 was significantly overexpressed in ATII cells after stable transfection, $^{***}P<0.001$. C. Overexpression of miR-21-5p promoted ATII cell proliferation after treatment with 50 μM H_2O_2 at 24, 36 and 48 h, $^{*}P<0.05$. D. Overexpression of miR-21-5p suppressed the rates of ATII cell apoptosis after treatment with 50 μM H_2O_2 at 24 and 48 h, $^{**}P<0.01$.

ARDS cell model and investigate the role of miR-21-5p in ATII cell apoptosis.

Materials and methods

Cell culture

Human type II alveolar epithelial cells were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in alveolar epithelial cell medium (Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FBS) and an antibiotic mixture of 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin). Cells were incubated in a humidified incubator at 37°C in 95% air and 5% CO_2. To build the cell apoptosis model, cells were treated with 0.5 μM H_2O_2 for different time periods. This study was conducted in accordance with the declaration of Helsinki, and with the approval of the Ethics Committee of the Zunyi Medical College Affiliated Hospital.

Cell transfection

Lentivirus-mediated miR-21-5p overexpression vectors and the negative control (NC) were obtained from Genechem Biotech (Shanghai, China). The Multiplicity of infection (MOI) method was performed to determine the most effective transfection concentration of the lentivirus. Based on the results (**Figure 2A**), lentivirus concentration at MOI = 200 (20 μL 1×10^8 TU/mL lentivirus + 80 μL cells [1×10^4 cells]) was chosen for further experiments. The small interference RNAs (siRNAs) were transfected into ATII cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The siRNAs for targeting PDCD4 were obtained from Dharmacon (Lafayette, CO, USA).

Quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using Trizol regent (Invitrogen Inc., USA), according to the manufacturer's instructions. 2 μg of RNA was used for the reverse transcription reaction and complementary DNA (cDNA) synthesis by using the Reverse Transcription Kit (TaKaRa, Dalian, China). qPCR was performed with SYBR green real-time Master Mix (TOYOBO, Japan). GAPDH was selected as the reference gene for analyzing the relative expression of PDCD4. The relative expression of miR-21-5p was detected using a SYBR PrimeScript miRNA RT-PCR Kit (TAKARA, Dalian, China), according to the manufacturer's instructions and constitutive expression of gene U6 was used as an internal control. The primers were synthesized by Sangon (Shanghai, China) and the sequences are shown in **Table 1**. Applied Biosystems 7500 Sequence Detection system (ABI, USA) was used to perform qPCR and data collection. The relative expression levels of PDCD4 and miR-21-5p were determined according to the expression of $2^{-\Delta\Delta ct}$.

Western blot

Total protein was extracted from cultured cells using SDS lysis buffer (Beyotime, Shanghai, China) on ice for 30 min and centrifuged at 4°C, 12000×g for 15 min. Supernatant was collected and the concentration was determined using BCA protein assay kit (Pierce, USA). 20 μg of total protein was separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 110 V for 2.5 hours, and transferred to 0.22 μm polyvinylidene difluoride (PVDF) (Millipore, Massachusetts, USA) membranes. The membranes were incubated with PCDC4 antibody (1:1500, Abcam, ab51495) or GAPDH (1:2000, Abcam, ab8245) at 4°C overnight. The membranes were then washed with PBS and incubated with the secondary antibody for one hour at room temperature. Proteins were detected by enhanced chemiluminescence (ECL), according to the manufacturer's instructions (Beyotime, Shanghai, China) and the intensity of the bands were quantified by densitometry (Quantity One software; Bio-Rad, CA, USA).

CCK-8 assay

The CCK-8 method was performed to detect cell viabilities. Briefly, cells transfected with miR-21-5p or NC were cultured for 12, 24, 36 and 48 h. At each time-point, cells were collected and 50 μL of CCK-8 solution was added, then cultured at 37°C for 1.5 h. OD value at 450 nm was read using a microplate reader Thermo-Plate (Rayto Life and Analytical Science C. Ltd, Germany).

Apoptosis assay

Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (Beyotime, Shanghai, China) were used to detect cell apoptosis, according to the manufacturer's instructions. Briefly, 5×10^5 treated cells were harvested and stained using the Annexin-V FITC/PI apoptosis detection kit. These samples were analyzed using a flow cytometer (B&D, NJ, USA). Annexin-V(+)/PI(-) and Annexin-V(+)/PI(+) represented cells in early and late apoptosis and necrosis, respectively.

Dual-luciferase assay

The wild type 3'UTR of PDCD4 containing putative binding sites for miR-21-5p was cloned into the psi-CHECK2 vectors, named PDCD4-3'UTR-WT. PDCD4 mutant 3'-UTR (from ATAAGCUA to GCCTTAGT, the predicted miR-221 target binding sites) was generated by QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA), named as PDCD4-3'UTR-MUT. For dual-luciferase assays, 5×10^5 cells were seeded in a 24-well plate, one day before transfection. 50 ng of PDCD4-3'UTR-WT or -MUT vectors were co-transfected with 20 nM miR-21-5p mimics

Table 1. Primer sequences used for miRNA and mRNA expression analysis

Name	Primer sequence (5'-3')
miR-21-5p-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATGTTGA
U6-RT	CGCTTCACGAATTTGCGTGTCAT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
miR-21-5p-F	ACACTCCAGCTGGGTAGCTTATCAGACTGA
Universal-R	TGGTGTCGTGGAGTCG
PDCD4-F	GTGGAGTACCAGTGTTGGCA
PDCD4-R	GTGGAGTACCAGTGTTGGCA
GAPDH-F	ACACCCACTCCTCCACCTTT
GAPDH-R	TTACTCCTTGGAGGCCATGT

F forward primer, *R* reverse primer, *RT* reverse transcription primer.

or negative control into cells using Lipofectamine 2000 reagent. After 48 hours, luciferase activities were measured using Dual-Luciferase Reporter Assay System (Promega, USA), according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the SPSS 20.0 (SPSS Inc, USA). Statistical significance between groups was determined using a Student's *t*-test or one-way analysis of variance (ANOVA). And *P*-value of <0.05 was considered statistically significant.

Results

Oxygen suppressed ATII cell proliferation, induced ATII cell apoptosis and inhibited miR-21-5p expression

To investigate the role of oxygen in ATII cell functions, ATII cells were treated with 50 μM H_2O_2. CCK-8 assay showed that 50 μM H_2O_2 suppressed ATII cell proliferation at 24, 36 and 48 h (**Figure 1A**). Furthermore, flow cytometry showed that cell apoptosis rates were induced after treatment with 50 μM H_2O_2 for 24 and 48 h (**Figure 1B**). Moreover, qRT-PCR results indicated the expression of miR-21-5p was significantly suppressed after treatment with 50 μM H_2O_2 for 24 and 48 h (**Figure 1C**). These results suggest that miR-21-5p is involved in oxygen-induced cell apoptosis.

Overexpression of miR-21-5p suppresses oxygen-induced cell apoptosis and inhibits cell proliferation

To confirm whether miR-21-5p was involved in oxygen-induced cell apoptosis, miR-21-5p vectors were transfected in a stable manner in ATII cells. Stable transfection of lentivirus-mediated miR-21-5p vectors were detected using fluorescence microscope (MOI = 200) (**Figure 2A**). qRT-PCR results showed that miR-21-5p expression was significantly increased after stable transfection of lentivirus-mediated miR-21-5p vectors (**Figure 2B**). CCK-8 assay results showed that overexpression of miR-21-5p promoted ATII cell proliferation after treatment with 50 μM H_2O_2 for 24, 36 and 48 h (**Figure 2C**). Moreover, overexpression of miR-21-5p suppressed ATII cell apoptosis rates after treatment with 50 μM H_2O_2 for 24 and 48 h (**Figure 2D**). These results validated our finding that miR-21-5p is involved in oxygen-induced cell apoptosis.

PDCD4 is a target of miR-21-5p

To investigate how miR-21-5p antagonizes oxygen-induced apoptosis in ATII cells, miRNA-mRNA target experiments were performed. Western blot was performed to analyze the expression of programmed cell death 4 (PDCD4) protein. We found that the expression of PDCD4 protein was significantly increased after treatment with 50 μM H_2O_2 for 48 h, and overexpression suppressed miR-21-5p-inhibited PDCD4 expression (**Figure 3A** and **3B**). In order to determine whether miR-21-5p directly regulates PDCD4 expression, TargetScan (www.targetscan.org) miRNA target prediction database was used for computational analyses. The bioinformatics prediction results showed that miR-21-5p has one predictive target site in the human PDCD4 3'-UTR (**Figure 3C**). The miR-21-5p stable expression ATII cells were transfected with psi-CHECK2-PDCD4-3'UTR-WT vectors and showed a significant reduction in luciferase reporter gene activity (**Figure 3D**). These results confirmed that miR-21-5p directly targets the 3'UTR of PDCD4 and down-regulates PDCD4 expression.

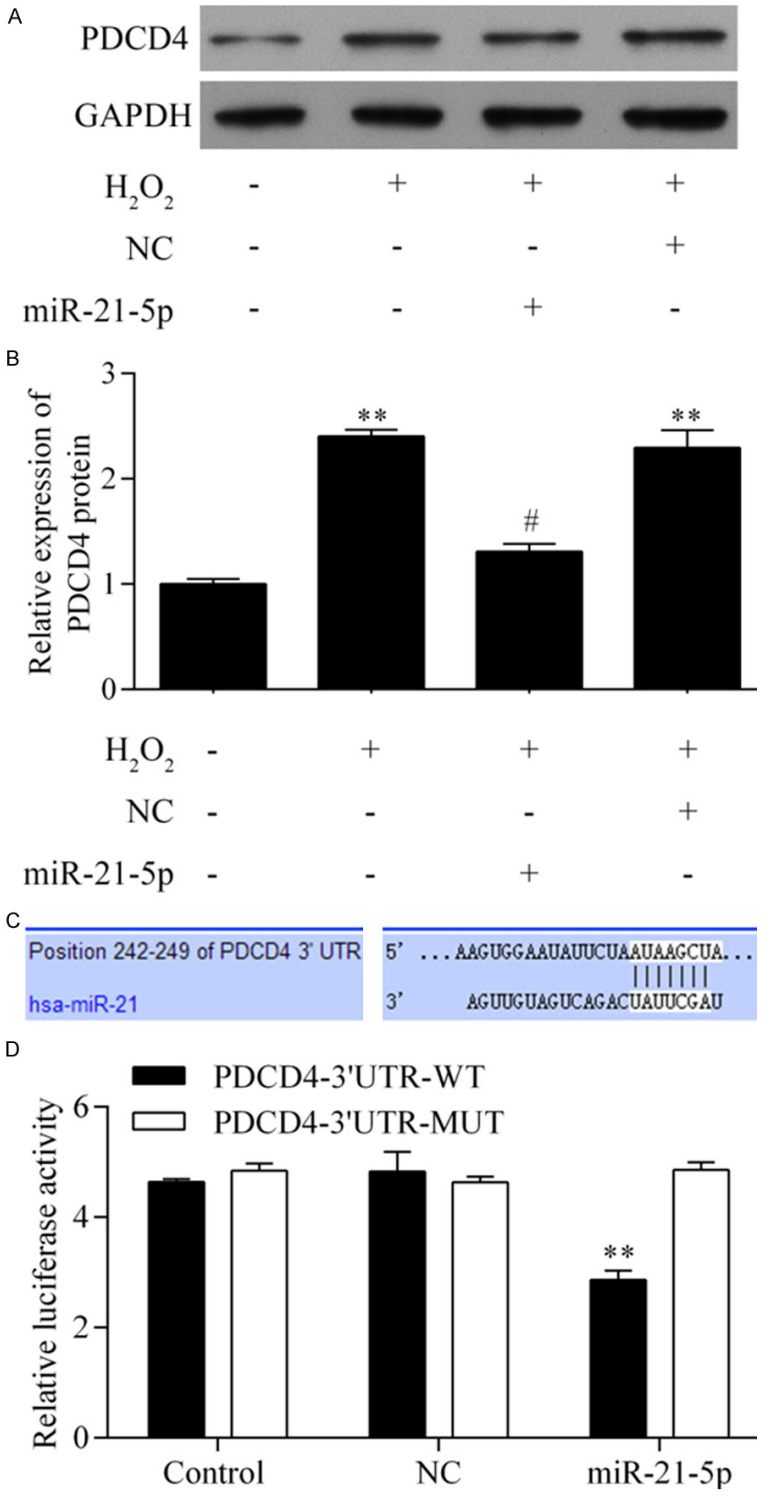

Figure 3. PDCD4 is a target of miR-21-5p. A. Western blot analyzed the protein expression of PDCD4 under different conditions. B. The quantity of PDCD4 protein in different conditions, $^{**}P<0.01$ *vs.* control group [H_2O_2 (-), NC (-), miR-21-5p (-)], $^{\#}P<0.05$ *vs.* H_2O_2 treat group [H_2O_2 (+), NC (-), miR-21-5p (-) or H_2O_2 (+), NC (+), miR-21-5p (-)]. C. Sequence alignment of miR-21 and 3'-UTR of *PDCD4* using TargetScan algorithm. D. Dual luciferase activity in miR-21 stable expression ATII cells upon transfection with MTDH-3'UTR-WT or -MUT psi-CHECK2 vectors. $^{**}P<0.01$.

Knockdown of PDCD4 suppresses oxygen-induced cell apoptosis and inhibits cell proliferation

To confirm whether PDCD4 is involved in oxygen-induced cell apoptosis, PDCD4 was knocked down in ATII cells. After transfection with PDCD4 siRNA, ATII cells were treated with 50 μM H_2O_2 for 48 h. Western blot results showed PDCD4 protein was significantly decreased by PDCD4 siRNA (**Figure 4A**). CCK-8 assay showed that knockdown of PDCD4 promoted ATII cell proliferation after treatment with 50 μM H_2O_2 for 24, 36 and 48 h (**Figure 4B**). Moreover, suppression of PDCD4 expression inhibited ATII cell apoptosis rates after treatment with 50 μM H_2O_2 for 24 and 48 h (**Figure 4C**). These results confirm that knockdown of PDCD4 suppresses oxygen-induced cell apoptosis and inhibits cell proliferation.

Discussion

Acute respiratory distress syndrome (ARDS) is a complicated condition of acute inflammatory lung injury resulting from a variety of predisposing conditions [2]. Inflammation-mediated injury involved in ARDS has been intensely investigated, and oxidant-mediated tissue injury is likely to be important in the pathogenesis of ARDS [27]. Reactive oxygen species (ROS) serve as signaling molecules for the evolution and perpetuation of the inflammatory process, which contributes to tissue damage [27]. Treatment with high concentrations of O_2 in ARDS patients is implicated in exacerbating

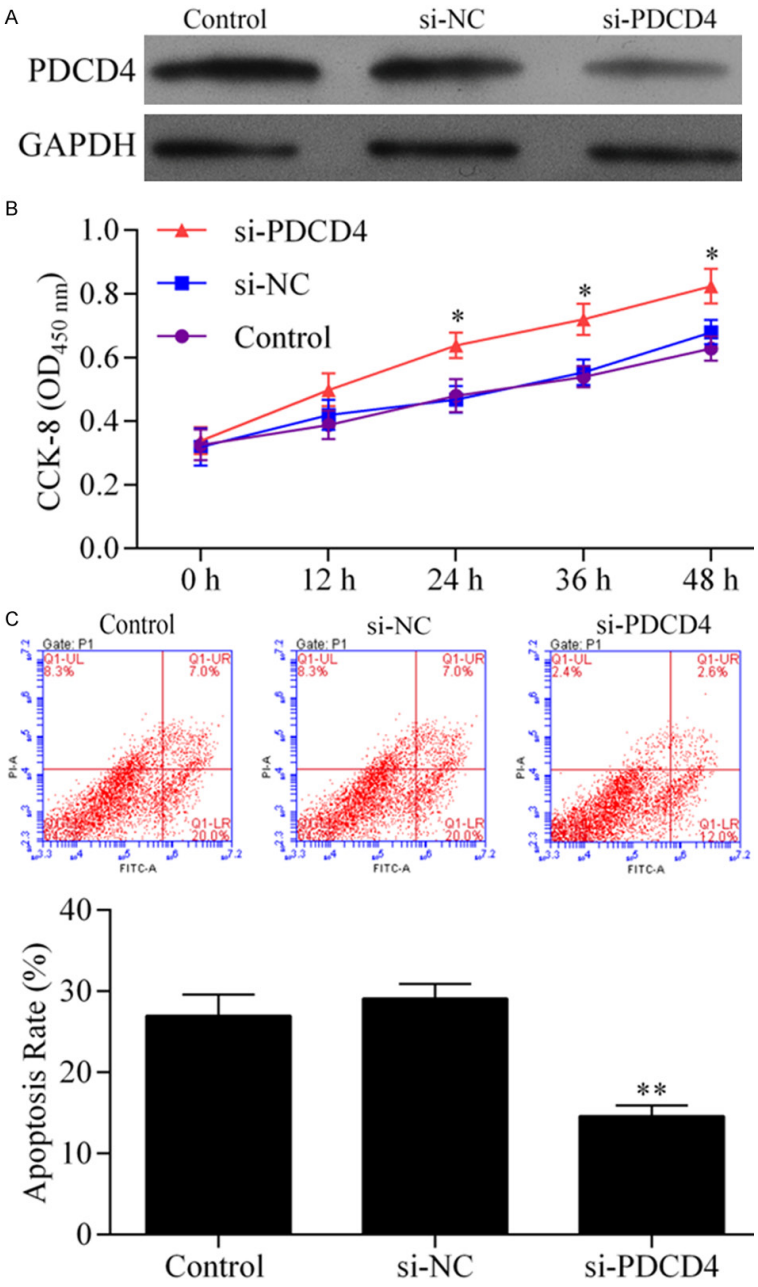

Figure 4. Knockdown of PDCD4 suppresses oxygen-induced cell apoptosis and inhibits cell proliferation. A. After transfection with PDCD4 siRNA, ATII cells were treated with 50 μM H_2O_2 for 48 h. Western blot results showed that PDCD4 protein was significantly decreased by PDCD4 siRNA. B. CCK-8 assay showed that knockdown of PDCD4 promoted ATII cell proliferation after treatment with 50 μM H_2O_2 for 24, 36 and 48 h, $^{*}P<0.05$. C. Flow cytometry showed that knockdown of PDCD4 expression was inhibited Rate of ATII cell apoptosis after treatment with 50 μM H_2O_2 for 24 and 48 h, $^{**}P<0.01$.

the primary injury [28]. We simulated ARDS *in vitro*, with 50 μM H_2O_2 to induce ATII cell damage and investigate the mechanism of oxidant-mediated lung injury.

miRNAs are a group of non-coding RNAs about 18-22 nt in length which play a pivotal role in virtually all cellular functions, including cell growth, cell apoptosis, and differentiation [15, 16]. miR-21 is located on the chromosome 17q23.2 locus [29], and shows increased expression in human cancers and other diseases [30-32]. Previous studies found that miR-21 plays important roles in the epithelial to mesenchymal transition (EMT) and in tumor metastasis [33, 34]. Yang *et al* found that overexpression of miR-21 exerts significantly protective effects on cardiac microvascular endothelial cell injury through the phosphatase and tensin homolog (PTEN) vascular endothelial growth factor (VEGF) signaling pathway [35]. Huang *et al* found that up-regulation of miR-21 is able to against cardiac hypoxia/reoxygenation-induced cell apoptosis and excessive autophagic activity in H9C2 cells through the regulation of the PTEN/Akt/mTOR signaling pathway [36]. In our present study, we found that lentivirus-mediated overexpression of miR-21 promoted ATII cell proliferation and suppressed the ATII cell apoptosis rate after treatment with H_2O_2. These results suggest that miR-21 antagonizes oxidant-mediated apoptosis in alveolar epithelial type II cells, which may provide new insight in understanding the molecular mechanisms underlying ARDS.

Programmed cell death 4 (PDCD4) is a well-known tumor suppressor, which is markedly induced by apoptosis and is being developed as a potential target in anticancer therapy [37]. Apart from the tumor studies, the biological functions of PDCD4 have also been investigated in other diseases. PDCD4 acts as a regulator of apoptosis by regulating the activator protein-1 (AP-1) in vascular smooth muscle

cells [38]. In addition, PDCD4 promotes the inflammatory response by activating the nuclear factor-κB (NF-κB) pathway and inhibiting the expression of interleukin (IL)-10 [39, 40]. Moreover, platelet-derived growth factor (PDGF)-BB promotes human orbital fibroblast cell proliferation by inhibiting PDCD4 expression via up-regulation of miR-21 [41]. However, the involvement of PDCD4 in ARDS has not been investigated, until recently. In the present study, we found that the expression of PDCD4 was induced after treatment with H_2O_2 in ATII cells. Furthermore, knockdown of PDCD4 promoted ATII cell proliferation and inhibited the rate of ATII cell apoptosis after treatment with H_2O_2. We also confirmed that PDCD4 is a direct target of miR-21. Although PDCD4 has been shown to be a direct target gene of miR-21 in other diseases, this is the first time it has been shown to regulate the relationship between miR-21 and PDCD4 in ARDS.

In the present study, we used H_2O_2 to induce ATII cell damage to simulate ARDS *in vitro*, and to investigate the mechanism of oxidant-mediated lung injury. We found that ATII cell apoptosis increased after treatment with H_2O_2. Furthermore, overexpression of miR-21 or knockdown of PDCD4 promoted ATII cell proliferation and inhibited the rate of ATII cell apoptosis after treatment with H_2O_2. In addition, we confirmed that miR-21 regulates PDCD4 expression by targeting its 3'UTR. Taken together, our data suggest that miR-21 antagonizes oxidant-mediated apoptosis in alveolar epithelial type II cells. This provides new insight in understanding the process of ARDS and provides potential new targets for the treatment of ARDS.

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Disclosure of conflict of interest

None.

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