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Original Article

miR-21-5p targets PTEN and reduces H_2O_2-induced apoptosis in rat AECII cells

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Abstract: Inhibiting apoptosis of type II alveolar epithelial cells (AECII) could effectively reduce hyperoxia-induced acute lung injuries (HALI). It was found that miR-21-5p reduced H_2O_2 induced AECII apoptosis, *in vitro*, but its specific target gene was not clear. In the present study, AECII cells were insulted with H_2O_2 to construct an *in vitro* HALI model. H_2O_2 then insulted AECII transfected with lentivirus expressing miR-21-5p or miR-21-5p inhibitors. AECII apoptosis rates were detected with flow cytometry at 0, 12, 24, and 48 hours. In addition, expression levels of miR-21-5p and PTEN in AECII were examined by quantitative real-time PCR or Western blotting 48 hours after transfection. It was found that miR-21-5p decreased and PTEN increased in HALI AECII cells, compared with that of normal ones. In addition, miR-21-5p overexpression reduced apoptosis and PTEN expression in HALI cells. Dual-luciferase reporter assay confirmed that PTEN is one of the target genes of miR-21-5p. In addition, PTEN inhibitors partially offset H_2O_2 induced AECII apoptosis. Present data indicates that miR-21-5p reduces H_2O_2 induced AECII apoptosis by targeting PTEN. miR-21-5p and PTEN could be targets for HALI treatment.

Keywords: Hyperoxia-induced acute lung injury (HALI), apoptosis, miR-21-5p, PTEN

Introduction

Hyperoxia-induced acute lung injury (HALI) is the most typical complication of oxygen therapy. It can further develop into acute respiratory distress syndrome (ARDS) or neonatal bronchopulmonary dysplasia. In addition, HALI is one of the leading causes of death and neonatal disabilities [1]. Studies have shown that hyperoxia could cause apoptosis and necrosis of alveolar epithelial cells (AEC). It has been reported that apoptosis is the key linking hyperoxia toxicity, lung injury, and survival rates in rats [2]. Type II alveolar epithelial cell (AECII) is the stem cell of AEC, prone to suffering hyperoxia attacks [3]. Inhibiting apoptosis of AECII could effectively reduce the degree of HALI in rats [4]. Therefore, regulation of AECII apoptosis might be an effective measure for prevention and treatment of HALI.

Hyperoxia-induced apoptosis is mainly mediated by reactive oxygen species (ROS). H_2O_2, a substance most suitable as a signal molecule in ROS, has been widely used to induce oxidative damage and to establish models of apoptosis. The current study found that 0.5 mmol/L H_2O_2 could successfully induce AECII apoptosis. As H_2O_2 insulting time goes on, cell survival rates decreased, while apoptosis rates increased [5].

MicroRNAs (miRNAs) are small noncoding RNAs containing 18-22 nucleotides, widely expressed in various tissues and organs of the body. They are involved in cell growth, proliferation, differentiation, apoptosis, metabolism, and other biological processes. miRNA-21 has been gradually favored in tumors [6, 7], cardiovascular [8-10], liver [11], lung [12, 13], renal [14], diabetes [15], and other aspects of research. In this study, using a high-throughput microarray method, it was found that miR-21-5p was highly expressed in AECII. Expression decreased after H_2O_2 insulting [5]. Overexpression of miR-21-5p in AECII significantly reduced its apoptosis rate [5]. In HALI rats, miR-21-5p overexpressed in the lung tissues of rats and effectively reduced HALI severity [5, 16]. Results suggest that miR-21-5p could alleviate AECII apoptosis in HALI situations and protect the lungs. However, the anti-apoptotic target genes and pathways remain unclear.

It has been reported that PTEN is one of the target genes of miR-21-5p [17]. In addition, PTEN-PI3K/Akt signaling pathways have been reported to inhibit cell cycle progression and promote apoptosis [18, 19]. The current study investigated the relationship between miR-21-5p, PTEN, and AECII apoptosis, speculating that miR-21-5p reduces AECII apoptosis by targeting PTEN.

Materials and methods

Materials

Materials included fetal bovine serum (Gibco, Cat. No. 10099-141), DMEM-low glucose medium (Gibco, Cat. No. 31600-034), penicillin-streptomycin (Hyclone Company), 0.25% trypsin (Hyclone, SH30043.01B), Annexin V-FITC/PI cell apoptosis detection kit (Kaikey biological, KGA106), TRIzol Reagent (Invitrogen Corporation), miR-21-5p overexpressing vector, miR-21-5p lentiviral vector, miR-21-5p negative lentiviral vector (Shanghai Jikai Biological Technology Co., Ltd), QuantiTect SYBR Green PCR kit (TaKaRa), PTEN antibody, horseradish peroxidase (HRP) labeled secondary antibody, rabbit anti-human β-actin (Santa Cruz Company), IP cell lysates (P0013), BCA protein quantification kit, SDS-PAGE gel preparation kit (Beyotime Biotechnology, Shanghai, China), PYr-Mirna target plasmid (Jikai Gene Biotechnology Co., Ltd, Shanghai, China), ECL kit, Liposome 2000 (Lipofectamine 2000), plasmid extraction kit, dual-luciferase assay kit (Invitrogen Company), miR-21-5p mimic, NC membrane (Shanghai Ji Ma company), and phen (PTEN inhibitor), a product of Merck (Germany). Primers were synthesized by Shanghai Sangon Biology (Shanghai, China). Unlisted reagents were of analytical grade.

Instruments

Instrumentation included a FACS Calibur flow cytometer (BD Biosciences, USA), inverted fluorescence microscope (Nikon, Japan), fluorescence quantitative PCR instrument (Bio-Rad, USA), fluorescence spectrophotometer (RF-5301PC, PerkinElmer, Japan), full-wavelength fluorescent microplate reader (TECAN Safire, Austria), enzyme-linked immunosorbent assay (Thermo Fisher Scientific, multi-scan MK3), gel image processing system (Image-Pro Plus 6.0, Media Cybernetics, USA), and dual-luciferase detection instrument (Berthold, Germany).

Cell culture and grouping

AECII cells were purchased from Shanghai Jikai Gene Biotechnology Co., Ltd. They were cultured in 10% fetal bovine serum DMEM-low glucose medium in a 37°C, 95% air humidity, and 5% CO_2 incubator. AECII cells were seeded into five six-well plates and incubated for 24 hours. AECII cells were randomly divided into 5 groups: Control: Cells were pre-incubated for 24 hours and the cells were incubated with an equal volume of PBS; H_2O_2: Cells were pre-incubated for 24 hours and added 0.5 mmol/L H_2O_2; H_2O_2 + miR-21-5p: After transfection of lentivirus with miR-21-5p over-expression vector for 24 hours, 0.5 mmol/L H_2O_2 was added; H_2O_2 + vector: After transfection of miR-21-5p lentiviral negative vector for 24 hours, 0.5 mmol/L H_2O_2 was added; H_2O_2 + miR-21-5p inhibitor: After transfection of lentivirus with miR-21-5p inhibition vector for 24 hours, 0.5 mmol/L H_2O_2 was added. Apoptosis rates of AECII were detected at 0, 12, 24, and 48 hours after H_2O_2 incubation.

AECII cell identification by transmission electron microscope

AECII cells were identified by a transmission electron microscope, as previously reported [5]. AECII cells were incubated for 36 hours and digested with 0.125% trypsin/EDTA. Cell suspension was collected and centrifuged at 1,000 rpm for 10 minutes at 4°C. The supernatant was removed and cells were fixed with 4% glutaraldehyde for 24 hours. The cell pellet was transferred to a vial and rinsed three times for 10 minutes at 4°C in PBS. It was fixed at 4°C for 30 minutes in 1% osmium tetroxide and rinsed three times with PBS. It was then observed using a transmission electron microscope (TEM).

Apoptosis detection by FCM

Flow cytometry with Annexin V-FITC/PI staining was used to evaluate early necrosis and apoptosis of AEC-II cells [10]. Annexin V- FITC and propidium iodide (PI) staining kit (BD Biosciences, USA) was used to estimate levels of phosphatidylserine on the surface of AEC-II cells, according to manufacturer instructions. Using a FACSCalibur flow cytometer (BD Biosciences, USA), necrosis and apoptosis of AEC-II cells were studied.

AEC-II cells were seeded in 6-well plates. The cells were digested with 1 mL 0.125% trypsin/EDTA for 2-3 minutes. Digested cells were centrifuged, removing their supernatant. Cell density was adjusted to 2 × 10^5/mL and 100 μL cell suspensions were added into 24-well plates and cultured in the incubator. The supernatant was discarded and the 100 μL medium was added. The virus solution valued MOI 60 was obtained using 12 μL 1 × 10^8 TU/mL lentivirus. It was mixed gently and added with 2 μL 5 μg/mL hexamethylenediamine bromide. After 24 hours of transfection of AECII, cell injury was induced with 0.5 mmol/L H_2O_2 and cell suspension was prepared at 0, 12, 24, and 48 hours after injury. Cells were collected by centrifugation (1,000 rpm, 5 minutes), washed twice with PBS (1,000 rpm, 5 minutes), and added with 500 μL binding buffer. Five μL Annexin V-FITC was added and mixed, then 5 μL Propidium Iodide was added and mixed at room temperature without light. It reacted for 5-15 minutes. Apoptosis was observed and detected by flow cytometry 1 hour later.

miR-21-5p and PTEN mRNA detection with real-time quantitative PCR

After incubation for 24 hours by H_2O_2, cells of each group were all washed twice with PBS buffer. Next, 500 μL TRIzol cytoplasm lysis solution was added into each well. It was incubated at room temperature for 5 minutes and centrifuged for 5 minutes (12,000 rpm, 4°C). The supernatant was transferred into the new tube and 0.1 mL chloroform was added and mixed. It stayed at room temperature for 5 minutes and was centrifuged for 15 minutes (12,000 rpm, 4°C). The supernatant was collected. An equal volume of isopropanol was added, slightly mixed by inverting. It stayed at room temperature for 10 minutes and was centrifuged for 10 minutes (12,000 rpm, 4°C). The supernatant was discarded and 1 mL 75% ethanol was used to wash precipitation. It was then centrifugated for 5 minutes (12,000 rpm, 4°C). The supernatant was discarded and an appropriate volume of DEPC water was added to dissolve the RNA pellet. Absorbance values were measured by an ultraviolet spectrophotometer at 260 nm and 280 nm (OD_{260} and OD_{280}). OD_{260}/OD_{280} values, as well as RNA concentrations, were then calculated. The real-time quantitative PCR (qPCR) system was as follows: 37°C, 15 minutes; 85°C, 5 seconds, stored at -20°C. The qPCR reaction system was as follows: miR-21-5p: 95°C, 5 minutes; 39 cycles, 95°C, 45 seconds, 60°C, 30 seconds, 72°C 45 seconds; 72°C 10 minutes. PTEN mRNA: 94°C. 5 minutes; 39 cycles, 94°C, 45 seconds, 51°C, 30 seconds, 72°C, 44 seconds; 72°C, 10 minutes. β-actin: 94°C, 5 minutes; 39 cycles, 94°C 45 seconds, 60°C, 30 seconds, 72°C, 44 seconds; 72°C 10 minutes. Ct values of each target gene and β-actin gene were determined. Three parallel wells were conducted in all samples to reduce the error of operation. Relative quantification of target genes was calculated by the $2^{-\Delta\Delta CT}$ method. Primer sequences were miR-21-5p: F: 5'-GTCAATAGCTTATCAGACTGA-3'; R: 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAACA-3'. PTEN: F: 5'-TTTGAAGACCATAACCCACCAC-3'; R: 5'-ATTACACCAGTTCGTCCCTTTC-3'; β-actin: F: 5'-TTCCTCCGCAAGGATGACACGC-3'; R: 5'-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACAAAAATAT-3'.

PTEN protein expression detection with Western blotting

Western blotting experiments were conducted with standard procedures, as previously reported [20]. The culture medium was removed. Cells in each plate were washed with 3 mL 4°C pre-cooled PBS 3 times for 1 minute and lysed using 400 μL lysis buffer containing 100 mmol/L PMSF under ice for 30 minutes. Cells were then collected and centrifuged at 4°C and 12,000 rpm for 5 minutes. The supernatant was obtained and stored at -20°C. The BCA method was used to detect protein concentrations, according to instructions. Protein was separated by 10% SDS-PAGE gel electrophoresis. Proteins were transferred to PVDF membranes and blocked with 5% skim milk at room temperature for 1 hour. They were then incubated with the primary antibody (1:2000) overnight at 4°C. They were washed with PBST for 10 minutes, a total of 3 times. They were then incubated with a secondary antibody (1:5000) at room temperature for 1 hour. Next, they were washed with PBST for 10 minutes, a total of 3 times. ECL was used to detect results. Strip optical density values were analyzed using a gel image processing system (Image-Pro plus 6.0).

Dual-luciferase gene assay

Dual-luciferase reporter assays were conducted, as previously reported [21], to observe if

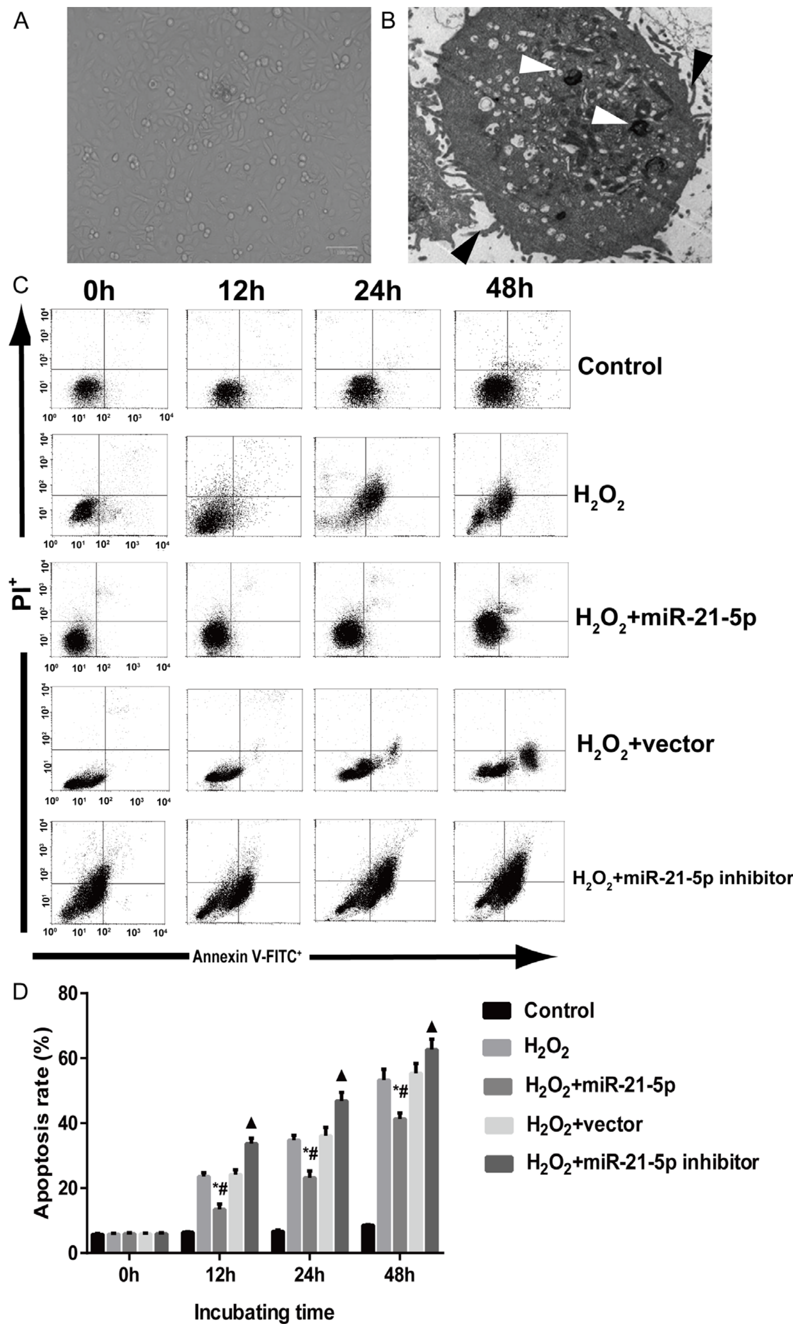

Figure 1. Morphology of AECII cells and miR-21-5p reduce H_2O_2 induced apoptosis in AECII cells. (A, B) AECII morphology from microscopy (A, ×200) and TEM (B, white arrow heads showed osmiophiiic multilamellar body of type II alveolar epithelial cells and black arrow heads showed the microvilli). (C, D) Flow cytometry shows that miR-21-5p reduce H_2O_2 induced apoptosis in AECII cells. H_2O_2 induced apoptosis in AECII cells and miR-21-5p treatment effectively reduced apoptosis rates (mean ± SD, n = 8). *, $P < 0.05$ compared with H_2O_2 group. #, $P < 0.05$ compared with H_2O_2 + vector group. ▲, $P < 0.05$ compared with H_2O_2 group.

miR-21-5p directly targets PTEN genes. PTEN-3U and PTEN-3U-M plasmid vectors were constructed by inserting them into the pMIR-luciferase vector. Primers were: PTEN 3' UTR wild type: F: 5'-TTGTGGCAACAGATAAGTTTGCAGTTGGCTAAGAGAGGTT-3'; R: 5'-CATTCCCCTAACCCGAATACATGCATTAGAATGTAGCAAA-3'; PTEN 3'UTR mutant: F: 5'-TTGTGGCAACAGCTGAATCTGCAGTTGGCTTAAGAGAGGTT-3'; R: 5'-ATGTAGCAAAACCCTTCGGAAACCTCTCTTAGCCAACTGC-3'. Synthesized plasmids were transformed into *E. coli* to obtain positive clones. Plasmids were extracted and purified for use.

HEK293T cells were used as the dual-luciferase reporter gene assay cell. Cells were cultured in DMEM medium (containing 10% fetal bovine serum, 2 mmol/L glutamine, 100 U streptomycin) at 5% CO_2 and 37°C incubation. PTEN-3U, miR-21-5p mimics, and internal control vectors transfected HEK293T cells separately, in which PTEN-3U-W, NC were used as control groups. The ratio of each well was: Firefly: Renilla: Transfection reagent = 0.1 μg: 0.01 μg: 0.25 μL. The transfection process was carried out according the instructions of Invitrogen Lipofectamine 2000. Later, 100 μL PLB solution was added into the transfected cells to lysis at room temperature for 15 minutes. Relative luciferase activity was measured and calculated.

Statistical analysis

SPSS 20.0 statistical software was used for analysis. Data with normal distribution are expressed as mean ± standard. One-way ANOVA and post-

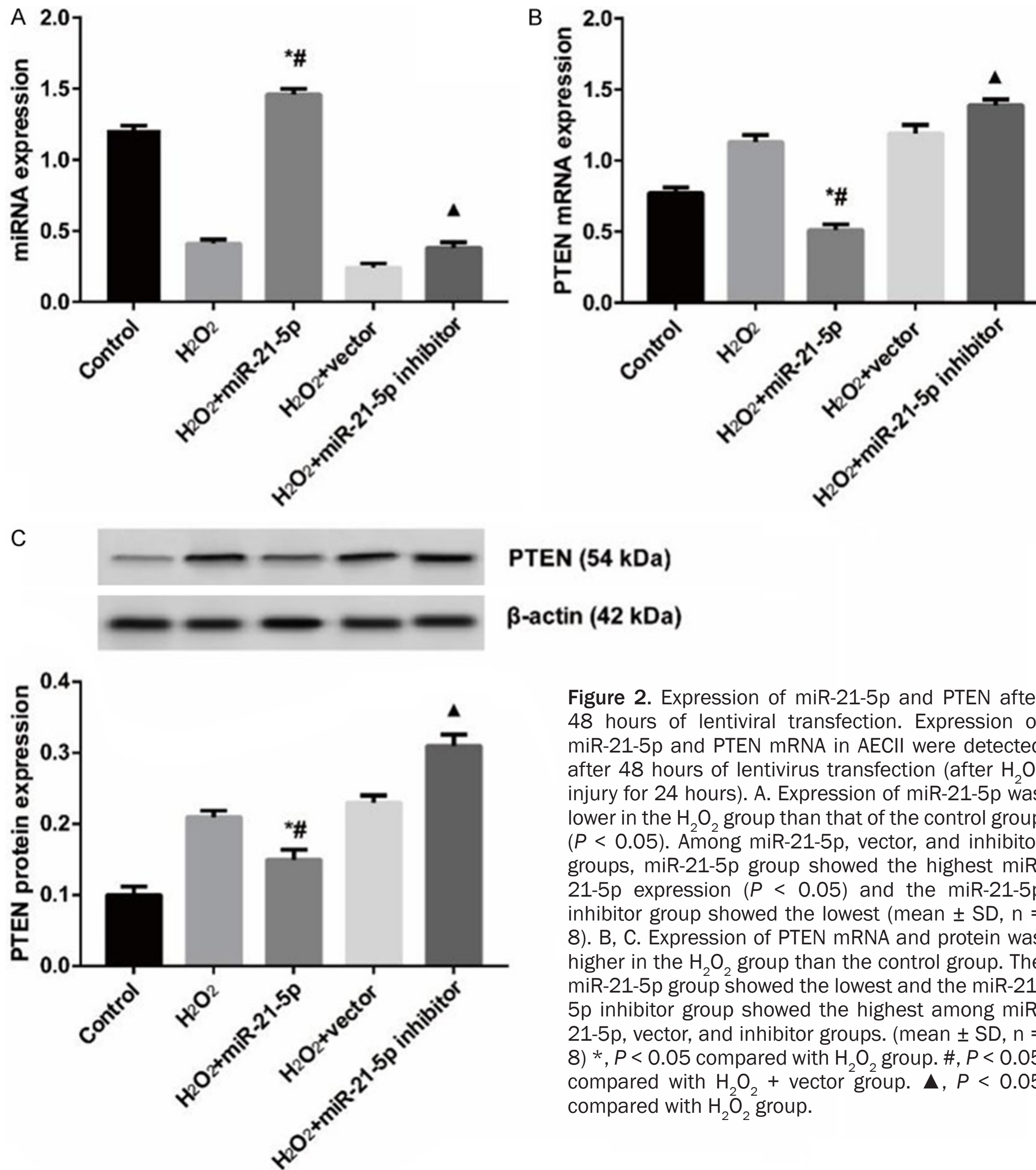

Figure 2. Expression of miR-21-5p and PTEN after 48 hours of lentiviral transfection. Expression of miR-21-5p and PTEN mRNA in AECII were detected after 48 hours of lentivirus transfection (after H_2O_2 injury for 24 hours). A. Expression of miR-21-5p was lower in the H_2O_2 group than that of the control group ($P < 0.05$). Among miR-21-5p, vector, and inhibitor groups, miR-21-5p group showed the highest miR-21-5p expression ($P < 0.05$) and the miR-21-5p inhibitor group showed the lowest (mean ± SD, n = 8). B, C. Expression of PTEN mRNA and protein was higher in the H_2O_2 group than the control group. The miR-21-5p group showed the lowest and the miR-21-5p inhibitor group showed the highest among miR-21-5p, vector, and inhibitor groups. (mean ± SD, n = 8) *, $P < 0.05$ compared with H_2O_2 group. #, $P < 0.05$ compared with H_2O_2 + vector group. ▲, $P < 0.05$ compared with H_2O_2 group.

hoc Dunnett's T3 method were conducted to compare differences among groups. Data of dual-luciferase experiment were analyzed with multivariate analysis of variance. $P < 0.05$ indicates statistically significant differences.

Results

Identification of AECII cells

As shown in **Figure 1A**, AECII cells showed a spindle-shape and adherent growth. TEM showed the characteristics of osmiophilic lamellar bodies and microvilli detected by TEM (**Figure 1B**).

H_2O_2 induced apoptosis in AECII and miRNA-21-5p expression affected apoptosis rates

Results showed that AECII apoptosis rates gradually increased with H_2O_2 incubation times ($P < 0.05$). The apoptotic rate of H_2O_2 insulted group was significantly higher than that of the control group ($P < 0.05$). The apoptosis rate of the miRNA-21-5p group was significantly lower than that of the vector group and miR-21-5p

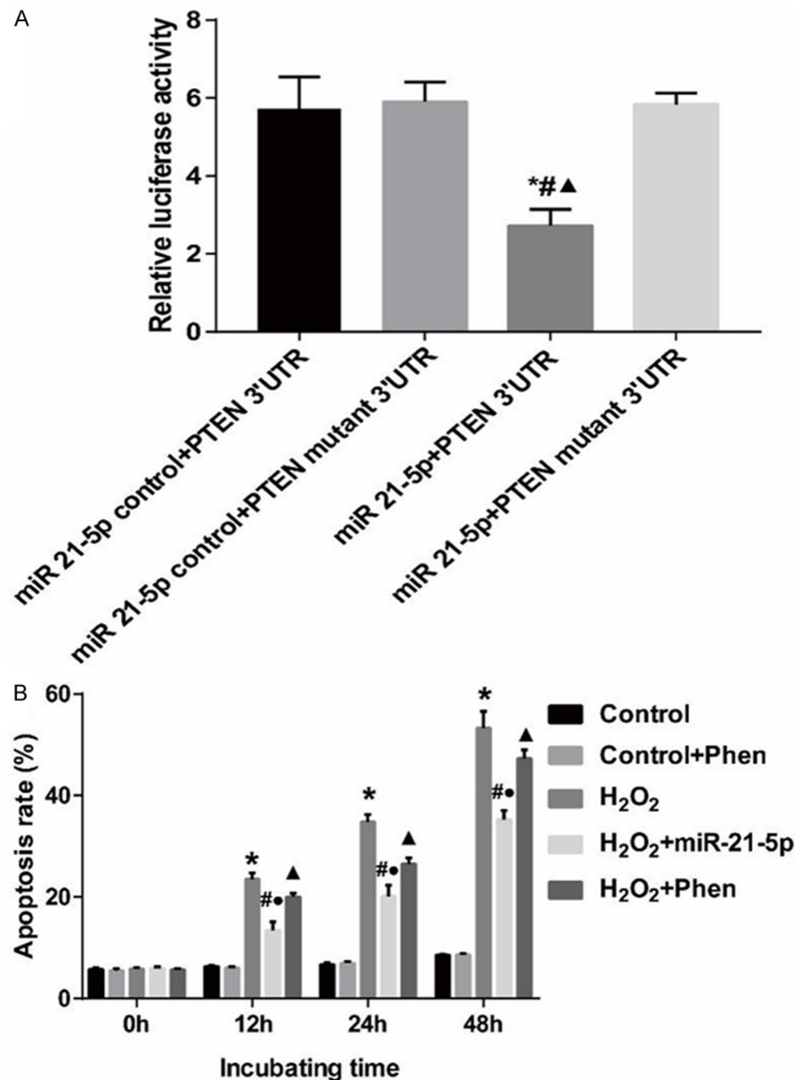

Figure 3. miR-21-5p targets PTEN and reduces H_2O_2 induced AECII apoptosis. A. The dual-luciferase reporter assay of mi-R21-5p. HEK293T cells were used in the dual-luciferase reporter gene assay. The relative ratio of luciferase was higher in NC + PTEN-3U group, NC + PTEN-3U-M group, and mimic + PTEN-3U-M group than the mimic + PTEN-3U group. Besides, there was no significant difference in the relative ratio of luciferase among NC + PTEN-3U group, NC + PTEN-3U-M group, and mimic + PTEN-3U-M group (mean ± SD, n = 5). B. PTEN inhibitor reduces apoptosis of H_2O_2 insulted AECII. PTEN was blocked with its specific inhibitors PHEN. PHEN administration partially reduced H_2O_2 induced apoptosis of AECII, but its anti-apoptosis effects were lower than those of miR-21-5p at the same time points (mean ± SD, n = 5).

inhibitor group ($P < 0.05$). Moreover, the apoptosis rate of miR-21-5p inhibitor group was higher than the vector group at the same time points ($P < 0.05$, **Figure 1D**).

HALI cells showed reduced miR-21-5p and increased PTEN expression and miR-21-5p expression affects PTEN expression

Expression of miR-21-5p and PTEN mRNA in AECII cells was detected after 48 hours of lentivirus transfection (after H_2O_2 injury for 24 hours). Results showed that expression of miR-21-5p was lower in the H_2O_2 group than that of the control group ($P < 0.05$). Concerning the miR-21-5p, vector, and inhibitor groups, the miR-21-5p group showed the highest miR-21-5p expression ($P < 0.05$), while the miR-21-5p inhibitor group showed the lowest ($P < 0.05$) (**Figure 2A**).

As shown in **Figure 2B**, expression of PTEN mRNA was higher in the H_2O_2 group than the control group ($P < 0.05$). In addition, expression of PTEN mRNA was the lowest in the miR-21-5p group ($P < 0.05$), while the miR-21-5p inhibitor group showed the highest ($P < 0.05$) among miR-21-5p, vector, and inhibitor groups.

miR-21-5p reduced PTEN protein expression

Results of expression of PTEN protein, detected by Western blotting after 48 hours of transfection (24 hours after H_2O_2 injury), showed that expression was higher in the H_2O_2 group than the control group ($P < 0.05$). Expression was the highest in the miR-21-5p group ($P < 0.05$), while expression in the miR-21-5p inhibitor group was the lowest ($P < 0.05$, **Figure 2C**).

Dual-luciferase reporter gene assay indicates miR-21-5p target PTEN

Relative luciferase activity was higher in the NC + PTEN-3U group, NC + PTEN-3U-M group, and mimic + PTEN-3U-M group than in the mimic + PTEN-3U group ($P < 0.05$). Moreover, there were no significant differences among NC + PTEN-3U, NC + PTEN-3U-M, and mimic + PTEN-3U-M groups ($P > 0.05$) (**Figure 3A**).

PTEN inhibitor reduces apoptosis of H_2O_2 insulted AECII

Aiming to examine the mechanisms responsible for miR-21-5p mediated anti-apoptosis effects in AECII, PTEN was blocked with its specific inhibitors. Phen administration partially reduced H_2O_2 induced apoptosis of AECII, but its anti-apoptosis effects were lower than those of miR-21-5p at the same time points (all $P < 0.05$, **Figure 3B**).

Discussion

It has been proven that miR-21-5p is highly expressed in AECII cells. Expression decreases when cells undergo H_2O_2 insult [5]. However, the specific mechanisms remain unclear. In the present study, miR-21-5p overexpression with plasmids expressing miR-21-5p in AECII cell lines reduced 0.5 mmol/L H_2O_2 induced apoptosis. Moreover, miR-21-5p overexpression reduced PTEN mRNA and protein expression. Dual-luciferase assay confirmed that PTEN is one of the target genes of miR-21-5p. Results indicate that miR-21-5p targets PTEN and reduces HALI *in vitro*.

The present study found that miR-21-5p expression decreased when ACEII cells underwent H_2O_2 insult, indicating that miR-21-5p may be correlated with H_2O_2 induced apoptosis in ACEII. PTEN mRNA and protein expression levels were negatively correlated with expression levels of miR-21-5p, indicating that PTEN could be one of the target genes of miR-21-5p. This study proved that miR-21-5p directly targets PTEN, according to the dual-luciferase reporter gene assay. Meng et al. found that, in human hepatocellular carcinoma cells, PTEN expression decreased and miR-21 increased, while downregulation of miR-21 could increase expression of PTEN, as well as decrease proliferation, invasion, and metastasis of hepato-carcinoma cells [22, 23]. Ou et al. [24] found that miR-21 could inhibit the apoptosis of nasopharyngeal carcinoma cells (CNE). Results of dual-luciferase reporter assay showed that miR-21-5p targeted PTEN mRNA 3' UTR.

The current study blocked PTEN with its inhibitors PHEN. Just like the anti-apoptotic effects of miR-21-5p, PHEN administration decreased H_2O_2 induced apoptosis in AECII (**Figure 3C**). The PTEN inhibitor did not completely offset anti-apoptotic effects of miR-21-5p. This is reasonable because miR-21-5p targets more than one gene and PTEN related pathways.

There has been much miRNA-21-related research related to gene therapy. It has been applied to clinical, but most studies were applied to tumor disease. James Devaney believed that gene therapy is an effective treatment for ARDS/ALI [25]. Alveolar epithelial cells could be targeted not only selectively through endotracheal intubation management [26], but also according to antibodies, pulmonary endothelial cell surface antigens, and gene carrier complex formation, selectively targeting intravenous injections [27]. This provides a theoretical basis for the operability of ARDS/ALI gene therapy. Although protective mechanisms of miR-21-5p on the lungs are still in the stage of laboratory research, it is believed that, with the elucidation of anti-apoptotic mechanisms of miR-21, miR-21 related molecular therapy might bring new opportunities in solving clinical problems.

The current study indicates that miR-21-5p reduces H_2O_2 induced AECII cell apoptosis by targeting PTEN. miR-21-5p and PTEN could be targets for HALI treatment.

Disclosure of conflict of interest

None.

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