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Original Article

microRNA-146a relieves smoking relative chronic obstructive pulmonary disease through targeting ATG12

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Abstract: Tobacco smoke is a primary risk factor of chronic obstructive pulmonary disease (COPD) through exacerbating local inflammation and oxidative stress response and impairing autophagy in the lung. MicroRNA-146a (miR-146a) has been identified to be lowly expressed in lung fibroblasts of smokers with COPD, resulting in chronic inflammation. Moreover, bioinformatics analysis shows that human autophagy related 12 gene (ATG12) is a predicted target of miR-146a. Therefore, the present study was performed to investigate the effect of miR-146a on the progression of COPD-induced by smoking, and explore whether ATG12 takes part in this process. The results show that miR-146a expression was decreased in the blood samples of COPD patients, which was positively associated with the value of FEV1/FVC%. Cigarette smoke extract (CSE) decreased the expression of miR-146a, while increased cleaved (C)-Caspase 3/9 expression and decreased cell viability, as well as promoted the production of reactive oxygen species (ROS) and malondialdehyde (MDA) in a concentration-dependent manner in lung epithelial BEAS-2B cells. In contrast, these effects were weakened when miR-146a was upregulated. miR-146a negatively regulated ATG12 expression in BEAS-2B cells via binding to the 3'UTR of ATG12 mRNA. ATG12 promoted cell viability and repressed cell apoptosis and oxidative stress mediated by miR-146a in BEAS-2B cells. Furthermore, miR-146a overexpression mitigated lung injury and reduced the production of ROS and MDA in smoking-induced COPD mice through down-regulating ATG12. Together, this study demonstrates that miR-146a is beneficial for the recovery of lung injury in smoking induced COPD through inhibiting the oxidative stress via ATG12 downregulation.

Keywords: miR-146a, ATG12, oxidative stress, smoking, chronic obstructive pulmonary disease

Introduction

COPD is a progressive disease with increasing morbidity and mortality rates, and is considered as the third leading cause of mortality worldwide [1, 2]. COPD is mainly triggered by cigarette smoking and is characterized by chronic inflammation, decreased lung function, and reduced airflow [3]. Evidence suggests that smoking initiates local inflammation and excessive oxidative stress response in the lung, leading to the production of a high concentration of oxidants and ROS, which is considered as one of the major mechanisms of COPD [4]. Notably, cells can be protected against oxidative stress by enzymatic and non-enzymatic antioxidant systems [5], such as carbocysteine, carotenoids, glutathione, vitamins C/D/E, peroxidases, catalases, and superoxide dismutases [6, 7]. Therefore, it is essential to decipher the mechanism by which ROS triggers the cytoprotective antioxidant responses and find out a potential method for this disease treatment [8].

MicroRNAs (miRs) are a class of non-protein coding RNAs with 18-25 nucleotides, that regulate approximately 30% of human protein-coding genes by directly combining with the 3' untranslated region (UTR) of the messenger RNA (mRNA) of their target genes, causing translational repression and/or mRNA degradation [9]. Through altering gene expression, miRs regulate multiple cellular activities such as survival, apoptosis, proliferation, cell cycle, and migration in many kinds of diseases including cancers, cardiovascular dysfunctions, and lung diseases [10-12]. miR-146a was recently reported to be downregulated in lung fibroblasts isolated from the parenchymal tissues of smokers with COPD compared with that of the non-COPD smokers, after stimulation with inflammatory cytokines, such as interleukin (IL)-1β and tumor

Table 1. Information of the participants

Group	Cases	Gender (F/M)	Age (year)	Smoking (year)	Course of disease (year)
Non-smoking COPD	1	F	68.6	0	3.1
	2	M	52.2	0	2.2
	3	F	46.0	0	7.0
	4	M	64.2	0	2.5
	5	F	48.9	0	6.1
	6	M	46.7	0	3.5
	7	M	68.0	0	4.0
	8	M	52.0	0	5.2
	9	F	46.5	0	4.5
	10	M	52.2	0	5.0
Smoking COPD	11	F	58.6	9.0	6.6
	12	M	56.3	5.8	5.8
	13	M	55.3	7.9	6.1
	14	M	55.5	9.3	4.3
	15	F	45.7	8.2	2.1
	16	M	49.9	6.3	3.2
	17	M	41.3	6.5	3.1
	18	F	58.4	8.5	6.5
	19	M	48.2	5.2	3.8
	20	M	55.7	8.3	7.4

F: Female; M: Male.

necrosis factor (TNF)-α [13]. Moreover, they also revealed that miR-146a downregulated the level of prostaglandin E2, an inflammatory mediator, through targeting cyclooxygenase-2 (COX-2) [14]. Additionally, Osei et al. [15] suggested that the low expression of miR-146a-5p in COPD fibroblasts was inclined to display a more pro-inflammatory phenotype, which then contributes to the pathogenesis of COPD. In this view, miR-146a might play a vital role in inhibiting the progression of COPD through repressing inflammatory reactions. In Addition, Xie et al. [16] recently proposed that the potential of miR-146a as a negative comprehensive indicator of inflammation and oxidative stress status in the brain of chronic type 2 diabetes mellitus rats, suggesting miR-146a exerted a inhibitory role for oxidative stress. However, the effects of miR-146a in the oxidative stress of smoking induced COPD remain largely unclear, as well as its underlying mechanism.

In addition, recent studies have suggested that cigarette smoke-induced ROS-activation impairs autophagy, leading to the accumulation of mis-folded or damaged proteins as peri-nuclear aggresome-bodies [17, 18] and then the occurrence and progression of COPD [19]. Using bioinformatics analysis, miR-146a was found to bind to the 3'UTR of the mRNA of ATG12 gene (**Figure 5C**), demonstrating that miR-146a might be involved in the smoking-induced COPD through regulating the expression of ATG12.

This study was performed to probe the effect of miR-146a in the progression of COPD induced by tobacco smoke *in vivo* and *in vitro* through measurement of the oxidative stress markers and to explore whether ATG12 is involved in.

Materials and methods

Patient

Ten patients with COPD diagnosed referring to the Global Initiative for COPD guideline criteria [3] were admitted in our hospital from May 2015 to May 2016 and were selected as the smoking COPD group. All of the 10 cases of COPD patients had smoking history for at least 5 years. Asthma patients were excluded from this study. Additionally, ten COPD patients without smoking history (non-smoking COPD group) were included as the control. There were no significant differences of age and gender between the two groups. All patients involved in the study completed the whole study. The plasma samples from smoking COPD patients and non-smoking COPD patients were collected at 8:00 in the morning on an empty stomach. The main information for smoking COPD patients and non-smoking COPD patients are listed in **Table 1**. Experiments in this study involving human samples were performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Sichuan Provincial People's Hospital.

Pulmonary function tests

Pulmonary function tests were performed as described in a previous study [20]. Total lung capacity and FEV1/FVC were measured referring to the Buxco resistance/compliance application manual.

Detection of oxidative stress reaction

The current study used MDA and ROS to evaluate the oxidative stress reaction of BEAS-2B cells and the patients with COPD or healthy individuals.

The malondialdehyde (MDA) content in cell lysis or blood samples of human was tested by thiobarbituric acid methods using kit provided by NanJing JianCheng Bio-engineering institute (Nanjing, Jiangsu, China). Absorbance value of each well was measured at 532 nm with a microplate reader purchased from Bio-Tek Instruments (VT, USA). ROS production in cells was detected by staining BEAS-2B cells with an oxidation-sensitive fluorescent probe 2, 7-dichlorodihydrofluorescein diacetate (DCFH-DA) in accordance with the manufacturer's description. BEAS-2B cells were given 1% or 5% CSE for 48 hours at 37°C. Then, cells were incubated with 1 ml of DCFH-DA (Sigma-Aldrich, MO, USA) solution at 37°C for 20 minutes. Subsequently, cells were collected and washed with PBS for twice. Finally, the fluorescence intensity was detected by a microplate reader at 488 nm excitation and 525 nm emission wavelengths.

Preparation of cigarette smoke extract (CSE)

CSE was obtained from cigarette according to previous study [21, 22]. In briefly, a 10% of CSE was prepared by bubbling smoke from one cigarette purchased from China Hunan Industrial Co., Lto (Hunan, China) (tar, 10 mg/cigarette; and nicotine, 1.0 mg/cigarette) into 10 ml of cell culture medium at a rate of one cigarette/2 min. Then the suspension was adjusted to PH value of 7.4 using 1 mol/L NaOH. Then the suspension was filtrated and sterilized with a 0.22 μm filter. CSE suspension was calibrated by assessing the absorbance (optical density (OD) = 0.86 ± 0.05) at 320 nm. CSE was freshly prepared for each experiment and used within 30 min. 1% CSE was prepared using 5% CSE with 5-fold dilution in culture medium.

Cell culture condition and treatment

Human normal lung epithelial cell line BEAS-2B was brought from American Type Culture Collection (ATCC, VA, USA) and cultured in BEGM (Kit Catalog No. CC-3170, Lonza/Clonetics Corporation, Basel, Switzerland) without the GA1000 (gentamycin-amphotericin B mix) and incubated in a humidified incubator filling with 5% CO_2 at 37°C. For CSE treatment, BEAS-2B cells were incubated with 1% or 5% CSE for 48 hours, then the cells were harvested for further study.

Cell transfection

Mimics or inhibitors targeting human miR-146a gene (Guangzhou RuiBo Company, Guangzhou, Guangdong, China) were transfected to BEAS-2B cells at 50-60% confluence with Lipofectamine 2000 (Invitrogen, CA, USA) to upregulate or downregulate expression of miR-146a. In the same way, overexpressing plasmid of ATG12 (OE-ATG12) and small interfering RNAs (siRNAs) (si-ATG12) obtained from OriGene (CA, USA) were used to up/down-regulate ATG12 expression.

Real-time PCR analysis

Total RNA samples were obtained from BEAS-2B cells or COPD patients' or healthy individuals' blood samples using GenElute™ Total RNA Purification Kit (Sigma-Aldrich, MO, USA). Then the total RNA samples were synthesized into the cDNA using Solar® mRNA Enrichment Kit (A&D Technology™, Beijing, China). Next, the cDNA was used to analyze the expression of miR-146a and U6 with TaqMan RT-PCR kit (Applied Biosystems, Foster City, CA, USA). Relative quantification of mRNA levels was evaluated by the $2^{-\Delta\Delta Ct}$ method [23]. The relative quantity of miR-146a was normalized by U6 level.

Western blotting analysis

Total protein was extracted from BEAS-2B cells by using radioimmunoprecipitation lysis buffer (KenGEN, Nanjing, Jiangsu, China) at 4°C. Then the mixed liquor was centrifuged for 30 minutes at 4°C at a speed of 12,000 g. After quantification with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, MA, USA), 30 μg of proteins from each sample were separated by 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Merck, Darmstadt, Germany). Subsequently, the membranes were sealed with 5% non-fat milk [diluted in Tris buffered saline with 0.1% Tween-20 (TBST)] for 1 hour at room temperature, and probed with the indicated primary antibodies against total caspase-3 (T-caspase-3) (1:1000

dilution; No. ab13847, Abcam, CA, USA), T-caspase-9 (1:500 dilution; No. ab25758, Abcam, CA, USA), cleaved capase-3 (C-caspase-3) (1:1000 dilution; No. #9661, Cell Signaling Technology, CA, USA) and C-caspase-9 (1:1000 dilution; No. ab2324, Abcam, CA, USA), ATG12 (1:2000 dilution; No. #2010, Cell Signaling Technology, CA, USA) and GAPDH (1:5000 dilution; Thermo Fisher Scientific, MA, USA) for whole night at 4°C. Next day, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The protein expression was determined by ECL regent (Millipore, CA, USA) and quantified with Image J software.

3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay

BEAS-2B cells were seeded overnight in 96 well-plates at a density of 3×10^3 cells/well, then the cells were treated with 1% CSE or 5% CSE for 48 hours. During the last 4 hours of incubation, cell culture medium was replaced by 100 μL complete cultural medium and 10 μL MTT solution (5 mg/mL) to make formazan form, then 100 μl DMSO was added to each well. At the end, cell viability was evaluated with the OD values measured at 570 nm with a microplate reader (Bio-Tek Instruments, VT, USA).

Flow cytometry assay

For cell apoptosis detection, Annexin V Apoptosis Detection kit (BD Biosciences, San Diego, CA, USA) was recruited. In detail, cells were trypsinized with 0.25% of EDTA free trypsin (Thermo Fisher Scientific, MA, USA) and incubated with 5 μl of Annexin V and 5 μl PI solution for 15 minutes diluted in 100 μl 1X binding buffer in the dark. Then, the cells were washed with 1X binding buffer for three times and resuspended with 500 μl of 1X binding buffer. Cells were measured with a Beckman FC500 flow cytometer (Beckman Coulter, Inc., Brea, California, USA) and analyzed by FlowJo 7.6 software.

Fluorescent gene reporter assay

BEAS-2B cells were transiently co-transfected with the luciferase reporter plasmid encoding the 3'UTR of ATG12 (wild type) or the plasmid with binding site mutated (MT) (GenePhar-ma, Shanghai, China) with mimics or mimic-NC. Then luciferase activities were determined after 48-hours of cell transfection using the Dual-Luciferase Reporter Assay System (Promega, WI, USA) in accordance with the manufacturer's description. Renilla luciferase activity was used to normalize the luciferase activity of firefly.

Construction of animal COPD model

C57BL/6 male mice purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were used to build in vivo COPD model, according to previous study [24]. The mice were divided into 4 groups: Control group, COPD group, COPD + mimic group and COPD + mimic + OE-ATG12 group, with 6 mice in each group. For COPD model construction, mice were exposed to smoke twice a day with 5 cigarettes at 8:00 am and 5 cigarettes at 4:00 pm, respectively. Smoking duration was more than 1 hour for a total of 5 weeks. The COPD + mimics group was intraperitoneally injected with 50 μM miR-146a (diluted in 200 μL PBS) for 2 times per week. COPD + mimic + OE-ATG12 group was given 50 μM mimics-miR-146a and 25 μM OE-ATG12 intraperitoneally injection (diluted in 200 μL PBS). Control group without passive smoking (n=6) and COPD group (n=6) was given 200 μL of PBS in the same way as control. All treatment was performed 1 hour after smoke exploration every week until mice were killed. All mice were sacrificed until 36 days, then the lung tissues and blood samples were collected for pathology and the examination of ROS and MDA, respectively. Protocols involving animals were performed in accordance with the principles and procedures of the NIH Guide and were approved by the Ethics Committee of Sichuan Provincial People's Hospital.

Histopathological analysis

Lung tissues obtained from mice were dehydrated, paraffin embedded and cut into 5 μm slices. After paraffin slices were dewaxed with xylene solution and alcohol solution, and sealed with 5% goat serum (Thermo Fisher Scientific, MA, USA), the slices were stained with hematoxylin and eosin (HE) (JK Chem Biomart, Shanghai, China), successively. Pathological results were judged with the staining results under a light microscope.

Immunohistochemistry (IHC)

Three procedures of immunohistochemical staining was used to assess the protein expression of ATG12 in the lung tissues from mice with different treatments. Briefly, paraffin-embedded tissues were cut into 4-μm sections and subjected to deparaffinization using xylene, rehydration with graded ethanol and antigen retrieval with Tris-EDTA and 3% H_2O_2 at room temperature for 10 minutes. After that, the slides were sealed by 5% goat serum for 1 hour at room temperature and then proved with antibody against ATG12 (No. GTX124181, GeneTex, CA, USA), overnight at 4°C, followed by being probed with secondary antibody. 3, 3' Diaminobenzidine (DAB; Thermo Fisher Scientific, MA, USA) was used as a chromogen for immunohistochemical staining, and harris haematoxylin solution was used to stain cell nuclei.

Data statistics

Data from at least three independent repeats were represent as the mean ± standard deviation (SD). Comparisons between two groups or >2 groups were performed by student's t test or one-way ANOVA followed by Bonferroni post hoc test by SPSS 18.0 software, respectively. Pearson's correlation coefficient was used to evaluate the correlation between miR-146a expression and FEV1/FVC (%) in COPD patients. *P* values less than 0.05 were regarded statistically significant.

Results

miR-146a expression is downregulated in COPD patients

To explore the function of miR-146a in the oxidative stress reaction of patients with smoking relative COPD, miR-146a expression was assessed in the serum samples of non-smoking COPD patients (n=10) and smoking COPD patients (n=10) who smokes for more than 5 years. The results showed that miR-146a expression in smoking COPD patients was obviously lower than that of non-smoking COPD patients (**Figure 1A**). Furthermore, the high expression of miR-146a was closely correlated with the low value of FEV1/FVC% of COPD patients (**Figure 1B**). Moreover, smoking COPD patients showed increased serum MDA level (**Figure 1C**). The results from this part suggested that miR-146a might play a role in the pathogenesis of COPD induced by smoking.

CSE promotes the apoptosis of human lung epithelial BEAS-2B cells

Then, the effects of CSE were explored on miR-146a expression and the apoptosis of lung epithelial cells *in vitro*. As **Figure 2A** shows, expression of miR-146a was decreased when BEAS-2B cells were treated with 1% or 5% CSE in a concentration dependent manner. CSE treatment also increased the expression of C-Caspase 3 and C-Caspase 9 (**Figure 2B**), induced cell apoptosis (**Figure 2C**) and inhibited cell viability in BEAS-2B cells (**Figure 2D**). Moreover, CSE enhanced the production of ROS (**Figure 2E**) and MDA (**Figure 2F**). These results indicate that CSE obviously accelerates cell injury and oxidative stress in lung epithelial cells.

Upregulation of miR-146a rescues cell injury induced by CSE in lung epithelial BEAS-2B cells

Next, the function of miR-146a in the pathogenesis of COPD was investigated *in vitro*. miR-146a expression was markedly elevated when BEAS-2B cells were transfected with mimics of miR-146a, while its expression was significantly decreased when BEAS-2B cells were treated with inhibitors (**Figure 3A**). Upregulation of miR-146a reduced expression of C-Caspase 3 and C-Caspase 9 in the presence of 5% CSE, whereas knockdown of miR-146a induced an increase in C-Caspase 3 and C-Caspase 9 expressions in the presence of 5% CSE in BEAS-2B cells (**Figure 3B**). Moreover, upregulation of miR-146a rescued cell apoptosis promotion (**Figure 3C**), cell viability reduction (**Figure 3D**) and the increases in ROS (**Figure 3E**) and MDA levels (**Figure 3F**) mediated by 5% CSE, whereas knockdown of miR-146a enhanced these effects (**Figure 3C-F**). These findings clarify that upregulation of miR-146a rescues the injury of lung epithelial cells induced by CSE.

ATG12 works in coordination with CSE in the promotion of BEAS-2B cell injury

Next, the function of AGT12 was probed in the pathogenesis of smoking related COPD. Transfection of cells with the si-2 targeting ATG12 significantly decreased the expression of ATG-12 while OE-ATG12 increased ATG12 expression both in mRNA and protein levels (**Figure 4A**, **4B**). Upregulation of ATG12 significantly repressed the viability of BEAS-2B cells (**Figure 4C**), and induced cell apoptosis (**Figure 4D**) and

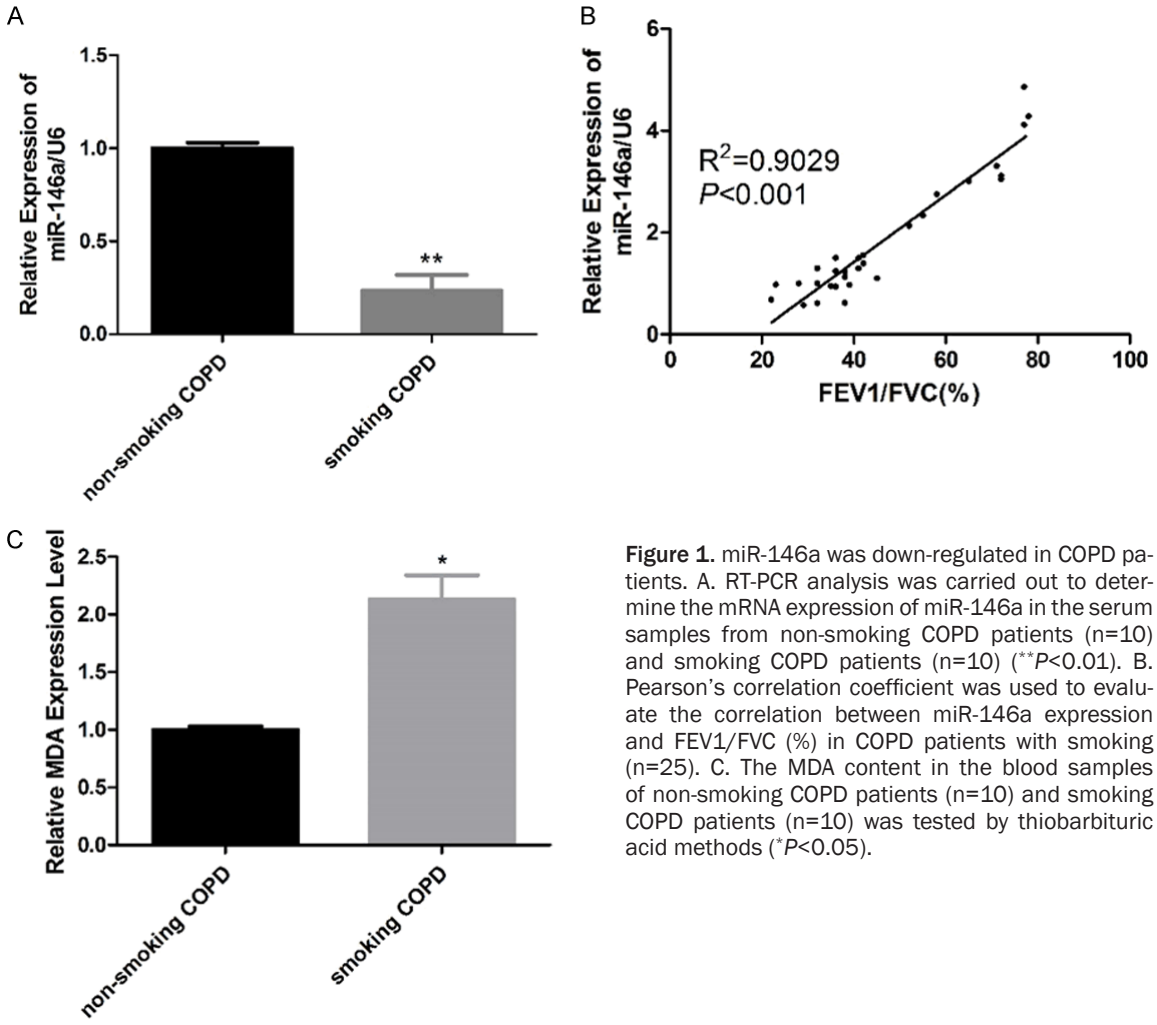

Figure 1. miR-146a was down-regulated in COPD patients. A. RT-PCR analysis was carried out to determine the mRNA expression of miR-146a in the serum samples from non-smoking COPD patients (n=10) and smoking COPD patients (n=10) (**P<0.01). B. Pearson's correlation coefficient was used to evaluate the correlation between miR-146a expression and FEV1/FVC (%) in COPD patients with smoking (n=25). C. The MDA content in the blood samples of non-smoking COPD patients (n=10) and smoking COPD patients (n=10) was tested by thiobarbituric acid methods (*P<0.05).

increased the expression of C-Caspase 3 and C-Caspase 9 (**Figure 4E**), as well as promoted production of ROS (**Figure 4F**) and MDA (**Figure 4G**). However, cell viability was increased (**Figure 4C**), cell apoptosis was repressed (**Figure 4D**), the expression of C-Caspase 9 and C-Caspase 3 was reduced (**Figure 4E**), and the content of ROS (**Figure 4F**) and MDA (**Figure 4G**) was decreased when knockdown of ATG12 on the base of 5% CSE in BEAS-2B cells. These results demonstrate that overexpression of ATG12 aggravates the injury of BEAS-2B cells induced by CSE.

Upregulation of miR-146a reduces cell injury induced by CSE via downregulating ATG12 expression in BEAS-2B cells

Subsequently, it was explored whether miR-146a regulated expression of ATG12 in BEAS-2B cells, which then assuaged cell injury mediated by CSE. As shown in **Figure 5A**, **5B**, upregulation of miR-146a significantly reduced expression of ATG12 not only in mRNA level but also in the protein level. Upregulation of miR-146a obviously decreased the transcriptional activity of ATG12. However, transcriptional activity of ATG12 showed no obvious change when the binding sites between ATG12 and miR-146a were mutated (**Figure 5C**). Furthermore, upregulation of ATG12 abolished the effects of miR-146a on cell growth promotion (**Figure 5D**), cell apoptosis inhibition (**Figure 5E**), expression of C-Caspase 3 and C-Caspase 9 reduction (**Figure 5F**), and production of ROS and MDA (**Figure 5G**, **5H**). Overall, these discoveries illustrated that upregulation of miR-146a assuages cell injury induced by CSE via downregulating ATG12 expression in BEAS-2B cells.

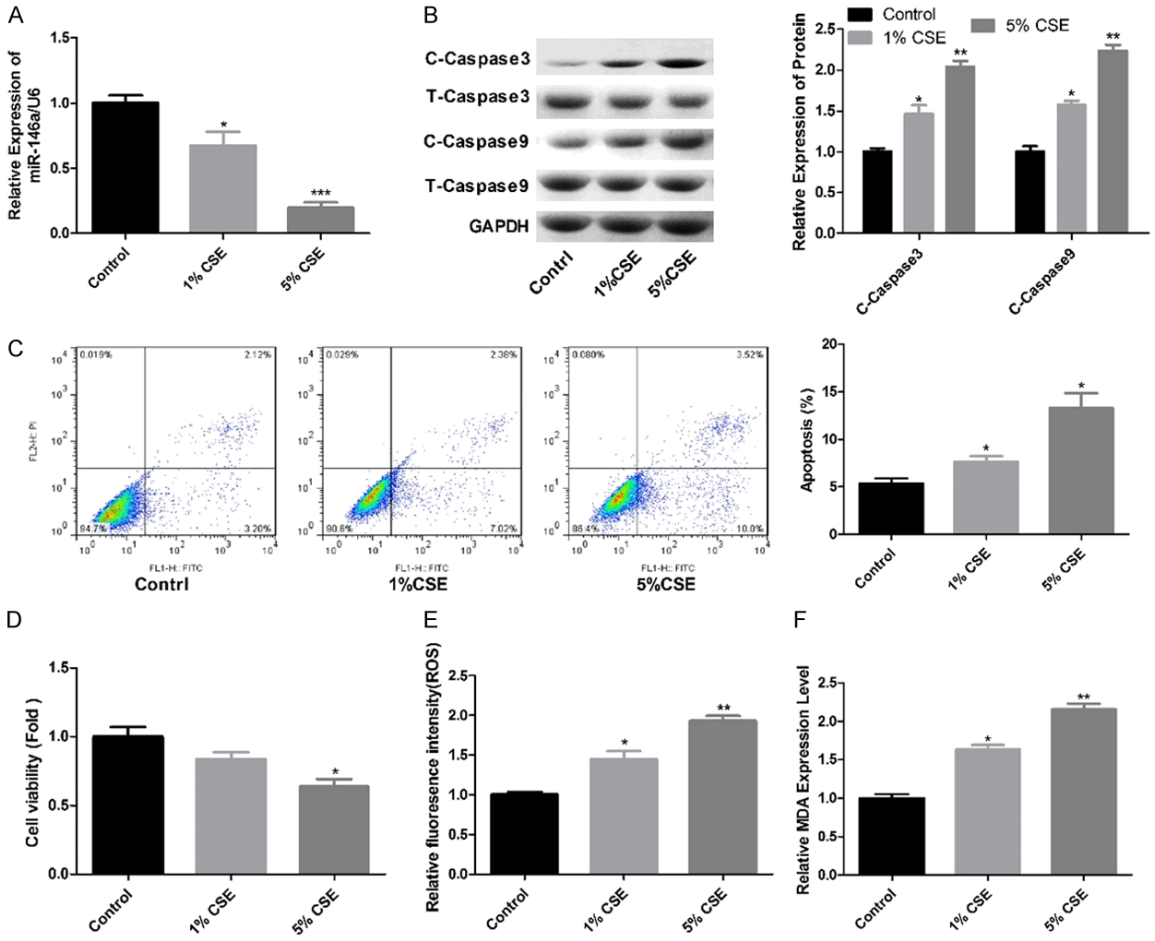

Figure 2. CSE induced the apoptosis of BEAS-2B cells. A. RT-PCR technology was executed to measure expression of miR-146a in BEAS-2B cells with 1% or 5% CSE treatment or not. B. Western blotting analysis of the expression of proteins representing cell apoptosis, such as T-Caspase 3, T-Caspase 9, C-Caspase 3 and C-Caspase 9, in BEAS-2B cells incubated with 1% CSE, 5% CSE or not. C. Flow cytometry was used to assess cell apoptosis in 1% CSE, 5% CSE, and control groups. D. MTT analysis of the viability of BEAS-2B cells in different groups: 1% CSE, 5% CSE and control. E. Oxidation-sensitive fluorescent probe DCFH-DA was used to examine activation of ROS in BEAS-2B cells with 1% or 5% CSE treatment or not. F. The MDA contents in 1% or 5% CSE treated BEAS-2B cells were tested by thiobarbituric acid method. ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, 1% CSE group or 5% CSE group compared to control group).

miR-146a alleviates lung injury and represses oxidative stress reaction via down-regulating ATG12 in vivo

Finally, the effects of miR-146a/ATG12 were investigated on the occurrence and development of COPD in vivo. **Figure 6A** showed the expression of miR-146a in the lung tissues of mice from control, COPD, COPD + mimics and COPD + mimics + OE-ATG12 groups. The morphometric quantification demonstrated that COPD + mimics group showed decreased mean linear intercepts (MLI) compared to COPD group (**Figure 6B** upper), as well as decreased expression level of ATG12 (**Figure 6B** Lower). Moreover, upregulation of miR-146a reduced the production of serum ROS and MDA in COPD mice induced by passive smoking (**Figure 6C**, **6D**). However, these relaxation effects of miR-146a upregulation were all weakened when ATG12 was overexpressed (**Figure 6B-D**). These results reveal that miR-146a alleviates the pathological lung injury and represses oxidative stress reaction smoking induced COPD mice models via downregulating ATG12.

Discussion

Although it has been widely documented that cigarette smoking serves as one of the main causes of COPD, the pathogenetic mechanisms of cigarette smoking in the occurrence and

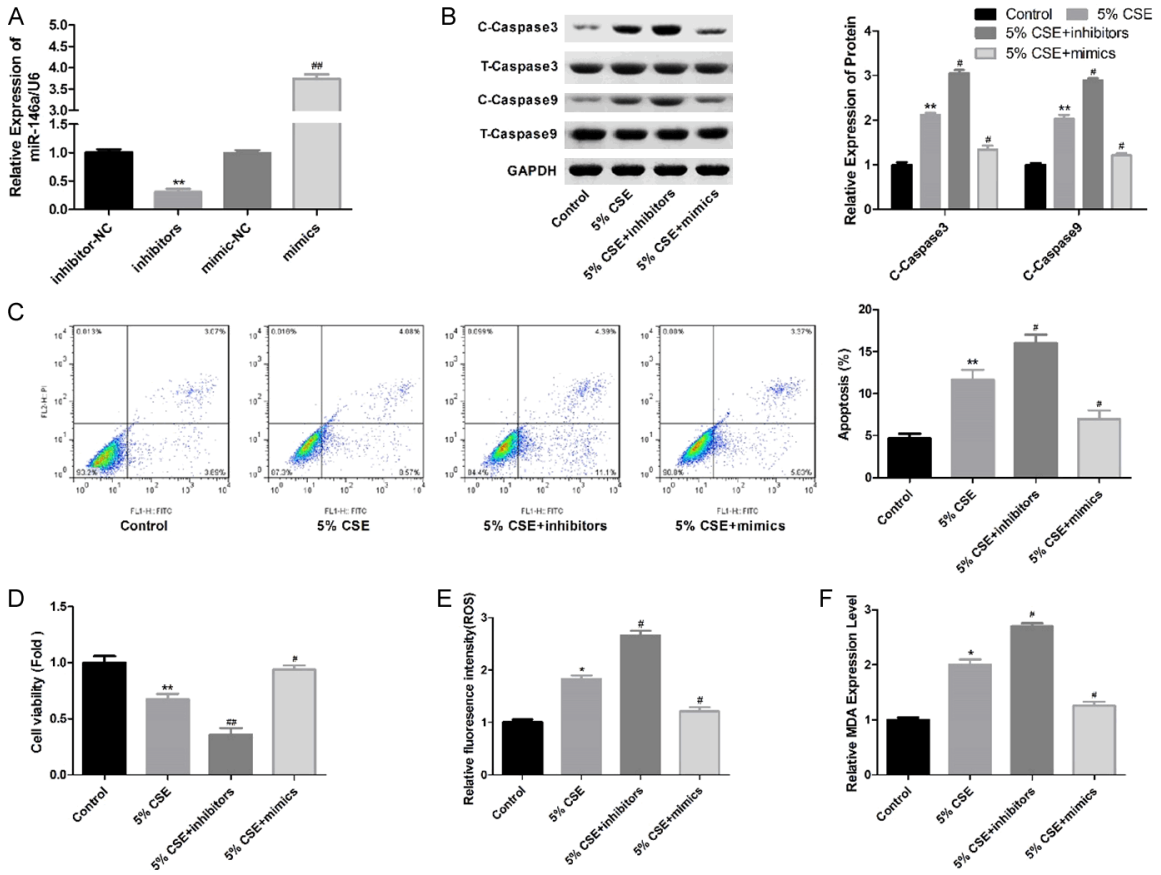

Figure 3. Upregulation of miR-146a rescued cell injury induced by CSE in lung epithelial BEAS-2B cells. (A) RT-PCR technology was used to assess the transfected efficiency of inhibitor ($^{**}P<0.01$, inhibitors group compared to inhibitor-NC group; $^{\#\#}P<0.01$, mimics group compared to mimic-NC group). (B) Western blotting analysis of the expression of T-Caspase 3, T-Caspase 9, C-Caspase 3 and C-Caspase 9 in BEAS-2B cells treated with 5% CSE, 5% CSE + inhibitors, 5% CSE + mimics or nothing. (C) Cell apoptosis was determined by flow cytometry in control, 5% CSE, 5% CSE + inhibitors, and 5% CSE + mimics groups. (D) MTT analysis of the viability of BEAS-2B cells in different groups: control, 5% CSE, 5% CSE + inhibitors, and 5% CSE + mimics. (E) Oxidation-sensitive fluorescent probe DCFH-DA was used to examine the activation of ROS in BEAS-2B cells treated with 5% CSE, 5% CSE + inhibitors, 5% CSE + mimics or nothing. (F) Thiobarbituric acid method was used to assess the MDA contents in BEAS-2B cells given 5% CSE, 5% CSE + inhibitors, or 5% CSE + mimics treatment. (B-F, $^{*}P<0.05$, $^{**}P<0.01$, 5% CSE group compared to control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, 5% CSE + inhibitors group or 5% CSE + mimics group compared to 5% CSE group).

development of COPD are poorly understood. Research indicates that gene regulation mediated by smoking may play a vital role in the pathogenesis of COPD. For instance, Schembri et al. [25] reported that cigarette smoking modulated airway epithelial gene expression through miR-218. Ezzie et al. [26] compared the miRNA expressing profile of lung tissues from non-COPD smokers and COPD patients and demonstrated that 70 miRNAs were differentially expressed. The present study explored the expression and effect of miR-146a in the smoking induced COPD, and found that miR-146 was decreased in the blood samples of smoking COPD patients compared with that of non-smoker COPD patients. Furthermore, it was observed that stimulation of lung epithelial BEAS-2B cells with CSE significantly decreased the level of miR-146a, as well as increased expression of C-Caspase 3/9, protein markers of cell apoptosis, and increased the production of ROS and MDA, markers of excessive oxidative stress, which suggested that the onset of COPD might be induced by tobacco smoke through dysregulation of miR-146a expression.

In recent years, the effect of miR-146a is discovered in COPD progression gradually. Perry et al. [27] found that miR-146a-5p downregulated the secretion of IL-1-induced inflammato-

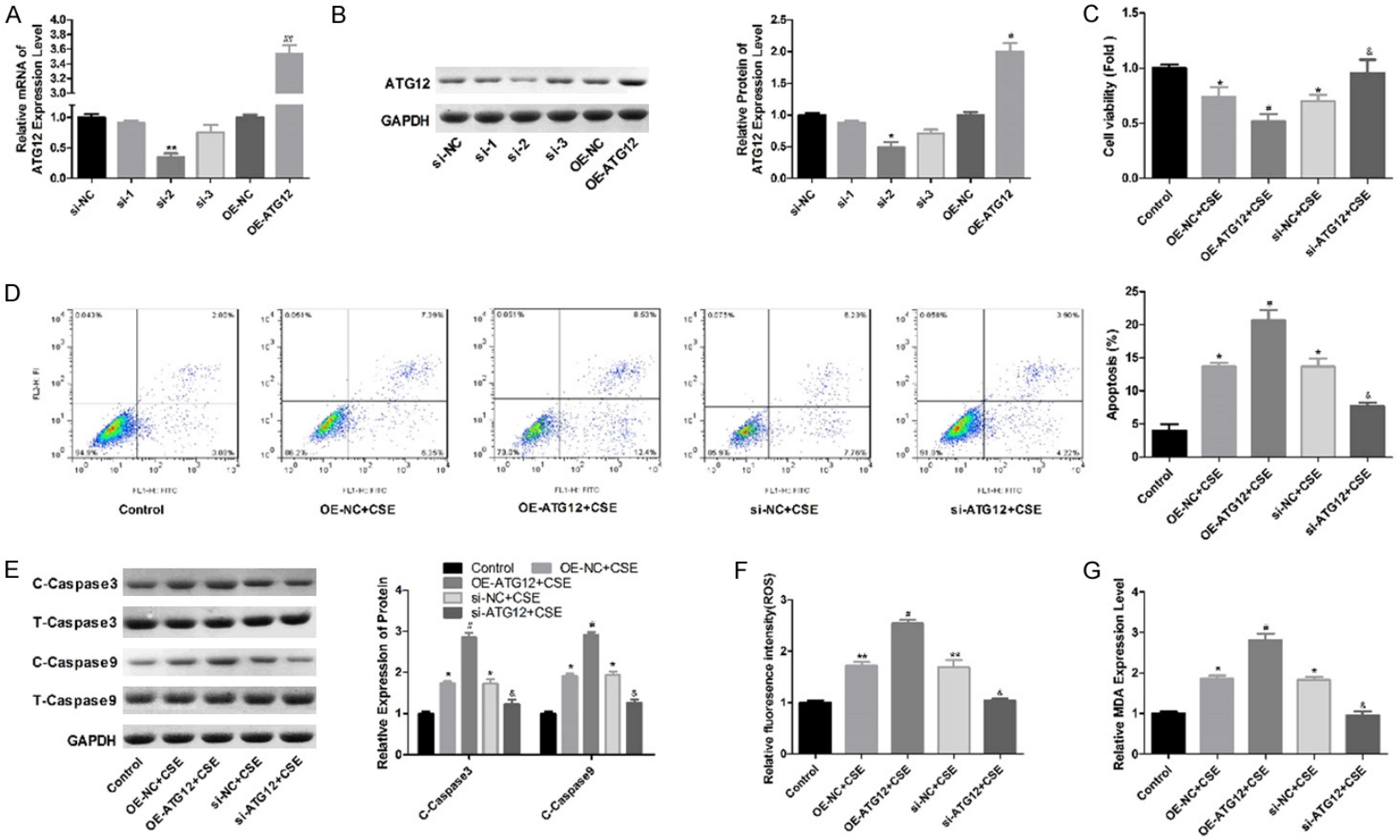

Figure 4. Upregulation of ATG12 exerts a synergistic role of CSE in BEAS-2B cells. (A, B) Cell transfected efficiencies were determined by RT-PCR and Western blotting technologies in mRNA and protein levels, respectively ($^{*}P<0.05$, $^{**}P<0.01$, compared with si-NC group, expression of ATG12 in the si-1 group was significantly reduced; $^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the OE-NC group, expression of ATG12 in OE-ATG12 group was significantly increased). After BEAS-2B cells were transfected with OE-ATG12, OE-NC, si-ATG12 or si-NC for 24 hours, cells were given 5% CSE treatment for 48 hours, then the cells were collected for (C) MTT assay to detect cell viability. (D) Cell apoptosis was determined by flow cytometry. (E) Western blotting analysis to examine the protein expression levels of T-Caspase 3, T-Caspase 9, C-Caspase 3, and C-Caspase 9. (F) oxidation-sensitive fluorescent probe DCFH-DA was used to examine the activation of ROS. (G) Thiobarbituric acid method was used to assess MDA content. (C-G, $^{*}P<0.05$, $^{**}P<0.01$, OE-NC + CSE group or si-NC + CSE group compared to control group; $^{\#}P<0.05$, OE-ATG12 + CSE group compared to OE-NC + CSE group; $^{\&}P<0.05$, si-ATG12 + CSE group vs si-NC + CSE group).

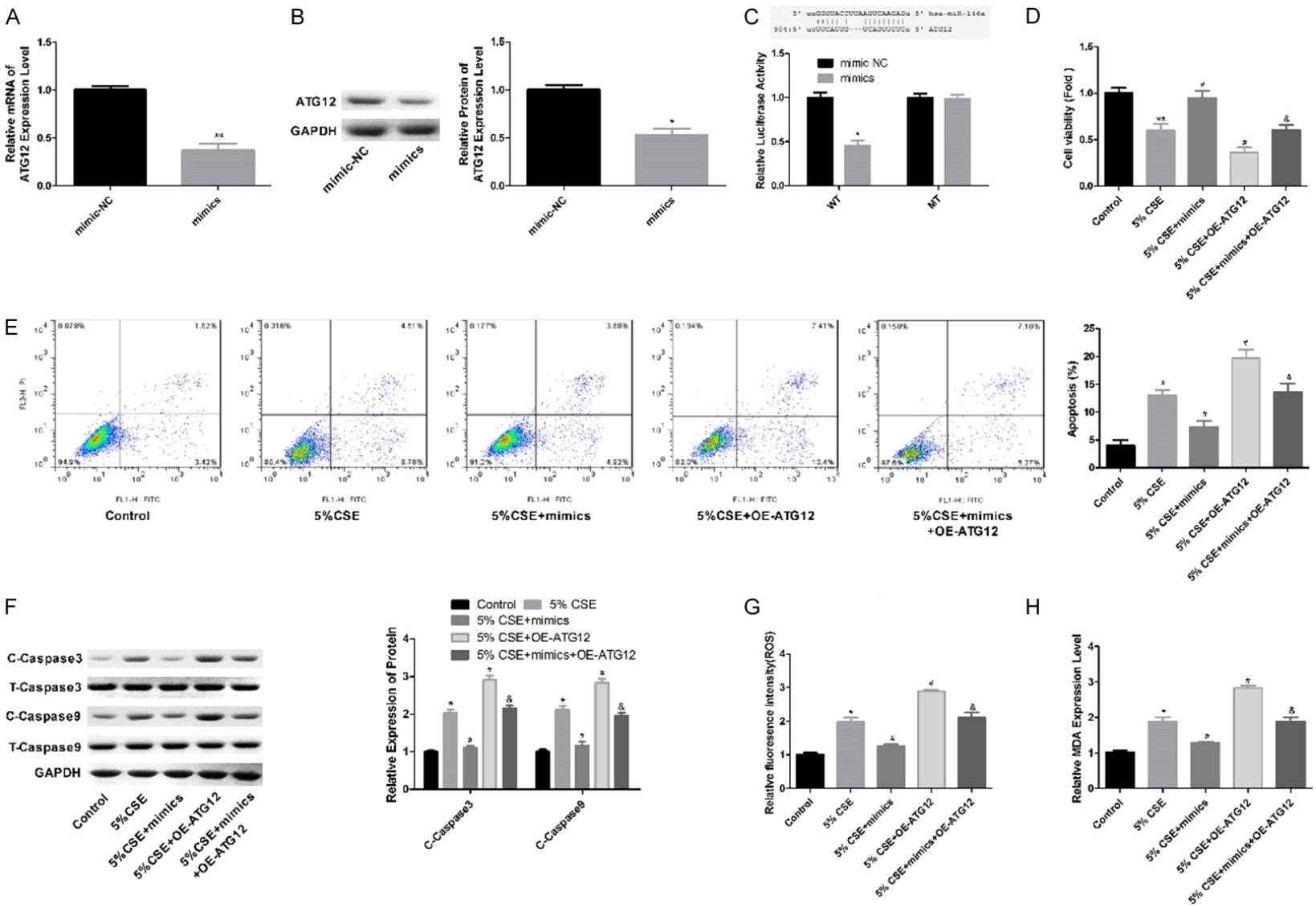

Figure 5. miR-146a combined with ATG12 and negatively regulate expression in BEAS-2B cells. (A, B) mRNA and protein levels of ATG12 were determined by using RT-PCR and Western blotting technology after BEAS-2B cells were transfected mimics or mimic-NC. (C) Fluorescent reporter gene assay was performed to assess the fluorescence activity of luciferase reporter plasmid after 48 hours of BEAS-2B cells were transfected with mimics or mimic-NC. (A-C, $^{*}P<0.05$, $^{**}P<0.01$, mimics group vs mimic-NC group). BEAS-2B cells were transfected with OE-ATG12, mimics, or OE-ATG12 + mimics for 24 hours, followed by incubated with 5% CSE for 48

hours, then the cells were collected for (D) MTT assay to detect cell viability. (E) Cell apoptosis was determined by flow cytometry. (F) Western blotting analysis to examine the protein expression levels of T-Caspase 3, T-Caspase 9, C-Caspase 3 and C-Caspase 9. (G) oxidation-sensitive fluorescent probe DCFH-DA to examine the activation of ROS. (H) Thiobarbituric acid method to assess the MDA contents. (D-H, $^{*}P<0.05$, $^{**}P<0.01$, 5% CSE group compared to control group; $^{\#}P<0.05$, 5% CSE + mimics group or 5% CSE + OE-ATG12 group compared to 5% CSE group; $^{\&}P<0.05$, 5% CSE + mimics + OE-ATG12 group compared to 5% CSE + mimics group).

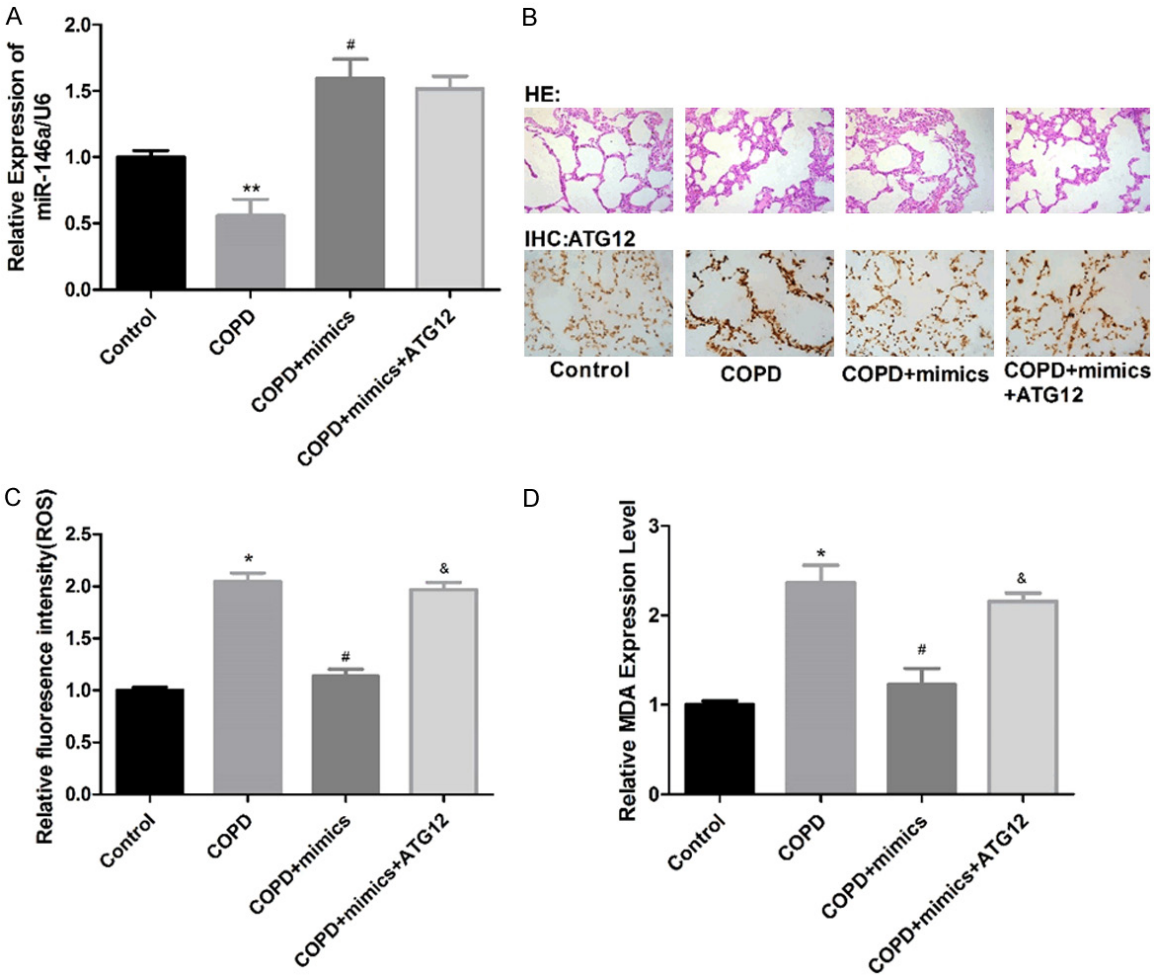

Figure 6. Upregulation of miR-146a alleviated pathological lung injury and repressed oxidative stress reaction of COPD mice through down-regulating ATG12. Twenty-four mice were divided into four groups: control, COPD, COPD + mimics and COPD + mimics + OE-ATG12 groups, 6 mice in each group. Mice were killed and lung tissues were taken out for the following research. A. RT-PCR was performed to test the mRNA level of miR-146a in the lung tissues. B. H&E staining used to evaluate the pathological change of lung tissues (Upper); IHC technology used to assess the express of ATG12 (Lower) (Magnification: 100×). C. Oxidation-sensitive fluorescent probe DCFH-DA was performed to examine the activation of ROS. D. Thiobarbituric acid method used to assess the MDA contents of the serum samples. ($^{*}P<0.05$, $^{**}P<0.01$, COPD group vs control group; $^{\#}P<0.05$, COPD + mimics group vs COPD group; $^{\&}P<0.05$, COPD + mimics + OE-ATG12 group vs COPD + mimics group).

ry mediators including IL-8 from pulmonary epithelial cells. Besides, miR-146a was also identified to deactivate nuclear factor (NF)-κB through inhibiting activation of NF-κB transducers IL-1 receptor-associated kinase and TNF receptor-associated factor 6 [28]. Emmanuel et al. [15] showed that miR-146a-5p has an anti-inflammatory role in lung fibroblasts through downregulating IRAK-1 expression and subsequently inhibition the production of IL-8. Furthermore, they also found that downregulation of miR-146a-5p in COPD fibroblasts was strongly implicated the single nucleotide polymorphism (SNP) rs2910164 (GG allele) in the

miR-146a-5p gene. Together, these findings suggest that miR-146a as a therapeutic target for alleviating the abnormal inflammatory reactions in COPD [13]. In addition to inflammatory response, the effects of miR-146a were explored in the oxidative stress induced by smoking. Upregulation of miR-146a significantly rescued the increased levels of ROS and MDA caused by CSE, suggesting that miR-146a could also alleviate the COPD through reduction of oxidative stress related to smoking.

In addition to oxidative stress, the deleterious effects of cigarette smoke exposure also include cell growth, apoptosis, protein-processing, anti-bacterial immune defense and autophagy [18, 29, 30]. Shivalingappa et al. [31] reported that pharmacological autophagy induction with carbamazepine or cysteamine (antioxidant) can control lung damage in the murine models of smoking induced COPD-emphysema. However, inhibition of autophagy was identified to ameliorate acute lung injury induced by avian influenza A H5N1 infection [32]. These findings indicate the important roles of autophagy in lung function. ATG12 is an autophagy related factor which is essential for LC3 lipidation and leads to autophagosome formation through conjugation with ATG5 [33, 34]. Yuwen et al. [35] showed that miR-146a-5p negatively regulates ATG12 expression in lung cancer, which was consistent with our study, that ATG12 expression is decreased when miR-146a was upregulated in BEAS-2B cells through binding to each other. Moreover, cell viability promotion and cell apoptosis inhibition and the reduction of ROS and MDA induced by miR-146a upregulation was impaired when AGT12 was upregulated. These results suggest that miR-146a assuaged cell injury induced by CSE via downregulation of ATG12 expression. Furthermore, the function of miR-146a/ATG12 axis was assessed in smoking induced mice COPD. The COPD group showed increased MLI, indicating that the COPD mouse model was successfully constructed [36]. Upregulation of miR-146a significantly decreased MLI and ATG12 expression, as well as the production of ROS and MDA.

In conclusion, this study demonstrates that miR-146a is beneficial to the recovery of lung injury in smoking induced COPD through inhibiting the oxidative stress via downregulation of ATG12 expression. The study provides more profound theoretical basis for serving miR-146a as a promising therapeutic target for COPD patients.

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Disclosure of conflict of interest

None.

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