Original Article

Tumor necrosis factor-α promotes airway mucus hypersecretion by repressing miR-146a-5p and miR-134-5p levels in human airway epithelial cells

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Background: Airway mucus acts as an indispensable protective component of innate immune response against invading pathogens. However, airway mucus hypersecretion, largely consisting of mucin 5AC (MUC5AC), is the leading cause of airflow obstruction and airway hyperresponsiveness that contributes to chronic obstructive pulmonary disease (COPD). MicroRNAs (miRNAs) are frequently dysregulated in the pathogenesis of COPD, but the definite role of miRNAs in airway mucus hypersecretion is not well understood.

Methods: A cell model of mucus hypersecretion was established in 16HBE cells by treatment with TNF-α. Cell viability and apoptosis were assessed using cell counting kit-8 (CCK-8) and flow cytometry, respectively. The aberrant expression of miR-146a-5p and miR-134-5p was assayed in TNF-α-treated 16HBE cells, and the effect of miR-146a-5p and miR-134-5p on regulating MUC5AC expression was evaluated using quantitative real-time PCR (qPCR) and Western blot analysis.

Results: TNF-α treatment resulted in a significant decrease of cell viability, and increase of cell apoptosis and MUC5AC expression in 16HBE cells. Additionally, the expression of miR-134-5p and miR-146a-5p was markedly decreased in the cell model. Importantly, forced expression of miR-134-5p and miR-146a-5p significantly repressed TNF-α-induced upregulation of MUC5AC. Mechanistically, although miR-134-5p did not affect 16HBE cells viability and apoptosis, miR-134-5p partially blocked TNF-α-induced MUC5AC expression by inhibiting the activation of NF-κB signaling. On the other hand, miR-146a-5p enhanced cell viability and reduced cell apoptosis. miR-146a-5p also repressed TNF-α-induced MUC5AC expression by inhibiting p38 MAPK (mitogen-activated protein kinase) signaling activation.

Conclusions: The current data demonstrated that both miR-134-5p and miR-146a-5p conferred protection against TNF-α-induced mucus hypersecretion through repressing NF-κB and p38 MAPK signaling, indicating that miR-134-5p and miR-146a-5p may serve as the biomarker for COPD.

Keywords: miR-134-5p; miR-146a-5p; chronic obstructive pulmonary disease (COPD); mucin 5AC (MUC5AC); NF-κB

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Introduction

Chronic obstructive pulmonary disease (COPD) is an airway obstructive pulmonary disease characterized by persistent airflow limitation (1). That air pollution and occupational exposures to dust and fumes are risk factors to induce COPD, and cigarette smoke is the main cause of COPD (2). The pathogenesis of COPD mainly includes airway inflammation, oxidative stress, protease/antiprotease imbalance, emphysema, and airway mucus secretion (3-5). In clinical treatment, glucocorticoids and bronchodilators are used to alleviate the symptoms of COPD but produce limited clinical efficacy (6,7). Mucus hypersecretion is associated with shortness of breath, impaired mucociliary clearance, infection, and chronic cough (8,9). Currently, the underlying mechanisms leading mucus hypersecretion are not well understood.

miRNAs are a kind of small non-coding RNAs, which play an important role in regulating gene expression (10). miRNAs are involved in various biological processes and in human diseases (11). Mounting evidence has indicated that miRNAs are closely related to the occurrence and development of COPD (12). Various miRNAs are differentially expressed in serum, sputum, and lung of COPD patients (13). For instance, the level of miR-15b is accumulated in COPD lung tissues, and miR-15b targets SMAD7 that participates in transforming growth factor β (TGF-β) signaling pathway to regulate COPD progression (14). In plasma and lung tissue from COPD, the level of miR-29b is reduced, and miR-29b regulates airway inflammation in COPD by targeting bromodomain protein 4 (BRD4) (15).

Tasena *et al.* has demonstrated that 20 differentially expressed miRNAs are associated with mucus hypersecretion in COPD (12). Out of the 20 miRNAs, miR-134-5p and miR-146a-5p are lower expressed in COPD patients, and are crucial miRNAs associated with several mucus hypersecretion-correlated potential targets (12). miR-134-5p is a potential marker for the clinical diagnose of acute exacerbation of COPD (16). Down-regulated miR-146a-5p is involved in the pathogenesis of COPD through inducing more proinflammatory phenotype (17-19). Given that NF-κB signaling acts as critical roles in facilitating mucin 5AC (MUC5AC) expression (20) and miR-134-5p possesses a potential effect of regulating NF-κB activation, we thus investigated whether miR-134-5p repressed MUC5AC expression by inhibiting NF-κB signaling in cell model of airway mucus hypersecretion. We also explored the role and underlying mechanism of miR-146a-5p in regulating

MUC5AC expression. Although several inflammatory cytokines (IL-1β, IL-5, IL-13, etc.) have been demonstrated to produce MUC5AC expression *in vitro* and *in vivo* (21,22), the effect of TNF-α on airway mucus hypersecretion has not been widely investigated, and thus TNF-α was used to establish the cell model of airway mucus hypersecretion.

We present the following article in accordance with the MDAR checklist (available at https://dx.doi.org/10.21037/tcr-20-3375).

Methods

Cell culture and TNF-α treatment

The human bronchial epithelial cells (16HBE), provided from ScienCell (Santiago, California, USA), were maintained in RPMI 1640 medium (Solarbio, Beijing, China) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and kept at temperature of 37 ℃ in an incubator with 5% CO_2. 16HBE cells were treated with TNF-α (R&D Systems, Minneapolis, MN, USA; 10 ng/mL) for 24 h to construct airway mucus hypersecretion cell model according to previous reports (23,24) and our preliminary experiments (Figure S1).

Flow cytometry

16HBE cell apoptosis was determined using Flow cytometry with an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). 16HBE cells ($1×10^6$ cells/mL) were treated with TNF-α (10 ng/mL) for 24h in the presence of indicated reagents. After washing with PBS, the cells were re-suspended with 500 μL 1× binding buffer and stained with 5 μL of Annexin V conjugated to FITC and 5 μL PI for 15 min. Cytometry data was analyzed using FlowJo software (Treestar, Ashland, OR).

miRNA mimics

miR-134-5p mimics (UGUGACUGGUUGACCAGAGG GG), miR-146a-5p mimic (UGAGAACUGAAUUCCAU GGGUU), and miRNA control (miRcont) were synthesized by Shanghai GenePharma Co., Ltd (Shanghai, China). 16HBE cells were transfected with 40 nmol/L miR-134-5p, miR-146a-5p mimic, or miRcont with Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA) and Opti-MEM-reduced serum medium (Solarbio) according to previous

Table 1 Oligonucleotide sequences used in the study

Primer	Sequences (5'-3')
hsa-miR-146a-5p	UGAGAACUGAAUUCCAUGGGUU
hsa-miR-146a-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACCC
hsa-miR-146a-5p-F	CGGCTGAGAACTGAATTCCAT
hsa-miR-146a-5p-R	GTGCAGGGTCCGAGGT
hsa-miR-134-5p	UGUGACUGGUUGACCAGAGGGG
hsa-miR-134-5p-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCCC
hsa-miR-134-5p-F	UGUGACUGGUUGACCAGA
hsa-miR-134-5p-R	GTGCAGGGTCCGAGGT
MUC5AC-F	CTCCTCTCCCACAACACCAAG
MUC5AC-R	TCCTCACAGATGCCAAAGCC
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
18S-F	AGGAATTCCCAGTAAGTGCG
18S-R	GCCTCACTAAACCATCCAA

reports and our preliminary experiments. Forty-eight h post-transfection later, cells were collected and qPCR was used to assess transfection efficiency.

Cell viability assay

Cell viability was evaluated with the Cell Counting Kit-8 (CCK-8, Solarbio). In brief, 16HBE cells were plated in 96-well plates (2×10^4 cells/well) for 24 h at 37 ℃. Then, 16HBE cells were treated with TNF-α (10 ng/mL) for different time (0, 24, 48, and 72 h). 10 μL CCK‐8 solution was added and incubated for another 3.5 h. The absorbance (value) at 450 nm was measured by a scanning microplate reader (Bio-Rad, Hercules, CA, USA).

Quantitative real-time PCR (qPCR)

Total RNA from 16HBE cells was isolated using TRIzol kit (Takara, Japan). Reverse transcription for complementary DNA (cDNA) synthesis was performed with Transcriptor First Strand cDNA Synthesis Kits (Roche, Basel, Switzerland). qPCR was carried out with SYBR Green qRT-PCR Master Mix (Enzyme, Nanjing, China) on the 7500 fast real-time PCR system (ThermoFisher Scientific, Waltham, MA, USA) as previously described (25). U6 and 18S was used as internal control for miRNA and mRNA,

respectively. Relative RNA expression was analyzed using $2^{-\Delta\Delta Ct}$ method and relative primer sequences were listed in *Table 1*.

Western blot analysis

Total protein from 16HBE cells was extracted using RIPA lysis buffer (Elabscience, Wuhan, China). After protein concentrations were determined by BCA Protein Assay Kit (Takara, Japan), equal amount of protein for each sample was resolved with SDS-PAGE (10%) and then immobilized onto PVDF membranes (Roche, Basel, Switzerland). The membranes were soaked in 5% non-fat dried milk in PBS and incubated with antibodies against MUC5AC (1:1,000, ab24071, Abcam), p65 (0.5 μg/mL, ab16502, Abcam), Lamin B1 (0.1 μg/mL, ab16048, Abcam), and β-actin (1:5,000; ab8226, Abcam) overnight at 4 ℃ respectively, and then incubation with corresponding secondary antibody at room temperature for 60 min. The blots were revealed with an ECL kit (Abcam) and analyzed by Image Software (NIH, Bethesda, MD, USA).

Statistical analysis

All data were reported as mean ± standard deviation (SD). Statistical analyses were performed by SPSS 13.0 (SPSS

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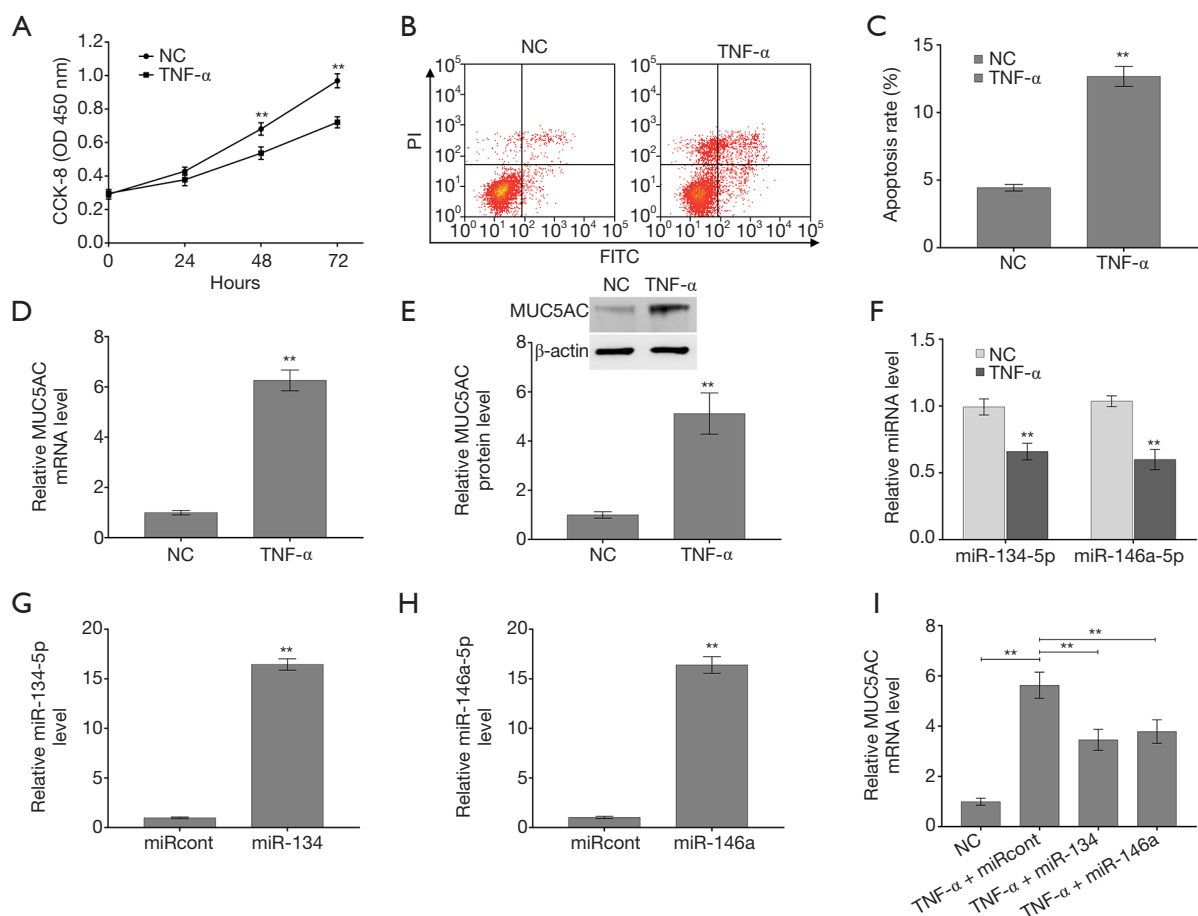Fu et al. TNF-α promotes airway mucus hypersecretion

Figure 1 TNF-α repressed 16HBE cell viability and increased MUC5AC expression. (A) Cell viability was assessed in 16HBE cells after treatment with TNF-α (10 ng/mL) for 0, 24, 48 or 72h using the CCK-8 assay. (B) 16HBE cells were treated with TNF-α (10 ng/mL) for 24 h, and cell apoptosis was assessed using flow cytometry. The proportion of apoptotic cells was showed in (C). Values are presented as mean ± SD of three independent experiments. The mRNA (D) and protein (E) expression levels of MUC5AC were assessed in 16HBE cells treated with or without TNF-α (10 ng/mL) for 24 h. (F) The expression level of miR-134-5p (miR-134) and miR-146a-5p (miR-146a) was assessed in 16HBE cells in the presence or absence of TNF-α (10 ng/mL). The expression level of miR-134 (G) and miR-146a (H) was assessed in 16HBE cells treated with miR-134 mimics and miR-146a mimics, respectively. (I) The mRNA level of MUC5AC was assessed in TNF-α-treated 16HBE cells in the presence of miR-134 or miR-146a. **, P<0.01.

Inc., Chicago, IL, USA). Differences between two groups were calculated with two-tailed student's *t*-test, or ANOVA followed by the Scheffé test for multiple groups. P<0.05 was considered statistically significant.

Results

TNF-α treatment increased MUC5AC expression, and repressed miR-134-5p (miR-134) and miR-146a-5p (miR-146a) expression in 16HBE cells

To explore the mechanisms underlying TNF-α-induced

airway mucus hypersecretion *in vitro*, 16HBE cells were treated with TNF-α (10 ng/mL) for 24 h to establish mucus hypersecretion cell model. CCK-8 assay was used to assess 16HBE cells viability after TNF-α treatment. As shown in *Figure 1A*, TNF-α treatment significantly repressed 16HBE cells viability compared to the control. The role of TNF-α in regulating 16HBE cells apoptosis was then assessed through Flow cytometry analysis. *Figure 1B,C* showed that the apoptosis rate of 16HBE cells was increased after TNF-α treatment. As expected, TNF-α treatment increased the mRNA and protein level of MUC5AC in 16HBE cells

(*Figure 1D,E*). The previous studies have demonstrated that miR-134-5p (miR-134) and miR-146a-5p (miR-146a) are involved in mucus hypersecretion in COPD (12,16-19). To explore the association of TNF-α-induced MUC5AC expression with these miRNAs, we first assessed whether TNF-α regulated the expression of these miRNAs. As shown in *Figure 1F*, the expression of miR-134 and miR-146a were significantly decreased in 16HBE cells after TNF-α treatment. More important, forced expression of miR-134 or miR-146a alleviated TNF-α-induced increase of MUC5AC (*Figure 1G,H,I*). These data suggest that TNF-α increased MUC5AC expression, at least in part by repressing miR-134 and miR-146a expression.

miR-134 partially blocked TNF-α-induced MUC5AC expression by repressing NF-κB activation

Figure 2A further showed that forced expression of miR-134 and miR-146a inhibited MUC5AC expression more effectively than miR-134 or miR-146a overexpression separately. Then we investigated the role of miR-134 and miR-146a in regulating TNF-α-induced MUC5AC expression, respectively. As shown in *Figure 2B,C,D*, miR-134 did not affect TNF-α-regulated 16HBE cells viability and apoptosis. However, miR-134 partially blocked TNF-α-induced MUC5AC expression (*Figure 2E*). Given the crucial role of NF-κB signaling in MUC5AC expression, we next whether miR-134 repressed TNF-α-induced activation of NF-κB signaling. The results from Western blot analysis showed that miR-134 markedly inhibited TNF-α-induced nuclear translocation of NF-κBp65 (*Figure 2F*), indicating that miR-134 blocked TNF-α-induced MUC5AC expression, at least in part by repressing NF-κB signaling activation.

miR-146a partially blocked TNF-α-induced MUC5AC expression by repressing p38MAPK activation

We next investigated the effect of miR-146a on regulating TNF-α-induced MUC5AC expression. To this end, 16HBE cells were treated with TNF-α in the presence or absence of miR-146a mimics, and cell viability and apoptosis were assessed using CCK-8 assay and flow cytometry, respectively. The results from CCK-8 assay showed that miR-146a significantly increased cell viability in 16HBE cells treated with TNF-α (*Figure 3A*). *Figure 3B,C* showed that miR-146a effectively inhibited TNF-α-induced cell apoptosis. Furthermore, MUC5AC level was assessed on both mRNA and protein in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. The qPCR analysis demonstrated that miR-146a markedly up-regulated the mRNA expression of MUC5AC compared with the miRcont (*Figure 3D*). Similarly, the result of western blot showed that the protein levels of MUC5AC were boosted by miR-146a mimics treatment (*Figure 3E,F*).

Previous studies demonstrated that p38MAPK signaling is correlated with MUC5AC expression in airway epithelial cell after TNF-α treatment (23). We thus investigated the association of miR-146a with p38MAPK signaling activation. As shown in *Figure 4A*, TNF-α treatment resulted in a significant increase of p38MAPK activation, whereas miR-146a partially inhibited TNF-α-induced p38MAPK activation. miR-146a did not affect the activation of NF-κB signaling in TNF-α-treated 16HBE cells (*Figure 4B*). These results demonstrate that miR-146a partially blocked TNF-α-induced MUC5AC expression, at least in part by repressing p38MAPK signaling activation.

Discussion

In the present study the role of miR-134 and miR-146a on repressing TNF-α-induced airway mucus hypersecretion was revealed and its underlying mechanism was further verified. The current data demonstrate that (I) TNF-α treatment increased MUC5AC expression, and repressed miR-134 and miR-146a expression in 16HBE cells; (II) miR-134 blocked TNF-α-induced MUC5AC expression by repressing NF-κB activation; (III) miR-146a blocked TNF-α-induced MUC5AC expression by repressing p38MAPK activation. These data uncovered the important role of miR-134 and miR-146a in regulating mucus hypersecretion by regulating NF-κB and p38MAPK, and may provide a therapeutic opportunity for patients with airway mucus hypersecretion.

COPD is chronic pulmonary disorder, characterized by persistent inflammatory response to inhaling cigarette smoke (26). Bronchial epithelial cells and macrophages are mainly responsible for chronic inflammatory response (26). These cells could discharge proteases which lead to elastin degradation and emphysema, and thus facilitate inflammatory response (27,28). Epithelial cells also release TGF-β to induce tissue remodeling (29). Based on these facts, we established a cell model of mucus hypersecretion using the human bronchial epithelial cells (16HBE) by TNF-α treatment. As expected, TNF-α treatment increased the mRNA and protein level of MUC5AC in 16HBE cells
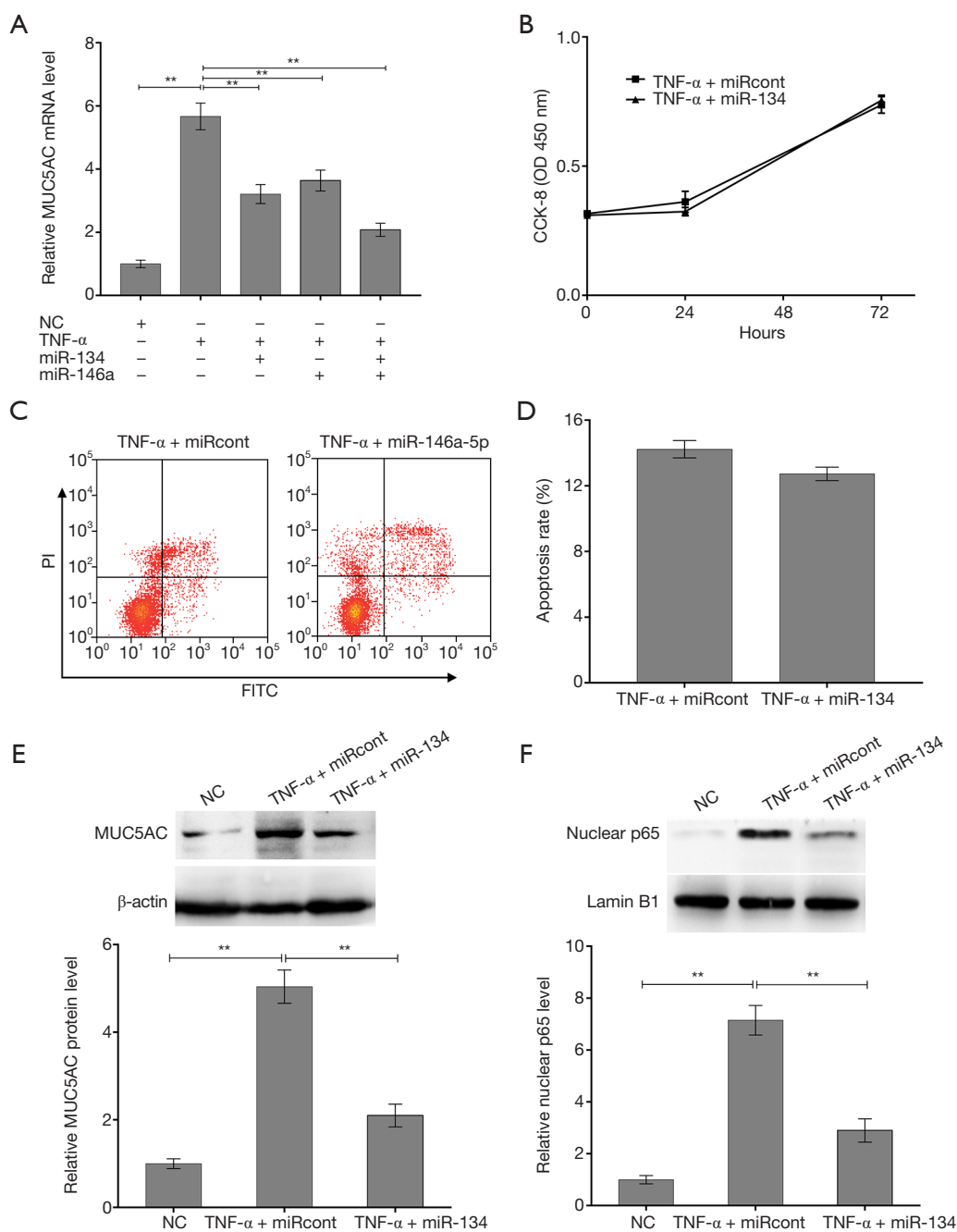
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Figure 2 miR-134 blocked TNF-α-induced MUC5AC expression by repressing NF-κB activation. (A) The mRNA level of MUC5AC was assessed using qPCR analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-134 or/and miR-146a. The characters "+" and "–" indicate the presence and absence of indicated reagents, respectively. (B) Cell viability was assessed using CCK-8 assay in TNF-α-treated 16HBE cells in the presence or absence of miR-134. (C,D) Cell apoptosis was assessed using flow cytometry in TNF-α-treated 16HBE cells in the presence or absence of miR-134. (E) The protein expression of MUC5AC was assessed using western blot analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-134. (F) The protein expression of nuclear p65 was assessed using western blot analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-134. Lamin B1 was used to an internal control. **, P<0.01.
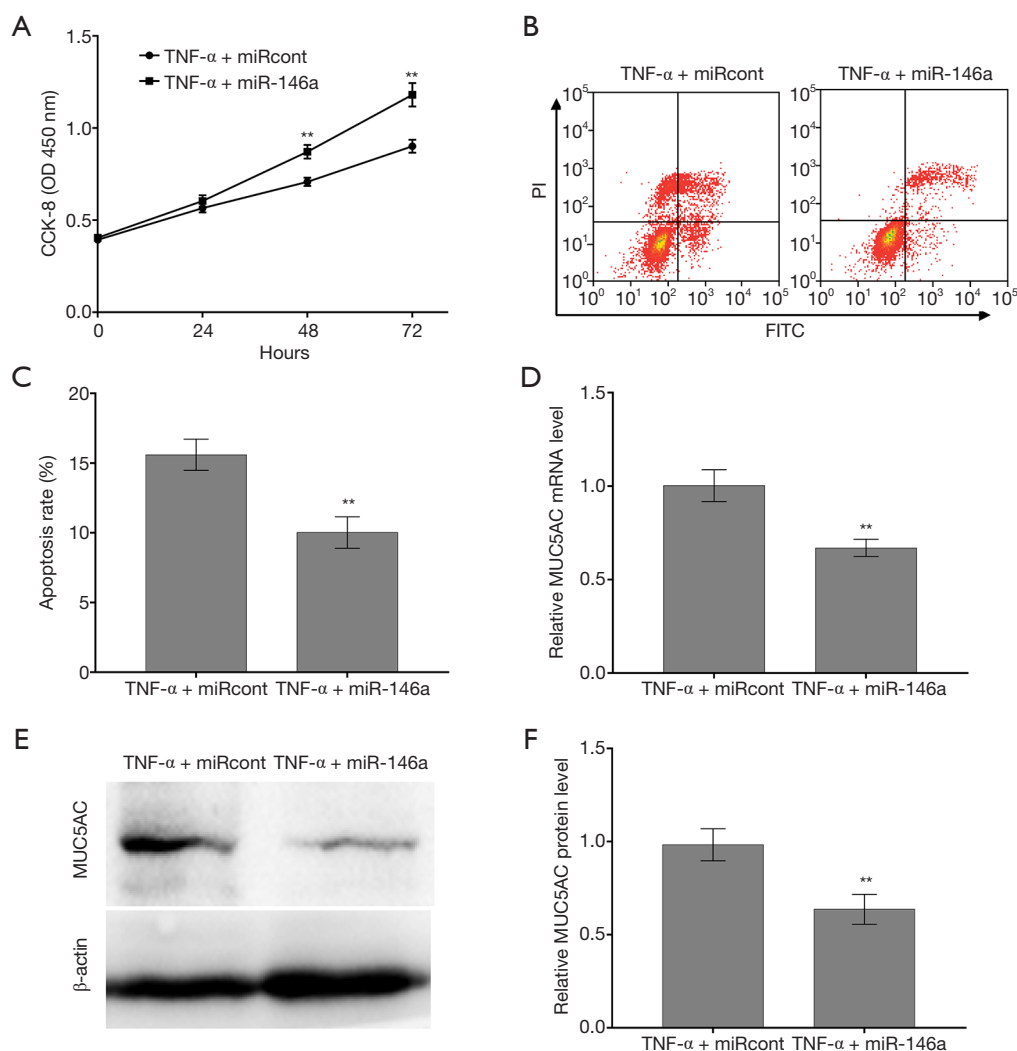
Figure 3 miR-146a blocked TNF-α-induced MUC5AC expression. (A) Cell viability was assessed using CCK-8 assay in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. (B,C) Cell apoptosis was assessed using flow cytometry in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. (D) The mRNA expression of MUC5AC was assessed using qPCR analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. (E,F) The protein expression of MUC5AC was assessed using western blot analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. **, $P<0.01$.

through qPCR and western blot analysis.

The pathological role of miRNAs in COPD has been verified in many studies, in which aberrant miRNAs expression has been revealed in lung cell or airway epithelial cell (30,31). Cao *et al.*, showed that the miR-183 level is upregulated in patients with COPD, and miR-183 is associated with the severity of COPD through repressing Ca^{2+}-activated K^+ channels β1 subunit (BKCaβ1) expression (32). Additionally, the plasma levels of miR-133, miR-499, and miR-206 are increased in COPD patients compared to the healthy controls (33). On the contrary, several miRNAs are downregulated in COPD patients such as miR-134, miR-146a, miR-500a, and miR-1207 (12). The miR-146a level is further decreased in COPD fibroblasts after cytokine (IL-1β, or TNF-α) treatment (19). In the study, we assessed the effect of TNF-α on regulating miR-146a expression in airway epithelial cell, and data showed that the expression of miR-146a is decreased in 16HBE cells following TNF-α treatment. miR-146a contributes to alleviate TNF-α-

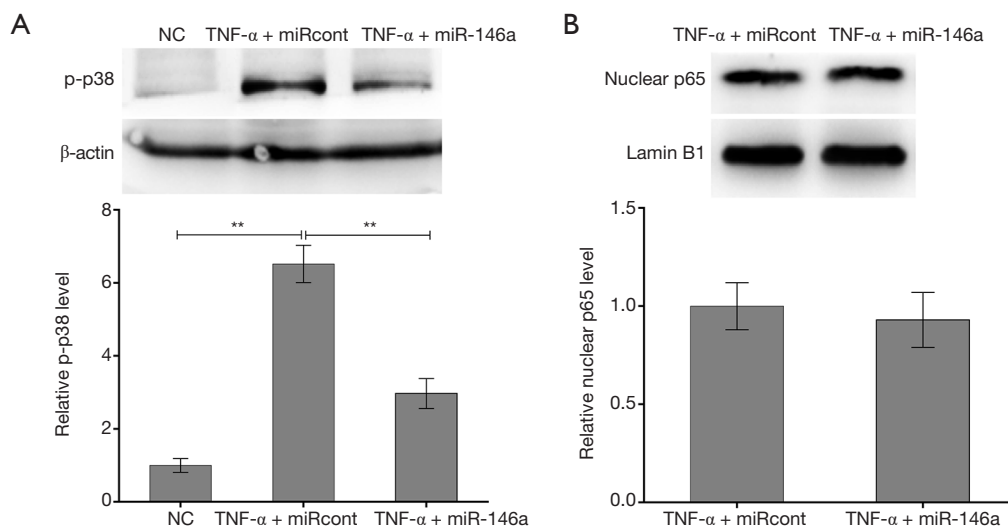**Figure 4** miR-146a repressed TNF-α-induced p38MAPK activation. (A) The phosphorylated p38 (p-p38) protein expression was assessed using western blot analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. β-actin was used to an internal control. (B) The protein expression of nuclear p65 was assessed using western blot analysis in TNF-α-treated 16HBE cells in the presence or absence of miR-146a. Lamin B1 was used to an internal control. **, P<0.01.

induced increase of MUC5AC. Although previous studies have demonstrated the negative association of miR-146a with NF-κB activation in small intestine ischemia (34) and cancer cells (35), the current results did not observe the effect of miR-146a on regulating NF-κB activation in airway epithelial cells. Our data revealed that TNF-α results in an increase of p38MAPK activation, whereas miR-146a partially inhibited TNF-α-induced p38MAPK activation, indicating that miR-146a blocks TNF-α-induced MUC5AC expression, at least in part by repressing p38MAPK signaling activation.

miR-134 is the strongest decreased miRNA in mild COPD patients with chronic mucus hypersecretion (12), but the potential function of miR-134 on mucus hypersecretion remains unknown. In the study, we found that the expression of miR-134 is decreased significantly in 16HBE cells after TNF-α treatment. More important, forced expression of miR-134 inhibits TNF-α-induced MUC5AC expression although miR-134 did not affect TNF-α-regulated 16HBE cells viability and apoptosis. Mechanistically, miR-134 represses TNF-α-induced NF-κB activation, indicating that miR-134 blocks TNF-α-induced MUC5AC expression, at least in part by repressing NF-κB activation.

The limitations of the present study lie in the following aspects: (I) the direct target gene of miR-134 in regulating

NF-κB signaling in airway epithelial cells could not be identified; (II) the direct target gene of miR-146a in regulating p38 signaling in airway epithelial cells could not be identified; (III) the effect of miR-134 and miR-146a on repressing cytokines-induced mucin hypersecretion has not been verified *in vivo*.

Conclusions

In conclusion, the current data demonstrated that both miR-134-5p and miR-146a-5p conferred protection against TNF-α-induced mucus hypersecretion through repressing NF-κB and p38 MAPK signaling, indicated that miR-134-5p and miR-146a-5p may provide a therapeutic opportunity for patients with airway mucus hypersecretion.

Acknowledgments

Footnote

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