Clusterin as a serum biomarker candidate contributes to the lung fibroblasts activation in chronic obstructive pulmonary disease

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Abstract

Background: Fibrosis in the peripheral airways contributes to airflow limitation in patients with chronic obstructive pulmonary disease (COPD). However, the key proteins involved in its development are still poorly understood. Thus, we aimed to identify the differentially expressed proteins (DEPs) between smoker patients with and without COPD and elucidate the molecular mechanisms involved by investigating the effects of the identified biomarker candidate on lung fibroblasts.

Methods: The potential DEPs were identified by isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis. The messenger RNA and protein levels of clusterin (CLU) in COPD patients and 12% cigarette smoke extract (CSE)-treated human bronchial epithelial cells were determined at the indicated time points. Furthermore, an *in vitro* COPD model was established via the administration of 8% CSE to normal human lung fibroblasts (NHLFs) at indicated time points. The effects of CSE treatment and *CLU* silencing on proliferation and activation of lung fibroblasts were analyzed.

Results: A total of 144 DEPs were identified between COPD patients and normal smokers. The iTRAQ-based proteomics and bioinformatics analyses identified CLU as a serum biomarker candidate. We also discovered that CLU levels were significantly increased ($P < 0.0001$) in Global Initiative for Obstructive Lung Disease II, III, and IV patients and correlated ($P < 0.0001$) with forced expiratory volume in 1 s ($R = -0.7705$), residual volume (RV) ($R = 0.6281$), RV/total lung capacity ($R = 0.5454$), and computerized tomography emphysema ($R = 0.7878$). Similarly, CLU levels were significantly increased in CSE-treated cells at indicated time points ($P < 0.0001$). The CSE treatment significantly inhibited the proliferation, promoted the inflammatory response, differentiation of NHLFs, and collagen matrix deposition, and induced the apoptosis of NHLFs; however, these effects were partially reversed by *CLU* silencing.

Conclusion: Our findings suggest that CLU may play significant roles during airway fibrosis in COPD by regulating lung fibroblast activation.

Keywords: Chronic obstructive pulmonary disease; Clusterin; Cigarette smoke extract; Airway fibrosis; Lung fibroblasts

Introduction

Chronic obstructive pulmonary disease (COPD) is the main cause of human morbidity and mortality worldwide, resulting in a huge economic and social burden to the healthcare system.[1] COPD is characterized by a sustained decrease in airflow and poorly reversible airflow limitation, which are usually associated with a progressive inflammatory response in the airways caused by tobacco exposure.[2] Airway remodeling is well known as one of the chief pathologies in COPD patients.[3] Nonetheless, the mechanisms underlying this process are complicated and remain unelucidated. Airway remodeling reportedly involves significant pathological variations, such as abnormalities of the endothelial cells, fibrosis of the airway wall, and hypertrophy and hyperplasia of the smooth muscle.[4] In particular, peribronchial and subepithelial fibrosis are considered as the key changes in airflow limitation. Therefore, preventing airway fibrosis may be beneficial in treating COPD.[5,6]

Clusterin (CLU), also known as apolipoprotein J, is an extremely conserved and multifunctional glycoprotein, which is ubiquitously expressed in many tissues.[7] The *CLU* gene has two alternatively spliced isoforms that encode for either a nuclear (50 kDa) or a secreted protein (75–80 kDa).[8] The nuclear form plays a significant role in regulating deoxyribonucleic acid (DNA) repair, whereas the secreted form has various biological functions, including the modulation of protein homeostasis/proteostasis and the pro-survival signalling and cell death pathways.[9] Furthermore, CLU has been confirmed to

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contribute in the development of many diseases, such as Alzheimer's disease,[10] renal fibrosis,[11] diabetes,[12] and cancer.[13] Recently, emerging evidence suggests that CLU is also responsible for several types of lung injury and disease.[14] For example, secretory CLU is reportedly upregulated in a rat model of pulmonary hypertension and subsequently stimulated vascular remodeling.[15] In addition, CLU was found to be associated with oxidative stress in asthma[16,17] and idiopathic pulmonary fibrosis.[14] Furthermore, the increased serum concentration of CLU has been suggested to be a potential biomarker of cognitive dysfunction in COPD patients,[18] whereas CLU has been demonstrated to protect airway fibroblasts from cigarette smoke extract (CSE)-induced oxidative stress injury *in vitro*.[19] However, the specific role of CLU in COPD is not well understood.

In the present study, we performed isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomics and bioinformatics analyses to examine the differentially expressed proteins (DEPs) between patients with tobacco-induced COPD and normal smokers. We detected the serum concentration and expression levels of the identified biomarker candidate, CLU, in COPD patients at different stages. We further investigated the effects of CSE treatment and *CLU* silencing on lung fibroblasts to determine the function of CLU in COPD progression. Therefore, our findings may provide novel therapeutic targets for the treatment of COPD.

Methods

Ethics approval

Our study was approved by the Ethics Committee of Shengjing Hospital of China Medical University (No. 2020PS025K) and performed in accordance with the 1964 *Declaration of Helsinki* and its later amendments. Written informed consent was obtained from each participant.

Study subjects

We enrolled two subject groups from the Shengjing Hospital of China Medical University, namely, the discovery group with six male subjects ($n=3$, smokers without COPD; and $n=3$, smokers with COPD) and the verification group with 68 male subjects ($n=26$, smokers without COPD; $n=8$ Global Initiative for Obstructive Lung Disease [GOLD] I; $n=12$ GOLD II; $n=14$ GOLD III; and $n=8$ GOLD IV, smokers with COPD). The first group was recruited from April to July 2019 for the detection of potential serum biomarker candidates via iTRAQ-based proteomics, whereas the second group was recruited from February to August 2020 for the validation of serum biomarker candidates by enzyme-linked immunosorbent assay (ELISA).

The inclusion criteria for COPD patients consisted of the following conditions: (1) male patients aged 50 to 70 years; (2) patients were diagnosed according to the GOLD guidelines and must be in a stable stage[20]; and (3) a smoking history of ten or more pack-years and a smoking cessation duration of five or more years. The exclusion criteria comprised the following: (1) patients presenting with unstable cardiovascular and cerebrovascular diseases, significant impairment of liver and kidney function, and mental diseases; (2) patients diagnosed with other lung diseases within 2 months, such as asthma, pulmonary tuberculosis, and pneumonia; and (3) patients taking prescribed immuno-suppressive medications, including hormonal drugs (eg, methylprednisolone), cytotoxic drugs (eg, azathioprine), calmodulin inhibitors (eg, cyclosporine), biologics (eg, antilymphocyte globulin), and new generation monoclonal antibodies. In addition, male smokers without COPD aged 50 to 70 years were enrolled as a control group. Basic information of the discovery and verification groups is presented in Tables 1 and 2, respectively.

Sample collection

Plasma samples were collected from each subject after overnight fasting. The samples were centrifuged for 30 minutes at room temperature and temporarily stored at −80°C until use.

iTRAQ-based proteomic analysis

Equal amounts of plasma were mixed into two samples per group. The highly abundant proteins were removed using ProteoMiner kits (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's protocol. After quantification via the Bradford method, the protein (100 μg) was centrifuged at 14,000 ×*g* for 40 minutes at 4°C, added with 200 μL 50% triethyl ammonium bicarbonate (TEAB; Sigma-Aldrich, St. Louis, MO, USA), and then centrifuged again. The proteins were digested with 1 μg/μL trypsin (Promega Corp., Madison, WI, USA) at 37°C for 24 hours. The peptides were processed using iTRAQ® Reagent-8Plex Multiplex Kit (AB Sciex, Foster City, CA, USA) and subsequently labeled with different iTRAQ tags, incubated at room temperature for 1 hour, added with 8 μL 5% hydroxylamine, and incubated again at room temperature for 15 minutes. Next, the labeled samples were pooled and dried by vacuum centrifugation. The peptides were separated via strong cation exchange chromatography (SCX) using Luna SCX column (250 mm × 4.60 mm, 100 Å; Phenomenex, Torrance, CA, USA) and ion-pair reversed-phase high-performance liquid chromatography, followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using Q-Exactive (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the obtained proteins were searched against the *Homo sapiens* database using Proteome Discoverer 1.4 (Thermo Fisher Scientific) and Mascot version 2.3 (Matrix Science, London, UK). All proteomics and bioinformatics analyses were conducted by Beijing Protein Innovation (Beijing, China).

Bioinformatics analysis

Functional characterization of the identified DEPs was performed by Gene Ontology (GO) annotation[21,22] using UniProt-GO database (http://www.ebi.ac.uk/GOA/). The DEPs were assigned to biological pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG)[23,24] database (http://www.genome.jp/kegg/). Based on the

enriched GO terms, the DEPs were classified into three functional categories, namely, biological process (BP), cellular component (CC), and molecular function (MF).

Cell culture

Human bronchial epithelial (HBE) cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific) with 10% fetal bovine serum (FBS; Gibco) at 37°C with 5% CO_2. Normal human lung fibroblasts (NHLFs) were purchased from Cambrex Bio Science Walkersville (Walkersville, MD, USA) and cultured in Dulbecco's Modified Eagle Medium (Gibco) containing 10% FBS, 100 U/mL streptomycin, and 100 μg/mL penicillin (Gibco).

CSE preparation and cell treatment

The CSE was prepared as previously described.[25] In brief, the cigarettes were burned, and the mainstream of the cigarette smoke was continuously aspirated through a suction filter at a constant flow rate of 1.050 L/min to remove the tar and nicotine. The remaining cigarette smoke gas phase was soaked into 15 mL phosphate buffered saline. The filter was air-dried for 12 hours at room temperature, and the amount of tar was calculated as the increase in the dry weight of the filter. The 100% CSE solution, which was considered as the smoke from one cigarette, was slowly bubbled through 10 mL RPMI 1640 medium and then sterilized, aliquoted, and stored at −80°C until use. The HBE cells were incubated with 12% CSE at different durations (0 h, 12 h, 24 h, 36 h, and 48 h),[26] whereas the NHLFs were exposed to 8% CSE at indicated time points (0 h, 2 h, 4 h, 6 h, and 8 h) in the absence of serum.[27]

Cell transfection

The cells were transfected using Lipofectamine® 3000 reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Briefly, the NHLFs (1×10^4 cells/ well) were plated in 96-well plates and allowed to reach 70% to 80% confluency. The cells were subsequently transfected with 50 nmol/L small interfering (si)-*CLU* (5′-GCAGCAGAGUCUUCAUCAU-3′) or negative controls (si-NCs) (GenePharma, Shanghai, China).

Cell proliferation assay

After the CSE treatment or cell transfection, the NHLFs were seeded into 96-well plates (3×10^3 cells/well) and allowed to grow for 24 hours before treatment with Hyclone RPMI 1640 medium (Invitrogen) supplemented with 10% FBS. The proliferation ability of NHLFs was detected using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan), according to the manufacturer's instructions.

Table 1: Baseline data of the enrolled subjects in the discovery group (*n*=6).

Variables	Control (*n*=3)	COPD (*n*=3)	*t*	*P* values
Age (years)	63±4	62±5	0.2705	0.8002
BMI (kg/m^2)	25±3	22±3	1.2247	0.2879
Smoking history (pack-years)	35±4	36±5	−0.2705	0.8002
Smoking cessation duration (years)	7±2	9±1	−1.5492	0.1963
FEV_1 (%pred)	83±15	25±3	6.5672	0.0028
RV (%pred)	65±12	272±49	−7.1070	0.0021
RV/TLC (%)	28±7	66±13	−4.4578	0.0112
FEV_1/FVC (%)	83±6	57±6	5.3072	0.0061
CT emphysema (%)	1±1	12±8	2.1060	0.0029

Data are presented as means±SDs. BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; CT: Computerized tomography; FEV_1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; %pred: Percent predicted; RV: Residual volume; SD: Standard deviation; TLC: Total lung capacity.

Table 2: Baseline data of the enrolled subjects in the verification group (*n*=68).

Variables	Control (*n*=26)	GOLD I (*n*=8)	GOLD II (*n*=12)	GOLD III (*n*=14)	GOLD IV (*n*=8)
Age (years)	62±3	64±2	65±4	64±3	64±3
BMI (kg/m^2)	24±4	25±2	23±5	25±3	23±4
Smoking history (pack-years)	33±5	37±7	36±8	40±7	39±8
Smoking cessation duration (years)	8±3	9±3	10±4	11±5	11±4
FEV_1 (%pred)	85±13	83±3	70±2*	41±4*	23±5*
RV (%pred)	66±11	67±38	116±36*	168±26*	266±42*
RV/TLC (%)	26±8	32±12	43±11*	54±8*	74±5*
FEV_1/FVC (%)	82±7	63±6	58±5*	52±4*	46±7*
CT emphysema (%)	1±1	4±2	5±4	13±4*	30±10*

Data are presented as means ± SDs. *$P < 0.0001$ *vs.* control. BMI: Body mass index; CT: Computerized tomography; FEV_1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; GOLD: Global Initiative for Obstructive Lung Disease; %pred: Percent predicted; RV: Residual volume; SD: Standard deviation; TLC: Total lung capacity.

Table 3: Information of the primers used for qRT-PCR.

Genes	Direction	Primer sequences
CLU	Forward (5′→3′)	CGAGAAGGCGACGATGAC
	Reverse (5′→3′)	GGTGGAACAGTCCACAGACA
GAPDH	Forward (5′→ 3′)	TTTGGTATCGTGGAAGGACTC
	Reverse (5′→3′)	GTAGAGGCAGGGATGATGTTCT

CLU: Clusterin; *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: Quantitative reverse-transcription PCR.

Real-time quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was extracted from HBE cells or NHLFs using TRIzol® reagent (Invitrogen), following the manufacturer's protocol, and reverse transcribed into complementary DNA (cDNA) using Omniscript Reverse Transcription Kit (Qiagen, Hilden, Germany). The expression level of *CLU* mRNA was measured using Takara TP960 PCR Thermal Cycler Dice Real Time System (Takara Bio Inc., Otsu, Japan), with glyceraldehyde-3-phosphate dehydro-genase (*GAPDH*) as an internal control. The primer sequences of *CLU* and *GAPDH* are listed in Table 3. The thermal cycling conditions were as follows: an initial denaturation at 94°C for 30 s, 40 cycles at 95°C for 15 s, 60°C for 30 s, and 68°C for 20 s, and a final extension at 72°C for 90 s. The relative gene expression levels were quantified using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) method.[28]

Western blot analysis

Total protein was extracted from HBE cells or NHLFs using Radio-Immunoprecipitation Assay lysis buffer (Beyotime, Shanghai, China) supplemented with protease inhibitors (Beyotime), following the manufacturer's manual. The protein concentrations were measured using Bicinchoninic Acid Assay protein assay kit (Beyotime). The proteins were separated using 10% to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose filter membranes, and blocked with 5% nonfat milk in Tris-buffered saline–Tween 20. Then, the membranes were incubated overnight at 4°C with primary antibodies against CLU (ab185957; Abcam, Cambridge, MA, USA); α-smooth muscle actin (α-SMA, ab5694; Abcam); fibronectin (FN, 15613-1-AP; Proteintech, Chicago, IL, USA); collagen I (ab34710; Abcam); collagen III (ab184993; Abcam); matrix metalloproteinase 2 (MMP-2, SAB4501891; Sigma-Aldrich); MMP-9 (AV33090; Sigma-Aldrich); B-cell lymphoma-2 (Bcl-2, PRS3335; Sigma-Aldrich); Bcl-2-associated X (Bax, sc7480; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); pro-caspase-3 (ab32499; Abcam); cleaved-caspase-3 (ab2302; Abcam); p53 (#2527; Cell Signaling Technology [CST], Inc., Danvers, MA, USA); p21 (#2947; CST); and GAPDH (ab8227; Abcam). The blots were subsequently incubated with secondary antibodies for 1 hour at room temperature and visualized using electro-generate chemiluminescence Western blotting KIT (Solarbio, Beijing, China). Protein quantification was performed using Image-Pro Plus 6.0 Software (Media Cybernetics, Silver Spring, MD, USA).

Enzyme-linked immunosorbent assay

The serum CLU levels were evaluated using ELISA kits (ab174447; Abcam), following the manufacturer's instructions. The levels of interleukin (IL)-6 (ab178013; Abcam), IL-8 (ab214030; Abcam), and tumor necrosis factor-α (TNF-α, ab181421; Abcam) in the NHLFs were also determined using commercial ELISA kits.

Statistical analysis

The continuous data with normal distribution are presented as means ± standard deviations. Normal distribution was confirmed by Shapiro-Wilk test. Student's *t*-test and one-way analysis of variance with a Tukey's *post hoc* test were performed to analyze the differences between two groups and among three or more groups using GraphPad Prism 8.0 statistical software (GraphPad, San Diego, CA, USA). Pearson correlation analysis was used to assess the clinical data relative to the CLU serum levels. Data with P values < 0.05 were considered statistically significant.

Results

CLU is identified as a serum biomarker candidate for COPD

To identify the potential biomarker candidates for COPD, we analyzed the discovery group ($n = 3$ for male smokers with or without COPD, respectively) and discovered no significant differences in the ages ($t = 0.2705$, $P = 0.8002$), body mass index (BMI, $t = 1.2247$, $P = 0.2879$), smoking histories ($t = -0.2705$, $P = 0.8002$), and smoking cessation durations ($t = -1.5492$, $P = 0.1963$) between smokers with and without COPD [Table 1]. In contrast, significant differences were observed in the forced expiratory volume in 1 second (FEV_1, $t = 6.5672$, $P = 0.0028$), residual volume (RV, $t = -7.1070$, $P = 0.0021$), RV/total lung capacity (TLC, $t = -4.4578$, $P = 0.0112$), FEV_1/forced vital capacity (FVC, $t = 5.3072$, $P = 0.0061$), and CT emphysema ($t = 2.1060$, $P = 0.0029$) values between the two subgroups.

The results of the iTRAQ-based proteomic analysis are presented in Figure 1. We successfully identified 144 DEPs, including 80 upregulated and 64 downregulated proteins, between the COPD patients and controls [Figure 2A]. Functional characterization of the identified DEPs via bioinformatics analysis revealed their corresponding GO annotations and KEGG pathway associations [Figure 2B–D]. The GO annotation showed that the most highly enriched GO terms were extracellular region, "immune response," and "molecular function regulator" under the CC, BP, and

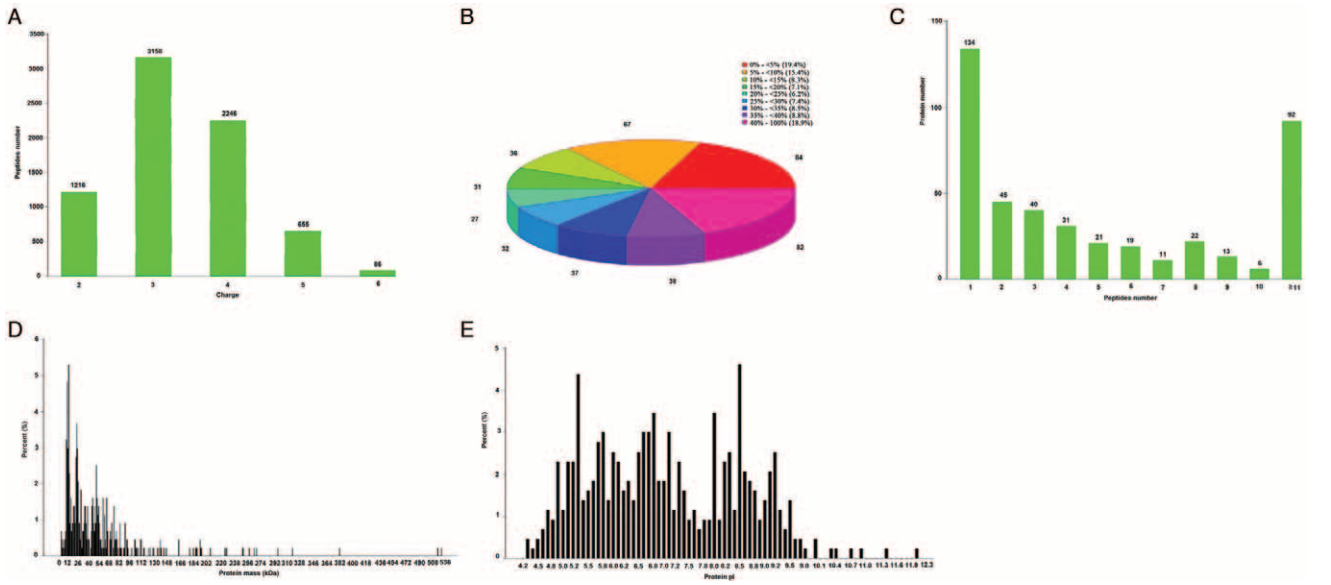

Figure 1: Results of the iTRAQ-based proteomic analysis. (A) Distribution of peptide charges. (B) Statistics of protein sequence coverages. (C) Distribution of the protein-peptide matches. (D) Distribution of protein motifs. (E) Distribution of protein isoelectric points. iTRAQ: Isobaric tags for relative and absolute quantitation; pI: Isoelectric point.

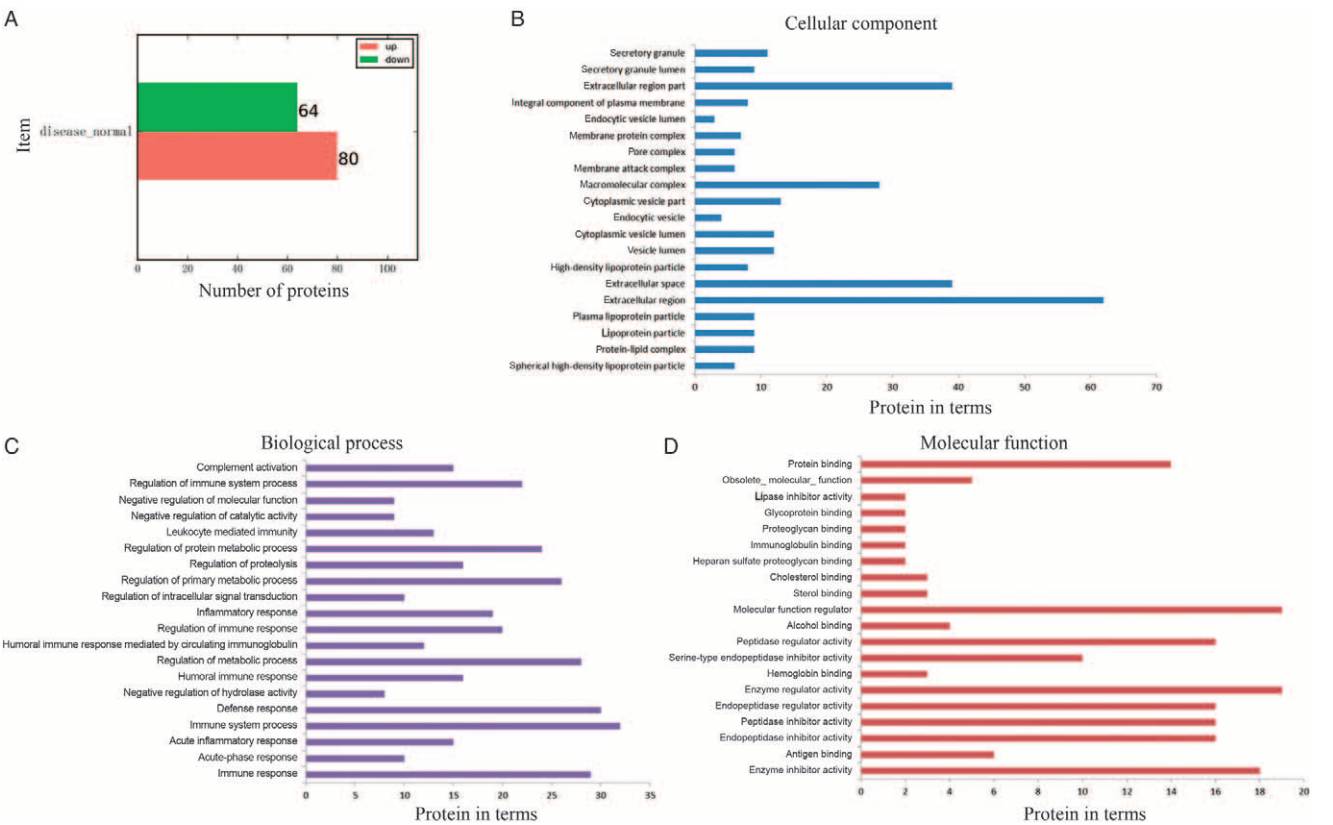

Figure 2: Functional characterization of DEPs using GO annotation and KEGG pathway analysis. (A) A total of 144 DEPs, including 80 upregulated and 64 downregulated proteins, were identified. (B) Number of DEPs enriched with GO terms under the CC category. (C) Number of DEPs enriched with GO terms under the BP category. (D) Number of DEPs enriched with GO terms under the MF category. BP: Biological process; CC: Cellular component; DEPs: Differentially expressed proteins; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: Molecular function.

MF categories, respectively. For the KEGG pathway analysis, the identified DEPs were found to be closely associated with "complement and coagulation cascades" [Supplementary Figure 1, http://links.lww.com/CM9/A983]. In addition, a total of 15 proteins, including prothrombin, fibrinogen α chain, coagulation factor XIII A chain, α-1-antitrypsin, complement component C6, complement component C8 γ chain, CLU, cDNA FLJ60818 (highly similar to

complement C3), α-2-macroglobulin, complement C5, heparin cofactor 2, C4b-binding protein β chain, fibrinogen β chain, complement C4A, and complement factor H, were highly enriched in this specific pathway. Notably, the CLU protein was observed to be highly enriched under the CC and BP categories and was closely related to the identified KEGG pathway; hence, CLU was selected for further analysis.

CLU is significantly upregulated in COPD patients and correlated with clinical variables

To detect the expression profile of CLU in COPD patients, we analyzed the verification group ($n = 68$ smokers with and without COPD) and found that RV and RV/TLC values were significantly higher and FEV_1 and FEV_1/FVC were significantly lower in the GOLD II, III, and IV COPD patients than in the controls ($P < 0.0001$), whereas CT emphysema was significantly higher in the GOLD III and IV patients than in the controls ($P < 0.0001$) [Table 2]. The ELISA results confirmed that the levels of CLU in the blood sera were significantly increased in the GOLD II, III, and IV COPD patients compared to the controls ($P < 0.0001$) [Figure 3A]. Furthermore, the serum levels of CLU were significantly higher in GOLD III ($P = 0.0216$) and IV ($P = 0.0127$) COPD patients than in GOLD I COPD patients. To determine the correlations between CLU levels and selected clinical variables, we performed Pearson correlation analysis and discovered that the CLU serum concentration was negatively correlated with FEV_1 (percent predicted, % pred) ($R = -0.7705$, $P < 0.0001$) and positively correlated with RV (%pred) ($R = 0.6281$, $P < 0.0001$), RV/TLC (%) ($R = 0.5454$, $P < 0.0001$), and CT emphysema (%) ($R = 0.7878$, $P < 0.0001$) [Figure 3B–E]. These findings imply that the elevated levels of CLU in the blood sera are significantly associated with disease severity in COPD patients.

CLU is upregulated in CSE-treated HBE cells

In addition, the mRNA and protein levels of CLU in CSE-treated HBE cells at different time points were analyzed. The qRT-PCR results demonstrated that the relative expression levels of *CLU* mRNA were significantly upregulated in CSE-treated HBE cells at 24 hours ($P = 0.005$), 36 hours ($P < 0.0001$), and 48 hours ($P < 0.0001$) [Figure 4A]. Similarly, the expression levels of CLU protein in CSE-treated HBE cells at 12 hours ($P = 0.002$), 24 hours ($P < 0.0001$), 36 hours ($P < 0.0001$), and 48 hours ($P < 0.0001$) were significantly increased [Figure 4B]. These results indicate that CLU may be involved in the development of COPD.

CSE treatment significantly promotes human lung fibroblast activation

We established an *in vitro* COPD model via administration of CSE to NHLFs at different time points and investigated the effects of CSE treatment on cell proliferation, inflammatory response, apoptosis, differentiation, and collagen deposition of NHLFs. The results showed that the cell viability of NHLFs was significantly decreased after CSE treatment at 4 hours ($P = 0.0061$), 6 hours ($P = 0.0017$), and 8 hours ($P < 0.0001$) [Figure 5A]. We also observed that the expression of proapoptotic proteins, including Bax ($P = 0.0143$ at 2 h, $P = 0.0112$ at 4 h, $P = 0.0007$ at 6 h, and $P < 0.0001$ at 8 h), cleaved/procaspase 3 ($P = 0.0020$ at 2 h, $P = 0.0015$ at 4 h, $P = 0.0004$ at 6 h, and $P = 0.0001$ at 8 h), p53 ($P = 0.0098$

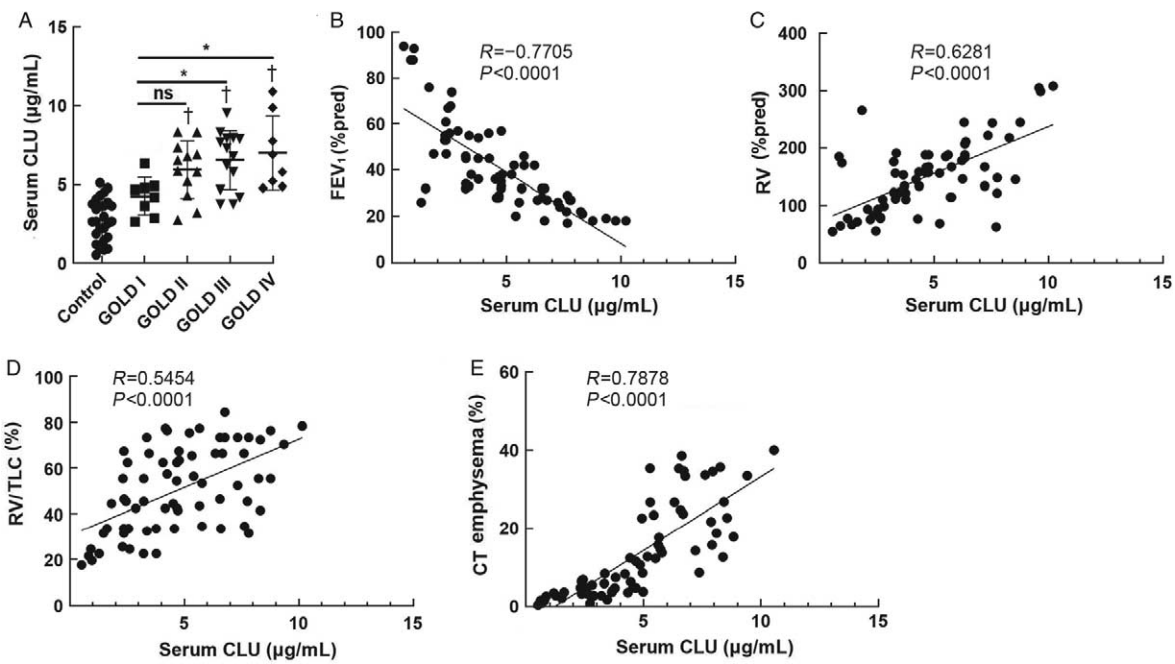

Figure 3: CLU serum levels were significantly upregulated in the COPD patients and correlated with key clinical variables. (A) Concentration of CLU In the blood serum samples of normal controls and GOLD I–IV patients in the verification group. (B) Correlation between CLU and FEV_1 (%pred). (C) Correlation between CLU and RV (%pred). (D) Correlation between CLU and RV/TLC(%). (E) Correlation between CLU and CT emphysema (%). $n = 68$. CLU: Clusterin; COPD: Chronic obstructive pulmonary disease; CT: Computerized tomography; FEV_1: Forced expiratory volume in 1 s; GOLD: Global Initiative for Obstructive Lung Disease; %pred: Percent predicted; ns: Not significant; RV: Residual volume; TLC: Total lung capacity. $^*P < 0.05$ *vs.* GOLD I; $^\dagger P < 0.0001$ *vs.* control.

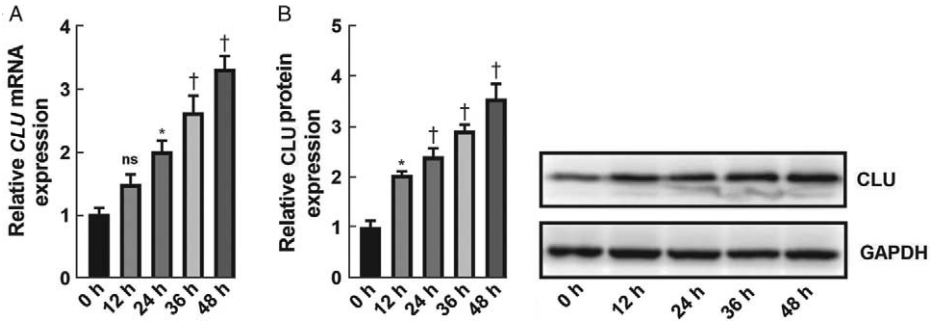

Figure 4: CLU expression is upregulated in CSE-treated HBE cells at different time points (0 h, 12 h, 24 h, 36 h, and 48 h). (A) The relative expression levels of *CLU* mRNA. (B) The relative expression levels of CLU protein. Data are presented as means ± SDs of three independent experiments, with GAPDH as an internal control. CLU: Clusterin; CSE: Cigarette smoke extract; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HBE: Human bronchial epithelial; mRNA: Messenger RNA; ns: Not significant; SD: Standard deviation. $^{*}P < 0.01$ *vs.* 0 h; $^{\dagger}P < 0.0001$ *vs.* 0 h.

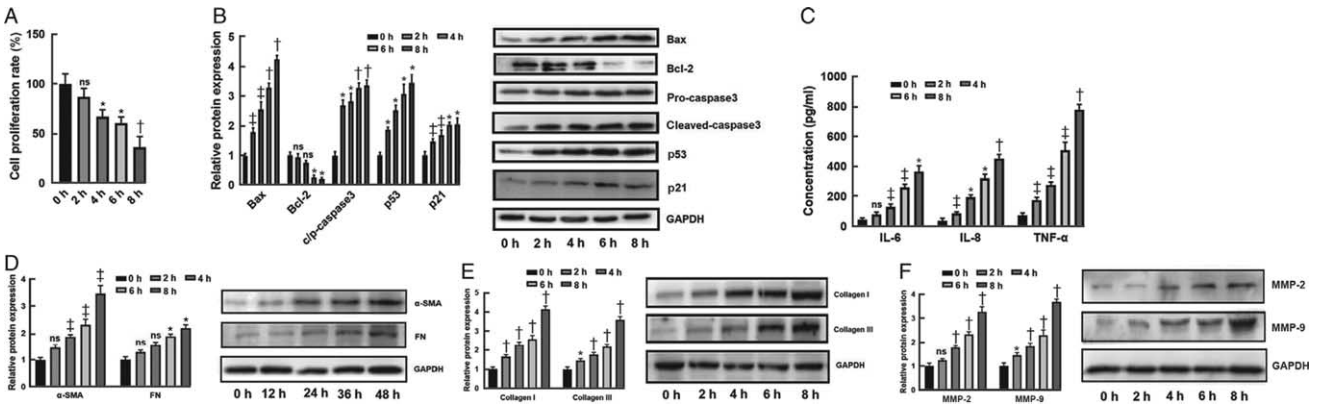

Figure 5: Effects of CSE treatment on proliferation and activation of human lung fibroblasts. (A) Effects of CSE treatment on cell proliferation. (B) Effects of CSE treatment on the expression of apoptosis-related proteins Bax, Bcl-2, caspase3, p53, and p21. (C) Effects of CSE treatment on the expression of inflammatory cytokines IL-6, IL-8, and TNF-α. (D) Effects of CSE treatment on α-SMA and FN expression. (E) Effects of CSE treatment on the expression of collagens I and III. (F) Effects of CSE treatment on MMP-2 and MMP-9 expression. Data are presented as means ± SDs of three independent experiments, with GAPDH as an internal control. α-SMA: α-Smooth muscle actin; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; CSE: Cigarette smoke extract; FN: Fibronectinj; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; MMP: Matrix metalloproteinase; ns: Not significant; SD: Standard deviation; TNF-α: Tumor necrosis factor α. $^{*}P < 0.01$, $^{\dagger}P < 0.001$, $^{\ddagger}P < 0.05$ *vs.* 0 h.

at 2 h, $P = 0.0093$ at 4 h, $P = 0.0087$ at 6 h, and $P = 0.0081$ at 8 h), and p21 ($P = 0.0392$ at 2 h, $P = 0.0325$ at 4 h, $P = 0.0029$ at 6 h, and $P = 0.0011$ at 8 h), was significantly upregulated in a time-dependent manner, whereas the expression of anti-apoptotic protein Bcl-2 ($P = 0.0054$ at 6 h, and $P = 0.0037$ at 8 h) was markedly downregulated after CSE administration [Figure 5B]. Similarly, the CSE treatment significantly increased the expression of inflammatory factors IL-6 ($P = 0.0292$ at 4 h, $P = 0.0168$ at 6 h, and $P = 0.0075$ at 8 h), IL-8 ($P = 0.0128$ at 2 h, $P = 0.0092$ at 4 h, $P = 0.0079$ at 6 h, and $P = 0.0008$ at 8 h), and TNF-α ($P = 0.0469$ at 2 h, $P = 0.0311$ at 4 h, $P = 0.0224$ at 6 h, and $P < 0.0001$ at 8 h) in a time-dependent manner [Figure 5C]. Furthermore, the expression of α-SMA ($P = 0.0387$ at 4 h, $P = 0.0334$ at 6 h, and $P = 0.0243$ at 8 h) and FN ($P = 0.0027$ at 6 h, and $P = 0.0022$ at 8 h), which are characteristic markers during the differentiation of lung fibroblasts into myofibroblasts, was notably enhanced after CSE treatment [Figure 5D]. We also observed that the expression levels of collagen I ($P = 0.0001$ at 2 h, $P < 0.0001$ at 4 h, 6 h, and 8 h), collagen III ($P = 0.0060$ at 2 h, $P < 0.0001$ at 4 h, 6 h, and 8 h), MMP-2 ($P < 0.0001$ at 4 h, 6 h, and 8 h), and MMP-9 ($P = 0.0063$ at 2 h, $P < 0.0001$ at 4 h, 6 h, and 8 h) were greatly elevated after CSE treatment [Figure 5E and 5F]. From these data, we confirmed that the maximum effect was achieved at 8 hours; therefore, we selected 8 hours as the duration of CSE treatment for subsequent analyses. Collectively, these findings suggest that CSE treatment simultaneously inhibited the proliferation, promoted the inflammatory response, differentiation, and collagen deposition, and induced the apoptosis of NHLFs.

si-CLU is successfully transfected into NHLFs

To further explore the function of CLU in COPD, the *CLU* gene was silenced, and the si-*CLU* was transfected into NHLFs. The transfection efficiency of *CLU* silencing was detected by qRT-PCR and Western blot experiments. Our results exhibited that both the mRNA and protein levels of CLU were significantly reduced in CSE-treated NHLFs ($P < 0.0001$) [Figure 6A and 6B], indicating that si-CLU was successfully transfected into the NHLFs.

CLU silencing significantly inhibits human lung fibroblast activation

Subsequently, we analyzed the effects of *CLU* silencing on the proliferation, inflammatory response, apoptosis, differ-

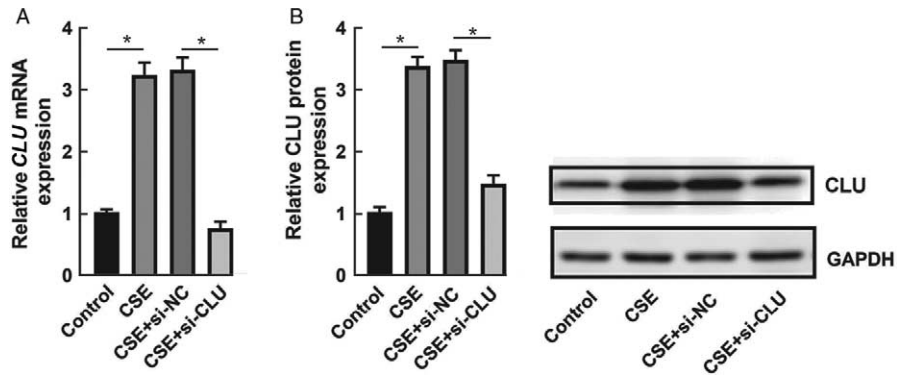

Figure 6: CLU expression in CSE-treated NHLFs transfected with *si-CLU* or si-NC. (A) The relative expression levels of *CLU* mRNA after transfection. (B)The relative expression levels of CLU protein after transfection. Data are presented as means ± SDs of three independent experiments, with GAPDH as an internal control. CLU: Clusterin; CSE: Cigarette smoke extract; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; mRNA: Messenger RNA; NC: Negative control; NHLFs: Normal human lung fibroblasts; SD: Standard deviation; si: Small interfering. *$P < 0.0001$.

entiation, and collagen deposition of NHLFs. The data showed that the combination of CSE treatment and *CLU* silencing (CSE + si-*CLU*) significantly increased the cell proliferation ability of NHLFs ($P = 0.0001$) [Figure 7A] and reduced the expression of proapoptotic proteins (Bax, $P = 0.0004$; c/p-caspase3, $P = 0.0005$; p21, $P = 0.0023$) [Figure 7B], proinflammatory factors (IL-6, $P = 0.0004$; IL-8, $P = 0.0042$; TNF-α, $P = 0.0023$) [Figure 7C], markers of differentiation (α-SMA, $P < 0.0001$; FN, $P = 0.0062$) [Figure 7D], markers of collagen deposition (collagen I, $P < 0.0001$; collagen III, $P < 0.0001$) [Figure 7E], and marker of matrix deposition (MMP-9, $P = 0.0114$) [Figure 7F], but significantly upregulated the expression of antiapoptotic protein Bcl-2 ($P < 0.0001$). However, no significant difference was observed in the expression of p53 and MMP-2. These data indicate that *CLU* silencing promoted the proliferation and inhibited the inflammatory response, differentiation, collagen matrix deposition, and apoptosis of CSE-treated lung fibroblasts.

Discussion

In the present study, we aimed to identify the key proteins involved in the development of tobacco smoke-induced COPD and to investigate the function of a selected biomarker candidate. We discovered that the serum concentration of CLU was significantly elevated in smoker patients with COPD and correlated with key clinical variables, including FEV_1, RV, RV/TLC, and CT emphysema. In addition, our results demonstrated that CLU expression was significantly upregulated in CSE-treated HBE cells, whereas *CLU* silencing inhibited lung fibroblast activation, specifically by enhancing the proliferation ability and inhibiting the inflammatory response, differentiation, collagen matrix deposition, and apoptosis of NHLFs. Taken together, our results suggest that CLU is potentially involved in the development of airway fibrosis in COPD patients.

Biomarkers are critical for the diagnosis and treatment of complex diseases.[29] For example, fetuin-B is a potential plasma biomarker associated with the severity of lung function abnormalities in COPD.[30] In addition, plasma fibrinogen is reportedly a relatively useful and prognostic marker for classifying the risks of future exacerbations and for identifying COPD patients with high mortality.[31] Furthermore, the club cell protein-16 can demonstrate the severity of COPD in former smokers.[32] Fibrinogen, C reactive protein, and white cell count were also associated with a higher risk of mortality in COPD patients.[33] Despite these reports, it is still difficult to ascertain COPD progression using available biomarkers. Hence, there is an urgent need to discover new biomarkers for the pulmonary function deterioration in COPD.[34] Unbiased and high-throughput techniques have been well acknowledged and well utilized for identifying biomarkers. Among these, proteomics is a powerful and promising tool for detecting novel prognostic proteins that has been extensively used for disease biomarker discovery.[35] Recently, an increasing number of studies have utilized proteomics for COPD biomarker discovery.[36–39] However, to our knowledge, this technique has not been applied to examine the serum biomarkers in smokers with and without COPD. Thus, we performed an iTRAQ-based proteomic analysis to identify the serum biomarkers in smoker patients with COPD and normal smokers. We discovered a total of 144 DEPs, including 80 upregulated and 64 downregulated proteins, between tobacco-induced COPD patients and normal smokers. Fundamentally, these common DEPs were considered as smoke-sensitive proteins in COPD patients.

We identified CLU as a biomarker candidate through functional characterization of the identified DEPs via GO annotation and KEGG pathway analysis and validated its serum concentration using the blood plasma from the verification group by ELISA. The results were consistent with the proteomic analysis data, which demonstrated the highly accurate quantification of the proteomics technique used. Furthermore, we confirmed that the serum levels of CLU were negatively correlated with FEV_1 and positively correlated with RV, RV/TLC, and CT emphysema. Therefore, CLU may be used as an auxiliary biomarker in the diagnosis and treatment of COPD. Our data also revealed that the CLU levels in the GOLD II, III, and IV COPD patients were significantly higher than in the controls and GOLD I COPD patients, suggesting that CLU may be

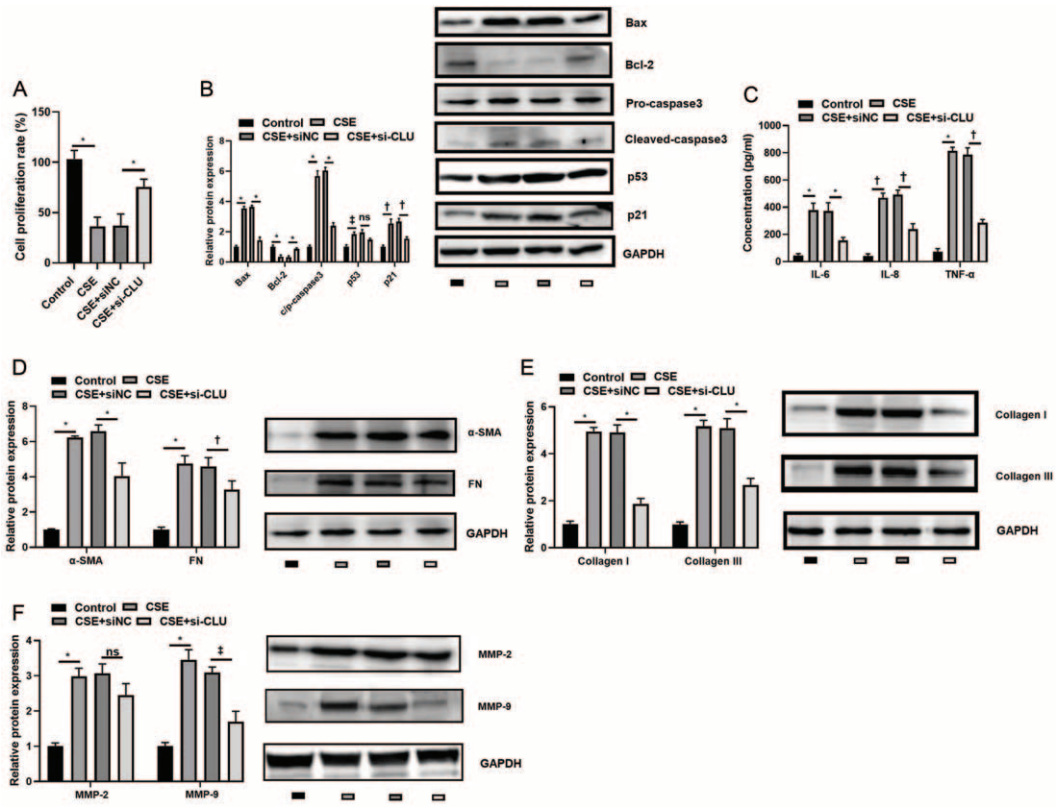

Figure 7: Effects of combined CSE treatment and *CLU* silencing on proliferation and activation of human lung fibroblasts. (A) Effects of CSE treatment and *CLU* silencing on cell proliferation. (B) Effects of CSE treatment and *CLU* silencing on the expression of apoptosis-related proteins Bax, Bcl-2, caspase3, p53, and p21. (C) Effects of CSE treatment and *CLU* silencing on the expression of inflammatory cytokines IL-6, IL-8, and TNF-α. (D) Effects of CSE treatment and *CLU* silencing on α-SMA and FN expression. (E) Effects of CSE treatment and *CLU* silencing on the expression of collagens I and III. (F) Effects of CSE treatment and *CLU* silencing on MMP-2 and MMP-9 expression. Data are presented as means ± SDs of three independent experiments, with GAPDH as an internal control. α-SMA: α-Smooth muscle actin; Bax: Bcl-2-associated X protein; Bcl-2: B-cell lymphoma-2; *CLU*: Clusterin; CSE: Cigarette smoke extract; FN: Fibronectin; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; MMP: Matrix metalloproteinase; NC: Negative control; ns: Not significant; SD: Standard deviation; TNF-α: Tumor necrosis factor α. $^{*}P < 0.001$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.05$.

useful in detecting the more severe grades of COPD and in screening the COPD progression in GOLD I patients. Our *in vitro* experiments confirmed that the mRNA and protein levels of CLU were significantly elevated in CSE-treated HBE cells in a time-dependent manner, which were in line with the serum concentrations of CLU in COPD patients. However, the exact role of CLU in COPD was not clearly understood. Therefore, we evaluated the effects of *CLU* silencing on lung fibroblast activation.

Lung fibroblasts, which exist within the pulmonary airway and parenchyma, are involved in the development of airway fibrosis and abnormal deposition of extracellular matrix in COPD. Hence, the activation of lung fibroblasts is a key process during airway fibrosis in COPD. It can occur under diverse pathological circumstances, such as during collagen matrix deposition and during differentiation of fibroblasts to myofibroblasts.[4] Therefore, we used CSE-treated NHLFs to mimic the COPD fibrosis model and investigated the effects of CSE treatment on the proliferation, inflammatory response, apoptosis, differentiation, and collagen matrix deposition of NHLFs. Similar to the results of previous studies, we confirmed that CSE treatment markedly inhibited the proliferation[40] and induced the apoptosis,[41,42] differentiation,[43] inflammatory response,[44] and collagen matrix deposition of lung fibroblasts.[45,46] These results indicated that CSE successfully induced the activation of lung fibroblasts. Notably, *CLU* silencing partly reversed the observed effects of CSE treatment.

The reported biological functions of CLU are still unclear. For example, CLU can reportedly improve the proliferation of primary astrocytes,[47] whereas lentivirus-mediated short hairpin (sh)-*CLU* can inhibit the proliferation of ovarian cancer cells.[48] Additionally, CLU can suppress apoptosis in cardiomyocytes and human retinal pigment epithelial cells. By contrast, overexpression of *CLU* can reduce the cell proliferation ability of simian virus (SV40)-immortalized human prostate epithelial cells.[49] In the present study, we discovered that *CLU* silencing significantly improved the proliferation but suppressed the apoptosis of lung fibroblasts. The differences in cell/tissue types or sources may account for the observed discrepancies in the data. Interestingly, CLU has been confirmed as a multi-faceted protein that functions in inflammation and autoimmunity.[50] For example, plasma CLU level is a potential biomarker of the inflammatory process in Alzheimer's disease and a surrogate marker of obesity-associated systemic inflammation. [51] In addition, CLU can regulate allergic airway inflammation by suppressing the recruitment of C-C motif chemokine ligand 20-

mediated dendritic cells.[52] However, we found that *CLU* silencing suppressed the inflammatory response by reducing the expression of inflammatory factors IL-6, IL-8, and TNF-α in lung fibroblasts. Furthermore, *CLU* silencing inhibited differentiation by decreasing the expression of α-SMA and FN and hindered collagen matrix deposition by down-regulating the expression of collagens I and III and MMP-9 in lung fibroblasts. However, these results contradict those of a previous study reporting that *CLU* knockdown enhanced renal inflammation and aggravated tissue fibrosis after ischemia-reperfusion injury in the kidney.[53] Hence, these discrepancies should be explored and further research must be performed to fully elucidate the role of CLU during the development of airway fibrosis in COPD.

In conclusion, our findings suggest that elevated serum CLU levels are associated with the disease severity in COPD patients. Furthermore, *CLU* silencing inhibited lung fibroblast activation, demonstrating that CLU may be responsible for the development of airway fibrosis in COPD.

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Conflicts of interest

None.

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