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ORIGINAL RESEARCH

Downregulation of Lysosome-Associated Membrane Protein-2A Contributes to the Pathogenesis of COPD

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Background: Macroautophagy plays an important role in the pathogenesis of chronic obstructive pulmonary disease (COPD), but the role of chaperone-mediated autophagy (CMA) has not been investigated. We investigated if and how CMA is involved in the pathogenesis of COPD.

Methods: We measured the level of lysosome-associated membrane protein-2A (LAMP-2A), which is a critical component of CMA that functions as a receptor for cytosolic substrate proteins, in total lung tissues and primary human bronchial epithelial cells (HBECs) from healthy never smokers, smokers, and COPD patients. We assessed the effects of LAMP-2A knock-down on cigarette smoke extract (CSE)-induced aging, cell cycle arrest, and apoptosis in BEAS-2B cells and the expression levels of apoptosis hallmarks in primary HBECs and lung tissue sections.

Results: We found that the protein levels of LAMP-2A in lung homogenates and primary HBECs from smokers and COPD patients were lower than those from never smokers. In addition, its level in primary HBECs was negatively correlated with years of smoking. CSE caused degradation of LAMP-2A protein via the lysosomal pathway by activating macroautophagy. Knock-down of LAMP-2A markedly enhanced CSE-induced expression of senescence markers such as p16, p21, p27, and p53. G2/M cell cycle arrest, up-regulation of cyclin B1, and apoptosis in BEAS-2B cells. Apoptosis was increased in CSE-treated primary HBECs and in lung tissues from smokers and COPD patients.

Conclusion: Cigarette smoke-induced down-regulation of LAMP-2A is involved in acceleration of aging and apoptosis of lung epithelial cells, which might at least partially contribute to COPD pathogenesis.

Keywords: COPD, LAMP-2A, lung epithelial cells, aging, apoptosis

Introduction

Chronic obstructive pulmonary disease (COPD) affects more than 210 million people,[1] thus representing a major health and economic burden worldwide. The most important identifiable risk factor for COPD is cigarette smoke (CS). CS causes inflammation, oxidative stress, and an imbalance between proteases and anti-proteases, which have been suggested as the pathogenic triad in the pathogenesis of COPD.[2–4] Recent evidence supports the role of cellular senescence, apoptosis, and autophagy in the development of COPD.[5]

Autophagy is a lysosomal degradation pathway for cytoplasmic materials,[6] which is induced by diverse stimuli including nutrient starvation, cytokines, and oxidative stress. Three main types of autophagy have been identified in mammals: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). These 3 forms of autophagy differ in their delivery methods to lysosomes. In macroautophagy, cargo is sequestered inside autophagosomes for delivery to lysosomes through vesicular fusion. In microautophagy, cytosolic material is internalized for degradation in

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single-membrane vesicles that form through invaginations on the surface of lysosomes or late endosomes. In contrast, vesicles are not necessary in CMA, in which a cytosolic chaperone identifies substrate proteins and delivers them to the surface of lysosomes for internalization through a translocation complex. This complex is formed by the multimerization of the CMA receptor protein, lysosome-associated membrane protein-2A (LAMP-2A).[7] LAMP-2A is a critical component of CMA that functions as a receptor for cytosolic substrate proteins. The level of LAMP-2A is known to correlate with CMA activity.

The essential function of autophagy is to maintain cellular homeostasis and adapt to adverse environments. However, when not regulated, persistence of inefficient autophagy may be detrimental to lung epithelial cells, leading to apoptosis through a cell-autodigestive process. Dysfunction of the autophagy-lysosomal pathway has recently been implicated in respiratory diseases such as interstitial lung disease, asthma, cystic fibrosis, and COPD.[8] Numerous reports have documented aberrant activation of autophagy in lung epithelial cells and lung tissues of COPD patients, murine models, and cell culture model systems.[9–15] Moreover, knockout of light chain-3B (LC3B, a marker of macroautophagy) reduces lung apoptosis and airspace enlargement in mice and inhibits the cleavage of poly ADP-ribose polymerase (PARP), indicating that macroautophagy contributes to apoptosis in human bronchial epithelial cells, causing emphysematous changes.[16,17] Thus, it is likely that macroautophagy is involved in the pathogenesis of COPD. However, it has not been clear if and how CMA contributes to the pathogenesis of COPD. In the present study, we first investigated the aberrant expression of LAMP-2A in lung parenchyma and bronchial epithelial cells in COPD, and then the contribution of CMA to CS-induced bronchial epithelial cell senescence and death.

Methods

Cells and Human Lung Tissues

Normal human bronchial epithelial cells (BEAS-2B, from American Type Culture Collection, Manassas, VA, USA) were maintained in defined keratinocyte serum-free medium (GIBCO) at 37°C and 5% CO_2. Primary human bronchial epithelial cells (HBEC) were obtained according to the ethical guideline following the declaration of Helsinki and given approval by the Seoul National University Hospital Institutional Review Board (SNUH IRB number: H-1602-108-742) and an informed consent was obtained from each subject. Primary HBECs were isolated from bronchial brushing samples during bronchoscopy. Airway brushings were obtained from the anterior basal segmental bronchus of right lower lobe. The brush was immediately immersed in a tube containing 10 mL of ice-cold RPMI containing 20% fetal bovine serum. Within a few minutes, the cells were centrifuged and resuspended in defined keratinocyte serum free medium. The epithelial lineage was verified by immunocytochemical staining. Cultured HBECs were stained intensely and exclusively for the epithelial specific markers (cytokeratin and E-cadherin) but not for macrophage and endothelial lineage markers (CD11b and CD31). Submerged cells were grown as monolayers to 80–100% confluence and then used for experiments. All human lung tissues were obtained from lung resection of thoracic surgical cases, most of which were lung cancer surgery. After review and approval by the Seoul National University Hospital Institutional Review Board (SNUH IRB Number: H-1309-073-521), the specimens were collected from non-cancerous parts of resected lung tissue.

CSE Preparation

Commercial cigarettes (THIS; 84 mm long with a diameter of 8 mm, purchased from Korea Tomorrow & Global Corp.) were smoked continuously using a bottle system connected to a vacuum machine. The smoke from 20 cigarettes was bubbled in 60 mL of PBS (GIBCO). The nicotine content was 0.65 mg per cigarette and the tar content was 6.5mg per cigarette. The puff duration was 22 ~ 24 seconds per cigarette. The large insoluble particles contained in the resulting suspension were removed by filtering the solution through a 0.22 μm filter.

Protein Extraction and Western Blot Analysis

Total cellular extracts were prepared in 1X cell lysis buffer (Cell Signaling Technology). Frozen lung tissues were homogenized in tissue extraction buffer (Life Technologies) containing a protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentration was measured using the Bradford protein assay (Bio-Rad).

Proteins were resolved by 4–12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1 h before being incubated overnight at 4°C with primary antibodies. The membranes were washed with washing buffer and incubated with secondary antibodies for 1 h. After successive washes, the membranes were developed using the SuperSignal West Pico Chemiluminescent kit (Thermo Fisher Scientific). The following antibodies were used for protein detection: anti-LAMP-2A and anti-p16 (Abcam); anti-phosphorylated-p21 (p-p21) at Ser146, anti-p21, anti-p27, anti-p-p53 at Ser15, anti-p53, anti-poly (ADP-ribose) polymerase (PARP), anti-GAPDH (Santa Cruz Biotechnology Inc.); anti-active caspase-3, anti-cyclin B1, and anti-LC3B (Cell Signaling Technology).

Transfection of siRNA

Transfection of siRNAs (control, LAMP-2A, and LC3B siRNA) was performed using the Neon Transfection System. After 48 h, the cells were used in the experiments. Control and LAMP-2A siRNAs were purchased from Santa Cruz Biotechnology Inc. LC3B siRNA was obtained from Cell Signaling Technology.

Real-Time PCR

Total RNA was isolated using the RNeasy kit (Qiagen). cDNA was synthesized from 1 μg of total RNA using the Reverse Transcription system (Promega). PCR amplification was performed using SYBR Green (Applied Biosystems). The primers used were listed in Table 1.

Immunohistochemistry

Lung tissues were fixed, embedded, cut, and placed on slides using the Discovery XT automated immunohistochemistry stainer (Ventana Medical Systems, Inc.). Tissue sections were deparaffinized and rehydrated. Cell conditioning 1 (CC1) standard (pH 8.4 buffer containing Tris/Borate/EDTA) was used for antigen retrieval. The sections were incubated with primary antibody for 32 min at 37°C, washed, and incubated with a secondary antibody for 20 min at 37°C. After successive washes, slides were incubated with DAB H_2O_2 substrate for 8 min at 37°C. Stained cells were observed under a microscope (EVOS XL Core Cell Imaging System, Thermo Fisher Scientific). The slide micrographs were taken using a pathology slide scanner (SCN400 F, Leica Microsystems).

Cell Cycle Assay

Cells were fixed with 70% ethanol at −20°C for 24 h. The fixed cells were washed with 1X PBS and then added to 200 μL propidium iodide (PI) solution containing RNase A (1 mg/mL), 0.1% Triton X-100, and PI (50 μg/mL). Following incubation at room temperature for 30 min, cell cycles were analyzed using a FACScan flow cytometer (BD Biosciences).

Table 1 List of Primer Sequences of Quantitative Real Time-PCR

Primer Name	Primer Sequence (5`→ 3`)
LAMP-2A Fwd	TAT GTG CAA CAA AGA GCA GA
LAMP-2A Rev	CAG CAT GAT GGT GCT TGA G
LC3B Fwd	GAG AAG CAG CTT CCT GTT CTG G
LC3B Rev	GTG TCC GTT CAC CAA CAG GAA G
GAPDH Fwd	GAA GGT GAA GGT CGG AGT C
GAPDH Rev	GAA GAT GGT GAT GGG ATT TC

Cell Viability Assay

MTT solution was added to the cells to a final concentration of 0.5 mg/mL, and cells were incubated at 37°C for 1 h. After removing cell culture media, 50 µL of DMSO was added, and the optical density of each well was read at 570 nm.

TUNEL Assay

Cells were fixed with 1% paraformaldehyde for 15 min on ice. After washing the cells with PBS, the TUNEL assay was performed using the APO-BrdU TUNEL assay kit (Life Technologies) according to the manufacturer's protocol. The cells were analyzed with a fluorescent microscope (Nikon ECLIPSE TE300, Nikon Corporation).

Statistical Analysis

Statistical analysis was performed using GraphPad software. Differences between means were explored using Kruskal–Wallis test by Dunn's multiple comparison post-hoc test. Data were analyzed using a two-tailed unpaired *t*-test or Mann–Whitney *U*-test, as appropriate, to determine statistical significance. Data are presented as mean ± SD. To evaluate correlations in the human samples, Pearson correlation coefficient was calculated. A p-value of < 0.05 was considered significant.

Results

LAMP-2A Expression is Decreased in Human Lung Tissue from Smokers and COPD Patients

We first evaluated the expression of LAMP-2A in the protein extracts of lung tissues from healthy controls (never smokers) (n=12), smokers (n=12), and COPD patients (n=12) (Table 2). The protein expression of LAMP-2A was significantly lower in smokers and COPD patients compared with never smokers (Figure 1A and B). To examine whether the decreased level of LAMP-2A is due to regulation at the transcription step, mRNA level of LAMP-2A in lung tissues was determined by quantitative real time-PCR. The level of LAMP-2A mRNA was not significantly altered between groups (Figure 1C). Immunohistochemical staining for LAMP-2A in formalin-fixed and paraffin-embedded human lung tissues showed that LAMP-2A expression was lower in the lung epithelial cells of smokers and COPD patients compared to never smokers (Figure 1D and E).

Primary HBECs from Smokers and COPD Patients Have Lower Expression of LAMP-2A

To confirm the lower expression levels of LAMP-2A in lung epithelial cells from smokers and COPD patients, primary HBECs were collected from never smokers (n=5), smokers (n=6), and COPD patients (n=5) using a bronchial brush

Table 2 Clinical Characteristics of Subjects of Lung Tissues

	Never Smokers (n = 12)	Smokers (n = 12)	COPD(n = 12)	*p* value
Age, yr	67.2 ± 8.1	58.3 ± 11.5	71.9 ± 4.9	0.004**
Male, % of group	16.7	83.3	91.7	<0.001*
Smoking index (pack-yr)	0	33.9 ± 28.8	47.0 ± 23.5	<0.001**
Lung function				
FEV1/FVC (%)	78.7 ± 5.0	78.0 ± 5.0	61.5 ± 4.6	<0.001**
%FEV1	119.3 ± 12.4	108.0±13.5	92.4 ±13.1	0.001**
GOLD stage				
Stage 0 (%)	100	100	0	
Stage 1 (%)	0	0	100	<0.001*

Notes: Data are the mean ± SE. Values are mean ± SD. *$\chi 2$ test for independence. **Kruskal Wallis Test.
Abbreviations: FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity.

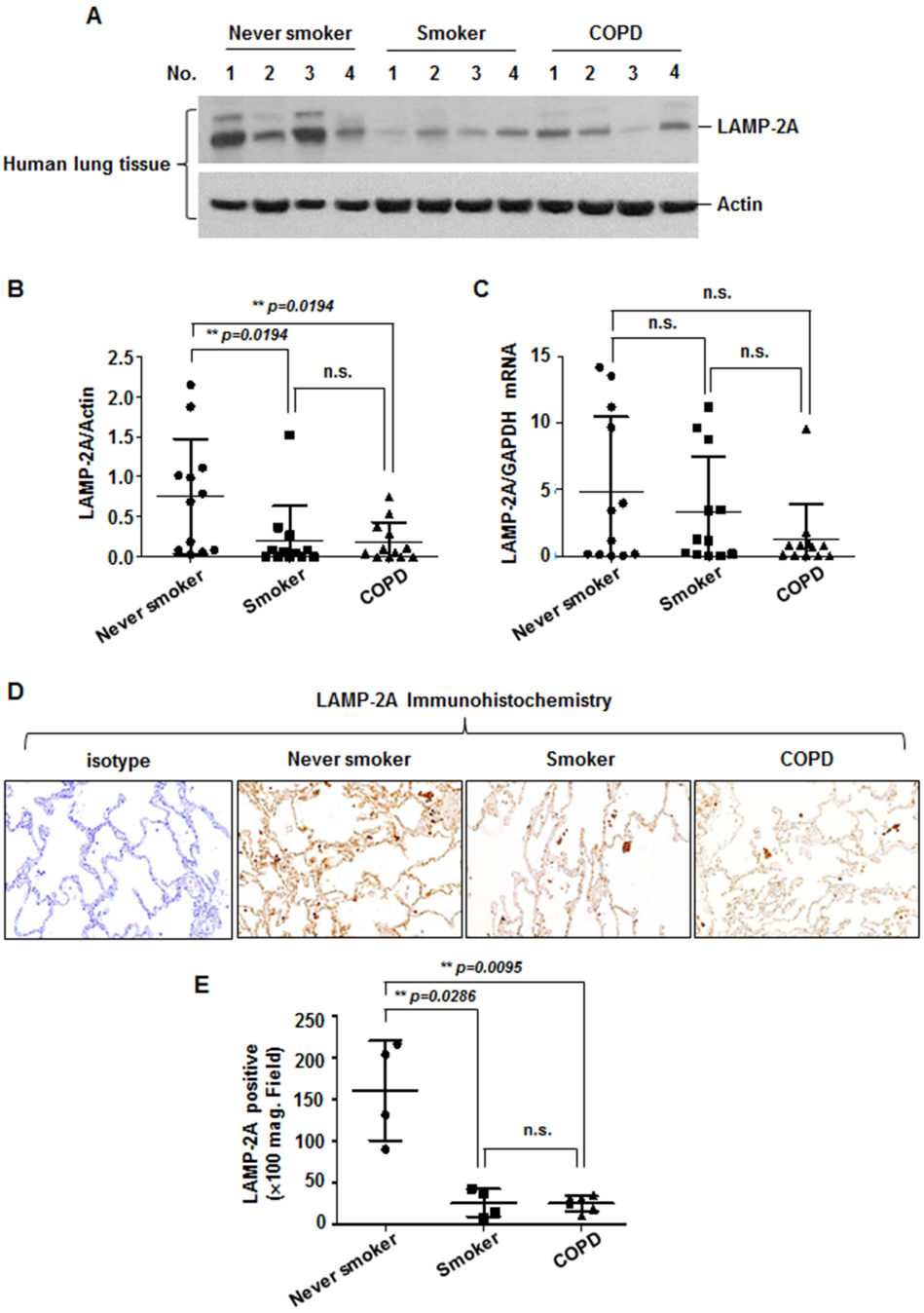

Figure 1 LAMP-2A expression is decreased in human lung tissue from smokers and COPD patients. (**A**) Lung homogenates from never smokers (n = 12), smokers (n = 12), and COPD patients (n = 12) were subjected to Western blot analysis for LAMP-2A and actin. (**B**) Gel data were quantified using Scion image densitometry. Data represent the mean ± SD. Normal versus smoker: $^{**}p = 0.0194$, normal versus COPD: $^{**}p = 0.0194$. (**C**) Real-time PCR analysis of LAMP2A and GAPDH expression. Data represent the mean ± SD. n.s., not-significant (**D**) LAMP-2A immunohistochemistry in lung tissues from never smokers (n = 4), smokers (n = 4), and COPD patients (n = 6). Original magnifications, ×100. (**E**) Quantity of LAMP-2A positive cells in ×100 field of the whole group. Data represent the mean ± SD. Normal versus smoker: $^{**}p = 0.0286$, normal versus COPD: $^{**}p = 0.0095$.

Abbreviation: n.s, not-significant.

(Table 3). LAMP-2A expression in primary HBECs was significantly lower in smokers and COPD patients compared to never smokers (Figure 2A and B). The level of LAMP-2A mRNA was not significantly altered between groups in primary HBECs (Figure 2C).

Table 3 Clinical Characteristics for Subjects of Bronchial Epithelial Cells

	Never Smokers (n = 5)	Smokers (n = 6)	COPD (n = 5)	*p* value
Age, yr	62.8 ± 13.0	63.0 ± 10.1	66.0 ± 7.4	NS
Male, % of group	0	100.0	100.0	<0.001*
Smoking index (pack-yr)	0	25.2 ± 19.0	45.3 ± 29.4	0.001**
Lung function				
FEV1/FVC (%)	78.4 ± 5.0	83.2 ± 6.4	50.6 ± 16.4	0.001**
%FEV1	118.0 ± 23.3	110.7 ± 13.6	74.6 ± 34.7	0.102**
DLco/VA (%)	93.2 ± 12.2	97.0 ± 24.8	71.8 ± 20.0	0.181**
GOLD stage				
GOLD 0 (%)	100	100	0	<0.001*
GOLD 1 (%)	0	0	60	
GOLD 2 (%)	0	0	0	
GOLD 3 (%)	0	0	40	

Notes: Data are the mean ± SE. Values are mean ± SD. *χ2 test for independence. **Kruskal Wallis Test.
Abbreviations: DLco/VA, diffusion capacity measured with carbon monoxide adjusted for the alveolar volume ventilated; FEV1, forced expiratory volume in 1 s; FVC, forced vital capacity; NS, not statistically.

LAMP-2A Expression is Negatively Correlated with Years of Smoking

To evaluate the correlation between expression level of LAMP-2A and the clinical parameters of 16 subjects including never smokers (n=5), smokers (n=6), and COPD patients (n=5), the Pearson correlation coefficient was calculated.

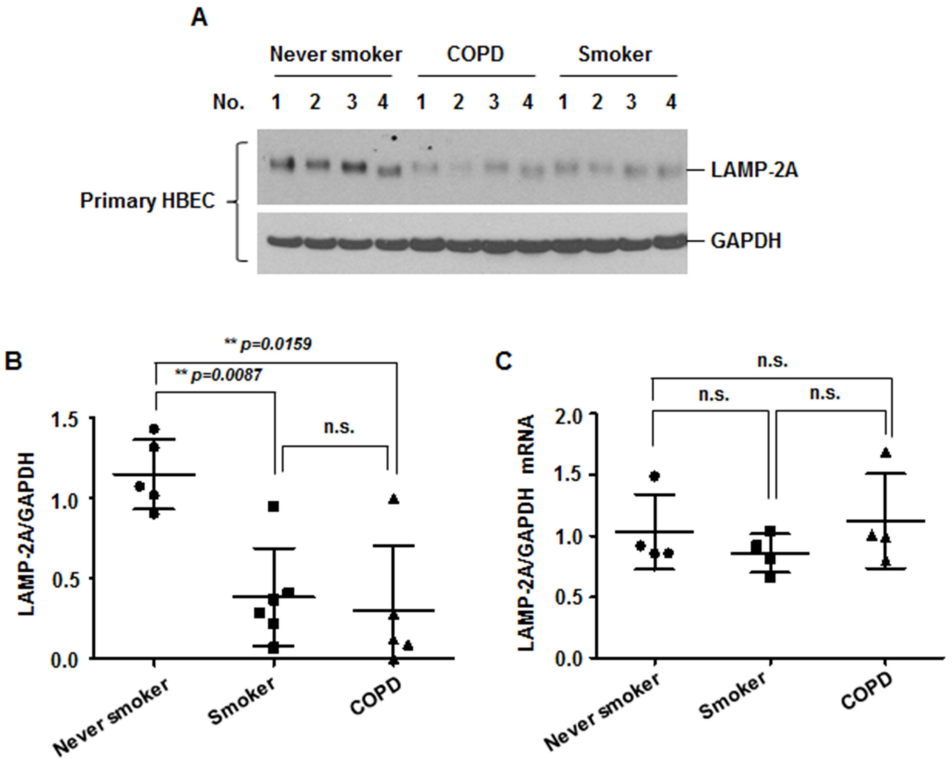

Figure 2 Primary HBECs from smokers and COPD patients have lower expression of LAMP-2A. (**A**) Primary HBECs were isolated from normal (n = 5), smoker (n = 6), and COPD (n = 5) patients. Cell lysates were subjected to Western blot analysis for LAMP-2A and GAPDH. (**B**) Gel data were quantified using Scion image densitometry. Data represent the mean ± SD. Normal versus smoker: **p = 0.0087, normal versus COPD: **p = 0.0159. (**C**) Real-time PCR analysis of LAMP-2A and GAPDH expression. Data represent the mean ± SD.
Abbreviation: n.s, not-significant.

LAMP-2A expression levels were not significantly correlated with lung function, including predicted post-bronchodilator forced expiratory volume in 1 s (FEV1% pred.) and diffusion capacity measured with carbon monoxide adjusted for the alveolar volume ventilated (DLCO/VA %) (Figure 3A and B). However, the expression levels of LAMP-2A were negatively correlated with the number of years of smoking (Figure 3C). Patient age was not correlated with LAMP-2A expression (Figure 3D).

Down-Regulation of LAMP-2A is Mediated by CSE-Induced Activation of Macroautophagy

As LAMP-2A expression was lower in primary HBECs of smokers and COPD patients, we next evaluated whether CSE treatment mediated the down-regulation of LAMP-2A. As CSE have been shown to be cytotoxic, we first evaluated the concentration-dependent effect of CSE on the cell viability. Primary HBECs were treated with CSE (0, 0.5, 1, 2, or 4%) for 24 h and MTT cell viability assay was performed. CSE at a concentration of 2% or less did not affect cell viability and CSE (> 4%) induced cell death (data not shown). Based on this result, we used 2% or less concentration of CSE. Primary HBECs were isolated from healthy never smokers. Verified HBECs (passage No. 2) were treated with CSE for 0, 4, 8, and 24 h. The expression of LAMP-2A protein was lower 24 h after CSE treatment (Figure 4A). In contrast, the expression of LAMP-2A mRNA did not change even after 2, 4, 6, and 24 h of CSE exposure (Figure 4B). This indicates that downregulation of LAMP-2A is regulated at the posttranslational level. The level of LAMP-2A is modulated by two different mechanisms: the lysosomal degradation of LAMP-2A and its dynamic distribution between matrix and lysosomal membrane.[18] Considering the post-translational regulation of LAMP-2A, its down-regulation might be due to degradation in lysosomes. Cathepsin B is a cysteine protease found mainly in the lysosome. The cathepsin B/L inhibitor, z-FA-FMK, suppressed the CSE-induced degradation of LAMP-2A (Figure 4C).

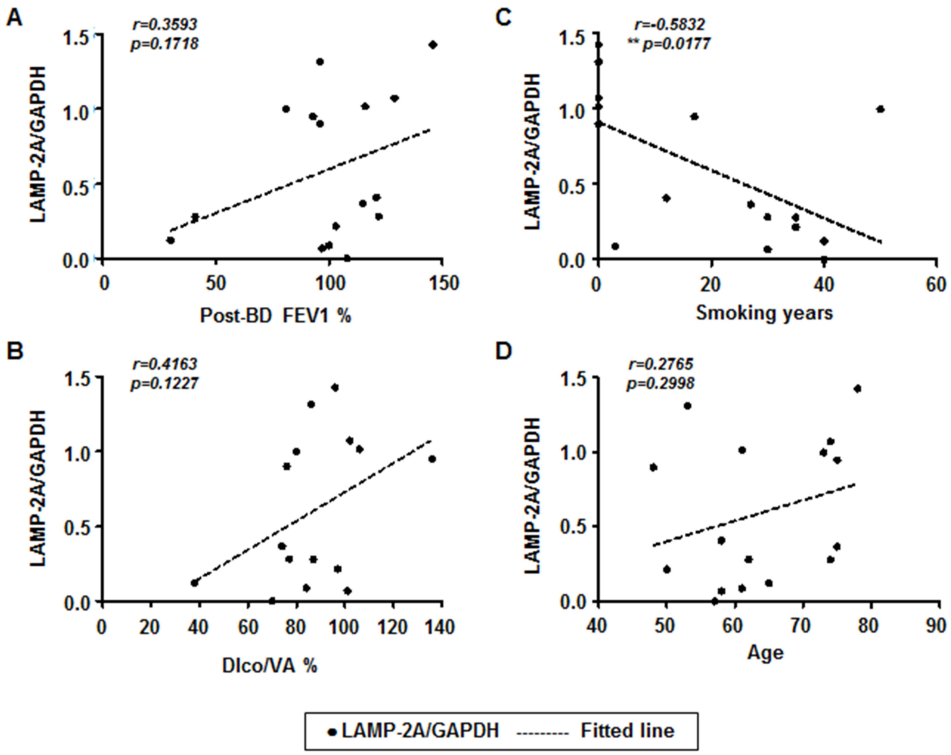

Figure 3 LAMP-2A expression is negatively correlated with years of smoking. Pearson correlation coefficient, r, was calculated for the expression level of LAMP-2A of primary HBECs (y-axis) and clinical parameters (x-axis) (**A–D**) of 16 subjects including normal (n = 5), smokers (n = 6), and COPD patients (n = 5). (**A**) Post-bronchodilator forced expiratory volume in 1 s (FEV1% pred.) (r = 0.3593, *p* = 0.1718) (**B**) Diffusion capacity measured with carbon monoxide adjusted for the alveolar volume ventilated (DLCO/VA %) (r = 0.4163, *p* = 0.1227) (**C**) Years of smoking (r = −0.5832, **p = 0.0177) (**D**) Aging (r = 0.2765, *p* = 0.2998).

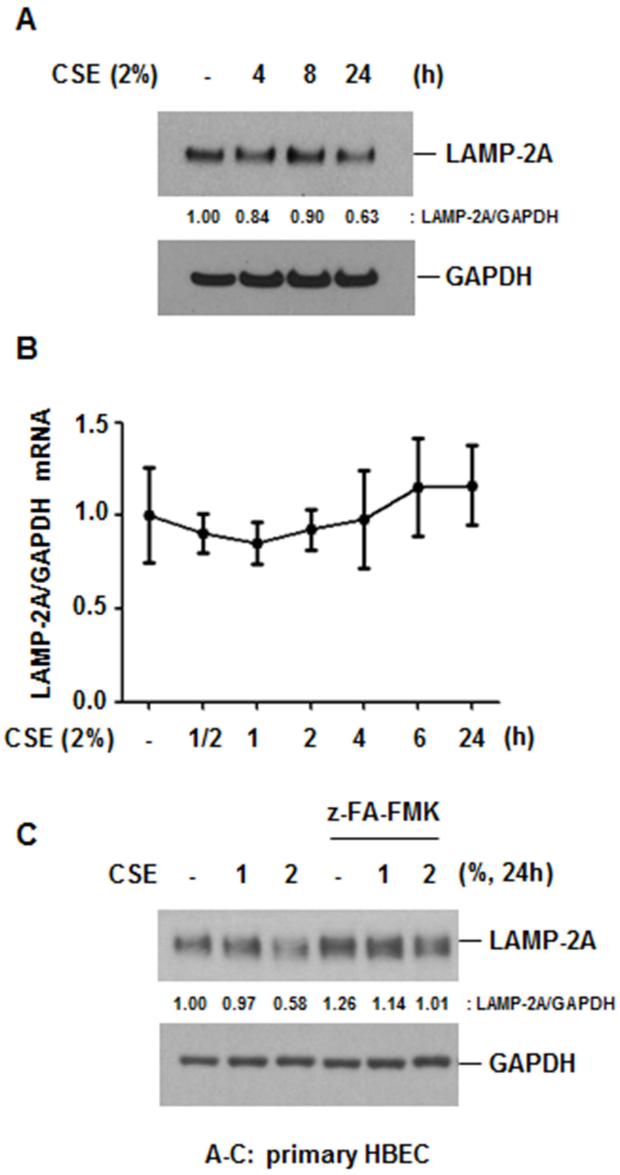

Figure 4 CSE degrades LAMP-2A via the lysosomal pathway. (**A** and **B**) Primary HBECs were treated with CSE (2%) for the indicated times. Total cellular extracts were subjected to Western blot analysis for LAMP-2A and GAPDH. The expression of LAMP-2A mRNA was measured by quantitative real-time PCR. Data were normalized to the expression of GAPDH. Data represent the mean ± SD. (**C**) HBECs were pretreated with z-FA-FMK (50 μM) for 1 h and then stimulated with CSE (1 or 2%) in the presence or absence of z-FA-FMK for 24 h. Total cellular extracts were subjected to Western blot analysis for LAMP-2A and GAPDH. Gel data were quantified using Scion image densitometry.

As macroautophagy is a well-known lysosomal degradation pathway, we evaluated whether it is involved in LAMP-2A down-regulation. Contrary to LAMP-2A, LC3B protein (a macroautophagy marker) was upregulated in lung homogenates from COPD patients compared with never smokers (Figure 5A and B). Both LC3B mRNA and protein were upregulated in primary HBECs treated with CSE (Figure 5C and D). We next evaluated whether LC3B knockdown blocked LAMP-2A degradation in human lung epithelial cell line (BEAS-2B). Knockdown of LC3B did not affect the expression of LAMP-2A mRNA (Figure 5E). CSE-mediated degradation of LAMP-2A was inhibited in LC3B siRNA-transfected BEAS-2B cells (Figure 5F). These results suggest that increased expression of LC3B mediates the down-regulation of LAMP-2A, indicating that macroautophagy may negatively regulate CMA.

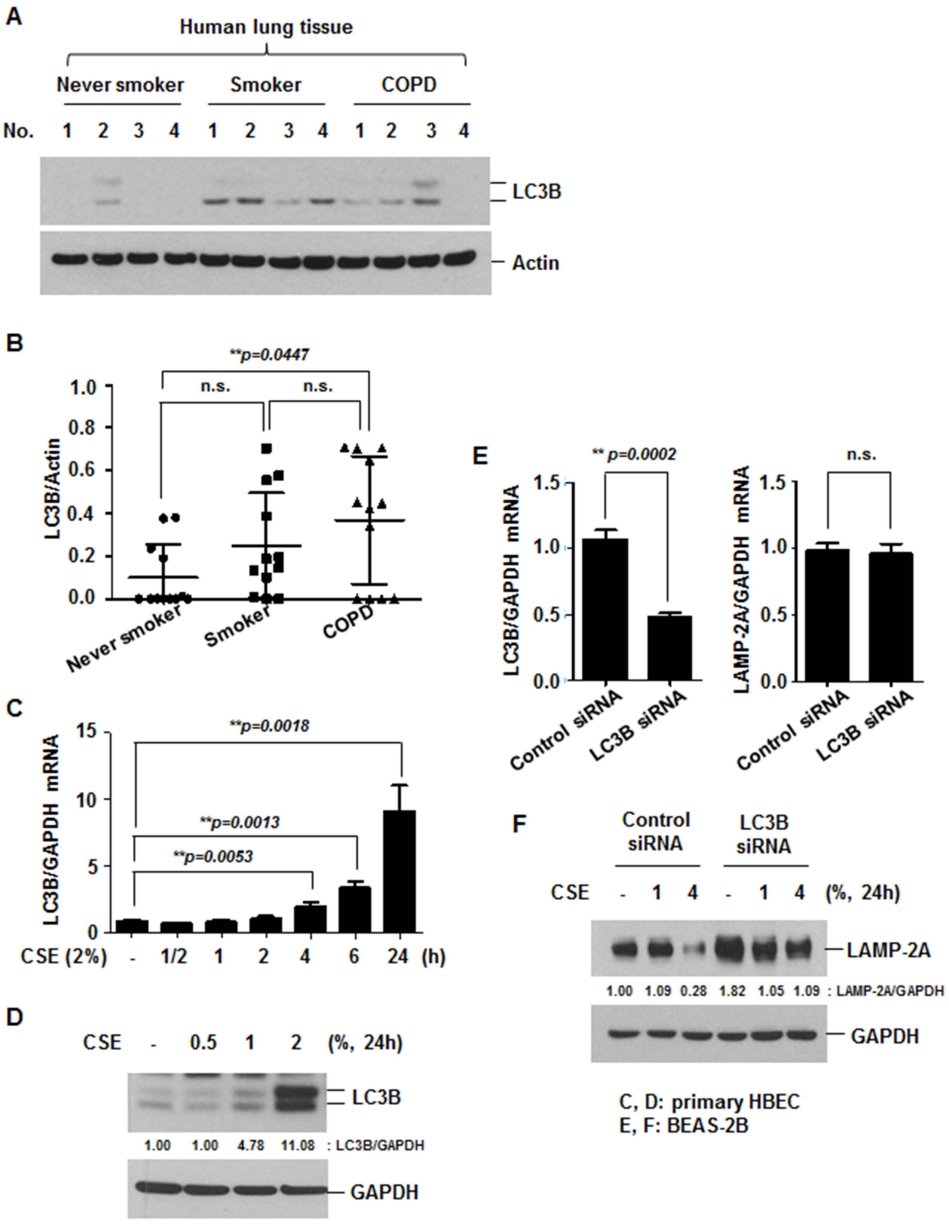

Figure 5 Downregulation of LAMP-2A is mediated by CSE-induced activation of macroautophagy. (**A** and **B**) Lung homogenates from never smokers (n = 12), smokers (n = 12), and COPD patients (n = 12) were subjected to Western blot analysis for LC3B and Actin (**A**). Gel data were quantified using Scion image densitometry (**B**). Data represent the mean ± SD. Normal versus smoker: $p = 0.0827$, normal versus COPD: $^{**}p = 0.0447$. (**C**) Primary HBECs were stimulated with CSE (2%) for the indicated times. (**D**) HBECs were treated with CSE (0.5, 1 or 2%) for 24 h. The expression of LC3B was measured by quantitative real-time PCR. Data were normalized to the expression of GAPDH. Data represent the mean ± SD. Total cell lysates were subjected to Western blot analysis for LC3B and GAPDH. (**E** and **F**) BEAS-2B cells were transiently transfected with control siRNA and LC3B siRNA. Forty-eight hours after transfection, the cells were stimulated with CSE (1 or 4%) for 24 h. The expression of LC3B and LAMP-2A was measured by quantitative real-time PCR. Data were normalized to the expression of GAPDH. Data represent the mean ± SD (**E**). Total cell lysates were subjected to Western blot analysis for LAMP-2A and GAPDH (**F**).

Downregulation of LAMP-2A Causes Bronchial Epithelial Cell Death by Inducing Cellular Senescence and Cell Cycle Arrest

CSE exposure is known to induce cellular senescence, which is associated with the pathogenesis of COPD.[19] To investigate the effect of decreased levels of LAMP-2A on CSE-induced cellular senescence, BEAS2-B cells were transiently transfected with control siRNA and LAMP-2A siRNA and the cells were treated with CSE for 8 or 24 h. The expression of LAMP-2A mRNA and protein was effectively suppressed in LAMP-2A siRNA-transfected cells (Figure 6A and B). As expected, CSE increased the levels of senescence markers such as p16, p-p21, p21, p27, p-p53,

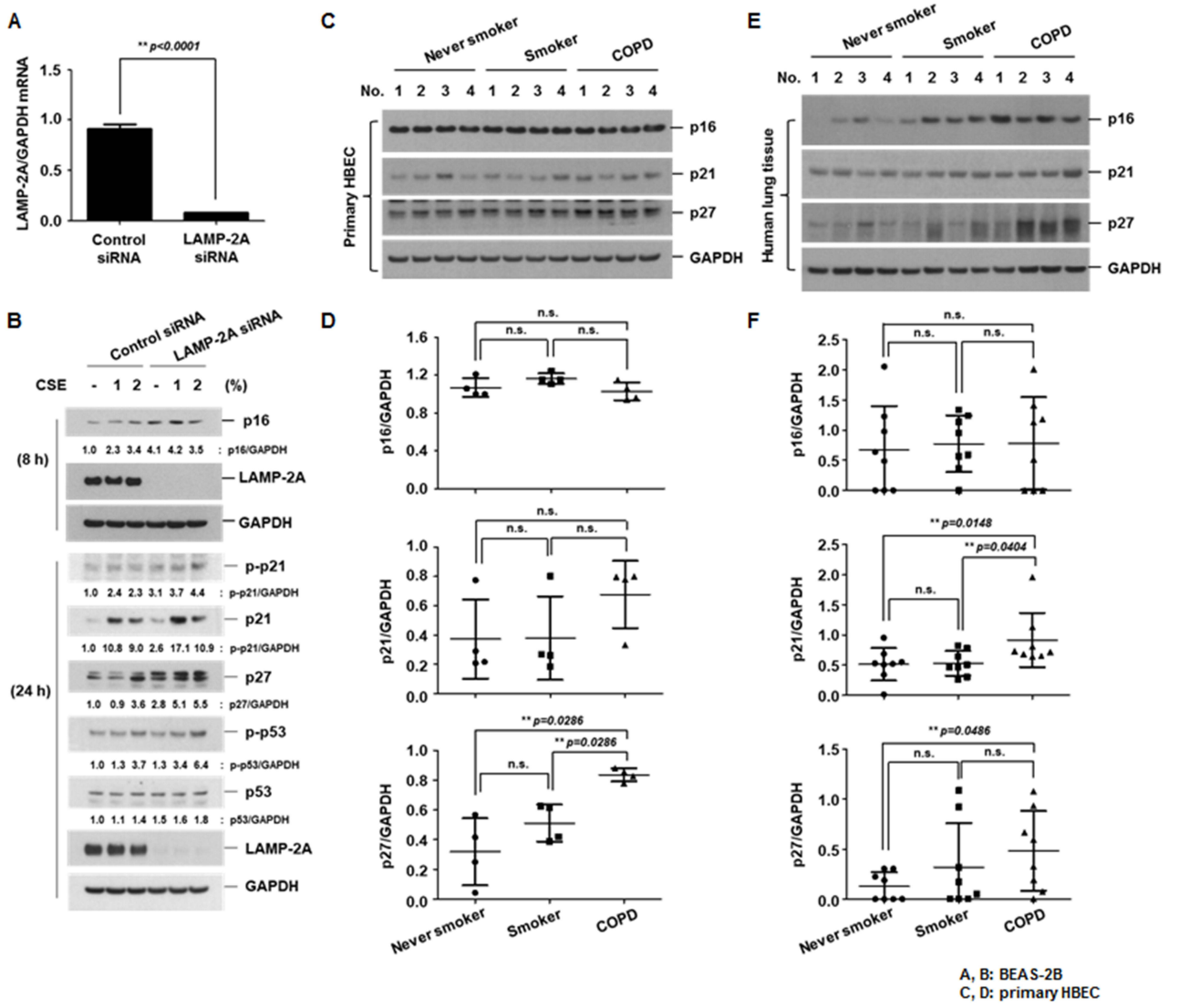

Figure 6 Knockdown of LAMP-2A enhanced CSE-induced cellular senescence. (**A** and **B**) BEAS-2B cells were transiently transfected with control siRNA and LAMP-2A siRNA. Forty-eight hours after transfection, the cells were treated with CSE (1 or 2%) for 8 or 24 h. The expression of LAMP-2A mRNA was measured by quantitative real-time PCR. Data were normalized to the expression of GAPDH. Data represent the mean ± SD. $^{**}p < 0.0001$ (**A**). Total cellular extracts were subjected to Western blot analysis for p16, p-p21, p21, p27, p-p53, p53, LAMP-2A, and GAPDH (**B**). (**C**) Whole cell lysates from primary HBECs of never smokers (n = 4), smokers (n = 4), and COPD patients (n = 4) were subjected to Western blot analysis for p16, p21, p27, and GAPDH. (**D**) Gel data were quantified using Scion image densitometry. Data represent the mean ± SD. (**E**) Lung protein extracts from never smokers (n = 8), smokers (n = 8), and COPD patients (n = 8) were subjected to Western blot analysis for p16, p21, p27, and GAPDH. (**F**) Gel data were quantified using Scion image densitometry. Data represent the mean ± SD.

and p53 in a dose-dependent manner (Figure 6B). Interestingly, knockdown of LAMP-2A enhanced CSE-induced expression of p16, p-21, p21, p27, p-p53, and p53 (Figure 6B). The level of senescence marker (p27) was higher in primary HBECs and human lung tissue from COPD patients (Figure 6C–F). As cellular senescence is characterized by a stable cell cycle arrest that is triggered by a variety of stress stimuli,[20] we next evaluated whether lower expression of LAMP-2A affected the cell-division cycle. Treatment with CSE induced G2/M arrest in BEAS-2B cells (Figure 7A). Cyclin B1 is a regulatory protein involved in mitosis and is known to accumulate at the G2/M phase. CSE increased the level of cyclin B1 (Figure 7B). Knockdown of LAMP-2A increased the proportion of cells in G2/M and enhanced CSE-induced arrest of G2/M (Figure 7A). Moreover, both basal and CSE-induced cyclin B1 expression were notably upregulated in LAMP-2A siRNA-transfected BEAS-2B cells (Figure 7B).

Cellular senescence and defects in the G2/M checkpoints may mediate a loss of cell viability. To assess the effect of decreased levels of LAMP-2A on the viability of CSE-treated cells, BEAS-2B cells were transiently transfected with

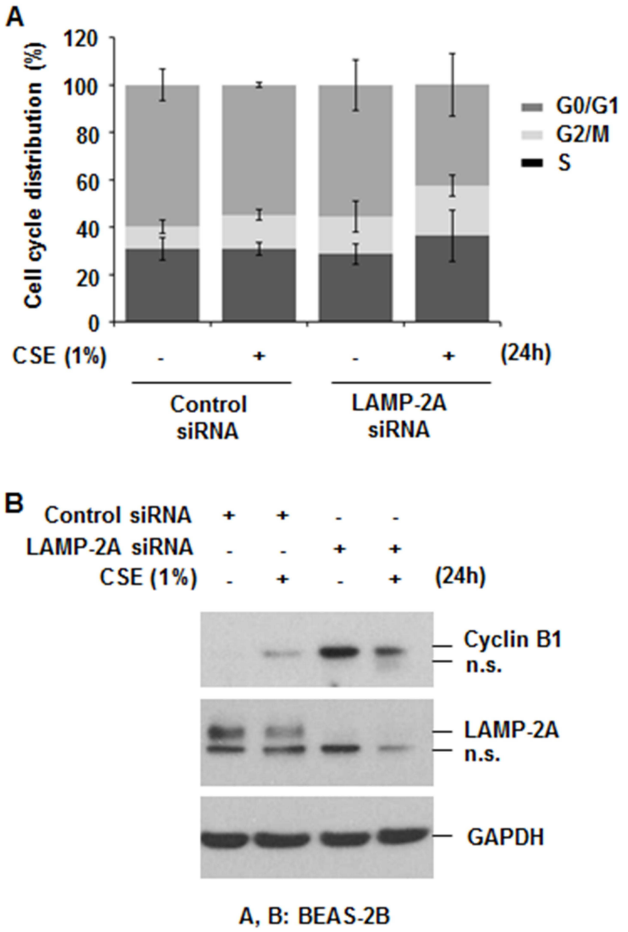

Figure 7 Knockdown of LAMP-2A enhanced CSE-induced G2/M cell cycle arrest. (**A** and **B**) BEAS-2B cells were transiently transfected with control siRNA or LAMP-2A siRNA. Forty-eight hours after transfection, the cells were treated with CSE (1%) for 24 h. The cells were harvested and stained with propidium iodide (PI) for 30 min and then subjected to flow cytometric analysis to determine the cell distribution at each phase of cell cycle (**A**). Data represent the mean ± SD. Control siRNA-CSE G2/M versus LAMP-2A siRNA-CSE G2/M: $^{**}p = 0.0351$ Total cellular extracts were subjected to Western blot analysis for cyclin B1, LAMP-2A, and GAPDH (**B**).

control siRNA or LAMP-2A siRNA and then the cells were treated with different concentrations of CSE for 24 h. Compared to primary HBECs, BEAS-2B cells were more sensitive in response to CSE stimulation. In BEAS-2B cells, CSE at a concentration of 1% or less did not affect cell viability and CSE (> 2%) induced cell death (data not shown). Therefore, we used 1 and 2% CSE to test the effect of LAMP-2A knockdown on cell death. Cell viability was determined using an MTT assay. We observed that the growth rate of cells transfected with LAMP-2A siRNA was slightly decreased. CSE exposure reduced cell viability in a dose-dependent manner. Cell death was accelerated in LAMP-2A siRNA-transfected cells upon CSE treatment (Figure 8A). CSE has been reported to induce apoptosis.[21] To investigate the effect of decreased levels of LAMP-2A on CSE-induced apoptotic cell death, TUNEL staining was performed. Apoptotic (TUNEL positive) cells were observed in the CSE-treated group, and knockdown of LAMP-2A potentiated CSE-induced apoptosis (Figure 8B). In addition, the levels of active caspase-3 and cleaved PARP (apoptosis hallmarks) were greater in CSE-treated primary HBECs from smokers and COPD patients than in cells from never smokers (Figure 8C and D). Epithelial cells from smokers and COPD patients seem to be susceptible to CSE-induced apoptotic cell death, which was supported by TUNEL staining of human lung tissue sections. There were more apoptotic alveolar and bronchial epithelial cells in human lung tissue from smokers and COPD patients than in never smokers (Figure 8E and F). These results indicate that downregulation of LAMP-2A leads to acceleration of cell senescence, cell cycle arrest, and subsequent apoptotic cell death of lung epithelial cells.

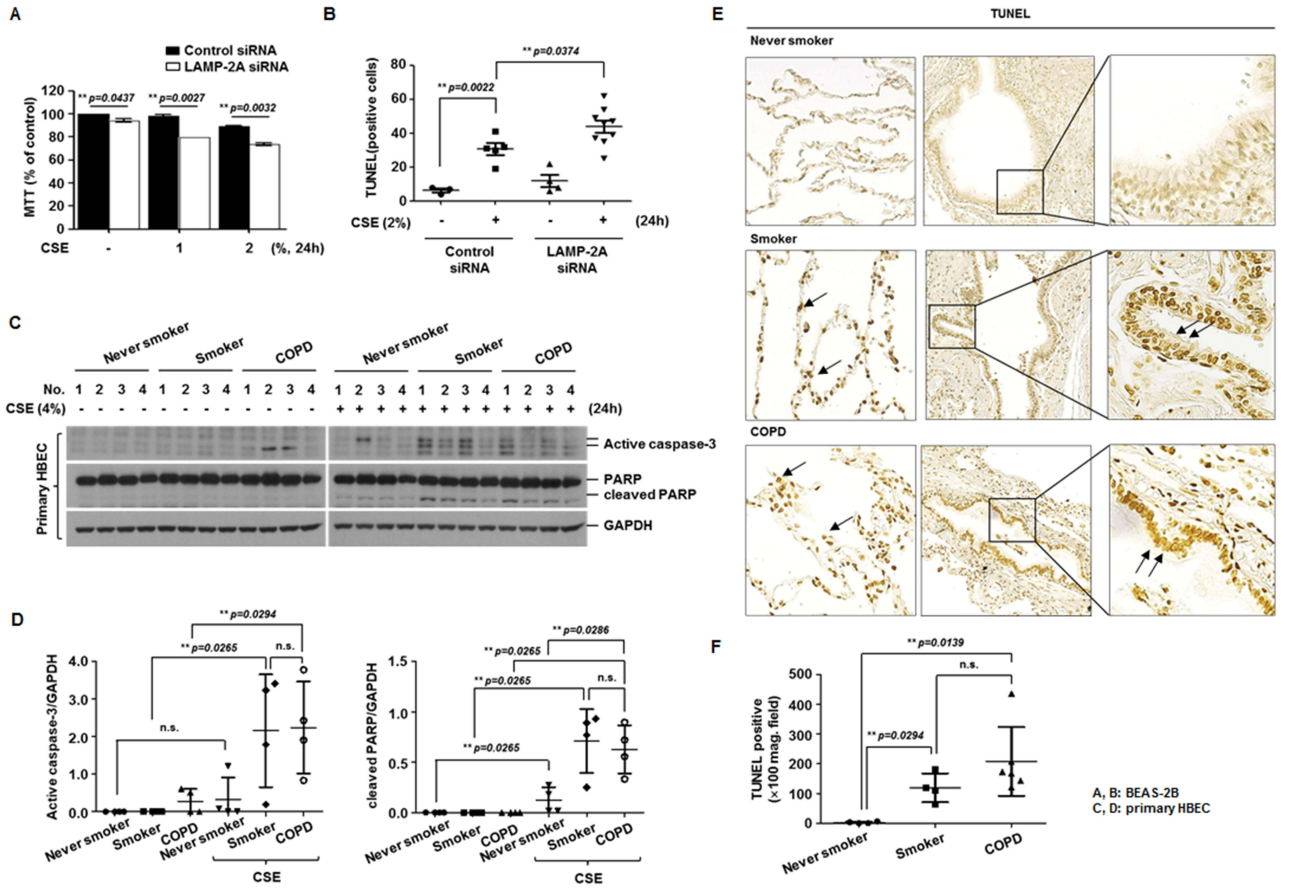

Figure 8 Knockdown of LAMP-2A enhanced CSE-induced cell death. (**A** and **B**) BEAS-2B cells were transiently transfected with control siRNA or LAMP-2A siRNA. Forty-eight hours after transfection, the cells were treated with CSE (1 or 2%) for 24 h. Cell viability was determined by MTT assay (**A**). TUNEL staining was performed according to the manufacturer's protocol (**B**). Data represent the mean ± SD. (**C**) Primary HBECs from never smokers (n = 4), smokers (n = 4), and COPD patients (n = 4) were treated with CSE (4%) for 24 h. Total cell extracts were subjected to Western blot analysis for active caspase-3, PARP, and GAPDH. (**D**) Gel data were quantified using Scion image densitometry. Data represent the mean ± SD. (**E**) TUNEL staining using human lung tissue sections of never smokers (n = 4), smokers (n = 4), and COPD patients (n = 6). Original magnifications, ×200 (left, right), ×100 (middle). (**F**) Quantity of TUNEL positive cells in ×100 field of the whole group. Data represent the mean ± SD. Normal versus smoker: $^{**}p = 0.0294$, normal versus COPD: $^{**}p = 0.0139$.

Abbreviations: n.s, not-significant.

Discussion

The role of CMA in COPD pathogenesis has not been fully elucidated. The experiments presented here show that decreased CMA mediated acceleration of cellular senescence and apoptosis in human lung epithelial cells. The evidence is that the protein levels of LAMP-2A, a marker of CMA, in lung homogenates from smokers and COPD patients were lower than those from never smokers. Downregulation of LAMP-2A was evident in lung epithelial cells upon immunohistochemical staining of lung tissue. This was confirmed in primary human bronchial epithelial cells isolated from smokers and COPD patients. Therefore, it seems likely that decreased CMA in human lung epithelial cells might at least partially play a role in the pathogenesis of COPD.

While the protein levels of LAMP-2A in primary HBECs from smokers and COPD patients were lower than those from never smokers, it is unclear how this downregulation occurs. Although a previous report has shown that LAMP-2A protein expression is decreased as part of the aging process,[22] age was not correlated with the levels of LAMP-2A protein in primary HBECs in our results. A likely explanation for this finding is that factors other than aging are required for the decrease in LAMP-2A expression. Since the duration of smoking was negatively correlated with the levels of LAMP-2A in primary HBECs in our experiments, we hypothesized that smoking might cause down-regulation of LAMP-2A. As expected, our results showed that CSE exposure decreased the level of LAMP-2A protein; however, LAMP-2A mRNA was unchanged in primary HBECs, which is inconsistent with previous study.[23] In previous study, CSE induces LAMP-

2A expression through a Nrf2-dependent manner in HBECs. Knockdown of Nrf2 reduces CSE-induced expression of LAMP-2A mRNA and protein,[23] which might be due to use of different cigarettes and different methods of CSE preparation. Our study showed that treatment with an inhibitor (z-FA-FMK) of lysosomal proteases, cathepsin B/L, suppressed CSE-induced downregulation of LAMP-2A. These results indicate that CSE causes downregulation of LAMP-2A by lysosomal degradation. This is in accordance with a previous report that the decrease in levels of LAMP-2A in aging did not result from age-dependent transcriptional down-regulation, but instead originated from abnormal degradation of LAMP-2A in the lysosomal lumen.[24]

Although CSE causes lysosomal degradation of LAMP-2A, it is not clear whether macroautophagy, which is a well-known lysosomal degradation pathway, is involved. In our experiments, the macroautophagy marker (LC3B) was higher in lung homogenates from COPD patients than in those from never smokers. This is in accordance with previous studies showing that the expression levels of LC3B and autophagy-associated proteins such as Atg4, Atg5, Atg12, and Atg7 were higher in the lungs of COPD patients than in lungs of healthy individuals.[12] In addition, the expression of LC3B mRNA and protein was upregulated in primary HBECs treated with CSE in this study. These findings suggest that activation of macroautophagy might be involved in CSE-induced lysosomal degradation of LAMP-2A.

In order to evaluate the relationship between LC3B and LAMP-2A, we determined the effect of LC3B knockdown on the CSE-induced degradation of LAMP-2A. The expression level of LAMP-2A mRNA was unchanged by LC3B knockdown, but CSE-induced downregulation of LAMP-2A was suppressed. These results indicate that macroautophagy mediates the down-regulation of LAMP-2A by lysosomal degradation. Moreover, it has been reported that knockdown of LAMP-2A led to activation of macroautophagy, but overexpression inhibited LC3B.[24,25] Therefore, there is crosstalk between LC3B and LAMP-2A, resulting in mutual inhibition.

CSE-induced activation of macroautophagy leads to lysosomal degradation of LAMP-2A. How might the decrease in LAMP-2A be involved in the pathogenesis of COPD? Our results indicate that decreased level of LAMP-2A enhances cellular senescence and apoptosis in lung epithelial cells. The evidence showed that knockdown of LAMP-2A enhanced CSE-induced expression of senescence markers such as p16, p21, p27, and p53. Consistent with our observation, a previous study reported that CMA impairment is sufficient to induce cellular senescence.[26] Cellular senescence is characterized by stable cell cycle arrest.[20] Once DNA damage is too great to be repaired, cells lose the potential to replicate, resulting in cell cycle arrest. In this study, flow cytometric analysis showed that CSE induced G2/M arrest, which was augmented by knockdown of LAMP-2A. Although there is little agreement over sequential links from cell cycle arrest to cell death, excessive damage/stress can induce cell cycle arrest and result in apoptosis. Thus, many anticancer compounds trigger apoptosis accompanied by G2/M arrest.[27–29] As expected, our results showed that inhibition of LAMP-2A enhanced CSE-induced apoptotic cell death. Consistent with our results, while LAMP-2A knockdown enhances CSE-induced apoptosis, overexpression of LAMP-2A suppresses apoptosis in CSE treated cells.[23] Moreover, we observed that apoptotic cell death was increased in CSE-treated primary HBECs and lung tissue sections of both smokers and COPD patients. Therefore, a likely explanation for our results is that decreased level of LAMP-2A accelerates aging and apoptotic cell death in lung epithelial cells. However, in this study, we used primary HBECs cultured in monolayer. To reflect more physiological condition, using HBECs differentiated at air liquid interface is required.

Conclusion

CSE inhibits CMA by lysosomal degradation of LAMP-2A, which is mediated by macroautophagy. Decreased LAMP-2A is involved in acceleration of aging and apoptotic cell death in lung epithelial cells. Our findings suggest that blocking lysosomal degradation of LAMP-2A or boosting LAMP-2A expression might be beneficial to COPD management.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no potential conflicts of interest in this work.

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