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

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Research

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Abstract

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 (HBEC) from healthy never smokers, healthy 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, and p27, 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.

Conclusions: Cigarette smoke-induced down-regulation of LAMP-2A is involved in the pathogenesis of COPD by accelerating aging and apoptosis of lung epithelial cells.

Background

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 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.

Materials And Methods

Cells

Normal human bronchial epithelial cells (BEAS-2B) were maintained in defined keratinocyte serum-free medium (GIBCO) at 37°C and 5% CO_2. Primary human bronchial epithelial cells (HBEC) were obtained after review and approval by the Seoul National University Hospital Institutional Review Board (SNUH IRB number: H-1602-108-742). Primary HBECs were isolated from bronchial brushing samples during bronchoscopy. 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. Submerged cells were grown as monolayers to 80–100% confluence and then used for experiments.

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 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 (SNUH IRB Number: H-1309-073-521) 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-p21, anti-p27, anti-poly (ADP-ribose) polymerase, 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 as follows: human LAMP-2A primers (fwd 5’-TAT GTG CAA CAA AGA GCA GA-3’, rev 5’-CAG CAT GAT GGT GCT TGA G-3’), LC3B primers (fwd 5’-GAG AAG CAG CTT CCT GTT CTG G-3’, rev 5’-GTG TCC GTT CAC CAA CAG GAA G-3’), and GAPDH (fwd 5’-GAA GGT GAA GGT CGG AGT C-3’, rev 5’-GAA GAT GGT GAT GGG ATT TC-3’).

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).

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).

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. Data were analyzed using a two-tailed unpaired t-test or Mann-Whitney U test, as appropriate, to determine statistical significance. Data from *in vitro* cell experiments are presented as mean ± SD. Data from experiments using human lung tissues are expressed as the mean ± SE. 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). The protein expression of LAMP-2A was significantly lower in smokers and COPD patients compared with never smokers (Fig. 1A, 1B). 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 (Fig. 1C).

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. Airway brushings were obtained from the anterior basal segmental bronchus of right lower lobe. 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) (data not shown). Verified HBECs were used in the experiment. LAMP-2A expression in primary HBECs was significantly lower in smokers and COPD patients compared to never smokers (Fig. 2A, 2B).

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. 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 %) (Fig. 3A, 3B). However, the expression levels of LAMP-2A were negatively correlated with the number of years of smoking (Fig. 3C). Patient age was not correlated with LAMP-2A expression (Fig. 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. 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 (Fig. 5A). In contrast, the expression of LAMP-2A mRNA did not change even after 2, 4, 6, and 24 h of CSE exposure (Fig. 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 (Fig. 4C).

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 smokers and COPD patients compared with never smokers (Fig. 5A, 5B). Both LC3B mRNA and protein were upregulated in primary HBECs treated with CSE (Fig. 5C, 5D). We next evaluated whether LC3B knockdown blocked LAMP-2A degradation. Knockdown of LC3B did not affect the expression of LAMP-2A mRNA (Fig. 5E). CSE-mediated degradation of LAMP-2A was inhibited in LC3B siRNA-transfected cells (Fig. 5F). These results suggest that increased expression of LC3B mediates the down-regulation of LAMP-2A, indicating that macroautophagy may negatively regulate CMA.

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,

bronchial epithelial cells (BEAS2-B) 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 (Fig. 6A, 6B). As expected, CSE increased the levels of senescence markers such as p16, p21, and p27 in a dose-dependent manner (Fig. 6B). Interestingly, knockdown of LAMP-2A enhanced both basal and CSE-induced expression of p16, p21, and p27 (Fig. 6B). The level of senescence marker (p27) was higher in primary HBECs and human lung tissue from COPD patients (Fig. 6C-6F). 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 (Fig. 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 (Fig. 7B). Knockdown of LAMP-2A increased the proportion of cells in G2/M and enhanced CSE-induced arrest of G2/M (Fig. 7A). Moreover, both basal and CSE-induced cyclin B1 expression were notably upregulated in LAMP-2A siRNA-transfected cells (Fig. 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 control siRNA or LAMP-2A siRNA and then the cells were treated with different concentrations of CSE for 24 h. 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 (Fig. 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 (Fig. 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 (Fig. 8C, 8D). 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 (Fig. 8E). These results indicate that downregulation of LAMP-2A leads to cell senescence, cell cycle arrest, and subsequent apoptotic cell death of lung epithelial cells.

Discussion

The role of CMA in COPD pathogenesis has not been elucidated. The experiments presented here show that decreased CMA contributes to COPD pathogenesis by accelerating cellular senescence and inducing 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 bronchial epithelial cells plays 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 in COPD patients. 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. In addition, 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 [22].

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 smokers and 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 protein expression of LAMP-2A was reduced. 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 [23, 24]. 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 levels of LAMP-2A lead to 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, and p27. Consistent with our observation, a previous study reported that CMA impairment is sufficient to induce cellular senescence [25]. 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 [26–28]. As expected, our results showed that inhibition of LAMP-2A enhanced CSE-induced apoptotic cell death. 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 levels of LAMP-2A contribute to COPD by accelerating aging and inducing apoptotic cell death in lung epithelial cells.

To the best of our knowledge, this is the first study to elucidate the role of CMA in COPD pathogenesis. In conclusion, CSE inhibits CMA by lysosomal degradation of LAMP-2A, which is mediated by macroautophagy. Decreased LAMP-2A is involved in the pathogenesis of COPD by accelerating aging and inducing apoptotic cell death in lung epithelial cells. Our findings suggest that blocking lysosomal degradation of LAMP-2A or boosting LAMP-2A expression might be a novel therapeutic approach in the management of COPD.

Abbreviations

CMA
Chaperone-mediated autophagy
COPD
Chronic obstructive pulmonary disease
CSE
cigarette smoke extract
DLCO/VA
diffusion capacity measured with carbon monoxide adjusted for the alveolar volume ventilated
FEV1
forced expiratory volume in 1 s
HBEC
Human bronchial epithelial cells
LAMP-2A
lysosome-associated membrane protein-2A
LC3B
light chain-3B
mRNA
messenger RiboNucleic Acid
PARP
Poly ADP-ribose polymerase

Declarations

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None.

Authors' contributions

Y.J., K.H.L., C.H.L., and C.G.Y. conceived the study and contributed to the study design. K.H.L., C.H.L., J.W., and J.Y.K. performed the laboratory analysis. K. H. L, J.W., C. H. L., and C.G.Y. performed the data analysis. Y.J., K.H.L., and C.G.Y. wrote the first draft of the article; all authors critically reviewed the manuscript and approved the final version.

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Availability of data and materials

All data generated or analysed during this study are included in this article. Data could be obtained upon request to the corresponding author.

Ethics approval and consent to participate

All human subject sample acquisitions and experiments were conducted with the appropriate approval from the Seoul National University Hospital Institutional Review Board (SNUH IRB Number: H-1602-108-742, H-1309-073-521).

Consent for publication

All of the authors have consented to publication of this research.

Competing interests

The authors report no potential conflicts of interest relevant to this article.

References

1. Mathers CD, Loncar D: **Projections of global mortality and burden of disease from 2002 to 2030**. *PLoS Med* 2006, **3**:e442.
2. O'Donnell R, Breen D, Wilson S, Djukanovic R: **Inflammatory cells in the airways in COPD**. *Thorax* 2006, **61**:448–454.
3. Rahman I: **Oxidative stress in pathogenesis of chronic obstructive pulmonary disease: cellular and molecular mechanisms**. *Cell Biochem Biophys* 2005, **43**:167–188.

4. Abboud RT, Vimalanathan S: **Pathogenesis of COPD. Part I. The role of protease-antiprotease imbalance in emphysema**. *Int J Tuberc Lung Dis* 2008, **12**:361–367.
5. Bodas M, Vij N: **Augmenting autophagy for prognosis based intervention of COPD-pathophysiology**. *Respir Res* 2017, **18**:83.
6. Levine B, Kroemer G: **Autophagy in the pathogenesis of disease**. *Cell* 2008, **132**:27–42.
7. Schneider JL, Cuervo AM: **Autophagy and human disease: emerging themes**. *Curr Opin Genet Dev* 2014, **26**:16–23.
8. Racanelli AC, Kikkers SA, Choi AMK, Cloonan SM: **Autophagy and inflammation in chronic respiratory disease**. *Autophagy* 2018, **14**:221–232.
9. Mizumura K, Cloonan SM, Nakahira K, Bhashyam AR, Cervo M, Kitada T, Glass K, Owen CA, Mahmood A, Washko GR, et al: **Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD**. *J Clin Invest* 2014, **124**:3987–4003.
10. An CH, Wang XM, Lam HC, Ifedigbo E, Washko GR, Ryter SW, Choi AM: **TLR4 deficiency promotes autophagy during cigarette smoke-induced pulmonary emphysema**. *Am J Physiol Lung Cell Mol Physiol* 2012, **303**:L748-757.
11. Chen ZH LH, Jin Y: **Autophagy protein microtubule-associated protein 1 light chain-3B (LC3B) activates extrinsic apoptosis during cigarette smoke-induced emphysema**. *Proc Natl Acad Sci U S A* 2010, **107**:18880–18885.
12. Chen ZH KH, Sciurba FC: **Egr-1 regulates autophagy in cigarette smoke induced chronic obstructive pulmonary disease**. *Plos one* 2008, **3**:e3316.
13. Ito S, Araya J, Kurita Y, Kobayashi K, Takasaka N, Yoshida M, Hara H, Minagawa S, Wakui H, Fujii S, et al: **PARK2-mediated mitophagy is involved in regulation of HBEC senescence in COPD pathogenesis**. *Autophagy* 2015, **11**:547–559.
14. Lam HC, Cloonan SM, Bhashyam AR, Haspel JA, Singh A, Sathirapongsasuti JF, Cervo M, Yao H, Chung AL, Mizumura K, et al: **Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction**. *J Clin Invest* 2013, **123**:5212–5230.
15. Tran I, Ji C, Ni I, Min T, Tang D, Vij N: **Role of Cigarette Smoke-Induced Aggresome Formation in Chronic Obstructive Pulmonary Disease-Emphysema Pathogenesis**. *Am J Respir Cell Mol Biol* 2015, **53**:159–173.
16. Lee CH, Lee KH, Jang AH, Yoo CG: **The Impact of Autophagy on the Cigarette Smoke Extract-Induced Apoptosis of Bronchial Epithelial Cells**. *Tuberc Respir Dis (Seoul)* 2017, **80**:83–89.
17. Ryter SW, Lam HC, Chen ZH, Choi AM: **Deadly triplex: smoke, autophagy and apoptosis**. *Autophagy* 2011, **7**:436–437.
18. Cuervo AM, Dice JF: **Regulation of lamp2a levels in the lysosomal membrane**. *Traffic* 2000, **1**:570–583.
19. Kuwano K, Araya J, Hara H, Minagawa S, Takasaka N, Ito S, Kobayashi K, Nakayama K: **Cellular senescence and autophagy in the pathogenesis of chronic obstructive pulmonary disease (COPD)**

and idiopathic pulmonary fibrosis (IPF). *Respir Investig* 2016, **54**:397–406.

20. van Deursen JM: **The role of senescent cells in ageing**. *Nature* 2014, **509**:439–446.
21. Slebos DJ, Ryter SW, van der Toorn M, Liu F, Guo F, Baty CJ, Karlsson JM, Watkins SC, Kim HP, Wang X, et al: **Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death**. *Am J Respir Cell Mol Biol* 2007, **36**:409–417.
22. Kiffin R, Kaushik S, Zeng M, Bandyopadhyay U, Zhang C, Massey AC, Martinez-Vicente M, Cuervo AM: **Altered dynamics of the lysosomal receptor for chaperone-mediated autophagy with age**. *J Cell Sci* 2007, **120**:782–791.
23. Massey AC, Kaushik S, Sovak G, Kiffin R, Cuervo AM: **Consequences of the selective blockage of chaperone-mediated autophagy**. *Proc Natl Acad Sci U S A* 2006, **103**:5805–5810.
24. Han Q, Deng Y, Chen S, Chen R, Yang M, Zhang Z, Sun X, Wang W, He Y, Wang F, et al: **Downregulation of ATG5-dependent macroautophagy by chaperone-mediated autophagy promotes breast cancer cell metastasis**. *Sci Rep* 2017, **7**:4759.
25. Kang HT, Lee KB, Kim SY, Choi HR, Park SC: **Autophagy impairment induces premature senescence in primary human fibroblasts**. *PLoS One* 2011, **6**:e23367.
26. Zhang L, Zheng Y, Deng H, Liang L, Peng J: **Aloperine induces G2/M phase cell cycle arrest and apoptosis in HCT116 human colon cancer cells**. *Int J Mol Med* 2014, **33**:1613–1620.
27. Zhang Z, Wang CZ, Du GJ, Qi LW, Calway T, He TC, Du W, Yuan CS: **Genistein induces G2/M cell cycle arrest and apoptosis via ATM/p53-dependent pathway in human colon cancer cells**. *Int J Oncol* 2013, **43**:289–296.
28. Lv C, Hong Y, Miao L, Li C, Xu G, Wei S, Wang B, Huang C, Jiao B: **Wentilactone A as a novel potential antitumor agent induces apoptosis and G2/M arrest of human lung carcinoma cells, and is mediated by HRas-GTP accumulation to excessively activate the Ras/Raf/ERK/p53-p21 pathway**. *Cell Death Dis* 2013, **4**:e952.

Figures

Figure 1

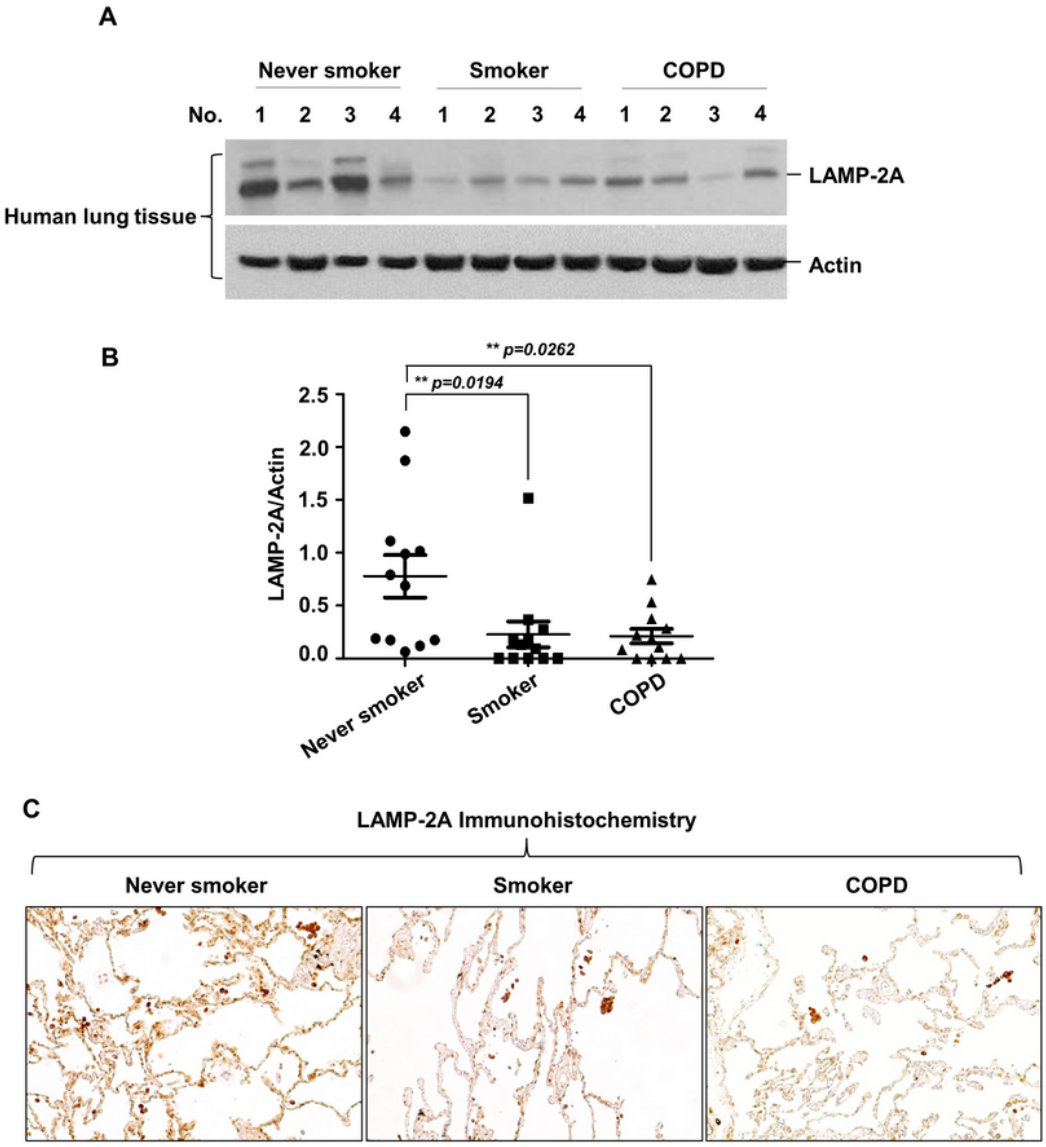

Figure 1

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 ± SE. Normal versus smoker: **p = 0.0194, normal versus COPD:

**p = 0.0262. (C) LAMP-2A immunohistochemistry in lung tissue from human patients. Original magnifications, X100.

Figure 2

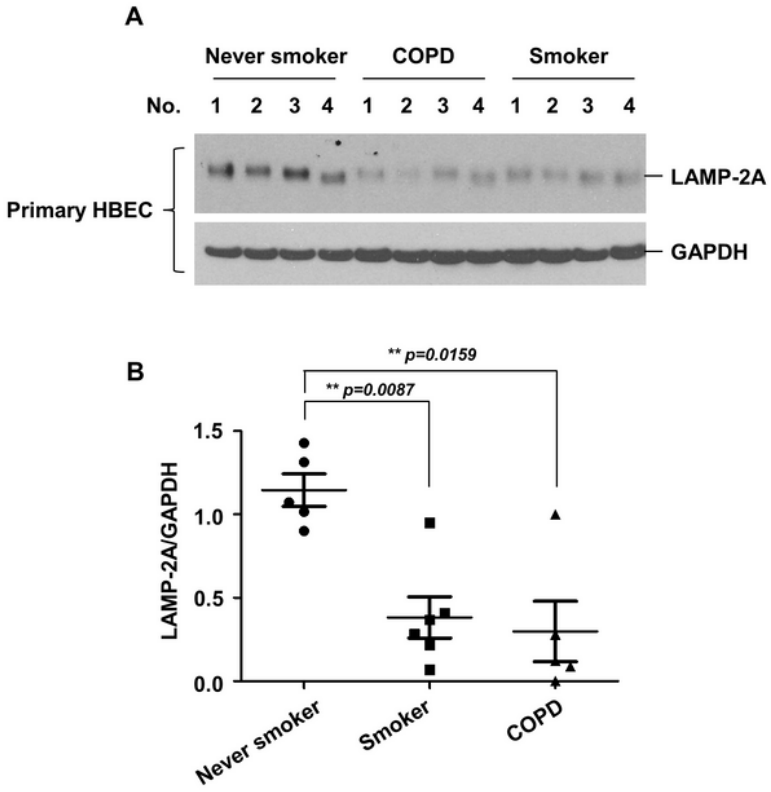

Figure 2

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 ± SE. Normal versus smoker: **p = 0.0087, normal versus COPD: **p = 0.0159.

Figure 3

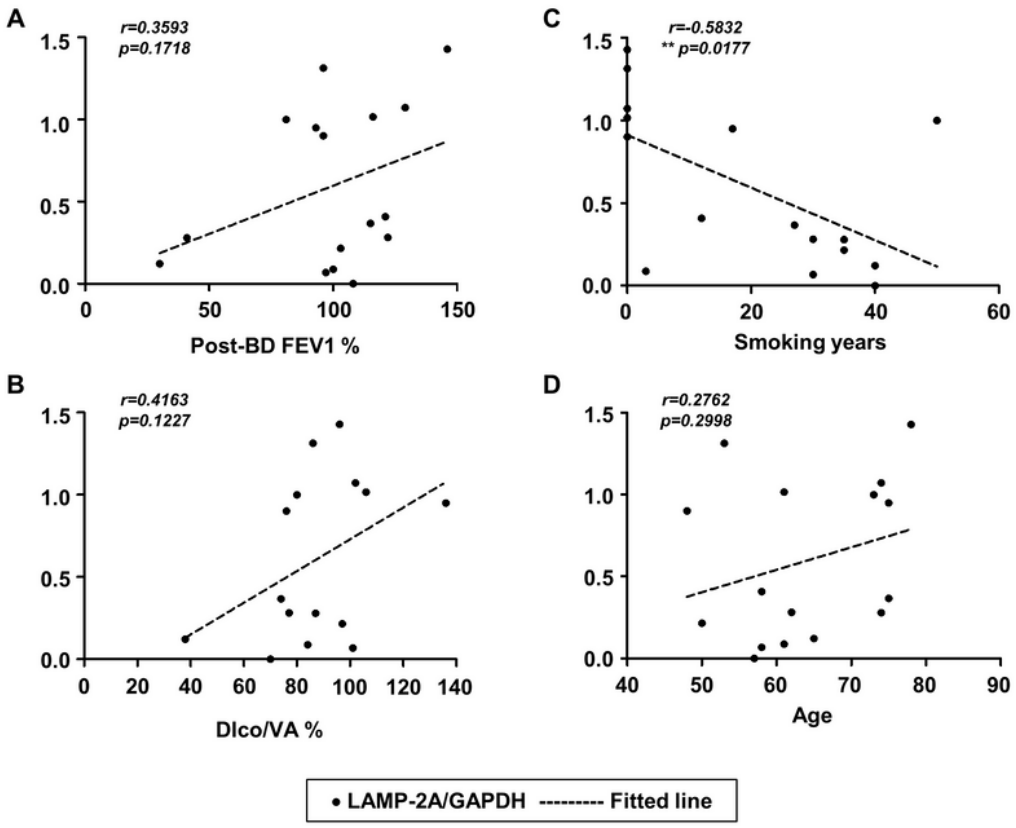

Figure 3

LAMP-2A expression is negatively correlated with years of smoking. Pearson correlation coefficient, r, was calculated for the expression level of LAMP-2A (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

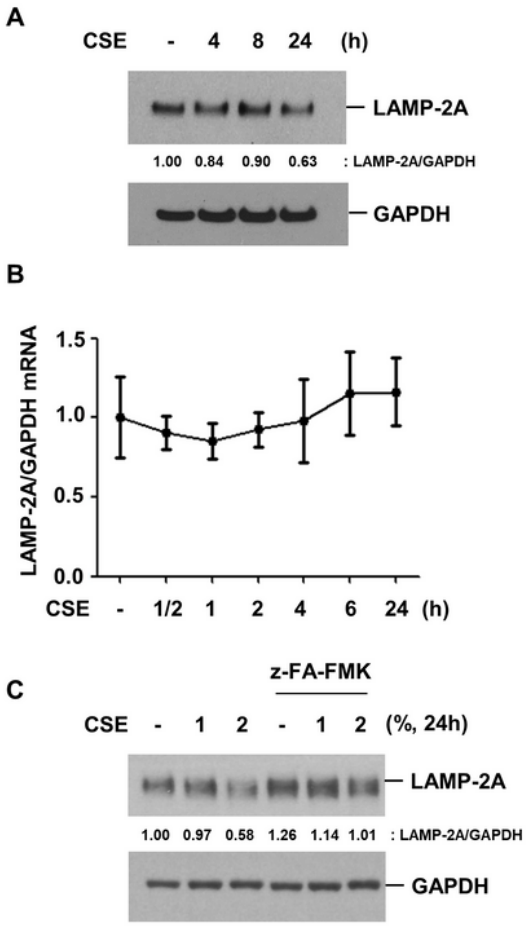

Figure 4

CSE degrades LAMP-2A via the lysosomal pathway. (A-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 (2 or 4%) 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.

Figure 5

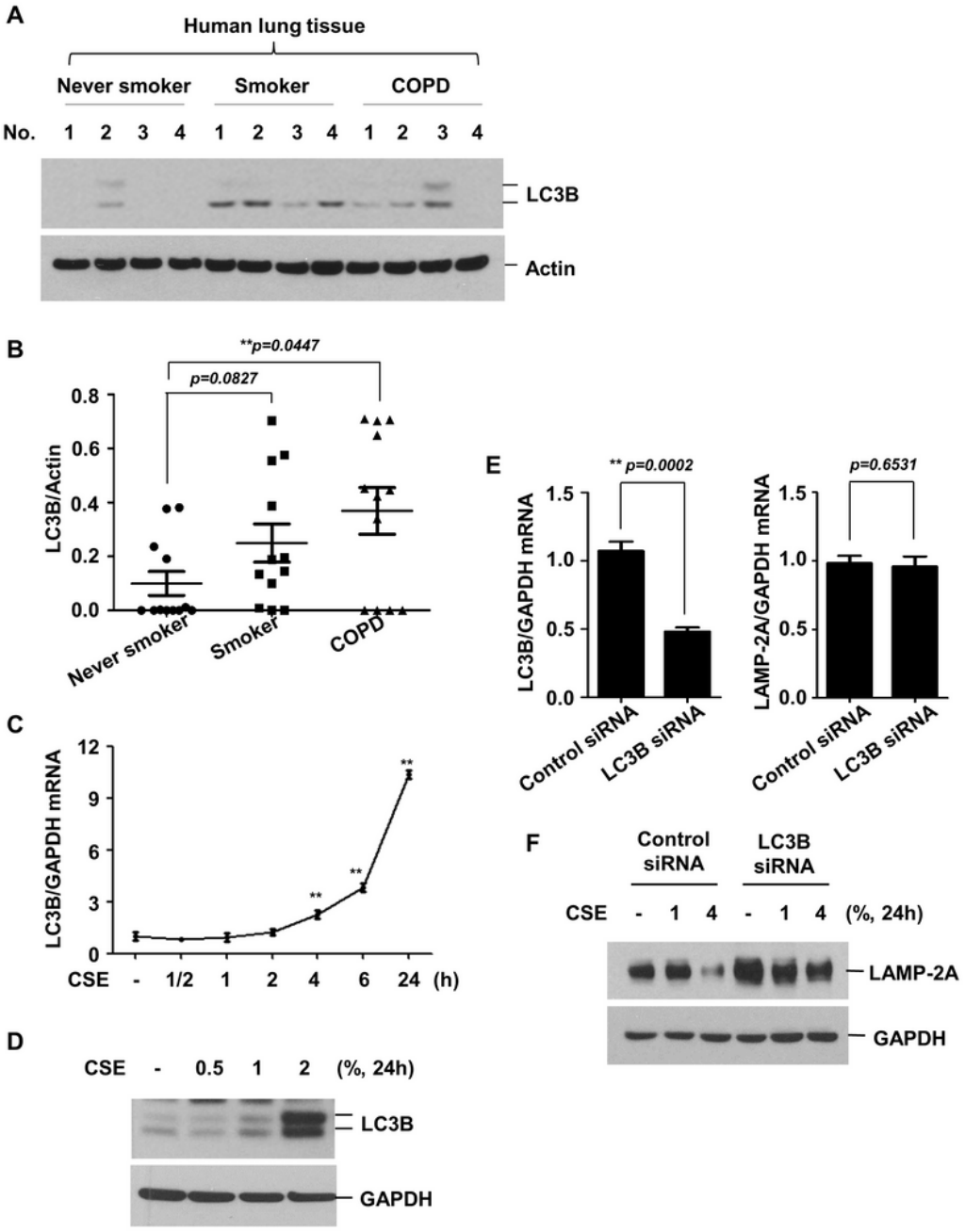

Figure 5

Downregulation of LAMP-2A is mediated by CSE-induced activation of macroautophagy. (A-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 ± SE. 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-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).

Figure 6

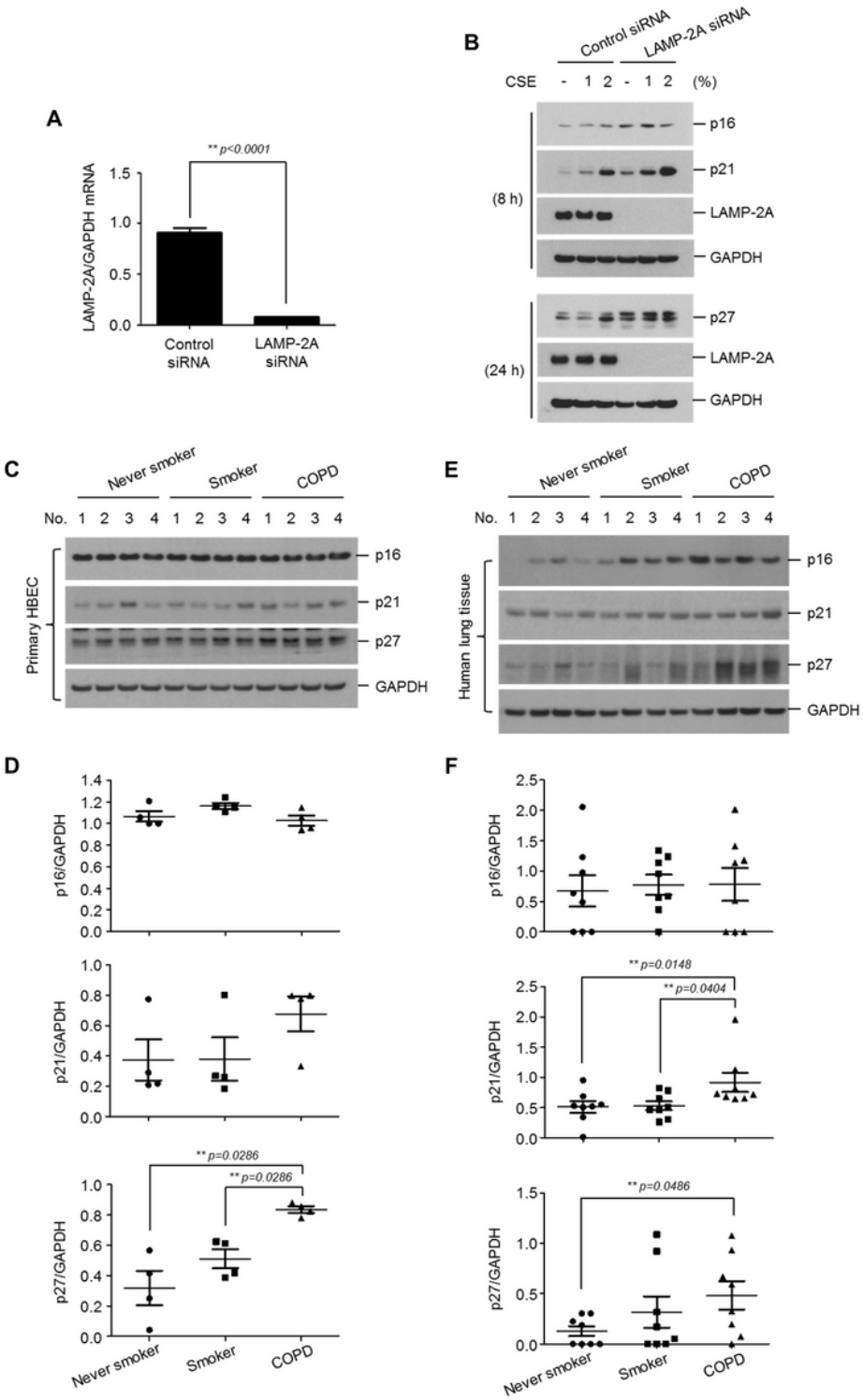

Figure 6

Knockdown of LAMP-2A enhanced CSE-induced cellular senescence. (A-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 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, p21, p27, 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 ± SE. (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 ± SE.

Figure 7

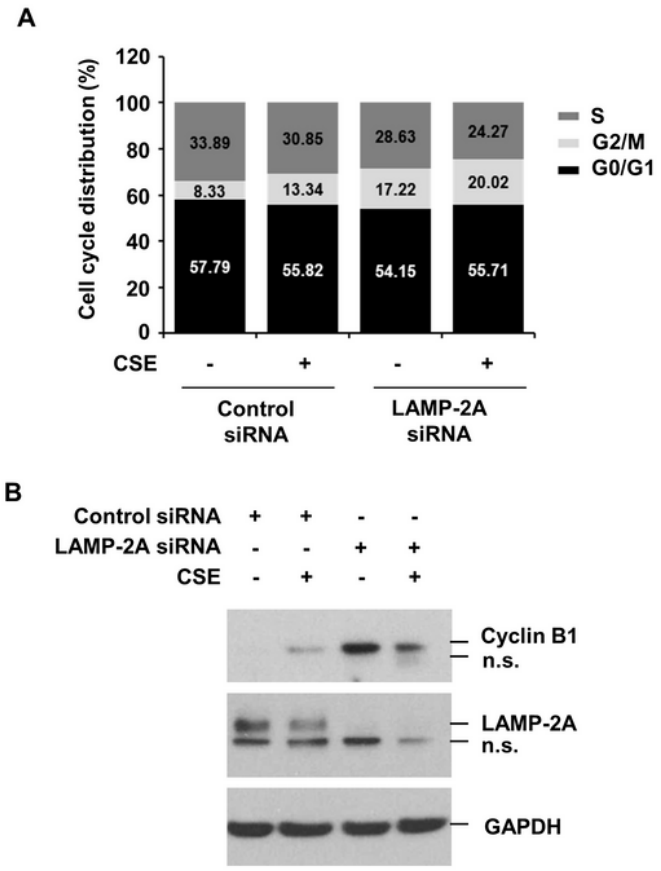

Figure 7

Knockdown of LAMP-2A enhanced CSE-induced G2/M cell cycle arrest. (A-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 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). Total cellular extracts were subjected to Western blot analysis for cyclin B1, LAMP-2A, and GAPDH (B).

Figure 8

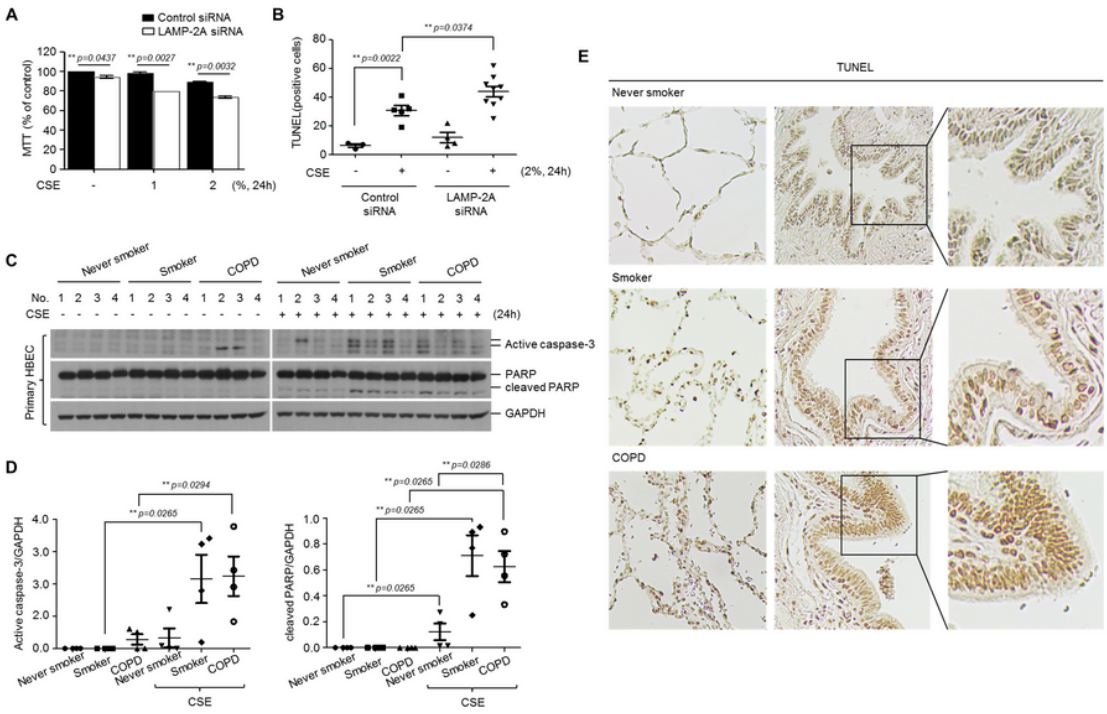

Figure 8

Knockdown of LAMP-2A enhanced CSE-induced cell death. (A-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 ± SE. (E) TUNEL staining using human lung tissue sections of never smokers (n = 4), smokers (n = 4), and COPD patients (n = 6). Original magnifications, X100 (left, middle), X200 (right)