Inhibition of E3 ligase Pellino-1 attenuates chronic obstructive pulmonary disease and lung cellular senescence by promoting p21 degradation

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June 29, 2022

Abstract

Background and Purpose Chronic obstructive pulmonary diseases (COPD) are age-related, airflow-obstruction diseases mostly caused by cigarette smoke. However, the relationship between COPD and lung cellular senescence is still not fully understood. Here, we investigated how E3 ligase Pellino-1 mediated COPD and lung cellular senescence. Experimental Approach We used western blot, qPCR and co-IP assays to analyze the correlation of Pellino-1 and P21 in cells with or without silencing Pellino-1. Then we used flow cytometry, immunofluorescence staining and β- galactosidase assay to analyze the influences of silencing Pellino-1. Furthermore, we constructed COPD and aging models in vivo. Adenovirus of knock-down and overexpression Pellino-1 was used to infected mice. Immunohistochemistry and HE staining were used to analyze the lung pathology. Key Results Here, we first found that the E3 ubiquitin ligase Pellino-1 could bind to senescence marker p21 and modify p21 by K63-site ubiquitination and verified with silencing Pellino-1. Furthermore, we found that p21-mediated lung cellular senescence could be inhibited by silencing Pellino-1. Moreover, by constructing an adenovirus mouse model, we found that silencing Pellino-1 could inhibit COPD and inflammation via reduction of SASPs regulated by p21. Resistomycin, a potential Pellino-1 inhibitor, interrupts the interaction between Pellino-1 and p21, which accelerates the ubiquitin-dependent degradation of p21 and consequently inhibits lung cellular senescence and COPD progression. Conclusion and Implications Our study elucidated that inhibition of E3 ligase Pellino-1 exhibits therapeutic potential for treatment to attenuate the progression of lung cellular senescence and COPD.

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Abbreviations

COPD: Chronic obstructive pulmonary diseases; CS: cigarette smoke; SASPs: senescence-associated secretory phenotypes; SOD: superoxide dismutase; MDA: malondialdehyde; CSE: cigarette smoke extract; FRC: functional residual capacity; FEV1: forced expiratory volume in one second; FVC: forced vital capacity; GFP: green fluorescent proteins.

Acknowledgments

Funding: This work was supported by grants from the National Natural Science Foundation of China (No. 82073870) and the Shandong Provincial Natural Science Foundation (No. ZR2019MH001)

Author contributions

Conceptualization: J.M. and X.L.; methodology: J.M., Y.Z., L.W. and X.L.; investigation: J.M., Y.Z., L.W., H.Z., J.L., N.H., Y.Z. and X.X..; and visualization: J.M., Y.Z., and L.W. Funding acquisition: X.L. Project administration: J.M. and X.L. Supervision: X.L. Writing – original draft: J.M., Q.S. and L.W. Writing – review & editing: J.M., Q.S. and X.L.

Conflicts of interest

The authors declare no competing interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract

Background and Purpose

Chronic obstructive pulmonary diseases (COPD) are age-related, airflow-obstruction diseases mostly caused by cigarette smoke. However, the relationship between COPD and lung cellular senescence is still not fully understood. Here, we investigated how E3 ligase Pellino-1 mediated COPD and lung cellular senescence.

Experimental Approach

We used western blot, qPCR and co-IP assays to analyze the correlation of Pellino-1 and P21 in cells with or without silencing Pellino-1. Then we used flow cytometry, immunofluorescence staining and β-galactosidase assay to analyze the influences of silencing Pellino-1. Furthermore, we constructed COPD and aging models *in vivo* . Adenovirus of knock-down and overexpression Pellino-1 was used to infected mice. Immunohistochemistry and HE staining were used to analyze the lung pathology.

Key Results

Here, we first found that the E3 ubiquitin ligase Pellino-1 could bind to senescence marker p21 and modify p21 by K63-site ubiquitination. Furthermore, we found that p21-mediated lung cellular senescence could be inhibited by silencing Pellino-1. By constructing adenovirus mouse models, we found that silencing Pellino-1 could inhibit COPD and inflammation via reduction of SASPs regulated by p21. Resistomycin, a potential Pellino-1 inhibitor, interrupts the interaction between Pellino-1 and p21, which accelerates the ubiquitin-dependent degradation of p21 and consequently inhibits lung cellular senescence and COPD progression.

Conclusion and Implications

Our study elucidated that inhibition of E3 ligase Pellino-1 exhibits therapeutic potential for treatment to attenuate the progression of lung cellular senescence and COPD.

Keywords: COPD, lung senescence, Pellino-1, P21, ubiquitin

Introduction

Chronic obstructive pulmonary disease (COPD) is regarded as a chronic disease characterized by airway obstruction, which is largely irreversible (Hogg & Timens, 2009). Despite progress in the treatment of COPD symptoms, few advances have been made to ameliorate disease progression or mortality. The lung-cancer-related mortality of patients with COPD for longer than 5 years is increased 191% over that of patients without COPD (Hong et al., 2018). Furthermore, the morbidity of lung cancer in COPD patients is increased more than five times (Keller et al., 2018). Bronchodilation is the main treatment method used on COPD patients at present. Clinically, tiotropium bromide, an anticholinergic bronchodilator, most effectively improved the early stage of COPD patients (Zhou et al., 2017). Those patients who could not benefit from bronchodilators were treated with roflumilast or macrolides, which could cause severe side effects. The lack of effective drugs for COPD is an urgent matter and still presents a challenge.

The mechanisms leading to COPD mainly include inflammation, the imbalance of proteases and anti-proteases, oxidative stress, and cigarette smoke (CS) (Barnes, 2017; Fischer, Pavlisko, & Voynow, 2011; Fischer, Voynow, & Ghio, 2015). Among these mechanisms, CS is known as the primary risk factor of COPD and leads to premature lung cellular senescence (Ito & Barnes, 2009; MacNee, 2009; Tuder, Kern, & Miller, 2012). Long-term CS accelerates COPD and the process of lung cellular senescence, resulting in increased damage to lung bubbles, airflow limitation, and a significant decline in lung function (Lange et al., 2015). CS exposure destroys the epithelial barrier, leading to exposure of subepithelial layers to inspired air, which contributes to the progression of COPD (Aghapour, Raee, Moghaddam, Hiemstra, & Heijink, 2018). Damage to the epithelial barrier would further lead to abnormal epithelial-mesenchymal transition (EMT), which could also promote lung cancer progression in COPD patients (Hou et al., 2019).

COPD is considered to accelerate the senescence process in the lung, and several senescence mechanisms are also applicable to COPD patients (MacNee, 2016). The relationship between COPD and lung cellular senescence is widely known, as oxidative stress causes cell cycle arrest and DNA damage mostly through the PI3K-mTOR or P38-SIRT1-P53/P16 pathways (Ascher, Elliot, Rubio, & Glassberg, 2017; Bowdish, 2019; Hecker, 2018). Reports have shown that p21 can regulate lung cellular senescence and COPD by inhibiting the phosphorylation of Rb (Tchkonia, Zhu, van Deursen, Campisi, & Kirkland, 2013). Another important bridge linking COPD and lung cellular senescence is senescence-associated secretory phenotypes (SASPs). Senescent cells still maintain their metabolic functions and secrete SASPs, which alert their environments and senescent neighboring cells (Beghe, Cerri, Fabbri, & Marchioni, 2021; Salama, Sadaie, Hoare, & Narita, 2014). The SASPs, including IL-6, IL-8, and TNF-α, in COPD patients were significantly higher than those in the healthy (Hacievliyagil, Mutlu, & Temel, 2013). Such SASPs, including certain cytokines, chemokines, proteases, and growth factors, are overexpressed in CS-induced COPD tissues, which could be mediated by p21 (Barnes, Baker, & Donnelly, 2019; Hecker, 2018). However, the type of modification of p21 that accelerated the senescence process and enhanced secretion of SASPs was still unknown.

Pellino proteins, as a family of E3 ubiquitin ligases, include Pellino-1, 2, 3 and play critical roles in regulating toll-like receptors and inflammatory pathways (Hughes et al., 2019; R. Lim, Barker, & Lappas, 2018; Medvedev, Murphy, Zhou, & Li, 2015). A study reported that Pellino-1 regulated the responses of the airway to viral infection but did not explain why this phenomenon occurred (Marsh et al., 2020). There are also two reports showing that Pellino-1 could mediate lung cancer, but they did not report if Pellino-1 could regulate the transformation of inflammatory carcinoma, which might contribute to the research on Pellino-1 in lung cellular senescence and COPD (Jeon et al., 2017; Jeon, Kim, Koh, Chung, & Ha, 2016). Pellino-1, as an immuno-regulator, could enhance the secretion of many SASPs (Bennett et al., 2012). Interestingly, a review raised an opinion that Pellino-1 might be a regulator of lung injury through immuno-signaling (Wang et al., 2021). However, there is still no research combining Pellino-1 with lung cellular senescence or COPD.

Due to the relationship between COPD and lung cellular senescence, we studied the interaction of Pellino-1 and p21 and how they influence COPD and lung cellular senescence. Here, we found that ubiquitination of p21 was a critical senescence marker of COPD caused by CS. Further, we found that resistomycin could block the interaction of Pellino-1 and p21, which exhibited potential anti-senescence ability. This research provided a new mechanism to prevent COPD and lung cellular senescence.

Materials and Methods

Cell lines

Human normal lung cell lines BEAS-2B (ATCC) were cultured in RPMI-1640 medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) at 37°C in a 5% CO2 incubator. Human kidney epithelial cell 293T was cultured in DMEM medium (Hyclone, Logan, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA) at 37°C in a 5% CO2 incubator. All cell lines culture condition contained 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Mice and Experiment Models

For studies of lung senescence and COPD, we used 6-week-old BALB/c mice in the beginning of this study. In order to investigate the role of PELI1 in lung senescence, as shown in Fig.3A, we divided mice into younger group, aging model group and natural aging group. Mice in aging model group were treated with D-Galactose (100mg/kg) per day i.h. and sacrificed at the end of 9 week. Younger and natural aging group were treated with equal volume of PBS. Younger group was sacrificed at the end of 9 week and natural aging group was sacrificed at the end of 18 week. Each group was treated with sh-PELI1 or EGFP adenovirus at beginning of experiment and the end of 6 week respectively, i.v. And in order to avoid losing effects of adenovirus, natural aging group was injected additionally at the end of 12 week.

In order to investigate the role of PELI1 in COPD, as shown in Fig.4A, we divided mice into air group and cigarette-smoking (CS) group. Mice in CS group were treated with 5 burned cigarette per day in a self-made smoke-exposure device for 18 weeks. Mice in Air group were not treated with CS. Mice were injected with sh-PELI1 or EGFP adenovirus at beginning of experiment and the end of 9 week respectively, i.v. Mice were sacrificed at the end of 18 week.

To explore the anti-lung senescence and anti-COPD effects of PELI1 inhibitor resistomycin, we divided mice similarly as shown in Fig. 6A and 7A. Resistomycin (1.25, 2.5, 5 mg/kg) per day or equal volume of PBS was given to mice in COPD experiments, i.g. Resistomycin (5 mg/kg) group was injected with overexpression PELI1 adenovirus additionally at beginning of experiment and the end of 9 week. While other groups were injected with EGFP adenovirus. For lung senescence experiments, older and younger mice were treated with or without resistomycin (5 mg/kg) for 18 weeks and injected with sh-PELI1 or EGFP adenovirus at beginning of experiment and the end of 6 and 12 week respectively, i.v. Mice in these experiments were sacrificed at the end of 18 week.

Mice experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The study procedures were approved by the Institutional Animal Care and Use Committee of Shandong University, School of Medicine, Shandong University.

MTT assay

MTT assay was used to analyze cell viability. Cells were seeded in 96-well plates and after incubation and treatment 15 μL MTT (5 g/L, Sigma) were added to wells for 4h. Then DMSO were added after removing liquid. The absorbance of 570nm were measured with a microplate reader (Molecular Devices, USA). IC50 values were calculated by Graphpad Prism 9.2 software.

Transfection of siRNA

PELI1 siRNA and siRNA control was transfected into cells with Lipofectamine® 2000 (Hanbio Biotechnology Co., Ltd, Shanghai, China). Cells were seeded in 6-well plates and cultured for 24h after transfection

to be measured.

RT-qPCR

Total RNA was extracted with Trizol (Invitrogen, Cat# 15596026), and cDNA was synthesized with fast reverse transcription kit (Sparkjade, Qingdao, China, Cat# AG0304). RT-qPCR was performed with SPARKscript II RT Master Mix (Sparkjade, Qingdao, China, Cat# AG0202). ΔΔCT method was used to analyze the fold of mRNA expression of GAPDH. RT-qPCR primers were shown in Table. S1.

Western Blot

RIPA buffer-medium (Beyotime, Cat# P0013C) was used to cells lysis. Lung tissue of mice was treated with a tissue grinder and lysis by RIPA buffer-strong (Beyotime, Cat# P0012). Total protein was quantified using a BCA Protein Assay Kit (Beyotime, Cat# P0012). Proteins were separated by SDS–PAGE gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, #1704271). Primary and secondary antibodies were listed in Table. S2. Membrane was treated with Sparkjade ECL super kit (Sparkjade, Qingdao, China, Cat# ED0015). Blot membrane were captured with a Fluor Chem M system (Protein Simple, CA). Blot images were analysed using Image J Software.

Cell Cycle Distribution

Cells stained with PI (Beyotime, Cat# C1052) were analyzed with flow cytometry (BD C6 Accuri, USA) to measure cell cycle arrest condition. Results were analyzed with winMDI software.

Immunofluorescence Staining Assay

BEAS-2B cells were seeded on 6-well plates. Cells were permeabilized in 1% Triton X-100 for 15 min, and then blocked in 5% BSA for 1h, washed twice before incubated with anti-PELI1 and anti-P21 antibodies overnight at 4. Then goat anti-rabbit secondary antibody was used for 1h at 37°C and then washed and stained by DAPI for 10 min. Immunofluorescence images were capture by ZEISS Axio Observer (Oberkochen, Germany). Images were analyzed and treated with ZEISS ZEN lite software.

Immunoprecipitation

BEAS-2B cells were transfected with the indicated plasmids or siRNA and were cultured for 24 h. The cells were collected and lysed in 550 μL Co-IP lysis buffer including 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mg/mL aprotinin, 1 mg/mL pepstatin, 1% NP-40, 1 mM EDTA and 0.25% deoxycholate for 30 min. After centrifuge for 30 min at 12000 rpm, 10% of soluble lysates were divided as input, the remainder was incubated with specific antibody at 4°C overnight, followed by incubation with Protein A/G Plus–Agarose (Santa Cruz) beads at 4°C for 2 h. Then the immunocomplexes were mixed with 5x loading buffer and boiled for 10 min at 98°C. The precipitated proteins were subjected to SDS-PAGE gel and analyzed with corresponding antibody.

Degradation of P21

To determine protein degradation, cells were incubated with the protein synthesis inhibitor cycloheximide (CHX, 10 μg/mL) for the indicated times and the expression of indicated proteins were evaluated by immunoblotting and quantitative analyses.

Preparation of Cigarette Smoking Extraction (CSE)

Preparation of CSE were reported in our previous study (Sun et al., 2018). Ten cigarettes (Taishan Brand, Shandong, People's Republic of China) burned were collected in PBS by a vacuum pump. Then CSE was filtrated by 0.22 μm filter. And the CSE was mixed with medium containing 1% FBS before use.

Immunohistochemistry (IHC) staining

Lung tissues were fixed in 3.7% formaldehyde diluted by PBS for 24 h and sliced by a Leica CM1520 freezing microtome. IHC was performed on 5-μm tissue sections using the ABC Kit after adding antibodies of P21. Diaminobenzidine (DAB) was used to dye the sections.

Adenovirus and Plasmids Construction

Plasmid of K48 and K63 ubiquitin were purchased from Addgene (K. L. Lim et al., 2005). Control siRNA and PELI1-siRNA were constructed by Gene Pharma Company, Shanghai, China. Empty control EGFP, shPELI1 and PELI1 overexpression adenovirus were constructed by Shanghai Hanbio Company, Shanghai, China.

Quantification and statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Student's t test (2-tailed) was used to compare difference between two groups. One-way ANOVA test was used to analyze difference among multiple groups. $P < 0.05$ was considered statistically significant. Analyses were performed using Graphpad Prism 9.2 software.

Results

E3 ligase Pellino-1 ubiquitylates p21 at the K63 site

Pellino-1 (PELI1), an E3 ubiquitin ligase, had shown the ability to adjust immunosignals and metabolism (Choi et al., 2006; Z. F. Jiang et al., 2003; Smith et al., 2009). In order to determine if PELI1 could regulate signals in the COPD process, we firstly screened a series of genes associated with COPD and found a positive correlation between Pellino-1 and p21 expression in the GTEx-lung database and TCGA para-carcinoma lung tissue database using GEPIA online tools (Fig. 1A and S1). Using pulmondb online analysis tools and GSE1650 datasets, we analyzed the relationship between PELI1 and p21 in COPD lung tissues (Fig. 1B). The column similarity matrix showed that a huge discrepancy in PELI1 and p21 between COPD patients and healthy people, while patients in same state of health showed obvious similarities. These bioinformatical analysis implied that PELI1 had a strong relationship with p21. Then, we investigated whether Pellino-1 participated in a protein-protein interaction with p21. The result of a co-IP assay showed that Pellino-1 could directly bind to p21 in both homo normal lung epithelial cell lines (BEAS-2B) and homo normal kidney epithelial cell lines (293T) (Fig. 1C). Then, we used two kinds of siRNA to knockdown Pellino-1. In both BEAS-2B and 293T cell lines, knockdown Pellino-1 led to a decrement of p21 and an increment of p-Rb and Cyclin E (Fig. 1D). Notably, except p21, the protein expression level of typical senescence marker P16 and P53 did not change significantly. We also found that the half-life of p21 was shortened by silencing Pellino-1 by treatment with 10 μg/mL of the protein synthesis inhibitor CHX (Fig. 1F). However, we found that mRNA levels of p21 did not decrease after Pellino-1 knockdown in both BEAS-2B and 293T cells, which implied that pellino-1 may regulate p21 by degradation (Fig. 1E). Moreover, we found after treatment with 5 μg/mL MG132 (an inhibitor of the 26S proteasome) and silencing of PELI1 that the protein level of p21 did not significantly change while that of p-Rb did increase, which enhanced the hypothesis that pellino-1 may regulate p21 by degradation (Fig. 1G). Thus, to explain how Pellino-1 down-regulated the protein level of p21, we performed a series of assays to find the specific combination mode between them. SKP2, known as an E3 ligase bound to p21, was reported to regulate the p21 degradation via ubiquitin at the K48-Ub site (Bornstein et al., 2003; Yu, Gervais, & Zhang, 1998). After Pellino-1 silencing and MG132 treatment, we found that the binding of p21 and SKP2 was increased (Fig. 1H). Furthermore, we established two kinds of BEAS-2B cells which stably expressed HA-targeted ubiquitin at K63 and K48 sites. In K63-Ub-type cells, treated with MG132, the results of co-IP assay showed that silencing Pellino-1 led to a decrease in HA-targeted K63-ubiquitin complexed with p21 (Fig. 1I). While in K48-Ub-type cells, silencing Pellino-1 led to an increase in HA-targeted K63-ubiquitin complexed with p21 (Fig. 1J). In this part, we clarified that the E3 ligase Pellino-1 could bind to p21 and link ubiquitin at the K63 site, which decreased SKP2-mediated K48-Ub-linked p21 and avoided the degradation of p21.

Silencing Pellino-1 inhibits senescence by decreasing the p21 level

D-Galactose is widely used for constructing senescence models, which causes oxidative stressed senescence in mice (Azman & Zakaria, 2019; Chen, Chen, & Zhou, 2019). After treatment with 5% cigarette smoke extract (CSE) and D-Galactose (20 μg/mL), the cell viability of BEAS-2B was significantly decreased and the effect

reversed by silencing Pellino-1 (Fig. 2A). Additionally, we added MG132 to examine the influence of p21 degradation. MG132 treatment did not significantly influence cell viability. Silencing Pellino-1 decreased the overexpression of p21 protein caused by D-Galactose, which led to an increase in p-Rb and Cyclin E (Fig. 2B). Silencing PELI1 did not change the p21 level but did alter p-Rb and Cyclin E levels in the presence of MG132, indicating that silencing PELI1 regulated p-Rb and Cyclin E via degradation of p21. The results of immunofluorescence staining showed the same tendency in the p21 protein level (Fig. 2D). Moreover, CSE treatment showed the same tendency. Cyclin E and p-Rb were key regulators that kept the cell cycle stepping into S phase. Based on the change in p-Rb and Cyclin E, we used flow cytometry to investigate the cell cycle state. Treatment with D-Galactose and CSE arrested cells stepping into S phase and increased the ratio of G1 phase cells (Fig. 2C). Then, we found that silencing Pellino-1 could inhibit G1 phase arrest and rebalance the cell cycle state. Moreover, using a β-Galactosidase staining assay, we found that silencing Pellino-1 could inhibit the senescence caused by D-Galactose, CSE, and MG132 (Fig. 2D). With MG132 treatment, silencing Pellino-1 did not change the protein level of p21 but inhibited G1 phase arrest and cellular senescence, which indicated that Pellino-1 mediated cellular senescence through p21 degradation and its downstream effects on p-Rb and Cyclin E.

Thus, we used adenovirus to infect shPELI1 plasmid and compared D-Galactose-mediated senescence in mice with natural senescence in younger and older mice to investigate the influence of Pellino-1 and senescence *in vivo* (Fig. 3A). In the D-Galactose group and 18-week-old group, the movement and food-intake of mice was obviously reduced, while the younger group and shPELI1 group did not show the same phenomenon. Comparing older and younger mice, the protein expression level of p21 was significantly elevated in older mice and could be reduced by shPELI1 (Fig. 3B). The D-Galactose group showed a similar tendency. We also detected the expression of p-Rb and Cyclin E, which implied that silencing PELI1 would decrease the expression of p21 and lead to the recovery of the cell cycle regulators p-Rb and Cyclin E to inhibit the senescence process. We also found by immunohistochemistry assay that the p21 protein was overexpressed in the bronchia of the D-Galactose and older group (Fig. 3D). Silencing PELI1 could reduce the expression of p21 and caused thinner bronchial walls. Then, using the HE staining assay, we found significant inflammatory infiltration in D-galactose-treated mice (Fig. 3C). The older group showed less inflammatory infiltration but still more than younger group, which could be relieved by silencing PELI1. To further investigate why silencing PELI1 could relieve the inflammatory infiltration in senescent mice, we determined the level of superoxide dismutase (SOD) and malondialdehyde (MDA) in lung tissue (Fig. 3E, F). The results showed that silencing PELI1 could relieve the reduction in SOD and overexpression of MDA caused by senescence. SASPs not only regulate the senescence process but also play a critical part in inflammation. Thus, we examined the mRNA expression of a series of SASPs and found that interleukin (IL)-6, IL-1α, matrix metalloproteinase (MMP)-9, MMP-12, tumor necrosis factor (TNF)-α, and chemokine CCL2 were significantly elevated in senescent mice and reduced by silencing PELI1 (Fig. 3G). These results explained that PELI1 regulated lung cellular senescence through the oxidative stress signaling cycle of SASPs-p21-SASPs.

Silencing Pellino-1 inhibits COPD and SASP

In a COPD mouse model, we also used adenovirus to infect shPELI1 plasmid to investigate the influence of Pellino-1 on COPD caused by cigarette smoke (Fig. 4A). Functional residual capacity (FRC), forced expiratory volume in one second (FEV1), and forced vital capacity (FVC) were important parameters to evaluate patients' lung function. COPD patients had a higher FRC and a lower ratio of FEV1/FVC. Before sacrificing the mice, we firstly gave 10 mg/kg budesonide, a bronchodilator, and investigated the difference in FRC and FEV1/FVC between CS and non-CS mice (Fig. 4B). The results showed that silencing PELI1 could restore FRC and FEV1/FVC to a relatively normal level. Patients with COPD also showed symptoms of lung inflammation, which caused changes in the number of white cells. Then, total white cell and differential cell counts in bronchoalveolar lavage fluid (BALF) were performed (Fig. 4C). We found that total white cells, macrophages, and neutrophils were significantly elevated in BALF of CS mice and could be reduced by silencing PELI1. Then, we used ELISA assay to check IL-6 and TNF-α levels in BALF and found that high levels of IL-6 and TNF-α caused by CS could also be reduced by silencing PELI1 (Fig. 4D). Then, we found that the protein level of p21 in lung tissue was significantly higher in CS mice and could be reduced

by silencing PELI1 (Fig. 4D). Meanwhile, we determined the mRNA expression of Cyclin E (Fig. 4E). Furthermore, using immunohistochemistry staining and HE staining, we found that silencing PELI1 could reduce inflammatory infiltration and the p21 expression level in bronchia and thinned bronchial walls caused by CS (Fig. 4F, G). We also checked the mRNA levels of SASPs and MDA and SOD levels and found a similar tendency (Fig. 4H-J). Such results of senescence-related protein expression levels and pathological features in senescence and CS mice after silencing of PELI1 implied that PELI1 regulated COPD through the senescence signaling pathways.

Resistomycin blocks Pellino-1 and inhibits lung cellular senescence and COPD

Our former study had reported that resistomycin, a natural compound (Fig. 5A), could bind to the F137 site of Pellino-1 and inhibited the binding of Pellino-1 and Snail/Slug (Liu et al., 2020). However, as a potential anti-cancer compound, resistomycin had a certain cytotoxicity. Thus, we performed an MTT assay and decided to use 0.2 μM resistomycin in CSE-treated cellular experiments (Fig. 5B, C and S2). Then, we found that resistomycin could reduce the overexpressed p21 level caused by D-Galactose and CSE, which led to an increase in p-Rb and Cyclin E (Fig. 5D). The results of immunofluorescence staining showed the same tendency (Fig. 5I). Meanwhile, the mRNA levels of Cyclin E were significantly increased by resistomycin under D-Galactose and CSE treatment (Fig. 5E). Through co-IP assay, we found that resistomycin could block the binding of Pellino-1 and p21 (Fig. 5F). We also found that resistomycin enhanced the binding of p21 and SKP2 by blocking Pellino-1 (Fig. 5G). Furthermore, using the K48-Ub-HA- and K63-Ub-HA-targeted cells mentioned above, we found that resistomycin enhanced p21 ubiquitination at the K48 site mediated by SKP2 (Fig. 5H). Using the β-Galactosidase staining assay, we found that resistomycin could reduce the senescence caused by D-Galactose and CSE (Fig. 5I). These results indicated that resistomycin could block the binding of Pellino-1 and p21, which enhanced the binding of p21 and SKP2 and showed potential anti-senescence ability.

In order to investigate the anti-senescence and anti-COPD ability of resistomycin *in vivo* , we performed further mouse experiments. We treated CS and non-CS mice with resistomycin in three dose (1.25, 2.5, and 5 mg/kg·d) and used adenovirus to infect overexpressed PELI1 plasmid (Fig. 6A). Firstly, we checked the FEV1/FVC and FRC parameters of each group (Fig. 6B). The results showed that resistomycin could relieve COPD dose-dependently, while overexpressed PELI1 reduced such effects. Then, we found that resistomycin could reduce the increase in total white cells, macrophages, and neutrophils in BALF caused by CS, which was reversed by overexpressed PELI1 (Fig. 6C). ELISA showed that the protein expression level of IL-6 and TNF-α in BALF had a similar tendency (Fig. 6D). Meanwhile, the CS-induced inflammatory infiltration observed by the HE staining assay was reduced by resistomycin dose-dependently (Fig. 6E). Furthermore, the result of immunohistochemistry staining showed that p21 levels in the bronchial walls were reduced by resistomycin (Fig. 6F). Then, we checked the protein expression of senescence-related proteins and the mRNA expression of Cyclin E and SASPs, and we found that resistomycin could inhibit the process of senescence by reducing the protein level of p21 and SASPs (Fig. 6G, H, and S3). These results implied that resistomycin could inhibit COPD and inflammation in an anti-senescent manner.

Thus, we further examined the anti-senescence ability in a mouse model of senescence. Likewise, we compared the younger and older model treated with resistomycin with 5 mg/kg·d and using overexpressed PELI1 adenovirus as reversing verification (Fig. 7A). In a comparison of older and younger mice, the protein expression level of p21 was significantly elevated in older mice and could be reduced by resistomycin treatment (Fig. 7B). Unsurprisingly, the results of other senescence-related proteins implied that resistomycin had such anti-senescence ability. This was confirmed again by the changes in mRNA expression of SASPs (Fig. 7C). The HE staining assay showed that the inflammatory infiltration and collapse of pulmonary alveoli had been improved by resistomycin (Fig. 7D). Besides, immunohistochemistry showed that resistomycin could reduce the bronchial wall thickening caused by senescence and reduce p21 levels, which could be reversed by PELI1 overexpression (Fig. 7E). From the results above, we concluded that resistomycin could inhibit COPD and lung cellular senescence through reduction of PELI1-regulated p21 and the secretion of SASPs.

Discussion

Despite the infinite efforts of researchers and medical staff, COPD is still an incurable disease. Clinical medications for COPD are separated into three major classes: bronchodilators, anti-inflammatory agents, and antioxidants (Halpin et al., 2021; Lozano et al., 2012). However, these treatments are only relievers, and relapse occurs easily. To date, the most effective COPD drugs are the inhaled dosage forms, which means that finding effective oral or i.v. drugs for COPD remains a challenge. One of the greatest challenges is the irreversibility of COPD, not only because of its associated pathological changes but also because of its enhancement effects on premature lung cellular senescence (Cohen et al., 2017; Hogg et al., 2004; Lozano et al., 2012). Cigarette smoking, the primary risk factor in COPD, could also lead directly to lung cellular senescence, which influences metabolism, oxidant stress, and endocrine signaling (Karrasch, Holz, & Jörres, 2008). Meanwhile, COPD caused by cigarette smoke leads to significant overexpression of senescence markers, including p21, a CDK inhibitor. Regarding lung cellular senescence, p21 plays a critical part in regulating oxidative stress, the cell cycle, inflammation, and signal transduction (Abbas & Dutta, 2009; Sherr & Roberts, 1995, 1999). Several E3 ligases can regulate the ubiquitination of p21, including SKP2, FBXO22, TRIM27, etc. (Xing et al., 2020; Zhang et al., 2019). However, none of these E3 ligases ubiquitinate p21 at the K63 site. Thus, in our research, Pellino-1 was unique for its ability to regulate p21. Due to a lack of animal experiments on COPD, which number far fewer than anti-cancer experiments, any progress on the mechanism of COPD is extremely valuable. Finding the combination of Pellino-1 and p21 might give us a new direction to design anti-senescence and anti-COPD medicine via suppression of the function of Pellino-1.

Moreover, reports show that the irreversibility of COPD is closely associated with EMT leading to abnormal MMPs, growth factors, airway destruction, and remodeling (Eapen et al., 2019; B. Jiang et al., 2018). These results were in close agreement with those of our former study that Pellino-1 could combine and regulate the EMT transcription factors snail/slug. That might, from another perspective, explain why pellino-1 could regulate COPD and lung cellular senescence.

In our former study, we found a natural anti-cancer compound named resistomycin, which could combine with the FHA domain of Pellino-1 and block the interaction of Pellino-1 and target proteins (Liu et al., 2020). Another report showed that resistomycin could prevent hepatoma through moderate immunoreactivity, which indicated that resistomycin might influence the lung in similar way (Alazzouni et al., 2021). Thus, we used resistomycin to verify our findings, and the results showed that resistomycin could block the combination of p21 and Pellino-1. Also, we found that resistomycin inhibited COPD and lung cellular senescence. Here, we firstly found the interaction of Pellino-1 with p21 and its inhibition by resistomycin, but a limitation of this paper was that we did not determine which molecule mediated this interaction, and further studies are warranted.

In summary, long-term cigarette smoke caused COPD and lung inflammation, which promoted the release of SASPs. Overexpressed SASPs led to the increment of P16, P53, and p21 protein levels. In the non-senescent condition, p21 was ubiquitinated by SKP2 and degraded by the 26S proteasome. In the senescent condition, p21 was ubiquitinated by Pellino-1 at the K63 site and inhibited the phosphorylation of Rb, which could be blocked by resistomycin. Then, unphosphorylated Rb combined with E2F to inhibit the expression of Cyclin E, which led to G1 cell cycle arrest and accelerated cellular senescence. This signaling cycle explains how Pellino-1 regulates lung cellular senescence to a certain extent. The results of the present study provide the first evidence that the E3 ligase Pellino-1 could combine with p21 and regulate its K63-site ubiquitination.

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