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Resveratrol attenuates cigarette smoke extract induced cellular senescence in human airway epithelial cells by regulating the miR-34a/SIRT1/NF-κB pathway

Xiao-li Zeng, MD[a,b], Xin-na Yang, MD[a,b], Xiao-ju Liu, PhD[a,b,*]

Abstract

Chronic obstructive pulmonary disease (COPD) is characterized by accelerated lung aging. Smoking is the critical risk factor for COPD. Cellular senescence of airway epithelial cells is the cytological basis of accelerated lung aging in COPD, and the regulation of microRNAs (miRNAs) is the central epigenetic mechanism of cellular senescence. Resveratrol (Res) is a polyphenol with anti-aging properties. This study investigated whether Res attenuates cigarette smoke extract (CSE)-induced cellular senescence in human airway epithelial cells (BEAS-2B) through the miR-34a/SIRT1/nuclear factor-kappaB (NF-κB) pathway. BEAS-2B cells were treated with Res, CSE and transfected with miR-34a-5p mimics. Cellular senescence was evaluated by senescence -related β-galactosidase (SA-β-gal) staining and expression of senescence-related genes (p16, p21, and p53). The expressions of miR-34a-5p, SIRT1, and NF-κB p65 were examined using quantitative real time polymerase chain reaction and western blotting. The senescence-associated secretory phenotype (SASP) cytokines (IL-1β, IL-6, IL-8, TNF-α) were assessed by enzyme-linked immunosorbent assay. The binding between miR-34a-5p and SIRT1 was confirmed by dual-luciferase reporter assay. The results showed that CSE dose-dependently decreased cell viability and elevated cellular senescence, characterized by increased SA-β-gal staining and senescence-related gene expressions (p16, p21, and p53). Further, CSE dose-dependently increased the expression of miR-34a-5p and SASP cytokines (IL-1β, IL-6, IL-8, TNF-α) in BEAS-2B cells. Pretreatment with Res inhibited CSE-induced cellular senescence and secretion of SASP cytokines (IL-1β, IL-6, IL-8, TNF-α) in a dose-dependent manner. Moreover, Res reversed the CSE-induced down-regulation of SIRT1 and up-regulation of miR-34a-5p and NF-κB p65. SIRT1 is a target of miR-34a-5p. Overexpression of miR-34a-5p via transfection with miR-34a-5p mimic in BEAS-2B cells attenuated the inhibitory effect of Res on cellular senescence, accompanied by reversing the expression of SIRT1 and NF-κB p65. In conclusion, Res attenuated CSE-induced cellular senescence in BEAS-2B cells by regulating the miR-34a/SIRT1/NF-κB pathway, which may provide a new approach for COPD treatment.

Abbreviations: 3'UTR = 3'untranslated region, COPD = chronic obstructive pulmonary disease, CS = cigarette smoke, CSE = cigarette smoke extract, ELISA = enzyme linked immunosorbent assay, miRNA = microRNA, NF-κB = nuclear factor-kappaB, qRT-PCR = quantitative real time polymerase chain reaction, Res = resveratrol, SASP = senescence-associated secretory phenotype, SA-β-gal = senescence-associated β-galactosidase, SIRT = Sirtuin.

Keywords: cellular senescence, cigarette smoke extract, miR-34a, resveratrol, SIRT1

1. Introduction

Chronic obstructive pulmonary disease (COPD) is the leading cause of morbidity and mortality worldwide. COPD is characterized by airway obstruction and chronic inflammation in the lungs, which drive airway remodeling, emphysema and chronic bronchitis. Cigarette smoke (CS) is the most well-established risk factor for COPD.[1] The underlying mechanisms of COPD remain poorly understood, and current treatments primarily focus on relieving symptoms without reducing disease progression and mortality.[2] Therefore, novel insights into the pathogenesis and treatment of COPD are required.

COPD is a disease that results in accelerated aging of the lungs.[3] Cellular senescence is the cytological basis for

The authors have no conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval was not required as the human airway epithelial cells used in this study were obtained from Fu Heng Biology (Shanghai, China).

[a] The First School of Clinical Medicine, Lanzhou University, Lanzhou, China, [b] Department of Gerontal Respiratory Medicine, The First Hospital of Lanzhou University, Lanzhou, China.

**Correspondence: Xiao-ju Liu, The First School of Clinical Medicine, Lanzhou University, Lanzhou, China; Department of Gerontal Respiratory Medicine, The First Hospital of Lanzhou University, Lanzhou 730000, China (e-mail: liuxiaoju835@126.com).*

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accelerated aging in COPD.[4] Cellular senescence is an irreversible cell cycle arrest condition in which cells are still metabolically active.[5] Senescence of airway epithelial and endothelial cells has been found to accumulate in lungs of COPD patients.[6] Consequently, senescent cells lose regenerative capacity and disturb cellular repair in COPD lungs, leading to emphysema and deteriorating lung function.[7] The expressions of senescence-related biomarkers such as p16, p21, p53, and senescence-associated β-galactosidase (SA-β-gal) can be used to identify senescent cells.[8] Senescent cells also secrete excessive amounts of proinflammatory cytokines, chemokines, growth factors, and proteases, termed the senescence-associated secretory phenotype (SASP), which is a critical difference from terminally differentiated cells.[9] SASP may exert harmful effects on the tissue microenvironment of neighboring cells.[10] In COPD, CS has been identified as a significant inducer of cellular senescence. Cigarette smoke extract (CSE) induces growth arrest and increase the expression of SA-β-gal, p16 and p21 in alveolar epithelial cells.[11] Similar results were reported in which CSE exposure led to enhanced SA-β-gal activity, increased p16 expression and cellular senescence in bronchial epithelial cells.[12]

Sirtuins are conserved NAD(+)-dependent deacetylases that play vital roles in the promotion of metabolism and longevity.[13] There are 7 Sirtuins (SIRT1-7) in mammals, and SIRT1 is a critical factor that inhibits cellular senescence and prolongs organismal lifespan by regulating multiple cellular processes. SIRT1 has been shown to be down-regulated during aging, and up-regulation of SIRT1 can decrease CS-induced cellular senescence.[14] Yao et al found that SIRT1 protects against cellular senescence and inflammation in lungs induced by CS.[15] Nuclear factor-kappaB (NF-κB) is an important transcription factor that is activated in various diseases and has been implicated in SASP and inflammation. SIRT1 has been found to negatively regulate NF-κB signaling.[16] Therefore, activation of SIRT1 inhibits NF-κB activity and reduces inflammation levels, reduces inflammation levels, and subsequently ameliorates vascular endothelial dysfunction with aging.[17]

Micro-RNAs (miRNAs) are a class of small endogenous non-coding RNAs consisting of 18 to 25 nucleotides that negatively regulate target genes.[18] MiRNAs bind the 3'untranslated region (3'UTR) of target messenger RNAs (mRNAs), leading to direct degradation of mRNA and inhibition of protein translation. Increasing evidence indicate that miRNAs including miR-34a, miR-24, miR-146 play important roles in the aging process by regulating target genes involved in senescence.[19,20] MiR-34a, a key regulator of SIRT1, is increased in airway epithelial cells from patients with COPD.[21] An antagonist of miR-34a restored SIRT1 expression in airway epithelial cells, reduce markers of cellular senescence, decrease the SASP response, and promote cell proliferation by reversing cell cycle arrest.[21] Collectively, these suggest that miR-34a promotes cellular senescence and that miR-34a antagomirs could be employed as anti-aging therapies.

Resveratrol (Res) is a polyphenol found in various plants such as grapes, strawberries, tomatoes, peanuts, cocoa, and sugar cane.[22] Several previous studies demonstrated that Res has many bioactivities, including anti-carcinogenic, anti-inflammatory, cardiovascular -protection, and anti-aging effects.[23] The anti-aging properties of Res have been attributed to several complex mechanisms involving various signaling pathways. Hsu et al reported that Res exerts anti-aging effects by increasing the expression of the anti-aging gene Klotho in the kidney.[24] Csiszar et al demonstrated Res up-regulated the activity of Nrf2 in aged primary vascular smooth muscle cells to decrease secretion of SASP.[25] Furthermore, studies have confirmed that Res administration significantly down-regulates the levels of miR-34a.[26] However, it is still unclear whether Res exerts anti-aging effects by regulating the miR-34a/SIRT1/NF-κB pathway. Therefore, this study aimed to investigate the effect of Res on CSE-induced cellular senescence in airway epithelial cell line (BEAS-2B) and the underlying mechanisms.

2. Methods

2.1. Cell culture and treatment

BEAS-2B cells were purchased from Fu Heng Biology (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (Biological Industries, Beit Haemek, Israel) containing 10% fetal bovine serum (Biological Industries), 1% penicillin/streptomycin at 37°C in an incubator with 5% CO_2. BEAS-2B cells were then treated with various concentrations of CSE (2.5%–15%) and/or Res (10, 25 and 50 μM, Sigma-Aldrich, USA) for the indicated time points.

2.2. CSE preparation

CSE was prepared following the methods of Li et al[27] One cigarette without filter (Lanzhou Brand; Gansu Tobacco Industry Co, Ltd, China) was aspirated using a vacuum pump. The smoke was drawn into 50 mL of PBS for 2 to 3 minutes. The CSE solution was sterilized through a 0.22-μm filter, and considered as 100% CSE. CSE (100%) was diluted to the desired concentrations with culture medium for each experiment and used within 30 minutes of preparation. The optical densities of CSE solutions prepared in this manner were constant.

2.3. Cell transfection

MiR-34a-5p mimic and scrambled mimic control (miR-NC) were obtained from GenePharma (Shanghai, China). All oligomers were transfected into BEAS-2B cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. Further experiments were conducted 24 hours after transfection. The expression of miR-34a-5p was determined by quantitative real-time polymerase chain reaction (qRT-PCR).

2.4. Cell viability assay

BEAS-2B cells were seeded in 96-well plates at a density of 8×10^3 cells/well. The cells were exposed to 0, 2.5, 5, 7.5, 10, and 15% CSE for 24 hours or 7.5% CSE for 0, 6, 12, 24 and 48 hours. Then, MTT reagent (Sigma) was added and incubated at 37°C for 4 hours in the dark. Next, the supernatants were discarded, and 150 μL DMSO was added to each well. A microplate reader (Synergy H1, Biotek, VT) was used to measure the absorbance at 490 nm. According to this formula, the cell survival rate was calculated as $(OD_{treatment} - OD_{blank})/OD_{control} - OD_{blank}) \times 100\%$.

2.5. SA-β-gal staining

The SA-β-gal activity was determined using an SA-β-gal staining kit (Beyotime, Shanghai, China). BEAS-2B cells were plated in 24-well plates at a density of 4×10^4 cells/well. After treatment with CSE, Res, and/or transfection, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature, and then incubated in SA-β-gal staining solution overnight at 37°C. Finally, the cells were observed using a microscope (IX73, Olympus, Tokyo, Japan) to capture images, in which the senescent cells were colored blue.

2.6. qRT-PCR assay

Total RNA was extracted from BEAS-2B cells using RNAiso Plus reagent (Takara, Dalian, China). RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit or Mir-X™ miRNA First-Strand Synthesis Kit (Takara). Primers for mRNA and miRNA were obtained from Accurate Biotechnology (Hunan, China). The primers used are listed in Table 1. qRT-PCR

was performed using the SYBR® Premix Ex Taq™ II kit (Takara) and a Mx3000P Real-Time Thermal Cycler (Agilent, Santa Clara, CA). The relative expression of mRNA or miRNA was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to GAPDH or U6, respectively.

2.7. Western blot analysis

Total protein was extracted from BEAS-2B cells using RIPA lysis buffer (Solarbio, Beijing, China). Each protein sample (40 μg) was resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were blocked with 5% skimmed milk for 1 hour and then incubated overnight at 4°C with primary antibodies against SIRT1 (BOSTER, Wuhan, China), NF-κB p65 and *β*-actin (Signalway Antibody, College Park, MD, USA). After overnight incubation, the membranes were washed with TBST buffer and incubated with goat anti-rabbit IgG (Signalway Antibody) for 2 hours at 1:3000 dilution at room temperature. Finally, blots were quantified using an enhanced chemiluminescence kit and *β*-actin was used as an internal control.

2.8. Cytokine measurements

The SASP cytokines (IL-1*β*, IL-6, IL-8, TNF-*α*) in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (Elabscience Biotechnology Co, Ltd, Wuhan, China) according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader (Synergy H1, Biotek, VT).

2.9. Dual-luciferase reporter assay

The binding sites of miR-34a-5p and SIRT1were predicted using TargetScan. The wild (wt) or mutant (mut) 3'UTR sequence of SIRT1 was inserted into the GP-miRGLO vector (GenePharma). Then, SIRT1-3'UTR-wt vector or SIRT1-3'UTR-mut vector was co-transfected with miR-34a-5p mimic or miR-NC into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen). After 24 hours, the transfected cells were lysed and luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Fitchburg, WI).

2.10. Statistical analysis

All experiments were performed in at least 3 independent assays and are presented as mean ± standard deviation. Statistical analyses were performed using SPSS 21.0 software (SPSS Inc., Chicago, IL). One-way ANOVA was used to compare mean data among multiple groups, and a post hoc LSD test was used for inter-group comparisons. Statistical significance was set at $P < .05$.

Table 1

Primer sequences.

Gene	Primer sequence (5′–3′)
p16	Forward: CTCTGAGAAACCTCGGGAAACT
	Reverse: AACTACGAAAGCGGGGTGG
p21	Forward: TTCCTCATCCACCCCATCC
	Reverse: CCCTGTCCATAGCCTCTACTGC
p53	Forward: CTTCCATTTGCTTTGTCCCG
	Reverse: TCTTACATCTCCCAAACATCCCT
SIRT1	Forward: ATGCCAGAGTCCAAGTTTAGAAGA
	Reverse: CTCCATCAGTCCCAAATCCAG
NF-κB p65	Forward: TACGGATTCTGGTGGGGTGT
	Reverse: CCATCAGGACAGGGGAAAAGT
GAPDH	Forward: GCACCGTCAAGGCTGAGAAC
	Reverse: TGGTGAAGACGCCAGTGGA
miR-34a-5p	TGGCAGTGTCTTAGCTGGTTG
U6	Forward: GGAACGATACAGAGAAGATTAGC
	Reverse: TGGAACGCTTCACGAATTTGCG

NF-κB = nuclear factor-kappaB, SIRT1 = Sirtuin1.

3. Results

3.1. CSE inhibited BEAS-2B cell viability

To evaluate the effect of CSE exposure on cell viability, BEAS-2B cells were exposed to 0, 2.5, 5, 7.5, 10 and 15% CSE for 24 hours or 7.5% CSE for 0, 6, 12, 24 and 48 hours. The MTT assay indicated that the cell viability was decreased in a dose- and time-dependent manner (Fig. 1). When the concentration of CSE reached 10% and 15%, or when cells were exposed to 7.5% CSE for 48 hours, the survival rate of BEAS-2B cells was < 50%. The concentrations of CSE (2.5%–7.5%) were used to treat cells for the following experiments, and experimental times were set at 24 hours as determined by these results.

3.2. CSE exposure induced cellular senescence and increased level of miR-34a in BEAS-2B cells

SA-*β*-gal is a verified marker of cellular senescence. Senescent cells are stained blue using SA-*β*-gal staining. To investigate the effect of CSE on cellular senescence, BEAS-2B cells were exposed to various CSE concentrations for 24 hours followed by SA-*β*-gal staining. As shown in Figure 2A and B, the percentage of SA-*β*-gal positive cells increased with increasing concentrations of CSE. The senescence-related markers p16, p21, and p53 were measured using qRT-PCR. The results showed that CSE exposure dose-dependently increased the gene expression of p16, p21, and p53 (Fig. 2C-E). Additionally, SASP cytokines including IL-1*β*, IL-6, IL-8, and TNF-*α* in cell media were measured by ELISA. Compared to the control group, the levels of these cytokines were elevated after CSE exposure (Fig. 2F–I). These results support the promoting effects of CSE on cellular senescence.

We also evaluated the effect of CSE exposure on miR-34a-5p expression. qRT-PCR assay showed that CSE dose-dependently increased the expression of miR-34a-5p in BEAS-2B cells (Fig. 2J). The results indicate that miR-34a might participate in CSE-induced cellular senescence.

3.3. Res attenuated cellular senescence and regulated miR-34a/SIRT1/NF-κB pathway in CSE-stimulated BEAS-2B cells.

To investigate whether Res alleviated CSE-induced cellular senescence, BEAS-2B cells were pretreated with 10, 25 and 50 μM Res for 6 hours followed by exposure to 7.5% CSE for another 24 hours. As shown in Figure 3A and B, the percentage of SA-*β*-gal positive cells induced by CSE was reduced by Res pretreatment in a dose-dependent manner. Further, pretreatment with Res dose-dependently attenuated CSE-induced expression of senescence-related markers, including p16, p21, and p53 (Fig. 3C–E). In addition, pretreatment with Res dose-dependently attenuated the levels of IL-1*β*, IL-6, IL-8, and TNF-*α* in CSE-treated cells (Fig. 3F–I). These results indicate that Res protected BEAS-2B cells against CSE-induced cellular senescence.

To investigate whether the anti-aging efficacy of Res is associated with the miR-34a/SIRT1/ NF-κB pathway, we measured the miR-34a-5p, SIRT1, NF-κB p65 expression using qRT-PCR and western blotting. As shown in Figure 3J, pretreatment with Res decreased the expression of miR-34a-5p in CSE-treated cells. Further, SIRT1 expression was reduced and NF-κB p65

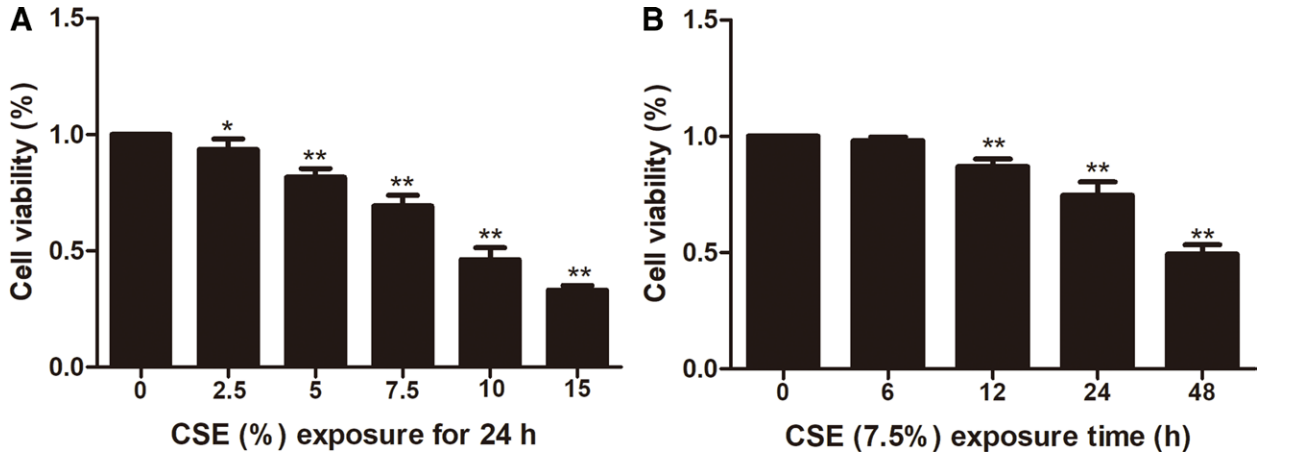

Figure 1. CSE inhibited BEAS-2B cells viability. BEAS-2B cells were treated with 0%, 2.5%, 5%, 7.5%, 10% and 15% CSE for 24 hours (A) or 7.5% CSE for 0, 6, 12, 24 hours and 48 hours (B). MTT assay was measured to evaluate the cell viability. Data were presented as means ± SD (n = 5). $^{**}P < .01$, $^{*}P < .05$ versus control. CSE = cigarette smoke extract, MTT = methyl thiazolyl tetrazolium.

expression was increased after CSE exposure. In contrast, Res pretreatment dose-dependently increased SIRT1 expression and decreased NF-κB p65 expression (Fig. 3K–O). Altogether, these observations suggest that the miR-34a/SIRT1/NF-κB pathway may be implicated in the protective effect of Res on CSE-induced cellular senescence in BEAS-2B cells.

3.4. Overexpression of miR-34a reversed the effects of Res on cellular senescence in CSE-stimulated BEAS-2B cells

A dual-luciferase reporter assay was performed to investigate the target of miR-34a. As shown in Figure 4A and B, miR-34a-5p had sequences complementary to SIRT1. The fluorescence intensity decreased in cells co-transfected with SIRT1-3'UTR-wt vector and miR-34a-5p mimic, however fluorescence intensity showed no significant changes in cells transfected with SIRT1-3'UTR-mut vector. This result implied that SIRT1 is a target of miR-34a.

To determine the function of miR-34a in the inhibitory effect of Res on cellular senescence, we increased its expression by transfecting BEAS-2B cells with miR-34a-5p mimic for 24 hours, then pretreated with 50 μM Res for 6 hours, followed by exposure to 7.5% CSE for another 24 hours. As shown in Figure 4C, the expression of miR-34a-5p was significantly increased in BEAS-2B cells transfected with miR-34a-5p mimic compared in Res + CSE or Res + CSE + miR-NC groups. Furthermore, compared to the Res + CSE group, overexpression of miR-34a-5p enhanced the percentage of SA-β-gal positive cells and elevated the expression of senescence-related markers p16, p21, and p53 (Fig. 4D–H). Additionally, ELISA revealed that overexpression of miR-34a-5p increased the secretion of IL-1β, IL-6, IL-8, and TNF-α (Fig. 4I–L). Thus, overexpression of miR-34a-5p abrogated the inhibitory effect of Res on CSE-induced cellular senescence in BEAS-2B cells. These results confirm that miR-34a is implicated in the inhibitory effect of Res on CSE-induced cellular senescence and secretion of SASP cytokines in BEAS-2B cells.

Next, we investigated whether Res mediates the SIRT1/NF-κB signaling pathway via miR-34a. As shown in Figure 4M–Q, compared to the Res + CSE group, miR-34a-5p overexpression by transfection of BEAS-2B cells with miR-34a-5p mimic decreased the mRNA and protein expression of SIRT1, whereas these effects increased the expression of NF-κB p65. These data indicate that miR-34a overexpression reversed the expression of SIRT1 and NF-κB p65 in BEAS-2B cells treated with Res + CSE, simultaneously abrogating the inhibitory effect of Res on cellular senescence caused by CSE. Collectively, these data suggest that Res attenuates CSE-induced cellular senescence via the miR-34a/SIRT1/NF-κB pathway.

4. Discussion

Airway epithelial cells are the first line of defence against environmental factors and pathogens. Senescence of airway epithelial cells plays an essential role in COPD progression.[28] Cellular senescence impairs the regenerative capacity of stem cells, and senescent cells release SASP cytokines, which contribute to airway inflammation and remodeling in COPD.[29] Cellular senescence is an irreversible process that is typically identified by the increased expression of SA-β-gal and up-regulation of senescence-related markers, including p16, p21, and p53.[8] Senescent cells express β-galactosidase enzyme, which is detectable at a pH of 6 and termed SA-β-gal. Additionally, this enzyme is widely used as a biomarker to identify senescent cells.[8] An important mechanism by which cells maintain the senescent state is to alter expression of senescence-related genes, such as p16, p21, p53, and retinoblastoma protein (pRB).[30] The transcriptional regulators p53 and pRB are the most important factors; p21 works downstream of p53, whereas p16 works upstream of pRB.[30] These components are cyclin-dependent kinase inhibitors that regulate cellular senescence, which can be induced by overexpression of any of these components. In addition, senescent cells secrete multiple inflammatory proteins termed SASP, which cause persistent inflammation and further accelerate senescence.[10] NF-κB dependent proinflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α, are critical components of SASP, and are suggested to be highly conserved and robustly expressed cytokines.[30] Furthermore, a large array of stressors can induce cellular senescence. In vitro and vivo, CS exposure has been found to be sufficient to induce cellular senescence as a stressor. CSE exposure activates senescence-related pathways in human lung fibroblasts, such as p53 and p16-pRB pathways.[31] In the present study, CSE dose-dependently increased the percentage of SA-β-gal stained cells and the expression of p16, p21, and p53 in BEAS-2B cells, accompanied by increased secretion of SASP cytokines including IL-1β, IL-6, IL-8, and TNF-α. Overall, these data indicate that CSE promotes senescence of airway epithelial cells.

Res, a polyphenol found in various plants, has anti-aging properties and prolongs lifespan of several species by targeting multiple cellular processes.[32] Res reduces SASP cytokines in rats induced by CS exposure and suppresses lung aging in mouse models of accelerated aging.[33,34] In vitro, Res inhibits the release of SASP cytokines and the expression of senescence-related markers such as p53, p16, p21 in bone marrow stromal stem cells stimulated by tert-butyl hydroperoxide and promotes osteoblastic differentiation.[35] Similar to these findings, our data showed that Res dose-dependently alleviated CSE-induced

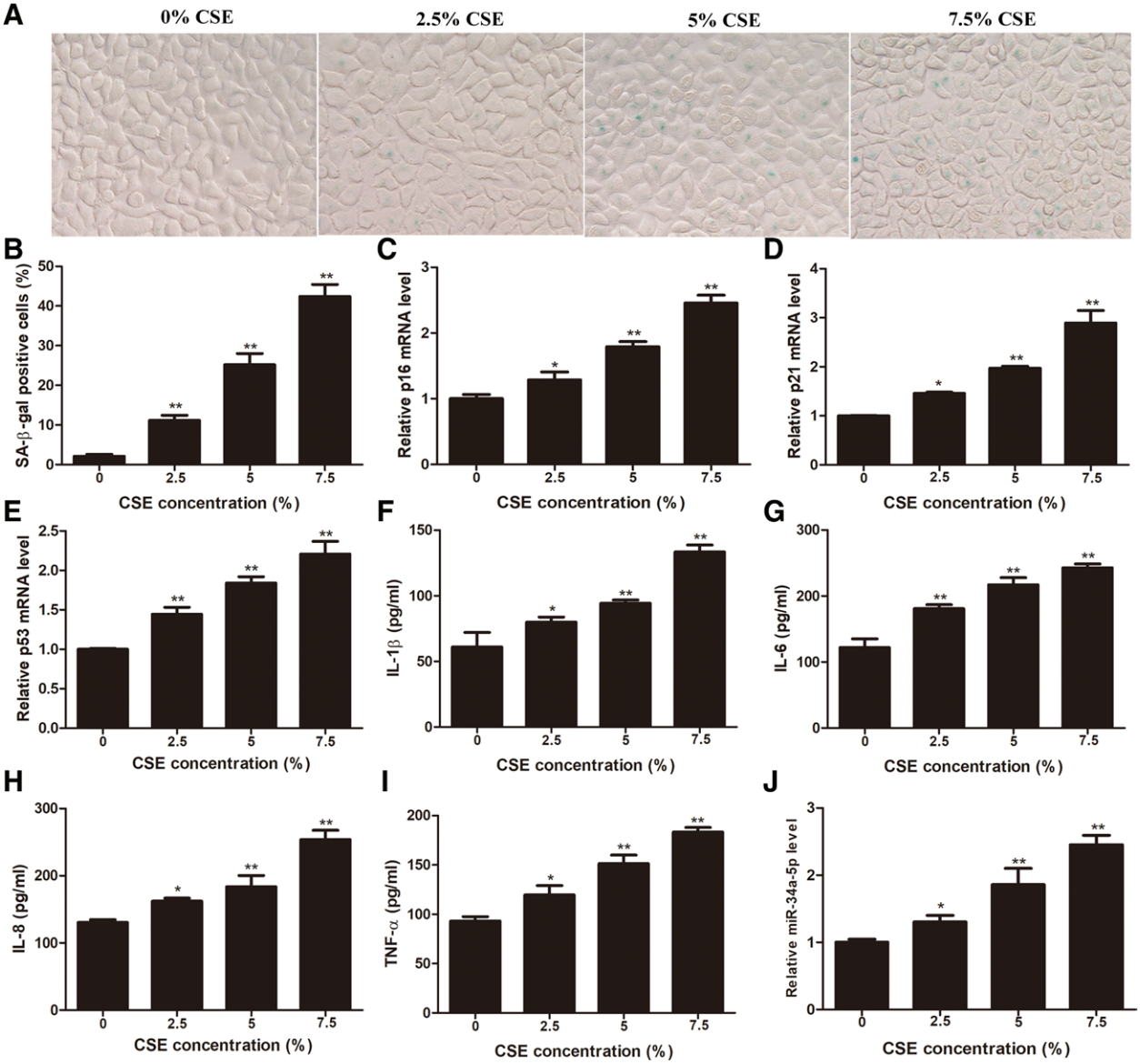

Figure 2. CSE increased senescence-related markers, SASP cytokines and miR-34a-5p in BEAS-2B cells. Cells were treated with 0%, 2.5%, 5%, and 7.5% CSE for 24 hours. (A) Images of SA-β-gal staining (400×). (B) The percentages of SA-β-gal positive cells. (C–E) The mRNA expressions of senescent genes p16, p21, and p53 were tested by qRT-PCR. (F–I) The levels of IL-1β, IL-6, IL-8, and TNF-α in supernatants were assessed by ELISA. (J) The expression of miR-34a-5p was assessed by qRT-PCR. Data are presented as means ± SD (n = 3-5). $^{**}P < .01$, $^{*}P < .05$ versus control. CSE = cigarette smoke extract, ELISA = enzyme-linked immunosorbent assay, SASP = senescence-associated secretory phenotype, SA-β-gal = senescence-associated β-galactosidase, qRT-PCR = quantitative real time polymerase chain reaction.

increase in the percentage of SA-β-gal positive cells and increased expression of p16, p21, and p53. In a addition, Res preconditioning decreased CSE-induced SASP secretion. These results provide direct evidence that Res attenuates CSE-induced senescence in airway epithelial cells. However, the mechanism underlying the anti-aging properties of Res has not been clarified. Therefore, we investigated whether Res attenuates CSE-induced cellular senescence by regulating the miR-34a/ SIRT1/NF-κB pathway.

A variety of miRNAs, termed senescence-related miRNAs, can act as senescence inducers.[36] Philipot et al identified that miR-24 negatively regulates p16 expression and promotes cellular senescence in human chondrocytes.[37] Afanasyeva et al reported that miR-885-5p exertes antitumor effects by interfering with cell cycle progression and cell survival.[38] Additionally, miR-146a has been demonstrated to negatively regulate the expression of TNF-α and IL-8.[39] Meanwhile, miR-34a has been found to act as a senescence promoter that can reduce SIRT1 expression by binding to its 3'UTR and is likely involved in vascular endothelial progenitor cell senescence.[40] Consistent with the above research, dual-luciferase report assays in this study demonstrated that SIRT1 is a direct target of miR-34a. Yamakuchi et al reported that miR-34a inhibits SIRT1 expression and activated p53 to exert antitumor effects.[41] Disayabutr et al showed that miR-34a expression was increased in alveolar epithelial cells of idiopathic pulmonary fibrosis patients and was positively correlated with SA-β-gal expression.[42] NF-κB, a key transcription factor that regulates proinflammatory genes, has been previously found to control SASP release from senescent alveolar epithelial cells.[43] In relation to this, SIRT1 interacts with the RelA/p65 subunit of NF-κB and deacetylates lysine 310, enhancing the expression and activity of NF-κB.[44] Furthermore, SIRT1 knockout or knockdown enhanced NF-κB expression and activity, as well as proinflammatory cytokine release, whereas up-regulation of SIRT1 inhibited NF-κB-mediated inflammatory mediator release.[45,46] This study showed that CSE dose-dependently

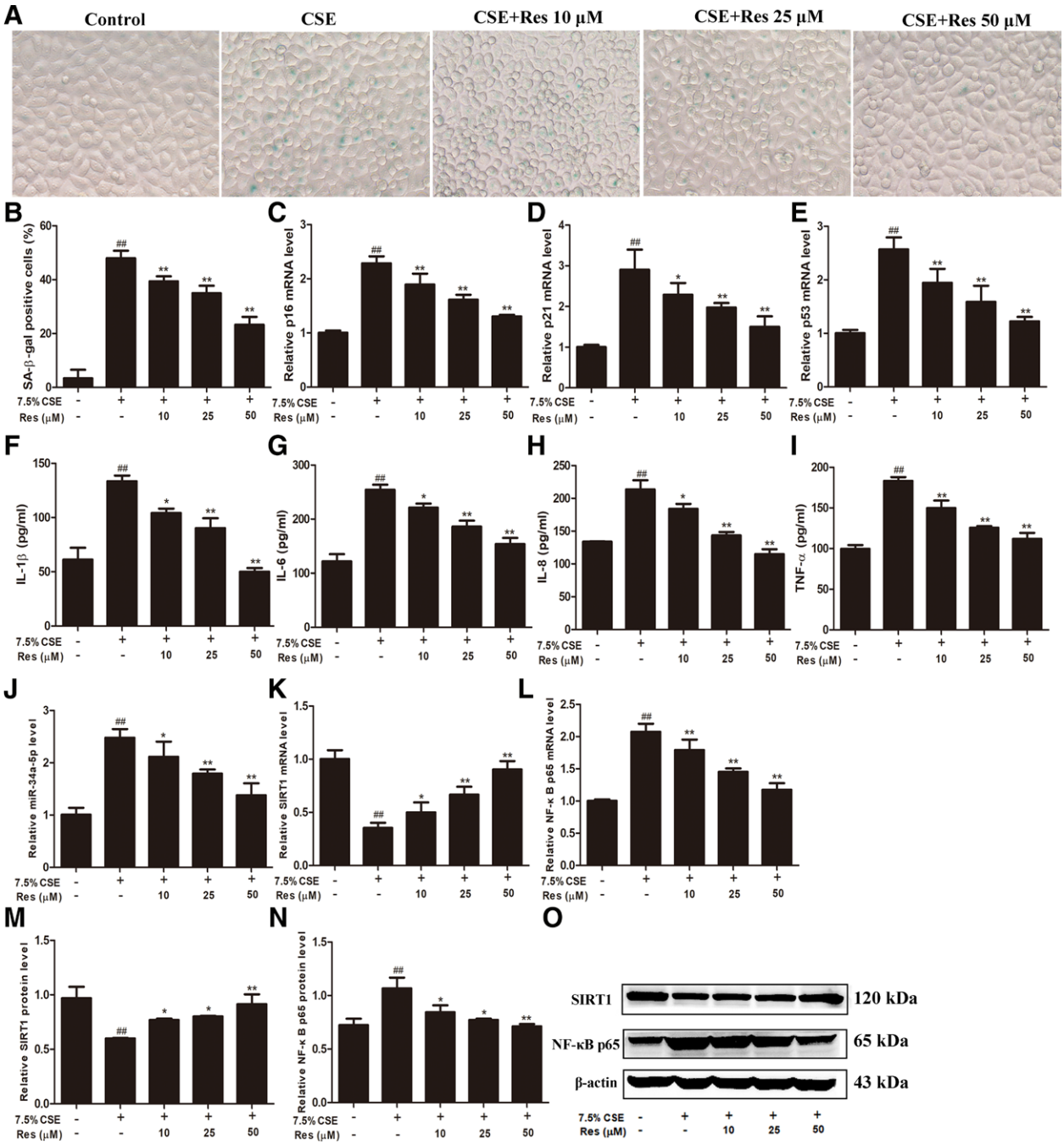

Figure 3. Effect of Res on CSE-induced cellular senescence and miR-34a/SIRT1/NF-κB pathway in BEAS-2B cells. Cells were pretreated with 10 μM, 25 μM and 50 μM Res for 6 hours, then exposed to 7.5% CSE for another 24 hours. (A) Images of SA-β-gal staining (400×). (B) The percentages of SA-β-gal positive cells. (C–E) The mRNA expressions of senescent genes p16, p21, and p53 were measured by qRT-PCR. (F–I) The levels of IL-1β, IL-6, IL-8, and TNF-α in supernatants were assessed by ELISA. (J) The expression of miR-34a-5p was assessed by qRT-PCR. (K–O) The mRNA and protein expressions of SIRT1 and NF-κB p65 were quantified by qRT-PCR and western blot. Data are presented as means ± SD (n = 3-5). $^{\#\#}P < .01$ versus control, $^{**}P < .01$, $^{*}P < .05$ versus CSE group. CSE = cigarette smoke extract, ELISA = enzyme-linked immunosorbent assay, NF-κB = nuclear factor-kappaB, Res = resveratrol, SA-β-gal = senescence-associated β-galactosidase, qRT-PCR = quantitative real time polymerase chain reaction.

increased miR-34a-5p and NF-κB p65 expression but decreased SIRT1 expression in BEAS-2B cells. Furthermore, Res pretreatment decreased the levels of miR-34a and NF-κB p65 and enhanced SIRT1 expression in CSE-treated BEAS-2B cells. These observations suggest that the miR-34a/SIRT1/NF-κB pathway may be implicated in the protective effect of Res on CSE-induced cellular senescence in BEAS-2B cells.

Following these findings of Res pretreatment, we next investigated whether Res exerted anti-aging effect by inhibiting miR-34a. To confirm this, we transfected BEAS-2B cells with miR-34a-5p mimic to increase miR-34a expression, followed by treatment with Res and CSE. The results showed that miR-34a overexpression abrogated the inhibitory effect of Res on CSE-induced cellular senescence. Simultaneously, the increased expression of SIRT1 and the decreased expression of NF-κB accompanied by decreased secretion of SASP cytokines caused by Res were reversed by miR-34a overexpression. These data indicate that Res

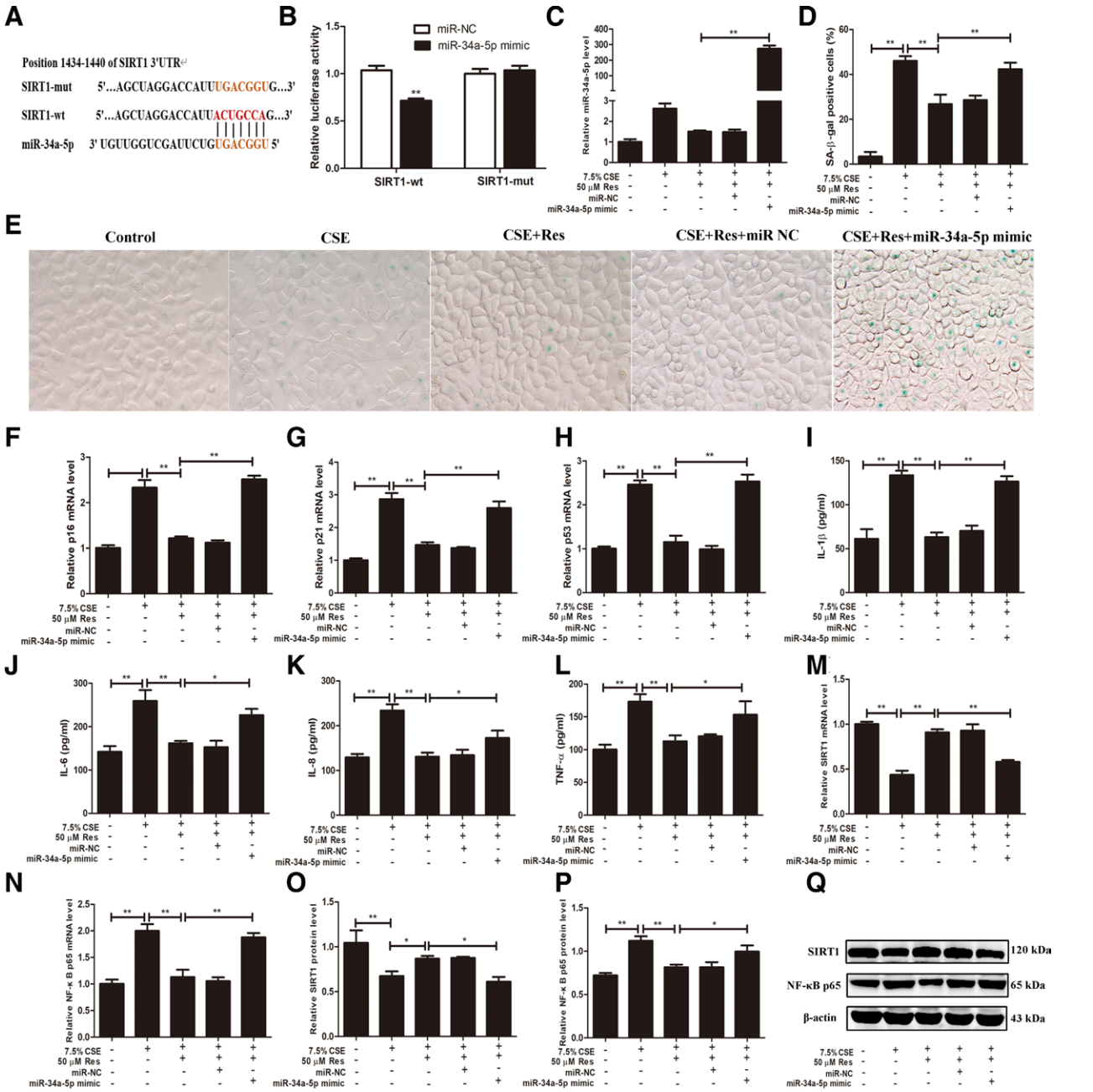

Figure 4. MiR-34a overexpression reversed the effects of Res on the cellular senescence induced by CSE in BEAS-2B cells. Cells were transfected with miR-34a-5p mimic or miR-NC for 24 hours, then pretreated with 50 μM Res for 6 hours, following exposure to 7.5% CSE for another 24 hours. (A) The binding sites between miR-34a and 3′UTR of SIRT1. (B) Dual-luciferase reporter assay. (C) The expression of miR-34a-5p was assessed by qRT-PCR. (D) The percentages of SA-β-gal positive cells. (E) Images of SA-β-gal staining (400×). (F–H) The mRNA expressions of senescent genes p16, p21, and p53 were measured by qRT-PCR. (I–L) The levels of IL-1β, IL-6, IL-8, and TNF-α in supernatants were assessed by ELISA. (M–Q) The mRNA and protein expressions of SIRT1 and NF-κB p65 were quantified by qRT-PCR and western blot. Data are presented as means ± SD (n = 3-5). $^{**}P < .01$, $^{*}P < .05$. CSE = cigarette smoke extract, ELISA = enzyme-linked immunosorbent assay, NF-κB = nuclear factor-kappaB, Res = resveratrol, SA-β-gal = senescence-associated β-galactosidase, qRT-PCR = quantitative real time polymerase chain reaction.

targets miR-34a to regulate the SIRT1/NF-κB pathway and reduce CSE-induced cellular senescence and SASP secretion in BEAS-2B cells.

5. Conclusion

In summary, our results indicate that CSE induces senescence in airway epithelial cells. Additionally, Res attenuates CSE-induced cellular senescence through the miR-34a/SIRT1/NF-κB pathway. These results provide novel research targets and potential treatment strategies for COPD.

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Author contributions

Conceptualization: Xiao-li Zeng, Xin-na Yang, Xiao-ju Liu.
Data curation: Xiao-li Zeng, Xin-na Yang.
Formal analysis: Xiao-li Zeng.

Methodology: Xiao-li Zeng, Xin-na Yang.
Supervision: Xiao-li Zeng, Xiao-ju Liu.
Writing – original draft: Xiao-li Zeng.
Writing – review & editing: Xiao-li Zeng, Xiao-ju Liu.

References

[1] Duffy SP, Criner GJ. Chronic obstructive pulmonary disease: evaluation and management. Med Clin North Am. 2019;103:453–61.
[2] Woldhuis RR, de Vries M, Timens W, et al. Link between increased cellular senescence and extracellular matrix changes in COPD. Am J Physiol Lung Cell Mol Physiol. 2020;319:L48–60.
[3] Barnes PJ. Senescence in COPD and its comorbidities. Annu Rev Physiol. 2017;79:517–39.
[4] Chilosi M, Carloni A, Rossi A, et al. Premature lung aging and cellular senescence in the pathogenesis of idiopathic pulmonary fibrosis and COPD/emphysema. Transl Res. 2013;162:156–73.
[5] Birch J, Barnes PJ, Passos JF. Mitochondria, telomeres and cell senescence: implications for lung ageing and disease. Pharmacol Ther. 2018;183:34–49.
[6] Birch J, Anderson RK, Correia-Melo C, et al. DNA damage response at telomeres contributes to lung aging and chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol. 2015;309:L1124–37.
[7] Easter M, Bollenbecker S, Barnes JW, et al. Targeting aging pathways in chronic obstructive pulmonary disease. Int J Mol Sci . 2020;21:6924.
[8] González-Gualda E, Baker AG, Fruk L, et al. A guide to assessing cellular senescence in vitro and in vivo. FEBS J. 2021;288:56–80.
[9] van Deursen JM. The role of senescent cells in ageing. Nature. 2014;509:439–46.
[10] Coppé JP, Desprez PY, Krtolica A, et al. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol. 2010;5:99–118.
[11] Du Y, Ding Y, Shi T, et al. Suppression of circXPO1 attenuates cigarette smoke-induced inflammation and cellular senescence of alveolar epithelial cells in chronic obstructive pulmonary disease. Int Immunopharmacol. 2022;111:109086.
[12] Liu A, Wu J, Li A, et al. The inhibitory mechanism of Cordyceps sinensis on cigarette smoke extract-induced senescence in human bronchial epithelial cells. Int J Chron Obstruct Pulmon Dis. 2016;11:1721–31.
[13] Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol. 2010;5:253–95.
[14] Zhang Y, Huang W, Zheng Z, et al. Cigarette smoke-inactivated SIRT1 promotes autophagy-dependent senescence of alveolar epithelial type 2 cells to induce pulmonary fibrosis. Free Radic Biol Med. 2021;166:116–27.
[15] Yao H, Sundar IK, Ahmad T, et al. SIRT1 protects against cigarette smoke-induced lung oxidative stress via a FOXO3-dependent mechanism. Am J Physiol Lung Cell Mol Physiol. 2014;306:L816–28.
[16] Edwards JR, Perrien DS, Fleming N, et al. Silent information regulator (Sir)T1 inhibits NF-κB signaling to maintain normal skeletal remodeling. J Bone Miner Res. 2013;28:960–9.
[17] Gano LB, Donato AJ, Pasha HM, et al. The SIRT1 activator SRT1720 reverses vascular endothelial dysfunction, excessive superoxide production, and inflammation with aging in mice. Am J Physiol Heart Circ Physiol. 2014;307:H1754–63.
[18] Cieśla M, Kolarz B, Majdan M, et al. Plasma micro-RNA-22 is associated with disease activity in well-established rheumatoid arthritis. Clin Exp Rheumatol. 2022;40:945–51.
[19] Ma X, Zheng Q, Zhao G, et al. Regulation of cellular senescence by microRNAs. Mech Ageing Dev. 2020;189:111264.
[20] Munk R, Panda AC, Grammatikakis I, et al. Senescence-associated MicroRNAs. Int Rev Cell Mol Biol. 2017;334:177–205.
[21] Baker JR, Vuppusetty C, Colley T, et al. Oxidative stress dependent microRNA-34a activation via PI3Kα reduces the expression of sirtuin-1 and sirtuin-6 in epithelial cells. Sci Rep. 2016;6:35871.
[22] Reinisalo M, Kårlund A, Koskela A, et al. Polyphenol stilbenes: molecular mechanisms of defence against oxidative stress and aging-related diseases. Oxid Med Cell Longev. 2015;2015:340520.
[23] Braidy N, Jugder BE, Poljak A, et al. Resveratrol as a potential therapeutic candidate for the treatment and management of Alzheimer's disease. Curr Top Med Chem. 2016;16:1951–60.
[24] Hsu SC, Huang SM, Chen A, et al. Resveratrol increases anti-aging Klotho gene expression via the activating transcription factor 3/c-Jun complex-mediated signaling pathway. Int J Biochem Cell Biol. 2014;53:361–71.
[25] Csiszar A, Sosnowska D, Wang M, et al. Age-associated proinflammatory secretory phenotype in vascular smooth muscle cells from the non-human primate Macaca mulatta: reversal by resveratrol treatment. J Gerontol A Biol Sci Med Sci. 2012;67:811–20.
[26] Alharris E, Alghetaa H, Seth R, et al. Resveratrol attenuates allergic asthma and associated inflammation in the lungs through regulation of miRNA-34a that targets FoxP3 in mice. Front Immunol. 2018;9:2992.
[27] Li T, Fanning KV, Nyunoya T, et al. Cigarette smoke extract induces airway epithelial cell death via repressing PRMT6/AKT signaling. Aging (Albany NY). 2020;12:24301–17.
[28] Tam A, Sin DD. Pathobiologic mechanisms of chronic obstructive pulmonary disease. Med Clin North Am. 2012;96:681–98.
[29] Bagdonas E, Raudoniute J, Bruzauskaite I, et al. Novel aspects of pathogenesis and regeneration mechanisms in COPD. Int J Chron Obstruct Pulmon Dis. 2015;10:995–1013.
[30] Kumari R, Jat P. Mechanisms of cellular senescence: cell cycle arrest and senescence associated secretory phenotype. Front Cell Dev Biol. 2021;9:645593.
[31] Nyunoya T, Monick MM, Klingelhutz A, et al. Cigarette smoke induces cellular senescence. Am J Respir Cell Mol Biol. 2006;35:681–8.
[32] Agarwal B, Baur JA. Resveratrol and life extension. Ann N Y Acad Sci. 2011;1215:138–43.
[33] Wang XL, Li T, Li JH, et al. The effects of resveratrol on inflammation and oxidative stress in a rat model of chronic obstructive pulmonary disease. Molecules. 2017;22:1529.
[34] Navarro S, Reddy R, Lee J, et al. Inhaled resveratrol treatments slow ageing-related degenerative changes in mouse lung. Thorax. 2017;72:451–9.
[35] Ali D, Chen L, Kowal JM, et al. Resveratrol inhibits adipocyte differentiation and cellular senescence of human bone marrow stromal stem cells. Bone. 2020;133:115252.
[36] Wu H, Ma H, Wang L, et al. Regulation of lung epithelial cell senescence in smoking-induced COPD/emphysema by microR-125a-5p via Sp1 mediation of SIRT1/HIF-1a. Int J Biol Sci. 2022;18:661–74.
[37] Philipot D, Guérit D, Platano D, et al. p16INK4a and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodeling in osteoarthritis. Arthritis Res Ther. 2014;16:R58.
[38] Afanasyeva EA, Mestdagh P, Kumps C, et al. MicroRNA miR-885-5p targets CDK2 and MCM5, activates p53 and inhibits proliferation and survival. Cell Death Differ. 2011;18:974–84.
[39] Ong SM, Hadadi E, Dang TM, et al. The pro-inflammatory phenotype of the human non-classical monocyte subset is attributed to senescence. Cell Death Dis. 2018;9:266.
[40] Sun X, Huang K, Haiming X, et al. Connexin 43 prevents the progression of diabetic renal tubulointerstitial fibrosis by regulating the SIRT1-HIF-1α signaling pathway. Clin Sci (Lond). 2020;134:1573–92.
[41] Yamakuchi M, Ferlito M, Lowenstein CJ. miR-34a repression of SIRT1 regulates apoptosis. Proc Natl Acad Sci USA. 2008;105:13421–6.
[42] Disayabutr S, Kim EK, Cha SI, et al. miR-34 miRNAs regulate cellular senescence in type II alveolar epithelial cells of patients with idiopathic pulmonary fibrosis. PLoS One. 2016;11:e0158367.
[43] Tian Y, Li H, Qiu T, et al. Loss of PTEN induces lung fibrosis via alveolar epithelial cell senescence depending on NF-κB activation. Aging Cell. 2019;18:e12858.
[44] Lin J, Sun B, Jiang C, et al. Sirt2 suppresses inflammatory responses in collagen-induced arthritis. Biochem Biophys Res Commun. 2013;441:897–903.
[45] Rajendrasozhan S, Yang SR, Kinnula VL, et al. SIRT1, an antiinflammatory and antiaging protein, is decreased in lungs of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med. 2008;177:861–70.
[46] Deng HJ, Zhou CH, Huang LT, et al. Activation of silent information regulator 1 exerts a neuroprotective effect after intracerebral hemorrhage by deacetylating NF-κB/p65. J Neurochem. 2021;157:574–85.