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ITGB4 deficiency induces senescence of airway epithelial cells through p53 activation

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Abbreviations:

ITGB4: integrin β 4

HBE: Human Bronchial Epithelial

SA- β -gal: senescence-associated- β -galactosidase

SASP: senescence-associated secretory phenotype

MAPK: mitogen-activated protein kinase

FAK: focal adhesion kinase

EGFR: epidermal growth factor receptor

Keywords: senescence, asthma, airway epithelial cells, integrin β 4, p53

Abstract

Aging is characterized by a progressive loss of physiological integrity, leading to impaired organ function and, ultimately, increased vulnerability to death. Many complex diseases are related to aging, including asthma. In the lung, the airway epithelium serves as the first barrier to prevent the access of inspired external stimuli and dictates the initial stress responses. Notably, in the airway mucosa of asthma patients an increase of senescent airway epithelial cells has been detected. Although it has been speculated that the senescence of

airway epithelial cells could increase asthma susceptibility and aggravate asthma severity, the role of cell senescence in the development of asthma remains unclear. Integrin $\beta 4$ (ITGB4) is a structural adhesion molecule with complex physiological functions that is down-regulated in airway epithelial cells of asthma patients. This study demonstrates that the expression of ITGB4 in airway epithelial cells is down-regulated significantly under oxidative stress or upon inflammatory stimulation. Moreover, we show that ITGB4 deficiency induces the senescence of airway epithelial cells through the activation of p53 pathway both *in vitro* and *in vivo*. Together, our results demonstrate that airway epithelial senescence induced by ITGB4 deficiency after oxidative stress or inflammatory stimulation may be involved in the pathogenesis of asthma. Understanding the contribution of ITGB4 deficiency to the senescence of airway epithelial cells in asthma patients may provide new therapeutic approaches for the treatment of asthma.

Introduction

Biological aging includes various cellular, molecular and structural changes based on several theories [1]. Although usually age-related, biological aging can also occur in early life, partially independent of age [2]. In addition to replicative aging, aging is also induced under various stimulation conditions, such as DNA damage, oncogene activation and so on [3]. Of note, more and more studies have verified that oxidative stress and chronic inflammatory stimuli are also common factors that could induce aging [4, 5]. Such stress-induced aging is also known as pathological aging or premature aging [2]. In the article, we use the term

“senescence” to describe aging specifically at the cellular level (cellular aging), which includes a range of alterations in cell morphology and function [6]. It refers to the decline of cell proliferation and differentiation, detachment from the cell cycle, and the decline of the ability to proliferate upon stimulation by growth factors or mitogens [7].

There are strong evidences that the dysfunction of airway epithelial cells plays a key role in the pathogenesis of asthma [8, 9]. Cell senescence in the lung is an important risk factor for the development of asthma [10, 11]. Meanwhile, increased aging airway epithelial cells have been detected on the airway mucosa of asthma patients [10]. The role of airway epithelial senescence in the pathogenesis of asthma has drawn a lot of attention [12]. More importantly, aging airway epithelial cells have been verified to be associated with the development of airway remodeling which may be further involved in the aggravation of asthma [10, 13]. However, the specific mechanism of aging in airway epithelial cells is still unclear.

As the first barrier cells to exogenous stimuli airway epithelial cells receive the most oxidative and chronic inflammatory stimuli [14, 15]. Our previous studies found that ITGB4 was down-regulated on the airway epithelial cells of asthma patients [16]. ITGB4 is located at the basal surface of airway epithelial cells in hemi-desmosome structures which mediate the stable adhesion of airway epithelial cells to the underlying basement membrane component laminin-5 [17]. The long cytoplasmic domain of ITGB4 subunit could recruit a series of signaling molecules and thereby influences the proliferation, damage repair, and oxidative stress of epithelial cells [18, 19]. Besides, it's also demonstrated that, after HDM stress, ITGB4 defects in airway epithelial cells conferred increased airway

hyper-responsiveness and aggravated airway inflammation compared with that of the wild-type mice [20]. Notably, ITGB4 has also been shown to be associated with senescence in many different cells [21-23]. Senescence is controlled by two major senescence pathways, p53-p21^{WAF1/CIP1} and Rb-p16^{INK4a13} [24]. P53 is a transcriptional activator and repressor that could control the expression of genes that cause cell cycle arrest in response to genomic damage [25, 26]. Besides, p16^{INK4a13} is a cyclin-dependent kinase inhibitor that leads to pRb hypophosphorylation which is crucial for long-term maintaining of senescence [27]. It has also been verified that ITGB4 could promote the activation of p53 through different signaling pathways [21, 28-30]. Then, we further hypothesized that ITGB4 deficiency in airway epithelia might induce the senescence of airway epithelial cells in asthma patients. In this paper, we firstly detect the expression of ITGB4 in airway epithelial cells after oxidative stress or inflammatory stimulation, respectively. Then, we observe the senescence phenotype and probe the mechanism for the senescence in ITGB4 defective airway epithelial cells.

Results

ITGB4 expression was decreased in airway epithelial cells under different stress conditions

To observe the expression of ITGB4 in airway epithelial cells under oxidative stress or inflammatory stimulation, normal primary Human Bronchial Epithelial (HBE) cells were treated with TNF α or O₃ and detected by western blotting analysis (Figure 1A, B). The result showed that, at the same concentration, the expression of ITGB4 is negatively related to the

duration of stimulation no matter stimulated with TNF α or O₃. Besides, we detected ITGB4 expression and senescent cells in different stressed mice models by immunohistochemistry staining and senescence-associated- β -galactosidase (SA- β -gal) staining, respectively (Figure 1C, D). Our results demonstrated that the expression of ITGB4 on airway epithelial cells was both down-regulated in LPS stimulation model and O₃ stressed model. Meanwhile, after LPS stimulation or O₃ stress, more senescent cells were detected on the airway epithelial cells of model mice.

ITGB4 deficiency caused the senescence of airway epithelial cells in vivo

To avoid lethal effect of ITGB4 null mice, we constructed a conditional ITGB4 deficiency model: CCSP-rtTA^{tg/-}/TetO-Cre^{tg/-}/ITGB4^{fl/fl}. In the model, ITGB4 was deleted only in the airway epithelial cells [20]. Then, SA- β -gal was used to detect the senescence in airway epithelial cells [31]. Our results showed that ITGB4 deficient airway epithelial cells presented typical premature senescence. The rate of positive SA- β -gal staining in airway epithelial cells of ITGB4^{-/-} mice was significantly higher than that in control mice (Figure 2A). Meanwhile, the immunohistochemistry of Ki67 was performed to measure cellular proliferative capacity in vivo. The cellular proliferative capacity of the airway epithelial cells in ITGB4^{-/-} mice was decreased obviously compared with control mice (Figure 2B). It is generally verified that p53/p21 signaling pathway participates in the process of cell senescence in vitro and in vivo [6, 32]. Our results also demonstrated that both of p53 and

p21 expression in airway epithelial cells were significantly upregulated in ITGB4^{-/-} mice.

However, there was no significant difference in p16 expression (Figure 2C, D, E).

ITGB4 deficiency caused the senescence of airway epithelial cell in vitro

To further verify the senescence effect caused by ITGB4 deficiency, ITGB4 silenced HBE cells were constructed by ITGB4 silencing siRNA. The silencing efficiency was validated by qPCR and western blotting, respectively (Figure 3A, B). Our results showed that ITGB4-silenced cells had longer and larger cell shapes. The SA-β-gal staining positive rate in ITGB4-silenced group was significantly higher than the control group 10 days after ITGB4 defects (Figure 4A). In premature aging, cell growth is arrested in G1 phase and the cells could not enter the S phase as usual. Meanwhile, compared to the control group, cell cycle was significantly inhibited after ITGB4 silencing. In ITGB4 silenced cells, the G1 phase cell number was significantly increased and S/G2 phase cell number was reduced (Figure 4B). These results suggested that ITGB4 deficiency can arrest cells in G1 phase. Decrease in mitochondrial membrane potential is also an important sign of cell senescence [33]. Our results also demonstrated that JC-1 monomers increased in ITGB4 silenced cells, which presented a drop in mitochondrial membrane potential (Figure 4C). Moreover, a DCHF-DA assay was used to analyze the anti-oxidative activity of HBE cells (Figure 4D). The anti-oxidative activity was significantly reduced when ITGB4 was silenced. Specifically, ITGB4 deficient cells had more ROS fluorescence compared with control cells. Furthermore, we detected the senescence-associated secretory phenotype (SASP) in ITGB4 deficient cells.

Consistent with previous results, the results showed that SASP related genes IL-6 and MMP2 increased obviously in ITGB4 deficiency cells than in control cells (Figure 4E).

P53/p21 signaling pathway was activated after ITGB4 silencing

P53/p21 is a classic signaling pathway of aging [24, 34]. Also, we have previously found increased expression of p53 and p21 in airway epithelia of ITGB4^{-/-} mice (Figure 2C, D).

Therefore, we further detect the activation of p53/p21 signaling pathway by qPCR and western blotting in HBE cells. We found that p-p53 (the activated form of p53), p53 and p21 were significantly upregulated in ITGB4-silenced HBE cells (Figure 5A, B, D). The upregulation of p-p53 was the most pronounced. However, there was a slightly increase in p16 expression with no statistical difference (Figure 5C, D).

ITGB4 deficiency induced senescence of airway epithelial cells through activation of p53

ITGB4 deficiency induced senescence-like changes and activated aging related p53/p21 signals in airway epithelial cells. These results strongly suggested that ITGB4 deficiency may induce the senescence of the airway epithelial cells through activation of p53/p21 signaling pathway. To further verify the involvement of p53/p21 signaling pathway in ITGB4 deficiency induced senescence-like changes, we silenced p53 by siRNA (Figure 6A, B). After p53 were silenced, the increase of SA-β-Gal positive staining ratio and the loss of mitochondrial membrane potential in ITGB4 deficiency HBE cells were reversed obviously (Figure 6C, D).

DISCUSSION

Aging is a complex biological process that involves various cellular, molecular and structural changes based on several mechanisms [1]. Accumulating studies show that aging of functional cells was detected in the lungs of asthma patients [11, 35]. It has also been demonstrated that the aging process could affect the pathogenesis of asthma and is related to the severity of asthma [11]. The incidence of asthma is getting higher with the increase of age [36]. In addition, compared with younger asthma adults, older asthma patients had poorer clinical response to treatment, higher rates of near-fatal asthma-related events, and higher mortality [37, 38]. As the first barrier to external stress including oxidative stress or inflammatory stimulation, airway epithelial cells play a key role in the pathogenesis of asthma [39, 40]. Notably, increased senescent airway epithelial cells are also found at the airway mucus of asthma patients [10]. However, the specific mechanism for the senescence of airway epithelial cells in asthma is still unclear.

We have previously reported that ITGB4 is downregulated in the airway epithelial cells of asthma patients [16]. It also has demonstrated that oxidative stress or inflammatory stimulation reduced the expression of ITGB4 on airway epithelial cells [19, 41]. In this study, our results further found that ozone stress and TNF- α stimulation decreased the expression of ITGB4 on the airway epithelial cells. ITGB4 is a structural adhesion molecule which anchors airway epithelial cells on the basement membrane by forming "hemidesmosome" structure [17]. The long cytoplasmic domain of ITGB4 subunit can recruit a series of signaling molecules that influence the functional conditions of airway epithelial cells [21, 41, 42]. By RNAi, ITGB4 deficiency inhibits cell division cycle and proliferation in vitro [18, 19].

ITGB4 has also been shown to be associated with aging in many different cells [22, 23].

Here, our results also verified that ITGB4 deficiency induced senescence-like changes of airway epithelial cells in vivo and in vitro. Cell senescence in the lung is closely related to the pathogenesis asthma. The senescence of airway epithelial cells is associated with the development of asthma such as airway remodeling [10, 11, 13]. Thus, our results strongly suggest that senescence of airway epithelial cells after ITGB4 deficiency may have a close relationship to the pathogenesis of asthma.

P53-p21^{WAF1/CIP1} and Rb-p16^{INK4a13} are the two major senescence related pathways [24]. P53 can regulate several cell-cycle checkpoints that could cause senescence in response to genomic damage, oncogene expression and telomere attrition et al. [25, 26]. While, p16 is a cyclin-dependent kinase inhibitor that could cause pRb hypophosphorylation which is crucial for long-term maintaining of senescence [27, 43]. As mentioned above, ITGB4 is a complex structural adhesion molecule that could interacts complexly with mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), epidermal growth factor receptor (EGFR) and many other signaling molecules [21, 44, 45]. In this paper, our study further showed that ITGB4 deficiency in airway epithelial cells could activate p53 signaling pathway. P53 is located at a key position in many stress signal pathways which is also regulated and modified by numerous factors [6, 34]. Abnormal DNA damage, oncogene expression and telomere attrition lead to cell senescence through the p53 signaling pathway [6, 46]. P53 is one of the key genes engaged in aging which could activate p21. Then, p21 binds to cyclin CDK2, Cyclin D and Cyclin E, and regulates the expression of Rb and E2F protein eventually plays a role in inhibiting the cell cycle [25, 26]. It has been verified that

the integrin could regulate the expression of p53 signaling pathway [21, 28, 30]. There is also accumulating evidence for pathways linking ITGB4 to activation of p53 [29, 47]. This is further supported by our data that ITGB4 deficiency induces the activation of p53 signaling pathway in airway epithelial cells. While, these effects of integrin on p53 signaling pathway are sometimes opposite to each other due to the different conditions and cell types [29, 30, 48]. As we all know, a great number of known pathways that could activate p53 signaling pathway. However, there is still no convincing evidence to verify that these distinct effects are related. Then, we think the regulation mechanism of p53 pathway by ITGB4 still need more study to verify (possibly PC-PLC or FAK1). In order to further confirm the participation of p53 in senescence induced by ITGB4 deficiency, we blocked p53 expression on ITGB4 silenced airway epithelial cells. Consistent with our speculation, p53 inhibition could reverse the senescence-like changes induced by ITGB4 deficiency in airway epithelial cells.

Overall, this study demonstrated that expression of ITGB4 in airway epithelial cells decreased significantly after oxidative stress and chronic inflammatory stimulation.

Moreover, we also verified that ITGB4 deficiency induced the senescence of airway epithelial cells through the activating of p53 pathway. These results explain that oxidative stress or inflammatory stimuli may contribute to the senescence of airway epithelial cells.

While, after ITGB4 deficiency, the influence of senescent airway epithelial cells on the lung inflammation and airway remodeling in asthma patients still need further studies.

MATERIALS AND METHODS

Mouse models: LPS stimulation and O₃ stress

All animal studies were approved by the No.201803079 Central South University at XiangYa Animal Care and Use Committee. All the methods were carried out in accordance with the relevant guidelines and regulations. Male C57BL/6 mice, 8 weeks of age and free of murine specific pathogens, were obtained and housed under barrier conditions in air-filtered, temperature-controlled units under a 12-hour light-dark cycle and with free access to food and water. LPS stressed mice were instilled with LPS (50 ng/mouse; Sigma-Aldrich) intratracheally to construct the LPS stressed mice, whereas the control mice received an equal volume instillation of sterile saline solution [49]. Then, they were euthanized 16 hours after LPS administration. Ozone challenged mice were stressed with ozone (2.0 ppm, 30min) for four days and euthanized 12 hours after the last exposure, whereas the control mice were treated with fresh air [19]. Furthermore, the conditional ITGB4 knockout mice were constructed according to our previous procedures [20].

Senescence-Associated- β -galactosidase Staining

Frozen sections of lungs or cells were washed twice with PBS and fixed for 6-7 minutes in fixation buffer at room temperature. Then, cells was washed three times again and incubated with fresh senescence-associated- β -gal stain solution at 37°C without CO₂ overnight (CS0030, Sigma, USA). After washed with PBS, the cells staining positive for SA- β -gal were counted as a percentage of the total cell number. Positive cells had blue sedimentation in their cytoplasm.

Immunohistochemistry of lung tissue

Lungs were inflated, fixed in 4% paraformaldehyde, and embedded in paraffin blocks and cut into 5 μ m sections. Immunohistochemistry staining was performed on mouse lung paraffin sections with the use of the following antibodies: ITGB4 (Ab182120, Abcam), Ki67 (ARG53222, Arigo), p53 (Ab31333, Abcam), p21 (Ab109199, Abcam), p16 (Ab51243, Abcam). For microscopy, we employed Zeiss Axio Scope.A1 or Zeiss Discovery.V8 Stereo microscopes (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) integrated with an Axio-Cam ICc3 camera (Spectra Service, Ontario, NY). Images were obtained by AxioVision Rel. 4.7 software from Zeiss. Negative controls included the omission of the primary antibody. To avoid the impact of altered airways after LPS or Ozone treatment, we detected ITGB4 expression based on the same epithelial cell number from different groups.

Cell cultures

Normal Primary Human bronchial Epithelial (HBE) cells from healthy non-smoking donors purchased from Lifeline Cell Technology (Frederick, MD, USA) were incubated at 37 °C in a humidified 5% CO₂/95% air atmosphere and the medium was changed every other day.

Oxidative stress and inflammatory stimulation on HBE cells

HBE cells were seeded into 6-well tissue culture plates at an appropriate density and were cultured in conditioned medium until sub-confluence. The cells were then washed twice with PBS and the medium was replaced with DMEM containing 0.5 % FBS. After culture for 24

h, this medium was replaced with DMEM containing 10 % FBS. Simultaneously, the culture system was treated with TNF- α (20ng/ml) or O₃ (1.5 ppm) for 0.5 h, 1 h, 2 h and 3 h respectively [50, 51]. The culture system of the control group was not treated with TNF- α or O₃. Then, the previous medium was replaced with DMEM containing 10% FBS again and the cells were cultured for another 24 h.

ITGB4 and p53 siRNA synthesis and transfection

The effective ITGB4 siRNA (5'-CAGAAGAUGUGGAUGAGUU-3') [52], p53 siRNA (5'-GACTCCAGTGGTAATCTACT-3') [53], and the nonsense siRNA (5'-UUCUCCGAACGUGUCACGU-3') were designed and synthesized by Guangzhou RiboBio (RiboBio Inc, China). HBE cells were transfected by negative control siRNA and effective silencing siRNA respectively. And transfections were conducted with the LipofectAMINE 3000 (Invitrogen, USA) under instruction.

Western blotting analysis

Western blotting analysis was performed according to previous procedures [54]. Briefly, 50 μ g protein from bronchial epithelial cells was separated by 8% or 10% SDS-PAGE and then transferred to a polyvinylidene difluoride (PVDF) membrane. Next, the PVDF membrane was incubated with the antibody overnight and then incubated with HRP conjugated secondary antibody. The following antibodies were used in western blotting analysis: ITGB4 (Ab182120, Abcam), p53 (Ab31333, Abcam), p-p53 (Ab33889, Abcam), p21 (Ab109199, Abcam), p16 (Ab51243, Abcam).

Quantitative RT-PCR

Total RNA was prepared from different groups collected in Trizol (Invitrogen, USA) according to the manufacturer's protocol [19]. 1 µg mRNA purified from transfected cells was reversely transcribed into cDNA by using AMV reverse transcriptase (Qiagen, Netherlands) with oligo d(T) primer and RNase inhibitor at 40° C for 50 min followed by heating at 90°C for 5 min. Next, 1 µl reverse transcriptase (RT) was added into the 30 µl PCR mixture for 40 cycles. Each cycle contained 93°C for 30 s and 58°C for 60 s by using Taq polymerase. Negative controls consisted of an equal volume of water to substitute for the RNA volume in the RT reaction. By comparing the copy numbers of the target gene and β-actin, we managed to normalize the mRNA expression data for sample-to-sample variability in RNA input, RNA quality and the reverse transcription efficiency.

Cell cycle analysis by propidium iodide

Cell cycle analysis was carried out 48 hours after siRNA transfection. 5×10^5 HBE cells from ITGB4-silenced group and control group were harvested by brief trypsinization, washed twice with PBS, fixed in 70% ethanol for 2h at 4°C and washed with PBS again. Next, transfected cells were stained with 20 mg/ml propidium iodide (PI) containing 10 mg/ml RNase (Beyotime Biotechnology, China). After protection from light and incubation at 37°C for 30 min, these samples were analyzed on a FACS caliber flow cytometer. Then, the populations of G1, S and G2 cells were quantified with ModFit LT software.

Mitochondrial membrane potentials assay

JC-1 probe was used to measure mitochondrial depolarization in HBE cells. The mitochondrial membrane potential was detected with a Mitochondrial Membrane Potential Assay Kit (Beyotime biotechnologies, China). In short, 5×10^5 cells were collected, incubated with JC-1 at 37°C for about 20 minutes in the dark and then rinsed twice with ice-cold working solution. Next, 20,000 cells were analyzed by flow cytometry (FACS Calibur) in each measurement. Mitochondrial depolarization is indicated by JC-1 transition from red fluorescence to green fluorescence.

Anti-oxidation assay

Anti-Oxidation ability was evaluated by the dichlorofluorescein diacetate (DCFH-DA) method. 48 hours after transfection, HBE cells were harvested, diluted in DCFH-DA to 10^6 - 10^7 cells/ml and incubated for 20 min at 37°C, rocked every 5 min (Beyotime biotechnologies, China). Then, the samples were washed three times with serum-free medium. Concurrently, it was of great importance to generate a positive control by stressing cells with reactive oxygen for 30 min. The cells were detected by a Microplate Reader (Thermo, Varioskan Flash) to assess the reactive oxygen by measuring DCF fluorescence.

Statistical analysis

All experiments were run at least three times and analyses were performed using Prism (GraphPad, La Jolla, CA) statistical software. All data are presented as the mean values $\pm$ standard deviation (SD). An unpaired t-test was used for comparisons between the two groups. For all values, significance is denoted by $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

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Author contributions

Chi Liu and Lin Yuan conceived and designed this study. Lin Yuan, Xizi Du and Sha Tang carried out the experiments. Lin Yuan, Shuangyan Wu and Leyuan Wang contributed to the interpretation of the results. Lin Yuan, Chi Liu, Yang Xiang, Xiangping Qu, Huijun Liu and Xiaoqun Qin wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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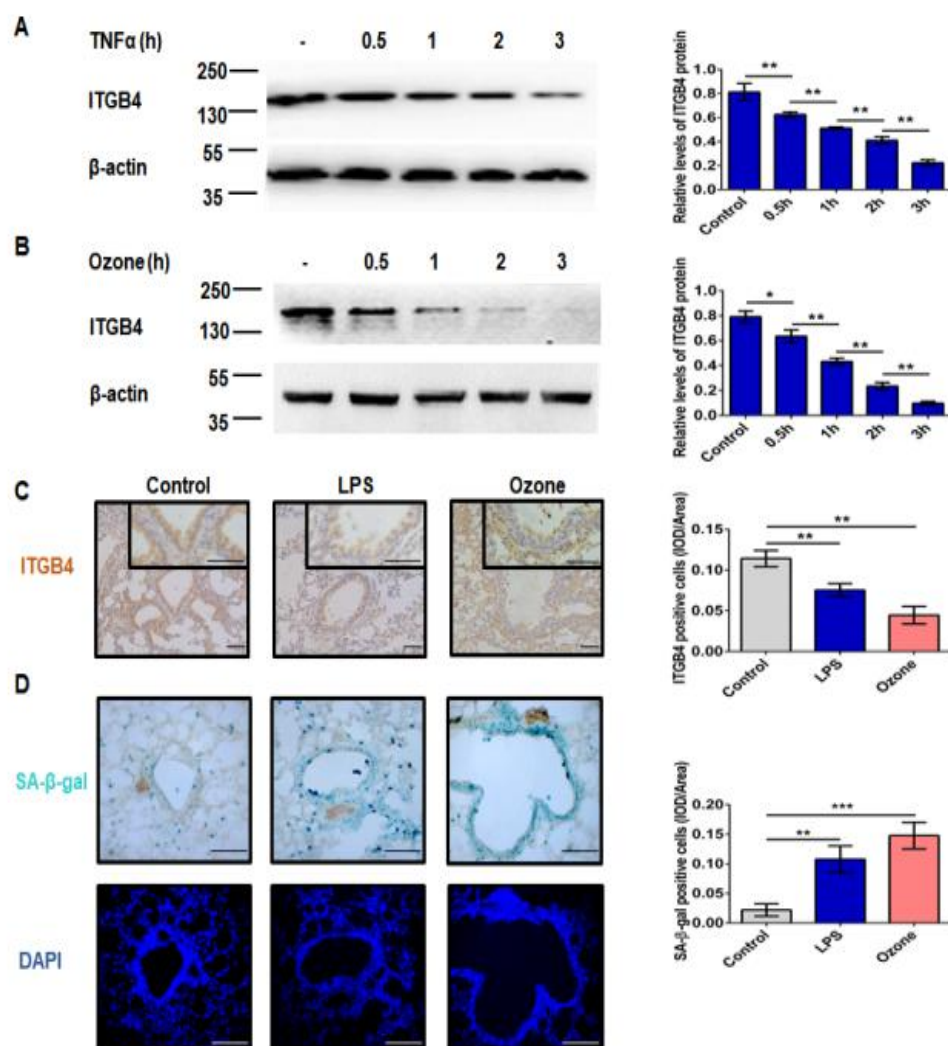


Figure 1. ITGB4 expression was decreased under oxidative stress or inflammatory stimulation. (A, B) ITGB4 expression in HBE cells was measured by western blotting after stressed with $\text{TNF}\alpha$ or O_3 , respectively. Quantification of western blotting was normalized to the level of β -actin ($n = 6$). (C, D) ITGB4 expression and senescent cells on airway epithelial cells in LPS stressed mice model and O_3 stressed mice model were detected by immunohistochemistry staining and SA- β -gal staining, respectively. DAPI was used to stain cell nuclei (blue). ($n = 5$; Magnification: C, 100 $\times$; Insets, 200 $\times$; Magnification: D, 200 $\times$; Scale bar: 50 μm). Error bars show means $\pm$ SD for treatments tested in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

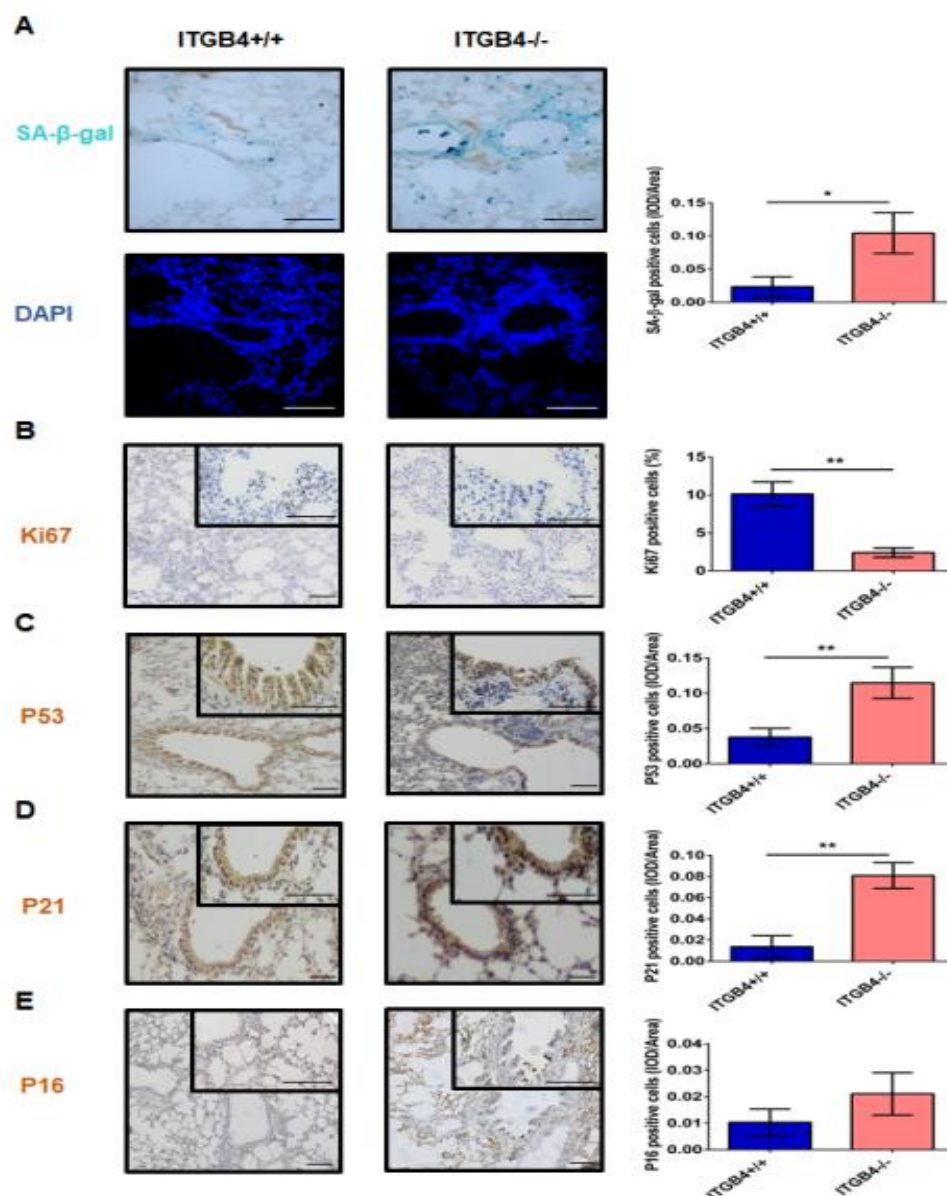


Figure 2. ITGB4 deficiency caused the senescence of airway epithelial cells in vivo. (A, B, C, D, E) SA-β-gal staining and immunohistochemistry of Ki67, p53 expression, p21 expression, p16 expression were performed in ITGB4^{-/-} mice. DAPI was used to stain cell nuclei (blue). (n = 6; Magnification: A, 200×; Magnification: B, C, D and E, 100×; Insets, 200×; Scale bar: 50 μm). Data are shown as means ± SD from four independent experiments. * $p < 0.05$, ** $p < 0.01$.

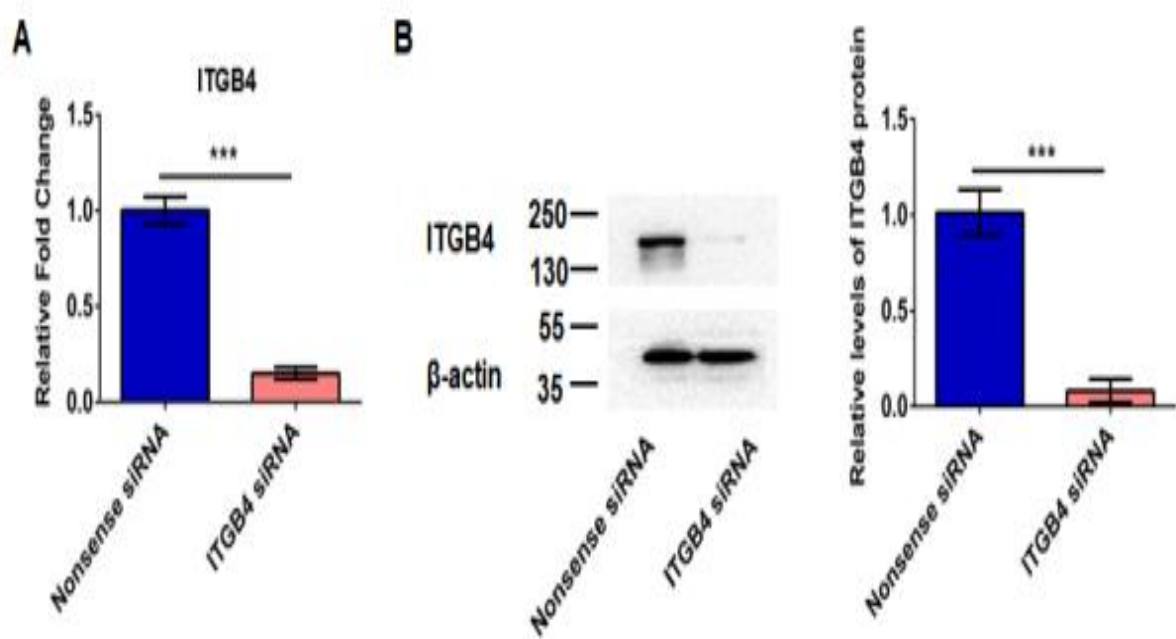


Figure 3. Silencing efficiency of ITGB4 siRNA. (A) The expression of ITGB4 mRNA was detected by qPCR. Results of ITGB4 silencing group and the control group were quantified relative to mRNA of β -actin ($n = 8$). (B) ITGB4 protein expression was observed by western blotting. Quantification of western blotting normalized to the level of β -actin ($n = 5$). The experiments are performed thrice and the error bars represent means $\pm$ SD. *** $p < 0.001$.

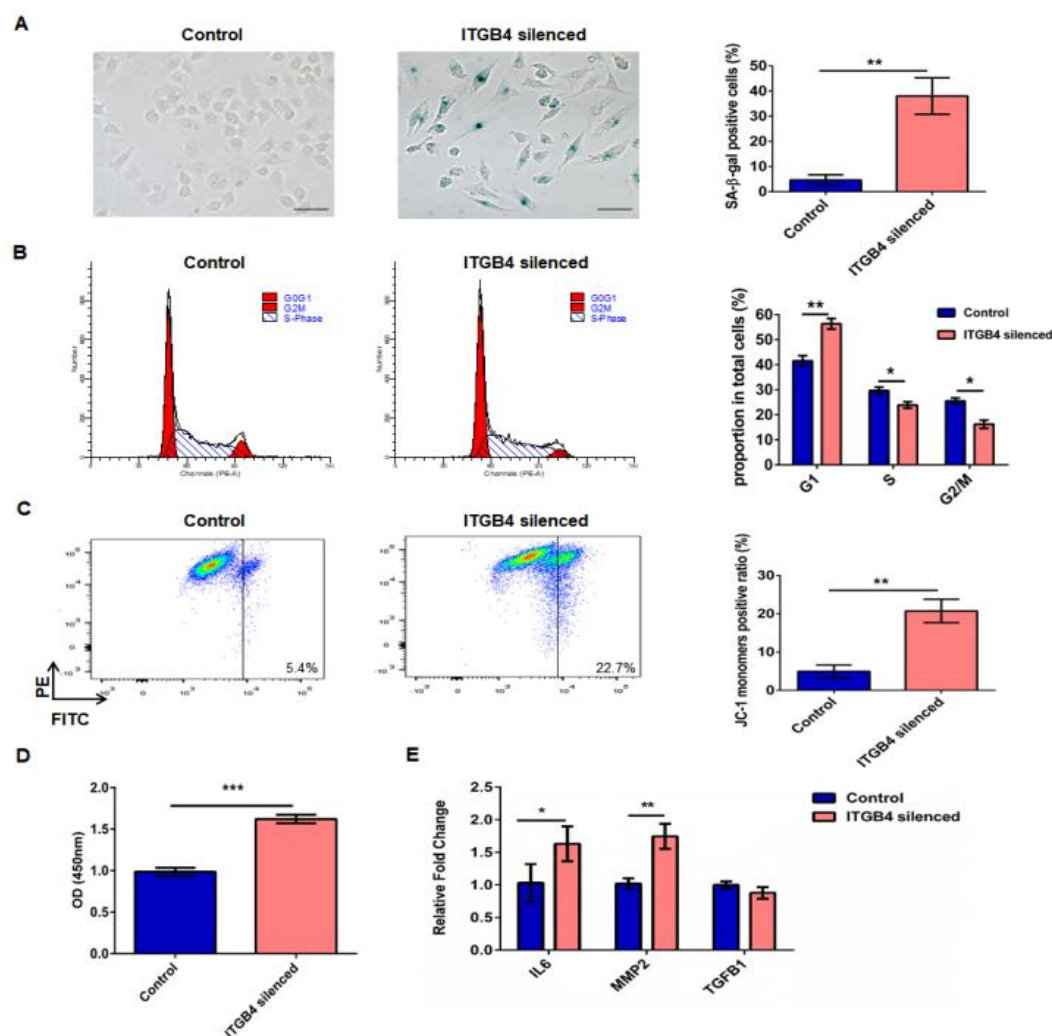


Figure 4. ITGB4 deficiency caused the senescence of HBE cells in vitro. (A) SA-β-gal staining. Cells staining positive for SA-β-gal showed blue color sedimentation (n = 6; Magnification: 200×; Scale bar: 50 μm). (B) Cell cycle analysis by propidium iodide (n = 6). (C) Mitochondrial membrane potentials assay (n = 6). (D) Effects of ITGB4 silencing on ROS generation. HBE cells were stained with DCFH-DA and later analyzed by Varioskan Flash 48 h after transfection. More ROS were induced in ITGB4 silencing group (n = 6). (E) qPCR was performed to measure the mRNA expression of SASP related genes (n = 5). Error bars show means ± SD from five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

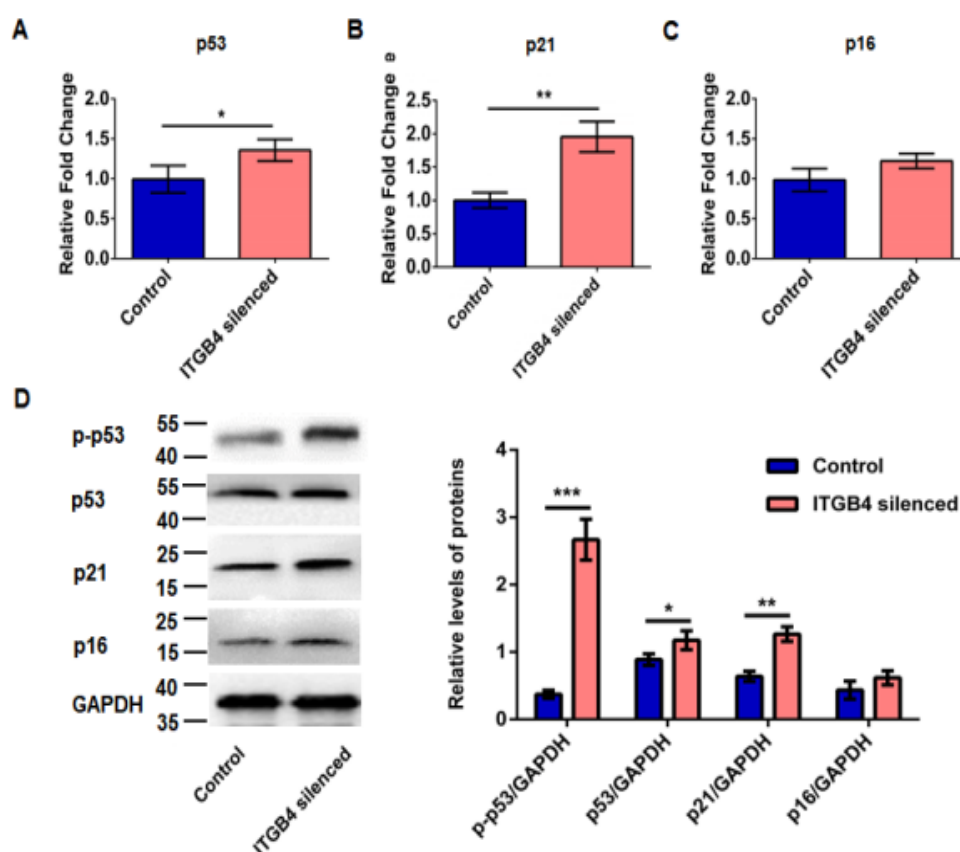


Figure 5. P53/p21 signaling pathway was activated when ITGB4 was silenced in HBE cells.

(A, B, C) qPCR was used to measure the mRNA expression of p53 and p21 and p16 (n = 5).

(D) The protein expression level of p-p53, p53, p21 and p16 was detected by western

blotting. The quantification of western blotting normalized to the level of GAPDH (n = 5).

The experiments are performed three times and the error bars represent means $\pm$ SD. * $p <$

0.05, ** $p <$ 0.01, *** $p <$ 0.001.

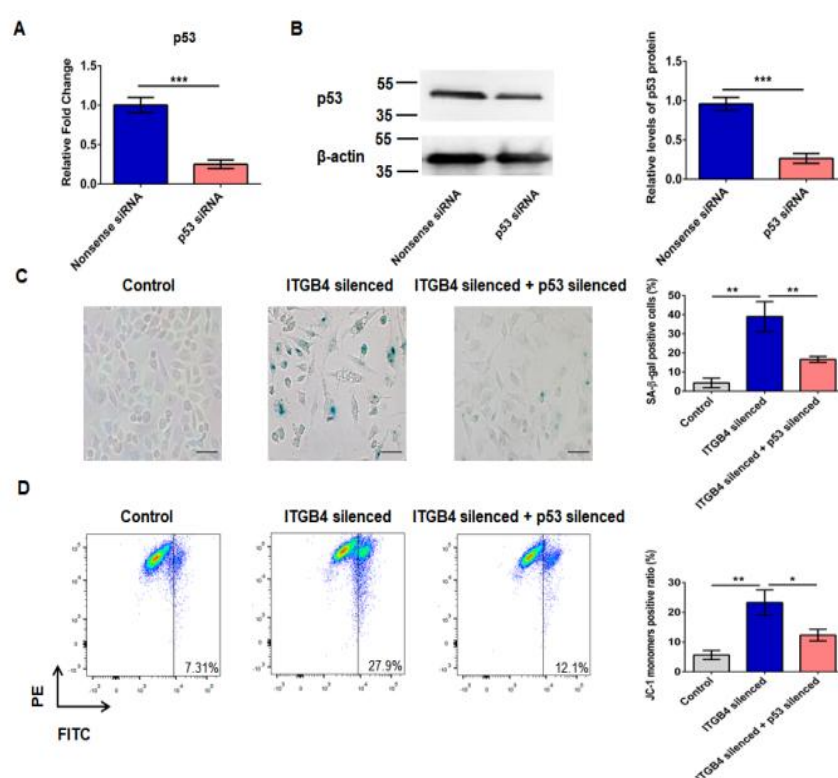


Figure 6. Blockade of p53 reversed the senescence phenotype induced by ITGB4 deficiency. (A, B) Silencing efficiency of p53 siRNA. P53 expression was detected by qPCR and western blotting, respectively. Quantification of qPCR and western blotting normalized to the level of β -actin ($n = 5$). (C) SA- β -gal staining ($n = 6$; Magnification: 200 $\times$; Scale bar: 50 μ m). (D) Mitochondrial membrane potentials assay. JC-1 transition from green fluorescence to red fluorescence ($n = 6$). Error bars show means $\pm$ SD from five independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.