

Wound repair and anti-oxidative capacity is regulated by ITGB4 in airway epithelial cells

Chi Liu · Hui-jun Liu · Yang Xiang · Yu-rong Tan ·
Xiao-lin Zhu · Xiao-qun Qin

Received: 22 January 2010 / Accepted: 25 March 2010
© Springer Science+Business Media, LLC. 2010

Abstract Integrin beta 4 (ITGB4) is a structural adhesion molecule which engages in maintaining the integrity of airway epithelial cells. Its specific cytomembrane structural feature strongly indicates that ITGB4 may engage in many signaling pathways and physiologic processes. However, in addition to adhesion, the specific biologic significance of ITGB4 in airway epithelial cells is almost unknown. In this article, we investigated the expression and functional properties of ITGB4 in airway epithelial cells in vivo and in vitro. Human bronchial epithelial cell line (16HBE14O-cells) and primary rat tracheal epithelial cells (RTE cells) were used to determine ITGB4 expression under ozone stress or mechanical damage, respectively. An ovalbumin (OVA)-challenged asthma model was used to investigate ITGB4 expression after antigen exposure in vivo. In addition, an ITGB4 over-expression vector and ITGB4 silence virus vector were constructed and transfected into RTE cells. Then, wound repair ability and anti-oxidation capacity was evaluated. Our results demonstrated that, on the edge of mechanically wounded cell areas, ITGB4 expression was increased after mechanical injury. After ozone stress, upregulation expression of ITGB4 was also detected. In the OVA-challenged asthma model, ITGB4 expression was decreased on airway epithelial cells accompanying with structural disruption and damage of anti-oxidation capacity. Besides, our study revealed that upregulation of ITGB4 promotes wound repair ability and anti-oxidative ability, while such abilities were blocked when ITGB4 was silenced. Taken together, these results showed that ITGB4 was a new interesting molecule

involved in the regulation of wound repair and anti-oxidation processes for airway epithelial cells.

Keywords Integrin $\beta 4$ · Airway epithelial cells · Wound repair · Anti-oxidation

Introduction

Airway epithelial cell is the first direct barrier which forms the initial defense to inhaled air and other substances. Under normal circumstances, injurious chemicals, particulates, and biological insults can be removed effectively by structural integrity and functional homeostasis of airway epithelial cells. However, destroy of airway microenvironment homeostasis by structural disruption or functional breakdown could induce or intensify many lung diseases [1, 2]. Asthma, COPD, and bronchitis have been classified as epithelial diseases for the typical pathology phenotypes on airway epithelial cells [3–6]. It is likely that lose of structural integrity plays a very important role during the initiation and development of many lung diseases [3, 4].

A highly regulated and almost impermeable barrier of airway epithelium is maintained partly through cell–cell and cell–extracellular matrix interactions. Adhesion molecules, especially for integrin beta 4 (ITGB4), play a key role in this structural adhesion process [7]. ITGB4 is a laminin receptor that mediates the stable adhesion of epithelial cells to the basal membrane through hemidesmosomes architecture [8]. The distinguishing structural feature of ITGB4, i.e., the typical cytoplasmic domain, is different from any other integrin subunits in both size ($\sim 1,000$ amino acids) and structure. This feature of cytoplasmic domain indicated that the possible complex signaling pathway and multiple functions of ITGB4 [9]. Progress has been made in identifying

C. Liu · H. Liu · Y. Xiang · Y. Tan · X. Zhu · X. Qin (✉)
Physiology Department, Xiangya Medical School, Central South
University, Changsha, Hunan 410078, People's Republic of
China
e-mail: liuchi7669@gmail.com

specific regions and motives within the ITGB4 cytoplasmic domain that mediate the cytoskeleton interactions and signaling properties of ITGB4, but much remains to be determined [10]. In order to airway epithelial cells, the precise physiologic function of ITGB4 was almost unknown.

In this study, we detected that expression of ITGB4 increased after mechanical injury on rat tracheal epithelial cells (RTE cells) and human bronchial epithelial cell line (16HBE14O-cells) separately. By ozone stress, ITGB4 expression was also enhanced for both 16HBE14O-cells and RTE cells. Further, an OVA-challenged asthma model was constructed to investigate the expression of ITGB4 after antigen exposure in vivo. The results showed that, in the OVA-challenged asthma model, ITGB4 was down regulated on airway epithelia accompanying with obvious structural disruption. At the same time, the anti-oxidation capacity of RTE cells in the OVA-challenged group was impaired. In order to interpret the concrete physiologic functional properties of ITGB4 on airway epithelial cells, ITGB4 overexpression vector, and silence vector were constructed and transfected into RTE cells. We found that wound repair ability and anti-oxidative ability were enhanced after ITGB4 was overexpressed, while such abilities were blocked when ITGB4 was silenced. Furthermore, our results also showed that proliferation ability was promoted by ITGB4 overexpression, and inhibited when ITGB4 was silenced. The expression of three important antioxidases was increased after ITGB4 was overexpressed, while the expression was decreased by ITGB4 silence. These results collectively showed that, besides acting as an adhesion protein, ITGB4 was an important functional molecule for airway epithelial cells in regulating wound repair and anti-oxidation processes.

Materials and methods

Cell cultures

An immortalized human bronchial epithelial cell line (16HBE14O-) presented by Professor Gruenert of the University of California at San Francisco was maintained in a mixture medium of DMEM:F12 (1:1) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, and 10% heat inactivated fetal bovine serum (FBS) and incubated at 37°C in 5% CO₂.

Cultured RTE cells were prepared according to the methods previously described [11, 12], with some modifications. All procedures were reviewed and approved by the Institution Animal Care and Use Committee. Briefly, male Sprague-Dawley (SD) rats (body weight, 300–350 g) were killed by CO₂ asphyxiation. Then, the tracheas were removed under sterile conditions and incubated overnight at 4°C in

DMEM F-12 medium (Gibco BRL, Grand Island, New York, USA) with 0.05% type XIV protease (Sigma, St. Louis, MO, USA). Fetal bovine serum (Gibco) was then added to the incubation medium (DMEM/F-12) to a final concentration 10%. RTE cells were then flushed out and collected by centrifugation (150 g at 4°C for 1 min), and washed twice with DMEM/F-12. The RTE cells were plated onto the apical (gelled) surface and incubated at 37°C in 5% CO₂.

Ozone stress in vitro and flow cytometry

In this ozone stress array, 16HBE14O-cells or RTE cells were stressed with ozone (1.5 ppm) for 30 min [13]. Then, cells were harvested and washed twice in PBS 12 h after ozone stress. Cell surface ITGB4 was localized by binding of anti-rat antibodies (Ab29044, 1:250 dilution; Abcam, Cambridge, UK) or anti-human antibody (Ab63438, 1:250 dilution; Abcam). Then, a phycoerythrin (PE) fluorescence second antibody was used to label ITGB4 expression by flow cytometry. For each sample, 0.5–1 × 10⁶ cells were stained; comparable numbers of cells were used for each independent test.

SDS-PAGE and Western blotting

For the preparation of cell extracts, cells from different groups were washed thrice with ice-cold PBS, and lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 100 µg/ml PMSF) on ice for 20 min. After centrifugation at 16,000×g for 2 min at 4°C, the supernatants were analyzed by 10% SDS-PAGE and then electrophoretically transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA, USA). After blocking for 2 h with 5% fat-free milk at room temperature, the membranes were incubated with either anti-human ITGB4 monoclonal antibody (Ab29044, 1:1000 dilution; Abcam) or anti-rat ITGB4 monoclonal antibody (M126, 1:1,000 dilution; Abcam), and then reacted with HRP-conjugated secondary antibody. Protein bands were visualized with an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA).

Mechanical injury and immunocytochemistry assay

A mechanical scrape injury was induced by creating a wound with a pipette tip across the surface of confluent 16HBE14O-cells and RTE cells layer on the cell plates separately. Following the injury, the apical surface was washed with DMEM to remove denuded epithelial cells, and areas of the injury were examined to assure uniformity. After 12 h injury, expression of ITGB4 on the epithelial cells was assessed by immunocytochemistry. Briefly, the cell plates were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and blocked with 5% goat serum prior to

incubation with ITGB4 anti-rat antibodies (M126, 1:250 dilution; Abcam) or anti-human antibody (Ab29044, 1:250 dilution; Abcam). Species appropriate secondary antibodies conjugates were used to visualize ITGB4. Images were obtained using a microscope (Nikon Ti 108, Japan). In this study, ITGB4 expression was tested and the staining intensity was evaluated using the following semiquantitative scale of 0–4 (0—being no staining and 4—being maximum staining) depending on staining intensity.

OVA-challenged asthma model to rats

Briefly, 30 SD rats were randomly divided into five experiment groups and one control group. The asthma models were induced by intraperitoneal injection with 1 mg of OVA (grade V; Sigma) and 200 µg of aluminium hydroxide (EM Industries, Hawthorne, NY, USA) on day 1, and then boosted with a second injection 7–10 days later. On day 21, the animals were challenged via the intratracheal route with 50 mg of antigen in 30 ml aliquots. The challenge was repeated on days 22 and 23. Control rats were sensitized or challenged with saline, respectively.

Immunohistochemistry of lung tissue

Lungs were removed and fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5 µm sections. Rat monoclonal antibodies to ITGB4 were used for the detection of ITGB4 (H-101; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Briefly, endogenous peroxidase is inhibited by soaking tissue sections in 3% H₂O₂. After rinsing in PBS, sections were incubated with goat serum to block non-specific binding of antibodies, and then incubated overnight at 4–8°C with primary ITGB4 anti-rat antibodies H101 (1:150). After washing in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG for 1 h and washed again. Streptavidin biotin peroxidase complex (SABC) was incubated with the sections for 60 min at room temperature. After washing in PBS, the signal was detected with 3,3'-diaminobenzidine. Omission of primary antibody was included as a method of providing a control for each biopsy. Images were taken and staining intensity of ITGB4 was computed with a method as described above.

Real-time PCR measurement for ITGB4 mRNA in rat lung tissues

RNA was extracted from rat lung tissues and reverse transcription was performed by AMV reverse transcriptase (Qiagen, Hilden, Germany). PCR was then carried out using primers and taqman probes (TaKaRa, Dalian, China). Taqman probes were labeled at the 5' end with the reporter dye, 5-carboxyfluorescein (FAM), and at the 3' end with the

quencher, 6-carboxyltetramethyl rhodamine (TAMRA). The primers and Taqman probes were synthesized as follows: ITGB4, forward: 5'-CCTCATGGATGGTCTAACCC-3', reverse: 5'-TGAGTGCTGAGAGAGCCACT-3'; FAM + CTCCCTCTCACCCGGCATGTGA + TAMRA, GAPDH forward: 5'-GTGTCCGTCGTGGATCTGA-3', reverse: 5'-CCTGCTTCACCACCTTCTTGA-3', FAM + CCGCCTG GAGAAACCTGCCAAGTATG + TAMRA. Briefly, 1 µl of the reverse-transcripts was added to a 30 µl PCR mixture for 40 cycles. Each cycle included 93°C for 30 s, and 60°C for 60 s by using Taq polymerase. Negative controls consisted of an equal volume of water substituted for the volume of RNA in the RT reaction. Normalization of mRNA expression data for sample-to-sample variability in RNA input, RNA quality, and reverse transcription efficiency was achieved by comparing the copy numbers of target mRNA with that of rat GAPDH for each run.

Measurement of anti-oxidation capacity of primary RTE cells from OVA-challenged model

Primary RTE cells were obtained following the instructions as described previously. Then, the RTE cells from different groups were treated according to the instructions of the anti-oxidation assay, as described above [14]. Briefly, RTE cells were harvested and diluted in DCFH-DA (approximately 10⁶–10⁷ cells/ml), and incubated for 20 min at 37°C. The incubated cells were mixed well with a tip every 5 min and washed thrice with serum-free medium. Then, the cells were stressed with reactive oxygen species (ROS) for 30 min. The anti-oxidation capacity were analyzed by flow cytometry (BD FACS Calibur, Becton–Dickinson, San Jose, CA) to survey the total reactive oxygen in the cell by detecting the DCF fluorescent.

Isolation of ITGB4 cDNA by RT-PCR

Total cellular RNA was extracted using Trizol reagent (Invitrogen) and first-strand cDNA prepared with AMV reverse transcriptase (Promega, Madison, WI, USA) using an oligo(dT)12–18 primer. RT-PCR reactions with the ITGB4-specific primers: forward, 5'-AACCGCTGTAAGA AGGCTCA-3', reverse, 5'-AGAGAGCCACTGGTGGTCA G-3'. PCR reaction was subjected to 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s for 30 cycles. The PCR product covered the entire ORF of ITGB4 and was confirmed by DNA sequencing.

Construction of ITGB4 expression vector and stable transfection

The full length coding region of ITGB4 cDNA was inserted into a pcDNA3.1 expression vector (Genechem, Shanghai,

China) to generate a pcDNA3.1/ITGB4 (+) overexpression vector, with transcription initiated by the CMV promoter. For stable transfection, RTE cells were transfected with pcDNA3.1/ITGB4 or mock control pcDNA3.1 using Lipofectamine reagent (Invitrogen) and screened under 800 µg/ml G418 (Merck, Darmstadt, Germany) for approximately 3 weeks. Cellular clones of stably transfected cells were obtained by limiting dilution and confirmed by Western blot analysis and real time RT-PCR.

Rat ITGB4 small miRNA synthesis

Candidate miRNAs directed against ITGB4 mRNA were designed. Three potential miRNA and one control miRNA were selected using the prediction of single strand domains within the secondary mRNA structure and subsequent negative BLAST analyses (Table 1). miRNA were ligated into a pcDNATM 6.2-GW/EmGFPmiR vector (Invitrogen). And the candidate silence vector was packaged to recombinant virus pLenti6-V5-Dest Lentivector (Invitrogen). Then, three miRNA vectors were transfected into RTE cells using the Silencer miRNA construction kit (Invitrogen). Under our experimental condition, to silence ITGB4, RTE was infected with ITGB4 miRNA pLenti6-V5-Dest Lentivector by addition of lentivirus into the cell culture at an MOI of 10. Real time RT-PCR and Western blotting were used to screen the effective miRNA. The interference efficiency was detected in every silenced experimental group.

Wound repair assay

This assay was used to demonstrate the effect of ITGB4 on epithelial cell wound repair. We have previously published details of this method [15]. Briefly, RTE cells were grown until confluent in 12-well plates with DMEM: F12, and a small wound was made in the confluent monolayer with a rubber styllet. The edge of the wound was recognized and the remaining wound area was measured serially every 4 h in 24 h by video microscopy (Olympus, Japan). A linear regression equation of the remaining wound area to time was

obtained. The repair index (RI), equal to the absolute value of the slope, was used to judge the repair speed of RTE.

Cell proliferation assay

Twenty-four hour following transfection, cells were trypsinized, and 2×10^3 cells/well were seeded into 96-well plates in triplicate. Briefly, 10 µl of MTT (5 µg/µl in PBS) was added to each well, and the cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. The cells were lysed at 24, 48, 72, and 96 h using cell lysis reagent (20% SDS and 50% DMSO [pH 4.7]) and absorbency was measured at 570 nm with an ELISA Reader (BIO-Tek ELx800, Winooski, VT, USA). The cellular proliferation curve was delineated by the mean absorbency at each time point.

Biochemical assays

In order to avoid the intervention of GFP after transfection, the total antioxidant capacity (T-AOC) of RTE cells was determined according to a commercial test kit (Jiancheng, Nanjing, China). Briefly, T-AOC of different groups was measured using the Azinodiethyl-benzthiazoline sulfate (ABTS) method. In this assay, incubation of ABTS with H₂O₂ and a peroxidase (metmyoglobin) resulted in the production of the blue-green radical cation ABTSt, which was measured at 600 nm. Besides, the activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase in tests were also detected using commercial assay kits (Jiancheng) following the manufacturer's instructions.

Statistical analysis

All data are presented as the mean $\pm$ standard error of the mean (SEM). * $P < 0.05$ was considered to be significant. Comparison between two different groups was conducted using Student's *t*-test. The effectiveness of the different miRNA groups was analysed by one-way ANOVA.

Table 1 Candidate miRNA sequences for ITGB4

Candidate	Detailed sequence
miRNA-1F	TGCTGTATTCAAGGCTGCTCAAAGGACGTTTTGGCCACTGACTGACGTCCTTTGCAGCCTGAATA
miRNA-1R	CCTGTATTCAAGGCTGCAAAGGACGTCAGTCAGTGGCCAAAACGTCCTTTGAGCAGCCTGAATAC
miRNA-2F	TGCTGACACCTTGAACACAAAGTTGCGTTTTGGCCACTGACTGACGCAACTTTGTTC AAGGTGT
miRNA-2R	CCTGACACCTTGAACAAAGTTGCGTCAGTCAGTGGCCAAAACGCAACTTTGTGTTC AAGGTGTC
miRNA-3F	TGCTGTCCACAAGCACCTTCTTCATAGTTTTGGCCACTGACTGACTATGAAGAGTGCTTGTGGA
miRNA-3R	CCTGTCCACAAGCACTCTTCATAGTCAGTCAGTGGCCAAAACATGAAGAAGGTGCTTGTGGAC
Control miRNA F	CTATTACCATGGTGATGCGTTTTGGCAGTACATCAATGGGCGTGGATAGCGTTTTGACTCACGGT
Control miRNA R	ATTCCAATCTCCACCCCATTTGACGTCAATGGGAGTTTGTTTTGCACCAAATCAACGGGACGTG

Differences among ITGB4-silenced group, control miRNA group, pcDNA/ITGB4 group, and control pcDNA 3.1 group was performed by means of one-way ANOVA followed by Dunnett's or LSD's post-hoc test

Results

Mechanical injury and ozone stress upregulates the expression of ITGB4

As demonstrated above, ozone is a common outer pollution to the airway. Our previous study showed that ozone stress to airway epithelial cells can induce significant oxidative damage [13]. This suggests that the exposure to outer oxidation stress will cause a detriment to airway epithelial cells. In this study, both 16HBE14O and RTE cells were stressed by ozone exposure for 30 min. After oxidation damage by ozone stress, a significant increase of ITGB4 expression was observed, as demonstrated by Western blotting detection (Fig. 1a, b, d, e). As ITGB4 is a typical transmembrane protein, we also detect ITGB4 surface protein by flow cytometry. The results showed that expression of ITGB4 surface protein was increased significantly after ozone stress both in 16HBE14O-cells (388 ± 29 vs. 172 ± 31 , $P = 0.0007$, $n = 3$, unpaired t test) and RTE cells (404 ± 17 vs. 239 ± 21 , $P = 0.0004$, $n = 3$, unpaired t test; Figs. 1c, f).

Besides, more susceptible to outer oxidation damage, it is increasingly apparent that structural interrupt and shedding of airway epithelium were common in many lung diseases, such as asthma. However, the inner mechanism and regulation factor was not fully interpreted. In order to study the expression of ITGB4 under structural damage, a wound area on the confluence epithelial cells was made by mechanical damage. In parallel to the up-regulation of ITGB4 expression after ozone stress, immunocytochemistry results showed that expression of ITGB4 was also increased on the edges of wounds 12 h after injury both in 16HBE14O-cells (2.23 ± 0.02 vs. 3.19 ± 0.03 , $P = 0.003$, $n = 3$, unpaired t test) and RTE cells (2.36 ± 0.04 vs. 3.27 ± 0.06 , $P = 0.003$, $n = 3$, unpaired t test; Fig. 2).

OVA challenge decreased ITGB4 expression and attenuated anti-oxidation capacity

As OVA-challenged rats model showed that typical asthma phenotype corresponding to gross changes in epithelial phenotype and function, we examined rat lung tissue to investigate the effects of OVA stimuli on ITGB4 expression. We found that ITGB4 protein expression was decreased significantly on airway epithelia of OVA-challenged rats (Fig. 3a) by semiquantitative evaluation (2.74 ± 0.04 vs. 1.49 ± 0.05 , $P = 0.006$, $n = 4$, unpaired t test; Fig. 3b). Besides, mRNA expression of ITGB4 was also significantly decreased in the lung tissue of

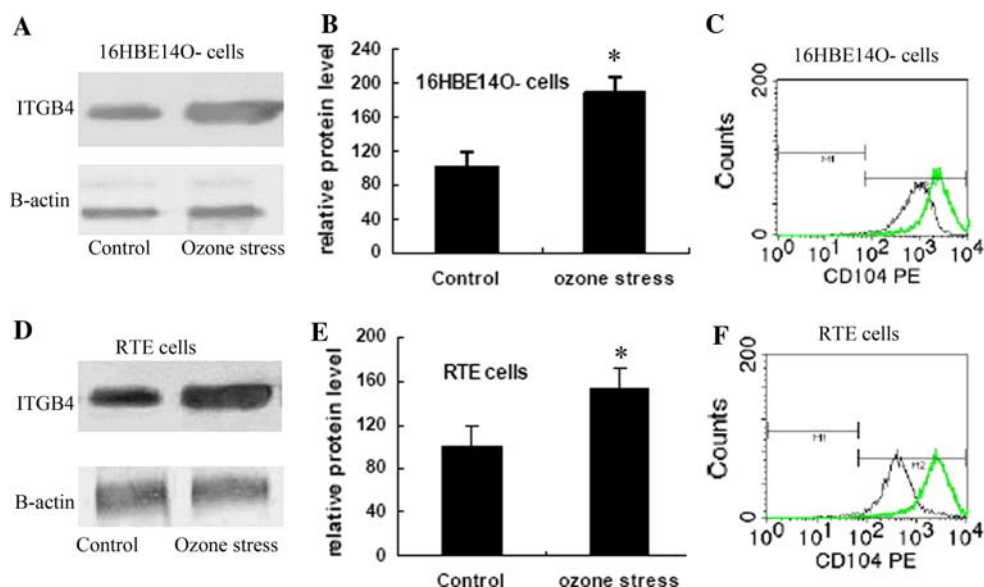
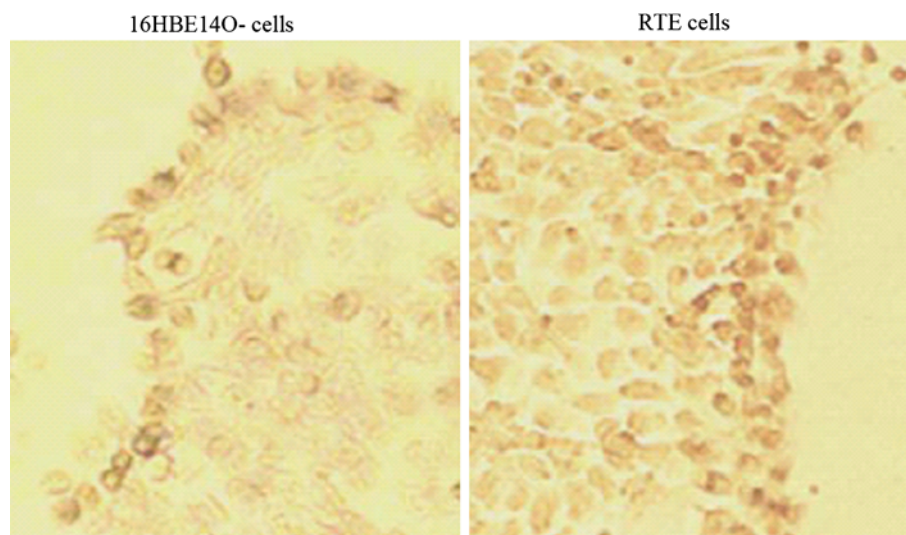


Fig. 1 Expression of ITGB4 after ozone stress was detected by western blotting and flow cytometry in two kinds of airway epithelial cells. **a** ITGB4 protein expression increased after ozone stress by Western blot analysis of 16HBE14O-cells. **b** Quantification of Western blots normalized to the level of β -actin in **a**. **c** Flow cytometry results showed that cell surface expression of ITGB4 after

ozone stress of 16HBE14O increased. **d** For RTE cells, after ozone stress, ITGB4 protein expression was enhanced. **e** Quantification of Western blots normalized to the level of β -actin in **d**. **f** ITGB4 surface protein expression was increased significantly after ozone stress of RTE cells by flow cytometry detection

Fig. 2 Wound was created on the surface of confluent 16HBE14O-cells (*left*) and a RTE cell (*right*) layer. 12 h after injury, ITGB4 expression was detected by immunocytochemistry. Semiquantitative evaluation of ITGB4 staining intensity showed that expression of ITGB4 significantly increases on the wound edges for both 16HBE14O and RTE cells



OVA-challenged asthma model by real time RT-PCR detection (Fig. 3b).

We further determined the structural alteration and anti-oxidation capacity of RTE cells in the OVA-challenged asthma model. By immunohistochemistry, the sections from control group showed that bronchi walls and pulmonary alveoli were intact and regular. However, inflammatory

infiltration, bronchial epithelial denudation, and abnormal wound repair were observed in the OVA-challenged rats. Besides, after OVA challenge, anti-oxidation capacity of RTE cells was also detected. Compared with control rats, RTE cells of the OVA-challenged group contained significantly increased reactive oxygen content (878 ± 29 vs. 431 ± 36 , $P = 0.0006$, $n = 4$, unpaired t test; Fig. 3c).

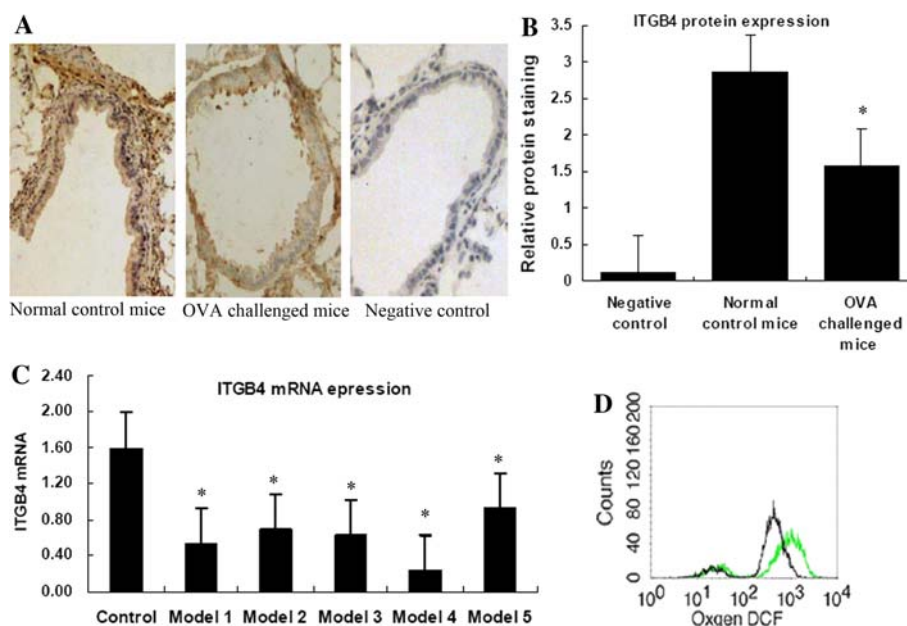


Fig. 3 The expression of ITGB4 on the airway mucus of OVA-challenged rat model was detected by immunohistochemistry and real time RT-PCR. **a** ITGB4 is mainly expressed on bronchial epithelial cells. Compared with the control rats, ITGB4 expression on the airway biopsies from OVA-challenged rats decreased obviously by semiquantitative evaluation. **b** Semiquantitative evaluation of ITGB4 staining on sections of **a**. **c** Real time RT-PCR results also showed

that ITGB4 mRNA expression of OVA-challenged rats decreased compared to control rats. **d** Anti-oxidation ability was assessed in RTE cells of OVA-challenged rats. The dark line and the green line represented the content of DCF in the control rats and the OVA-challenged rats, respectively. The RTE cells of OVA-challenged rats contained more reactive oxygen which suggested that the anti-oxidation ability was inhibited

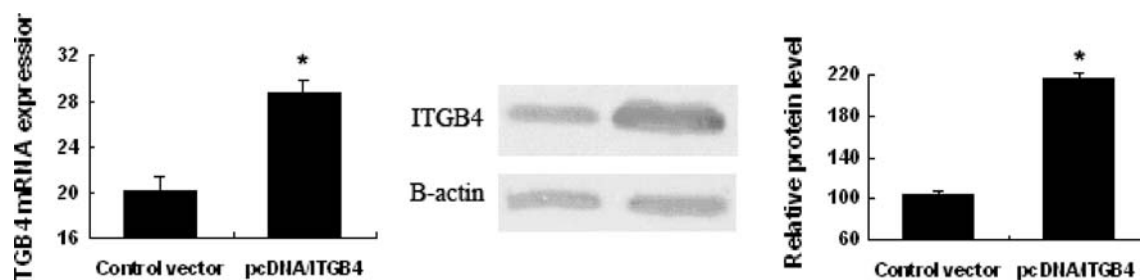


Fig. 4 ITGB4 mRNA expression and protein expression after pcDNA3.1/ITGB4 transfection were detected by real time RT-PCR and Western blotting. **a** ITGB4 mRNA expression can increase at least 50% after transfection with pcDNA3.1/ITGB4 compared with

control vector. **b** Western blotting results also showed significantly increased expression of ITGB4 protein after transfection with pcDNA3.1/ITGB4. **c** Quantification of Western blots normalized to the level of β -actin

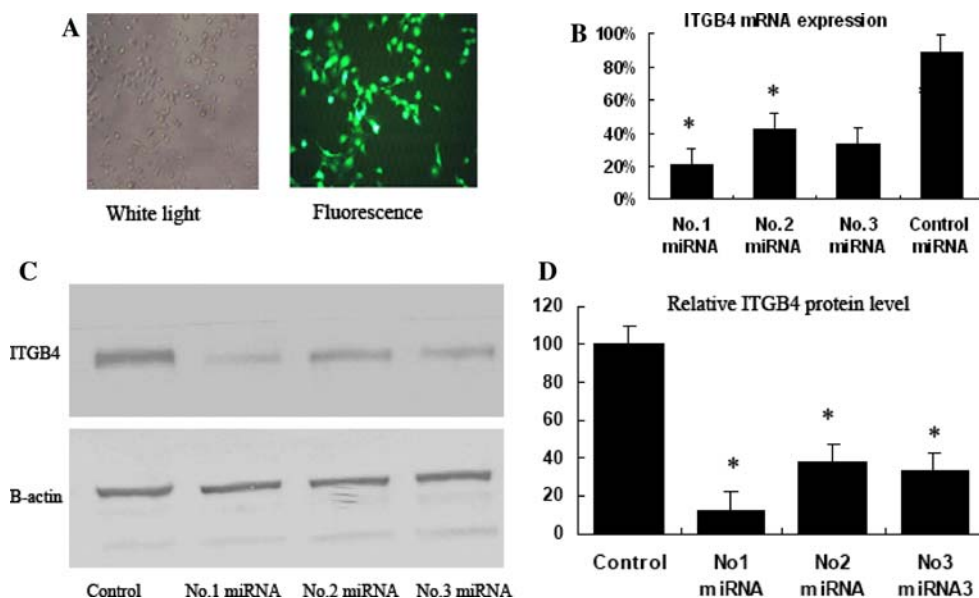
Effects of ITGB4 on wound repair and proliferation

Tracheal epithelial (RTE) cells were transfected with a pcDNA3.1 plasmid-containing full length cDNA sequence of ITGB4. The pcDNA3.1 vector alone was used as control vector. From the multiple independent clones obtained, one clone from the pcDNA3.1/ITGB4 transfection group and one clone from the control vector transfection group were selected for further study. Transfection of pcDNA3.1/ITGB4 vector resulted in 50% up-regulation of ITGB4 mRNA detected by real time RT-PCR ($*P < 0.05$, Fig. 4a). Consistent with the increased mRNA level of ITGB4 after transfection, Western blotting result also showed that a remarked upregulation of ITGB4 protein level after pcDNA3.1/ITGB4 transfection ($*P < 0.05$; $n = 5$, unpaired t test; Fig. 4b, c). In addition, RNA interference technology was used to silence ITGB4. Cells were transfected with virus vectors containing three different candidate miRNA sequences and control miRNA sequences separately. Two days after virus transfection,

more than 95% cells were effectively transfected (Fig. 5a). 48 h after transfection, all three candidate ITGB4 miRNA vector could silence ITGB4 mRNA expression effectively ($*P < 0.05$; $n = 5$, ANOVA; Fig. 5b). Western blotting results also showed that No.1 ITGB4 miRNA can silence ITGB4 protein expression effectively ($*P < 0.05$, $n = 5$, ANOVA; Fig. 5c, d). We decided to use No.1 miRNA as effective miRNA for its optimized silence efficiency.

Tracheal epithelial (RTE) cells were separately transfected with pcDNA3.1/ITGB4 vector, control pcDNA3.1 vector, ITGB4 miRNA lentivirus, and control lentivirus. A small wound was made in each well with an area of approximately $2.817 \pm 0.029 \text{ mm}^2$. Satisfactory correlation was detectable between the time and the remaining wound area ($r = -0.943$). RI was computed by use of the method described in described before [15]: RI (pcDNA3.1 control) = 1.31 and RI (miRNA control) = 1.32. Analysis of the wound repair ability was assessed by comparing the RIs of different groups. The results showed that would repair of RTE cells was inhibited after ITGB4 silence

Fig. 5 Validation of miRNA targeting ITGB4 gene expression. Three candidate miRNA vectors and control miRNA vector were constructed and transfected with RTE cells. **a** All three miRNA vector can inhibited ITGB4 mRNA expression significantly 48 h after transfection. **b** Western blotting results indicated that No. 1 miRNA vector can inhibit ITGB4 protein expression obviously. **c** Quantification of Western blots normalized to the level of β -actin



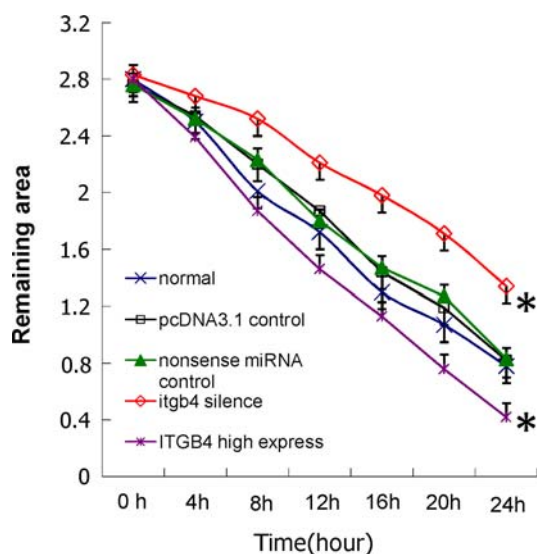


Fig. 6 The effect of ITGB4 on wound repair. RI was used to evaluate the speed of wound repair. Compared with the non-targeted miRNA-transfected control cells, the repair of RTE cells was inhibited after ITGB4 silence. In contrast, wound repair of ITGB4 overexpression cells was accelerated significantly compared with pcDNA3.1 control transfected cells

(RI = 1.07, * $P < 0.05$) compared with control miRNA-transfected cells. In contrast, cells with high expression of ITGB4 showed increased repair ability (RI = 1.47, * $P < 0.05$; Fig. 6).

As proliferation is a critical and important step to the wound repair process, we detect the effect of ITGB4 on cell proliferation by MTT incorporation assays. The results demonstrated that that proliferation of RTE cells was inhibited after ITGB4 silence compared with control miRNA-transfected cells (* $P < 0.05$, $n = 10$, ANOVA). In contrast, proliferation of RTE cells transfected with pcDNA3.1/ITGB4 vector was promoted compared with pcDNA 3.1 control transfected cells (* $P < 0.05$, $n = 10$, ANOVA; Fig. 7).

T-AOC of RTE cells and expression of the three antioxidantases after transfection

In order to investigate the effect of ITGB4 on total anti-oxidation ability of RTE cells, the anti-oxidation ability of RTE cells from different groups was assessed by T-AOC detection. The data showed that T-AOC levels of the ITGB4-silenced groups were significantly decreased compared with control miRNA-transfected cells (* $P < 0.05$, $n = 6$, ANOVA). T-AOC content in ITGB4 overexpressed cells was higher than that in the pcDNA3.1 control-transfected cells (* $P < 0.05$, $n = 6$, ANOVA; Fig. 8).

The anti-oxidation capacity of airway epithelial cells was strongly correlated with the expression of antioxidant

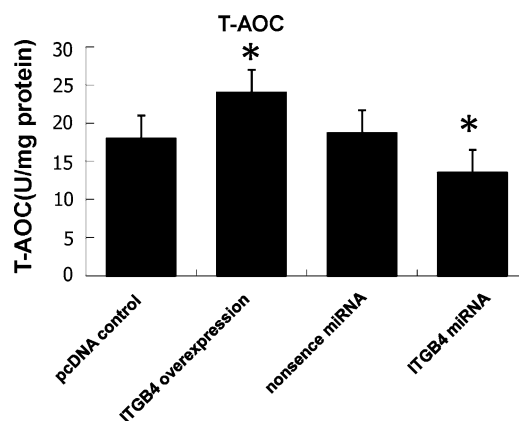


Fig. 7 Proliferation of stably transfected RTE cells was examined by MTT assay. From 24 to 96 h after integrin 4 was silenced, overexpression of ITGB4 promoted proliferation of RTE cells, while ITGB4 silence inhibited proliferation significantly

enzymes. Therefore, we further analyzed the expressions of three major antioxidant enzymes on transfected RTE cells. Similar trend was observed for all of the three antioxidant enzymes. First, expression of GPx was reduced after ITGB4 silence (606 ± 56 vs. 720 ± 64 , * $P < 0.05$, $n = 10$, ANOVA; Fig. 9a) and was promoted after ITGB4 overexpression (806 ± 61 vs. 717 ± 59 , * $P < 0.05$, $n = 10$, ANOVA). Second, SOD expression was also inhibited after ITGB4 silence (16.53 ± 1.43 vs. 10.86 ± 1.31 , * $P < 0.05$, $n = 10$, ANOVA; Fig. 9b), which was promoted when ITGB4 was overexpressed (20.12 ± 1.56 vs. 16.27 ± 1.41 , * $P < 0.05$, $n = 10$, ANOVA, Fig. 9b). Finally, in the ITGB4 silenced group, catalase expression was decreased significantly compared with miRNA control group (3.37 ± 0.89 vs. 6.01 ± 0.95 , $n = 10$, * $P < 0.05$,

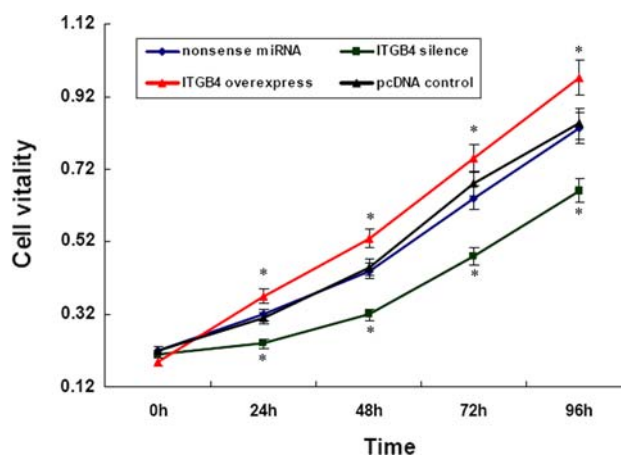
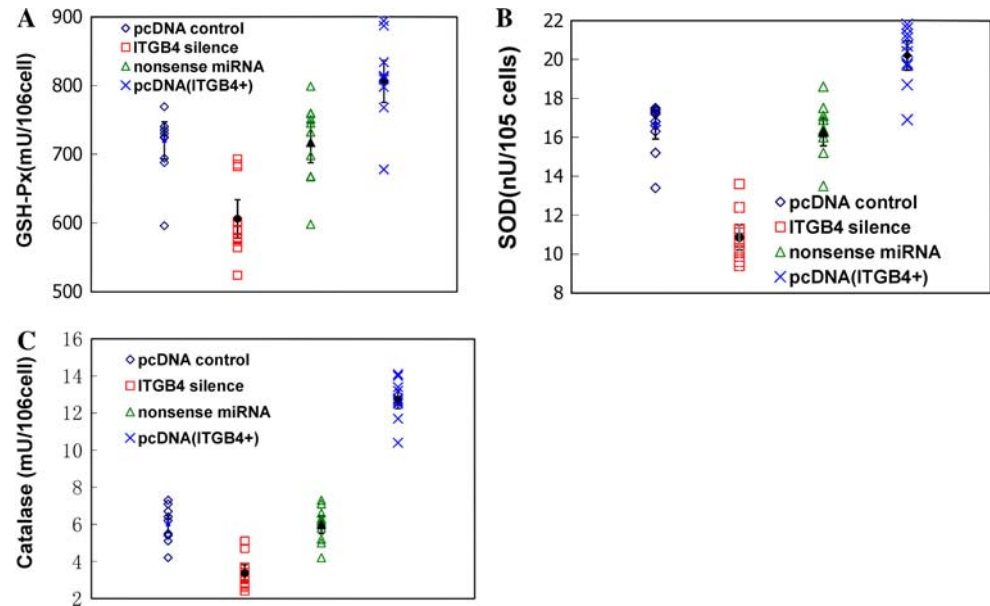


Fig. 8 T-AOC of RTE cells is detected by a T-AOC assay kit. The results showed that T-AOC of ITGB4 overexpressed cells increased compared with control cells after stressed with the same active oxygen. In contrast, ITGB4 silenced group showed that a decreased T-AOC ability

Fig. 9 Expression of three antioxidases was detected using commercial assay kits. **a** Assays for GSH-Px revealed that a significant decrease in ITGB4 silenced cells. ITGB4 overexpressed RTE cells showed that a significantly increased GSH-Px content. **b** Expression of SOD decreased obviously in ITGB4-silenced cells. After ITGB4 was overexpressed, SOD activity was enhanced. **c**: Expression of catalase decreased in ITGB4-silenced group and enhanced significantly in ITGB4 overexpression group. *Black markers* represent the mean value of every group



ANOVA; Fig. 9c). In contrast, in the ITGB4 overexpressed group, catalase was upregulated obviously compared with pcDNA3.1 control group (12.74 ± 1.01 vs. 5.98 ± 0.96 , $n = 10$, $* P < 0.05$, ANOVA; Fig. 9c).

Discussion

As the first interface to the outer space, airway epithelial cells are continuously subjected to environmental pollutants and rely on several intrinsic mechanisms to maintain barrier integrity and homeostasis of the airway microenvironment following injury [16]. For its barrier function, it is becoming clear that structural integrity of airway epithelial cells may also influence or orchestrate many lung diseases, such as asthma and COPD [17]. ITGB4, as a membrane receptor, is expressed on airway epithelial cells and mediates cell substratum attachment in a hemidesmosome style [18]. However, besides adhesion, the specific functions of ITGB4 on airway epithelial cells have not yet been fully explored, although the specific intermembrane structure has been involved in many different physiological processes.

We at the first time demonstrated that ITGB4 expression was upregulated on the edge of mechanical wounds for 16HBE14O and RTE cells separately. Therefore, it is likely that ITGB4 expression has a close relationship to the structural condition of airway epithelial cells. Structural disruption and shedding of airway epithelial cells are main pathological character of asthma patients. In this study, in the OVA-challenged asthma model, we further showed that both mRNA and protein expression of ITGB4 decreased compared to normal control. Areas of epithelial cell shedding and thickening of the basement membrane was

obvious on bronchial biopsies of the asthma model. These results suggested that ITGB4 may engage in the wound repair process of airway epithelial cells.

In addition, using ITGB4 overexpression vector and ITGB4 silence vector, we found that upregulation of ITGB4-promoted wound repair, and silence of ITGB4 inhibited. This is a new physiology function of ITGB4 on airway epithelial cells. Wound repair is an important and supplicated process. It has been verified that wound repair process of airway epithelial cells connects with many airway diseases. However, the molecules that engaged in the regulation of wound repair are unclear. The regulation function of ITGB4 on wound repair could improve our understanding of pathology phenotype of asthma and other lung diseases.

In wound repair processes, the proliferative ability is an important regulator factor [19]. Our results showed that proliferation of RTE cells was inhibited after ITGB4 silence and promoted when ITGB4 was overexpressed. Therefore, the regulation function of ITGB4 on wound repair may be partly through the modification of proliferation ability. Such regulation of proliferation and wound repair by ITGB4 on airway epithelial cells may provide advanced therapy in many epithelial diseases. It is of critical importance in rapid reepithelialization after epithelial shedding to maintain airway mucosal barrier properties.

Epithelial cells lining the respiratory airway are also the first cells to encounter high concentrations of inspired oxygen. Therefore, airway epithelial cells are in a uniquely vulnerable position for oxidative damage. Oxidation damage is a typical feature of many lung diseases, including asthma, emphysema, and hyperoxia [20]. An imbalance between the production of ROS and antioxidant capacity

leads to oxidative stress which causes a series of lung diseases by damaging lipids, protein, and DNA [21]. Animals of asthma model and in vitro studies indicated that ROS can reproduce many of the features of asthma [22]. Our previous study showed that ozone is a common oxidation injury to airway epithelial cells. Rats that exposures to continuous ozone stress displayed obvious airway inflammation and airway hyper-responsiveness [23]. Here, this study demonstrated that ITGB4 expression increased conspicuously after ozone stress in vitro. It suggested that ITGB4 may be sensitive to the oxygen stress. In the OVA-challenged model, ITGB4 was downregulated, at the same time, anti-oxidation ability of RTE cells were attenuated. These results all intensely suggested a biological role of ITGB4 in anti-oxidation processes.

Accordingly, we further investigated the role of ITGB4 on anti-oxidation ability of airway epithelial cells. The results demonstrated that upregulation of ITGB4 promoted the antioxidation ability of RTE cells and ITGB4 silence inhibited. Expression of anti-oxidation enzyme had a close relationship to the anti-oxidation ability of airway epithelial cells. Normally, airway epithelial cells contain large numbers of antioxidation enzymes. SOD, catalase, and GSH-Px are the most important antioxidant enzymes of airway epithelial cells. SOD is a ubiquitous enzyme with an essential function in protecting aerobic cells against oxidative stress [24] that catalyzes the dismutation of superoxide anion to oxygen and hydrogen peroxide (H_2O_2). The latter two enzyme systems are used in cell defence against common oxidants. Catalase is most effective in the presence of high H_2O_2 concentrations. While, in the presence of low concentrations of either H_2O_2 or other peroxides, the glutathione system plays a critical role [25]. In order to explore the possible mechanism of ITGB4 on anti-oxidation ability of airway epithelial cells, the expression of three most important antioxidation enzymes is detected. The data showed that ITGB4 overexpression can significantly increase the expression of all three enzymes, while ITGB4 silence cells showed that a decreased regulation. These findings indicated that ITGB4 could regulate the antioxidation ability, partly through regulating the expression of SOD, catalase, and GSH-Px.

In conclusion, this study determines the expression and functional properties of ITGB4 on airway epithelial cells. It suggested that ITGB4 involves in regulating the ability of wound repair and anti-oxidation in airway epithelial cells. These new physiological functions of ITGB4 may be of value in studying the pathogenesis of many airway diseases.

Acknowledgments The authors sincerely appreciate the editor and reviewers for their positive and insightful comments. We thank Professor Gruenert from the San Francisco Branch Campus of the

University of California for the gift of the immortalized human bronchial epithelial cell line, 16HBE14O. This study was supported by grant #30800504 from the National Natural Science Foundation of China, the key projects #2007FJ3009 from Hunan Science and Technology plans, and the construct program of the key discipline in Hunan province.

References

1. Busse W, Elias J, Sheppard D, Banks-Schlegel S (1999) Airway remodeling and repair. *Am J Respir Crit Care Med* 60: 1035–1042
2. Holgate ST (2007) Epithelium dysfunction in asthma. *J Allergy Clin Immunol* 120:1233–1244
3. Hargreave FE, Parameswaran K (2006) Asthma, COPD and bronchitis are just components of airway disease. *Eur Respir J* 28:264–267
4. Holgate ST (2007) The epithelium takes centre stage in asthma and atopic dermatitis. *Trends Immunol* 28:234–251
5. Bergeron C, Boulet LP (2006) Structural changes in airway diseases characteristics, mechanisms, consequences, and pharmacologic modulation. *Chest* 129:1068–1087
6. Trautmann A, Kruger K, Akdis M, Müller-Wening D, Akkaya A, Bröcker EB, Blaser K, Akdis CA (2005) Apoptosis and loss of adhesion of bronchial epithelial cells in asthma. *Int Arch Allergy Immunol* 138:142–150
7. Sheperd D (2003) Functions of pulmonary epithelial integrins: from development to disease. *Physiol Rev* 83:673–686
8. Sheppard D (1998) Airway epithelial integrins: why so many? *Am J Respir Cell Mol Biol* 19:349–351
9. Mercurio AM, Rabinovitz I, Shaw LM (2001) The $\alpha 6 \beta 4$ integrin and epithelial cell migration. *Curr Opin Cell Biol* 13:541–545
10. Dowling J, Yu QC, Fuchs E (1996) $\beta 4$ integrin is required for hemidesmosome formation, cell adhesion and cell survival. *J Cell Biol* 134:559–572
11. Davenport EA, Nettesheim P (1996) Regulation of mucociliary differentiation of rat tracheal epithelial cells by type I collagen gel substratum. *Am J Respir Cell Mol Biol* 14:19–26
12. Matsuura S, Shirakami G, Iida H, Tanimoto K, Fukuda K (2006) Tracheal epithelial cells: a comparison with isoflurane and halothane. *Anesth Analg* 102:1703–1708
13. Qin XQ, Xiang Y (2000) Fibronectin or RGD peptide promotes nitric oxide synthesis of rabbit bronchial epithelial cells. *Acta Physiol Sin* 52:519–521
14. Sakon S, Xue X, Takekawa M, Sasazuki T, Okazaki T, Kojima Y, Piao JH, Yagita H (2003) NF- κ B inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. *EMBO J* 22:3898–3909
15. Tan YR, Qi MM, Qin XQ, Xiang Y, Li X, Wang Y, Qu F, Liu HJ, Zhang JS (2006) Wound repair and proliferation of bronchial epithelial cells enhanced by bombesinreceptor subtype 3 activation. *Peptides* 27:1852–1858
16. Parks WC, Wilson CL, Lopez-Boado YS (2004) Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* 4:617–629
17. Hackett TL, Knight DA (2007) The role of epithelial injury and repair in the origins of asthma. *Curr Opin Allergy Clin Immunol* 7:63–68
18. Rousselle P, Lunstrum GP, Keene DR, Burgeson RE (1991) Kalinin: an epithelium-specific basement membrane adhesion molecule that is a component of anchoring filaments. *J Cell Biol* 114:567–576
19. Tesfaigzi Y (2003) Processes involved in the repair of injured airway epithelia. *Arch Immunol Ther Exp* 51:283–288

20. Rahman I, Biswas SK, Kode A (2006) Oxidant and antioxidant balance in the airways and airway diseases. *Eur J Pharmacol* 533:222–239
21. Mak JC, Chan-Yeung MM, Moira MW (2006) Reactive oxidant species in asthma. *Curr Opin Pulm Med* 12:7–11
22. Bucchieri F, Lordan J, Richter A, Buchanan D (2000) Increased sensitivity of asthmatic bronchial epithelial cells to oxidant-induced injury. *Am J Respir Crit Care Med* 161:A153
23. Wu H, Guan C, Qin XQ, Xiang Y, Qi MM, Luo ZQ, Zhang CQ (2007) Upregulation of substance P receptor expression by calcitonin gene-related peptide, a possible cooperative action of two neuropeptides involved in airway inflammation. *Pulm Pharmacol Ther* 20:513–524
24. Larivee P, Levine SJ, Martinez A, Logun C, Shelhamer JH (1993) Protein kinase C 4 (zeta) isozyme mediates the airway mucin secretory effect of platelet activating factor. *Am Rev Respir Dis* 147:A934
25. Cantin PA (1999) Oxidant and antioxidants in lung injury. In: Moss J (ed) *Lam and other diseases characterized by smooth muscle proliferation*. Dekker, New York, pp 519–531