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Original Article

Integrin β4 was downregulated on the airway epithelia of asthma patients

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The shedding of airway epithelial cells and loss of epithelial functional homeostasis are major pathological characteristics of asthma; however, the mechanism underlying these pathologies remains obscure. Our previous work showed that there were three variation sites in 5′ flanking region of integrin β4 in asthma patients, which was correlated with decreased expression of integrin β4 in peripheral leukocytes. Integrin β4 is an important structural adhesion molecule on airway epithelia to keep the structural adhesion of epithelial cells. In this work, we further demonstrated that integrin β4 expression was downregulated in airway epithelia of asthma patients. To probe the relationship between imbalanced expression of integrin β4 and dysfunction of the airway epithelial cells in asthma, integrin β4 was silenced in human bronchial epithelium cells (16HBE14O) by integrin β4 small-interfering RNA lentivirus vector. Upon silencing of integrin β4, 16HBE14O cells showed reduced proliferation and wound repair. Most cells were shown to be arrested in G1 phase after integrin β4 silencing, and increased apoptosis was induced in the integrin β4-silenced cells. In summary, our results provided compelling evidence that integrin β4 was involved in the structural integrity and functional homeostasis of airway epithelial cells. It is likely that downregulation of integrin β4 on asthma airway epithelia contributes to the structural disruption and dysfunction of airway epithelial cells.

Keywords integrin β4; airway epithelia; asthma; siRNA

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Introduction

Inspection of the airway epithelia of asthma patients revealed a damaged structure and abnormal physiological function, including epithelial shedding and metaplasia, thickening of the basal membrane, increased susceptibility to outer stimuli, and aberrant repair after damage [1–3]. As the first barrier to environmental pollutants and allergens, airway epithelia could recognize and receive stimuli from the outside and react appropriately. Barbato *et al*. [4] found that the airways of asthmatic children were remodeled in the early stage of asthma, and this remodeling could be detected even prior to the onset of symptoms and diagnosis. Isolation, characterization, and culture of primary epithelial cells from asthma airway showed that in asthma patients, airway epithelial cells were less differentiated and secreted an abnormal set of cytokines and pro-inflammatory mediators [5]. The ability to repair tissue after injury was also compromised in asthma patients, a phenotype that might be closely connected to asthma pathogenesis [6,7]. Later studies also indicated that airway epithelial cells in asthma patients had more severe reactions to IL-3 and IL-4, oxidants, and allergy stressors; these data further supported the importance of airway epithelia in asthma pathogenesis [8,9]. At the same time, lots of studies have focused on the underlying mechanisms to theses pathological changes of asthma airway epithelia, and accumulating evidence showed that impaired structural integrity and loss of functional homeostasis in asthma airway epithelia might attribute to the imbalanced expression of some critical genes [10,11]. However, the determination of these potential genes and the possible pathogenesis are not fully understood.

Proteins of adhesion molecule family were expressed on the surface of many cell types and could engage in multiple physiological and pathological processes. Many lung diseases have been investigated closely relating to adhesion molecules [11]. Asthma is a chronic inflammatory disorder/disease characterized by typical structural damage and dysfunction of airway epithelial cells. It is likely that imbalanced adhesion molecule expression can induce some phenotypes and pathological abnormalities. To investigate the correlation between the imbalanced expression of adhesion molecules and asthma pathogenesis, our previous work identified genes that were differentially expressed in the peripheral leukocytes of asthma patients by using cDNA microarray analysis. The results

demonstrated that integrin β4 is significantly downregulated in asthma patients, and three variation sites were acquired in 5′ flanking region, which was correlated with downexpression of integrin β4 [12].

Integrin β4 was mainly expressed on basal cells to adhere them to the basement membrane, as in hemidesmosome structure [13–15]. Damage of airway epithelial cells was common on asthma airway epithelial cells. In this study, we further observed that integrin β4 was downregulated in the airway epithelia of asthma patients. As the airway epithelia is the first barrier to the outer environment and allergen stimulation, we suspected that the epithelial dysfunction and pathological phenotype of asthma might be related to the decreased expression of integrin β4. In order to understand the possible correlation between downregulation of integrin β4 and pathological phenotype of asthma airway epithelia, integrin β4 small-interfering RNA (siRNA) virus vectors were used to silence integrin β4 on bronchial epithelial cells (16HBE14O). After integrin β4 was silenced, proliferation, wound repair, and apoptosis of 16HBE14O cells were investigated.

Materials and Methods

Subjects

Approval for the use of human tissues was granted by the Ethical Committee of China. Healthy control subjects ($n = 6$) and adult atopic asthma subjects ($n = 6$) with age ranging from 28 to 72 were recruited. Atopy was defined as a positive skin prick test response (wheal of ≥ 3 mm diameter larger than that produced by the negative control wheal) to common aeroallergens: *Dermatophagoides pteronyssinus*, dander, dog, and mixed grasses (ALK). Asthma was diagnosed in accordance with American Thoracic Society criteria. All of the adult asthma patients (diagnosed separately by Xiangya Hospital, Hunan People's Hospital, and Xiangtan Central Hospital) displayed significant airway hyperresponsiveness that was detected by the method of Niimi A *et al*. [16]. In order to avoid any interference from medications or other factors, asthma patients were enrolled at the time of their initial asthma diagnosis. The patients had not received any medical treatment for the condition prior to enrolling in the study. Healthy control subjects were defined as individuals with normal pulmonary function and negative skin prick test results who were not taking any medication, and did not have lung or systemic diseases. Bronchial biopsy specimens of asthma patients and control subjects were fixed in 4% paraformaldehyde and embedded in paraffin.

Immunohistochemistry for integrin β4

The protein expression of integrin β4 in the airway mucus of human asthma patients was detected by immunohistochemistry. To obtain accurate and objective results, two integrin β4 antibodies were used. One antibody was a rabbit antibody that recognizes amino acids 28–128 within an extracellular domain of the human integrin β4 (H-101; Santa Cruz, USA) and the other was a mouse monoclonal antibody that recognizes amino acid residues in the cytoplasmic region of human integrin β4 (M126; Abcam, Cambridge, UK).

Briefly, endogenous peroxidase was inhibited by soaking tissue sections in 3% H_2O_2. After rinsing in PBS, sections were incubated with goat serum to block nonspecific binding of antibodies and incubated overnight at 4–8°C with primary antiserum (H-101, 1:100 dilution; M126, 1:200 dilution). After being washed in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG for 1 h and washed again. The sections were then incubated with SABC for 1 h at room temperature. After being washed in PBS, the signal was detected with 3,30-diaminobenzidine. Staining with omission of primary antibodies was included as a negative control for each biopsy.

Quantification of integrin β4 staining

Integrin β4 staining intensity was determined using a color video camera (Sony DXC-950P; Sony, Tokyo, Japan). The camera was connected to a Leica Imaging Workstation with imaging software (Leica Q500IW; Leica, Cambridge, UK). A semi-quantitative scoring method was used by three independent blinded observers to record integrin β4 staining expression by scoring from scale of 0 to 4 (0, being no staining, and 4, being maximum staining) depending on the staining intensity in circular airways.

Cell culture

The immortalized human bronchial epithelial cell line 16HBE14O (a kind gift from Prof. Gruenert, University of California, San Francisco, USA) was maintained at 37°C with 5% CO_2, in a mixture of DMEM/F12 (1:1) media supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% heat-inactivated fetal bovine serum. Throughout this study, the cells were always grown on the coated culture plates, which were coated with fibronectin-coating solution containing 0.01 mg/ml of fibronectin, 0.029 mg/ml of collagen for at least 1 h before culturing the cells.

siRNA synthesis and transfection

Candidate siRNAs directed against integrin β4 mRNA were designed according to the criteria designed by Elbashir *et al*. [17]. Three potential siRNAs were selected according to the prediction of single-strand domains within the mRNA secondary structure (**Table 1**). BLAST analyses were performed to ensure that no additional significantly matching human

Table 1 The siRNA sequences and properties

siRNA	Position	Sequences	GC%
No. 2973	Exon 10	5′-GGUCACCUCCAAGAUGUUC-3′	48
		3′-GAACAUCUUGGAGGUGACC-5′	52
No. 2881	Exon 11	5′-GGGCAACAUCCAUCUGAAA-3′	43
		3′-UUUCAGAUGGAUGUUGCCC-5′	43
No. 2988	Exon 10	5′-GGACUGGGUCCUUUCACAU-3′	48
		3′-AUGUGAAAGGACCCAGUCC-5′	52
Nonsense siRNA	None	5′-UUCUCCGAACGUGUCACGU-3′	44
		3′-ACGUGACACGUUCGGAGAA-5′	56
GAPDH siRNA	Exon 3	5′-GUGGAUAUUGUUGCCAUCA-3′	44
		3′-GAUGGCAACAAUAUCCAG-5′	56

transcripts would be targeted by these siRNAs. Then, the three siRNAs used for integrin β4 silencing, the siRNA used for GAPDH silencing, and the nonsense siRNA were prepared by using the Silencer siRNA construction kit (Ambion, Austin, USA). Transfections were conducted with the siPORT NeoFection (Ambion). Briefly, siRNA was diluted into free DMEM/F12 without serum or antibiotics and mixed in siPORT NeoFection. Cells were then transfected at 37°C with 5% CO_2 for 6 h with different siRNA concentrations and changed with DMEM/F12 containing 10% fetal calf serum without antibiotics.

Efficiency of silencing after siRNA transfection

Real-time RT-PCR was used to detect *integrin β4* mRNA expression after siRNA transfection. The sequences of the primers and Taqman probes used are as follows: *integrin β4* forward: 5′-CACCGCGTGCTAAGCACAT-3′, *integrin β4* reverse: 3′-TGTGGTCGAGTGTGAGTGTTCTG-5′; Taqman probe: FAM + ACCCTCACACGGGACTACAAC TCACTG + TAMRA; *GAPDH* forward: 5′-CCACTCCTC CACCTTTGAC-3′, *GAPDH* reverse: 3′-ACCCTGTTGCT GTAGCCA-5′; Taqman probe: FAM + TTGCCCTCAAC GACCACTTTGT + TAMRA). Total RNA (1 μg) purified from siRNA transfected cells was reverse transcribed into cDNA using AMV reverse transcriptase (Qiagen, Hilden, Germany) with RNase inhibitor and oligo d(T) primer at 40°C for 50 min followed by heating at 90°C for 5 min. Then, 1 μl of the reverse-transcript product was added to a 30-μl PCR mixture for 40 cycles. Each cycle included 93°C for 30 s, 54°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 the RNA input, RNA quality, and reverse transcription efficiency was achieved by comparing the copy numbers of target mRNAs with that of human *GAPDH*.

Integrin β4-specific siRNA vector construction and transfection

The effective No. 2973 siRNA and control siRNA were annealed and ligated into the pGCSIL-GFP vector to create integrin β4 siRNA expression vectors and control expression vectors (GeneChem, Shanghai, China). All plasmids were sequenced to confirm that they were correct. Then, the integrin β4 silence and control vectors were packaged into recombinant lentivirus vectors using Lentivector expression system (GeneChem) and titered to 10^9 TU/ml.

Before transfection, 16HBE14O cells were plated in 24-well plates (2×10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 ml (10^8 TU/ml) with complete medium containing polybrene (8 mg/ml) and added to the cells for 1 h incubation at 37°C, followed by incubation in 0.3 ml of fresh prepared polybrene-DMEM/F12 for another 24 h, which was replaced with fresh DMEM/F12 medium. Then the cells were cultured for another 48 h. A high percentage (more than 90%) of transfectants-expressed GFP was detected at 72 h after lentivirus vector transfection (data not shown).

Western blot analysis after vector transfection

Three days after transfection, proteins from parental 16HBE14O cells, control siRNA virus vector transfected cells, integrin β4 siRNA virus vector transfected cells were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes (Millipore, Bedford). Membranes were blocked and then probed with antibodies against integrin β4 (1:1000 dilution; Santa Cruz), GAPDH (1:5000 dilution; Sigma, St. Louis, USA). After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution; Biyuntian, Ningbo, China) and visualized by super ECL detection reagent (Applygen, Beijing, China).

Proliferation assay

Three days after vector transfection, 16HBE14O cells from different groups were seeded in 96-well plates at a density of 10^4 cells/well separately. The medium was replaced with serum-free medium that containing 0.05 g/L epithelial growth factor for another 24, 48, 72, and 96 h to synchronize cell growth. Briefly, 15 μl of 0.5% MTT was added to each well and incubated for 4 h. Then, the medium and MTT were removed, 150 μl of DMSO was added to each well and the plates were shaken for 10 min. At all time points, the OD value of each well was determined at 570 nm using a microplate reader (Tecan, Australia).

Cell cycle analysis by propidium iodide

Cell cycle analysis was carried out 72 h after transfection. 16HBE14O cells (5×10^5) of different groups were harvested by brief trypsinization, washed twice with PBS, fixed in 70% ethanol overnight and stained with propidium iodide (PI; 20 μg/ml of final concentration)/Triton X-100 containing 10 mg/ml RNase (DNase free). After incubation at 37°C for 30 min, the samples were analyzed on an FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, USA), and the populations of G1, S, and G2/G1 cells were quantified.

Monolayer wound repair assay

This assay was used to demonstrate the effect of integrin β4 on epithelial cell migration and wound repair, which has been reported in our previous study [18]. Briefly, 72 h after transfection, cells from different groups were grown to confluence in 12-well plates with DMEM/F12 (1:1) separately. A small wound was made in the confluent monolayer with a rubber stylet. The edge of the wound was recognized, and the remaining wound area was measured serially every 4 h by video microscopy (Olympus Company, Tokyo, Japan) for 24 h. The linear relationship between the wound surface area and time was used to calculate the wound repair ability, corresponding to the decrease in wound surface per hour. Therefore, a linear regression equation of the remaining wound area per time elapsed was obtained. The repair index (RI), equal to the absolute value of slope, was used to score the repair speed of transfected cells.

Apoptosis detection

The Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, USA) was used to stain apoptotic cells. Briefly, 72 h after transfection, the transfected cells from different groups were harvested and washed twice with cold PBS, trypsinized, and resuspended in 1× binding buffer at a concentration of 1×10^5 cells/ml. Annexin V-FITC (9×10^{-3} μg/μl) and PI (2.27×10^{-3} μg/μl) were added to the cell suspension (100 μl), followed by incubation for 15 min at room temperature. Four hundred microliters of 1× binding buffer was added to the stained cell suspension. Then, the cells were identified as living cells [annexin V-FITC (−), PI (−)], early apoptotic cells [annexin V-FITC (+), PI (−)], late apoptotic cells [annexin V-FITC (+), PI (+)], and dead cells [annexin V-FITC (−), PI (+)] by different staining using flow cytometry (Becton Dickinson).

Statistical analysis

All experiments were run at least five times. All data are presented as the mean ± standard error of the mean. For all determinations, the differences were considered significant when $*P < 0.05$ or $**P < 0.01$. The unpaired *t*-test was used for two groups' comparison. Comparisons of the effectiveness of the different siRNA groups or the differences between integrin β4-silenced group and control groups were performed by means of two-way ANOVA or one-way ANOVA, followed by Dunnett's or LSD's *post hoc* test.

Results

Integrin β4 expression was reduced in the airway mucosa of asthma patients

Two antibodies were used to detect integrin β4 expression on the airway mucus of normal control individuals and asthma patients. **Figure 1(A,B)** showed the sections stained with cytoplasmic region antibody M126 and extracellular domain antibody H101, respectively. The semi-quantitative scoring [**Fig. 1(C)**] showed that, compared with normal person, integrin β4 staining in the airway mucosa of asthma patients reduced significantly in both the extracellular domain (1.83 ± 0.01 vs. 3.49 ± 0.03, $P = 0.006$, $n = 3$, unpaired *t*-test) and the cytoplasmic region (1.87 ± 0.08 vs. 3.68 ± 0.07, $P = 0.002$, $n = 3$, unpaired *t*-test).

Integrin β4 mRNA and protein expression levels were inhibited by effective siRNA

To verify the silencing efficiency of the three-candidate siRNAs, we detected the remaining expression of *integrin β4* mRNA in transfected cells by real-time RT-PCR. Both No. 2973 and No. 2988 siRNA inhibited *integrin β4* mRNA expression significantly at a concentration range from 5 to 100 nM ($P < 0.01$; $n = 5$, two-way ANOVA) as shown in **Fig. 2(A)**. Besides, comparing with No. 2988 siRNA, No. 2973 siRNA showed more significant silence efficiency on *integrin β4* mRNA expression within the concentrations range from 5 to 100 nM. When the concentration of No. 2973 siRNA was 25 nM, the silence efficiency is higher than 80%. Increased silence efficiency was shown with the increasing of No. 2973 siRNA concentration. But light increased silence efficiency was observed

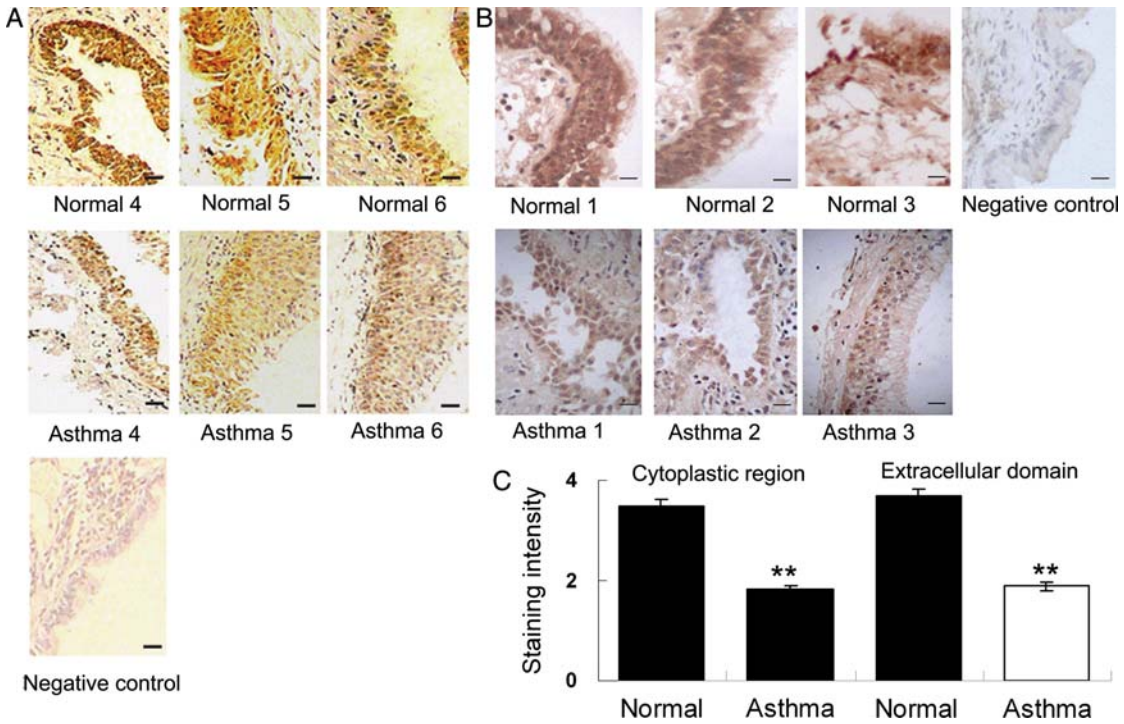

Figure 1 Expression of integrin β4 in the airway mucus of asthma patients and normal person was detected by immunohistochemistry (A) and (B) show staining of cytoplasmic region and an extracellular domain of integrin β4 detected by two separate antibodies (bar = 100 μm). (C) Semi-quantitative scoring was utilized to determine the expression of integrin β4. Staining intensity was determined according to the indicated scoring scale and shown using a bar graph. $^{**}P < 0.01$.

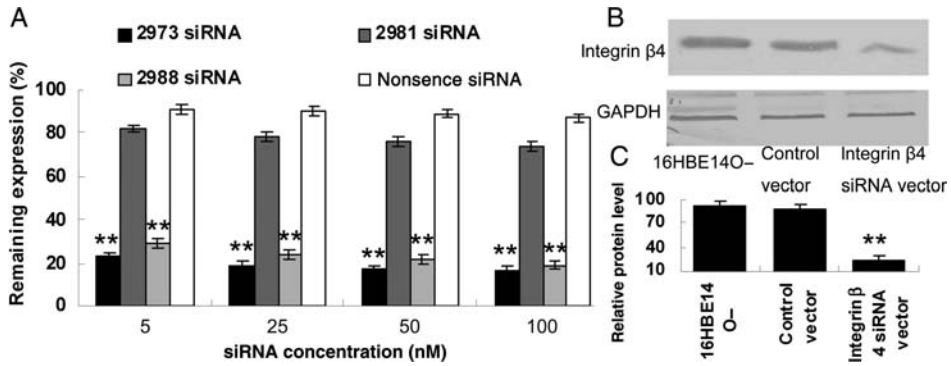

Figure 2 Silencing efficiency of *integrin β4* siRNAs (A) Silencing of *integrin β4* mRNA on 16HBE14O cells. Results were quantified relative to *GAPDH* mRNA 48 h after siRNA transfection. Three candidate integrin β4 siRNAs (No. 2973, No. 2888, No. 2981) and a non-silencing control siRNA were transfected into 16HBE14O cells in triplicate at four different concentrations. (B) The effective No. 2973 siRNA lentivirus vector and control siRNA lentivirus vector were prepared and transfected into 16HBE14O cells. Three days after lentivirus vector transfection integrin β4 protein expression was detected by western blot analysis. (C) Quantification of western blot was normalized to the level of GAPDH in **Fig. 1(B)**. $^{**}P < 0.01$.

when the siRNA concentrations are 50 and 100 nM. These results indicated that optimized silence efficiency could be achieved when the siRNA concentration is 25 nM. Therefore, we considered No. 2973 siRNA as specific effective siRNA sequence for *integrin β4* gene. Then, we ligated No. 2973 siRNA sequence into pGCSIL-GFP vector and packaged into recombinant lentivirus vectors for its optimized silence efficiency and minimized cytotoxicity.

Next, to determine whether integrin β4 siRNA virus vector could also decrease integrin β4 protein expression, we performed western blot to investigate the protein expression of integrin β4 after transfection [**Fig. 2(B)**]. The results demonstrated that, 72 h after transfection, the integrin siRNA virus vector could inhibit the integrin β4 protein expression significantly ($P = 0.0001$, $n = 5$, ANOVA). More than 85% integrin β4 protein expression was inhibited in the 16HBE14O cells transfected with integrin β4 siRNA virus vector [**Fig. 2(C)**]. While compared with the control siRNA vector, there was no significant influence on the integrin β4 protein expression ($P > 0.5$, $n = 5$, ANOVA).

Proliferation and cell cycle progression are restrained by integrin β4 silencing

MTT incorporation assays for measuring cell proliferation were performed after integrin β4 silencing (**Fig. 3**). During 24–96 h period after integrin β4 was silenced, growth curves obtained from control siRNA vector transfected cells were similar to that from the parental 16HBE14O cells. However, integrin β4-silenced 16HBE14O cells proliferated slowly compared with nonsense siRNA transfected cells ($P < 0.01$, $n = 8$, ANOVA). Meanwhile, the cells proliferation activity was also measured by PI staining after integrin β4 silencing [**Fig. 4(A)**]. In agreement with the inhibitory effects of integrin β4 on cell proliferation, after integrin β4 was silenced, there were more than 60% cells in the G1 phase and <20% cells in the S phase. While for both parental 16HBE14O cells and control siRNA vector transfected cells, there were only ~40% cells in the G1 phase but more than 30% in the S phase [**Fig. 4(B)**]. Taken together, our results strongly suggested that downregulation of integrin β4 inhibited cell cycle and therefore restrained proliferation of 16HBE14O cells.

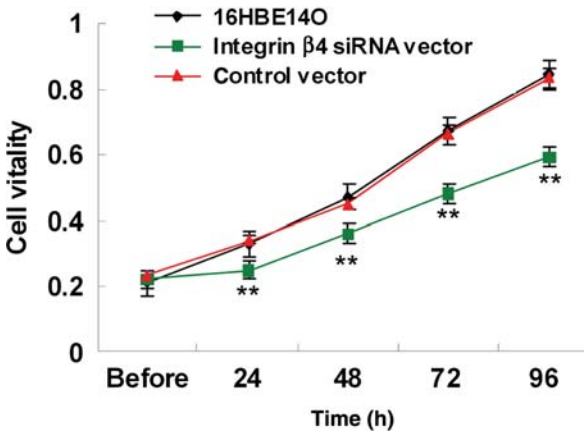

Figure 3 Proliferation after integrin β4 silencing was evaluated by MTT assay Normal 16HBE14O cells, control siRNA vector transfected cells, and integrin β4 siRNA vector transfected cells were all tested with the ELISA reader at 570 nm after transfection. Cell number was evaluated by the absorbance at the indicated time points with eight independent experiments. $^{**}P < 0.01$.

Effects of integrin β4 silencing on wound repair ability

A small wound was mechanically made in each well in areas of approximately of 30.769 ± 0.083 μm^2. Twenty-four hours after wounding, the wound area in the parental 16HBE14O group and control siRNA vector transfected group had a

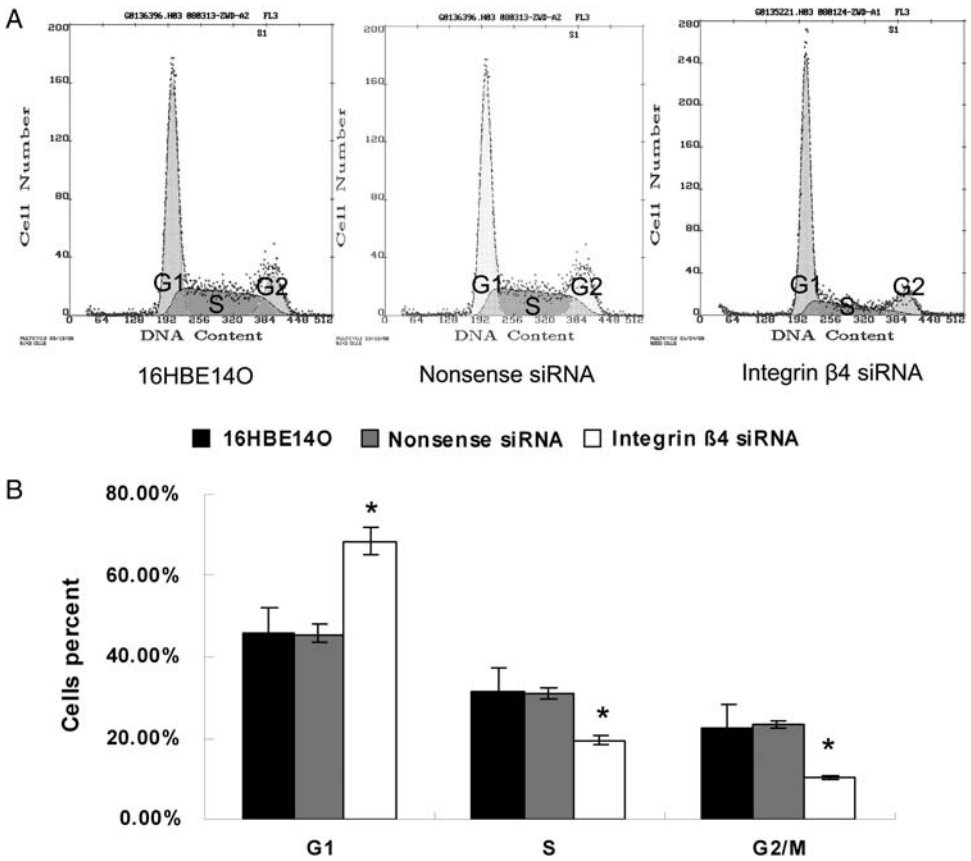

Figure 4 Cell cycle after integrin β4 silencing was detected with PI staining (A) Seventy-two hours after vector transfection, the populations of G1, S, and G2/M from different groups were separated and signed. (B) Cell cycle populations in G1, S, and G2/M phases were quantified from the DNA histograms with the ModFit LT v3.0 software package (Verity Software House). $^{*}P < 0.01$.

similar repair area, which has became almost confluent, whereas the integrin β4-silenced cells migrated only 47.69 ± 7.45% of the wound area. Satisfactory correlation was detectable between the time and the remaining wound area ($R = 0.963$, $P < 0.01$). For the control siRNA vector transfected cells and parental 16HBE14O cells, a relative rapid wound repair speed was calculated with the RI of 0.41 and 0.42, respectively. In contrast, wound repair in integrin β4-silenced cells was blocked with the RI of 0.24 ($P < 0.05$) (**Fig. 5**).

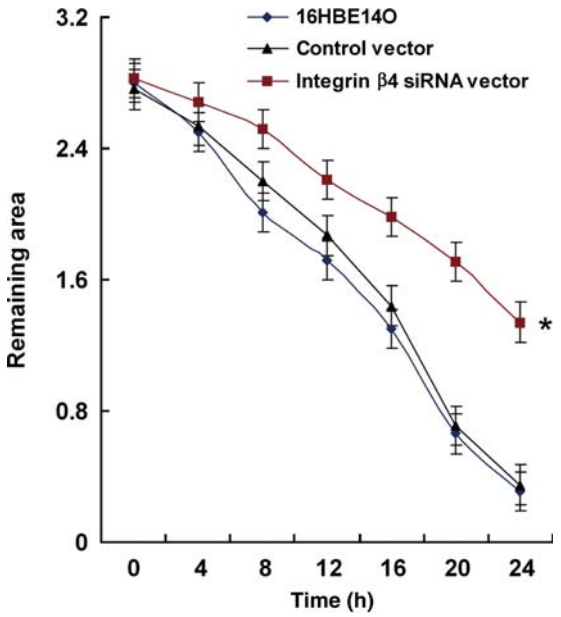

Figure 5 The effect of integrin β4 silencing on wound repair Monolayer images were digitized for 0, 4, 8, 12, 16, 20, and 24 h after a small wound was made and the wound area was calculated by image pro-plus system automatically. Closure of monolayer wounds in 16HBE14O cells from different groups was recorded. The RI was calculated from the linear regression equation and used to compare the repair speed.

Integrin β4 silencing induced apoptosis in 16HBE14O cells

To assess whether integrin β4 silencing contributed to the apoptosis of 16HBE14O cells, we performed annexin V/PI staining followed by fluorescence-activated cell sorting analysis to detect cell apoptosis, as shown in **Fig. 6(A)**. The subpopulations of live, early apoptotic, late apoptotic, and dead cells were separated and calculated by different cell staining as described above. For the parental 16HBE14O cells and control siRNA vector transfected cells, 80% are live; whereas for integrin β4-silenced cells, the percentage was <50%. Furthermore, there were more early and late apoptotic cells in the integrin β4-silenced group, i.e. 1.69 and 3.58 fold compared with that in the control siRNA vector transfected group, respectively. For all three groups, there were little cells that belong to the dead subpopulation [**Fig. 6(B)**]. To better understand the long-term effects of integrin β4 silencing on cell apoptosis, the percentage of later apoptotic cells along with the time after integrin β4 silencing was plotted. For the parental

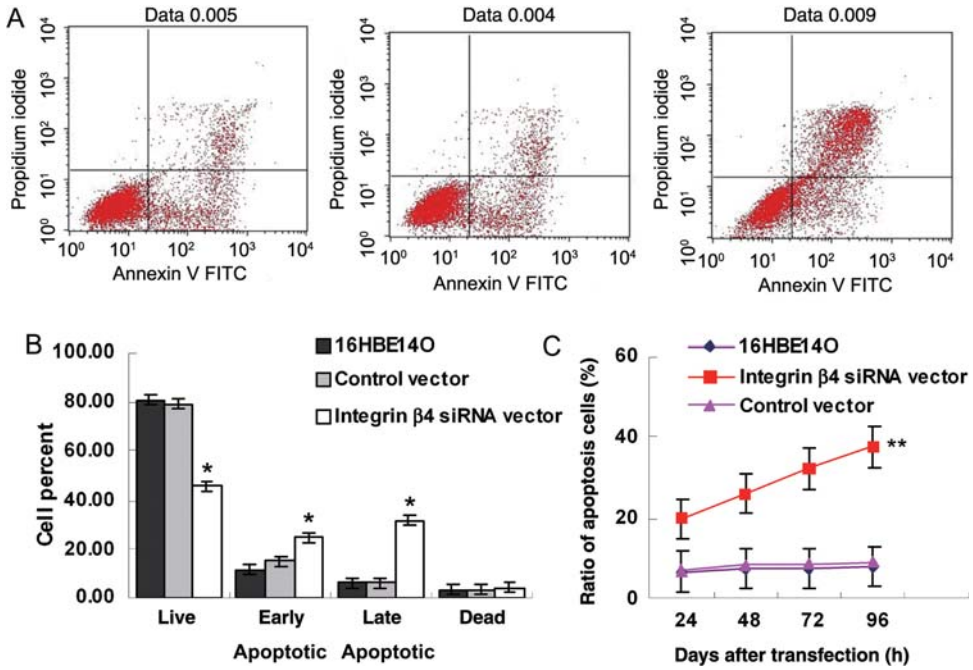

Figure 6 Integrin β4 silencing mediate apoptosis (A) Annexin V/FITC and PI staining by flow cytometry analysis. The subpopulations of live, early apoptotic, late apoptotic, and dead cells are stained differently and calculated. (B) The proportion of different staining cells was shown. (C) The time course of late apoptosis was taken after integrin β4 was silenced. The cell percent of late apoptosis was recorded at 24, 48, 72, and 96 h after *integrin β4* was silenced. $^{**}P < 0.01$; $^{*}P < 0.05$.

16HBE14O cells and the control siRNA vector transfected cells, the percentage of later apoptotic cells was always <10% at all time points. In contrast, for integrin β4-silenced cells, the percentage increased from 20 (24 h after integrin β4 silencing) to 40% (96 h after integrin β4 silencing). In summary, stronger late apoptosis signals were detected in the integrin β4-silenced cells after serial subcultivation ($P < 0.01$, $n = 5$, ANOVA) [**Fig. 6(C)**].

Discussion

Asthma was characterized with significant structural damage and disordered physiological functions of airway epithelia [19,20]. Structural damage, exaggerated reactions to allergens, differential cytokine expression and irregular airway remodeling, and repair were common in airway epithelial cells of asthma patients [8,21,22]. However, the mechanism to the pathological alteration of asthma airway epithelia was still unclear.

Adhesion molecules were important proteins that expressed on many epithelial cells to maintain structural and chemotactic adhesion. Previous studies have identified the possible correlation between adhesion molecules and lung diseases such as asthma [11]. Our previous work used a cDNA microarray analysis to survey genes that were differentially expressed in the peripheral leukocytes of asthma patients. The results showed that integrin β4 was downregulated significantly in adult atopic asthma patients [12]. Further, amplification and sequencing study found that there were three variation sites in 5′ flanking region of *integrin β4* in asthma patients, which were correlated with decreased expression of integrin β4. Integrin β4 was a member of integrin proteins family that plays an important role in keeping the structural adhesion and integrity of airway epithelial cells [13]. In general, each integrin is composed of a single α-subunit and a single β-subunit. The α6 subunit is the only ligand for the β4 integrin that was expressed as integrin α6β4 heterodimer on airway epithelia. Structural adhesion of airway epithelial cells relied on the expression of integrin β4 that can 'anchor' epithelial cells on extracellular matrix (ECM) in a hemidesmosome style [15]. Therefore, the interaction between ECM and integrins played an important role in maintaining the adhesion and structural integrity of airway epithelial cells, while the abnormal expression of integrins could result in the pathogenesis of many lung diseases. For example, in asthma patients, ECM protein alteration has been observed to be contributed to the airway remodeling and decreased pulmonary function of asthma patients [21,22].

In this study, we further detected that in both extracellular and cytoplasmic domains, integrin β4 expression decreased significantly on the airway mucus of asthma patients. Integrin β4 has traditionally been deemed an important adhesion protein for epithelial cells. In addition, the β4 subunit is unique among the other integrin β subunits because of its very long cytoplasmic domain. Progress has been made in identifying specific regions and motives within the integrin β4 cytoplasmic domain that mediate the cytoskeleton interactions and signaling properties of integrin β4, but much remains to be determined on airway epithelial cells [23]. So we sought to explore the relationship between reduced expression of integrin β4 and the pathological phenotypes of the airway epithelial cells on asthma airway. Accordingly, a specific integrin β4 siRNA lentivirus vector was constructed and transfected into 16HBE14O cells to silenced integrin β4.

Upon silencing of integrin β4, 16HBE14O cells showed reduced proliferation ability, and cell cycle analysis also revealed G1 phase arrest. These results strongly suggested that integrin β4 was an important regulator of the cell cycle for airway epithelial cells. It has been shown that primary epithelial cells from asthma airway were less differentiated and their proliferation was inhibited [24]. Our study observed a decreased integrin β4 expression, and inhibited proliferation ability of airway epithelial cells was induced by integrin β4 silencing. Therefore, we further suspect that decreased expression of integrin β4 may be responsible for the inhibited proliferation ability of epithelial cells on asthma airway. It brought a new possible mechanism to the inhibited proliferation ability of epithelial cells on asthma airway.

Meanwhile, after integrin β4 was silenced, the wound repair ability of airway epithelial cells was blocked. Repair of the damaged epithelia was a crucial step in maintaining the structural integrity and functional homeostasis of airway epithelia. In the airway epithelia of healthy adults, expression of β4 was mainly restricted to basal cells. However, after injury, integrin β4 expression was clearly elevated and could be detected in many cell types, which suggest that integrin β4 might be involved in the repair processes of airway epithelia [13]. Our results further demonstrated that integrin β4 was an important participant in the damage/repair cycle of the airway epithelia cells. It is increasingly apparent that normal repair processes in the asthmatic epithelia were also compromised, inducing a disrupted epithelial structure [2–4,6,7]. However, the molecule mechanism for it was not understood thoroughly. Our study indicated that decreased expression of integrin β4 was related to the damaged wound repair process of epithelial cells on asthma airway. It is a meaningful result to understand the function properties of airway epithelial cells and pathogenesis for asthma.

Moreover, our results showed that integrin β4 silencing could induce more early and late apoptosis signals. After serial subcultivation, stronger later apoptosis signals were

induced in the integrin β4-silenced cells. Trautmann *et al*. [25] first suggested that the loss of integrin β4 may induce apoptosis. Integrin β4-knockout keratinocytes were not only weakly adhesive, but also susceptible to degeneration and, at least in some cases, apoptotic cell death. Freyer and coauthors [26] have also done a nice work on emphasizing the importance of interaction between integrins and ECM. Changes in the matrix factors present within the airway wall during inflammation may alter survival signaling for airway epithelial cells. In asthma patients, using markers of earlier apoptotic events (caspase-3, DNA repair enzymes), some investigators found higher levels of epithelial cell apoptosis than normal controls [27]. However, the mechanism by which apoptosis was induced in asthma was still unclear. Consistent with previous studies, in the integrin β4-silenced 16HBE14O cells, a significantly greater number of early and late apoptosis signals were detected. Our results further verified that the decreased interaction between integrin β4 and ECM could induce the apoptosis of airway epithelial cells. This finding also provided evidence that apoptosis of airway epithelial cells on asthma patients was partly related to the decreased expression of integrin β4. It partly interpreted the functional alteration on airway epithelial cells of asthma patients and hinted an inner connection between structural abnormalities and epithelial apoptosis in asthma airway.

In summary, the mechanisms underlying the fragility and dysfunction of asthma airway epithelia were the main focus of this work. Our study provided interesting evidence that integrin β4 was downregulated on the airway mucus of asthma patients. In addition, the downregulation of integrin β4 on airway epithelia appeared to be correlated with asthma pathogenesis and phenotype. These finding improves our interpretation of asthma pathogenesis, though additional results will be needed to verify these models.

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