Overexpression of ATP5b promotes cell proliferation in asthma

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Abstract. Asthma is a complicated systemic disease of the airways, which is characterized by variable symptoms, including bronchial hyper-responsiveness, inflammation and airflow obstruction. The prevalence of asthma has increased 2-3-fold over recent decades in developed countries; however, the molecular mechanism of asthma remains unclear. In the current study, the expression of recombinant protein *Dermatophagoides farinae*I (Derf I) was induced by isopropyl β-D-1-thiogalactoside (IPTG) and purified using Ni-NTA. Derf I, an important antigen of asthma, was used to establish the animal model of asthma. Airway hyper-responsiveness was measured using unrestrained whole-body plethysmography with a four-chamber system. Immunoglobulin (Ig)E, IgG and IgG2a were analyzed using indirect enzyme-linked immunosorbent assay (ELISA). Proteomic technology was applied to detect the difference between the normal lung tissue and asthma lung tissue samples of the asthma model. Cytokines in bronchoalveolar lavage fluid and the splenocyte culture medium were measured by ELISA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to detect the mRNA expression of ATP synthase, H+ transporting, mitochondrial F1 complex, β polypeptide (ATP5b). In addition, cell growth of arterial smooth muscle cells (ASMCs) was evaluated by MTT assay. In the current study, Derf I was successfully used to construct the animal model of asthma. Out of 23 proteins that exhibit 3-fold upregulation or downregulation, ATP5b was chosen for further investigation. The data indicated that ATP5b was overexpressed in the asthma lung tissue when compared with the normal lung tissue. However, when ATP5b was knocked down, cell growth decreased. Therefore, overexpressed ATP5b leads to airway smooth muscle cell (ASMC) proliferation and finally to ASM thickening. Thus, to the best of our knowledge, this is the first study to report that the expression level of ATP5b was markedly increased in lung tissue samples of an asthma model compared with the tissue samples from normal lungs, which promoted ASMC proliferation and contributed to airway remodeling.

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Introduction

Asthma is a complicated systemic disease of the airways, which is characterized by variable and recurring symptoms, including bronchial hyper-responsiveness, inflammation and airflow obstruction. When an asthma exacerbation occurs, certain symptoms, such as shortness of breath, wheezing, coughing, and chest tightness are uncontrolled (1). Together, the symptoms eventually impair lung function by decreasing the flow of gases to and from the alveoli in the distal lung (2). The prevalence of asthma has increased 2-3-fold over recent decades in developed countries (3-5). It is estimated that asthma affects ~300 million people in the world and causes a quarter of a million mortalities (6). Globally, asthma accounts for approximately one in every 250 mortalities (7), which is a significant cause of morbidity and mortality in developed countries (8). In America, 9-20% of children and 1-3% of adults suffer from asthma (8,9), and the financial burden of asthma is estimated to be >$10 billion (10). Furthermore, there is a high prevalence of ~5% in China (11). Numerous factors, including improved epidemiological reporting, medical care and increased environmental allergens contribute to the increased prevalence. However, the molecular mechanism of asthma remains poorly understood.

Asthma is a chronic airway inflammatory disease, where persistent inflammation in the airway results in structural changes termed airway remodeling (12). Airway smooth muscle cells (ASMCs) confer an abnormally exaggerated bronchoconstriction in asthma, and the phenomenon is commonly referred to as airway hyper-responsiveness (AHR), which is a hallmark characteristic of asthma (13). Airway remodeling encompasses subepithelial fibrosis, deposition of extracellular

matrix (ECM) proteins, increased smooth muscle mass and mucus gland hyperplasia. Generation of growth factors from patients with asthma drive mesenchymal cell proliferation and differentiation toward increased matrix deposition and smooth muscle production (14-16). Airway remodeling is particularly observed in patients with refractory asthma and progressive decline in lung function (17).

Studies demonstrate that airway remodeling is closely connected with the progression of AHR (18).

It has previously been demonstrated that asthma markedly increases Th2-mediated responses and decreases Th1-mediated responses (19-21). Clinically, the majority of asthma patients show a significant increase in ASM bundles, primarily due to increases in cell number, collectively contributing to airway remodeling (12). Furthermore, airway remodeling is progressive and the degree of structural changes correlates with disease severity (18). In this sense, ASMC hyperplasia has been postulated as the predominant mechanism of ASM thickening (22). Simultaneously, increased proliferation results in decreased pulmonary function in asthmatic patients (23). It is critical to further understand the mechanism of airway remodeling in patients with asthma; however, the molecular mechanisms remain unclear. Thus novel targets must be identified to prevent airway remodeling and thus, treat asthma (24).

In the current study, proteomic technology was applied to analyze the difference between normal lung and asthmatic lung tissue samples. In addition, the molecular mechanisms of asthma were detected.

Materials and methods

Animals and materials. A total of 20 female BALB/c mice (specific pathogen-free; aged 5-6 weeks; weight, 16-20 g) were purchased from the Animal Center of Guangdong Province (Guangzhou, China) and fed in a specific pathogen-free grade breeding room (separated into two groups of ten, bred separately and conventionally in cages, at a temperature of 18-22˚C, 50-60% humidity and 10-14 h lighting with an air flow of 10-25 cm/min).

The present study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Shenzhen University (Shenzhen, China). Reagents and chemicals were analytical grade (Sigma-Aldrich, St. Louis, MO, USA) and solvents were HPLC grade (Mallinckrodt Australia Pty Ltd., NSW, Australia). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed using a Mini Protean II apparatus (Bio-Rad Laboratories, Inc., Hercules, California, USA) with 12% gels and a Tris/Tricine buffer system. Two-dimensional PAGE was performed using Immobiline IPG strips (13 cm) with a pH range of 3-10 (GE Healthcare Life Sciences, Shanghai, China). $Al(OH)_3$ and methacholine were purchased from Sigma-Aldrich. Biotin-labeled goat anti-mouse immunoglobulin IgE was purchased from Novus Biologicals, (Colorado, USA), horseradish peroxidase (HRP)-labeled goat anti-mouse IgG and IgG2a were obtained from eBioscience, Inc. (San Diego, CA, USA). Mouse interleukin [IL; IL-4 (catalog no. 431104) and IL-10 (catalog no. 431414)], and interferon (INF)-γ enzyme-linked immunosorbent assay (ELISA) kits (catalog no. 430801) were obtained from BioLegend, Inc. (San Diego, CA, USA). The vector of pET-28b/*Dermatophagoides farinae*I (Derf I) was constructed in the State Key Laboratory of Respiratory Disease for Allergy, Shenzhen University (Shenzhen, China).

Expression and purification of recombinant protein Derf I. Expression of recombinant protein, Derf I was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside at mid-log phase at 37˚C and the samples were collected 4 h post-induction. Harvested induced cells were lysed with 10 mg/ml lysozyme in Tris buffer (100 mM NaH_2PO_4 and 10 mM Tris-Cl) and homogenized by sonication. Inclusion bodies were collected by centrifugation at 12,000 x g for 20 min at 4˚C and then washed three times with Tris buffer containing 0.5% (v/v) Triton X-100. Inclusion bodies, solubilized in 6 M GuHCl, were purified by Ni-NTA at 4˚C. The recombinant protein was electrophoresed (110 V) on 12% (w/v) SDS polyacrylamide gels and its protein concentration was determined using the bicinchoninic acid assay (AppliChem GmbH, Darmstadt, Germany) according to the manufacturer's protocol.

Sensitization and asthma challenge. The animal sensitization model was designed for the following experiment. Briefly, the mice were randomly divided into two groups (20 mice per group). The model groups were sensitized intraperitoneally (I.P.) with 50 μg Derf I with 4 mg Al $(OH)_3$ on days 1, 3 and 7, while the normal group was sensitized, challenged and treated with phosphate-buffered saline (PBS) and 4 mg $Al(OH)_3$. The model groups were induced with 50 μg/ml Derf I every day for one week, which was substituted with 50 μg/ml PBS in the normal group.

Assessment of AHR to methacholine challenge. Twenty-four h after the final challenge, AHR was measured using unrestrained whole-body plethysmography with a four-chamber system (Buxco Research Systems, Wilmington, NC, USA) as previously described (25).

PAGE and mass analysis. The recombinant protein Derf I was diluted with an identical volume of lysis buffer (0.5L) as follows: 9 mol/l Urea, 0.8% IPG buffer (pH, 3-10), 1% dithiothreitol (DTT) and 2% 3-[(3-cholamidopropyl) dimethylammonio] propane-1-sulphonic acid. Subsequently, PAGE and mass analysis were performed as described by Cheng *et al* (26) and Li *et al* (27).

Determination of allergen-specific IgE, IgGand IgG2a antibodies in serum. Blood samples (1 ml) were obtained from the eyes of the mice, and centrifuged at 4˚C and 324 x g for 10 min. The supernatant serum was collected and the IgE, IgG, and IgG2a were subsequently analyzed using an indirect ELISA protocol according to the manufacturer's protocol.

Bronchoalveolar lavage fluid (BALF) and differential cell counts. BALF was collected as previously described (28) with

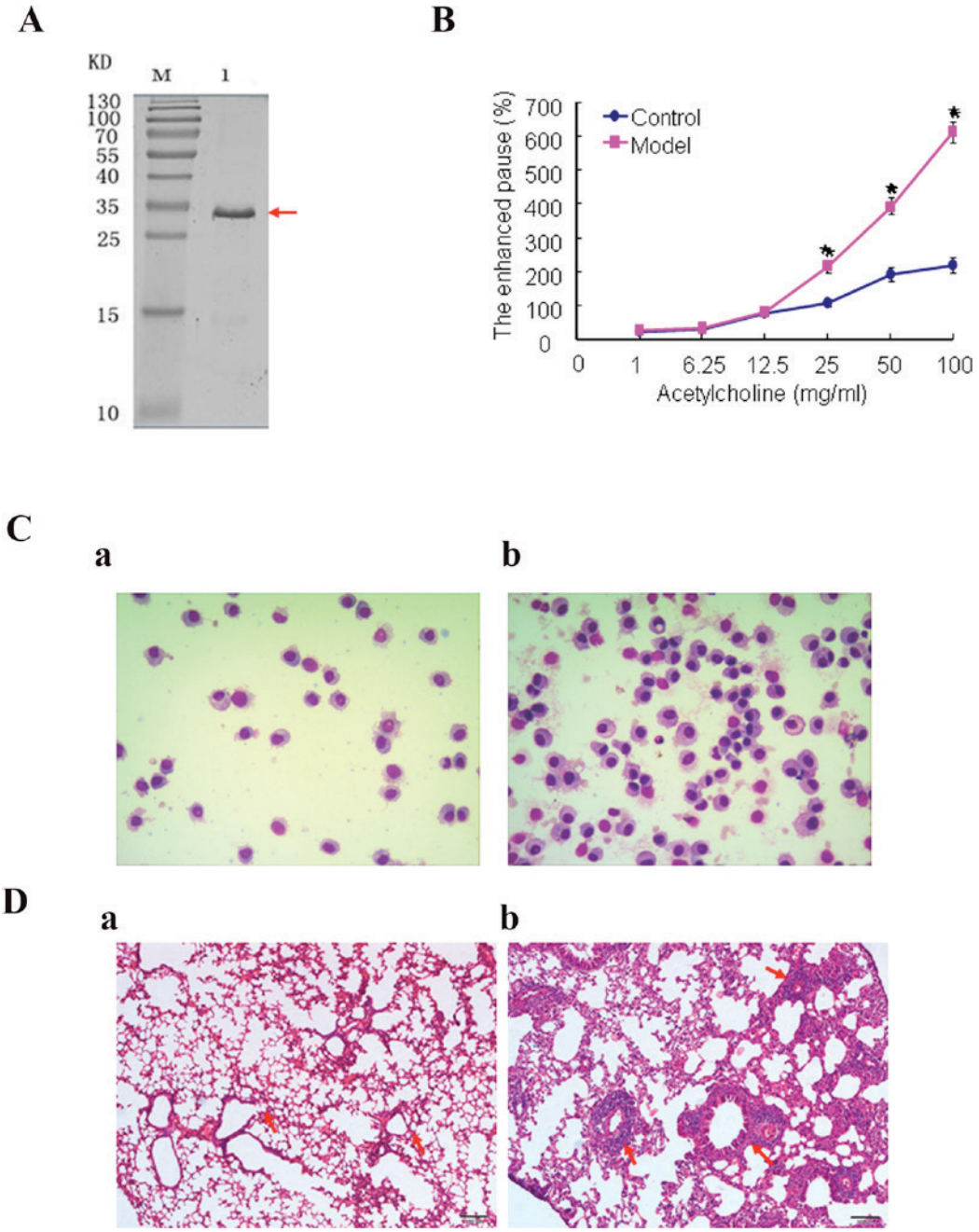

Figure 1. Identification of the murine model of allergic asthma induced by the Derf I protein. (A) 12% SDS-PAGE analysis of the purified Derf I protein (lane M, protein marker). (B) Airway hyper-responsiveness assay for the mice (*P<0.05 vs. control). (C) Representative images of total inflammatory cells and eosinophils in bronchoalveolar lavage fluid in the (Ca) normal and (Cb) asthma model groups. (D) Formalin-fixed lung sections were stained with hematoxylin and eosin in the (Da) normal and (Db) asthma model groups. Red arrows indicate inflammatory cells (Scale bar, 100 μm). Derf I, *Dermatophagoides farinae* I.

slight modifications. Briefly, mice were sacrificed by euthanasia, and BALF was obtained using 1 ml PBS containing protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). Cytospin preparations were prepared and counted using Liu's stain under an optical microscope. Cells were classified as eosinophils according to morphologic and histologic criteria (29).

Measurement of cytokines in BALF and the splenocyte culture medium. The splenocyte cells from the mice were cultured in RPMI-1640 medium with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 24-well plates at a density of $5x10^4$/well, stimulated with the recombinant Derf I protein (200 μg/well), and incubated at 37˚C for 72 h. The IL-4, IL-10 and INF-γ levels in the splenocyte culture medium and BALF were detected using an ELISA kit (catalog no. 421701, Biolegend, Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was performed to detect the mRNA expression of ATP5b on an Applied Biosystem 7000 Sequence Detection System (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which was performed as described by Ding *et al* (28) and Zuo *et al* (30). Five micrograms of total RNA from each sample was reverse transcribed to cDNA using an A3500 RT system (Promega Corporation, Madison, WI, USA). The following

Table I. Total cell number and number of eosinophils in bronchoalveolar lavage fluid.

Cell (x10^4/ml)	Control group	Model group
Total cells	20.30±3.35	64.50±5.08[a]
Eosinophils	0	15.32±3.04[a]

[a]P<0.05.

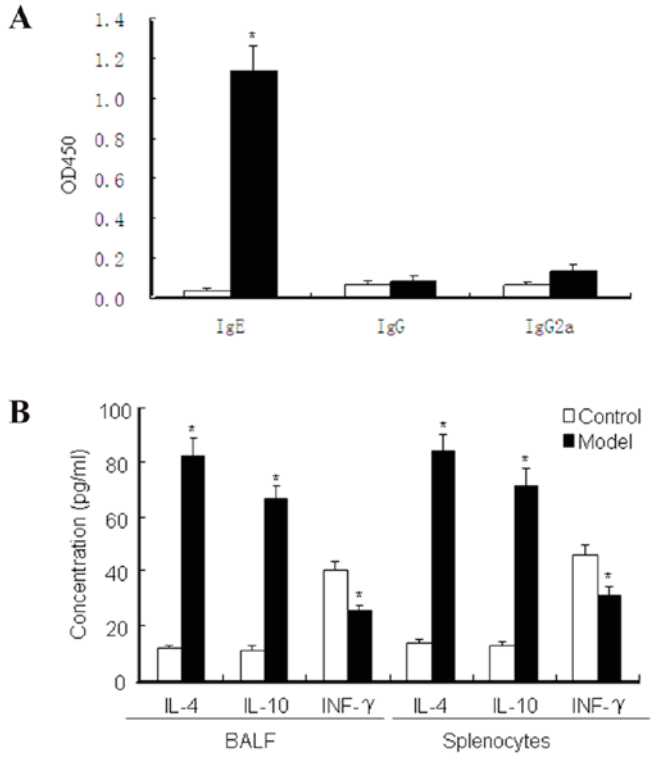

Figure 2. Allergic asthma model group induced with the *Dermatophagoides farinae*I protein exhibited an allergen-specific Th2 immune responses. (A) Allergen-specific antibodies in serum. (B) Cytokine production in spleen cell cultures and BALF, *P<0.05 vs. control. OD, optical density; Ig, immunoglobulin; IL, interleukin; INF, interferon; BALF, bronchoalveolar lavage fluid.

forward and reverse primers were used: Forward, AGTTGC TGAGGTCTTCACGG and reverse, CTTTGCCACGGCTTC TTC for ATP5b; and forward, TGGCAGAGATGCGTGGAGA and reverse, GGCAAGTCTTCCGAGTAGTTTT for GAPDH. The PCR conditions were as follows: 5-mindenaturation at 95˚C followed by 40 cycles at 95˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 30 sec. Amplification of the target gene was monitored as a function of increased SYBR-Green. I (Qiagen GmbH, Hilden, Germany) fluorescence. An analysis threshold was set, and the cycle threshold (Cq) was computed for each sample (31). The comparative difference in gene expression was then determined.

RNA interference. siRNA targeting human ATP5b was delivered into ASMCs using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. In addition, a non-targeting siRNA pool (Dharmacon, Inc., Lafayette, CO, USA) was used at the same concentration as a control for the RNA interference assays. After 48 h of transfection, cells were subjected to MTT assays or RNA were collected and analyzed by RT-qPCR.

MTT assay. ASMCs were seeded into96-well culture plates at adensity of 500 cells/well with scramble siRNA-ATP5b and siRNA-ATP5b. After 48 h, the cell culture was removed and 20 μl of MTT was added to each well. The cells were further cultured at 37˚C for 4 h, and the cell culture was removed and 150 μl DMSO was added to dissolve crystals completely by pipetting up and down. A microplate reader (SynergyH1; Bio-Tek Instruments, Inc., Winooski, VT, USA) measured the corresponding absorption value at a wavelength of 562 nm indicated cell proliferation as the number of live cells was proportional to the optical density value.

Statistical analysis. The data are expressed as the mean ± standard deviation. Student's t-test was used for the statistical analysis of interval data and P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of the murine model of allergic asthma induced by DerfI. In the current study, DerfI as an important antigen of asthma was applied to construct the asthma model. Purified DerfIprotein was obtained (Fig. 1A). AHR changes were assessed by methacholine challenge 24 h after the final allergen challenge. The results (Fig. 1B) demonstrated that AHR in the model groups was significantly greater (P<0.05) than in the normal control group. In addition, the AHR of the mice increased with the increasing concentration of methacholine. Fig. 1C and Table I showed that the total counts of cells and inflammatory cells in the BALF sampled from the model groups were significantly greater than those in the normal group (P<0.05), particularly the eosinophils. As shown in Fig. 1D, lung tissue samples from the model group were damaged by internal hemorrhage and edema. The bronchial and vascular walls became thickened and infiltrated by a marked number of inflammatory cells. In addition, the trachea in the models was filled with markedly more mucus than the normal control group. However, lung tissue structures in the normal group were well defined without discernible damage or edema, and the trachea and blood vessels were not infiltrated peripherally by inflammatory cells. Thus, the asthma model had been successfully induced.

Allergen-specific T helper (Th)2 immune responses induced by the DerfI protein. Serum IgE, IgG and IgG2a levels were measured by ELISA to evaluate the asthma model. The results (Fig. 2A) showed that the levels of serum IgEin the asthma model group were significantly higher than those in the normal control group (P<0.05). Furthermore, the concentrations of IL-4, IL-10 and IFN-γ in the splenocyte culture medium supernatant and BALF were detected by ELISA. The asthma model groups (Fig. 2B) exhibited allergen-specific Th2 immune responses, where by IL-4 and IL-10 levels were evidently upregulated compared with the normal control group (P<0.05). However, the IFN-γ level in the asthma model

Table II. Analysis of mass spectral data.

Spots	Master no.	Score	NCBInr no.	Protein name	Expression
1	701	382	gil7331218	TPA exp: keratin Kb40	-
2	1103	240	gil13775198	SH3 domain-binding glutamic acid-rich-like protein 3	+
3	2304	269	gil55291	Alpha-tubulin isotype M-alpha-2	+
4	2602	612	gil26344461	Unnamed protein product	+
5	2606	1106	gil23272966	Atp5b protein	+
6	3302	500	gil6678281	Indolethylamine N-methyltransferase	-
7	3401	391	gil2437840	Annexin III	-
8	3604	646	gil22164798	Selenium-binding liver protein	+
9	3701	306	gil191765	Alpha-fetoprotein, partial	-
10	3702	711	gil26340966	Alpha-fetoprotein, partial	+
11	3803	94	gil26080429	Aldehyde dehydrogenase family 16 member A1	-
12	4605	459	gil468546	CCT (chaperonin containing TCP-1) beta subunit	-
13	4805	643	gil199765	Moesin	-
14	5307	135	gil7242156	Acyl-protein thioesterase 2	+
15	6304	163	gil54855	Triosephosphate isomerase	-
16	6307	167	gil6680121	Glutathione S-transferase Mu 2	+
17	6405	274	gil293317	Adenylyl cyclase-associated protein	+
18	6702	590	gil62653546	Glyceraldehyde-3-phosphate dehydrogenase-like	+
19	6703	859	gil6678359	Transketolase	+
20	8303	125	gil424143	Heat shock protein HSP27	-
21	8403	569	gil62653546	Glyceraldehyde-3-phosphate Dehydrogenase	+
22	8408	186	gil1125026	3-hydroxyacyl CoA dehydrogenase	+
23	9301	507	gil6671688	Carbonyl reductase [NADPH] 2	+

Table III. Mascot scores of representative ATP5b protein matched peptides.

1	MLSLVGRVAS	ASASGALRGL	SPSAALPQAQ	LLLRAAPAGV	HPARDYAAQA
51	SAAPKAGTAT	GRIVAVIGAV	VDVQFDEGLP	PILNALEVQG	RDSR**LVLEVA**
101	**QHLGESTVR**T	IAMDGTEGLV	RGQK**VLDSGA**	**PIKIPVGPET**	**LGRIMNVIGE**
151	**PIDER**GPIKT	KQFAPIHAEA	PEFIEMSVEQ	EILVTGIKVV	DLLAPYAKGG
201	KIGLFGGAGV	GKTVLIMELI	NNVAK**AHGGY**	**SVFAGVGER**T	REGNDLYHEM
251	IESGVINLKD	ATSK**VALVYG**	**QMNEPPGAR**A	R**VALTGLTVA**	**EYFR**DQEGQD
301	VLLFIDNIFR	FTQAGSEVSA	LLGR**IPSAVG**	**YQPTLATDMG**	**TMQER**ITTTK
351	KGSITSVQAI	YVPADDLTDP	APATTFAHLD	ATTVLSR**AIA**	**ELGIYPAVDP**
401	**LDSTSRIMDP**	**NIVGNEHYDV**	**AR**GVQKILQD	YKSLQDIIAI	LGMDELSEED
451	KLTVSRARKI	QRFLSQPFQV	AEVFTGHMGK	LVPLKETIKG	FQQILAGEYD
501	HLPEQAFYMV	GPIEEAVAKA	DKLAEEHGS		

Matched peptides presented in bold. The peptide sequences obtained by mass spectrometry analysis were compared with the representative ATT5b.

group was significantly lower than that in the normal control group (P<0.05), which enhances inflammatory and immunosuppressive function. These data demonstrated that Derf I protein disturbed the Th1/Th2 balance, which promoted the Th2 immune response and suppressed the Th1 response.

Mass spectral analysis and identification of ATP5b. A total of 23 monoisotopic peaks were input into the Mascot search engine to search the Swiss-Prot database (http://www.gpmaw.com/html/swiss-prot.html). The annotation of all the identified proteins is summarized in Table II. The query result showed that protein spot 2606 is the ATP5b protein. In addition, the database query result and mascot score of a representative ATP5b protein are demonstrated in Table III and Fig. 3.

ATP5b was identified as an overexpressed protein and is involved in cell proliferation. It was further verified by RT-qPCR that ATP5b was expressed in the asthma model

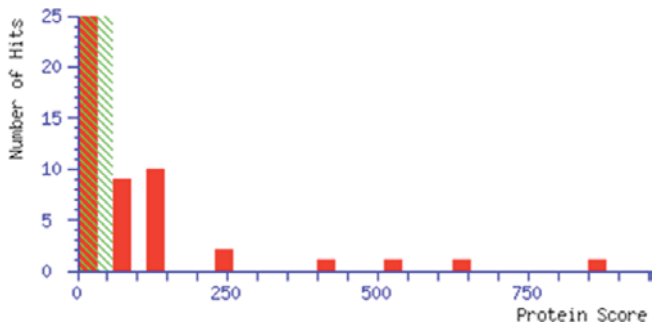

Figure 3. Mass spectral analysis of protein spot 2606. Database query result and score of protein spot 2606. Mascot Score Histogram. Ions score is-10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores >58 indicate identity or extensive homology (P<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

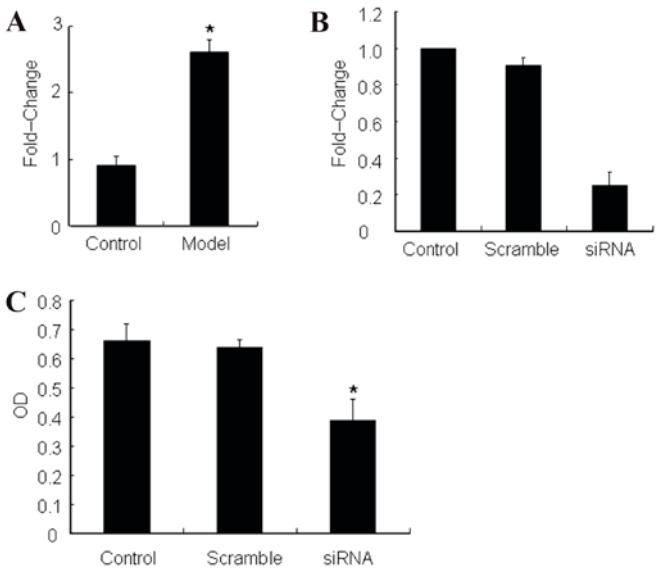

Figure 4. Overexpression of ATP5b protein was observed and ATP5b silencing decreased cell proliferation. (A) ATP5b was verified to be highly expressed in the asthma model using reverse transcription-quantitative polymerase chain reaction (*P<0.05 vs control). (B) ATP5b was knocked down in ASMCs. (C) Silencing of ATP5b decreased cell proliferation of ASMCs (*P<0.05 vs.control). ATP5b, ATP synthase, H^+ transporting, mitochondrial F1 complex, β polypeptide; OD, optical density.

mice to a significantly greater extent when compared with the normal control mice (Fig. 4A). In order to confirm the role and function of ATP5b, the experiments were designed to silence ATP5b expressionin ASMCs and detect its effect on cell proliferation (Fig. 4B). The data indicated that silencing of ATP5b inhibited ASMC growth by MTT (Fig. 4C). Thus, overexpression of ATP5b enhances ASMC proliferation.

Discussion

Asthma is an inflammatory disease of the airways. Sensitivity to asthma is determined by many factors, such as multigenic predisposition, aberrant immune response and environmental factors (32).

More than 50% of asthma cases are caused by allergies to environmental allergens (33). Clinicians may effectively intervene in these factors to prevent the development of asthma (34). One of the most frequent allergens associated with asthma is dust mite, as 10% of individuals with asthma are allergic to it (35). Therefore, dust mite is often used to induce experimental asthma in mice models that closely resemble human asthma (36,37). Therefore, in the current study, Derf I was selected as the important dust mite antigen to construct the asthma model.

In the present study, the recombinant protein, Derf I was used to create the asthma model. The results demonstrate that construction of the animal model was successful, as the typical symptoms, including persistent inflammation, hyper-reactivity and remodeling of the airways (involving smooth muscle thickening, mucus overproduction and sub epithelial thickening) were observed. There are certain characteristics of airway inflammation in asthma resulting from bronchial wall infiltration due to eosinophils, T lymphocytes and activated mast cells. The current data show that the model groups had specific Th2 immune responses, where IL-4 and IL-10 levels were evidently upregulated compared with the normal control group (P<0.05). However, the IFN-γ level in the model mice was significantly lower than that of the control mice (P<0.05). The above data indicated that the DerfI protein promoted the Th2 immune response and suppressed the Th1 response. Our results are consistent with those of Mihaltan reports that cytokines from Th2 cells are key in mediating airway inflammation (38).

In the current study, ATP5b was observed to be overexpressed in the asthma model. Recent studies show that ATP5b is a subunit of a mitochondrial ATP synthase complex (39). ATP5b, as a receptor for various ligands, is involved in biological processes, such as metabolism of lipid formation, regulation of proliferation, differentiation and recognition of immune responses of tumor cells (39,40). The present study indicated that ATP5b overexpression leads to ASMC proliferation, since rapidly proliferating cells have a high demand for energy and glycolytic intermediates for anabolic processes. Furthermore, certain glycolytic enzymes directly participate in pathways of proliferation as ATP5b increases ATP extracellularly. However, when the ATP5b was knocked down in ASMCs, decreased cell growth was observed. The present results are consistent with the study by Huang *et al* (40), which reports that targeting breast cancer cell lines with ATP5b inhibitor, aurovertin B decreases proliferation (40,41).

In the present study. The results of proteomics and RT-qPCR demonstrated that, compared with normal control lung tissue samples, the lung tissue samples of the asthma mouse models overexpressed ATP5b, leading to ASMC proliferation and ultimately to ASM thickening. Thus, to the best of our knowledge, this is the first study to report that ATP5b overexpression promoted the proliferation of ASMCs and contributed to airway remodeling.

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