Characterization of the human mucin 5AC promoter and its regulation by the histone acetyltransferase P300

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Abstract. Histone acetylation is important in the modification of gene transcription in asthma and is regulated by histone acetyltransferases (HATs). P300 (P300 HAT) is an enzyme that is able to acetylate a wide variety of proteins. The modification of core histones can further regulate gene transcription, cell proliferation and other cell processes. Airway mucus hypersecretion is one of the most serious pathophysiological symptoms of chronic airway inflammatory diseases, and the human mucin 5AC (MUC5AC) gene has been reported to be a major component of respiratory secretions related to asthma and chronic obstructive pulmonary disease. In the present study, the 5' sequence of the human MUC5AC gene with a 1,348-bp DNA sequence was amplified from human A549 cells genomic DNA by polymerase chain reaction (PCR), and the product of the PCR was sequenced. By promoter deletion analysis, five promoter segments with different lengths were amplified by PCR. The products were identified by DNA sequencing and the six promoter segments were inserted into pGL3-enhancer vectors. The core promoter area was identified with a series of 5' deletion promoter plasmids using luciferase reporter assays. MUC5AC promoter activity, and the mRNA and protein expression levels of MUC5AC were observed in P300 wild-type, P300 mutant, P300 small interfering RNA and P300 control groups. The results showed that the core promoter area of MUC5AC was located within the -935/+48 region and that P300 reduced the expression of MUC5AC in A549 cells.

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Introduction

Asthma is a chronic airway disease that affects >300,000,000 individuals worldwide (1). The prevalence of atopy and asthma has markedly increased in developing countries in recent years (2). The annual worldwide mortality rate of asthma has been reported to be ~250,000 and it is also a substantial economic burden (1).

Failure to clear bronchial secretions is the main pathological reason for asthmatic lung disease. The primary cause for asthma-associated mortality is intraluminal airway asphyxiation of mucus plugs (3,4). Defective mucociliary clearance is observed even in mild stable asthma (5-7). A decrease in clearance causes acute exacerbation (8).

In total, 21 genes are reported to encode mucins in the human genome. Mucin 5AC (MUC5AC) is expressed at high levels in the airway system (9,10). The expression of MUC5AC varies in airway diseases, including asthma, with an increase in the number of goblet cells. Mucus may alter the normal structure and status of goblet cells after failing to incorporate with MUC5AC. Without the normal reaction between MUC5AC and mucus, the airway viscoelasticity becomes vulnerable to plugging (11-13).

Current evidence shows that MUC5AC is regulated by several factors. Previous studies have found that interleukin-13 can increase the expression of MUC5AC in epithelial cell lines and in murine models (14-17). Epidermal growth factor receptor (EGFR) signaling increases the expression of MUC5AC and the expression of MUC5AC can be inhibited with an EGFR tyrosine kinase inhibitor (17). Previous studies have also shown that toll-like receptor 2 is related to the expression of MUC5AC in asthmatic models (18) and corticosteroids have been demonstrated to decrease mucus in airways and further decrease the expression of MUC5AC (19).

In the pathogenesis of asthma, epigenetic modifications are important environmental and genetic factors. In particular, the reversible process of histamine acetylation is mediated by histone acetyltransferases (HATs) and histone decarboxylases (HDACs); histone acetylation is closely associated with gene transcription and controlled by HATs.

P300 (EP300 or KAT3B) consists of 2,414 amino acids and its full length is 300 kDa. It was first cloned in 1994 (20,21) in studies aimed at identifying proteins that bind E1A, an

adenoviral oncogenic transcription factor. P300 was found to have HAT activity and to acetylate a number of proteins (22). Studies have shown that the gain-of-function in P300 can be induced by mutation, the overexpression of P300 or several disease etiology conditions, such as diabetes, obesity, thrombocytopenia or hypertrophic heart diseases. Although, a P300 inhibitor has not been used in clinical trials (23), P300 inhibitors are considered potential therapeutic applications in several diseases.

In the present study, it was hypothesized that P300 may suppress the production of MUC5AC. The MUC5AC promoter was constructed and its activity was investigated with dual-luciferase assays. The study then observed the effects of the HAT protein P300 on the transcription of MUC5AC in adenocarcinomic human alveolar basal epithelial cells and examined the associated mechanisms.

Materials and methods

Cell culture. The A549 lung carcinoma cell line and Beas-2b cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (both Thermo Fisher Scientific, Inc., Waltham, MA, USA) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). The cells were maintained at 37˚C in a 5% CO_2 humidified chamber.

Plasmids and small interfering RNA (siRNA). The DNA sequence (-1,300 to +48) of the MUC5AC promoter region was amplified by polymerase chain reaction (PCR) and digested with *Kpn*I and *Bgl*II (Thermo Fisher Scientific, Inc.). PCR was performed by using genomic DNA which was extracted from the A549 cells using a TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd., Beijing, China), DNA polymerase (LA Taq), dNTPs, GC Buffer (Thermo Fisher Scientific, Inc.) and forward and reverse primers. The forward primers contained a *Kpn*I restriction site, and the reverse primer contained a *Bgl*II site. Sequences of primers were as follows: Forward, 5'-CGGGGTACCCTA CCCATTCACATTTTCCCCATCC-3' and reverse, 5'- GGA AGATCTGGGACCAAGCTGAGCTCTGC-3'. PCR was performed with the following thermocycling conditions: 94˚C for 5 min followed by 30 cycles of amplification (94˚C, 30 sec; 60˚C, 30 sec; 72˚C, 2 min) and followed by a final extension at 72˚C for 10 min. Subsequently, it was subcloned into the promoter-less luciferase expression plasmid, pGL3-Basic (Promega Corporation, Madison, WI, USA). The resulting plasmid was termed pGL-1300/+48. Truncated plasmids of the MUC5AC promoter were constructed using pGL-1300/+48 as a template. *Kpn*I and *Xho*I double digestions were performed on the MUC5AC promoter fragments of different lengths obtained from the above PCR reaction, and pGL3-enhancer vector. The vector and the target fragments were linked using a T4 ligase; competent DH5α cells (Tiangen Biotech Co., Ltd.) were transformed, positive clones were selected, and verification was conducted using restriction endonuclease analysis and DNA sequencing. The successfully constructed plasmids were named pGL-1300/+48, P2: pGL-935/+48, P3: pGL-583/+48, P4: pGL-205/+48, P5: pGL-116/+48, and P6: pGL-23/+48. The siRNAs were synthesized and purified (Shanghai GenePharma, Co. Ltd., Shanghai, China). The targeted sequence was designed to silence P300 gene transcription with the sequence 5'-GUCCUGGAUUAGGUU UGAUTT-3'. The control siRNA sequence was 5'-AUCAAA CCUAAUCCAGGACTT-3'.

Transient transfection and luciferase assays. The p300 mutant (p300 mut; P300Δ1472-1522) and wild-type (p300 wt) plasmids were provided by Professor Zhou Guoping (Fourth School of Clinical Medicine, Nanjing Medical University, Nanjing, China) and were originally described by Boyes *et al* (24). siRNA transfection in the A549 cells was performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Transient transfection was performed following 24 h cell adaptation on 96-well plates ($1.5x10^4$ cells/well).

In the ectopic overexpression experiments, the cells were co-transfected with the P300 wt or P300 mut plasmids, and the pGL-935/+48 reporter plasmid. Following transfection for 24 h, the cells were collected for the luciferase assays. For the P300 siRNA and P300 overexpression experiments, reporter plasmids containing siRNA for P300 or the P300-overexpression plasmid were co-transfected into A549 cells and harvested after 24 h. Subsequently, the luciferase activity was measured with the Dual Reporter assay system (Promega Corporation). All experiments were conducted independently in triplicate.

RNA extraction and reverse transcription-quantitative (RT-q)PCR analysis. RT-qPCR analysis was performed to confirm the expression of MUC5AC in A549 cells. Following transfection for 24 h, the total R NA was extracted from the A549 cells using TRIzol®. The RT-qPCR analysis was performed with the SYBR PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and ABI 7500 Fast system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were synthesized by Changzhou Bo Hong Biological Engineering Co., Ltd. (Changzhou, China). MUC5AC primer sequences were as follows: Forward, 5'-CTGTGAAGGTGGCTGACCAAG A3' and reverse, 5'AAGGTGTAGTAGGTGCCGTCG AA-3'; GAPDH was selected as the control reference with the following primer sequences: Forward, 5'-TGGTATCGT GGAAGGACTCATGAC3' and reverse, 5'TGCCAGTGA GCTTCCCGTTCAGC-3'. cDNA was reverse transcribed by primers using the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) according to the manufacturer's protocol. The reverse transcription cDNA products were stored at -20˚C for PCR amplification. According to the instruction of the SYBR Premix Ex Taq II (Tli RnaseH Plus), the following reagents were added to the PCR reaction mixture: 2 μl cDNA template, 0.8 μl forward primer (10 μM), 0.8 μl reverse primer (10 μM), 10 μl SYBR® Premix EX Taq™ II (2X), 0.4 μl ROX Reference Dye II (50X) and 6 μl ddH2O to make a 20-μl total reaction system. Detection and quantification were performed as follows: Pre-denaturation, 95˚C for 30 sec; 40 cycles of denaturation at 95˚C for 5 sec; and extension, 60˚C for 34 sec. Fluorescence data was collected at the extension step. The relative expression of the MUC5AC gene was analyzed with the $2^{-\Delta\Delta Cq}$ method (25).

Immunofluorescence. The A549 cells were seeded on glass coverslips in 6-well plates ($5x10^4$ cells/well). When the cells reached ~50% confluence, the cells were washed twice with PBS and fixed with 4% paraformaldehyde. Prior to staining with antibodies for immunofluorescence, the cells were blocked with 0.2% Triton, 1% bovine serum albumin and 1% goat serum (Sangon Biotech, Co., Ltd., Shanghai, China) for 30 min at room temperature. Following blocking, the fixed cells were washed twice with PBS and incubated with MUC5AC primary antibody (EPR16904; cat. no. ab198294; 1:250) rabbit anti human monoclonal antibody (Abcam, Cambridge, UK) at 4˚C in a humid chamber overnight. The following morning, the cells were incubated with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (cat. no. ab150077; 1:500; Abcam) for 1 h at room temperature and washed three times prior to observation under a confocal laser fluorescent microscope (Zeiss 710; Carl Zeiss AG, Oberkochen, Germany).

Statistical analysis. Statistical analysis was performed with SPSS software (version 12.0; SPSS, Inc., Chicago, IL, USA). All data are presented as the mean ± standard deviation. Student's t-test was used to analyze the statistical significance between groups. Differences between multiple groups were tested by one-way analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of recombinant plasmid pGL-1300/+48. The pGL-1300/+48 gene fragment was amplified by PCR. The product and the pGL vector were digested using restriction endonucleases *Bgl*II and *Kpn*I. The recombinant plasmid pGL-1300/+48 was constructed by connecting the digested products with DNA ligase. The recombinant plasmid was then identified by PCR. The endonuclease digestion result is presented in Fig. 1. The PCR product and the digested product had a size of ~1,350 bp, as expected. The results indicated that the recombinant plasmid pGL-1300/+48 was successfully constructed. Through agarose gel electrophoresis, the PCR products showed bands at ~983, 631, 253, 164 and 71 bp (Fig. 2). The electrophoresis results of the PCR products were as predicted.

Verification of the recombinant luciferase reporter plasmid. The electrophoresis results of the recombinant luciferase reporter plasmid containing the MUC5AC promoter fragments with restriction enzyme digestions are shown in Fig. 2. The results were as expected, and further sequencing was conducted. The sequencing results were consistent with the designed DNA fragment sequences, which further confirmed the successful construction of a recombinant plasmid of MUC5AC promoter fragments (Fig. 3).

Transcription activation activity of the expression of MUC5AC. The reporter gene plasmids containing regulatory sequences of different lengths of the human MUC5AC promoter region were co-transfected with the internal control plasmid -TK into A549 and Beas-2b cells,

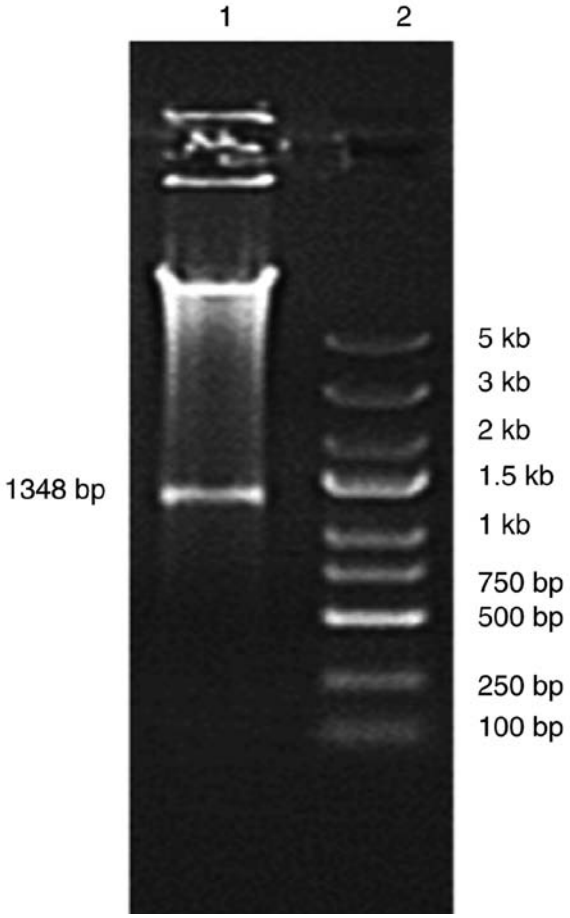

Figure 1. Promoter structure of the mucin 5AC. 1, DL5kb marker; 2, promoter structure.

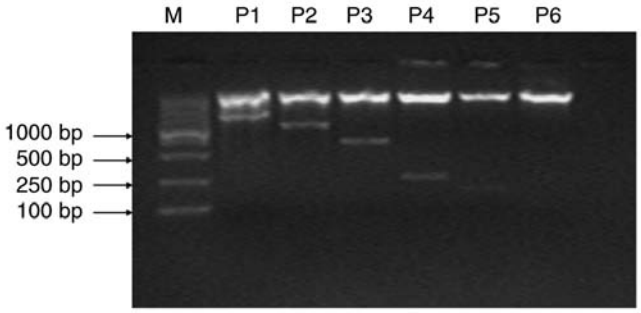

Figure 2. Electropherogram of plasmids containing different lengths of the mucin 5AC gene promoter digested with the restriction enzyme. M, DL5000 marker; P1, pGL-1300/+48; P2, pGL-935/+48; P3, pGL-583/+48; P4, pGL-205/+48; P5, pGL-116/+48 P6, pGL-23/+48.

respectively. The transfected cells were stimulated for 30 min, and the specific luciferase activity was detected. The results showed that the luciferase activity of the recombinant pGL-MUC5AC-935/+48 plasmid was significantly induced (Fig. 4A and B).

mRNA expression of P300. To demonstrate transfection success, the empty vector control, the P300 expression plasmid (P300 wt) and the P300 siRNA, in addition to the control plasmids, were transfected into A549 cells respectively. The transfected cells were stimulated for 30 min and

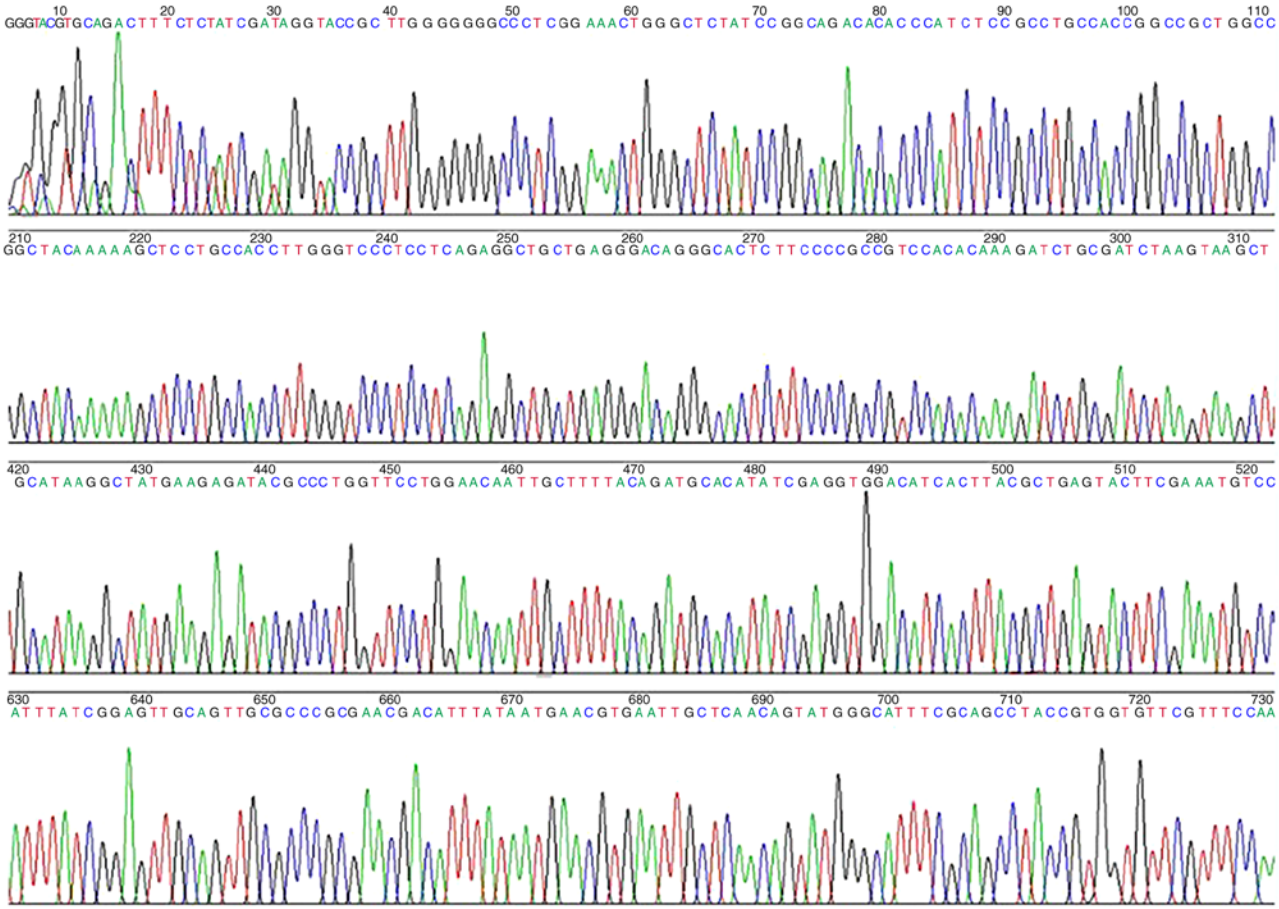

Figure 3. Sequencing analysis of pGL-MUC5AC-1 300/+48.

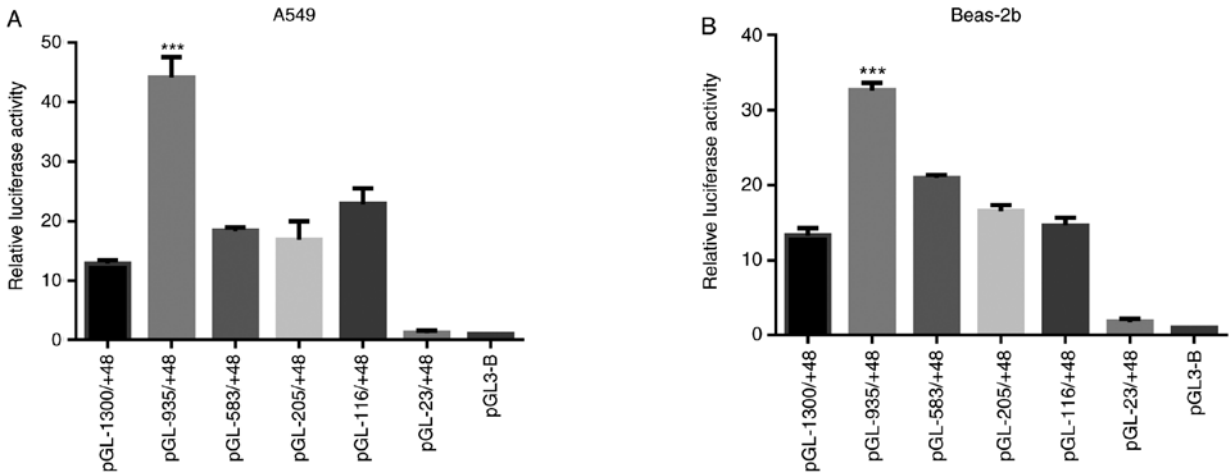

Figure 4. Effect of MUC5AC promoter sequences on luciferase activity in A549 and Beas-2b cells. The human MUC5AC promoters were transfected into (A) A549 and (B) Beas-2b cells. After 24 h, the cells were collected for the luciferase assay. Data were statistically analyzed using a one-way analysis of variance, followed by Tukey's post hoc test. ***P<0.001 between pGL-935/+48 and other MUC5AC promoters. The fold-change of each MUC5AC promoter was determined relative change to PGL3-B. MUC5AC, mucin 5AC; PGL3-B, PGL3-Basic.

the mRNA expression of P300 was detected. The results showed that P300 wt markedly increased the mRNA expression of P300, whereas P300 siRNA inhibited the expression of P300 (Fig. 5A and B).

P300 inhibits MUC5AC promoter activity. In order to determine how P300 regulates MUC5AC promoter activity, the MUC5AC plasmid pGL-935/+48, the empty vector control, P300 wt, P300 siRNA, and the control plasmids were co-transfected into A549 cells, respectively (Fig. 6). By overexpressing P300, the promoter activity was significantly decreased in the A549 cells (Fig. 6A). However, P300 siRNA markedly increased the promoter activity of MUC5AC (Fig. 6B). Taken together, P300 inhibited the expression of MUC5AC by suppressing its promoter activity.

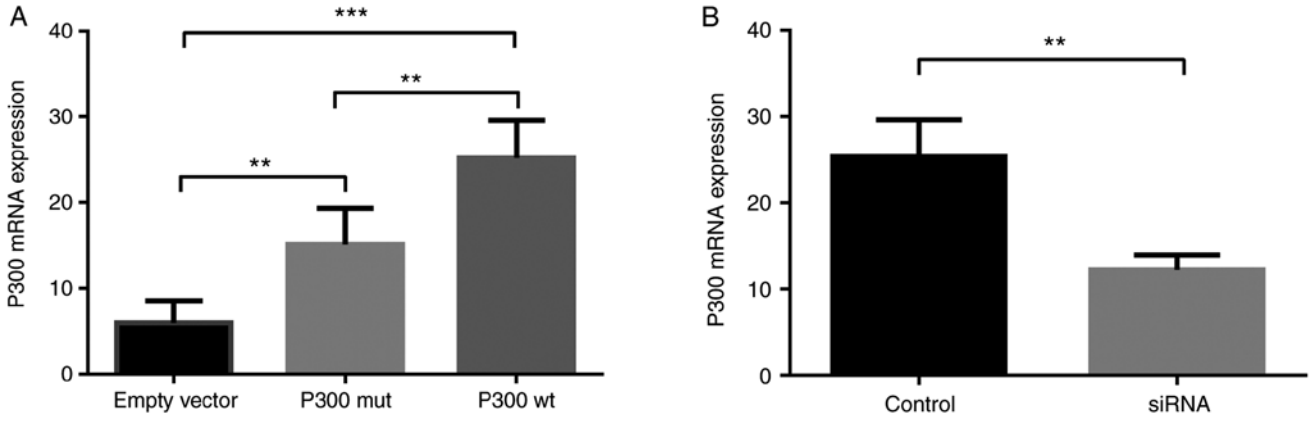

Figure 5. mRNA expression of P300. (A) Luciferase activities were detected following transfection with the empty vector control, P300 wt plasmid or P300 mut plasmid in A549 cells. Data were statistically analyzed using a one-way analysis of variance, followed by Tukey's post hoc test. (**P<0.01; ***P<0.001). (B) Luciferase activities were detected following transfection with P300 siRNA or control siRNA. Data were tested with Student's paired t-test. (**P<0.01). wt, wild-type; mut, mutant; siRNA, small interfering RNA.

P300 decreases the mRNA level of MUC5AC. To determine whether P300 affects MUC5AC gene transcription, the co-transfected A549 cell RNA was isolated and the expression of MUC5AC was detected by RT-qPCR analysis (Fig. 7A and B). The mRNA expression of MUC5AC was significantly decreased following co-transfection with the P300 wt expression plasmid compared with the control. By contrast, P300 siRNA increased the mRNA levels of MUC5AC, which suggests that P300 decreased the mRNA expression of MUC5AC.

P300 decreases the protein expression level of MUC5AC. The empty vector, untransfected control, the P300 expression plasmid and P300 siRNA, together with their control plasmids, were transfected into A549 cells. Immunofluorescence was then performed to detect the protein expression of MUC5AC in each group. The A549 cells were stained with immunofluorescent MUC5AC (green) antibody, and the nuclei were stained with DAPI (blue). The results showed that the P300 expression plasmid downregulated the protein levels of MUC5AC, whereas P300 siRNA upregulated the protein levels of MUC5AC (Fig. 8).

Discussion

Mucosal airway hypersecretion is one of the most serious pathophysiological features of chronic airway inflammatory diseases. In cystic fibrosis, the thick and dehydrated airway mucus, which is difficult to clear, makes patients prone to chronic inflammation and bacterial infections. It is beneficial to reduce the impact of lung infections and improve lung function by clearing the airways of mucus (26). Excessive mucus production and secretion in airways are associated with disabling symptoms (cough and sputum), decline in lung function, exacerbations and mortality in patients with chronic obstructive pulmonary disease (COPD) (27). With an increasing number of individuals suffering from mucosal airway hypersecretion, it is becoming increasingly urgent to develop an effective treatment. It is well known that mucus plugging is the main cause of acute asthma (3). In patients with asthma, bronchial secretion deposits are frequently observed (28). At present, anticholinergics, corticosteroids or other medicines are applied to decrease mucus hypersecretion in the clinical setting. However, these treatments have a number of side effects. Therefore, attempts to identify novel efficient therapeutic methods for airway inflammatory diseases are ongoing (29).

Mucins are high-molecular-weight glycoproteins secreted by goblet cells, which cover the airways, the esophagus and other glandular organs (30). At present, 21 mucin-like genes have been identified and sorted into two categories; membrane-bound and secreted mucins (31). Secreted mucins have terminal cysteine-rich domains and form disulfide bonds for polymers that may be detected on gels (9,10,32).

MUC5AC and MUC5B are two typical polymers of airway mucins (9,10). MUC5B, a tenacious secretory mucin, is produced in the lung airway system in normal and pathological conditions. MUC5AC is also a gel-forming mucin and secreted at high levels in asthmatic inflammation or COPD. Increasing evidence has demonstrated that MUC5AC may have a less important role in human airway systems in normal conditions. Therefore, suppression of the production of MUC5AC may be a therapeutic method for secretory chronic airway inflammatory diseases (4). In our previous studies, mucosal airway hypersecretion was investigated in A549 lung adenocarcinoma cells, human NCI-H292 airway epithelial cells and normal human bronchial epithelial (NHBE) cells (33-35). In the present study, A549 alveolar only basal epithelial cells were used to investigate mucosal airway hypersecretion, owing to their high stability, medium culture conditions and low cost. Furthermore, this cell line has been investigated widely and it is known to express MUC5AC, MUC1 and MUC5B (36).

Histone acetylation, particularly by HATs, is essential for gene transcription and protein expression in asthmatic diseases. The decreased activity of HDACs is likely to cause increased secretion of inflammatory factors, thus inducing asthma (37). P300 has HAT activity, which can acetylate a number of proteins that regulate lung function and glandular secretion. Additionally, P300 exerts its effect through its binding domains with different proteins. P300 is able to interact with >411 proteins, to induce a signal

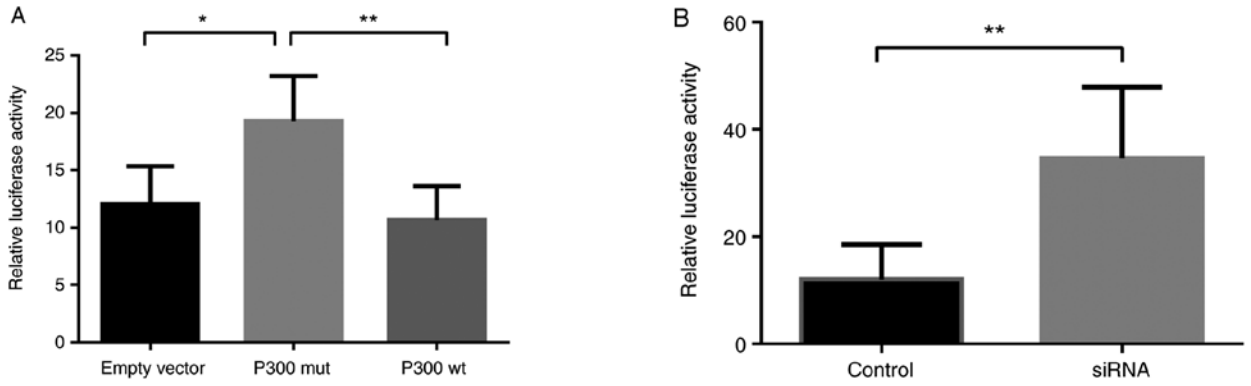

Figure 6. P300 inhibits MUC5AC promoter activity. (A) Luciferase activities were detected following co-transfection with the luciferase reporter plasmid with promoter (pGL-935/+48) and the empty vector control, P300 wt plasmid or P300 mut plasmid in A549 cells. Data were statistically analyzed using a one-way analysis of variance, followed by Tukey's post hoc test. (*P<0.05; **P<0.01). (B) Luciferase activities were detected following co-transfection with MUC5AC promoter (pGL-935/+48) and P300 siRNA or control siRNA, Data were tested with Student's paired t-test. (**P<0.01). MUC5AC, mucin 5AC; wt, wild-type; mut, mutant; siRNA, small interfering RNA.

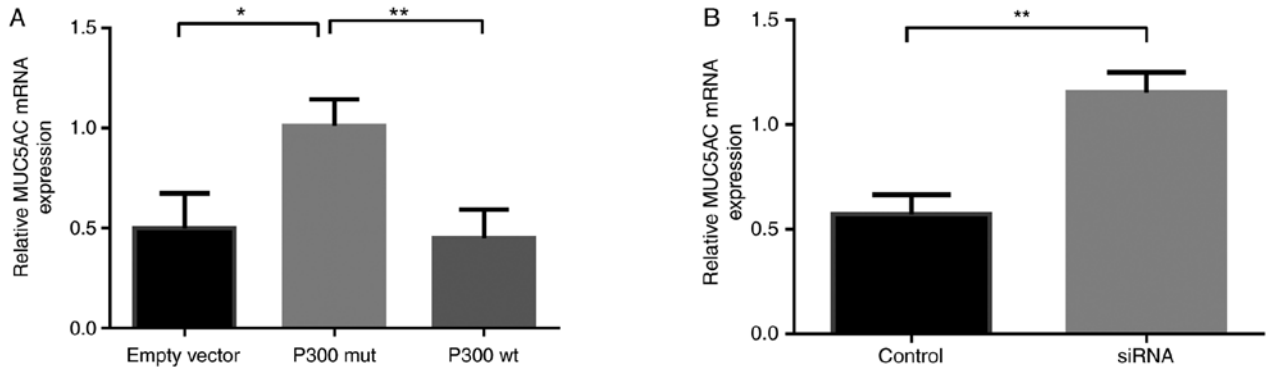

Figure 7. P300 inhibits mRNA expression levels of MUC5AC. (A) mRNA levels of MUC5AC in A549 cells were detected by RT-qPCR analysis following transfection with the P300 wt and P300 mut plasmid for 24 h. Data were statistically analyzed using one-way analysis of variance, followed by Tukey's post hoc test. (*P<0.05; **P<0.01). (B) mRNA levels of MUC5AC in A549 cells following transfection with P300 siRNA and control siRNA by RT-qPCR analysis. Data were tested with Student's paired t-test. (**P<0.01). MUC5AC, mucin 5AC; wt wild-type; mut, mutant; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA.

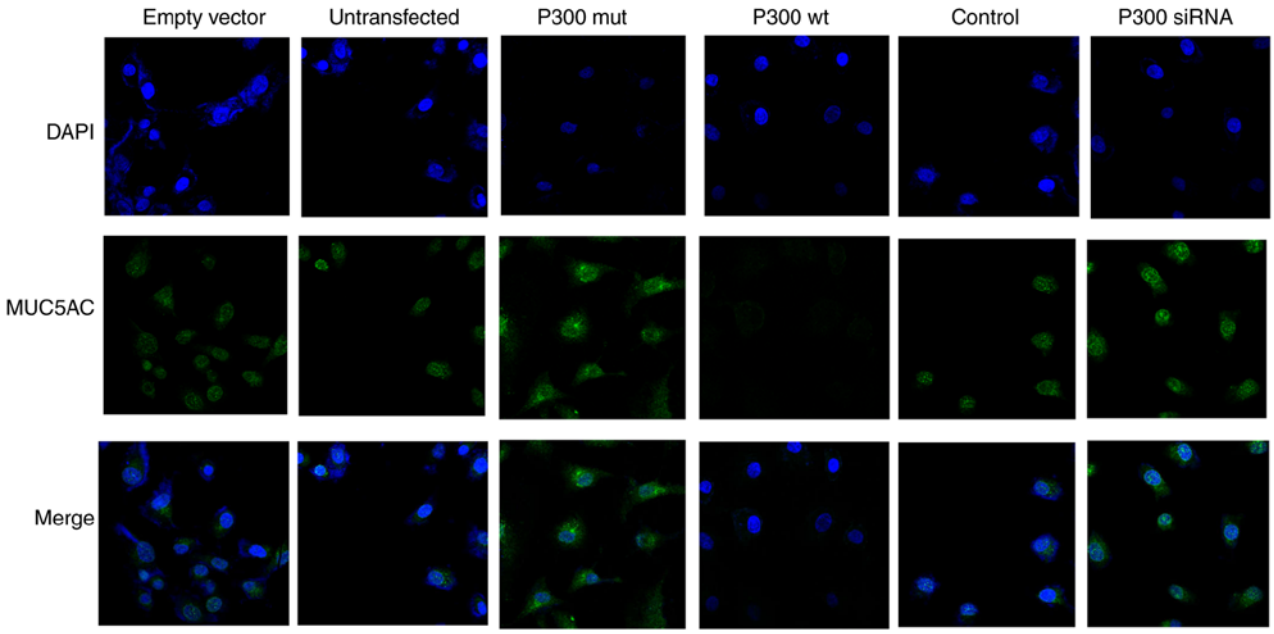

Figure 8. P300 downregulates protein expression levels of MUC5AC in A549 cells. Following co-transfection with the empty vector, untransfected control, P300 wt plasmid, P300 mut plasmid, P300 siRNA and control siRNA for 24 h, protein expression levels of MUC5AC were detected by immunofluorescence. MUC5AC, mucin 5AC; wt wild-type; mut, mutant; siRNA, small interfering RNA.

response or influence gene expression. In addition, P300 and other acetyltransferases can influence protein-coupled structures by protein lysine acetylation. P300 is involved in the integration of protein complexes and regulating DNA element functions (3).

In the present study, the MUC5AC gene promoter was cloned and a luciferase reporter with different lengths was successfully constructed. The results demonstrated that the core promoter area was in the region of -935 to -583 bp upstream of the MUC5AC gene. In addition, the expression of MUC5AC was reduced by P300. Through a literature review and analyses using relevant software, it was demonstrated that the region of the core promoter contained nuclear factor (NF)-κB, and transcription factor Sp1 (Sp1) binding sites. Each binding site can bind with different transcription factors, which influence MUC5AC gene transcription (38). There are several studies showing that Sp1 and NF-Kb (39,40) are important ubiquitous transcription factors, which regulate the transcription of MUC5AC and mucous metaplasia by interacting with gene promoters (19). Furthermore, studies have shown that glucocorticoids can influence gene expression and activity by integrating with the ligand-activated glucocorticoid receptor and activating glucocorticoid-responsive elements (GREs) in the promoter region. The MUC5AC gene also has GRE regions in its 5' sequence and may be similarly influenced by glucocorticoids. All signals influencing P300 (activity, structure or recruitment), may have an impact on gene transcription to further affect the gene expression of MUC5AC. P300 is ubiquitously expressed in multiple tissues/organs and is involved in numerous physiological processes. Studies on P300 have mainly focused on viral infections, cancer and neurodegenerative diseases (41). Consistent with these diseases, P300 is important in mucosal airway hypersecretion. Differences in the mechanisms of P300 in these diseases remain to be elucidated. Therefore, the task of investigating the clinical applicability of targeting the expression of P300 as a potential treatment for mucosal airway hypersecretion involves understanding how the protein receives signals in cells, what induces its recruitment in a given signal transduction pathway, and what determines the final outcome of its individual activity. Therefore, further investigations are required focusing on the pathways that regulate the P300 pathway, and investigating the effects of MUC5AC in lung inflammatory secretion diseases.

In conclusion, the findings of the present study indicate that P300 inhibited the gene expression of MUC5AC in A549 cells, which may be a novel therapeutic target for chronic airway inflammatory diseases. However, further investigations are required to identify the signal pathways influencing P300.

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Availability of data and materials

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

Authors' contributions

SX carried out the experiments, collected and analyzed the data and wrote the manuscript. YH designed the experimental study and assisted with revising the manuscript. JS was involved in experiments and analysis. LL and JQ evaluated the data, and revised and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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