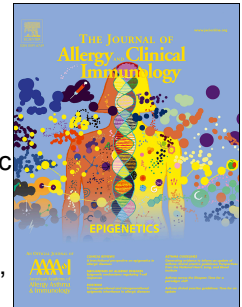


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Keywords: Allergic rhinitis; MUC1; Airway epithelial barrier; Tight junction proteins.

Declaration of interest: The authors declare that they have no relevant conflicts of interest.

Capsule Summary

- MUC1 gene and protein expression was significantly decreased in nasal mucosal tissues in AR patients.
- MUC1-deficiency is associated with epithelial barrier dysfunction in AR by regulating claudin-1 and occludin expression.

To the Editor,

The epithelial barrier dysfunction plays a critical role in the pathogenesis of allergic airway inflammation¹. Disrupted nasal epithelial barrier is observed in allergic rhinitis (AR) and asthma patients². For example, Steelant et al³ reported decreased epithelial barrier function in AR patients and found decreased expression of the tight junction proteins occludin and zonula occludens 1 (ZO-1). Another study showed that the levels of junction proteins, E-cadherin and ZO-1, were reduced in the nasal mucosal tissues of AR patient⁴. However, the etiology of airway epithelial barrier dysfunction remains to be further investigated. It is known that the mucus on the surface of the airway is a protective barrier for the airway⁵. MUC1 is one of the major components of the mucus, and MUC1-deficiency was found involving the pathogenesis of many inflammatory diseases⁶⁻⁸. However, the role of MUC1 in maintaining the nasal epithelial barrier functions and the role of MUC1-deficiency in the pathogenesis of AR are still unknown. Therefore, this study aims to elucidate the role of MUC1 in maintaining nasal epithelial barrier functions and the role of MUC1-deficiency in the development of AR.

MUC1 expression is decreased in nasal mucosa in both AR patients and AR rats.

We firstly evaluated the MUC1 expression in the nasal mucosa of AR patients and control subjects. All the patients had typical perennial AR symptoms and positive serum specific IgE (Table S1). The results showed that the expression of MUC1 was significantly less in AR patients compared to healthy controls (Fig. 1A-D). An AR rat

model was generated (Fig S1A), and the MUC1 expression in nasal mucosa of rats was analyzed by immunohistochemical staining, western blotting and RT-qPCR. The results showed that the MUC1 expression was also decreased in AR rats compared with control rats (Fig. 1E-H). The results suggest that MUC1 may be involved in the pathogenesis of AR.

MUC1-deficiency aggravates nasal epithelial barrier dysfunction in AR rats

To explore the role of MUC1 in AR, we developed an AR model with MUC1 KO rats and wild type (WT) rats with ovalbumin (OVA) as the specific antigen. We observed that the nasal epithelial permeability was higher in WT AR rats than that in naïve control rats, which was significantly increased in MUC1 KO AR rats (Fig. 2A). The results indicate that MUC1-deficiency can aggravate nasal epithelial permeability of AR rats.

The nasal mucosal tissues were collected; the expression of epithelial connection proteins was analyzed by Western blotting, immunohistochemistry and RT-qPCR. The results showed that the expression of occludin, claudin-1 and E-cadherin significantly decreased in MUC1 KO rats compared with WT rats. The results suggest that MUC1-deficiency may down-regulate the expression of claudin-1, occludin and E-cadherin. The results also showed that the expression of occludin, claudin-1 and E-cadherin was significantly decreased WT AR rats than naïve control rats, which was further decreased in MUC1 KO AR rats. The results demonstrate that

MUC1-deficiency aggravates the reduction of nasal epithelial connection proteins in AR rats (Fig 2).

MUC1-deficiency aggravates experimental AR.

After final intranasal OVA challenge, as compared to naïve control rats, the number of sneezing and nasal scratching was increased, the number of neutrophils and eosinophils, as well as the levels of IL-4, IL-5, IL-13, IL-17a and TNF- α were increased in nasal lavage of AR rats (Fig. S1E-K), which were further increased in MUC1 KO AR rats (Fig.S1 E, F, I). The results indicate that MUC1-deficiency aggravates AR.

MUC1 inhibition reduces occludin and claudin-1 expression in BEAS-2B cells

We knocked down MUC1 expression in BEAS-2B cells by transfecting siRNA (Fig. S2A-C). Western blotting showed that the occludin and claudin-1 protein levels were significantly decreased after knocking down MUC1 expression (Fig. S2D-F). Confocal images showed that the positive staining of occludin and claudin-1 was also significantly reduced after knocking down MUC1 expression (Fig. S2G, H). The results indicate that MUC1 inhibition reduces occludin and claudin-1 protein production in BEAS-2B cells (Fig. S2).

In summary, the present data show that MUC1 is significantly less in the nasal epithelium of AR patients and AR rats. MUC1-deficiency aggravates AR symptoms

and AR-related nasal epithelial barrier functions. Depletion of MUC1 suppresses the expression of epithelial cell connection protein. Our findings suggest that MUC1-deficiency is associated with in the pathogenesis of AR, which may be considered as a promising therapeutic target in AR patients.

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Figure legends

Figure1: MUC1 expression is decreased in nasal mucosa both in AR patients and rat models.

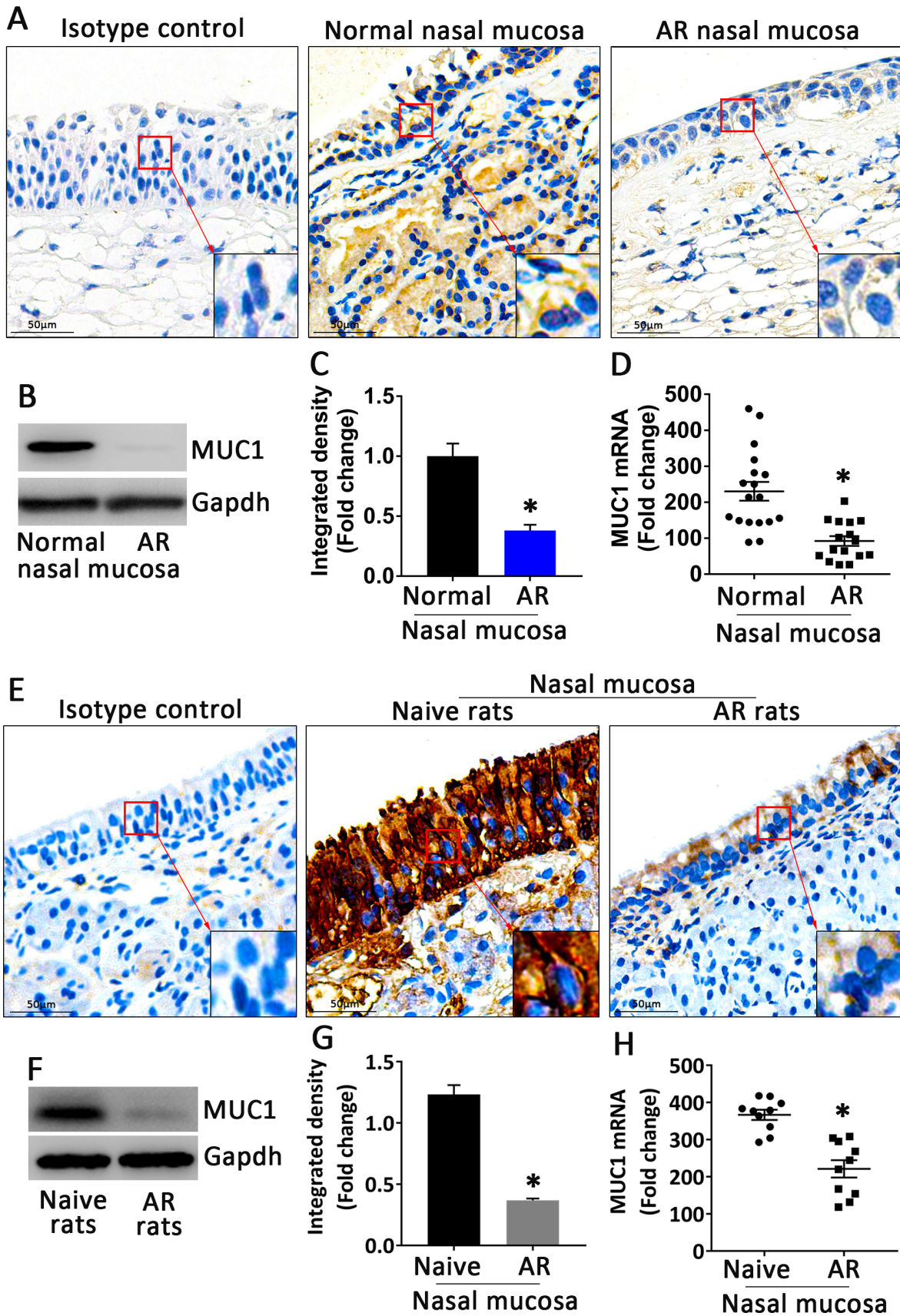
Nasal mucosal samples were collected from healthy subjects (n=11) and AR patients (n=20). (A) MUC1 expression (in brown) in the nasal mucosa. Scale bar: 50 μ m. (B) MUC1 protein in the nasal mucosal extracts. (C) Densitometric analysis of immunoblots of panel B. (D) MUC1 mRNA levels in the nasal mucosa. Data of bars are presented as mean $\pm$ SEM. *P<0.05 versus normal.

E-H, nasal mucosal samples were excised from AR rats. (E) MUC1 expression (in brown) in the nasal mucosa. (F) MUC1 protein levels in the nasal mucosa. (G) Densitometric analysis of immunoblots of panel F. (H) MUC1 mRNA levels in the nasal mucosa. Data of bars are presented as mean $\pm$ SEM. *P<0.05 versus naive rats. (n=8-10).

Figure2: MUC1-deficiency is associated with nasal epithelial barrier dysfunction.

(A) Epithelial permeability to FITC-dextran (a tracer). (B) Occludin, claudin-1(C) and E-cadherin (D) mRNA expression in the nasal mucosa. (E) Protein levels of occludin, claudin-1 and E-cadherin in the nasal mucosa. Bars show occludin (F), claudin-1 (G) and E-cadherin (H) protein levels as determined by densitometry normalized to GAPDH. (I) Representative confocal images of tight junction proteins (occludin and claudin-1) in the nasal mucosa. Scale bars: 25 μ m. (J) Representative

immunohistochemical staining of E-cadherin in the nasal mucosa of rats. Original magnification was 400×. Data are presented as mean $\pm$ SEM. * indicate $P < 0.05$ (n=8-10).



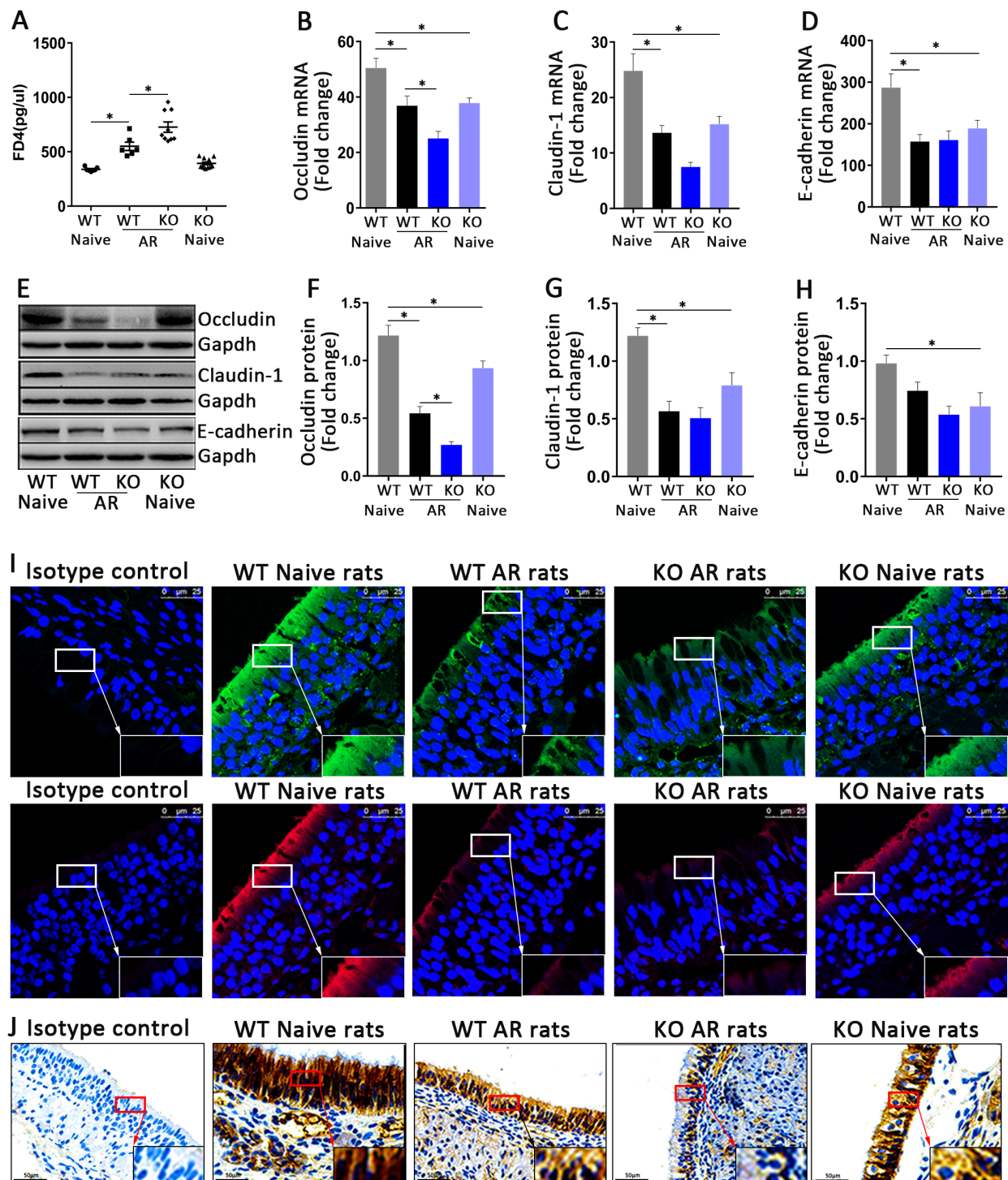


Table S1 Patient information

Group	Allergy (n=20)	Normal (n=11)
Average age (range)	51.1(22-63)	57.8(32-69)
Male : female	3:1	8:3
Symptom of AR	20	0
Rhinitis duration, y, mean $\pm$ SD	7.2 $\pm$ 7.8	0
sIgE positive	20	0
Serum sIgE was defined as positive when the serum level of sIgE was ≥ 0.35 kU/L		

Table S2 Primer sequences

Gene	Species	Forward	Reverse
MUC1	Rat	5-CATTCACATTGACTTCCGAGA-3	5-TAGCATCGACTGTGATTTTCC-3
Occluding	Rat	5- CGTGTAGTCGGTTTCATAGTG-3	5-GGAGATTCCTCTGACCTTGTC-3
E-cadherin	Rat	5-CTGGCTCAAATCAAAGTCCTG-3	5-TCCTGCTCCTACTGTTTCTACG-3
Claudin-1	Rat	5-ACATGCCTCCAATGCCGTTC-3	5-AAGATGCAAATCCAGGTCTACCC-3
Gapdh	Rat	5-CAACGGGAAACCCATCACCA-3	5-ACGCCAGTAGACTCCACGACAT-3
MUC1	Human	5-TGCCGCCGAAAGAACTACG-3	5-TGGGGTACTCGCTCATAGGAT-3
Gapdh	Human	5-ACAACCTTTGGTATCGTGGAAGG-3	5-GCCATCACGCCACAGTTTC-3

Supplemental materials

MUC1-deficiency promotes nasal epithelial barrier dysfunction in subjects with allergic rhinitis

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Patients

The inferior turbinates were collected from AR patients (n=20) and healthy controls (n=11). Control subjects did not report any nasal symptoms of AR, and did not have a history of AR or rhinosinusitis. All the patients had typical perennial AR symptoms and positive serum specific IgE. Serum sIgE against 31 common allergens was measured using the Pharmacia Immuno-CAP system according to the manufacturer's instructions (Pharmacia Diagnostics, Clayton, NC, USA). The analysis for the presence of a sIgE was defined as positive when the serum level of sIgE was ≥ 0.35 kU/L. Patient information can be found in Table S1. This study was approved by the Medical Ethics Committee of the first Affiliated Hospital of Guangzhou Medical University. We obtained written informed consent from all participants.

Cell culture

The BEAS-2B cell cultured in DMEM-F₁₂ media with supplements, including 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine, at 37°C with 5% CO₂ in humidified air. The culture medium was replaced every 48 h.

Animals.

Generation and genotyping of MUC1 transgenic rats. MUC1 knockout (KO) Sprague Dawley (SD) rats were produced by targeted gene mutation at Cyagen Biosciences Inc. (Guangzhou, China). The deletion was made by microinjection of TALENs and located in the exon 1 of rat MUC1 gene (Gen Bank: NM_012602.1). Genotyping was performed by PCR of tail DNA using primers specific for the rat MUC1 gene with

forward primer 5'-CTAGCAAGCCTAAAAGGTGAGAGGT-3' and reverse primer 5'-ACGAAGAGCATTTCCTACTC-3', followed by DNA sequencing analysis. PCR protocols included 95°C 2min and 30 cycles of 95°C-15sec, 57°C-30sec, 72°C-45sec, 72°C-10min. PCR product (250bp) was analyzed on 1.5% agarose gel.

Adult female SD rats (250 ± 20 g) were purchased from the Guangdong Medical Laboratory Animal Center (Guangdong, China). This study was approved by the Ethics Committee of Guangzhou Medical University, and all procedures were conducted in accordance with the experimental animal guidelines of Guangzhou Medical University (Guangzhou, China). Rats were housed in the SPF Experimental Animal Center of Guangzhou Medical University. All rats had free access to standard food and water and allowed to be acclimated for seven days before experiment.

AR development in rats.

AR rat model was developed with OVA as the specific antigen following published procedures¹ (Figure S1). Briefly, rat were sensitized with an intraperitoneal injection of 1000 µg of ovalbumin (OVA; grade V; Sigma, St. Louis, MO, USA) and 20 mg of aluminum hydroxide gel (Sigma Aldrich, St Louis, MO) on days 1, 8, and 15. One week after the last intraperitoneal injection, rat were intranasally challenged with OVA diluted in sterile normal saline (300 µg OVA/20 µl per nostril) on days 22-36. The control (normal) group was given saline. After the final intranasal OVA challenge, the AR was confirmed using a symptom score calculated from the total number of sneezing and nasal scratching during a 30-min period.

Enzyme-linked immunosorbent assay (Elisa)

Cytokine levels were determined by ELISA with commercial reagent kits following the manufacturer instructions (Thermo Scientific, America).

Tissue preparation and histopathological examination.

On day 36 (after the final antigen challenge), rats were deeply anesthetized and sacrificed. Nasal mucosa samples were excised and fixed in 4% paraformaldehyde (Biosharp, Hefei, Anhui, China) for 24 h, and then embedded in paraffin. Paraffin-embedded tissue samples were cut into 3 μ m thick sections and stained with hematoxylin and eosin (HE). Histopathological changes were evaluated and photographed using an orthorhombic optical photomicroscope (Eclipse Ci, Leica, Germany).

Immunohistochemistry and immunofluorescence analysis

For immunohistochemistry, sections were deparaffinized, then submerged in citrate buffer (pH 6.0) for antigen retrieval. Samples were treated with H₂O₂ for 15 min to block endogenous peroxidase, and then incubated overnight at 4 °C in recommended dilutions of anti-occludin, anti-E-cadherin and anti-claudin-1 antibodies (Invitrogen, Carlsbad, CA, USA). After washing with PBS, sections were incubated with a secondary antibody for 30 min at room temperature. Signals were

visualized with a diaminobenzidine (DAB) peroxidase substrate kit (Zhong shan Jin qiao, Beijing, China).

For immunofluorescence staining, sections were incubated with appropriate Alexa Fluor-conjugated secondary antibodies and cell nuclei were stained with DAPI (Invitrogen, Carlsbad, CA, USA).

Western blot analysis

Tissue samples and BEAS-2B cell were lysed in RIPA buffer (Beyotime Biotechnology Institute, China) and lysates subjected to SDS-PAGE and Western blotting for detection of the following antigens: MUC1(Abcam, Cambridge, MA), occludin (Invitrogen, CA, USA), claudin-1 (Invitrogen, CA, USA), E-cadherin (Invitrogen, CA, USA), β -catenin (Cell Signaling Technology, Danvers, MA, USA). After incubation with a secondary antibody (Cell Signaling Technology, Danvers, MA, USA), immunoreactive bands were captured on the Gel DOC XR Gel Imaging System (BIO-RAD) and quantified with image lab v5.2 software.

Quantitative PCR and siRNA transfection.

Total RNA was isolated from the nasal mucosa and BEAS-2B by using a Total RNA Extraction Kit (Takara, Tokyo, Japan). RNA samples were then reverse transcribed into first-strand cDNA using the PrimeScriptTM RT reagent kit from Takara. RT-qPCR was performed on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies, CA, USA). The amplification protocol was set as follows: 95 °C

denaturation for 30 s followed by 40 cycles of 5 s denaturation at 95 °C, 30 s of annealing/extension and data collection at 60 °C. The primer sequences of MUC1, occludin, claudin-1, E-cadherin, and gapdh (internal reference) are listed in TableS2.

A MUC1 small interfering RNA (siRNA) kit was purchased from Huiyuan Biotech (Guangzhou, China). BEAS-2B cells were transfected with siRNA in serum and antibiotic-free medium. After 8h, the medium was aspirated and replaced with medium containing serum for a further 48 h. Lipofectamine-2000 (Invitrogen, Paisley, UK) was used at a final concentration of 2 µg/mL for the transfection.

Statistical Analysis

The results of the four experiments were analyzed using SPSS for Windows version 23.0 (SPSS, Chicago, IL, USA) and expressed as mean ± Standard Error (SEM). Between-groups comparisons were performed using one-way ANOVA and analyzed by LSD t-test (when equal variances are assumed) or Tamhane's T2 test (nonparametric test; when equal variances are not assumed), and $p < 0.05$ indicated statistical significance.

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Fig. S1. MUC1-deficiency is associated with aggravated sneezing and nasal inflammation of AR rat model.

(A) The protocol of OVA-induced rat model of allergic rhinitis. (B) Histopathological scoring of airway inflammation in H&E-stained nasal mucosa sections. (C) Records of nasal rubbing and sneezing of rats. (D) Bars show the weight of rhinorrhea of rats. (E) Total number of inflammatory cells in nasal lavage fluid. (F) Differential cell percentages in nasal lavage fluid. Results are expressed as percentages of total cells by counting in total of 200 cells in H&E-stained cytospin samples. Levels of IL-5 (G), IL-13 (H), IL17A (I), IFN- γ (J) and TNF- α (K) in nasal lavage fluid. Data are presented as mean $\pm$ SEM. * $P < .05$ versus WT naive rats; # $P < .05$ versus WT AR rats. (n=8-10).

Fig. S2. MUC1 inhibition reduces occludin and claudin-1 protein production in BEAS-2B cell.

We knocked down MUC1 expression in BEAS-2B cells by transfecting siRNA. BEAS-2B cells were transfected with non-silencing siRNA (Control-siRNA) and MUC1-silencing siRNA (MUC1-siRNA) (A) MUC1 mRNA levels in BEAS-2B cells (B) MUC1 protein levels in BEAS-2B cells. (C) MUC1 protein levels as determined by densitometry normalized to GAPDH. (D) Immunoblots show levels of occludin and claudin-1 proteins in BEAS-2B cells. (E-F) Densitometric analysis of the blots of panel D. (G) Representative confocal images of the tight junction protein occludin in BEAS-2B cells. Scale bars: 25

μm. (H) Representative confocal images of the tight junction protein occludin in BEAS-2B cells. Scale bars: 25 μm. Data of bars are presented as mean ± SEM. *P<.05 versus Control-siRNA. (n≥3).

