

Original Article

KyoT2 downregulates airway remodeling in asthma

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Abstract: The typical pathological features of asthma are airway remodeling and airway hyperresponsiveness (AHR). KyoT2, a negative modulator of Notch signaling, has been linked to asthma in several previous studies. However, whether KyoT2 is involved in the regulation of airway remodeling or the modulation of airway resistance in asthma is unclear. In this study, we aimed to evaluate the therapeutic potential of KyoT2 in preventing asthma-associated airway remodeling and AHR. BALB/c mice were used to generate a mouse model of asthma. Additionally, the expression of Hes1 and Notch1 in airway was analyzed using Immunofluorescence examination. The asthmatic mice were intranasally administered adenovirus expressing KyoT2 and were compared to control groups. Furthermore, subepithelial fibrosis and other airway remodeling features were analyzed using hematoxylin and eosin staining, Van Gieson's staining and Masson's trichrome staining. AHR was also evaluated. This study revealed that KyoT2 downregulated the expression of Hes1, repressed airway remodeling, and alleviated AHR in asthmatic mice. It is reasonable to assume that KyoT2 downregulates airway remodeling and resistance in asthmatic mice through a Hes1-dependent mechanism. Therefore, KyoT2 is a potential clinical treatment strategy for asthma.

Keywords: KyoT2, airway remodeling, airway hyperresponsiveness (AHR)

Introduction

Asthma is characterized by chronic airway inflammation, airway hyperresponsiveness (AHR), and airway wall remodeling [1, 2]. Airway remodeling, the most typical pathological feature of asthma, involves subepithelial fibrosis, smooth muscle hypertrophy, and collagen deposition [3]. The Notch signaling pathway is a fundamental, conserved signaling system that controls cellular development, proliferation, and apoptosis. Previous studies have shown that Notch is associated with airway inflammation, AHR [4], and airway remodeling in asthma [5]. The Notch signaling pathway also downregulates mucin 5AC (MUC5AC) expression in epithelial cells in the airways of asthmatic subjects [6].

A ternary complex, consisting of the Notch intracellular domain (NICD), C-promoter binding factor-1/suppressor of hairless/Lag-1 (CSL), and mastermind-like family (MAML) [7], initiates the transcription of target genes. The LIM protein KyoT2, encoded by KyoT, competes with

NICD for binding to CSL. It negatively regulates transcription by competing with other CSL-binding transcriptional activators and displacing CSL from DNA [8]. Thus, KyoT2 is a repressor of Notch signaling. Another Notch signaling blocker, γ -secretase inhibitor, was shown to have beneficial effects in asthmatic mice when administered intranasally [4]. Previously, we showed that NICD upregulates the activity of the type I collagen gene by upregulating Hes1. Therefore, repressing Hes1 could downregulate subepithelial fibrosis of the airways of asthmatic subjects [9]. Here, we hypothesize that KyoT2, a negative modulator of Notch signaling, downregulates remodeling of airways in asthmatic subjects and has therapeutic effects.

Materials and methods

Animals

Healthy male BALB/c mice, with ages ranging from 6 to 8 weeks and an average body weight of 24 ± 3 g, were obtained from the animal center of the Fourth Military Medical University. The

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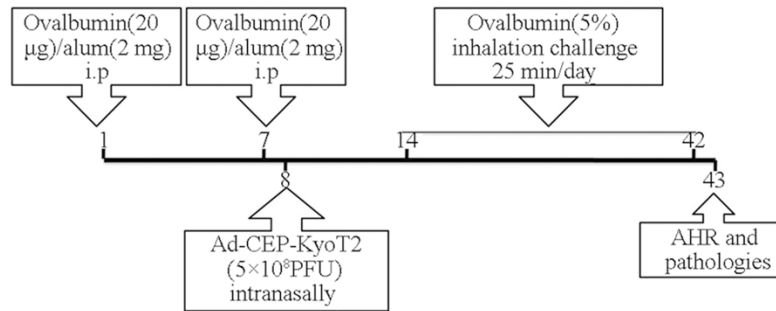


Figure 1. Flowchart for mice that were sensitized with OVA, challenged with OVA, treated with adenoviral vectors, and sacrificed for pathology analysis.

on day 8. The mice in groups 2 and 3 were intranasally administered 5×10^8 plaque forming units (PFUs) of mock adenovirus and Ad-CEP-KyoT2, respectively, on day 8 [11] (**Figure 1**). Mice from each group were sacrificed on day 43 for histological analysis.

Measurement of airway responsiveness

mice were maintained in an animal facility under specific pathogen-free standard laboratory conditions. This study was conducted in accordance with the principles of the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Fourth Military Medical University. Written informed consent was obtained from all participants' guardians.

Experimental groups

The BALB/c mice were divided into 4 groups, and each group included 6-12 mice. Group 1 (Control) was the normal control. Group 2 (Asthma + KyoT2) was the chronic asthma model treated with the target adenovirus (over-expressing KyoT2, Ad-CEP-KyoT2). Group 3 (Asthma + Mock) was the chronic asthma model treated with mock adenovirus. Group 4 (Asthma) was the chronic asthma model treated with only phosphate-buffered saline (PBS).

Allergen sensitization and challenge protocol

The mice included in the asthma model were sensitized with ovalbumin (OVA) as described previously [10]. From day 14, the mice were challenged with nebulized OVA solution in a transparent chamber for 25 minutes per day for 4 weeks (**Figure 1**). Group 1 mice were sensitized with PBS and challenged using nebulized PBS instead of OVA.

Adenoviruses treatment

The KyoT2 plasmid was a kind gift from Professor Hua Han of the Fourth Military Medical University. The recombinant adenovirus vectors containing KyoT2 (Ad-CEP-KyoT2, PSB805) were constructed by Sunbio (Shanghai, China). Group 1 and 4 mice were only exposed to PBS

Twenty-four hours after the last challenge with OVA, the mice treated with adenovirus expressing KyoT2 and mock adenovirus were anesthetized using 1% pentobarbital sodium (P3761; Sigma, USA), which was intraperitoneally injected at a dose of 50 mg/kg. A cannula was inserted via tracheotomy and connected to a rodent ventilation machine (AniRes 2005 v. 3.0; Best lab International Inc. China) set at 120 breaths/min. The ratio of inspiratory to expiratory beats (I:E) was 2:1. After recording a stable baseline airway pressure, methacholine (A2251; Sigma, USA) was infused intravenously over 1 s via the tail vein [12]. Recordings were taken every 3 minutes. The airway responsiveness procedures have been described in detail previously [13]. When the mouse respiratory pressure reached 2 times the baseline pressure, the methacholine challenge was stopped. The methacholine gradient infused via the mouse's tail vein, designed according to the instructions provided in the manual of the AniRes2005 system, was as follows: 0.008 µg/g, 0.016 µg/g, 0.032 µg/g, and 0.064 µg/g.

Immunofluorescence examination

The mice that did not undergo AHR measurements from the four groups were sacrificed. The lung specimens were fixed and embedded in paraffin, and then sectioned into 5-µm-thick slices. The sections were stained as described previously [14]. Following antigen retrieval, the specimens were blocked using nonimmune serum, and they were then incubated in primary and secondary antibodies. Anti-Notch1 (#4380S; CST, USA) and anti-Hes1 (SC-13844; Santa Cruz Biotechnology, USA) were used as the primary antibodies, and the second antibodies were goat anti-mouse IgG (red, A-11005; Invitrogen, China) and rabbit anti-goat IgG

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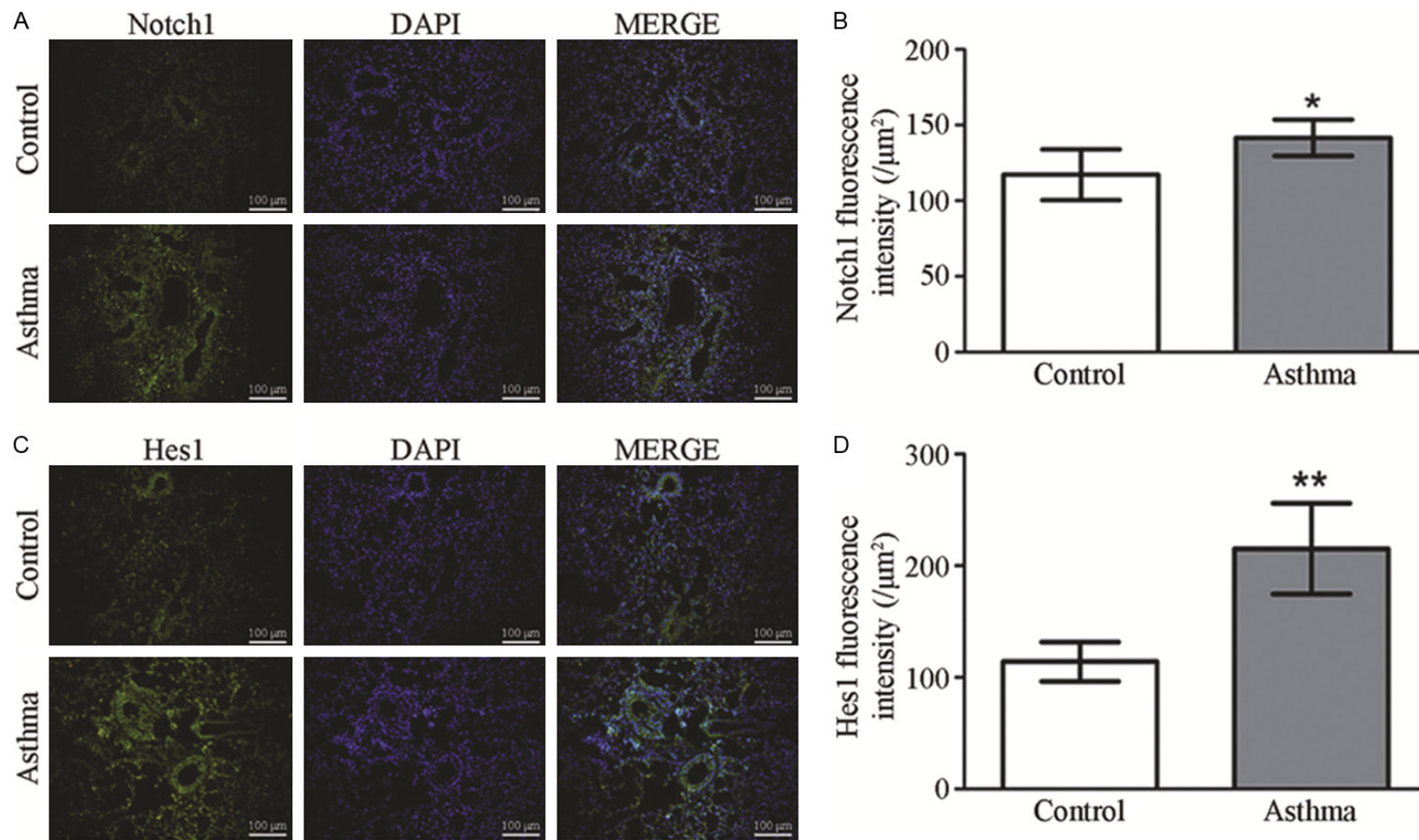


Figure 2. Immunofluorescence imaging of Notch signaling components in asthmatic mice and in the control group. A, B. The expression of Notch1 in mice sensitized with OVA was greater than that in mice treated with PBS (ANOVA and LSD; *, $P < 0.05$). C, D. The expression of Hes1 in mice sensitized with OVA was greater than that of mice treated with PBS (ANOVA and LSD; **, $P < 0.01$).

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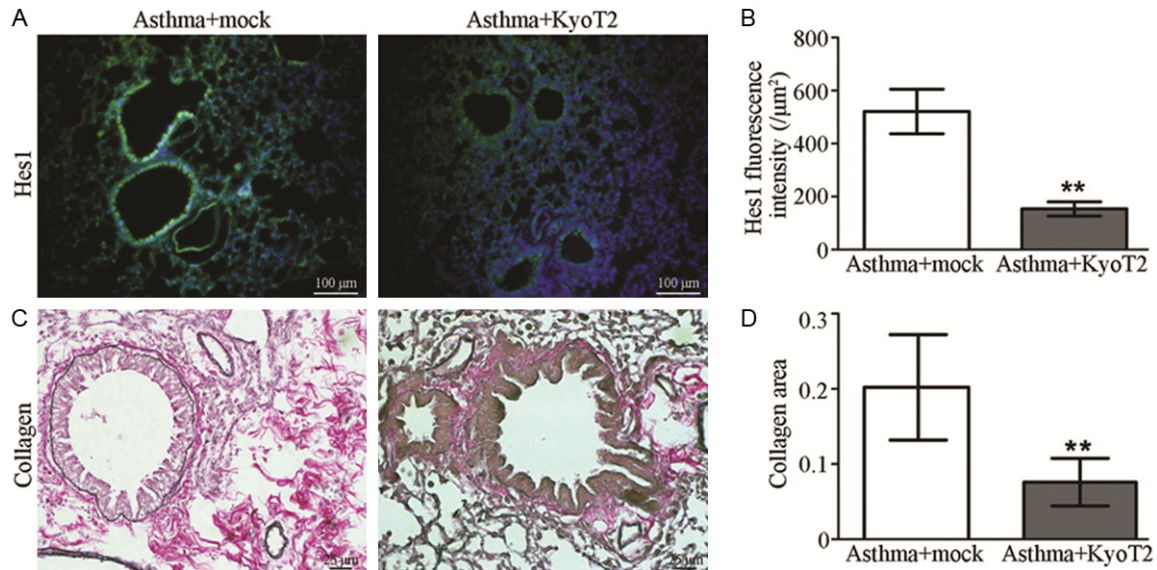


Figure 3. Histological analysis. A. Immunofluorescence staining of lung tissues of asthma models that were treated with the mock adenovirus or adenovirus overexpressing KyoT2. The expression of Hes1 (green) was greater in the former specimens than in the latter. B. KyoT2 downregulated Hes1 expression *in vivo* (ANOVA and LSD; **, $P < 0.01$). C. VG staining of lung tissue sections from asthmatic mice treated with KyoT2 adenovirus and control asthma groups treated with the mock adenovirus. The KyoT2 adenovirus downregulated the inflammation and the collagen deposition (red) in asthmatic mice. D. KyoT2 downregulated collagen synthesis in the asthma mouse models (ANOVA and LSD; **, $P < 0.01$).

(green, 02-13-06; KPL, USA). We added 50 μ l of 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA), mounted the specimens in mounting medium, examined them under a microscope, and analyzed the images by using Image J software.

Hematoxylin and eosin (HE), Van Gieson's (VG) stain and Masson's trichrome stain

Sections were stained with hematoxylin (32-897-6X250ML; Sigma, USA) and eosin (E4009-5G; Sigma, USA), and with VG stain according to the manufacturer's instructions (BA4083A; Baso, Zhuhai, China) to analyzing airway inflammation and pathological changes. Furthermore, some sections were processed for Masson's trichrome stain (HT15; Sigma, USA) to visualize collagen fibers and smooth muscles.

Observation of the airway remodeling

Slides were examined by microscope and photographed at 400 \times magnification. Bronchiole collagen area, smooth muscle layer and base membrane thickness were measured using NIS-Elements system. The measurements were performed in 6 fields per slide. 3 to 6 bronchi-

oles were observed for each section, and 3 mouse lung tissues were examined for each group. The mean values for each animal were calculated.

Statistical analysis

The statistical analyses were performed by using the SPSS 19.0 program. Comparisons between groups were analyzed using the least significant difference test (LSD) and analysis of variance. All data were expressed as mean \pm standard deviation. Differences with p values < 0.05 were considered statistically significant.

Results

Expression of Notch signaling components

To investigate the effects of Notch signaling on the airways of asthmatics *in vivo*, we generated a mouse asthma model. The lung tissue sections of these model mice were examined using immunofluorescence. **Figure 2** shows that the fluorescence intensities of Notch1 and Hes1 immunostaining in the asthmatic groups were stronger than those in the normal control group (* $P < 0.05$; ** $P < 0.01$).

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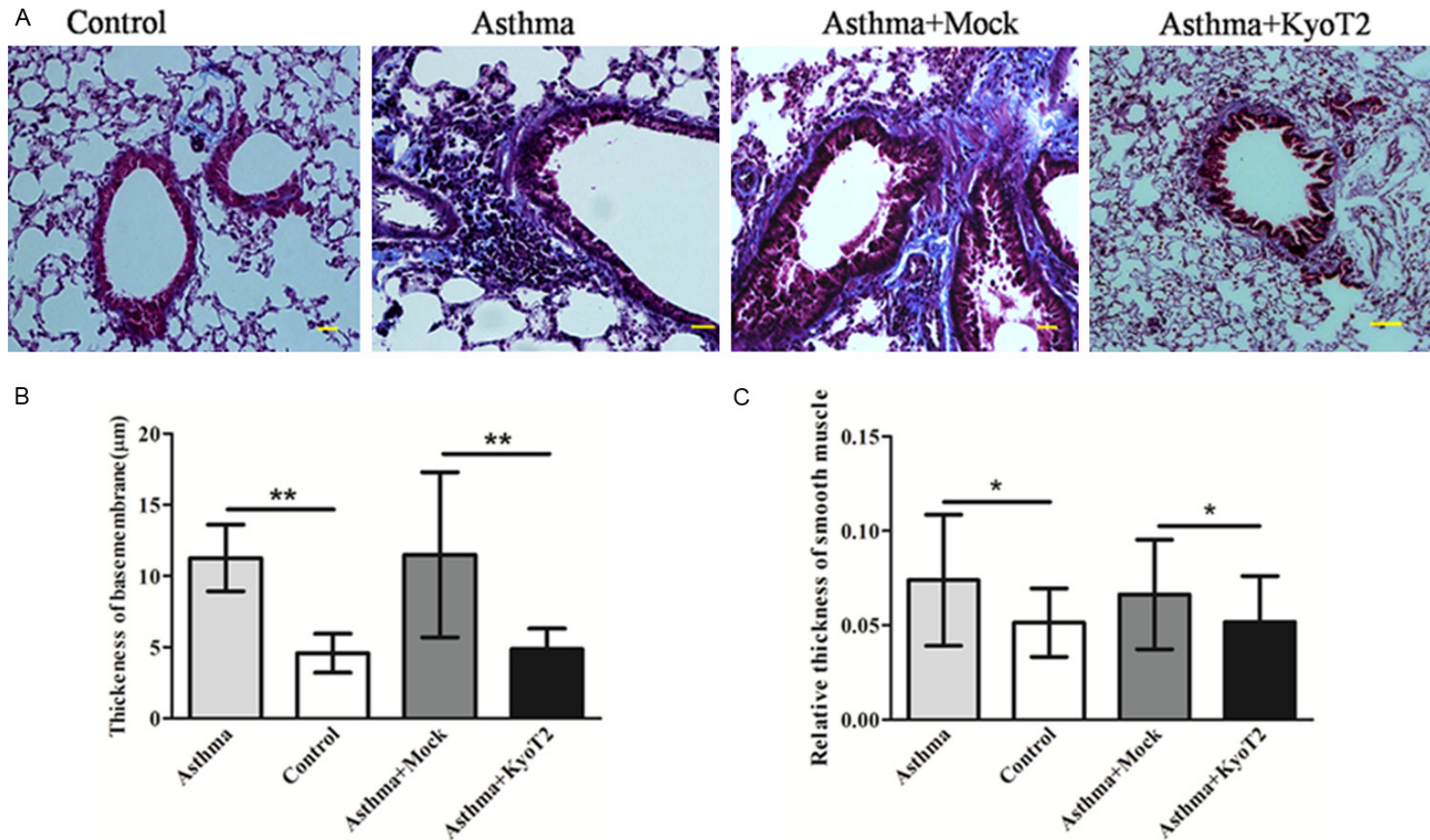


Figure 4. Masson's trichrome staining of lung tissue sections showed. A. Normal control mice (Control) had normal airway, asthmatic mice (Asthma) had more severe airway remodeling and greater number blue collagen fibers than the former. Asthmatic mice treated with mock adenovirus (Asthma + Mock) had more severe airway remodeling and greater number blue collagen fibers than asthmatic mice treated with KyoT2 adenovirus (Asthma + KyoT2). B. Control group and Asthma + KyoT2 group had better thickness of airway basement membrane (ANOVA and LSD; **, $P < 0.01$; *, $P < 0.05$). C. Control group and Asthma + KyoT2 group had better thickness of airway smooth muscles layers (ANOVA and LSD; **, $P < 0.01$; *, $P < 0.05$).

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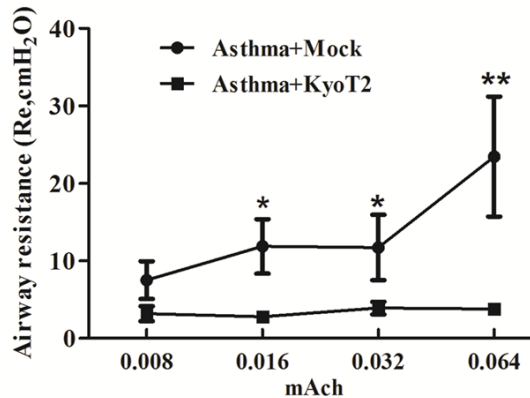


Figure 5. The AHR assay indicates that KyoT2 repressed the AHR of asthmatic mice when contrasted to the control group (two-way ANOVA and LSD; **, $P < 0.01$; *, $P < 0.05$).

Ad-CEP-KyoT2 downregulates Hes1 expression

We then determined whether the Lim protein KyoT2, a putative repressor of Notch, regulated Hes1 expression in vivo. To achieve this, we examined lung sections of BABL/c asthmatic mice that were administered the Ad-CEP-KyoT2 adenovirus intranasally. We found that Ad-CEP-KyoT2 lowered Hes1 expression when compared to mice that were administered the mock adenovirus (**Figure 3A** and **3B**).

Ad-CEP-KyoT2 represses subepithelial fibrosis and other typical forms of airway remodeling

To evaluate the effects of KyoT2 on asthma, we studied the lung sections of the asthmatic BALB/c mice treated with Ad-CEP-KyoT2 adenovirus intranasally and those treated with Mock adenovirus by using HE, VG staining and Masson's trichrome staining. We found that the asthmatic mice treated with the Ad-CEP-KyoT2 adenovirus had less inflammation and less airway wall remodeling than those treated with the mock adenovirus (**Figures 3** and **4**). The VG staining (**Figure 3C**) and Masson's trichrome staining (**Figure 4A**) showed a decrease in the amount of collagen fibers and smooth muscle layers in the asthmatic mice that were treated with the Ad-CEP-KyoT2 adenovirus when compared to the mock adenovirus-treated mice. The Masson's trichrome staining also showed the asthmatic mice had more severe inflammation and airway remodeling when compared to the normal control mice (**Figure 4A-C**). The statistical analysis also showed that the collagen

(**Figure 3D**), basement membrane (**Figure 4B**), and airway smooth muscles (**Figure 4B**) were all thinner in the asthmatic mice treated with the Ad-CEP-KyoT2 adenovirus than in those treated with the mock adenovirus. Thickness of epithelium was no significantly different between the Asthma + Mock group and the Asthma + KyoT2 group (data not shown).

KyoT2 improves the lung function

To examine the effect of KyoT2 on the responsiveness of asthmatic airways, we measured the AHR of the asthmatic mice that were administered adenoviral vectors overexpressing KyoT2. We found that KyoT2 repressed the AHR of the asthmatic mice, as shown in **Figure 5**.

Discussion

To explore the effects of KyoT2 on asthmatic airways, we generated an asthma mouse model and treated them with adenoviral vectors overexpressing KyoT2. We measured the change in the expression levels of Hes1, a key downstream component of the Notch signaling pathway, in asthmatic mice after treatment with adenoviral vectors overexpressing KyoT2. Subsequently, we analyzed the lung sections of the mice by using histological measurements. We also measured the airway resistance in asthma mouse models treated with adenoviral vectors overexpressing KyoT2 and in the control group.

The relative expression level of important Notch signaling components such as Hes1 and Notch1 were found to be upregulated in asthmatic mice. These results were in general agreement with the finding that Notch signaling plays a critical role in airway tissue development [15]. Measurements of Notch components expression levels in Keloid disease and extralesional tissues showed that these tissues had differing expressional features of Notch components [16].

In a study examining the effects of relaxin, Notch signaling downregulated myocardial fibrosis [17]. In contrast, our study showed that Notch signaling was upregulated in asthma and that repressing Notch signaling would be beneficial as asthma therapy. These results indicate that Notch signaling has tissue-specific features.

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We showed that Hes1 expression in the asthmatic mouse lung tissues treated with adenovirus expressing KyoT2 was decreased when compared to those treated with the mock adenovirus. This result suggests that the LIM protein KyoT2 repressed Notch signaling by competing with NICD for binding to CSL [8], subsequently downregulating Hes1. Hes1 is a core downstream molecule of the Notch signaling pathway. In previous studies, we showed that Notch downregulated MUC5AC and collagen type I expression in cells in the airways of asthmatic mice through Hes1-dependent mechanisms [6]. Hes1 is typically considered an inhibitory molecule; However, Hes1 was found to promote the activity of the type I collagen genes A1 and A2 (COL1A1 and COL1A2) in our previous experiments [9]. In the present study, Hes1 expression was increased in the asthmatic mouse lung specimens. Moreover, KyoT2 decreased Hes1 expression and led to a decline in the levels of collagen in the airways of asthmatic mice. Two previous studies demonstrated that Hes1 had similar features [18, 19]. Therefore, KyoT2 could regulate airway remodeling through a Hes1-dependent mechanism.

In a previous study, the efficacy of recombinant adenoviral vectors towards several respiratory infections in mice was investigated [20]. Another study by Chuang et al. [11] showed that intranasal administration of Ad-FasL vectors abolished AHR and induced apoptosis of lymphocytes and eosinophils in asthmatic mice. Moreover, Fu et al. [21] used an adenovirus expressing IL-10 to decrease AHR in asthmatic mice. Together, these studies showed that adenoviral vectors were safe. To the best of our knowledge, our study is the first to use adenoviral overexpression of KyoT2 to treat asthmatic mice. Histological staining showed that less inflammatory cells were present in the lung tissues of the asthmatic mice after administering adenoviral vectors expressing KyoT2. VG staining and Masson's trichrome staining of the lung tissues also showed that there was more deposition of collagen in the asthmatic mice treated with the mock adenoviral vectors than in those treated with the adenoviral vectors expressing KyoT2. The decreasing thickness of the airway basement membranes and smooth muscles in the asthmatic mice treated with the adenoviral vectors expressing KyoT2 suggested that KyoT2

is effective in treating asthma. These results demonstrate that KyoT2 can alleviate allergic inflammation and reduce airway remodeling caused by asthma.

Asthma is a chronic inflammatory disease characterized by AHR. Strong allergies to pollen or house dust mites trigger asthmatic symptoms. We investigated the AHR of asthmatic mice in response to methacholine. The results demonstrated that adenoviral vectors expressing KyoT2 abolished the AHR of the asthmatic mice. Existing clinical therapeutic approaches used in asthmatic subjects have focused on the use of corticosteroids and bronchodilators [22], and human clinical trials involving biological therapies have not produced satisfactory results. Recently, novel therapies have been proposed that are directed towards cytokines, including Toll-like receptor agonists, bitter taste and EP4 receptor, nuclear factor-kappa B, and the anti-IgE monoclonal antibody [23]. Notch was found to be associated with asthma in some studies [24, 25], and Huang et al. [5] proposed that regulator T cells downregulated Delta4-mediated vascular remodeling in asthmatic airways. However, all of these existing therapies are shown to have unsatisfactory curative effects in clinical applications [26].

This study examined the use of adenoviral vectors expressing KyoT2 to improve lung function in asthmatic mice. This innovative therapeutic strategy has the potential to be adopted in clinical treatments for asthma. In conclusion, KyoT2-targeted treatment may be used as a novel treatment approach for subepithelial fibrosis and other types of airway remodeling in asthmatic subjects.

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Disclosure of conflict of interest

None.

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