

Airway epithelial overexpressed CTSK induces airway remodeling through EMTU activation in asthma

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Abstract

Accumulating studies in recent years have revealed that airway remodeling is involved in the occurrence, development, and treatment sensitivity of asthma. Airway epithelial cells (AECs) regulate the activation of epithelial-mesenchymal trophic units (EMTUs) during airway remodeling through secretion of a series of signaling mediators. However, the major trigger and the intrinsic pathogenesis of airway remodeling is still obscure. Here, we show that the expression of CTSK in airway epithelia increased significantly along with the development of airway remodeling in HDM-stressed asthma model. Increased secretion of CTSK from airway epithelia induced the activation of EMTU through the activation of PAR2-mediated pathway. We found that CTSK is a potential biomarker of airway remodeling for asthma patients that can reflect the degree of airway remodeling and the severity of asthma. Blockade of CTSK inhibits EMTU activation and alleviate airway remodeling effectively that is an effective intervention target of airway remodeling. Thus, our findings provide that CTSK is a potential biomarker for airway remodeling which may also be a useful target for the targeted intervention of airway remodeling in asthma patients.

Airway epithelial overexpressed CTSK induces airway remodeling through EMTU activation in asthma

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Abstract

Background and Purpose

Airway epithelial cells (AECs) regulate the activation of epithelial-mesenchymal trophic units (EMTUs) during airway remodeling through secretion of a series of signaling mediators. However, the major trigger and the intrinsic pathogenesis of airway remodeling is still obscure.

Experimental Approach

The differential expressed genes in airway epithelia related to airway remodeling was screened and verified by RNA-sequencing and signaling pathway analysis. Then, the effect of increased CTSK in airway epithelia on airway remodeling and EMTU activation was identified both *in vitro* and *in vivo* and the molecular mechanism was elucidated in EMTU model. Moreover, the potential of CTSK as an effective biomarker of airway remodeling was analyzed in asthma cohort with different severity. Finally, the inhibitor of CTSK was administered to pursue the potential therapeutic interventions for airway remodeling of asthma.

Key Results

The expression of CTSK in airway epithelia increased significantly along with the development of airway remodeling in HDM-stressed asthma model. Increased secretion of CTSK from airway epithelia induced the activation of EMTU through the activation of PAR2-mediated pathway. Blockade of CTSK inhibits EMTU activation and alleviate airway remodeling effectively that is an effective intervention target of airway remodeling.

Conclusion and Implications

Increased expression of CTSK in airway epithelia is involved in the development of airway remodeling in asthma through EMTU activation, which was mediated partly through PAR2-mediated signaling pathway. CTSK is a potential biomarker for airway remodeling which may also be a useful target for the targeted intervention of airway remodeling in asthma patients.

Keywords: airway remodeling, airway epithelial cell, CTSK, EMTU

What is already known

Airway remodeling is closely involved in the pathophysiological progression of asthma.

Airway epithelial cells play a central role in the pathogenesis of asthma.

What this study adds

Expression of CTSK in airway epithelia is closely related to the severity of airway remodeling.

Increased expression of CTSK promotes EMTU activation through PAR2 pathway.

What is the clinical significance

CTSK is a promising target of airway remodeling in asthma patients.

Introduction

Asthma is a complex clinical syndrome that is characterized by wheezing, coughing, variable airflow limitation, and airway hyperresponsiveness (AHR) [1]. Although asthma is always viewed as a chronic airway inflammatory disease, the main traditional anti-inflammatory glucocorticoid therapy for asthma has limited clinical effects in improving lung function, preventing disease exacerbations and delaying the natural pathological process of asthma [2]. Notably, accumulating studies in recent years have revealed that airway remodeling is involved in the occurrence, development, and treatment sensitivity of asthma. Concretely, both our previous work and peer studies have demonstrated that airway remodeling occurs in the absence of airway inflammation which further contributes to airway obstruction and AHR directly. Thus, airway remodeling is closely involved in the pathophysiological progression of asthma that further influence the treatment response of asthma [3]. However, the pathogenesis of airway remodeling in asthma is still obscure. Therefore, the major trigger and the intrinsic pathogenesis of airway remodeling is urgently needed further work to be addressed.

Airway epithelial cells is the first cell barrier to contact the outside hazardous stimuli that play a central role in the pathogenesis of asthma [4]. It is particularly noteworthy that the structural and functional instability of airway epithelia have been identified as the main incentive of airway remodeling in asthma patients [5]. In detail, after stimulated by noxious stress, damaged airway epithelia can activate resident submucosal mesenchymal cells by releasing various inflammatory factor, growth factors (eg, transforming growth factor beta 1 [TGFB1]) and exosomes, etc., ultimately leading to airway remodeling [6]. On this basis, the cross talk between airway epithelia and submucosal mesenchymal cells is regarded as the activation of epithelial-mesenchymal trophic units (EMTUs), which finely interpreted the pathogenesis of inflammation-independent airway remodeling [7,8]. Parallel studies have also verified that EMTU is activated in asthmatic patients that is accompanied by increased activation of fibroblasts [8]. However, the underlying mechanism of AECs-regulated EMTU activation during airway remodeling in asthma patients remain obscure.

To further clarify the involvement of airway epithelia in the activation of EMTU and the development of airway remodeling, asthma model with different degrees of airway remodeling was constructed and the differential expressed genes in airway epithelia were screened. Interestingly and noticeably, the expression of CTSK increased significantly in the airway epithelia of asthma patients that is highly correlated with the degree of airway remodeling [9]. Then, to further clarify the possible feasibility of CTSK as a biomarker for airway remodeling in asthma patients, the correlation between the expression of CTSK and lung function parameters in asthma patients was analyzed. On this basis, the influence of CTSK on EMTU activation and subsequent airway remodeling was addressed both *in vitro* and *in vivo*. Moreover, the underpinning molecular mechanism of CTSK-mediated airway remodeling was also unraveled in the co-culture model of EMTU. Finally, the targeted intervention effect of CTSK in airway remodeling was explored through corresponding inhibitor treatments.

Materials and Methods

Animals

The animal studies were approved by the Central South University at XiangYa Animal Care and Use Committee (No. 201803079). All the methods were carried out in accordance with the relevant guidelines and regulations. BALB/c mice (male, 8 weeks, SPF) were obtained and housed under barrier system with air-filtered, temperature-controlled units under a 12 h light-dark cycle and with free access to food and water. For the construction of HDM-stressed model, mice were intranasally (i.n) stimulated with 50ug HDM (Sigma-

Aldrich, St. Louis, MO, USA) for 3 weeks, 5 weeks and 7 weeks, separately. The control mice received an equal volume instillation of PBS [10]. Some of HDM-stressed mice were intraperitoneally treated with CTSK inhibitor, Odanacatib (3.606 mg/kg, Selleck), 1 time per week over the period of HDM treatment [11].

RNA sequencing and data analysis

Primary airway epithelial cells from HDM stressed mice were extracted according to previous description [12]. The construction of sequencing library, computer sequencing and data analysis were completed by Beijing Nohe Zhiyuan Technology Co., LTD. The sequencing data were analyzed for differences. Among them, there were 170 differentially expressed genes between the 3-week HDM-stressed group and control group, and 639 differentially expressed genes between the 7-week HDM-stressed and control group. Then, the differentially expressed genes from the two groups were intersected. 560 nonoverlapping genes were selected as differential genes associated with airway remodeling.

RNA extraction, RT-PCR and qPCR

Total RNA was prepared from lung tissue samples with Trizol (Invitrogen) according to protocol and quantified on a Varioskan microplate reader (Thermo Scientific, USA). RT-PCR was conducted according to the PrimeScript RT Master Mix Kit (Takara, Japan). Quantitative PCR (qPCR) was performed on a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-rad, USA) by the use of TB Green® Premix Ex Taq (Takara, Japan) with thermal cycling conditions [13]. Primer sequences were shown in Table 1.

Histopathology and immunohistochemistry of lung tissue

Lungs were inflated, fixed in 4% paraformaldehyde, embedded in paraffin blocks, and cut into 5 μ m sections. Hematoxylin and eosin staining was performed to evaluate inflammatory infiltration and basement membrane thickness [14]. Periodic acid-Schiff staining (Shanghai Sun Biotechnology, Pudong, Shanghai, China) was used to detect mucus secretion [15] and Masson Trichrome staining was used to measure collagen deposition [16]. Immunofluorescence analysis was performed as previously described [17]. In brief, the paraffin sections were performed with the following antibodies: CTSK (sc-48393, Santa Cruz), PAR2 (sc-365992, Santa Cruz), KI67 (ARG53222, Arigo Biolaboratories), E-CAD (ab76055, Abcam), VIMENTIN (#5741, Cell Signaling Technology) and α -SMA (ab5694, Abcam). For microscopy, we employed Zeiss Axio Scope.A1 or Zeiss Discovery.V8 Stereo microscopes (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) integrated with an Axio-Cam ICc3 camera (Spectra Service, Ontario, NY). Images were obtained by AxioVision Rel.4.7 software from Zeiss. Negative controls included the omission of the primary antibody. The quantification of immunofluorescence intensity was performed by Image J (version 1.52; National Institutes of Health, Bethesda, Md).

Patient populations and sample collection

This study was approved by the Institutional Review Board of School of Basic Medical Science of Central South University (No. 2020KT-54). All necessary informed consents in writing were obtained from all patients for permission to use their clinical information and samples for analysis. The 2022 Global Strategy for Asthma Management and Prevention was employed for asthma diagnosis [18]. Asthma was categorized as mild-moderate asthma (MMA) and severe asthma (SA) by disease severity described as previous study [19]. Sputum samples, pulmonary function tests (within 14 days before and after sample collection) and medication records were processed or collected from 21 healthy controls (HCs), 44 patients with MMA and 8 patients with SA at Xiangya Hospital of Central South University (Changsha, Hunan, China). The demographic and clinical characteristics of those patients are shown in Table 2.

Induced sputum samples were collected from the enrolled 21 HCs and 44 asthma patients. For sputum induction, each volunteer inhaled a 4.5% saline atomized solution three times for 5 min each time and coughed sputum into a separate cup. Then, four-parts 0.1% dithiothreitol was added to one-part sputum and mixed for 15 min before adding four-parts phosphate-buffered saline. Finally, sputum cells were pelleted by centrifugation and used for RNA extraction after filtering [20].

Quantitative CT assessment of airways

The computed tomography (CT) scans of asthma patients were obtained from the Imaging Department of Xiangya Hospital of Central South University. The Synapse 3D 4.4 System (Fujifilm Medical System, Tokyo, Japan) was employed for conducting 3-dimensional reconstruction of airway trees and assessing the airway dimensions. The study workflow is shown in Figure S1, with procedures described in detail elsewhere [21]. The Right upper lobe apical segment (RB1), right lower lobe posterior basal segment (RB10), left upper lobe apical segment (LB1) and left lower lobe posterior basal segment (LB10) were selected as the target bronchial tube for detailed analysis. Airway dimensional measurements (ie, airway wall thickness (AWT), the percentage of wall thickness, airway wall area and AWT-Pi10) were also obtained by the software at the midpoint of the third to fifth generation of airways as previous describe [22].

Cell culture and treatment

Human bronchial epithelial (HBE) cells and human lung fibroblasts (HLF-1), which were purchased from Lifeline Cell Technology (Frederick, MD, USA), were cultivated in DMEM with 10% FBS at 37 in 5% CO₂. Conditioned medium-exposure experimental model was carried out to assess epithelial-mesenchymal cross talk according to the previous method with minor modifications [23]. Cell-debris-free culture medium from HBE cells was harvested and incubated with HLF-1. This enables the assessment of the effect of soluble mediators released from HBE cells on the phenotype of HLF-1. To construct CTSK-overexpressed HBE cells, cells were transfected with plasmid for 48 hours. Meanwhile, primary mouse airway epithelial cells were prepared according to our previously procedures [12].

ELISA assay

Cell conditioned medium was collected and processed as previously described [10]. Levels of CTSK (430804, BioLegend) were determined with ELISA assays according to the manufacturer's protocols.

Western blot analysis

Western blot was performed as described previously [10]. Briefly, 50 ug protein from lung tissue, HBE cells or primary airway epithelial cells from mice was prepared and separated by 10% or 12% SDS/PAGE. Then, the separated protein was transferred to a polyvinylidene difluoride (PVDF) membrane. Next, the PVDF membrane was incubated with antibody overnight and then incubated with horseradish peroxidase-conjugated secondary antibody. The following antibodies were used to determine the expression of corresponding protein: CTSK (sc-48393, Santa Cruz), PAR2 (sc-365992, Santa Cruz), E-CAD (ab76055, Abcam), α -SMA (ab5694, Abcam) and COL-1 (ab34710, Abcam). B-actin (ab8226, Abcam) was used as corresponding controls.

Statistical analysis

All data were analyzed with GraphPad Prism Software (version 6; GraphPad Inc, San Diego, Calif). Characteristics of asthma patients were analyzed using chi-squared test, Fisher exact test, or Mann-Whitney U test. Comparisons between 2 groups were performed with Mann-Whitney U test. Differences in means between multiple groups were examined by 1-way ANOVA followed by Tukey post hoc test or 2-way ANOVA followed by Tukey post hoc test. Spearman correlation was used to assess the associations among the expression of CTSK and lung function, or airway CT parameters. Differences were considered statistically significant for *P < 0.05, **P < 0.01, or ***P < 0.001.

RESULTS

The expression of CTSK in airway epithelia increased significantly along with the development of airway remodeling in the mice model of asthma

To explore the molecular events of AECs involving in the development of airway remodeling, mouse model of asthma with different degrees of airway remodeling was constructed by HDM stress (Figure 1A). HE, PAS and Masson staining results showed that the degree of airway remodeling (shown as the level of airway inflammation, mucus production and collagen deposition) increased significantly with the duration of HDM

stress (Figure 1B-D). Next, RNA-Seq expression profiling of primary airway epithelial cells was performed from control, 3-week, 5-week and 7-week HDM-stressed group, respectively. The differential expression genes in the 7-week HDM-stressed group were intersected with the differential expression genes in the 3-week HDM-stressed group. Then, 560 differential expression genes were obtained that is associated with asthma airway remodeling (Figure S2A). On this basis, 17 candidate genes were screened that have been previously confirmed to be related to airway remodeling through KEGG and GO enrichment analysis screened (Table 3). qPCR results further showed that, among the 17 differentially expressed genes, the expression of CTSK increased gradually along with the development of airway remodeling after HDM stress (Figure S2B). The IHC staining results also showed the consistent results of CTSK expression in the HDM-stressed models (Figure 1B-1E). Not only that, the expression of CTSK was positively correlated with mucus production and collagen deposition which indicated the potential of CTSK as a biomarker of airway remodeling in asthma model (Figure 1F and 1G).

CTSK is a potential biomarker of airway remodeling for asthma patients

To further verify the potential of CTSK as a biomarker of airway remodeling in asthma patients with variable severity, the expression of CTSK in the induced-sputum samples of HCs ($n = 21$) and asthma patients (including 44 MMA and 8 (SA)) were detected. The demographic characteristics were shown in Table 1 and there were no significant differences in age or gender between the groups. The expression of CTSK increased significantly in the asthma group compared to HC group (Figure 2A). More than this, stratification in this cohort based on disease severity identified that the elevated CTSK in asthma patients were primarily driven by the severe asthma subjects (Figure 2B). Considering the obvious discrimination of CTSK level in asthma patients, especially the differences between HC group and MMA group, the predictive values of CTSK for the classification of asthma was analyzed using nominal logistic regression and showed as areas under the ROC curves. Consistent with our prediction, CTSK level yield excellent predictive value for distinguishing asthma patients from HC subjects (area under the ROC curve, > 0.9667). Moreover, CTSK level can also distinguish different asthma subgroups with different severity effectively, such as MMA patients and SA patients (area under the ROC curve, > 0.819) (Figure 2C). Moreover, the correlation between the level of CTSK in the sputum samples and the lung function parameters in asthma patients were also analyzed. Among all asthma patients, the level of CTSK was negatively correlated with FEV1 ($r=-0.433$, $p= 0.0074$), FEV1 percentage ($r=-0.4247$, $p= 0.0098$) and FEV1/forced vital capacity ($r=-0.4771$, $p= 0.0033$) (Figure 2D). Due to the more obvious high expression of CTSK, the correlation analysis was also conducted in SA group. In SA group, there were more significant negative correlations between CTSK levels and FEV1 ($r=-0.5042$, $p= 0.0489$), FEV1 percentage ($r=-0.7093$, $p= 0.0045$), FEV1/forced vital capacity ($r=-0.7695$, $p= 0.0013$) (Figure 2E). In addition, CTSK is also positively correlated with FEV1 ($r=-0.3770$, $p= 0.0366$) and FEV1 percentage ($r=-0.5133$, $p= 0.0219$) in MMA subjects (Figure S3A). Also of note is that, the level of CTSK in the sputum samples positively correlated with ACT score in all asthma subjects ($r=-0.3628$, $p= 0.0232$) (Figure 2F), SA subjects ($r=-0.8194$, $p= 0.002$) (Figure 2G) and MMA subjects ($r=-0.2560$, $p= 0.2351$) (Figure S3B).

The above results have demonstrated that the level of CTSK in the sputum samples is strongly associated with lung function parameter of asthma patients with different severity. On this basis, the possibility of CTSK as a biomarker of airway remodeling was further explored through evaluating the correlation between the level of CTSK and airway CT parameters in asthma patients. To account for the differences in airway size, the airway CT parameters was calculated for the labeled airways in each asthma subject (Table 2). Analysis results demonstrated that the level of CTSK was positively correlated with airway wall thickness ($r=0.3770$, $p= 0.0366$), percentage of wall thickness ($r=0.6538$, $p= 0.0013$), airway wall area ($r=0.5506$, $p= 0.0065$) and AWT-Pi10 ($r=0.3770$, $p= 0.0366$) in asthma patients (Figure 3A). Especially, in SA subjects with higher CTSK expression, the correlation between the level of CTSK and airway wall thickness ($r=0.8660$, $p= 0.0117$), the percentage of wall thickness ($r=0.7302$, $p= 0.0429$) or AWT-Pi10 ($r=0.6477$, $p= 0.0429$) is more obvious (Figure 3B). In addition, the level of CTSK is also positively correlated with airway wall thickness ($r=0.6739$, $p= 0.0011$), percentage of wall thickness ($r=0.5829$, $p= 0.0070$) and AWT-Pi10 ($r=0.5279$, $p= 0.0116$) in MMA subjects (Figure S3C). These results demonstrate that the level of CTSK can reflect the

degree of airway remodeling in asthma patients with varying severity. Taken together, these results strongly suggest the potential of CTSK as an effective biomarker of airway remodeling for asthma patients.

Increased secretion of CTSK from airway epithelial cells induced the activation of EMTU

Our previous study has shown that EMTU was activated in HDM-stressed asthma model which contributes to the pathogenesis of airway remodeling [24]. Here, our results further showed that, with the development of airway remodeling, the expression of Ki67 and E-cad in HBE cells decreased gradually and the expression of VIMENTIN and α -SMA in fibroblasts progressively around the bronchia in HDM-stressed asthma model, indicating the activation of EMTU (Figure S4). To further study the molecular mechanism of CTSK promoting EMTU activation, the co-culture models of EMTU *in vitro* was constructed. After HDM stress, the expression of CTSK in HBE cells increased significantly (Figure 4A). Specifically, the secretion of CTSK from HBE cells was induced significantly after HDM stress (Figure 4B). It is also worth noting that, the expression of α -SMA and collagen 1 in HLFs was also promoted in the co-culture models which indicated the activation of EMTU (Figure 4C, D and Figure S5).

To further verify the critical role of CTSK in EMTU activation, CTSK high-expressed HBE cells was constructed through CTSK-high expression plasmid (Figure S6). Expression of CTSK in HBE cells increased obviously both in cellular and extracellular after plasmid transfection (Figure 4E and F). Consistent with previous results, the expression of α -SMA and collagen 1 in HLFs increased significantly after co-culturing with HBE cells with high CTSK expression (Figure 4G, H and Figure S5). Taken together, these results verify that the increased expression of CTSK in airway epithelia induce the activation of EMTU effectively after HDM stress.

CTSK regulates the activation of EMTU through PAR2-mediated pathway

Previous studies have confirmed that protease-activated receptors (PARs) 2 is a major receptor of CTSK that is involved in the pathogenesis of pulmonary diseases by regulating downstream signaling molecules such as p85 and p110 [25-27]. Thus, the influence of CTSK on PAR2-mediated pathway in HLFs after EMTU activation was detected. In our co-culture model, the interaction between CTSK and PAR2 was confirmed by COIP detection (Figure 5A). Besides, both HDM-stress HBE cells and CTSK-overexpressed HBE cells could activate PAR2-mediated pathway in HLFs significantly after co-culture (Figure 5B and C). Consistent with previous results, PAR2 silence after siRNA transfection in HLFs inhibited the activation of EMTU (Figure 5D and E). These results further demonstrated that the activation of EMTU induced by CTSK was mediated party through PAR2-related signaling pathway. **Blockade of CTSK inhibits EMTU activation and alleviates airway remodeling in asthma mice model**

To further confirm the possibility of CTSK as a potential therapeutic target for airway remodeling in asthma, Odanacatib (a CTSK specific inhibitor) was used to block the expression of CTSK. The expression of CTSK in the lung of HDM-stressed asthma model was inhibited significantly after Odanacatib treatment (Figure 6A). HE, PAS and MASSON staining revealed that CTSK blockage alleviated the mucus production and collagen deposition significantly after HDM stress (Figure 6B, C and D). Moreover, lower levels of VIMENTIN and α -SMA were detected in the lung of HDM-stressed model after Odanacatib treatment which indicated the inhibition of EMTU activation (Figure 7A). Similar results were also detected in the immunohistochemistry images in which Odanacatib treatment reduced the expression of VIMENTIN and α -SMA around the bronchia and increased the expression of Ki67 and E-CAD in AECs (Figure 7B-E). These results corroborate the notion that CTSK blockade targeting airway epithelia prevent EMTU activation and alleviates airway remodeling in asthma.

DISCUSSION

Asthma is a common irreversible chronic pulmonary disease that is associated with increasing global health burden [28]. Besides airway inflammation, airway remodeling, the major pathological feature of asthma determines the pathology progression and treatment sensitivity of asthma. In this study, we demonstrated that the expression of CTSK in airway epithelia increased significantly along with the exacerbation of airway

remodeling in HDM-stressed asthma model. More than that, the expression of CTSK is negatively associated with the lung function parameters of asthma patients, which is a potential biomarkers of airway remodeling in asthma patients. In addition, the increased secretion of CTSK from AECs led to EMTU activation through PAR2-mediated signaling pathway after HDM exposure. Consequently, targeted block of CTSK can inhibit EMTU activation and alleviate airway remodeling in HDM-stressed mice. It is traditionally deemed that airway inflammation drives airway remodeling which further induce AHR and fixed airflow obstruction during the development of asthma. However, more and more evidences demonstrated that airway remodeling and airway inflammation may occur in parallel in childhood asthma [29]. Moreover, airway remodeling continued to progressed that is independent of airway inflammatory even after allergen is removal [30]. In this study, our results also confirmed that mucus secretion and collagen deposition was induced in the early stage after HDM stimulation which is accompanied with the onset of airway inflammation. Together, these results indicate that airway remodeling and airway inflammation can occur in parallel during the development of asthma.

As the interface between the host and external environment, airway epithelia perform an important role in the pathogenesis of asthma though receiving, integrating, and responding to local and environmental signals [31-33]. Consequently, the structural damage and abnormal repair of airway epithelia can induce airway remodeling directly even before the onset of asthma [34]. However, the specific mechanism of airway remodeling induced by damaged epithelia remain to be elucidated. Our results demonstrated that the expression of CTSK in airway epithelia increased significantly along with the exacerbation of airway remodeling after HDM stress. More than this, the increased expression of CTSK is negatively associated with the lung function parameters of asthma patients which also closely related to the degree of airway remodeling. This correlation between CTSK expression and lung function parameters of asthma patients indicated the potential of CTSK as an efficient biomarker of airway remodeling in asthma patients.

The lack of accessible and effective clinical biomarkers remains a major obstacle to the progress of airway remodeling in asthma patients. The biomarkers in asthma can be obtained in various sources (e.g., urine, blood, bronchoalveolar lavage fluid, induced sputum) [35,36]. Among them, induced sputum enables us to analyze the expression level of secreted factors from airway epithelial cells more directly, which is relatively easy to manipulate [37]. Here, our results demonstrated that the increased expression of CTSK in the induced sputum is negatively correlated with the lung function, and positively correlated with the degree of airway remodeling and the severity of asthma in the cohort of asthma patients. Especially, asthma patients with variable severity were enrolled in our present study that can assess the progression of asthma more comprehensively. Thus, the level of CTSK in the induced sputum could indicate the severity and pathology phenotype of asthma (such as decreased lung function and airway remodeling) which also indicated the potential of CTSK as effective biomarker of airway remodeling in asthma patients. However, there are also some limitations in this study. The first one is that this is a cross-sectional and a snapshot of asthma cohort in a short time window and a longer time span (e.g., one to several years). Measurements to the evolution of asthma over time as well as comparison between stable phase and asthma exacerbation phase would be conducted in our subsequent study. Besides, the relationship between circulating CTSK and airway remodeling or asthma severity also need further analysis.

Equally valuable and interesting is the application of an automated, quantitative software program in this study to measure and analyze the degree of airway remodeling in asthma patients, such as airway wall thickness, the percentage of wall thickness, and airway wall area and AWT-Pi10. The automated technique used eliminated selection bias by segmenting, labeling and measuring all of the proximal airways and allowing comparison of those airways that may have been excluded using previous methods. Imaging examinations, especially CT, have been prove to be a useful way to assess airway remodeling in previous studies [38]. Kasahara et al. found that the radiographic measurement of WT and WA increased significantly in asthma patients [39]. Therefore, the detection and evaluation method of airway remodeling in this study is an accurate and useful supplement to the current study of airway remodeling in asthma patients.

EMTU activation has been proved to play a critical role in the occurrence and development of airway

remodeling, although the initiation of EMTU activation is still unclear [40,41]. Our results demonstrate that EMTU was activated by epithelia-released CTSK after HDM stress. CTSK is a cysteine protease member of the cathepsin lysosomal protease family that regulates the dynamic balance of airway extracellular matrix by affecting the function of mesenchymal cells [42,43]. Moreover, CTSK is also a kind of secretory protein that can degrade collagen fibers during tissue remodeling through interacting with target cells in a paracrine manner [44,45]. The interaction between CTSK and TGF- β 1 in silica-induced pulmonary fibrosis leads to the activation of lung fibroblasts and airway smooth muscle cell which strongly indicated the involvement of CTSK in airway remodeling through activation of EMTU. Consistent with previous study, our results proved that CTSK secreted from AECs bind to the membrane receptor PAR2 on pulmonary fibroblasts that further induced the activation of fibroblasts. Similarly, Vemuri B. Reddy, et al. also reported that CTSS, a member of cysteine cathepsins, could cleave PAR2 in human keratinocytes which is consistent with the possible pruritic role of CTSS in inflammatory skin disease [46]. Meanwhile, previous studies have demonstrated that PAR2 was involved in tryptase-induced phenotypic conversion in atrial fibroblasts through p38/JNK pathway [47]. These evidences implicated the engagement of CTSK in fibroblasts activation through PAR2-mediated signaling pathway. However, the specific internal mechanism between CTSK and PAR2 during airway remodeling still needs in-depth study [48]. On this basis, our results further found that blockade of CTSK by Olanacatib inhibits the activation of EMTU both *in vitro* and *in vivo*. Accordingly, both airway inflammation and airway remodeling were also restrained in the HDM-stressed mouse model after Olanacatib treatment. Taken together, the current data indicate that CTSK is a potential treatment target to inhibit airway remodeling in asthma patients, although the specific role of CTSK in other cells cannot be excluded. More than this, CTSK was identified as a EMTU activation-related gene for the first time that is a vital mediator between airway epithelia and lung fibroblasts during EMTU activation. Different from other biomarkers, secretory CTSK can be released from damaged airway epithelia and act on target cells at a distance, which reflects the changes of pulmonary microenvironment in asthma patients more accurately.

Conclusions

In summary, we have demonstrated that the increased expression of CTSK in airway epithelia induces airway remodeling in asthma patients. Furthermore, the increased secretion of CTSK from airway epithelia activate fibroblasts through PAR2-mediated signaling pathway which is critical for EMTU activation and subsequent airway remodeling. These results provide some useful insights into the molecular mechanisms of airway remodeling which may provide new therapeutic approaches for airway remodeling in asthma.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that they have no competing interests.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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Figure Legends

Figure 1. The expression of CTSK in AECs increased significantly in the asthma models with different degrees of airway remodeling. (A) Model of asthma with different degrees of airway remodeling. Mice were instilled with HDM for 3-week, 5-week and 7-week, respectively. Control mice were treated with PBS. (B-D) HE, PAS, Masson staining in mice model. (E) Immunohistochemistry of CTSK in lung tissue of HDM-stressed mice. (F) Correlation analysis between CTSK mRNA expression and mucus production, collagen deposition in HDM-stressed mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 2. The expression of CTSK is related to the severity of asthma in patients. (A) The mRNA expression of CTSK in the induced sputum of asthma patients and HCs. (B) The mRNA expression of CTSK in the induced sputum of asthma patients with variable severity. (C) ROC analysis of CTSK and asthma severity. (D) Correlation analysis between the mRNA expression of CTSK and pulmonary function in asthma patients. (E) Correlation analysis between the mRNA expression of CTSK and pulmonary function in severe asthmatic patients. (F) Correlation analysis between the mRNA expression of CTSK and ACT score in asthma patients. (G) Correlation analysis between the mRNA expression of CTSK and ACT score in severe asthmatic patients. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. The expression of CTSK is related to the degree of airway remodeling in asthma patients. (A) Correlation analysis between the mRNA expression of CTSK and CT parameters in asthma patients. (B) Correlation analysis between the mRNA expression of CTSK and CT parameters in severe asthmatic patients. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 4. Secretory CTSK in airway epithelia is involved in the activation of EMTU in co-culture models. (A) The mRNA expression of CTSK in airway epithelial cells. (B) ELISA for the secretory of CTSK in the conditioned medium of airway epithelial cells. (C-D) The mRNA and protein expression of α -SMA and Collagen 1 in lung fibroblasts in co-culture model. (E) The mRNA expression of CTSK in CTSK-overexpressed airway epithelial cells. (F) ELISA for the secretory of CTSK in in the conditioned medium of CTSK-overexpressed airway epithelial cells. (G-H) The mRNA and protein expression of α -SMA and Collagen 1 in HLFs in the co-culture model with high expression of CTSK in airway epithelial cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 5 . Secretory CTSK from HBE cells regulated the activation of PAR2-mediated pathway in HLFs. (A) The combination of CTSK and PAR2 was detected by COIP. (B) The mRNA expression of PAR2 in lung fibroblasts after co-culture. (C) The mRNA expression of PAR2-mediated downstream molecules in HLFs after co-culture. (D) The mRNA expression of α -SMA, Collagen 1 and PAR2 signaling pathway in lung fibroblasts in co-culture model with PAR2 silence in HLFs. (E) The protein expression of α -SMA and Collagen 1 in HLFs co-culture model with PAR2 silence in HLFs. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 6 . CTSK blockage alleviated airway remodeling in HDM-stressed mice. (A) The protein expression of CTSK in lung tissue of HDM-stressed mice after CTSK inhibition. (B-D) HE, PAS, MASSON staining in HDM-stressed mice after CTSK inhibition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 7 . CTSK blockage reduced EMTU activation in HDM-stressed mice. (A) Western blot staining for α -SMA, Vimentin and E- cadherin in the lung tissue of HDM-stressed mice after CTSK inhibition. (B-E) Immunohistochemistry of α -SMA, Vimentin, E- cadherin and Ki67 in the lung tissue of HDM-stressed mice after CTSK inhibition. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 1 . TaqMan primers for RT-qPCR.

Primer genus	Gene	Primer
Human	CTSK	forward

Primer genus	Gene	Primer
Human	PAR2	reverse
Human Human Human Human Human Mouse	Collagen1 α -SMA PIK3R1 PIK3CA β -actin CTSK	forward reverse forward reverse
Mouse	β -actin	forward reverse

Table 2 . Characteristics of HCs and asthma patients.

	HCs	HCs	Asthma	MMA	SA
n	n	21	52	44	8
Age	Age	40.15±13.48	47.58±12.27	47.64±12.41	44.25±9.98
Height, m	Height, m	1.63±0.07	1.59±0.07	1.61±0.74	1.61±0.08
Weight, kg	Weight, kg	57.36±9.87	60.91±8.70	64.13±11.29	61.83±4.63
BSA	BSA	1.58±0.16	1.61±0.05	1.60±0.18	1.62±0.09
BMI	BMI	21.21±2.18	24.01±2.48	23.49±3.54	24.00±2.76
FEV ₁	FEV ₁	3.26±0.66	1.81±0.42	2.16±0.82	1.26±0.07
FVC	FVC	3.89±0.05	2.59±0.82	3.07±0.81	2.41±0.41
FEV ₁ /FVC	FEV ₁ /FVC	0.86±0.07	0.57±0.08	0.68±0.15	0.50±0.09
FEV ₁ %	FEV ₁ %	1.03±0.01	0.61±0.15	0.77±0.22	0.52±0.08
PEF	PEF	7.44±1.26	5.44±1.94	5.61±1.79	3.73±0.84
MEF ₇₅	MEF ₇₅	6.60±1.38	3.54±2.21	3.83±2.18	1.42±0.38
MEF ₅₀	MEF ₅₀	4.31±1.47	1.84±1.35	1.93±1.29	0.64±0.13
MEF ₂₅	MEF ₂₅	1.84±0.08	0.62±0.62	0.69±0.570	0.24±0.03
MEF _{75/25}	MEF _{75/25}	3.59±1.29	1.40±1.09	1.50±1.04	0.52±0.05
ACT Score	ACT Score	/	17.00±4.09	17.36±3.28	13.00±1.12

Table 3. Log2 fold change of candidate genes.

gene-name	Control/3week	Control/5week	Control/7week	3week/5week	3week/7week	5week/7week
CTSK	0.595991782	1.982547459	2.87461985	-1.3611171	-2.2806328	-0.918703563
CTSS	0.281391646	1.208747723	1.41780772	-0.90463727	-1.1406661	-0.236359941
ACP5	0.55870883	1.100538338	1.58466872	-0.52221907	-1.032937	-0.511772273
CCL17	0.755633893	1.667827507	1.64041994	-0.88427153	-0.8853444	0.001048479
CCL12	1.168826673	1.628109896	1.97093531	-0.43438794	-0.8056629	-0.370599527
CD36	0.772274544	0.659487551	1.16961136	0.132588878	-0.403337	-0.536156815
CTSL	0.206127716	0.496918133	1.62907313	-0.27127296	-1.4308174	-1.161733753
ITGB2	-0.46365558	0.835587521	1.20412345	-1.27888136	-1.6730303	-0.393942218
CXCL12	0.031382976	0.732768763	1.43106331	-0.67855008	-1.4046587	-0.72771829
IL-18	0.5914566	-1.19132404	-1.3241934	1.804377305	1.91249468	0.108921487
S100A8	-0.00408659	-0.896642874	-0.8966429	0.921750875	1.31593464	0.395925462
CSF1	-0.68694331	-0.507880055	-1.0937697	-1.02952878	0.40267288	0.562563231
IL-13	1.17006761	3.009048362	3.67109875	-1.81821076	-2.5104307	-0.688586945
S100A9	0.040350886	-0.851710518	-1.0665055	0.921063034	1.1087594	0.189434012
ATP60DV2	0.258433761	0.778531451	1.64192451	-0.49577905	-1.3864479	-0.890681598
CCL7	0.852039517	0.546161113	1.58273592	0.331117276	-0.7305785	-1.060802904
CLEC7a	0.086187392	0.551473599	1.28124353	-0.43890005	-1.1961617	-0.75668141

Figure S1. The study workflow of 3D Imaging-based Stereology.

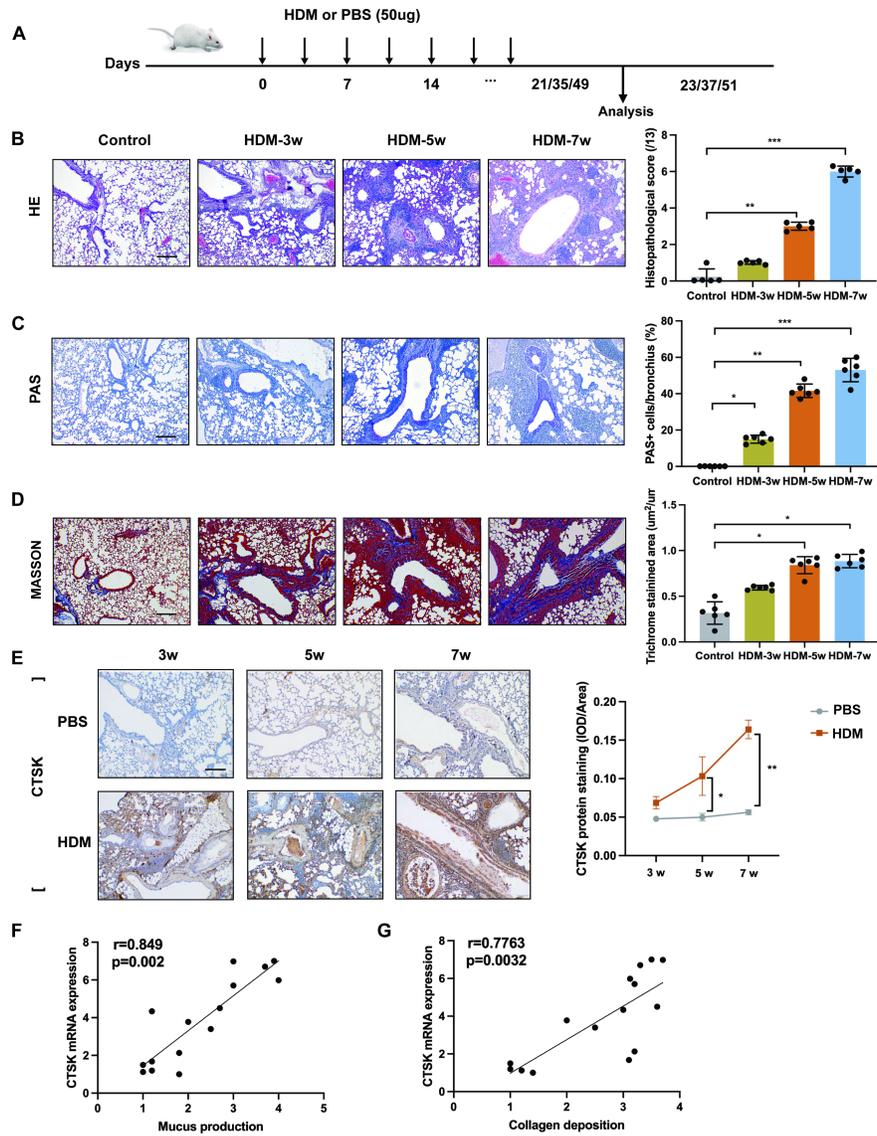
Figure S2. The expression of remodeling candidate genes in HDM-stressed mice. (A) Differential genes indistinct HDM stimulation groups. (B) The mRNA expression of candidate genes in the lung tissue of HDM-stressed mice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

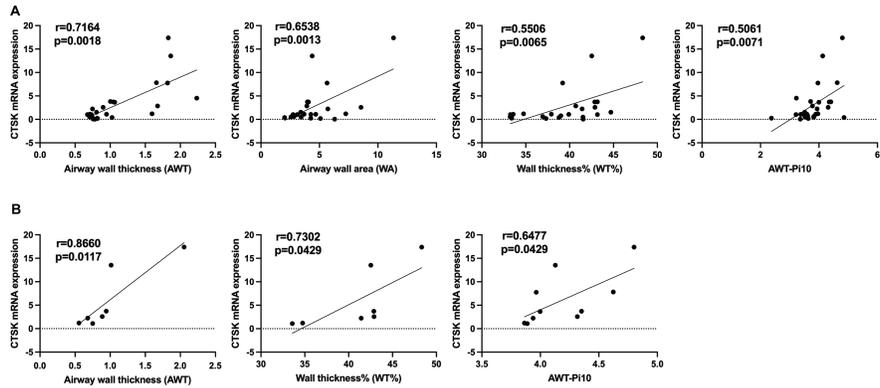
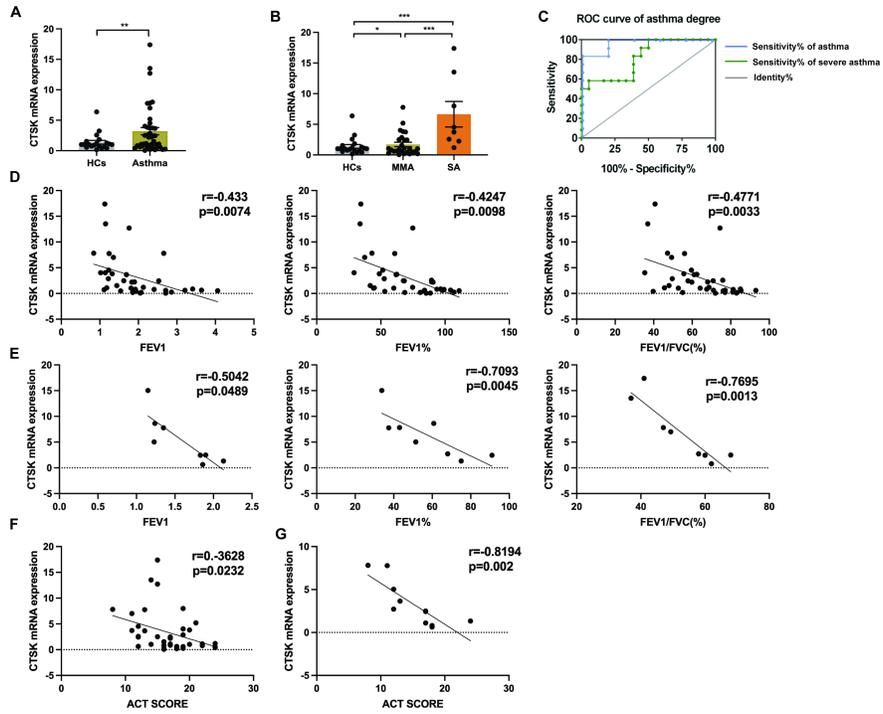
Figure S3. The expression of CTSK is related to lung function and airway CT parameters in MMA patients. (A) Correlation analysis between CTSK expression and lung function in MMA subjects. (B) Correlation analysis between CTSK expression and ACT score in MMA subjects. (C) Correlation analysis between CTSK expression and airway CT parameters in MMA subjects. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

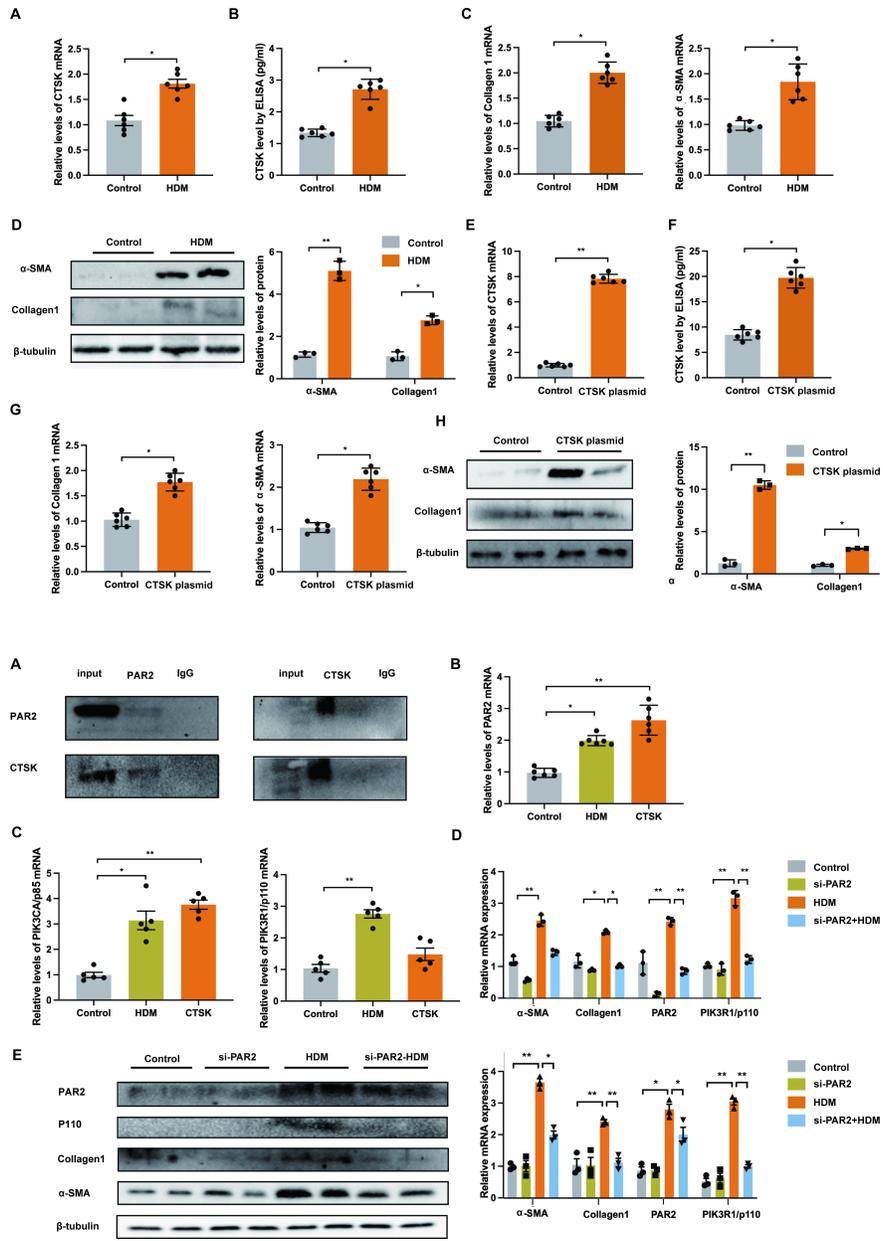
Figure S4. EMTU activation in the asthma models with different degrees of airway remodeling. (A-D) Immunohistochemistry of α -SMA, Vimentin, E-cadherin and Ki67 in the lung tissue of HDM-stressed asthma model with different degrees of airway remodeling e. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

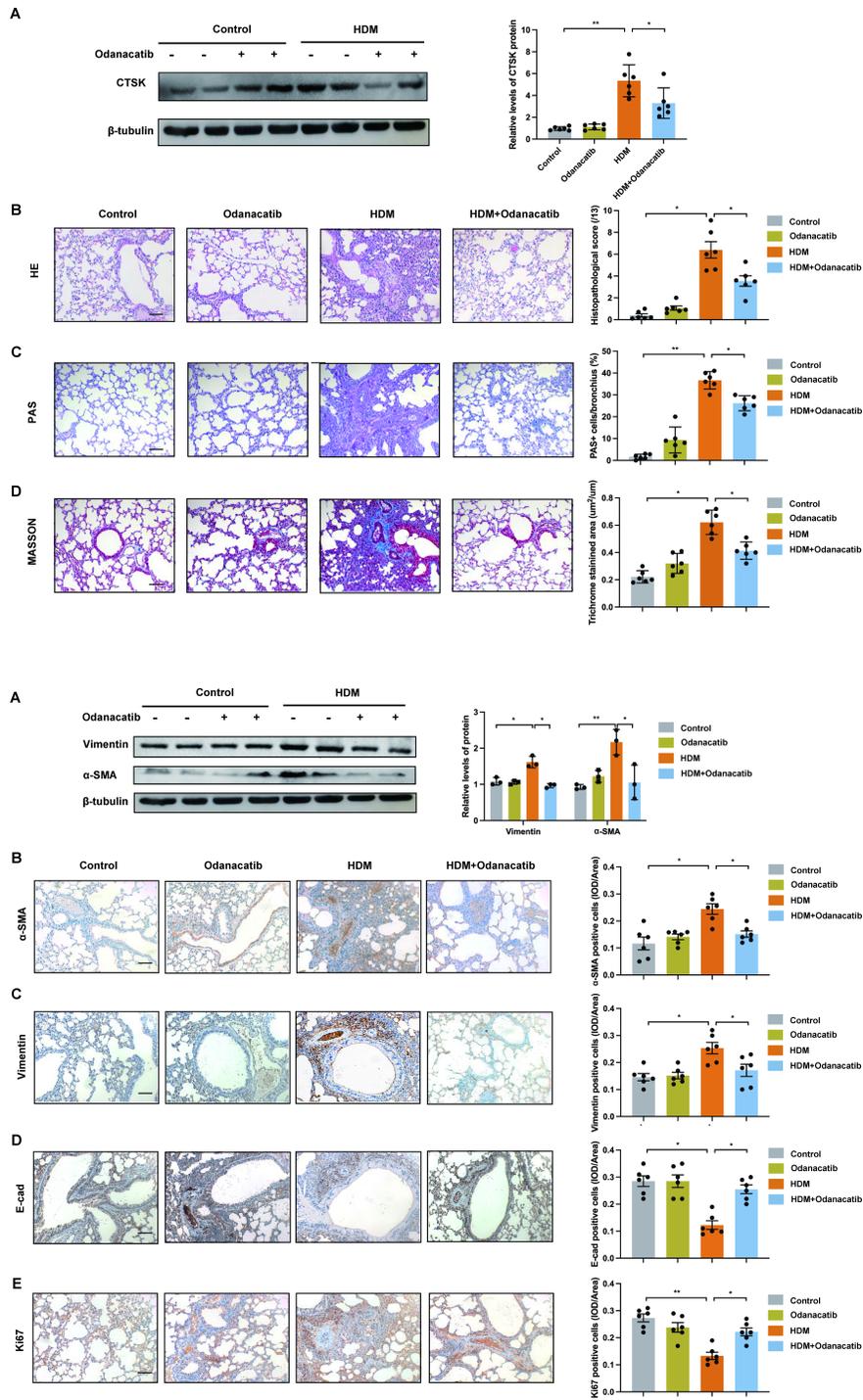
Figure S5. EMTU activation in co-culture model. (A-B) Immunofluorescent staining for α -SMA and Collagen 1 in lung fibroblasts in EMTU model.

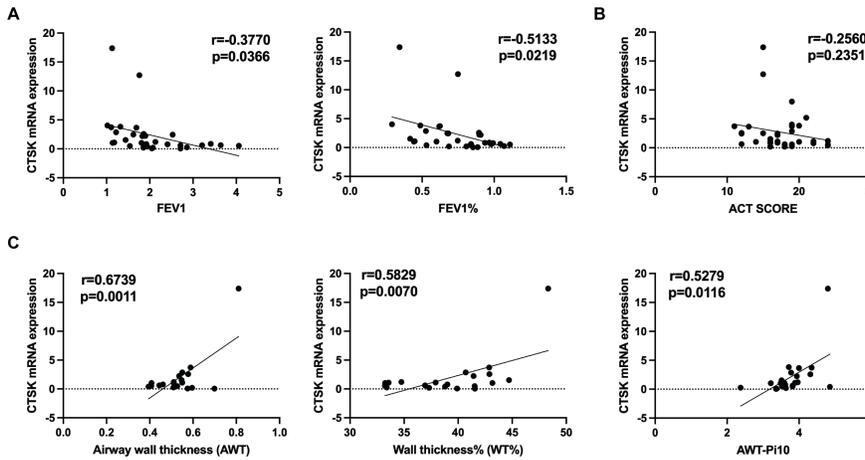
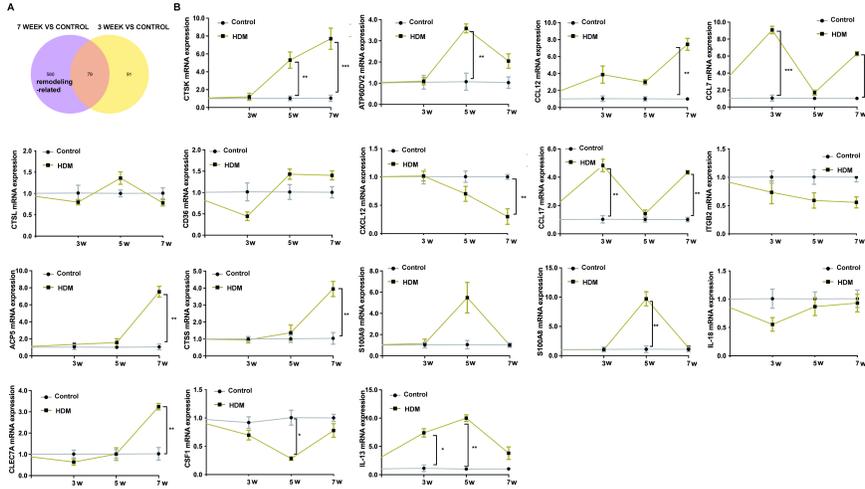
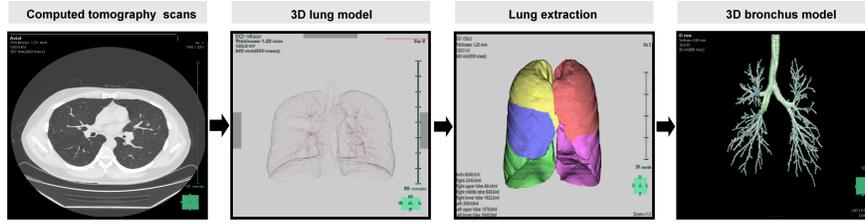
Figure S6. Construction of HBE cells with high expression of CTSK. (A-B) The mRNA and protein expression of CTSK in airway epithelial cells after transfection with CTSK-overexpressed plasmid t. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

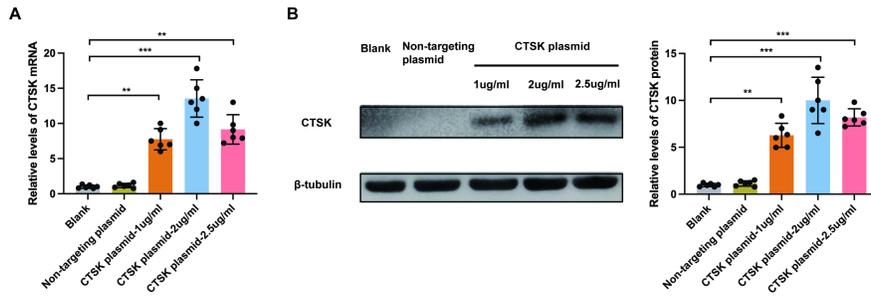
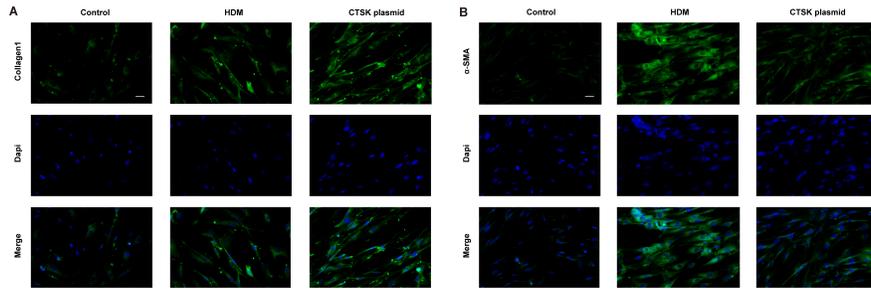
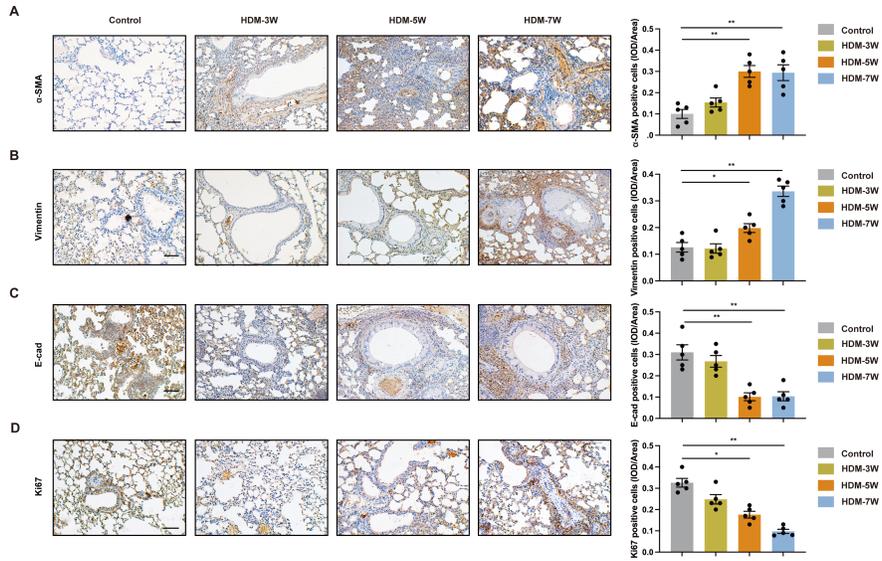












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