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Expression and localization of the MMP inhibitor RECK in a rat model of COPD: its involvement in the development of COPD

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Abstract. – OBJECTIVE: To unravel the potential function of reversion-inducing cysteine-rich protein with Kazal motifs (RECK) as the matrix metalloproteinase (MMP) inhibitor in the development of chronic obstructive pulmonary disease (COPD).

MATERIALS AND METHODS: Twelve specific pathogen-free Sprague Dawley rats were randomly assigned to the control cohort (n = 6) or the COPD cohort (n = 6). COPD model was developed by tobacco smoke exposure. Functional residual capacity (FRC), static lung compliance (Cchord), ratio of forced expiratory volume in 0.1 s to forced vital capacity (FEV0.1/FVC), and peak expiratory flow (PEF) were detected by respiratory function tests. Immunohistochemistry was performed to determine the pathological changes as well as the expression and localization of RECK in pulmonary tissue. RECK expression was further quantified by real-time polymerase chain reaction (PCR) and Western blot assays.

RESULTS: COPD rats had significantly reduced FEV0.1/FVC% and PEF values but increased FRC and Cchord levels, as compared to the control cohort ($p < 0.05$). Hematoxylin and eosin (HE) staining indicated typical COPD pathological changes, including leukocyte infiltration, airway thickening, alveoli fusion, etc., in the COPD rats. IHC indicated reduced expression of RECK in the COPD cohort, which was mainly expressed on the epithelium and partly expressed on subepithelial cells and inflammatory cells. Real-time PCR and Western blot assays further revealed the significantly lower expression of RECK in lung tissue from the COPD cohort.

CONCLUSIONS: RECK is mainly expressed on airway epithelial cells. COPD rats expressed significantly lower RECK levels, indicating that RECK exhibits a protective function in the development of COPD.

Key Words:
MMP inhibitor, RECK, COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most common chronic diseases in the world, with a prevalence of approximately two hundred million people and the fourth highest mortality rate worldwide[1]. Airway remodeling is the most common pathological change induced by COPD, featured as goblet cell hyperplasia, subepithelial fibrosis, airway thickening, vascular proliferation, and hyperplasia of smooth muscle cells, etc[2,3]. Matrix metalloproteinases (MMPs) play a pivotal role in airway remodeling. Significant elevation of MMP expression, including MMP-1, -2, -3, -8, -9, -10, and -12, has been observed in bronchoalveolar lavage fluid and induced sputum in COPD patients[4,5]. MMP-9 also has been found to be increased in biopsied lung tissue and serum from COPD patients[6-8]. The abnormally increased expression of these MMPs can result in extracellular matrix degradation, alveolar space enlargement, reduced alveolar elastic recoil, and increased leukocyte infiltration, thus leading to the eventual development of COPD[9].

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Reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is an MMP inhibitor that contains the structure of serine protease inhibitors and specifically inhibits membrane-anchored MMPs[10]. RECK is physiologically expressed in multiple organs and tissues, including the lung[10]. Significantly reduced expression of RECK has been observed in malignant tumor tissues as well as in malignant cell lines; therefore, it may contribute to the invasiveness of malignant diseases[11,12]. RECK also has been demonstrated to be involved in the development of many nonmalignant diseases, including asthma, myocardial fibrosis, coronary atherosclerosis, etc. Compared to normal individuals, it has been shown that asthmatic patients have a significantly reduced RECK level in induced sputum that is positively correlated with the forced expiratory volume in 1 s (FEV1)[13]. A previous study also has demonstrated that an animal model of myocardial fibrosis had lower levels of RECK in myocardial tissue, compared to controls. Moreover, angiotensin II (Ang-II) can suppress the expression of RECK in cardiac fibroblasts and promote their migration. In contrast, the overexpression of RECK can inhibit the migration of cardiac fibroblasts mediated by Ang-II[14]. Furthermore, it has been shown that RECK is capable of inhibiting various MMPs, including MMP-2, -7, and -9, among which MMP-9 has been found to be associated with the development of COPD[15,16]. However, the relevance of RECK in the development of airway remodeling in COPD has yet to be clarified. Therefore, utilizing a rat model of COPD, we sought to determine the expression and localization of RECK in lung tissue so that we could eventually decipher its involvement in the development of airway remodeling and COPD.

Materials and Methods

Animals

Healthy adult male Sprague Dawley rats (n = 12; weight, 200-250 g) were purchased from Guangdong Medical Laboratory Animal Center and housed at the Animal Center of the Guangzhou Respiratory Institute. The rats were randomly divided into the control cohort (n = 6) or the COPD cohort (n = 6). Rats of COPD cohort were exposed to smoking in a cigarette smoke chamber for one hour each time and twice per day. The interval between every two times must be no less than 6 hours. A total of 10 cigarettes (without filters) were used per hour. To make it easy for the rats to adapt to the smoking exposure, it started with 5 cigarettes each time at the beginning and added one more cigarette every two days until it reached 10 cigarettes each time. The smoking exposure lasts for 24 weeks before the COPD rat model was fully developed. No special treatment was applied to the control cohort. The pulmonary function test was performed at the end of the smoke exposure for all the rats in the control cohort and the COPD cohort. After that, all of the rats were euthanatized and sampled for further analysis. This study was approved by the Ethics Committee of Guangzhou Medical University.

Pulmonary Function Test

After anesthetization with an intraperitoneal injection of pentobarbital sodium (1 g/dL, 100 mg/kg), the rats were fixed and underwent tracheal intubation through the incision between the second and third cricoid cartilage. The function of the lung in terms of the functional residual capacity (FRC), static lung compliance (Cchord), ratio of forced expiratory volume in 0.1 s to forced vital capacity (FEV0.1/FVC), and peak expiratory flow (PEF) were determined by an invasive lung function apparatus during mechanical ventilation.

Immunohistochemical Staining

Excised lung tissue was fixed with 4% paraformaldehyde for 24 h, followed by immersion in 70% ethanol, paraffin-embedding, and sectioning. After deparaffinization with xylene, hematoxylin and eosin (HE) staining was performed, and the tissue sections were examined under an optical microscope to detect pulmonary pathological changes. For immunohistochemistry, the slides were deparaffinized and hydrated. After incubation with 3% hydrogen peroxide for 5 min and rinsing with phosphate-buffered saline (PBS), the slides were incubated at 4 °C with 1:200-diluted anti-RECK antibody (rabbit-anti-rat; PLLABS, Richmond, BC, Canada) overnight. Next, the slides were rinsed with PBS three times and incubated with biotinylated goat-anti-rabbit IgG at room temperature for 45 min. Finally, the slides were rinsed three times and stained with 3,3'-diaminobenzidine (DAB). Restaining with HE was then performed. PBS was used for a negative control instead of the primary antibody, and the remaining conditions were the same. After cov-

ering the slides with general clarity gum, the expression and localization of RECK were observed under a microscope.

Real-Time Polymerase Chain Reaction (PCR)

One milliliter of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to lung tissue homogenate (roughly 100 mg), and the mixture was allowed to stand for 15-30 min; then, 0.2 mL of chloroform was added for RNA extraction. cDNA was produced using a RevertAid RT Reverse Transcription Kit (Thermo Scientific™, Waltham, MA, USA). The corresponding gene sequence was attained from a gene database, and the primer sequences were designed with Primer Premier 5 software. The primers for RECK were 5′-GGTTGGCTTAGGCTGCTGTGAG-3′ and 5′-GGTCCAGGAGAGGAGTCTGTTCG-3′, and the primers for β-actin were 5′-ATCACTATCGGCAATGAGCGGTTC-3′ and 5'-CTCCTGCTTGCTGATCCACATCTG-3′. Real-time PCR was performed on an ABI7500 real-time PCR machine (Applied Biosystems, Foster City, CA, USA), using the dsDNA dye SYBR-green (Thermo Fisher Scientific, Waltham, MA, USA) for real-time DNA quantification. The two-step PCR expansion procedure was applied. The conditions for reverse transcription were 42°C for 60 min and 70°C for 5 min. The PCR conditions consisted of an initial denaturation step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s.

Western Blot

After rinsing twice with PBS, roughly 50-100 mg of lung tissue was homogenized. The lung homogenate was transferred to an Eppendorf (EP) tube and thoroughly mixed with precooled lysis buffer (containing phenylmethylsulfonyl fluoride) for protein extraction. After standing on ice for 30 min, the homogenate mixture was centrifuged (1200 rpm, 10 min). The supernatant was stored at -80°C for further analysis. The protein concentration of each sample was determined by the bicinchoninic acid (BCA) assay. Each protein sample (40 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, and blocked with 5% skimmed milk at 4°C overnight. After incubation with anti-rabbit primary antibody (RECK, diluted at 1:1000, CST; β-actin, diluted at 1:1000, Sino Biological Inc.) at 4°C overnight, the membrane was rinsed twice with Tris-Buffered Saline containing Tween 20 (TBST-20) and incubated with secondary antibody diluted in skimmed milk at room temperature for 2 h. After rinsing twice, enhanced chemiluminescence reagents (ECL; Absin) were applied, and images were collected with Gel-pro software.

Statistical Analysis

The SPSS software package 16.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data were denoted as the median ± standard deviation. Comparison of continuous parameters with equal variances between the two groups was performed with the independent-samples *t*-test. For the comparison of numerical data with heterogenous variance, Dunnett's test was performed. For all statistical analyses, p-values < 0.05 were considered statistically significant.

Results

Respiratory Functional and Pathological Changes in COPD Rats

The pulmonary function test was performed to validate the establishment of the COPD model. Compared with the control rats, the rats in the COPD cohort manifested significantly deteriorated pulmonary function, featured as a significant reduction of FEV0.1/FVC% and PEF, as well as elevation of FRC and Cchord. The differences between the two cohorts were statistically significant (Figure 1). HE staining of samples from the COPD cohort also demonstrated pathological changes characteristic of COPD. As shown in Figure 2, lung tissue from the control rats had an intact alveolar structure, normal alveolar septum and bronchial wall, and mild infiltration of inflammatory cells. On the contrary, lung tissue from the COPD cohort had an enlarged alveolar space, thickened alveolar septum, entrilobular emphysema, bulla derived from partial alveolar fusion, hyperplasia of bronchial smooth muscle, and significant infiltration of inflammatory cells around the bronchial wall. Both the pulmonary function test and the pathological test confirmed the successful development of COPD in the COPD cohort.

Expression and Localization of RECK in Lung Tissue

As demonstrated by the immunohistochemical staining, RECK was mainly expressed on airway epithelial cells in normal lung tissue. Partial ex-

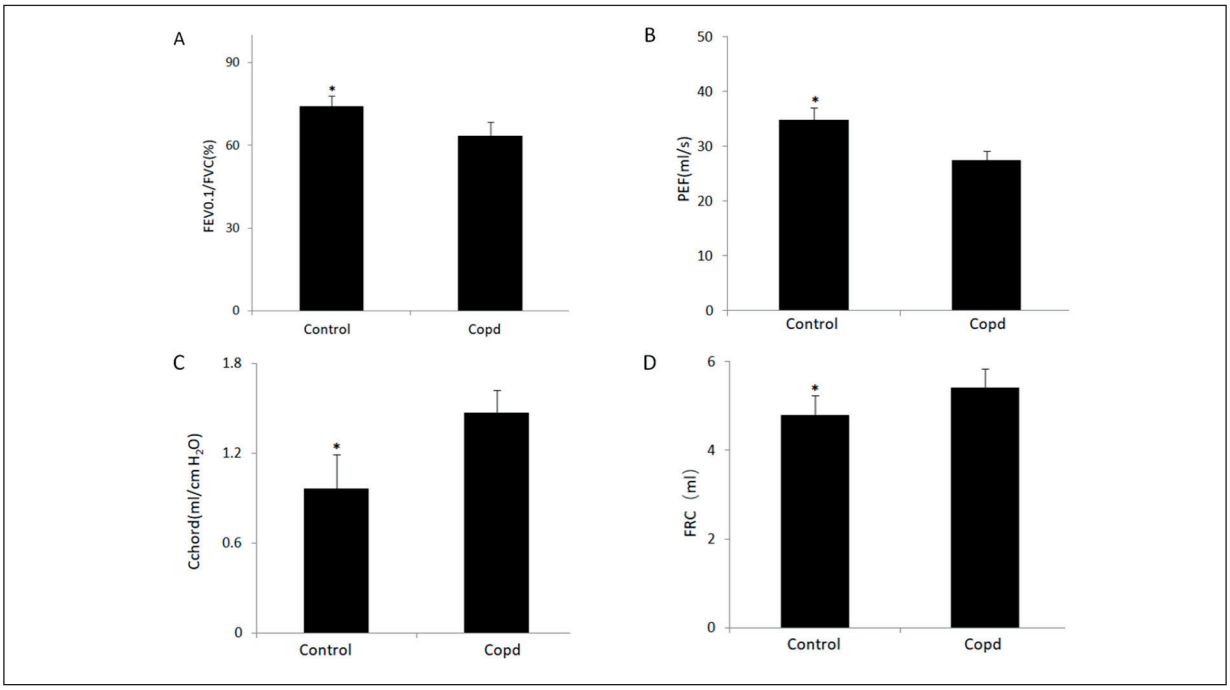

Figure 1. Pulmonary function tests. **A**, Comparison of FEV0.1/FVC% between the control cohort and the COPD cohort. **B**, Comparison of PEF between the control cohort and the COPD cohort. **C**, Comparison of Cchord between the control cohort and the COPD cohort. **D**, Comparison of FRC between the control cohort and the COPD cohort. Abbreviations: PEF, peak expiratory flow; FEV0.1/FVC%, ratio of forced expiratory volume in 0.1 s to forced vital capacity; Cchord, static lung compliance; FRC, functional residual capacity.

pression was also observed on subepithelial cells and infiltrating immune cells. As for the COPD rats, RECK expression on airway epithelial cells was significantly diminished when compared to the normal rats (Figure 3).

COPD Rats Expressed Lower RECK Levels in Lung Tissue

Real-time PCR and Western blot assays were performed to further show the changes of RECK expression in lung tissue from COPD rats. As

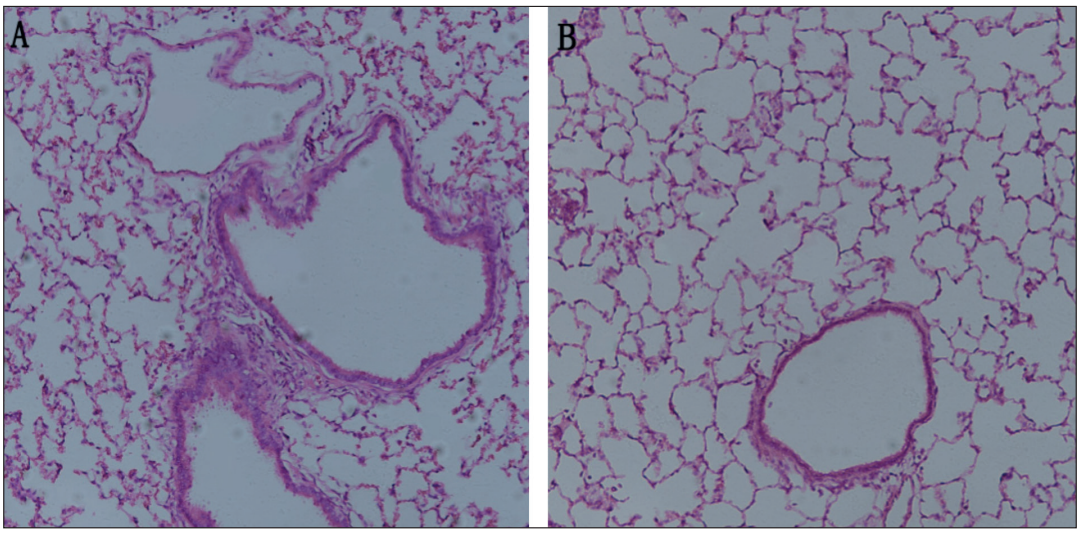

Figure 2. Macroscopic images (200× magnification) of lung tissue (HE staining). **A**, COPD rats: significant infiltration of inflammatory cells, hyperplasia of smooth muscle cells around the bronchial wall, and thickening of the airway wall and alveolar sputum. **B**, Control rats: intact alveolar structure, normal bronchial wall, and mild infiltration of inflammatory cells.

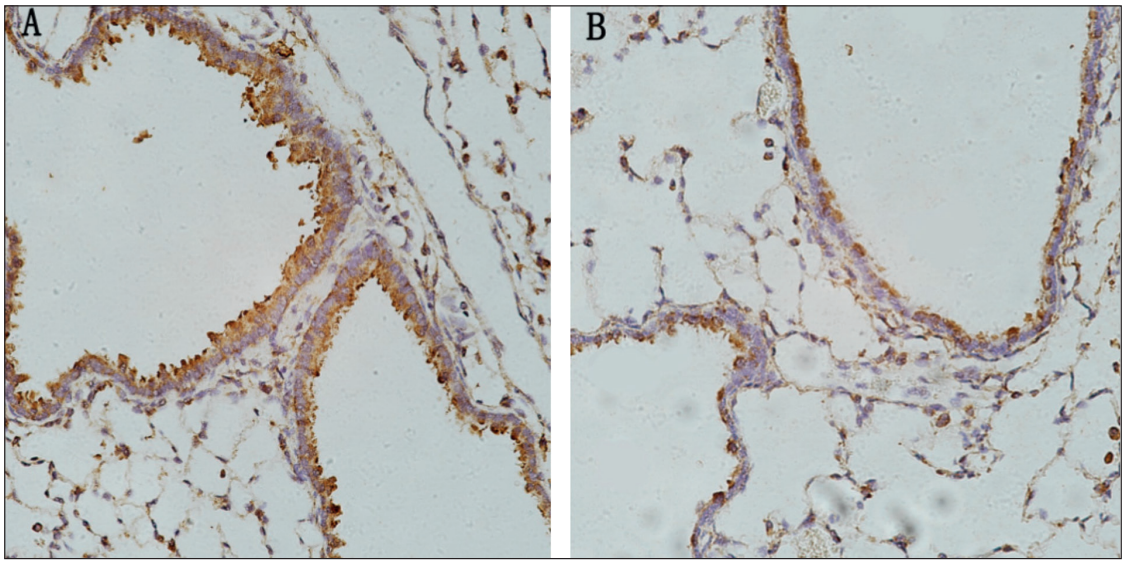

Figure 3. RECK expression and localization based on immunohistochemical staining (400× magnification). **A**, Control rats: RECK expression was highly abundant on airway epithelial cells. **B**, COPD rats: RECK expression on airway epithelial cells was significantly diminished.

shown in Figure 4, both the absolute and relative levels of RECK mRNA in lung tissue were significantly reduced in the COPD cohort as compared to the control cohort ($p < 0.01$). Western blot assays also indicated a significantly reduced protein expression of RECK in the COPD cohort as compared to the control cohort. Altogether, these results indicated that RECK expression was reduced at both the transcriptional level and the translational level in COPD lung tissue.

Discussion

Our study successfully showed the abundant expression of RECK on airway epithelial cells.

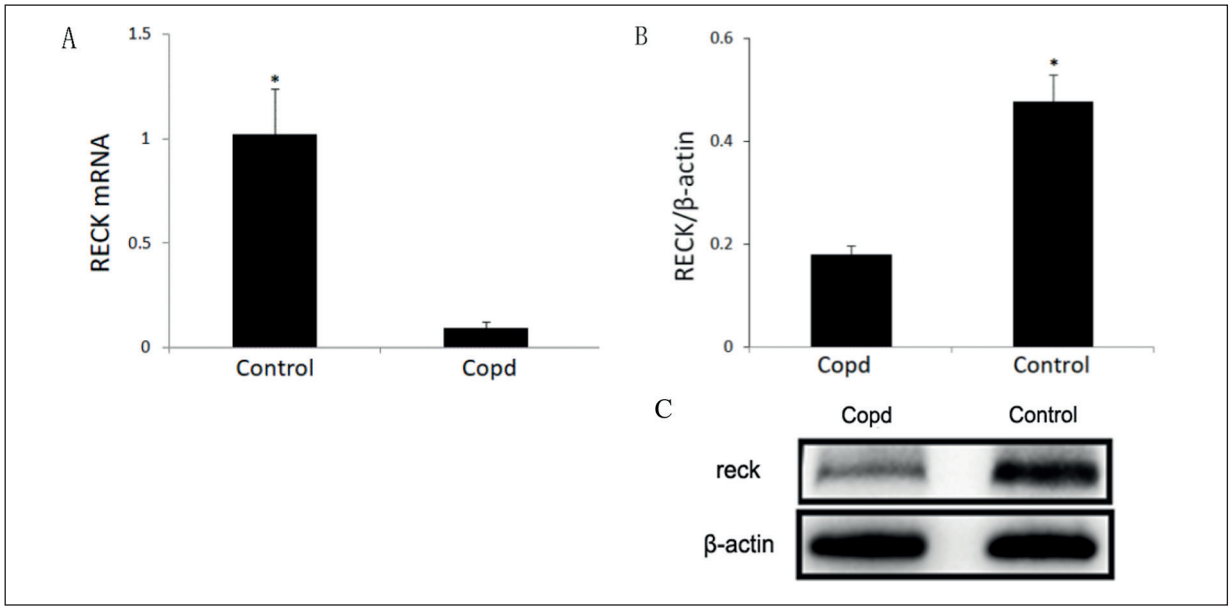

Figure 4. **A**, Absolute level of RECK mRNA by real-time PCR analysis, *$p < 0.01$. **B**, Relative level of RECK protein by western blot analysis, *$p < 0.01$. **C**, Western blot image of the RECK protein level.

Utilizing the COPD model established by smoke exposure, we first demonstrated that RECK expression on airway epithelial cells was significantly reduced in COPD rats. Our findings indicated that RECK may play an important role in the maintenance of the airway structure, which may be diminished during the development of airway remodeling and COPD. To the best of our knowledge, this is the first study to analyze the relevance of RECK in the development of COPD.

RECK was first discovered by Takahashi et al[10] during v-Ki-ras transfection in NIH3T3 cells. The encoding gene of RECK is located on chromosome 9p12-p13, with a gene length of 87 kb and containing 21 exons and 20 introns; it yields a protein that is a membrane-anchored glycoprotein, with 13 single nucleotide polymorphisms discovered to date[10]. RECK is widely expressed on multiple tissues as well as on undifferentiated cells[10]. Further studies have revealed a decreased expression of RECK on many types of malignant cell lines and malignant tumors, including colorectal cancer, lung cancer, pancreatic cancer, gastric cancer, liver cancer, cholangiocarcinoma, breast cancer, etc[17-24]. Accompanied with a reduced RECK expression, tumor cells have shown enhanced invasiveness and metastasis. Likewise, the restoration of RECK expression on tumor cells has resulted in the suppression of tumor progression, accompanied by reduced expression of MMP-9[10]. MMP-9, one of the most common matrix metalloproteinases, has been demonstrated to be associated with the pathological process of many malignant diseases, cardiovascular disease, COPD, etc[25-27]. As a MMP inhibitor, RECK has been found to participate in the regulation of vascularization and extracellular matrix by inhibiting MMPs, including MMP-1, -2, -3, -8, -9, -10, -12, etc[10,15,16,28]. While the involvement of RECK in the development of malignant diseases and cardiovascular disease has been verified by published studies[12,14], no studies have ever analyzed the involvement of RECK in the development of COPD.

Smoke exposure-induced COPD in rat is a well-developed COPD model that is widely applied to COPD-related studies[29]. In this study, both the pulmonary function test and pathological evaluation verified the successful establishment of a rat model of COPD. Immunohistochemical staining, as well as real-time PCR and Western blot assays indicated the reduced expression of RECK in lung tissue of COPD rats as compared to control rats. Previously, a lower RECK level was detected in induced sputum of asthmatic patients as compared to normal individuals[13]. The RECK level in induced sputum was also positively correlated with FEV1[13]. These findings show that RECK plays a protective role in the airway. As mentioned before, RECK functions as an MMP inhibitor. Many MMPs have been associated with the development of COPD, especially MMP-9. It has been observed that MMP-9 can promote the development of emphysema by degrading extracellular matrix[9]. An additional study also found that RECK is a major inhibitor of MMP-9 and that it can regulate its expression at the transcriptional level[15]. Therefore, reduction of RECK expression may promote the overexpression of MMP-9 in lung tissue and thus lead to the development of COPD.

It has been speculated that RECK is mainly expressed on inflammatory cells and participates in the development of asthma by inflammatory regulation[13]. Yet, our study demonstrated that RECK was mainly expressed on airway epithelial cells rather than on inflammatory cells, fibroblasts, or smooth muscle cells. As a membrane-anchored protein, RECK only exerts its function locally. The reduced expression of RECK on epithelial cells may be a secondary modification of the inflammatory response rather than the initiation of inflammation itself.

Herein, we report the first study to unravel the correlation between RECK expression in lung tissue and COPD. However, the limitations of this study should be objectively addressed. First of all, we only presented the expressional changes of RECK in a COPD rat model and did not dig deep into the underlying mechanism. The simultaneous changes of MMP-9 expression in the COPD model along with the reduced expression of RECK have yet to be verified. Also, the impact of RECK expression on pulmonary function should also be addressed in a future study. Last but not least, this was only a preliminary study using an animal model. These findings should be further verified in clinical patients.

Conclusions

In summary, this is the first report on the expression and localization of RECK in lung tissue and its correlation with COPD. Our findings shed light on the development of a therapeutic strat-

egy against COPD by modifying RECK-related pathways. Further studies are still warranted to verify these findings and to unravel the underling mechanism.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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Authors' Contribution

Conceived and designed the experiments: Zhenxing Li, Xiao Wang, Xuguang Guo,Taoping Li. Performed the experiments: Zhenxing Li, Xiao Wang, Hanzhen Xiong, Xuguang Guo. Analyzed the data: Zhenxing Li, Xiao Wang, Xuguang Guo, Taoping Li. Wrote the paper: Zhenxing Li, Xiao Wang, Taoping Li.

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