

Transcriptome-wide profiling discover: PM2.5 aggravates airway dysfunction through epithelial barrier damage regulated by Stanniocalcin 2 in an OVA-induced model

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ABSTRACT

Background: Epidemiologic evidence suggests that PM2.5 exposure aggravates asthma, but the molecular mechanisms are not fully discovered.

Methods: Ovalbumin (OVA)-induced mice exposed to PM2.5 were constructed. Pathological staining and immunofluorescence were performed in *in vivo* study. Gene set enrichment analysis (GSEA) was performed to identify the pathway involved in asthma severity by using U-BIOPRED data (human bronchial biopsies) and RNA-seq data (Beas-2B cells treated with PM2.5). Lentiviruses transfection, Real-time qPCR, immunofluorescence staining and trans-epithelial electrical resistance (TEER) measurement were performed for mechanism exploration *in vitro*.

Results: PM2.5 exposure aggravated airway inflammation and mucus secretion in OVA-induced mice. Based on transcriptome analysis of mild-to-severe asthma from human bronchial biopsies, gene set enrichment analysis (GSEA) showed that up-regulated reactive oxygen species (ROS) pathway gene set and down-regulated apical junction gene set correlated with asthma severity. Consistent with the analysis of mild-to-severe asthma, after PM2.5 exposure, the ROS pathway in Beas-2B cells was up-regulated with the down-regulation of apical junction. The expression levels of genes involved in the specific gene sets were validated by using qPCR. The mRNA levels of junction genes, *ZO-1*, *E-cadherin* and *Occludin*, were significantly decreased in cells exposed to PM2.5. Moreover, it confirmed that inhibition of ROS recovered the expression levels of E-cadherin, Occludin and ZO-1, and ameliorated inflammation and mucus secretion in airway in OVA-induced mice exposed to PM2.5. Meanwhile, ROS level was elevated by PM2.5. By checking trans-epithelial electrical resistance (TEER) value, we also found that epithelial barrier was damaged after PM2.5 exposure. Importantly, *Stanniocalcin 2* (*STC2*) was identified as a key gene in regulation of epithelial barrier. It showed that *STC2* expression was up-regulated by PM2.5, which was recovered by NAC as well. Over-expression of *STC2* could decrease the expression levels of ZO-1, Occludin and E-cadherin. Contrarily, suppression of *STC2* could increase the expression levels of ZO-1, Occludin and E-cadherin reduced by PM2.5.

Conclusions: By using transcriptome analysis, we revealed that *STC2* played a key role in PM2.5 aggravated airway dysfunction through regulation of epithelial barrier in OVA-induced mice.

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1. Introduction

Asthma is a common chronic airway disease worldwide. Both genetic and environment are risk factors contribute to asthma. Single cell RNA sequencing indicated that apparently unrelated environmental risk factors like infection with influenza virus or exposure to ozone can recruit lung neutrophils to promote the initiation of allergic asthma (Tibbitt et al., 2019).

Over the last decades, epidemiological studies discovered that the prevalence of allergies caused by environmental risk factors has been increasing worldwide (Li et al., 2019; Perez et al., 2013; Radermecker et al., 2019). In China, ambient particulate matter (PM) pollution is one of the top four risk factors for both number of deaths and percentage of disability-adjusted life-years (DALYs) at the national level (Zhou et al., 2019). It has been shown that exposure to inhalable PM including PM₁₀ (aerodynamic diameter of 10 µm or less) and PM_{2.5} (aerodynamic diameter of 2.5 µm or less) lead to a major public-health problem with high socio-economic impact. The rise of respiratory mortality was associated with the increased concentration of PM₁₀ and PM_{2.5} (Liu et al., 2019). Air pollutants triggered airway injury, which exhibited disruption of airway epithelial barrier, inflammatory cell infiltration and oxidative stress (Guan et al., 2016). A study conducted that PM_{2.5} aggravated asthma through disturbing the balance of TH17/regulatory T cells (Sun et al., 2019).

Airway epithelial cell is an important part of the innate immune system in the lung. The barrier function of the epithelial layer is maintained through formation of epithelial junctions. E-cadherin, ZO-1, OCLN, and CLDN have been shown to be critical for formation of epithelial junctions (Aghapour et al., 2018). The transmembrane protein, E-cadherin, is critical for maintaining apical-basolateral polarization and adhesion to neighboring cells (Nawijn et al., 2011). The epithelial barrier predominantly consists of tight junctions (TJs), which are located at the most apical part of the intracellular junctional complex between adjacent epithelia (Le, 2012). TJs are comprised of proteins such as occludin, and claudins, junctional adhesion molecules (JAM) and zonula occludens (ZO)-1 (Kazunari and Kenji, 2020). In this study, by using gene set enrichment analysis (GSEA), we found that STC2, which is usually used as a prognostic marker in cancer, played a novel role in regulation of epithelial barrier in OVA-induced mice exposed to PM_{2.5}.

2. Material and methods

2.1. Reagents

PM_{2.5} collection and extraction were performed as described according to the previous publication (Xiang et al., 2020). Briefly, PM_{2.5} samples were collected for 1 year from March 2018 to February 2019 using an air sampler on the open-air roof of a ten-story building of the third people's Hospital of Chengdu. Quartz fiber filters were used to collect PM_{2.5}. To eliminate the contamination, the filters were pre-heated at 550 °C. Then balanced and stabilized before sampling (20 ± 0.5 °C, 40 ± 5% RH). The samples were collected every weekday for 23hrs. The filter was cut into 1 cm² area of pieces, immersed in sterilized water and sonicated 3 times to extract the water-soluble components following lyophilization, and stored at -80 °C until use. Prior to use, the sample was diluted with sterilized phosphate buffer saline (PBS, pH 7.2) and sonicated for 30 min to disperse possible aggregates.

We purchased the following supplies from different companies. NAC (sigma-Aldrich, USA), 2 × Taq SYBR Green qPCR Mix (Vazyme, SQ101), HiScript IIQ RT SuperMix for qPCR (Vazyme, R223-01), FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, RC101-01), Ovalbumin (Solarbio, #326A0519), Methacholine (Sigma-Aldrich, #108K1232), Reactive Oxygen Species Assay Kit (Applygen Technologies Inc, C1300).

2.2. Antibodies

We purchased the following antibodies from different companies. Anti-ZO-1 (Thermo, SH252320), Anti-Ecadherin (Cell Signaling, 24E10). Secondary Abs used: Alexa Fluor 488-conjugated goat anti-mouse IgG H&L (Abcam, ab150113), Alexa Fluor 555-conjugated goat anti-Rabbit IgG H&L (Abcam, ab150078).

2.3. Animals and experimental protocol

6–8-week-old female C57BL/6 J mice were obtained from Tengxin Biotechnology Co., (Ltd.: SCXK (Chongqing) 2017-0001). All animal experiments were conducted and approved in accordance with the guidelines of the Committee on the Protection and Use of Animals in the ChongQing Medical University. After adaptive feeding for 1 week, C57BL/6 J mice were randomly divided into control group (PBS group), asthma model group (OVA group), PM_{2.5}-exposed asthma group (PM_{2.5} + OVA group) and NAC group (PM_{2.5} + OVA + NAC group). The asthma model was established by using classical asthma model construction. 0.2 ml OVA (0.1 mg/ml) + Al(OH)₃ (5 mg/ml) was injected intraperitoneally on the 1st and 7th day, and 50 µl OVA (1 µg/µl) was given nasal stimulation on the 15th to 21st day for 7 days. Before challenge, 50 µl PM_{2.5} (10 mg/ml) suspension was injected into the airway in PM_{2.5} + OVA group. NAC (500 mg/Kg) was given 200ul intragastrically before 2 h of stimulation, in the PM_{2.5} + OVA group for 7 days in succession. The control group was treated with PBS. 24 h after the last stimulation, mice were anesthetized, neck trachea was isolated, chest cavity was exposed, left lung was ligated, 0.5 ml cold PBS was extracted with 1 ml syringe and slowly injected into left lung through trachea. after repeated aspiration for 3 times, mouse bronchoalveolar lavage fluid (BALF) was collected (recovery rate ≥ 80%). The BALF 1500 r/min was centrifuged for 8 min at 4 °C, and the heavy suspension precipitation was performed for cell count. The total cell number in BALF was counted under the microscope after HE staining.

2.4. Histology

After the tissue of lung was isolated, it was immersed in a 10% formaldehyde solution for fixation, alcohol dehydration, paraffin embedding, and slicing (5 µm), and the procedure was stained with the routine Hematoxylin-Eosin staining and the periodate-Schiff's staining reagent instructions. The pathological changes of lung airway, alveolar wall thickness and inflammatory cell infiltration were observed under a 200 × optical microscope (DM4000B, Leica, Germany), and the severity of peribronchial and perivascular inflammation was scored using previously described methods (Xie et al., 2015). Each bronchus observed was scored from 0 to 5, with approximately 10 areas scored in total. The expression of glycogen in the airway epithelium of the mouse was observed by an optical microscope (IX73, Olympus, Japan), and the cases of goblet cell proliferation and mucus secretion were assessed.

2.5. Data curation and pre-processing

We searched Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) for available gene expression profiling datasets for asthma. GSE76225 contains bronchial biopsies samples of 91 moderate and severe asthmatic patients. Affymetrix U133 Plus 2.0 PM only arrays were used to explore the gene expression profile. Clinical information of 91 participants with moderate-to-severe asthma and GSE76225 series matrix files from the unbiased biomarkers in prediction of respiratory disease outcomes (U-BIOPRED) were downloaded from GEO2R in GSE76225. The probe sets of Affymetrix U133 Plus 2.0 Array were annotated to genes based on the annotation platform GPL4133-12599. All corresponding probe set signals were averaged to produce a single expression value. Finally, the expression data of 19,922 genes was obtained.

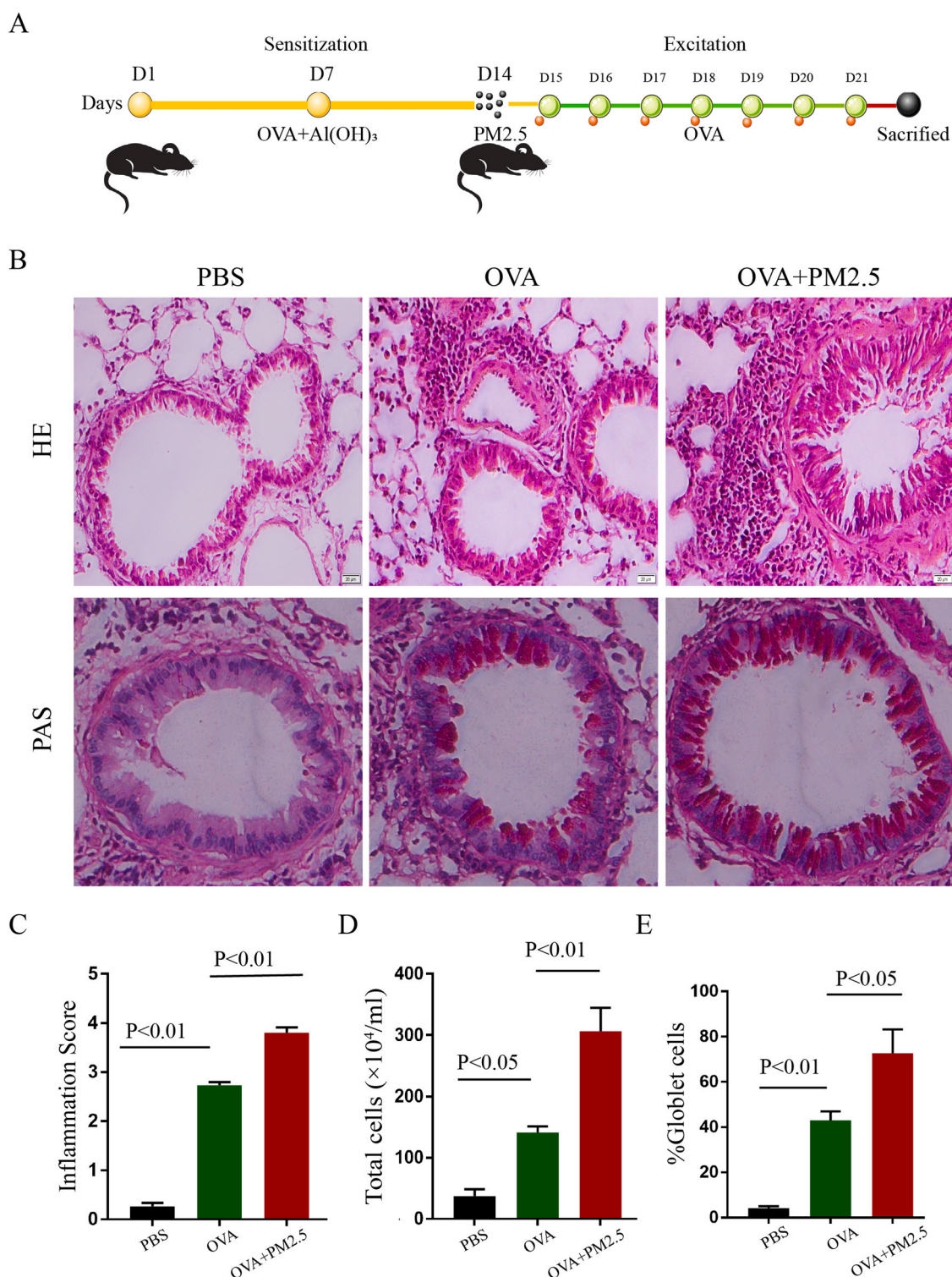


Fig. 1. PM2.5 exposure aggravated airway inflammation and mucus secretion in an OVA-induced murine model. A. Asthma models used in our study (schematic). B. Lung section is stained with hematoxylin and eosin [H&E], periodic acid-Schiff [PAS]. C. Statistic data of inflammation score in different groups. D. Amounts of total cells in each group. E. Statistic data of globule cells. The data represent means±s.d. All data are representative of three experiments.

2.6. Gene set enrichment analysis (GSEA)

The fold changes of severe asthma *versus* moderate asthma were calculated for GSEA analysis by using the limma R package, and a data matrix including genes log2 fold change values were used as the input data. The version 6.0 annotated gene sets (h.all.v6.2.symbols.gmt) was downloaded from the MsigDB database. According to previous studies

(Zhao et al., 2018), GSEA was performed using the clusterProfiler and GSVA package. Significantly enriched gene sets were ranked according to enrichment score, top 10 representative gene sets selected for each subtype (*p*-value less than 0.05) were used for the visualization heatmap of enriched gene sets using pheatmap R package. In this study, we used boxplot to show the gene expression in enriched gene sets, and the statistical significance between severe asthma and moderate asthma was

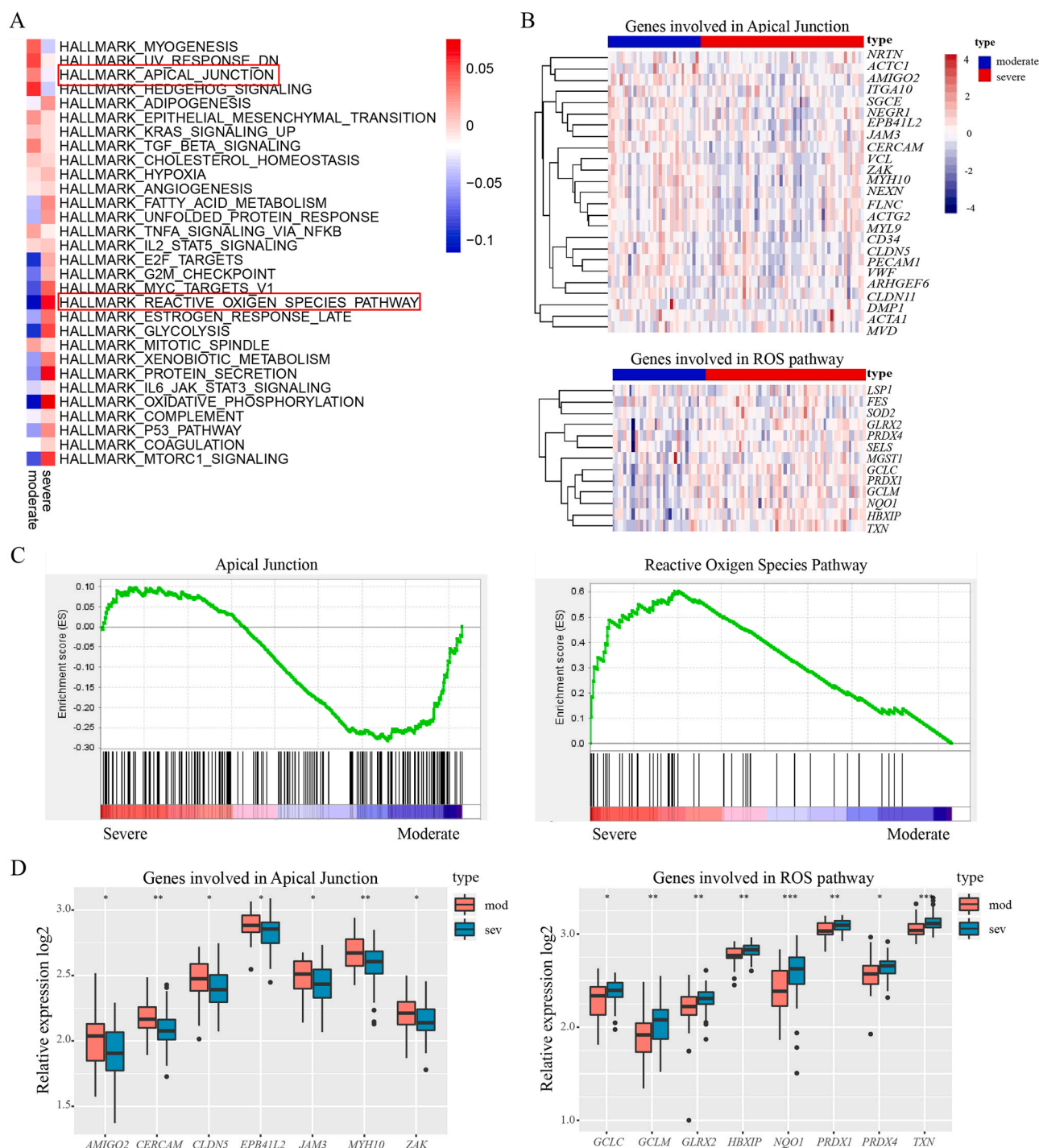


Fig. 2. GSEA enrichment in moderate and severe asthma. A, C. GSEA analysis of severe asthma versus moderate asthma, apical junction is down-regulated and ROS pathway is up-regulated in severe asthma, high score is in red and low score in blue. B. Heat map of genes involved in apical junction and ROS pathway, High expression levels are in red and low levels in blue. D. Relative expression of genes involved in apical junction and ROS pathway.

analyzed by one-way ANOVA.

2.7. Cell culture and transfection

Human normal lung epithelial cell (Bease-2B) was purchased from Shanghai Jikai gene Chemistry Technology Co., Ltd. The Bease-2B cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) culture medium containing 10% fetal bovine serum (FBS) (Gibco, USA), and 1% penicillin-streptomycin mixed solution, grown to 70% confluence at 37 °C in with 5% CO₂ condition. The culture medium was

changed every 1–2 days. After the Bease-2B cells reached 80% confluence in 6-well plastic plates, the cells were pretreated with serum-free DMEM culture medium. According to 2×10^5 cells / well, the cells were adherent inoculated in 6-well plate. After 2 h of NAC (10 mM) pretreatment, PM2.5 was added to culture for 24 h, and the follow-up experiment was carried out. OE-STC2 and shSTC2 lentiviruses were packed by Genechem and transduced into BEAS-2B at a final concentration of 80 PFU/cell (after cells had grown to 30–50% confluence). After 72 h, the STC2 expression was evaluated by qPCR.

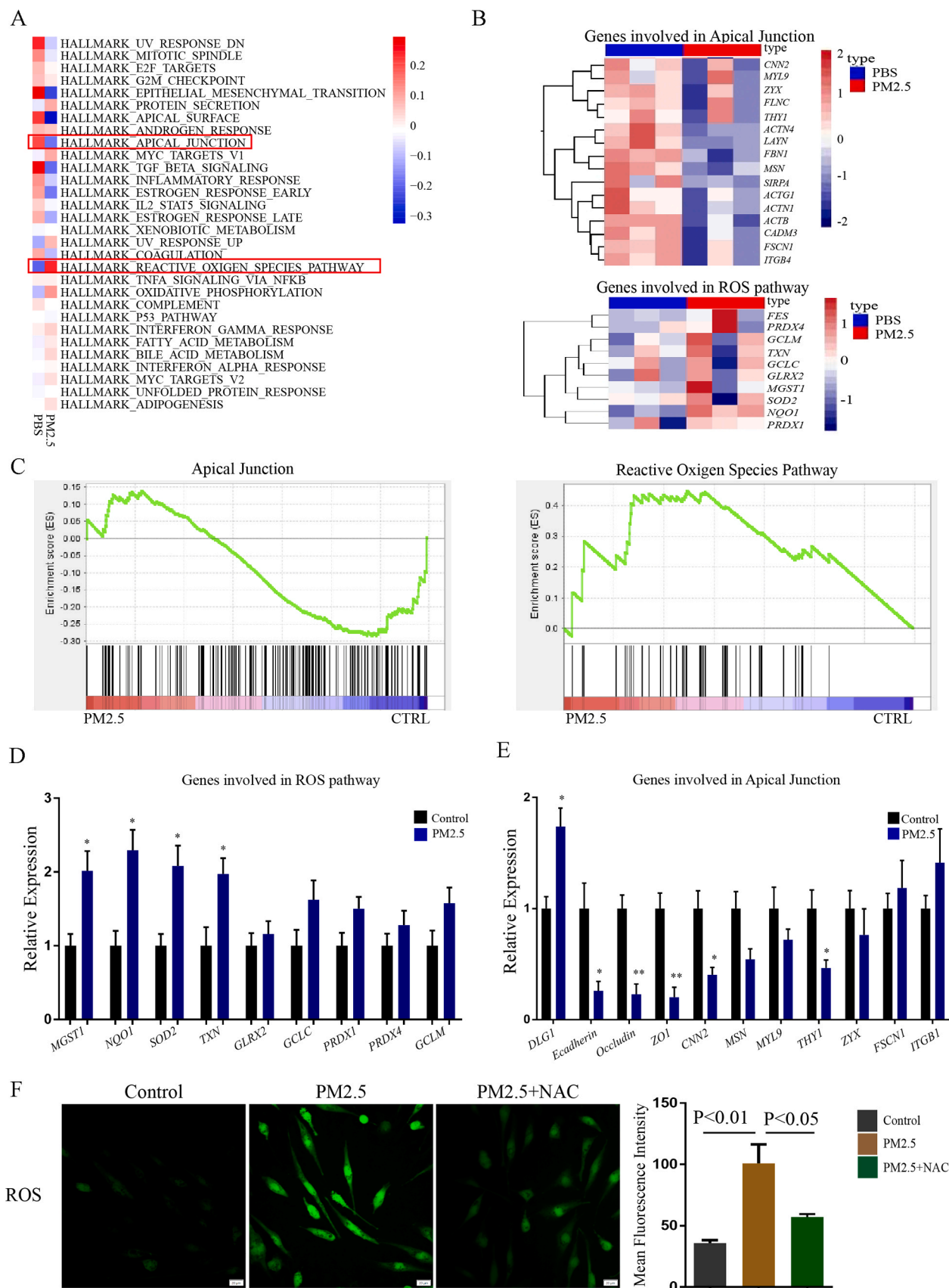


Fig. 3. *In vitro* PM2.5 exposure induced oxidative stress and down-regulated apical junction in epithelial cells. A, C. GSEA analysis of RNA-seq data in epithelial cells expose to PM2.5, apical junction is down-regulated and ROS pathway is up-regulated. B. Heat map of genes involved in apical junction and ROS pathway, High expression levels are in red and low levels in blue. D, E. Relative expression of genes involved in apical junction and ROS pathway are validated by qPCR. F. ROS level in Control, PM2.5 and PM2.5 +NAC group. The data represent means $\pm$ s.d. All data are representative of three experiments.

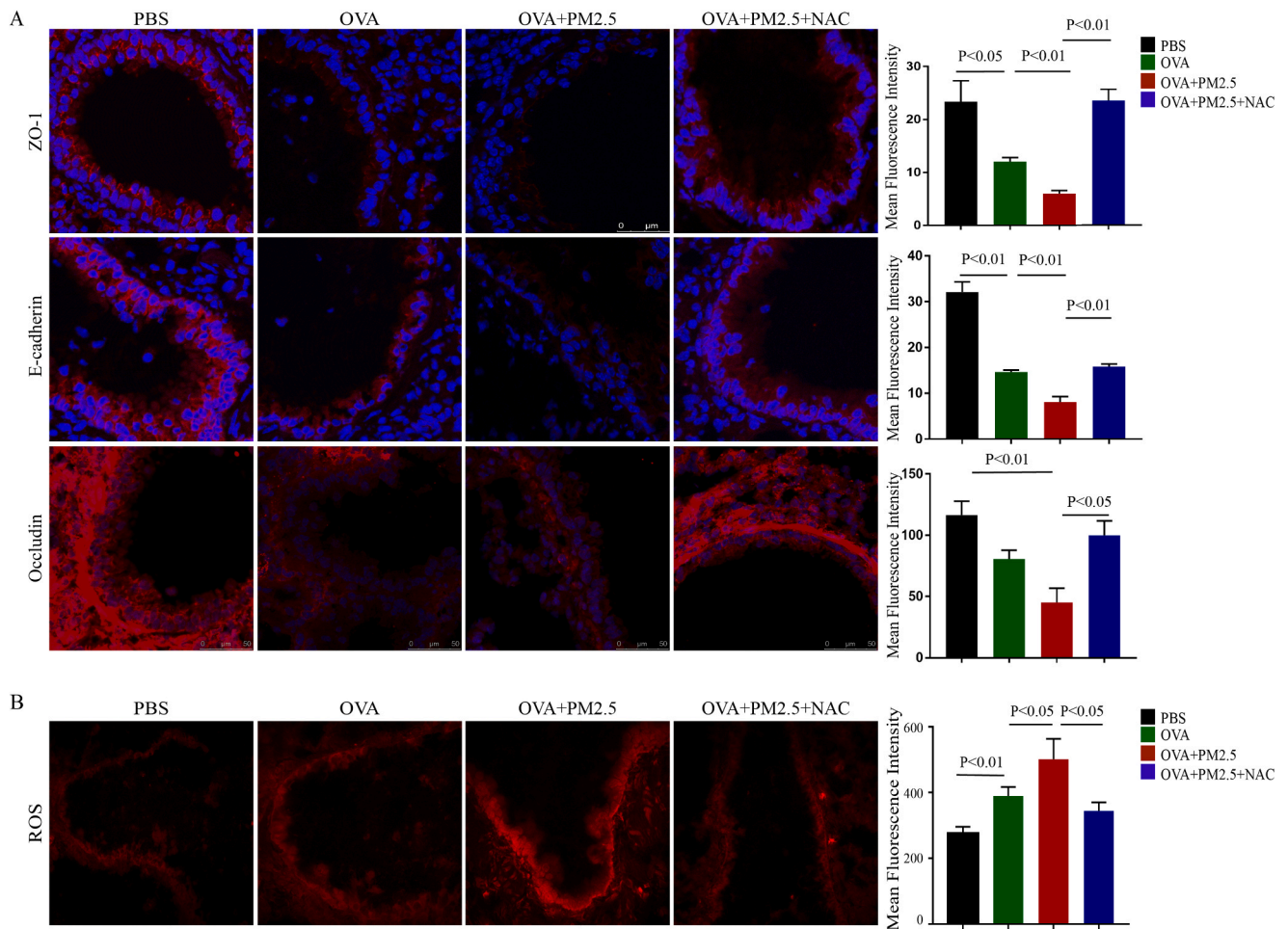


Fig. 4. PM_{2.5} exposure led to epithelial barrier damage in an OVA-induced murine model through oxidative stress. A. Immunofluorescence imaging showing ZO-1, E-cadherin and Occludin expression in different groups. B. ROS level in different groups. The data represent means $\pm$ s.d. All data are representative of three experiments.

2.8. RNA isolation and real-time PCR

Total RNA was extracted from tissues and cells using the FastPure Cell/ Tissue Total RNA Isolation Kit (Vazyme, RC101-01). RNA was quantified using the Epoch multi-volume spectrophotometer system (biotechnology, USA). The purified RNA was synthesized using HiScript IIQ RT SuperMix for qPCR (Vazyme, R223-01) for the first strand cDNA. Real-time PCR analysis was performed using 2 $\times$ Taq SYBR Green qPCR Mix (Vazyme, SQ101). The data was calculated by using $2^{-\Delta\Delta CT}$, ACTIN as the internal reference gene.

2.9. Immunofluorescence

Immunofluorescence staining was performed as described according to the previous publication (Liang et al., 2019). Frozen sections (thickness 5 μ m) and cells in culture dishes were fixed with ice methanol and permeabilized with PBS containing 0.1% Triton X-100 for 10 min at room temperature separately. Specimens were blocked with 10% goat serum in PBS for 30 min at 37 $^{\circ}$ C, then incubated with ZO-1, Occludin and E-cadherin antibodies, at 4 $^{\circ}$ C overnight. Alexa Fluor 488-conjugated goat anti-mouse IgG H&L or Alexa Fluor 555-conjugated goat anti-Rabbit IgG H&L were used to bind primary Abs. Nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI). The specimens were observed by CSU-W1 confocal microscopy (OLMPUS, Japan) analyzed with CellSens Application Suite Software.

2.10. Impedance spectroscopy

The cells were seeded on polycarbonate Transwell inserts (24 well, 8.0 μ m pore size), placed in the CellZscope module and cultured in a humidified incubator (37 $^{\circ}$ C, 5% CO₂). Resistance values were recorded using an automated cell monitoring system, CellZscope (nanoAnalytics, UK) until a plateau was reached wherein PM_{2.5} was added on top of the monolayer. TER values, expressed in $\Omega \cdot \text{cm}^2$, were recorded in real-time over a 24-hours period post-addition of PM_{2.5}. All experiments were carried out in triplicate and repeated at least three times.

2.11. Statistical analysis

All statistical data were analyzed by the SPSS 19.0 and GraphPad Prim7.0 softwares. The results of the experiment presented as means $\pm$ s.d. Significant differences between two groups were determined with the Tukey-Kramer post-test or Dunnett's T3 method. Differences among multiple groups were measured by using one-way ANOVA, and *P* values < 0.05 was of statistical significance.

3. Results

3.1. PM_{2.5} exposure aggravated airway inflammation and mucus secretion in an OVA-induced murine model

Previous epidemiological study showed that long-term exposure to

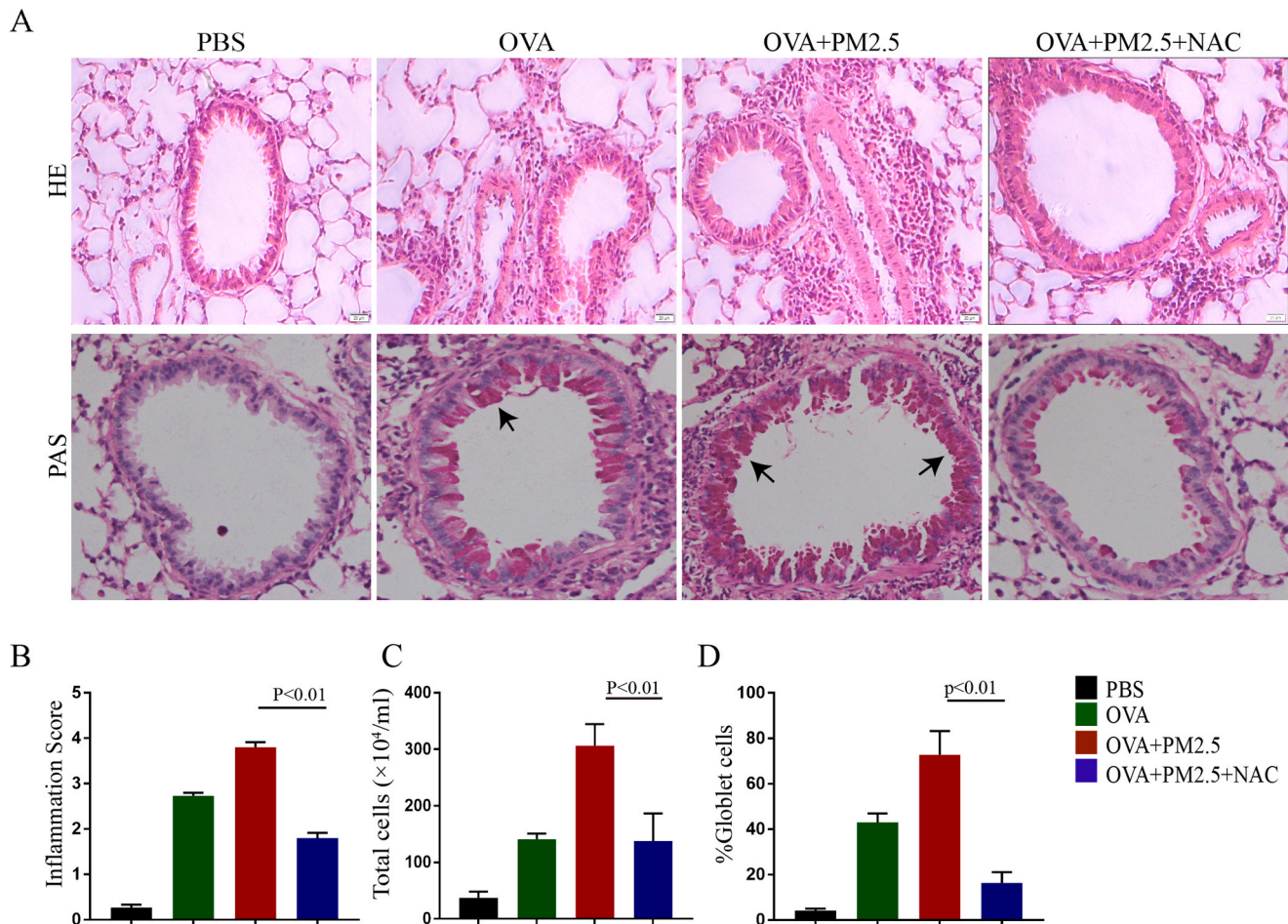


Fig. 5. PM2.5 exposure led to epithelial barrier damage in an OVA-induced murine model through oxidative stress. A. Lung section is stained with hematoxylin and eosin [H&E], periodic acid–Schiff [PAS]. B. Statistic data of inflammation score in different groups. C. Amounts of total cells in each group. D. Statistic data of goblet cells. The data represent means $\pm$ s.d. All data are representative of three experiments.

air pollution was associated with asthma severity (Havet et al., 2018). In our present studies, an asthma model exposed to PM2.5 was built (Fig. 1A). Female C57BL/6 mice were challenged intratracheally with PBS, OVA, or OVA plus PM2.5, respectively. H&E staining showed that PM2.5 exposure significantly aggravated airway inflammation in OVA-induced mice, as evidenced by increased inflammation score in the lungs and the number of total cells in BALFs (Fig. 1B, C and D). Meanwhile, PAS staining showed that PM2.5 exposure enhanced mucus secretion, and increased the amount of goblet cells by 1.69-fold compared to OVA-induced mice (Fig. 1B and E).

3.2. GSEA enrichment in moderate and severe asthma

Expression data of bronchial biopsies from U-BIOPRED project is useful to analyze the pathogenesis of asthma due to integration of clinical and multi-omics data (Riley et al., 2018). To figure out the key factors associated with asthma severity, we analyzed a dataset of moderate-to-severe asthma from U-BIOPRED project (GSE76225). To characterize transcriptional changes associated with asthma severity, GSEA was employed to determine distinct gene set in bronchial biopsies from 91 patients (moderate: 35, severe: 56). Pathway heat map visualization of GSEA enrichment scores demonstrated that the signatures for ROS pathway were highly enriched in severe asthmatic patients compared to those in moderate asthmatic patients (enrichment score = 0.607, $p = 0.015$; Fig. 2A and C). In contrast, we observed that the enrichment score for genes in apical junction gene set was down-regulated in severe asthmatic patients compared to those in

moderate asthmatic patients (enrichment score = 0.335, $p = 0.03$; Fig. 2A and C). The genes involved in apical junction and ROS pathway were showed in the heat-map (Fig. 2B). Furthermore, in apical junction gene set, the expression of *AMIGO2*, *CERCAM*, *CLDN5*, *EPB41L2*, *JAM3*, *MYH10* and *ZAK* showed significant decreases in severe asthma compared to those in moderate asthma (Fig. 2D). *GCLC*, *GCLM*, *GLRX2*, *HBXIP*, *NQO1*, *PRDX1*, *PRDX4*, and *TXN* in ROS pathway gene set were significantly up-regulated in severe asthmas compared to those in moderate asthmas (Fig. 2D). These results indicated that up-regulated oxidative stress and down-regulated apical junction might be associated with asthma severity.

3.3. In vitro PM2.5 exposure induced oxidative stress and down-regulated apical junction in epithelial cells

The airway epithelium is the first line defenses against air pollution (Hirota et al., 2015). To investigate the transcriptional changes of airway epithelium after PM2.5 exposure, RNA-seq analysis was performed *in vitro*. Human bronchial epithelial cells were treated with 62 $\mu\text{g}/\text{cm}^2$ of PM2.5 for 24 h, and then GSEA was also employed to determine the enrichment of relative pathways. Consistent with the analysis of the asthmatic patients data, GSEA enrichment scores demonstrated that ROS pathway was up-regulated after PM2.5 exposure with a decreased enrichment in apical junction gene set (ROS pathway enrichment score = 0.46, $p = 0.05$; apical junction enrichment score = 0.42, $p = 0.03$; Fig. 3A and C). The relative expression levels of the genes involved in the ROS pathway and apical junction were visualized

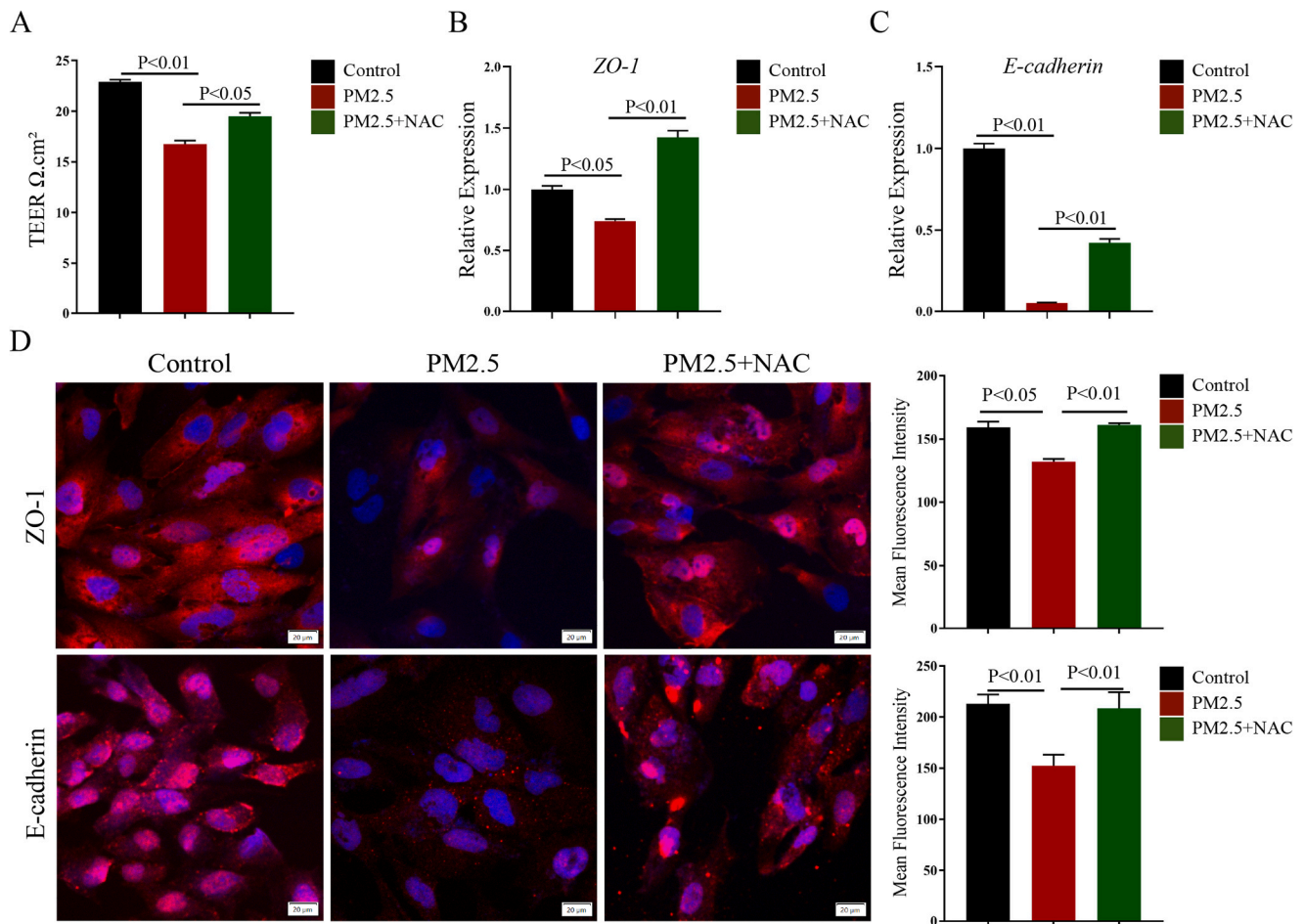


Fig. 6. PM2.5 destroyed epithelial barrier by up-regulation of STC2. A. Value of TEER in each group. B. Relative expression of ZO-1 in Control, PM2.5 and PM2.5 +NAC group. C. Relative expression of *E-cadherin* in Control, PM2.5 and PM2.5 +NAC group. D. Immunofluorescence imaging with ZO-1 and E-cadherin in red in different treatments. Statistic data of each indicated genes in immunofluorescence analysis. The data represent means $\pm$ s.d. All data are representative of three experiments.

in heat-map (Fig. 3B). Meanwhile, the mRNA levels of the genes in human bronchial epithelial cells were validated by quantitative real-time PCR. The mRNA levels of *MGST1*, *NQO1*, *SOD2* and *TXN* involved in ROS pathway were significantly up-regulated in cells exposed to PM2.5 compared to those in control cells (Fig. 3D). We also observed robust decreases in mRNA levels of *E-cadherin*, *Occludin*, *ZO-1*, *CNN2*, *THY1*, and *ZYX* after PM2.5 exposure (Fig. 3E). To further evaluate the role of oxidative stress in human bronchial epithelial cells after PM2.5 exposure, we examined ROS level (Fig. 3F). Confocal microscope analysis revealed that the fluorescence intensities of ROS probe were significantly increased by 2.43-fold after PM2.5 exposure. NAC, the antioxidants, decreased the fluorescence intensities of ROS in cells exposed to PM2.5.

3.4. PM2.5 exposure led to epithelial barrier damage in an OVA-induced murine model through oxidative stress

Consistent with our present results *in vitro*, PM2.5 exposure reduced the fluorescence intensities of E-cadherin, ZO-1, and Occludin in OVA-induced mice (Fig. 4A). The results indicated that PM2.5 exposure further impaired the apical connections of epithelial cells in mice with allergic airway inflammation. Meanwhile, ROS levels were examined in the lung tissue as well. PM2.5 exposure increased the fluorescence intensities of ROS in OVA-induced mice (Fig. 4B). Furthermore, we found that NAC recovered the fluorescence intensities of ROS in mice treated with OVA plus PM2.5 exposure. In the meantime, it showed that NAC

restored the expression levels of E-cadherin, ZO-1, and Occludin in mice (Fig. 4A). To further explore the role of epithelial barrier in OVA-induced mice exposed to PM2.5, we observed the airway inflammation and mucus secretion in lungs after NAC administration. We also found that NAC also significantly decreased the characteristic features of airway inflammation and mucus secretion (Fig. 5).

3.5. PM2.5 destroyed epithelial barrier by up-regulation of STC2

Trans-epithelial electrical resistance (TEER) is a widely accepted quantitative technique to measure the integrity of tight junction dynamics in cell culture models. To determine how PM2.5 exposure influenced epithelial barrier, we measured TEER value in cells exposed to PM2.5. It showed that PM2.5 decreased TEER value, which indicated that the integrity of the cellular barriers was damaged (Fig. 6A). Moreover, the transcript and protein levels of E-cadherin and ZO-1 were determined. Compared with control group, the expression levels of E-cadherin and ZO-1 were decreased in cells exposed to PM2.5, and recovered by NAC treatment (Fig. 6B, C and D).

In the meantime, we identified *STC2* as a key gene involved in epithelial barrier based on RNA-seq data. First of all, we found the expression of *STC2* in lung negatively correlated with *E-cadherin* and *Occludin* in the Genotype - Tissue Expression (GTEx) Project database (Fig. 7A). Secondly, the *STC2* expression was up-regulated by PM2.5 exposure, whereas NAC reduced the increased *STC2* expression (Fig. 7B). To further investigate the effect of *STC2* on epithelial barrier,

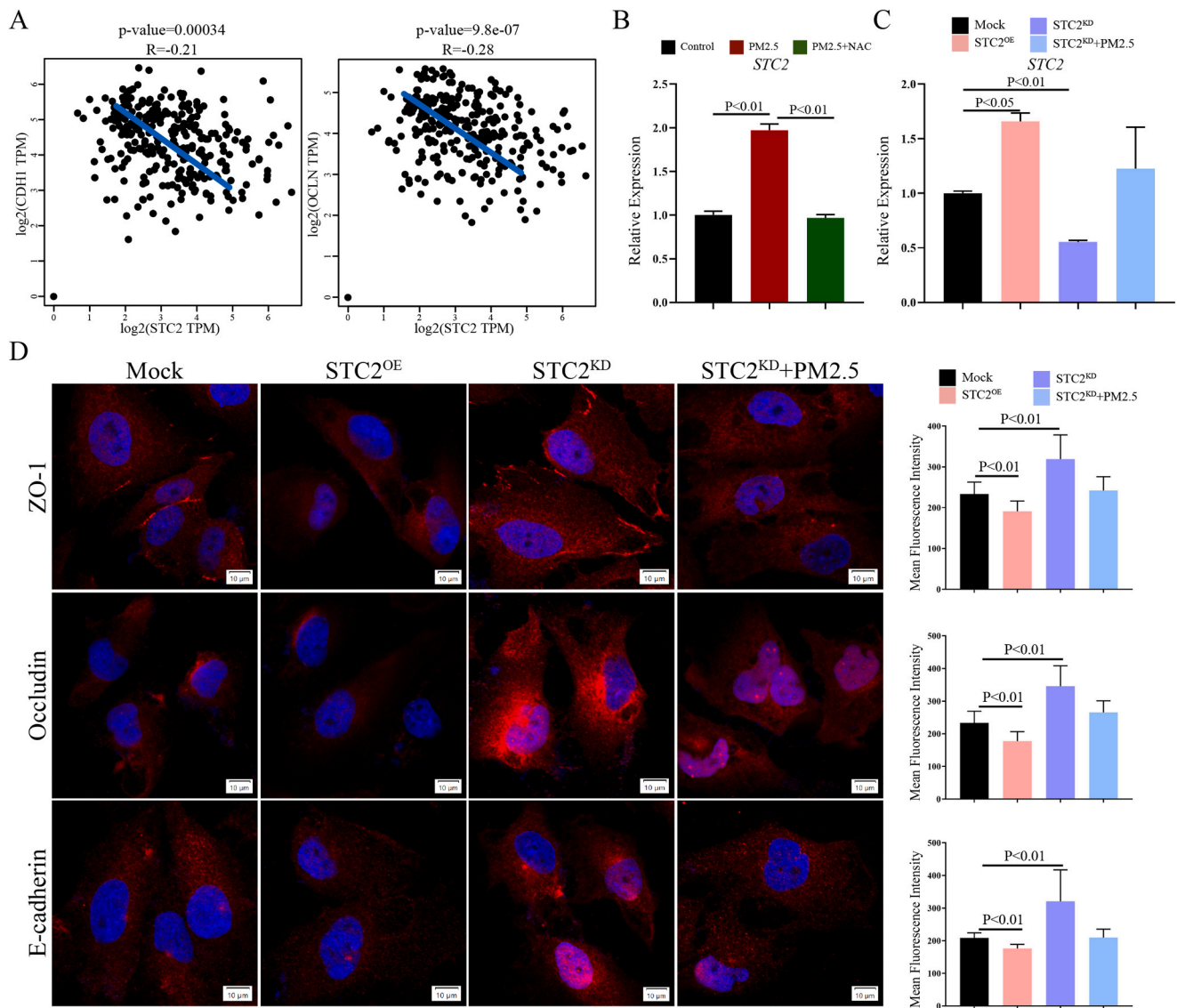


Fig. 7. PM_{2.5} destroyed epithelial barrier by up-regulation of STC2. **A.** The expression of *STC2* in lung negatively correlated with *E-cadherin* and *Occludin* in GTEx database. **B.** Relative expression of *STC2* in Control, PM_{2.5} and PM_{2.5} +NAC group. **C.** Relative expression of *STC2* in different group. Mock: vector, STC2^{OE}: over-expression of *STC2*, STC2^{KD}: knock-down of *STC2*, STC2^{KD}+PM_{2.5}: knock-down of *STC2* cells exposed PM_{2.5}. **D.** Immunofluorescence imaging showing ZO-1, E-cadherin and Occludin expression in different groups. Statistic data of each indicated genes in immunofluorescence analysis. The data represent means±s.d. All data are representative of three experiments.

over-expression *STC2* and knock-down *STC2* lentiviruses were transduced into HBE cells. The expression levels of *STC2* in different group were validated by qPCR. It showed that *STC2* expression was increased in over-expression group, and decreased in knock-down group (Fig. 7C). PM_{2.5} failed to increase the *STC2* expression in knock-down group. In addition, the expression levels of E-cadherin, Occludin and ZO-1 were measured by immunofluorescence (Fig. 7D). It showed that Over-expression of *STC2* decreased the expression levels of ZO-1, Occludin and E-cadherin. Contrarily, suppression of *STC2* increased the expression levels of ZO-1, Occludin and E-cadherin. Consistent with the *STC2* expression, PM_{2.5} failed to alter the expression levels of ZO-1, Occludin and E-cadherin in knock-down group as well.

4. Discussion

U-BIOPRED has been used to identify asthma phenotypes using an integration of clinical and multi-omics approach. Cohorts with severe asthma enrolled in U-BIOPRED is characterized by poor symptom

control, increased comorbidity and airway inflammation, despite high levels of treatment (Shaw et al., 2015). To figure out the key factors associated with asthma severity, we analyzed a dataset of moderate-to-severe asthma from U-BIOPRED project, in which it contains 91 patients (moderate: 35, severe: 56). Reactive oxidative species (ROS) are essential for biological processes. However, long-term chronic exposure to ROS leads to serious health threats. Excessive production of ROS plays an important role in the pathogenesis of asthma, and it has been found that exhaled levels of ROS positively correlate with the severity of asthma (Jarjour and Calhoun, 1994; Kirkham and Rehman, 2006). In our present studies, GSEA analysis showed the cohorts with severe asthma exhibited highly enriched signatures for ROS pathway compared with patients with moderate asthma (enrichment score =0.607, $p = 0.015$). In the meantime, compared with cohorts with moderate asthma, there were fewer apical junctions in cohorts with severe asthma (enrichment score =0.335, $p = 0.03$). Therefore, we speculated that enhanced oxidative stress and dysfunctional epithelial apical junction might be two key factors associated with asthma

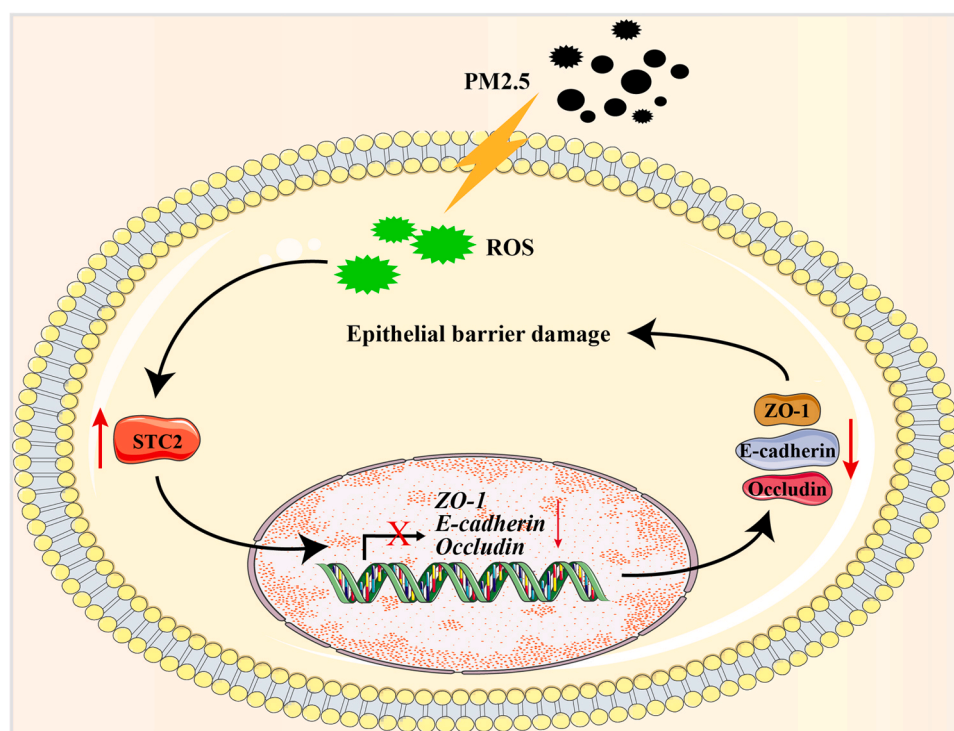


Fig. 8. Schematic of PM2.5 destroyed epithelial barrier. PM2.5 exposure induces ROS level in epithelial cells. The elevated ROS level inhibits ZO-1, E-cadherin and Occludin expression through up-regulation of STC2. It leads to epithelial barrier damage eventually.

severity. Additional *in vivo* and *in vitro* studies are needed to confirm it.

PM2.5 is listed as the fifth major risk factor for death. Host defense dysfunction caused by PM2.5 exposure may be crucial to susceptibility to respiratory infections (Yang et al., 2020). A cross-sectional association between long-term personal exposure to air pollution and the severity of rhinitis suggested that rhinitis patients in areas with higher levels of air pollution had severe nasal symptoms (Burte et al., 2020). We also observed that PM2.5 aggravated the symptoms of OVA-induced model, as evidenced by significantly enhanced airway inflammation and mucus production. In the meantime, we sought to assess the relationship between PM2.5 exposure and changes in airway epithelial transcriptomics. Consistent with the U-BIOPRED data, GSEA showed that airway epithelial cells also exhibited highly enriched signatures for ROS pathway with lower enrichment in apical junction after PM2.5 exposure (ROS pathway enrichment score = 0.46, $p = 0.05$; apical junction enrichment score = 0.42, $p = 0.03$). Structural changes in the airways are features of severe asthma. It has been reported that dysfunctional epithelial apical junction could promote the permeability of inhaled particles and allergens into the sub-epithelial space, and increase the risk for infections, which may contribute to the progression of asthma (Gras et al., 2012; Sweerus et al., 2017). Our data showed that the expression levels of the junction proteins, ZO-1, E-cadherin and Occludin, were decreased by PM2.5 through oxidative stress both in OVA-induced asthma model and *in vitro* assay. Furthermore, we confirmed that PM2.5 exposure increased permeability of epithelial *in vitro* by using TEER measurement.

Stanniocalcin-2 (STC2) is a secreted, homodimeric glycoprotein, which expresses in a variety of tissues and has autocrine or paracrine functions, involved in regulatory effects on calcium and phosphate transport. Its C-terminal contains a cluster of histidine residues that may interact with metal ions. Studies have been reported that STC2 plays an important role in the development of cancers. Increased cytoplasmic STC2 expression correlated with conventional indicators of aggressiveness of renal cell carcinoma (Meyer et al., 2009). STC2 promotes cell EMT and glycolysis in nasopharyngeal carcinoma (Li et al., 2021). We speculated STC2 would be involved in epithelial barrier damage after

PM2.5 exposure. In our present study, we discovered a novel role of STC2 in epithelial barrier. Its expression negatively correlated with ZO-1, E-cadherin and Occludin expression. After knock-down of STC2, PM2.5 failed to alter the expression levels of junction proteins.

5. Conclusions

Collectively, our results provide new insights into understanding the regulatory mechanism of PM2.5 aggravates airway dysfunction in an OVA-induced model and suggest that the STC2 played a novel role in regulation of epithelial barrier damage (Fig. 8).

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CRediT authorship contribution statement

Lei Zhang: Writing - original draft, Investigation. **Xiang He:** Conceptualization, Writing - review & editing, Funding acquisition. **Ying Xiong:** Supervision. **Guoping Li:** Supervision, Funding acquisition. **Anying Xiong:** Investigation. **Qin Ran:** Investigation. **Junyi Wang:** Data curation. **Dehong Wu:** Data curation. **Bin Niu:** Data curation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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