

ORIGINAL ARTICLE

Clinical Mechanisms in Allergic Disease

Inhibition of H3K27me3 demethylases attenuates asthma by reversing the shift in airway smooth muscle phenotype

Qijun Yu¹ | Xiaowei Yu² | Wenxue Zhao³ | Manni Zhu¹ | Zhengxia Wang¹ |
Jiaxiang Zhang¹ | Mao Huang¹  | Xiaoning Zeng¹

¹Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China

²Department of Respiratory Medicine, Changzhou Second People's Hospital, Nanjing Medical University, Changzhou, Jiangsu, China

³Department of Medicine, Lung Biology Center, University of California San Francisco, San Francisco, California

Correspondence

Mao Huang and Xiaoning Zeng, Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China. Emails: huangmao6114@126.com (M.H.); zengxiaoning@hotmail.com (X.Z.)

Funding information

the Jiangsu Province Special Program for Young Medical Talent, Grant/Award Number: QNRC2016599; the Precision Medicine Research of the National Key Research and Development Plan of China, Grant/Award Number: 2016YFC0905800; the Jiangsu Province Special Program of Medical Science, Grant/Award Number: BL2012012; the Changzhou International Science and Technology Collaboration Program, Grant/Award Number: CZ20110021; the Priority Academic Program Development of Jiangsu Higher Education Institutions, Grant/Award Number: PAPD; JX10231802; the National Natural Science Foundation of China (NSFC), Grant/Award Number: 81700028

Summary

Background: The shift in airway smooth muscle cells (ASMCs) phenotype between proliferation and contraction during asthma has been reported recently, highlighting a role of ASMCs plasticity in the pathophysiology of asthma. As an event involved in epigenetic post-translational modification, histone H3 lysine27 (H3K27) demethylation has attracted significant attention with respect to the epigenetic changes in diverse cells; however, little is known about its contribution to the switching of ASMCs phenotype in asthma.

Objective: To investigate the role of trimethylated H3K27 (H3K27me3) demethylation in ASM remodelling as well as the underlying mechanism.

Methods: Mice were exposed five times a week to house dust mite (HDM) extract for 5 weeks. Lung function was measured following the final HDM challenge. Airway inflammation and remodelling were then assessed in lungs of individual mice. Human ASMCs were purchased from ScienCell Research Laboratories. Proliferation, synthesis, migration and contraction of ASMCs were analysed, respectively.

Results: We observed demethylation at H3K27me3 sites in lungs harvested from mice exposed to HDM extract. Administration of a selective inhibitor of H3K27 demethylase (GSK-J4) could ameliorate the classical hallmarks of asthma, such as airway hyperresponsiveness, airway inflammation and remodelling. We established a proliferative as well as a contractile model of human ASMCs to explore the impacts of H3K27 demethylase inhibition on ASMCs phenotype. Our results indicated that GSK-J4 decreased ASMCs proliferation and migration elicited by PDGF through the Akt/JNK signalling; GSK-J4 also prevented the upregulation of contractile proteins in ASMCs induced by TGF- β through the Smad3 pathway.

Conclusions: Inhibition of H3K27me3 demethylation alleviated the development of asthmatic airway disease in vivo and modulated ASMCs phenotype in vitro. Collectively, our findings highlight a role of H3K27me3 demethylation in experimental asthma and ASMCs phenotype switch.

KEYWORDS

airway smooth muscle cells, asthma, GSK-J4, H3K27me3, histone demethylase

1 | INTRODUCTION

As effector cells involved in abnormal contraction and stenosis in asthmatic airways,^{1–3} airway smooth muscle cells (ASMCs) are believed to be important in airway remodelling. Apart from their contractile function, ASMCs also have potential proliferative and secretory capacities. Recent studies have revealed two phenotypes of ASMCs: the contractile phenotype and the proliferative/synthetic phenotype. ASMCs cultured *in vitro* switch from one phenotype to the other depending on the stimuli they are exposed to.^{4,5} *In vivo*, different phenotypes may coexist in the same set of asthmatic airways, forming heterogeneous populations of ASMCs.^{6,7} In the presence of diverse stimuli, ASMCs may alter their phenotype and exhibit diverse biological functions, contributing to the perpetuation of airway inflammation and/or remodelling. As components serving multiple and diverse roles in airways, ASMCs are considered promising targets for asthma management, which may be achieved by modulating ASMCs phenotype.

Epigenetic modifications of histone, such as acetylation or methylation, have been reported to be involved in the pathogenesis of various diseases, including asthma.⁸ Histone 3 Lys27 (H3K27) trimethylation (H3K27me3) is believed to dynamically repress gene transcription, whereas H3K27me3 demethylation by specific demethylases, Jumonji C domain containing protein 3 (JMJD3, also termed KDM6B) and ubiquitously transcribed X chromosome tetratricopeptide repeat protein (UTX), is thought to activate gene transcription.^{9–13} Previous studies have demonstrated the important role of H3K27me3 demethylation in the differentiation and reprogramming of numerous cells, including immune cells (dendritic cells and macrophages)^{14,15} and structural cells (neurons and epithelial cells).^{16–18} This event has also been confirmed to have a role in the cellular phenotype transformation known as epithelial–mesenchymal transition (EMT).¹⁹ However, its contribution to the phenotype switch of ASMCs observed in asthma remains obscure.

In this study, we focused on the potential role of H3K27me3 demethylation in a house dust mite (HDM)-induced murine model of asthma treated with a selective demethylase inhibitor (GSK-J4). Furthermore, the effect of GSK-J4 was also assessed on ASMCs phenotype switch *in vitro*.

2 | MATERIALS AND METHODS

2.1 | Animals

Specific pathogen-free (SPF) female BALB/c mice (18 to 22 g), 6–8 weeks of age, were purchased from Vital River Laboratories (Beijing, China). The mice were housed in a temperature-controlled room under a 12-hour/12-hour dark/light cycle and were provided with food and water *ad libitum*. All experimental protocols for animals and tissue samples were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Nanjing, China).

2.2 | Antigen and drug administration

A total of 40 SPF female BALB/c mice were randomly divided into five groups (control, HDM, HDM + GSK-J4, HDM + dexamethasone (DEX) and HDM + dimethylsulfoxide (DMSO)). Mice were intranasally given droplets containing purified HDM extract (Greer Laboratories, Lenoir, NC, USA) (25 µg of protein solubilized in 25 µL of phosphate-buffered saline (PBS)) for 5 days/wk for up to five consecutive weeks. During the last two consecutive weeks, GSK-J4 (Tocris Bioscience, Bristol, UK) (2.5 mg/kg body weight), dexamethasone (Sigma-Aldrich; St. Louis, MO) (1 mg/kg body weight) or a vehicle (DMSO) (Sigma-Aldrich) was administered intraperitoneally 1 hour before HDM challenge. All treatments were administered under isoflurane anaesthesia and ended 24 hours before sacrifice.^{20,21}

2.3 | Measurement and analysis of airway responsiveness

Airway hyperresponsiveness (AHR) was induced with methacholine (Mch) (Sigma-Aldrich; St. Louis, MO) 24 hours after the final HDM challenge. Lung function was assessed by direct measurement of lung resistance and dynamic compliance (C_{dyn}). Measurements of lung resistance and compliance were conducted with the FinePointe RC System (Buxco Research Systems, Wilmington, NC), in which mice were mechanically ventilated. Mice were sequentially challenged with aerosolized PBS (baseline), followed by increasing doses of methacholine ranging from 3.125 to 50 mg/mL. Maximum resistance and average compliance values were recorded during a 3-minute period after each challenge. We continuously computed RL and dynamic compliance (C_{dyn}) by fitting flow, volume and pressure to an equation of motion.²²

2.4 | BALF collection and differential cell counts

Following measurement of AHR, bronchoalveolar lavage fluid (BALF) was collected and processed for cell counts as previously described.²⁰ The BALF supernatants were stored at -80°C until further evaluation.

2.5 | Immunohistochemistry and immunofluorescence

Lung tissues fixed in 4% paraformaldehyde and embedded in paraffin were sectioned into 5-µm thick slices and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS), Masson's trichrome (MT) stain or were immunostained for α -smooth muscle actin (α -SMA) (1:200) (Abcam, Cambridge, UK), proliferating cell nuclear antigen (PCNA) (1:10,000) (Abcam) and H3K27me3 (1:200) (Cell Signaling Technology Inc, Danvers, Massachusetts, USA). Frozen lung sections were stained with antibodies against H3K27me3 (1:200). The degree of inflammation was measured as previously described.^{23,24} The peribronchial smooth muscle layer was outlined and quantified using ImageJ (National Institutes of Health, Bethesda, MD). The results are

expressed as the area of α -SMA-positive staining (square micrometres) per micrometre of length of the bronchiole basement membrane, as previously described.²⁴

2.6 | ELISA

Total IgE was measured in BALF and serum (MultiSciences, Hangzhou, Zhejiang, China). Levels of IL-4, IL-5, IL-13 and eotaxin-1 (Raybiotech, Atlanta, USA) in BALF and lung homogenates were quantified by ELISA. Levels of VEGF (eBioscience, San Diego, CA) and eotaxin-1 in the ASMCs supernatant were also measured by ELISA according to the manufacturer's protocols. Absorbance was measured at 450 nm using an ELISA reader (CANY, Shanghai, China).

2.7 | Cell culture and treatment

Normal human ASMCs were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured at 37°C and 5% CO₂ in smooth muscle cell medium (SMCM) (ScienCell) supplemented with 20 U/L penicillin, 20 µg/mL streptomycin, 1% smooth muscle cell growth supplement and 2% foetal bovine serum (ScienCell). Cells at passages 3-8 were used for the experiments. Cells at 70%-80% confluence were synchronized by withdrawing serum for 12 hours. Following serum starvation, ASMCs were stimulated with 20 ng/mL PDGF or 4 ng/mL TGF- β (Peprotech, Rocky Hill, USA) alone or in combination with 7.5 µmol/L GSK-J4. Next, the cells were further cultured for the indicated durations.

2.8 | Cell viability assay and cell proliferation assay

ASMCs were cultured in a 96-well plate at a density of 5×10^3 cells per well and treated with GSK-J4 at concentrations of 0, 1, 2, 5, 7.5, 15 and 20 µmol/L for 24 hours or 48 hours. Then, cells were incubated in 10% CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for an additional 4 hours at 37°C. Optical density (OD) was measured on a microplate reader (CANY, Shanghai, China). CCK-8 signal was calculated using the following formula: Cell viability = $[\text{OD}_{\text{GSK-J4}} - \text{OD}_{\text{blank}}] / [\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}]$, where blank represents wells containing cell culture medium and CCK-8 solution but no cells; control represents wells containing cell culture medium, CCK-8 solution and cells; and GSK-J4 represents wells containing cell culture medium, CCK-8 solution, cells and GSK-J4. The experiment was repeated three times in four samples from each group per experiment under identical experimental conditions. The cytotoxicity of GSK-J4 and the effects of PDGF and GSK-J4 on ASMCs were examined using the CCK-8 assay. The 5-ethynyl-2'-deoxyuridine (EdU) assay was used to determine the impact of GSK-J4 on the proliferation of ASMCs, and cells were cultured using the aforementioned procedure. After 48 hours of treatment, an EdU assay kit (Ribobio, Guangzhou, China) was used for labelling according to the manufacturer's instructions. Images were obtained using a fluorescence microscope (Olympus IX71, Japan).

2.9 | Flow cytometry

Subconfluent cells were treated with PDGF in the presence or absence of GSK-J4 for 48 hours. Cells were then harvested and fixed with ice-cold 70% (v/v) ethanol for 24 hours. Following centrifugation, cells were washed with PBS and resuspended in propidium iodide (PI) solution (BD Biosciences, San Jose, CA) containing PI (50 mg/mL), Triton X-100 (0.1%, v/v) and RNaseA (50 mg/mL). Cells were then incubated for 30 minutes in the dark at 37°C. DNA content was determined using a FACScan flow cytometer (Beckman Coulter, CA, USA).

2.10 | Transwell assay

Migration experiments were performed using Corning Transwell 8-µm pore polycarbonate membrane cell culture inserts. ASMCs were digested by means of trypsinization (trypsin/0.25% EDTA solution for about 1-2 minutes, neutralized by SMCM medium containing 2% FBS), and then seeded into the upper chambers of Corning Transwell 8-µm pore polycarbonate membrane cell culture inserts at a concentration of $2-3 \times 10^4$ cells/well. Each lower chamber contained 500 µL of medium containing 2% FBS with or without PDGF/GSK-J4. After incubation at 37°C for 24 hours, the membrane with cells was fixed in 4% paraformaldehyde for 30 minutes. Non-migrated cells were gently removed with a cotton swab, and the cells that had migrated onto the lower surface of the membrane were stained with crystal violet. One random microscopic field of each group at a magnification of 10 $\times$ was observed. Six random microscopic fields at a magnification of 20 $\times$ were observed for subsequent cell counts.

2.11 | Wound healing assay

The wound healing assay was carried out using the ibidi-µ-Dish^{35mm}, high and ibidi Culture-Insert 2 Well (Ibidi GmbH, Martinsried, Germany). An ASMCs suspension was prepared at a concentration of 6×10^5 cells/mL in SMCM, and 70 µL of the suspension was seeded onto each well. Cells were incubated at 37°C with 5% CO₂. After 24 hours, the Culture-Insert 2 Well was gently removed using sterile tweezers, and the dish was filled with new cell free SMCM with or without PDGF/GSK-J4. Wounds were photographed at baseline and 12 hours later. The wound healing area was quantified by means of ImageJ, and the percentage of wound healing area was calculated using the following formula: $[\text{Area}_{\text{baseline}} - \text{Area}_{12\text{hour}}] / \text{Area}_{\text{baseline}} \times 100\%$.

2.12 | Immunofluorescence

ASMCs were fixed with 4% paraformaldehyde for 30 minutes; non-specific binding was blocked by incubating cells in a solution containing 5% BSA and 0.1% Triton X-100 for 1 hour at room temperature. ASMCs were incubated overnight at 4°C with the α -SMA (1:200) and MLCK (1:250) primary antibodies (Abcam). After three

washes with PBS, ASMCs were incubated with the corresponding FITC-conjugated goat anti-rabbit IgG (1:200) (Abcam) for 2 hours at room temperature, and the nuclei were stained with DAPI. Images were taken using a fluorescence microscope (Olympus IX71, Japan).

2.13 | Western blotting

Whole cell extractions from mice lung tissues and ASMCs were harvested using RIPA lysis buffer in the presence of protease inhibitors and a phosphatase inhibitor. Protein concentrations were measured using the BCA protein assay (Beyotime, Shanghai, China). Equal amounts of total protein samples were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and electroblotted to a polyvinylidene fluoride membrane. After blocking with 5% non-fat milk in TBS Tween-20 (TBST; 25 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, and 0.1% Tween-20), the membrane was incubated overnight at 4°C with antibodies against α -SMA (1:2000), MLCK (1:5000) (Abcam), H3K27me3 (1:1000), phospho-Akt (1:1000), Akt (1:1000), phospho-p38 (1:1000), anti-p38 (1:1000), phospho-ERK (1:1000), ERK (1:1000), phospho-JNK (1:1000), JNK (1:1000), Smad2/3 (1:1000), phospho-Smad3 (1:1000) (Cell Signaling Technology Inc, Danvers, Massachusetts, USA) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000) (Peprotech, Rocky Hill, USA). After washing, the membrane was incubated with HRP-conjugated secondary antibody (1:5000) (Peprotech, Rocky Hill, USA) for 1 hour at room temperature. Signals were detected using an ECL kit (Pierce, Thermo Scientific, Waltham, USA), and the binding of specific antibodies was visualized using a Bio-Rad Gel Doc/Chemi Doc Imaging System and analysed by ImageJ software.

2.14 | Data analysis and statistics

All experiments were performed in triplicate, and data were presented as the mean $\pm$ SEM. Results were then plotted using Prism software (Graphpad Software, San Diego, CA, USA). Statistical comparisons were performed using Student's *t* test or one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test. *P* values <0.05 were considered statistically significant.

3 | RESULTS

3.1 | H3K27me3 demethylation in a HDM-induced murine asthma model

We first established a HDM-induced murine asthma model as shown in Figure 1A. The expression of H3K27me3 was downregulated in the lungs of HDM-exposed mice compared to the lungs of control mice (Figure 1B,C). We further confirmed this observation by immunocytochemistry and immunofluorescence staining, as evidenced by the decreased number of H3K27me3-positive cells (Figure 1D,E). The effect of GSK-J4 on H3K27me3 expression was also confirmed by western blotting analysis. As expected, GSK-J4

treatment inhibited HDM-elicited H3K27me3 demethylation (Figure 1G,H).

3.2 | Impacts of GSK-J4 treatment on HDM-induced AHR and airway inflammation

Higher lung resistance and lower C_{dyn} were observed in HDM-exposed mice compared to control mice (Figure 1I,J). As with DEX, GSK-J4 treatment suppressed lung resistance in asthmatic mice (Figure 1I). In agreement with lung-resistance findings, the results of C_{dyn} showed an improved response in GSK-J4-treated mice compared with HDM-treated mice (Figure 1J). We also observed robust airway inflammation in mice from the HDM group by evaluating the infiltration of inflammatory cells into the BALF. In contrast, treatment with GSK-J4 induced decreases in the total cell counts and the number of eosinophils, neutrophils and lymphocytes (Figure 1K). GSK-J4-treated mice also exhibited diminished inflammatory infiltration around airway lumens and vessels compared to that observed in mice in the HDM group (Figure 2A,B). Moreover, total IgE levels were increased in the BALF and serum after HDM exposure (188.2 ± 30.5 and 640.2 ± 41.9 ng/mL, respectively), and this elevation was abolished by GSK-J4 administration (88.9 ± 28.5 and 340.2 ± 48.2 ng/mL, respectively) (Figure 2C). We also observed that HDM-treated mice had increased amounts of IL-4, IL-5, IL-13 and eotaxin-1 in the BALF (33.5 ± 2.0 , 45.24 ± 1.7 , 129.5 ± 10.4 and 68.32 ± 5.23 pg/mL, respectively) as well as in lung homogenates (74.1 ± 6.1 , 317.8 ± 29.2 , 190.2 ± 19.9 and 333.5 ± 36.7 pg/mL, respectively). Elevation of IL-5, IL-13 and eotaxin-1 induced by HDM exposure was attenuated by GSK-J4 treatment (28.7 ± 2.3 , 94.1 ± 9.2 pg/mL and 36.54 ± 6.1 in the BALF, respectively, and 169.1 ± 20.7 pg/mL, 120.8 ± 14.0 and 199.7 ± 13.4 pg/mL in lung homogenates, respectively; Figure 2D,E). However, IL-4 levels did not differ significantly between mice in the HDM group and those in the GSK-J4 group (GSK-J4 group: 30.4 ± 1.3 pg/mL in the BALF and 71.49 ± 9.8 pg/mL in lung homogenates) (Figure 2D,E).

3.3 | Impacts of GSK-J4 on HDM-induced airway remodelling

The percentage of airway epithelium cells that stained positively with PAS was reduced in the GSK-J4-pretreated group compared to the HDM group (Figure 3A). The extent of collagen deposition around the interstitia of the airways and vessels was also decreased in the lung tissues of the GSK-J4-pretreated mice compared to those of the HDM-exposed mice (Figure 3A). To evaluate contractile protein expression, we examined α -SMA levels. Reduced α -SMA-stained cells in the peribronchial area were observed in mice from the GSK-J4 group compared to those in the HDM group (Figure 3A), indicating a thinner peribronchial smooth muscle layer. Furthermore, as is depicted in Figure 3A, the percentage of PCNA-positive lung mesenchymal cells was increased in response to HDM, whereas GSK-J4 administration partly inhibited this increase in PCNA expression.

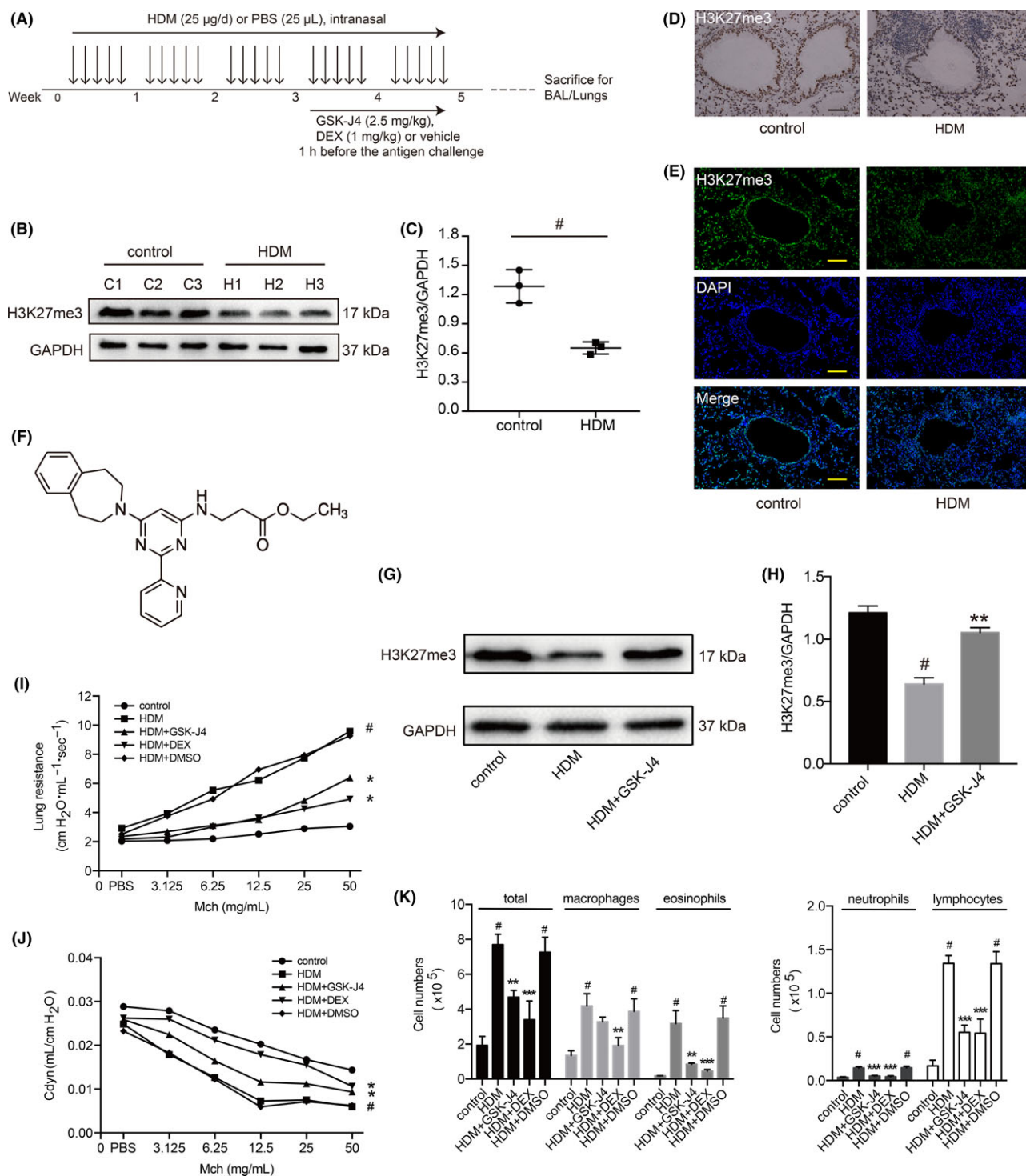


FIGURE 1 Chemical structure of GSK-J4 and the experimental protocol for asthma model. H3K27me3 was demethylated in a HDM-induced murine asthma model. Effects of GSK-J4 treatment on AHR and inflammatory cell infiltration in the BALF. A, HDM model timeline with GSK-J4, DEX or vehicle (DMSO) administration for 5 days/wk for five consecutive weeks. B, Total H3K27me3 in lung tissues was measured by western blotting. C, The density quantification of H3K27me3 was expressed as a ratio relative to GAPDH. D, Immunohistochemical staining for H3K27me3 in lung sections from mice in the control group (left) and the HDM group (right). E, Immunofluorescent staining for H3K27me3 in lung sections from mice in the control group (left) and the HDM group (right) (magnification, 40×; scale bar, 50 µm). F, General chemical structure of GSK-J4. G, The effect of GSK-J4 on H3K27me3 level was measured by western blotting. H, The density quantification of H3K27me3 was expressed as a ratio relative to GAPDH. I, J, Mice inhaled increasing doses of methacholine (3.125–50 mg/mL), and lung resistance and C_{dyn} were measured. K, Inflammatory cell numbers and differentiation counts in the BALF were determined using a haemocytometer, and at least 200 cells were counted. Data represent the mean ± SEM. (n = 8 per group). #P < 0.05 compared to the control group. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the HDM group

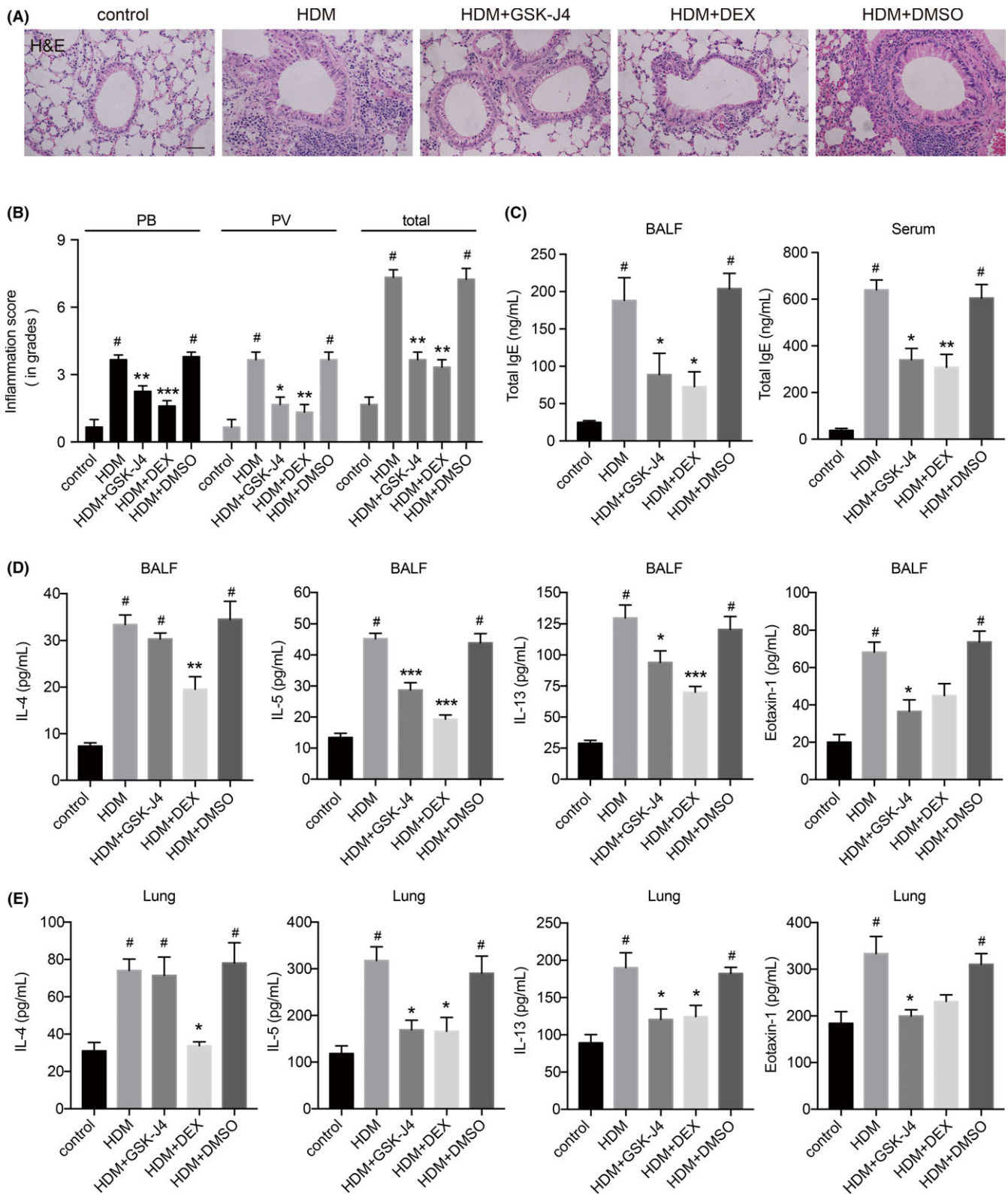


FIGURE 2 Effects of GSK-J4 treatment on HDM-induced airway inflammation and levels of total inflammation and individual cytokines. A, H&E-stained lung sections for analysis of inflammatory cell infiltration (magnification, 40 $\times$; scale bar, 50 μ m). B, Layers of inflammatory cells were counted, and the total inflammation score was summed using the peribronchial (PB) and perivascular (PV) inflammation scores. C, Total IgE in the BALF (left) and serum (right). D, E, Concentrations of Th2 cytokines (IL-4, IL-5 and IL-13) and eotaxin-1 in the BALF (D) and lung homogenates (E) were measured by ELISA. Data represent the mean $\pm$ SEM. ($n = 7$ per group). # $P < 0.05$ compared to the control group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the HDM group

3.4 | Impacts of GSK-J4 on ASMCs proliferation

First, the toxicity of GSK-J4 on ASMCs was measured. Cell viability was observed to be $88\% \pm 1\%$ and $84\% \pm 4\%$ in the $7.5 \mu\text{mol/L}$ group at 24 and 48 hours, respectively (Figure 4A). PDGF stimulated ASMCs proliferation compared to the control group in a dose-dependent manner (Figure 4B), whereas pretreatment with GSK-J4 ($7.5 \mu\text{mol/L}$) significantly reduced the PDGF (20 ng/mL)-induced ASMCs proliferation from $130.4\% \pm 2.8\%$ to $110.4\% \pm 3.3\%$

($P < 0.05$) (Figure 4C). Furthermore, our results demonstrated that the number of EdU-positive cells treated with PDGF alone was significantly elevated compared to that in the control group, and pretreatment with GSK-J4 distinctly inhibited PDGF-induced proliferation (Figure 4D,E).

To further elucidate the effects of GSK-J4 on PDGF-induced ASMCs proliferation, we also examined cell cycle events by flow cytometry. As depicted in Figure 4F,G, PDGF stimulation resulted in a higher synthesis phase (S phase) population ratio compared to that

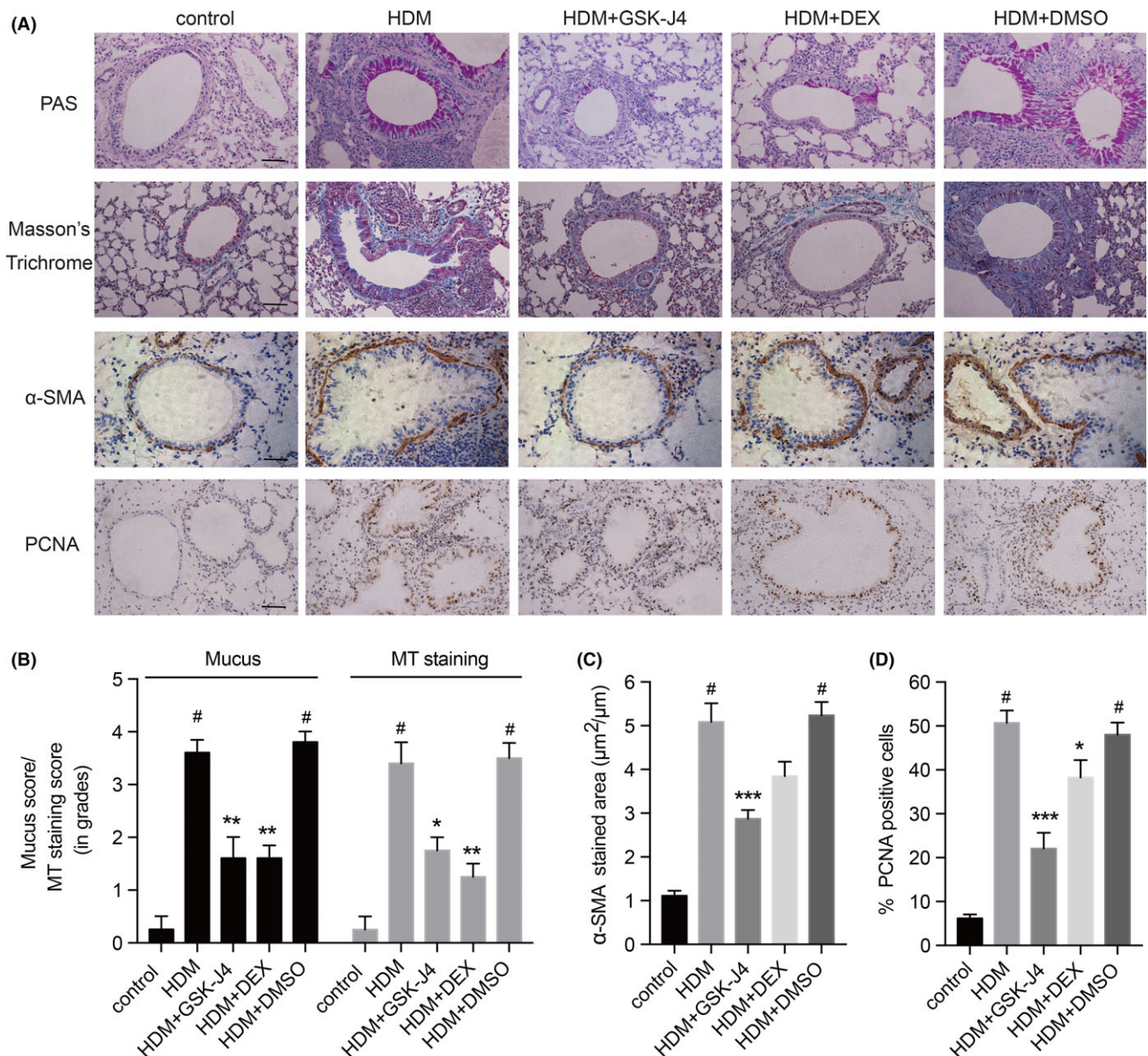


FIGURE 3 Effects of GSK-J4 on airway remodelling. A, Reduced production of mucus, peribronchial collagen deposition, area of peribronchial α-SMA staining and PCNA-positive cells in the airways of mice in the GSK-J4 group compared to those in the HDM group (magnification, $40\times$; scale bar, $50 \mu\text{m}$). B, Bar graph shows the mucus score and the MT staining score quantified by multiplying the percentage of positive cells and the staining intensity, respectively. C, Bar graph shows the quantitative analysis of the area of α-SMA-positive staining. D, Bar graph shows the percentage of PCNA-positive peribronchial mesenchymal cells. Data represent the mean $\pm$ SEM. ($n = 8$ per group). # $P < 0.05$ compared to the control group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to the HDM group [Colour figure can be viewed at wileyonlinelibrary.com]

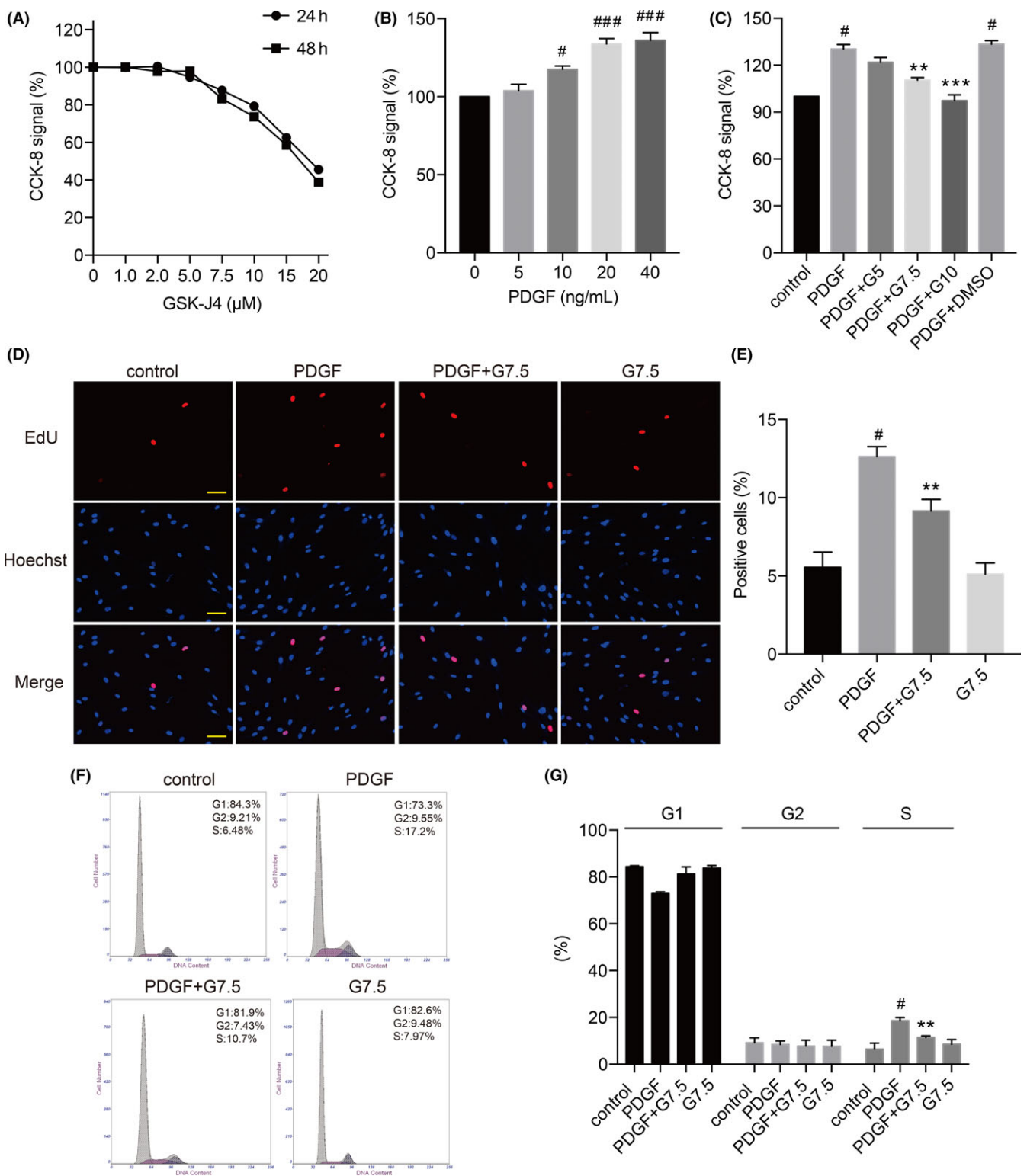


FIGURE 4 Effects of GSK-J4 on ASMCs proliferation. A, The effect of GSK-J4 on the viability of ASMCs was assessed by CCK-8 assay. B, The effect of PDGF on the proliferation of ASMCs was assessed by CCK-8 assay (48 h). C, Effect of GSK-J4 on PDGF-stimulated human ASMCs as assessed by the CCK-8 assay. D, E, PDGF-induced Edu-positive cells were decreased by GSK-J4 treatment (magnification, 20×; scale bar, 100 μm). F, G, GSK-J4 pretreatment blocked PDGF-induced S phase entry of ASMCs. Data represent the mean ± SEM of at least three independent experiments. #*P* < 0.05, ##*P* < 0.01 and ###*P* < 0.001 compared to the control group. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared to the PDGF group

in the control mice ($18.6\% \pm 1.6\%$ vs $6.5\% \pm 1.2\%$ in the control group, $P < 0.05$). In contrast, the effect of PDGF was partly suppressed by GSK-J4 pretreatment ($11.5\% \pm 0.9\%$ vs $18.6\% \pm 1.2\%$ in the PDGF group, $P < 0.05$).

3.5 | Impacts of GSK-J4 on ASMCs synthesis and migration

PDGF stimulation caused significant VEGF release from ASMCs at 24 and 48 hours, respectively (114.4 ± 10.4 to 326 ± 21.6 pg/mL and 149.3 ± 9.8 to 479.8 ± 41.5 pg/mL, respectively), and GSK-J4 treatment did not exhibit a significant effect on PDGF-induced VEGF synthesis (Figure 5A). Moreover, PDGF stimulation did not induce significant synthesis of eotaxin-1 from ASMCs in our study (Figure 5B). However, the results from the migration assay and wound healing assay suggested that GSK-J4 suppressed PDGF-induced ASMCs migration (Figure 5C-F).

3.6 | Impacts of GSK-J4 on PDGF-induced activation of the Akt/MAPKs pathway

PDGF treatment caused decreased H3K27me3 level while GSK-J4 induced recovery of H3K27me3 level (Figure 5G,J). To further elucidate the underlying mechanism of the inhibitory effect GSK-J4 has on ASMCs functions, we investigated the phosphorylation of Akt and mitogen-activated protein kinases (MAPKs) (JNK, p38 and ERK). As shown in Figure 5G, GSK-J4 decreased PDGF-induced phosphorylation of Akt and JNK in ASMCs without impinging on p38 or ERK phosphorylation. These data revealed that the Akt and JNK signalling pathways were involved in the inhibitory effect of GSK-J4 on PDGF-mediated ASMCs proliferation and migration.

3.7 | Impacts of GSK-J4 on ASMCs contraction

Based on our previous findings in mice, we assessed the expression of contractile proteins in human ASMCs. ASMCs may switch to a hypercontractile phenotype in response to TGF- β .^{5,7} ASMCs were incubated with TGF- β (4 ng/mL) in the absence and presence of GSK-J4 (7.5 μ mol/L). In accordance with previous studies, TGF- β increased α -SMA and MLCK expression compared to that in the control group, as demonstrated by both immunofluorescence staining and western blot analysis. In contrast, treatment with GSK-J4 inhibited these effects (Figure 6A,B). Likewise, TGF- β treatment led to a slight decrease in H3K27me3 level, and GSK-J4 suppressed this change in part (Figure 6D). Furthermore, we assessed the phosphorylation of Smad3 in the different groups. Western blotting of ASMCs extracts demonstrated an increase in the phosphorylation of Smad3 following TGF- β stimulation. Moreover, this increase was blocked by pretreatment of ASMCs with GSK-J4 (Figure 6D). These results indicated that the decrease in TGF- β -induced contractile protein expression conferred by GSK-J4 treatment was partly dependent on the Smad3 signalling pathway.

4 | DISCUSSION

Using a HDM-mediated allergic asthma model, we observed a significant loss of H3K28me3-positive cells in lungs from asthmatic mice. Furthermore, inhibiting H3K27me3 demethylase activity with GSK-J4 alleviated the classical symptoms in asthmatic mice, such as AHR, airway inflammation and remodelling. As accumulating evidence has revealed an important role of ASMCs in asthma,^{5,25,26} we especially focused on the ASMCs phenotypic transformation elicited by H3K27me3 demethylation. Our results demonstrated, for the first time, that important cytokines in asthma, such as PDGF and TGF- β , switched the phenotypes of ASMCs, and the selective inhibitor GSK-J4 reduced both the proliferative/synthetic and contractile functions of ASMCs, highlighting a role of H3K27me3 demethylation in ASMCs plasticity.

As the specific demethylases targeting the trimethyl groups on histones, JMJD3 and UTX are responsible for H3K27me3 demethylation, which removes the inhibition of the transcription of multiple genes.²⁷ GSK-J4, a selective inhibitor of JMJD3 and UTX, has been reported to maintain the H3K27me3-driven repression of gene expression.²⁸ Most of the synthesized inhibitors targeting demethylases have presented poor specificity²⁹; however, GSK-J4 has been demonstrated to be the most specific inhibitor for JMJD3 and UTX.³⁰ Thus, we used GSK-J4 as an effective tool to determine the contribution of H3K27me3 demethylation to ASMCs plasticity.

In asthma, ASMCs is considered to be important in the development of AHR, airway inflammation and airway remodelling. For example, ASMCs proliferation leads to increased ASMCs mass, secretion of inflammatory mediators perpetuates airway inflammation, and contraction induces bronchoconstriction.^{5,7} Besides, ASMCs migration may partially contribute to airway remodelling.³¹ Studies have also confirmed that ASMCs phenotype switching exerts a functional influence on ASMCs from human origin.^{32,33} Due to phenotype plasticity, ASMCs may possess diverse biological functions in the presence of multiple stimuli. Exposure of ASMCs to mitogens, such as PDGF, results in a proliferative/synthetic phenotype (modulation), while TGF- β induces a switch to a hypercontractile phenotype by upregulation of contractile proteins (maturation).^{5,7}

PDGF, which is associated with those changes in airway structure and function in asthma,³⁴ has been confirmed as an important factor for ASMCs proliferation, synthesis and migration.⁵ A previous study demonstrated that GSK-J4 led to decreased proliferation and migration of fibroblast-like synoviocytes,³⁵ our study confirmed that GSK-J4 pretreatment partially counteracted PDGF-induced ASMCs proliferation and migration as well. Considering that targeting H3K27me3 demethylation in lung cancer is promising,^{36,37} further studies on the effect of H3K27me3 demethylation in asthma are also of great interest. However, the impact of GSK-J4 pretreatment on ASMCs synthesis was not significant. As important factors involved in angiogenesis in asthmatic airways^{38,39} and eosinophil inflammation, VEGF and eotaxin-1 derived from ASMCs have been confirmed to be overexpressed in asthmatic airways.^{40,41} However, in our study, no notable decrease in PDGF-induced VEGF production

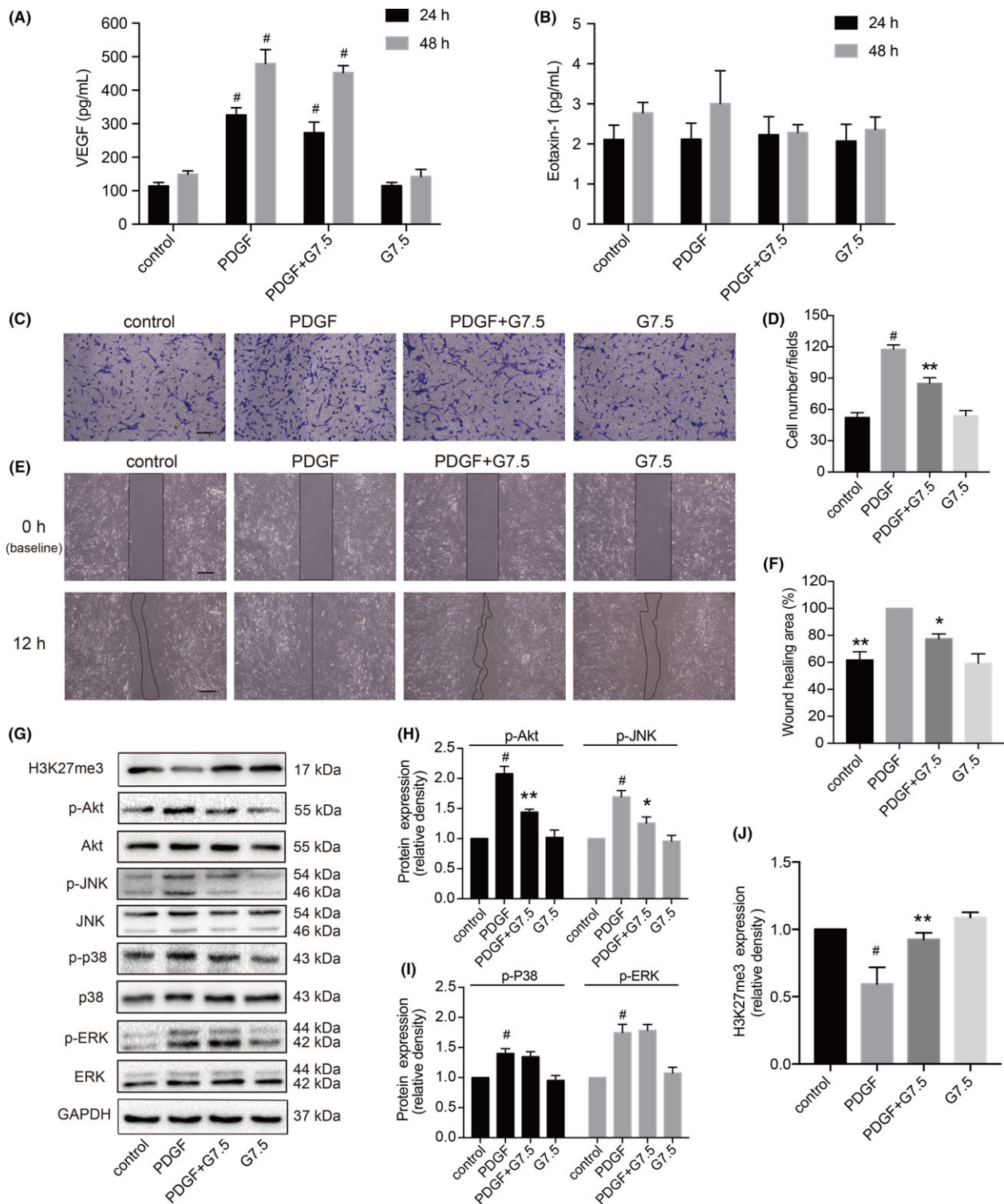


FIGURE 5 Effects of GSK-J4 on PDGF-induced synthesis and migration of ASMCs and the activation of the Akt/MAPKs pathway. A, B, Effects of GSK-J4 on PDGF-induced VEGF and eotaxin-1 synthesis from ASMCs at 24 and 48 hours. C, D, GSK-J4 treatment notably suppressed PDGF-induced ASMCs migration as determined by the transwell assay. The cells that migrated across the membrane were counted from six microscopic fields of each membrane after 24 hours, and the bar graph shows the average (magnification 20 $\times$). One representative random microscopic field of each group is shown (magnification 10 $\times$; scale bar, 200 μ m). E, F, GSK-J4 treatment remarkably inhibited PDGF-induced ASMCs migration as tested by a wound healing assay. Wounds were photographed at baseline and 12 hours later (magnification 10 $\times$; scale bar, 200 μ m). G, H3K27me3 expression, phosphorylation and total protein levels of Akt, JNK, p38 and ERK (1 h) were measured by western blotting. H, I, Relative density quantification of phosphorylated proteins relative to total proteins. (J) The density quantification of H3K27me3 was expressed as a ratio relative to GAPDH. Data represent the mean $\pm$ SEM of at least three independent experiments. $^{\#}P < 0.05$ compared to the control group. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ compared to the PDGF group

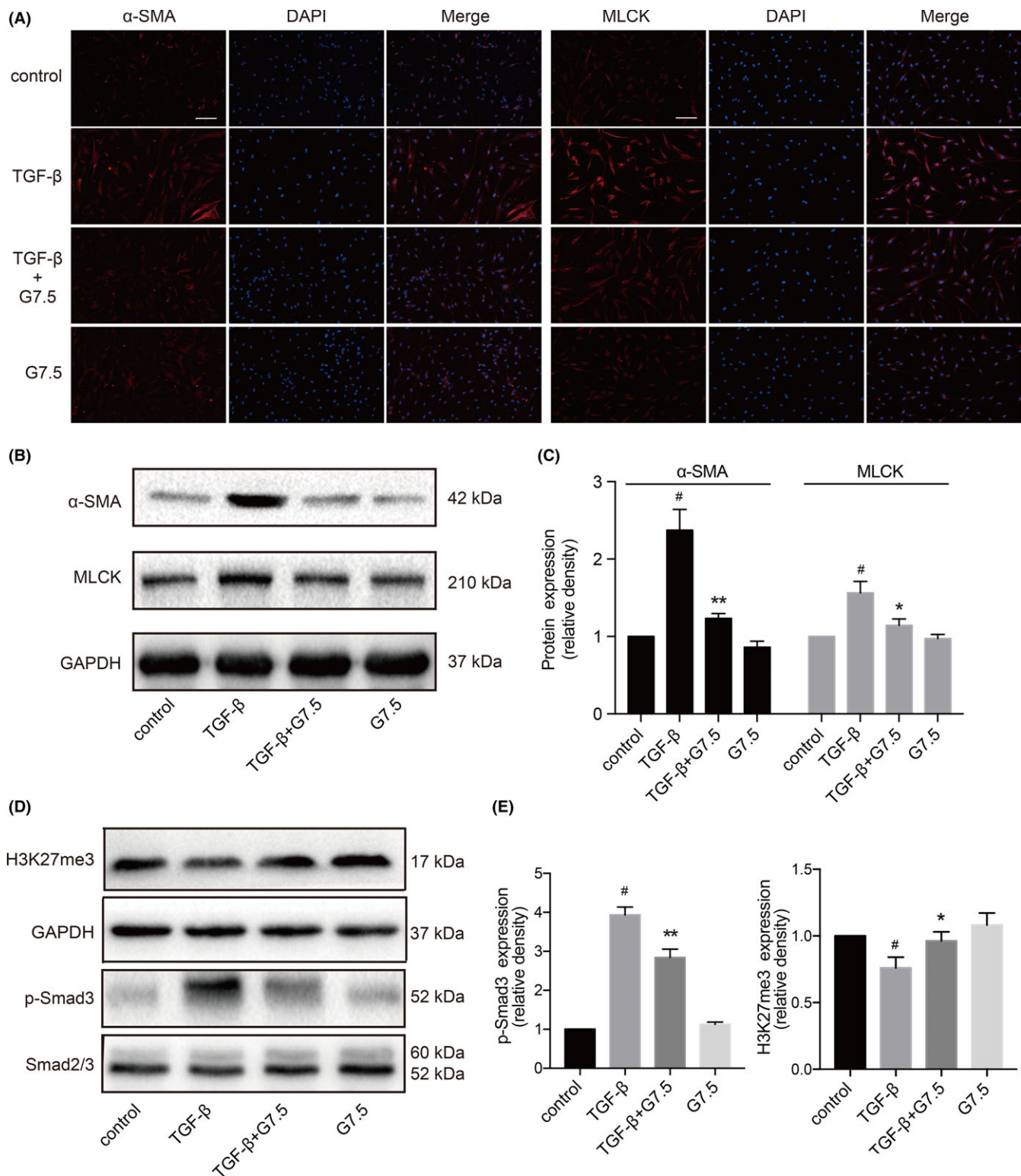


FIGURE 6 Effects of GSK-J4 on TGF- β -induced accumulation of contractile proteins in ASMCs and the activation of the Smad3 pathway. A, Immunofluorescence staining of α -SMA (red), MLCK (red) and nuclei (blue) in ASMCs (magnification, 40 $\times$; scale bar, 50 μ m). B, Protein levels of α -SMA and MLCK (48 h) were measured by western blotting. C, The density quantification of α -SMA and MLCK was expressed as a ratio relative to GAPDH. D, Protein levels of H3K27me3, Smad3 and the phosphorylation of Smad3 (1 h) were measured by western blotting. E, The relative density quantification of phospho-Smad3 was expressed as phosphorylated proteins relative to total proteins. The density quantification of H3K27me3 was expressed as a ratio relative to GAPDH. Data represent the mean $\pm$ SEM of at least three independent experiments. # P < 0.05 compared to the control group. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to the TGF- β group

was observed following GSK-J4 pretreatment, and no PDGF-elicited upregulation of eotaxin-1 was observed. Considering the inhibitory effect of GSK-J4 on eotaxin-1 in the BALF and lung homogenates from HDM-exposed mice, we hypothesize that H3K27me3 demethylation is involved in the regulation of eotaxin-1 synthesis via other factors *in vivo*. Further studies are needed to illustrate the underlying mechanisms.

Akt (also termed protein kinase B) and MAPKs regulate a vast array of cellular processes, such as cell proliferation and migration. MAPKs comprise a family of protein kinases, including ERK, JNK and p38, whose functions and regulation have been conserved across organisms. ERK functions in the control of cell division, JNK is a critical regulator of transcription and p38 is a key modulator of inflammatory cytokines.⁴² Bao et al¹⁶ have confirmed that GSK-J4 administration inhibited ERK signalling in neuromast cells, while Turgeon et al⁴³ demonstrated that decreased H3K27 di-trimethylation affected JNK and p38 phosphorylation in response to IL-1 β in intestinal epithelial cells. Herein, we demonstrated that GSK-J4 treatment suppressed phosphorylation of Akt and JNK in PDGF-treated ASMCs, whereas no impact was observed on the phosphorylation of p38 or ERK. Our results reveal that H3K27me3 demethylation plays a role in PDGF-induced ASMCs proliferative-synthetic phenotype transformation, which is probably mediated by Akt/JNK signalling, thereby highlighting this signalling pathway as a promising target for ASMCs modulation.

As a pleiotropic cytokine contributing to airway remodelling,^{44,45} TGF- β has been reported to show increased expression in asthmatic airways.^{46,47} As mentioned above, TGF- β treatment can induce a hypercontractile phenotype of ASMCs by upregulation of contractile proteins. α -SMA and MLCK, which phosphorylates the myosin light chain and increase ASMCs contraction,⁴⁸ are typical indicators of contractile tension. Therefore, we investigated whether GSK-J4 affected TGF- β -triggered ASMCs maturation. Stimulation with TGF- β resulted in a significant increase in α -SMA and MLCK levels in ASMCs, whereas GSK-J4 administration abolished this effect. Moreover, TGF- β -induced EMT is also associated with airway remodelling,⁴⁹ and previous study revealed that GSK-J4 significantly inhibited TGF- β induced EMT process.¹⁹ Therefore, we speculated that H3K27me3 demethylation might play a role TGF- β signalling pathway.

Among the Smad proteins, Smad3, an important downstream signalling molecule of the TGF- β pathway, serves as a multifaceted regulator and has been implicated in various diseases, including diabetes and carcinoma.^{50,51} TGF- β stimulation has been suggested to induce significant phosphorylation of Smad3 in ASMCs in COPD.⁵² Consistent with the results from a previous study, our data indicated that the inhibitory role of GSK-J4 in TGF- β signalling was also Smad3 dependent. Considering the small magnitude of TGF- β -induced H3K27me3 level change, it would seem that TGF- β might contribute slightly to H3K27me3 demethylation. However, different incubating time of TGF- β may also exert an effect on H3K27me3 level (1 hour in our study), which deserves further investigation.

We found that the Akt/JNK and Smad3 pathways were part of the mechanisms implicated in H3K27me3 inhibition to modulate

ASMCs phenotype switch. Treatment with GSK-J4 impaired the phosphorylation of Akt/JNK and Smad3, without impinging on the expression of total proteins. It can be inferred that, upon PDGF/TGF- β stimulation, Akt/JNK and Smad3 transduce signalling into nucleus, respectively, leading to more H3K27me3 demethylases on target gene promoter to initiate gene transcription. However, inhibition of H3K27me3 demethylases with GSK-J4 may block this change, reciprocally affecting transcription of relevant proteins involved in PDGF/TGF- β signalling activation. Previous studies have revealed a role of phosphatase and tensin homolog (PTEN) in PDGF/TGF- β signalling and H3K27me3 regulation, respectively.⁵³⁻⁵⁶ Thus, we speculate that PTEN may be a potential player, and further studies are needed to explore the detailed mechanism.

Altogether, our results revealed that inhibition of H3K27me3 demethylases with GSK-J4 alleviated asthma by preventing the induction of both a proliferative/synthetic and a hypercontractile phenotype. Our study not only provided novel insight into the epigenetic regulatory mechanisms of ASMCs phenotype plasticity but also identified an exploitable epigenetic target in asthma.

ACKNOWLEDGEMENTS

This work was supported by the Jiangsu Province Special Program for Young Medical Talent (QNRC2016599), the Precision Medicine Research of the National Key Research and Development Plan of China (2016YFC0905800), the Jiangsu Province Special Program of Medical Science (BL2012012), the Changzhou International Science and Technology Collaboration Program (CZ20110021), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD; JX10231802) and the National Natural Science Foundation of China (NSFC) (Grant No. 81700028).

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ETHICS

All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (IACUC1709030).

ORCID

Mao Huang  <http://orcid.org/0000-0001-6901-8434>

REFERENCES

1. James AL, Wenzel S. Clinical relevance of airway remodelling in airway diseases. *Eur Respir J*. 2007;30:134-155.
2. Lambert RK, Wiggs BR, Kuwano K, Hogg JC, Pare PD. Functional significance of increased airway smooth muscle in asthma and COPD. *J Appl Physiol*. 1993;74:2771-2781.

3. Oliver MN, Fabry B, Marinkovic A, Mijailovich SM, Butler JP, Fredberg JJ. Airway hyperresponsiveness, remodeling, and smooth muscle mass: right answer, wrong reason? *Am J Respir Cell Mol Biol*. 2007;37:264-272.
4. Halayko AJ, Tran T, Gosens R. Phenotype and functional plasticity of airway smooth muscle: role of caveolae and caveolins. *Proc Am Thorac Soc*. 2008;5:80-88.
5. Wright DB, Triant S, Siddiqui S, et al. Phenotype modulation of airway smooth muscle in asthma. *Pulm Pharmacol Ther*. 2013;26:42-49.
6. Sukkar MB, Stanley AJ, Blake AE, et al. 'Proliferative' and 'synthetic' airway smooth muscle cells are overlapping populations. *Immunol Cell Biol*. 2004;82:471-478.
7. Zuyderduyn S, Sukkar MB, Fust A, Dhaliwal S, Burgess JK. Treating asthma means treating airway smooth muscle cells. *Eur Respir J*. 2008;32:265-274.
8. Yang IV, Schwartz DA. Epigenetic mechanisms and the development of asthma. *J Allergy Clin Immunol*. 2012;130:1243-1255.
9. Agger K, Cloos PA, Christensen J, et al. UTX and JMJD3 are histone H3K27 demethylases involved in HOX gene regulation and development. *Nature*. 2007;449:731-734.
10. Cao R, Wang L, Wang H, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science*. 2002;298:1039-1043.
11. Lan F, Bayliss PE, Rinn JL, et al. A histone H3 lysine 27 demethylase regulates animal posterior development. *Nature*. 2007;449:689-694.
12. Muller J, Hart CM, Francis NJ, et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. *Cell*. 2002;111:197-208.
13. Zhou VW, Goren A, Bernstein BE. Charting histone modifications and the functional organization of mammalian genomes. *Nat Rev Genet*. 2011;12:7-18.
14. Donas C, Carrasco M, Fritz M, et al. The histone demethylase inhibitor GSK-J4 limits inflammation through the induction of a tolerogenic phenotype on DCs. *J Autoimmun*. 2016;75:105-117.
15. Satoh T, Takeuchi O, Vandenbon A, et al. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. *Nat Immunol*. 2010;11:936-944.
16. Bao B, He Y, Tang D, Li W, Li H. Inhibition of H3K27me3 histone demethylase activity prevents the proliferative regeneration of zebrafish lateral line neuromasts. *Front Mol Neurosci*. 2017;10:51.
17. Jepsen K, Solum D, Zhou T, et al. SMRT-mediated repression of an H3K27 demethylase in progression from neural stem cell to neuron. *Nature*. 2007;450:415-419.
18. Sen GL, Webster DE, Barragan DI, Chang HY, Khavari PA. Control of differentiation in a self-renewing mammalian tissue by the histone demethylase JMJD3. *Genes Dev*. 2008;22:1865-1870.
19. Durham AL, Heineman L, Chung KF, Barnes PJ, Adcock IM. Histone demethylation and acetylation are involved in the epigenetic regulation of epithelial-mesenchymal transition (EMT). *Eur Respir J*. 2013;42:P666.
20. Gregory LG, Causton B, Murdoch JR, et al. Inhaled house dust mite induces pulmonary T helper 2 cytokine production. *Clin Exp Allergy*. 2009;39:1597-1610.
21. Zaffini R, Di Paola R, Cuzzocrea S, Menegazzi M. PARP inhibition treatment in a nonconventional experimental mouse model of chronic asthma. *Naunyn-Schmiedeberg's Arch Pharmacol*. 2016;389:1301-1313.
22. Kerzerho J, Maazi H, Speak AO, et al. Programmed cell death ligand 2 regulates TH9 differentiation and induction of chronic airway hyperreactivity. *J Allergy Clin Immunol*. 2013;131:1048-1057.
23. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. *Cancer Res*. 1998;58:1808-1812.
24. Hou C, Kong J, Liang Y, et al. HMGB1 contributes to allergen-induced airway remodeling in a murine model of chronic asthma by modulating airway inflammation and activating lung fibroblasts. *Cell Mol Immunol*. 2015;12:409-423.
25. Siddiqui S, Redhu NS, Ojo OO, et al. Emerging airway smooth muscle targets to treat asthma. *Pulm Pharmacol Ther*. 2013;26:132-144.
26. Dekkers BG, Pehlic A, Mariani R, Bos IS, Meurs H, Zaagsma J. Glucocorticosteroids and β_2 -adrenoceptor agonists synergize to inhibit airway smooth muscle remodeling. *J Pharmacol Exp Ther*. 2012;342:780-787.
27. Kumar D, Lassar AB. Fibroblast growth factor maintains chondrogenic potential of limb bud mesenchymal cells by modulating DNMT3A recruitment. *Cell Rep*. 2014;8:1419-1431.
28. Hubner MR, Spector DL. Role of H3K27 demethylases Jmjd3 and UTX in transcriptional regulation. *Cold Spring Harb Symp Quant Biol*. 2010;75:43-49.
29. King ON, Li XS, Sakurai M, et al. Quantitative high-throughput screening identifies 8-hydroxyquinolines as cell-active histone demethylase inhibitors. *PLoS ONE*. 2010;5:e15535.
30. Kruidenier L, Chung CW, Cheng Z, et al. A selective jumoni H3K27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature*. 2012;488:404-408.
31. Gizycki MJ, Adelroth E, Rogers AV, O'Byrne PM, Jeffery PK. Myofibroblast involvement in the allergen-induced late response in mild atopic asthma. *Am J Respir Cell Mol Biol*. 1997;16:664-673.
32. Roscioni SS, Prins AG, Elzinga CR, et al. Protein kinase A and the exchange protein directly activated by cAMP (Epac) modulate phenotype plasticity in human airway smooth muscle. *Br J Pharmacol*. 2011;164:958-969.
33. Dekkers BG, Bos IS, Zaagsma J, Meurs H. Functional consequences of human airway smooth muscle phenotype plasticity. *Br J Pharmacol*. 2012;166:359-367.
34. Hirota JA, Ask K, Farkas L, et al. In vivo role of platelet-derived growth factor-BB in airway smooth muscle proliferation in mouse lung. *Am J Respir Cell Mol Biol*. 2011;45:566-572.
35. Jia W, Wu W, Yang D, et al. Histone demethylase JMJD3 regulates fibroblast-like synovial cell-mediated proliferation and joint destruction in rheumatoid arthritis. *FASEB J*. 2018;26:fj201701483R.
36. Chen X, Song N, Matsumoto K, et al. High expression of trimethylated histone H3 at lysine 27 predicts better prognosis in non-small cell lung cancer. *Int J Oncol*. 2013;43:1467-1480.
37. Dalvi MP, Wang L, Zhong R, et al. Taxane-platin-resistant lung cancers co-develop hypersensitivity to jumoni demethylase inhibitors. *Cell Rep*. 2017;19:1669-1684.
38. Hoshino M, Nakamura Y, Hamid QA. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol*. 2001;107:1034-1038.
39. Hurst SM, McLoughlin RM, Monslow J, et al. Secretion of oncostatin M by infiltrating neutrophils: regulation of IL-6 and chemokine expression in human mesothelial cells. *J Immunol*. 2002;169:5244-5251.
40. Faffe DS, Flynt L, Mellema M, et al. Oncostatin M causes eotaxin-1 release from airway smooth muscle: synergy with IL-4 and IL-13. *J Allergy Clin Immunol*. 2005;115:514-520.
41. Faffe DS, Flynt L, Mellema M, et al. Oncostatin M causes VEGF release from human airway smooth muscle: synergy with IL-1 β . *Am J Physiol Lung Cell Mol Physiol*. 2005;288:L1040-L1048.
42. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002;298:1911-1912.
43. Turgeon N, Blais M, Delabre JF, Asselin C. The histone H3K27 methylation mark regulates intestinal epithelial cell density-dependent proliferation and the inflammatory response. *J Cell Biochem*. 2013;114:1203-1215.
44. Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF- β in airway remodeling in asthma. *Immunol Cell Biol*. 2007;85:348-356.

45. Oenema TA, Smit M, Smedinga L, et al. Muscarinic receptor stimulation augments TGF-beta1-induced contractile protein expression by airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*. 2012;303:L589-L597.
46. Redington AE, Madden J, Frew AJ, et al. Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med*. 1997;156:642-647.
47. Vignola AM, Chanez P, Chiappara G, et al. Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med*. 1997;156:591-599.
48. Gawaziuk JP, Ma X, Sheikh F, Cheng ZQ, Cattini PA, Stephens NL. Transforming growth factor-beta as a differentiating factor for cultured smooth muscle cells. *Eur Respir J*. 2007;30:643-652.
49. Zhang M, Zhang Z, Pan HY, Wang DX, Deng ZT, Ye XL. TGF-beta1 induces human bronchial epithelial cell-to-mesenchymal transition in vitro. *Lung*. 2009;187:187-194.
50. Tang YN, Ding WQ, Guo XJ, Yuan XW, Wang DM, Song JG. Epigenetic regulation of Smad2 and Smad3 by profilin-2 promotes lung cancer growth and metastasis. *Nat Commun*. 2015;6:8230.
51. Yadav H, Quijano C, Kamaraju AK, et al. Protection from obesity and diabetes by blockade of TGF-beta/Smad3 signaling. *Cell Metab*. 2011;14:67-79.
52. O'Leary L, Sevinc K, Papazoglou IM, et al. Airway smooth muscle inflammation is regulated by microRNA-145 in COPD. *FEBS Lett*. 2016;590:1324-1334.
53. Takahashi Y, Morales FC, Kreimann EL, Georgescu MM. PTEN tumor suppressor associates with NHERF proteins to attenuate PDGF receptor signaling. *EMBO J*. 2006;25:910-920.
54. Zhang H, Bajraszewski N, Wu E, et al. PDGFRs are critical for PI3K/Akt activation and negatively regulated by mTOR. *J Clin Invest*. 2007;117:730-738.
55. Yu M, Trobridge P, Wang Y, et al. Inactivation of TGF-beta signaling and loss of PTEN cooperate to induce colon cancer in vivo. *Oncogene*. 2014;33:1538-1547.
56. Zhou X, Zang X, Ponnusamy M, et al. Enhancer of zeste homolog 2 inhibition attenuates renal fibrosis by maintaining Smad7 and phosphatase and tensin homolog expression. *J Am Soc Nephrol*. 2016;27:2092-2108.

How to cite this article: Yu Q, Yu X, Zhao W, et al. Inhibition of H3K27me3 demethylases attenuates asthma by reversing the shift in airway smooth muscle phenotype. *Clin Exp Allergy*. 2018;48:1439-1452. <https://doi.org/10.1111/cea.13244>