

Novel insights into the role of BRD4 in fine particulate matter induced airway hyperresponsiveness

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ABSTRACT

Epidemiological research has identified that exposure to fine particulate matter (PM2.5) can increase airway hyperresponsiveness (AHR) which is considered a typical characteristic of asthma. Although the effect of PM2.5 on AHR has been elucidated to a certain degree, its exact mechanism remains unclear. Bromodomain-containing protein 4 (BRD4) is recognized as a member of the bromodomain and extraterminal (BET) family, with the ability to maintain higher-order chromatin configuration and regulate gene expression programs. The primary objective of our study was to examine the role of BRD4 in AHR triggered by PM2.5, and to elucidate its possible molecular mechanism. A mouse model with AHR was established using a nose-only PM2.5 exposure system. We observed that PM2.5 enhanced AHR in the experimental group compared to the control group, and this alteration was accompanied by increased lung inflammation and BRD4 expression in bronchi-lung tissue. However, the BRD4 inhibitor (ZL0420) could alleviate the aforementioned alterations in the mouse model with PM2.5 exposure. To explore the exact molecular mechanism, we further examined the role of BRD4 in human airway smooth muscle cells (hASMCs) after exposure to PM2.5 DMSO extracts. We found that PM2.5 DMSO extracts, which promoted the contraction and migration of hASMCs, was accompanied by an increase in the levels of BRD4, kallikrein 14 (KLK14), bradykinin 2 receptor (B2R), matrix metalloproteinases2(MMP-2), matrix metalloproteinases9(MMP-9), vimentin and bradykinin (BK) secretion, while ZL0420 and BRD4 gene silencing could reverse this response. In summary, these results demonstrate that BRD4 is an important player in AHR triggered by PM2.5, and BRD4 inhibition can ameliorate AHR induced by PM2.5. In addition, PM2.5 DMSO extracts can promote the contraction and migration of hASMCs by increasing BRD4 expression.

1. Introduction

Accumulating evidence supports that fine particulate matter (PM2.5) is a serious threat to patients with asthma, as evidenced by their increased hospitalization rates and mortality (Liu et al., 2019; Atkinson et al., 2014). In addition, Loftus showed that a 6.7 $\mu\text{g}/\text{m}^3$ increase in PM2.5 could lead to a decline of 0.9% in forced expiratory volume in the first second (FEV1) in children due to asthma (Loftus et al., 2015). Currently, PM2.5, which mediates various molecular mechanisms, including oxidative stress, the inflammatory response, immune system imbalance, apoptosis, genotoxicity and autophagy, has been shown to increase the occurrence of airway hyperresponsiveness (AHR) (Lu et al., 2021). However, its exact mechanism of operation remains unclear.

Bromodomain-containing protein 4 (BRD4) belongs to a member of the bromodomain and extraterminal (BET) family and contains bromodomains (BRDs) that can recognize and bind to acetylated lysine residues on histone tails. Evidence supports that BET can drive a Th17-bias immune response to increase Th17 cytokine levels (Mele et al., 2013; Cheung et al., 2017). Moreover, the inhibition of the BET protein has been shown to ameliorate AHR by inhibiting the Th2 immune response and airway inflammation in a mouse model of asthma (Kersch et al., 2019; Nguyen et al., 2016).

BRD4, a member of the BET family, has the ability to recruit the positive transcription elongation factor b and trigger RNA polymerase II-dependent transcription, thereby regulating the expression of numerous genes in mammals. Although BRD4 is involved in the pathological process of many

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diseases, most studies exploring the role of BRD4 have mainly focused on the occurrence of cancer. Currently, there are limited studies on the role of BRD4 in the pathological process of asthma, despite mediating airway inflammation and airway remodeling. For instance, BRD4 is correlated with increased levels of inflammatory molecules, such as interleukin-6 (IL-6), interleukin-1 β (IL-1 β), interleukin-8 (IL-8), and interleukin-9 (IL-9) (Wei et al., 2015; Huang et al., 2009; Perry et al., 2015; Xiao et al., 2018). BRD4 can enhance nitric oxide (NO) synthesis by coupling with NOS2 promoter regions (Wienerroither et al., 2014). NO, as the end product of nitrosative stress, is positively correlated with the enhancement of eosinophilic airway inflammation (Zuo et al., 2014). In addition, BRD4 is correlated with the activation of oxidative stress, which has been supported by the fact that BRD4 inhibition could activate Nrf2 signaling and ameliorate cell death induced by oxidative stress (Hussong et al., 2014). An asthma characteristic, apart from inflammation and oxidative stress, is airway remodeling, which eventually causes airflow obstruction and a rapid decline in lung function (Bousquet et al., 2000). To date, BRD4 has been shown to participate in airway remodeling, as evidenced by the induction of epithelial mesenchymal transformation (EMT), the promotion of myofibroblast differentiation and the TGF- β -induced airway smooth muscle cell (ASMC) proliferation, thereby leading to airway narrowing (Tian et al., 2016; Ijaz et al., 2017; Perry et al., 2015). The migration of ASMCs has been recognized as a key player in the induction of airway remodeling. Interestingly, evidence supports that BRD4 knockdown inhibits vascular smooth muscle cell proliferation and migration (Wang et al., 2015). However, the role of BRD4 in human airway smooth muscle cell (hASMC) migration has not yet been elucidated. Therefore, we uncovered the role of BRD4 in regulating the migration of ASMCs induced by PM2.5 DMSO extracts. In summary, BRD4 either directly promotes proinflammatory cytokine expression correlated with AHR or indirectly induces airway remodeling to enhance AHR.

In this study, we aimed to elucidate the role of BRD4 in PM2.5-induced AHR for the first time. Our previous study showed that PM2.5 contributed to an enhancement in AHR, with the ability to upregulate kallikrein 14 (KLK14) expression to increase the level of bradykinin (BK) (Cao et al., 2020). Considering that BRD4 could regulate the expression of numerous inflammatory cytokines, we further examined whether BRD4 was located upstream of KLK14 and bradykinin 2 receptor (B2R), influencing the secretion of BK, thereby accounting for the pathogenesis of AHR. In addition, we explored the role of BRD4 in airway remodeling induced by PM2.5. Airway remodeling can lead to airway narrowing, thereby causing the occurrence of AHR. In short, this study aimed to elucidate the relationship between BRD4 and AHR induced by PM2.5, in an attempt to provide a novel therapeutic target for AHR induced by PM2.5.

2. Methods

2.1. Animals and ethics statement

In total, 40 male C57BL/6 J mice (wild-type, 6–7-week-old, 20 ± 2 g) were obtained from Beijing Sibeifu Biotechnology Co., Ltd. All animals were housed in individually ventilated cages and provided with water and standard mouse chow. The air in the individually ventilated cages was purified using filter. None of the animals fell ill or died before the experimental endpoint. All animal experiments were approved by the Ethical Committee of the Second Hospital of Hebei Medical University, China (approval number: 2020-AE011).

2.2. Experimental groups of animals and PM2.5 exposure

As described in our previous work (Cao et al., 2020), a mouse model with AHR was established using a nose-only PM2.5 exposure system (Huironghe Technology Co., Beijing, China) from December 25, 2019, to March 18, 2020, in Shijiazhuang, Hebei Province. The PM2.5 exposure system mainly contains an ambient fine particle concentrator and a

separate 30-port nose-only inhalation exposure chamber for toxicology studies. The PM2.5 online concentration, enrichment and exposure system can collect PM2.5 from the atmosphere, and concentrate PM2.5, with a maximum 10-fold concentration by cutting, filtration, and condensation. Thus, the major physicochemical features of PM2.5 used in the laboratory are the same as those of PM2.5 collected from the atmosphere. The mice then were exposed to PM2.5 through the nose-only inhalation exposure chambers. After being acclimatized to the laboratory environment for one week, all animals were randomly assigned to four groups ($n = 10/\text{group}$):

FA (filtered air): the mice received filtered air, and were restrained in animal restraining devices for 4 h per day, 5 days per week, for 12 weeks. The mice receive air purified using filter device. This filter device consists of primary filter screen and advanced filter.

PM2.5: the mice were exposed to PM2.5 for 4 h per day, 5 days per week, for 12 weeks.

PM2.5 + ZL0420: one hour before PM2.5 exposure, the mice were treated with 10 mg/kg BRD4 inhibitor (ZL0420) by intraperitoneal injection, once a day, 5 days per week, for 12 weeks. According to the manufacturer's instructions, ZL0420 was dissolved in DMSO and 20% SBE- β -cyclodextrin sequentially, and the SBE- β -cyclodextrin solvent was phosphate-buffered saline (PBS).

PM2.5 + Vehicle: one hour before PM2.5 exposure, the mice were treated with the same amount of vehicle (PBS + DMSO + SBE- β -cyclodextrin) by intraperitoneal injection, once a day, 5 days per week, for 12 weeks.

2.3. Measurement of lung function

Pentobarbital (50 mg/kg) was used to anesthetize the mice via intraperitoneal injection. As described in our previous studies (Cao et al., 2020; Zhao et al., 2019), mice were endotracheally intubated and connected to a flexivent system using an endotracheal cannula (SCIREQ Inc. Montreal, Canada). After initiating mechanical ventilation, the mice were subjected to deep lung inflation (DI; slow inflation to a pressure of 30 cmH₂O held for 3 s). The tidal volume of mechanical ventilation in mice was 10 mL/kg, and the respiratory rate was 150 breaths per min during the entire mechanical ventilation process. The mice were challenged with different concentrations of methacholine (Mch: 0, 3.125, 6.25, 12.5, 25 and 50 mg/mL) 24 h after the last exposure to PM2.5, and the values of airway resistance (RN), respiratory system resistance (Rrs), and tissue damping (G) were measured and recorded.

2.4. Bronchoalveolar lavage fluid collection

Bronchoalveolar lavage fluid (BALF) was obtained 24 h after the last exposure to PM2.5. The mice were anesthetized, endotracheally intubated, and BALF was collected via lavaging airways thrice using 1 mL of normal saline. The recovered volume of lavage fluid was over 80% that of instilled fluid, followed by centrifugation at 3000 rpm at 4 °C for 10 min. Finally, the supernatants were collected for the measurement of cytokine levels.

2.5. Measurement of cytokines in BALF and hASMC supernatant

According to the manufacturer's instructions, individual ELISA kits were used to measure cytokine levels in BALF and the supernatant of hASMCs, including BK (Elabscience Biotechnology Co., Ltd) and IL-1 β , IL-6, and IL-8 (Neobioscience Co., Ltd).

2.6. Hematoxylin-eosin staining of lung tissue

The lung tissue was fixed using 4% neutral formaldehyde solution for 72 h. Tissue sections were stained with hematoxylin-eosin (HE) to visualize the histopathological abnormalities of lung tissue.

2.7. Immunohistochemistry (IHC)

The immunohistochemical assay was adapted to assess the expression of BRD4 in the lung tissue of mice. The Section (4 μ m in thickness) were routinely dewaxed and rehydrated with graded ethanol. The sections were incubated with 3% H₂O₂ at 37 °C for 1 h to eliminate endogenous peroxidase activity. Then, they were washed with PBS three times and subsequently incubated in goat serum for 1 h at 37 °C. The sections were then incubated with anti-BRD4 rabbit monoclonal antibody (1:400, Abcam, UK) for 15 h at 4 °C. The next day, the sections were incubated with a secondary antibody at 37 °C for 1 h. Next, they were washed with PBS three times and stained with DAB. Finally, a light upright microscope (Zeiss, Germany) was used to capture all the images.

2.8. Western blotting

Western blotting was used to further assess the relative protein levels. The proteins were extracted separately from hASMCs and lung tissue with cold RIPA buffer (Beijing Solarbio Science and Technology Co., Ltd., China) containing protease and phosphatase inhibitors (Beijing Solarbio Science and Technology Co., Ltd., China), followed by the quantification of protein concentration using the BCA assay. Proteins were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, MA, USA) at 200 mA for 2 h at 4 °C. The membranes were blocked in 5% bovine serum albumin for 1.5 h, and subsequently incubated with the primary antibodies at 4 °C for 15 h. The primary antibodies used were as follows: BRD4 (rabbit monoclonal antibody, dilution: 1:800, Abcam, UK), KLK14 (rabbit monoclonal antibody, dilution: 1:500, Abcam, UK), B2R (mouse monoclonal antibody, dilution: 1:500, Santa Cruz, USA), B2R (rabbit monoclonal antibody dilution: 1:500, Abcam, UK), MMP2 (rabbit polyclonal antibody, dilution: 1:800, Proteintech Group, Inc., USA), MMP9 (rabbit polyclonal antibody, dilution: 1:800, Abcam, UK), vimentin (mouse monoclonal antibody, dilution: 1:500, Santa Cruz, USA) and GAPDH antibody (rabbit polyclonal antibody, dilution: 1:5000, Proteintech Group, Inc., USA). The next day, blotting bands were incubated with an appropriate conjugated secondary antibody (goat anti-rabbit IgG, dilution: 1:5000 and goat anti-mouse IgG, dilution: 1:3000) at 25 °C for 1 h. The blotting bands were then washed with TBST 4 times for 5 min each time. Finally, the bands were detected with ECL Plus detection reagents and quantified on a Bio-Rad ChemiDoc imaging system (Bio-Rad, USA). The bands were analyzed using Image J software (National Institutes of Health, USA).

2.9. Cell culture

HASMCs were purchased from the BeNa Culture Collection (Beijing, China). HASMCs were cultured in nutrient media containing Dulbecco Modified Eagle Medium (DMEM), 1% penicillin-streptomycin antibiotics (Sigma-Aldrich, USA), and 10% fetal bovine serum (FBS) (Gibco, USA), and maintained at 37 °C with 5% CO₂. Twenty-four hours prior to PM2.5 DMSO extracts exposure, the hASMCs were plated in a 6-well plate at such a concentration that the next day the wells reached 70% confluency. The hASMCs were then treated with different concentrations of PM2.5 DMSO extracts (0, 3.125, 6.25, 12.5, and 25 μ g/mL) for 24 h. Notably, the PM2.5 powder used in this study was the same as that used in our previous study (Cao et al., 2020). Thus, the components of PM2.5 DMSO extracts, and the content of each component, were the same as those in our previous study (Cao et al., 2020). The PM2.5 powder was dissolved in DMSO (5 mg/mL), followed by filtration through a 0.22- μ m filter in order to eliminate some insoluble matter. Finally, PM2.5 DMSO extracts was obtained and diluted in standard culture medium to obtain the aforementioned concentrations.

2.10. Immunofluorescence

Immunofluorescence was performed to examine the localization of BRD4 in hASMCs. HASMCs were fixed with 4% formaldehyde at 25 °C

for 25 min. After being washed with PBS three times, the cells were incubated with 0.5% Triton-100 for 25 min. The cells were then incubated in 10% goat serum at room temperature for 1 h, followed by incubation with anti-BRD4 (dilution: 1:200) at 4 °C for 15 h. The next day, the cells were incubated with FITC-conjugated secondary antibody (Bioss, Woburn, MA, USA, dilution: 1:500) for 1 h at 25 °C. The nuclei of hASMCs were stained with DAPI for 6 min. Finally, the localization of BRD4 was observed using a fluorescence microscope (Zeiss Inc., Germany).

2.11. BRD4 siRNA transfection of hASMCs

Twenty-four hours prior to transfection, the hASMCs were plated in a 6-well plate at a concentration that reached 60% confluence the next day. Three separate BRD4-specific siRNA sequences and a negative control siRNA sequence (RiboBio, China) were transfected using Lipofectamine®RNAiMAX Reagent (Thermo Fisher Scientific, Waltham, MA, USA). During transfection, hASMCs were cultured in nutrient media without FBS and penicillin-streptomycin antibiotics. After 5 h of transfection, the culture medium was replaced with standard nutrient media. The expression of BRD4 was assessed by western blotting 48 h after transfection.

2.12. BRD4 inhibition of hASMCs

Twenty-four hours prior to PM2.5 DMSO extracts exposure, the hASMCs were plated in a 6-well plate at a concentration that reached 50–60% confluence the next day. The next day, hASMCs were cultured in the nutrient media containing 2% FBS (Gibco, USA), and maintained at 37 °C with 5% CO₂ for 24 h. One hour before exposure to PM2.5 DMSO extracts, hASMCs were cultured in the nutrient media that containing 10% FBS (Gibco, USA), and were treated with 40 mmol/L BRD4 inhibitor (ZL0420) according to a recent study (Tian et al., 2019), followed by exposure to PM2.5 DMSO extracts for 24 h. The expression of associated proteins was assessed by western blotting 24 h after exposure to PM2.5 DMSO extracts.

2.13. Cell contraction assays

The cell contraction assay kit (Cell Biolabs, Inc.) was used to assess the contractile function of hASMCs. In this process, hASMCs were cultured in a 25 T culture bottle. After BRD4 knockdown or BRD4 inhibition, hASMCs were treated with PM2.5 DMSO extracts (12.5 μ g/mL) for 24 h. Then, 50 μ L of cell solution (approximately 2×10^6 cells) mixed with 200 μ L cold gel working solution was added into each well in a 48-well plate for 1.5 h. After collagen polymerization, 500 μ L standard nutrient media containing PM2.5 DMSO extracts (12.5 μ g/mL) was added on top of the collagen of the PM2.5 group for 24 h, whereas as a negative control, 500 μ L standard nutrient media containing the same amount of DMSO solution was added on top of the collagen for 24 h. Notably, one hour prior to exposure to PM2.5 DMSO extracts, ZL0420 solution was added on top of the collagen of the BRD4 inhibition group. Finally, the diameter of each collagen gel was measured using a ruler 24 h after PM2.5 DMSO extracts exposure.

2.14. Cell migration assays

The transwell chamber method was used to evaluate cell migration. Corning cell culture inserts (6.5 mm diameter, 8- μ m pore size) were selected to perform cell migration assays. The cells were pretreated with PM2.5 DMSO extracts (0, 3.125, 6.25, 12.5, and 25 μ g/mL) for 24 h prior to cell migration assays. HASMCs (1×10^4) resuspended in DMEM containing 2% FBS were added to the upper chamber, and DMEM containing 20% FBS was added to the bottom chamber for 24 h. The next day, the cells were fixed and stained with 0.1% crystal violet solution. After removing the cells from the upper side of the membrane, the

migrating cells were quantified by calculating the average number of cells in five random fields under a microscope equipped with a $\times 10$ objective lens.

2.15. Statistical analysis

The results are presented as the mean $\pm$ SEM and analyzed using IBM SPSS Statistics for Windows, version 19.0 (IBM Corp., Armonk, NY, USA). Comparisons between two groups were evaluated using the two-sample Student's *t*-test. The statistical significance was set at $p < 0.05$.

3. Results

3.1. PM 2.5 concentration

During the PM2.5 exposure period, the average concentration of daily ambient PM2.5 was $90 \mu\text{g}/\text{m}^3$. The data were provided by the Shijiazhuang Environmental Protection Bureau. The PM2.5 online exposure system can collect PM2.5 from the atmosphere and provide a maximum 10-fold PM2.5. The average concentration of the daily PM2.5 used in laboratory was $820 \mu\text{g}/\text{m}^3$. The maximum and minimum concentrations of PM2.5 exposure were $2000 \mu\text{g}/\text{m}^3$ and $500 \mu\text{g}/\text{m}^3$, respectively. The exposure concentration of PM2.5, which we aimed to achieve in the vivo experiment in mice, was 800–1000 $\mu\text{g}/\text{m}^3$.

3.2. BRD4 inhibition contributed to alleviating PM2.5-induced AHR

The role of BRD4 in AHR induced by PM2.5 was elaborated next. As presented in Fig. 1. B–D, PM2.5 had an adverse effect on lung function. The Rrs, RN, and G values in the PM2.5 group were much higher than those in the FA group under the stimulation of Mch at certain concentrations. Compared with the PM2.5 + vehicle group, the PM2.5 + ZL0420 group showed an apparent trend of decreased lung function (i.e., Rrs, RN, and G). Moreover, under the stimulation of the 12.5 mg/mL Mch, the Rrs, RN, and G values of the PM2.5 group were more than two times higher than the baseline values. However, the concentration of Mch that caused a two fold increase in airway resistance (G, RN, Rrs), was much higher in the FA and PM2.5 + ZL0420 groups. In brief, PM2.5 could enhance AHR, and BRD4 inhibition could improve PM2.5-induced AHR.

3.3. BRD4 inhibition could alleviate lung histopathology induced by PM2.5

There were indications that PM2.5 could result in alveolar space widening, airway wall thickening and increased inflammatory cytokines. Compared with the FA group, the PM2.5 group showed increased IL-1 β , IL-6, IL-8, and BK levels in BALF. Our results showed that BRD4 played a critical role in lung damage induced by PM2.5. As presented in Fig. 2. A, compared with the PM2.5 + vehicle group, BRD4 inhibition ameliorated lung pathology, as evidenced by the improvement of alveolar structure and amelioration of airway wall thickening. As presented

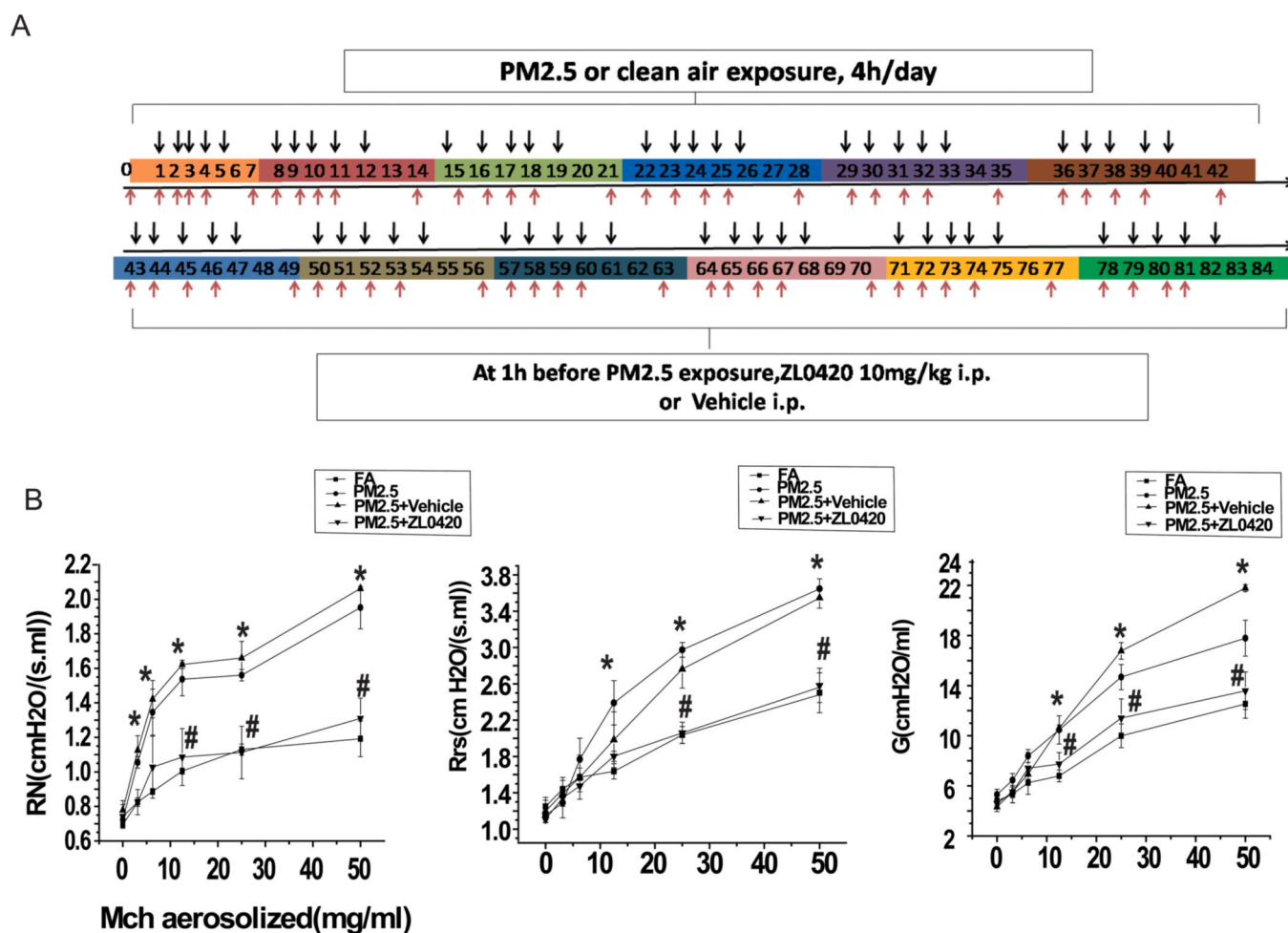


Fig. 1. Protocol for an animal model with PM2.5 exposure and measurement of AHR. A. Protocol for an animal model with PM2.5 exposure. B–D. The measurement of AHR (RN, Rrs, G) in four groups ($n = 4$ per group). The results are presented as the mean $\pm$ SE. Differences are indicated by $p < 0.05$. * $p < 0.05$, PM2.5 group vs. FA group. # $p < 0.05$, PM2.5 + ZL0420 group vs. PM2.5 + vehicle group.

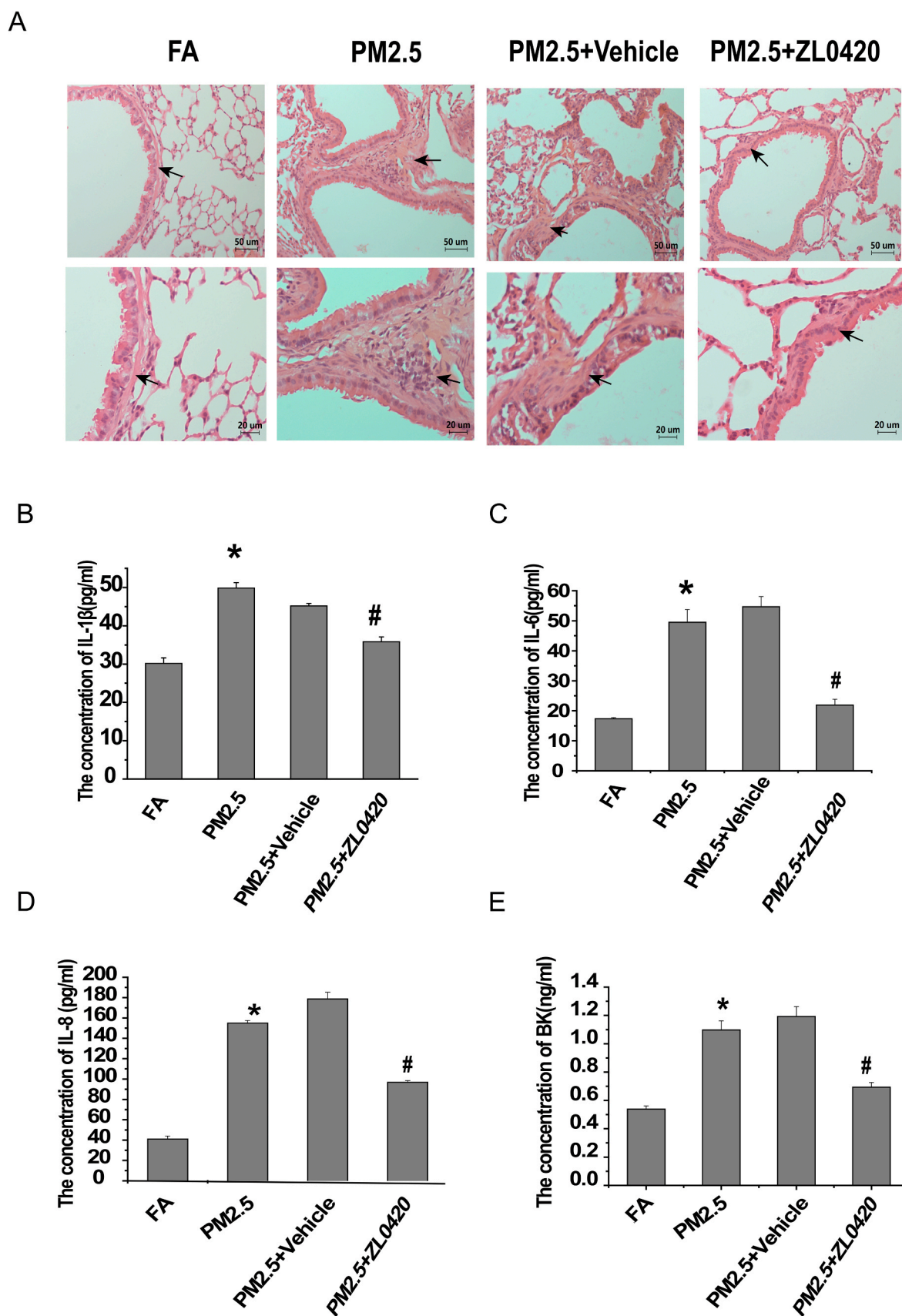


Fig. 2. HE staining (200 $\times$ and 400 $\times$ magnification) and measurement of inflammatory cytokines. A. H&E staining (200 $\times$ and 400 $\times$ magnification) (n = 3 per group). The arrows in the figure point at airway wall thickening. B-E. Assessment of IL-1 β , IL-6, IL-8, and BK levels in BALF (n = 3 per group). The results are presented as the mean $\pm$ SE. Differences are indicated by $p < 0.05$. * $p < 0.05$, PM2.5 group vs. FA group. # $p < 0.05$, PM2.5 + ZL0420 group vs. PM2.5 + vehicle group.

in Fig. 2. B–E, PM2.5 upregulated IL-1 β , IL-6, IL-8, and BK levels in BALF, whereas BRD4 inhibition could downregulate the aforementioned inflammatory cytokine levels.

3.4. Assessment of BRD4 expression levels in lung tissue with immunohistochemistry

To explore the exact mechanisms by which PM2.5 exacerbated AHR, immunohistochemistry was used to assess BRD4 protein levels in lung tissue cells. Positive signs of the BRD4 antibody were visualized as brownish yellow granules. As is shown in Fig. 3A, the results showed that the lung tissue of the PM2.5 group had a higher number of brown areas than the FA group. Compared with the PM2.5 + vehicle group, the BRD4 inhibitor significantly reduced the positive staining areas of BRD4 in the lung tissue.

3.5. Verification of BRD4, KLK14, B2R, MMP2, MMP9, and vimentin levels in lung tissue by western blotting

The protein levels of BRD4, KLK14, B2R, MMP2, MMP9, and vimentin in lung tissue were measured by western blotting. As is shown in Fig. 3. B–C, the BRD4, KLK14, B2R, MMP2, MMP9, and vimentin levels in the PM2.5 group were upregulated in comparison with those in the FA group. The expression of BRD4 was downregulated in the PM2.5 + ZL0420 group compared to that in the PM2.5 + vehicle group, and the reduction of BRD4 was accompanied by a decrease in KLK14, B2R, vimentin, MMP2 and MMP9 levels. In other words, BRD4 participates in regulating the above protein expression in the lung tissue.

3.6. PM2.5 DMSO extracts could promote BRD4 expression in hASMCs by western blotting and immunofluorescence

Thus far, we demonstrated that BRD4 is essential for PM2.5 -induced AHR. Next, we assessed the BRD4 expression in hASMCs using western blotting and immunofluorescence. As is shown in Fig. 4. A–B, the

western blotting results showed that compared with the control group, the BRD4 expression in hASMCs increased 24 h after the treatment with different PM2.5 DMSO extracts concentrations (3.125, 6.25, 12.5, and 25 μ g/mL). Following the treatment with 12.5 μ g/mL PM2.5 DMSO extracts, the expression level of BRD4 in hASMCs was the highest. The immunofluorescence results showed that the expression of BRD4 increased after exposure to 12.5 μ g/mL PM2.5 DMSO extracts for 24 h, which was consistent with the results of western blotting. Moreover, the immunofluorescence analysis confirmed that BRD4 was located in the nucleus rather than in the cytoplasm of hASMCs, as is shown in Fig. 4.E. In addition, as is shown in Fig. 4. C–D, both B siRNA and C siRNA knocked down the BRD4 gene by transfection, thereby inhibiting the BRD4 expression in hASMCs.

3.7. BRD4 gene knockdown inhibited the contractile function of hASMCs

Thus far, we showed that BRD4 is required for AHR triggered by PM2.5. We then explored the underlying mechanism by which BRD4 mediated PM2.5-induced AHR. The results of our previous study showed that a treatment with 12.5 μ g/mL PM2.5 DMSO extracts resulted in the highest hASMCs viability (Cao et al., 2020). Notably, the PM2.5 powder used in this study was the same as that used in our previous study (Cao et al., 2020). Thus, we first detected the role of BRD4 in the contractile function of hASMC before and after exposure to PM2.5 DMSO extracts (12.5 μ g/mL) using the Cell Contraction Assay Kit. As presented in Fig. 5. A–B, BRD4 gene knockdown inhibited hASMC contraction before and after exposure to PM2.5 DMSO extracts, as reflected by the inhibition of the reduction of gel diameter. In addition, as presented in Fig. 5. F–G, ZL0420 inhibited hASMC contraction before and after exposure to PM2.5 DMSO extracts, which was the consistent with the results of BRD4 gene silencing. Taken together, the contraction of hASMCs was dependent on BRD4 expression to a certain degree.

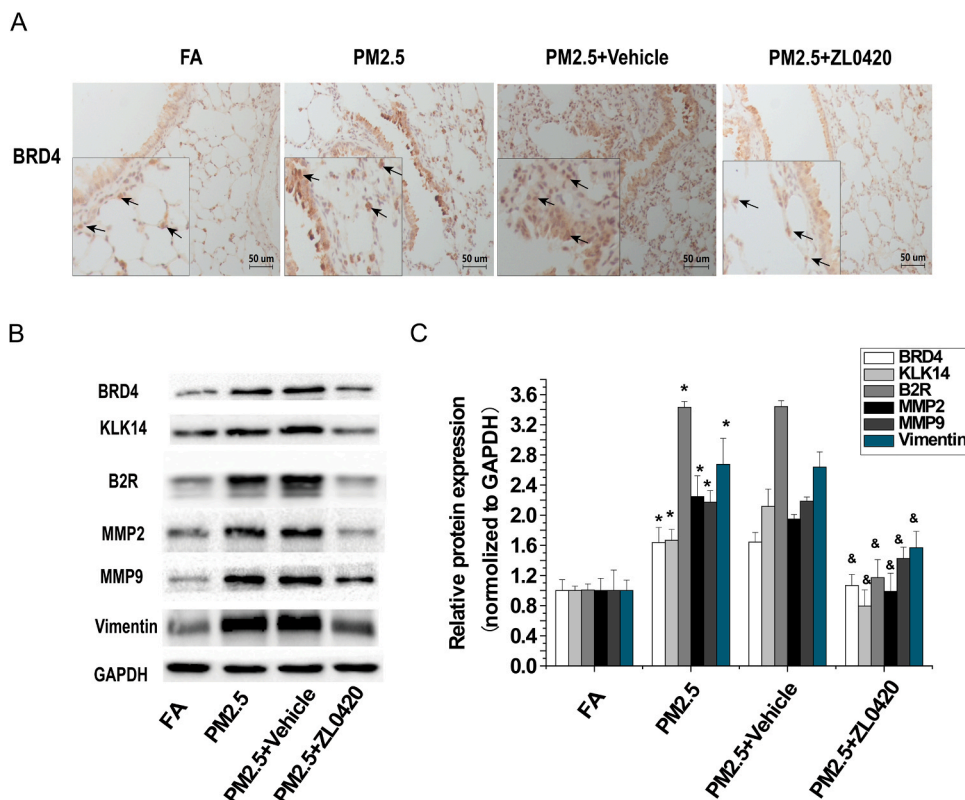


Fig. 3. Assessment of BRD4 expression levels in lung tissue cell using immunohistochemistry, as well as quantification of BRD4 and related protein expressions in lung tissue using western blotting. As is shown in figure A, the lung tissue of the PM2.5 group had a higher number of brown areas than the FA group. In turn, the positive staining areas in the lung tissue of the PM2.5 + BRD4 inhibitor group were reduced compared to those of the PM + vehicle group (n = 3 per group). B–C Assessment of BRD4 and related protein expression levels using western blotting. Protein levels are quantified as relative protein levels (normalized to GAPDH). The results are presented as the mean $\pm$ SE. *p < 0.05, PM2.5 group vs. FA group. &p < 0.05, PM2.5 + ZL0420 group vs. PM2.5 + vehicle group (n = 3 per group).

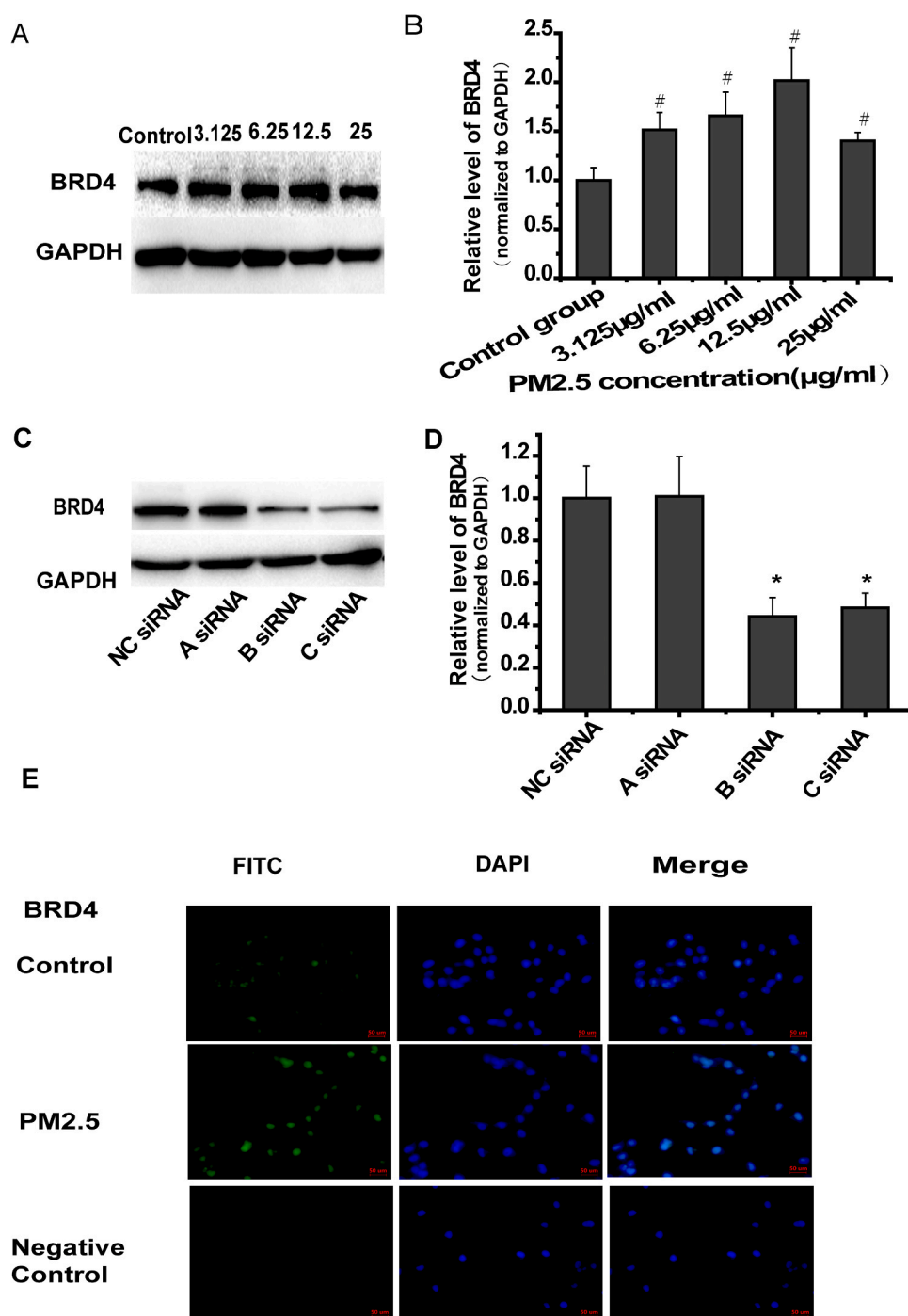


Fig. 4. Quantification of BRD4 levels in hASMCs using western blotting and confirmation of BRD4 localization in hASMCs with cell immunofluorescence. As is shown in figure. A-B, the quantification of BRD4 expression in hASMCs was performed using western blotting. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. # $p < 0.05$, vs. control group. C-D. Measurement of BRD4 protein level in hASMCs after transfection with targeting siRNAs. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. * $p < 0.05$, vs. NC siRNA group. E. Verification of BRD4 localization in hASMCs using cell immunofluorescence, DAPI: blue; BRD4: green.

3.8. BRD4 could increase AHR by regulating the KLK14 and B2R expression and bradykinin secretion in hASMCs

Next, we explored the exact molecular mechanisms of BRD4 in AHR triggered by PM2.5 DMSO extracts. The results of our previous study revealed that PM2.5 enhanced AHR by promoting KLK14 and B2R protein expression (Cao et al., 2020). Thus, we examined whether BRD4 was located upstream of KLK14 and B2R to mediate PM2.5 DMSO extracts-induced AHR. As is shown in Fig. 5., BRD4 knockdown and ZL0420 could lead to the reduction of KLK14 and B2R expression, thereby inhibiting BK secretion in the supernatant of cells, thus further supporting the view that BRD4 could mediate PM2.5 DMSO extracts-induced hASMC contraction by regulating the expression of

KLK14 and B2R.

3.9. PM2.5 was able to promote the migration of hASMCs

The migration of hASMCs plays a key role in airway remodeling. We selected the transwell chamber method to assess the migration ability of hASMC before and after PM2.5 DMSO extracts exposure. As presented in Fig. 6. A–B, PM2.5 DMSO extracts could promote hASMC migration 24 h after the treatment with different PM2.5 DMSO extracts concentrations (3.125, 6.25, 12.5, and 25 µg/mL), which was assessed by calculating the average number of cells in five random fields under a microscope equipped with a $\times 10$ objective lens. Following a treatment with 6.25 µg/mL PM2.5 DMSO extracts, the amount of hASMC migration was the highest.

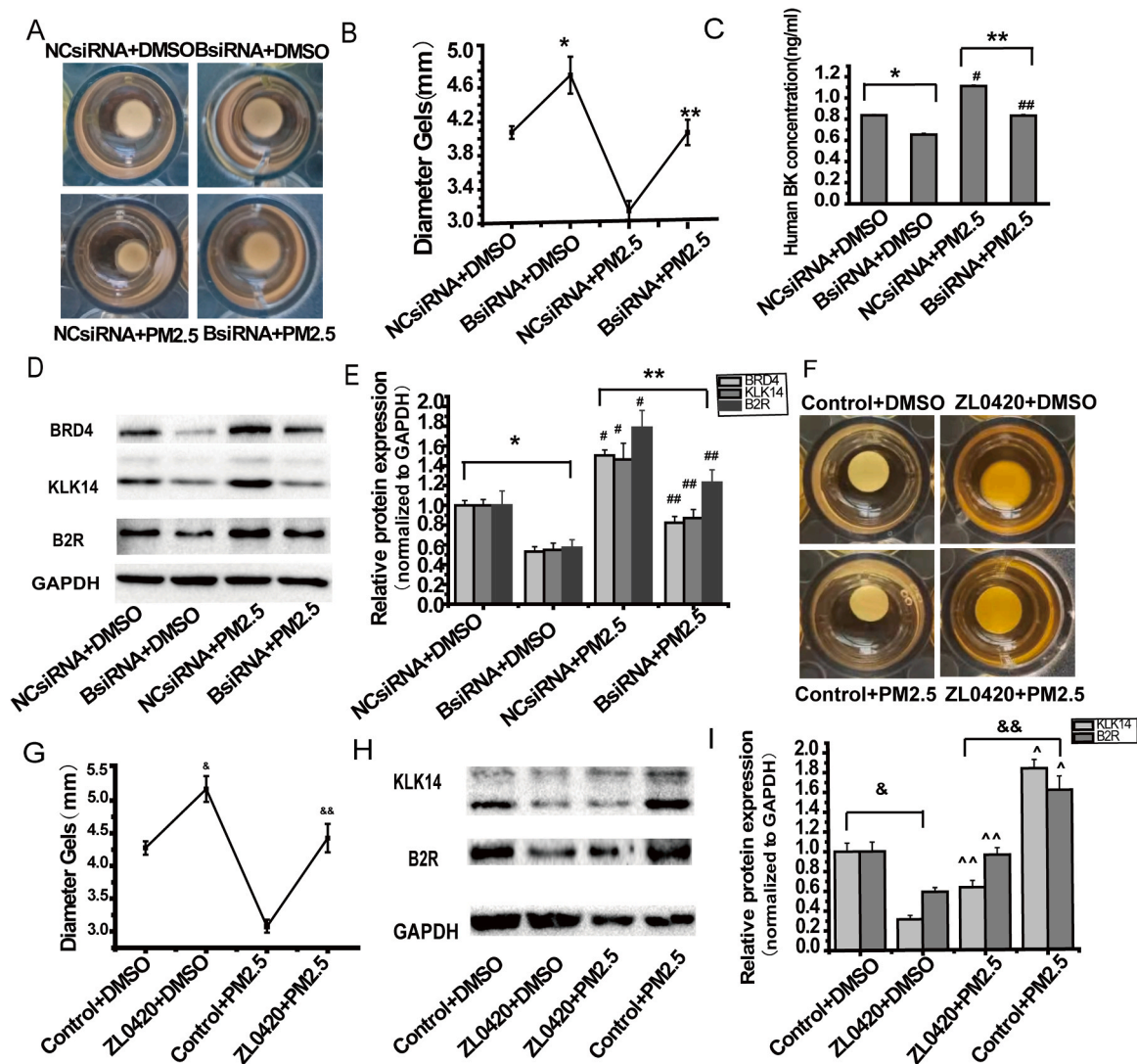


Fig. 5. The underlying mechanism of BRD4 in mediating the contraction of hASMCs. Figure A-B. BRD4 gene knockdown inhibited the contractile function of hASMCs before and after PM2.5 DMSO extracts exposure. The results are presented as the mean $\pm$ SE. * $p < 0.05$, BsiRNA + DMSO vs. NCsiRNA + DMSO group. * $p < 0.05$, BsiRNA + PM2.5 vs. NCsiRNA + PM2.5. C. The measurement of BK levels in the presence or absence of BRD4 gene knockdown. D-E. Quantification of KLK14 and B2R levels in the presence or absence of BRD4 gene knockdown. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. * $p < 0.05$, BsiRNA + DMSO vs. NCsiRNA + DMSO group. * $p < 0.05$, BsiRNA + PM2.5 vs. NCsiRNA + PM2.5. # $p < 0.05$, NCsiRNA + PM2.5 vs. NCsiRNA + DMSO group. ## $p < 0.05$, BsiRNA + PM2.5 vs. BsiRNA + DMSO. F-G. ZL0420 inhibited the contractile function of hASMCs before and after PM2.5 DMSO extracts exposure. The results are presented as the mean $\pm$ SE. & $p < 0.05$, ZL0420 + DMSO vs. Control + DMSO group. && $p < 0.05$, ZL0420 + PM2.5 vs. Control + PM2.5. H-I. Quantification of KLK14 and B2R levels in the presence or absence of BRD4 inhibition. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. & $p < 0.05$, ZL0420 + DMSO vs. Control + DMSO group. && $p < 0.05$, ZL0420 + PM2.5 vs. Control + PM2.5. ^ $p < 0.05$, Control + PM2.5 vs. Control + DMSO. ^^ $p < 0.05$, ZL0420 + PM2.5 vs. ZL0420 + DMSO group.

Meanwhile, as is shown in Fig. 6. C-D, this alteration was often accompanied by increased MMP2, MMP9, and vimentin expression in hASMCs. MMP2, MMP9, and vimentin represent the ability of hASMCs to migrate to a certain degree, and are positively correlated with the migration ability of hASMCs (Xu et al., 2019). The western blotting results showed that compared with the control group, the MMP2 expression in hASMCs increased 24 h after the treatment with different PM2.5 DMSO extracts concentrations (3.125, 6.25, 12.5, and 25 $\mu\text{g/mL}$), whereas the expression of MMP9 and vimentin in hASMCs increased 24 h after the treatment with different PM2.5 DMSO extracts concentrations (3.125, 6.25 and 12.5 $\mu\text{g/mL}$). Following a treatment with 6.25 $\mu\text{g/mL}$ PM2.5 DMSO extracts, the aforementioned migration-related protein levels in hASMC were the highest.

3.10. BRD4 could promote hASMC migration by regulating the MMP2, MMP9, and vimentin expression in hASMCs

Next, we aimed to further verify whether BRD4 gene knockdown and BRD4 inhibition could significantly inhibit hASMC migration and elucidate its molecular mechanisms. The concentration of PM2.5 DMSO extracts adopted in the migration experiments with transfected cells was 12.5 $\mu\text{g/mL}$. As is shown in Fig. 7. A-B, we found that BRD4 gene knockdown inhibited hASMC migration, as demonstrated by an apparent decrease in the amount of hASMC migration. Meanwhile, as is shown in Fig. 7. C-D, BRD4 gene knockdown inhibited the expression of MMP2, MMP9, and vimentin in hASMCs. In addition, as is shown in Fig. 7. E-H, ZL0420, a BRD4 inhibitor, inhibited hASMC migration by inhibiting the expression of MMP2, MMP9, and vimentin, which was consistent with the results of BRD4 gene silencing, thus further supporting the view that BRD4 participates in PM2.5 DMSO extracts -induced hASMC migration.

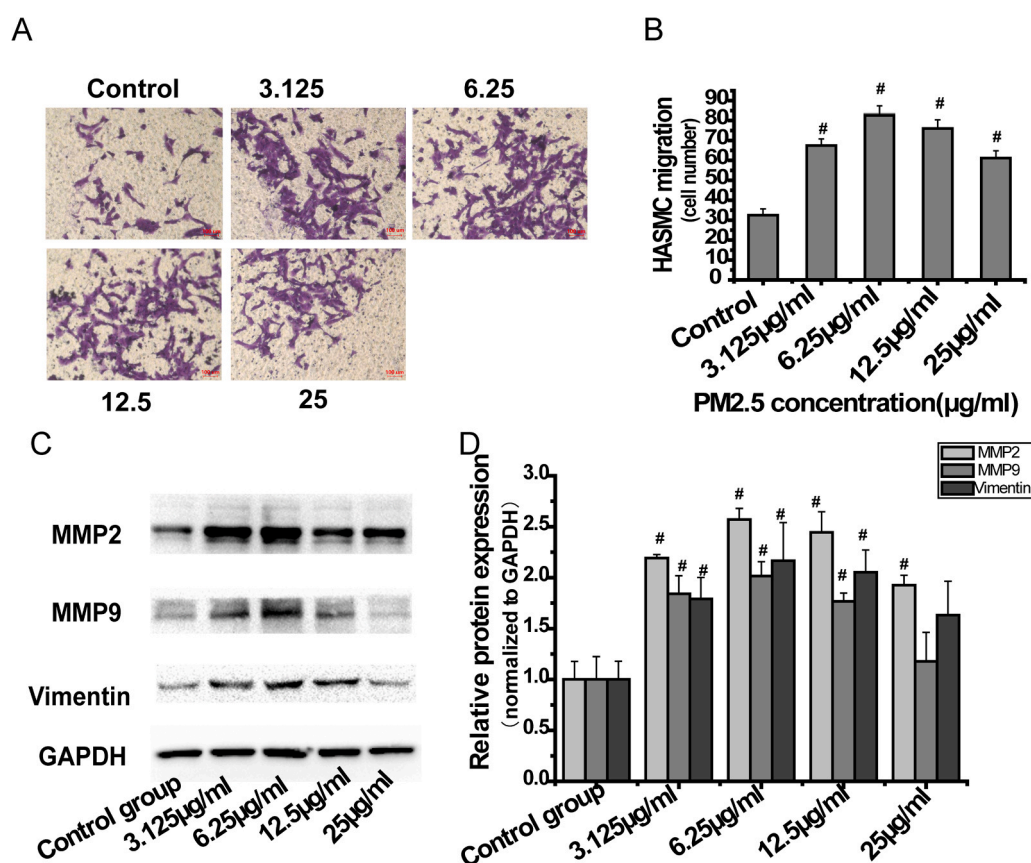


Fig. 6. PM2.5 DMSO extracts promoted the migration of hASMCs. Figure A-B. Assessment of the hASMC migration function after exposure to PM2.5 DMSO extracts. The results are presented as the mean $\pm$ SE. C-D. Quantification of MMP2, MMP9, and vimentin levels in hASMCs exposed to PM2.5 DMSO extracts. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. [#] $p < 0.05$, vs. control group.

4. Discussion

With the development of urban industrialization, air pollution, and PM2.5 in particular, has become a serious threat to human health. Compared with coarse particles, the small size of PM2.5 particles facilitates their suspension for a relatively longer time in the air and makes them easy to be inhaled and deposited into alveoli in the respiratory tract, thereby inducing or exacerbating lung diseases. Accumulating evidence has demonstrated that PM2.5 could induce asthma exacerbation. A previous study showed that ambient PM2.5 exposure had an adverse impact on asthma-related emergency department (ED) visits, as evidenced by a 1.5% increase in ED visits owing to asthma in response to each increase of 10 $\mu\text{g}/\text{m}^3$ PM2.5 (Fan et al., 2016). Similarly, a recent study in Shijiazhuang showed that approximately 92% of asthma hospitalizations during the heating season were attributable to PM2.5 exposure (Liu et al., 2019). Currently, PM2.5 has shown its potential to enhance AHR by activating various signaling pathways (Lu et al., 2021). Among the underlying mechanisms by which PM2.5 evokes AHR, we found that most researchers focused on inflammation, airway remodeling and immune system imbalance. For instance, PM2.5 exposure promotes the production of proinflammatory cytokines (i.e., TNF- α , IL-1 β , IL-6, and IL-8) in human bronchial epithelial cells (Cachon et al., 2014). In addition, the enhancement of AHR induced by PM2.5 exposure has been attributed to the immune system imbalance to a certain degree, reflected by shifting the balance toward an increase in Th17 and Th2 immune responses after PM2.5 exposure (Sun et al., 2020; Zhao et al., 2012). PM2.5 can induce EMT to cause airway remodeling (Cochard et al., 2020). However, considering the heterogeneity of PM2.5 in different countries and regions, the adverse effects of PM2.5 on AHR, and its possible molecular mechanisms still need to be further explored.

To observe PM2.5-induced lung injuries, a mouse model with AHR was established using a nose-only PM2.5 exposure system. The major physicochemical features of PM2.5 used in the laboratory are the same as those of PM2.5 collected from the atmosphere. None of the animals fell ill or died before the experimental endpoint. We observed that PM2.5 could enhance AHR in mouse models, accompanied by inflammatory cell infiltration, alveolar structure damage, and airway wall thickening, which was consistent with previous studies (Ogino et al., 2017; Zhao et al., 2019). Notably, we found that PM2.5 could promote BRD4 protein expression, whereas the BRD4 inhibitor (ZL0420) could alleviate AHR and ameliorate the pathological damage of lung tissue, thus identifying the pivotal role of BRD4 in AHR evoked by PM2.5. BRD4, a member of the BET family, maintains higher-order chromatin configuration and participates in the formation of super-enhancers with the ability to regulate the expression of numerous inflammatory cytokines, such as IL-6, IL-8, IL-9 and NO (Perry et al., 2015; Xiao et al., 2018; Wienerroither et al., 2014). Although some studies have explored the correlation between BRD4 and AHR, the exact mechanism by which BRD4 participates in regulating AHR remains unclear. In this study, we observed that exposure to PM2.5, upregulated the IL-1 β , IL-6, and IL-8 levels in the BALF of mice, which was attributed to the infiltration of numerous inflammatory cells, including eosinophils, neutrophils, and macrophages (Zhao et al., 2019). However, BRD4 inhibition could downregulate the aforementioned inflammatory cytokine levels, further implying that a key role was played by BRD4 in modulating the aforementioned inflammatory cytokine levels (i.e., IL-1 β , IL-6, IL-8), which was consistent with the results of previous studies (Perry et al., 2015; Xiao et al., 2018). Among these cytokines, IL-6 has the potential to stimulate the proliferation of ASMCs and participate in the differentiation of Th2 and Th17 cells to a certain degree (Knobloch et al., 2016; Diehl et al., 2002; Veldhoen et al., 2006). Both IL-1 β and TNF- α can

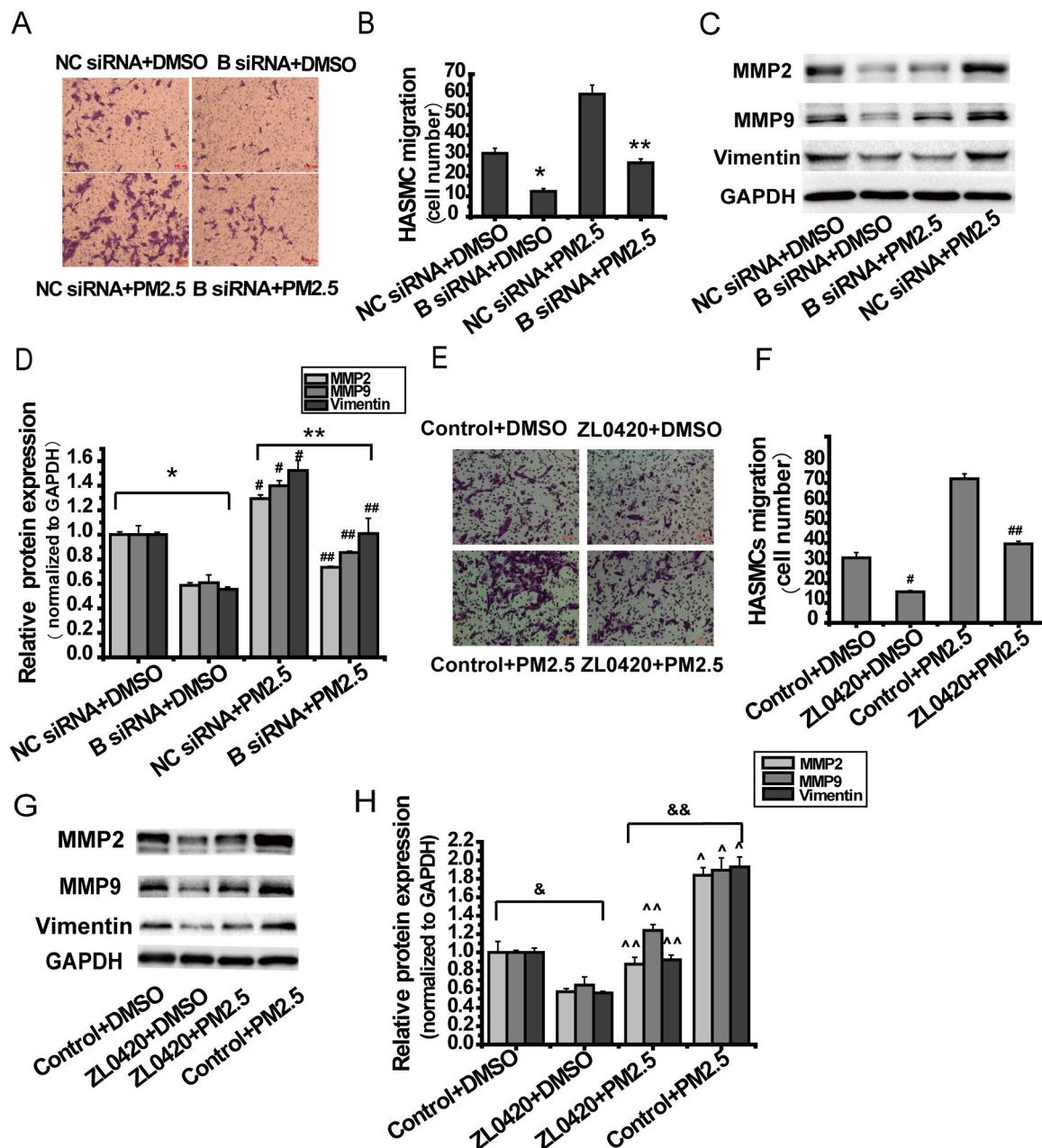


Fig. 7. Confirmation of the role of BRD4 in PM2.5 DMSO extracts-induced hASC migration. A-B. The ability of hASC to migrate was measured following transfection by targeting BRD4 siRNAs. The results are presented as the mean $\pm$ SE. * $p < 0.05$, BsiRNA+DMSO vs. NCsiRNA+DMSO group. ** $p < 0.05$, BsiRNA+PM2.5 vs. NCsiRNA+PM2.5. C-D. Quantification of MMP2, MMP9, and vimentin levels in hASCs transfected with targeting BRD4 siRNAs. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. * $p < 0.05$, BsiRNA+DMSO vs. NCsiRNA+DMSO group. ** $p < 0.05$, BsiRNA+PM2.5 vs. NCsiRNA+PM2.5. # $p < 0.05$, NCsiRNA+PM2.5 vs. NCsiRNA+DMSO group, ## $p < 0.05$, BsiRNA+PM2.5 vs. BsiRNA+DMSO. E-F. ZL0420 inhibited the ability of hASC to migrate. The results are presented as the mean $\pm$ SE. # $p < 0.05$, ZL0420+DMSO vs. Control+DMSO group, ## $p < 0.05$, ZL0420+PM2.5 vs. Control+PM2.5. G-H. Quantification of MMP2, MMP9, and vimentin levels in hASCs in the presence or absence of BRD4 inhibition. Protein levels are quantified as relative protein level (normalized to GAPDH). The results are presented as the mean $\pm$ SE. & $p < 0.05$, ZL0420+DMSO vs. Control+DMSO group. && $p < 0.05$, ZL0420+PM2.5 vs. Control+PM2.5. ^ $p < 0.05$, Control+PM2.5 vs. Control+DMSO. ^^ $p < 0.05$, ZL0420+PM2.5 vs. ZL0420+DMSO group.

enhance airway responsiveness by increasing airway inflammation and inducing immune system imbalance (Mahmutovic et al., 2018; Lee et al., 2016). IL-8 is largely involved in the contraction and migration of ASMCs, as well as in the chemotaxis of neutrophils and eosinophils (Govindaraju et al., 2006; Nakagome and Nagata, 2018). Furthermore, our previous study has demonstrated that PM2.5 can upregulate BK levels by promoting KLK14 and B2R expression, thereby enhancing airway responsiveness in mice (Cao et al., 2020). Given the important role of BRD4 in regulating the expression of proteins related to inflammation, we hypothesized that BRD4 regulates KLK14 and B2R protein levels in lung

tissue, thereby modulating the secretion of BK. BK, as an important inflammatory molecule, can influence the concentration of cytosolic calcium by binding to B2R, thereby causing hASC contraction (Cao et al., 2020). As predicted, we found that ZL0420 could downregulate the expression of KLK14 and B2R to inhibit the secretion of BK in BALF. Based on these results, we propose that BRD4 is located upstream of KLK14 and B2R, thereby modulating the secretion of BK in BALF.

Apart from inflammation, airway remodeling as a key characteristic of asthma could result in the obstruction and stenosis of the airway to induce the occurrence of AHR. Airway remodeling is characterized by airway

structure changes in response to chronic inflammatory stimulation, reflected by airway matrix collagen deposition, epithelial interstitial fibrosis, hypertrophy and hyperplasia of airway smooth muscle, myofibroblast proliferation, epithelial reticulointerstitial thickening and microvascular formation. Evidence supports that the NF- κ B-BRD4 signaling pathway is required for modulating allergen-induced inflammation, EMT, extracellular matrix production, and myofibroblast differentiation. In turn, ZL0454, a novel BRD4 inhibitor, disrupts BRD4 coupling with the NF- κ B-RNA polymerase II complex and inhibits its histone acetyltransferase activity, thereby alleviating the aforementioned response (Tian et al., 2019). Therefore, we further explored the role of BRD4 in PM2.5-induced airway remodeling. First, we demonstrated that the chronic inhalation of PM2.5 could induce airway remodeling, as evidenced by airway wall thickening, which was consistent with the results of previous studies (Churg et al., 2003). In addition, as predicted, BRD4 inhibition could alleviate airway wall thickening in mice exposed to PM2.5. We further explored the exact molecular mechanisms of BRD4 in PM2.5-induced airway remodeling. Matrix metalloproteinases (MMPs) are a zinc-dependent proteases, which are closely related to the cell migration ability by degrading extracellular matrix proteins (Pittayapruerk et al., 2016; Page-McCaw et al., 2007), especially MMP2, MMP9, and MMP12 (Srivastava et al., 2007). Vimentin, an important type III intermediate filament protein, participates in the formation of the cytoskeleton and regulates cell adhesion and contraction. Furthermore, vimentin plays a key role in maintaining cell integrity and regulating cell migration, apoptosis, angiogenesis, and EMT (Terriac et al., 2017). In short, MMP2, MMP9 and vimentin are closely associated with airway remodeling. In this study, we observed that BRD4 inhibition downregulated MMP2, MMP9 and vimentin protein expression in lung tissue, thus indicating that BRD4 is involved in PM2.5-induced airway remodeling by regulating the expression of these proteins. Taken together, BRD4 actively participated in PM2.5-induced AHR, while the treatment of the BRD4 inhibitor reversed the aggravation of lung function by inhibiting inflammation and alleviating airway remodeling. Notably, the concentration of PM2.5 in the atmosphere is hard to attain the level indicated in the experiment. However, it is certain that PM2.5 is harmful to human respiratory system, as evidenced by the aggravation of lung function, and an increase in lung inflammation, which is consistent with the results of our research (Xing et al., 2016). Of course, whether the molecular mechanism in the study would also occur in humans at real-world exposure levels still need to be further explored, and it provides us with a direction for further investigation.

hASMCs, as important effector cells, can perform contractile function, thereby regulating airway caliber and influencing AHR. In addition, the enhancement of hASMC migration could cause airway remodeling, resulting in obstruction and stenosis of the airway. Therefore, we selected hASMCs to observe the role of BRD4 in PM2.5 DMSO extracts -induced cell contraction and migration. Notably, the present study has its limitation. In vitro experiments we did not use genuine PM2.5 but PM2.5 DMSO extracts. By using DMSO extracts, the effects of insoluble particulate matter are absent and bioavailability of the PM components is modified. Consequently, the vitro effects of PM2.5 in DMSO should be attributed to PM2.5 DMSO extracts, rather than genuine PM2.5. Meanwhile, the PM2.5 DMSO extracts, used in the vitro experiment, was the same as in our previous study (Cao et al., 2020). Thus, the components of PM2.5 DMSO extracts, and the content of each component, were the same as in our previous study. The elemental carbon (EC) and organic carbon (OC) contents of the particles were relatively high. Other elements including Na, Mg, Al, P, S, K, Ca, Cr, Fe, Zn, and Cl were also detected in the particles (Cao et al., 2020). We observed that the level of BRD4 in hASMCs was the highest after exposure to 12.5 μ g/mL PM2.5 DMSO extracts. The BRD4 protein expression levels were not positively correlated with PM2.5 DMSO extracts concentration. This result could be explained by the fact that PM2.5 DMSO extracts could affect cell viability, and too high concentrations of PM2.5 DMSO extracts might induce cell necrosis and apoptosis, thereby inhibiting BRD4 protein expression (Cao et al., 2020; Zhao et al., 2019). However, the expression of BRD4 in lung tissue increased after exposure to PM2.5, and the inhalation

of high concentrations of PM2.5 did not inhibit BRD4 expression in the lung tissue of mice. The different results between the cell level and whole level could be interpreted by the fact that the effective concentrations used in vitro experiments were higher than those locally reached in vivo experiments. Besides the dose amount, the difference could be also due to the different chemical nature of the stimulus, because the DMSO extract used in vitro experiments may have increased the bioavailability of PM2.5 organic components to a dramatic extent. BRD4 gene knockdown and ZL0420 inhibited hASMC contraction after exposure to 12.5 μ g/mL PM2.5 DMSO extracts, as demonstrated by the inhibition of the reduction of gel diameter, thus further demonstrating the key role of BRD4 in PM2.5 DMSO extracts -induced hASMC contraction. Meanwhile, we found that BRD4 gene silencing and BRD4 inhibition could interfere with KLK14 and B2R protein expression, thus further demonstrating that BRD4 was located upstream of KLK14 and B2R, which was consistent with the results of animal experiments. The association between BRD4 and KLK14 was proposed for the first time and was bi-directionally verified at the cellular and whole levels. In addition to the contraction of hASMC, the migration of hASMC plays a pivotal role in airway remodeling. To date, most studies exploring the role of BRD4 in cell migration have focused on tumor cells rather than ASMCs (Xu et al., 2020; Hu et al., 2015). Interestingly, evidence supports that BRD4 knockdown inhibits vascular smooth muscle cell proliferation and migration (Wang et al., 2015). Therefore, we uncovered the role of BRD4 in regulating the migration of ASMCs induced by PM2.5 DMSO extracts. In this study, the correlation between PM2.5 DMSO extracts and the migration of hASMCs was determined using the transwell chamber method. We observed that PM2.5 DMSO extracts promoted the migration of hASMCs, which was consistent with a previous study (Ye et al., 2018). However, the concentration of PM2.5 DMSO extracts significantly promoting hASMC migration was 6.25 μ g/mL, which was different from that in the aforementioned study (Ye et al., 2018). This result could be attributed to the heterogeneity of PM2.5 in different countries and regions. In addition, the enhancement of hASMC migration was dependent on an increase in migration-related protein expression levels (i.e., MMP-2, MMP-9 and vimentin). We found that the aforementioned migration-related protein levels in hASMCs were the highest in response to exposure to 6.25 μ g/mL PM2.5 DMSO extracts, which was consistent with the result of transwell experiment. It is generally considered that MMPs including MMP2 and MMP9 are essential for cell motility and extracellular matrix (ECM) degradation, as represented by the promotion of cell migration due to the overexpression of MMPs (Deryugina and Quigley, 2006). Therefore, MMP-2, MMP-9 and vimentin protein levels represent the migration function of cells to a certain degree. Notably, BRD4 knockdown and ZL0420 inhibited the migration of hASMC, as evidenced by a decrease in cell migration and the reduction of MMP-2, MMP-9 and vimentin protein levels. These data indicated that BRD4 had the ability to regulate MMP-2, MMP-9 and vimentin protein expression, thereby influencing hASMC migration, which was consistent with the results of previous animal experiments.

This project showed the key role of BRD4 in PM2.5-induced AHR at animal levels, and elucidated the molecular mechanism of BRD4 in PM2.5 DMSO extracts -induced contraction and migration of hASMCs at cellular levels. These research results are of great significance for further elucidating the exact mechanisms by which PM2.5 induces AHR and provide a novel target for the treatment of PM2.5-induced AHR. In addition, the enhancement of AHR induced by PM2.5 is associated with the interaction of various cells to a certain degree. Therefore, whether BRD4 regulates the expression of other molecules associated with AHR in other cell types remains to be clarified.

5. Conclusion

The results of this study identified BRD4 as a pivotal player in PM2.5-induced AHR. The contraction and migration of hASMC was dependent on BRD4 expression to a certain degree. In brief, BRD4 may be a novel therapeutic target for AHR induced by PM2.5.

CRediT authorship contribution statement

Xi Lu: Methodology, Software, Data curation, Writing - original draft. **Huiran Zhang:** Methodology, Data curation. **Min Wang:** Methodology. **Jingwen Li:** Methodology. **Fangfang Qu:** Methodology. **Rongqin Li:** Project administration. **Xixin Yan:** Methodology, Supervision.

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Declaration of Competing Interest

The authors do not have competing interests.

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