



RESEARCH ARTICLE



# Nrf2 regulates downstream genes by targeting miR-29b in severe asthma and the role of grape seed proanthocyanidin extract in a murine model of steroid-insensitive asthma

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## ABSTRACT

**Context:** Grape seed proanthocyanidin extract (GSPE) is effective in treating severe asthma (SA).

**Objective:** To examine the relationship between Nrf2-miR-29b axis and SA, and to detect whether preventive use of GSPE relieves SA via it.

**Materials and methods:** We recruited 10 healthy controls, 10 patients with non-severe asthma (nSA), and 9 patients with SA from February 2017 to December 2017. Peripheral blood mononuclear cells from these volunteers were extracted. A murine model of steroid-insensitive asthma was established in six-week-old female BALB/c mice that were sensitised and challenged with OVA, Al(OH)<sub>3</sub> and LPS for 31 days. Mice in the treated groups were injected with DXM (5 mg/kg/d), with or without GSPE (100 mg/kg/d). Control group received PBS. We performed quantitative real-time PCR, western blot and luciferase reporter assay in animal and cell models.

**Results:** SA group demonstrated significantly lower concentrations of Nrf2 protein, Nrf2 mRNA, and miR-29b than nSA group and control group. Conversely, higher levels of platelet derived growth factor C (PDGFC), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), and collagen type III alpha 1 (COL3A1) were measured in SA than in the other two groups. PDGFC, PIK3R1, and COL3A1 were the target genes of miR-29b. GSPE + DXM significantly elevated the expression of Nrf2 (+188%), Nrf2 mRNA (+506%), and miR-29b (+201%), and significantly reduced the expression of PDGFC (−72%), PIK3R1 (−40%), and COL3A1 (−65%) compared with OVA + LPS.

**Conclusions:** Nrf2-miR-29b axis is involved in the pathogenesis of SA. GSPE, as an adjuvant drug, maybe a potential therapeutic agent for SA.

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Steroid insensitivity; nuclear factor erythroid 2-related factor 2; airway inflammation



## Introduction

Severe asthma (SA) is defined as asthma that requires high dose inhaled corticosteroids plus a second controller or treatment with systemic corticosteroids to control, or which remains uncontrolled despite this therapy (Chung et al. 2014). The molecular mechanisms that contribute to SA are complicated and have not been fully clarified. The underlying mechanisms include, but are not limited to, airway inflammation, airway remodelling, and steroid resistance, which interact each other.

Grape seed proanthocyanidin extract (GSPE), a class of flavonoid compounds with a unique molecular structure, has beneficial features such as anti-inflammation, antioxidation, anti-neoplastic, and antibiosis (de la Iglesia et al. 2010). Our previous study indicated that GSPE could modulate the inflammatory response, reduce airway remodelling, and restore sensitivity to glucocorticoid treatment by regulating Nrf2-iNOS-HDAC2 axis, thus having the pharmacological effect of treating severe asthma (Cao et al. 2016). However, the specific molecular mechanism and whether there are other signalling pathways are still unclear.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor generally expressed in various organisms and

cellular, and is a key regulator of antioxidation. Oxidative stress triggers the dissociation of Nrf2 from Kelch-like ECH-associated protein 1 and translocation into the nucleus to form a heterodimer together with small Maf protein (Kim and Vaziri 2010). This heterodimer then initiates transcription of genes encoding many antioxidant enzymes and phase II detoxifying enzymes by binding to the antioxidant reaction element (Lu et al. 2016). Currently, more and more attention has been paid to the role of Nrf2 in the occurrence and development of asthma. A recent study showed that activation of Nrf2 pathway effectively attenuated ovalbumin (OVA)-induced allergic airway inflammation (Ye et al. 2017). Conversely, inhibition of Nrf2 with small interfering RNA (siRNA) increased airway inflammation, Th2 cytokines and adhesion molecules in BALF and lung tissue of asthmatic mice (Ci et al. 2015). Nrf2 knockout mice were more sensitive to cigarette smoke and showed more significant airway inflammation and airway responsiveness dominated by neutrophils (Ying et al. 2014). Likewise, another study described abnormal Nrf2 function as closely related to the severity of childhood asthma *in vitro* (Fitzpatrick et al. 2011). Our previous study also confirmed that the activity of Nrf2 in steroid-insensitive asthmatic mice was lower than that in steroid-sensitive asthmatic mice (Cao et al.

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2016). Taken together, these studies suggested that Nrf2 might play an important role in the development of asthma, especially SA.

The direct binding of Nrf2 to the Mir29ab1 gene could activate the expression of miR-29b (Kurinna et al. 2014). MiR-29b has been shown to prevent the pathogenesis of many diseases such as cancer metastasis, pulmonary fibrosis, diabetic kidney disease, vascular disease, and airway inflammation (Cushing et al. 2015; Guo et al. 2016; Langsch et al. 2016; Dua et al. 2017; Li et al. 2017). It is worth mentioning that miR-29b has an effect on epithelial-mesenchymal transformation, secretion of inflammatory cytokines, collagen deposition, cytoskeletal changes, DNA methylation, and other processes in various airway inflammatory diseases (Salama et al. 2014). Recently, the miR-29b expression was found to be significantly decreased not only in the asthmatic mice challenged and sensitised with OVA (Garbacki et al. 2011), but also in the peripheral blood of asthmatic patients (Panganiban et al. 2016). Therefore, we speculate that there are strong associations between miR-29b and asthma. However, little is known about the underlying mechanisms. Then we predicted the downstream genes of miR-29b by TargetScan and found that its possible target genes were involved in multiple signalling pathways which played critical roles in asthma.

The purposes of this study were as follows: (1) Determining that the expression level of miR-29b and Nrf2 were related to the severity of asthma. (2) Identifying target genes of miR-29b which were expressed differentially in patients with SA from aspects of inflammatory reaction, extracellular matrix, and cellular skeleton. (3) Investigating the regulation role of Nrf2-miR-29b axis on the downstream genes using both animal and cell models. (4) Evaluating the effect of prophylactic use of GSPE on restoring steroid sensitivity and relieving asthma via Nrf2-miR-29b axis. These findings may have implications in elucidating the pathogenesis, searching for new biomarkers and therapeutic targets of SA.

## Materials and methods

### Patients and samples

We recruited 10 healthy controls and 19 well characterised adult patients with asthma from the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University. Nine Patients were diagnosed as severe asthma (SA) based on the European Respiratory Society (ERS)/American Thoracic Society (ATS) guidelines (Chung et al. 2014). The specific inclusion criteria of SA were as follows: (1) forced expiratory volume in 1 second ( $FEV_1$ )  $\geq 40\%$  predicted, and post-bronchodilator  $FEV_1 \geq 55\%$  predicted; (2) less than 6 times of acute attacks in the last

6 months and no acute attack of asthma during the month of preceding enrolment; (3) no hospitalisation for asthma exacerbation in the last 6 months; (4) no asthma-related endotracheal intubation within the last 1 year; (5) oral doses of prednisolone  $\leq 20$  mg/day; (6) non-smokers. Ten Patients not meeting the above criteria were considered to be non-severe asthma (nSA). The detailed clinical data of asthmatics were presented in Table 1. There was no difference in age, gender and body mass index (BMI) between control group and asthmatic groups. Participants with cardiac insufficiency, abnormal liver function, pulmonary embolism, coinfection, tuberculosis and blood system diseases were not included. At the period of drawing blood, all enrolled patients were in a stable clinical condition without an acute attack of asthma. The blood samples were stored in the sterile PAXgene blood RNA tube for further detection or long-term preservation. Human peripheral blood mononuclear cells (PBMCs) were isolated from the blood of participants as previously described (Corkum et al. 2015). This study was approved by the ethics institute of Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University ([2017] KY028-01) and the signed informed consent was obtained from all participants.

### Chemicals

GSPE we used was purchased from Dalian Jianfeng Pharmaceutical Co., Ltd., China, and its specification showed that its purity was more than 99.9%. Gas chromatography-mass spectrometry and high-performance liquid chromatography analysis indicated that it contained 56% dimeric proanthocyanidins, 12% trimeric proanthocyanidins, 6.6% tetrameric proanthocyanidins, and small amounts of monomeric and high-molecular-weight oligomeric proanthocyanidins and flavonoids (Zhou et al. 2011).

### RNA isolation, reverse transcription PCR and quantitative real-time PCR analysis

Total RNA from mice frozen tissues and human PBMCs was extracted using a TRIzol reagent (Invitrogen, Carlsbad, USA). MiR-29b was reverse transcribed into cDNA with the Transcriptor first-strand cDNA synthesis kit (Vazyme, Nanjing, China). The general RNA extraction was reverse transcribed into cDNA using the HiScript II Q RT SuperMix kit (Vazyme, Nanjing, China). The expression of RNA was measured by quantitative real-time PCR (qRT-PCR, ABI PRISM7500, USA) with AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China) under recommended reaction conditions: 95 °C for 5 min, the 40 cycles of 95 °C for 10 sec, and 60 °C for 30 sec. Each reaction

**Table 1.** Subjects characteristics.

Variabiles	Healthy control (n = 10)	Non-severe asthma (n = 10)	Severe asthma (n = 9)	p value
Age	53.00 ± 2.46	43.70 ± 4.47	53.00 ± 2.93	0.103
Female/male	5/5	6/4	3/6	0.505
Smoker/Non-smoker	3/7	3/7	0/9	0.818
BMI	24.27 ± 1.27	25.96 ± 2.24	24.79 ± 0.62	0.737
EOS ( $\times 10^9/L$ )	0.18 ± 0.04	0.33 ± 0.10	0.23 ± 0.11	0.464
FeNO	15.20 ± 1.48	58.60 ± 11.70**	30.89 ± 5.34 <sup>†</sup>	0.001
FEV <sub>1</sub> (% of predicted)	103.20 ± 2.62	83.32 ± 5.54*	58.46 ± 6.15*** <sup>††</sup>	<0.001
FEV <sub>1</sub> /FVC%	104.50 ± 2.24	72.82 ± 4.28***	46.67 ± 3.93*** <sup>†††</sup>	<0.001

Data presented as mean ± SEM.

BMI: body mass index; EOS: eosinophils; FeNO: fractional exhaled nitric oxide; FEV<sub>1</sub>(% of predicted): percent of predicted forced expiratory volume in 1 second; FEV<sub>1</sub>/FVC%: FEV<sub>1</sub> as a percentage of forced vital capacity.

\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to health control.

<sup>†</sup> $p < 0.05$ , <sup>††</sup> $p < 0.01$ , <sup>†††</sup> $p < 0.001$  compared to non-severe asthma group.

**Table 2.** Primer sequences used for gene amplification in human PBMCs.

Primer name	Forward sequence	Reverse sequence
β-Actin	CGTGGACATCCGCAAGA	GAAGGTGGACAGCGAGGC
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGCATC
Nrf2	CCAGCCCAGCACATCCAG	AACTCATTGTCTATACAACCG
miR-29b	CAGACCTGTAGCACCATTGAA	TATCCTTGTTCAGACTCCTCAC
IFNG	ATCGTTTTGGTCTCTTGG	ATTATTTTTCTGTCACTCTCTCTT
PDGFC	TCATTCTCCCTCGCCTT	ACCTGAACCAGTTTCCCAA
TRAF4	GCCCATTCAAAGACTCCG	TTCCAGATGAGCACGCCA
PIK3R1	AGTGAGGCTGTGATTGGGCGT	GGATCTGTTTTGGCTGAAAGGTGT
LAMA2	AAAGCGGCAACGACAAA	TCCTCAGACATAGGTGGCAAT
COL1A1	GCGTGTACCCCACTCAGC	CCGAACCAGACATGCCTC
COL3A1	TGGAATCTGTGAATCATGCCCTACT	CTGCGAGTCTCTACTGTACT
PPIC	TGACCGTCCAATCAACAAC	AATCAGCGTCTCAACCACAA
MMP16	AGTAACATACCCGTAAACCTT	TATCAGATCGCAAGACCCA
ELN	CTTCGGATTGTCTCCATT	TCCACCAAGCAGTAGCACC

**Table 3.** Primer sequences used for gene amplification in the murine model.

Primer name	Forward sequence	Reverse sequence
β-Actin	ACGGCCAGGTCATCACTATTG	CAAGAAGGAAGGCTGGAAAAGA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGCATC
Nrf2	GAAAAAGAAGTGGGCAACTGTGG	GGTGGGATTTGAGTCTAAGGAGGT
miR-29b	CAGACCTGTAGCACCATTGAA	TATCCTTGTTCAGACTCCTCAC
PDGFC	AAGGAACAGAACGGAGTGCAA	ACTGCAACTAATCTCCACCCA
PIK3R1	CCCACTACTGTAGCCAAACAAC	CGTACCAAAAAGGTCCCATCA
COL3A1	CTGTAACATGGAACTGGGAAA	CCATAGCTGAACTGAAAACCAC

**Table 4.** The predicted target genes of miR-29b.

Function	Target genes	Gene encoding production
Inflammation	IFNG	Interferon gamma
	PDGFC	Platelet derived growth factor C
	TRAF4	TNF receptor-associated factor 4
	PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1
Extracellular matrix	LAMA2	Laminin, alpha 2
	COL1A1	Collagen, type I, alpha 1
	COL3A1	Collagen, type III, alpha 1
Cytoskeleton	PPIC	Cyclophilin C
	MMP16	Matrix metalloproteinase 16
	ELN	Elastin

was run in duplicate, and the average cycle threshold (Ct) value was calculated for analysis. The primer sequences were purchased from Suzhou GenePharma Co., Ltd. (Suzhou, China) and presented in Tables 2 and 3.

### Prediction of possible target genes of miRNA-29b

We predicted the putative target genes of miR-29b by the online bioinformatics' tool TargetScan ([http://www.targetscan.org/vert\\_71/](http://www.targetscan.org/vert_71/)). Ten potential target genes were selected and listed in Table 4, i.e., IFNG, PDGFC, TRAF4, PIK3R1, LAMA2, COL1A1, COL3A1, PPIC, MMP16 and ELN, which were associated with inflammation (IFNG, PDGFC, TRAF4 and PIK3R1), extracellular matrix (LAMA2, COL1A1 and COL3A1) and cytoskeleton (PPIC, MMP16 and ELN).

### Protein extraction and western blot

We obtained total protein from PBMCs and mice lung tissue using RIPA (Beyotime Institute of Biotechnology, Shanghai, China) together with protease inhibitors and phosphatase inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). Accordingly, the nuclear protein was extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo

Scientific, Rockford, USA). Protein concentrations were detected by the BCA protein assay (Thermo Scientific, Rockford, IL, USA). We loaded 20 µg samples per lane on 10% SDS-PAGE gel for separation and then transferred them to polyvinylidene difluoride membranes (Millipore, Billerica, USA). The membranes were blocked with 5% non-fat milk in TBS Tween 20 at room temperature for 2 h, and then probed with anti-Nrf2 antibody (Abcam, Cambridge, UK), anti-histone H3 antibody (Beyotime Institute of Biotechnology, Shanghai, China), anti-PDGFC antibody (Abclonal Inc., Wuhan, China), anti-PIK3R1 antibody (Abclonal Inc., Wuhan, China), anti-COL3A1 antibody (Abclonal Inc., Wuhan, China) and anti-GAPDH antibody (Abcam, Cambridge, UK) overnight at 4 °C, respectively. At last, the membranes were washed three times with TBST followed by incubation for 2 h at room temperature with goat anti-rabbit horseradish peroxidase-conjugated IgG (Cell Signalling Technology Inc., MA, USA). The intensity of each protein band was visualised by FlourChemQ system (Proteinsimple, CA, USA).

### Luciferase reporter assay

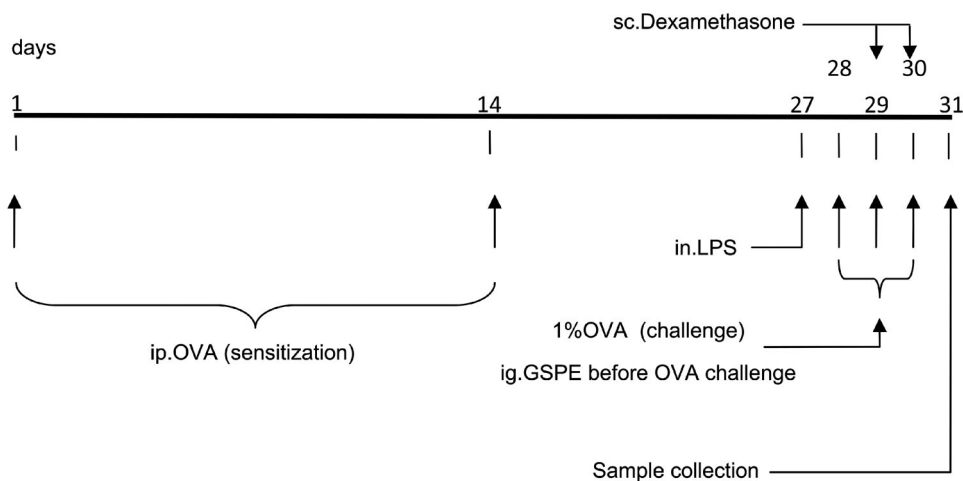
Luciferase Assay System (GenePharma Co., Ltd., Shanghai, China) was used to confirm the target relationship between miR-29b and the potential downstream target genes. Briefly, wild type 3'-UTR sequence of target genes containing miR-29b binding site and mutant 3'-UTR sequence of those target genes were inserted into Pmir-GLO luciferase reporter vectors (GenePharma Co., Ltd., Shanghai, China). These Pmir-GLO vectors were co-transfected with miR-29b mimic/inhibitor or mimic/inhibitor control (GenePharma Co., Ltd., Shanghai, China) into human bronchial epithelial cells (16HBE cells). The cells were harvested and relative luciferase activity (activity firefly/activity renilla) was detected.

### Cell culture and transfection

16HBE cells were purchased from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China) and cultured in RPMI1640 medium (Gibco-BRL, Gaithersburg, USA) at 37 °C, 5% CO<sub>2</sub> in a humidified incubator. Cells between passages 4 and 10 were used for all experiments. MiR-29b mimic, miR-29b inhibitor and their negative controls were transfected into 16HBE cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA). We then used qRT-PCR and Western blot to detect the effects of miR-29b on its downstream target genes.

### Animals

Six-week-old female BALB/c mice (each weighing 18–22 g) were purchased from Lingchang Biotechnology Co., Ltd. (Shanghai, China). A murine model of steroid-insensitive asthma was established in accordance with the method described by Komlósi et al. (2006). Briefly, 20 mice were randomly categorised into control, OVA + LPS, dexamethasone (DXM) and GSPE + DXM groups (n = 5 per group). On days 1 and 14, the mice in OVA + LPS, DXM and GSPE + DXM groups were sensitised by intraperitoneal injection of 20 µg OVA (Grade V; Sigma, USA) emulsified in 2.25 mg aluminium hydroxide (Thermo Fisher Scientific, Waltham, MA, USA) in a total volume of 0.1 mL phosphate-buffered saline (PBS). On days 28, 29 and 30, the mice were placed in a plastic chamber and started to receive



**Figure 1.** Establishment of a mouse model of steroid-insensitive asthma. We randomly assigned all BALB/c mice to four groups: control group ( $n=5$ ), OVA + LPS group ( $n=5$ ), DXM group ( $n=5$ ) and GSPE + DXM group ( $n=5$ ). The mice were sensitised with 20  $\mu\text{g}$  OVA and 2.25 mg aluminium hydroxide gel in a total volume of 100  $\mu\text{L}$  by intraperitoneal injection on days 0 and 14. Mice were intranasally sensitised with 10  $\mu\text{g}$  (60  $\mu\text{L}$ ) LPS on day 27 and then challenged with OVA from day 28 to day 30. The DXM and GSPE + DXM groups mice were subcutaneously injected with DXM (5 mg/kg/day) on days 29 and 30. The mice in GSPE + DXM group were fed daily with GSPE (100 mg/kg/day) by oral gavage 1 h before the OVA challenge per day from days 28 to 30. Control mice were sensitised and challenged using the same protocol but using only PBS. All mice were sacrificed on day 31. ip.: intraperitoneal; in.: instil; ig.: intragastric; sc.: subcutaneous; OVA: ovalbumin, LPS: Lipopolysaccharide, DXM: dexamethasone; GSPE: grape seed proanthocyanidin extract; PBS: phosphate-buffered saline.

aerosol inhalation of 1% aerosolized OVA at a flow rate of 3 mL/min for 20 min by an ultrasonic nebuliser (Jiangsu Yuyue Medical Equipment & Supply Co. Ltd., China). On day 27, the day before the first OVA challenge, the mice were instilled intranasally with 10  $\mu\text{g}$  (60  $\mu\text{L}$ ) lipopolysaccharide (LPS) (Beyotime Institute of Biotechnology, Shanghai, China). On days 29 and 30, the mice in DXM and GSPE + DXM groups were injected subcutaneously with DXM (Fengyuan Inc., Ma'anshan, China), 5 mg/kg/day with a total volume of 0.1 mL. Other groups received 0.1 mL normal saline as placebo. The mice in GSPE + DXM group were given GSPE via intragastric administration (100 mg/kg/day) 1 h before the OVA challenge per day from days 28 to 30 (Figure 1). Other groups received PBS as placebo. The mice in control group received the same schedule for sensitisation and challenge with an equivalent amount of PBS instead of OVA and LPS. All animal protocols were reviewed and approved by the Animal Care and Use Committee of Nanjing Medical University (IACUC-1709011).

### Analysis of bronchoalveolar lavage fluid (BALF) and plasma

We collected blood samples from the mice by the retro-orbital puncture method. The samples were then centrifuged (2,000 g, 10 min, 4 °C), and the plasma was stored at -80 °C until analysis. Besides, we collected BALF by lavaging the lungs 3 times with 0.5 mL of ice-cold 0.9% sodium chloride. The BALF was centrifuged (1,000 g, 15 min, 4 °C), and the supernatant was stored at -80 °C for further detection. The resulting pellets were resuspended in PBS for total inflammatory cell counts with a haemocytometer and a microscope. The levels of interleukin (IL)-4, IL-13 and interferon- $\gamma$  (IFN- $\gamma$ ) in BALF and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) in plasma were detected by enzyme-linked immunosorbent assay (MultiSciences, Hangzhou, China) according to the manufacturer's instructions.

### Lung histology

The left lobe of the lung was obtained from the sacrificed mice and fixed in 10% neutral-buffered formalin. The lung was

thoroughly dehydrated prior to embedding in paraffin. Paraffin sections (5  $\mu\text{m}$ ) were then stained with either hematoxylin-eosin (HE) or periodic acid-Schiff (PAS). The severity of inflammatory cell infiltration in the peribronchial areas of mice from different groups was performed in a blinded manner based on a 5-point scoring system: 0, none (no inflammation); 1, minimal (occasion ruffing with inflammatory cells); 2, mild (1–3 cells); 3, moderate (4–5 cells); and 4, severe (more than 5 cells) (Lin et al. 2014). To quantify airway goblet cells, we used a 5-point grading system in a blinded setting: 0, no goblet cells; 1, <25% of the epithelium; 2, 25%–50% of the epithelium; 3, 50%–75% of the epithelium; 4, >75% of the epithelium (Bao et al. 2009). Scoring of goblet cells was performed in at least three different fields for each lung section.

### Statistical analysis

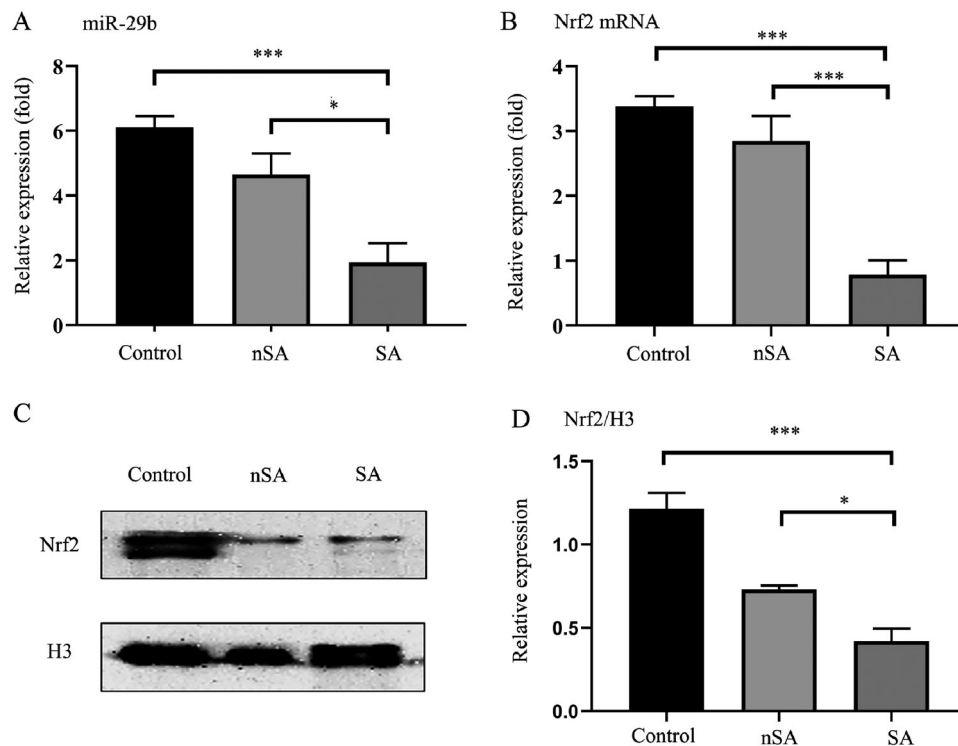
Differences between two groups in the cell experiment were analysed by paired *t*-test. Differences among more than two groups were accessed by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison tests. Data are expressed as mean  $\pm$  SEM. All statistical analyses were conducted using GraphPad Prism 8 software (La Jolla, CA, USA). A *p* value of <0.05 was considered statistically significant.

## Results

### Characteristics of asthmatic patients

As shown in Table 1, there were no statistically significant differences in age, gender, smoking status, BMI, and peripheral eosinophils among the three groups. Patients with nSA had fractional exhaled nitric oxide (FeNO) values significantly higher than the other two groups ( $p < 0.05$ ). Both FEV1/FVC% and FEV1 (% of predicted) were significantly lower in the two asthmatic groups compared to the control group ( $p < 0.05$ ), but there were significantly greater reductions in SA compared to nSA group ( $p < 0.01$ ).





**Figure 2.** MiR-29b and Nrf2 expression is associated with severe asthma in human PBMCs. (A,B), The relative RNA expression levels of miR-29b (A) and Nrf2 (B) was examined using RT-PCR in the control group ( $n = 10$ ), nSA group ( $n = 10$ ) and SA group ( $n = 9$ ); (C), protein expression of Nrf2 was evaluated using Western blot in the nucleus of the control group, nSA group and SA group. The intensity of the band was determined by quantitative analysis. H3 was used as an internal control. Data were presented as mean  $\pm$  SEM. PBMCs: Human peripheral blood mononuclear cells; nSA: non-severe asthma; SA: severe asthma; Nrf2: nuclear factor erythroid 2-related factor 2; H3: histones 3. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

### MiR-29b and Nrf2 expressions in human PBMCs

To confirm the relationship between miR-29b and SA, qRT-PCR was used to detect the expression of miR-29b in human PBMCs. As expected, SA group showed a significant decreased expression of miR-29b in PBMCs, when compared with a control group and nSA group (Figure 2A,  $p < 0.001$  and  $p < 0.05$ , respectively). Similarly, as shown in Figure 2B, we also observed a substantial reduction in the expression of Nrf2 mRNA in SA group compared with the other two groups ( $p < 0.001$ ), while no such effect was observed between nSA group and control group. Western blot analysis confirmed the marked reduction of Nrf2 expression in SA group compared to nSA group and control group (Figure 2C,  $p < 0.05$  and  $p < 0.001$ , respectively). Therefore, Nrf2 expression was positively correlated with miR-29b in the occurrence of severe asthma. Since Nrf2 was found to activate miR-29b expression by using sequencing (ChIP-Seq) (Kurinna et al. 2014), our finding implied that the Nrf2-miR-29b axis appeared to play a role in the pathogenesis of SA.

### Target genes of miR-29b were screened in human PBMCs

Analysis of the target genes of miR-29b would aid our understanding of the regulatory role of miR-29b in SA. We selected 10 putative target genes of miRNA-29b by TargetScan. To verify which genes were regulated by miR-29b, qRT-PCR and Western blot were performed in PBMCs. We observed COL3A1 mRNA expression was notably upregulated in SA group compared with a control group and nSA group (Figure 3G,  $p < 0.001$ ,  $p < 0.01$ , respectively). Accordingly, COL3A1 protein expression was markedly higher in SA group than in the control and nSA group (Figure 3K,  $p < 0.05$ ). Both transcription and translation levels of

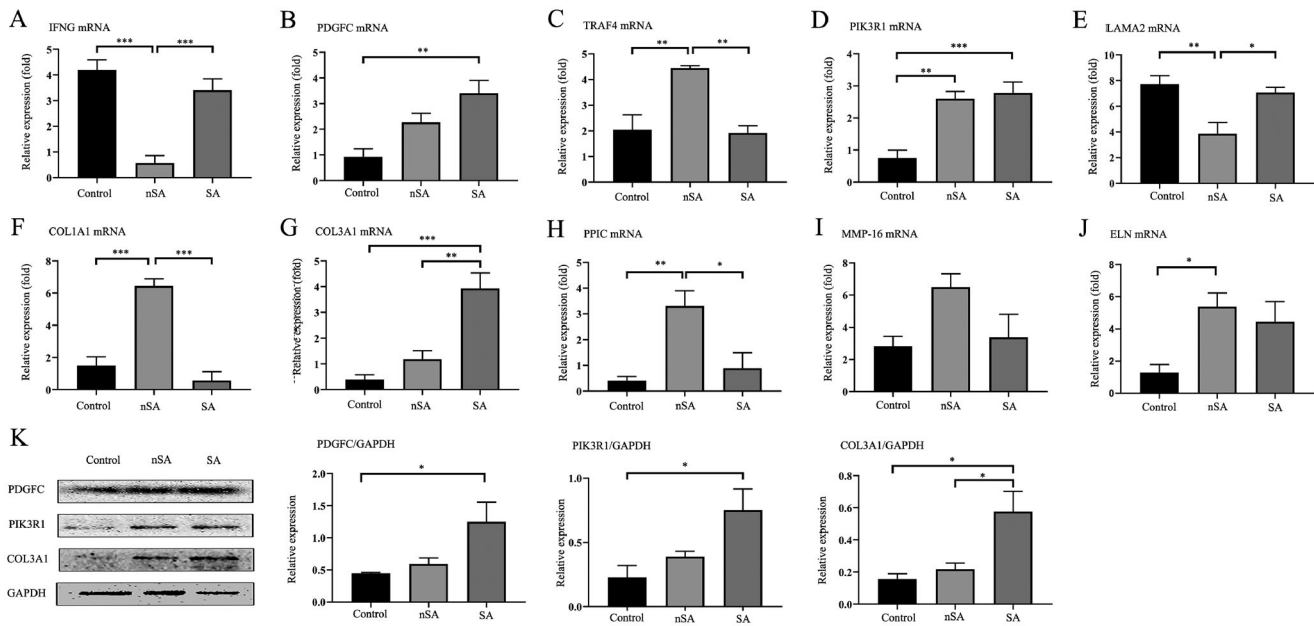
the other two predicted target genes, i.e., PDGFC and PIK3R1 were also significantly elevated in SA group when compared with a control group, but when compared to nSA group, there was no statistically significance (Figure 3B,D,K). Although the transcription levels of IFNG, TRAF4, LAMA2, COL1A1 and PPIC were significantly different between nSA group and SA group (Figure 3A,C,E,F,H), their expression levels showed no linear correlation with the severity of the disease. These data suggested that PDGFC, PIK3R1 and COL3A1 might be the downstream targets of miR-29b.

### Target genes of miR-29b were confirmed using dual-luciferase reporter assay

The predictions for miR-29b targets were subjected to further verification via dual-luciferase reporter assay. As presented in Figure 4, lower luciferase activities were only observed in 16HBE cells co-transfected with miR-29b mimics and PDGFC-miR-29b WT/PIK3R1-miR-29b WT/COL3A1-miR-29b WT reporter plasmid (Figure 4A-C,  $p < 0.01$ ,  $p < 0.05$  and  $p < 0.01$ , respectively). However, miR-29b had no effect on the luciferase activity of PDGFC-Mut, PIK3R1-Mut and COL3A1-Mut (Figure 4A-C,  $p > 0.05$ ). These results indicated that PDGFC, PIK3R1 and COL3A1 were the direct targets of miR-29b.

### MiR-29b directly regulated the expressions of PDGFC, PIK3R1 and COL3A1 in 16HBE cells

To confirm whether miR-29b could regulate the expression of the target genes that we tested above, we used 16HBE cells to conduct experiments *in vitro*. As shown in Figure 5A-C,



**Figure 3.** Ten putative miR-29b target genes are screened in human PBMCs. mRNA expression of IFNG (A), PDGFC (B), TRAF4 (C), PIK3R1 (D), LAMA2 (E), COL1A1 (F), COL3A1 (G), PPIIC (H), MMP16 (I) and ELN (J) were detected by RT-PCR in control ( $n = 10$ ), nSA ( $n = 10$ ) and SA ( $n = 9$ ) groups respectively; (K), protein expression was measured by Western blot, and GAPDH was used as an internal control, and quantitative analysis of PDGFC, PIK3R1 and COL3A1 protein expression was performed. Data were presented as mean  $\pm$  SEM. PBMCs: Human peripheral blood mononuclear cells; nSA: non-severe asthma; SA: severe asthma; IFN: interferon gamma; PDGFC: platelet derived growth factor C; TRAF4: TNF receptor-associated factor 4; PIK3R1: phosphoinositide-3-kinase, regulatory subunit 1; LAMA2: Laminin, alpha 2; COL1A1: Collagen, type I, alpha 1; COL3A1: Collagen, type III, alpha 1; PPIIC: Cyclophilin C; MMP16: Matrix metalloproteinase 16; ELN: Elastin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

overexpression of miR-29b could decrease the mRNA levels and protein levels of PDGFC, PIK3R1 and COL3A1 in 16HBE cells ( $p < 0.05$ ). On the contrary, inhibiting miR-29b increased the transcription and translation levels of these three target genes ( $p < 0.05$ ).

### GSPE attenuated airway inflammation in a murine model of steroid-insensitive asthma

We assessed the level of airway inflammation in murine lung tissue by HE staining. Under a light microscope, we directly observed that the OVA + LPS group showed an obvious peribronchial and perivascular inflammatory cell infiltration, which mainly was eosinophils when compared with the control group. DXM treatment non-significantly attenuated inflammatory cell infiltration, while adding with GSPE had an inhibiting effect on the number of inflammatory cells (Figure 6A,C,  $p < 0.05$ ). Mucus hypersecretion also could be inhibited only in the GSPE + DXM group (Figure 6B,D,  $p < 0.05$ ). The number of total inflammatory cells in BALF was significantly increased in OVA + LPS group compared with the control group (Figure 6E,  $p < 0.001$ ). Both GSPE + DXM and DXM-only treatment indeed led to a markedly decreased level of total cells in OVA + LPS challenged mice (Figure 6E,  $p < 0.001$ ). However, GSPE + DXM treatment could reduce the cells number more efficiently than DXM only (Figure 6E,  $p < 0.05$ ).

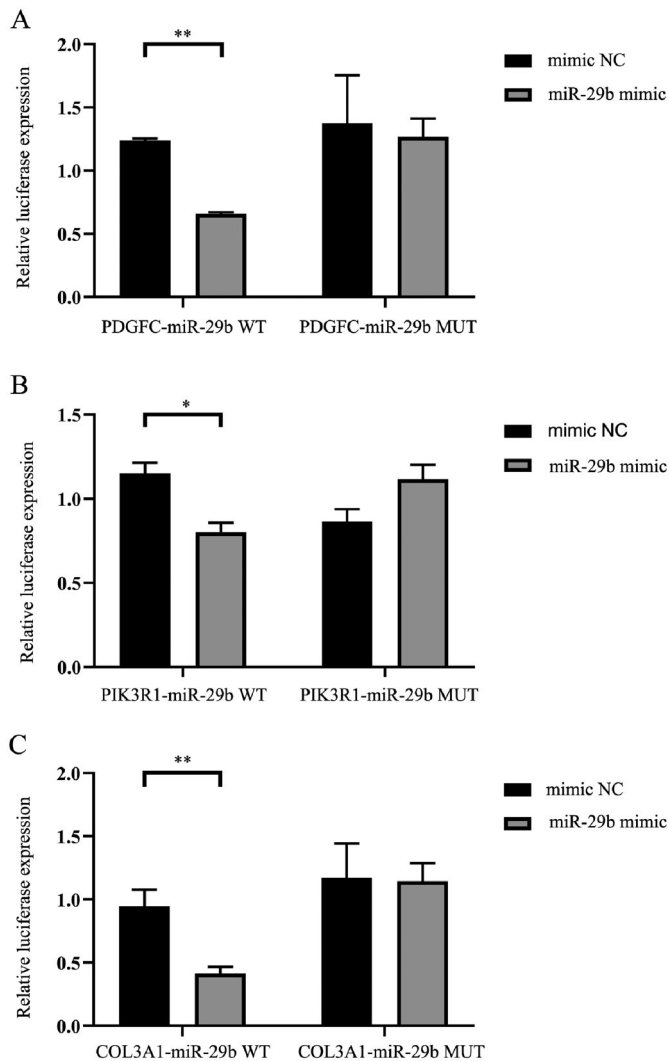
### GSPE decreased IL-4, IL-13 and TGF- $\beta$ 1 and increased IFN- $\gamma$ expressions in a murine model of steroid-insensitive asthma

To evaluate whether GSPE had an impact on Th1/Th2 balance, we detected the levels of Th1 cytokines (IFN- $\gamma$ ) and Th2 cytokines (IL-4 and IL-13) in BALF. The lower level of IFN- $\gamma$  and a higher level of IL-4/IL-13 in BALF were observed in the

OVA + LPS group compared to the control group (Figure 7A-C,  $p < 0.01$ ). GSPE + DXM treatment exhibited a significant promotion of IFN- $\gamma$  and suppression of IL-4 and IL-13 in BALF compared with the OVA + LPS group (Figure 7A-C,  $p < 0.05$ ). However, there was no significant difference between the DXM group and the OVA + LPS group. We observed that the level of TGF- $\beta$ 1 in plasma in the OVA + LPS group was significantly higher than that in the control group (Figure 7D,  $p < 0.01$ ). Likewise, treatment with GSPE + DXM rather than DXM only could markedly decrease the TGF- $\beta$ 1 level in plasma (Figure 7D,  $p < 0.05$ ).

### GSPE promoted Nrf2-miR-29b axis activity in a murine model of steroid-insensitive asthma

We next validated whether the Nrf2-miR-29b axis worked in a murine model of steroid-insensitive asthma and whether GSPE affected this axis activity specifically. We examined miR-29b, Nrf2 mRNA and Nrf2 protein expression in mice lung tissues obtained 24 h after the final challenge. As shown in Figure 8A,B, the OVA + LPS mice group manifested a remarkable reduction in expression of miR-29b and Nrf2 mRNA compared with the control group ( $p < 0.05$ ). Western blot analysis also revealed a significant decrease of Nrf2 expression in mice treated with OVA + LPS (Figure 8C,  $p < 0.05$ ). Accordingly, OVA + LPS exposure was found to increase mRNA and protein expression of PDGFC, PIK3R1 and COL3A1 in lung tissue, reaching a statistical significance in comparison with control mice (Figure 8D-G,  $p < 0.05$ ). These findings implied that Nrf2-miR-29b axis activity played an important role in a steroid-insensitive asthmatic mouse model. Furthermore, we found that DXM only treatment had no effect on the transcription levels of Nrf2, miR-29b, PDGFC, PIK3R1 and COL3A1, whereas GSPE + DXM treatment significantly increased Nrf2 mRNA and miR-29b expression and



**Figure 4.** PDGFC, PIK3R1 and COL3A1 are validated as the direct target genes of miR-29b in 16HBE cells. Luciferase reporter assays confirmed PDGFC (A), PIK3R1 (B) and COL3A1 (C) were the direct targets of miR-29b. Data were expressed as mean  $\pm$  SEM of three independent experiments. PDGFC: platelet derived growth factor C; PIK3R1: phosphoinositide-3-kinase, regulatory subunit 1; COL3A1: Collagen, type III, alpha 1; 16HBE cells: human bronchial epithelial cells; NC, negative control; WT, wild-type; MUT, mutated. \* $p < 0.05$ , \*\* $p < 0.01$ .

decreased PDGFC, PIK3R1 and COL3A1 mRNA expression (Figure 8A,B,D,E,F,  $p < 0.05$ ). In addition, our results showed the differences in protein expression of Nrf2, PDGFC, PIK3R1 and COL3A1 were significant between the mice treated with GSPE + DXM and the ones treated with OVA + LPS, which were not observed between the DXM group and OVA + LPS group (Figure 8C,G,  $p < 0.05$ ). Taken together, these results indicated that pre-treatment of GSPE might ameliorate steroid resistance via activating Nrf2-miR-29b axis.

## Discussion

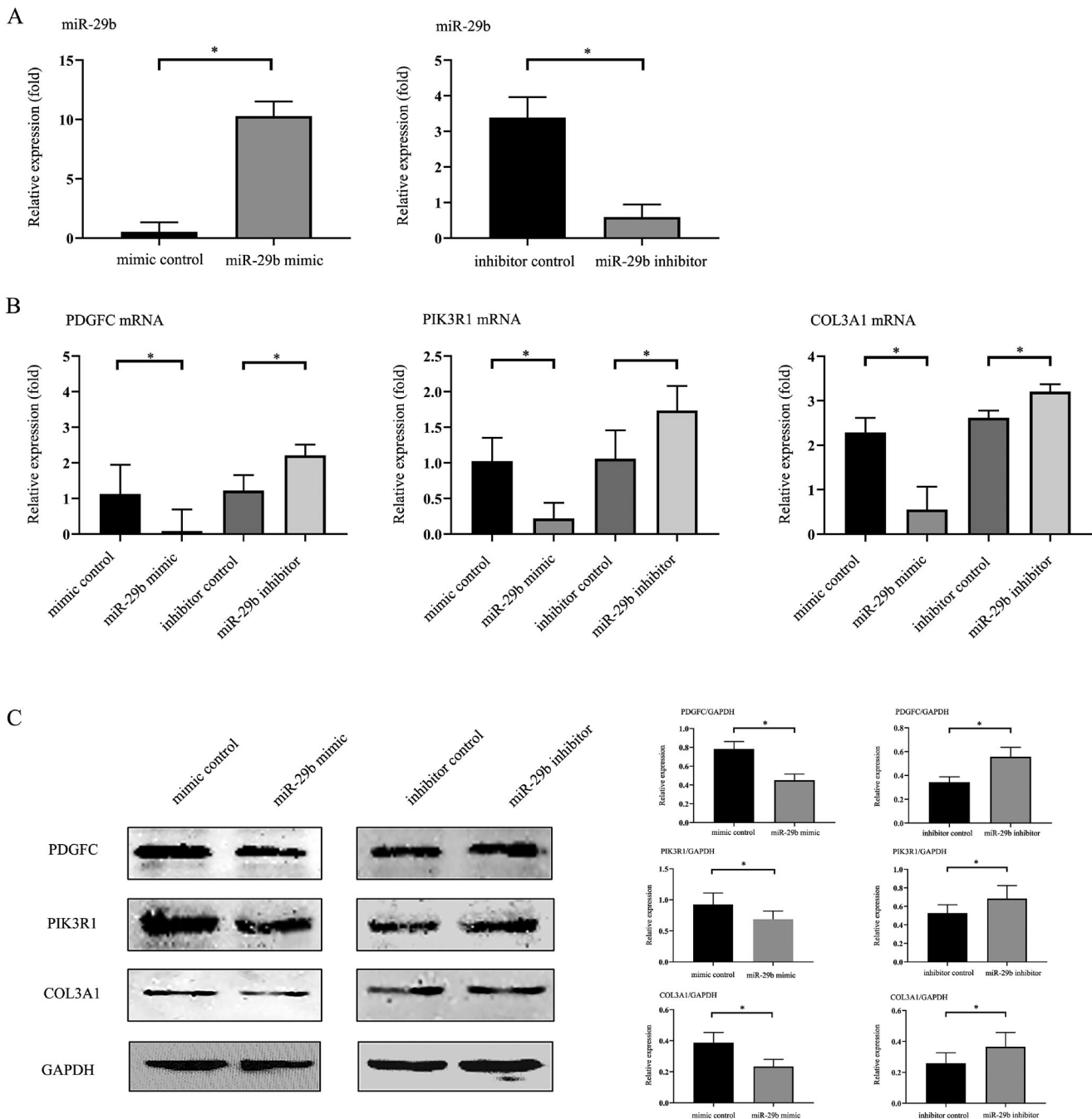
Nowadays we are increasingly aware of the importance of treating SA, but relatively little is known about the underlying mechanisms. In the present study, we showed that the decreased expression of miR-29b was related to the occurrence of SA and maybe was regulated by Nrf2. Moreover, we confirmed PDGFC, PIK3R1 and COL3A1 as miR-29b targets, and their transcription and translation were regulated by miR-29b. Additionally, our

results suggested that pre-treatment of GSPE reduce the severity of asthma via activating Nrf2-miR-29b axis in a steroid-insensitive asthma model.

The reasons for the ineffective therapeutical strategies for SA remain largely elusive. From clinical data of participants, it was becoming evident that individuals with SA have lower FEV<sub>1</sub> (% of predicted) and FEV<sub>1</sub>/FVC than those with nSA and healthy control, suggesting more obvious airway obstruction in SA patients (Svenningsen et al. 2019). Svenningsen et al. (2019) suggested that the increase of mucus and eosinophils was the main cause of airway obstruction.

A recent study showed that the expression of Nrf2 in patients with SA was significantly lower than that in patients with nSA, and those with low expression had a higher risk of relapse (Hirai et al. 2019). This was consistent with our findings. Nrf2 dysfunction may reflect the progression of airway inflammation and play a key role in the development of steroid resistance (Hirai et al. 2019). Besides, the Nrf2 pathway has an antioxidant function (Fitzpatrick et al. 2011), which is probably the main determinant of allergen-mediated asthma susceptibility (Rangasamy et al. 2005). It has been reported that Nrf2 activates the expression of miR-29b in keratinocytes (Kurinna et al. 2014). Based on a previous study, which demonstrated that miR-29b could regulate IFN- $\gamma$  production and helper T cell differentiation (Steiner et al. 2011), we speculated that the Nrf2-miR-29b axis might be implicated in the pathogenesis of SA. As expected, we showed that miR-29b consistent with Nrf2 in SA were significantly lower than that in controls and nSA. Accordingly, the Nrf2-miR-29b axis was also verified as being involved in a mouse model of steroid-insensitive asthma. These findings reinforced the potential of the Nrf2-miR-29b axis as a target in the management of SA.

We then selected 10 possible target genes of miR-29b and found that the expression levels of 3 of them (PDGFC, PIK3R1, and COL3A1) were increasing with the severity of asthma in PBMCs. It should be pointed out that there was a trend to have higher PDGFC and PIK3R1 levels in SA patients when compared to nSA patients, although the difference did not reach statistical significance. It was presumed that it might be related to the small number of participants. We further determined that PDGFC, PIK3R1 and COL3A1 were the target genes of miR-29b by luciferase reporter assay. PDGFC, a member of the PDGF family, shares common functions with other members of the PDGF family (Fang et al. 2004). Recent studies have shown that PDGFC was involved in the pathogenesis of asthma (Bosse et al. 2006; Ingram et al. 2006). PDGFC could be notably activated by inflammatory cytokines (Bruland et al. 2009). Early growth response-1 (Egr-1), an important transcription factor that regulated pulmonary inflammation and might participate in the process of airway remodelling (Ingram et al. 2006), induced the expression of PDGFC by binding to DNA (Midgley and Khachigian 2004). In addition, PDGFC could be up-regulated by TGF- $\beta$ 1 (Bosse et al. 2006) and IL-13 (Ingram et al. 2006), and acted as an autocrine growth factor to induce proliferation and migration of airway smooth muscle cells by nuclear factor- $\kappa$ B pathway (Bosse et al. 2006; Ito et al. 2009; Cheng et al. 2018; Dai et al. 2018), as well as the expression of matrix metalloproteinase (MMP) (Kurinna et al. 2014). PIK3R1 gene was responsible for encoding the regulatory p85 $\alpha$  subunit of phosphatidylinositol 3-kinase (PI3K). PI3K blockers could significantly reduce airway inflammation (Jiang et al. 2017), suppress eosinophil and lymphocyte migration to the airway (Myou et al. 2003), and inhibit epithelial-mesenchymal transition (EMT) and TGF- $\beta$ 1-



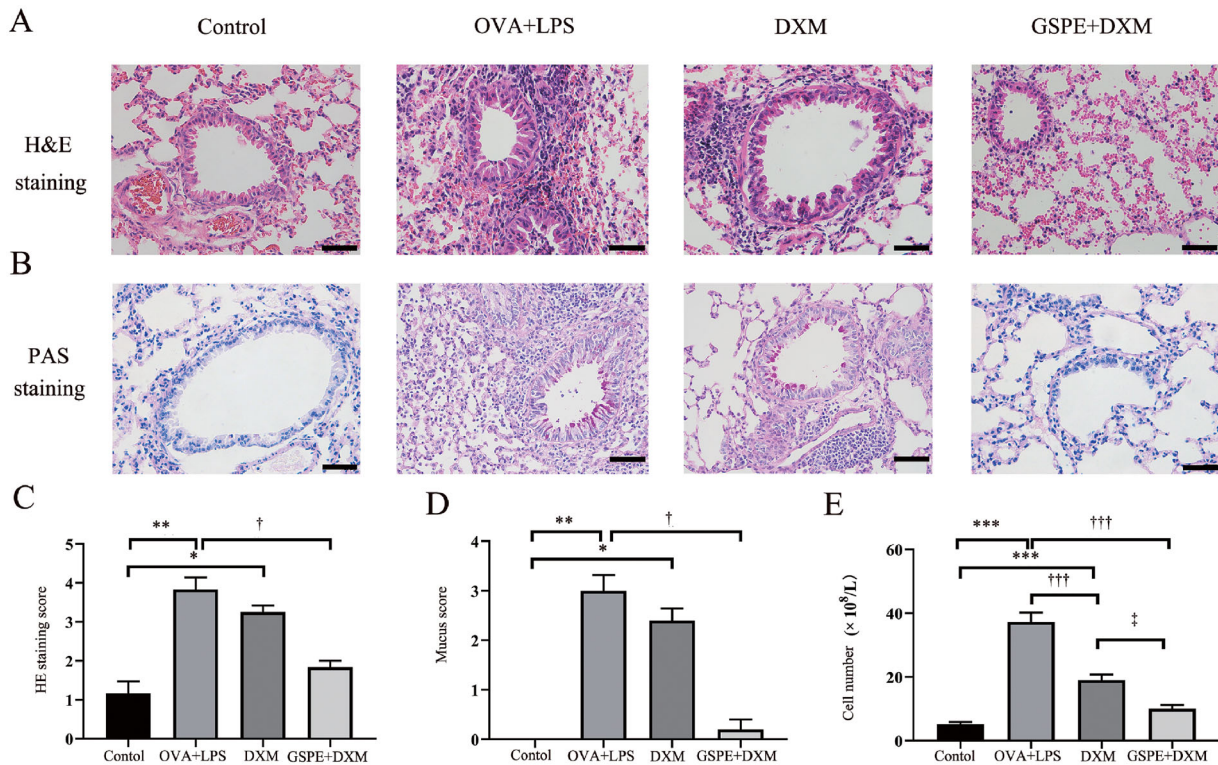
**Figure 5.** MiR-29b targets and modifies the expression of PDGFC, PIK3R1 and COL3A1 in 16HBE cells. (A), The expression of miR-29b was examined using RT-PCR; (B), the transcription levels of PDGFC, PIK3R1 and COL3A1 were detected by RT-PCR; (C), the protein expression levels of PDGFC, PIK3R1 and COL3A1 were assessed by western blot. The intensity of bands was determined by quantitative analysis. GAPDH was used as an internal control. Data were presented as mean  $\pm$  SEM of three independent experiments. PDGFC: platelet derived growth factor C; PIK3R1: phosphoinositide-3-kinase, regulatory subunit 1; COL3A1: Collagen, type III, alpha 1; 16HBE cells: human bronchial epithelial cells; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

induced migration of 16HBE cells (Yang et al. 2018) in OVA-challenged mice. PI3K/Akt pathway was proved to regulate airway high reactivity (AHR), recruit a variety of inflammatory cells and promote airway remodelling (Lee et al. 2006; Kim et al. 2010; Yang et al. 2018). COL3A1 was associated closely with asthma severity (Reeves et al. 2014), and corticosteroids were ineffective in reversing COL3A1-related airway remodelling (Chakir et al. 2003). Pascoe et al. (2017) extracted mRNA from donor lungs of patients with asthma (mainly fatal asthma) and non-asthma, respectively. By detecting the difference of gene expression between the two groups and using a targeted multiplex array, it was found that the level of COL3A1 in patients

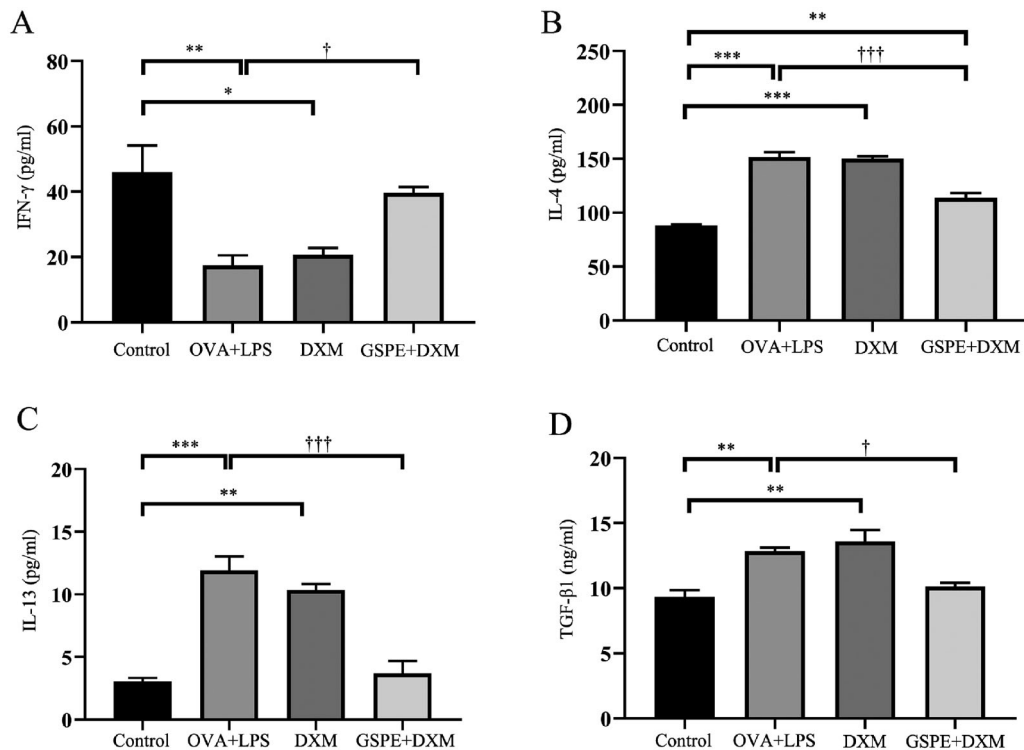
with asthma was significantly increased, and it was proved to regulate cell proliferation and inflammation (Pascoe et al. 2017). All these studies support our observations of the expression level of PDGFC, PIK3R1 and COL3A1 elevated in SA patients and in the murine model of steroid-insensitive asthma. Overall, Nrf2-miR-29b axis, targeting these downstream genes, played an important role in the mechanism of SA, due to its properties of anti-inflammatory, anti-airway remodelling and improvement of steroid resistance. We reasonably speculated Nrf2-miR-29b axis was defective in adults with SA.

Airway inflammation often determines the characteristics and severity of asthma. Type (T) 2 inflammation dominates SA,

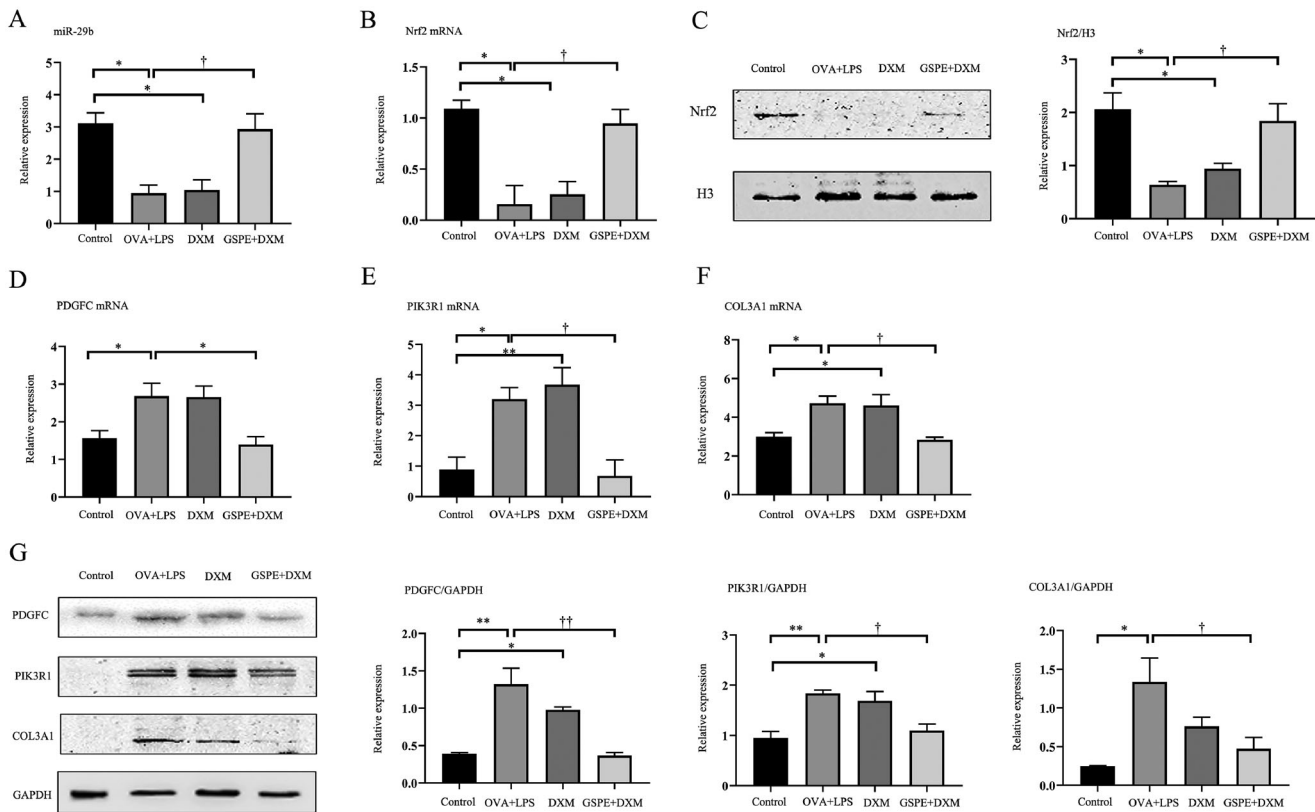




**Figure 6.** GSPE reduces airway inflammation in a murine model of steroid-insensitive asthma. (A), HE staining of lung tissue sections, scale bars = 100  $\mu$ m; (B), PAS staining of lung sections, scale bars = 100  $\mu$ m; (C), HE staining score; (D), mucus score; (E), total cell counts in BALF. Data are expressed as the mean  $\pm$  SEM ( $n = 5$  per group). HE: hematoxylin-eosin; PAS: periodic acid-Schiff; OVA: ovalbumin; LPS: Lipopolysaccharide, DXM: dexamethasone; GSPE: grape seed proanthocyanidin extract. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to the control group. † $p < 0.05$ , †† $p < 0.01$ , ††† $p < 0.001$  compared to OVA + LPS group. ‡ $p < 0.05$  compared to the DXM group.



**Figure 7.** GSPE regulated the expression of Th1/Th2 cytokines in a murine model of steroid-insensitive asthma. Mouse lung lavage fluid and plasma were obtained on day 31, and then enzyme-linked immunosorbent assay was used to detect the levels of IFN- $\gamma$  (A), IL-4 (B), IL-13 (C) in BALF and TGF- $\beta$ 1 in plasma (D). Data were presented as the mean  $\pm$  SEM ( $n = 5$  per group). IL: interleukin; OVA: ovalbumin; LPS: lipopolysaccharides; DXM: dexamethasone; GSPE: grape seed proanthocyanidin extract; IFN: interferon; TGF: transforming growth factor; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  compared to control group. † $p < 0.05$ , †† $p < 0.01$ , ††† $p < 0.001$  compared to OVA + LPS group.



**Figure 8.** GSPE ameliorates Nrf2-miR-29b axis in a murine model of steroid-insensitive asthma. Lung tissues were obtained on day 31, total RNAs, nuclear proteins and total cellular proteins from the four groups were extracted. (A,B,D,E,F), the relative mRNA expression levels of miR-29b, Nrf2, PDGFC, PIK3R1 and COL3A1 were detected by an RT-PCR method ( $n = 5$  per group); (C,G), the protein expression of Nrf2, PDGFC, PIK3R1 and COL3A1 were detected by Western blot. H3 and GAPDH were used as internal controls ( $n = 5$  per group). Data were expressed as the mean  $\pm$  SEM. OVA: ovalbumin; LPS: Lipopolysaccharide, DXM: dexamethasone; GSPE: grape seed proanthocyanidin extract; Nrf2: nuclear factor erythroid 2-related factor 2; PDGFC: platelet derived growth factor C; PIK3R1: phosphoinositide-3-kinase, regulatory subunit 1; COL3A1: Collagen, type III, alpha 1; H3: histones 3; GAPDH: glyceraldehyde-3-phosphate dehydrogenase. \* $p < 0.05$ , \*\* $p < 0.01$  compared to the control group. † $p < 0.05$ , †† $p < 0.01$  compared to OVA + LPS group.

including multiple pathways: (a) IgE, (b) eosinophils, and (c) the IL-4/IL-13 pathway (Busse 2019). We observed that only treatment with GSPE + DXM resulted in a sharp decrease of IL-4 and IL-13 in BALF, and eosinophils in lung sections when compared with the OVA + LPS group in mice, indicating that the preventive administration of GSPE could alleviate T2 inflammation *in vivo*. IL-4 caused the conversion of the antibody isotype to IgE, which in turn caused allergen sensitisation (Busse 2019). Meanwhile, IL-13 promoted the process of airway remodelling by inducing smooth muscle contraction (Cao et al. 2016). The findings in the present study were in accordance with our previous study (Zhou et al. 2015) and further supported the idea that GSPE might act as T2 inflammation inhibitor in SA to restore Th1/Th2 balance.

The related study reported that under a Th2-dominant micro-environment, *Chlamydomydia pneumoniae* infection was more likely to induce airway remodelling (Park et al. 2010), which might be related to the production of TGF- $\beta$  and vascular endothelial growth factor (VEGF) enhanced by Th2 cytokines (Wen et al. 2003). We also observed that TGF- $\beta$ 1 in plasma and goblet cell hyperplasia in lung sections were significantly increased after OVA + LPS exposure, which concurred with our previous results (Zhou et al. 2015). As we know, TGF- $\beta$  and VEGF played important roles in the development of airway remodelling (Wen et al. 2003). Expression level of TGF- $\beta$ 1 was positively correlated with the severity of asthma (Al-Alawi et al. 2014). TGF- $\beta$ 1 mediated asthma pathogenesis by elevating migratory capacity of fibroblasts (Itoigawa et al. 2015), increasing deposition of

extracellular matrix (Itoigawa et al. 2015) and participating in the synthesis and secretion of MMP-9 (Ohbayashi and Shimokata 2005). IFN- $\gamma$  could selectively inhibit the production of VEGF, as well as reduce the production induced by Th2 cytokines and TGF- $\beta$  (Wen et al. 2003). IFN- $\gamma$  was also involved in the pathogenesis of SA with fixed airway obstruction, although the exact mechanism was unknown. In our study, only GSPE + DXM treatment could remarkably downregulate TGF- $\beta$ 1 and goblet cell hyperplasia and upregulate IFN- $\gamma$  expression, therefore, it was reasonable to believe that preventive use of GSPE may reduce later airway remodelling effectively.

Steroid resistance is another important contributory factor in the development of SA. Our *in vivo* experiment further showed that LPS could induce steroid-insensitive asthma, and GSPE + DXM treatment led to an increased expression of Nrf2 and recovery of sensitivity to DXM. Previous studies showed oxidative stress in LPS-treated animals played a key role in the pathophysiology of SA exacerbation (Xiaojuan et al. 2012). Further study supported that GSPE protected human lens epithelial B3 cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative stress by reducing the production of reactive oxygen species (Jia et al. 2011). Adenuga et al. (2010) found that Nrf2 deficiency resulted in steroid resistance potentially in the lungs of mice following LPS exposure. Moreover, Nrf2 nuclear translocation could protect against oxidative stress triggered by *tert*-butyl hydroperoxide (Bautista-Expósito et al. 2019). In this study, we showed that GSPE + DXM treatment rather than DXM only could increase the expression of Nrf2 and miR-29b and inhibited the expression

of downstream targets of miR-29b in mice with steroid-insensitive asthma. The results indicated that preventive use of GSPE could upregulate steroid sensitivity and alleviate asthma severity via Nrf2-miR-29b axis.

However, our study had limitations. First, the sample size of SA was relatively small, which may be prone to false negatives due to low statistical power or fortuitous false-positive results. Second, data on lung function and airway collagen level in mice were not available. Third, the ineffectiveness of dexamethasone cannot be absolutely concluded because of the short treatment duration in the murine model of steroid-insensitive asthma. We intend to explore the longer-term effects of dexamethasone in follow-up work.

## Conclusions

We conclude that Nrf2 may regulate downstream genes by targeting miR-29b and play a critical role in the pathogenesis of SA. In addition, our results suggest that prophylactic administration of GSPE alleviates airway inflammation and restores sensitivity to corticosteroids by activating the Nrf2-miR-29b axis in a mouse model of steroid-insensitive asthma. Therefore, our study provides considerable insight into a novel target for the treatment of SA and a basis for GSPE as an adjuvant drug for SA in the future.

## Disclosure statement

The authors have no other relevant affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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