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REGULAR ARTICLE

Interference of miR-943-3p with secreted frizzled-related proteins4 (SFRP4) in an asthma mouse model

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Abstract

The aim of this study is to investigate the potential roles of miR-943-3p and its target gene secreted frizzled-related proteins4 (SFRP4) in allergic asthma and elucidate its underlying mechanism, which may prompt a new clue about developing novel treatments of this disease. An allergic asthma mouse model was generated by challenging with ovalbumin (OVA); lung pathological features of mice were viewed using H&E staining; thickness of subepithelial fibrosis and smooth muscle was measured using Masson's trichrome staining. Inflammatory cells from bronchoalveolar lavage fluid (BALF) were counted based on Diff-Quik staining and morphometric analysis. Expressions of miR-943-3p, SFRP4 and Wnt signal pathway-associated proteins were detected using RT-PCR or immunoblotting, respectively. SFRP4 was downregulated in the bronchial biopsies of allergic asthma patients and represented a unique intersection between differentially expressed genes (DEGs) and genes in the Wnt signal pathway. Both miR-943-3p upregulation and SFRP4 downregulation were detected in allergic asthma patients and OVA-induced mice. Besides, OVA-induced mice possessed more inflammatory cells in BALF including macrophage (mac), eosinophil (eos), lymphocyte (lym) and neutrophil (neu), higher expression of collagen, β-catenin and c-Myc as well as thicker subepithelial fibrosis and smooth muscle in lung than control mice. In vivo delivery of miR-943-3p agomir worsened these symptoms, while both miR-943-3p antagomir and Ad-SFRP4 administration effectively alleviated this disease. Taken together, miR-943-3p accelerated the progression of airway inflammation and remodeling in allergic asthma via suppressing the activity of SFRP4 through Wnt signaling pathway in asthma patients and OVA-induced mice.

Keywords Allergic asthma · SFRP4 · miR-943-3p · Wnt signaling pathway · Secreted protein

Introduction

Asthma is a chronic airway disease characterized by respiratory-related symptoms such as expiratory airflow limitations; the total number of asthma-affecting patients is anticipated to reach upwards of 400 million by 2025 (Ober and Yao 2011; Reddel et al. 2015). Although a high prevalence of asthma has been reported from all age groups, older adult patients significantly exhibited more severe symptoms (Zein et al. 2015). Until now, several contributors including genetics and environment have been involved in the progression of asthma but the details of pathogenesis of asthma are largely unknown (Ober and Yao 2011). Allergic inflammation and airway remodeling are two essential characteristics of asthma (Schatz and Rosenwasser 2014). Infiltration of inflammatory cells (mainly including eosinophil and neutrophils) into the bronchial airway is important for the initiation of asthma (Chauhan and Ducharme 2012), therefore allergic asthma is probably the commonest phenotype of asthma in the general population of patients (Schatz and Rosenwasser 2014). Airway remodeling was evident during the histological evaluation of chronic asthma, as demonstrated by the structural changes in the airway, represented by smooth muscle hyperplasia, mucous metaplasia and subepithelial fibrosis (Hirota and Martin 2013; Munakata 2006). Accumulating evidences suggest that airway remodeling does not only aggravate the progression of asthma but is also associated with the

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asthma-related progressive loss of lung function (Fehrenbach et al. 2017).

Current studies in mice have shown that overexpression of the Wnt/β-catenin signaling pathway can accelerate and worsen the progression of airway remodeling in chronic asthma (Kwak et al. 2015). The Wnt signaling pathway can be activated through the canonical pathway, planar cell polarity (PCP) pathway and Wnt/Ca^{2+} pathway. As the important extracellular signaling receptors of Wnt ligands, the secreted frizzled-related protein family (SFRPs, consisting of five members: SFRP1–SFRP5) acts as the indispensible gatekeeper and limiter in the canonical and non-canonical Wnt signaling pathway (Bafico et al. 1999); secreted frizzled-related proteins4 (SFRP4) has exhibited efficient inhibition on tumor proliferation (Carmon and Loose 2008) and ischemic injury (Matsushima et al. 2010). Interestingly, downregulation of SFRP4 was published from microarray of allergic asthma established by Chamberland et al. (2009), which hints that the downregulation of SFRP4 is possibly involved in the development of asthma. Nevertheless, reports about the roles of SFRP4 in asthma are still absent.

Another imperative question is what causes the downregulation of SFRP4 in asthma. In the past few years, a microRNA-mRNA interaction network has been observed and reported as an important pathway for the regulation of gene expression. MicroRNAs (miRNA, 18~22 nt), a class of the endogenous single-stranded noncoding small RNA, could directly bind to the 3′-untranslated region (3′-UTR) of targeted mRNAs through complementary pairing and subsequently induce mRNA degradation and inhibit translation (Bijkerk et al. 2016). Until now, more and more miRNAs were demonstrated to be involved in airway remodeling in asthma. For instance, miRNA-34/449 attenuates airway remodeling by suppressing Nur77-mediated autophagy (Yin et al. 2017); Collison et al. (2011) found a significantly changed profile of miRNA expression in the airway wall of ovalbumin (OVA)-induced mice; treatment with the antagomir of miR-126 could effectively reduce the recruitment of eosinophils. However, whether the downregulation of SFRP4 in asthma was mediated by miRNAs is still unknown.

Here, based on previous reports and an in-depth analysis of a published gene expression microarray of allergic asthma, the SFRP4 and its upstream regulator miR-943-3p were considered as promising targets for asthma treatment. Therefore, an asthma mouse model was established through OVA induction and then the role of the miR-943-3p/SFRP4 axis in airway inflammation and remodeling of allergic asthma was validated. In conclusion, our present study demonstrates that the miR-943-3p/SFRP4 axis is involved in the progression of airway inflammation and remodeling in allergic asthma, through inactivation of the Wnt signaling pathway.

Materials and methods

Microarray data

Microarray of gene expression in allergic asthma patients was retrieved from the Gene Expression Omnibus (GEO) datasets (https://www.ncbi.nlm.nih.gov/geo/); GSE41649 was selected for further analysis, which was constructed from bronchial biopsies of patients with allergic asthma ($n = 4$) and healthy counterparts ($n = 4$). Expression profile of GSE41649 was analyzed on the GPL96 Affymetrix Human Genome U133A Array platform, differential expression was defined as P value < 0.05 and the fold-change value > 2. Furthermore, the enrichments of differentially expressed genes (DEGs)-associated KEGG pathways were analyzed using DAVID web server (https://david.ncifcrf.gov/), the activation of the Wnt signal pathway was further validated using GSEA version 3.0 Intersections of DEGs and the modules of the Wnt signal pathway were screened using online BioVenn analysis (www.biovenn.nl/).

Identification of the upstream regulator of SFRP4

The upstream miRNA regulator of SFRP4 was screened using TargetScan web server (http://www.targetscan.org/vert_72/); miR-943-3p exhibited the highest potential. Then, the 3′-UTR wild-type sequence (containing the binding site of miR-943-3p) and mutated sequence (specifically mutated at the binding sites of miR-943-3p) of SFRP4 were subcloned into pGL3 firefly luciferase reporter plasmid (Promega, Madison, WI, USA), respectively. MiR-943-3p agomir and scrambled oligonucleotide control (agomir-NC) were commercially obtained from GenePharma (Shanghai, China). For the luciferase assay, HEK293T cells were pre-seeded in 24-well plates for 12 h and when the confluence of cells reached 50%, the constructed reporter plasmids, miR-943-3p agomir or agomir-NC were co-transfected into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, Firefly and *Renilla* luciferase activities were measured using a PHERAstar FSX plate reader (BMG-Labtech, Ortenberg, Germany) and relative activity of Firefly luciferase was normalized to *Renilla.*

Clinical samples collection

Sample collection was approved by the Shuguang Hospital Affiliated to Shanghai Traditional Chinese Medical University; all patients provided written informed consent and the characteristics of subjects are shown in Table 1. For the induction of sputum, we used the scheme described by Yoshikawa et al. (Sol et al. 2016; Yoshikawa et al. 1998). All patients are required to wash their mouths thoroughly with

Table 1 Characteristics of subjects

Type	Age	Sex	FVC* 1(%)	FEV1* 1(%)	PC_{20} mg/ml †	Allergy‡	ICS-treatment(μg/day)
Healthy controls	(1) 21	F	3.33 (97)	2.73 (91)	70.9	None	None
	(2) 20	M	6.02 (107)	5.10 (105)	>128	None	None
	(3) 20	M	5.52 (98)	4.43 (101)	53.3	None	None
	(4) 29	F	2.84 (80)	2.59 (86)	61.1	None	None
Asthmatic subjects	(1) 21	F	3.60 (91)	3.25 (96)	6.12	C, D, DPt, Du, G, Rw, T	500
	(2) 20	F	3.50 (99)	2.80 (90)	2.53	C, D, DPt, G, Du, Rw, T	300
	(3) 23	F	3.84 (100)	3.07 (96)	1.01	C, D, G, DPt, T	500
	(4) 39	F	3.96 (112)	3.21 (110)	2.23	C, Du, G, Rw, T	500

* Forced vital capacity (FVC) and pre-bronchodilator forced expiratory volume in one second (FEV1) expressed in liter and (%) of predicted. † Concentration of methacholine inducing a 20% fall in FEV1. ‡ Skin response read at 10 min if mean wheal diameter ≥ 3 mm; C: cat, D: dog, DPt: D. Pteronyssinus, Du: dust, G: grass pollens, M: mold, Rw: ragweed, T: tree pollens. A wheal response to histamine ≥ 3 mm served as a positive control. ICS: Inhaled corticosteroid

water. They then inhaled a 3% saline solution nebulized in an ultrasonic atomizer (Ne-U12; Omron Co., Tokyo, Japan); maximum output at room temperature. The patients were then encouraged to cough every 3 min. Sputum samples were kept at 4 °C for no more than two hours before further processing. The 10 mmol/L phosphate buffer solution diluted part of the sputum of each group and swirled gently at room temperature for 20 min. After 400 *g* centrifugation for 10 min, the cell ball was suspended again. Two mL of sputum was extracted for RNA extraction and detection of SFRP4 and miR-943-3p mRNA expression.

Mouse model of allergic asthma

Establishment of the allergic asthma model in mice and the subsequent experiments were approved by the Shuguang Hospital Affiliated to Shanghai Traditional Chinese Medical University. Male BALB/c mice (6–8 weeks, specific-pathogen-free) were commercially obtained from the medical animal center of Sichuan University (Sichuan, China) and housed in a humidity- and temperature-controlled room (22 ± 1 °C) under a 12-h light. The allergic asthma mouse model was established according to the published protocol of Long et al. (2009) with some modification. In brief, the mice were sensitized on days 1 and 7 by an intraperitoneal injection of OVA reagent (75 μg per mouse, dissolved in 100 μL phosphate-buffered saline and potentiated by 7.5 mg aluminum hydroxide) (Sigma-Aldrich, St. Louis, MO, USA). From day 14 to day 27, the mice were challenged with intranasal administration of 20 μL OVA (50 μg) dissolved in 0.9% NaCl solution. The control mice received the same volume of sterile saline. Seven days before OVA induction, commercial Ad-SFRP4 (1×10^9 pfu, Cyagen Biosciences, Suzhou, China) was intratracheally instilled into the mice; the control mice were treated with sterile saline. Briefly, mice were anesthetized with an intraperitoneal injection of 0.1 mL pentobartibal sodium and placed in a supine position head up on a board tilted at a 50° angle, orally intubated into the trachea with a sterile plastic catheter connected to a 1-mL syringe and instilled with Ad-SFRP4 or sterile saline. Additionally, miR-943-3p agomir and miR-943-3p antagomir were dissolved in endotoxin-free water, followed by transfection using an Entranster™-in vivo transfection reagent (Engreen Biosystem, Beijing, China). The efficiency of in vivo transfection was detected using real-time PCR. Each experimental group included six or more mice.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from the sputum of patients or lung tissue of mice and carried out using TRIzol® reagent (Invitrogen), according to the manufacturer's instructions. After quantification using spectrophotometry, 2 μg of RNA was reversely transcribed into cDNA using ABScript II cDNA first-strand synthesis kit (ABclonal, Cambridge, Boston, USA). Subsequently, real-time PCR was performed using SYBR real-time PCR Kit (GenePharma). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression of miR-943-3p and mRNA SFRP4 with the endogenous control of U6 (for miRNA) and GAPDH (for mRNA). The specific primers used are presented in Table 2.

Analysis of inflammatory cells from bronchoalveolar lavage fluid

BALF was performed according to the methods of Ye et al. (2017); 1 mL of cold sterile phosphate-buffered saline per mice was used. Then, the collected BALF was centrifuged at 3000 rpm for 15 min and the cell pellet was re-suspended in 100 μL of phosphate-buffered saline at 4 °C. The total number of cells was determined with a hemocytometer. The cell smears were prepared by cytocentrifugation (Cytospin 3;

Table 2 Primers for qRT-PCR

Gene	Sequence(5′-3′)
miR-943-3p	
Forward primer	CTCCTGACTGTTGCCGTC
Reverse primer	GAATACCTCGGACCCTGC
SFRP4	
Forward primer	CGGGATAAATAGGGTCCCGC
Reverse primer	GCAGGAGAGTTTCTTCCCCC
GAPDH	
Forward primer	AGCCACATCGCTCAGACAC
Reverse primer	GCCCAATACGACCAAATCC
U6	
Forward primer	ATTGGAACGATACAGAGAAGATT
Reverse primer	GGAACGCTTCACGAATTTG

Thermo Scientific, Pittsburgh, PA, USA) and visualized using a Diff-Quik staining kit (Sysmex Co., Kobe, Japan). At least 200 cells per slide were counted under a light microscope, inflammatory cells including macrophage (mac), eosinophil (eos), neutrophil (neu) and lymphocyte (lym) were identified and counted according to the morphological criteria and staining features.

Histological examination and collagen measurement

Mice were anesthetized with an intraperitoneal injection of 0.1 mL pentobartibal sodium and the left lung of mice was excised followed by the conventional procedures of fixation (4% paraformaldehyde for 48 h), dehydration (gradient ethanol), transparent (exylene) and embedding (paraffin). Sagittal sections were cut to a thickness of 3 μm, deparaffinized, rehydrated and subjected to hematoxylin and eosin staining for general morphology (Koh et al. 2010), Masson's trichrome staining for subepithelial fibrosis (Andersson et al. 2017) as well as immunohistochemical staining for α-smooth muscle actin and collagen (Kwak et al. 2015). Morphological features were observed under a light microscope (BX61, Olympus, Tokyo, Japan). Lung collagen content was quantified using the commercial sircol soluble collagen assay (Biocolor; Carrickfergus Co., Antrim, UK); lung samples were collected as previously described (Kwak et al. 2015).

Immunoblotting

The collected lung samples were also subjected to western blot analysis for c-Myc and β-catenin quantification. RIPA lysate was used to obtain total protein; proteins were segregated using SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes. Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk was used for membrane incubation for 1 h. Then, the membranes experienced incubation with the following primary antibodies including Dvl-1 (1:2000, Abcam, Cambridge, MA, USA), non-phospho (active) β-catenin (1:1000, #8814S, Cell Signaling Technology, MA, USA), total β-catenin (1:500, #9562S, Cell Signaling Technology), TCF7 (1: 2000, #2203S, Cell Signaling Technology) and c-Myc (1:2000, #9402S, Cell Signaling Technology) at 4 °C overnight. The membranes were washed in TBST three times and incubated with anti-rabbit IgG H&L (HRP) secondary antibody (ab6721, 1:2000) at room temperature for 1.5 h. After washing using TBST thrice, the membranes were subjected to color reaction by ECL Plus from Life Technology and GAPDH was detected as control groups.

Statistical analysis

All results are expressed as mean ± standard deviation; differences between groups were analyzed using the independent sample *t* test (for two-group comparison) and one-way ANOVA (for the comparison more than two groups). The level of statistically significant was set at a two-tailed *P* value less than 0.05.

Result

Significantly changed genes and KEGG pathways in allergic asthma

GSE41649 microarray of bronchial biopsies from four allergic asthma patients and four healthy counterparts was achieved form GEO datasets. In this published microarray, multiple genes presented a significant expression differentiation in patients compared with healthy counterparts. Twenty representative genes (10 of the upregulated genes such as MUC5AS, SLC6A14 and KCNJ16; 10 of the downregulated genes such as ERAP4, CFD and SFRP4) with the most significant differences are shown in Fig. 1(a). Then, the abundance of the KEGG pathway was counted using DAVID web server and six pathways including the Wnt signaling pathway, dilated cardiomyopathy and focal adhesion were activated in allergic asthma patients. Among these pathways, the Wnt signaling pathway exhibited the highest gene ratio and the biggest changes ($P < 0.001$, Fig. 2a, b). Furthermore, GSEA analysis also showed that allergic asthma patients tend to express more in the Wnt signaling pathway compared with healthy counterparts (Fig. 2c). A Venn plot showed that SFRP4 is the unique intersection

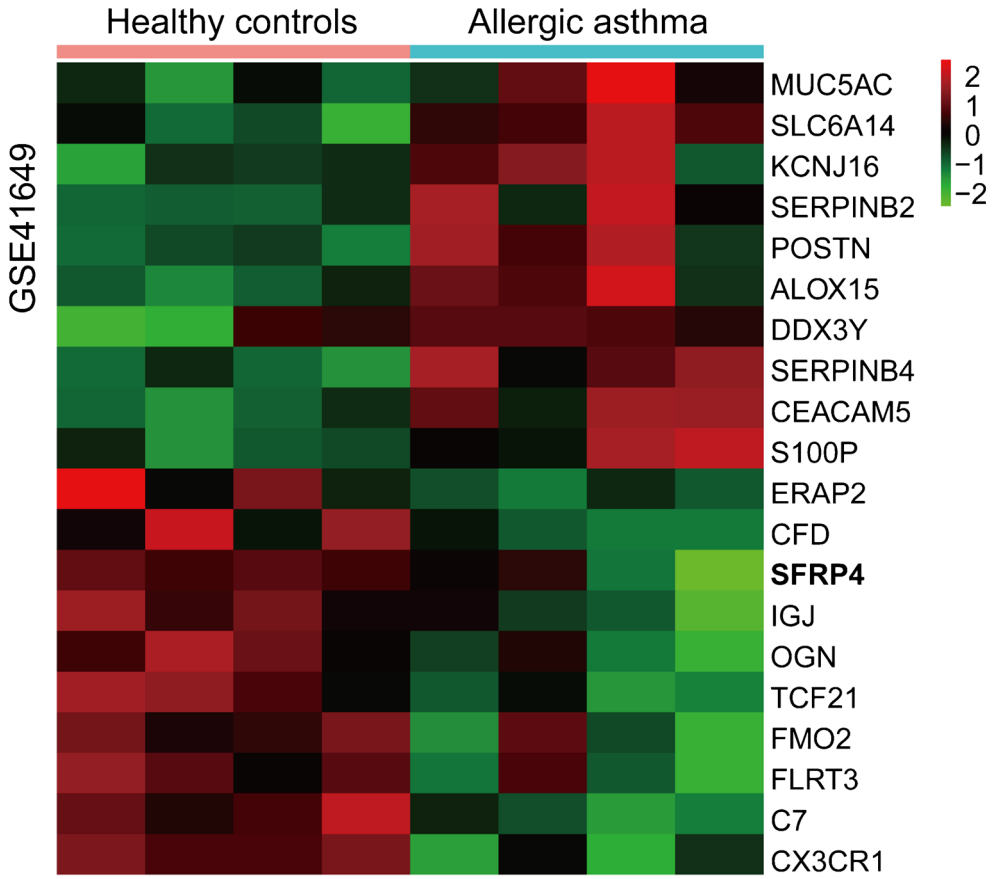

Fig. 1 Information of GSE41649 microarray. **a** GSE41649 microarray was constructed from the bronchial biopsies of 4 allergic asthma patients and 4 healthy counterparts. In this published microarray, multiple genes presented a significant expressive differentiation in patients compared with the healthy counterparts. Twenty represented genes with the most significant differences are presented, 10 of the upregulated genes such as MUC5AS, SLC6A14 and KCNJ16; 10 of the downregulated genes such as ERAP4, CGD and SFRP4

of DEGs ($n = 155$) and genes in the Wnt signaling pathway ($n = 7$) (Fig. 2d).

3′-UTR of SFRP4 is the direct target of miR-943-3p

The upstream regulator of SFRP4 was predicted using the bioinformatics tool TargetScan, miR-943-3p showed the biggest potential due to the highest complementarity with the 3′-UTR of SFRP4, a binding site containing 7 consecutive nucleotides existing between SFRP4 and miR-943-3p (Fig. 3a). The predicted relationship between SFRP4 and miR-943-3p was further validated using a dual-luciferase reporter assay, as shown in Fig. 3(b); miR-943-3p agomir could effectively attenuate the expression of reporter plasmid carrying wild-type 3′-UTR of SFRP4 but failed to trigger any influence to reporter plasmid carrying mutated 3′-UTR of SFRP4 (Fig. 3b). In addition, expression of miR-943-3p and SFRP4 mRNA in sputum from allergic asthma patients or OVA-induced mice were measured using real-time RT-PCR (Fig. 3c–f). Both SFRP4 downregulation and miR-943-3p upregulation were observed not only in the induced sputum of allergic asthma patients compared with healthy participants ($P < 0.01$, Fig. 3c, e) but also in OVA-induced mice compared with sham-operated mice ($P < 0.01$, Fig. 3d, f).

The effect of miR-943-3p/SFRP4 dysregulation on airway inflammation

The in vivo transfection efficiency of miR-943-3p antagomir, miR-943-3p agomir and Ad-SFRP4 was determined by real-time RT-PCR. As shown in Fig. 4(a, b), in vivo transfection of miR-943-3p agomir showed robust miR-943-3p overexpression and caused an evident decrease of SFRP4 expression compared with the agomir-NC group ($P < 0.01$), while miR-943-3p antagomir transfection resulted in a significant reduction of miR-943-3p and an increase of SFRP4 ($P < 0.01$). In vivo delivery of Ad-SFRP4 significantly increased the expression of SFRP4 while there is no significant difference between miR-943-3p agomir + Ad-SFRP4 group and Ad-NC group. All these results showed that the expression of SFRP4 was negatively regulated by miR-943-3p.

Airway inflammation of asthmatic mice was evaluated based on the abundance of inflammatory cells in BALF. Cells in BALF were visualized using Diff-Quik staining kit. As shown in Fig. 5(a–j), the total number of cells was significantly increased in the OVA-induced mice compared with the normal controls ($P < 0.01$); this increase was further expanded in miR-943-3p agomir-treated mice ($P < 0.05$). However, both miR-943-3p antagomir and Ad-SFRP4 transfection caused a potent decrease in the total number of cells ($P < 0.01$).

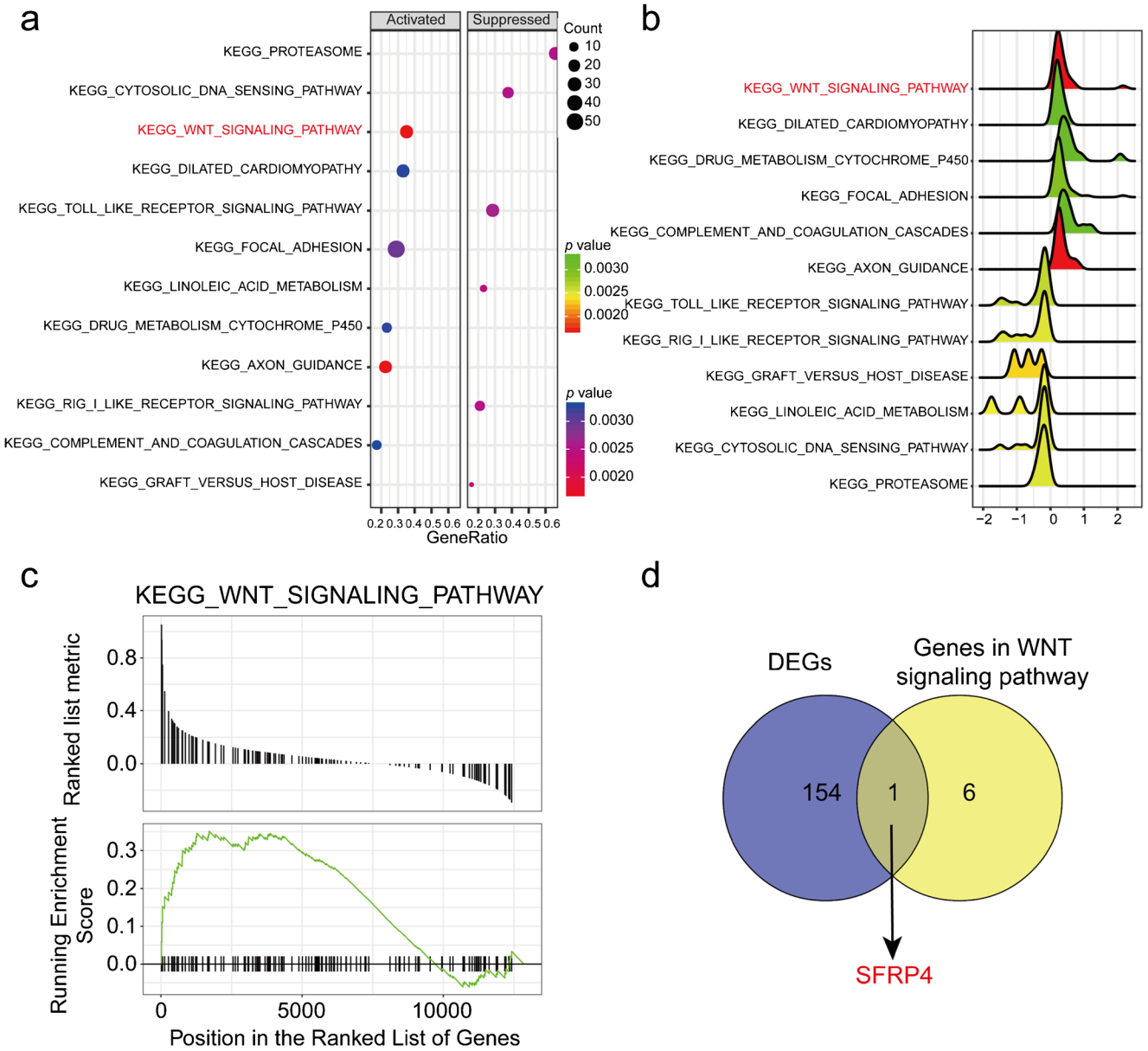

Fig. 2 Enrichment analysis of the KEGG pathway. **a** The gene ratio plot of the KEGG pathway activated or suppressed in allergic asthma. Circle size is scaled to the gene count. **b** Ridgeplot of the changed KEGG pathways gene sets. **c** Gsea plot showing the disruption of Wnt signal pathway-associated transcript genes. The ranked list metric and running enrichment score of most genes in the Wnt pathway were more than 0, indicating that most involved genes were upregulated in allergic asthma samples. **d** Venn plot showing that SFRP4 was the unique intersection of DEGs and genes in Wnt signaling pathway

According to the different staining manifestation and morphological features, the number of inflammatory cells including mac (Fig. 5k), eos (Fig. 5l), neu (Fig. 5m) and lym (Fig. 5n) were counted, respectively. Interestingly, they exhibited a similar variation pattern; total cell numbers increased in allergic asthma mice compared with normal controls and were further enriched with miR-943-3p agomir treatment but decreased with miR-943-3p antagomir and Ad-SFRP4 treatment. These data confirmed that miR-943-3p suppresses the SFRP4-mediated improvements of airway inflammation.

The effect of miR-943-3p/SFRP4 dysregulation on airway remodeling

In order to explore the role of miR-943-3p/SFRP4 in airway remodeling of allergic asthma, subepithelial fibrosis and smooth muscle were histologically examined in OVA-induced mice with miR-943-3p or SFRP4 gain-and-loss of function treatment (Fig. 6a–n). Firstly, OVA-induced mice exhibited notable airway remodeling represented by the increased thickness of subepithelial fibrosis and smooth muscle as well as elevated contents of collagen ($P < 0.01$).

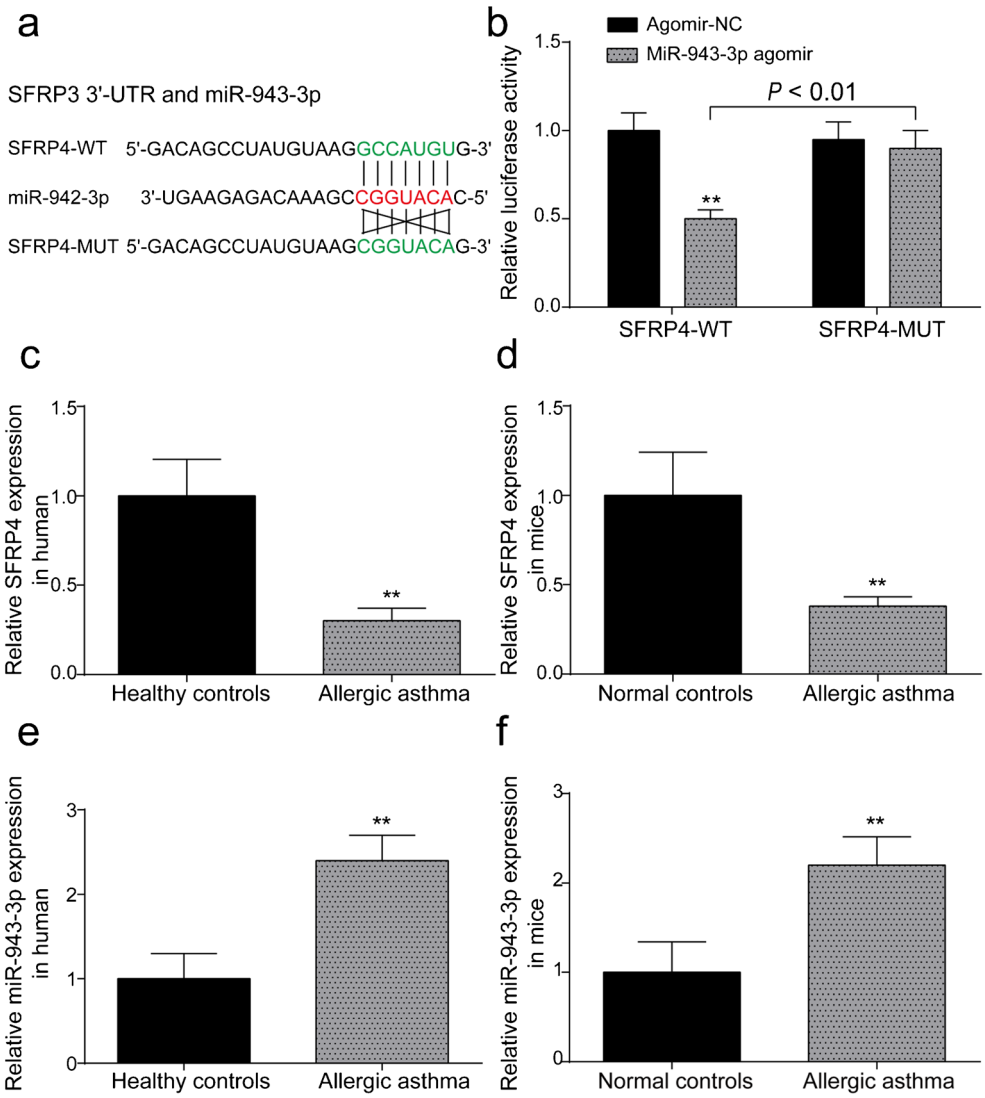

Fig. 3 MiR-943-3p directly targets SFRP4. **a** Binding sites of miR-943-3p targeted to 3′-UTR of SFRP4. MiRNA-mRNA interaction network was predicted using bioinformatics (TargetScan 7.2). **b** Validation of the predicted target relationship using luciferase reporter assay. $^{**}P < 0.01$ compares with the agomir-NC group. Expression of SFRP4 mRNA in **c** sputum samples and **d** lung tissue. Expression of miR-943-3p in **e** sputum samples and **f** lung tissue. Sputum samples were collected from patients with allergic asthma and healthy counterparts ($n = 6$ for each group); lung tissues were collected from OVA-induced mice and control mice ($n = 6$ for each group). $^{**}P < 0.01$ compares with the control group

Furthermore, the thickness of subepithelial fibrosis and smooth muscle as well as the contents of collagen in OVA-induced mice was markedly increased with the treatment of miR-943-3p agomir ($P < 0.01$), while it was decreased with miR-943-3p antagomir and Ad-SFRP4 ($P < 0.01$). By these morphological analyses, miR-943-3p-induced suppression of SFRP4 was demonstrated to promote airway remodeling in OVA-induced mice.

The effect of miR-943-3p/SFRP4 on the canonical Wnt pathway activation

The SFRP4-involved Wnt signal pathway has been proved to regulate the progression of airway remodeling in asthma (Ogawa et al. 2011). Therefore, it is reasonable to hypothesize that miR-943-3p can modulate Wnt signal pathway activation through suppressing SFRP4 expression. Here, expressions of Dvl-1, β-catenin, TCF7 and c-Myc, proteins related to the Wnt signal pathway, were detected using immunoblotting. As shown in Fig. 7(a–e), compared to sham-operated mice, expression of Wnt signal pathway-associated proteins were significantly increased in OVA-induced mice and reached its highest level if the OVA-induce mice were treated with miR-943-3p agomir. Notably, both the miR-943-3p antagomir and Ad-SFRP4 transfection could effectively inhibit the expression of these proteins. These results suggested that miR-943-3p downregulation and SFRP4 upregulation could potently inhibit the activation of the canonical Wnt signal pathway.

Discussion

Allergic asthma is a well-known chronic respiratory and inflammatory disease of the airways, which is usually triggered by allergen exposure, such as pollen, dust, or animal dander. For those patients suffering from allergic asthma, allergen

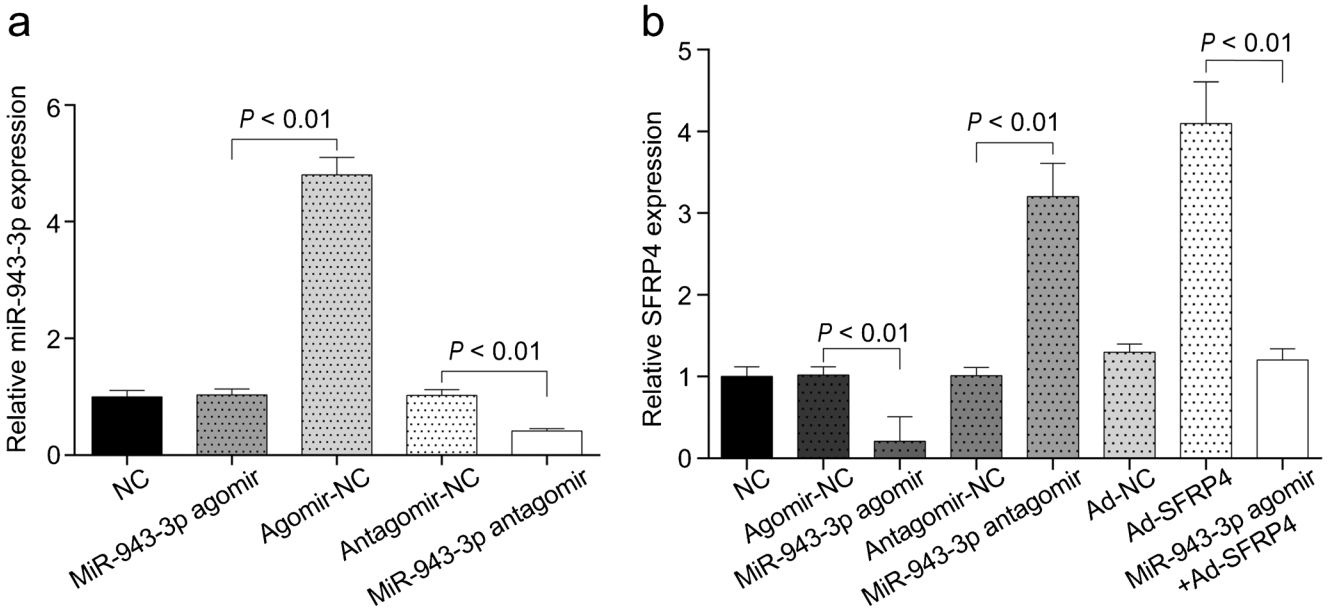

Fig. 4 MiR-943-3p inhibits the expression of SFRP4 in vivo. **a** MiR-943-3p agomir and antagomir regulate miR-943-3p expression in vivo. **b** Expression of SFRP4 in asthmatic mice transfected with miR-943-3p antagomir, miR-943-3p agomir, Ad-SFRP4, or co-transfected with miR-943-3p antagomir and Ad-SFRP4

avoidance has been investigated as a way to reduce allergen levels and symptoms. The Global Initiative for Asthma (GINA) guideline recommends a stepwise approach to symptom control and risk reduction. One or more daily controller medications provide regular maintenance treatment and ongoing treatment adjustments may involve an increase in daily dose or adding additional controller treatment if the patient is not well controlled. Similarly, ongoing treatment adjustments may involve a step-down in treatment once good asthma control has been maintained for 3 months. Inhaled corticosteroids (ICS) represent the initial and dominant controller option, yet inevitable side effects still exist. Until now, the details of pathogenesis of asthma is largely unknown and inhaled glucocorticoids is still being used as the first-line treatment for asthma but its side effects such as growth stunting in children, suppression of the adrenal axis and osteopenia may be inevitable (Chauhan and Ducharme 2012).

In the present study, we revealed the significantly altered profiles of gene expression and the KEGG pathway in asthma, represented by downregulated SFRP4 and activated Wnt signaling pathway. The Wnt signal pathway has been reported as the important mediator for cellular proliferation, differentiation, apoptosis and survival (Van Scoyk et al. 2008), and increasing evidence has also uncovered its underlying associations with lung diseases including asthma in the past few years (Cohen et al. 2009; Kwak et al. 2015; Van Scoyk et al. 2008). However, the role of the Wnt signaling pathway in asthma still remains a controversial topic. Bartel et al. found that specific targeting of Apc by miR-142-3p in murine embryo lungs led to the activation of Wnt signaling and enhanced proliferation of airway smooth muscle cells, thus promoting the development of asthma. (Beckert et al. 2018; Carraro et al. 2014). Blocking of the Wnt/β-catenin signaling pathway by a β-catenin-targeting siRNA greatly attenuated the symptoms of airway remodeling including smooth muscle hyperplasia and subepithelial fibrosis through downregulating TGF-β and tenascin C/PDGFR (Kwak et al. 2015). Small molecule inhibitors of β-catenin, XAV-939 and ICG-001, also exhibited a similar effect (Yao et al. 2017). On the other hand, Sebastian Reuter and his colleagues revealed that activation of the Wnt/β-catenin pathway could evidently ameliorate the development of allergic airway disease in an acute mouse model (Reuter et al. 2014). Yang et al. found that curcumin effectively reversed OVA-induced alternations through the activation of the Wnt/β-catenin signaling pathway (Yang et al. 2017). Thus, to fully understand the aberrant Wnt/β-catenin signal pathway in asthma could provide a huge potential for asthma intervention.

SFRPs act as the extracellular inhibitor of the canonical and non-canonical Wnt signaling pathway through binding to Wnt proteins and Frizzled receptors, SFRP4, which have the largest structural difference compared with the other family members (Sandsmark et al. 2017). Here, in-depth analysis of microarray GSE41946 (generated from the bronchial biopsies of patients with allergic asthma) (Chamberland et al. 2009) and the KEGG pathway showed that SFRP4 is the unique intersection of DEGs and genes in the Wnt signal pathway in asthma, this hints that the activation of the Wnt signal pathway in asthma; critically depends on the downregulation of SFRP4. Further in vivo studies showed that the upregulation of SFRP4 using a recombinant adenovirus system effectively suppressed allergic asthma-induced airway remodeling,

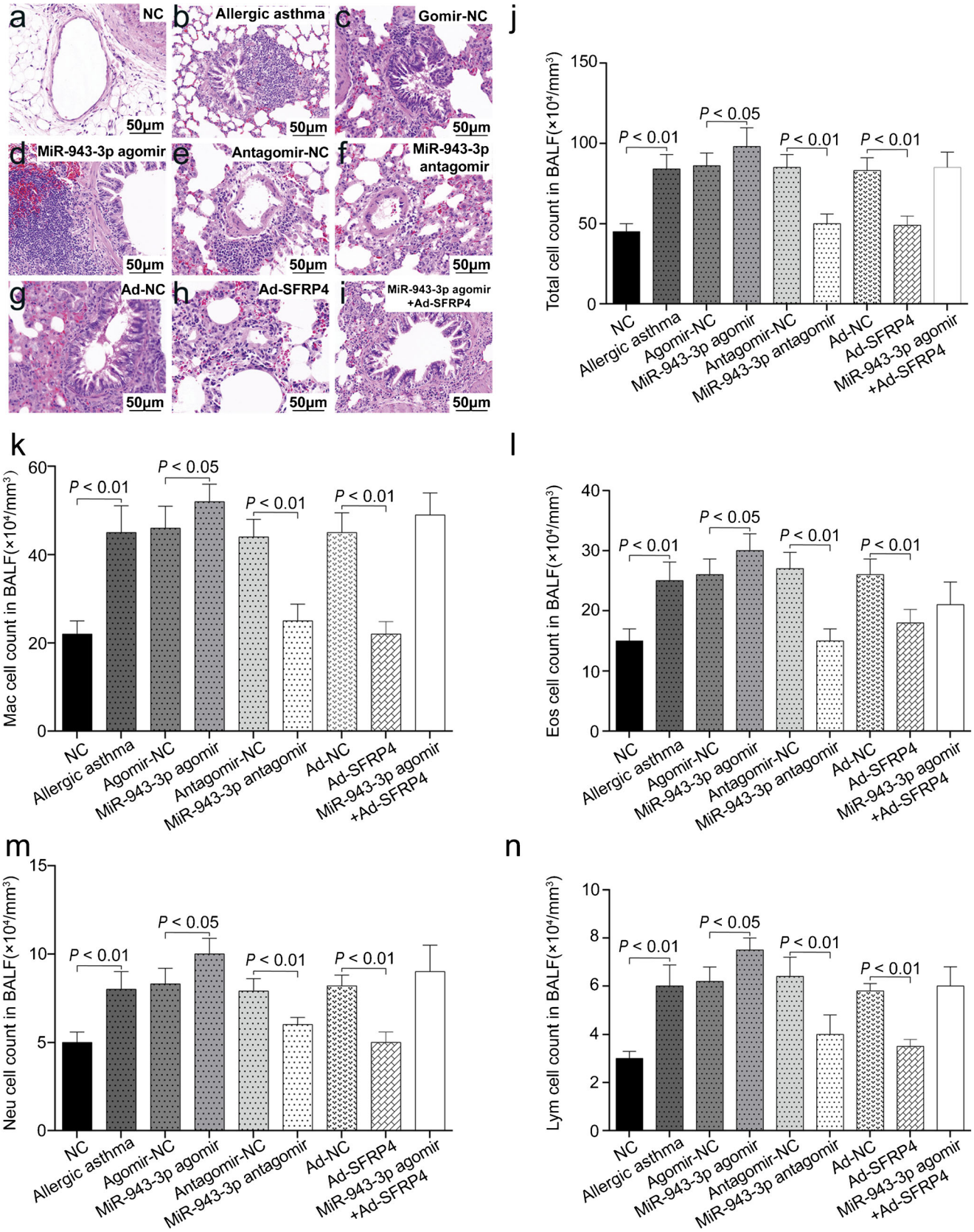

Fig. 5 Inflammatory cells in BALF. **a–i** Representative pictures of H&E staining. Results of Diff-Quik staining for BALF. **j** Total number of inflammatory cells in BALF. **k** Number of macrophage cells in BALF. **l** Number of eosinophil cells in BALF. **m** Number of neutrophil cells in BALF. **n** Number of lymphocyte cells in BALF

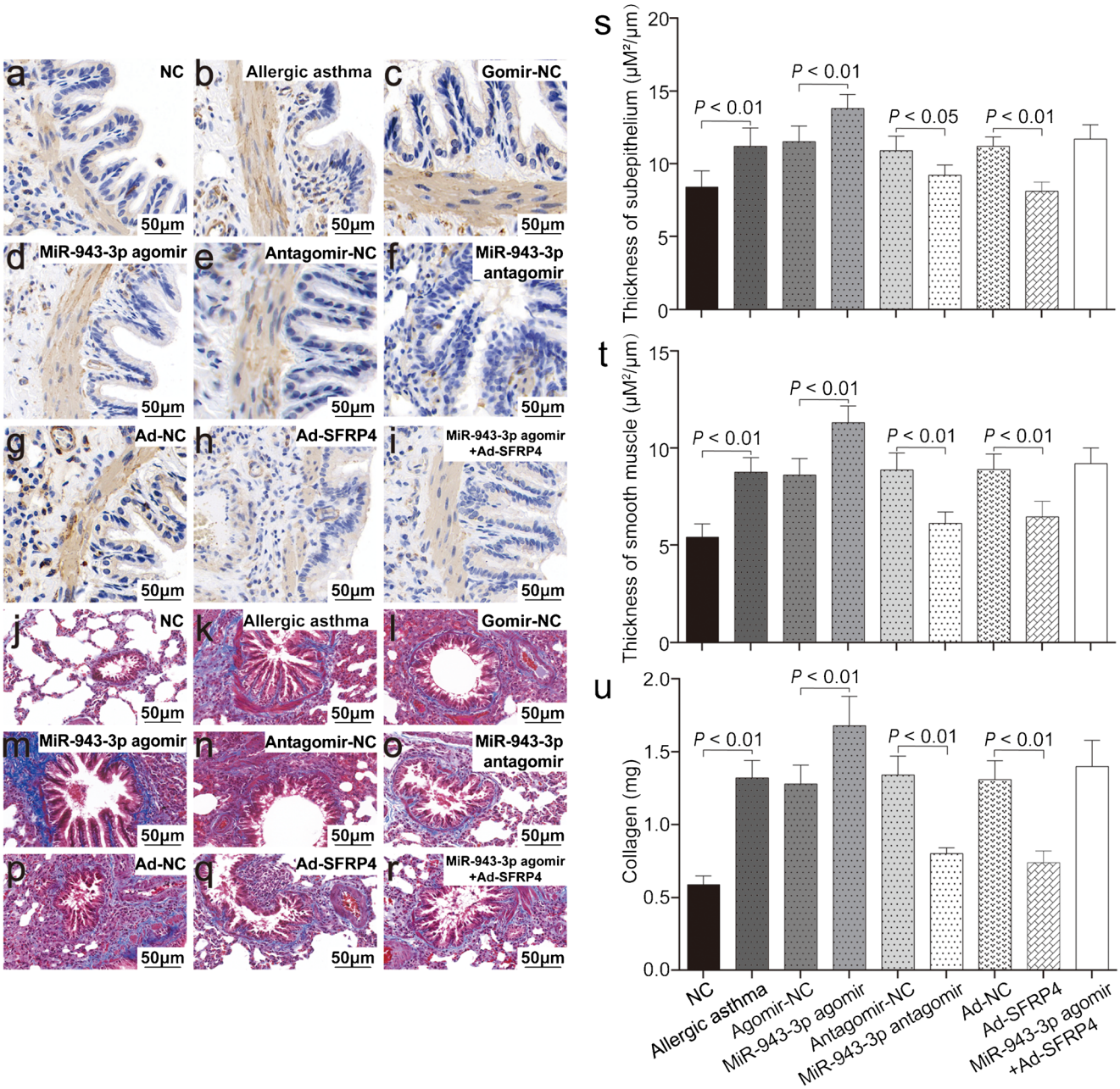

Fig. 6 Influence of miR-94-3p/SFRP4 on the airway modeling progression of allergic asthma. **a**–**i** Representative immunohistochemical staining for α-smooth muscle actin in asthma mouse models and normal mice. **j**–**r** Representative Masson's trichrome staining for subepithelial fibrosis and collagen in asthma mouse models and normal mice. **s** Effects of miR-943-3p and SFRP4 on subepithelial fibrosis shown in immunohistochemical staining for α-smooth muscle actin were quantified. **t** Effects of miR-943-3p and SFRP4 on smooth muscle hyperplasia shown in immunohistochemical staining for α-smooth muscle actin were quantified. **u** Effects of miR-943-3p and SFRP4 on collagen in lung tissue shown in Masson's trichrome staining were quantified

including the decreased thickness of the subepithelial fibrosis and smooth muscle as well as reduced lung content of collagen. Besides the airway remodeling, SFRP4 upregulation also improved airway inflammation, in which the airway infiltrations of inflammatory cells including macrophage, eosinophil, lymphocyte and neutrophils were greatly reduced in the Ad-SFRP4-treated allergic asthma mice. Given that the activated WNT/β-catenin signaling pathway has also been detected in multiple processes of inflammation, oxidative stress and even cancers (Ma and Hottiger 2016; Vallee and Lecarpentier 2018), whether the remarkable effects of SFRP4 exhibited in allergic asthma treatment are also efficacious to other diseases will be the emphases for our subsequent studies.

On the other hand, to the best of our knowledge, questions such as how the SFRP4 was downregulated in allergic asthma and whether the progressions of airway inflammation and

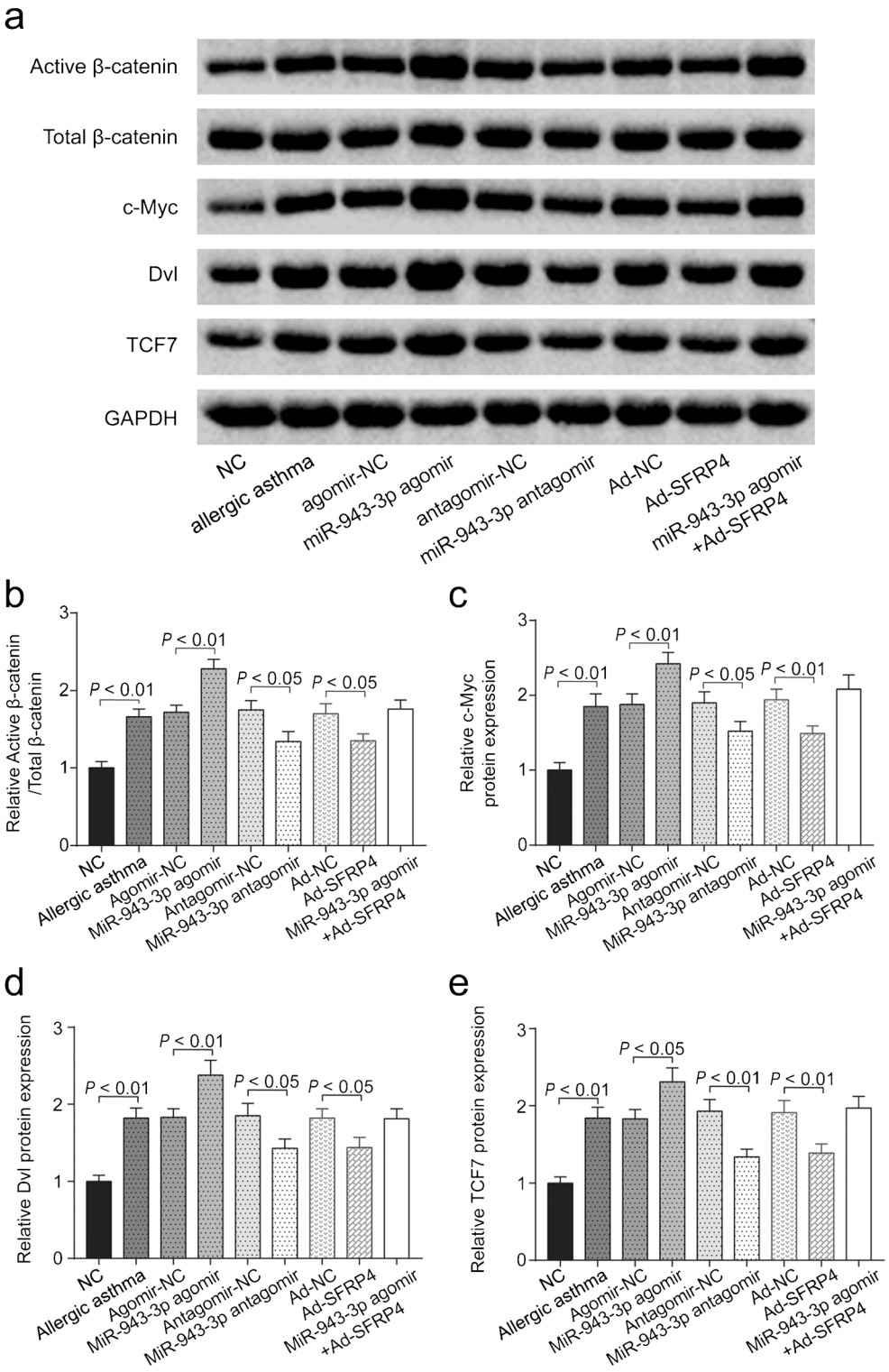

Fig. 7 Influence of miR-94-3p/SFRP4 dysregulation on the activation of Wnt signal pathway. **a** Protein levels of active-β-catenin, total β-catenin, c-Myc, Dvl-1, and TCF7 in allergic asthma mice with abnormal expression of miR-943-3p and SFRP4. **b** Protein level ratio of the active-β-catenin to total β-catenin in allergic asthma mice with abnormal expression of miR-943-3p and SFRP4. **c** Protein levels of c-Myc in allergic asthma mice with abnormal expression of miR-943-3p and SFRP4. **d** Protein levels of Dvl-1 in allergic asthma mice with abnormal expression of miR-943-3p and SFRP4. **e** Protein levels of TCF7 in allergic asthma mice with abnormal expression of miR-943-3p and SFRP4

remodeling were initiated or worsened by the downregulated SFRP4 through the Wnt signal pathway-dependent manner remain unsolved. According to the estimation, expression of about 60% human genome and nearly all major genes were regulated by microRNA at the post-transcriptional level; dysfunction of miRNAs has been involved in many human diseases including cancer, cardiovascular diseases and immune system disorders (Bartel 2009; Laffont and Rayner 2017). In our present study, in vitro and in vivo studies showed that miR-943-3p can negatively regulate the expression of SFRP4, in which transfection of miR-943-3p agomir could effectively suppress the expression of SFRP4 but miR-

943-3p antagomir largely promoted the expression of SFRP4 close to the Ad-SFRP4 level. Corresponding to the downregulation of SFRP4, expression of miR-943-3p was significantly increased not only in the sputum of allergic asthma patients but also in the lung tissue of OVA-induced mice. Treatment of miR-943-3p agomir caused worsened airway inflammation and remodeling in OVA-induced mice but miR-943-3p antagomir caused improved airway inflammation and remodeling in OVA-induced mice as Ad-SFRP4 exhibited. In addition to miR-943-3p, miR-135-5p can also suppress the expression of SFRP4 and subsequently induce unfavorable clinical manifestations and prognosis to pancreatic cancer; miR-517 has been predicted to regulate the expression of SFRP4 and thus is associated with various pregnancy-related complications such as abnormal NT thickness and preeclampsia (Hromadnikova et al. 2014). These results suggest that expression of SFRP4 can be regulated by more than one miRNA but whether these miRNAs were dysregulated and subsequently caused the abnormal expression of downstream SFRP4 in allergic asthma needs further investigation.

Although the high expression of SFRP4 expectedly showed beneficial effects against the processes of allergic asthma-induced airway inflammation and remodeling as mentioned above, the adverse of SFRP4 overexpression has also been documented. For example, functional loss of SFRP4 and the subsequent activation of the Wnt signaling pathway have favorable effects on naturally aged bone loss (Haraguchi et al. 2016); epicardial adipose tissue-derived SFRP4 upregulation was independently associated with the presence of coronary artery disease (Ji et al. 2017). Also, although SFRP4 acted as a tumor suppressor and reduced its expression in several types of cancer (Pohl et al. 2015), an increased expression of SFRP4 has also been observed in prostate cancer (Sandsmark et al. 2017). It has been suggested that these oncogenic properties and adverse effects of SFRP4 may partly be associated with biphasic modulation to the Wnt signaling pathway as well as undiscovered interactions with other signaling pathways. Previous reports have showed the discordant roles of SFRP4 in prostate cancer; patients with an overexpression of membranous SFRP4 have a good prognosis (Horvath et al. 2004; Horvath et al. 2007) but cytoplasmic SFRP4 causes a worse prognosis (Mortensen et al. 2015). Therefore, the side effect of long-term overexpression of SFRP4 induced by Ad-SFRP4 and miR-943-3p agomir should be constantly monitored. In conclusion, downregulation of SFRP4 and the subsequent activation of the Wnt signaling pathway were positively associated with the worsened progress of allergic asthma-related airway inflammation and remodeling. MiR-943-3p, as the upstream negative regulator of SFRP4, was significantly downregulated in allergic asthma patients and OVA-induced mice. Upregulation of SFRP4 induced by Ad-SFRP4 or miR-943-3p antagomir presented favorable effects, for example, SFRP4 upregulation inhibited the infiltration of inflammatory cells, thinned the thickness of subepithelial fibrosis and smooth muscle as well as reduced the lung content of collagen, through the suppression of the Wnt signaling pathway.

However, there are still some limitations to this study. Firstly, the animal disease model used for the present research was a mouse, which possesses huge differences in the anatomical and physiological respiratory system compared with a human. Besides, the respiratory rate of a mouse is much higher than that of human beings and they didn't develop chronic airway inflammation and epithelial changes typical of human asthma. Thus, further investigation in a mouse model might be needed in future studies for comprehensively uncovering the pathogenesis of asthma.

In conclusion, our study uncovered a potential but vital role of miR-943-3p and its target SFRP4 in allergic asthma, which, to be more specific, was that miR-943-3p targeted SFRP4 and suppressed the activity of SFRP4 in the Wnt signaling pathway, thus accelerated the progression of airway inflammation and remodeling in allergic asthma.

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Compliance with ethical standards

Ethics approval and consent to participate This study was authorized by the Shuguang Hospital Affiliated to Shanghai Traditional Chinese Medical University, who obtained written informed consents from all the participants.

Informed consent Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflicts of interest.

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