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Fisetin reduces ovalbumin-triggered airway remodeling by preventing phenotypic switching of airway smooth muscle cells

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Abstract

Background The transformation of airway smooth muscle cells (ASMCs) from a quiescent phenotype to a hypersecretory and hypercontractile phenotype is a defining feature of asthmatic airway remodeling. Fisetin, a flavonoid compound, possesses anti-inflammatory characteristics in asthma; yet, its impact on airway remodeling and ASMCs phenotype transition has not been investigated.

Objectives This research seeked to assess the impact of fisetin on ovalbumin (OVA) induced asthmatic airway remodeling and ASMCs phenotype transition, and clarify the mechanisms through network pharmacology predictions as well as in vivo and in vitro validation.

Methods First, a fisetin-asthma-ASMCs network was constructed to identify potential targets. Subsequently, cellular and animal studies were carried out to examine the inhibitory effects of fisetin on airway remodeling in asthmatic mice, and to detemine how fisetin impacts the phenotypic transition of ASMCs.

Results Network analysis indicated that fisetin might affect asthma via mediating the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (AKT) pathway. Intraperitoneal administration of fisetin in vivo reduced airway inflammation and remodeling, as shown by reduced inflammatory cells, decreased T helper type 2 (Th2) cytokine release, diminished collagen accumulation, mitigated airway smooth muscle thickening, and decreased expression of osteopontin (OPN), collagen-I and α-smooth muscle actin (α-SMA). Moreover, fisetin suppressed the PI3K/AKT pathway in asthmatic lung tissue. According to the in vitro data, fisetin downregulated the expression of the synthetic phenotypic proteins OPN and collagen-I, contractile protein α-SMA, and inhibited cellular migration, potentially through the PI3K/AKT pathway.

Conclusion These results suggest that fisetin inhibits airway remodeling in asthma by regulating ASMCs phenotypic shift, emphasizing that fisetin is a promising candidate for the treatment of airway smooth muscle remodeling.

Keywords Fisetin, Airway remodeling, Phenotypic switching, OPN, Collagen-I, α-SMA

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Background

Asthma is a long-lasting, multifaceted disease with the pathological features such as airway inflammation and remodeling. Airway remodeling involves subepithelial fibrosis, increased smooth muscle bulk, and extracellular matrix aggregation [1]. Airway structural remodeling causes airway stenosis, restricted airflow, and decreased lung function, and severely affects the quality of life [2]. Glucocorticoids are widely used as first-line drugs for asthma, but are associated with a range of adverse drug reactions, including psychiatric symptoms, growth retardation, abnormalities of the teeth and rashes [3]. Thus, novel and affective anti-asthma agents are urgently needed.

Impaired airway smooth muscle cells (ASMCs) play important roles in airway structural remodeling. Extracellular matrix proteins and transforming growth factors, promote the shift of ASMCs from a dormant to a synthetic stage in vitro [4, 5]. ASMCs from asthmatic patients exhibit a preference for a synthetic phenotype compared to those from non-asthmatic donors [6]. ASMCs with a synthetic phenotype display features such as hypertrophy, migration, proliferation and parasecretion, leading to increased airway tension, worsened mechanics and exacerbated airway hyperresponsiveness [7]. Additionally, ASMCs contribute to airway stenosis and airway hyperresponsiveness by modulating cell contractility [8]. The expression of the contractile protein α-smooth muscle actin (α-SMA) in the airway is related to airway function in asthma [9]. Therefore, it is essential to develop treatments targeting ASMCs dysfunction.

The compound 3,3',4',7-tetrahydroxyflavone (fisetin) is a natural bioactive flavonoid widely derived from fruit and vegetable sources [10]. Fisetin has numerous biological functions, involving anti-aging, anti-cancer, anti-inflammatory, and anti-fibrotic properties [11–14]. Pervious research revealed that fisetin suppresses airway inflammation in an asthma model [15, 16]. Fisetin is known to have potent effects on a range of airway cell types. Fisetin inhibits lipopolysaccharide-induced neutrophil and macrophage infiltration and attenuates acute lung injury [17]. Fisetin significantly inhibits tumour necrosis factor alpha-induced Interleukin (IL)-8 levels in human airway epithelial cells, suggesting that fisetin is a potential agent for the treatment of inflammatory lung diseases [18]. Fisetin has the potential ability to inhibit cigarette smoke extract-induced epithelial-mesenchymal transition in airway epithelial cells [19]. However, its role in regulating airway remodeling and ASMCs function is still unknown.

Herein, our study focused on the impacts of fisetin in a murine model of asthma. The phenotypic switching and biological functions of ASMCs were examined to elucidate the potential mechanisms.

Materials and methods

Reagents

Fisetin was obtained from Beyotime (Shanghai, China). Ovalbumin (OVA) was acquired from Sigma-Aldrich (Taufkirchen, Germany). Antibodies against β-actin, GAPDH, Phosphoinositide 3-kinase (PI3K), p-PI3K, and the PI3K inhibitor LY294002 were obtained from Beyotime (Shanghai, China). Antibodies against Protein Kinase B (AKT), p-AKT, collagen-I, osteopontin (OPN) and α-SMA were acquired from Cell Signaling Technology (Beverly, USA).

Animals

Female BALB/c mice (6-weeks-old, weighing 18–20 g) were provided by Gem Pharmatech Company (Nanjing, China) and kept in standard laboratory conditions (22–24 °C, 49–51% humidity). Water and food were freely available to all animals. All animal studies were conducted following National Institutes of Health (NIH) guidelines and authorized according to the Ethics Committee of the Second Affiliated Hospital of Shandong First Medical University.

Network pharmacology

The simplified molecular input line entry system (SMILES) structure for fisetin was retrieved from PubChem website. The structure date file (SDF) format were submitted into the Swiss Target prediction database (http://www.swisstargetprediction.ch/) to facilitate further targeting prediction [20]. Targets related to asthma and ASMC were gathered from the GeneCards (https://www.genecards.org/) and Online Mendelian Inheritance in Man (OMIM) databases (https://omim.org/) [21, 22]. The overlapping genes related to fisetin, asthma, and ASMC were considered potentially valuable for asthma treatment. Metascape was implemented to perform gene functional enrichment analysis (https://metascape.org/) [23]. Protein-protein interaction (PPI) networks were created and further analyzed to classify hub genes via the Search Tool for the Retrieval of Interaction Genes/Proteins (STRING) (https://string-db.org/) [24]. Topological analysis and visualization were conducted using the Cytoscape plug-in NetworkAnalyzer.

Asthmatic model establishment and fisetin treatment

Mice were randomly assigned to the following five groups ($n=10$ per group): (1) control group, (2) OVA group, OVA-sensitized/OVA-exposed; (3) OVA+FL group, OVA-sensitized/OVA-exposed+low dose of fisetin (2.5 mg/kg); (4) OVA+FH group, OVA-sensitized/OVA-exposed+high dose of fisetin (5 mg/kg); (5) OVA+DEX group, OVA-sensitized/OVA-exposed+1 mg/kg dexamethasone. The mice were administered 100 μL of OVA mixed with 2 mg of aluminum hydroxide

intraperitoneally on days 1 and 8. During days 15–43, the animals were nebulized using 2.5% OVA for 30 min/day, and were then administered three times weekly for 4 weeks using an ultrasonic nebulizer. The drug-treated groups received fisetin or dexamethasone 30 min before each OVA challenge. Dexamethasone was used as a positive control because it is an established first-line treatment of asthma. Figure 1 reveals the experimental strategies.

Examination of bronchoalveolar lavage fluid (BALF)

The animals were intratracheally intubated, and 750 μL of saline was administered three times after euthanasia. The BALF was spun at 2000 rpm and 4 ℃ for 15 min. The pellet was reconstituted and counted with a hemocytometer. Smears were Giemsa-stained and screened under light microscopy. The BALF supernatants were used for cytokine analysis. IL-4, IL-5, and IL-13 levels in BALF were measured with enzyme-linked immunosorbent assay (ELISA) panels. Five mice from each group were randomly selected for cell counts and cytokine analysis.

Lung histopathology

Lung samples were paraffin-embedded and split to 5-μm slices. Hematoxylin and eosin (H&E) staining was employed for assessing pulmonary pathological changes, whereas Masson staining was employed to analyze peribronchial collagen deposition. Periodic acid-Schiff (PAS) staining was used to examine goblet cell hyperplasia. Image-Pro Plus software was used to quantify the bronchial smooth muscle area (WAm), Masson stained area, and perimeter of the bronchial basement membrane (Pbm). Analyses were performed to determine the bronchial smooth muscle thickness (WAm/Pbm), Masson stained area/Pbm and PAS stained area/Pbm. Images were photographed under light microscopy at 400× magnification. Five mice from each group were randomly selected for histological examination, and five bronchioles per mouse were quantified.

Immunohistochemistry

Paraffin samples were dewaxed, rehydrated, subjected to antigen retrieval, and blocked with serum. Afterward, the sections were treated with primary antibodies targeting collagen-I (1:200), OPN (1:100), and α-SMA (1:200) overnight at 4 ℃, and then exposed to secondary antibodies for 1 h. Freshly prepared DAB was applied for chromogenic detection. Photographs of the sections were obtained using a light microscope at 400× magnification. Five mice from each group were randomly selected for immunohistochemical analysis, and five bronchioles per mouse were quantified.

Cell cultivation

Mouse airway smooth muscle cells (MASMCs) were obtained from Otwo Biotech (Shenzhen, China). MASMCs were cultivated in Dulbeccos modified Eagle medium (DMEM) containing 10% fetal bovine serum, and 1% penicillin/streptomycin in 5% CO2 environment.

Cell cytotoxicity assays

Fisetin at 0, 12.5, 25, 50, 100, and 200 μM was applied to the MASMCs for 24 h. Subsequently, a cell counting kit-8 (CCK-8) test was used to evaluate cellular cytotoxicity. This procedure was performed as follows: MASMCs were placed in 96-well dishes (3000 cells per well) overnight and exposed to various doses of fisetin. After the supernatant was discarded, CCK-8 solution was applied to stain the cells for 30 min. Absorbance was analyzed via a spectrophotometer (CANY, Shanghai, China). The cell cytotoxicity assays were repeated thrice.

Wound healing assays

The MASMCs were placed in 6-well dishes with a seeding density of 5×10^5 cells/well. Upon reaching 90–100% confluence, a wound was simulated by creating a straight scratch with a 200 μL micropipette tip. Cell debris from the scratches was rinsed with PBS, and the cells were exposed to serum-free DMEM. Images at 0 and 24 h were observed using an inverted microscope. The scratch area

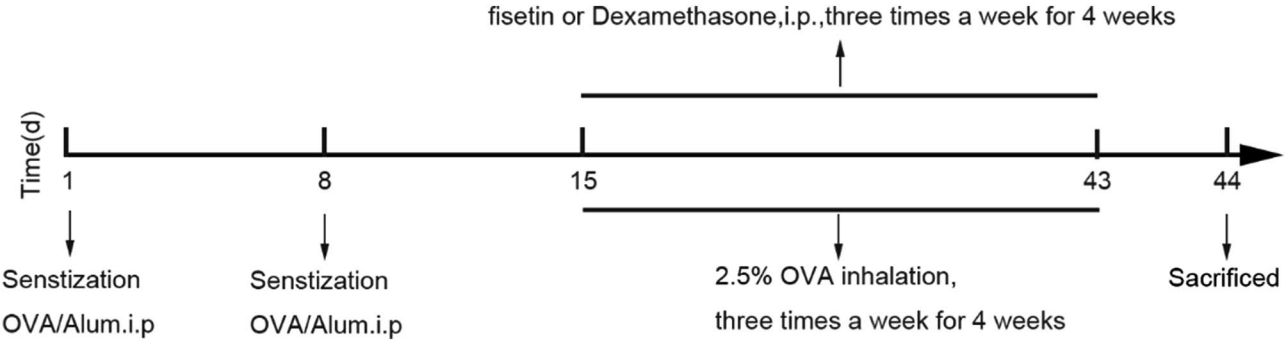

Fig. 1 Experimental strategies and schedule

was measured using ImageJ software. The wound healing assay was performed in triplicate.

Western blotting

Lung tissues and MASMCs protein were homogenized and treated with a protease inhibitor cocktail in RIPA. After 5 min spinning at 12,000 rpm, the protein supernates were gathered. Protein samples (20 μg) were resolved through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently electroblotted on polyvinylidene fluoride (PVDF) membranes. Following a 2-hour blocking step, the PVDF membranes were then exposed to primary antibodies overnight with PI3K (1:1000), p-PI3K (1:1000), AKT (1:1000), p-AKT (1:1000), collagen-I (1:1000), OPN (1:1000), α-SMA (1:1000), β-actin (1:1000) and GAPDH (1:1000). Following incubation with secondary antibodies, the protein bands were revealed with Tanon High-sig ECL Western blotting assay reagent (Tanon, Shanghai, China). Three mice from each group were randomly selected for Western blotting analysis, and the cell Western blotting experiments were performed in triplicate.

Immunofluorescence

MASMCs were cultured on slides and treated according to the established experimental protocol. After being subjected to cellular fixation, membrane breaking, and serum closure, cell slides were exposed to collagen-I (1:200), OPN (1:100), and α-SMA (1:300) at 4 °C. Thereafter, the slides were subjected to fluorescent-labeled secondary antibodies for 2 h in a dark chamber. Finally, the nuclei were stained using DAPI, and samples were sealed with an antifade medium. Images were taken under fluorescent light and processed using ImageJ software. The cell immunofluorescence experiments were performed in triplicate.

Statistical analysis

All experiments were performed with three to five biological replicates and three experimental replicates. GraphPad Prism 8 was utilized to construct graphs and perform data analysis. Results are displayed as mean ± SEM. One-way ANOVA followed by Tukey's test was implemented to compare groups. $P<0.05$ was deemed significantly different.

Results

Network pharmacology identified prospective target genes for fisetin in treating asthma

100 targets of fisetin were obtained from Swiss Target Prediction. Following the elimination of redundant targets, 2551 asthma-associated targets and 5153 ASMC-associated targets were collected from the GeneCards (relevance score > 1) and OMIM databases. Then, 34 intersecting targets were identified through a Venn diagram (Fig. 2A). These overlapping targets are considered as possible targets for the application of fisetin in asthma therapy. Next, enrichment analysis was conducted using Metascape. The primary biological activities focused on biological activities such as controlling inflammation and cell growth, IL-4 and IL-13 signaling, and white blood cell movement, all of which are strongly associated with asthma. Additionally, the data showed significant enrichment in the PI3K/AKT signaling pathway. The top 20 significant genes are shown in Fig. 2B and C.

Analysis of the network structure of 34 common targets indicated that fisetin could potentially targets AKT1, matrix metalloprotein 9 (MMP9), epidermal growth factor receptor (EGFR), and other genes for asthma treatment (Fig. 2D). In the interaction network constructed using Cytoscape software, AKT1, which exhibited the highest maximal clique centrality (MCC), betweenness centrality, and degree values, is considered the predominant node (Fig. 2E). These findings suggest that the PI3K/AKT pathway appears to be enriched and may be involved in the mechanism of fisetin therapy for asthma.

Fisetin alleviated airway inflammation in asthmatic mice

To ascertain the protective role of fisetin in asthmatic mice, we examined its influence on airway inflammation. Airway inflammation is driven by aberrant Th2 cells that generate cytokines, including IL-4, IL-5, and IL-13 [25]. Our results displayed that mice exposed to OVA exhibited higher levels of aforementioned cytokines. In comparison to mice challenged by OVA, administration of fisetin or dexamethasone diminished these cytokines levels (Fig. 3A-C). The findings are aligned with the network analysis results.

The analysis of pulmonary inflammation was conducted using BALF cell countsas well as peribronchial and perivascular inflammatory cell infiltration. Our results showed that BALF from saline control mice contained no inflammatory cells. OVA-treated mice had higher BALF counts of neutrophils, eosinophils, and total cells. Fisetin or dexamethasone reduced the inflammatory cell counts compared to asthmatic mice (Fig. 3D-F).

Fisetin alleviated airway remodeling in asthmatic mice

H&E staining revealed more peribronchial and perivascular inflammatory lesions in asthmatic mice than in control mice. Fisetin or dexamethasone decreased these inflammatory cell infiltration in the lungs (Fig. 4A). Similarly, fisetin prevented airway structural remodeling in OVA-treated mice. Airway remodeling was assessed using WAm/Pbm, Masson stained area/Pbm and PAS stained area/Pbm. As shown by H&E staining, OVA challenge caused a notable rise in peribronchial smooth muscle thickness compared with that of the controls, and the

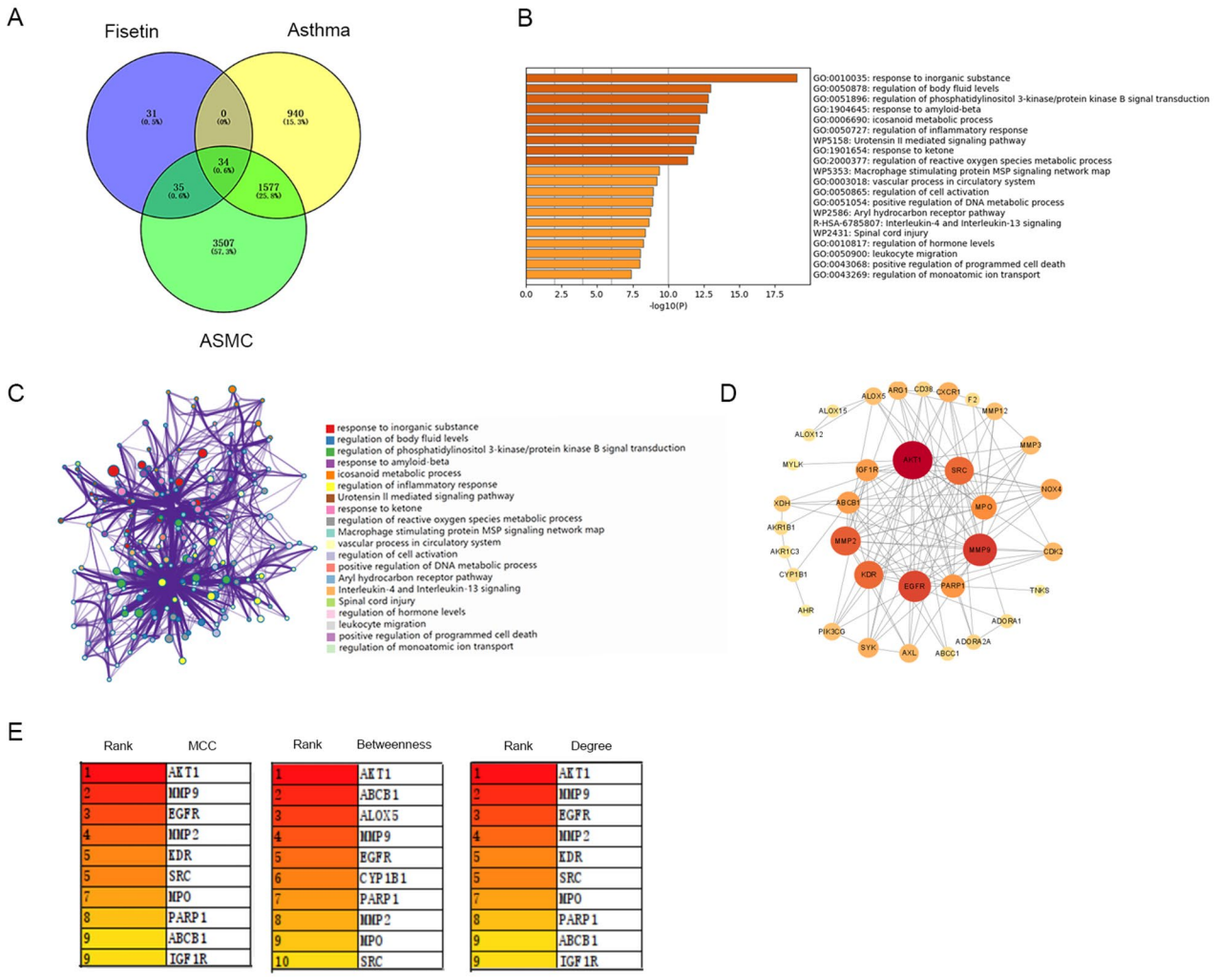

Fig. 2 Performing enrichment analysis, constructing PPI networks, and analyzing modules. (**A**) Venn diagram of fisetin targets, asthma-related genes, and ASMC-related genes. (**B**) Metascape bar graph of enriched terms showing the top 20 targets. The enhanced terms were prioritized according to p-values, with more vibrant colors representing smaller p-values. (**C**) Metascape visualization of the networks of the top 20 clusters, with each cluster distinguished by a unique color based on its cluster ID. Nodes with the same cluster ID exhibit stronger interconnections. (**D**) Construction of a PPI network for genes with increased expression. (**E**) The top 10 targets were ranked according to MCC, betweenness centrality, and degree, as performed using the plug-in of Cytoscape

levels of thickness were mitigated by treatment with fisetin or dexamethasone (Fig. 4A and B). Masson staining showed that the increased peribronchial collagen deposition induced by OVA was inhibited by fisetin or dexamethasone (Fig. 4C and D). PAS staining showed that OVA challenges resulted in marked goblet cell hyperplasia and mucus plugging, which were inhibited by fisetin or dexamethasone (Fig. 4E and F).

Fisetin alleviated OPN, collagen-I and α-SMA levels in asthmatic mice

Asthma biomarkers such as OPN, collagen-I and α-SMA which are also considered novel therapeutic targets, are related to asthma severity and airflow limitations [26, 27]. Immunohistochemical analyses were employed to evaluate OPN expression. Bronchiolar and pulmonary OPN expression was significantly increased in OVA-treated mice, whereas fisetin or dexamethasone treatment decreased OPN expression (Fig. 5A and B). Immunohistochemistry assays also showed an increase in collagen-I and α-SMA staining surrounding the bronchioles in OVA-challenged mice compared with the controls. Fisetin or dexamethasone treatment reversed these increases (Fig. 5C – 5 F).

Western blotting analysis yielded consistent results (Fig. 5G). OPN, collagen-I and α-SMA levels of OVA-challenged mice were much higher than those of the controls. Fisetin or dexamethasone reduced OPN, collagen-I and α-SMA expression compared to OVA-challenged mice (Fig. 5H – 5 J).

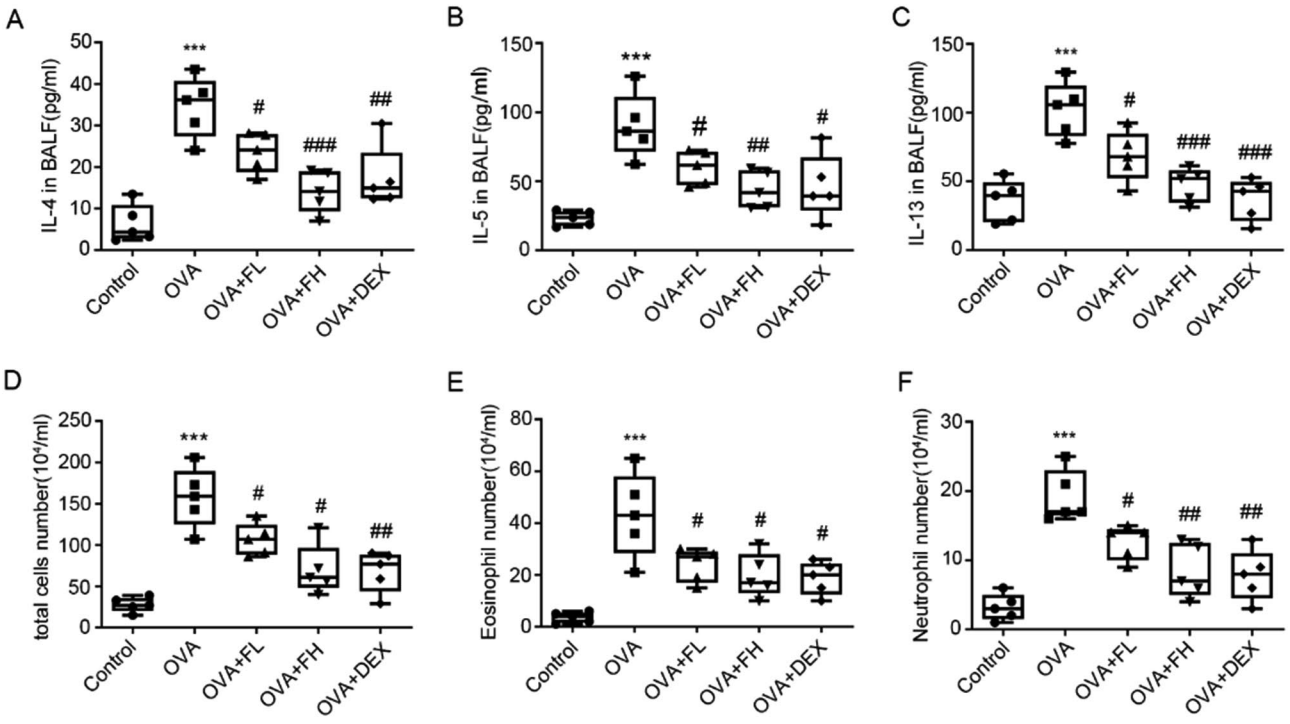

Fig. 3 Fisetin attenuated OVA-challenged airway inflammation. IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**) in BALF were quantified using ELISA ($n=5$). Total cells (**D**), eosinophils (**E**), and neutrophils (**F**) in BALF were counted ($n=5$). Results are displayed as mean ± SEM. $^{***}P<0.001$ vs. control, $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$ vs. OVA

Fisetin inactivated the PI3K/AKT pathway in asthmatic mice

Western blotting was employed to analyze the PI3K/AKT pathway in the lungs. Our findings exhibited that PI3K and AKT phosphorylation levels were greatly raised in OVA-induced mice, whereas fisetin or dexamethasone notably prevented their phosphorylation. There was no discernible variation in total PI3K and AKT expression between the groups (Fig. 6).

Effect of fisetin on MASMCs cytotoxicity

The cytotoxic effects of fisetin on MASMCs were evaluated in vitro using the CCK-8 assay. The findings indicated that fisetin did not display any noticeable toxicity at low doses ranging from 0 μM to 100 μM. In contrast, 200 μM fisetin inhibited cell viability after 24 h (Fig. 7). Thus, we selected fisetin concentrations below 100 μM for subsequent experiments.

Effects of fisetin on MASMCs phenotypic switching and migration

Increasing evidence suggests that ASMCs in individuals with asthma tend to display a synthetic and contractile phenotype [28]. The increased expression of OPN and collagen-I which are extracellular matrix components, indicates ASMC transformation to a synthetic phenotype. To ascertain whether fisetin regulates synthetic phenotype switching, we investigated its impact on the expression of OPN and collagen-I. According to our findings, fisetin treatment for 24 h displayed a dose-dependent decline in OPN and collagen-I levels (Fig. 8A-C). These findings indicate that fisetin prevents MASMCs from transitioning to a synthetic phenotype. As a marker of ASMCs, α-SMA plays a key role in cell proliferation, migration and contraction [29, 30]. In our study, we investigated the effect of fisetin on α-SMA. We found that fisetin treatment for 24 h reduced α-SMA levels in a dose-dependent manner (Fig. 8A and D).

Prior research has indicated that the change of ASMCs to a synthetic type can enhance cell migration [31, 32]. Subsequently, a wound closure test was performed to evaluate migratory capacity. Our findings indicated that fisetin treatment for 24 h inhibited MASMCs migration in a dosage-dependent way (Fig. 8E and F).

Effects of fisetin on the PI3K/AKT pathway in MASMCs

The PI3K/AKT pathway regulates many cellular processes, including phenotypic switching and migration [33, 34]. Therefore, we sought to determine whether fisetin modulates the PI3K/AKT pathway. After 1 h of fisetin treatment, our investigation indicated a dosage-dependent decline in p-PI3K and p-AKT levels in MASMCs. However, the PI3K and AKT levels remained unchanged (Fig. 9).

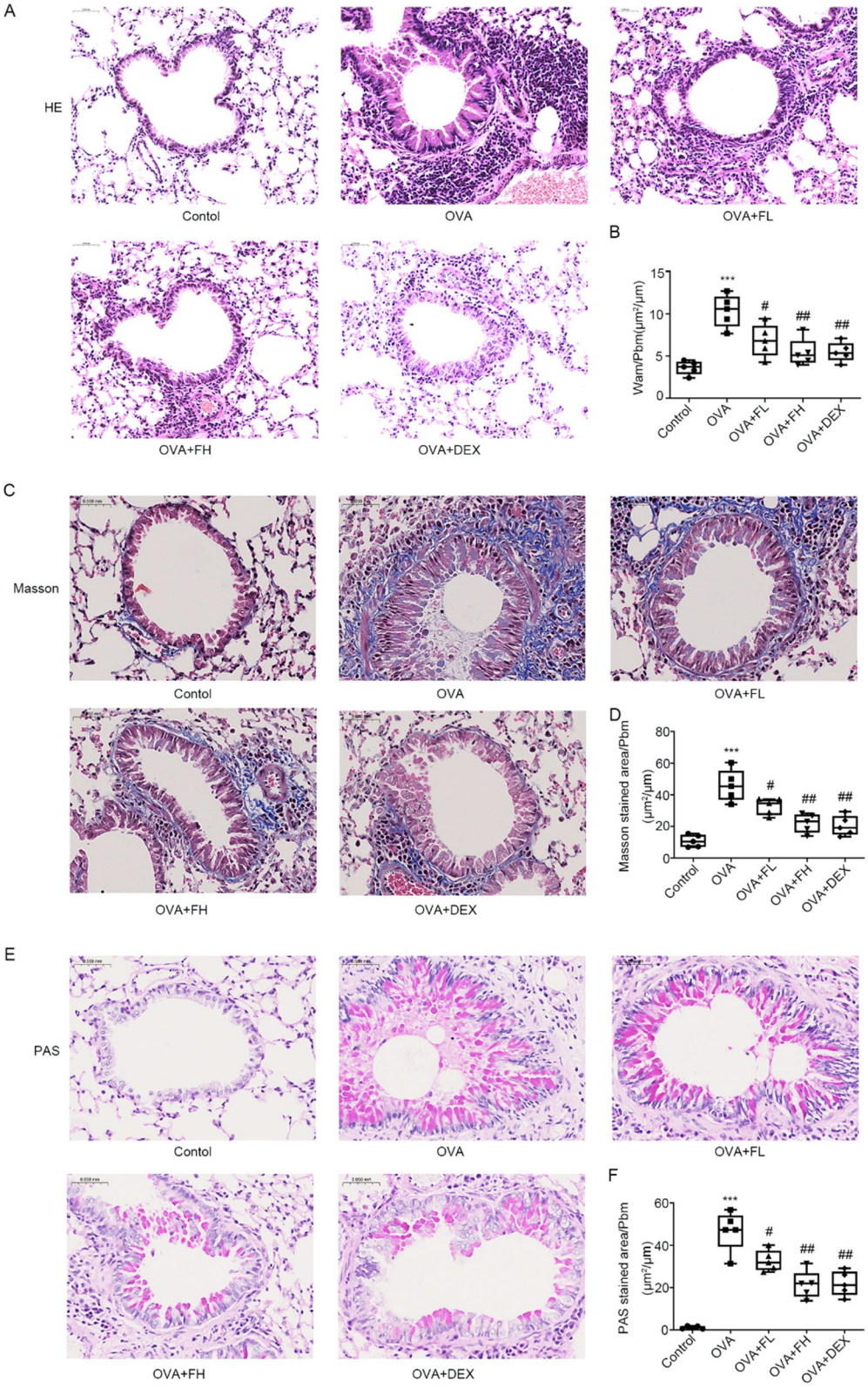

Fig. 4 Fisetin alleviated OVA-challenged airway remodeling. (**A**) Histopathological analysis was performed using H&E staining. (**B**) Quantification of WAm/Pbm ($n=5$). (**C**) Collagen accumulation was detected through Masson staining. (**D**) Quantification of Masson stained area/Pbm ($n=5$). (**E**) Goblet cell hyperplasia was detected using PAS staining. (**F**) Quantification of PAS stained area/Pbm ($n=5$). Results are represented as the mean ± SEM. $^{***}P<0.001$ vs. control, $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. OVA

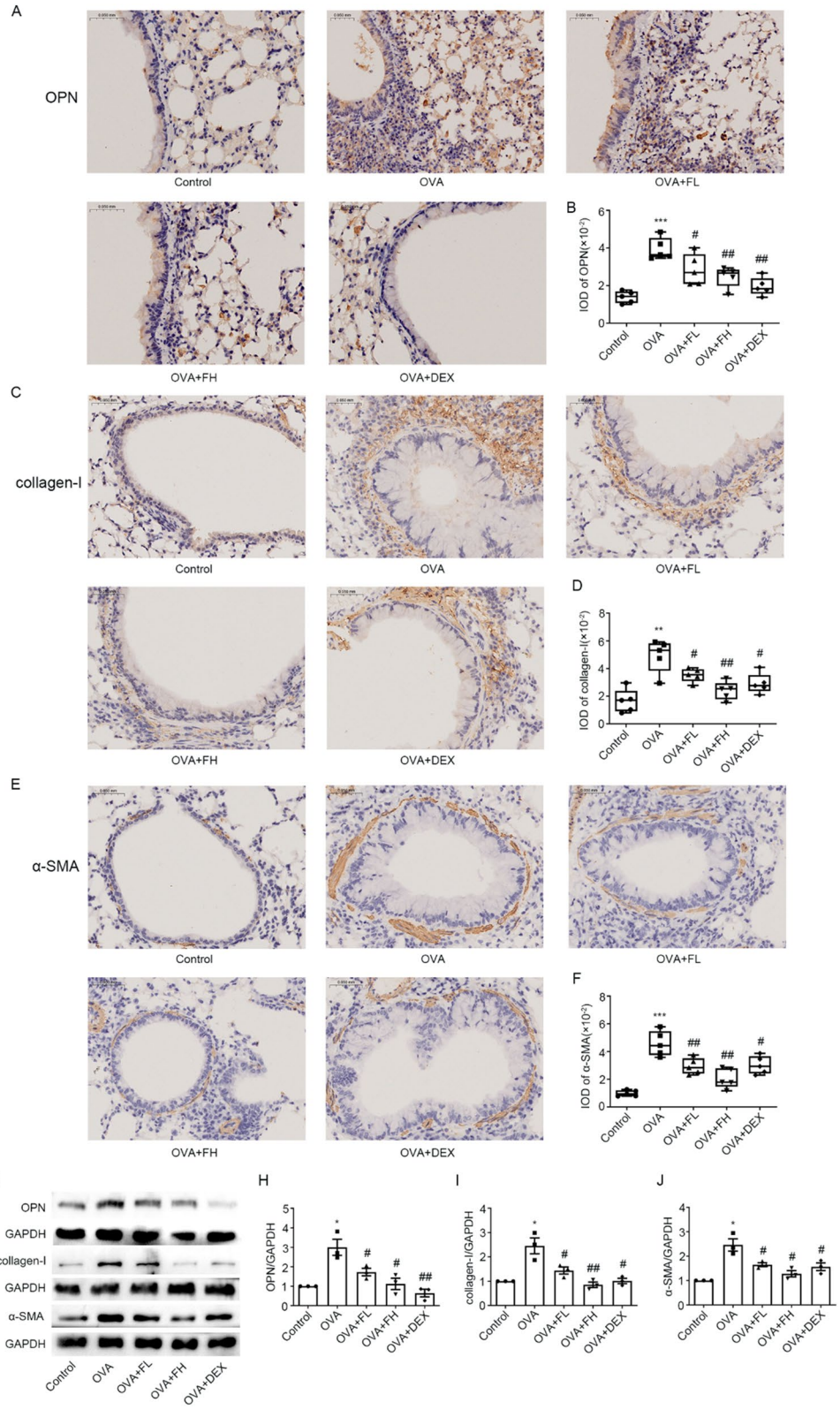

Fig. 5 Fisetin suppressed OPN, collagen-I and α-SMA levels in asthmatic mice. (**A**, **C**, **E**) Immunohistochemical staining was performed on lung sections to detect OPN, collagen-I and α-SMA expression. (**B**, **D**, **F**) Integrated optical density (IOD) detection of OPN, collagen-I and α-SMA immunohistochemical staining ($n=5$). (**G**) Western blotting images of OPN, collagen-I and α-SMA. (**H**, **I**, **J**) Quantification of protein expression ($n=3$). Results are represented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs. control; $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. OVA

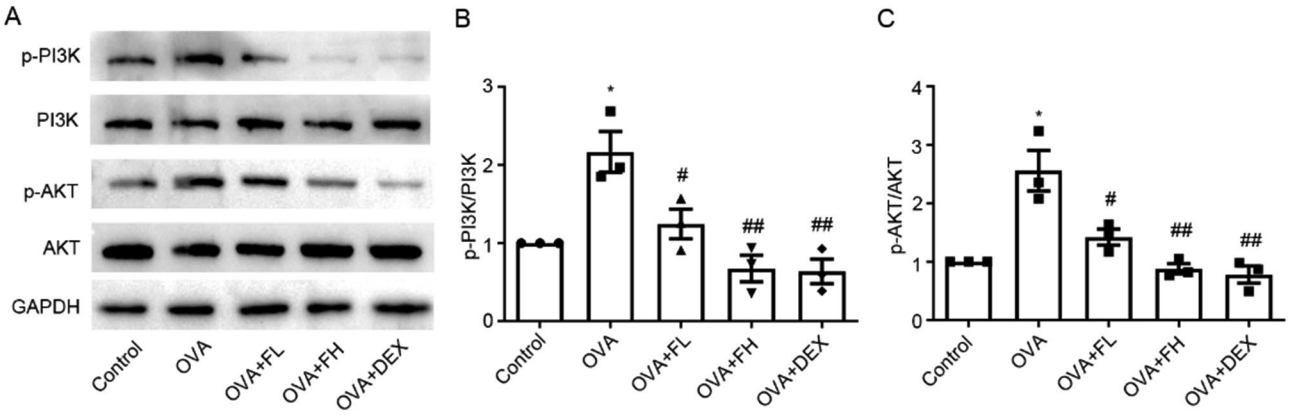

Fig. 6 Fisetin inactivated the PI3K/AKT pathway in asthmatic mice. (**A**) Western blotting detection of p-PI3K, PI3K, p-AKT, AKT, and GAPDH. (**B**, **C**) Quantification of protein expression ($n=3$). Results are represented as mean ± SEM. $^{*}P<0.05$ vs. control; $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. OVA

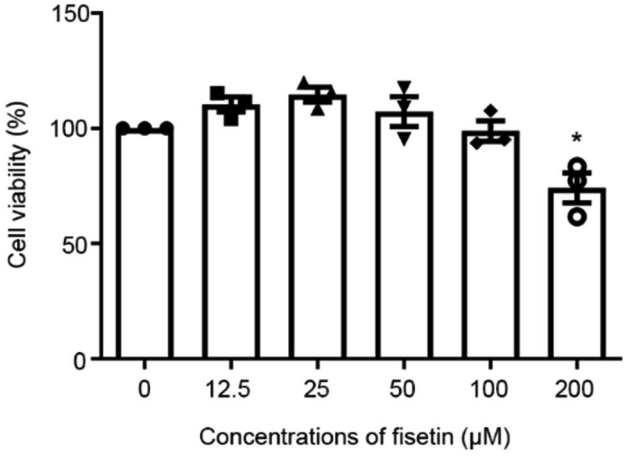

Fig. 7 Effects of fisetin on MASMCs cytotoxicity ($n=3$). Results are represented as mean ± SEM. $^{*}P<0.05$ vs. control

Fisetin altered the MASMCs phenotype via the PI3K/AKT pathway

To elucidate the impact of the PI3K/AKT pathway on fisetin-induced phenotypic switching, a PI3K inhibitor (LY294002) was selected to disrupt the PI3K/AKT pathway [35]. After 30 min of preincubation with 5 μM LY294002, MASMCs were cultured with or without 100 μM fisetin for 24 h. Subsequently, OPN, collagen-I and α-SMA expression were assessed via Western blotting and immunofluorescence. As displayed in Fig. 10, Western blotting assays showed that the levels of OPN, collagen-I and α-SMA were reduced by fisetin or LY294002. Compared to LY294002 treatment, cells treated with both LY294002 and fisetin showed further reductions in OPN, collagen-I and α-SMA levels. Similarly, immunofluorescence staining showed that fisetin or LY294002 decreased levels of OPN, collagen-I and α-SMA compared to the controls. Combined treatment with fisetin and LY294002 further downregulated the expression of OPN, collagen-I and α-SMA compared with LY294002 treatment alone. These results imply that fisetin-regulated phenotypic switching in MASMCs may be mediated by the PI3K/AKT pathway.

Discussion

Airway remodeling involves the abnormal restructuring of cells and molecules in asthmatic airways, and is characterized by goblet cell hyperplasia, subepithelial fibrosis, hyperproliferation, and hypertrophy of ASMCs [36]. ASMCs from asthmatic donors tend to shift towards a hypersecretory and hypercontractile phenotype compared to those from normal donors [6, 28]. The phenotypic switching of ASMCs can lead to airflow limitation and airway remodeling [37]. Thus, regulating ASMC phenotypic switching may be a novel approach for controlling airway remodeling.

Fisetin is a bioactive polyphenol flavonoid derived from various vegetables, fruits, and nuts [12]. It possesses diverse pharmacological characteristics, involving anticancer, anti-aging, and anti-inflammatory actions [38, 39]. Huang W and Wu S at el. revealed the advantages of fisetin in reducing airway inflammation in asthmatic mice [15, 16]. Nevertheless, the contributions of fisetin to airway remodeling are not well understood. Besides, fisetin has been demonstrated the capability to relax ASMCs and prevent airway smooth muscle constriction [40]. Whether fisetin regulates the synthetic ability of ASMCs is unknown. Given its widespread role as a senotherapeutic drug, discovering the effects of fisetin on asthma could expand its therapeutic potential.

Our experiments evaluated the impact of fisetin on asthmatic mice, focusing on the phenotypic switching of MASMCs. In this study, we identified 2551 asthma-related targets, 5153 ASMC-related targets, and 100 fisetin-related targets, yielding 34 common targets. A bar graph summary was generated using Metascape Gene List Analysis. Functional enrichment analysis discovered that these targets primarily participate in controlling cell

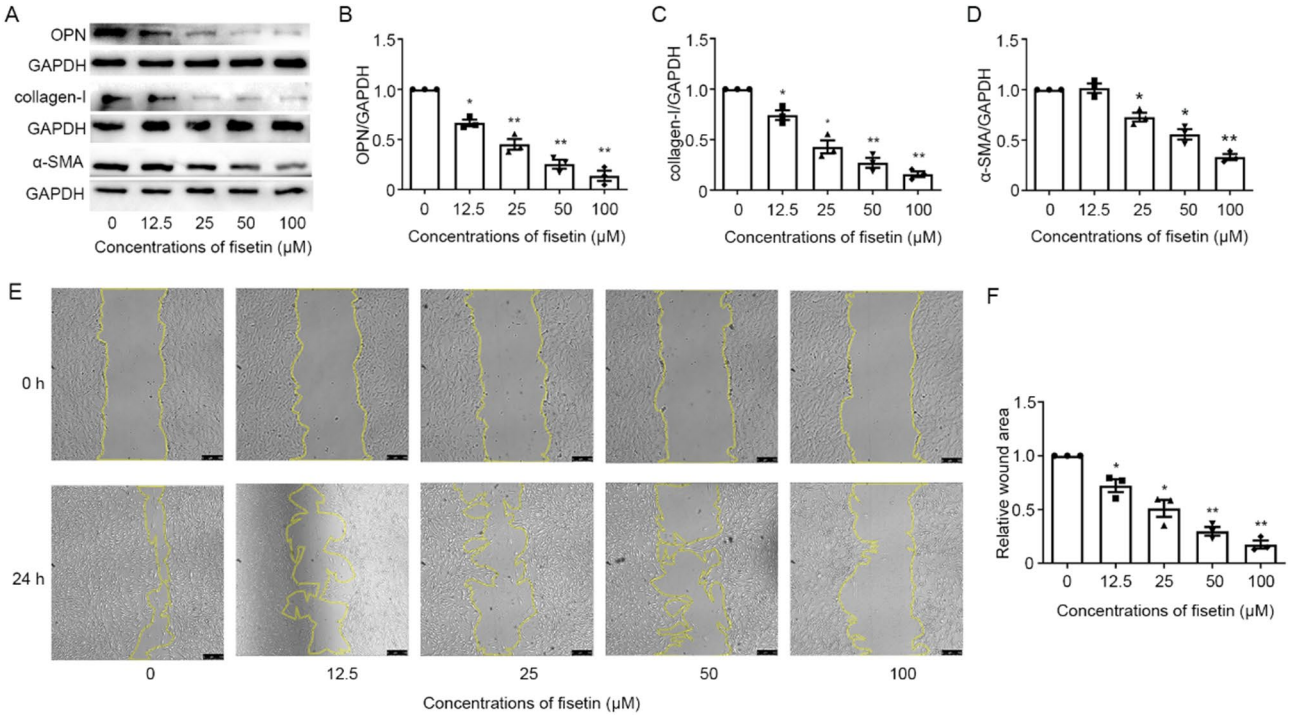

Fig. 8 Effects of fisetin on MASMCs phenotypic switching and migration. (**A**) Western blotting bands of OPN, collagen-I, α-SMA and GAPDH. (**B**, **C**, **D**) Quantification of protein expression ($n=3$). (**E**) Wound healing assay. (**F**) Relative wound area ($n=3$). Results are represented as mean ± SEM. $^{*}P<0.05$, and $^{**}P<0.01$ vs. control

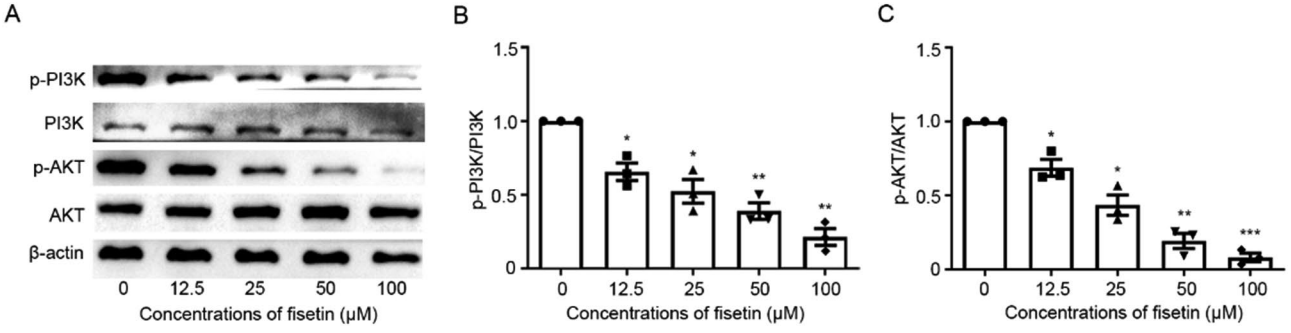

Fig. 9 Effect of fisetin on the PI3K/AKT pathway in MASMCs. (**A**) Western blotting bands of p-PI3K, PI3K, p-AKT, AKT and GAPDH. (**B**, **C**) Quantification of expression ($n=3$). Results are represented as mean ± SEM. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs. control

movement and tissue remodeling, which were partly confirmed in experiments using mouse models of asthma and MASMCs. We found that treatment with fisetin significantly suppressed airway inflammation and remodeling. Severe inflammation, characterized by increased Th2 cytokine levels and elevated inflammatory cell recruitment, was observed in mice challenged with OVA. Fisetin markedly attenuated the resulting airway inflammation. Additionally, fisetin significantly alleviated the pathological characteristics caused by OVA, such as airway smooth muscle thickening, subepithelial collagen accumulation and goblet cell hyperplasia. Given the significant role of enhanced airway smooth muscle mass in airway remodeling [28], we investigated how fisetin impacts MASMCs. Our results showed that fisetin inhibited the phenotypic switching and migration of MASMCs. This discovery offers new perspectives on the ability of fisetin to prevent remodeling, particularly its effects on ASMC phenotypic switching.

Smooth muscle cells transitioning to a synthetic phenotype generate more extracellular matrix components, which causes airway remodeling in asthma. OPN, also termed secreted phosphoprotein 1, functions as an extracellular matrix ingredient as well as a pro-inflammatory cytokine [41]. Asthmatic individuals exhibit elevated OPN levels in their lungs, particularly in ASMCs, epithelial cells, and myofibroblasts [42]. In addition, OPN subepithelial expression levels are related to disease

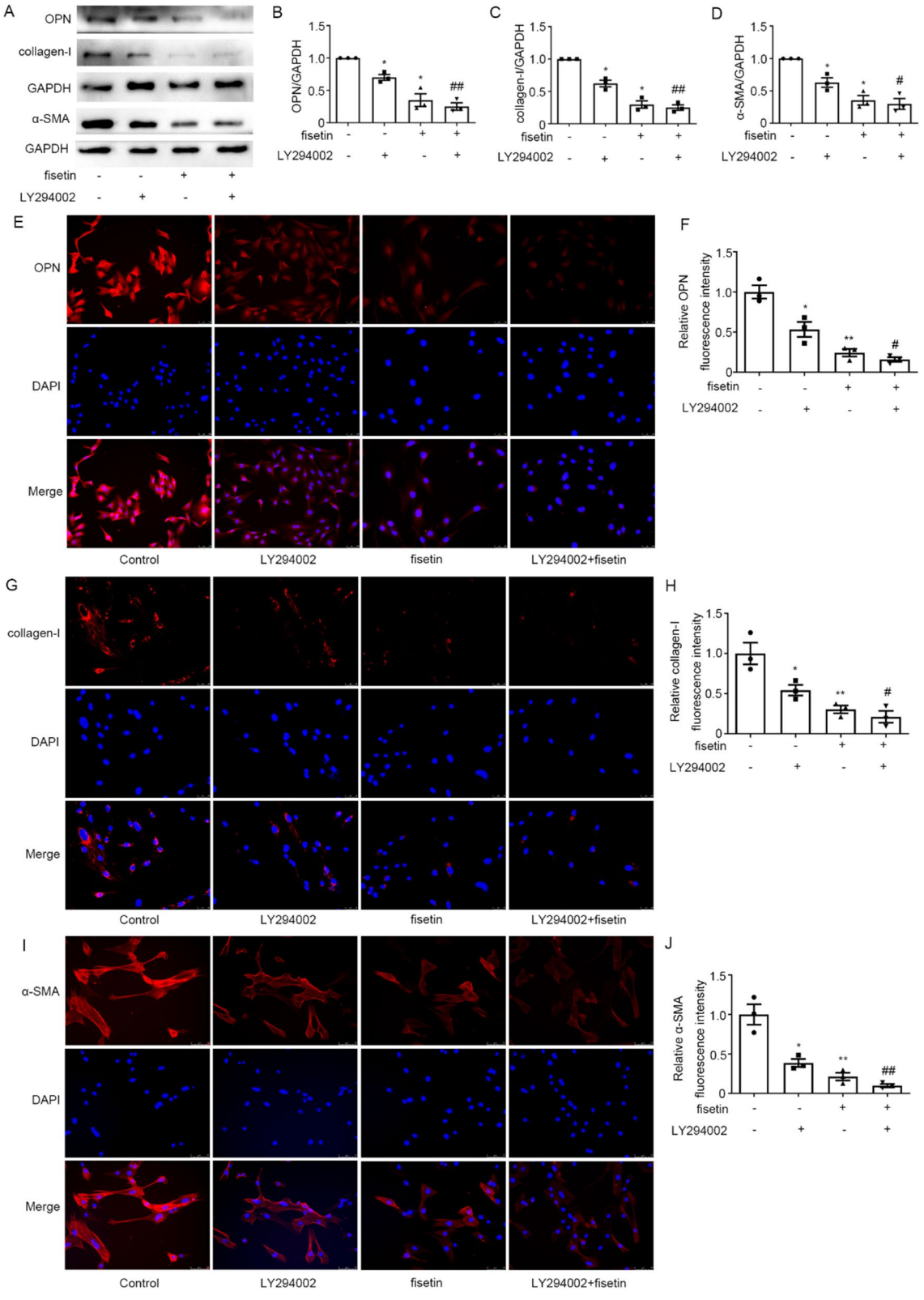

Fig. 10 Fisetin altered the MASMC phenotype by regulating the PI3K/AKT pathway. (**A**) Western blotting bands of OPN, collagen-I, α-SMA and GAPDH. (**B**, **C**, **D**) Quantification of expression ($n=3$). (**E**, **G**, **I**) Representative immunofluorescence of OPN, collagen-I and α-SMA (magnification ×200). (**F**, **H**, **J**) Relative immunofluorescence intensity of OPN, collagen-I and α-SMA ($n=3$). Results are represented as mean ± SEM. $^{*}P<0.05$ and $^{**}P<0.01$ vs. control; $^{\#}P<0.05$, and $^{\#\#}P<0.01$ vs. LY294002

severity [42]. Consistent with these findings, OPN levels are elevated in house dust mites and OVA-challenged asthmatic mice. Furthermore, OPN contributes to eosinophil recruitment by binding to eosinophil-recruiting chemokines [43]. OPN also promotes airway remodeling by enhancing the proliferation, migration, and collagen secretion of pulmonary fibroblasts [44]. Thus, we investigated this novel asthma biomarker in our study. We found that OPN levels were elevated in mice treated with OVA, but significantly decreased after administration of fisetin or DEX. Consistent results were obtained in vitro, showing that fisetin inhibited OPN expression in a dose-dependent manner. Collagen-I is not only a key marker of airway remodeling, but also an indicator of the synthetic phenotype of ASMCs [45]. In this study, fisetin inhibited the upregulation of collagen-I expression in both cell and animal experiments. Our findings suggest that fisetin may inhibit the transformation of synthetic phenotypes.

As one of the main contractile proteins, α-SMA is considered an important indicator of airway remodeling. Exorbitant α-SMA expression serves to accelerate cell proliferation, migration and contraction, resulting in airway remodeling and airway.

hyperresponsiveness [9, 29, 30]. In our study, the results demonstrated that α-SMA levels were elevated in mice treated with OVA, but significantly decreased after administration of fisetin or DEX. Further in vitro studies also revealed that fiestin inhibited α-SMA expression. These results indicate that fisetin may regulate the contractile phenotype of ASMC.

We further examined the effect of fisetin on the migration ability of MASMCs, and the results revealed that fisetin impeded MASMCs migration in a dose-dependent manner. Previous studies have shown that MASMCs characterised by the synthetic phenotype exhibit enhanced cell migration [31, 32]. Owing to the regulation effect of fisetin on the MASMC phenotype, we speculate that the inhibitory effects of fisetin on migration are related to its ability to regulate phenotypic switching.

Based on this, the molecular mechanisms of fisetin in asthma therapy are worthy of in-depth exploration. Network pharmacology analysis revealed that the enriched pathways were also linked to the PI3K/AKT pathway. According to the values obtained from Cytoscape software, 10 core targets were identified. Among them, AKT1 has the highest degree and median centrality values, prompting us to investigate the PI3K/AKT pathway.

The PI3K/AKT pathway is a critical signaling axis that coordinates cellular growth, mobility, and differentiation [46–48]. Multiple lines of evidence support that fisetin could suppress the PI3K/AKT pathway in different cells [49–51]. Our conclusions are consistent with previous studies. We found that fisetin dosage-dependently blocked PI3K and AKT phosphorylation in ASMCs. In addition, it was noted that the combination of fisetin and LY294002 suppressed OPN, collagen-I and α-SMA expression more effectively than either fisetin or LY294002 individually. These findings suggest that the ability of fisetin to inhibit the phenotypic switching of ASMCs may be mediated by the PI3K/AKT pathway. Research indicates that inhibiting PI3K/AKT signal transduction can attenuate airway remodeling and airway inflammation in asthma [52, 53]. We found that fisetin downregulated p-PI3K and p-AKT levels compared with those in asthmatic mice, indicating that the PI3K/AKT pathway is indispensable for the protective effect of fisetin on asthmatic airway remodeling.

Conclusion

Overall, our research demonstrated that fisetin regulates the phenotype switching of MASMCs and alleviates asthmatic airway remodeling by targeting the PI3K/AKT signaling pathway. Our study indicates that fisetin might be utilized as a treatment for asthma.

Abbreviations

ASMCs	Airway smooth muscle cells
OVA	Ovalbumin
PI3K	Phosphoinositide 3-kinase
AKT	Protein Kinase B
Th2	T helper type 2
OPN	Osteopontin
α-SMA	α-smooth muscle actin
NIH	National Institutes of Health
BALF	Bronchoalveolar lavage fluid
DEX	Dexamethasone
IL	Interleukin
ELISA	Enzyme linked immunosorbent assay
H&E	Hematoxylin and eosin
WAm	Bronchial smooth muscle area
Pbm	Bronchial basement membrane
MASMCs	Mouse airway smooth muscle cells
DMEM	Dulbeccos modified Eagle medium
CCK-8	Cell counting kit-8
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
SMILES	Simplified molecular input line entry system
SDF	Structure data file
OMIM	Online Mendelian Inheritance in Man
PPI	Protein-protein interaction
STRING	Search Tool for the Retrieval of Interaction Gene/Proteins
MMP9	Matrix metalloprotein 9
EGFR	Epidermal growth factor receptor
MCC	Maximal clique centrality

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-024-03005-8.

Supplementary Material 1: Additional file 1: Fig S1. The acquisition of genes related to ASMC, asthma and fisetin. (A) ASMC-related genes extracted from Genecards database. (B) Asthma-related genes extracted from Genecards database. (C) ASMC-related genes extracted from OMIM database. (D) Asthma-related genes extracted from OMIM database. (E) Fisetin-related genes extracted from Swiss Target Prediction database.

Supplementary Material 2: Additional file 2. Fig S2. All Western blotting images.

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Not applicable.

Author contributions
LYY and YQL designed the study and wrote the article. LB conducted the network analysis. LZ performed the animal experiments. LMJ conducted the cell experiments. ML and HC organized the experimental scheme. CJ revised the manuscript. All authors have approved the submitted version.All authors have agreed both to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Data availability
The data supporting the current study's findings are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
All animal experiments were approved by the Ethics Committee of the Second Affiliated Hospital of Shandong First Medical University (approval no: 2023-A-061).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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