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Scopoletin inhibits PDGF-BB-induced proliferation and migration of airway smooth muscle cells by regulating NF-κB signaling pathway

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Abstract
Background: Asthma is a common chronic inflammatory disease of the airway, and airway remodeling and the proliferation mechanism of airway smooth muscle cells (ASMCs) is of great significance to combat this disease.
Objective: To assess possible effects of scopoletin on asthma and the potential signaling pathway.
Materials and methods: ASMCs were treated PDGF-BB and scopoletin and subjected to cell viability detection by CCK-8 assay. Cell migration of ASMCs was determined by a wound closure assay and transwell assay. The protein level of MMP2, MMP9, calponin and α-SMA were measured using western blot. The levels of NF-κB signaling pathway were detected by Western blotting.
Results: Scopoletin inhibited proliferation of PDGF-BB - induced ASMCs. Also it suppressed the migration and invasion of PDGF-BB - induced ASMCs. We further showed that Scopoletin regulated phenotypic transition of ASMCs. Mechanically, Scopoletin inhibited proliferation and invasion of ASMCs by regulating NF-κB signaling pathway.
Conclusions: We therefore thought Scopoletin could serve as a promising drug for the treatment of asthma.

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Introduction

Asthma is a common chronic inflammatory disease of the airway.[1] It is characterized by inflammation, reversible airway obstruction, hyperresponsiveness, and airway remodeling. Common symptoms include wheezing, cough, chest tightness, and dyspnea, which are caused by a combination of genetic and environmental factors.[2,3] The exact pathogenesis remains to be studied. At present, asthma cannot be cured completely. Airway remodeling is one of the key features of asthma and is characterized by increased airway wall thickness, so abnormal proliferation of airway smooth muscle cells (ASMCs) is thought to contribute to airway remodeling.[4] Platelet-derived growth factor (PDGF)-BB is a cytokinin which is a key regulator stimulating multiple types of cells, and could induce the proliferation as well as motility of ASMCs, ultimately leading to asthma. Therefore, the study of airway remodeling and the proliferation mechanism of ASMCs is of great significance in the prevention and treatment of asthma.

Scopoletin is a phenolic coumarin isolated from a variety of plants and foods.[5] Scopoletin, also known as 6-methoxy-7-hydroxycoumarin, has obvious pharmacological activities, such as analgesia, anti-inflammatory, hypotensive and spasmolysis, especially antitumor and prevention and treatment of hyperuricemia.[6] In addition, its antioxidant activities have been indicated in multiple studies.[7,8] Scopoletin could inhibit the proliferation as well as motility of cervical cancer cells and promote the apoptosis of prostate cancer cells by regulating the PI3K/AKT signaling pathway.[8] Scopoletin also inhibits the proliferation of immature dendritic cells (DC) and has anti-angiogenic effects. In addition, scopoletin inhibits dendritic cell activation and the pathogenesis of autoimmune encephalomyelitis via nuclear factor kappa B (NF-κB) pathway.[9]

An important role of scopoletin in inflammatory response has been revealed widely. In diabetes, scopoletin inhibits inflammation and steatosis by modulating the TLR4–MyD88–NF-κB signaling pathway. It could also inhibit the expressions of Interleukin 4 (IL-4), IL-5, IL-10, and other allergic factors. Notably, scopoletin regulates the NF-κB signaling pathway, which has a key role in inflammation and cell growth. Therefore, we speculate that scopoletin may have an anti-asthmatic effect; however, the specific mechanism of action is not clear.

This study investigates the role of scopoletin in the progression of asthma. We constructed a PDGF-BB-induced ASMCs and provided the hypothesis that scopoletin affected the proliferation and invasion of ASMCs. We further investigated the possible regulatory mechanism and confirmed that scopoletin could serve as a promising drug for asthma.

Materials and methods

Cell culture

Human ASMCs were obtained from CHI Scientific Inc. (Jiangsu, China) and maintained in Dulbecco's modified eagle medium (DMEM; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Hyclone, UT, USA) and 100-U/mL penicillin–streptomycin in a humidified incubator hood aspirated with 5% CO_2 and incubated at 37°C. For PDGF-BB treatment, PDGF-BB (Sigma, St. Louis, MO, USA) was added at a concentration of 10 ng/mL to ASMCs for 24 h. Scopoletin (CAS: 92-61-5), bought from Sigma (S2500), was dissolved in dimethyl sulfoxide (DMSO) at 10 mM as a stock solution.[10]

Cell viability assay

ASMCs viability was measured through the Cell Counting Kit-8 (CCK-8) assay (Beyotime, China).[11] Briefly, ASMCs at a density of 2×10^3 cells for each well were seeded in 96-well plate and maintained in complete growth media for 24 h at 37°C. After indicated treatment, cells were removed from culture medium and exposed to CCK-8 reagent at 37°C for 1.5 h. The relative cell viability was assessed with microplate spectrophotometer at 450 nm (Bio-Rad, Hercules, CA, USA).

Wound healing assay

ASMCs upon indicated treatment were subjected to mechanical wound with a 20-μL pipette tip.[12] Afterwards, ASMCs were wiped with culture medium to remove cell debris. Then the cells were treated with complete culture medium to induce wound healing. Photographs were taken at 0 h and 24 h of scratch wound, and the relative distance of wound was measured.

Transwell assay

Briefly, about 3×10^4 ASMCs as indicated treatment were cultured in serum-free medium supplied with 20% Matrigel.[13] Then cells were plated in upper chambers containing filters (8.0-μm membrane pores), and complete culture medium supplied with 10% FBS was aspirated into lower chambers. After induction for 36 h, cells in upper chambers migrated into lower chambers. In order to measure the cell quantity in lower chambers, cells in bottom chambers were fixed and incubated with 0.5% crystal violet and cell colonies were counted manually.

Immunoblot

Proteins were subjected to lysis with RIPA buffer (Cell Signaling). The cell samples were then collected and subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene fluoride (PVDF) membranes, followed by blocking with 5% bovine serum albumin (BSA) in TBST buffer. Subsequently, membranes were conjugated with primary antibodies targeting MMP2 (1:1000; Abcam, Cambridge, UK), α-SMA (1:1000; Abcam), MMP9 (1:1000; Abcam), calponin (1:1000; Sigma), anti-p65 (1:2000; Abcam), anti-p-p65 (1:1000; Abcam), anti-IKKα (1:1000; Abcam), anti-p-IκBα (1:1000; Santa Cruz), anti-IκBα (1:1000; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:10000; Abcam) for 2 h at room temperature. Subsequently, the

membranes were incubated with specific secondary antibodies at room temperature for 1 h. The blots were analyzed with ECL kit.

Enzyme-linked-immunosorbent serologic assay (ELISA)

The concentration of vascular endothelial growth factor (VEGF) was quantified by ELISA kit following the manufacture's booklet.[14] Briefly, 50-ng/mL VEGF standard samples or cell lysate samples (about 1 mg of protein) were added to each well. Incubate at room temperature and add prepared biotin antibody to each well at room temperature. After addition of prepared Streptavidin solution, the TMB one-step development solution was added to each well. Incubate at room temperature following by stop solution to each well. Read at 450 nm immediately.

Statistical analysis

Data were displayed as mean ± SD. Statistical analysis was performed using SPSS. The statistical significance of the difference among more than two groups was analyzed using one-way ANOVA and Turkey's post-hoc test. $P < 0.05$ was considered as a level of significance.

Results

Scopoletin treatment reduces PDGF-BB-induced increased ASMC viability

In order to detect the role of scopoletin (Figure 1A) in the survival of ASMCs, CCK-8 assay was performed. Scopoletin alone at indicated concentration caused no obvious change in cell viability, suggesting that the use of scopoletin at these concentrations caused no harm on to cell viability (Figure 1B). PDGF-BB stimulation significantly induced growth of ASMCs growth to about 30%, compared with the control group. However, scopoletin treatment inhibited the cell growth in the PDGF-BB-induced ASMC in a dose-dependent manner (significant inhibition at a concentration of 20 μM) (Figure 1C). These data suggested that scopoletin is effective in improving PDGF-BB- suppressed cell growth.

Scopoletin suppresses ASMC migration induced by PDGF-BB

As previously reported, ASMC migration is considered as a key factor in the pathogenesis of asthma.[15] Therefore, the effect of scopoletin treatment on ASMC migration when stimulated with PDGF-BB was analyzed. The results of wound closure assay revealed that the increased wound closure ability induced by PDGF-BB was reversed by scopoletin treatment (Figure 2A). Moreover, transwell migration assays indicated that PDGF-BB stimulation greatly induced the migration of ASMCs, compared with the control group (Figures 2A and 2B). However, scopoletin partially inhibited cell migration promoted by PDGF-BB in a dose-dependent manner (Figures 2C and 2D).

Scopoletin regulated phenotypic transition of ASMCs

As previously demonstrated, the phenotypic modulation of ASMCs play a vital role in the pathogenesis of airway remodeling in chronic asthma. Thus, we evaluated the influence of scopoletin in the phenotypic transition of ASMCs. As shown in Figure 3A, the induced MMP-2 and MMP-9 expressions by PDGF-BB were greatly suppressed in ASMCs. As a critical component of contractile apparatus of ASMCs, α-SMA expression is closely associated with the progression of asthma.[16] Subsequently, the effect of scopoletin on the contractile phenotype switching of ASMCs was examined. The expression of calponin and α-SMA was reduced in PDGF-BB-induced ASMCs (Figure 3A). VEGF level

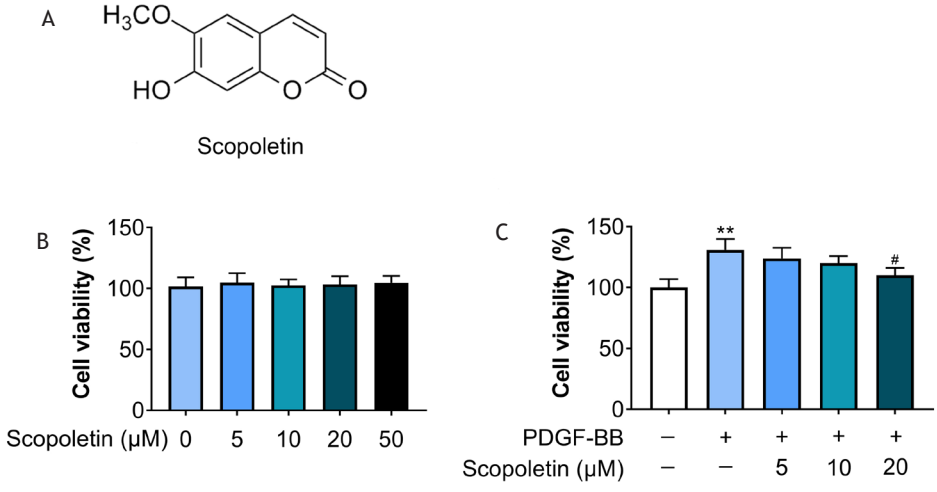

Figure 1 Scopoletin treatment suppresses PDGF-BB-induced ASMC growth. (A) Chemical structure of scopoletin. (B) CCK-8 assay detected the impact of scopoletin on ASMCs survival. (C) CCK-8 assay detected the impact of scopoletin on PDGF-BB-induced ASMCs survival. **$P < 0.01$, ***$P < 0.001$ vs. control, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. PDGF-BB.

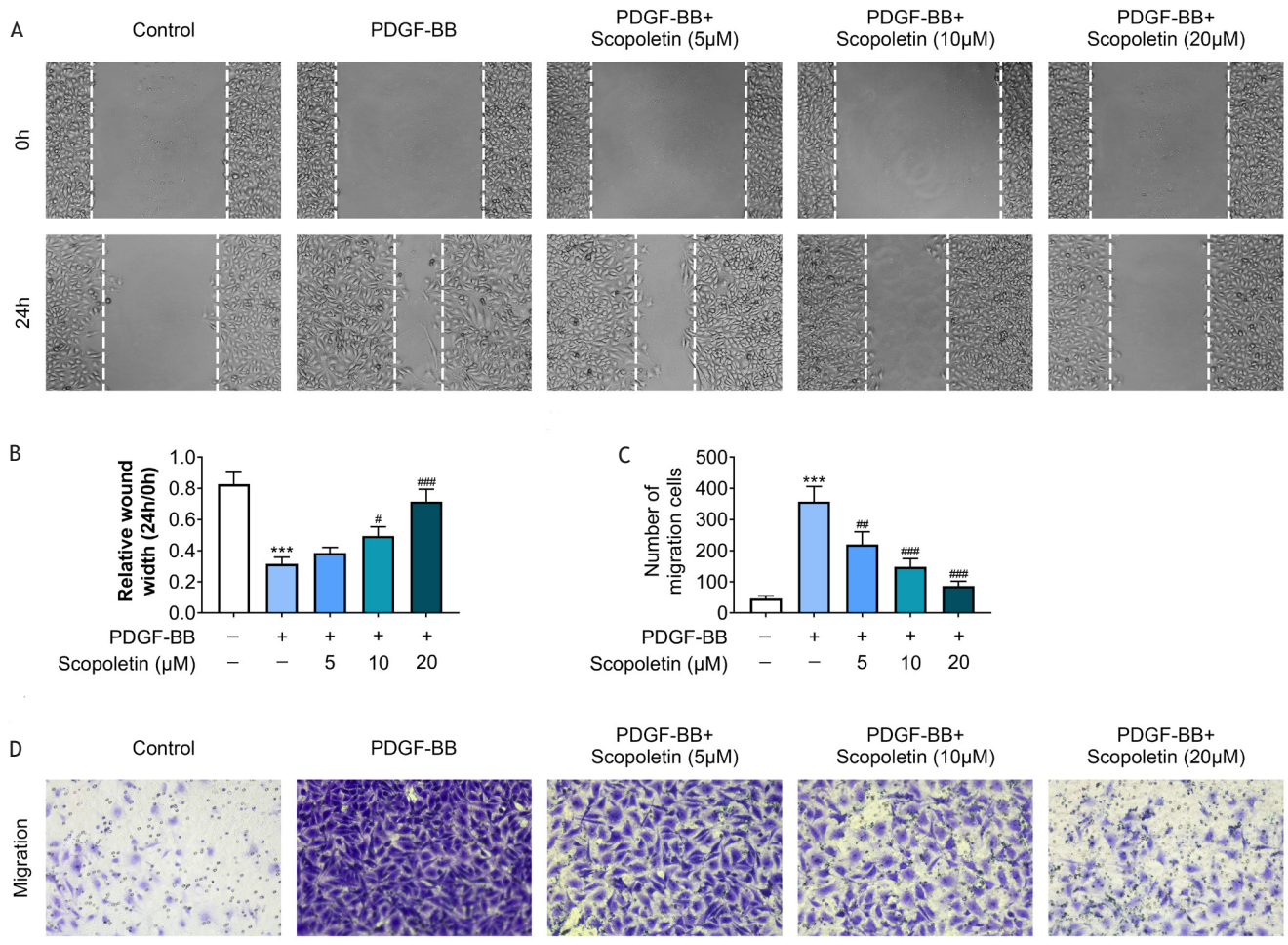

Figure 2 Scopoletin suppresses ASMC migration induced by PDGF-BB. (A) and (B) Wound closure assay revealed reduced cell migration in PDGF-BB-induced ASMCs. (C) and (D) Transwell assay detected cell migration in scopoletin-treated ASMCs induced by PDGF-BB. **P < 0.01, ***P < 0.001 vs. control, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. PDGF-BB.

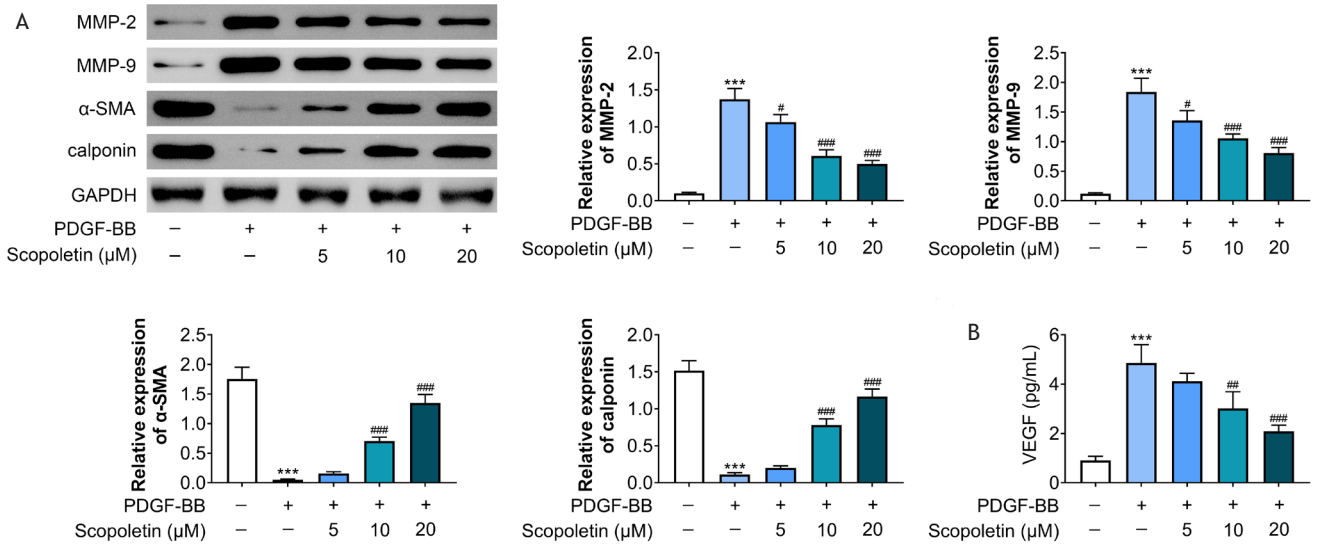

Figure 3 Scopoletin regulates phenotypic transition of ASMCs. (A) Expression of MMP-2, MMP-9, a-SMA, and calponin in scopoletin-treated ASMCs induced by PDGF-BB. (B) Level of VEGF in scopoletin-treated ASMCs induced by PDGF-BB. **P < 0.01, ***P < 0.001 vs. control, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. PDGF-BB.

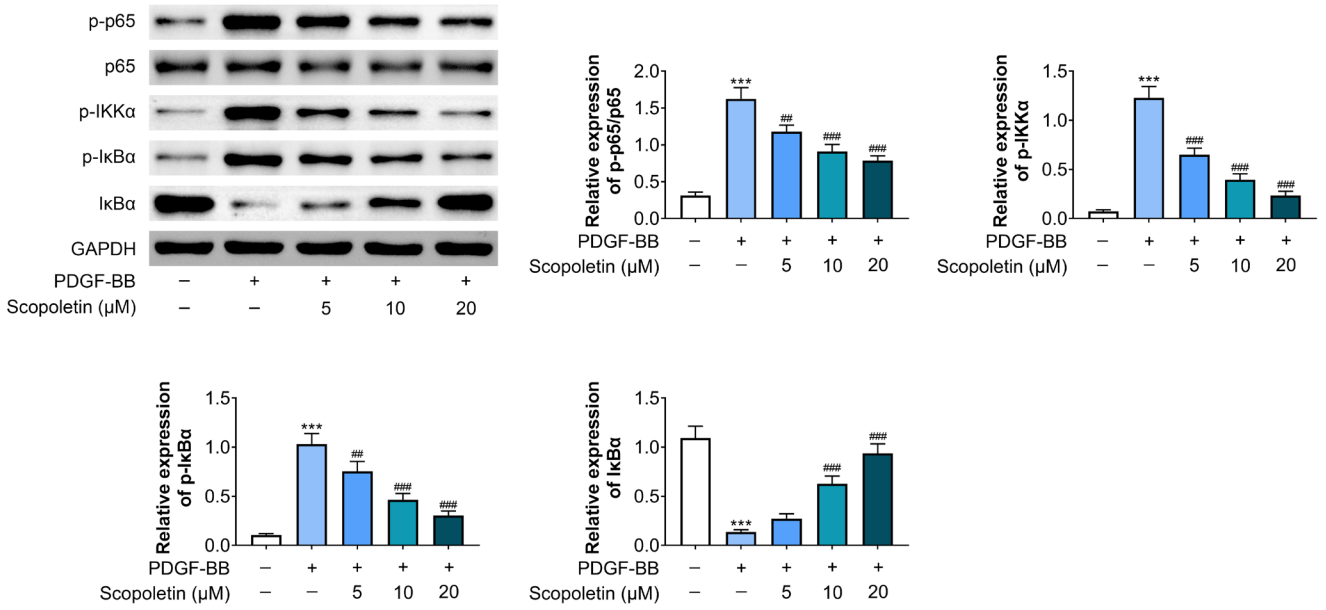

Figure 4 Scopoletin inhibits proliferation and invasion of ASMCs by regulating NF-κB signaling pathway. Expression of p-p65, p65, p-IKKa, p-IkBa, and IkBa in scopoletin-treated ASMCs induced by PDGF-BB. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. PDGF-BB.

was enhanced in PDGF-BB-induced ASMCs, but addition of scopoletin reversed its effect (Figure 3B).

Scopoletin inhibits proliferation and invasion of ASMCs by regulating NF-κB signaling pathway

NF-κB signaling plays an important role in regulating proinflammatory cytokines production, and recruiting eosinophils, which promotes allergic inflammation.[17] Activation of NF-κB pathway has been implicated in allergic asthma. To reveal the potential mechanism, the activation of NF-κB signaling pathway was analyzed (Figure 4). We also observed the enhanced p-p65, p-IKKα, p-IκBα, and reduced IκBα in PDGF-BB-treated cells (Figure 4). Scopoletin treatment partially relieved these altered proteins in a dose-dependent manner, implying that scopoletin inhibits proliferation and invasion of ASMCs by regulating NF-κB signaling pathway.

Discussion

Asthma is a common and frequently occurring disease that seriously endangers the health of young people. Asthma was once considered as a disorder of the immune system, but now it is more recognized as a disease of airway wall.[18] Airway remodeling plays a dominant role in the pathological development of asthma. The abnormal proliferation of ASMCs is considered to contribute to airway remodeling.[19] PDGF is a cytokinin which is a key regulator stimulating fibroblasts, glial cells, smooth muscle cells as well as stagnant cells.[16] Notably, PDGF-BB could induce the proliferation and motility of ASMCs, eventually leading to asthma.[20] Therefore, the study of airway remodeling and the growth of ASMCs are very important for treating asthma. In this study, we found that scopoletin inhibited proliferation of PDGF-BB-induced ASMCs. In addition, it suppressed the motility of PDGF-BB-induced ASMCs. We further demonstrated that scopoletin regulated phenotypic transition of ASMCs. Therefore, we thought that scopoletin could serve as a promising drug for treating asthma. However, the precise regulatory mechanism needs further study.

Scopoletin has a series of biological activities, such as antitumor and anti-inflammatory.[21] Previous study revealed the effects of scopoletin on growth performance as well as antioxidant activity in chickens. Scopoletin could attenuate intracerebral hemorrhage-induced brain injury in rats.[22] In addition, another study indicated that scopoletin induced cell death via the mitochondrial apoptosis pathway as well as cell cycle arrest.[7] Performing CCK-8, wound closure, and transwell assays, we here found that scopoletin suppressed the viability and motility of PDGF-BB-induced ASMCs.[23] ASMC migration is considered as a key factor in the pathogenesis of asthma. Therefore, we thought the effects of scopoletin on the viability and motility of PDGF-BB-induced ASMCs could affect progression of asthma. Similarly, scopoletin also inhibited the proliferation of immature dendritic cells, and suppressed the proliferation as well as motility of multiple types of tumor cells. These studies confirmed the critical functions of scopoletin in different types of diseases.

Previous studies have confirmed that scopoletin inhibits NF-κB signaling pathway to inhibit the activation of dendritic cells and the pathogenesis of experimental autoimmune encephalomyelitis.[24] Similarly, we here also revealed the effects of scopoletin on NF-κB pathway in PDGF-BB-induced ASMCs.[25,26] However, both the viability and motility of ASMCs were affected by scopoletin via this pathway, and this pathway has been confirmed to affect the proliferation and migration of multiple types of cells. In fact, the key role of NF-κB pathway in the pathology of asthma has been revealed widely.[27] NF-κB pathway is involved in

infection, inflammation, immune response, apoptosis, and other pathological processes.[28] NF-κB activation is closely related to asthma and other diseases. Inhibition of NF-κB has important clinical significance in the treatment of asthma.[27,29] In addition, multiple drugs treat or improve asthma through this signaling pathway. For example, Korean Red ginseng affected the ovalbumin (OVA)-induced asthma by modulating the NF-κB pathway.[7] Schisandrin B could also attenuate airway inflammation by mediating the NF-κB pathway in asthma animal model.[17] These studies confirmed that this pathway could serve as a promising target for asthma.

Conclusion

We established scopoletin inhibited proliferation and invasion of PDGF-BB-induced ASMCs, and regulated the phenotypic transition of ASMCs. Mechanically, scopoletin could mediate the NF-κB pathway, therefore inhibited PDGF-BB-induced proliferation as well as motility in ASMCs. We therefore believed that scopoletin could serve as a promising drug for asthma.

Competing interests

The authors state that there were no conflicts of interest to disclose.

Contribution of authors

Zhongxiang Fan and Dan Tang designed the experiments and Qiang Wu carried them out. Qun Huang analyzed and interpreted the data. Jie Song and Qiping Long prepared the manuscript with contributions from all coauthors.

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