Nuclear factor-κB mediates the phenotype switching of airway smooth muscle cells in a murine asthma model

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Abstract: Airway smooth muscle cells (ASMCs) phenotype modulation, characterized by reversible switching between contractile and proliferative phenotypes, is considered to contribute to airway proliferative diseases such as allergic asthma. Nuclear Factor-κB (NF-κB) has been reported as a key regulator for the occurrence and development of asthma. However, little is known regarding its role in ASM cell phenotypic modulation. To elucidate the role of NF-κB in regulating ASM cells contractile marker protein expression, and its impact on proliferation and apoptosis. We found that chronic asthma increased the activation of NF-κB in the primary murine ASM cells with a concomitant marked decrease in the expression of contractile phenotypic marker protein including smooth muscle alpha-actin (α-SMA). Additionally, we used the normal ASM cells under different processing to build the phenotype switching when we found the activation of NF-κB. Meanwhile, the expression of α-SMA in asthma was significantly increased by the NF-κB blocker. NF-κB blocker also suppressed asthma mouse ASM cell proliferation and promoted apoptosis. These findings highlight a novel role for the NF-κB in murine ASM cell phenotypic modulation and provide a potential target for therapeutic intervention for asthma.

Keywords: Asthma, airway smooth muscle cells, phenotype, NF-κB

Introduction

Asthma is one of the most common chronic diseases in the world, affecting over 300 million people. It is characterized by bronchoconstriction and airway hyper-responsiveness (AHR), followed by inflammatory manifestations in the respiratory system [1]. Airway smooth muscle cells (ASMs) play a key role in the development of asthma by converting from contractile phenotype to proliferation phenotype [2], which is the typical feature of ASM cells, called phenotypic modulation referring to the capacity of cells to exhibit reversible switching between contractile and proliferative phenotypes in response to different stimuli [3]. In vitro, modulation to a proliferative phenotype results from exposure of ASMCs to mitogenic stimuli, for instance PDGF, leading to increased proliferative activity and decreased contractile function [4]. Removal of growth factors, for example by serum deprivation or in the absence of PDGF, results in maturation of the cells to a contractile phenotype, characterized by increased expression of contractile protein marker such as smooth muscle alpha-actin (α-SMA), increased contractile function [4].

ASMCs phenotype modulation may contribute to the pathogenesis of asthma [3]. Proliferative ASMCs have an increased proliferative capacity, become mitotically active and exhibit a diminished abundance of contractile apparatus-associated proteins with a concomitant attenuation of responsiveness to contractile agonists [5]. Increased ASM mass may contribute substantially to AHR and declining lung function [5]. Therefore, identification of the mechanisms that underlie ASM phenotypic modulation may provide important pharmacological targets to prevent the altered proliferative and inflammatory responses of ASMCs.
The transcription factor nuclear factor-κB (NF-κB) has pivotal roles in inflammatory disorders [6]. There are several NF-κB family members: NF-κB1 (also called p50), NF-κB2 (p52), RelA (p65), c-Rel, and RelB [7]. The best-characterized NF-κB inhibitor is IκBα, which binds avidly to RelA/p65 [7]. Therefore, NF-κB has been described as an IκB-independent repression mechanism of NF-κB. Numerous studies have confirmed that NF-κB, especially p65, is over-activated in asthma [8, 9]. During the pathogenesis of asthma, NF-κB p65 may act at different levels of airway remodeling, such as regulating ASMCs proliferation and apoptosis. Results of previous studies about vascular smooth muscle cells showed that the expression and activation of NF-κB p65 was related to the expression of α-SMA, contributing to phenotypic switching of differentiated smooth muscle cells into the inflammatory state and enhancing the smooth muscle cell proliferation [10].

In this study, we present evidence that the NF-κB p65 is required for mouse ASMCs phenotypic modulation. We found that asthma ASMCs increased the expression of NF-κB p65 with a concomitant marked decrease in the expression of contractile phenotypic marker protein (α-SMA) and promoted murine ASM cells proliferation and decreased apoptosis. These changes were changed by pharmacological inhibitor (PDTC). PDTC was ispotenitly regarded to be a potent inhibitor of nuclear factor-κB (NF-κB). In addition, serum deprivation suppressed mouse ASM cell phenotypic modulation by inhibiting PDGF-induced NF-κB p65 and phosphorylation NF-κB p65 activation in normal murine ASM cells [4].

Materials and methods

Animals

Six-week-old female BALB/c mice (18-20 g) were obtained from Hunan SJA Laboratory Animal Co. in Changsha Hunan province and maintained in a pathogen-free environment at 22°C with 12 h dark/light cycle and free access to water and food in the animal facilities of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. All procedures were performed according to the guide line of Guangdong province and approved protocol from the IRB committee of SIAT.

Animal sensitization and allergen exposure

Female BALB/c mice were randomly divided into the asthma group and the control group (n=15), the protocol was shown in Figure 1. Mice in asthma group were sensitized with subcutaneous injections of 20 μg grade V ovalbumin (Sigma, USA) mixed in 50 ul of 36 mg/ml Imject ® Alum (Thermo Fisher Scientific, USA) on days 0, 14, 28, and or 42. Then from day 21, mice were challenged three different days a week in 25 cm×20 cm×20 cm container. The mice were sacrificed and sampled on day 51, as described in Materials and methods.

Lung plethysmography

Airway hyperresponsiveness (AHR) in mice was evaluated at day 51, by using an noninvasive whole-body plethysmograph (Buxco Electronics, USA) [11], registering the responses to rising doses of the bronchoconstrictor methacholine.
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**Figure 2.** Manifests and airway hyperresponsiveness of murine asthma. Noninvasive whole-body plethysmograph analysis of AHR (A). Airway inflammation was assessed based on HE staining. OVA-induced mice showed extensive infiltration by inflammatory cells around the pulmonary blood vessels and airways (C), while such effects were not observed in control animals (B). In the asthma group, there was an apparent increase in the thickness of smooth muscle layer (D) and airway wall (E). Results show the relative area of airway wall and airway smooth muscle based on the airway basement perimeter. n=10 mice; \(^*\) P < 0.01 versus control mice. Representative immunohistochemical staining of α-SMA in the lung tissue of airway smooth muscle layer (F and G).
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(0-100 mg/ml) (Sigma, USA). Airway hyperresponsiveness was analyzed as enhanced pause (Penh).

Murine ASMC culture

ASMCs were isolated with modification of previously reported method [12]. Briefly, twenty-four hours after the last challenge, the tracheas and lungs were completely isolated from sacrificed mice and then the extraneous membranes and connective tissues were carefully dissected away, cleaned of serosa, vasculature, and epithelia at room temperature, and washed three times in Hanks’ balanced salt solution (HBSS) (Hyclone, USA). The trachea and bronchus were grinded until the color of the tissue turned white, therefore, the remaining tissue mainly dominated by medium and small airway through this way. then the bronchus was cut into 1 mm×1 mm blocks which were tiled on 25 cm² flask and immersed into DMEM (Hyclone, USA) containing 20% FBS (Gibco, USA) with 1% penicillin-streptomycin (Gibco, USA). The cultures were incubated in a CO₂ incubator at 37°C. Medium were changed every two days. About one month the cells were 80-90% confluent, then they were detached with trypsin 0.25%-EDTA (Gibco, USA), and transferred to a new flask, and then non-attached cells were removed after 30 minutes. After this purifica-
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In the fifth to the eighth generation of passage cells were used for experiments. The cells were identified by morphology and anti-α-SMA (1:500, Abcam, USA) immune staining.

Hematoxylin and eosin staining and immunohistochemistry

The right middle lung from each mouse was fixed in 10% formalin, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin (HE). For immunohistochemistry analysis, tissue sections were deparaffinised and rehydrated. Then sample was treated at 95°C with Target Retrieval (Beyotime Biotechnology, Shanghai, China), blocked at room temperature using 1% BSA (Sigma) in DPBS (Gibco), and incubated with anti-α-actin (1:100) (Abcam), anti-NF-κB p65 (1:50) (Cell Signaling Technology, USA), then keeping the sections in 4°C refrigerator overnight. On the second day, after washing, sections were incubated for 30 min at room temperature with HRP-conjugated anti mouse (1:200) and with HRP-conjugated anti rabbit (1:200) (Abcam). Biotinylation was detected using DAB Color Developing Reagent Kit (Dako, USA). Negative control staining was performed by using DPBS instead of primary antibody.

Immunocytofluorescence and immunocytochemistry for murine ASM cells

Murine ASMCs were grown on glass coverslips in 24-well plates with DMEM containing 10% FBS with 1% penicillin-streptomycin for 2 days, in the same way, cells were directly grown in 24-well plates. Cells were exposed to primary mouse monoclonal antibody, α-SMA (1:100, dilution), anti-NF-κB p65 (1:50, dilution) and...
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anti-pho-NF-κB p65 (1:50, dilution) (Cell signaling technology) overnight, next morning the cells were incubated with Alexa 488-labeled secondary antibody and HRP-conjugated antibody (Abcam) respectively. Negative control staining was performed by using DPBS instead of primary antibody.

**Real-time polymerase chain reaction**

Cultured murine ASM cells were frozen in TRIzol solution (Invitrogen, USA). Total RNA was isolated according to the protocol for TRIzol and cDNA was transcribed from total RNA using ReverTra qPCR RT Master Mix gDNA remover. The primer sequences were as follows: for smMHC, 5'-GTGTGTTGTGCACCCCCCTC-3' (sense) and 5'-GATGTGAGCGCATCTCAT-3' (antisense); for calponin, 5'-TCTGCACATTTTAACGGTGTC-3' (sense) and 5'-GCCAGCTTGTTCTTACTTAC-3' (antisense); for α-SMA; 5'-GGCACCACTGAAACCTAGGC-3' (sense) and 5'-ACATACCGGTGTGTCACGAGG-3' (antisense); and for β-actin 5'-GTATCCATGAAATAGTTGACGGG-3' (sense) and 5'-GCAGTACAATTTACACAGGCAAT-3' (antisense). Quantitative real-time PCR

Figure 5. NF-κB p65 high expression with contractile marker protein decreased expression in asthma. (A) (Calmodulin), (B) (smMHC) and (C) (α-SMA) mRNA expressions in ASMcs were analyzed by Q-PCR, the contractile markers increased in control group (A and B), but α-SMA had no differences in statistics between two groups (C). α-SMA protein expression (30 µg whole-cell lysates) decreased in murine normal ASMcs (D), NF-κB p65 activation in each sample was expressed as the ratio of phospho-NF-κB p65 level to total NF-κB p65 level. NF-κB p65 activation increased significantly in murine ASMcs (E). Data shown are means ± SD of 10 mice. **P < 0.01 compared with the control group.
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A

\[ \text{α-SMA} \quad \text{Merge} \]

1dSFM

6dSFM

PDGF

B

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was performed with the use of QuantiFast SYBR Green PCR Kit (Invitrogen, USA) on Roche LightCycler 480II system (USA). Target gene expression was normalized to β-actin using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Murine ASMCs were harvested and lysed in lysis buffer (Beyotime, Shanghai, China). Protein extracts were then subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred to polyvinylidene fluoride (PVDF) (Sigma, USA) membranes. After incubation with a blocking buffer [5% non-fat milk in TBST] for 1 h, the membranes were first incubated with antibody against anti-NF-κB p65, anti-p-NF-κB p65 (1:1000 dilution), α-SMA (1:500 dilution), followed by incubation in 4°C refrigeration overnight, the next day the membranes were washed with TBST three times and incubated with the appropriate secondary antibody conjugated to HRP (Abcam, USA). Detection was performed by enzyme-linked chemiluminescence (ECL, Thermo, USA). Antibodies against GAPDH (1:1000) (Kangchen, Shanghai, China) were used for detecting the protein loading control. The gray value of NF-κB p65, pho-NF-κB p65, α-SMA and GAPDH was analyzed with Image J software.

Cell proliferation assay by MTT

Cells were cultured in 96-well plates at a density of 10,000 cells/well. The quantity of viable cells was estimated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, USA). MTT (20 µl of 5 mg/ml solution, Sigma, USA) was added to each well and incubated for 4 h at 37°C. The cells were then treated with 150 µl/well dimethyl sulfoxide (DMSO) (Sigma, USA) and the plates were vibrated on the shaking table. The absorbance of each well was determined in Multiskan Spectrum (Thermo Scientific) using an activation wavelength of 490 nm.

Flow cytometric analysis of apoptosis

An Annexin V-Alexa Fluor 488/PI Apoptosis Detection Kit (Beijing 4A Biotech Co., Ltd, China) was used to identify the translocation of phosphatidylserine (PS). Cells were harvested. the cell pellet was incubated in diluted binding buffer (containing 4 ml binding buffer with 12 ml DEPC-treated water (Invitrogen, USA)) to regulate the cell concentration of 1×10^6/ml. then 100 ul cell suspension was taken into 5 ml flow tube with 5 µl Annexin V/FITC and 10 µl PI (20 µg/ml) for 15 min in the dark. Control groups used DPBS instead of dyestuff. Apoptosis was determined by flow cytometry (BD Accuri C6, USA) and analyzed with BD C Sample flow cytometer (Becton-Dickinson). At least 10,000 events were analyzed for each sample.

Statistical analysis

All datas presented are expressed as the mean ± standard deviation of experiments repeated three or more times. The statistical analysis was performed by SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Student’s t-test (unpaired, two-tailed) or repeated measures data of ANOVA was performed to compare the means between two groups. The means of the different groups were compared using one-way analysis of variance. P < 0.05 was used to indicate a statistically significant difference.

Results

Establishment of murine asthma model

The feeding, activity and respiratory rhythm in the control group looked normal with no signs...
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Figure 7. Inhibitor of NF-κB increased ASMCs contractile marker protein expression. (A) phospho-NF-κB p65 expression in murine ASMCs treated with PBS (control), asthma or 100 µmol PDTC for 30 min were analyzed by Immunocytofluorescence. ***P < 0.01 versus control (normal group). ###P < 0.01 versus asthma group. Western blot analysis of effects of different doses of PDTC (0, 1, 25, 50, 100 µM) increased the expression of α-SMA (B) and decreased the ratio of phospho-NF-κB p65 level to total NF-κB p65 level (C) on murine asthma ASMCs in dose-dependent manner. GAPDH served as a loading control. *P < 0.05 versus asthma group, **P < 0.01 versus 1 µmol PDTC group. ***P < 0.01 versus 25 µmol PDTC group. PDTC suppressed proliferation (D, MTT, right; n=3). The early apoptosis cells in (E) (Flow cytometry: n=3) were at the bottom of right-hand side. Stimulation with 100 µM PDTC increased the number of early apoptosis cells.
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of asthma. In the asthma group, there was evidence of shortness of breath, irregular respiratory rhythm, a slight tremor of the limbs, irritability, cough, camponotus, cyanosis of lips and a reduction in activity. At higher levels of allergens, the mouth and nose appeared cyanotic. Firstly, we found that AHR was significantly increased in asthma mice (Figure 2). Meanwhile, OVA-induced mice showed extensive infiltration by inflammatory cells around the pulmonary blood vessels and airways, while such effects were not observed in control animals (Figure 2). The thickness of both the airway wall and the smooth muscle layer normalized by airway basement perimeter was also substantially greater in the chronic asthmatic mice than in the control mice (Figure 2), the smooth muscle layer with α-SMA was shown the obvious thickening in asthma mice (Figure 2).

Morphology and identification of ASM cells

ASMCs were observed under an inverted microscope. ASMCs presented fusiform or polygon shapes. There were 1-2 nuclei in the center of cells. There was a fascicular arrangement of cells in one region, which displayed a “peak-like” morphological feature (Figure 3). α-SMA immunofluorescent staining was used for identification of ASM cells. Under a laser scanning confocal microscope (leica tsc sp5, German) with a wavelength of 488 nm, FITC-labeled α-SMA appeared green, and they were uniformly distributed in the cytoplasm for the most part. DAPI-labeled nuclei exhibited blue fluorescence at a wavelength of 358 nm (Figure 3). α-SMA immunohistochemical staining was also used for identification of ASM cells. HRP-conjugated α-SMA appeared brown (Figure 3).

NF-κB p65 activation in asthma group

We further proved that NF-κB p65 activated in asthma group, immunohistochemical was performed to demonstrate marked induction of NF-κB p65 expression (Figure 4) in murine lung tissue. Immunofluorescence was also performed to demonstrate marked induction of NF-κB p65 (Figure 4) expression in murine ASM cells, NF-κB p65 exhibited green florescence after staining. The florescence signal intensity and density were significantly higher in asthmatic group relative to the control group. Compared with control group, murine lung tissue and ASMCs in asthma group proved higher activation of NF-κB p65.

NF-κB p65 high expression with contractile marker protein decreased expression in asthma

To investigate the role of NF-κB p65 in asthma group, we grouped and used QPCR to show the differences between contractile phenotype markers (including smMHC, calmodulin and α-SMA) of ASM cells (Figure 5). However, compared with control group, α-SMA protein expression in asthma decreased obviously, coupling with the high protein expression of NF-κB p65 and pho-NF-κB p65 (Figure 5).

Building the phenotype switching process with normal ASMCs can be found the activation of NF-κB

To further confirm the effects of NF-κB p65 in the mediation of phenotype, firstly, we used immunofluorescence which performed to demonstrate marked induction of α-SMA (Figure 6) protein in murine normal ASM cells that were growth arrested in serum-free media for 6 days (6dSFM) compared with 1dSFM (Figure 6), then 6dSFM murine normal ASM cells in culture were exposed to PDGF (20 ng/ml) for 24 h, 48 h and 72 h respectively. Normal ASM cells treated with PDGF (20 ng/ml) had decreased α-SMA expression by immunofluorescence and western blot. In contrast, the ratio of phospho-NF-κB p65 level to total NF-κB p65 protein expression was decreased in 6dSFM murine normal ASM cells. PDGF-induced normal ASMCs had increased the ratio of pho-NF-κB p65 level to total NF-κB p65 protein expression and the effects decreased in a time-dependent fashion.

Inhibitor of NF-κB increased ASM contractile marker proteins expression

We next asked whether targeting NF-κB p65 in ASMCs would also affect phenotype and function, PDTC was regarded to be a potent inhibitor of Nuclear factor-κB (NF-κB), which suppressed the production of the pho-NF-κB p65, murine asthma ASMCs in culture were exposed in the presence of different concentrations of PDTC (1 µmol, 25 µmol, 50 µmol, 100 µmol) for 30 min. As shown in Figure 7, we explored the effects of PDTC on suppressing the expression of pho-NF-κB p65, which was demonstrated by Immunocytofluorescence. With the different concentrations of PDTC, α-SMA protein expres-
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...sion increased in a dose-dependent fashion, however, the ratio of pho-NF-κB p65 level to total NF-κB p65 protein expression was decreased with PDTC in a dose-dependent manner.

To determine direct evidence that asthma cells were in pathological state, we then explored the role of NF-κB p65 in the regulation of cell state during proliferation and apoptosis, which was analyzed by MTT and flow cytometry after Annexin V/PI double staining. As shown in Figure 7, the proliferation of murine ASM cells was significantly enhanced in asthma group compared with the control, but these changes were reversed obviously in the presence of PDTC (100 µmol). As shown in Figure 7. The percentage of murine ASM cells in early apoptosis reduced in asthma group, but this reduction was also prevented by PDTC.

Discussion

Airway smooth muscle (ASM) responses play an important role in asthma, which is evident in the key hallmarks of asthma. Modulation towards a synthetic phenotype has an increased proliferative capacity and a decreased apoptotic capacity, exhibit a diminished abundance of contractile apparatus-associated protein (α-SMA) with a concomitant attenuation of responsiveness to contractile agonists, which contributed to the development of asthma. The major finding of this study was that compared with normal group, activation and high expression of NF-κB p65 concomitant with down-regulation of contractile marker protein (α-SMA) has been found in murine asthma lung tissue and ASMCs. Additionally, after serum deprivation of normal ASM cells, PDGF-induced down-regulation of ASMCs contractile marker protein was regulated by NF-κB p65. Pharmacological blockade of NF-κB p65 (PDTC) suppressed down-regulation of murine asthma ASM cells contractile marker in a dose-dependent manner, and PDTC inhibited proliferation and promoted apoptosis in asthma ASM cells. These findings suggest that NF-κB p65 activation may affect the expression of contractile marker protein and regulate phenotype of ASM cells, as Tadashi Yoshida and his colleagues demonstrated that results of the study provided clear evidence showing that NF-κB p65 activation within SMCs caused SMC phenotypic switching and NF-κB p65 activation within smooth muscle cells (SMCs) played a critical role in SMC phenotypic switching and neointima formation following vascular injury [10].

Molecular mechanisms that control the phenotype switching of ASMCs have not yet been identified. The ASMCs are believed to be remarkably plastic in that it can undergo rapid and reversible changes of its phenotype in response to a variety of different stimuli. Our results demonstrated that the differentiated phenotype of cultured ASMCs could be induced and maintained as the result of serum deprivation. To further confirm the relationship between NF-κB p65 and ASMCs contractile marker pro-
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As is well known, NF-κB, a multiprotein complex, is involved in early cellular defense reactions in higher organisms and plays a pivotal role in immune and inflammatory responses [22]. The present study is the first to show a requisite role for NF-κB p65 in modulating murine ASM cell phenotype by demonstrating that blockade of phospho-NF-κB p65 with PDTC prevented reductions of murine asthma ASMCs contractile marker. PDTC is a widely used pharmacological agent in molecular and cell biology and it has been reported to have both pro-oxidant and antioxidant properties. Using a molecular biology approach, we demonstrated that PDTC augmented α-SMA protein expression in dose-dependent manner. Antony Leonard et al reported that dynamic changes in the actin cytoskeleton played an important role in NF-κB signaling, and a functional and dynamic actin cytoskeleton as regulated by coordinate action of LIMK1 and SSH-1L was necessary for RelA/p65 nuclear translocation [23]. We also found that murine ASMCs proliferation was increased in asthma group and was antagonized by the selective NF-κB p65 blocker PDTC. Li J et al. demonstrated that inhibition of NF-κB activation using NF-κB decoy nanoparticles in vitro could attenuate proliferation of human PASM cells, they demonstrated a potential new link between inflammation (as reflected by high CRP plasma levels) and cell proliferation in hPASMCs [24]. Whereas murine ASMCs apoptosis was decreased in asthma group and the effects could be reversed by PDTC. Hyun Jeong Kwak et al. reported that PDTC exerts protective effects against airway inflammation, and that HO-1 induction may be at least partly responsible for its action [25]. Our data demonstrated that NF-κB p65 up-regulation was required for ASMCs dedifferentiation, proliferation, and growth, leading to airway remodeling and airway inflammation development. Therefore, investigating the pathways responsible for NF-κB p65 up-regulation is vital to understanding the progression of airway disease. Blockade of NF-κB p65 represents a potential treatment for reducing airway remodeling and possibly the progression of chronic asthma. Tadashi Yoshida et al proved that NF-κB activation within SMCs causes SMCs phenotypic switching and neointima formation in concert with Klf4. NF-κB inhibitors exhibiting an affinity for SMCs would be a candidate for treatment of vascular diseases including atherosclerosis [10, 26].

In conclusion, the principal finding of this work are findings of this work as follows: (a) The phenotype of asthma ASMCs is synthetic-based, and the asthma group can be found the activation and high expression NF-κB. (b) In asthma group, NF-κB inhibitor can induce the synthetic phenotype to contractile phenotype, which suggests that NF-κB is a key factor in the phenotypic transformation of ASMCs. (c) NF-κB takes part in the phenotype switching of ASMCs, proliferation and apoptosis in asthma group, so it is expected to become a novel target for asthma treatment.

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Disclosure of conflict of interest

None.

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