

Wnt/ β -catenin signaling pathway regulates asthma airway remodeling by influencing the expression of c-Myc and cyclin D1 via the p38 MAPK-dependent pathway

XIAO-XIAO JIA^{*}, TING-TING ZHU^{*}, YUE HUANG, XIN-XIN ZENG, HONG ZHANG and WEI-XI ZHANG

Department of Pediatric Allergy and Immunology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325027, P.R. China

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Abstract. Airway remodeling is the main characteristic of asthma; however, the mechanisms underlying this pathophysiological change have not been fully elucidated. Previous studies have indicated that the Wnt/ β -catenin and mitogen-activated protein kinase (MAPK) signaling pathway are involved in the development of airway remodeling during asthma. Therefore, the present study established an airway remodeling rat model, after which β -catenin, cyclin D1 and c-Myc protein expressions were analyzed via western blotting in the lung tissue and airway smooth muscle cells (ASMCs) of rats. The mRNA expression of the aforementioned proteins were evaluated via reverse transcription-quantitative PCR. β -catenin, cyclin D1 and c-Myc are core transcription factors and target genes of the Wnt/ β -catenin and MAPK signaling pathways. Furthermore, β -catenin, c-Myc and cyclin D1 protein expression were determined following blocking of the p38 MAPK signaling pathway *in vitro*. The results demonstrated that higher expressions of β -catenin, cyclin D1 and c-Myc were detected in lung tissues and ASMCs in the asthma group compared with the control. Blocking the p38 MAPK signaling pathway with a specific inhibitor SB203580 also downregulated the expressions of β -catenin, cyclin D1 and c-Myc *in vitro*. Taken together, these results indicated that the Wnt/ β -catenin signaling pathway

may regulate the process of airway remodeling via the p38 MAPK-dependent pathway.

Introduction

Airway inflammation and remodeling are the main pathological features of asthma (1). Airway remodeling is regulated by various cells and cellular elements, including airway smooth muscle cells (ASMCs), transforming growth factor- β , matrix metalloproteinase-9, interleukin (IL)-4 and IL-13. Although asthma can be controlled by medication, the complicated mechanisms underlying this pathophysiological process are not fully understood. Currently, there are no treatments that can effectively inhibit the airway remodeling process. It is therefore important to clarify the mechanism of asthmatic airway remodeling to develop better clinical approaches for the prevention and treatment of this condition.

The Wnt/ β -catenin signaling pathway participates in various biological activities and is considered to serve an essential role in many pulmonary diseases, including pulmonary fibrosis, lung cancer and pulmonary hypertension (2,3). It has been demonstrated that dysregulation of the Wnt/ β -catenin signaling pathway is inextricably linked to the pathogenesis of asthmatic airway remodeling (4). β -catenin is a key nuclear effector of canonical Wnt signaling, which is closely associated with asthma pathogenesis by modulating the development of airway remodeling (4-6). Mitogen-activated protein kinase (MAPK) is activated in response to many extracellular stimuli, including growth factors, cytokines and environmental stress, and it is also hypothesized to be integral to asthma pathogenesis (7,8). Previous studies have identified a potential interaction amongst certain signaling pathways, including the Wnt/ β -catenin and MAPK pathways (9-11). The p38 MAPK pathway is one of the important branching pathways of MAPK that is likely activated by Wnt proteins to regulate the development of mesenchymal cells into osteoprogenitors (12). Given that Wnt/ β -catenin and MAPK are associated with asthma, it was hypothesized that the interaction of Wnt/ β -catenin and MAPK may influence the process of asthma airway remodeling. The protein product of the proto-oncogene c-Myc is a critical downstream effector of cellular proliferation induced by the Wnt/ β -catenin and MAPK signaling pathways (13). Similarly

Correspondence to: Dr Wei-Xi Zhang, Department of Pediatric Allergy and Immunology, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, 109 West Xueyuan Road, Wenzhou, Zhejiang 325027, P.R. China
E-mail: zhangweixi112@163.com

^{*}Contributed equally

Abbreviations: ASMC, airway smooth muscle cell; MAPK, mitogen-activated protein kinase; Wam, area of smooth muscle; Wat, area of airway wall; Pbm, perimeter of basement membrane

Key words: airway remodeling, asthma, β -catenin, Wnt, p38 mitogen-activated protein kinase

to c-Myc, cyclin D1 is a target gene for the Wnt/ β -catenin and MAPK pathways (14,15).

Therefore, the present study assessed whether the Wnt/ β -catenin signaling pathway participated in the development of airway remodeling during asthma and determined the molecular signaling pathways involved in the process.

Materials and methods

Rat asthma models. All animal experiments were conducted in accordance with the standards set by the Chinese Academy of Experimental Animal Management Practices and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The present study was approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University & Laboratory Animal Centre of Wenzhou Medical University (approval no. wydw 2015-0039). A total of 48 healthy specific pathogen-free (SPF) grade male Sprague-Dawley rats (6-8 weeks old; weight, 180-200 g) were purchased from Shanghai Slack Experimental Animal Company and kept in an SPF animal facility at the Laboratory Animal Centre of Wenzhou Medical University. All animals were housed in an environmental temperature of $22\pm 1^\circ\text{C}$, relative humidity of $50\pm 1\%$ and a 12 h light/dark cycle with free access to food and water. A rat asthma model was established by sensitization and stimulation with chicken egg ovalbumin (OVA; Thermo Fisher Scientific, Inc.) as previously described (16). Intraperitoneal injection of OVA (1 mg) and aluminum hydroxide (100 mg; Beyotime Institute of Biotechnology) in 1.5 ml normal saline was administered on the 1st and 8th day of the sensitization period. Subsequently, rats were challenged with OVA (0.1 mg/ml) aerosol via a jet nebulizer (PARI, GmbH) for 30 min every alternate day for 60 days starting from day 15. Rats in the control group were sensitized and challenged with normal saline (17). This experimental procedure was performed in a plastic box with a hole through which the OVA aerosol was transported. The canonical Wnt/ β -catenin pathway was blocked by recombinant rat Dickkopf-related protein (DKK)-1 (rrDKK-1; 10 $\mu\text{g}/\text{ml}$ diluted in phosphate buffer saline (PBS), R&D Systems, Inc.). In the rrDKK-1 group, rrDKK-1 was injected intraperitoneally 30 min before each inhalation of OVA aerosol. Rats were subsequently treated the same as those of the asthma group. Finally, all rats in experimental groups were euthanized using 3% isoflurane gas mixed with air, after which lung tissue was removed. Death was confirmed after 1-2 mins of cardiac and respiratory observation.

Morphological changes of airways. Lung tissue was carefully collected under sterile conditions. The upper and lower lobes of the right lung hilar segment were clipped, and then fixed using 4% paraformaldehyde immersion for 4 h at room temperature. The solid lung tissue was routinely embedded in paraffin and cut into 4 μm thick slices. Subsequently, sections were stained with hematoxylin for 5 min and eosin for 2 min at room temperature using a hematoxylin and eosin staining kit (Beijing Solarbio Science & Technology Co., Ltd.). Masson's trichrome staining was performed using a kit (Beijing Solarbio Science & Technology Co., Ltd.) in accordance with the manufacturers' protocol to observe subepithelial collagen

deposition at room temperature for a total of 15 min (Weigert's iron hematoxylin, 8 min; bieberich scarlet, 5 min; aniline blue, 2 min). The pathological structure of airway remodeling in lung tissue was observed and quantified under a light microscope (Nikon Corporation; magnification, $\times 200$) with an image analysis system. The area of smooth muscle (Wam) and area of airway wall (Wat) were determined by morphometric analysis (Image-Pro Plus 6.0; Media Cybernetics Inc.). The perimeter of basement membrane (Pbm) was used to normalize Wat and Wam. Thus, the ratios of Wat to Pbm (Wat/Pbm) and Wam to Pbm (Wam/Pbm) were used to evaluate airway remodeling. Bronchioles with 150-200 μm inner diameter were selected for observation.

Isolation and culture of ASMCS. Rats were sacrificed after the last nebulization, and their thoracic cavities were opened with surgical scissors. The trachea and lung were separated rapidly and transferred to PBS at 4°C . The lung tissue, blood vessels and adipose tissue that adhered to the trachea were removed under the microscope. Next, the trachea and bronchi were dissected longitudinally, and the adventitia and intima were scraped. The remains of bronchi were cut into small pieces that were < 1 mm in diameter. Trypsin was used to digest and remove impurities such as epithelial cells and fibroblasts, and ASMCS of high purity were obtained then subcultured. Samples were then cultured in DMEM (Thermo Fisher Scientific, Inc.) containing 20% fetal bovine serum (Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37°C . ASMCS were identified by observing the typical 'hill and valley' growth pattern (18) and via the immunocytochemistry staining of α -smooth muscle actin (1:100; Abcam; cat. no. ab32575). Passage 4-6 of ASMCS were used for the experiments.

ASMC identification. Adherent ASMCS were collected and made into a suspension prior to inoculation into a sterile cell smear on a 12-well plate. At 70-80% confluence, cells were washed three times with PBS then fixed in 4% paraformaldehyde for 15 min at room temperature. Following washing and sealing, cells were incubated in rabbit monoclonal anti- α -smooth muscle actin antibodies (as aforementioned) at 4°C overnight. Next, all the cells were incubated with a species-specific secondary antibody treatment (sheep anti-rabbit; 1:100; Beijing ZhongshanJinqiao Biotechnology Co., Ltd.; cat. no. PV-9001) at room temperature for 1 h. Following washing, drying, and H&E staining, microscopic examination was used to confirm whether the cells were ASMCS.

Western blot analysis. The inhibitor for p38 MAPK, SB203580 (Selleck Chemicals), was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C . The inhibitor was diluted to the specified concentrations with culture medium before use for western blot experiments. Protein expressions of β -catenin, phosphorylated (p) β -catenin, cyclin D1, and c-Myc in lung tissues and ASMCS were assessed by western blot analysis. Total protein was separated from the right lung of rats and also from ASMCS. The protein concentration was measured using the bicinchoninic acid method. Proteins (20 μg) were separated via 12% SDS-PAGE (300 mA) on a gel. Proteins were then transferred onto PVDF membranes then blocked for 2 h in 5% skimmed milk at room temperature.

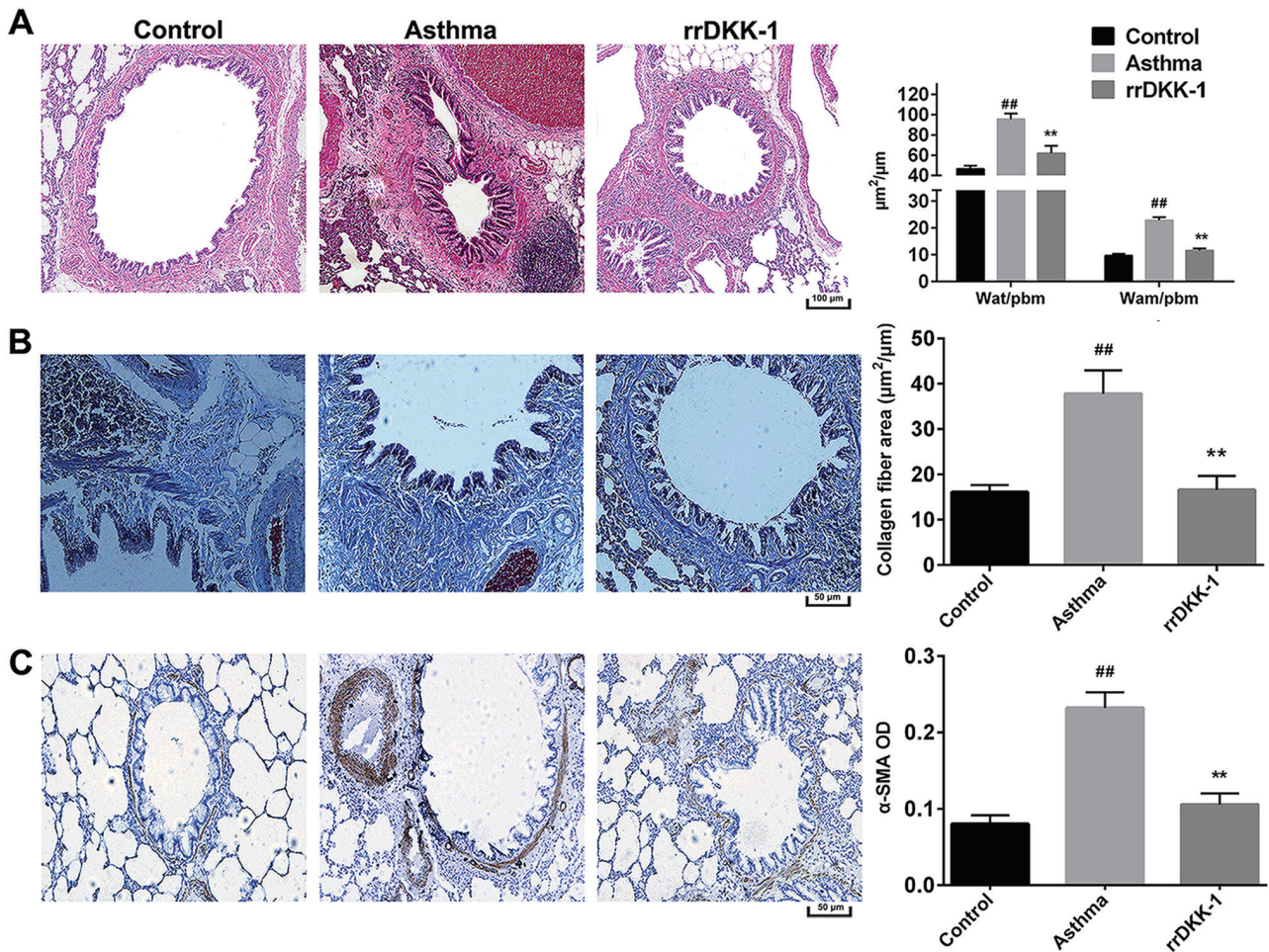


Figure 1. Inhibition of the Wnt signaling pathway using rrDKK-1 reduces airway remodeling in chronic asthma rats. (A) Hematoxylin and eosin staining of lung tissue and the quantitative analysis of the airway wall and smooth muscle layer thickness (magnification, $\times 100$). (B) Masson's trichrome staining of lung tissue demonstrating collagen deposition and the quantitative analysis of collagen fiber area (magnification, $\times 200$). (C) Immunohistochemistry α -SMA staining and the semi-quantitative analysis of α -SMA expression using Image-Pro Plus software (magnification, $\times 200$). Data were expressed as the mean \pm standard deviation ($n=6$). ^{##} $P<0.01$ vs. control group; ^{**} $P<0.01$ vs. asthma model group. rrDKK-1, recombinant rat Dickkopf-related protein-1; α -SMA, α -smooth muscle actin; OD, optical density.

Membranes were incubated overnight with the corresponding monoclonal primary antibodies against β -catenin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 2677S), phosphorylated (p) β -catenin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 9561S), β -actin (1:1,000; Abcam; cat. no. ab8226), cyclin D1 (1:5,000; Abcam; cat. no. ab134175), c-Myc (1:1,000; Abcam; cat. no. ab190026), p-p38 MAPK (1:1,000; Affinity Biosciences; cat. no. AF4001) and p38 MAPK (1:5,000; Thermo Fisher Scientific, Inc.; cat. no. 33-1300) at 4°C . Membranes were washed three times in Tris-buffered saline and Polysorbate 20 then incubated in horseradish peroxidase-conjugated anti-rabbit IgG secondary antibodies (1:500; Jackson ImmunoResearch Laboratories, Inc.; cat. no. 111-035-003) for 2 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.), with β -actin as the loading control.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA in rat lung tissues and ASMCs were extracted by TRIzol (Takara Biotechnology Co., Ltd) according to the manufacturer's instructions. First-strand cDNA was generated from $4\ \mu\text{g}$ of total RNA using a Maxime

RT PreMix kit (Takara Bio, Inc.) to prime the reverse transcription reaction, according to the manufacturer's protocol. qPCR was carried out using a Real-Time PCR System 7500 (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR reaction mixture consisted of $10.4\ \mu\text{l}$ of 2xSYBR Green 1 Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), $0.4\ \mu\text{l}$ of both forward and reverse primers, $2\ \mu\text{l}$ of sample cDNA solution and distilled water, giving a final volume of $20\ \mu\text{l}$. Primer sequences were as follows: GAPDH forward, 5'-AGTGTGTTTTCCAGCTTGGGTAAT-3' and reverse 5'-CCACTCAGCTTTAAATCCGCT-3'; β -catenin forward, 5'-ACCATCGAGAGGGCTTGTG-3' and reverse, 5'-CGC ACTGCCATTTAGCTCC-3'; c-Myc forward, 5'-ACCATC GAGAGGGCTTGTG-3' and reverse, 5'-CGCACTGCC ATTTAGCTCC-3' and cyclin D1 forward, 5'-ACCATCGAG AGGGCTTGTG-3' and reverse, 5'-CGCACTGCCATTTA GCTCC-3'. Thermocycling conditions were as follows: 95°C preconditioning for 2 min, denaturation for 90 sec at 95°C , annealing for 5 sec at 95°C and amplification for 30 sec at 58°C for 40 cycles. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for normalization. The expression of target mRNA was determined using the $2^{-\Delta\Delta\text{Ct}}$ method (19).

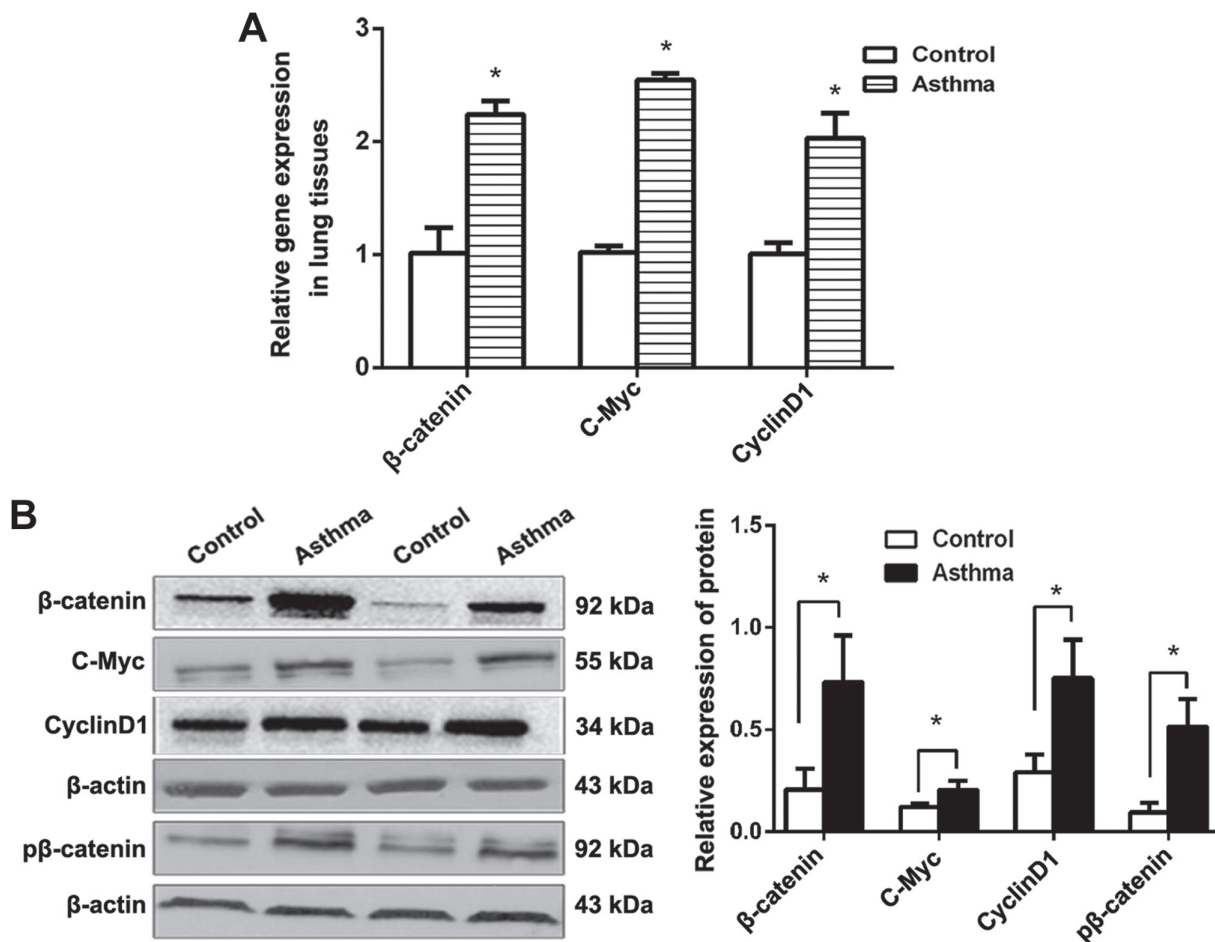


Figure 2. Expression of β -catenin, p- β -catenin/ β -catenin, c-Myc and cyclin D1 levels in the lung tissue of rats. (A) mRNA expression of β -catenin, c-Myc and cyclin D1 in lung tissue were higher in asthma group compared with the control group. (B) Protein expressions of β -catenin, p- β -catenin/ β -catenin, c-Myc and cyclin D1 were higher in asthma group (n=6). *P<0.05 vs. control group. p, phosphorylated.

Statistical analysis. Data were expressed as mean \pm standard deviation. Comparisons were performed utilizing one-way analysis of variance followed by Fisher's exact test. Significant differences between groups were tested with the unpaired t test, whereas linear correlation analysis was used for bivariate correlation. P<0.05 was considered to indicate statistical significance.

Results

Blocking the Wnt signaling pathway reduces asthma airway remodeling. A rat model of airway remodeling was established through repeated OVA sensitization, where rats were subjected to the OVA challenge every other day for 60 days. Obvious airway remodeling was observed through H&E staining of lung tissues. Compared to the control group, the asthma model rats developed an obvious increase of airway wall and airway smooth muscle thickness (Fig. 1). However, blocking the Wnt signaling pathway significantly reduced this effect. To assess whether the Wnt signaling pathway was involved in the pathogenesis of asthma airway remodeling, the airway structural changes following administration of rrDKK-1, a specific inhibitor of Wnt signaling pathway, was assessed. Administration of rrDKK-1 significantly inhibited the structural changes and lightened the collagen deposition compared

to the model group (P<0.01; Fig. 1A and B). In addition, blocking Wnt signaling by rrDKK-1 significantly decreased α -SMA expression (P<0.01; Fig. 1C).

β -catenin, c-Myc and cyclin D1 expression are elevated in the lung tissue of asthma group rats. In lung tissue, β -catenin, c-Myc and cyclin D1 mRNA expressions were all significantly higher in the asthma group compared with the control group (P<0.05; Fig. 2A). Similar results for β -catenin, p- β -catenin/ β -catenin, c-Myc and cyclin D1 protein expression were observed (P<0.05; Fig. 2B). In addition, the protein expression of β -catenin were positively associated with c-Myc and cyclin D1 (P<0.05; Fig. 3A).

β -catenin, c-Myc and cyclin D1 expression are elevated in ASMCs isolated from asthma group rats. ASMCs isolated from bronchial tissue were confirmed by 'hill and valley' exhibition of their features under phase-contrast microscopy (Fig. 4A) and by positive α -SMA expression determined via immunohistochemical staining (Fig. 4B). ASMCs in the asthma group exhibited higher mRNA (Fig. 4C) and protein (Fig. 4D) expression of β -catenin, p- β -catenin/ β -catenin, c-Myc and cyclin D1 compared with the control. The protein expression of β -catenin was positively associated with c-Myc and cyclin D1 (P<0.05; Fig. 3B).

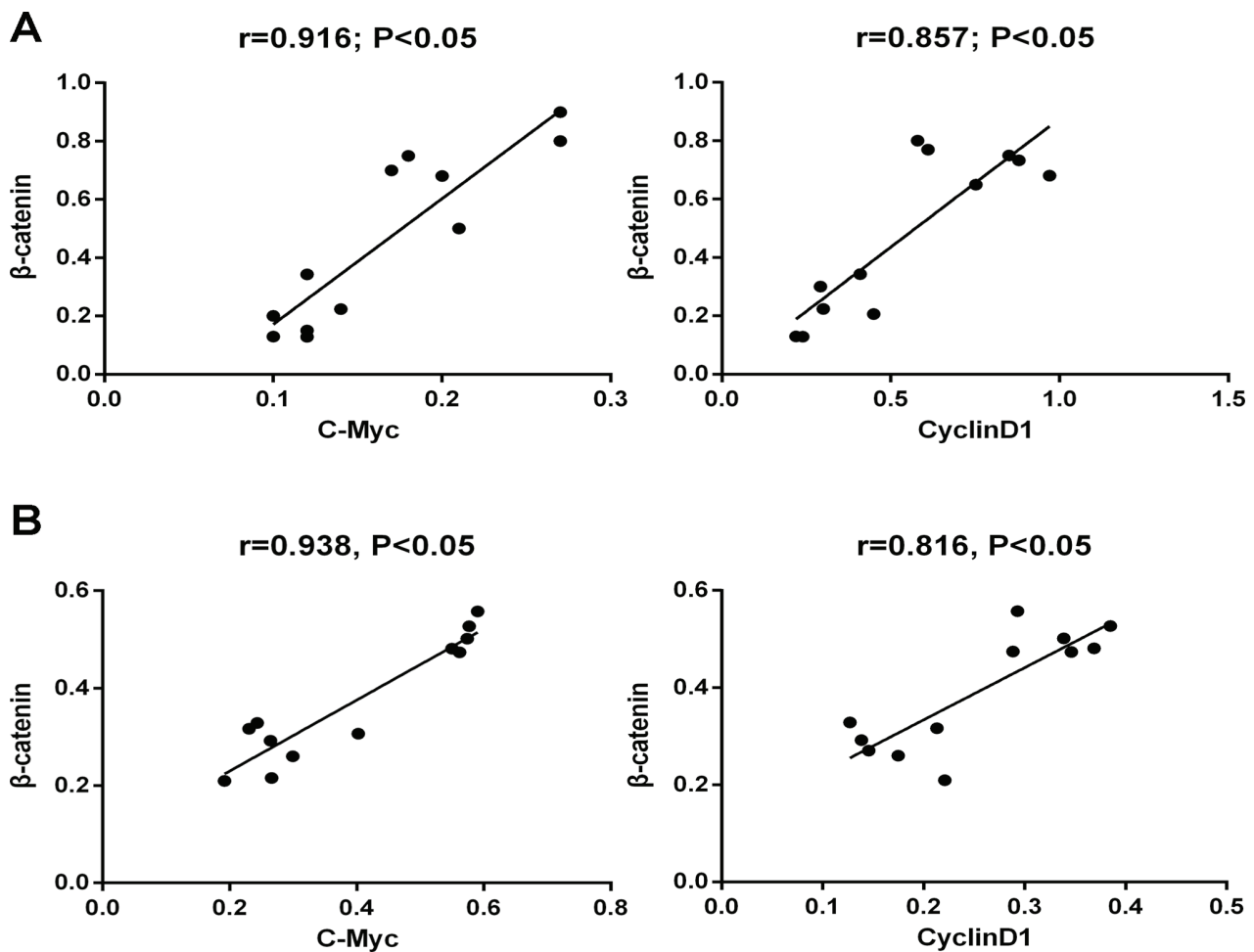


Figure 3. Linear correlation analysis of β -catenin, c-Myc and cyclin D1. Correlation of β -catenin, c-Myc and cyclin D1 in (A) lung tissue and (B) ASMCs (n=6). ASMC, airway smooth muscle cells.

The p38 MAPK signaling pathway is involved in the Wnt/ β -catenin-regulated airway remodeling. The present study investigated whether the p38 MAPK signaling pathway was implicated in β -catenin-regulated processes to elucidate the underlying molecular mechanisms of asthma airway remodeling. The results indicated that p38 MAPK expression was significantly higher in the asthma group compared with the control group (Fig. 5A). Blocking the p38 MAPK signaling pathway using SB203580 (5 and 10 μ M) significantly down-regulated the protein expressions of β -catenin, c-Myc and cyclin D1 in the ASMCs from asthma model rats (Fig. 5B). The results indicated that p38 MAPK serves a role in β -catenin regulated airway remodeling.

Discussion

In the present study, the connection between the Wnt/ β -catenin signaling pathway and asthma airway remodeling and its underlying mechanism were investigated. The findings determined that the interaction between Wnt and p38 MAPK may have an influence on the process of airway remodeling.

Thickened airway walls, collagen deposition and increased mass of α -SMA are the main features of airway remodeling. The present findings were consistent with previous studies where blocking of the Wnt signaling pathway significantly

inhibited airway structural changes and collagen deposition in an *in vivo* asthma model (4,20).

β -catenin, the core transcription factor of the Wnt/ β -catenin signaling pathway, is an essential factor that transmits signals to the nucleus, initiates transcription of Wnt-specific genes and determines the specificity of various cells and tissues (20). C-Myc and cyclin D1 participate in a broad range of cell and tissue development, and are the critical downstream effectors of cellular proliferation (15,21-23). The present study determined that β -catenin, c-Myc, and cyclin D1 mRNA and protein expression increased in the lung tissue of asthmatic rats and ASMCs compared with the control. The correlation analysis demonstrated that c-Myc and cyclin D1 expression levels in asthmatic rats positively correlated with β -catenin. These findings suggested that the Wnt/ β -catenin signaling pathway may affect the asthma airway remodeling by upregulating c-Myc and cyclin D1 expression.

Multiple signaling transduction pathways exist in cells. The biological functions of these signaling pathways are not independent and there are certain links that mediate mutual restraint and complementary internal relations. The MAPK family, includes p38 MAPK, extracellular signaling-related kinase (ERK) and c-Jun N-terminal kinase (JNK), which are hypothesized to be integral to asthma pathogenesis (8). p38 MAPK is activated by inflammation, stress

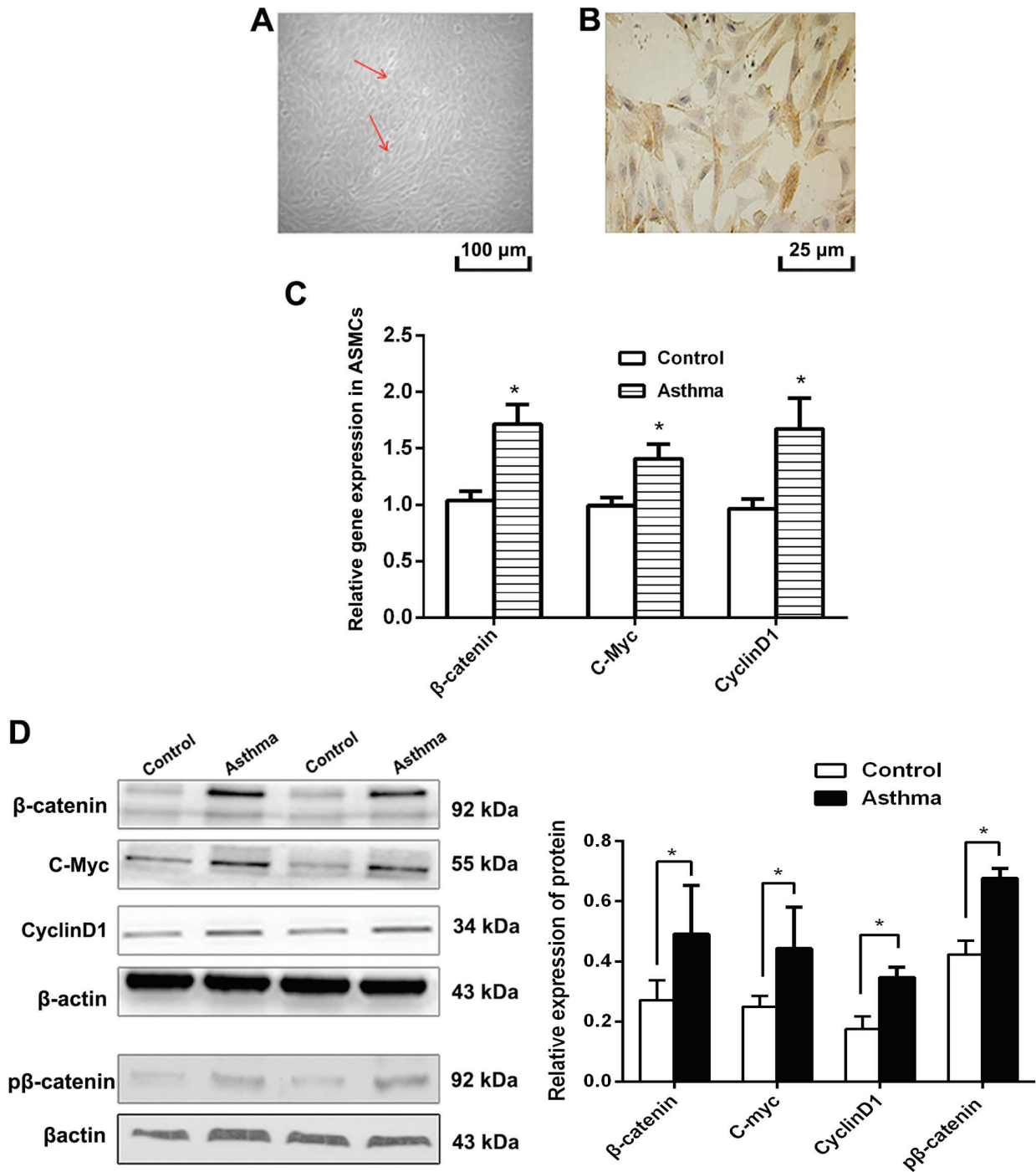


Figure 4. Expression of β -catenin, p- β -catenin/ β -catenin, c-Myc and cyclin D1 in ASMCs from asthma model rats. (A) ASMCs were observed under a microscope demonstrating typical ‘hill and valley’ features as indicated by red arrows. (B) Positive-smooth muscle actin expression was detected via immunocytochemistry, which confirmed the cells were ASMCs. (C) mRNA and (D) protein expression of β -catenin, p- β -catenin/ β -catenin, c-Myc and cyclin D1 in ASMCs were all higher in the asthma group compared with the control (n=6). *P<0.05 vs. control group. p, phosphorylated; ASMC, airway smooth muscle cells.

and injury (7,24,25). A previous study determined that activation of p38 MAPK results in increased β -catenin nuclear localization and Wnt-responsive gene activity (9). Furthermore, c-Myc and cyclin D1 are important p38 MAPK targets (13,26,27). The present study further investigated the coordination between the Wnt/ β -catenin signaling pathway and the p38 MAPK signaling pathway in ASMCs obtained from asthma model rats. β -catenin and p38 MAPK protein expression were significantly increased

in asthma rats, whilst blocking the p38 MAPK pathway downregulated β -catenin, c-Myc and cyclin D1 expressions. Given that c-Myc and cyclin D1 are the target genes of both the Wnt/ β -catenin and p38 MAPK signaling pathways, the present results indicated that the interaction between Wnt/ β -catenin and p38 MAPK may influence the airway remodeling process.

In conclusion, the present study determined that the Wnt/ β -catenin signaling pathway may affect the asthma

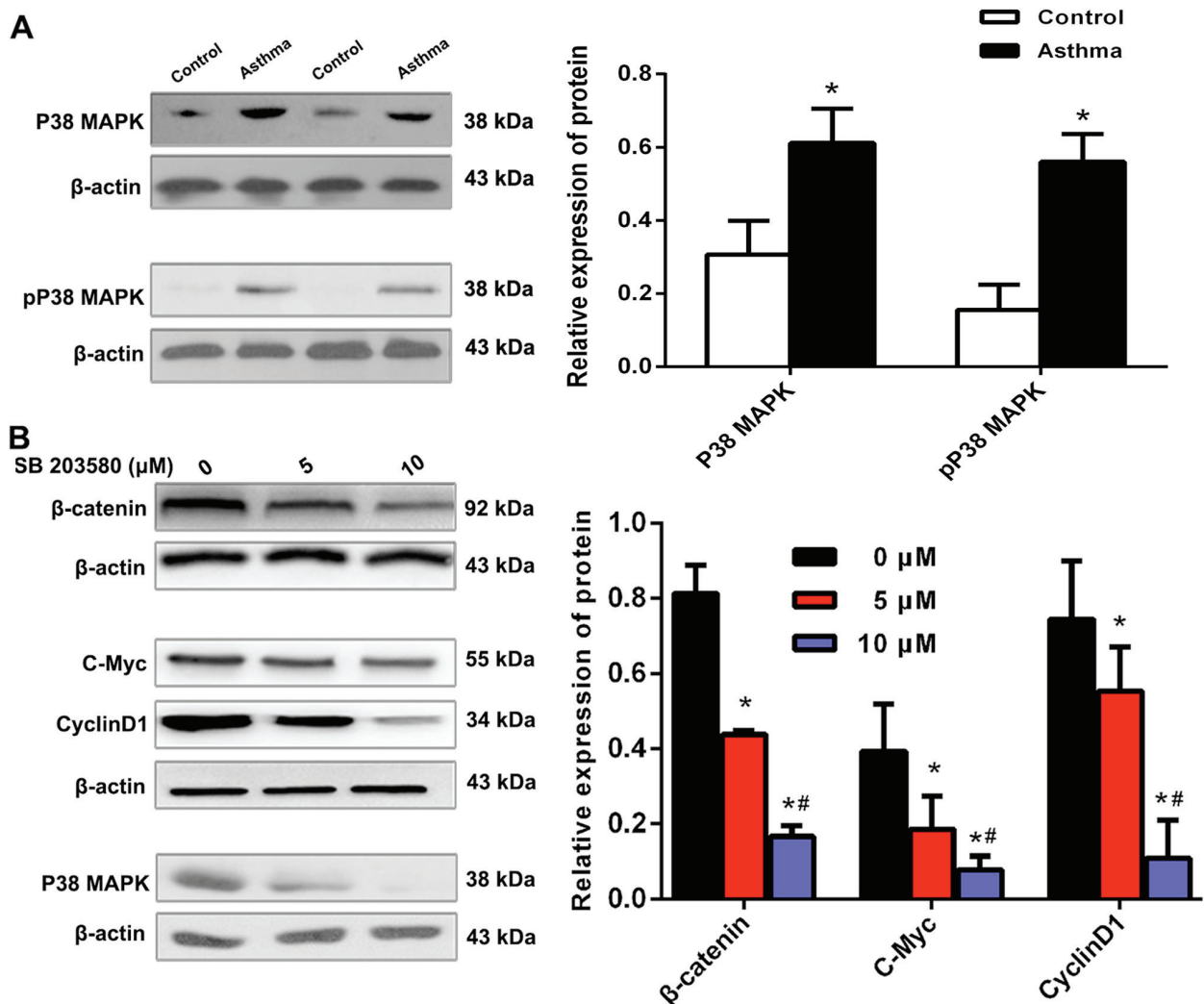


Figure 5. Blocking p38 MAPK downregulates the expression of β -catenin, c-Myc and cyclin D1 in ASMCs from asthma model rats. (A) Expression of p38 MAPK and p-p38 MAPK/p38 MAPK in ASMCs were higher compared with the control group. (B) ASMCs from asthma model rats were treated with various concentrations of inhibitor SB 203580. Following treatment, the expressions of p38 MAPK, β -catenin, c-Myc and cyclin D1 were downregulated in both the 5 and 10 μ M group (n=3). *P<0.05 vs. control group; **P<0.01 vs. 5 μ M group. MAPK, mitogen-activated protein kinase; p, phosphorylated.

airway remodeling process by upregulating c-Myc and cyclin D1 expression via the p38 MAPK-dependent pathway.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ designed the present study and reviewed the manuscript. XJ and TZ performed the experiments to acquire the data. YH, XZ and HZ analyzed and interpreted the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Laboratory Animal Ethics Committee of Wenzhou Medical University and the Laboratory Animal Centre of Wenzhou Medical University (approval no. wyd2015-0039).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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