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# MiR-23b controls TGF-β1 induced airway smooth muscle cell proliferation via direct targeting of Smad3☆

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ABSTRACT

*Background:* MicroRNAs are small yet versatile gene tuners that regulate a variety of cellular processes, including cell growth and proliferation. Here we report that miR-23b inhibited airway smooth muscle cells (ASMCs) proliferation through directly targeting of Smad3.

*Methods:* We obtained ASMCs by laser capture microdissection of normal and asthmatic mice lung tissues. Mice ASMCs were cultured and induced by TGF-β1. The implication between TGF-β1 and miR-23b in ASMCs were detected by RT-PCR. The effects of miR-23b on ASMCs proliferation and apoptosis were assessed by transient transfection of miR-23b mimics and inhibitor. The expression of Smad3 in ASMCs were detected by RT-PCR and Western blotting analysis. Dual-Luciferase Reporter Assay System will be applied to identify whether Smad3 is a target gene of miR-23b.

*Results:* TGF-β1 and miR-23b mRNA expression of in-situ bronchial ASMCs collected by laser capture microdissection were increased in asthmatic mice compared to non-asthma controls. This is accompanied by an increase in miR-23b mRNA expression in TGF-β1 induced ASMCs. miR-23b up-regulation significantly inhibited TGF-β1-induced ASMCs proliferation and promoted apoptosis. MiR-23b negatively regulates the expression of Smad3 in ASMCs. Dual-Luciferase Reporter Assay System demonstrated that Smad3 was a direct target of miR-23b.

*Conclusions:* MiR-23b may function as an inhibitor of asthma airway remodeling by suppressing ASMCs proliferation via direct targeting of Smad3.



Airway smooth muscle (ASM) is the critical effector tissue that maintains bronchomotor tone, and phenotypic changes in ASM play a pivotal role in the pathogenesis of a variety of lung diseases [1]. Indeed, an increase in ASM tissue mass is a major driver of airway remodeling associated with asthma [2–5]. The pathological change in ASM mass is a combined result of hypertrophy and hyperplasia.

Transforming growth factor β1 (TGF-β1), a key mediator, has been shown to participate in the development of lung fibrosis in severe asthma patients in previous studies [6,7]. It can also stimulate the proliferation and migration of smooth muscle cells in vitro [8,9]. Therefore TGF-β1 stimulation can be used in vitro as a model of inflammation and remodeling, due to its central effects in these processes [10].

It is well-established that the TGF-β/Smad signaling pathway is mediated through the phosphorylation and interaction of R-Smad (Smad2 and Smad3) and co-Smad (Smad4) to regulate gene transcription in the nucleus [11]. Among the Smad proteins, Smad3 plays a central role in regulating airway smooth muscle cell proliferation and airway remodeling.

MicroRNAs (miRNAs) are small noncoding RNAs that control the translation of mRNAs by promoting the degradation of target mRNAs or preventing their translation [12]. MiR-23b has been reported to be involved in many cell functions including cell proliferation, migration and differentiation [13–17]. And a recent study has shown that miR-23b served as a molecular switch in regulating

*Abbreviations:* ASMCs, airway smooth muscle cells; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; Smad, drosophila mothers against decapentaplegic protein; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; LCM, Laser capture microdissection; NC, negative control; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; TGF-β1, transforming growth factor-β1.

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TGF-β1 signaling by targeting Smad3 [18].

Considering the noteworthy role TGF-β1 plays during airway remodeling in asthma, the purpose of this study was to elucidate if and how miR-23b was involved in the proliferation of asthmatic mice ASMCs.

## 1. Material and methods

### 1.1. Animal experiment

Adult 6- to 8-week-old BALB/c mice (females) were obtained from Center of Experiment Animal of Sun Yat-sen University (Certificate of Conformity: Guangdong Experimental Animal Testing by certificate No. SCXK (粤) 2011-0029). All the experiments were performed in accordance with the regulations of the Center of Animal Experiments of Sun Yat-sen University. Ethical approval for this investigation was obtained from the Research Ethics Committee, Sun Yat-sen University. eighty female BABL/c mice were randomly divided into two groups with 40 mice in each group. (1) control group: mice were treated with saline; (2) asthma group: mice were sensitized and challenged with OVA mice were sensitized on days 0 and 14 by intraperitoneal injection of 10 μg OVA emulsified in 1 mg of aluminum hydroxide in a total volume of 200 μl. Seven days after the last sensitization, mice were exposed to ovalbumin aerosol (2.5% w/v diluted in sterile physiological saline). Mice were exposed for up to 30 min three times per week for 2, 4, 8weeks. The aerosol (particle size; 2.0–6.0 μm) was generated by a nebulizer (Ultrasonic nebulizer boy037G6000, Pari, Germen) driven by filling a perspex cylinder chamber (Diameter 50 cm, Height 50 cm) with a nebulized solution.

### 1.2. Laser capture microdissection and real-time PCR

To examine the expression of TGF-β1and miR-23b in ASM, bronchial biopsies were obtained from control group and asthma group challenged for 0, 2, 4, 8weeks. After the last challenge, lungs were removed from the mice after sacrifice. The left lung tissues were fixed with 10% neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 5 μm sections of fixed embedded tissues were cut on a rotary microtome, placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (H&E) to assess the airway remodeling. Single lobes of the right lung were inflated with 50% optimal cutting temperature (OCT) compound-50% PBS (Tissue-Tek OCT Compound, Sakura Finetek, Torrance, CA), and frozen away fully covered with OCT. Frozen sections were cut at 6 μm thickness and mounted on LCM slides (Arcturus). The slides were immediately stored on dry ice and then at −80 °C until used. Sections were fixed in 70% ethanol for 30 s, and stained and dehydrated in a series of graded ethanol followed by xylene using HistoGene LCM frozen section staining kit (Arcturus) according to the manufacturer's instruction. ASM cells were captured onto the CapSure HS LCM caps (Arcturus) by a Pixcell II Laser Capture Microdissection System (Arcturus, Mountain View, CA) and total RNA was extracted by using a PicoPure RNA isolation kit (Arcturus) according to the manufacturer's instructions.

### 1.3. Cell culture and treatments

ASMC was prepared by the explant method from trachea and bronchi of BALB/c mice. Briefly, Tracheas and bronchi were dissected and incubated in Hanks' balanced salt solution (HBSS) with 0.1% collagenase solution (Sigma Chemical Co., St. Louis, MO) at 37 °C for 20 min. The tissues were cut into 0.5 $mm^3$ pieces. They were then placed in DMEM medium containing 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ incubator at 37 °C. ASMCs were identified by the typical “hill and valley” growth pattern and immunocytochemistry staining for α-smooth muscle actin. ASMCs were then cultured in DMEM medium supplemented with penicillin, streptomycin in above concentration and 10% FBS. The medium was changed every 2 days. Confluent cells at passage numbers 4–6 were used for the experiments. Treatments with human recombinant TGF-β1 (R&D Systems, Minneapolis, MN) were performed after serum-free starvation of AMSCs for 12 h.

### 1.4. Transfection

The miR-23b mimics, mimics negative control (NC), inhibitor, inhibitor negative control (inhibitor NC) were purchased from Genepharma Inc (Shanghai, China). Cells were seeded at a density of $2–3 \times 10^5$ cells per well in six-well plates for 24 h. Cells were then transfected with oligonucleotides or plasmid, using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells that were treated with blank Lipofectamine 2000 alone were denoted the Mock control group. Cells were harvested at the indicated time for cell proliferation assays, protein analysis and migration assays.

### 1.5. Real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from ASMCs with Trizol (Invitrogen), according to the manufacturer's instructions. Two micrograms of large RNAs (larger than 200 nt) or 1 lg of small RNAs (smaller than 200 nt) were mixed with oligo (dT) or miRNA specific stem-loop RT primers (miR-23b RT primer and U6 RT primer, Table 1) respectively and reverse transcribed to cDNA using First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). These cDNAs were

**Table 1**
Quantitative polymerase chain reaction primers.

| Primer name | | Sequence 5′-3′ |
|---|---|---|
| TGF-β1 | F | ACCTGCAAGACCATCGACAT |
| TGF-β1 | R | GGTTTTCTCATAGATGGCGT |
| Smad3 | F | GAGAGGTGTGCGGCTCTACT |
| Smad3 | R | CTGGTTGCAGTTGGGAGACT |
| β-actin | F | CATTGCTGACAGGATGCAGA |
| β-actin | R | CTGCTGGAAGGTGGACAGTGA |
| miR-23b | | AUCACAUUGCCAGGGAUUACC |
| miR-23b | F | ACACTCCAGCTGGGATCACATTGCCAGG |
| miR-23b | RT | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGTAATCC |
| U6s | F | CTCGCTTCGGCAGCACA |
| U6 | R | AACGCTTCACGAATTTGCGT |
| All | R | CTCAACTGGTGTCGTGGA |