



MiR-23b controls TGF- β 1 induced airway smooth muscle cell proliferation via TGF β R2/p-Smad3 signals

Ming Chen^a, Linjie Huang^{a,1}, Wei Zhang^b, Jianting Shi^a, Xiaoling Lin^a, Zhiqiang Lv^a, Wei Zhang^a, Ruiyun Liang^a, Shanping Jiang^{a,*}

^a Department of Respiratory Medicine, Sun Yat-Sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong Province 510120, China

^b Department of Geratology, The Second People's Hospital of Shenzhen, Shenzhen 518000, China

ARTICLE INFO

Article history:

Received 4 November 2015

Received in revised form

14 December 2015

Accepted 19 December 2015

Available online 31 December 2015

Keywords:

miR-23b

TGF β R2/p-Smad3

Airway smooth muscle cells

ABSTRACT

Background: Abnormal proliferation of ASM (airway smooth muscle) directly contributes to the airway remodeling during development of lung diseases such as asthma. Here we report that a specific microRNA (miR-23b) controls ASMCs proliferation through directly inhibiting TGF β R2/p-Smad3 pathway.

Methods: The expression of miR-23b in ASMCs was detected by quantitative real-time polymerase chain reaction (RT-PCR). The effects of miR-23b on cell proliferation and apoptosis of ASMCs were assessed by transient transfection of miR-23b mimics and inhibitor. The target gene of miR-23b and the downstream pathway were further investigated.

Results: Overexpression of miR-23b significantly inhibited TGF- β 1-induced ASMCs proliferation and promoted apoptosis. RT-PCR and Western blotting analysis showed miR-23b negatively regulates the expression of TGF β R2 and p-Smad3 in ASMCs. Subsequent analyses demonstrated that TGF β R2 was a direct and functional target of miR-23b, which was validated by the dual luciferase reporter assay.

Conclusions: MiR-23b may function as an inhibitor of airway smooth muscle cells proliferation through inactivation of TGF β R2/p-Smad3 pathway.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Asthma is a complex respiratory disease characterized by chronic inflammation and remodeling of airway tissues. 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 asthma (Panettieri et al., 2008). Indeed, an increase in ASM tissue mass is a major driver of airway narrowing associated with asthma and chronic obstructive pulmonary disease (Fernandes et al., 2001; Stewart, 2001; Benayoun et al., 2003; Martin and Ramos-Barbon, 2003). The pathological change in ASM mass is a combined result of hypertrophy (increase in cell size) and hyperplasia (increase in cell number through proliferation).

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; NC, negative control; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; TGF- β 1, transforming growth factor- β 1; TGF β R2, transforming growth factor- β 1 receptor II.

* Corresponding author. Fax: +86 2081332853.

E-mail address: shanpingjiang@126.com (S. Jiang).

¹ Joint first authors.

<http://dx.doi.org/10.1016/j.molimm.2015.12.012>

0161-5890/© 2015 Elsevier Ltd. All rights reserved.

Transforming growth factor β 1 (TGF- β 1), a key mediator, has been shown to participate in the development of lung fibrosis in severe asthma patients in previous studies (Makinde et al., 2007; Brown et al., 2012). It can also stimulate the proliferation and migration of smooth muscle cells in vitro (Ma et al., 2007; Chen and Khalil, 2006). Therefore TGF- β 1 stimulation can be used in vitro as a model of inflammation and remodeling, due to its central effects in these processes (Ierodiakonou et al., 2013).

TGF- β mediates its effects by interacting with transmembrane receptors. There are three types of receptors for TGF- β , including type I (TGF β R1, 50–60 kDa), type II (TGF β R2, 75–85 kDa) and type III (TGF β R3, a 280 kDa proteoglycan with a 120 kDa core protein), in which only TGF β R2, which is autophosphorylated, can bind TGF- β . Upon binding with TGF- β , TGF β R2 recruits TGF β R1 and phosphorylates TGF β R1 which in turn initiates signal transduction mediated by the downstream Smad proteins (Degryse et al., 2011). 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 (Macfarlane and Murphy, 2010). MiR-23b has been reported to be involved in many cell func-

tions including cell proliferation, migration and differentiation (Li et al., 2014; Pellegrino et al., 2013; Donadelli and Palmieri, 2013; Ishteiwy et al., 2012; Au Yeung et al., 2011). And a recent study has shown that miR-23b served as a molecular switch in regulating Transforming growth factor- β 1 (TGF- β 1) signaling (Zhang et al., 2015a).

Considering the noteworthy role TGF- β 1 plays during the the stage of airway remodeling, the purpose of this study was to elucidate if and how miR-23b was involved in the stage of airway remodeling.

2. Material and methods

2.1. Culture of ASMCs

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 Centre of Animal Experiments of Sun Yat-sen University. Ethical approval for this investigation was obtained from the Research Ethics Committee, Sun Yat-sen University. 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 mm³ 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% CO₂ 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.

2.2. 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.

2.3. Stimulation with TGF- β 1

Six- or 96-well tissue culture plates were coated with 40 μ g/ml rat tail collagen I or left uncoated, then incubated at 4 °C overnight. Coating solutions were then removed, and wells were rinsed with sterile PBS. ASM cells were seeded on these treated surfaces at $2-4 \times 10^3$ cells/cm² in 5% (v/v) FBS in DMEM and allowed to attach for 24 h. To avoid the influence of serum on expression and production of factors, cells were synchronized in 0.1% BSA (w/v) in DMEM for 24 h (Satoh et al., 1997) then stimulated for up to 48 h with 10 ng/mL TGF- β 1 in 0.1% (w/v) BSA in DMEM. Unstimulated cells were maintained in 0.1% (w/v) BSA in DMEM.

Table 1

Quantitative polymerase chain reaction primers.

Primer name	Sequence 5'-3'
TGF β R2 F	TCGTTCAAGCAGACGGATGT
TGF β R2 R	CTTCACTTCTCCCACAGCAT
β -actin F	CATTGCTGACAGGATGCAGA
β -actin R	CTGCTGGAAGGTGGACAGTGA
miR-23b	AUCACAUUGCCAGGGAUUAACC
miR-23b F	ACACTCCAGCTGGGATCATTGCCAGG
miR-23b RT	CTCAACTGGTGTCTGTGGAGTCGGCAATTCAGTTGAGGGTAATCC
U6 F	CTCGCTTCGGCAGCACACA
U6 R	AACGCTTCACGAATTTGCGT
All R	CTCAACTGGTGTCTGTGGA

2.4. 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 μ g 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 subjected to qPCR using SYBR premix Ex Taq kit (TaKaRa, Dalian, China) to detect the expression of TGF β R2 mRNA or miR-23b respectively. For TGF β R2 mRNA expression, paired primers (TGF β R2-Fwd and TGF β R2-Rev) were used with the level of β -actin mRNA as an endogenous reference gene. For miR-23b expression, miRNA specific forward primer (miR-23b-Fwd) and the universal reverse primer (Rev) were used with U6 snRNA for normalization. All reactions were performed using ABI Prism 7900HT (Applied Biosystems, Foster City, California, USA) in triplicate and the levels of gene expression were calculated by relative quantification method. All primers used were purchased from SBS Gentech (Shanghai, China) and shown in Table 1.

2.5. Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Corporation, Kumamoto, Japan). The principle of the kit relies on the phenomenon that in the presence of electron coupling reagent, WST-8 can be deoxidated by dehydrogenase in mitochondria and generate a highly water-soluble orange product (formazan). The color depth generated is proportional to the cell proliferation. Fibroblasts were seeded into 96-well plates at a density of 1×10^5 cells/well in 200 μ l medium. 10 μ l of CCK-8 solution were added to 90 μ l of culture medium following treatment at the indicated time and incubated at 37 °C for 2 h. The optical density was measured at 450 nm. Each group included at least 6 replicate wells, and all of the experiments were performed in triplicate.

2.6. Western blot analysis

Cells were harvested on ice, lysed with RIPA buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄ and 1 μ g/ml leupeptin. Proteinase inhibitor cocktail tablets and phosphatase inhibitor cocktail tablets were also added. BCA method was used to determine the protein concentrations. Equal amount of protein was separated by 10% SDS-PAGE gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then blocked for 1 h at room temperature in 5% non-fat dry milk and incubated overnight at 4 °C with the special antibody. After washing in TBS-T solution, membranes were

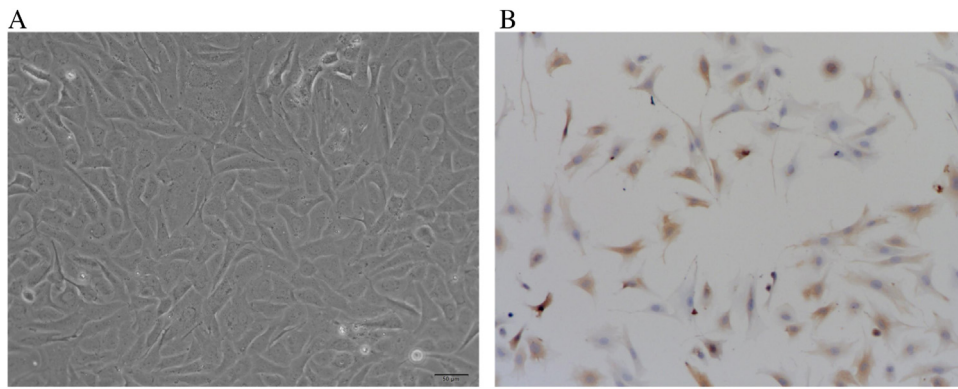


Fig. 1. Culture and identification of ASMCs isolated from mice. (A) ASMCs displayed the characteristic “hill and valley” appearance (phase contrast microscope $\times 100$). (B) Positive expression of α -actin in ASMCs (immunocytochemical staining $\times 100$).

incubated further with horseradish peroxidase-conjugated IgG secondary antibody. The blots were then washed three times in TBS-T, and antibody-bound protein was visualized with an Enhanced Chemiluminescence (ECL) kit.

2.7. Flow cytometry analysis of cell apoptosis

Above-mentioned cell samples were trypsinized and then centrifuged at 1000 rpm for 5 min. The cells were resuspended with 500 μ l binding buffer at a concentration of 10^6 cells/ml, after washing two times with PBS at 1000 rpm for 5 min. Then, 5 μ l FITC-conjugated AnnexinV and 5 μ l PI were added to the cells and incubated at room temperature for 15 min in the dark. The samples were analyzed within 1 h post-staining. Experiments were repeated three times.

2.8. Target prediction and luciferase activity assay

MiRNAs targeting TGF β R2 mRNA was predicted by a combination of TargetScan (www.targetscan.org) and microrna.org (<http://www.microrna.org>).

The sequence of mice TGF β R2 3'UTR was amplified with the primer (restriction sites were included): TGF β R2-wt: 5'-CCGctcgag AGATTCCCCAATAAGCAGAGGA-3' (Forward)/5'-ATAAGAATgcgccgcCTGCGGACTTTGGATTATTTG-3' (Reverse). TGF β R2-mut: 5'-CCGctcgagAGATTCCCCAATAAGCAGAGGA-3' (Forward)/5'-ATAAGAATgcgccgcCTGCGGACTTTGGATTATTTG-3' (Reverse). The PCR product was introduced downstream of the Renilla luciferase stop codon at the XhoI/NotI cloning sites of the psiCHECK vector (Promega, Madison, WI, USA). Mutation of the miR-23b binding site in TGF β R2 3'UTR was generated using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). One day before transfection, 1×10^5 293T cells were cultured in 24-well plates, the miR-23b mimic or negative control was transfected along with wild- or mutant-type TGF β R2-3'UTR psiCHECK vector, respectively. Luciferase activity was measured as previously described.

2.9. Statistical analysis

Statistical analyses were carried out using SPSS/Windows (SPSS Science, Chicago, IL) software. Statistical significances were evaluated by one-way ANOVA.

3. Result

3.1. Identification of primary mice airway smooth muscle cells

Primary cultures of ASMCs obtained from tissue explants were identified by characteristic “hill and valley” appearance under phase contrast microscope and positive immunofluorescence staining of α -smooth muscle actin. Both results showed all the cells were smooth muscle cells and the cell morphology was identical (Fig. 1).

3.2. Transfection of the miR-23b inhibitor sequence inhibits miR-23b expression, while transfection of the miR-23b mimics sequence increases miR-23b expression in ASMCs

ASMCs transfected with miR-23b emitted green fluorescence under fluorescence microscopy (Fig. 2(A)). The quantitative PCR results indicated that miR-23b expression decreased significantly in the group transfected with the miR-23b inhibitor sequence when compared with the blank control group and the group transfected with the inhibitor NC sequence, demonstrating that transfection with the miR-23b inhibitor inhibited miR-23b expression effectively. By contrast, miR-23b expression increased significantly in the group transfected with the miR-23b mimics sequence when compared with the blank control group and the mimics NC group, indicating that transfection with miR-23b mimics promoted miR-23b expression ($P < 0.05$; Fig. 2(B) and (C)).

3.3. MiR-23b negatively regulates the expression of TGF β R2 mRNA in ASMCs

The mRNA expression level of TGF β R2 increased significantly at 48 h after TGF- β 1 stimulation in the ASMCs transfected with the inhibitor sequence ($P < 0.05$); however, the expression level decreased significantly in the cells transfected with the miR-23b mimics sequence ($P < 0.05$; Fig. 3(A) and (B)).

3.4. MiR-23b influenced ASMCs proliferation

MiR-23b was transfected into ASMCs to determine its effects in vitro. As shown in Fig. 3, the enforced expression of miR-23b significantly decreased the growth rate of ASMCs. Therefore, these results suggest that the overexpression of miR-23b inhibits airway remodeling (Fig. 4).

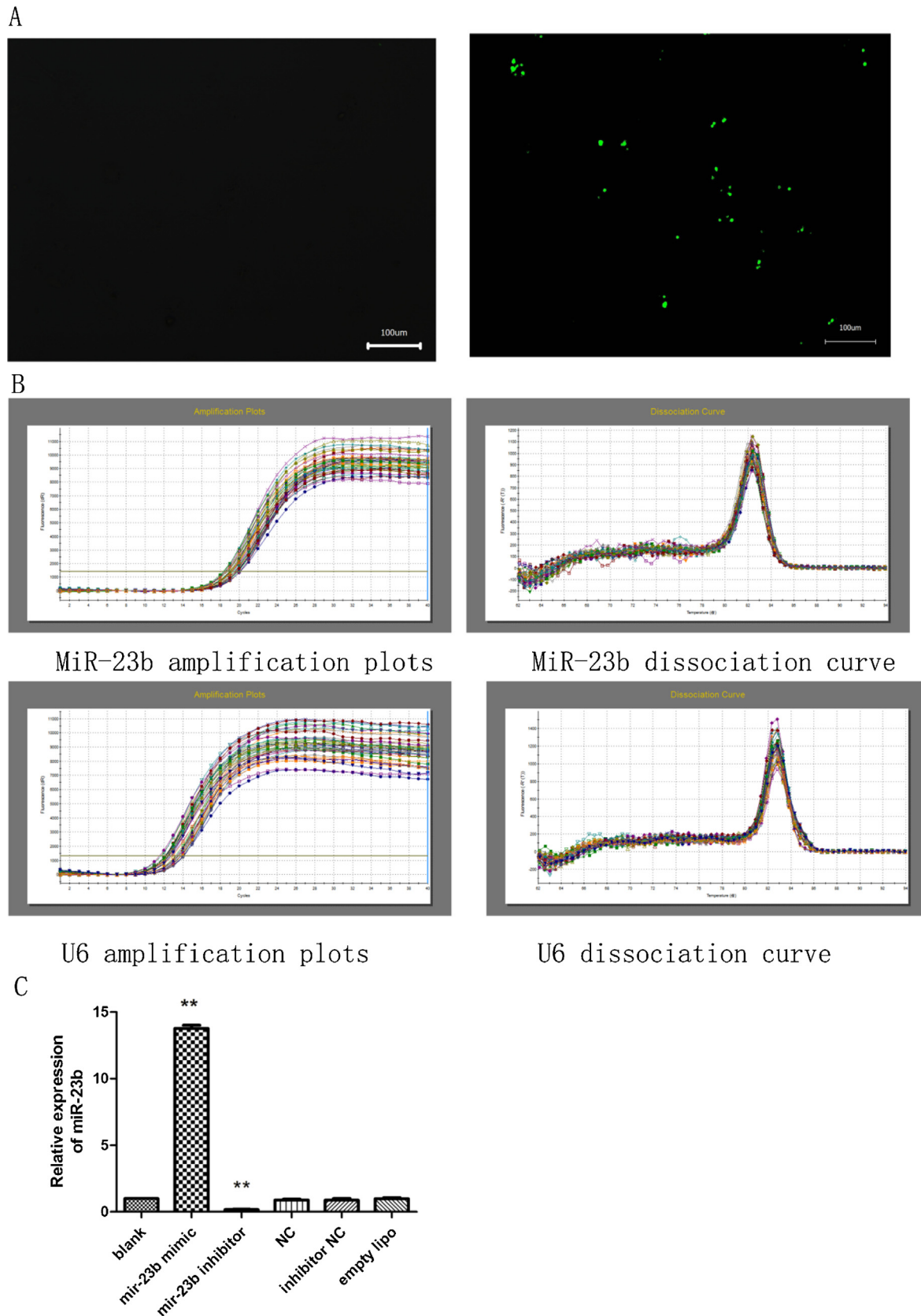


Fig. 2. Transfection of the miR-23b inhibitor sequence inhibits miR-23b expression, while transfection of the miR-23b mimics sequence increases miR-23b expression in ASMCs. (A) Under fluorescence microscopy, ASMCs transfected with miR-23b emitted green fluorescence (magnification, $\times 100$). (B and C) Fluorescence quantitative polymerase chain reaction assays revealed that the miR-23b inhibitor sequence significantly inhibited miR-23b expression; however, the miR-23b mimics sequence promoted miR-23b expression. * $P < 0.05$, ** $P < 0.01$, mimics versus control, inhibitor versus inhibitor control. Each experiment was performed in triplicate.

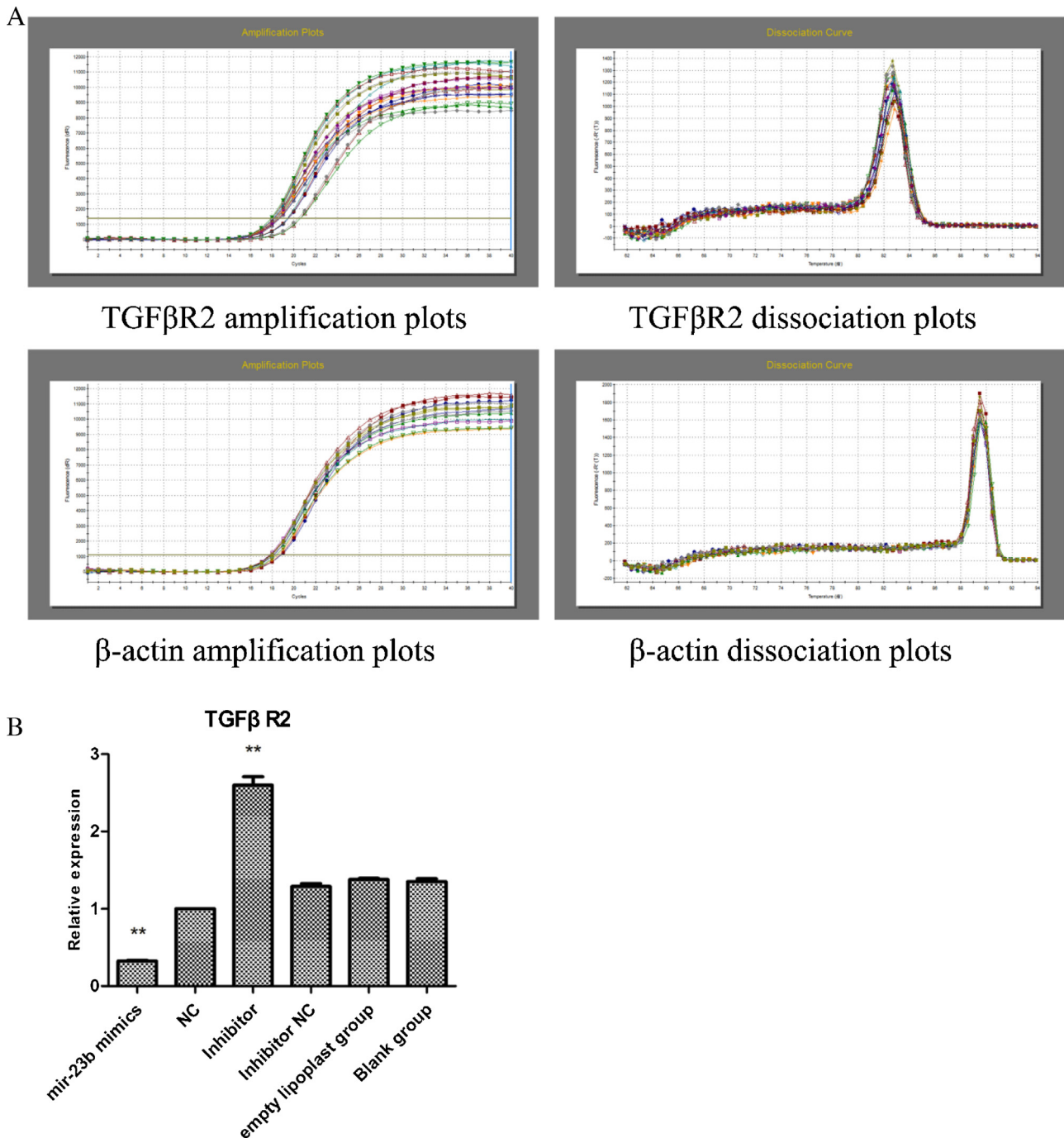


Fig. 3. MiR-23b negatively regulates the expression of TGFβR2 mRNA in ASMCs. The mRNA expression level of TGFβR2 increased significantly at 48 h after TGF-β1 stimulation in the ASMCs transfected with the inhibitor sequence ($P < 0.05$); however, the expression level decreased significantly in the cells transfected with the miR-23b mimics sequence ($P < 0.05$).

3.5. MiR-23b promotes cell apoptosis

We further explored the role of miR-23b on ASMCs apoptosis. ASMCs were treated with miR-23b and negative control, respectively; and the cells were subsequently stained with Annexin V-FITC/PI, followed by flow cytometry analyses. As shown in Fig. 4, the enforced expression of miR-23b significantly reduced the fraction of living cells and boosted apoptotic cells (Fig. 5; $P < 0.05$).

3.6. MiR-23b controls TGF-β1 induced airway smooth muscle cell proliferation via TGFβR2/p-Smad3 signals

Western blot analysis revealed that the protein expression level of TGFβR2 and p-Smad3 were significantly lower in the miR-23b mimics group when compared with the inhibitor group after 48 h of TGF-β1 stimulation ($P < 0.05$). As expected, TGF-β1 enhanced TGFβR2 and p-Smad3 expression (Fig. 6) could be partially reversed after addition of miR-23b mimic in ASMCs.

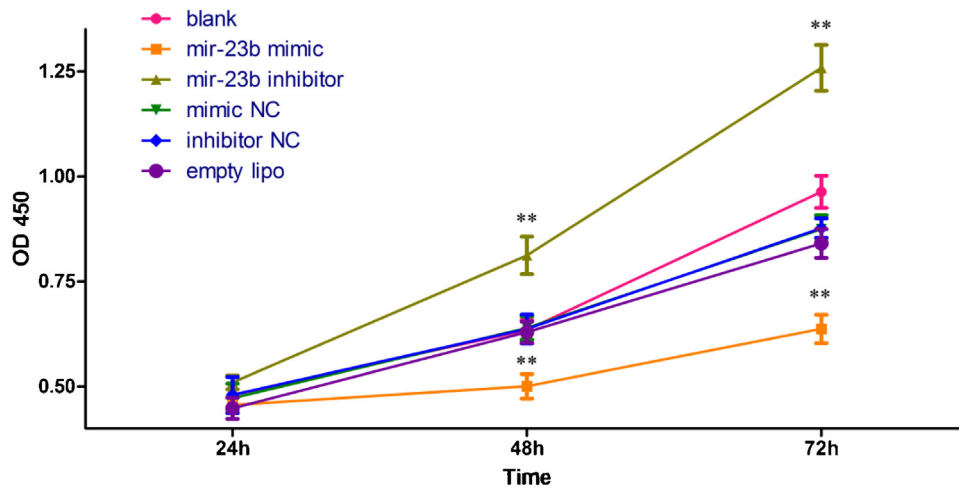


Fig. 4. MiR-23b influenced cell proliferation in ASMCs. Cells were seeded into a 96-well plate at 5,000 cells/well and examined at 0, 24, 48 and 72 h after transfection. MiR-23b overexpression inhibited cell proliferation in ASMCs. * $P < 0.05$, ** $P < 0.01$, mimics versus control, inhibitor versus inhibitor control. Each experiment was performed in triplicate.

3.7. MiR-23b directly targets *TGFβR2*

To well underlying molecular mechanism of miR-23b suppresses the proliferation of ASMCs, bioinformatic analysis of two publicly available algorithms (TargetScan and miRBase) was performed to explore the target gene of miR-23b. Results revealed complementarity between *TGFβR2* 3'UTR and miR-23b (Fig. 7(A)). To test whether *TGFβR2* can be regulated by miR-23b, the protein level of *TGFβR2* in ASMCs transfected with miR-23b mimics or inhibitor was determined by Western blotting. The results showed that miR-23b mimics dramatically decreased the protein level of *TGFβR2*, while miR-23b inhibitor increased the *TGFβR2* protein level in ASMCs.

To validate that *TGFβR2* is a direct target of miR-23b, *TGFβR2* luciferase reporters were constructed with wild-type and mutated 3'UTR of *TGFβR2*. These reporters were co-transfected with miR-23b mimics or inhibitor into ASMCs. Luciferase activity was measured by dual luciferase assays. As shown in Fig. 7 B, the luciferase activity was significantly decreased or increased compared to either mutant or empty controls when co-transfected with miR-23b mimics or inhibitor, respectively. These results demonstrate that miR-23b binds directly to the 3'UTR of *TGFβR2* to repress its expression in ASMCs.

4. Discussion

Recent studies have shown that miRNAs, which mediate post-transcriptional gene silencing, are involved in the proliferation stage of ASMCs (Mohamed et al., 2010; Hu et al., 2014). In the present study, we found that up-regulation of miR-23b inhibits cell proliferation, as shown in Fig. 3. Therefore, up-regulation of miR-23b may contribute to the termination of ASMCs, which is characterised by reduced proliferation and enhanced cellular apoptosis.

In our study, CCK-8 assay was used to test cell proliferation rate. Notably, as CCK-8 assay tested the activity of the mitochondrial enzyme, succinyl dehydrogenase, which is expressed only in living cells, it actually measures total viable cell number. Thus the increased cell number of ASMCs measured by CCK-8 assay may reflect an increased rate of proliferation and/or a decreased rate of apoptosis. Cells were transfected with MiR-23b mimics or NC or inhibitor or inhibitor NC or empty lipoplast for 48 h, and then, subjected to cell apoptosis (stained with PI and FITC-Annexin

V). MiR-23b overexpression caused an increase in early and late apoptotic cells in ASMCs. The exact effect of miR-23b on ASMCs proliferation and/or apoptosis needs further exploration.

MiR-23b is a multifunctional miRNA that contributes to the regulation of multiple signaling pathways, affecting cell proliferation, differentiation, apoptosis and adhesion (Zhang et al., 2015b; Begum et al., 2015; Mu et al., 2015; Iaconetti et al., 2015). Moreover, the functions and underlying mechanisms are currently under investigation. It has been reported that miR-23b prevents multiple autoimmune diseases through the regulation of inflammatory cytokine pathways, in which the molecule regulates a number of inflammatory cytokines, such as TGF-β1, NF-κB, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-17.

In this study, TGF-β1 was used to induce ASMCs proliferation and migration. Transforming growth factor β1 (TGF-β1) is a potent fibrotic factor responsible for the synthesis of extracellular matrix. In recent years, a large number of studies were carried out on the relationship between TGF-β1 and airway remodeling. The studies demonstrated that TGF-β1 is an important cytokine in airway remodeling. TGF-β superfamily members signal through transmembrane Ser-Thr kinase receptors that directly regulate the intracellular Smad pathway. *TGFβR2* expression is required for TGF-β signaling to regulate the specificity of its signaling pathway activation and its biological effects Smads are a unique family of signal transduction molecules that can transmit signals directly from the cell surface receptors to the nucleus. Smad3, together with transcriptional co-factors, activates downstream TGF-β signaling components, and finally results in growth suppression, cell cycle arrest and apoptosis. Thus, we evaluated the functional phosphorylated form of

Smad3 after miR-23b modulation and TGF-β1 treatment. Our data showed a reduced level of the phosphorylated activated form of Smad3 after miR-23b overexpression whereas miR-23b repression increased the phospho-Smad3 level. Therefore, a down-regulation of *TGFβR2* and phosphorylated form of Smad3, as shown in ASMCs, indicates cell cycling or proliferation.

To further investigate the specific role of miR-23b, it is very important to identify the target gene of miR-23b. MiR-23b targeting *TGFβR2* was predicted by a combination of TargetScan (www.targetscan.org) and microrna.org (<http://www.microrna.org>). Therefore, we speculate miR-23b may be involved in the termination of ASMCs by targeting *TGFβR2*. We used the dual-luciferase reporter system to explore the relationship between miR-23b and *TGFβR2*. Our results showed that the miR-23b was

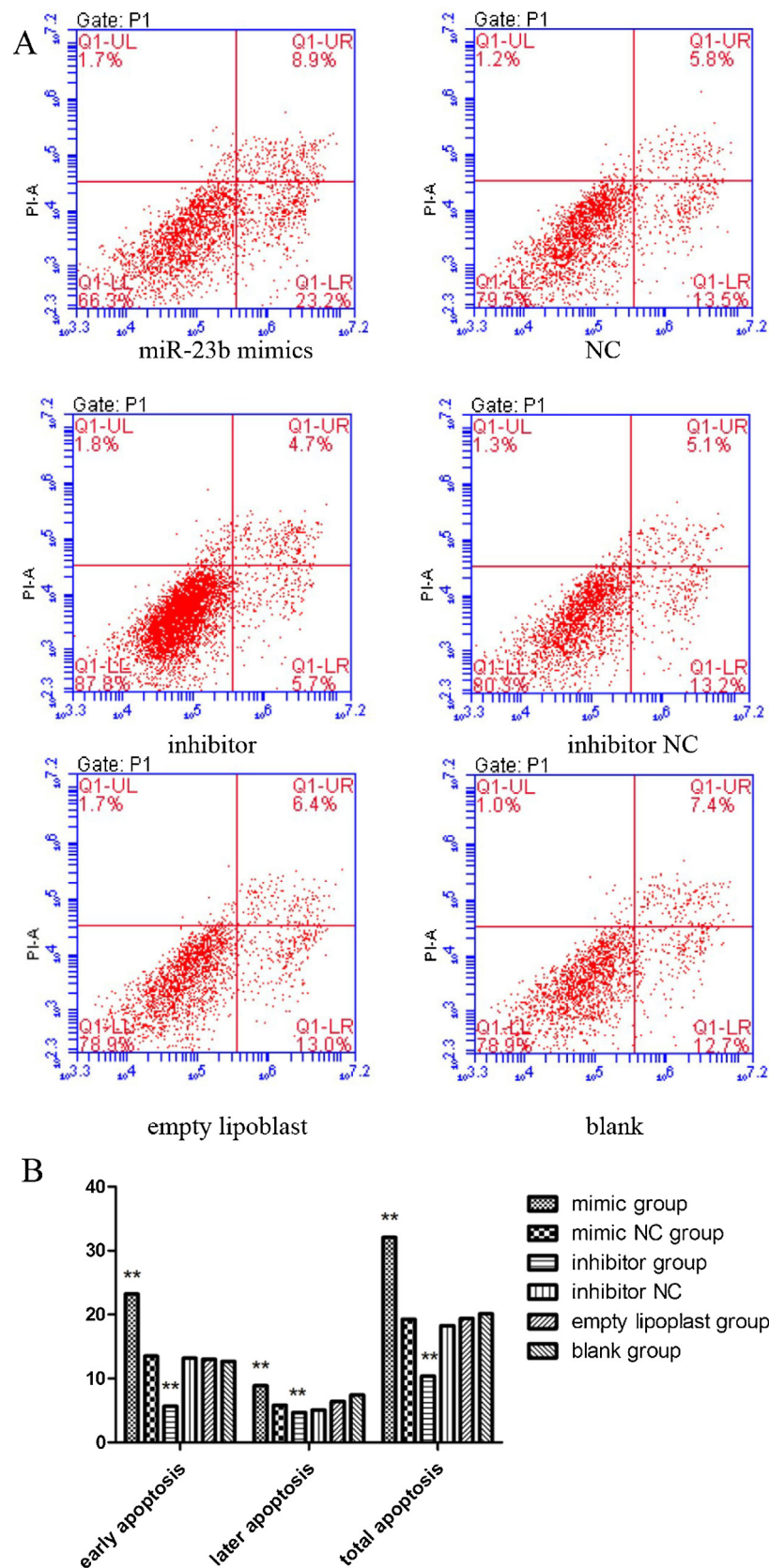


Fig. 5. MiR-23b promoted ASMCs apoptosis. Cells were transfected with MiR-23b mimics or NC or inhibitor or inhibitor NC or empty lipoplast for 48 hours, and then, subjected to cell apoptosis (stained with PI and FITC-Annexin V). MiR-23b overexpression caused an increase in early and late apoptotic cells in ASMCs. * $P < 0.05$, ** $P < 0.01$, mimics versus control, inhibitor versus inhibitor control. Each experiment was performed in triplicate.

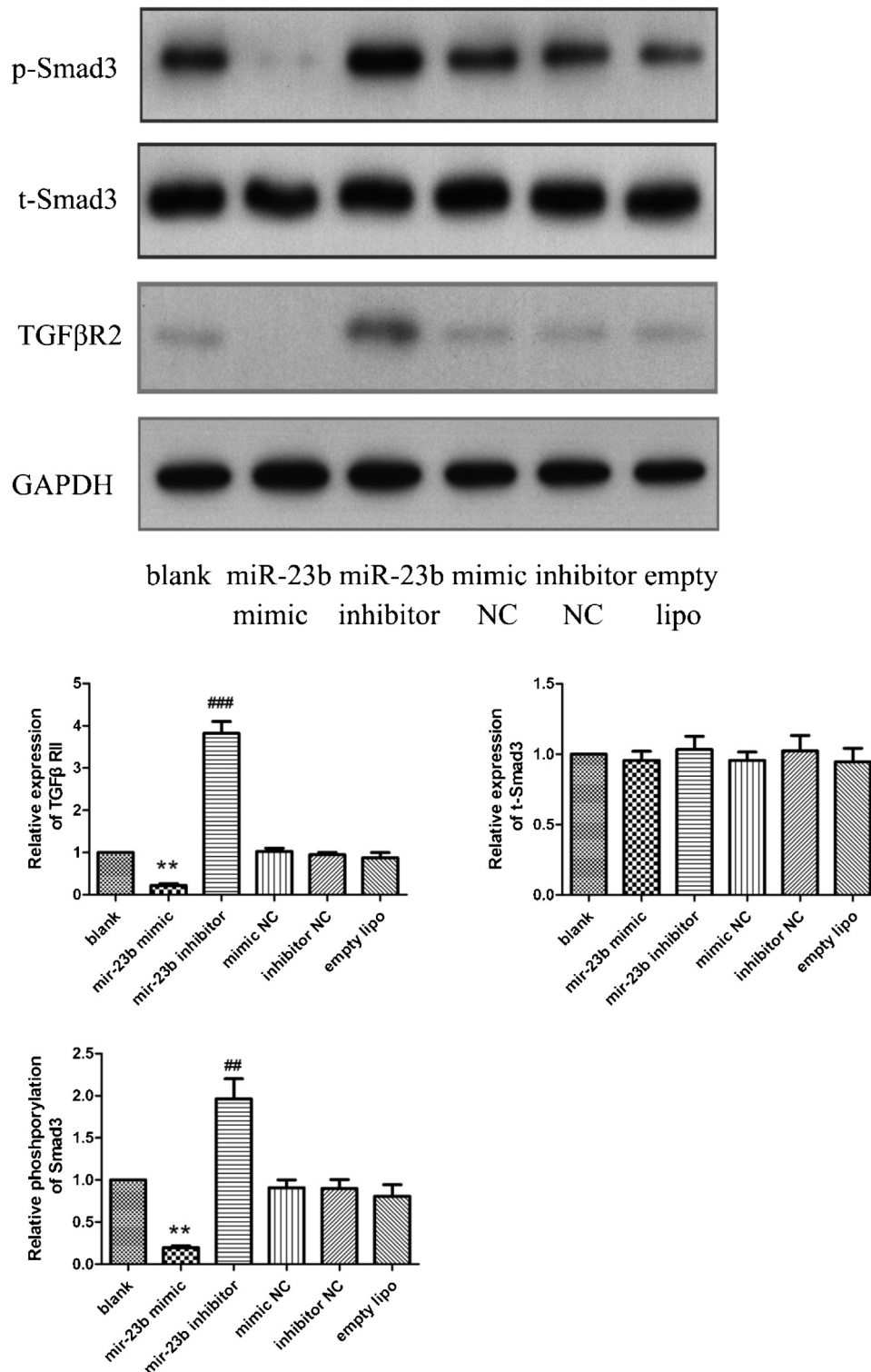


Fig. 6. Western blot analysis revealed that the protein expression levels of p-Smad3 and TGFβR2 were significantly lower in the miR-23b mimics group when compared with the inhibitor group after 48 h of TGF-β1 stimulation ($P < 0.05$). $*P < 0.05$, $**P < 0.01$, mimics versus control, inhibitor versus inhibitor control. Each experiment was performed in triplicate.

a repressor for TGFβR2. In this study, it was found that miR-23 directly targets and regulates TGFβR2 expression in ASMCs. Our study has confirmed that TGFβR2 are direct target gene of miR-23b in airway smooth muscle cell proliferation. In addition, p-Smad3 was upregulated, which was in agreement with the downregulation of miR-23b in ASMCs. Our data demonstrates that targeting TGFβR2 is one of the mechanisms by which miR-23b

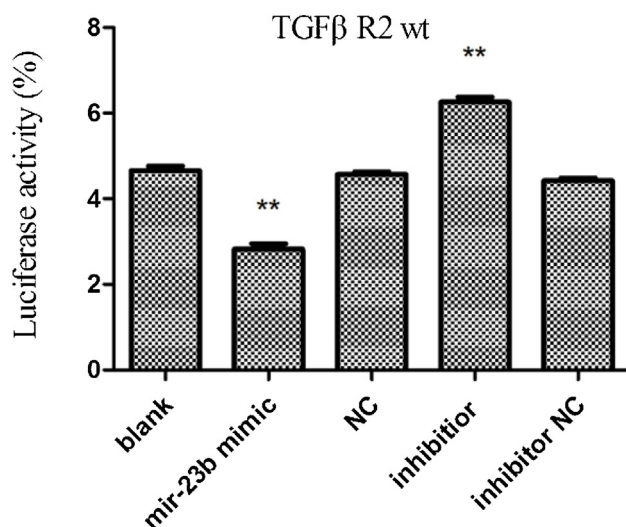
controls the activity of many intracellular transduction pathways. These findings suggest that miR-23b promotes the growth and migration of ASMCs by activating TGF-β signaling pathways. Our Studies show that TGFβR2/p-Smad3 plays important role in TGF-beta signaling in airway smooth muscle cells.

In conclusion, our study established miR-23b as a novel regulator of TGF-β1/Smad signaling and demonstrated that miR-23b

A



B



C

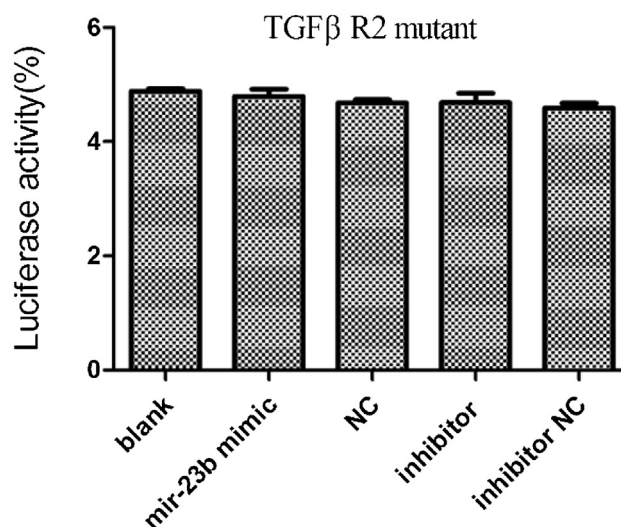


Fig. 7. MiR-23b directly targeted TGFβR2. (A) The binding site of MiR-23b to the 3'-UTR of TGFβR2. (B and C) Dual luciferase assays showed an increase after transfection of miR-23b inhibitor or a decrease after transfection of miR-23b mimics. * $P < 0.05$, ** $P < 0.01$, mimics versus control, inhibitor versus control. Each experiment was performed in triplicate.

inhibits ASMCs proliferation by suppressing TGFβR2/p-Smad3 signaling through direct targeting of the central TGF-β1/Smad pathway component, TGFβR2. Our findings raise the possibility of using miR-23b as a novel therapeutic agent to suppress TGFβR2/p-Smad3 signaling in order to prevent abnormal cell proliferation including that of the ASM.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81370120) and by the Guangdong Natural Science Foundation (S2013010014803).

References

- Panettieri Jr., R.A., Kotlikoff, M.J., Gerthoffer, W.T., Hershenson, M.B., Woodruff, P.G., Hall, I.P., Banks-Schlegel, S., National Heart Lung, and Blood Institute, 2008. Airway smooth muscle in bronchial tone, inflammation, and remodeling: basic knowledge to clinical relevance. *Am. J. Respir. Crit. Care Med.* 177 (February (3)), 248–252.
- Fernandes, D.J., Xu, K.F., Stewart, A.G., 2001. Antiremodelling drugs for the treatment of asthma: requirement for animal models of airway wall remodeling. *Clin. Exp. Pharmacol. Physiol.* 28, 619–629.
- Stewart, A.G., 2001. Airway wall remodeling and hyperresponsiveness: modelling remodelling in vitro and in vivo. *Pulm. Pharmacol. Ther.* 14, 255–265.
- Benayoun, L., Druihe, A., Dombret, M.C., Aubier, M., Pretolani, M., 2003. Airway structural alterations selectively associated with severe asthma. *Am. J. Respir. Crit. Care Med.* 167, 1360–1368.
- Martin, J.G., Ramos-Barbon, D., 2003. Airway smooth muscle growth from the perspective of animal models. *Respir. Physiol. Neurobiol.* 137, 251–261.
- Makinde, T., Murphy, R.F., Agrawal, D.K., 2007. The regulatory role of TGF-beta in airway remodeling in asthma. *Immunol. Cell Biol.* 85, 348–356.
- Brown, S.D., Baxter, K.M., Stephenson, S.T., Esper, A.M., Brown, L.A., Fitzpatrick, A.M., 2012. Airway TGF-beta1 and oxidant stress in children with severe asthma: association with airflow limitation. *J. Allergy Clin. Immunol.* 396, 391–396.
- Ma, J., Wang, Q., Fei, T., Han, J.D., Chen, Y.G., 2007. MCP-1 mediates TGF-beta-induced angiogenesis by stimulating vascular smooth muscle cell migration. *Blood* 109, 987–994.
- Chen, G., Khalil, N., 2006. TGF-beta1 increases proliferation of airway smooth muscle cells by phosphorylation of map kinases. *Respir. Res.* 7, 2.
- Ierodiakonou, D., Postma, D.S., Koppelman, G.H., Gerritsen, J., Ten, H.N., Timens, W., Boezen, H.M., Vonk, J.M., 2013. TGF-beta1 polymorphisms and asthma severity, airway inflammation, and remodeling. *J. Allergy Clin. Immunol.* 131, 582–585.
- Degryse, A.L., Tanjore, H., Xu, X.C., Polosukhin, V.V., Jones, B.R., Boomershtine, C.S., Ortiz, C., Sherrill, T.P., McMahon, F.B., Gleaves, L.A., Blackwell, T.S., Lawson, W.E., 2011. TGFβ signaling in lung epithelium regulates bleomycin-induced alveolar injury and fibroblast recruitment. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 300 (June (6)), L887–L897.
- Macfarlane, L.A., Murphy, P.R., 2010. MicroRNA: biogenesis, function and role in cancer. *Curr. Genomics* 11, 537–561.
- Li, W., Liu, Z., Chen, L., Zhou, L., Yao, Y., 2014. MicroRNA-23b is an independent prognostic marker and suppresses ovarian cancer progression by targeting runt-related transcription factor-2. *FEBS Lett.* 588, 1608–1615.
- Pellegrino, L., Krell, J., Roca-Alonso, L., Stebbing, J., Castellano, L., 2013. MicroRNA-23b regulates cellular architecture and impairs motogenic and invasive phenotypes during cancer progression. *Bioarchitecture* 3, 119–124.
- Donadelli, M., Palmieri, M., 2013. Roles for microRNA 23b in regulating autophagy and development of pancreatic adenocarcinoma. *Gastroenterology* 145, 936–938.
- Ishteiwy, R.A., Ward, T.M., Dykxhoorn, D.M., Burnstein, K.L., 2012. The microRNA -23b/-27b cluster suppresses the metastatic phenotype of castration-resistant prostate cancer cells. *PLoS One* 7, e2106.

- Au Yeung, C.L., Tsang, T.Y., Yau, P.L., Kwok, T.T., 2011. Human papillomavirus type 16 E6 induces cervical cancer cell migration through the p53/microRNA-23b/urokinase-type plasminogen activator pathway. *Oncogene* 30, 2401–2410.
- Zhang, X., Yang, J., Zhao, J., Zhang, P., Huang, X., 2015a. MicroRNA-23b inhibits the proliferation and migration of heat-denatured fibroblasts by targeting Smad3. *PLoS One* 10 (July (7)), e0131867.
- Mohamed, J.S., Lopez, M.A., Boriek, A.M., 2010. Mechanical stretch up-regulates microRNA-26a and induces human airway smooth muscle hypertrophy by suppressing glycogen synthase kinase-3 β . *J. Biol. Chem.* 285 (September (38)), 29336–29347.
- Hu, R., Pan, W., Fedulov, A.V., Jester, W., Jones, M.R., Weiss, S.T., Panettieri Jr., R.A., Tantisira, K., Lu, Q., 2014. MicroRNA-10a controls airway smooth muscle cell proliferation via direct targeting of the PI3 kinase pathway. *FASEB J.* 28 (May (5)), 2347–2357.
- Zhang, X., Yang, J., Zhao, J., Zhang, P., Huang, X., 2015b. MicroRNA-23b Inhibits the Proliferation and Migration of Heat-Denatured Fibroblasts by Targeting Smad3. *PLoS One* 10 (July (8)), e0131867.
- Begum, S., Hayashi, M., Ogawa, T., Jabboure, F.J., Brait, M., Izumchenko, E., Tabak, S., Ahrendt, S.A., Westra, W.H., Koch, W., Sidransky, D., Hoque, M.O., 2015. An integrated genome-wide approach to discover deregulated microRNAs in non-small cell lung cancer: clinical significance of miR-23b-3p deregulation. *Sci. Rep.* 5, 13236.
- Mu, W., Wang, X., Zhang, X., Zhu, S., Sun, D., Ka, W., Sung, L.A., Yao, W., 2015. Fluid shear stress upregulates E-Tmod41 via miR-23b-3p and contributes to F-actin Cytoskeleton Remodeling during erythropoiesis. *PLoS One* 10 (August (8)), e0136607.
- Iaconetti, C., De Rosa, S., Polimeni, A., Sorrentino, S., Gareri, C., Carino, A., Sabatino, J., Colangelo, M., Curcio, A., Indolfi, C., 2015. Down-regulation of miR-23b induces phenotypic switching of vascular smooth muscle cells in vitro and in vivo. *Cardiovasc. Res.* 107 (September (4)), 522–533.