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# MicroRNA-744 Inhibits Proliferation of Bronchial Epithelial Cells by Regulating Smad3 Pathway via Targeting Transforming Growth Factor-β1 (TGF-β1) in Severe Asthma

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Data Collection B
Statistical Analysis C
Data Interpretation D
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**Background:** Bronchial epithelial cells proliferation plays a pivotal role in airway remodeling in children with severe asthma. MicroRNAs (miRNAs) have gained great attention for many diseases, including asthma. The purpose of this study was to explore the mechanisms that underlie miR-744 modulating bronchial epithelial cells proliferation in severe asthma in children.

**Material/Methods:** Bronchial epithelial cells were isolated from bronchial biopsies of normal controls and asthmatic subjects. miR-744 and transforming growth factor-β1 (TGF-β1) expressions were measured by quantitative reverse transcription PCR (qRT-PCR). Proliferating cell nuclear antigen (PCNA), phosphorylation or total of mothers against decapentaplegic homolog3 (Smad3) and production of Smad anchor for receptor activation (SARA) were measured via Western blot analysis. A link between miR-744 and TGF-β1 was probed by luciferase activity and RNA immunoprecipitation. Cell proliferation was evaluated using the Cell Proliferation Assay Kit.

**Results:** Severe asthma showed a significantly elevated cell proliferation rate and reduced abundance of miR-744, which in turn inhibited cell proliferation of bronchial epithelial cells. In particular, TGF-β1 might be a candidate target of miR-744, and enrichment of miR-744 lowered the expression of TGF-β1 at mRNA and protein levels. Indeed, overexpression of miR-744 lowered the proliferation rate of bronchial epithelial cells via driving TGF-β1. Moreover, addition of miR-744 limited phosphorylation of Smad3 but reversed SARA protein abundance by regulating TGF-β1.

**Conclusions:** The presence of miR-744 repressed bronchial epithelial cells proliferation through mediating the Smad3 pathway by modulating TGF-β1, providing a promising therapeutic approach for epithelial function in severe asthma.

**MeSH Keywords:** Bronchial Arteries • Cell Proliferation • MicroRNAs • Severe Acute Respiratory Syndrome • Transforming Growth Factor beta

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## Background

Asthma is a complex airway disease characterized by chronic inflammation and airway remodeling [1], threatening the health of many people in all age groups worldwide, with an increased risk of sudden death in children [2,3]. In recent years, great advances have been made in pediatric asthma care, but effective strategies are still lacking [4]. Bronchial epithelium is proving to playing an essential role in airway remodeling in asthma [5]. Notably, cell proliferation has an impact on the outcome in many cases of damages or diseases, which is manifested by altered level of cell cycle-related proteins and is associated with autophagy, metabolism, inflammation, apoptosis, and metastasis by regulating molecular pathways [6]. Epithelial cell proliferation may account for the production of airway remodeling in severe asthma [7]. Therefore, we hypothesized that targeting inhibition of bronchial epithelial cells proliferation would be effective in treating severe asthma in children.

Of note, microRNAs (miRNAs), a class of small noncoding RNAs involved in gene regulation, contribute to many chronic respiratory diseases, including asthma in children [8,9]. miRNAs are reported to be essential for asthma and their dysregulation in bronchial epithelial cells can cause different effects in asthma [10,11]. miR-19a has been reported to be enhanced and to be associated with cell proliferation in bronchial epithelial cells from patients with severe asthma [12]. Additionally, miR-146a has been found to contribute to airway remodeling after cytokine stimulation in asthma patients, which may result from human bronchial epithelial cell survival and cell proliferation [13]. miR-21 has been reported to be a promising biomarker for diagnosis and treatment of asthma in children [14], and miR-744 has been suggested to inhibit cellular proliferation and invasion in colorectal cancer [15]. Meanwhile, the abundance of miR-744 has been reported to be lowered in severe asthma, suggesting miR-744 may be a promising modulator for asthma [16]. However, the mechanisms underlying the interaction of miR-744 with proliferation of bronchial epithelial cell in severe asthma remain unclear.

miRNAs have been implicated in cell migration, invasion, and other processes by regulating the expression of target genes. With intensive efforts, hundreds of potential genes have been reported for asthma, including transforming growth factor-β1 (TGF-β1) [17]. TGF-β is a tissue growth factor, and its dysregulation is essential for airway remodeling [18]. TGF-β1 has been reported to be elevated and involved in airway remodeling or shortening in patients with asthma [19]. A recent study revealed that introduction of TGF-β1 facilitates proliferation of bronchial epithelial cells and smooth muscle cells in asthma [20,21]. Since TGF-β1 opens up the possibility of cell proliferation, we hypothesized TGF-β1 is required for miR-744-mediated progression in severe asthma. In the present study, we investigated the effect of miR-744 on cell proliferation and probed a link between miR-744 and TGF-β1 in bronchial epithelial cells from severe asthmatic subjects and normal controls. These observations of miR-744 may contribute to development of effective epithelial repair in children with severe asthma.

## Material and Methods

### Specimens and histological assessment

All subjects with mild or severe asthma and normal subjects were recruited from the Respiratory Medicine Department of Children's Hospital Affiliated of Zhengzhou University according to the Global Initiative for Asthma criteria for asthma [22]. The study was approved by the Institutional Research Ethics Committee of Children's Hospital Affiliated of Zhengzhou University, and written informed consent was obtained from all subjects. Endobronchial biopsies were obtained from normal controls (N=45), mild asthmatic subjects (N=45), and severe asthmatic subjects (N=45). Normal controls had no history of lung disease, allergy, respiratory infection, or exposure to tobacco smoke. Mild asthmatic subjects had response to common allergens and had symptoms 3 to 6 times weekly, and severe asthmatics met the American Thoracic Society refractory asthma definition [23]. Clinical characteristics of all subjects are summarized in Table 1.

### Cell culture

Normal bronchial epithelial cells (NBEC), asthmatic bronchial epithelial cells (ABEC), and severe asthmatic bronchial epithelial cells (SABEC) were isolated from bronchial biopsies as described previously [24]. BEAS-2B cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA, USA). All cells were maintained at 37°C in 5% $CO_2$ using Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA).

### Cell transfection

NBECs or SABECs were plated into 6-well plates at a density of $5\times10^4$ cells/well and cultured in a humidified incubator with 5% $CO_2$. miR-744 mimic, miR-744 inhibitor, negative control (NC), and TGF-β1 overexpression vector were synthesized by Genepharma (Shanghai, China). The transfection was performed using Lipofectamine 2000 (Invitrogen) using the manufacturer's protocol. Transfection efficiencies were analyzed by qRT-PCR after 24 h.





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**Table 1.** Clinical characteristics of healthy controls and asthmatic subjects.

| Parameter | | Healthy control subjects | Subjects with mild asthma | Subjects with severe asthma |
|---|---|---|---|---|
| Age (years) | | 8.2±2.6 | 7.6±3.7 | 8.5±3.8 |
| Sex | Male | 24 | 16 | 28 |
| | Female | 21 | 29 | 17 |
| FEV1 | | 95.8±1.5 | 86±8.6 | 61.5±8.2 |
| $PC_{20}$ $FEV_1$ (mg/mL) | | >16 | 1.5±1.2 | 0.2±0.6 |
| Blood eosinophils (%) | | 4.1±4.2 | 3.8±1.6 | 5.22±2.63 |
| Sputum eosinophils (%) | | ND | ND | 52.4±25.2 |
| Atopic (%) | | - | 100 | 76.5 |
| ICS dose (μg) | | - | - | 1320.2±530.6 |

FEV1 – forced expiratory volume in 1 s; PC20 FEV1 – provocative concentration producing a 20% fall in FEV1; ND – not determined; ICS – inhaled corticosteroid.

### Cell proliferation

Cell proliferation was measured using the CyQUANT® Cell Proliferation Assay Kit (Invitrogen). Briefly, NBECs, ABECs, and SABECs ($1\times10^4$/well) were seeded in 96-well plates and incubated for 24 h. Each sample was prepared 6 times and the entire experiment was repeated 3 times. In order to assess the effect of miR-744 on cell proliferation, NBECs and SABECs were transfected with miR-744 mimics, inhibitors, or negative control and cultured in 96-well plates for 24 h. After removal of the medium, CyQUANT® GR dye was added to each well and kept for 5 min without light. The optical density was detected by a fluorescence microplate reader (Molecular Devices, Palo Alto, CA, USA) with excitation and emission at 480 and 520 nm. The percentage of proliferation was calculated by comparison with proliferation of epithelial cells from controls.

### RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) following the manufacturer's instructions. Samples were prepared 6 times and the entire experiment was repeated 3 times. The quality of RNA was determined by a NanaDrop Spectrophotometer (NanoDrop, Wilmington, DE, USA). Subsequently, first-strand cDNA was synthesized with a TaqMan Reverse Transcription Kit or microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The abundances of miR-744 and TGF-β1 were measured by qRT-PCR using SYBR green (Toyobo, Tokyo, Japan) detection with the amplification protocol. The following primers were designed from Invitrogen: TGF-β1 (Forward, 5'-GCCCTGGACACCAACTATT-3'; Reverse, 5'-AGGCTCCAAATGTAGGGG-3'), β-actin (Forward, 5'-AGCAGCATCGCCCCAAAGTT-3'; Reverse, 5'-GGGCACGA AGGCTCATCATT-3'), miR-744 (Forward, 5'-AATGCGGGGCT AGGGCTA-3'; Reverse, 5'-GTGCAGGG TCCGAGGT-3'), U6 (Forward, 5'-GCGCGTCGTGAAGCGTTC-3'; Reverse, 5'-GTGCAGGGTCCGAGGT-3'). Results were analyzed with $2^{-\Delta\Delta Ct}$ method using U6 small RNA and β-actin as housekeeping gene for normalization of miR-744 and TGF-β1, respectively.

### Western blots (WB)

Cell proteins were prepared using RIPA lysis buffer with 1% protease inhibitor and quantified using bicinchoninic acid (BCA) assay kit (Thermo Fisher) following the manufacturer's instructions. Samples were prepared 6 times and the entire experiment was repeated 3 times. Denatured samples at 98°C for 5 min were loaded onto a 10% SDS-PAGE gel and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk for 2 h at room temperature and then incubated overnight at 4°C with primary monoclonal antibodies against proliferating cell nuclear antigen (PCNA), TGF-β1, phosphorylated mothers against decapentaplegic homolog3 (p-Smad3), Smad3, and Smad anchor for receptor activation (SARA) or β-actin (Cell Signaling Technology, Danvers, MA, USA). Following 3 washes in Tris-buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated with secondary antibodies (CST) for 2 h at room temperature and then washed 3 times in TBST. Enhanced chemiluminescence (ECL) chromogenic substrate (GE Healthcare, Amersham, UK) was used for visualization of immunoreactivity, and densitometry analysis was conducted using Image Lab software (Bio-Rad, Hercules, CA, USA).

### Luciferase assays

Putative binging sites between miR-744 and 3' untranslated regions (3'-UTR) sequences of TGF-β1 were assessed using the TargetScan online software. The wild or mutant sequence of





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3'-UTR of TGF-β1 were amplified and cloned into pGL3 luciferase reporter vector or control vector (Promega, Madison, WI, USA) to generate the wide-type plasmid (TGF-β1-WT) or mutant-type plasmid (TGF-β1-MUT), respectively. WT or MUT luciferase reporter plasmids with miR-744 mimics or inhibitors were co-transfected in BEAS-2B cells using Lipofectamine 2000 according to the manufacturer's protocols. Samples were prepared 6 times and the entire experiment was repeated 3 times. Then, lysed cells were collected for the luciferase activities analysis using a Luciferase Assay Kit (GeneCopoeia, Rockville, MD, USA) after 48 h.

### RNA immunoprecipitation (RIP)

RIP of Argonaute 2 (Ago2) was used to probe the interaction between RNA and miRNA according to the protocol of the Magna RIP Kit (Millipore). In brief, NBECs and SABECs with miR-744 transfection were lysed in cell lysis buffer. Then, cell lysates were added to Anti-Ago2- or IgG-bound beads for 2 h at 4°C. Then, RNA-protein-beads complexes were washed in PBS and isolated using Trizol reagent. The expression of TGF-β1 in extracted products was analyzed by qRT-PCR. Samples were prepared 6 times and the entire experiment was repeated 3 times.

### Statistical analysis

All experiments were repeated independently more than 3 times. Data are presented as the mean ± standard error of mean. The statistical analysis was performed using GraphPad Prism 5 (GraphPad, Inc., La Jolla, CA, USA). The *t* test was used to assess significant differences between groups. The level of statistical significance in all graphs was $p<0.05$.

## Results

### miR-744 inhibits bronchial epithelial cells proliferation

Baseline proliferation of epithelial cells has an impact on the poor outcome of asthma. Hence, we analyzed the effect of miR-774 on bronchial epithelial cell proliferation. We collected the specimens and isolated cells from mild asthmatics (ABEC), severe asthmatics (SABEC), and normal controls (NBEC). The characteristics of asthmatics are shown in Table 1, indicating that asthma was associated with forced expiratory volume in 1 s (FEV1), provocative concentration producing a 20% fall in FEV1 (PC20 FEV1), blood eosinophils, sputum eosinophils, and atopic and inhaled corticosteroid (ICS) dose. Compared with NBEC, ABEC showed a strong decrease of proliferation rate, whereas SABEC displayed a progressive increase of proliferation rate (Figure 1A). To investigate whether miR-744 is required for severe asthma, the abundance of miR-744 was measured in severe asthma, mild asthma, and controls, showing that the abundance of mir-744 was altered in each subject (N=45), as revealed by an aberrantly reduced expression in SABECs compared to NBECs (0.66±0.09 compared to 0.99±0.07, $p=0.0055$) or ABECs (0.66±0.09 compared to 1.59±0.12, $p<0.0001$), respectively (Figure 1B). To validate whether miR-744 is critical for the proliferation of bronchial epithelial cells, transfection was carried out in NBECs and SABECs with miR-744 mimic or inhibitor. As expected, elevated abundance of miR-744 was observed in miR-744 mimic-transfected SABECs, while a loss of miR-744 level was shown in miR-744 inhibitor-transfected cells (Figure 1C). Furthermore, addition of miR-744 led to a lower proliferation rate in NBECs and SABECs, whereas abrogation of miR-744 facilitated cell proliferation compared with the NC group (Figure 1D). Overexpression of miR-744 reduced PCNA protein expression, while knockdown of miR-744 induced PCNA abundance in NBECs and SABECs (Figure 1E, 1F).

### miR-744 negatively regulates the abundance of TGF-β1

Because miR-744 was found to regulate bronchial epithelial cell proliferation in severe asthma, we probed a target gene of miR-744. Interestingly, bioinformatics analysis provided potential binding sites of miR-744 at position 17-23 within the 3'-UTR of TGF-β1 as determined using TargetScan (Figure 2A). Luciferase assay was performed to validate the interaction, showing a large reduction in luciferase activity in BEAS-2B cells co-transfected with TGF-β1 WT and miR-744 mimic, but little effect was shown in response to TGF-β1 MUT (Figure 2B). Conversely, abrogation of miR-744 had the opposite effect on luciferase activity (Figure 2C). Accumulation of miR-744 led to a significant decrease of TGF-β1 mRNA abundance in NBEC (Figure 2D) as well as in SABEC (Figure 2E). In contrast, miR-744 depletion caused the opposite effect on TGF-β1 mRNA level. Similarly, the abundance of TGF-β1 protein was markedly reduced by introduction of miR-744, and elevated by the absence of miR-744 in NBECs and SABECs (Figure 2F). In addition, Ago2 RIP assay showed that the abundances of TGF-β1 were significantly increased in bronchial epithelial cells with miR-744 mimic transfection compared with those in NC-treated cells, but IgG failed to show an effect (Figure 2G).

### TGF-β1 is essential for miR-744 modulating cell proliferation

To assess whether miR-744 drives cell proliferation by TGF-β1, we measured the cell proliferation in NBECs and SABECs transfected with TGF-β1 overexpression vector and/or miR-744 mimic or miR-NC. Overexpression of TGF-β1 promoted cell proliferation compared with the control group, whereas introduction of miR-744 mimic reversed TGF-β1-induced proliferation in NBECs and SABECs (Figure 3A, 3B). Addition of TGF-β1 enhanced the expression of PCNA protein in NBEC and SABEC compared with





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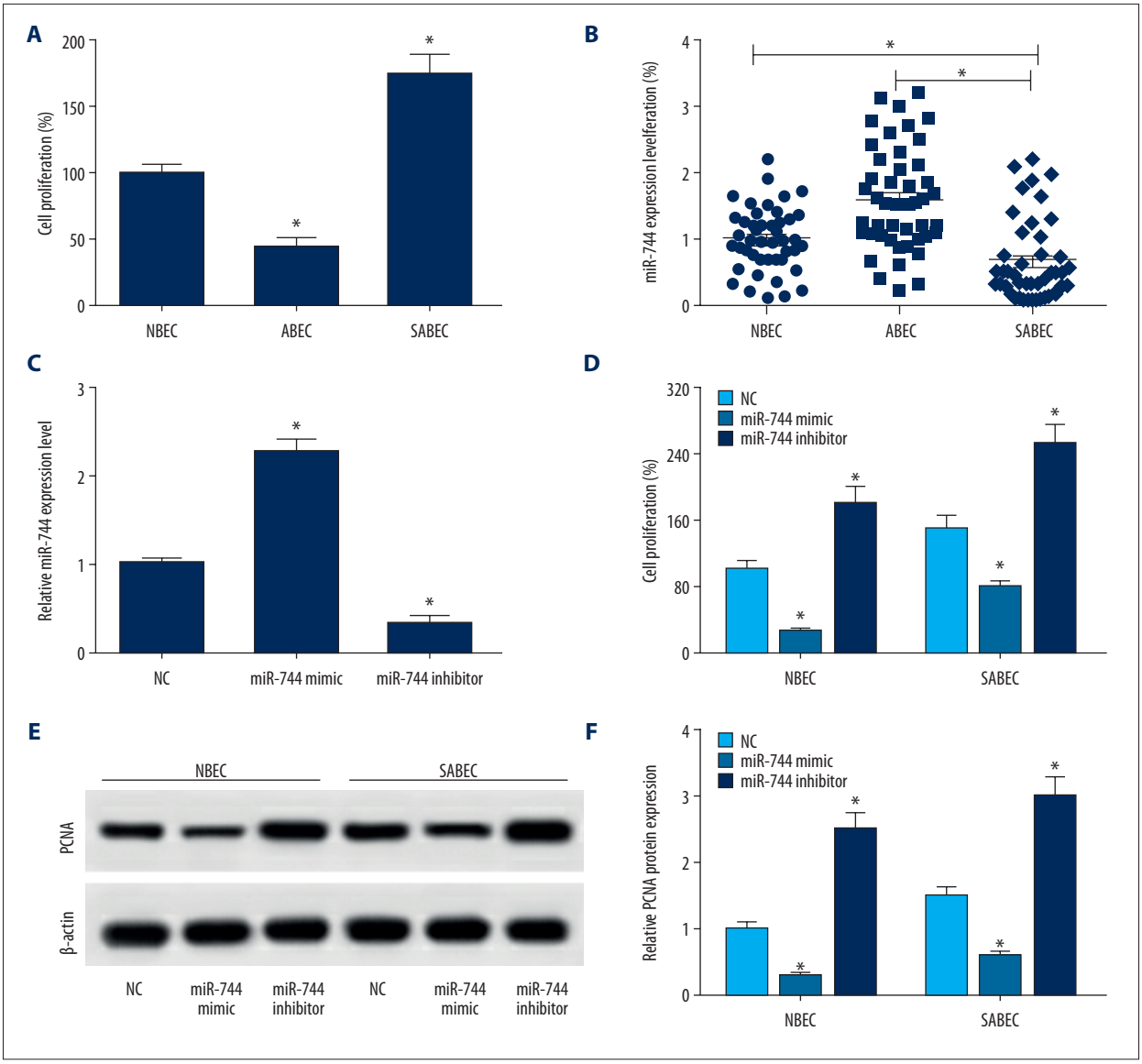


**Figure 1.** Addition of miR-744 reduced bronchial epithelial cells proliferation. (**A**) Cell proliferation was detected in bronchial epithelial cells from normal (NBEC), mild (ABEC), and severe (SABEC) asthmatic subjects. (**B**) The abundance of miR-744 was detected in bronchial epithelial cells of patients with severe asthma. (**C**) Alteration of miR-744 expression was detected in SABEC after miR-744 mimic or inhibitor treatment. (**D**) Cell proliferation rate was measured in bronchial epithelial cells using the Cell Proliferation Assay Kit. (**E, F**) The expression of PCNA protein was measured in NBEC and SABEC cells with miR-744 mimic or inhibitor transfection. * *P*<0.05 versus NC.

the control group (Figure 3C, 3D). However, the presence of miR-744 attenuated the abundance of PCNA caused by TGF-β1 in NBECs and SABECs (Figure 3C, 3D).

### miR-744 modulates phosphorylation of Smad3 and SARA production by regulating TGF-β1

To explore the potential mechanism by which miR-744 is involved in severe asthma progression, we assessed whether miR-744 alters the abundances of related proteins in the Smad pathway. We found that overexpression of TGF-β1 induced phosphorylation of Smad3 in NBECs and SABECs, whereas introduction of miR-744 protected against changes in the p-Smad3 level (Figure 4A, 4B). However, miR-744 and TGF-β1 failed to show an effect on Smad3 (Figure 4A, 4C). Addition of TGF-β1 resulted in loss of SARA production in NBECs and SABECs compared with control treatment (Figure 4A, 4D). However, accumulation of miR-744 restored the expression of SARA compared with miR-NC treatment (Figure 4A, 4D).





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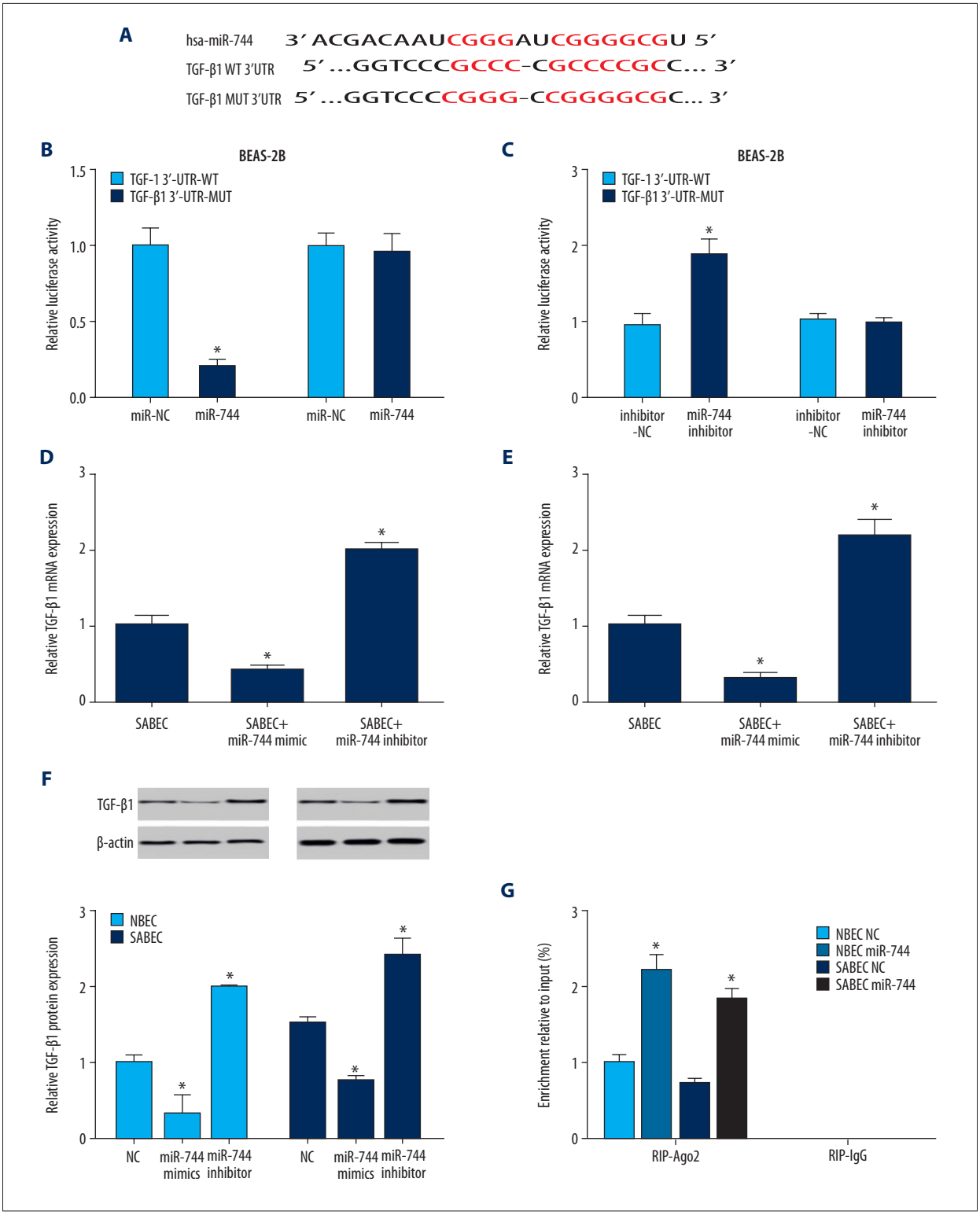


**Figure 2.** miR-744 directly targeted the 3'-UTR of TGF-β1. (**A**) Putative targeting sequences of miR-744 and 3'-UTR of TGF-β1 were provided by TargetScan. (**B, C**) Luciferase assays were used to probe the interaction between miR-744 and TGF-β1 in BEAS-2B cells with miR-744 mimic or inhibitor transfection. (**D–F**) Effects of miR-744 on TGF-β1 mRNA and protein expressions were evaluated in bronchial epithelial cells. (**G**) Interaction between miR-744 and TGF-β1 was analyzed by Ago2 RIP. * $P<0.05$ versus NC.





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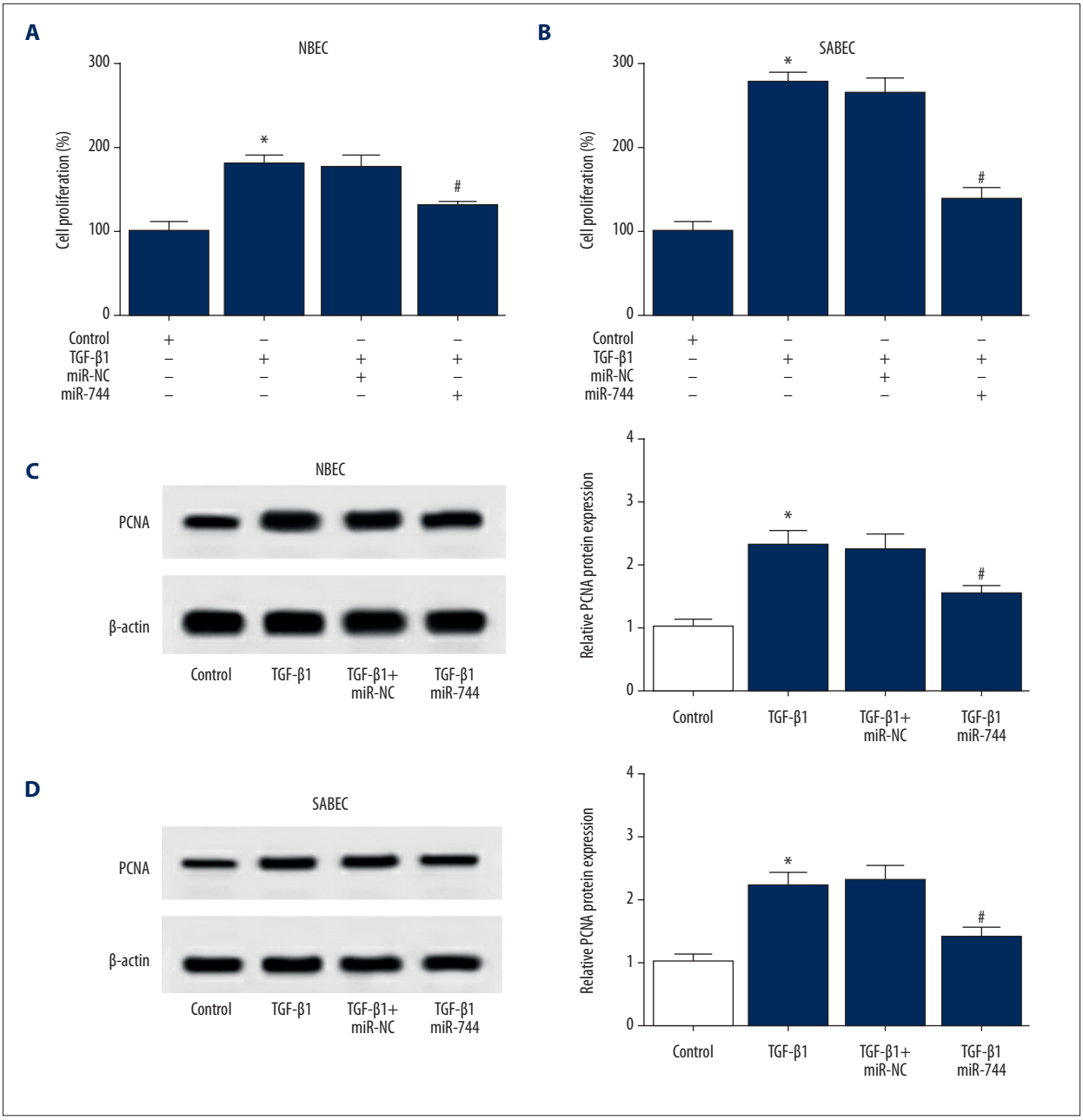


**Figure 3.** TGF-β1 was required for miR-744-mediated cell proliferation in bronchial epithelial cells from normal or severe asthma subjects. (**A, B**) Cell proliferation was measured in NBEC and SABEC cells transfected with TGF-β1 overexpression vector or (and) miR-744 mimic or miR-NC. (**C, D**) The abundances of PCNA were detected in transfected NBEC and SABEC cells. * $P<0.05$ versus control, # $p<0.05$ versus TGF-β1+miR-NC.

## Discussion

We found that miR-744 blocked proliferation of bronchial epithelial cells in severe asthma in children through regulating the Smad3 pathway by mediating the expression of TGF-β1. Previous studies showed epithelial damage in mild asthma and heightened epithelium damage in severe asthma, indicating the importance of epithelial function [25,26]. A higher rate of cell proliferation was observed in bronchial epithelial cells from severe asthmatic subjects in our study, in agreement with previous research [12]. We hypothesized that epithelium dysregulation in severe asthma requires epithelial proliferation for epithelium remodeling. However, the host effect on cell proliferation in severe asthma is elusive.





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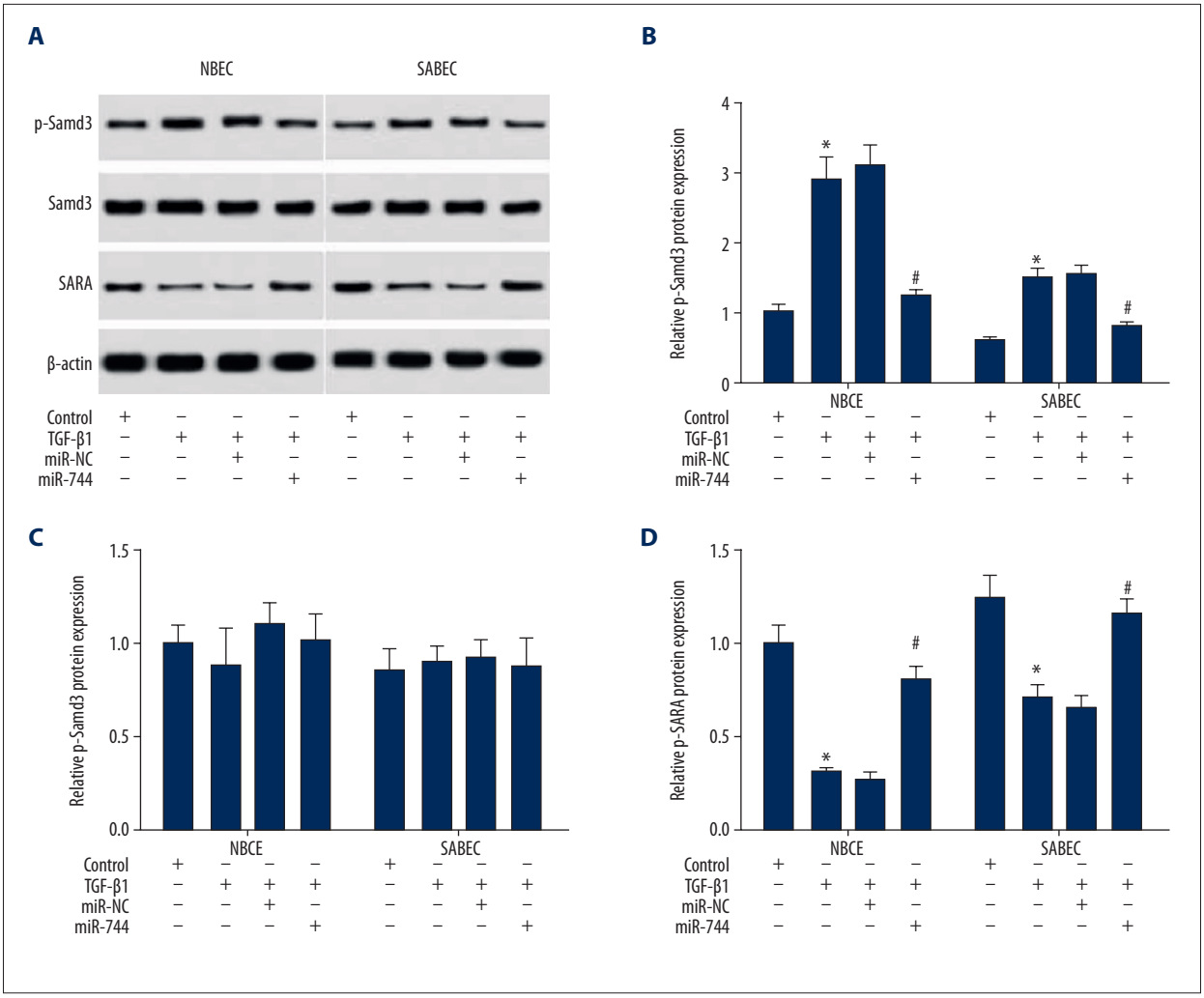


**Figure 4.** Enrichment of miR-744 reduced phosphorylation of Smad3 and increased the production of SARA in epithelial cells from normal or severe asthma subject. (**A**) Western blot analysis was conducted to detected total and phosphorylated Smad3 or SARA expression in NBECs and SABECs with TGF-β1 overexpression vector or (and) miR-744 mimic or miR-NC transfection. (**B–D**) Relative densitometry analyses of proteins were conducted using Image Lab software. * $P<0.05$ versus control, # $p<0.05$ versus TGF-β1+miR-NC.

Altered expression levels of miRNAs are required for severe asthma, as shown by the present study and in other research [16]. Here, we found miR-744 was lower in severe asthma, showing great promise for treatment of severe asthma. miR-744 has been reported to play essential roles in renal inflammation [27], neural differentiation [28], and cell proliferation [29]. Importantly, we showed that miR-744 limited the proliferation rate of bronchial epithelial cells in severe asthma. Likewise, miR-744 inhibits cell proliferation and invasion in colorectal cancer [15]. Introduction of miR-744 can activate the apoptotic pathway to limit cell proliferation in ovarian cancer cells [30]. This is also similar to findings that other miRNAs play important roles in asthma, and miR-139 and miR-34/449 were previously reported to inhibit proliferation and airway remodeling [31,32]. These finding suggest that miR-744 has a potentially profound effect on the progression of severe asthma. However, more information on how miR-744 affects severe asthma is required.

Generally, functional miRNAs are studies by modulating mRNA, usually inducing a reduced abundance of related protein [33]. As expected, using TargetScan software, we found some binding sites between miR-744 and 3'-UTR of TGF-β1. Using another approach to analyze the prediction of TGF-β1 as a target of miR-744, supported by luciferase activity assays and RIP, we next demonstrated that addition of miR-744 suppressed the abundance of TGF-β1 mRNA and protein in bronchial epithelial cells from severe asthmatics or normal controls. In recent years, most attention has been paid to TGF-β1, which was thought to promote proliferation of hepatic stellate cells





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and renal interstitial fibroblasts [34,35]. Previous studies suggested that the abundance of TGF-β1 was elevated in asthma patients [36,37]. In mild asthma, elevated TGF-β1 induced a loss of proliferation rate in asthmatics epithelial cells [36]. Nevertheless, TGF-β1 enhanced proliferation of fibroblasts to mediate airway remodeling in severe asthma [37]. In the present study, we found enhanced TGF-β1 facilitated bronchial epithelial cells proliferation in severe asthma. Emerging evidence suggests that miRNAs play a pivotal role in endothelial cells and detrusor fibrosis through the TGF-β1/Smad signaling pathway [38,39]. However, there is no direct evidence in support of the interaction between miR-744 and TGF-β1/Smad pathway.

The available evidence indicates that dysregulation of the TGF-β1/Smad pathway is essential for bronchial epithelial cell proliferation in patients with asthma [36]. TGF-β1 initiates cellular response, inducing phosphorylation of Smad2/Smad3 [40]. SARA was suggested to be required for Smad2/Smad3 signaling, and introduction of TGF-β1 lowered the SARA level in bronchial epithelial cells [6,41]. A similar trend was observed in our study, and further molecular assays showed that addition of miR-744 inhibited the abundances of p-Smad3 and increased SARA in bronchial epithelial cells by regulating TGF-β1. However, a previous study suggested that phosphorylation of Smad3 is negatively associated with cell proliferation [12]. These discrepancies may result from differences in sample sizes and cell sources, or from the different microenvironments in severe asthma between children and adults. Notably, we also found that miR-744 was ectopic in mild asthma in children and its abundance was higher than that in severe asthma. However, the mechanism and process involved in these differences are still elusive, and differences in results may arise from the disorganization or thickening of epithelium in asthma at different stages. Hence, the role and potential mechanism of miR-744 in mild asthma requires further investigation.

## Conclusions

The present study provides insights into the interaction between miR-744 and severe asthma in children. We found that patients with severe asthma have bronchial epithelial cells with high proliferation rate, miR-744 abundance was decreased in severe asthma patients, and addition of miR-744 inhibited bronchial epithelial cell proliferation by targeting TGF-β1. We3 also determined that miR-744 reduced Smad3 phosphorylation and elevated SARA levels by targeting TGF-β1 in cells of severe asthma patients. Our findings indicate that miR-744 inhibits cell proliferation through regulating the Smad3 signaling pathway by targeting TGF-β1, suggesting that miR-744 has therapeutic potential by targeting epithelial repair in children with severe asthma.

### Conflicts of interests

None.

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