



Long non-coding RNA TUG1 promotes airway remodeling and mucus production in asthmatic mice through the microRNA-181b/HMGB1 axis

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ABSTRACT

MicroRNA-181b (miR-181b) has been well noted with anti-inflammatory properties in several pathological conditions. It has also been suggested to be downregulated in patients with asthma. In this study, we explored the function of miR-181b in airway remodeling in asthmatic mice and the molecular mechanism. A mouse model with asthma was induced by ovalbumin (OVA) challenge, and miR-181b was found to be downregulated in lung tissues in the OVA-challenged mice. Overexpression of miR-181b was introduced in mice, after which the respiratory resistance, inflammatory infiltration, mucus production, and epithelial-mesenchymal transition (EMT) and fibrosis in mouse airway tissues were decreased. The integrated bioinformatics analysis suggested long non-coding RNA (lncRNA) TUG1 as a sponge for miR-181b. miR-181 directly targeted high mobility group box 1 (HMGB1) mRNA. HMGB1 was suggested to enhance activation of the nuclear factor kappa B (NF-κB) signaling. Further upregulation of lncRNA TUG1 blocked the protective functions of miR-181b in asthmatic mice. To conclude, this study evidenced that lncRNA TUG1 reinforces HMGB1 expression through sequestering microRNA-181b, which activates the NF-κB signaling pathway and promotes airway remodeling in asthmatic mice. This study may provide novel ideas in asthma management.

1. Introduction

Affecting approximately 340 million people worldwide annually, asthma is a frequent chronic inflammatory disease of the airways which characterized by airway inflammation and the subsequent symptoms including wheezing, chest tightness, cough, and breathlessness [1]. Airway narrowing, variable airflow obstruction, increased mucus production, airway hyperresponsiveness, and airway remodeling that involves epithelial damage and sub-epithelial fibrosis are hallmark events during asthma pathogenesis [2–4]. Asthma is a heterogeneous disease which mainly arises from the interactions between genetics and environmental exposure [5]. This allergic disease has been a global pandemic persistently since the existing treatments cannot effectively control the symptoms without side effects [6]. The need for novel options has aroused both basic and pharmacological researches to identify the inflammatory background of asthma and the molecular mechanism involved in the disease development.

The versatile and potent functions of non-coding RNAs (ncRNAs) in respiratory diseases have aroused increasing concerns, and microRNAs

(miRNAs) represent the mostly studied ncRNAs which govern mRNA translation through the RNA interference axis [7]. miRNAs are short RNAs comprising 18–25 nucleotides that induce mRNA degradation, and they are key players in asthma due to their regulation in inflammatory response in tissues [8]. Importantly, other transcripts, including long ncRNAs (lncRNAs), may sequester a miRNA and block its repressive functions on other transcripts, which are termed competitive endogenous RNAs (ceRNAs) [9,10]. Such ceRNAs mutually alter each other's abundance by competing for a limited pool of common miRNAs [11]. The lncRNA-miRNA-mRNA interactions have been validated in asthma as well [12]. miR-181b, a dominant miR-181 family member, has been well established with anti-inflammatory functions in several pathological conditions [13–15]. Since chronic inflammation and the subsequent airway remodeling are key attributes to allergic asthma [16], we therefore aimed to explore the potential function of miR-181b in asthma.

Our integrated bioinformatics analyses suggested that miR-181b owned binding relationships with lncRNA taurine upregulated gene 1 (TUG1) and high mobility group box 1 (HMGB1). LncRNA TUG1 has

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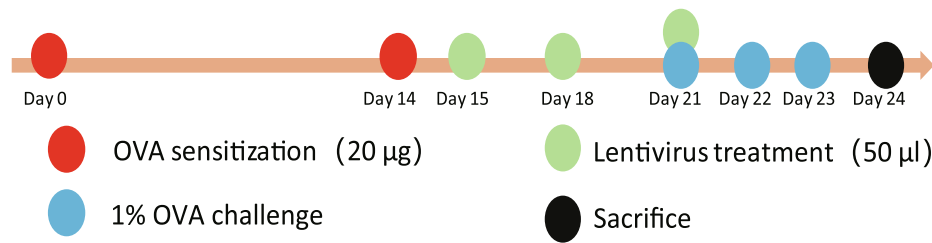


Fig. 1. A diagram for the establishment of the mouse model with asthma induced by OVA challenge.

shown pro-inflammatory and pro-fibrotic functions in endometrium [17]. Importantly, TUG1 was reported to promote airway remodeling in cigarette smoke-induced chronic obstructive pulmonary disease (COPD) [18]. As for HMGB1, it is a highly conserved DNA-binding protein and serves as an endogenous 'danger signal' for the initiation of innate immunity [19]. Importantly, HMGB1 has been documented as a contributor to allergen-induced airway remodeling and inflammation in asthmatic mice [20]. We therefore speculated that there is a lncRNA TUG/miR-181b/HMGB1 ceRNA axis in asthma progression, and gain-and loss-of-function studies of these molecules were performed in a mouse model to validate this hypothesis.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Animal Ethics Committee of Nanfang Hospital, Southern Medical University. All animal procedures were performed in line with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH, Bethesda, Maryland, USA). Great attempts were made to reduce the number and suffering of animals.

2.2. Animal model with asthma

A mouse model with asthma was induced by ovalbumin (OVA)

challenge. Female mice that are more susceptible to allergic airway inflammation were used in the present study [21]. Specific-pathogen-free grade female BALB/c mouse (6–8 weeks old, 20 ± 2 g) acquired from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) were used for model establishment. The animals were acclimatized for one week. After that, on day 0 and day 14, the animals were sensitized through an intraperitoneal injection of 2 mg aluminum hydroxide and 20 µg OVA. From day 21 to day 23, the airway of mice was challenged by 1% OVA in aerosol using an ultrasonic nebulizer (PARI GmbH, Starnberg, Germany). The control mice were treated with an equal volume of phosphate-buffered saline (PBS) instead at the same time points. The lentivirus was administrated into mice on day 15, day 18, and day 21 (before OVA challenge) through caudal veins. The miR-181b-overexpressing lentivirus (LV-mimic) and TUG1-overexpressing lentivirus (LV-TUG1) were provided by GenePharma Co., Ltd. (Shanghai, China). The virus titer was 1×10^9 TU/mL with 50 µL lentivirus injected each time. A diagram for these procedures is shown in Fig. 1.

2.3. Evaluation of respiratory resistance (Rrs) in mouse airway

Mice were anaesthetized by 1% pentobarbital sodium 24 h after the last OVA challenge (on day 24). Then, the AHR of mice was measured using an AniRes2005 animal pulmonary function system (Bestlab, Beijing, China) as per the manufacturer's protocols. The mice were allowed to inhale an ascending series of methacholine (0–4 mg/mL), and then the change in Rrs of mouse was evaluated.

2.4. Bronchoalveolar lavage fluid (BALF) analysis

After AHR measurement, the mice were given tracheal cannula, and 0.8 mL cold PBS was loaded into mouse lung and recovered through the cannula for lung lavage. After three repetitions, the lavage fluid was collected, and the cell counts of total karyocytes, eosinophils, macrophages and lymphocytes were calculated using a Qi3538 differential blood-cell calculator (Yunyan Instrument Co., Ltd., Shanghai, China).

2.5. Histological examination of lung

Lungs were fixed by an injection of 4% buffered paraformaldehyde into the tracheal cannula at a pressure of 20 cm H₂O, and then the lung tissue was removed from the chest cavity. Then, the lungs were immersed in paraformaldehyde for 24 h, embedded in paraffin, and cut into 5-µm sections. The sections were stained by the hematoxylin and eosin (HE) staining and periodic acid schiff (PAS) staining kits (Solarbio Science & Technology Co., Ltd., Beijing, China) to evaluate the inflammatory infiltration and mucus production in airway tissues. The inflammation and the enrichment of mucous cells was scored by three group-blinded pathologists. The HE inflammation scoring standards were as follows: 0, normal; 1, a few cells; 2, a ring of inflammatory cells, one cell layer deep; 3, a ring of inflammatory cells, 2–4 cells deep; 4, a ring of inflammatory cells, > 4 cells deep. The PAS staining scoring standards were as follows: 0, < 5% PAS-positive cells; 1, 5%–25% PAS-positive cells; 2, 25%–30% PAS-positive cells; 3, 50%–75% PAS-positive

Table 1
Primer sequences for RT-qPCR.

Gene	Primer sequence (5'-3')
miR-181b	F: TTCATTGCTGTCGGTGGG R: GAACATGTCTGCGTATCTC
lncRNA-TUG1	F: CTGAAGAAAGCAATCCATC R: GTAGGCTACTACAGGTCAATTTG
HMGB1	F: CCAAGAAAGTCTCAGAGAGGTG R: GTCCTTGAACCTCTTTTGGTCTC
ARNT2	F: GAAGACGCTGATGTCGGACAAG R: CAGAGTTGTGCCGTGACAGGAA
NR6A1	F: CCCTCTGATGAAGAACTCCACAG R: ACTCCTCGTTGCTGACCTTCAG
MYCBP2	F: CCTACTGTGCAAACTGGACTCC R: CTTGGCTTGACTAGCTGAGTC
TNIK	F: ACCTGTGATGGCTGTGGCAAGA R: TCTGAACAGCAACGGTGTGGA
CLASP1	F: CCTGGTTCTGTGAACGCCATGA R: GGCTCGTATCTCCTTCTCACAG
GAPDH	F: ATCACTGCCACCCAGAAAGAC R: TTTCTAGACGGCAGGTCTAGG
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT

Note: RT-qPCR, reverse transcription quantitative polymerase chain reaction; miR, microRNA; lncRNA-TUG1, long non-coding RNA taurine upregulated gene 1; HMGB1, high mobility group box 1; ARNT2, aryl hydrocarbon receptor nuclear translocator 2; NR6A1, nuclear receptor subfamily 6 group A member 1; MYCBP2, MYC binding protein 2; TNK, TRAF2 and NCK interacting kinase; CLASP1, cytoplasmic linker associated protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F: forward; R, reverse.

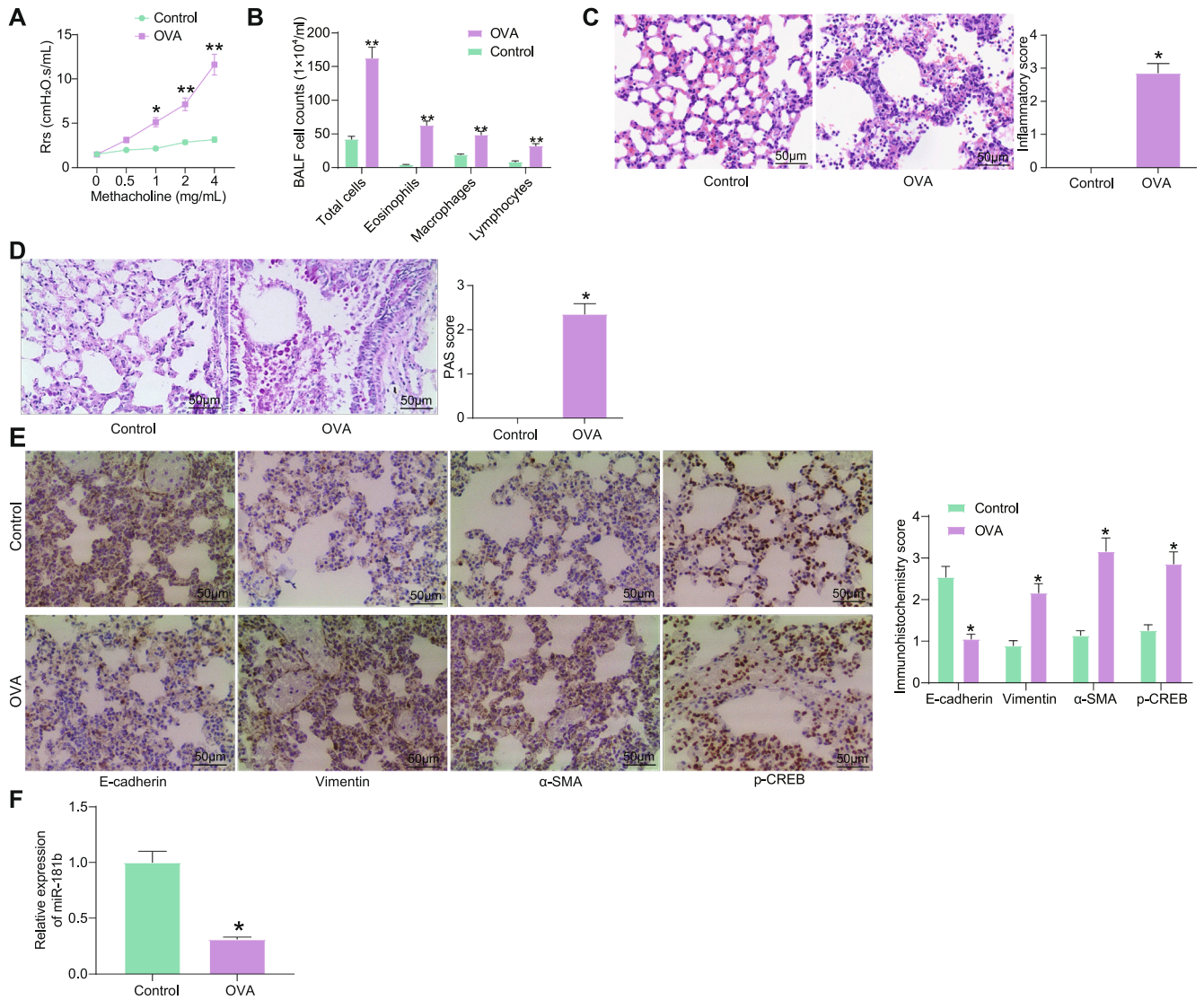


Fig. 2. miR-181b is poorly expressed in asthmatic mice. A, Rrs in mouse airway induced by different concentrations of methacholine; B, number of total cells, eosinophils, macrophages and lymphocytes in BALF of the mice; C, inflammatory infiltration in mouse airway observed by HE staining; D, mucus production in mouse airway examined by PAS staining; E, expression of E-cadherin, Vimentin, α-SMA and p-CREB in mouse airway tissues determined by IHC staining; F, expression of miR-181b in mouse airway tissues determined by RT-qPCR. N = 10 in each group. Data were expressed as mean ± SEM from three independent experiments. Data were analyzed by the unpaired *t* test (C, D and F) and two-way ANOVA (A, B and E). * *p* < 0.05, ** *p* < 0.01 vs. control.

cells; 4, > 75% PAS-positive cells. In addition, the expression of epithelial-mesenchymal transition (EMT)-related factors E-cadherin (ab231303, Abcam Inc., Cambridge, MA, USA) and Vimentin (ab8978, Abcam), the expression of fibrosis-related factor α-smooth muscle actin (α-SMA, ab7817, Abcam), and the expression of mucus production-related phospho-cAMP responsive element binding protein (p-CREB, phospho S133) (1:100, ab32096, Abcam) were determined by immunohistochemical (IHC) staining as per a previous report [22]. The expression was quantified by the Image J software (NIH), and the IHC staining intensity was scored as per the same scoring standards as the PAS scoring above.

2.6. Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Tissue and cells were incubated with TRIzol Reagent (Invitrogen, Thermo Fisher Scientific Inc., Waltham, MA, USA) to collect total RNA. An All-in-One™ first-strand cDNA synthesis (kit Fugen, Guangzhou, Guangdong, China) was used for cDNA synthesis. Next, real-time qPCR

was performed using SYBR green (Applied Biosystems, Inc., Carlsbad, CA, USA). Relative gene expression was determined using the 2^{-ΔΔCt} method. U6 and GAPDH were set as the internal references. The primers are listed in Table 1.

2.7. Cell treatment

Mouse airway smooth muscle cells (ASMC, CP-M005, Procell Life Science & Technology Co., Ltd., Wuhan, Hubei, China) were used to validate the binding relationships among miR-181b, TUG1 and HMGB1. The cells were cultured in ASMC-specific medium (CM-M005, Procell) at 37 °C in air enriched with 5% CO₂. The miR-181b mimic and negative control (NC) mimic were acquired from GenePharma and transfected into ASMCs using the Lipofectamine 2000 kit (Invitrogen) according to the kit's instructions.

2.8. Dual-luciferase reporter gene assay

The binding sequences between lncRNA TUG1 and miR-181b, and

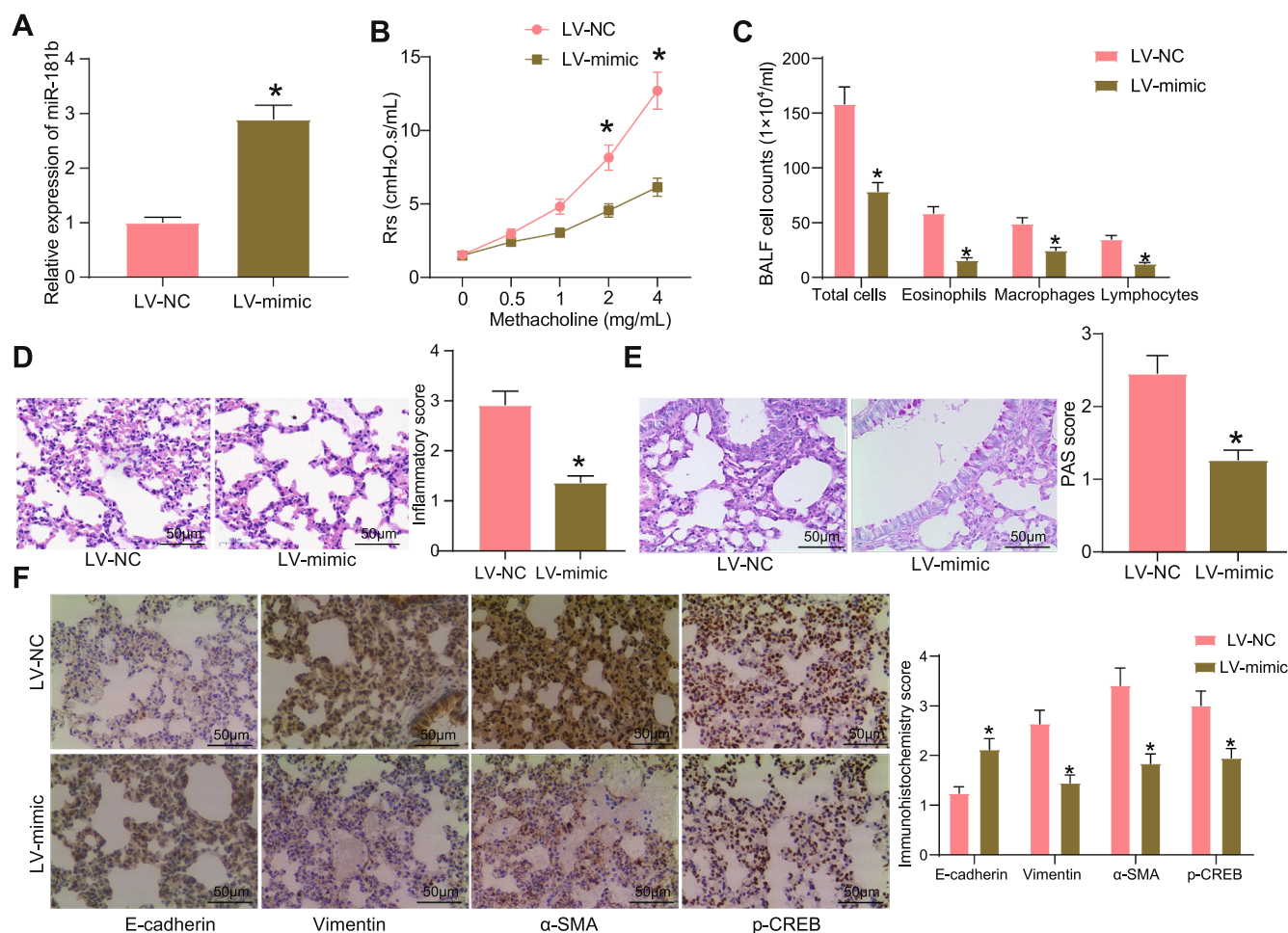


Fig. 3. Upregulation of miR-181b inhibits airway remodeling and mucus production in asthmatic mice. A, expression of miR-181b in mouse lung tissues determined by RT-qPCR; B, Rrs in mouse airway induced by different concentrations of methacholine; C, number of total cells, eosinophils, macrophages and lymphocytes in BALF of the mice; D, inflammatory infiltration in mouse airway observed by HE staining; E, mucus production in mouse airway examined by PAS staining; F, expression of E-cadherin, Vimentin, α -SMA and p-CREB in mouse airway tissues determined by IHC staining. N = 10 in each group. Data were expressed as mean $\pm$ SEM from three independent experiments. Data were analyzed by the unpaired *t* test (A, D and E) and two-way ANOVA (B, C and F). * *p* < 0.05 vs. LV-NC.

between miR-181b and HMGB1 were first predicted on StarBase (<http://starbase.sysu.edu.cn/>). The pmirGLO vector (Promega, Madison, WI, USA) containing the wild-type (WT) and mutant-type (MT) miR-181b binding sites within TUG1 sequence or HMGB1 3'UTR were designed and named TUG1-WT/MT and HMGB1WT/MT vectors, respectively. Well-constructed vectors were co-transfected with either miR-181b mimic or NC mimic into ASMCs for 48 h, and then the luciferase activity in cells was determined using a dual-luciferase reporter system (Promega).

2.9. RNA pull-down assay

The miR-181b and NC were labeled by biotin by RiboBio Co., Ltd. (Guangzhou, Guangdong China). Then, the Bio-miR-181b and Bio-NC were co-cultured with ASMC lysates and Dyna-beads (Invitrogen). After being washed, the RNA pulled down was quantified by RT-qPCR.

2.10. RNA immunoprecipitation (RIP)

A RIP method was applied to examine the RNA enrichment in cells. According to the instructions of a Magna RIPTM RNA-binding protein immunoprecipitation kit (Millipore, Bedford, MA, USA), ASMCs in RIP lysis buffer were co-incubated with antibodies against Ago2 or control IgG (Millipore) for immunoprecipitation. Then, magnet beads were further added, and the RNA in precipitates was determined by RT-qPCR.

2.11. Western blot analysis

The ASMCs and tissues were lysed in radio-immunoprecipitation assay cell lysis buffer (Beyotime Biotechnology Co., Ltd., Shanghai, China) containing protease inhibitor (Beyotime) to collect total protein, and the protein concentration was determined using a bicinchoninic acid (BCA) kit. Next, an equal volume of protein sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore). After that, the membranes were blocked by 5% non-fat milk (Beyotime) at room temperature for 10 min. Then, the membranes were co-cultured with the primary antibodies against HMGB1 (1:10,000, ab79823, Abcam), nuclear factor kappa B p65 (NF- κ B p65, 1:1,000, #8242, Cell Signaling Technology (CST), Beverly, MA, USA), Phospho-NF κ B p65 (Ser536, 1:1,000, #3033, CST) and GAPDH (1:1,000, ab181602, Abcam) at 4 °C overnight. Thereafter, the membranes were further incubated with the secondary antibody IgG H&L (HRP) at room temperature for 1 h. The protein signals were examined using an enhanced chemiluminescence (Beyotime) and analyzed by Image J.

2.12. Statistical analysis

All statistical analyses were performed using the GraphPad Prism 7 software (GraphPad Inc., San Diego, CA, USA). Data were in normal distribution and expressed as mean $\pm$ standard error of mean (SEM).

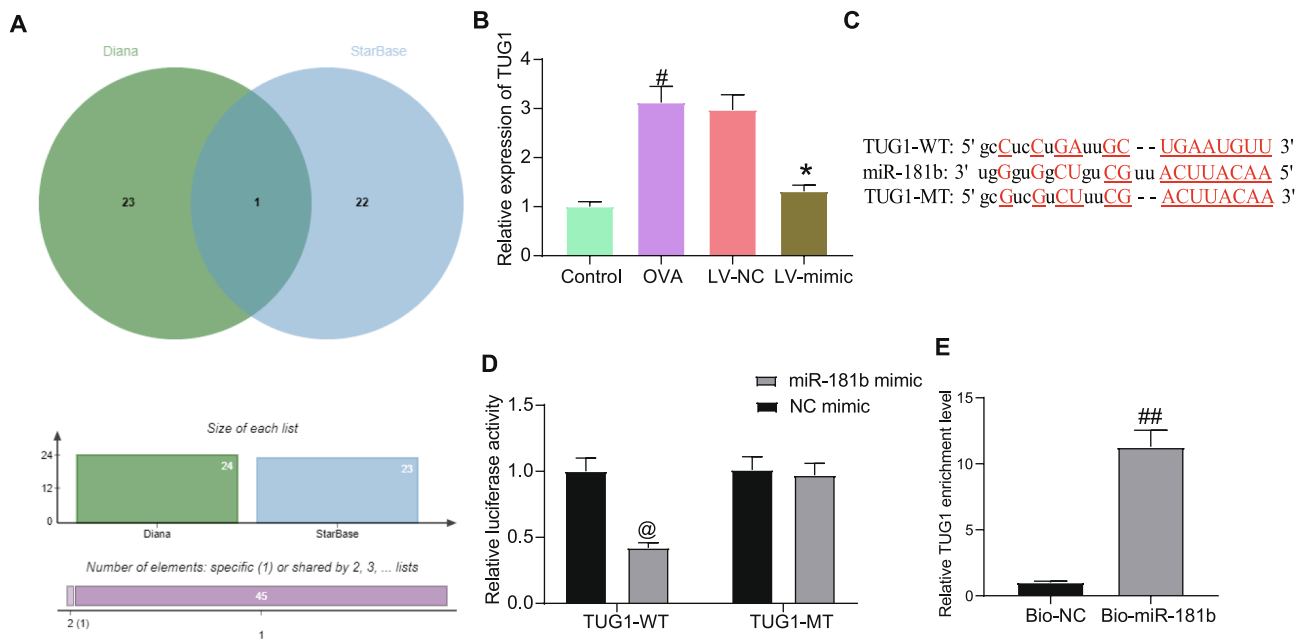


Fig. 4. LncRNA TUG1 serves as a sponge for miR-181b. A, potential lncRNAs that may bind to mmu-miR-181b predicted using StarBase and Diana bioinformatics systems; B, TUG1 expression in mouse lung tissues determined by RT-qPCR; C, putative binding sequence between TUG1 and miR-181b and the corresponding mutant sequence; D-E, binding relationship between miR-181b mimic and lncRNA TUG1 verified through dual luciferase reporter gene (D) and RNA pull-down (E) assays. Data were expressed as mean $\pm$ SEM from three independent experiments. Data were analyzed by the unpaired *t* test (E), one-way ANOVA (B), and two-way ANOVA (D). [#] *p* < 0.05 vs. control; ^{*} *p* < 0.05 vs. LV-NC; [@] *p* < 0.05 vs. NC mimic; ^{##} *p* < 0.01 vs. Bio-NC.

from three independent experiments. Differences were compared by unpaired *t* test (two groups) or one-way or two-way analysis of variance followed by Tukey's comparison test (ANOVA, more than two groups). *p* value < 0.05 (*p* < 0.05) represents a significant difference.

3. Results

3.1. miR-181b is poorly expressed in asthmatic mice

A mouse model with asthma was induced by OVA challenge, and mice treated with an equal volume of PBS were used as controls (Fig. 1). The Rrs in mouse airway was determined. It was found that the Rrs in OVA-challenged mice was notably enhanced as the increase of the methacholine (Fig. 2A). The cell counts in BALF was determined as well. It was found that the number of total cells, eosinophils, macrophages and lymphocytes in BALF of the OVA-challenged mice was significantly increased (Fig. 2B). The pathological changes in airway tissues was examined as well. The HE staining showed a significant inflammatory infiltration in the airway tissues of OVA-induced asthmatic mice (Fig. 2C), and the PAS staining results suggested an increased mucus production in the airway of the model mice (Fig. 2D). In addition, EMT and fibrosis are critical events during airway remodeling [23,24]. Thereafter, the IHC staining results suggested that OVA challenge led to a decline in the expression of epithelial marker E-cadherin while an increase in the expression of the mesenchymal marker Vimentin, indicating that the EMT process was triggered by OVA challenge. Likewise, the expression of the fibrosis marker α -SMA and the mucus production-related p-CREB in mouse airway was significantly increased as well (Fig. 2E). These results indicated that the mouse model was successfully established. In this setting, the miR-181b expression in the airway tissues of mouse was determined by RT-qPCR, which suggested that OVA challenge led to a notable decline in miR-181b expression in mouse (Fig. 2F).

3.2. Upregulation of miR-181b inhibits airway remodeling and mucus production in asthmatic mice

From the second day after the last OVA sensitization (day 15), LV-mimic overexpressing miR-181b was injected into the mice through caudal veins for three times at a three-day interval. Then, the mice were subjected to aerosol OVA challenge. The RT-qPCR suggested that the miR-181 expression in mouse lung tissues was notably increased after LV-mimic injection (Fig. 3A). Under this condition, it was found that the Rrs in mouse airway increased by OVA was then reduced by LV-mimic (Fig. 3B). Also, upregulation of miR-181b reduced the number of total cells, eosinophils, macrophages and lymphocytes in BALF of mice (Fig. 3C). The subsequent HE staining showed that overexpression of miR-181b reduced inflammatory infiltration, while the PAS staining suggested that the mucus production was reduced as well in this setting (Fig. 3D-E). Besides, upregulation of miR-181b further increased the expression of E-cadherin, while suppressed the expression of Vimentin, α -SMA and p-CREB in mouse airway tissues (Fig. 3F).

3.3. LncRNA TUG1 serves as a sponge for miR-181b

To explore the potential molecules involved in this cascade, we first predicted the potential lncRNAs that might bind to mmu-miR-181b using the bioinformatics systems StarBase and Diana (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php), and lncRNA TUG1 was confirmed as the only common outcome (Fig. 4A). We then explored TUG1 expression in the mouse lung tissues by RT-qPCR, which suggested that the expression of TUG1 was notably increased in model mice after OVA challenge but then reduced after LV-mimic injection (Fig. 4B).

To validate the binding relationship between lncRNA TUG1 and miR-181b, the putative binding site between lncRNA TUG1 and miR-181b was obtained from StarBase (Fig. 4C). Then, the TUG1-WT/TUG1-MT luciferase reporter vectors were constructed and co-transfected with NC mimic and miR-181b mimic into ASMCs. After 48 h, it was found that miR-181b mimic suppressed the luciferase activity of TUG1-WT in cells,

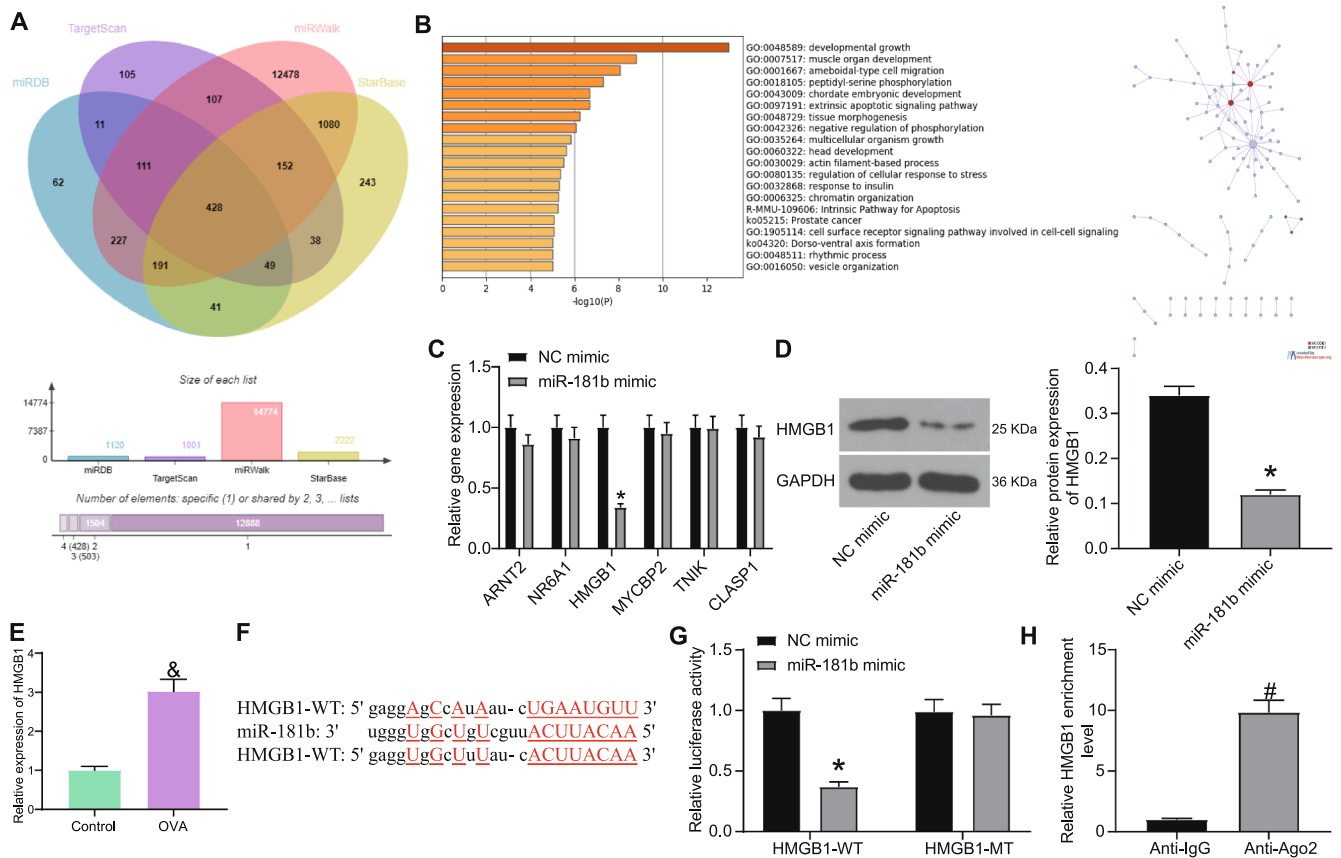


Fig. 5. miR-181b directly binds to HMGB1 mRNA. A, candidate downstream mRNAs of mmu-miR-181b predicted using four bioinformatics systems; B, a KEGG pathway enrichment analysis based on the screened genes; C, mRNA expression of these genes in ASMCs after miR-181b mimic transfection determined by RT-qPCR; D, protein expression of HMGB1 in ASMCs after miR-181b mimic transfection evaluated by western blot analysis; E, expression of HMGB1 mRNA in the lung tissues of OVA-challenged mice examined by RT-qPCR; F, putative binding sequence between miR-181b and HMGB1 mRNA and the corresponding mutant sequence; G-H, binding relationship between miR-181b mimic and HMGB1 mRNA verified through dual luciferase reporter gene (G) and RIP (H) assays. Data were expressed as mean $\pm$ SEM from three independent experiments. Data were analyzed by the unpaired t test (D, E and H) and two-way ANOVA (C and G). * $p < 0.05$ vs. NC mimic; # $p < 0.05$ vs. anti-IgG; & $p < 0.05$ vs. control.

whereas it showed little impact on the luciferase activity of TUG1-MT (Fig. 4D). In addition, the RNA pull-down assay performed using ASMCs confirmed an enrichment of TUG1 fragments in the compounds pulled down by Bio-miR-181b (Fig. 4E). These results verified that lncRNA TUG1 can specifically bind to miR-181b.

3.4. miR-181b directly binds to HMGB1 mRNA

The focus of the study then shifted to finding out the downstream target of miR-181b. Likewise, we explored the potential candidate downstream mRNAs of mmu-miR-181b on several bioinformatics systems including StarBase, TargetScan (http://www.targetscan.org/vert_72/), miRDB (<http://mirdb.org/>) and miRwalk (<http://mirwalk.umm.uni-heidelberg.de/>). Consequently, 428 common mRNAs were intersected (Fig. 5A). Subsequently, a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed to screen the enriched signaling pathways based on these genes, and the NF- κ B signaling pathway was found enriched (Fig. 5B).

To confirm the exact target mRNAs involved, we determined the expression of these mRNAs in ASMCs following miR-181b mimic transfection. The RT-qPCR results showed that only HMGB1 expression showed a decline after miR-181b upregulation (Fig. 5C). The western blot analysis also found that miR-181b mimic suppressed the protein expression of HMGB1 in ASMCs (Fig. 5D). In addition, we examined the expression of HMGB1 in lung tissues of the OVA-challenged mice and control mice. It was found that the HMGB1 level was significantly

increased in the asthmatic mice (Fig. 5E). Likewise, the putative binding site between miR-181b and HMGB1 was obtained from StarBase (Fig. 5F), and the following dual-luciferase reporter gene assay suggested co-transfection of miR-181b mimic and HMGB1-MT led to a notably decline in the luciferase activity in cells (Fig. 5G). In addition, the RIP assay suggested that the miR-181b and HMGB1 fragments were enriched by anti-Ago2 compared to anti-IgG (Fig. 5H).

3.5. lncRNA TUG1 upregulates HMGB1 expression in asthmatic mice through sponging miR-181b

Following the findings above, we further examined whether the TUG1/miR-181b/HMGB1 cascade affects asthma progression. The asthmatic mice administrated with LV-mimic were further injected with the LV-TUG1 vector to additionally upregulate the expression of lncRNA TUG1 in mice, and mice injected with LV-mimic + LV-NC were set as controls. The RT-qPCR results showed that LV-mimic alone led to a decline in the expression of TUG1 and HMGB1 in mouse lung tissues, while this trend was blocked by LV-TUG1 (Fig. 6A). The NF- κ B pathway was suggested to be enriched according to the above KEGG analysis, while HMGB1 has also been widely reported as a positive regulator of the NF- κ B signaling [25,26]. Activation of this signaling has been frequently confirmed in asthma pathogenesis [27,28]. We therefore explored the protein expression of HMGB1 and phosphorylation of NF- κ B p65 in mouse lung tissues. The western blot analysis results suggested that the protein level of HMGB1 and phosphorylation of NF- κ B p65 were

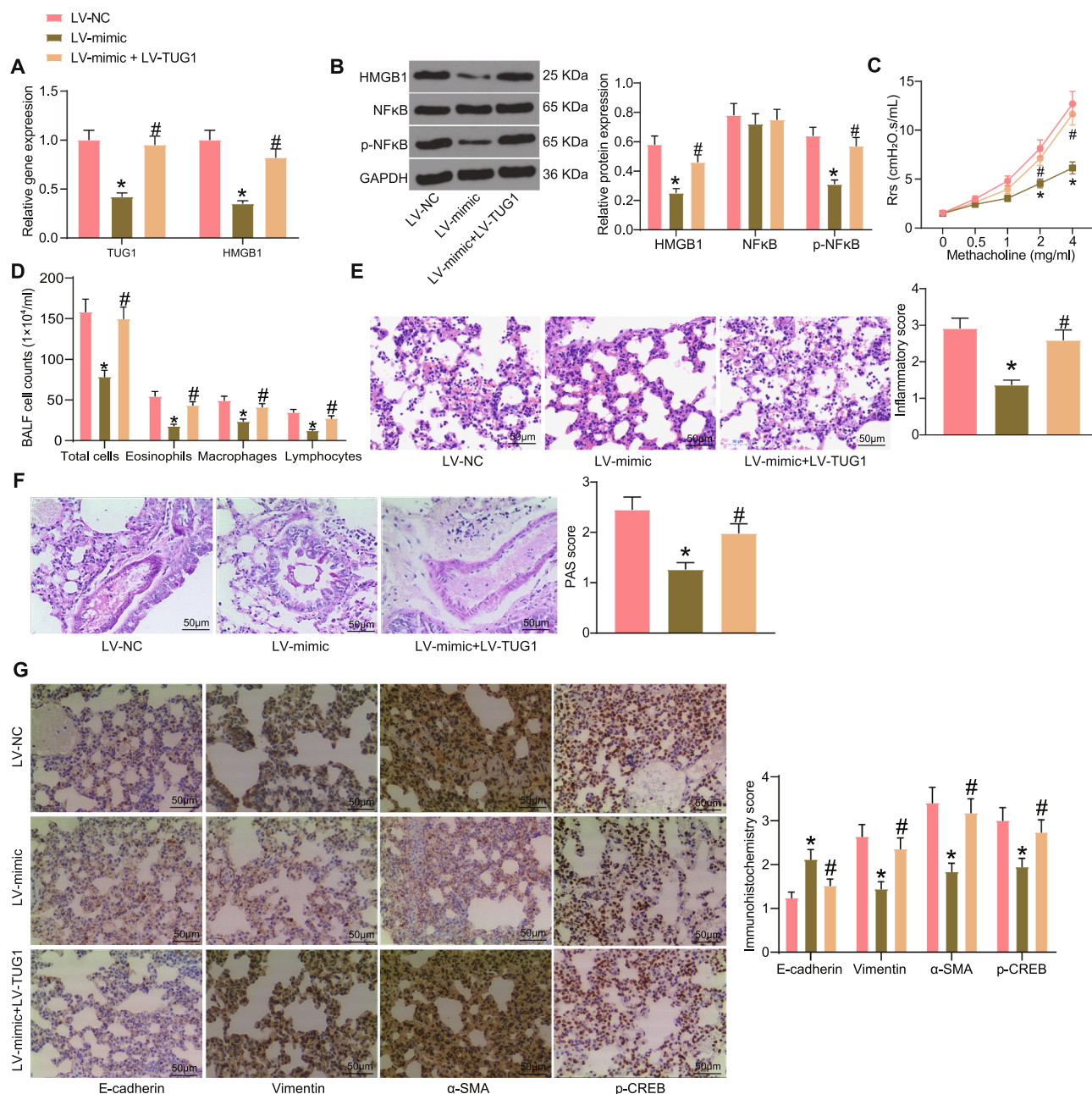


Fig. 6. LncRNA TUG1 upregulates HMGB1 expression in asthmatic mice through sponging miR-181b. A, expression of lncRNA TUG1 and HMGB1 mRNA in mouse lung tissues determined by RT-qPCR; B, protein level of HMGB1 and phosphorylation of NF-κB p65 in mouse lung tissues detected by western blot analysis; C, Rrs in mouse airway induced by different concentrations of methacholine; D, number of total cells, eosinophils macrophages and lymphocytes in BALF of the mice; E, inflammatory infiltration in mouse airway observed by HE staining; F, mucus production in mouse airway examined by PAS staining; G, expression of E-cadherin, Vimentin, α-SMA and p-CREB in mouse airway tissues determined by IHC staining. N = 10 in each group. Data were expressed as mean ± SEM from three independent experiments. Data were analyzed by one-way ANOVA (E and F) and two-way ANOVA (A, B, C, D and G). * $p < 0.05$ vs. LV-NC; # $p < 0.05$ vs LV-mimic.

initially suppressed by LV-mimic but then enhanced by LV-TUG1 (Fig. 6B).

The function of LV-TUG1 on the pathological presentations in mice was determined as well. First, the Rrs in mouse airway reduced by LV-mimic was aggravated after further TUG1 upregulation (Fig. 6C). Also, LV-TUG1 led to an increase in the number of total cells, eosinophils, macrophages and lymphocytes in BALF of mice (Fig. 6D). The HE staining results showed that upregulation of TUG1 increased inflammatory infiltration in mouse airway tissues (Fig. 6E), and the PAS staining exhibited that the mucus production was enhanced as well (Fig. 6F). In addition, LV-TUG1 blocked the functions of LV-mimic on EMT since it reduced the expression of E-cadherin, while increased the

expression of Vimentin, α-SMA and p-CREB in mouse airway tissues (Fig. 6G).

4. Discussion

Asthma still represents a worldwide challenge since the current treatment and overall management options are not satisfactory yet [29]. Emerging evidence has demonstrated that miRNAs play an extended array of functions in allergic diseases, including asthma [30]. In this study, we reported that miR-181b exerted anti-inflammatory and anti-airway remodeling effects in asthmatic mice. The lncRNA TUG/miR-181b/HMGB1 axis was dysregulated during asthma progression and

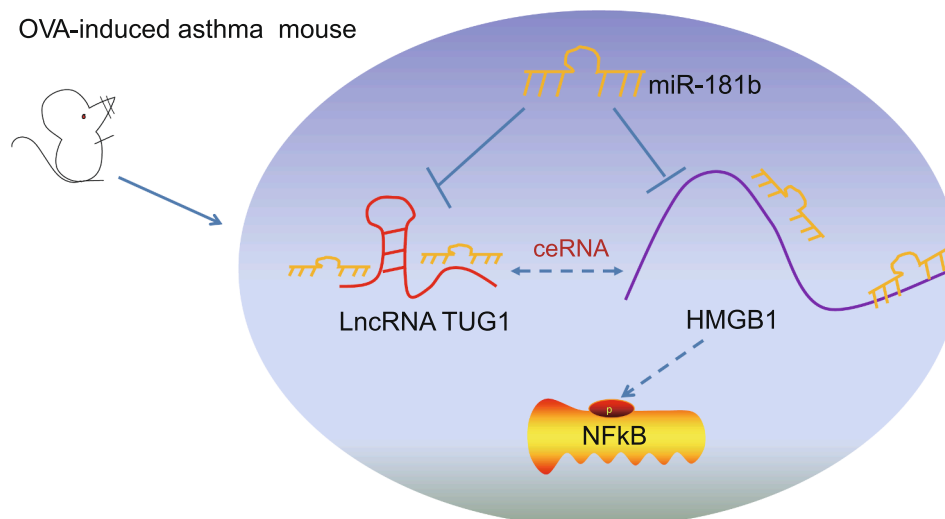


Fig. 7. A schematic diagram for the molecular mechanism. In OVA-challenged asthmatic mice, lncRNA TUG1 sequesters miR-181b and diminishes its inhibitory effect on HMGB1 mRNA, which enhances phosphorylation of NF- κ B p65 and aggravates airway remodeling and mucus production in mice.

mediated the activation of the NF- κ B signaling pathway.

After the successful model establishment of the murine model with asthma using OVA, we first confirmed a poor-expression profile of miR-181b in the airway tissues of mouse lungs. According to a previous study by Huo *et al.*, decreased expression of miR-181b was observed in the serum samples from asthmatic patients [31]. Although the functions of miR-181b on airway remodeling has not been investigated before, studies have reported its anti-inflammatory role in atherosclerosis [14], postoperative cognitive dysfunction [13], and vascular inflammation [32] and immunosuppressive role in sepsis [33]. Here in this study, upregulation of miR-181b was introduced in the model mice, after which the Rrs, inflammatory infiltration and mucus production were assessed. EMT and fibrosis are key events in airway remodeling [23,24], and we found that the abundance of EMT- and fibrosis-related factors in mouse airway tissues was reduced after miR-181b upregulation. In addition, the number of eosinophils in BALF of mice was reduced in this setting. Abundant existence of eosinophils is also crucial for airway inflammation and asthma progression [3]. In the study by Huo *et al.*, miR-181b expression was negatively correlated with the abundance of airway eosinophils in asthmatic patients as well [31]. Collectively, these results showed an alleviating role of miR-181b in the OVA-induced asthmatic symptoms in mice.

The relevant molecules involved in miR-181b regulation in asthmatic mice were then explored. The integrated bioinformatics analysis and the subsequent luciferase and RNA pull-down assays suggested lncRNA TUG1 as a lncRNA owning a binding relationship with miR-181b. Downregulation of lncRNA TUG1 has been documented to alleviate inflammation and airway remodeling in a murine model with COPD, and it reduced cigarette smoke extract-induced inflammation and collagen deposition in human bronchial epithelial cells and lung fibroblasts [18]. Likewise, lncRNA TUG1 was found to trigger proliferation and migration of ASMCs *in vitro*, which represents a key attribute to asthma pathogenesis [34]. In this study, we confirmed elevated expression of lncRNA TUG1 in mice after OVA challenge. Upregulation of lncRNA TUG1 was additionally administrated into asthmatic mice following miR-181b overexpression. Then, it was found that the protective effects of miR-181b on OVA-challenged mice were abrogated by further lncRNA TUG1 overexpression. Next, the target mRNAs of miR-181b were explored, and the KEGG pathway analysis based on the predicted genes suggested that the NF- κ B signaling pathway was mediated by miR-181b. Dysregulation or chronic activation of NF- κ B signaling is the critical contributor of pathogenesis in many diseases involving inflammation, immunity, cell proliferation, differentiation,

and survival [35]. miR-181b has been reported as a regulator of downstream NF- κ B signaling in vascular endothelium by targeting importin- α 3, a protein necessary for nuclear translocation of NF- κ B [32]. Similarly, miR-181b has been demonstrated as a positive regulator of the NF- κ B pathway in several pathological conditions [36,37]. In addition, among the candidate target genes, only HMGB1 showed a decline in cells overexpressing miR-181b. Elevated HMGB1 level has been seen in patients with asthma and COPD, which led to increased number of neutrophils and impaired lung function [38]. Importantly, increased HMGB1 was found to activate the NF- κ B pathway as well, which was responsible for the airway inflammation and airway hyperreactivity in mice induced by OVA [39]. In this study, we noticed that overexpression of miR-181b reduced the protein level of HMGB1 and the phosphorylation of NF- κ B p65 in mouse lung airway tissues, while further upregulation of lncRNA TUG1 reversed this trend.

Collectively, we concluded that lncRNA TUG promotes mucus production and airway inflammation and fibrosis, and airway remodeling in asthmatic mice by reinforcing HMGB1 expression through sequestering microRNA-181b, during which activation of the NF- κ B signaling pathway is implicated (Fig. 7). This study offered novel ideas in the molecular mechanism responsible for asthma progression. More studies are required to validate our findings and to provide new thoughts for the management of asthma.

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CRediT authorship contribution statement

Wufeng Huang: Conceptualization, Methodology, Software, Validation. **Changhui Yu:** Conceptualization, Methodology, Software. **Shixiu Liang:** Data curation. **Hong Wu:** Visualization. **Zili Zhou:** Visualization. **Aihua Liu:** Visualization. **Shaoli Cai:** Validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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