



MiRNA-192-5p attenuates airway remodeling and autophagy in asthma by targeting MMP-16 and ATG7



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ABSTRACT

Asthma is a chronic lung inflammatory disease with high incidence. MicroRNA-192-5p (miR-192-5p) was down-regulated in asthmatics. However, the role of miR-192-5p in asthma is still unclear. In current study, *in vitro*, the overexpression of miR-192-5p, matrix metalloproteinase (MMP)-16 and autophagy related 7 (ATG7) was conducted in airway smooth muscle cells (ASMCs). We found that miR-192-5p suppressed cell proliferation, and decreased MMP-16 and ATG7 expression. MMP-16 and ATG7 promoted cell proliferation, and further alleviated the down-regulation of miR-192-5p on proliferation of ASMCs. *in vivo*, miR-192-5p was down-regulated in asthma mice, and involved in improvement of asthma mice. MiR-192-5p was demonstrated to alleviate inflammation in asthma mice, including decreasing the level of ovalbumin (OVA)-specific IgE, interleukin (IL)-4, IL-5, IL-13, iNOS and COX-2. Moreover, the attenuation of airway remodeling induced by miR-192-5p in asthma mice were expressed by the reduction of fibroblast growth factor-23 (FGF-23) level, decrease in concentrations of MMP-2 and MMP-9 as well as down-regulation of collagen I deposition. Further, miR-192-5p also caused the suppression of autophagy in asthma mice, exhibiting a decrease in LC3II/I, beclin-1 and ATG7, and an increase in p62. Importantly, MMP-16 and ATG7 were confirmed to be targets of miR-192-5p. Therefore, our results indicate that miRNA-192-5p may attenuate airway remodeling and autophagy in asthma via targeting MMP-16 and ATG7.

1. Introduction

Asthma is a highly prevalent chronic lung inflammatory disease with increasing morbidity and mortality [1,2]. Asthma arises from interactions between genetic and environmental factors [3]. It is a complex syndrome and characterized by intermittent attacks of breathlessness, chest pressure and wheezing [4,5]. There are more than 300 million people being affected by asthma worldwide, and the number will further increase by a further 100 million until 2025 [1]. Currently, some options are applied into asthma treatment, such as bronchodilators, corticosteroids, a leukotriene receptor antagonist and anti-IgE therapy [6,7]. However, the prognosis is still poor, and potentially serious side effects of chemicals are existent. Therefore, it is necessary to explore the efficient molecular marker for asthma therapy.

MicroRNAs (miRNAs), 19–22 nucleotides in length, are single-stranded non-coding RNAs, and miRNAs inhibit gene translation and then reduce the expression of target genes through binding to 3'-untranslated regions (3'-UTR) of mRNAs [8,9]. The miRNAs are reported

to be involved in numerous biological processes, including differentiation, development, stress and regulation of inflammation in host immune cells [10,11]. Accumulating evidences have revealed that miRNAs also show a regulatory role in asthma [12,13]. Zhang et al. discovered that miR-192-5p blocked T follicular helper cell differentiation through targeting C-X-C chemokine receptor type 5, which was useful for childhood asthma improvement [14]. Yamamoto et al. found that miR-192-5p was remarkably decreased in asthmatics compared with healthy control subjects [15]. These findings suggest that miR-192-5p may be a reasonable target for asthma treatment. Hence, the study of mechanism underlying miR-192-5p involved in asthma is interesting.

Matrix metalloproteinase (MMP)-16 is a membrane-type metalloprotease and associated with proliferation, migration and invasion [16,17]. MMP-16 functions in cell development of some diseases by activating proMMP-2 (gelatinase A) [18,19]. MMP-2 is demonstrated to participate in the airway remodeling in bronchial asthma [20]. In addition, autophagy also plays an important role in airway remodeling

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and inflammation of asthma [21,22]. Interestingly, our present work has shown that miR-192-5p is predicted to bind to MMP-16 and autophagy related 7 (ATG7). Therefore, we hypothesize that miR-192-5p may take part in asthma through regulating MMP-16 and ATG7.

In current study, miR-192-5p was overexpressed in the airway smooth muscle cells (ASMCs) and asthma mice *in vitro* and *in vivo*. Then, we explored the effect of miR-192-5p on proliferation, airway inflammation, airway remodeling and autophagy. Our study suggested that miR-192-5p may be a new therapeutic target for asthma.

2. Materials and methods

2.1. Animals and treatments

Female BALB/c mice (18–22 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd (Liaoning, China). All mice were housed at $22 \pm 1^\circ\text{C}$ under a 12 h light/dark cycle. Relative humidity was maintained at 45–55 %. Mice were randomly separated into 4 groups (n = 12/group): (A) control; (B) asthma; (C) asthma + agomir negative control (agomir NC); (D) asthma + miR-192-5p agomir. The mice in group C or D were administered with 40 μl of 20 μg agomir NC or miR-192-5p agomir with by nasal instillation every seven days starting on the day 3. The mice in group A and B were administered with the same amount of normal saline only by nasal instillation. Additionally, on day 1, 7 and 14, the mice in group B, C and D were intraperitoneally injected with 0.2 ml of normal saline containing 50 μg of ovalbumin (OVA, Aladdin, Shanghai, China) and 4 mg of aluminum hydroxide. Starting on day 25, an airway challenge was performed daily for 30 min by aerosolized 2 % OVA normal saline for three weeks. The mice in group A were injected with the equal amount of normal saline. Then, the mice were sacrificed by intraperitoneal injection with pentobarbital (200 mg/kg), followed by collecting bronchoalveolar lavage fluid (BALF) and lung tissues for subsequent analysis. The lung tissues were fixed with 10 % neutral formaldehyde or stored at -80°C . All animal experiments were conducted in accordance with the Guideline for the Care and Use of Laboratory Animals and approved by The First Affiliated Hospital of Zhengzhou University.

2.2. Plasmid construction

The mouse native miR-192-5p precursor sequence was cloned into lentiviral vector pSico, named by Lenti-miR-192-5p. Moreover, the full-length CDS of mouse MMP-16 (NM_019724) or ATG7 (NM_001253717.1) were inserted into lentivirus vectors (pLJM1-EGFP or pLV-EF1a-IRES-NEO) to generate MMP-16 or ATG7 overexpression lentivirus particle (Lenti-MMP-16 or Lenti-ATG7).

2.3. Cell culture and infection

ASMCs were isolated from airway bronchus tissues of 4–6-week-old BALB/c mice (18–22 g) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum (FBS) at 37°C in 5 % CO_2 . The 293T cells were obtained from Procell Life Science & Technology Co., Ltd (Hubei, China) and grown in DMEM with 10 % FBS. Then, the ASMCs were infected with Lenti-miR-192-5p, Lenti-miR-control, Lenti-MMP-16, Lenti-NC1, Lenti-ATG7 or Lenti-NC2 for 24 h. Subsequently, the cells were used for subsequent analysis.

2.4. Immunofluorescence

After fixation, the lung tissues were cut into 5- μm slices, deparaffinized and hydrated. The cells were incubated with 0.1 % triton X-100 for 30 min. Then, the lung sections and cells were blocked with goat serum for 15 min at room temperature. The primary ATG7 antibody (1:100, Abclonal, Hubei, China) was used to incubate the lung sections overnight at 4°C . The primary α -actin antibody (1:200,

Proteintech, Hubei, China) was used to incubate the cells overnight at 4°C . Subsequently, they were covered with the secondary antibody Cy3-labeled goat anti-rabbit IgG (1:200, Beyotime, Shanghai, China) for 60 min at room temperature. The nuclei were counterstained with DAPI. The images were captured under a fluorescence microscope.

2.5. Real-time PCR

The total RNA from lung tissues and cells was extracted with RZ reagent (Tiangene, Beijing, China). The RNA was reversely transcribed into cDNA through M-MLV reverse transcriptase (Tiangene). Real-Time PCR was carried out with SYBR Green (Solarbio, Beijing, China). The levels of miR-192-5p, MMP-16, ATG7, iNOS and COX-2 were calculated using $2^{-\Delta\Delta\text{Ct}}$ method. U6 and GAPDH were internal controls. The primers were used in this study as follows:

Name	Sequence (5'-3')
miR-192-5p	RT primer: GTTGGCTCTGGTGCAGGGTCCGAGGTATTGCGACCAGAGCCA ACGGCTGT Forward: CGGTCCTGACCTATGAATTG Reverse: GTGCAGGGTCCGAGGTATTC
MMP-16	Forward: GGACCAACAGACCGAGATAA Reverse: CAATACAAGGAGGCATAAGG
ATG7	Forward: TGGGAGAAGAACCAGAAAGG Reverse: CAGGCACTTGACAGACACGA
iNOS	Forward: TTGGAGCGAGTTGTGGATTG Reverse: GTGAGGGCTTGGCTGAGTGA
COX-2	Forward: AAAACCTCGTCCAGATGCTA Reverse: TTGAGGAGAACAGATGGGAT

2.6. MTT assay

The cell proliferation was detected using MTT assay kit (Keygen, Jiangsu, China). Briefly, the 5×10^3 cells per well were seeded into 96-well plates. At indicated time (0, 12, 24, 48 or 72 h), the cells were incubated with 0.5 mg/ml MTT solution for 5 h at 37°C . The absorbance was measured at 570 nm using a microplate reader.

2.7. Enzyme-linked immunosorbent assay (ELISA)

The cell culture supernatant from ASMCs was collected, and then the fibroblast growth factor-23 (FGF-23) level was detected with an ELISA Kit (USCN, Hubei, China). The levels of FGF-23, interleukin (IL)-4, IL-5, IL-13, MMP-2 and MMP-9 from bronchoalveolar lavage fluid (BALF) were measured using according ELISA Kits (USCN or Multi sciences, Hubei or Shanghai, China). The serum samples for ovalbumin (OVA)-specific IgE was assessed through an ELISA Kit (CUSABIO, Hubei, China) according to the manufacturer's instructions.

2.8. Western blot analysis

Protein from lung tissues and cells were extracted with RIPA lysis buffer (Solarbio). The protein concentration was quantified by a BCA Protein Assay Kit (Solarbio). Equal quantities (10–20 μg) of protein sample were separated via SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5 % non-fat milk for 1 h, the membrane was incubated with primary antibodies overnight at 4°C and then incubated with the secondary antibodies (1:3000, Solarbio) for 1 h at 37°C . The bands were visualized using enhanced chemiluminescence reagents (Solarbio). The primary antibodies were used in this study as follows: MMP-16 (1:1000, Bioss, Beijing, China), MMP-2 (1:1000, CST, Danvers, MA, USA), MMP-9 (1:500, Proteintech), ATG7 (1:1000, Bioss), LC3II/1 (1:1000, CST), Beclin-1 (1:1000, Proteintech), p62 (1:2000, Proteintech), iNOS (1:500, Proteintech), COX-2 (1:500, Proteintech) and GAPDH (1:10000, Proteintech). GAPDH was used as an internal

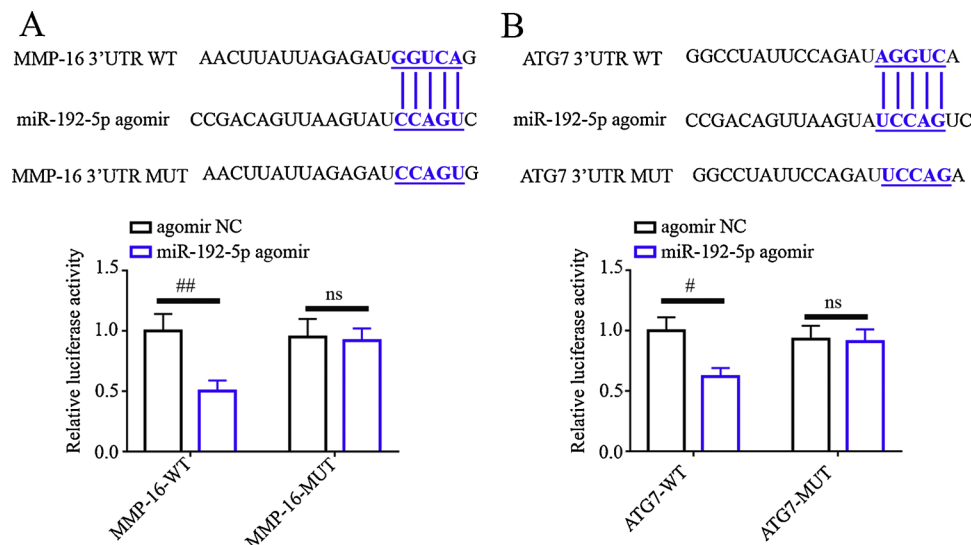


Fig. 1. MMP-16 and ATG7 were targets of miR-192-5p. (A, B) Bioinformatic analysis predicted that miR-192-5p could bind to MMP-16 and ATG7. The interaction between miR-192-5p and MMP-16 or ATG7 was confirmed using dual-luciferase reporter assay. $n = 3$. #, $p < 0.05$. ##, $p < 0.01$.

control.

2.9. Hematoxylin and eosin (HE) staining

After fixation, the lung tissues were embedded by paraffin and cut into 5- μ m sections. Then, the sections were stained with hematoxylin (Solarbio) and eosin (Sangon, Shanghai, China), respectively. Images were taken under a microscope. The sections were graded basing on inflammatory cell infiltration degree (0, no inflammatory cell infiltration; 1, a small number of infiltrated inflammatory cells; 2, an inflammatory cell monolayer around the airway; 3, inflammatory cell bilayer around the airway; 4, four layers of inflammatory cells around the airway).

2.10. Periodic acid-schiff (PAS) staining

The paraffin-embedded lung tissues were sliced into 5- μ m sections. After dewaxing, these sections were stained with PAS to evaluate goblet cell hyperplasia/submucosal gland hypertrophy in the epithelium, which was graded according to the percentage of goblet cells: no goblet cells (0); < 25 % (1); 25 %–50 % (2); 51 %–75 % (3); > 75 % (4). The images were taken under a BX53 microscope.

2.11. Masson staining and observation of structure

The mice were sacrificed and lung tissues were immediately collected. After fixation, the lung tissues were cut into 5- μ m sections, dewaxed and stained with hematoxylin (Solarbio, China) for 6 min. After reaction with 1 % hydrochloric acid-ethanol for 3 s, the sections were washed with water and then stained with Ponceau (Solarbio, China) for 1 min. Then, the sections were dehydrated, dried and sealed with neutral gum. The images were photographed under a BX53 microscope. The transverse section of the bronchial cross-section was visualized under a diameter of approximately 100 μ m. Image-Pro Plus 6.0 image analysis software was used to identify the inner perimeter of the bronchial wall (Pi), inner area of the bronchial wall (Wai), airway smooth muscle area (WAm) and the number of bronchial smooth muscle cells (N) as previously reported [23–25]. The measurement value was standardized as ratio of the Wai/WAm and N/Pi, representing the bronchial wall thickness (Wai/Pi) and the smooth muscle thickness (WAm/Pi) and smooth muscle nuclei (N/Pi). The experiment was repeated six times for each group.

2.12. Immunohistochemistry

The paraffin-embedded lung tissues were cut into 5- μ m sections, followed by deparaffinization and rehydration. The sections were incubated with 3 % H_2O_2 for 15 min at room temperature to inhibit endogenous peroxidase activities, and then blocked with normal goat serum for 15 min. Afterwards, the primary antibodies FGF-23 (1:500, RD, USA) and collagen I (1:200, Abcam, UK) were used to incubate these sections overnight at 4 $^{\circ}C$, respectively. The sections were covered with a secondary peroxidase-conjugated antibody (1:500, Beyotime or ThermoFisher) for 60 min at 37 $^{\circ}C$. Immunoreactivity was visualized via diaminobenzidine (DAB) solution (Solarbio). Hematoxylin (Solarbio) was used to counterstain the nuclei. Finally, slides were photographed under a BX53 microscope.

2.13. Dual-luciferase reporter assay

The fragments of wild-type 3'-UTR and the miR-192-5p "seed" mutant 3'-UTR of MMP-16 and ATG7 were synthesized and then inserted into pmirGLO luciferase reporter plasmid, which were named by MMP-16-WT, MMP-16-MUT, ATG7-WT and ATG7-MUT. The 293 T cells were co-transfected with recombinant luciferase reporter plasmid (WT or MUT plasmids) and miRNA (agomir NC or miR-192-5p agomir) via Lipofectamine 2000. After 48 h, the relative luciferase activity was assessed through Dual Luciferase Reporter Assay kit (Keygen).

2.14. Statistical analysis

Data were presented as the mean $\pm$ standard deviation and analyzed using GraphPad Prism version 7.0 software. Statistical significance was confirmed by one-way analysis of variance followed by Tukey's test. P values less than 0.05 were considered as statistically significant.

3. Results

3.1. MMP-16 and ATG7 were targets of miR-192-5p

As shown in Fig. 1A and B, we predicted that miR-192-5p could bind to MMP-16 and ATG7 by bioinformatic analysis. The dual-luciferase reporter assay identified that luciferase activity was decreased in 293 T cells co-transfected with miR-192-5p agomir and MMP-16-WT.

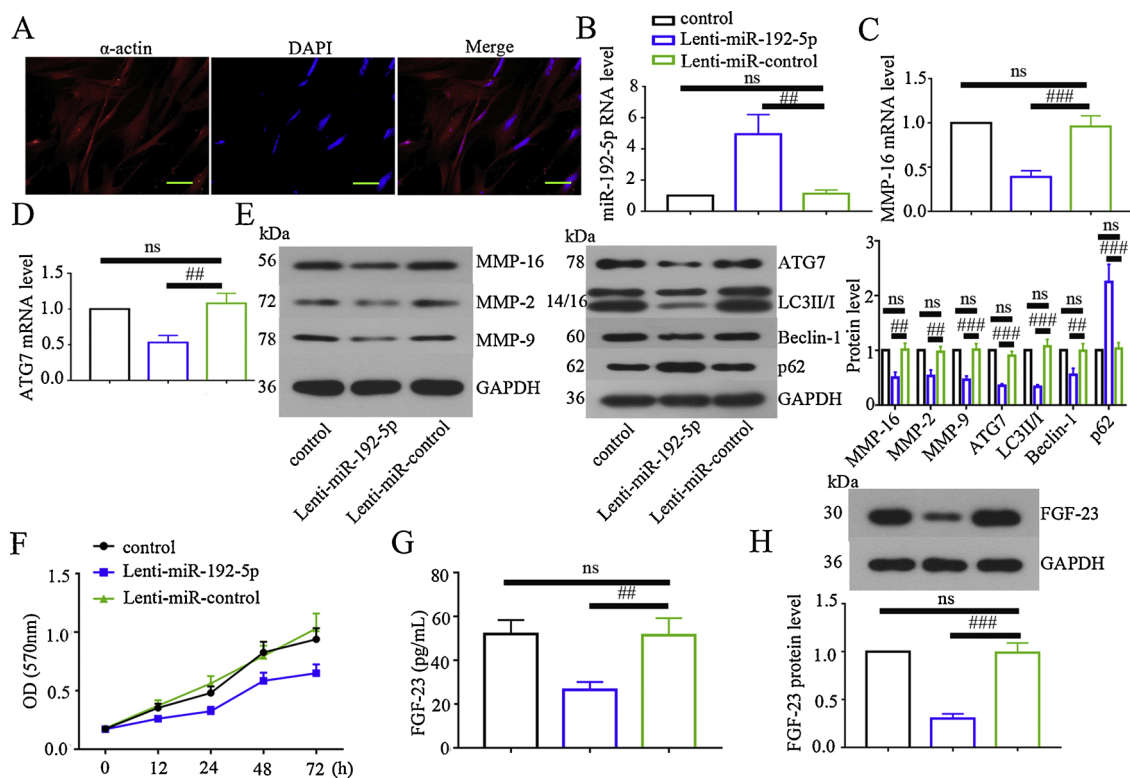


Fig. 2. MiR-192-5p suppressed cell proliferation, and down-regulated MMP-16 and ATG7 expression in ASMCs. (A) Immunofluorescence detected α -actin expression in ASMCs isolated from mice. Scale bar, 50 μ m. (B–D) Real-time PCR identified miR-192-5p, MMP-16 and ATG7 levels. (E) Western blot analysis determined MMP-16, MMP-2, MMP-9, ATG7, LC3II/I, beclin-1 and p62 protein levels. (F) MTT assay detected proliferation of ASMCs at indicated time (0, 12, 24, 48 or 72 h). (G) ELISA assay examined the FGF-23 level in ASMCs. (H) Western blot analysis revealed FGF-23 protein level of ASMCs. $n = 3$. ##, $p < 0.01$. ###, $p < 0.001$.

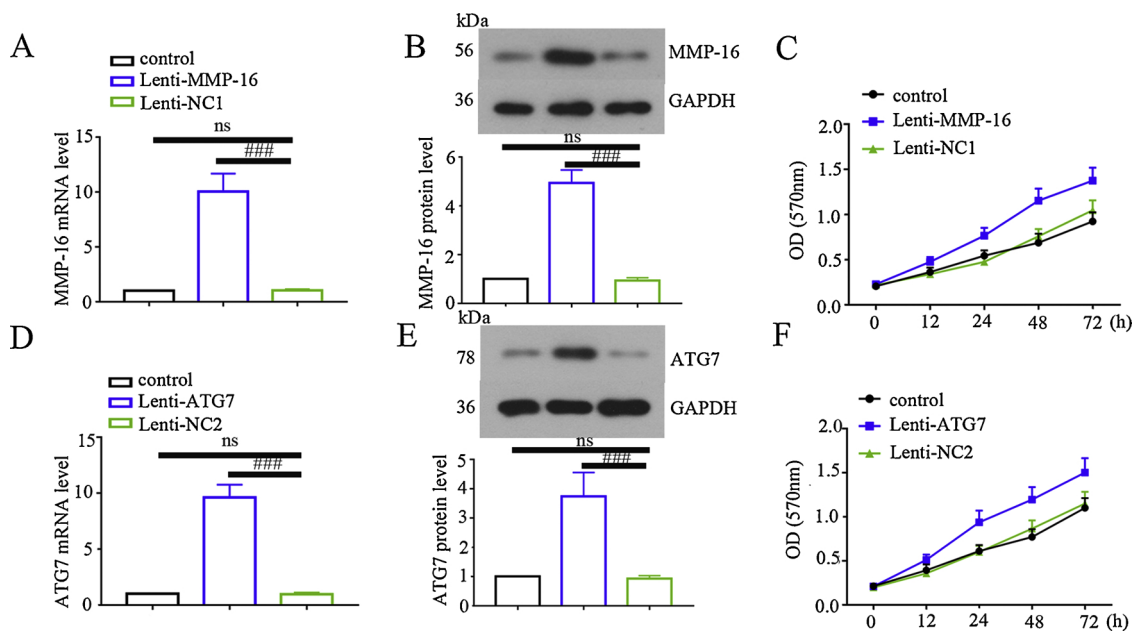


Fig. 3. MMP-16 and ATG7 promoted cell proliferation in ASMCs. (A, D) Real-time PCR measured the MMP-16 and ATG7 mRNA level in ASMCs, respectively. (B, E) Western blot analysis determined MMP-16 and ATG7 protein level in ASMCs, respectively. (C, F) MTT assay was used to detect cell proliferation at indicated time (0, 12, 24, 48 or 72 h). $n = 3$. ###, $p < 0.001$.

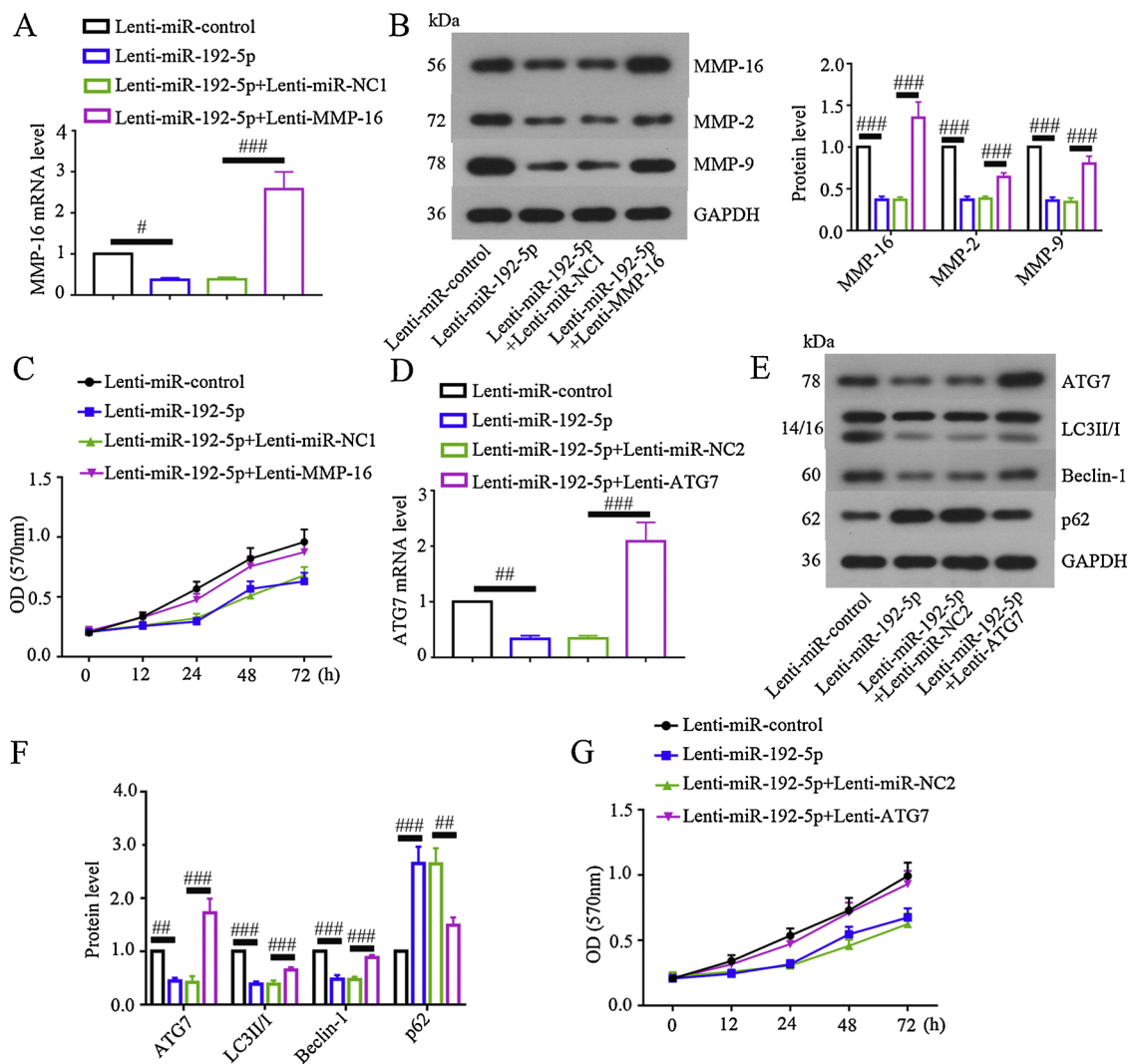


Fig. 4. MMP-16 and ATG7 alleviated the regulation of miR-192-5p on proliferation of ASMCs. (A, D) The mRNA level of MMP-16 and ATG7 was determined using real-time PCR. (B, E-F) The MMP-16, MMP-2, MMP-9, ATG7, LC3II/I, beclin-1 and p62 protein level was detected via western blot analysis. (C, G) Cell proliferation was identified with MTT assay at indicated time (0, 12, 24, 48 or 72 h). n = 3. #, p < 0.05. ##, p < 0.01. ###, p < 0.001.

However, no significant difference was detected in 293 T cells co-transfected with miR-192-5p agomir and MMP-16-MUT. Moreover, co-transfection of miR-192-5p agomir with ATG7-WT reduced the luciferase activity in 293 T cells, whereas there was no effect on luciferase activity in 293 T cells co-transfected with miR-192-5p agomir and ATG7-MUT. These results suggested that MMP-16 and ATG7 were confirmed to be targets of miR-192-5p.

3.2. MiR-192-5p suppressed cell proliferation, and down-regulated MMP-16 and ATG7 expression in ASMCs

To explore the effects of miR-192-5p on expression of MMP-16 and ATG7, α -actin, which is the marker of the contractile cell phenotype in differentiated ASMCs [26], was first testified in ASMCs isolated from mice using immunofluorescence (Fig. 2A). The miR-192-5p overexpression was conducted in ASMCs. Real-time PCR identified that miR-192-5p level was increased in the ASMCs infected with Lenti-miR-192-5p (Fig. 2B). Moreover, MMP-16 and ATG7 levels were reduced in miR-192-5p-overexpression ASMCs (Fig. 2C and D). Western blot analysis demonstrated that ectopic expression of miR-192-5p inhibited MMP-16, MMP-2, MMP-9, ATG7, LC3II/I and beclin-1 protein levels, and enhanced p62 protein level (Fig. 2E). MTT assay proved that miR-192-5p showed a suppressive role in proliferation of ASMCs (Fig. 2F). ELISA

assay and western blot analysis revealed that down-regulation of FGF-23 was caused by miR-192-5p overexpression (Fig. 2G and H). These findings indicated that miR-192-5p suppressed cell proliferation, and decreased MMP-16 and ATG7 expression in ASMCs

3.3. MMP-16 and ATG7 promoted cell proliferation in ASMCs

To detect the effect of MMP-16 and ATG7 on cell proliferation in ASMCs, MMP-16 or ATG7 overexpression was carried out respectively. Real-time PCR and western blot analysis showed that the MMP-16 or ATG7 level was increased in ASMCs infected with Lenti-MMP-16 (Fig. 3A and B) or Lenti-ATG7 (Fig. 3D and E). Moreover, MMP-16 or ATG7 overexpression facilitated cell proliferation (Fig. 3C and F). The data suggested that MMP-16 and ATG7 promoted cell proliferation in ASMCs.

3.4. MMP-16 and ATG7 alleviated the regulation of miR-192-5p on proliferation of ASMCs

To further investigate whether MMP-16 and ATG7 can be associated with proliferation in miR-192-5p-overexpression ASMCs, the ASMCs were co-infected with Lenti-miR-192-5p and Lenti-MMP-16 or Lenti-ATG7. An increase of MMP-16 or ATG7 was found in ASMCs co-infected

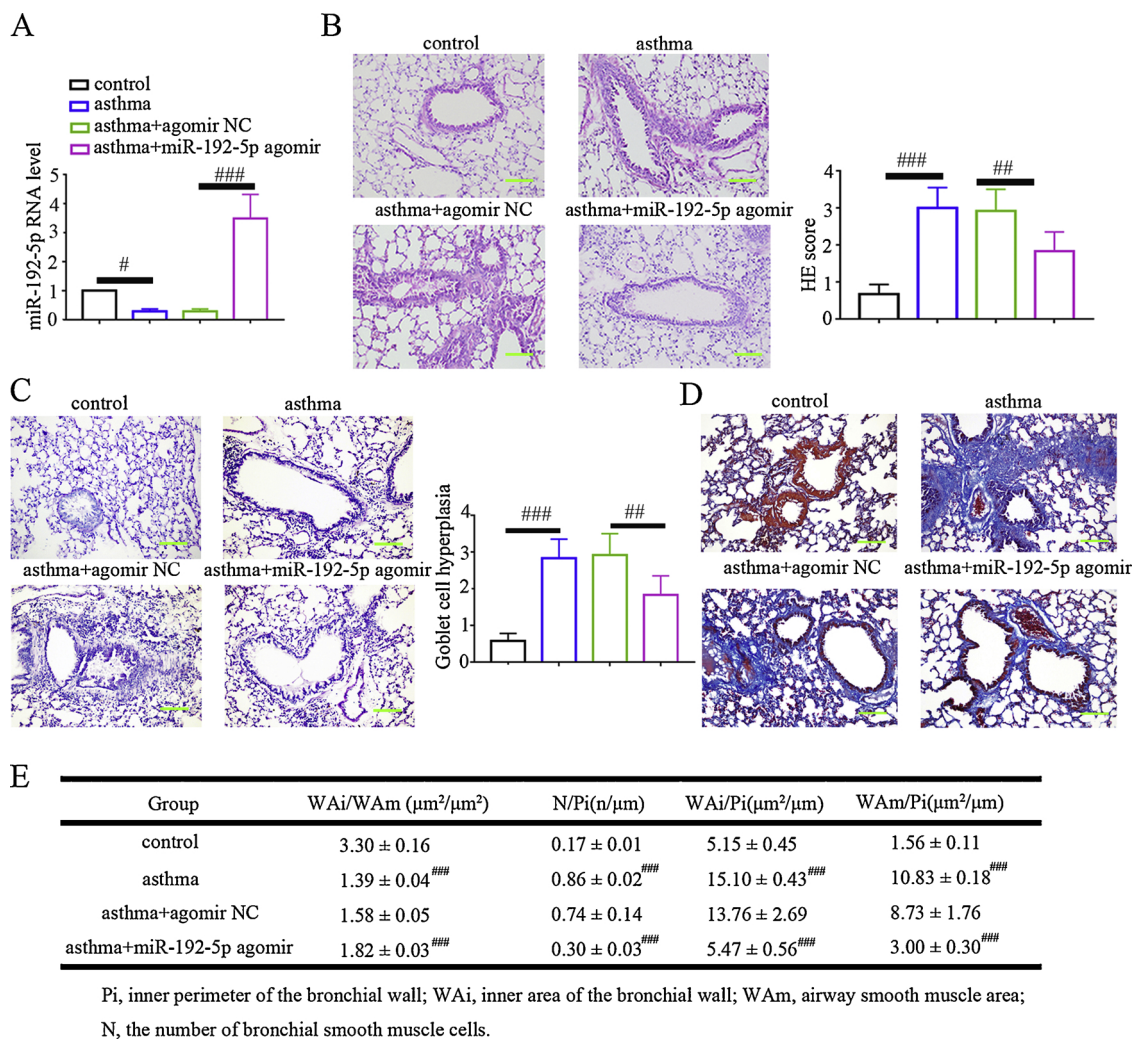


Fig. 5. Effect of miR-192-5p on lung tissues in asthma mice. (A) The miR-192-5p was measured via real-time PCR. (B) HE staining demonstrated the pathological morphology changes. Scale bar, 100 μm . (C) PAS staining detected goblet cell hyperplasia and airway mucus secretions. Scale bar, 100 μm . (D) Masson staining measured smooth muscle hyperplasia. Scale bar, 100 μm . (E) WAI/WAm, N/Pi, WAI/Pi and WAm/Pi level was calculated. $n = 6$. Pi, inner perimeter of the bronchial wall; WAI, inner area of the bronchial wall; WAm, airway smooth muscle area; N, the number of bronchial smooth muscle cells. #, $p < 0.05$. ##, $p < 0.01$. ###, $p < 0.001$.

with Lenti-miR-192-5p and Lenti-MMP-16 or Lenti-ATG7 using real-time PCR (Fig. 4A and D). Western blot analysis showed that miR-192-5p inhibited MMP-16, MMP-2, MMP-9, ATG7, LC3II/I and beclin-1 protein levels, and enhanced p62 protein level. However, MMP-16 or ATG7 overexpression reversed these miR-192-5p-induced protein levels (Fig. 4B, E and F). Moreover, MMP-16 or ATG7 overexpression promoted cell proliferation in miR-192-5p-overexpression ASMCS (Fig. 4C and G). These findings suggested that MMP-16 and ATG7 alleviated the down-regulation of miR-192-5p on proliferation of ASMCS.

3.5. Effect of miR-192-5p on lung tissues in asthma mice

To evaluate the role of miR-192-5p in lung tissues from asthma mice, the miR-192-5p was first measured using real-time PCR. The results showed that miR-192-5p was decreased in asthma mice, whereas miR-192-5p was significantly elevated in mice administered with miR-192-5p agomir (Fig. 5A). HE staining demonstrated that there were more serious pathological morphology changes been detected in asthma group compared to in control group, whereas pathological morphology changes were attenuated in asthma + miR-192-5p agomir group (Fig. 5B). PAS staining revealed that goblet cell hyperplasia and airway mucus secretions were detected in asthma group. However, a lesser

degree was testified in asthma + miR-192-5p agomir group (Fig. 5C). Masson staining identified that smooth muscle hyperplasia was observed in asthma group, whereas it was alleviated in asthma + miR-192-5p agomir group. Moreover, the inner perimeter of the bronchial wall (Pi), inner area of the bronchial wall (WAI), airway smooth muscle area (WAm) and the number of bronchial smooth muscle cells (N) were analyzed. The asthma group showed a decrease of WAI/WAm level, and an increase of N/Pi, WAI/Pi as well as WAm/Pi levels. However, asthma + miR-192-5p agomir group restored the levels of WAI/WAm, N/Pi, WAI/Pi and WAm/Pi (Fig. 5D and E). These results implied that miR-192-5p improved lung tissues in asthma mice.

3.6. MiR-192-5p attenuated inflammation in asthma mice

To examine the effect of miR-192-5p on inflammation in asthma mice, the BALF and serum from mice were collected. Total protein, inflammatory cells, eosinophils, neutrophils, lymphocytes and monocyte macrophage were enhanced in the BALF of mice in asthma group, whereas these were reduced in asthma + miR-192-5p agomir group (Fig. 6A). ELISA assay showed that asthma mice exhibited a massive elevation in serum OVA-specific IgE, whereas miR-192-5p agomir down-regulated OVA-specific IgE level in asthma mice (Fig. 6B). The

A

Group	Total protein ($\mu\text{g}/\mu\text{l}$)	Inflammatory cells ($\times 10^4/\text{ml}$)	Eosinophils ($\times 10^5/\text{ml}$)	Neutrophils ($\times 10^5/\text{ml}$)	Lymphocytes ($\times 10^4/\text{ml}$)	Monocyte macrophage ($\times 10^5/\text{ml}$)
control	0.40 ± 0.07	5.42 ± 1.32	1.62 ± 0.46	0.98 ± 0.30	1.50 ± 0.52	4.04 ± 0.96
asthma	$0.89 \pm 0.16^{###}$	$21.83 \pm 4.33^{###}$	$47.13 \pm 14.01^{###}$	$4.07 \pm 1.20^{###}$	$7.59 \pm 2.37^{###}$	$11.76 \pm 2.01^{###}$
asthma+agomir NC	0.87 ± 0.18	22.63 ± 5.77	53.27 ± 13.27	4.84 ± 1.13	7.04 ± 1.70	11.51 ± 3.80
asthma+miR-192-5p agomir	$0.60 \pm 0.15^{\#}$	$10.62 \pm 2.44^{###}$	$14.53 \pm 3.98^{###}$	$1.79 \pm 0.42^{###}$	$3.75 \pm 1.07^{###}$	$6.86 \pm 1.91^{\#}$

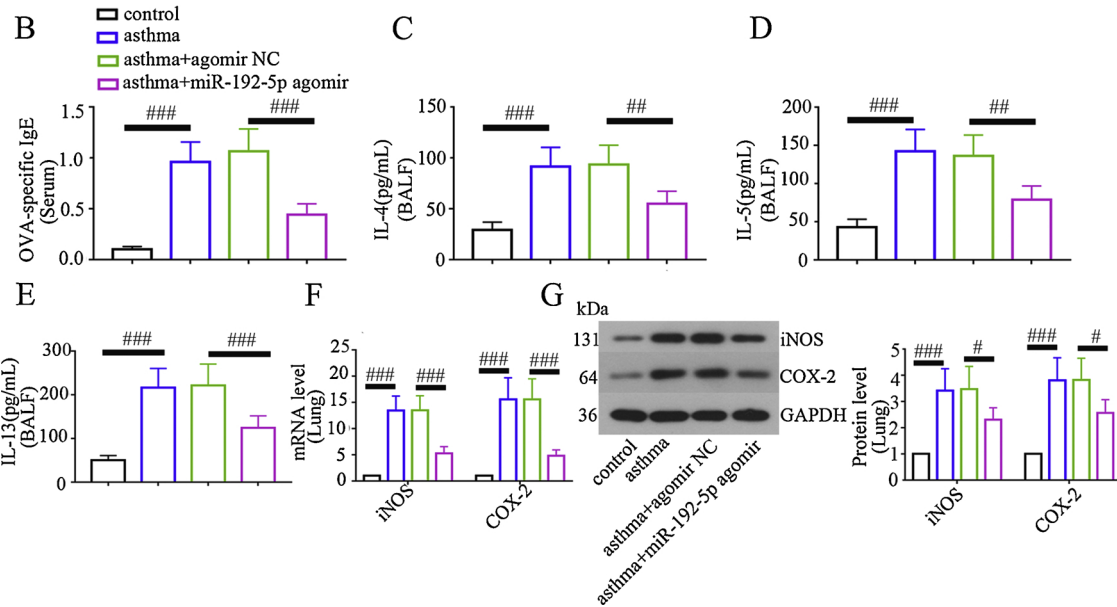


Fig. 6. MiR-192-5p attenuated inflammation in asthma mice. (A) Total protein, inflammatory cells, eosinophils, neutrophils, lymphocytes and monocyte macrophage were examined in the BALF of mice. (B) ELISA assay detected serum OVA-specific IgE. (C–E) ELISA assay measured the levels of IL-4, IL-5 and IL-13 in BALF of mice. (F–G) The iNOS and COX-2 level from lung tissue of mice was detected using real-time PCR and western blot analysis. $n = 6$. #, $p < 0.05$. ##, $p < 0.01$. ###, $p < 0.001$.

levels of IL-4, IL-5 and IL-13 in BALF of asthma mice were increased. Conversely, miR-192-5p agomir decreased asthma-caused IL-4, IL-5 and IL-13 levels (Fig. 6C–E). Real-time PCR and western blot analysis confirmed that asthma led to the increase of iNOS and COX-2, whereas miR-192-5p agomir alleviated iNOS and COX-2 level in lung tissue of asthma mice (Fig. 6F and G). The data implied that miR-192-5p attenuated inflammation in asthma mice.

3.7. MiR-192-5p decreased airway remodeling and autophagy in asthma mice

We explored the effect of miR-192-5p on airway remodeling and autophagy in asthma mice. ELISA assay and immunohistochemistry showed that FGF-23 was elevated in asthma group compared to in control group, whereas FGF-23 was reduced in asthma + miR-192-5p agomir group (Fig. 7A and B). Asthma mice exhibited higher concentrations of MMP-2 and MMP-9 in BALF than control mice. However, miR-192-5p agomir attenuated MMP-2 and MMP-9 levels in asthma mice (Fig. 7C and D). Further western blot analysis revealed that miR-192-5p agomir decreased MMP-2, MMP-9 and MMP-16 protein levels in lung tissues of asthma mice (Fig. 7E). The collagen I deposition was demonstrated to be down-regulated by miR-192-5p agomir in asthma mice using immunohistochemistry (Fig. 7F). Additionally, western blot analysis demonstrated that asthma mice had an increase of LC3II/I, beclin-1 and ATG7, and a decrease of p62. Conversely, miR-192-5p agomir reversed these protein levels in asthma mice (Fig. 8A). ATG7 expression was also reduced in asthma + miR-192-5p agomir group compared to asthma group via immunofluorescence (Fig. 8B). These results indicated that miR-192-5p decreased airway remodeling and

autophagy in asthma mice.

4. Discussion

In current study, MMP-16 and ATG7 were confirmed to be targets of miR-192-5p. *in vitro*, miR-192-5p inhibited cell proliferation, and decreased MMP-16 and ATG7 expression in ASMCs. Moreover, MMP-16 and ATG7 were found to promote cell proliferation, and further attenuate the down-regulation of miR-192-5p on proliferation of ASMCs. *in vivo*, miR-192-5p was down-regulated in asthma mice. MiR-192-5p overexpression was useful for improvement of asthma mice, and decreased airway remodeling and autophagy in asthma mice.

Increasing evidences have showed that miRNAs are involved in cell proliferation in asthma. For example, miR-221, targeting SIRT1, suppressed cell proliferation and promoted apoptosis in bronchial epithelial cells [27]. Moreover, miR-485 was found to reduce cell proliferation and elevate apoptosis in ASMC by targeting Smurf2 [28]. Like previous studies, our study also found that miR-192-5p showed a suppressive role in cell proliferation in ASMCs *in vitro*. Additionally, miR-192-5p participated in improvement of asthma mice. Importantly, our study confirmed that miR-192-5p could target MMP-16 and ATG7. Therefore, we hypothesize that miR-192-5p contributes to asthma by targeting MMP-16 and ATG7.

It is reported that miR-146a has been proved to repress infiltration of inflammatory cells, and suppress OVA-specific IgE level in OVA-induced asthmatic mice [29]. The production of IgE is associated with allergic airway inflammation in a murine asthma model. IgE stimulates eosinophils and mast cells to secrete cytokines, including IL-4, IL-5 and IL-13 [30]. Interestingly, in our study, miR-192-5p decreased OVA-

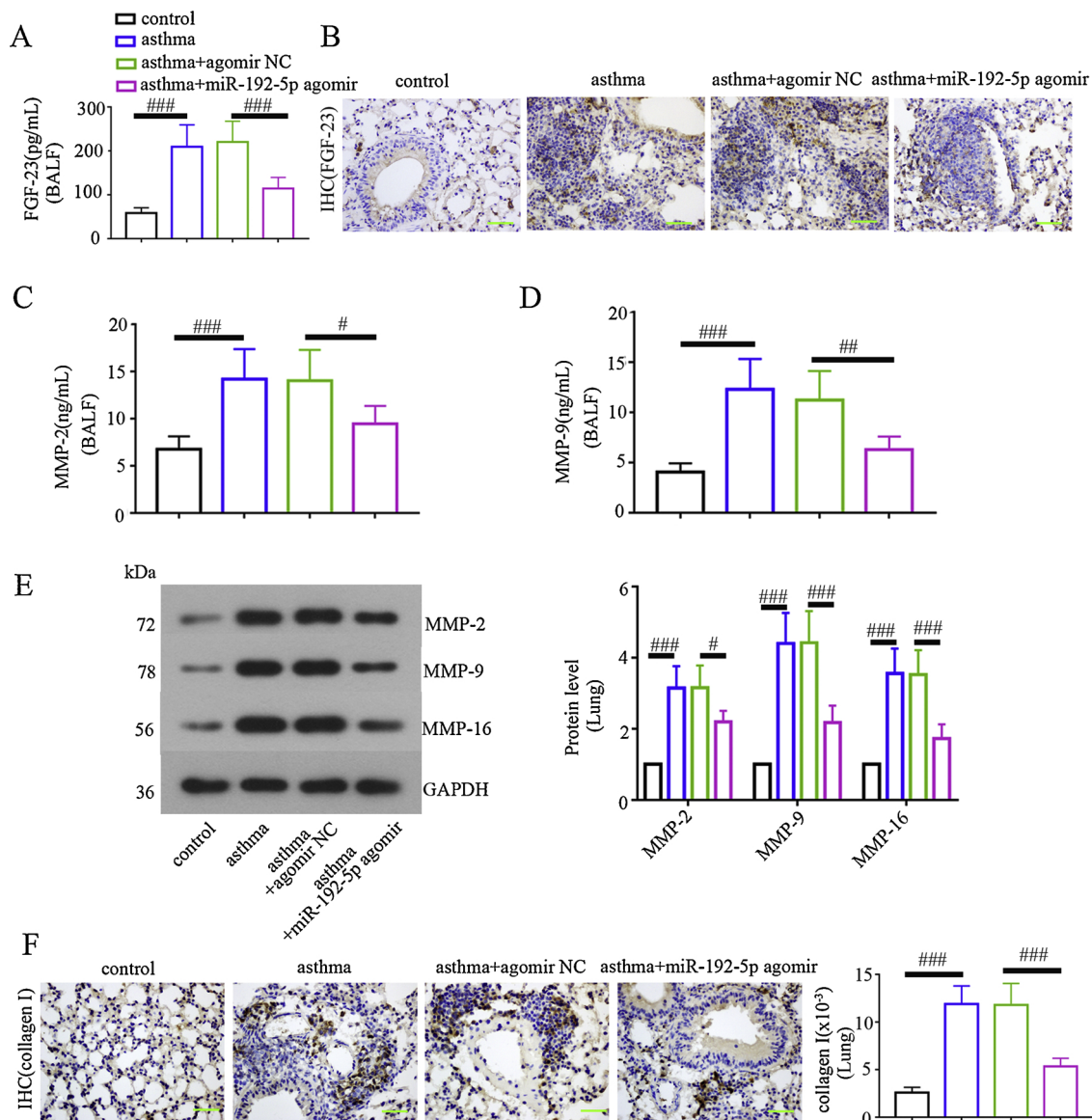


Fig. 7. MiR-192-5p decreased airway remodeling in asthma mice. (A) ELISA assay was used to detect FGF-23 level in BALF of mice. (B) Immunohistochemistry examined FGF-23 expression in lung tissue of mice. Scale bar, 50 μ m. (C–D) The concentrations of MMP-2 and MMP-9 in BALF of mice were measured using ELISA assay. (E) Western blot analysis was detected the MMP-2, MMP-9 and MMP-16 protein levels in lung tissues of mice. (F) The collagen I deposition was measured using immunohistochemistry. Scale bar, 50 μ m. n = 6. #, p < 0.05. ##, p < 0.01. ###, p < 0.001.

specific IgE, IL-4, IL-5 and IL-13 in asthma mice. In addition, nitric oxide (NO) is an inflammatory mediator and participates in the pathogenesis of airway inflammation in asthma. Inducible NO synthase (iNOS) could synthesize NO from L-arginine by NADPH and oxygen molecules [31]. It is reported that iNOS was elevated in asthmatic tissues and some inflammatory cells [32]. The inflammatory mediator prostaglandin E2 is synthesized by COX-2 and takes part in the inflammatory process [33]. From these, the production of iNOS and COX-2 was reduced by miR-192-5p overexpression in asthma mice. These findings suggest that miR-192-5p may alleviate inflammation in asthma. In addition to airway inflammation, airway remodeling is also one of pathological features in asthma. Eosinophils, neutrophils and other inflammatory cells were reported to take part in airway remodeling of asthma [34–36]. Our results showed that miR-192-5p decreased total protein, inflammatory cells, eosinophils, neutrophils, lymphocytes and monocyte macrophage in the BALF of asthma mice. Previous studies have identified that matrix metalloproteinases (MMPs), as a family of extracellular proteases, were associated with the degradation of the extracellular matrix in the process of tissue

remodeling [37]. For instance, Lin et al. discovered that MMP-2 and MMP-9 were elevated in BALF of OVA mice, and they participated in pathogenesis of airway remodeling of asthma [38]. Felsen et al. testified that MMP-2 and MMP-9 activities were increased around inflamed airways, which was the major site of tissue remodeling [39]. Interestingly, we found that miR-192-5p inhibited MMP-16, MMP-2 and MMP-9, down-regulated FGF-23 and collagen I deposition. Additionally, miR-192-5p alleviated pathological morphology changes, goblet cell hyperplasia, airway mucus secretions and smooth muscle hyperplasia in asthma mice. The N/Pi, WAI/Pi as well as WAm/Pi levels were decreased, and WAI/WAm level was increased in miR-192-5p overexpression asthma mice. Further, MMP-16 overexpression attenuated the down-regulation of miR-192-5p on proliferation of ASMCs. MMP-16 was a target of miR-192-5p. These results indicate that miR-192-5p may alleviate the airway inflammation and airway remodeling in asthma through targeting MMP-16.

It has been documented that autophagy inhibitor chloroquine exhibits effective preclinical efficacy, including reducing airway remodeling [40]. Accumulating evidences have showed that

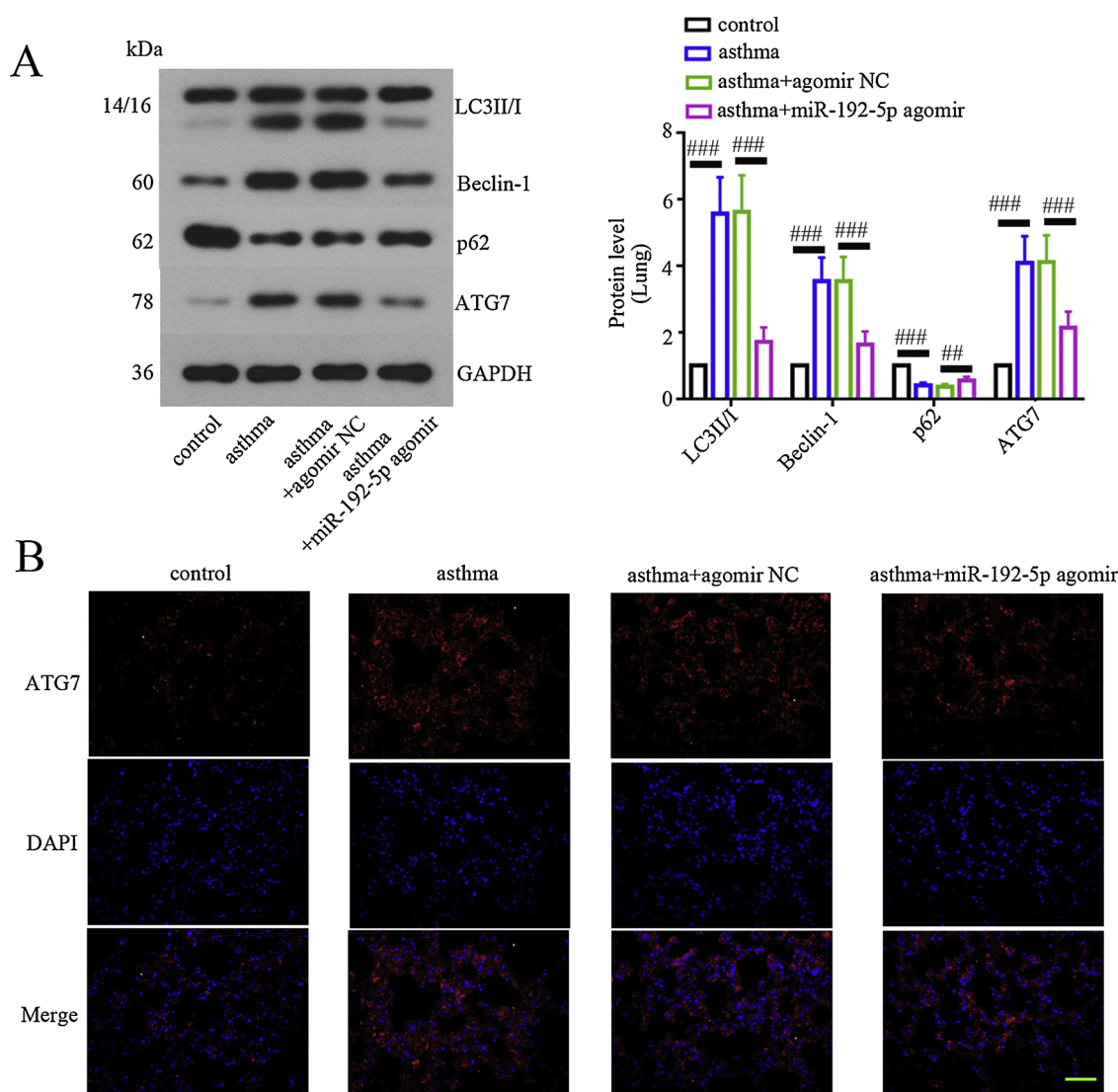


Fig. 8. MiR-192-5p decreased airway autophagy in asthma mice. (A) Western blot analysis was detected the LC3II/I, beclin-1, p62 and ATG7 protein levels in lung tissues of mice. (B) ATG7 expression was examined via immunofluorescence. Scale bar, 50 μ m. n = 6. ##, $p < 0.01$. ###, $p < 0.001$.

autophagosome formation is found to be increased in the airway in asthma [41,42]. Martin et al. determined that genetic variants in ATG5 was demonstrated to be related with childhood asthma [43]. Poon et al. revealed that autophagy was found to promote transforming growth factor- β 1 airway remodeling in asthma [44]. In our study, we discovered that the miR-192-5p led to inhibition of autophagy, evidenced by down-regulation of ATG7, LC3II/I and beclin-1, and up-regulation of p62. In addition, our results showed that ATG7 overexpression alleviated the decrease of miR-192-5p-caused proliferation in ASMCs. ATG7 was down-regulated by miR-192-5p, and identified to be a target of miR-192-5p. These findings imply that miR-192-5p may be involved in the inhibition of autophagy in asthma via targeting ATG7.

However, previous studies revealed that airway inflammation and airway remodeling could be alleviated through suppression of nuclear factor-kappa B (NF- κ B) signaling pathway in asthma [45]. Moreover, airway inflammation and airway remodeling were also reported to be aggravated through activation of JNK1/2-MMP-9 signaling pathway in asthmatic mice [46]. It means that miR-192-5p may attenuate the airway remodeling and autophagy by other signaling pathway in asthma. Reportedly, autophagy plays an important role in pro-apoptosis depending on cellular microenvironments [47]. The detection of autophagy-caused apoptosis change induced by miR-192-5p in asthma mice will be further explored. Besides, the correlation of clinic

outcomes of asthma patients with miR-192-5p expression was not analyzed. Therefore, to further investigate the mechanism of miR-192-5p in asthma, the asthma samples will be collected and the additional experiments will be conducted in the near future.

In conclusion, our current study demonstrated that miR-192-5p played a suppressive role in airway remodeling and reduced autophagy in asthma by targeting MMP-16 and ATG7. These finding will provide a new target for asthma treatment.

Declaration of Competing Interest

The authors have no declaration of interest.

Acknowledgments

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