



GAS5 promotes airway smooth muscle cell proliferation in asthma via controlling miR-10a/BDNF signaling pathway

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ABSTRACT

Aims: To explore the role of long non-coding RNA (lncRNA) growth arrest-specific transcript 5 (GAS5) in the cell proliferation of airway smooth muscle cells (ASMCs) in asthma.

Materials and methods: An asthma rat model was established by ovalbumin sensitization and challenge. The expression of GAS5, miR-10a and BDNF mRNA and protein was determined with qRT-PCR and western blot, separately. The targeting relationship between GAS5 and miR-10a was examined with RNA immunoprecipitation and RNA pull-down assay; the interaction between miR-10a and BDNF was evaluated by luciferase reporter assay. Cell Proliferation Assay (MTS) was used for ASMC proliferation detection. Knock-down of GAS5 was performed in asthmatic rats to determine the effects of GAS5 in vivo.

Key findings: Compared with control group, the inspiratory resistance and expiratory resistance were increased in asthma group; and the expression of GAS5, miR-10a and BDNF was higher, lower and higher, respectively. The expression of GAS5 and miR-10a was elevated and repressed, respectively, by platelet-derived growth factor-BB (PDGF-BB). GAS5 functioned as a bait of miR-10a. GAS5 regulates BDNF expression through miR-10a. PDGF-BB promotes the cell proliferation of ASMCs through miR-10a/BDNF. Knock-down of GAS5 significantly decreased airway hyperresponsiveness in asthmatic rats.

Significance: The lncRNA GAS5/miR-10a/BDNF regulatory axis played an important role in promoting ASMCs proliferation, thus contributing to asthma.

1. Introduction

Asthma is a kind of chronic disease, characterized by airway inflammation, airway hyperresponsiveness and airway remodeling [1,2]. Multiple factors were reported to cause asthma, such as infection, allergens, smoking, etc. [3–5]. It is well known that immunoglobulin E (IgE) is an essential mediator of allergic responses and plays a central role in allergic asthma pathophysiology [6]. As estimated, 5% of adults and 10% of children were diagnosed with asthma, with an increase in incidence [7,8]. Patients with asthma often have irreversible or partial irreversible airflow obstruction and continuous airway hyperresponsiveness. Currently, it is believed that the reason is that the airway has changed, namely, airway remodeling [9]. The proliferation of airway smooth muscle cells (ASMCs) plays an important role in the airway remodeling [10,11]. Therefore, fully exploring the signaling pathways regulating ASMC proliferation is of great clinical significance.

Numerous studies have found that a variety of stimulating factors,

including growth factors, hematogenic or inflammatory mediators, cytokines and extracellular matrix proteins, can induce ASMC proliferation [12,13]. Growth factor induced ASMC proliferation is considered the major cause of airway wall thickening in asthma, and increased level of platelet-derived growth factor (PDGF)-BB has been noted and it contributed to ASMC proliferation [14]. It has been reported that brain derived neurotrophic factor (BDNF) was highly expressed in ASMCs, and overexpression of BDNF could induce the cell proliferation of ASMCs [15,16]. However, the underlying mechanism is not clear.

Studies have demonstrated that multiple signaling pathways of asthma were regulated by abnormally expressed miRNAs, including miR-10a. As reported, miR-10a regulated ASMC proliferation through the direct targeting of PI3 kinase pathway. And overexpression of miR-10a decreased mitogen induced ASMC proliferation by 50%, while inhibition of miR-10a increased ASMC proliferation by 40% [17]. Bioinformatics analysis (<http://www.microrna.org/>) revealed the targeting relationship between miR-10a and BDNF, therefore, we focused

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on examining whether miR-10a can control ASMC proliferation through BDNF.

Long non-coding RNA (lncRNA), an important type of non-coding RNA, is involved in numerous pathophysiologic processes [18,19]. Researchers have found that lncRNA growth arrest-specific transcript 5 (GAS5) was expressed in airway epithelial cells, and inflammatory mediators could increase the expression levels of GAS5 in both ASMCs and airway epithelial cells [20]. Using bioinformatics software (DIANA TOOLS), we found that GAS5 could target miR-10a. Therefore, in this study, we established the asthma rat model and explored whether GAS5 regulated the ASMC proliferation via miR-10a and BDNF in asthmatic rats, uncovering a new regulatory mechanism of ASMC proliferation in asthma, and providing a new direction for the clinical treatment of asthma patients.

2. Materials and methods

2.1. Establishment of asthma rat model

Our study on animals was approved by the Animal Care Committee of the People's Hospital Affiliated to Zhengzhou University. All protocols of animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals proposed by the Chinese National Institutes of Health. The asthma rat model was established as previously described [21]. Twelve rats were randomly divided into 2 groups: control group (6 rats) and asthma group (6 rats). The rats in asthma group were intraperitoneally injected with 1 ml of 10% ovalbumin antigenic suspension (including 100 mg of ovalbumin and 100 mg of aluminum hydroxide in normal saline) on the 1st, 7th and 15th days, respectively, for the sensitization; on the 21st day, the rats were placed in sealed containers and then subjected to a 7-day aerosol inhalation of 2% ovalbumin antigenic suspension for 30 min daily; within 24 h after the final aerosol inhalation, the rats were anesthetized, endotracheally intubated, and then connected with the ventilator, and pulmonary function method was used to detect exhalation resistance and inhalation resistance, which were used as the indexes of airway responsiveness [22]. Similar procedures were performed on the rats in control group, however, the ovalbumin antigenic suspension was substituted by normal saline.

2.2. Enzyme-linked immunosorbent assay (ELISA)

The total serum immunoglobulin E (IgE) level in rats was determined with ELISA using an IgE Rat ELISA kit (ab157736, abcam, UK) according to the manufacturer's manual. All assays were repeated for triplicate, with the mean values used for statistical analysis.

2.3. Methacholine challenge test

Methacholine challenge test was performed to evaluate the airway hyperresponsiveness, with the inspiratory resistance and expiratory resistance calculated [22]. Rats were intravenously injected with methacholine (dissolved in 0.9% sodium chloride) at different concentration gradients from 0.0625 mg/kg to 1 mg/kg. They were injected at intervals of 5 min. Prior to the next methacholine injection, 55 μ l of methacholine was administered over 3–4 s according to the return of resistance curves to the pre-methacholine level. Subsequently, response was determined immediately as the peak increase above the baseline. Data were recorded and calculated using the dimensionless parameter, enhanced pause values.

2.4. Isolation and purification of ASMCs

The rat airway smooth muscle cells (ASMCs) were isolated and purified as previously reported [22,23]. Briefly, the rats were anesthetized and then sacrificed. Tracheas were resected and put into cold

phosphate buffered saline (PBS). Afterwards, epithelium plus fibrous tissue were removed under a microscope. The remaining tissue was cut into pieces and digested with collagenase I and elastase IV at 37 °C for 1 h. After centrifuged at 200g for 5 min, the pellet was washed twice using RPMI-1640 containing 10% fetal bovine serum (FBS). The cells were then seeded and cultured in a humidified incubator. Fresh medium was replaced every 3 days until they reached a confluence and passaged with 0.25% trypsin-EDTA solution. Passage 6–10 ASMCs were used in all experiments. For proliferation induction, ASMCs were stimulated with PDGF-BB (25 ng/ml) for 24 h followed by cell proliferation analysis [22].

2.5. qPCR

Total RNA was extracted from cells using TRIzol (Invitrogen). The first-strand cDNA was synthesized using M-MLV Reverse Transcriptase Kit (Fermentas) based on the protocol of manufacturer. Quantitative real-time PCR (qPCR) was performed with the SYBR Select Master Mix (Thermo Fisher) and analyzed on an ABI 7900-fast thermocycler (Applied Biosystems). Relative expression of long non-coding RNA (lncRNA) growth arrest-specific transcript 5 (GAS5) and miR-10a was normalized with U6, and the relative expression of brain derived neurotrophic factor (BDNF) was normalized with β -actin. The comparative Ct ($\Delta\Delta$ Ct) method was used for quantification. The primers used for qPCR were designed and synthesized by Sangon Biotech (Shanghai, China).

2.6. Western blot

To measure the pro-BDNF level, western blot determination was performed as previously reported [24]. Briefly, total proteins were extracted from ASMCs by using RIPA lysis buffer (containing a protease inhibitor cocktail). Then the protein extracts were subjected to SDS-PAGE and transferred to PVDF membrane. After blocked with 5% non-fat milk for 1 h at room temperature, the membrane was incubated using the antibodies including anti-BDNF (1:2000, Abcam), anti- β -actin (1:500, Abcam), and anti-AGO2 (1:1000, Abcam) at 4 °C overnight, followed by secondary antibody at room temperature for 2 h. Bands were visualized by ECL (GE Healthcare).

2.7. RNA immunoprecipitation (RIP)

The anti-AGO RIP was performed using the Imprint® RNA Immunoprecipitation kit (Sigma-Aldrich, US) according to the manufacturer's instructions. Briefly, the protein A magnetic beads were blocked with rabbit anti-rat IgG. After washing, rat IgG or monoclonal anti-AGO2 antibody was incubated with the magnetic beads, which were then resuspended in IP Buffer. The cell lysates of ASMCs were then incubated with the magnetic beads at 4 °C overnight. After excessive washing, the AGO2-RNA complexes were eluted. Finally, the western blot was used to examine the expression of AGO2, and qPCR was performed for the quantification of GAS5 and miR-10a.

2.8. RNA pull-down assay

The DNA probe for GAS5 was biotinylated by GenePharma (Shanghai) and incubated with M-280 Dynabeads Streptavidin for 2 h in binding buffer. The cell lysates of ASMCs were then incubated with the pretreated beads, with negative control (NC) served as control. After the beads were washed three times, the complexes of RNAs were eluted and examined with qPCR, and AGO2 was determined by western blot.

2.9. Cell transfection

To modulate the expression levels of GAS5, miR-10a and BDNF, ASMCs were transfected with GAS5 siRNA (si-GAS5), GAS5 overexpression vector (pcDNA-GAS5), miR-10a mimic or inhibitor, or BDNF

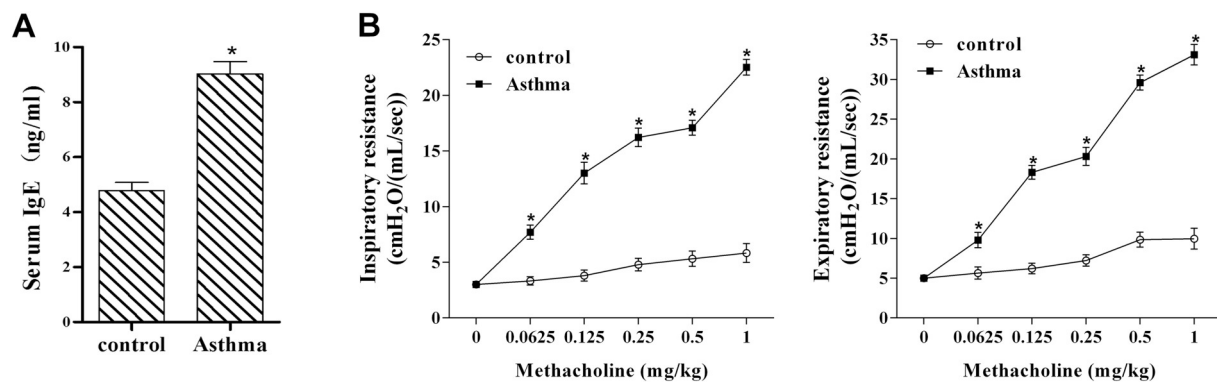


Fig. 1. Successful establishment of rat asthma model.

The asthmatic models in asthma group (n = 6) were successfully established, and (A) the serum IgE level was detected by ELISA, and (B) the inspiratory resistance and expiratory resistance were assessed between experimental and control group (n = 6). *P < 0.05 vs control group.

overexpression vector (pcDNA-BDNF). ASMCs were cultured in 6-well plates for 24 h, and si-GAS5, pcDNA-GAS5, miR-10a mimic or inhibitor, pcDNA-BDNF, or the individual corresponding control was transfected into the cells with Lipofectamine 2000 (Invitrogen, US) according to the manufacturer's instructions. After transfected for 48 h, the cells were collected.

2.10. Luciferase reporter assay

The luciferase reporter plasmids, BDNF 3'UTR-WT and BDNF 3'UTR-Mut were constructed. The ASMCs were cultured in 12-well plates for 24 h, and BDNF 3'UTR-WT and miR-10a mimic/inhibitor/negative control or BDNF 3'UTR-Mut and miR-10a mimic/inhibitor/negative control were transfected into the cells with Lipofectamine 2000. After 48 h, the cells were collected and the luciferase activity was examined by dual luciferase reporter assay system (Promega, US) according to the manufacturer's protocol.

2.11. Cell proliferation analysis

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, US) was used to detect viable cells according to manufacturer's protocol. Briefly, the ASMCs were seeded in a round-bottom 96-well microplate. After cultured in medium for 3 days, the cells were incubated with One Solution Reagent at 37 °C for 2 h followed by termination with SDS. The absorbance was measured at 490 nm with microplate reader. The amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

2.12. Injection of lentivirus

The lentivirus containing si-GAS5 or si-control was constructed by GenePharma (Shanghai). Thirty minutes before the establishment of asthma rat model, the rats received the tail intravenous injection of 100 µl of lentivirus containing si-GAS5 (n = 6) or si-control (n = 6) (5×10^7 TU/ml). Airway hyper-responsiveness was assessed within 24 h after the last aerosol inhalation.

2.13. Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc.). All cell-related assays for ASMCs such as gene expression test, cell proliferation, and luciferase activity were performed in triplicate, and data were generated with three replicates, which were shown as mean ± SD. The differences between groups were analyzed using Student's *t*-tests or analysis of variance (ANOVA) (for more than two groups). P < 0.05 was taken as statistically significant.

3. Results

3.1. Successful establishment of rat asthma model

Compared with control group (n = 6; 4.783 ± 0.706 ng/ml), the level of serum IgE was notably higher in asthmatic rats (Asthma, n = 6; 9.017 ± 0.123 , $P = 0.0022$) (Fig. 1A). The inspiratory resistance and expiratory resistance were examined with methacholine challenge test conducted. Compared with control group, the inspiratory resistances of rats injected with methacholine at 0.0625 mg/kg (3.33 ± 0.348 compared with 7.702 ± 0.582 , $P = 5.111\text{E}-08$), 0.125 mg/kg (3.780 ± 0.458 compared with 13.015 ± 0.894 , $P = 1.669\text{E}-09$), 0.25 mg/kg (4.785 ± 0.523 compared with 16.228 ± 0.757 , $P = 8.375\text{E}-11$), 0.5 mg/kg (5.312 ± 0.628 compared with 17.092 ± 0.617 , $P = 4.055\text{E}-11$), and 1 mg/kg (5.827 ± 0.771 compared with 22.523 ± 0.641 , $P = 4.671\text{E}-12$) were significantly increased in asthma group (Fig. 1B). The expiratory resistances of rats injected with methacholine at 0.0625 mg/kg (5.638 ± 0.693 compared with 9.8 ± 0.876 , $P = 8.266\text{E}-06$), 0.125 mg/kg (6.202 ± 0.615 compared with 18.312 ± 0.782 , $P = 1.039\text{E}-10$), 0.25 mg/kg (7.233 ± 0.670 compared with 20.312 ± 1.047 , $P = 4.365\text{E}-10$), 0.5 mg/kg (9.852 ± 0.863 compared with 29.603 ± 0.861 , $P = 4.365\text{E}-10$), and 1 mg/kg (9.973 ± 1.197 compared with 33.118 ± 1.187 , $P = 3.149\text{E}-11$) were also remarkably increased in asthma group. Our results demonstrated that the airway responsiveness was markedly increased in asthma group, indicating the successful construction of asthma model in rats.

3.2. Determination of lncRNA GAS5, miR-10a and BDNF in control and asthma groups

The airway smooth muscle cells (ASMCs) were isolated and purified from rats in control and asthma groups, and the expression of lncRNA GAS5, miR-10a and BDNF in ASMCs was then determined. As shown in Fig. 2, the relative levels of lncRNA GAS5 in asthma group (2.427 ± 0.052) were significantly higher than that in control group (1.00 ± 0.00) ($P = 2.74\text{E}-06$; Fig. 2A). The expression of miR-10a was significantly reduced in asthma group (0.39 ± 0.049) when comparing to control group (1.00 ± 0.00) ($P = 6.11\text{E}-05$; Fig. 2B). Besides, the mRNA and protein levels of BDNF were markedly increased in asthma group (1.987 ± 0.092) in comparison with control group (1.00 ± 0.00) ($P = 0.0001$; Fig. 2C). These results proved that lncRNA GAS5 was up-regulated in ASMCs of asthmatic rats.

3.3. GAS5 could function as a bait of miR-10a

Bioinformatics analysis showed that there were binding sites between GAS5 and miR-10a (Fig. 3A). To confirm the targeting

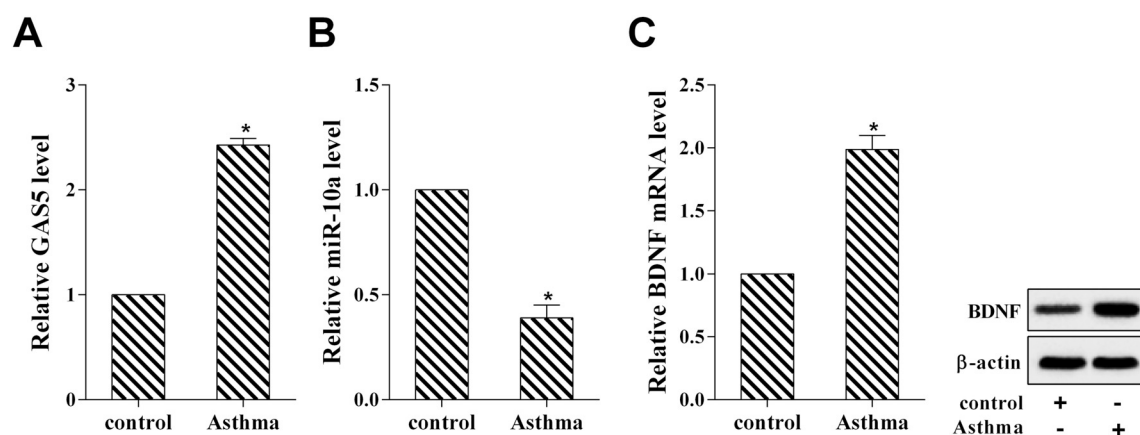


Fig. 2. Determination of lncRNA GAS5, miR-10a and BDNF in ASMCs isolated and purified from rats in control and asthma groups.

(A) lncRNA GAS5 levels in ASMCs isolated and purified from rats were determined by qPCR; (B) miR-10a expression in ASMCs isolated and purified from rats was determined by qPCR; (C) BDNF mRNA and protein levels in ASMCs isolated and purified from rats were examined by qPCR and western blot, respectively. β -Actin was used as the loading control. * $P < 0.05$ vs control group.

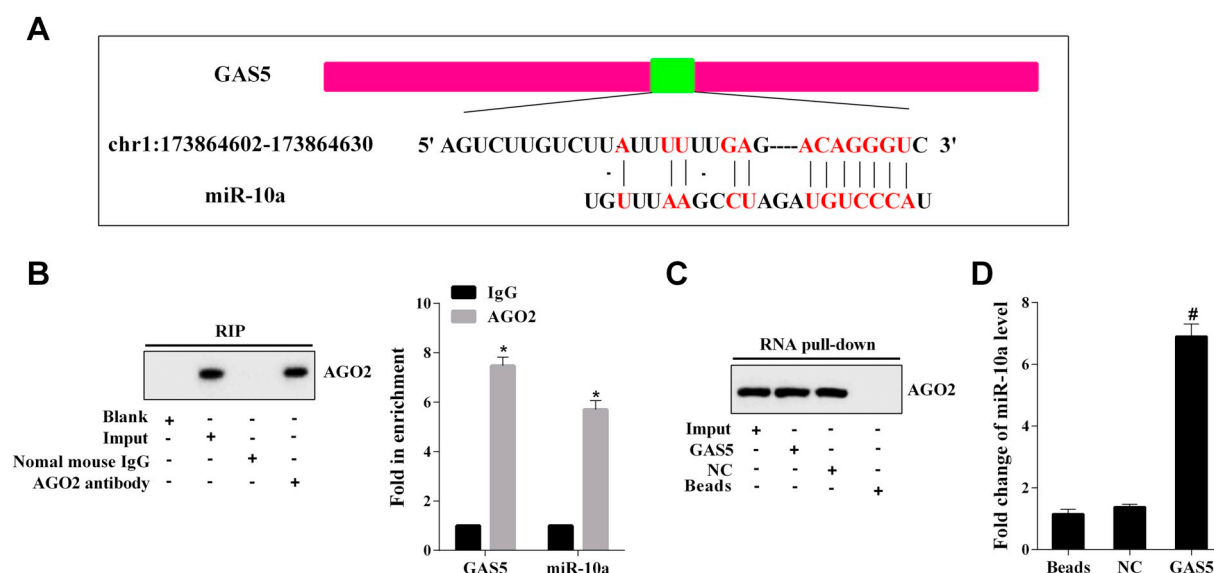


Fig. 3. GAS5 could function as a bait of miR-10a in ASMCs.

(A) The predicted miR-10a binding sites were shown in GAS5; (B) anti-AGO2 RNA immunoprecipitation assay was used to determine whether GAS5 and miR-10a present in the AGO2 protein complex; (C) RNA pull-down assay was performed to examine the interaction between GAS5 and miR-10a. (D) The miR-10a was estimated by qRT-PCR. * $P < 0.05$ vs IgG, # $P < 0.05$ vs NC.

relationship between GAS5 and miR-10a, a series of experiments were performed in ASMCs isolated and purified from normal rats. Anti-AGO2 RNA immunoprecipitation showed that GAS5 and miR-10a were pulled down by the AGO2 antibody. Compared with IgG group, the levels of GAS5 and miR-10a were significantly higher in AGO2 group (Fig. 3B). Besides, the RNA pull-down assay revealed that miR-10a was pulled down by the GAS5 probe. AGO2 was also pulled down by the GAS5 probe (Fig. 3C). Importantly, the miR-10a was more enriched in GAS5 group (6.9 ± 6.910) when comparing to NC group (1.377 ± 0.071) ($P = 0.001$; Fig. 3D). The assays proved the binding and interplay between GAS5 and miR-10a.

3.4. GAS5 regulates BDNF expression through miR-10a

The ASMCs were isolated and purified from normal rats, and cultured in the absence or presence of 25 ng/ml PDGF-BB for 24 h. As shown in Fig. 4A and B, the mRNA and protein levels of BDNF were increased when stimulated by PDGF-BB (1.00 ± 0.00 compared with 2.303 ± 0.078 , $P = 1.86E-05$). Knock-down of GAS5 dramatically

reduced PDGF-BB induced BDNF mRNA (2.223 ± 0.010 compared with 1.37 ± 0.054 , $P = 0.0004$) and protein expression. Further, inhibition of miR-10a could greatly reverse the decrease in BDNF mRNA (1.427 ± 0.031 compared with 1.83 ± 0.037 , $P = 0.0003$) and protein expression caused by GAS5 knock-down. It was illustrated that GAS5 regulated BDNF expression through miR-10a.

3.5. MiR-10a modulates the BDNF expression

Sequence alignment analysis demonstrated the presentation of miR-10a-binding sites on 3'UTR region of BDNF (Fig. 5A). In ASMCs, luciferase reporter assay revealed that miR-10a down-regulation with miR-10a inhibitor transfected dramatically enhanced the luciferase activity of BDNF 3'UTR-WT (0.96 ± 0.045 compared with 3.097 ± 0.092 , $P = 0.0013$) without affecting BDNF 3'-Mut (1.02 ± 0.021 compared with 0.97 ± 0.039 , $P = 0.353$). Further, the mRNA and protein levels of BDNF were markedly increased by miR-10a inhibitor transfection (1.00 ± 0.00 compared with 2.233 ± 0.109 , $P = 0.004$) (Fig. 5B). To the contrary, the up-regulation of miR-10a with miR-10a mimic

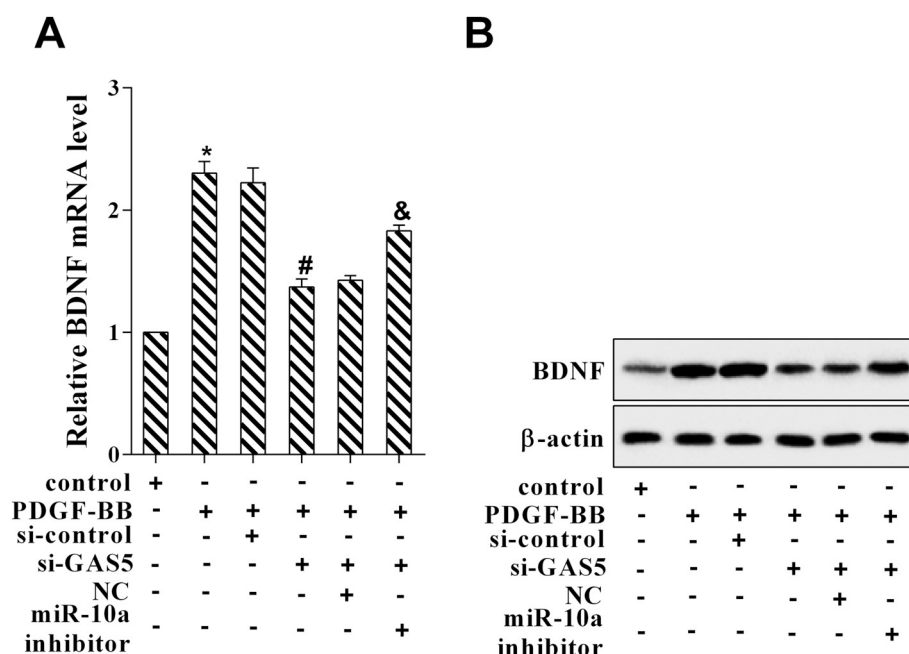


Fig. 4. GAS5 regulated BDNF expression through miR-10a.

The ASMCs were treated with si-GAS5 and/or miR-10a inhibitor in the absence or presence of 25 ng/ml PDGF-BB for 24 h. (A) The BDNF mRNA levels in ASMCs; (B) the BDNF protein levels in ASMCs. * $P < 0.05$ vs control, # $P < 0.05$ vs si-control, & $P < 0.05$ vs NC.

significantly suppressed the luciferase activity of BDNF 3'UTR-WT (0.947 ± 0.041 compared with 0.577 ± 0.034 , $P = 0.018$) without changing the BDNF 3'UTR-Mut luciferase activity (1.023 ± 0.031 compared with 0.98 ± 0.022 , $P = 0.096$), and also remarkably decreased the BDNF expression at both mRNA and protein levels (Fig. 5C). Here, we manifested that miR-10a negatively regulated BDNF expression.

3.6. PDGF-BB promotes the cell proliferation of ASMCs through miR-10a/BDNF

ASMCs were then treated with miR-10a mimic and/or pcDNA-BDNF in the absence or presence of PDGF-BB. As shown in Fig. 6A and B, the protein levels of BDNF and ASMCs proliferation (2.887 ± 0.084 compared with 5.237 ± 0.087 , $P = 0.0024$) were increased when stimulated by PDGF-BB, while miR-10a mimic dramatically reduced PDGF-BB induced BDNF protein expression and cells proliferation (5.153 ± 0.039 compared with 3.243 ± 0.095 , $P = 0.001$). Further, overexpression of BDNF could greatly reverse the decrease in BDNF protein expression and ASMCs proliferation (3.3 ± 0.041 compared with 4.377 ± 0.156 , $P = 0.006$) caused by miR-10a mimic. It was proven that PDGF-BB promotes the cell proliferation of ASMCs through miR-10a/BDNF.

3.7. PDGF-BB promotes the cell proliferation of ASMCs through GAS5/miR-10a/BDNF

To investigate the effect of GAS5 on ASMCs proliferation induced by PDGF-BB, ASMCs were assigned into 7 groups: control, PDGF-BB, PDGF-BB + si-control, PDGF-BB + si-GAS5, PDGF-BB + si-GAS5 + NC, and PDGF-BB + si-GAS5 + miR-10a inhibitor. Detection on cell proliferation indicated that PDGF-BB promoted the cell proliferation of ASMCs (3.107 ± 0.082 compared with 4.887 ± 0.067 , $P = 0.003$), but knock-down of GAS5 dramatically reduced PDGF-BB promoted cell proliferation of ASMCs (4.943 ± 0.050 compared with 3.33 ± 0.057 , $P = 0.001$); further, miR-10a inhibitor could greatly reverse the decrease in cell proliferation of ASMCs caused by knock-down of GAS5 (3.253 ± 0.037 compared with 4.183 ± 0.155 , $P = 0.008$) (Fig. 7). It

can be seen that PDGF-BB promotes the cell proliferation of ASMCs through GAS5/miR-10a/BDNF pathway.

3.8. Knock-down of GAS5 decreases airway hyperresponsiveness in asthmatic rats

We finally checked the GAS5/miR-10a/BDNF pathway in asthmatic rats. As shown above, the airway responsiveness was markedly increased in asthma group compared with control group. After receiving the tail intravenous injection of lentivirus containing si-GAS5 ($n = 6$) or si-control ($n = 6$), the inspiratory resistances of asthmatic rats injected with methacholine at 0.0625 mg/kg (7.002 ± 0.545 compared with 4.437 ± 0.394 , $P = 6.023\text{E}-07$), 0.125 mg/kg (10.907 ± 0.686 compared with 5.01 ± 0.420 , $P = 4.241\text{E}-10$), 0.25 mg/kg (14.51 ± 0.775 compared with 5.618 ± 0.574 , $P = 4.051\text{E}-09$), 0.5 mg/kg (20.182 ± 0.640 compared with 6.682 ± 0.753 , $P = 4.127\text{E}-08$), and 1 mg/kg (25.457 ± 0.750 compared with 9.545 ± 0.484 , $P = 2.701\text{E}-09$) were significantly decreased in si-GAS5 group when comparing to si-control group (Fig. 8A). Expiratory resistance of asthmatic rats was also dramatically decreased in si-GAS5 group when comparing to si-control group (Fig. 8A). These findings suggested that knock-down of GAS5 could decrease the airway hyperresponsiveness. The expression of GAS5, miR-10a and BDNF protein was also checked. We found that, compared with si-control group, GAS5 was down-regulated (1.00 ± 0.00 compared with 0.56 ± 0.041 , $P = 3.8\text{E}-10$), and the expression levels of miR-10a (1.00 ± 0.00 compared with 1.642 ± 0.076 , $P = 3.89\text{E}-09$) and BDNF protein were increased and decreased in si-GAS5 group, respectively (Fig. 8B).

4. Discussion

In this study, we found that the inspiratory resistance and expiratory resistance were increased in asthma group compared with control group. The expression of GAS5, miR-10a and BDNF was higher, lower and higher, respectively, in asthma group than that in control group. The expression of GAS5 and miR-10a was also significantly elevated and repressed, respectively, in the ASMCs treated by PDGF-BB. Knock-

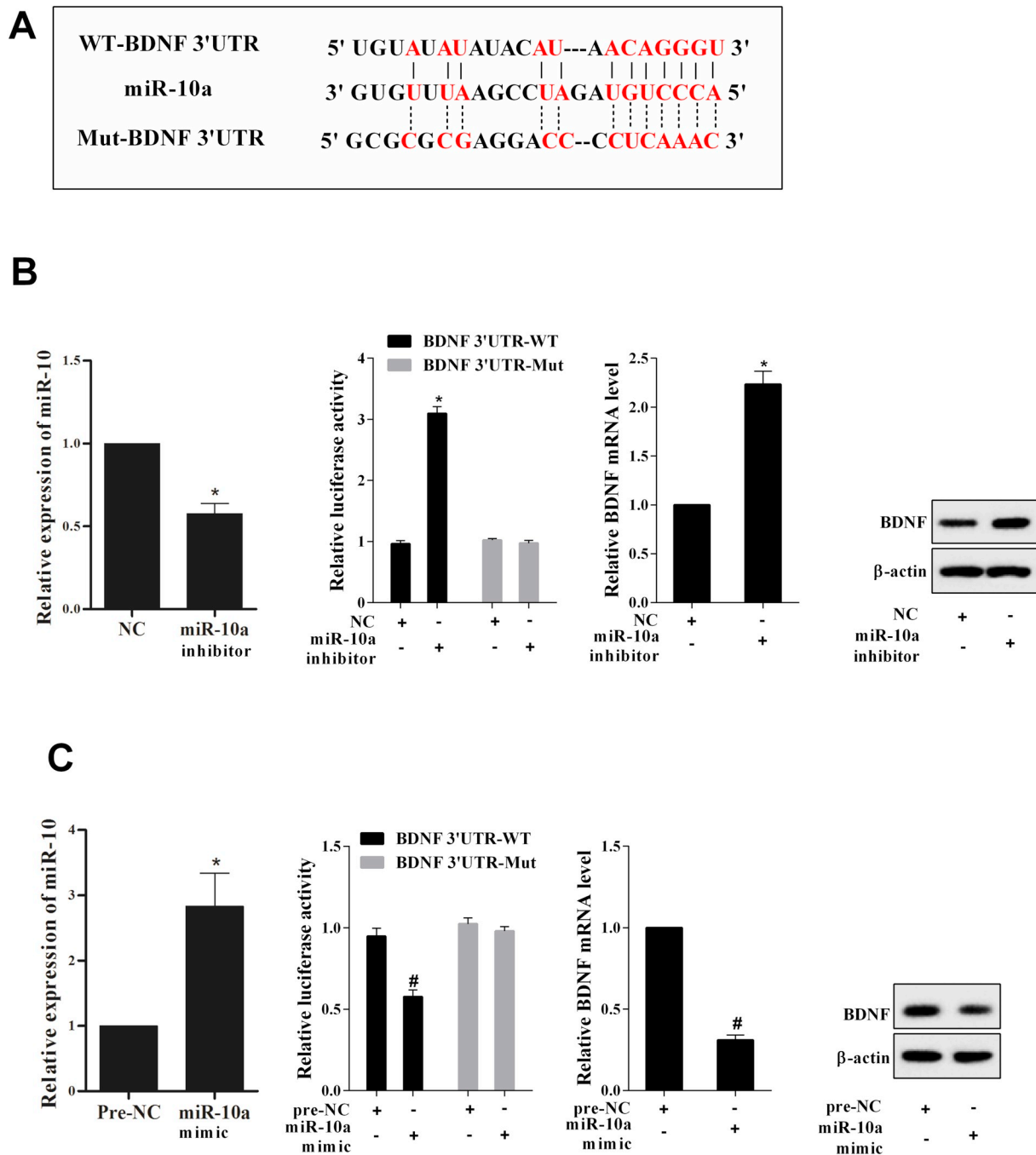


Fig. 5. miR-10a directly modulated the BDNF expression in ASMCs.

(A) The predicted miR-10a-binding sites on 3'UTR region of BDNF; (B) the effect of miR-10a inhibitor on miR-10a expression, BDNF 3'UTR activity, and BDNF mRNA and protein expression; (C) the effect of miR-10a mimic on miR-10a expression, BDNF 3'UTR activity, and BDNF mRNA and protein expression. * $P < 0.05$ vs NC, # $P < 0.05$ vs pre-NC.

down of GAS5 reduced PDGF-BB induced BDNF expression and ASMC proliferation, which was reversed by miR-10a inhibitor. miR-10a mimic reduced PDGF-BB induced BDNF expression and ASMC proliferation, which was reversed by overexpression of BDNF. Finally, knock-down of GAS5 significantly decreased airway hyperresponsiveness in asthmatic rats with increased miR-10a and decreased BDNF levels. Taken together, the results indicated that GAS5 regulated the ASMC proliferation via miR-10a/BDNF in asthmatic rats.

Airway remodeling can lead to irreversible or partial irreversible airflow obstruction and continuous airway hyperresponsiveness in patients with asthma [9]. Abnormal ASMC proliferation can result in the increased thickness of airway wall, contributing to the development of

airway remodeling of asthma [10,11]. Therefore, it is important to explore the underlying mechanism of ASMC proliferation.

As mentioned above, various stimulating factors can induce ASMC proliferation. For example, tumor necrosis factor α (TNF- α), transforming growth factor (TGF), basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), and interleukin 1 β (IL-1 β) were reported to induce ASMC proliferation via regulating the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signaling pathways [25–27]. BDNF was highly expressed in ASMCs, and overexpression of BDNF could induce the cell proliferation of ASMCs [15,16]. In this study, we also found the increased expression of BDNF in asthma group compared with control group, consistent with previous studies.

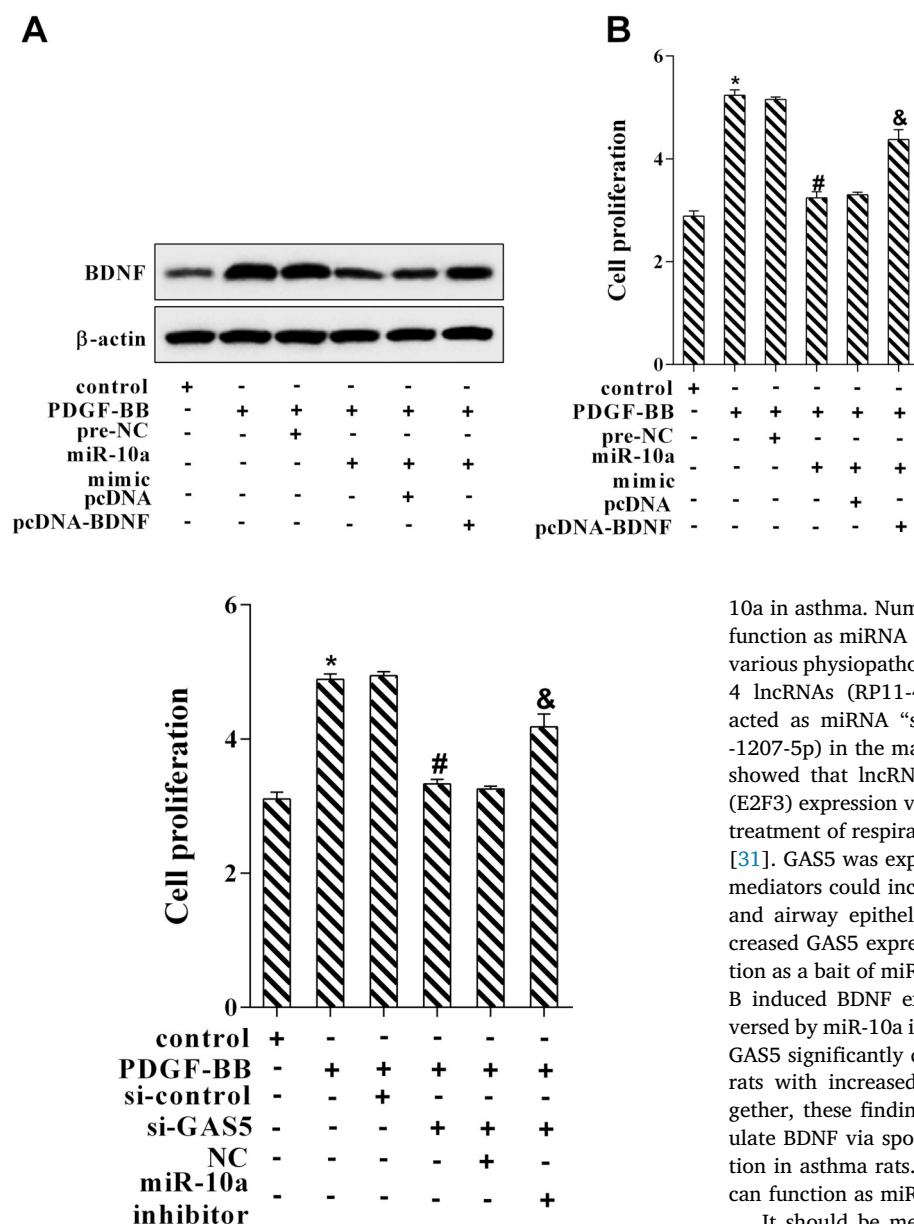


Fig. 7. PDGF-BB promoted the cell proliferation of ASMCs through GAS5/miR-10a/BDNF.

The cell proliferation in ASMCs treated with si-GAS5 and/or miR-10a inhibitor in the absence or presence of PDGF-BB. * $P < 0.05$ vs control, # $P < 0.05$ vs si-control, & $P < 0.05$ vs NC.

We next investigated the molecular mechanism of regulating BDNF in asthma. Studies have shown that overexpression of miR-10a decreased mitogen induced ASMC proliferation by 50%, while inhibition of miR-10a increased ASMC proliferation by 40% [17]. In this study, we found the decreased miR-10a expression in asthmatic rats, and that miR-10a can directly target the BDNF 3'UTR region. Moreover, overexpression of BDNF can reverse the inhibition of miR-10a mimic on PDGF-BB promoted ASMC proliferation. It has been reported that miR-10a suppressed the ASMC proliferation via directly inhibiting the catalytic subunit α of PI3K (PIK3CA), the central component of PI3K signaling pathway [17]. In our study, the findings suggested that miR-10a can decrease the ASMC proliferation by directly targeting BDNF. Therefore, our study uncovered a novel molecular target of miR-10a in the regulation of ASMC proliferation, further supporting the hypothesis that miR-10a was a potential therapeutic target of asthma.

We further explored the underlying mechanism of regulating miR-

Fig. 6. PDGF-BB promoted the cell proliferation of ASMCs through miR-10a/BDNF.

(A) The BDNF protein levels in ASMCs treated with miR-10a mimic and/or pcDNA-BDNF in the absence or presence of PDGF-BB; (B) the cell proliferation in ASMCs treated with miR-10a mimic and/or pcDNA-BDNF in the absence or presence of PDGF-BB. * $P < 0.05$ vs control, # $P < 0.05$ vs pre-NC, & $P < 0.05$ vs pcDNA.

10a in asthma. Numerous studies have demonstrated that lncRNAs can function as miRNA sponges to control the expression of target genes in various physiopathological processes [28,29]. It has been proposed that 4 lncRNAs (RP11-46A10.4, LINC00883, BCYRN1, and LINC00882) acted as miRNA “sponges” for 4 miRNAs (miR-150, -371-5p, -940, -1207-5p) in the maintenance of primary ASMCs [30]. A recent report showed that lncRNA-PVT1 controlled the E2F transcription factor 3 (E2F3) expression via sponging miR-203a, which was implicated in the treatment of respiratory syncytial virus caused asthma using α -asarone [31]. GAS5 was expressed in airway epithelial cells, and inflammatory mediators could increase the expression levels of GAS5 in both ASMCs and airway epithelial cells [20]. In this study, we observed the increased GAS5 expression in asthmatic rats, and that GAS5 could function as a bait of miR-10a. Besides, knock-down of GAS5 reduced PDGF-B induced BDNF expression and ASMC proliferation, which was reversed by miR-10a inhibitor. Importantly, we found that knock-down of GAS5 significantly decreased airway hyperresponsiveness in asthmatic rats with increased miR-10a and decreased BDNF levels. Taken together, these findings supported the hypothesis that GAS5 could regulate BDNF via sponging miR-10a, and thus promote ASMC proliferation in asthma rats. Our study for the first time discovered that GAS5 can function as miR-10a sponges to regulate BDNF expression.

It should be mentioned that there were some deficiencies in this study. The sample size of animal experiments was relatively small. And the roles of miR-10a and BDNF in ASMC proliferation in vivo were not fully explored. Therefore, the sample size would be increased to confirm the effects of miR-10a and BDNF on ASMC proliferation in vivo in the future work.

In conclusion, the GAS5/miR-10a/BDNF regulatory axis played an important role in the cell proliferation of ASMCs in asthmatic rats, and knock-down of lncRNA GAS5 could lower the airway hyperresponsiveness of asthmatic rats. These results uncovered a new regulatory mechanism of ASMC proliferation in asthma, and provided a new direction for the clinical treatment of asthma patients.

Conflict of interest

The authors declare no conflict of interest.

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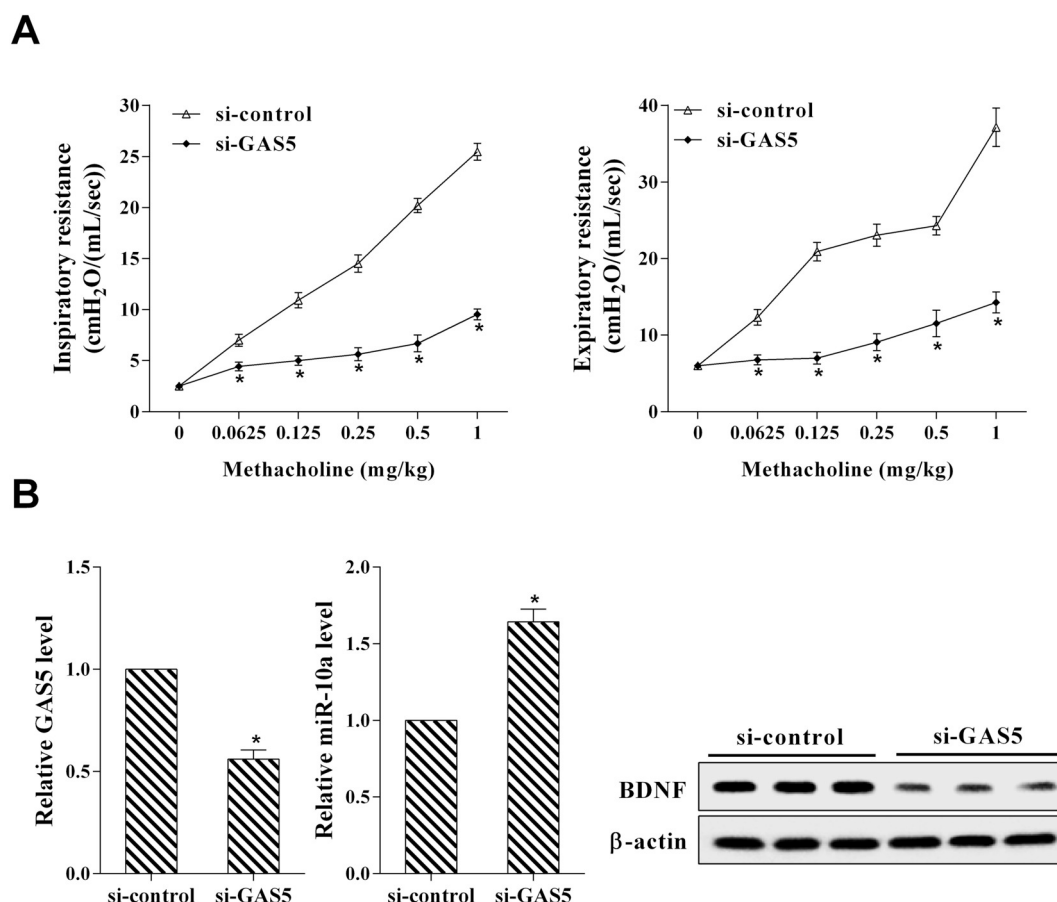


Fig. 8. Knock-down of GAS5 decreased airway hyperresponsiveness in asthmatic rats.

The lentivirus containing si-GAS5 or si-control was injected into asthmatic rats via caudal vein (n = 6/group). (A) The inspiratory resistance and expiratory resistance in asthmatic rats receiving the tail intravenous injection of lentivirus containing si-GAS5 or si-control; (B) the expression of GAS5, miR-10a and BDNF protein in asthmatic rats receiving the tail intravenous injection of lentivirus containing si-GAS5 or si-control. *P < 0.05 vs si-control.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.lfs.2018.09.002>.

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