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Effects of Nerve Growth Factor shRNA Inhibition on Asthma Phenotypes in a Mouse Model of Asthma

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ABSTRACT

Nerve growth factor (NGF) plays an important role in airway hyper-responsiveness (AHR). In this study, we aimed at investigating the effect of NGF inhibition on AHR and other asthma phenotypes in a mouse model of asthma.

12 mice in each group were injected with lentiviral vectors expressing non-targeting shRNA (sham shRNA), targeting NGF (shRNA-1 and shRNA-2), or normal saline for control before the asthma models were established. Peak inspiratory pressure (PIP), NGF levels in bronchoalveolar lavage fluid (BALF), and bronchoconstriction in response to acetylcholine (ACh) were measured. Immunohistochemistry semi-quantitative analysis of muscarinic acetylcholine receptor M3 (mAChR M3) and alpha-smooth muscle actin (a-SMA) were measured by Image Pro Plus (IPP), and qRT-PCR analysis of mRNAs of cholinergic receptors, muscarinic 3 (Chrm3), Ngf and Tropomyosin receptor kinase A (TrkA) were performed.

Immunohistochemistry showed mAChR M3 was overexpressed and a-SMA was hyperplasia in control and sham shRNA, semi-quantitative analysis revealed optical density (OD) values were significantly higher than shRNA-1 and shRNA-2, (p<0.001). BALF NGF levels were significantly higher in control and sham shRNA (457.16±45.32, 676.43±111.64) compared with shRNA-1 and shRNA-2 (261.56±25.81, 129.12±15.96 pg/mL) (p<0.001). PIP was significantly higher in control, compared with shRNA-1, shRNA-2, (p =0.045, 0.003), bronchoconstriction response to ACh was significantly higher in sham shRNA, compared with shRNA-1, shRNA-2, (p=0.02, 0.006). Expression of mRNAs of Chrm3, Ngf and TrkA genes in sham shRNA group were higher than shRNA-1 and shRNA-2.

Inhibiting NGF via NGF-targeting shRNAs appears to lessen the severity of asthma phenotypes in this mouse model of asthma.

Keywords: Asthma; Muscarinic receptors; Nerve growth factor; Respiratory hypersensitivity; Small interfering RNA; Smooth muscle

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INTRODUCTION

Asthma continues to be a major public health concern worldwide, with a global physician-diagnosed prevalence rate of 4.3%[1] in adults. Asthma affects 1-18% of the population in different countries.[2] The typical pathophysiological feature of asthma is airway hyper-responsiveness (AHR),[3] as a result of inflammatory cell infiltration,[4] B lymphocytes involved allergic response,[5,6] hyperplasia of fibrous tissue,[7] Airway smooth muscle (ASM) cell proliferation and hypertrophy and sensory nerve hyperinnervation.[8] Nerve growth factor (NGF) plays an important role in AHR through sensory nerve hyperinnervation.[9,10] A direct relationship has been found between peripherally administered NGF and airway hyper-responsiveness.[11,12] NGF inhibition can improve AHR;[10] and NGF inhibition via small interfering RNA (siRNA) alleviates allergic airway hyper-responsiveness in a mouse model of asthma.[13]

ASM is the effector of bronchial constriction. ASM cell hyperplasia and hypertrophy lead to abnormal constriction in asthma. ASM constriction and mucus secretion is traditionally associated with the parasympathetic nerve system in the airways. Parasympathetic activity is increased in airway inflammation[14]. mAChR M3 is expressed on epithelium and ASM and plays a key role in muscarinic constriction of small airways in both rodents and humans.[15,16] Muscarinic contraction in both segmental and subsegmental bronchi is mediated through mAChR M3.[15] mAChR M3 has been shown to regulate the effects of epithelial-derived chemokines on ASM cell migration, which results in lung remodeling. It has been suggested that mAChR M3 may be an important therapeutic target for obstructive airway diseases.[17] mAChR M2 is also involved in airway constriction. Blocking mAChR M2 action can inhibit relaxation.[18] mAChR M2 mediation of airway constriction may require the activation of both M2 and M3 receptors.

In inflammatory airway, epithelium and fibroblasts synthesize and release a multitude of inflammatory molecules,[19] including epidermal growth factor (EGF), platelet-derived growth factor (PDGF),[20] and transforming growth factor-β1 (TGF-β1),[21] which can induce ASM cell proliferation and hypertrophy in large airways in both nonfatal and fatal cases of asthma.[22] Immunocytochemical studies of lung sections from individuals without asthma showed that bronchial smooth muscles not only constitutively express NGF and other neurotrophins, but also responds to neurotrophins.[23] Rapid (non-genomic) modulation of airway smooth muscle function and genomic effects on muscle cell growth and proliferation can affect airway contractility.[24] Neurotrophins enhance intracellular calcium concentrations and force responses of human airway smooth muscle to agonists such as Ach.[25] Regardless of the source, neurotrophins can directly alter the contractility of airway smooth muscles, again via nongenomic mechanisms.[24] Antibody against NGF prevents atropine-enhanced, antigen challenge-induced airway hyper-reactivity and eosinophil activation in animals.[26] NGF is related to muscarinic receptors and asthma phenotypes and contributes to ASM cell hyperplasia and hypertrophy.

The aim of this study was to investigate the effect of NGF inhibition on AHR, preventing mAChR M3 overexpression, ASM cell hyperplasia and hypertrophy and other asthma phenotypes in a mouse model of asthma.

MATERIALS AND METHODS

Animals

In this study, 48 six-week-old male BALB/c mice were purchased from Slac Laboratory Animal Corporation Shanghai, China, and housed in the Department of Laboratory Animal Science of Fudan University, Shanghai, China. The study was done according to the Law of the People's Republic of China on the Protection of Wildlife. The protocol was approved by the central laboratory, center of animal experiments and ethics committee of the Eye, Ear, Nose, and Throat Hospital affiliated to Fudan University. The design of experiments is displayed in Figure 1.

Production of shRNA Lentivirus

Two shRNAs targeting the mouse NGF gene were used to inhibit NGF expression; shNGF-1: 5'-AGACTGTTTAAGAAACGGA-3' (designed by Jikai Gene Chemical Technology Co., Ltd., Shanghai, China: GC content 36.84%) and shNGF-2: 5'-AAGGCGTTGACAACAGATGAG-3' (GC content 47.62%).[27] A non-targeting control shRNA termed sham shNGF was also used: 5'-TTCTCCGAACGTGTCACGT-3' (Jikai Gene Chemical Technology Co., Ltd.). The sham shRNA

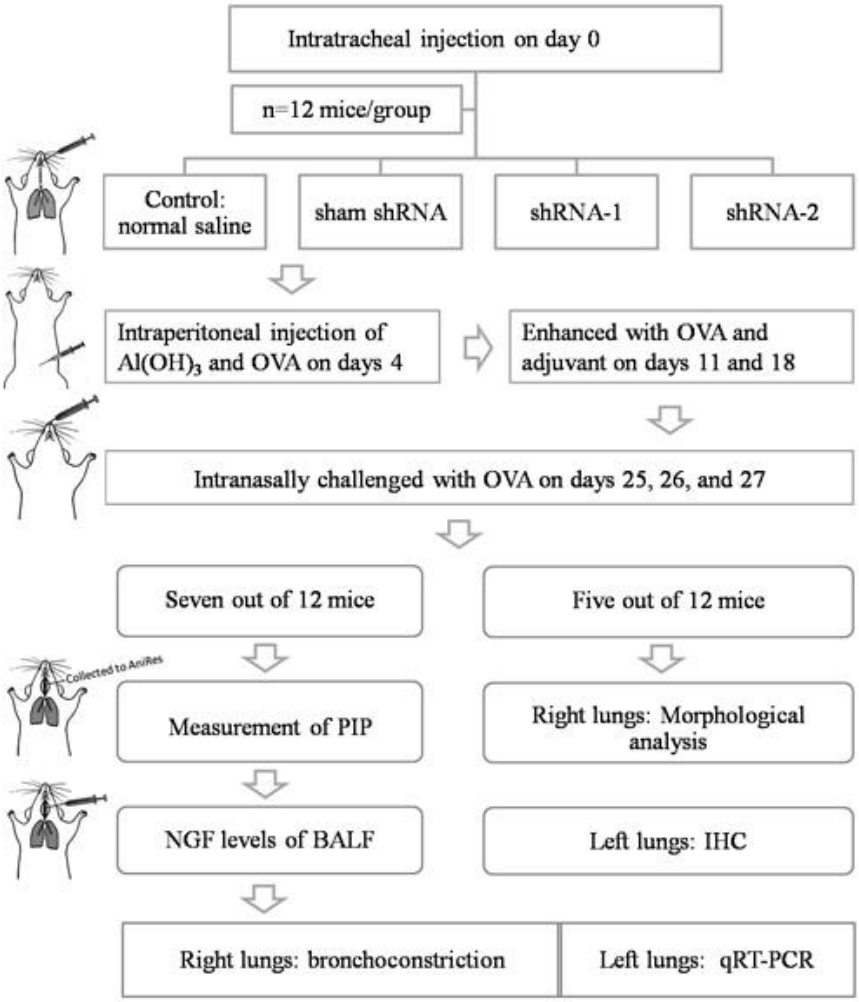

Figure 1. The mouse model of asthma used in this study to investigate effects of nerve growth factor shRNA inhibition on asthma phenotypes. After synthesis of RNAs, the mouse models were intratracheally pretreated with the RNAs, and asthma models were established with OVA and Al $(OH)_3$. Measurement of PIP, detection of BALF NGF levels, measurement of bronchoconstriction response to ACh and qRT-PCR analysis were performed for 7 mice randomly out of the 12 in each group. Morphological analysis and immunohistochemistry were done for remaining 5 mice in each group to investigate pathological features. Statistical analysis was performed at last.

Al $(OH)_3$, Aluminum hydroxide; OVA, Ovalbumin; PIP, peak inspiratory pressure; NGF, nerve growth factor; BALF, bronchoalveolar lavage fluid; IHC, immunohistochemistry; Ach, acetylcholine; qRT-PCR, quantitative real-time polymerase chain reaction

sequence was not homologous with any gene-coding sequence in the mouse genome.

The shRNA sequences were cloned into lentiviral vector GV118, which expresses the enhanced green fluorescent protein (EGFP) (Jikai Gene Chemical Technology Co., Ltd., China). The GV118-shRNA constructs were transformed into DH5α competent cells. Positive constructs were verified by high throughput sequencing. Lentiviral vectors carrying shRNAs as confirmed by sequencing were transfected into 293 T cells and lentivirus was produced following standard procedures, with the technical support of Jikai Gene Chemical Technology Co., Ltd, Shanghai, China. The viral titers were as follows: shNGF-1:3E+8 TU/mL, shNGF-2: 5E+8 TU/mL, and sham NGF shRNA: 1E+9 TU/mL.

Establishment of Mouse Model of Asthma

The 48 six-week-old male BALB/c mice were divided into control, sham shRNA, shRNA-1, and shRNA-2 groups (n=12 mice/group). On day 0, the mice in the sham shRNA, shRNA-1, and shRNA-2 groups were injected intratracheally with lentivirus containing sham shRNA, NGF shRNA-1, and NGF shRNA-2 (3E+6 TU each), respectively. Mice in the control group were injected with normal saline. Mice were anesthetized with chloral hydrate (0.5 mg/kg) plus fentanyl (10 μg/kg, Humanwell Healthcare (Group) Co., Hubei, China) for all injections. Mice in all groups were sensitized by intraperitoneal injection of aluminum hydroxide 2 mg (Thermo Fisher Scientific Inc., Waltham, MA, USA) containing ovalbumin 50 μg (OVA, Sigma-Aldrich Shanghai Trading Co Ltd, Shanghai, China) in a total volume of 200 μL PBS on day 4, and enhanced with 25 μg OVA in the same dosage of adjuvant on days 11 and 18. Finally, Mice in all groups were challenged with OVA (100 μg) by intranasal administration with a 24G intravenous needle catheter on days 25, 26, and 27.

Measurement of PIP

7 out of the 12 mice in each group were randomly anesthetized with 0.5 mg/kg chloral hydrate plus 10 μg/kg fentanyl. The trachea was exposed and a ventilation tube which was connected to an AniRes 2005 pulmonary function system (Beijing Bestlab High-Tech Co., Ltd., Beijing, China) was inserted. Default values: respiratory rate=120/min, inspiratory: expiratory ratio=1:1, tidal volume: 20 mL/kg. Tidal volume and PIP were recorded by the AniRes system software.

Detection of NGF Levels

Immediately after measurement of PIP, the 7 mice in each group were re-intubated with a venous needle catheter, the bronchoalveoli were lavaged with 0.4 mL normal saline for 3 times and bronchoalveolar lavage fluid (BALF) was collected. NGF levels in BALF were measured using enzyme-linked immunosorbent assay (ELISA) (Boster Biological Technology Co., Ltd., Wuhan, China).

Measurement of Bronchoconstriction

Low melting point agarose 5% (Sangon Biotech Co., Ltd., Shanghai, China) was prepared and maintained at 37°C. Seven right lungs in each group were inflated with the agarose and incubated at 37 °C in a salt bath (130 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM glucose, 10 mM sucrose, 10 mM HEPES 10, pH 7.4) until the agarose solidified. Agarose inflated lung was embedded in 5% low melting point agarose and 400 nm sections were prepared with a VT1000 S vibrating blade microtome (Leica Microsystems GmbH, Wetzlar, Germany). Constriction of lung tissue in the sections was stimulated by acetylcholine chloride (ACh) (Sangon Biotech Co., Ltd., Shanghai, China) at100 nmol/L, 300 nmol/L, 1 μmol/L, and 10 μmol/L and observed under a phase contrast microscope (Leica Microsystems GmbH, Wetzlar, Germany). After 100 nmol/L ACh was perfused into lung tissue at 10 mL/min for 30 minutes, lung tissue was then perfused with salt bath solution for 30 min, and then stimulation with the next concentration of ACh was performed. The area of bronchial lumen was measured using ImageJ analysis software (National Institutes of Health, MD, USA). Bronchoconstriction response was calculated using the following formula: $(S_0 - S_c)/ S_0*100\%$, with S_0 being the area of bronchial lumen in a resting state before stimulation and S_c being the area of bronchial lumen after ACh stimulation.

qRT-PCR Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure the mRNA expression of NGF, muscarinic receptors, β2-adrenergic and neurotrophic receptors. Total RNA was extracted from left lung in each group of the seven mice with Trizol reagent (Sigma-Aldrich Corporation, Shanghai, China) and reverse-transcribed with M-MLV Reverse Transcriptase (Promega (Beijing) Biotech Co., Ltd., Beijing, China). The primers (synthesized by Sangon Biotech Co., Ltd., Shanghai, China) used for amplification are listed in Table 1. qRT-PCR was carried out using a ViiA™ 7 qPCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the reaction system was composed of SYBR Premix Ex Taq 10 μl, ROX reference Dye II 0.4μl (TAKARA Biotechnology [Dalian] Co.,Ltd, Dlian, China), PCR forward primer 0.4 μl, PCR reverse primer 0.4 μl, DNA 2μl, ddH_2O 6.8 μl, and included 40 cycles of amplification. Expression of target genes ($2^{-\Delta\Delta Ct}$) was normalized against the gene for endogenous beta-actin, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{test group}} - (Ct_{\text{target gene}} - Ct_{\text{reference gene}})_{\text{control group.}}$

Table 1. Sequences of primers used to investigate the effects of nerve growth factor shRNA inhibition on asthma phenotypes in a mouse model of asthma

Symbol	GenBank number	Forward primers	Reverse primers
Chrm1	NM_007698.3	AGCAGCTCAGAGAGGTCACAGCCA	GGGCCTCTTGACTGTATTTGGGGA
Chrm2	NM_203491.1	CAAGACCCGGTGTCTCCCAGTCTA	TGACGATCCAACTAGTTCTACTGT
Chrm3	NM_033269.4	ACAGAAGCGGAAGCAGAAAACTTT	TTTGAAGGACAGAGGTAGAGCGGC
Adrb2	NM_007420.3	ATCTGAAGGAAGATTCCACGCCCA	AGAGGGTGAATGTGCCCATGATGA
Ngf	NM_013609.3	CCATGGGCCTGGAAGTCTAG	CAGACCCGGAACATCACTGTA
Actb	NM_007393	TTTGATGTCACGCACGATTT	AGCCATGTACGTAGCCATCC
TrkA	NM_001033124.1	CGTCATGGCTGCTTTTATGG	ACTGGCGAGAAGGAGACAG
p75 NTR	NM_033217.3	CAACCAGACCGTGTGTGAAC	GAGAACACGAGTCCTGAGCC

Abnormal expression of neuroreceptor mRNAs Chrm1, Chrm2, Chrm3 and Adrb2, and NGF, NGF receptors TrkA and p75 NTR mRNAs were analyzed by qRT-PCR. The sequences were primers for the genes.

Chrm1, Chrm2 and Chrm3: genes encoding muscarinic ACh receptors M1, M2 and M3, respectively; Adrb2: gene encoding β2-adrenergic receptor; Actb: gene encoding beta actin; TrkA: gene encoding neurotrophin receptor tropomyosin receptor kinase A; p75NTR: neurotrophin receptor p75; qRT-PCR, quantitative real-time polymerase chain reaction

Morphological Analysis

After photographing the gross pathology images, right lungs of the remaining five mice in each group were used for morphological analysis and the left lungs were used for immunohistochemistry evaluations. Tissues of five right lungs were fixed in 4% paraformaldehyde for 36 h, embedded in paraffin, and sectioned at 5 μm. Sections were air dried and then incubated at 45-50 ºC overnight. Paraffinized sections were dewaxed, then stained with hematoxalin and eosin, dehydrated and covered with neutral balsam.

Immunohistochemistry and Analysis

Tissues of the five left lungs were fixed and dehydrated in 15% and 30% sucrose solution both for 24 h at 4°C, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek USA, Inc., CA, USA) and frozen at -80°C. Frozen sections of 8 μm were prepared using a LEICA CM 1900 microtome (Leica Microsystems GmbH). Five discontinuous sections were selected from each sample and blocked with 10% goat serum for 30 min at 37°C. Sections were incubated with mouse anti-mouse α smooth muscle actin (α-SMA) (1:200) (Boster Biological Technology Co., Ltd.), rabbit anti-mouse mAChR M3 (1:200) (Boster Biological Technology Co., Ltd., China) and rabbit anti-mouse ADRB2 (1:200) (Boster Biological Technology Co., Ltd., China) at 4°C overnight and followed for one hour at 37°C. Sections were washed with PBS three times, incubated with rabbit anti-mouse or goat anti-rabbit IgG conjugated with CY3 (1:300) (Boster Biological Technology Co., Ltd., China). Sections that were incubated with secondary antibodies alone were used as controls. Nuclei were counterstained with 4', 6-Diamidino-2-phenylindole (DAPI, 1:1000; Sigma, St. Louis, MO, USA). After being mounted with glycerophosphate, the sections were observed under a fluorescent microscope (Nikon Corporation, Tokyo, Japan). IPP 6.0 (Media Cybernetics, CA, USA) was used to semi-quantitative analysis red fluorescence of α-SMA, mAChR M3 and ADRB2 expression levels. OD values of the five sections in each group were measure by IPP. Results were expressed as mean ± SD (standard deviation).

Statistical Analysis

Data are expressed as mean±standard deviation. To analyze the data statistically, we performed Student's t test (analysis of NGF levels and OD values) (IBM SPSS Statistics, version 21.0, IBM Corp., Armonk, New York, USA) and one-way analysis of variance (ANOVA) with Scheffe's post hoc multiple-comparison analysis (analysis of PIP, expression of cholinergic receptors mRNA and bronchoconstriction response). $p<0.05$ was considered as statistically significant and $p<0.001$ was considered to be very statistically significant.

RESULTS

PIP and BALF NGF Levels Are Lower in OVA-Challenged Mice Pretreated with NGF shRNA

PIP was measured two hours after the last challenge with OVA. PIP was 34.00±2.92, 45.20±6.30, 27.80±2.59 and 24.20±1.64 cmH_2O in control, sham shRNA, shRNA-1, and shRNA-2 groups, respectively (Figure 2A, B). As expected, the OVA sensitized and challenged mice of the control and sham shRNA groups showed high PIP. PIP was significantly higher in groups of in control and sham shRNA, control compared with shRNA-1, shRNA-2, $p=$ 0.045, 0.003, sham shRNA compared with shRNA-1, shRNA-2, p=0.11, 0.007. There was no significant difference in PIP between control and sham shRNA groups (Figure 2C).

Immediately following PIP measurements, lungs were lavaged and BALF was collected. NGF levels in BALF were determined by ELISA, three replicates and repeated three times. BALF NGF levels in control, sham shRNA, shRNA-1 and shRNA-2 groups were 457.16±45.32, 676.43±111.64, 261.56±25.81, and 129.12±15.96 pg/mL, respectively. Again, as expected, the OVA sensitized and challenged mice of the control and sham shRNA groups showed high BALF NGF levels. BALF NGF levels were significantly lower in shRNA-1 and shRNA-2 groups compared with control and sham shRNA (p<0.001). BALF NGF was also significantly lower in the control group compared with the sham shRNA group (p=0.001, Figure 2D).

Morphological Changes Associated with Asthma Are less Pronounced in OVA Challenged Mice Pretreated with NGF shRNA

Dissection and examination of lungs from mice in the different study groups revealed pulmonary consolidation in multiple lobes. The area of consolidation was smaller in lungs from shRNA-1 and shRNA-2 groups compared to lungs from control and sham shRNA groups (Figure 3 A-D).

HE staining showed ASM cell hyperplasia, loss of mucosal cell integrity, bronchial stenosis and inflammatory cell infiltration in lung tissue from control and sham shRNA mice (Figure 3E,F), with goblet cell hyperplasia in sham RNA tissue (Figure 3F). However, in tissue from shRNA-1 and shRNA-2, mice mucosal cells were integrated and no significant ASM cell hyperplasia, bronchial narrowing or inflammatory cell infiltration was observed (Figure 3G, H). Immuno-labeling of smooth muscle actin revealed ASM hypertrophy in lung tissue from control and sham

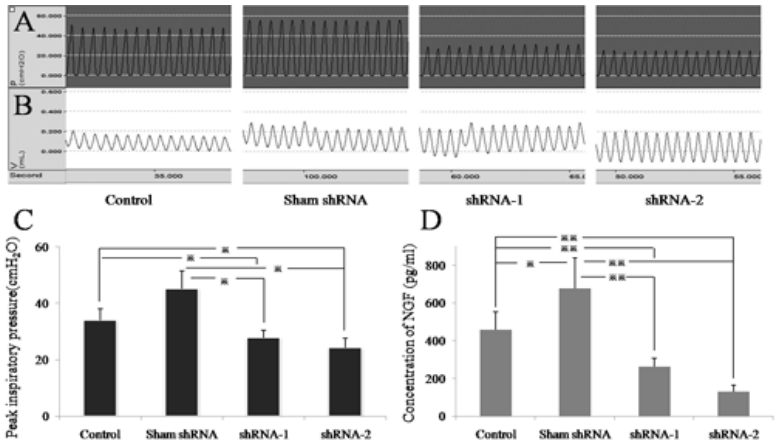

Figure 2. Effect of nerve growth factor (NGF) inhibition on peak inspiratory pressure (PIP) and NGF levels in bronchoalveolar lavage fluid (BALF)in the study of investigating the effects of nerve growth factor shRNA inhibition on asthma phenotypes. Pathophysiological characteristics were analyzed with PIP. PIP was measured two hours after the final OVA challenge. PIP (A) and tidal volume (B) are shown for individual representatives from control, sham shRNA, shRNA-1 and shRNA-2 groups. (C) Mean PIP in control, sham shRNA, shRNA-1 and shRNA-2 groups after statistical analysis. * $p < 0.05$. Immediately following PIP measurements, lungs were lavaged, BALF was collected, and NGF levels in BALF were detected by ELISA, 3 replicates and repeated 3 times. (D) Mean BALF NGF levels in control, sham shRNA, shRNA-1 and shRNA-2 groups. * $p < 0.05$, **$p < 0.001$.

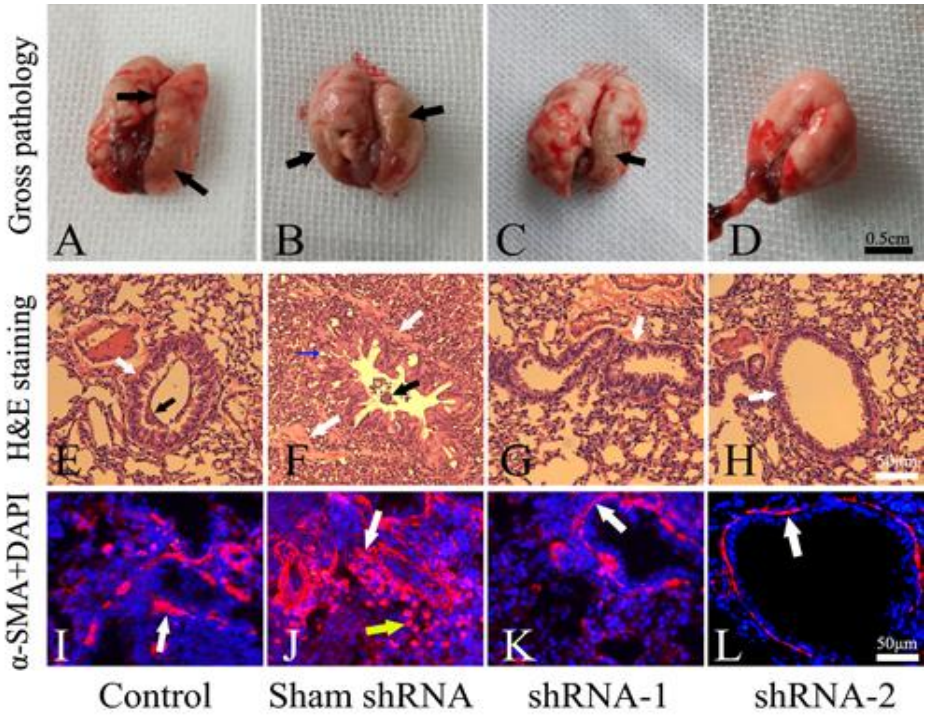

Figure 3. Effect of nerve growth factor (NGF) shRNAs on lung morphology. In order to investigate the effects of inhibiting NGF on pathological changes in the asthma mice, lung tissue from representative animals from control group (A, E, I), sham shRNA group (B, F, J), shRNA-1 group (C, G, K) and shRNA-2 group (D, H, L) was evaluated. (A-D) Gross pathology of whole lung. Black arrows indicate pulmonary consolidation. Scale bar = 0.5 cm. (E-H) H&E staining. Black arrows indicate bronchial stenosis and inflammatory cell infiltration; blue arrow indicates goblet cell hyperplasia; white arrows indicate airway smooth muscle (ASM) cell hyperplasia. Scale bar = 50 μm. (I-L) Immunolabeling of α-SMA. White arrows indicate ASM hyperplasia; the yellow arrow indicates diffuse focal hyperplasia.
Scale bar=50 μm. H&E, haematoxylin eosin; α-SMA, α-smooth muscle actin.

shRNA mice. ASM cell hypertrophy was apparent in tissue from both control and sham shRNA groups (Figure 3I, J), as well as hyperplasia, diffused focal hyperplasia and severe stenosis of bronchial lumen in the sham shRNA group (Figure 3J). In contrast, tissue from the shRNA-1 group showed only slight ASM cell hypertrophy (Figure 3K) and tissue from the shRNA-2 group had normal ASM cells and bronchial lumen (Figure 3L). OD values of a-SMA in red fluorescence in groups of control and sham shRNA (4.88±0.57, 5.94±0.97)*10^6 were significantly higher than shRNA-1 and shRNA-2 (3.14±0.56, 2.25±1.14)*10^6, (p<0.001).

ACh-Induced Bronchial Constriction Is Less Severe in OVA Challenged Mice Pretreated with NGF shRNA

Sections of bronchial lung tissue were treated with increasing concentrations of ACh, the area of bronchial lumen was measured and bronchoconstriction response was calculated relative to the resting state and repeated two times. At 300 nmol/L Ach, the constriction percent for control, sham shRNA, shRNA-1 and shRNA-2 groups was 37.63±3.65, 60.47±3.60, 37.65±5.93, and 27.29±3.05, respectively. Constriction in the sham shRNA group was significantly higher than that of the shRNA-1, shRNA-2, and control groups (p=0.009, 0.049 and 0.002). At the highest concentration of ACh (10 μmol/L), the constriction percent for control, sham shRNA, shRNA-1, and shRNA-2 groups was 74.66±6.52, 86.92±2.84, 59.26±5.16, and 43.31±5.29, respectively. At this concentration bronchial lumen constriction was significantly higher in both control and sham shRNA groups compared with shRNA-2 (p=0.02, 0.006) as well as in the sham shRNA group compared with shRNA-1 (p=0.02). There were no significant difference in constriction percent among the groups at 100 nmol/L and 1 μmol/L ACh (Figure 4).

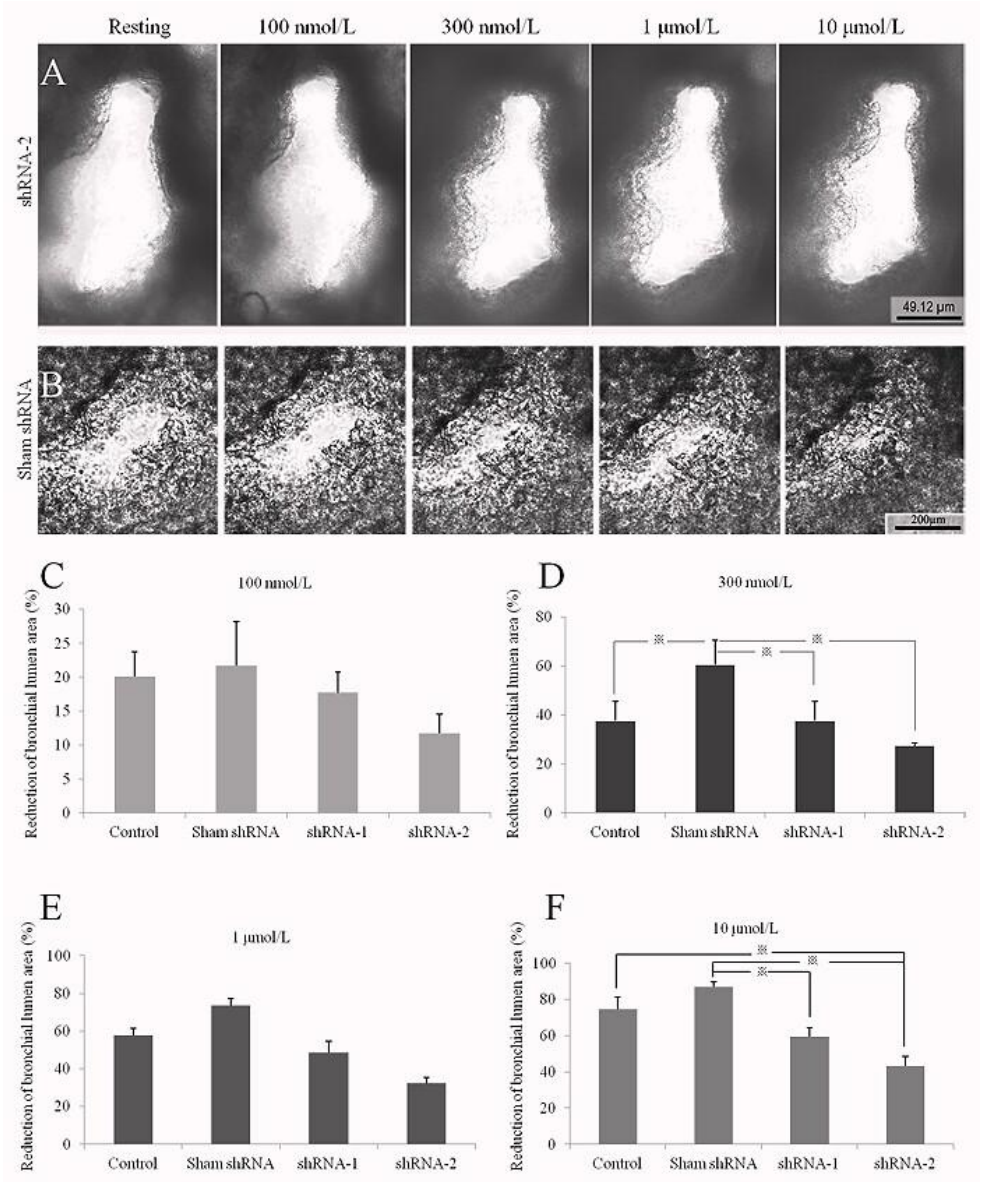

Figure 4. Bronchoconstriction in response to ACh. The effect of inhibiting nerve growth factor (NGF) on AHR in the asthma mice was analyzed by measurement of bronchoconstriction in response to ACh. Lung tissue was embedded in agarose and 400 nm sections were prepared. Sections were perfused with increasing concentrations of ACh to stimulate constriction, repeated 2 times. (A) Representative images of lung sections from mice in the shRNA-2 group (A) and from mice in the sham shRNA group (B) are shown. Lumen area was measured and percent reduction compared to resting state area was calculated for sections exposed to 100 nmol/L ACh (C), 300 nmol/L ACh (D), 1 μmol/L ACh (E) and 10 μmol/L ACh (F). * $p < 0.05$. Ach, acetylcholine; AHR, airway hyper-responsiveness.

Expression of Muscarinic, β2-Adrenergic and Neurotrophic Receptors is Affected by Pretreatment with NGF shRNA

Expression of mAChR M3 and ADRB2 in lung sections was investigated by immunofluorescent labeling and compared among the different study group.

mAChR M3 labeling was less intense in lung tissue from the shRNA-1 and shRNA-2 groups, with fewer epithelial cell layers being labeled, compared with tissue from control and sham shRNA groups (Figure 5). OD values of mAChR M3 in groups of control and sham shRNA (17.37±1.17, 20.81±1.70)*10^6 were

significantly higher than shRNA-1 and shRNA-2 (6.47±0.76, 6.05±0.47)*10^6, (p<0.001), and in sham shRNA was higher than control, (p=0.002). OD values of ADRB2 in groups of control, sham shRNA, shRNA-1, and shRNA-2 groups were (3.52±0.64, 4.44±0.83, 4.54±1.11 and 4.18±1.17)*10^6, respectively (Figure 6). ADRB2 was expressed nearly at the same levels and no significant differences among the groups.

Expression of mRNA from Chrm 1, 2 and 3 genes (the genes encoding mAChR M1, M2 and M3) in lungs of the studied animals, as well ADRB2 mRNA and neurotrophic receptors tropomyosin receptor kinase A (TrkA) and p75NTR mRNA, was measured by quantitative real-time PCR and compared among the different study groups (Figure 7), three replicates and repeated three times. Similar to M3 protein expression as observed by immunofluorescent labeling, Chrm3 mRNA expression was lower in shRNA1 and shRNA2 groups compared with the control and sham shRNA groups, with significant differences between each pair except for control vs shRNA1. Similar results were found for Chrm1 and Chrm2 expression. In contrast, ADRB2 expression, was higher in shRNA1 and shRNA2 groups compared with the control and sham shRNA groups. Expression of TrkA and p75NTR were lower in shRNA1 and shRNA2 groups compared with control and sham shRNA groups, but the only significant differences were in TrkA expression in shRNA1 and 2 groups compared to the sham shRNA group.

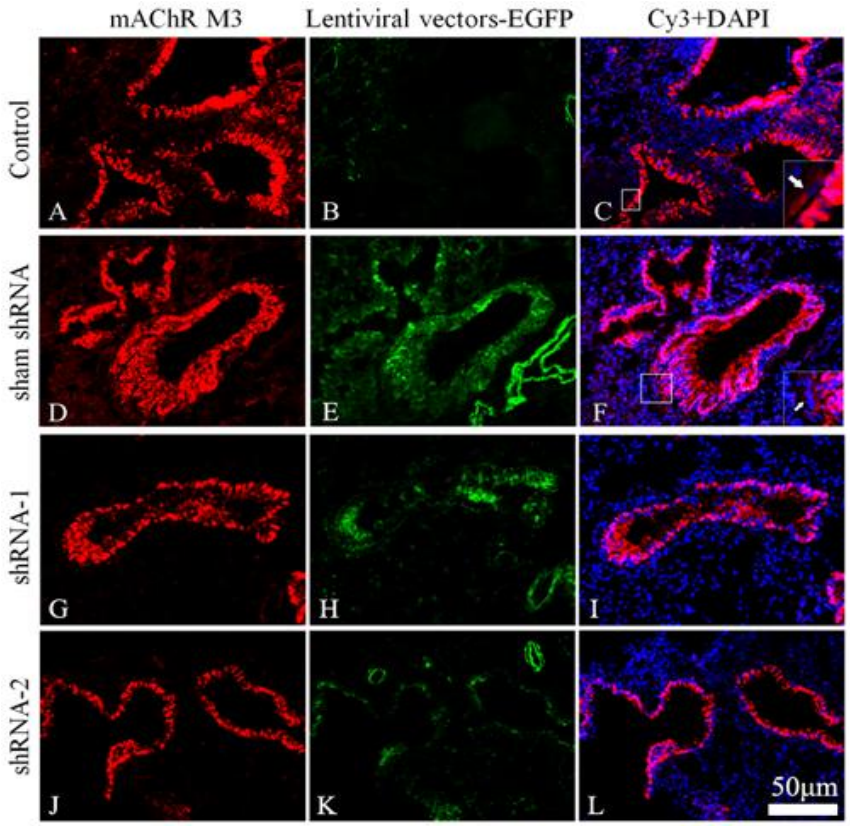

Figure 5. Targeting nerve growth factor (NGF) alleviating mAChR M3 overexpression in asthma mice. Expression of mAChR M3 was observed with immunohistochemistry. Lung sections were labeled with anti- mAChR M3 antibodies (A, D, G, J) and the nuclei were counterstained with DAPI (C, F, I, L). The presence of shRNA expressing lentivirus was indicated by EGFP (B, E, H, K). Representative sections from mice in the control group (A, B, C), sham shRNA group (D, E, F) shRNA-1 group (G, H, I) and shRNA-2 group (J, K, L) are shown. Insets in C and F show ASM cells. White arrows indicate mAChR M3 labeling. Scale bar = 50 μm.

NGF, nerve growth factor; mAChR M3, muscarinic acetylcholine receptor M3; EGFP, enhanced green fluorescent protein; Cy3, Cyanine-3; DAPI, 4',6-diamidino-2-phenylindole; ASM, airway smooth muscle.

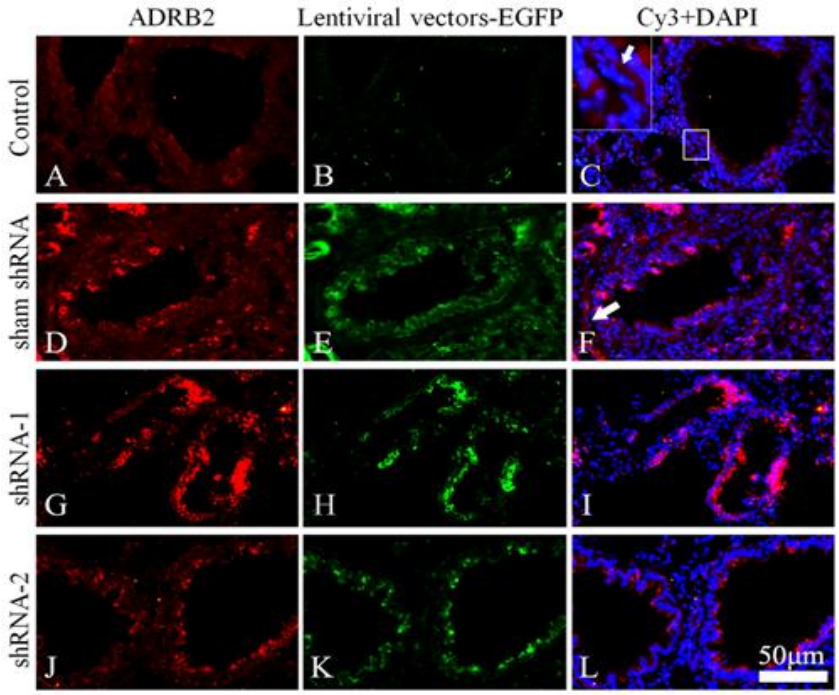

Figure 6. Expression of ADRB2 in asthma mice observed by immunohistochemistry. Lung sections were labeled with anti-ADRB2 antibodies (A, D, G, J) and the nuclei were counterstained with DAPI (C, F, I, L). Presence of shRNA expressing lentivirus was indicated by EGFP (B, E, H, K). Representative sections from mice in the control group (A, B, C), sham shRNA group (D, E, F) shRNA-1 group (G, H, I) and shRNA-2 group (J, K, L) are shown. Inset in C shows ASM cells. White arrows indicate smooth muscle cells. Scale bar = 50 μm.
ADRB2, beta-2 adrenergic receptor; EGFP, enhanced green fluorescent protein; Cy3, Cyanine-3; DAPI, 4',6-diamidino-2-phenylindole; ASM, airway smooth muscle.

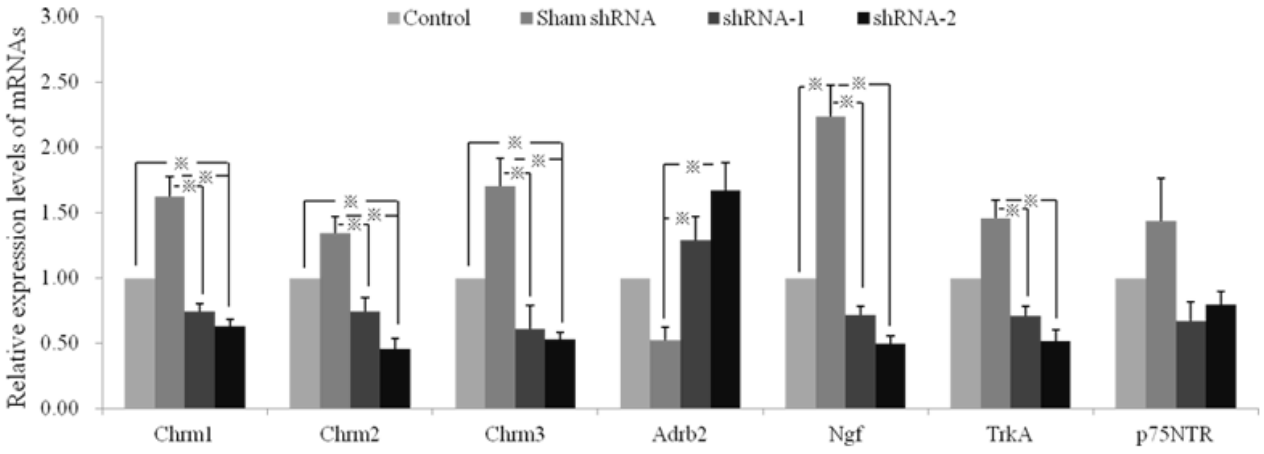

Figure 7. Expression of muscarinic, adrenergic and neurotrophic receptor mRNA in lung. To evaluate the effect of targeting NGF on mRNA expression of neuroreceptors, Chrm1, Chrm2, Chrm3 and Adrb2, NGF and NGF receptors, TrkA and p75 NTR mRNAs were measured by qRT-PCR, with normalization to beta actin expression, 3 replicates and repeated 3 times.
Chrm1, Chrm2 and Chrm3: genes encoding muscarinic ACh receptors M1, M2 and M3, respectively; Adrb2: β2-adrenergic receptor; TrkA: neurotrophin receptor tropomyosin receptor kinase A; p75NTR: p75 neurotrophin receptor. * p<0.05.

DISCUSSION

In this study, nerve growth factor shRNA alleviated airway hyper-responsiveness by preventing muscarinic acetylcholine receptor M3 overexpression and smooth muscle cell hyperplasia in a mouse asthma model. In the asthma model, ASM cell hyperplasia, loss of mucosal cell integrity, bronchial stenosis, inflammatory cell infiltration and goblet cell hyperplasia in lung tissue. PIP was increased in OVA sensitized and challenged mice, but this phenotype was attenuated by pretreatment with NGF shRNA. Airway resistance (Raw) is more commonly used to predict airflow limitation.[28] The use of inspiratory pressure instead of airway resistance (Raw) in this study may be a weakness. ACh-induced bronchoconstriction was less severe in OVA sensitized and challenged mice that had been pretreated with NGF shRNA's, particularly at higher doses of ACh. Brueggemann and colleagues reported ACh concentration-response curves were fitted by the Hill equation with a mean effective concentration 50 (EC50) of 248±49 nmol/L, and maximal constriction of 81±7% at 1 μmol/L.[29] But we did not find a significant difference in bronchoconstriction between OVA sensitized and challenged mice pretreated with sham shRNA or NGF shRNAs at 1 μmol/L ACh. Instead we found significant difference at 300 nmol/L and 10 μmol/L ACh, and maximal constriction of 86.92±2.84% in the sham shRNA group.

In addition, we found that mAChR M3 was strongly expressed on epithelial cells and ASM cells in the control and sham shRNA groups, and that expression of mRNA for mAChR M2 and M3 were significantly higher in control and sham shRNA groups compared with the shRNA-2 group. It was interesting that the sham shRNA group showed consistently more severe phenotypes than the control group. It is possible that lentiviral vectors might result in airway inflammation without carrying therapeutic components. Gao et al reported pulmonary interstitial hyperemia and edema, inflammatory cell infiltration, wide alveolar septa, and bronchial collapse and deformation was manifested in respiratory syncytial virus induced bronchiolitis.[30]

In this study, we focused on the effects of anti-NGF for asthma models, which prevented muscarinic receptors overexpression and improved asthma phenotypes. Many reports showed immune mechanism might involve the pathology of asthma.[31,32] An imbalanced ratio of T helper-17/regulatory T cells may contribute to the pathogenesis of paraquat-induced acute lung injury.[33] B2 lymphocyte numbers are increased in the allergic lung in a dynamic process, which could produce immunoglobulin E (Ig E).[5,6,34]

Similar with our results, Chen and co-workers reported that knockdown of NGF with siRNA significantly reduced airway resistance, NGF levels, proinflammatory cytokines, and eosinophil infiltration into airways in a mouse model of asthma[13]. But, the effects of NGF knock down on airway hyperresponsiveness, mAChR M3 expression and airway smooth muscle hyperplasia and hypertrophy were not investigated in that study. Recent data suggested that NGF is released by airway nerves in response to irritants or cigarette smoke, allergens, and infectious agents, contributing to irritability and enhanced contractility, and the presumed target of the released NGF is ASM, given that these cells express cognate TrkA receptors.[35] Inflammatory cytokines generally upregulate neurotrophin expression in airway smooth muscles, and in human bronchial smooth muscle cells, IL-1β-induced increases in NGF and BDNF, and cytokines on airway smooth muscle may be partly mediated by neurotrophins.[36] Immunohistochemistry semi-quantitative analysis of a-SMA in groups of control and sham shRNA were significantly higher than shRNA-1 and shRNA-2 in this study. Further research showed a potential synergistic effect of proinflammatory cytokines and growth factors such as neurotrophins on airway smooth muscle proliferation and survival has been suggested, since NTs and cytokines can share common signaling mechanisms by report.[24] In this study, we found obvious smooth muscle cell hyperplasia in the control and sham shRNA groups. The molecular mechanisms by which NGF regulates ASM cell proliferation such as specific to NGF signaling pathways and mAChR overexpression should be addressed in the future. Distribution of neuroreceptors under ultrastructure which was not investigated was a limitation of this study.

OVA sensitized and challenged mice pretreated with NGF shRNA had improved airway hyper-responsiveness, less airway inflammation and less ASM cell hyperplasia and hypertrophy compared with those not pretreated with NGF shRNA. Therefore, inhibiting NGF appears to lessen the severity of asthma phenotypes in this mouse model of asthma.

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