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miR-143 inhibits interleukin-13-induced inflammatory cytokine and mucus production in nasal epithelial cells from allergic rhinitis patients by targeting IL13Rα1

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ABSTRACT

Allergic rhinitis (AR) is a common chronic inflammatory condition of the nasal mucosal tissue. The interleukin-13 (IL-13) signaling pathway is of great importance in the pathogenesis of AR. However, how the signaling molecules in this pathway are regulated, particularly through microRNAs (miRNAs), remains unclear. In the present study, we investigated the regulatory role and mechanism of miRNA-143 (miR-143) in IL-13-induced inflammatory cytokine and mucus production in nasal epithelial cells (NECs) from AR patients. Our results showed that forced expression of miR-143 significantly decreased the mRNA and protein expression levels of granulocyte–macrophage colony-stimulating factor (GM-CSF), eotaxin and mucin 5AC (MUC5AC) in IL-13-stimulated NECs. Moreover, we confirmed that miR-143 directly targeted and significantly suppressed IL-13 receptor α1 chain (IL13Rα1) gene expression. This study thus suggests that miR-143 regulation of IL-13-induced inflammatory cytokine and mucus production in NECs from AR patients probably partly depends on inhibition of IL13Rα1. Therefore, the IL13Rα1 signaling pathway may be a potential target for the prevention and treatment of AR by miR-143.

1. Introduction

Allergic rhinitis (AR) is an immunoglobulin E (IgE)-mediated type I hypersensitivity disorder characterized by chronic inflammation of the nasal mucosal tissue. This condition considerably diminishes the quality of life of AR patients and increases their socioeconomic burden. The emergence of AR symptoms is closely linked to the infiltration and activation of mast cells, basophils, eosinophils, and CD4+ T helper type 2 (Th2) cells and to the local generation and release of various inflammatory mediators, which result in hypersecretion, hyperresponsiveness and remodeling of the nasal mucosa [1,2]. Additionally, as the first line of defense against inhaled antigens, nasal epithelial cells (NECs) play a pivotal role in the pathogenesis of AR. These cells can regulate the degree of the inflammatory reaction in the nasal mucosa by interacting with dendritic cells to induce antigen presentation, by releasing many chemokines to recruit effector cells, and by secreting various growth factors to promote airway remodeling [3–5]. Therefore, NECs act as critical initiators and mediators of nasal allergic inflammation and are considered as novel therapeutic targets for the development of anti-allergic medicines.

Interleukin-13 (IL-13), a typical Th2 cytokine, is the central mediator of IgE-mediated allergic diseases via activation of its receptor and the associated signal transducer and activator of transcription 6 (STAT6) [6]. Recent studies have indicated that IL-13 can lead to pathologic changes reminiscent of allergic airway diseases, such as eosinophil recruitment, mucus cell metaplasia, subepithelial fibrosis and smooth muscle hypertrophy [7–9]. Furthermore, IL-13 has been implicated in mucus hypersecretion and inflammatory mediator release by airway epithelial cells [10]. Hence, an increasing number of studies are beginning to focus on preventing and/or reducing allergic airway responses through targeting IL-13 and its signaling pathway. For example, Ramalingam et al. [11] found that IL-13 receptor α1 chain (IL13Rα1)-deficient mice failed to develop allergen-induced airway hyperresponsiveness and mucus hypersecretion, indicating that blockade of the IL-13 receptor may be effective in treating allergic disorders.

MicroRNAs (miRNAs) are a class of small, noncoding, single-stranded RNAs of approximately 22 nucleotides that regulate post-transcriptional protein-coding gene expression through binding to the 3′ untranslated region (UTR) of target mRNAs. Increasing evidence has demonstrated the regulatory functions of miRNAs in

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diverse immune responses and immunologic disorders [12–14]. Using a miRNA microarray assay, we previously showed that miRNA-143 (miR-143) was the most significantly downregulated miRNA in nasal mucosal tissues from AR patients compared with tissues from healthy control subjects, suggesting that miR-143 may be involved in the pathologic process of AR [15]. Further study is therefore required to elucidate how miR-143 regulates allergic inflammation of the nasal mucosa at the post-transcriptional level.

Based on these findings, we hypothesized that miR-143 may inhibit inflammatory cytokine and mucus production in NECs in AR. In the present study, we performed a series of in vitro experiments on the biological function and regulatory mechanism of miR-143 in AR and demonstrated that miR-143 plays an important role in repressing the secretion of inflammatory cytokines and mucus in IL-13-stimulated NECs from AR patients by targeting IL13Rα1. Thus, this receptor may be a potential target for the prevention and treatment of AR.

2. Materials and methods

2.1. Preparation of nasal mucosal specimens

Nasal mucosal samples were obtained from inferior turbinate sections from 23 patients with perennial AR (13 males and 10 females; mean age 33.9 years; range 21–61 years) and 18 patients with nonallergic rhinitis (NAR; 11 males and 7 females; mean age 31.4 years; range 23–57 years). All patients were diagnosed based on their medical history, nasal endoscopy, an allergen skin-prick test, and a specific IgE assay. Partial inferior turbinectomy was performed for nasal obstruction. No patient had received topical or systemic corticosteroid therapy for 4 weeks prior to study recruitment. The study protocol was approved by the Institutional Review Board of Huadong Hospital, Fudan University, and a written informed consent form was signed by each patient.

2.2. Isolation and cultures of primary NECs

As previously described [4], human primary NECs were isolated and established from the inferior turbinate tissues of 11 patients with AR. The cells were cultured in Bronchial Epithelial Growth Medium (BEGM, Lonza, Walkersville, MD, USA) under submerged conditions at 37 °C with 5% CO_2 in humidified air. When a 70–80% confluent monolayer appeared, the NECs were passaged. These cells (passage 2) were used for subsequent experiments.

2.3. Lentivirus production and infection

A recombinant lentiviral expression plasmid (pLVX-IRES-ZsGreen+miR-143) with green fluorescent protein (GFP) was constructed, and its identity was confirmed by DNA sequencing. To generate lentiviral particles, the recombinant expression plasmid was cotransfected with a packaging plasmid system (psPAX2 and pMD2G) into 293T cells, and the resultant viral particles were harvested 48 h after transfection. The NECs were then infected with the miR-143 lentiviral vector or with a negative-control vector without miR-143 (miR-NC) at a multiplicity of infection (MOI) of 60 and with polybrene (8 μg/ml) for 2–3 days. The infection efficiency was assessed in each experiment by observing the GFP expression, which usually ranged from 60% to 80%, under a fluorescence microscope.

2.4. IL-13 stimulation of NECs

After finishing the lentivirus infection experiment, the NECs were either unstimulated or stimulated with IL-13 (10 ng/ml or 50 ng/ml) for 24 h or 14 days in BEGM without hydrocortisone, and the medium was replaced twice per week. Cell pellets and supernatants were then collected for qRT-PCR and Western blot analysis.

2.5. Quantitative real-time reverse transcriptase-PCR (qRT-PCR)

Total cellular RNA, including miRNA, was extracted using Trizol reagent (Invitrogen) following the manufacturer's protocol. The RNA extracts were reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen). The synthesized cDNA was then quantified by SYBR Green assay on a CFX96 Touch™ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). U6 and β-actin were used as endogenous references for miRNAs and mRNAs, respectively. All primers were synthesized by Invitrogen (Shanghai, China), and their sequences are presented in Supplementary Table 1. Each assay was run in triplicate, and relative expression was calculated using the comparative cycle threshold method [16].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Granulocyte–macrophage colony-stimulating factor (GM-CSF), eotaxin, interleukin 1β (IL-1β) and mucin 5AC (MUC5AC) levels in the cell culture supernatants were measured using a GM-CSF Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), a CCL11/Eotaxin Quantikine ELISA Kit (R&D Systems) a Human Interleukin 1β ELISA Kit (BlueGene Biotech, Shanghai, China), and a Mucin-5 subtype AC ELISA Kit (BlueGene Biotech), respectively, in accordance with the manufacturers' instructions.

2.7. Prediction of miR-143 target genes

The target genes of miR-143 were predicted based on public bioinformatics databases, including TargetScan Human Release 6.2 (http://www.targetscan.org/), miRanda (http://www.microrna.org/) and PicTar (http://pictar.mdc-berlin.de/).

2.8. Luciferase reporter assay

The 3′-UTR of IL13Rα1, with wild-type (WT) or mutant (Mut) binding sites for miR-143, was amplified and cloned into the Xba I site of the pGL3 vector (Promega, Madison, WI, USA), which is immediately downstream of the luciferase gene, to generate the plasmid pGL3-WT-IL13Rα1-3′-UTR or pGL3-Mut-IL13Rα1-3′-UTR, respectively. The mutant 3′-UTR construct was prepared by inserting 5 mismatch mutations into the putative seed regions of IL13Rα1. For the luciferase reporter assay, NECs were cotransfected with the luciferase reporter vectors and miR-143 mimic or corresponding negative control (GenePharma, Shanghai, China). The pRL-TK plasmid (Promega) was used as a normalizing control. After 48 h of incubation, luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega).

2.9. Western blot analysis

NECs were harvested and centrifuged for 72 h after lentivirus infection. Western blot analysis was then conducted to assess IL13Rα1 and GAPDH expression, as we have previously described [17]. Anti-IL13Rα1 primary antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein bands were visualized using ECL detection reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA). Scanned images were quantified using

Quantity One software. The ratio of IL13Rα1/GAPDH is shown for semi-quantification.

2.10. Statistical analysis

Statistical analysis was done using SPSS software for Windows (version 16.0; SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean ± standard error of the mean (SEM). Differences were analyzed by a two-tailed *t* test or one-way analysis of variance (ANOVA), followed by post hoc Bonferroni tests. The predicted probability of being diagnosed with AR was used as a surrogate marker to construct receiver operating characteristic (ROC) curve. Pearson correlation analysis was used to determine the correlation between miR-143 and IL13Rα1 expression. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. miR-143 is strongly and specifically downregulated in nasal mucosal tissues from AR patients

To further analyze the expression levels of miR-143 between AR patients and NAR patients, we found that the expression of miR-143 was significantly decreased in nasal mucosal tissues from AR patients compared with tissues from NAR subjects (Fig. 1A). Furthermore, the ROC analysis revealed that miR-143 had moderate accuracy in discriminating AR from NAR (area under the curve [AUC] = 0.831; 95% confidence interval [CI], 0.714–0.948; sensitivity = 82.6%, specificity = 69.6%, Fig. 2B). These data suggest that miR-143 is a valuable diagnostic index for AR and may be related to the pathogenesis of AR.

3.2. The expression of miR-143 is upregulated in cultured NECs following lentivirus infection

In the current study, to elucidate whether miR-143 is involved in the biological functions of NECs in the development of AR, NECs from AR patients were infected with a lentiviral vector to force expression of miR-143. After approximately 48 h, the infection efficiency was 75%, as determined using an inverted fluorescence microscope (Fig. 1C). The expression levels of miR-143 in different groups were then further confirmed by qRT-PCR. We found that miR-143 expression was significantly upregulated in the NECs-miR-143 group compared with the NECs-miR-NC group (Fig. 1D). Taken together, these results suggest that the miR-143 lentiviral vector was successfully infected into NECs, allowing further exploration of the role of miR-143 in NECs exposed to IL-13.

3.3. miR-143 inhibits IL-13-induced GM-CSF, eotaxin and MUC5AC mRNA expression

To determine the effect of miR-143 on IL-13-induced GM-CSF, eotaxin, IL-1β and MUC5AC mRNA expression in the cultured primary NECs from AR patients, NECs from three different groups were stimulated with IL-13. As shown in Fig. 2A–C, the mRNA expression levels of GM-CSF, eotaxin and IL-1β were significantly increased in the NECs following IL-13 (50 ng/ml) stimulation for 24 h, whereas GM-CSF and eotaxin expression levels were significantly inhibited in miR-143-overexpressing NECs compared with the negative-control cells. In addition, MUC5AC mRNA expression was decreased following upregulation of miR-143 expression in the NECs exposed to IL-13 (10 ng/ml) for 14 days (Fig. 2D). Collectively, these data indicate that miR-143 overexpression can

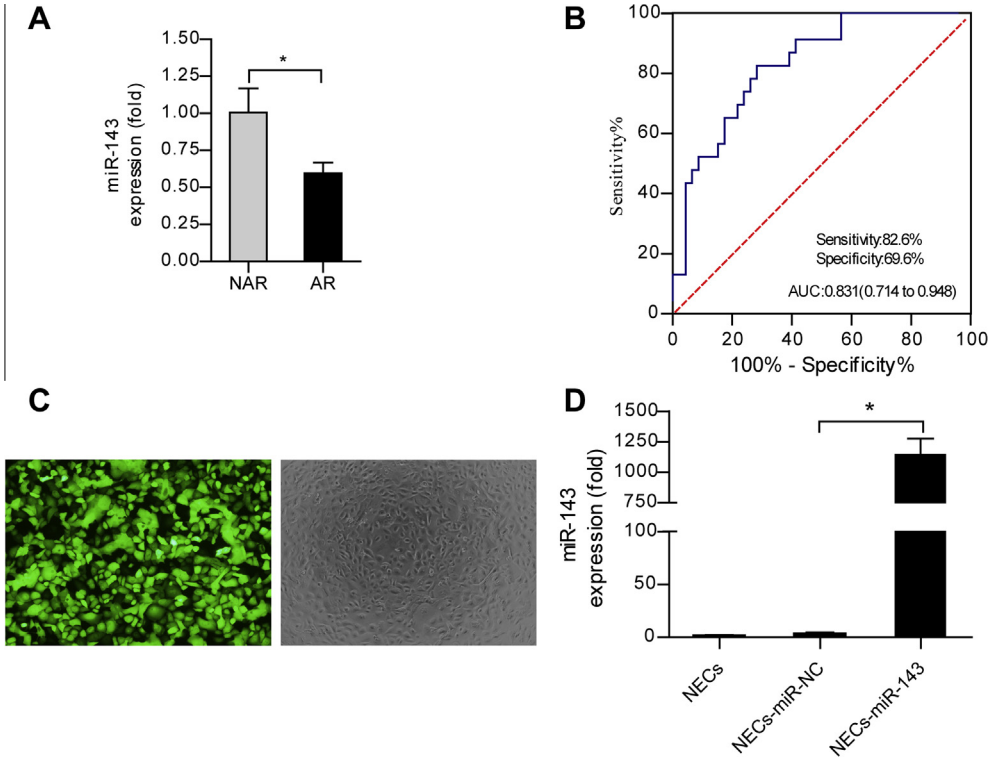

Fig. 1. Analysis of the expression level of of miR-143. (A) The miR-143 expression in nasal mucosal tissues from AR patients ($n = 23$) and NAR patients ($n = 18$). (B) ROC curve analysis used to discriminating AR group from NAR group. (C and D) Overexpression of miR-143 in primary NECs infected with the miR-143 lentiviral vector at a MOI of 60 for 48 h. (C) The ratio of GFP-positive cells in the NECs-miR-143 group was 75%, as determined using an inverted fluorescent microscope (original magnification 100×). (D) The expression level of miR-143 in different groups ($n = 11$) was determined by qRT-PCR. The data are presented as the mean ± SEM. $^*P < 0.05$.

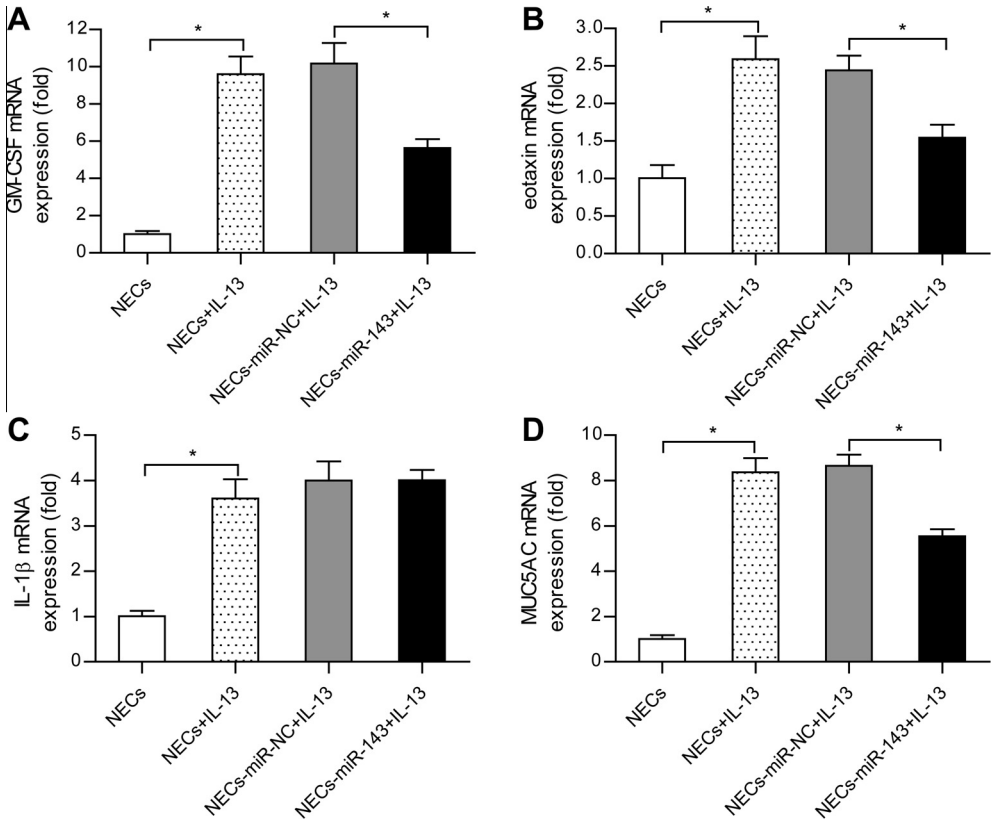

Fig. 2. Effects of miR-143 overexpression on IL-13-induced inflammatory cytokine and mucin mRNA expression in cultured NECs from AR patients ($n = 11$). The mRNA expression levels of GM-CSF (A), eotaxin (B), IL-1β (C), and MUC5AC (D) were measured by qRT-PCR. All data are presented as the mean ± SEM. $^{*}P < 0.05$.

effectively decrease IL-13-induced GM-CSF, eotaxin and MUC5AC mRNA stabilities in primary NECs from AR patients.

3.4. miR-143 suppresses the synthesis of inflammatory cytokines and MUC5AC protein in IL-13-stimulated NECs

To confirm whether miR-143 can regulate the synthesis of inflammatory cytokines and MUC5AC protein in IL-13-stimulated NECs from AR patients, we measured the concentrations of GM-CSF, eotaxin, IL-1β and MUC5AC in the culture supernatants of different groups using commercially available ELISA kits. We found that with forced expression of miR-143, the protein expression of GM-CSF and eotaxin was significantly inhibited in the NECs following IL-13 (50 ng/ml) stimulation for 24 h (Fig. 3A–C). Additionally, miR-143 overexpression significantly suppressed MUC5AC protein expression in the NECs exposed to IL-13 (10 ng/ml) for 14 days (Fig. 3D). These findings suggest that miR-143 can suppress the synthesis of pro-inflammatory cytokines and mucus in IL-13-stimulated NECs from AR patients.

3.5. miR-143 targets and inhibits IL13Rα1 gene expression

To determine whether IL13Rα1 is a direct target of miR-143 in NECs in AR, we first searched for the candidate target genes of miR-143 using three public miRNA databases, TargetScan, miRanda and PicTar. This search showed that the 3′-UTR of IL13Rα1 mRNA was a potential miR-143 binding site. Thus, the bioinformatics data suggested that IL13Rα1 may be a candidate target of miR-143 (Fig. 4A). Afterward, we analyzed the mRNA expression correlation between miR-143 and IL13Rα1. miR-143 showed a highly negative relationship with IL13Rα1 in nasal mucosal tissues from AR patients (Fig. 4B). We then conducted a dual-luciferase reporter assay to further validate whether miR-143 bound to the 3′-UTR of IL13Rα1 mRNA in NECs (Fig. 4C). The results showed that overexpression of miR-143 using miR-143 mimics led to remarkable suppression of IL13Rα1-3′-UTR reporter luciferase activity, whereas the mutant IL13Rα1-3'-UTR abrogated the suppressive effect of the miR-143 mimics (Fig. 4D). In addition to the luciferase activity analysis, IL13Rα1 mRNA and protein levels in NECs were assessed by qRT-PCR and Western blotting, respectively. We observed that miR-143 overexpression significantly inhibited endogenous IL13Rα1 mRNA and protein expression in NECs from AR patients (Fig. 4E and F), indicating that miR-143 directly regulates IL13Rα1 expression in NECs at the post-transcriptional level.

4. Discussion

Our study showed that miR-143 was strongly downregulated in nasal mucosal tissues from AR patients and had moderate diagnostic accuracy of AR. Importantly, we further demonstrated the effect of miR-143 on IL-13-induced inflammatory cytokine and mucus production in primary NECs from AR patients, and we suggest that the IL13Rα1 signaling pathway may be involved in this biological process. In particular, forced expression of miR-143 inhibited the mRNA and protein expression of GM-CSF, eotaxin and MUC5AC in IL-13-stimulated NECs. Moreover, miR-143 directly decreased IL13Rα1 mRNA stability and the IL13Rα1 protein expression level. To the best of our knowledge, this is the first report demonstrating the regulatory role of miRNAs in the inflammatory response of IL-13-stimulated NECs in AR.

Many studies have demonstrated that NECs are able to produce a variety of biologically critical cytokines in response to inflammatory stimulation or an allergen challenge [4,18]. For example, *Dermatophagoides pteronyssinus* 1 (Der p1) has been found to

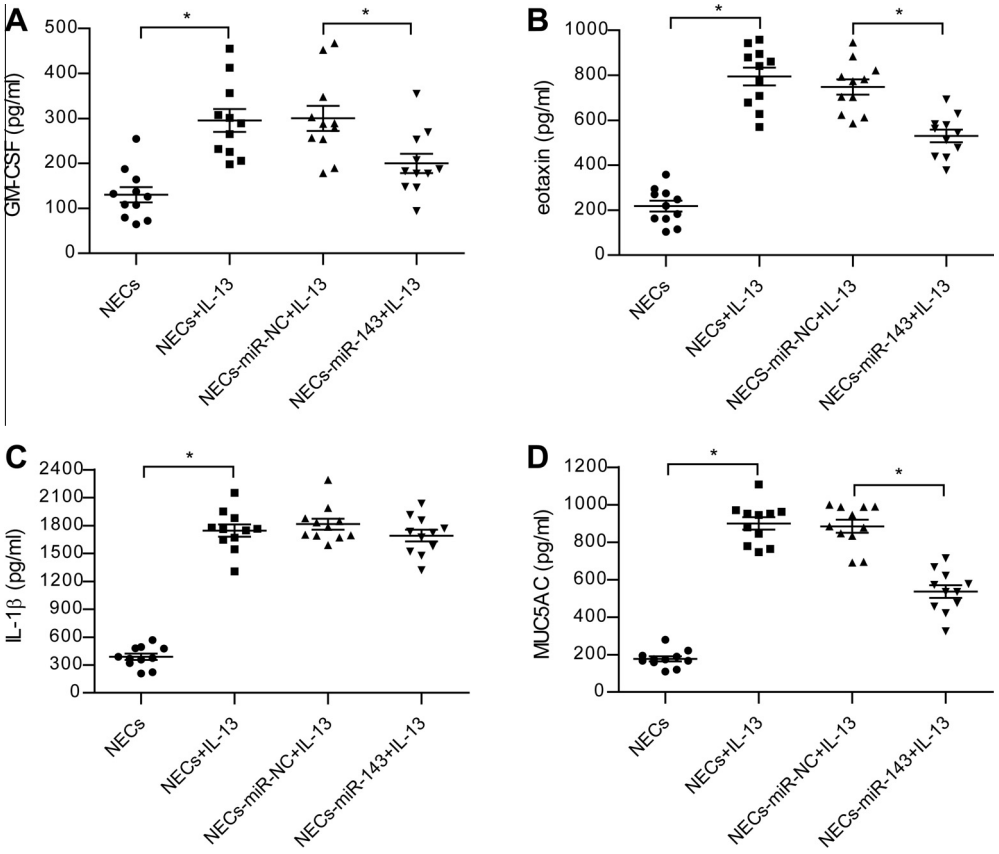

Fig. 3. Effects of miR-143 overexpression on the synthesis of inflammatory cytokines and MUC5AC protein in IL-13-stimulated NECs ($n = 11$). The protein levels of GM-CSF (A), eotaxin (B), IL-1β (C), and MUC5AC (D) were determined by ELISA. All data are presented as the mean ± SEM. $^*P < 0.05$.

promote the production of pro-inflammatory cytokines, such as IL-6, IL-8, IL-1β, GM-CSF, and TNF-α, in NECs from AR patients [4]. Our data also suggest that NECs obtained from AR patients can release GM-CSF, eotaxin, IL-1β and MUC5AC in vitro during IL-13 stimulation. Therefore, NECs might play an important role in the development of AR via secreting various inflammatory mediators and mucin, in addition to their traditional role as the first line of host defense.

GM-CSF and eotaxin are pro-inflammatory cytokines in allergic airway inflammation that are synthesized and released by airway epithelial cells, infiltrating leukocytes and fibroblasts in response to allergens and inflammatory mediators [19]. GM-CSF can also stimulate hematopoietic stem cells to differentiate into granulocytes and monocytes and can promote the survival and activation of airway inflammatory cells [20,21]. As a C–C chemotactic protein, eotaxin can recruit immune cells, such as eosinophils, Th2 cells, basophils, and mast cells, to inflammatory sites in allergic diseases by specifically binding to the chemokine receptor CCR3 [7,22]. Additionally, mucus hypersecretion (particularly MUC5AC expression) is a common feature of allergic airway disorders. Previous studies have provided evidence that airway epithelial cells secrete excessive mucins due to repeated exposure of the airway mucosa to allergens, pathogens, and/or pollutants [23]. Thus, inhibiting these pro-inflammatory cytokines and mucus production may at least partially contribute to blocking the pathologic process of allergic airway diseases.

IL-13 is considered to be a central mediator of the pathogenesis of allergic inflammation. Upon IL-13 binding to its receptor complex, composed of IL13Rα1 and IL4Rα, the STAT6 transcription factor is immediately phosphorylated and activated and then transferred into the nucleus, where it induces the expression of various genes. Target cells include B cells, macrophages, dendritic cells, eosinophils, basophils, airway epithelial cells, fibroblasts, and airway smooth muscle cells. Many studies from other groups and from our group have revealed that IL-3 specifically induces the production of various mediators (leukotrienes, GM-CSF, eotaxin, prostaglandin E2, and others) and mucus during airway inflammation [9,10]. Therefore, IL-13 production in the airway can promote IgE synthesis, eosinophil recruitment, mucus hyperproduction, subepithelial fibrosis, and airway hyperresponsiveness. Blocking the IL-13 signaling pathway may thus be a beneficial approach for controlling the pathologic process of allergic airway diseases.

Recent studies have confirmed that miR-143 acts as a tumor suppressor by targeting multiple anti-oncogenes and has a great ability to inhibit the proliferation, immigration and invasion of various tumor cells [24–27]. In addition, miR-143 has been found to be protective during vascular remodeling [28]. However, very little attention has been paid to the regulatory function of miR-143 in allergic inflammation. Our previous and current study showed that miR-143 was significantly and specifically downregulated in the nasal mucosal tissues in AR [15]. Subsequently, we investigated the role of miR-143 in the structural cells and immune cells associated with AR. We found that forced expression of miR-143 can decrease IL13Rα1 mRNA and protein expression in mast cells [17]. Moreover, the present study demonstrated that in cultured NECs from AR patients, miR-143 can suppress IL-13-induced inflammatory cytokine and mucus production as a consequence of miR-143 targeting IL13Rα1 expression. Thus, these studies provide valuable evidence that miR-143 plays a key role in the pathogenesis of AR.

In summary, the present study provides further insight into the role and molecular mechanism of a specific miRNA in the progression of AR and indicates that therapeutic upregulation of miR-143

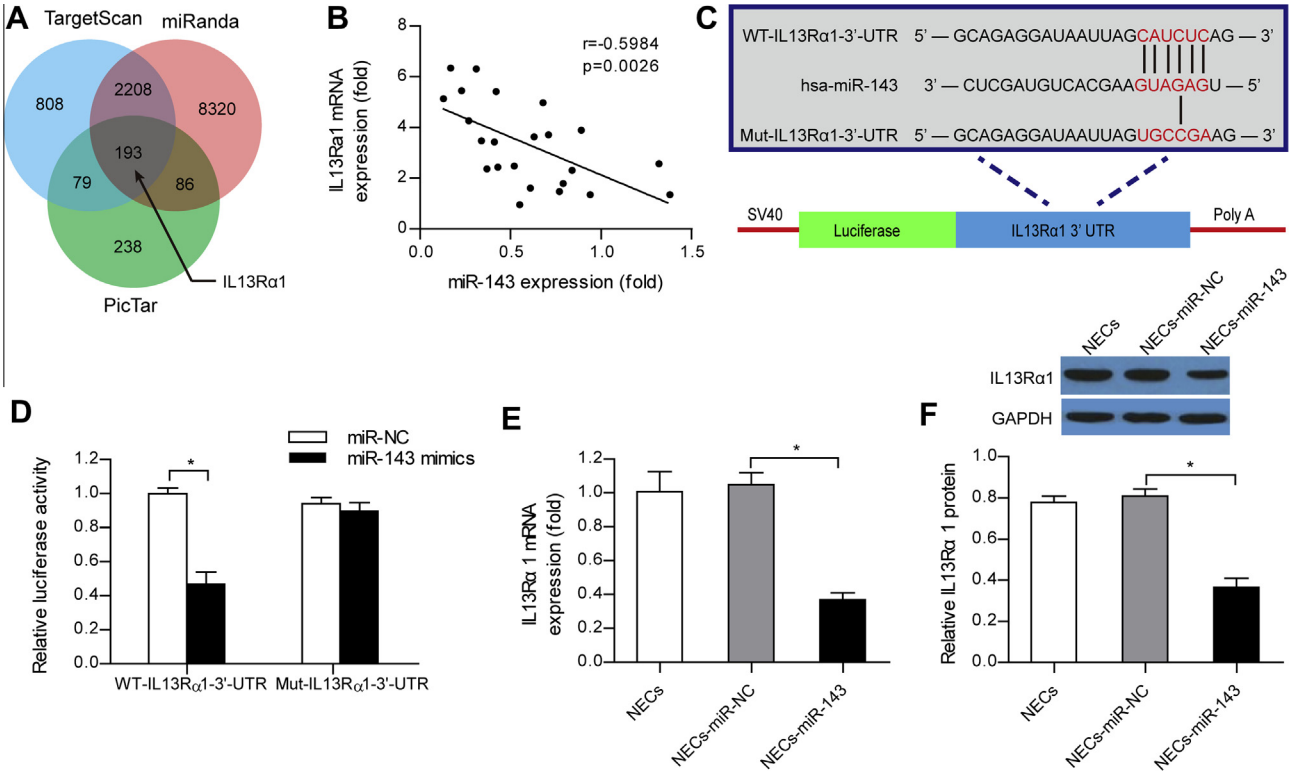

Fig. 4. miR-143 directly targets IL13Rα1 expression. (A) IL13Rα1 was predicted to be the target gene of miR-143 by three public miRNA databases. (B) Pearson's correlation analysis of the relative expression levels of miR-143 and IL13Rα1 in nasal mucosal tissues from AR patients ($n = 23$). (C) Schematic diagram of the design of the luciferase reporter system with WT-IL13Rα1-3′-UTR or Mut-IL13Rα1-3′-UTR. (D) A dual-luciferase reporter assay was performed in NECs ($n = 6$). (E and F) In NECs from AR patients ($n = 11$), miR-143 reduced the stability of IL13Rα1 mRNA and inhibited IL13Rα1 protein expression, as determined by qRT-PCR and Western blotting, respectively. Data are presented as the mean ± SEM. $^*P < 0.05$.

may be a feasible and effective approach for molecular targeted therapy for AR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.058.

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