MiR-375 is downregulated in epithelial cells after IL-13 stimulation and regulates an IL-13-induced epithelial transcriptome

TX Lu[1–3], E-J Lim[1], T Wen[1], AJ Plassard[4], SP Hogan[1], LJ Martin[5], BJ Aronow[4] and ME Rothenberg[1]

Interleukin 13 (IL-13)-induced epithelial gene and protein expression changes are central to the pathogenesis of multiple allergic diseases. Herein, using human esophageal squamous and bronchial columnar epithelial cells, we identified microRNAs (miRNAs) that were differentially regulated after IL-13 stimulation. Among the IL-13-regulated miRNAs, miR-375 showed a conserved pattern of downregulation. Furthermore, miR-375 was downregulated in the lung of IL-13 lung transgenic mice. We subsequently analyzed miR-375 levels in a human disease characterized by IL-13 overproduction—the allergic disorder eosinophilic esophagitis (EE)—and observed downregulation of miR-375 in EE patient samples compared with control patients. MiR-375 expression levels reflected disease activity, normalized with remission, and inversely correlated with the degree of allergic inflammation. Using a lentiviral strategy and whole-transcriptome analysis in epithelial cells, miR-375 overexpression was sufficient to markedly modify IL-13-associated immunoinflammatory pathways in epithelial cells *in vitro*, further substantiating interactions between miR-375 and IL-13. Taken together, our results support a key role of miRNAs, particularly miR-375, in regulating and fine-tuning IL-13-mediated responses.

INTRODUCTION

Interleukin 13 (IL-13) is an adaptive immune cytokine that is involved in mediating the effector functions of T helper type 2 (Th2) responses. The central role of IL-13 in allergic disorders has been demonstrated by the attenuation of experimental allergic diseases in animals with blockade and/or gene deletion of IL-13 and/or its receptor signaling components.[1–5] Furthermore, one of the critical functions of IL-13 is to modify epithelial gene expression at sites of inflammation. Notably, IL-13-induced gene expression changes in epithelial cells *in vitro* have been shown to significantly overlap with the gene expression changes seen in patients *in vivo*.[6–13] Recent early clinical studies with IL-13- neutralizing agents provide evidence that anti-IL-13 holds promise for the treatment of allergic disorders, especially in patient subgroups, based on various gene expression profiles (especially periostin).[14] Therefore, a better understanding of IL-13-mediated responses and the pathways that regulate IL-13-induced gene expression are likely to provide insight into therapeutic strategies, especially for allergic disorders characterized by IL-13 overproduction, such as eosinophilic esophagitis (EE).

MicroRNAs (miRNAs) are short single-stranded RNA molecules that regulate post-transcriptional gene silencing of target genes.[15] In animals, miRNAs base pair with the complementary regions in the 3′ untranslated regions of mRNA and induce translational repression and/or mRNA degradation depending on the degree of complementarity of the base pairing.[16] Recently, the miRNA let-7 has been shown to target IL-13 directly and miR-155 has been shown to target IL-13Rα1.[17–19] However, whether IL-13-induced miRNAs could regulate or fine-tune IL-13 mediated-responses has not been extensively explored. The epithelial cell is a particularly attractive model to investigate in this area as it has been shown to be a key target cell type for IL-13-mediated responses. For example, epithelial cells are

[1]Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. [2]Graduate Program of Molecular and Developmental Biology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. [3]Physician Scientist Training Program, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. [4]Division of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. [5]Division of Epidemiology and Biostatistics, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, Ohio, USA. Correspondence: ME Rothenberg (Rothenberg@cchmc.org)

Received 17 November 2011; accepted 8 February 2012; published online 28 March 2012. doi:10.1038/mi.2012.16

required for IL-13-induced airway hyperreactivity and mucus production,[20] and IL-13-induced epithelial cell gene expression changes have a critical role in the pathogenesis of EE.[9]

Here, we used miRNA array analysis to determine the differentially expressed miRNAs after IL-13 stimulation in two distinct human epithelial cell types: esophageal squamous cells and bronchial columnar cells. Among the IL-13-regulated miRNAs, miR-375 showed a conserved pattern of downregulation between these two epithelial cell types. Direct examination of human allergic tissue (esophageal biopsies from patients with EE) indicated that miR-375 was inversely related to the degree of allergic inflammation including esophageal eosinophil levels and gene expression levels of Th2 cytokine and mast cell–specific proteases. Functionally, miR-375 overexpression was sufficient to markedly modify IL-13-associated immunoinflammatory pathways in epithelial cells *in vitro*.

RESULTS

Expression profiling of miRNA in IL-13-stimulated epithelial cells

To identify miRNAs differentially expressed in epithelial cells in response to IL-13 stimulation, we profiled miRNA expression in IL-13-stimulated human bronchial and esophageal epithelial cells using miRNA microarrays. Comparing IL-13-treated and IL-13-untreated esophageal epithelial cells, we found six miRNAs that were differentially regulated in response to IL-13 (**Figure 1a**). These include four downregulated miRNAs—miR-375, miR-212, miR-181a-2*, and miR-145—and two upregulated miRNAs—miR-223 and miR-137. A similar analysis of IL-13-treated human bronchial epithelial cells found four downregulated miRNAs—miR-565, miR-7, miR-335, and miR-375—and two upregulated miRNAs—miR-146b and miR-203 (**Figure 1b**). MiR-375 was the only miRNA that was differentially regulated in both epithelial cell types after IL-13 treatment. We subsequently focused on miR-375 and validated its downregulation in both the human esophageal and human bronchial epithelial cells after IL-13 stimulation by quantitative reverse transcriptase-PCR (qPCR) (**Figure 2a,b**). Kinetic analysis of miR-375 expression in human esophageal epithelial cells and normal human primary bronchial epithelial cells indicated that miR-375 was downregulated after 24 and 48 h of IL-13 stimulation (**Figure 2c,d**).

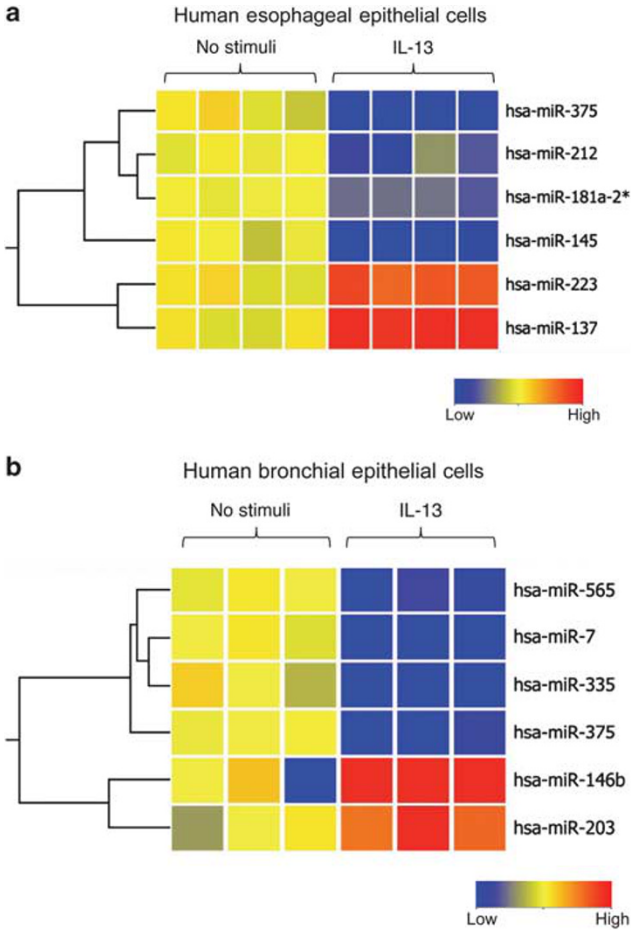

Figure 1 MicroRNA (miRNA) expression profile in human esophageal epithelial cells and human bronchial epithelial cells after 24 h of interleukin 13 (IL-13) stimulation. (**a**) Heat map of four downregulated and two upregulated miRNAs in IL-13-stimulated human esophageal epithelial cells compared with controls. (**b**) Heat map of four downregulated and two upregulated miRNAs in IL-13-stimulated human bronchial epithelial cells compared with controls. Red: upregulated in IL-13-stimulated cells compared with controls; blue: downregulated in IL-13-stimulated cells compared with controls.

Expression of miR-375 in IL-13 lung transgenic mice and in EE patients

To investigate the long-term effect of IL-13 exposure on miR-375 expression, we utilized an IL-13 lung transgenic mouse model where experimental asthma was induced by 4 weeks of doxycycline-induced IL-13 transgene expression. Compared with control mice that received no doxycycline, doxycycline-treated IL-13 transgenic mice had significant downregulation of miR-375 in the lungs (**Figure 3**). We also investigated whether miR-375 was downregulated in EE patients as EE has been reported to be a Th2-associated disease with IL-13 having a major role in its pathogenesis.[21,22] Compared with normal healthy controls, EE patients had significant downregulation of miR-375 in esophageal tissue. This downregulation was not evident in patients with chronic (noneosinophilic) esophagitis, which has a distinct etiology and pathogenesis from EE (**Figure 4a**).[23] We next investigated whether the expression levels of miR-375 were normalized in EE patients in remission. EE patients who responded to either fluticasone propionate therapy or diet modification had miR-375 levels comparable to normal healthy controls, whereas patients who did not respond to therapy continued to have repressed miR-375 levels (**Figure 4a**). As miR-375 downregulation fluctuated with disease activity, we further investigated the correlation of miR-375 expression with other markers of EE disease activity, including the high level of eosinophil infiltration observed in the esophageal biopsies of EE patients[23,24] and the expression of previously identified EE signature genes. Esophageal miR-375 expression exhibited a significant inverse correlation with the level of eosinophil infiltration in the esophageal biopsies as well as esophageal expression of genes involved in inflammation

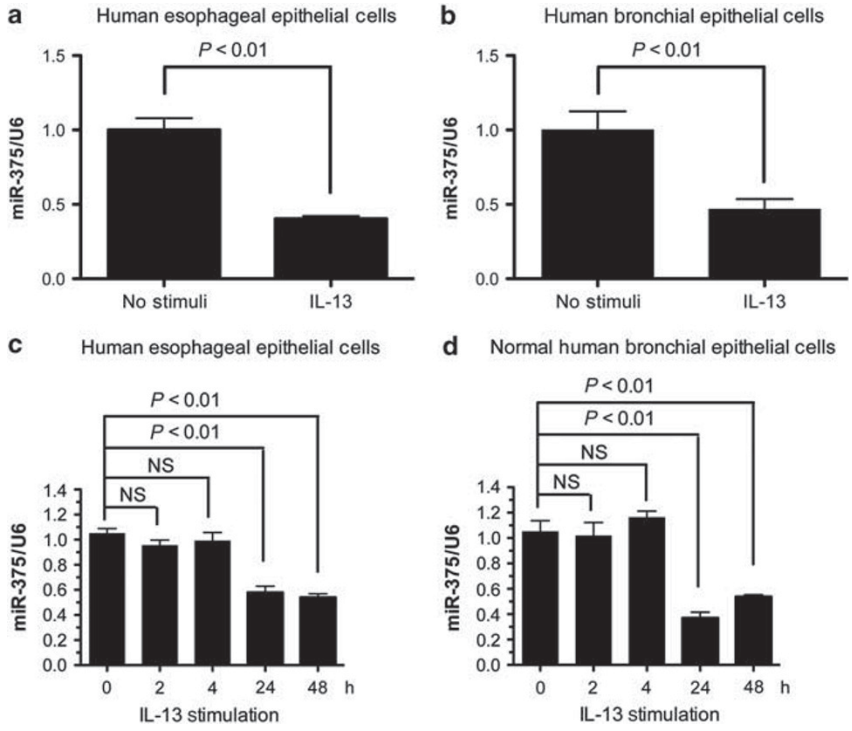

Figure 2 Quantitative reverse transcriptase-PCR (RT-PCR) verification of miR-375 expression in interleukin 13 (IL-13)-stimulated human esophageal epithelial cells and human bronchial epithelial cells. Expression of miR-375 was determined in (**a**) IL-13-stimulated human esophageal epithelial cells compared with controls and (**b**) IL-13-stimulated human bronchial epithelial cells compared with controls. (**c**) Kinetic analysis of miR-375 expression in IL-13-stimulated primary human esophageal epithelial cells. (**d**) Kinetic analysis of miR-375 expression in IL-13-stimulated normal human primary bronchial epithelial cells. The relative expression levels were normalized to U6 small nuclear RNA. *N* = 4 per group; data are represented as mean±s.e.m. NS, not significant.

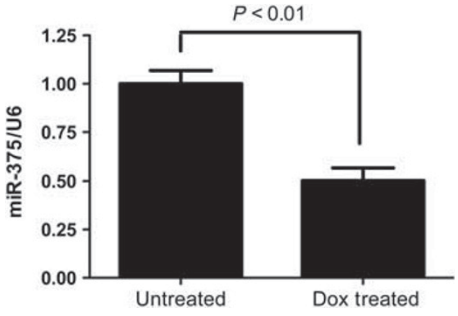

Figure 3 Expression of miR-375 in doxycycline (Dox)-induced interleukin 13 (IL-13) lung transgenic experimental asthma model. Relative expression level of miR-375 determined by qPCR normalized to U6. *N* = 6 mice per group; Data are represented as mean±s.e.m. qPCR, quantitative reverse transcriptase-PCR.

including CCL26 (eotaxin-3),[23] remodeling including POSTN (periostin),[25] Th2 cytokines including IL-5 and IL-13,[26] and cell-specific markers for eosinophils (CLC),[27] mast cells (CPA3 and TPSAB1),[28] and epithelial cells (FLG) (**Figure 4b,c**).[29]

MiR-375 is predominately expressed in the esophageal and bronchial epithelial cells

To determine the levels of miR-375 expression in different cell types, we performed qPCR analysis of miR-375 levels in primary esophageal epithelial cells, normal human primary bronchial epithelial cells, normal smooth muscle cells, neutrophils, eosinophils, monocytes, fibroblasts, and T cells. The esophageal epithelial cells had the highest miR-375 expression (**Figure 5**). We subsequently focused on the role of miR-375 in the esophageal epithelial cells.

MiR-375 regulates IL-13-regulated signature genes

Using a lentiviral vector, we stably overexpressed miR-375 in the esophageal epithelial cell line TE-7. We then treated the control-transduced and miR-375-transduced TE-7 cells with IL-13 to determine the effect of miR-375 on IL-13-induced esophageal epithelial transcriptome. MiR-375 was able to repress a large set of genes at baseline, consistent with the function of miRNAs as repressors of gene expression (**Figure 6a**). We also noted a smaller set of genes that were induced at baseline, most likely through miR-375-mediated repression of transcriptional repressors (**Figure 6a**). Interestingly, miR-375 was able to both potentiate and antagonize a subset of IL-13-mediated gene signatures, indicating a complex interaction between miR-375 and effects of IL-13 (**Figure 6a**). Functional analysis indicated that the pathways affected by miR-375 under IL-13-stimulated conditions were enriched for processes involved in extracellular matrix organization, cellular junctions, and inflammation (**Figure 6b**). The differentially regulated genes are listed in **Supplementary Table S1** online. An analysis of all miR-375-regulated genes indicated that inflammatory diseases and immunological diseases are the two most significantly overrepresented

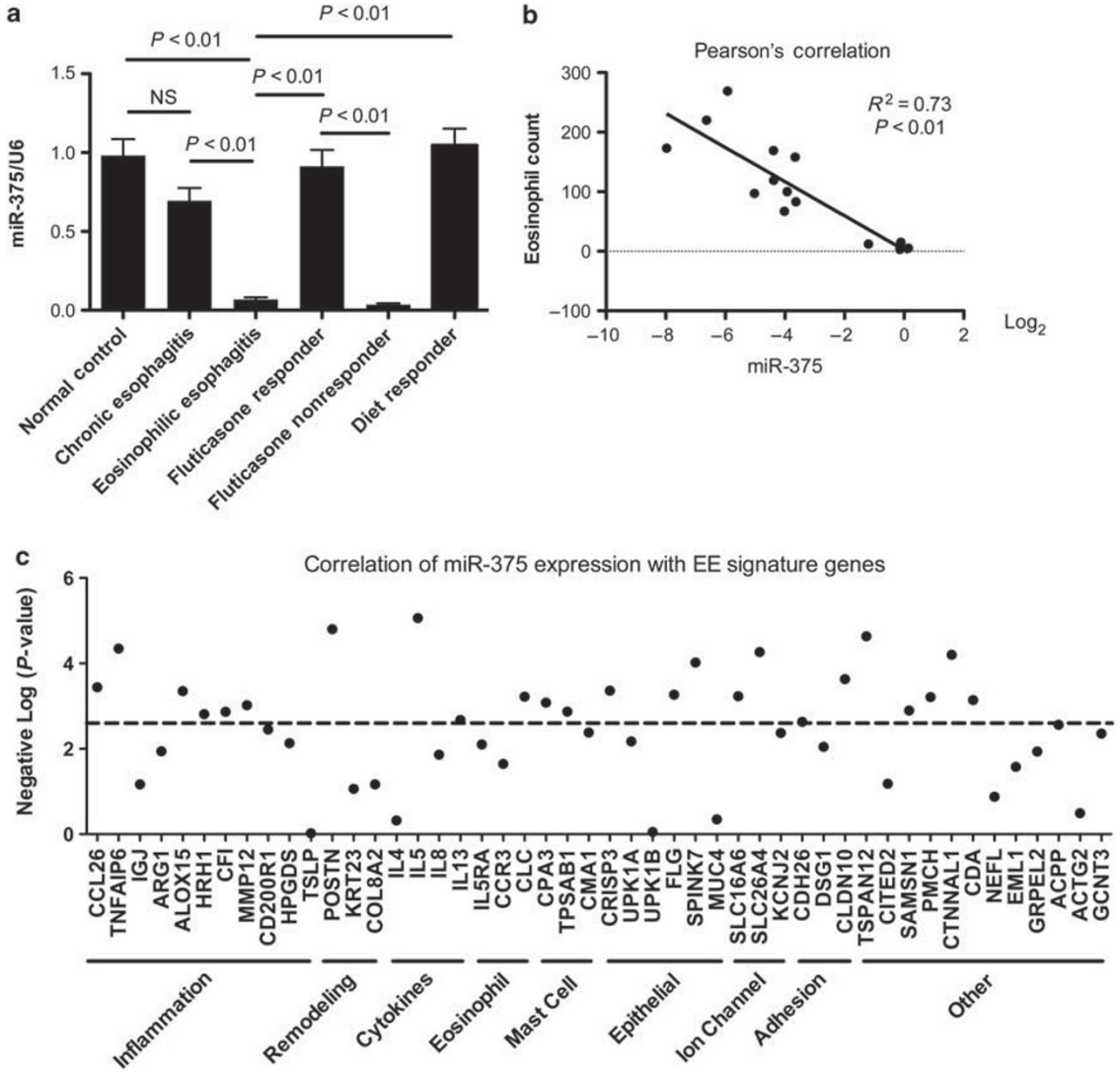

Figure 4 Expression of miR-375 in esophageal biopsies from eosinophilic esophagitis (EE) patients and its correlation with esophageal eosinophil counts and EE signature genes. (**a**) Expression of miR-375 in normal control, EE patients, chronic esophagitis patients, EE patients responsive to glucocorticoid therapy (fluticasone proprionate), EE patients unresponsive to glucocorticoid therapy, and EE patients responsive to diet modification. Expression levels were determined by qPCR normalized to U6 small nuclear RNA. *N*=8–15 patients per group; data are represented as mean±s.e.m. (**b**) Correlation between miR-375 expression and esophageal eosinophil counts. (**c**) Correlation between miR-375 expression and EE signature genes. The significance of the correlation was plotted as the negative log of *P*-value for each gene. The dashed line represents significance level after false discovery rate correction. qPCR, quantitative reverse transcriptase-PCR.

disease states (**Supplementary Figure S1** online). The miR-375 regulated genes in each of these disease processes are listed in **Supplementary Table S2** online.

DISCUSSION

Herein, we have identified miRNA changes induced by IL-13 stimulation in human bronchial and esophageal epithelial cells. In particular, we found that miR-375 was the only miRNA that was downregulated in both epithelial cell types after IL-13 stimulation. An analysis of different human cell types involved in allergic inflammation identified the highest expression of miR-375 in epithelial cells, strengthening the relevance of our findings. In addition, we found that miR-375 was inversely correlated with the level of esophageal eosinophils and expression of the mast cell–specific genes *CPA3* and *TPSAB1*. The downregulation of miR-375 was specific to EE patients as the chronic esophagitis patients have miR-375 expression levels comparable to normal controls. Disease remission with either fluticasone therapy or diet modification was associated with normalization of miR-375 levels, likely due to reduced IL-13, whereas patients who did not respond to fluticasone therapy continued to have repressed miR-375 levels. Furthermore, modulation of miR-375 levels was sufficient to regulate IL-13-mediated gene expression, particularly with pathways involved in immunoinflammatory processes. Indeed, levels of miR-375 markedly inversely correlated with a large set of

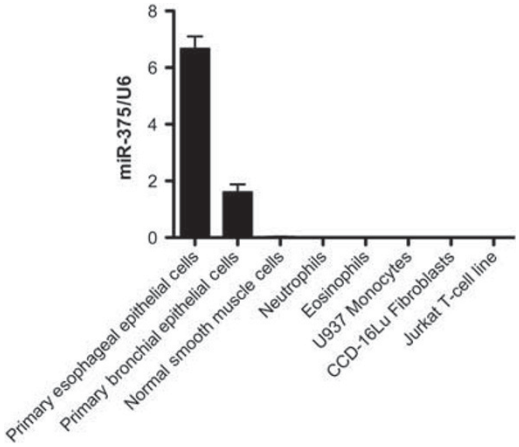

Figure 5 Expression level of miR-375 in different cell types. Relative expression level of miR-375 in different cell types determined by qPCR normalized to U6. *N*=3 per group; data are represented as mean±s.e.m. qPCR, quantitative reverse transcriptase-PCR.

immunoinflammatory genes (including IL-13) in the esophagus of patients with EE. Collectively, these findings provide multiple lines of evidence for a key role of miR-375 in epithelial cell–driven allergic inflammation.

Using a genome-wide transcriptome-based approach, we demonstrated that miR-375 could potentiate and repress IL-13-mediated effects, underlining the complex interaction between cytokine and miRNA-mediated gene regulation. The inflammatory diseases and immunological diseases are the two most significantly overrepresented disease states regulated by miR-375. These include allergy-associated genes such as *MMP12* and *MUC4* (**Supplementary Table S2** online).[30–33] Our results differed from those recently reported by Biton *et al.*[34] in that their results demonstrated upregulation of miR-375 after 2 h of IL-13 stimulation, whereas we found that miR-375 expression was unchanged after 2 h. However, they did demonstrate that miR-375 levels were at or below baseline after 16 h of IL-13 stimulation. This latter finding corresponds with our data that miR-375 is downregulated after 24 and 48 h of IL-13 stimulation. The disparity between our results at the 2-h time point could potentially be because of the different cell types used in the studies; they used the HT-29 human colon adenocarcinoma cell line, and we used human esophageal squamous cells and bronchial columnar cells. Notably, although miR-375 downregulation has been reported in patient samples from multiple Th2-associated diseases (e.g., atopic dermatitis and ulcerative colitis[35,36]) and hyperproliferative diseases (e.g., esophageal squamous carcinoma[37]), an upregulation of miR-375 in a Th2-associated disease in humans has yet to be reported. Thus, the long-term effect of IL-13 is likely to downregulate miR-375 expression.

MiR-375 has been previously shown to enhance goblet cell differentiation by repressing *KLF5* expression.[34] It has also been shown to attenuate cell proliferation by targeting *IGF1R*, *PDK1*, and *YWHAQ*.[37,38] However, neither of these pathways was affected in the EE patients or in our analysis of miR-375-regulated genes in the esophageal epithelial cells.[23] This suggests that the activity of miR-375 may be dependent on the cellular context, as indicated by previous reports.[38,39] MiR-375 has been previously reported to regulate *TSLP* expression in HT-29 human colonic adenocarcinoma cell line. *TSLP* and miR-375 were concomitantly induced by IL-13 in HT-29 cells and knockdown of miR-375 inhibited *TSLP* production. In addition, overexpression of miR-375 induced *TSLP* expression in HT-29 cells.[34] As *TSLP* has been reported to have an important role in EE pathogenesis,[40,41] we analyzed whether miR-375 was able to regulate *TSLP* expression in esophageal epithelial cells. We did not find any effect of miR-375 on *TSLP* production (**Supplementary Figure S2** online) and there was no correlation between miR-375 and *TSLP* in the esophageal samples (**Figure 4c**). Our control-transduced cells and pre-miR-375-transduced cells expressed *TSLP* at similar levels without stimulation and have similar levels of induction after polyinosinic: polycytidylic acid stimulation. This disparity could be because of the different cell types used in our studies and/or different mechanisms in *TSLP* induction in these cells, as IL-13 induced *TSLP* expression in HT-29 cells but not in esophageal epithelial cells according to previous reports.[9,34]

Although IL-13 downregulates miR-375 and miR-375 and could modulate IL-13-regulated gene expression, whether the overexpression of miR-375 could correct the allergic phenotype in asthma and EE remains to be investigated. This will likely be resolved in future studies utilizing miR-375 lung and/or esophageal epithelial–specific transgenic mice. In addition to miR-375, we identified 10 other miRNAs that were differentially regulated in either the human esophageal epithelial cells or the human bronchial epithelial cells. These likely reflect cell type–specific effects of IL-13 stimulation. Notably, previous reports indicated that the miRNAs miR-203 and miR-223 were differentially regulated in Th2-associated diseases.[35,36]

In summary, we report miRNA signatures of human esophageal and bronchial epithelial cells after IL-13 stimulation. We demonstrated that one epithelial-derived miRNA, miR-375, was downregulated in both epithelial cells types after IL-13 stimulation and was sufficient to regulate an IL-13-induced epithelial transcriptome. MiR-375 expression levels reflected disease activity, normalized with remission, and inversely correlated with the degree of allergic inflammation. It is notable that miR-375 was strongly associated with parameters germane to allergic responses including eosinophil levels, gene expression levels of the Th2 cytokines IL-5 and IL-13, the mast cell–specific enzymes CPA3 and TPSAB1, and *POSTN* (the gene that encodes periostin). It is notable that periostin has been demonstrated to have a key role in IL-13-associated remodeling responses,[42] and its level predicts responsiveness to anti-IL-13 therapy in humans,[14] highlighting the potential importance of our findings, as we have found that miR-375 strongly correlates with human *POSTN* levels *in vivo*. Using a lentiviral strategy and whole-transcriptome analysis in epithelial cells, miR-375 overexpression was sufficient to markedly modify IL-13-associated immunoinflammatory pathways in epithelial cells *in vitro*, further substantiating interactions between miR-375 and IL-13. Taken together, our results support a key role of miRNAs in regulating

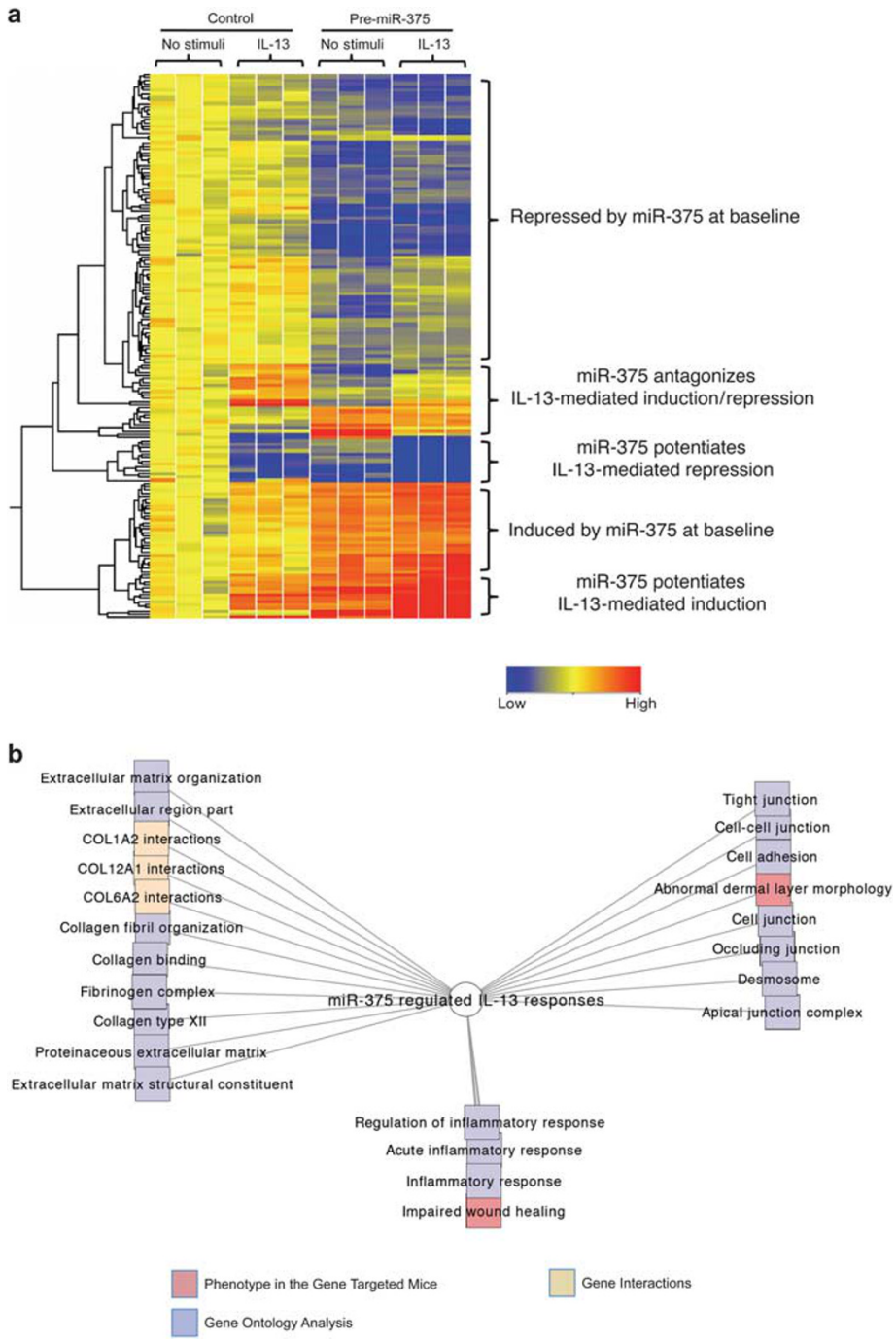

Figure 6 Genes differentially regulated by miR-375 in esophageal epithelial cells before and after interleukin 13 (IL-13) stimulation. (**a**) Heat map showing genes differentially expressed in esophageal epithelial cell line transduced with either control vector or pre-miR-375 expression vector before and after IL-13 stimulation. Red: upregulated compared with control-transduced unstimulated cells; blue: downregulated compared with control-transduced unstimulated cells. (**b**) Functional enrichment analysis of pathways affected by miR-375 under IL-13-stimulated conditions. The networks are shown as Cytoscape graph networks generated from ToppCluster network analysis.

and fine-tuning IL-13-mediated responses; we propose miR-375 is a key downstream mediator of IL-13-induced responses.

METHODS

Human esophageal tissues. Patients were selected without regard to age, race, or sex. Normal patients presented to the clinic with symptoms consistent with gastroesophageal reflux disease or EE but the endoscopic and histologic findings were normal. The active EE patients have a clinical diagnosis of EE and eosinophil counts of ≥24 per × 400 high-power field in the esophageal biopsies. The active chronic esophagitis patients have eosinophil counts of 1–15 per × 400 high-power field in the esophageal biopsies. Patients with systemic or swallowed topical glucocorticoid use were excluded from the selection

of active EE or active chronic esophagitis patients. The EE remission patients responding to steroid treatment have a clinical history of EE, treatment with swallowed topical glucocorticoids, and responsiveness as indicated by an eosinophil count of ≤1 per × 400 high-power field and normalization of histological features of the disease. The EE remission patients responding to diet treatment have a clinical history of EE, treatment with diet modification, and responsiveness as described above. The EE patients not responding to glucocorticoid treatment have a clinical history of EE, treatment with swallowed topical glucocorticoid, and nonresponsiveness as indicated by an eosinophil count of ≥ 24 per × 400 high-power field. This study was approved by the Institutional Review Board of the Cincinnati Children's Hospital Medical Center.

Cell culture. Human esophageal epithelial cells derived from human patient biopsies were cultured as previously described.[9] The human bronchial epithelial cell line HBEC was cultured as previously described.[43] The normal human primary bronchial epithelial cells were purchased from Lonza (Basel, Switzerland; catalog no. CC-2540) and cultured as previously described.[44] The U937 monocytes and Jurkat T cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. The CCD-16Lu fibroblasts were cultured in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin.

RNA extraction and miRNA microarray analysis. Human esophageal epithelial cells and bronchial epithelial cells were stimulated with media or 100 ng ml^{-1} IL-13 for 24 h. Total RNA including miRNA was isolated using miRNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and only samples with RNA integrity number > 8 were used. MiRNA expression from human bronchial epithelial cells was profiled using TaqMan Human MicroRNA Array v1.0 (Early Access), which includes probes for 365 human miRNAs, according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Data analysis was carried out using GeneSpring software (Agilent Technologies). To identify miRNAs differentially regulated between unstimulated and IL-13-stimulated samples, the expression data were normalized to the average of two endogenous control probes RNU44 and RNU48, then filtered on cycle threshold values < 35 and at least a twofold change between unstimulated and IL-13-stimulated samples. Statistical significance was determined at $P < 0.05$ with Benjamini–Hochberg false discovery rate correction. The list of differentially expressed miRNAs was clustered using hierarchical clustering and a heat map was generated. A similar analysis was carried out comparing unstimulated and IL-13-stimulated human esophageal epithelial cells, except that the TaqMan Human MicroRNA Array v2.0 was used, which includes probes for 667 human miRNAs. The microarray data have been deposited into the Array Express database (www.ebi.ac.uk/arrayexpress) with accession numbers E-MEXP-3351 and E-MEXP-3353 in compliance with minimum information about microarray experiment (MIAME) standards.

Experimental asthma induction in IL-13 bitransgenic mice. Bitransgenic mice bearing CCSP-rtTA and $(tetO)_7$CMV-IL-13 transgenes were previously described.[45] Experimental asthma was induced in IL-13 bitransgenic mice by feeding bi-transgenic mice doxycycline-impregnated food for 4 weeks as previously described.[45] All animals were housed under specific pathogen-free conditions in accordance with institutional guidelines. The use of animals in these experiments was approved by the institutional animal care and use committee of the Cincinnati Children's Hospital Medical Center.

Quantitative reverse transcriptase-PCR for miRNA. Levels of miRNA expression were measured quantitatively by using TaqMan MicroRNA Assays (Applied Biosystems) following the manufacturer's protocol. The expression levels were normalized to the U6 endogenous control. Relative expression was calculated as previously described.[45]

Quantitative reverse transcriptase-PCR for mRNA. Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). All primer/probe sets were obtained from Applied Biosystems. Samples were analyzed by TaqMan qRT-PCR for *TSLP* (Assay ID: Hs00263639_m1) and normalized to *HPRT1* (Assay ID: Hs01003267_m1). Relative expression was calculated as previously described.[45]

Correlation of miR-375 with major EE signature genes. Esophageal mRNA from EE patients was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's protocol. The TaqMan reagents for amplification of EE signature genes[9,23,24] were obtained from Applied Biosystems. TaqMan real-time PCR amplification was performed on an Applied Biosystems 7900HT Real-Time PCR System. The expression correlation study between miR-375 and 48 EE genes was performed in GraphPad Prism software (La Jolla, CA). Negative log of *P*-values from Pearson's correlation analysis were plotted to demonstrate correlation significance with EE genes. To control for the increased risk of false positives because of the number of statistical tests performed, we applied a Bonferroni correction based on the number of gene expression profiles compared. Because the average pairwise correlation between gene expression profiles was 0.54, we applied principal components analysis to determine the effective number of independent comparisons as previously described.[46] Using this approach, a *P*-value of 0.002 was required to achieve a family-wise error rate of 0.05.

Lentiviral transduction. The human esophageal epithelial cell line TE-7 cells were transduced with pmiRNA1-Pre-miR-375 vector or pmiRNA1-Control vector (System Biosciences, Mountain View, CA). The vectors include green fluorescent protein (GFP) and puromycin resistance genes as selection markers. At 3 days after transduction, cells were selected by fluorescence-activated cell sorting for GFP + cells and further cultured in media containing 4 μg ml^{-1} puromycin for 1 week. The cells were > 99% GFP + after selection.

Human genome-wide mRNA microarray. The Affymetrix (Santa Clara, CA) human gene 1.0ST array was used to compare gene expression profile of control-transduced TE-7 cells and pre-miR-375-transduced TE-7 cells before and after IL-13 treatment. Microarray data were analyzed using the GeneSpring software (Agilent Technologies) as previously described.[47] Global scaling was performed to compare genes from chip to chip, and a base set of probes was generated by requiring a minimum raw expression level of 20th percentile out of all probes on the microarray. The resulting probe sets were then baseline transformed and filtered on at least 1.2-fold difference between control-transduced and pre-miR-375-transduced cells with or without IL-13 treatment to identify miR-375-regulated genes. Statistical significance was determined at $P < 0.05$ with Benjamini–Hochberg false discovery rate correction. The resulting list of genes was clustered using hierarchical clustering and a heat map was generated. Biological functional enrichment analysis was carried out using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA) and Toppgene/Toppcluster.[46,48] The microarray data have been deposited into the Array Express database (www.ebi.ac.uk/arrayexpress) with accession number E-MEXP-3345 in compliance with MIAME standards.

Statistical analysis

Student's *t*-test was used to determine the significance between two groups. One-way analysis of variance with Tukey's *post hoc* test was used to determine the significance between more than two groups. Statistical significance and the *P*-values were indicated on the figures where appropriate. *P*-values of < 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS

This work was supported by the NHLBI Ruth L. Kirschstein National Research Service Award for individual predoctoral MD/PhD fellows F30HL104892 (to T.X.L.), the Ryan Fellowship from Albert J Ryan Foundation (to T.X.L.), and the Organogenesis Training grant (NIH T32HD046387 supporting T.X.L.). Additionally, this work was supported by the NIH R01DK076893 (to M.E.R.), the NIH R01AI073553 (to S.P.H.), the Campaign Urging Research for Eosinophilic Disease (CURED), the Buckeye Foundation, and the Food Allergy Initiative.

DISCLOSURE

M.E.R. has an equity interest in reslizumab, a drug being developed by Cephalon, and is a consultant for Immune Pharmaceuticals. The other authors declared no conflict of interest.

Author contributions

T.X.L. designed experiments, performed experiments, analyzed data, interpreted all results, and wrote the manuscript. E.-J.L. and T.W. performed experiments and analyzed data. A.J.P. and B.J.A. performed bioinformatics analysis. L.J.M. performed biostatistics analysis. S.P.H. analyzed data and contributed conceptually. M.E.R. designed experiments, interpreted all results, wrote the manuscript, and coordinated overall research efforts.

REFERENCES

1. Leigh, R. *et al.* Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *Am. J. Respir. Crit. Care. Med.* **169,** 860–867 (2004).
2. Yang, M. *et al.* Inhibition of arginase I activity by RNA interference attenuates IL-13-induced airways hyperresponsiveness. *J. Immunol.* **177,** 5595–5603 (2006).
3. Grunig, G. *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282,** 2261–2263 (1998).
4. Junttila, I.S. *et al.* Tuning sensitivity to IL-4 and IL-13: differential expression of IL-4Ralpha, IL-13Ralpha1, and gammac regulates relative cytokine sensitivity. *J. Exp. Med.* **205,** 2595–2608 (2008).
5. Lee, P.J. *et al.* ERK1/2 mitogen-activated protein kinase selectively mediates IL-13-induced lung inflammation and remodeling *in vivo*. *J. Clin. Invest.* **116,** 163–173 (2006).
6. Wills-Karp, M. Interleukin-13 in asthma pathogenesis. *Immunol. Rev.* **202,** 175–190 (2004).
7. Liacouras, C.A. *et al.* Eosinophilic esophagitis: updated consensus recommendations for children and adults. *J. Allergy Clin. Immunol.* **128,** 3–20 (2011).
8. Rothenberg, M.E. Biology and treatment of eosinophilic esophagitis. *Gastroenterology* **137,** 1238–1249 (2009).
9. Blanchard, C. *et al.* IL-13 involvement in eosinophilic esophagitis: transcriptome analysis and reversibility with glucocorticoids. *J. Allergy Clin. Immunol.* **120,** 1292–1300 (2007).
10. Lee, J.H. *et al.* Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. *Am. J. Respir. Cell Mol. Biol.* **25,** 474–485 (2001).
11. Laprise, C., Sladek, R., Ponton, A., Bernier, M.C., Hudson, T.J. & Laviolette, M. Functional classes of bronchial mucosa genes that are differentially expressed in asthma. *BMC Genomics* **5,** 21 (2004).
12. Woodruff, P.G. *et al.* Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. *Proc. Natl. Acad. Sci. USA* **104,** 15858–15863 (2007).
13. Zhen, G. *et al.* IL-13 and epidermal growth factor receptor have critical but distinct roles in epithelial cell mucin production. *Am. J. Respir. Cell Mol. Biol.* **36,** 244–253 (2007).
14. Corren, J. *et al.* Lebrikizumab treatment in adults with asthma. *N. Engl. J. Med.* **365,** 1088–1098 (2011).
15. Winter, J., Jung, S., Keller, S., Gregory, R.I. & Diederichs, S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat. Cell Biol.* **11,** 228–234 (2009).
16. Carthew, R.W. & Sontheimer, E.J. Origins and mechanisms of miRNAs and siRNAs. *Cell* **136,** 642–655 (2009).
17. Martinez-Nunez, R.T., Louafi, F. & Sanchez-Elsner, T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J. Biol. Chem.* **286,** 1786–1794 (2011).
18. Kumar, M. *et al.* Let-7 microRNA-mediated regulation of IL-13 and allergic airway inflammation. *J. Allergy Clin. Immunol.* **128,** 1077–1085 (2011).
19. Polikepahad, S. *et al.* Proinflammatory role for let-7 microRNAS in experimental asthma. *J. Biol. Chem.* **285,** 30139–30149 (2010).
20. Kuperman, D.A. *et al.* Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* **8,** 885–889 (2002).
21. Blanchard, C. & Rothenberg, M.E. Basic pathogenesis of eosinophilic esophagitis. *Gastrointest. Endosc. Clin. N. Am.* **18,** 133–143 (2008).
22. Mulder, D.J. & Justinich, C.J. Understanding eosinophilic esophagitis: the cellular and molecular mechanisms of an emerging disease. *Mucosal. Immunol.* **4,** 139–147 (2011).
23. Blanchard, C. *et al.* Eotaxin-3 and a uniquely conserved gene-expression profile in eosinophilic esophagitis. *J. Clin. Invest.* **116,** 536–547 (2006).
24. Abonia, J.P. *et al.* Involvement of mast cells in eosinophilic esophagitis. *J. Allergy Clin. Immunol.* **126,** 140–149 (2010).
25. Stansfield, W.E., Andersen, N.M., Tang, R.H. & Selzman, C.H. Periostin is a novel factor in cardiac remodeling after experimental and clinical unloading of the failing heart. *Ann. Thorac. Surg.* **88,** 1916–1921 (2009).
26. Kaiko, G.E. & Foster, P.S. New insights into the generation of Th2 immunity and potential therapeutic targets for the treatment of asthma. *Curr. Opin. Allergy Clin. Immunol.* **11,** 39–45 (2011).
27. Ackerman, S.J. *et al.* Charcot-Leyden crystal protein (galectin-10) is not a dual function galectin with lysophospholipase activity but binds a lysophospholipase inhibitor in a novel structural fashion. *J. Biol. Chem.* **277,** 14859–14868 (2002).
28. Xing, W., Austen, K.F., Gurish, M.F. & Jones, T.G. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. *Proc. Natl. Acad. Sci. USA* **108,** 14210–14215 (2011).
29. Blanchard, C. *et al.* Coordinate interaction between IL-13 and epithelial differentiation cluster genes in eosinophilic esophagitis. *J. Immunol.* **184,** 4033–4041 (2010).
30. Mukhopadhyay, S. *et al.* Matrix metalloproteinase-12 is a therapeutic target for asthma in children and young adults. *J. Allergy Clin. Immunol.* **126,** 70–76 (2010).
31. Lavigne, M.C. *et al.* Human bronchial epithelial cells express and secrete MMP-12. *Biochem. Biophys. Res. Commun.* **324,** 534–546 (2004).
32. Fahy, J.V. Remodeling of the airway epithelium in asthma. *Am. J. Respir. Crit. Care. Med.* **164** (10 Part 2), S46–S51 (2001).
33. Pouladi, M.A. *et al.* Interleukin-13-dependent expression of matrix metalloproteinase-12 is required for the development of airway eosinophilia in mice. *Am. J. Respir. Cell Mol. Biol.* **30,** 84–90 (2004).
34. Biton, M. *et al.* Epithelial microRNAs regulate gut mucosal immunity via epithelium-T cell crosstalk. *Nat. Immunol.* **12,** 239–246 (2011).
35. Wu, F. *et al.* MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha. *Gastroenterology* **135,** 1624–1635 (2008).
36. Sonkoly, E. *et al.* MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4. *J. Allergy Clin. Immunol.* **126,** 581–589 (2010).
37. Kong, K.L. *et al.* MicroRNA-375 inhibits tumour growth and metastasis in oesophageal squamous cell carcinoma through repressing insulin-like growth factor 1 receptor. *Gut* **61,** 33–42 (2012).
38. Tsukamoto, Y. *et al.* MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. *Cancer Res.* **70,** 2339–2349 (2010).
39. de Souza Rocha Simonini, P. *et al.* Epigenetically deregulated microRNA-375 is involved in a positive feedback loop with estrogen receptor alpha in breast cancer cells. *Cancer Res.* **70,** 9175–9184 (2010).
40. Rothenberg, M.E. *et al.* Common variants at 5q22 associate with pediatric eosinophilic esophagitis. *Nat. Genet.* **42,** 289–291 (2010).
41. Sherrill, J.D. *et al.* Variants of thymic stromal lymphopoietin and its receptor associate with eosinophilic esophagitis. *J. Allergy Clin. Immunol.* **126,** 160–165 (2010).
42. Blanchard, C. *et al.* Periostin facilitates eosinophil tissue infiltration in allergic lung and esophageal responses. *Mucosal Immunol.* **1,** 289–296 (2008).
43. Ramirez, R.D. *et al.* Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer Res.* **64,** 9027–9034 (2004).

44. Kariyawasam, H.H. *et al.* Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma. *J. Allergy Clin. Immunol.* **124,** 454–462 (2009).
45. Lu, T.X., Munitz, A. & Rothenberg, M.E. MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression. *J. Immunol.* **182,** 4994–5002 (2009).
46. Chen, J., Bardes, E.E., Aronow, B.J. & Jegga, A.G. ToppGene Suite for gene list enrichment analysis and candidate gene prioritization. *Nucleic Acids Res.* **37** (Web Server issue), W305–W311 (2009).
47. Lu, T.X. *et al.* MicroRNA-21 limits *in vivo* immune response-mediated activation of the IL-12/IFN-{gamma} pathway, Th1 polarization, and the severity of delayed-type hypersensitivity. *J. Immunol.* **187,** 3362–3373 (2011).
48. Kaimal, V., Bardes, E.E., Tabar, S.C., Jegga, A.G. & Aronow, B.J. ToppCluster: a multiple gene list feature analyzer for comparative enrichment clustering and network-based dissection of biological systems. *Nucleic Acids Res.* **38** (Web Server issue), W96–W102 (2010).