Airway Epithelial miRNA Expression Is Altered in Asthma

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Rationale: **Changes in airway epithelial cell differentiation, driven in part by IL-13, are important in asthma. Micro-RNAs (miRNAs) regulate cell differentiation in many systems and could contribute to epithelial abnormalities in asthma.**

Objectives: **To determine whether airway epithelial miRNA expression is altered in asthma and identify IL-13–regulated miRNAs.**

Methods: **We used miRNA microarrays to analyze bronchial epithelial brushings from 16 steroid-naive subjects with asthma before and after inhaled corticosteroids, 19 steroid-using subjects with asthma, and 12 healthy control subjects, and the effects of IL-13 and corticosteroids on cultured bronchial epithelial cells. We used quantitative polymerase chain reaction to confirm selected microarray results.**

Measurements and Main Results: **Most (12 of 16) steroid-naive subjects with asthma had a markedly abnormal pattern of bronchial epithelial miRNA expression by microarray analysis. Compared with control subjects, 217 miRNAs were differentially expressed in steroid-naive subjects with asthma and 200 in steroid-using subjects with asthma (false discovery rate < 0.05). Treatment with inhaled corticosteroids had modest effects on miRNA expression in steroid-naive asthma, inducing a statistically significant (false discovery rate < 0.05) change for only nine miRNAs. qPCR analysis confirmed differential expression of 22 miRNAs that were highly differentially expressed by microarrays. IL-13 stimulation recapitulated changes in many differentially expressed miRNAs, including four members of the miR-34/449 family, and these changes in miR-34/449 family members were resistant to corticosteroids.**

Conclusions: **Dramatic alterations of airway epithelial cell miRNA levels are a common feature of asthma. These alterations are only modestly corrected by inhaled corticosteroids. IL-13 effects may account for some of these alterations, including repression of miR-34/449 family members that have established roles in airway epithelial cell differentiation.**

Clinical trial registered with www.clinicaltrials.gov (NCT 00595153).

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Micro-RNAs (miRNAs) regulate post-transcriptional gene expression and have been implicated in regulating epithelial cell differentiation. Whether and how the expression of miRNAs is altered in the airway epithelium in asthma is not well understood.

What This Study Adds to the Field

In this study, we found that airway epithelial cells in human asthma have consistent and dramatic alterations in miRNA expression levels, including decreased expression of members of the miR-34/449 family, which are known to regulate differentiation of epithelial cells. In accompanying *in vitro* studies, we found that IL-13 has a strong and consistent effect on miRNA expression in airway epithelial cells and recapitulates the repression of miR34/449 family members that we observed in asthma.

Keywords: miRNA; asthma; airway epithelium

Asthma is an inflammatory condition of the lung characterized by episodic airway obstruction and remodeling of airway tissues (1). The airway epithelium is increasingly recognized as an important participant in asthma pathophysiology (2, 3). Airway epithelial cells produce mucus, which is a major contributor to airway obstruction in fatal asthma (4), and even patients with stable mild and moderate asthma have increased epithelial mucin stores (5). Previous human, animal model, and cell culture studies demonstrate that the Th2 cytokine IL-13 is a critical inducer of airway epithelial abnormalities in asthma (6–8). IL-13 stimulation of epithelial cells leads to an increase in the number of mucous cells (mucous metaplasia) and a decrease in the number of ciliated cells (9). IL-13 stimulation of airway epithelial cells activates signal transduction and transcription factor 6, leading to changes in expression of mRNAs that account for at least some of these phenotypic changes (10).

Micro-RNAs (miRNAs) are approximately 22-nucleotide RNAs that regulate post-transcriptional gene expression, generally by binding to the 3′ untranslated region (UTR) of target mRNAs and either triggering their degradation (11) or inhibiting translation (12). There are currently more than 800 human miRNAs described in mirBase (13) and more than 60% of human protein-coding genes have been under selective pressure to maintain pairing to miRNAs (14). Whether miRNAs help regulate

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changes in epithelial gene expression, cell differentiation, and function in asthma is uncertain. However, several miRNAs have been implicated in epithelial cell proliferation, differentiation, and apoptosis (15–17). Furthermore, miR-449, a member of the miR-34/449 family, was recently identified as a critical regulator of differentiation of nasal epithelial cells to ciliated cells by directly repressing transcript levels of *NOTCH1* and its ligand *DLL1* (18). A very recent study used microarrays to demonstrate differences in miRNA expression patterns in airway epithelial cells cultured from subjects with asthma versus healthy control subjects and confirmed by polymerase chain reaction (PCR) that three miRNAs were differentially expressed in these cultured cells (19). However, an earlier study of miRNA expression of freshly isolated airway tissue obtained by endobronchial biopsy found no differences between subjects with asthma and healthy control subjects (20). Further work is therefore required to determine whether airway epithelial miRNA expression is altered within the asthmatic airway, to characterize specific miRNA changes that may occur, and to identify mechanisms that contribute to these changes.

In this study, we used genome-wide profiling of miRNA expression in the bronchial epithelial compartment to identify miRNA expression changes associated with asthma. We also sought to identify the effect of inhaled corticosteroids (ICS) on bronchial epithelial miRNA expression because ICS are an effective and commonly used antiinflammatory therapy for asthma. We hypothesized that asthma is associated with widespread changes in miRNA expression that can be detected in freshly isolated airway epithelial cells and that IL-13 is an important contributor to these miRNA changes. To test this hypothesis, we studied subjects with asthma before and after treatment with ICS and analyzed the effects of IL-13 and corticosteroids on expression of miRNAs in cultured human bronchial epithelial cells.

METHODS

Subjects

We obtained bronchial cells by bronchoscopic brushing from three groups: (*1*) subjects with asthma who were not using ICS for 6 weeks before enrollment (labeled here as "steroid-naive"); (*2*) subjects with asthma who were using ICS continuously for at least 6 weeks before enrollment ("steroid-using"); and (*3*) healthy control subjects without asthma or lung disease (*see* Figure E1 in the online supplement). Steroid-naive subjects were studied at baseline and again after 8 weeks of treatment with budesonide, 200 μg twice a day. Steroid-using subjects were judged by their treating physician to require ICS for control of their asthma, although we did not formally assess their level of control without ICS. These subjects underwent bronchoscopy after standardizing their ICS regimen to budesonide, 200 μg twice a day for 8 weeks. All subjects were nonsmokers (defined as never-smoker or no smoking for 1 year and total smoking history <15 pack-years). Complete inclusion-exclusion criteria are provided in the METHODS section in the online supplement. Written informed consent was obtained from all subjects. All studies were approved by the University of California San Francisco (UCSF) Committee on Human Research and performed in accordance with the Declaration of Helsinki.

Microarray Analyses

After RNA extraction and sample quality assessment, labeling and array hybridizations for miRNAs were performed according to standard protocols from the UCSF Sandler Asthma Basic Research Center Functional Genomics Facility and Agilent Technologies (http://www.arrays.ucsf.edu and http://www.agilent.com) using Agilent custom UCSF miRNA multispecies 8 × 15K Ink-jet arrays. mRNA microarray analyses on epithelial brushings were performed using Affymetrix U133 Plus 2.0 arrays (Affymetrix, Inc., Santa Clara, CA) as described previously (21). Additional details are provided in the METHODS section in the online supplement. Microarray data may be found in the National Center for Biotechnology Information Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/, accession number GSE34466).

miRNA Expression Profiling Using Quantitative PCR

We performed quantitative PCR (qPCR) for selected miRNAs using methods described previously (22). In brief, we applied a multiplex quantitative reverse transcription PCR method involving the purification of multiplex PCR products followed by uniplex analysis on a microfluidics chip (Fluidigm, South San Francisco, CA). Data were normalized using the geometric mean value of miR-103 and miR-191 as "housekeeping" miRNAs (23) after demonstrating that these miRNAs were relatively invariantly expressed across our samples. The primers and probes used are listed in Table E1.

Human Bronchial Epithelial Cell Cultures

Normal human bronchial epithelial cells were harvested by standard techniques from airways of three lungs donated for lung transplantation and cultured at air-liquid interface (ALI) for 21 days. Details of culture conditions are conveyed in the METHODS section in the online supplement.

3′ UTR Analysis

A 301-bp fragment of the NOTCH1 3′ UTR and an irrelevant negative control sequence were cloned into the bdLV.ΔLNGFR.GFP sensor plasmid (24). BEAS-2B cells were transfected with plasmid and 10-nM miRIDIAN miR-449a mimic or negative control mimic (cel-miR-67; Thermo Scientific, Lafayette, CO), stained with Alexa Fluor 647-conjugated anti–LN-GFR antibody (UCSF Hybridoma Core), and analyzed by flow cytometry. The ratio of GFP to the LN-GFR reference reporter was calculated to determine the effects of the 3′ UTR sequences and miRNA mimics on sensor protein expression. Additional details are in the METHODS section in the online supplement.

Statistical Analysis

We compared subject group characteristics using Student *t* test, rank-sum test, and chi-square testing as appropriate. We assessed differential miRNA expression levels by microarray using linear models (R Bioconductor, limma package). We applied false discovery rate adjustment (FDR, Benjamini-Hochberg) (25) for multiple testing correction in microarray and qPCR analyses. In analyses restricted to the miR-34/449 family but across three groups, pair-wise group comparisons were corrected for multiple comparisons across groups using Tukey correction. In analyses of IL-13 exposure *in vitro*, selected miRNAs were assayed by qPCR across three donors in triplicate and the effect of IL-13 was tested using Student *t* test, with the donor as the unit of analysis. For budesonide experiments, miRNA levels in different culture conditions were compared using analysis of variance with Tukey correction. In 3′ UTR analysis, differences from control conditions were assessed using the Student *t* test. Visual presentation of enrichment for predicted miRNA targets among differentially expressed mRNA was performed by plotting enrichment within deciles of differential expressed mRNAs. Similarly, visual presentation of enrichment of miRNA targets among mRNAs highly correlated with miRNAs was performed by plotting deciles of correlation coefficients (26). Within each decile, the underrepresentation and overrepresentation of mRNAs that are predicted targets of a given miRNA were assessed using chi-square testing. The statistical significance of correlations between miRNAs and their predicted targets was also tested using permutation (10^4 permutations) (27). Pathway analyses were performed using the DAVID Gene Functional Classification Tool (http://david.abcc.ncifcrf.gov/) as described in the METHODS section in the online supplement. miRNA names and accession numbers provided here refer to miRBase 18, which forgoes the miR/miR* nomenclature in favor of the -5p/-3p nomenclature.

RESULTS

Subject and Epithelial Sample Characteristics

We studied 16 subjects with steroid-naive asthma. For all 16, epithelial samples were obtained by bronchial brushing before

TABLE 1. SUBJECT CHARACTERISTICS

	Healthy Control Subjects	Steroid-Naive Subjects with Asthma	Steroid-using Subjects with Asthma
N	12	16	19
Age	36 ± 11	32 ± 12	35 ± 12
Sex (M:F)	5:7	9:7	8:11
FEV_1 % predicted, mean ± SD	104 ± 9%	89 ± 14%*	83 ± 17%*
FVC % predicted, mean ± SD	107 ± 7%	101 ± 13%	96 ± 13%
PC_{20} methacholine, mg/ml (median, IQR)	10 (10–10)	0.56* (0.33–1)	0.81* (0.35–4.40)
Serum IgE (IU/ml unit)	19 (9–29)	301* (129–589)	131* (107–623)

Definition of abbreviations: IQR = interquartile range; PC_{20} methacholine = the provocative concentration of methacholine required to decrease the FEV_1 by 20%.
* $P < 0.05$ compared with healthy control subjects.

initiation of 8 weeks of treatment with inhaled budesonide, 200 μg twice a day. One subject withdrew during the ICS trial and the remaining 15 had a second epithelial sample obtained after treatment. One of the post-ICS samples did not pass quality control inspection on microarray data preprocessing, leaving 14 post-ICS samples for analysis. We also studied 19 subjects with steroid-using asthma who underwent bronchoscopy after standardization to inhaled budesonide, 200 μg twice a day for 8 weeks. Finally, we studied 12 nonasthmatic healthy control subjects without lung disease. These groups did not differ with respect to age or sex (Table 1). Both groups of subjects with asthma had airway obstruction compared with healthy control subjects with no asthma, as judged by FEV_1. Consistent with our inclusion criteria, both groups of subjects with asthma had airway hyperresponsiveness (lower provocative concentration of methacholine required to decrease the FEV_1 by 20%) compared with healthy control subjects. Only 2 of our 47 subjects had a greater than five pack-years smoking history; one steroid-using subject with asthma had a six pack-year history and one steroid-naive subject had a seven pack-year history. No subjects were active smokers (defined as smoking the last year). Epithelial sample cell counts and differentials were available from 55 of the 61 bronchial epithelial brushings performed in this study and, consistent with our previously published studies (21), these samples were predominantly epithelial cells (96.4 ± 6.1%), with 0.32 ± 1.1% squamous cells, 2.9 ± 5.8% macrophages, 0.19 ± 0.29% neutrophils, 0.07 ± 0.16% eosinophils, and 0.07 ± 0.18% lymphocytes. We further determined whether there were any differences in cell differentials among healthy control, steroid-naive asthma before ICS, steroid-naive asthma after ICS, and steroid-using asthma samples and found only one statistically significant difference in any cell type (lymphocyte percentages were lower in steroid-using subjects with asthma than in healthy control subjects, $P = 0.021$).

miRNAs Differentially Expressed in Steroid-Naive Subjects with Asthma

RNA was prepared from bronchial epithelial brushings from all subjects and analyzed using miRNA microarrays. Unsupervised hierarchical clustering using the 50 most variable miRNAs showed

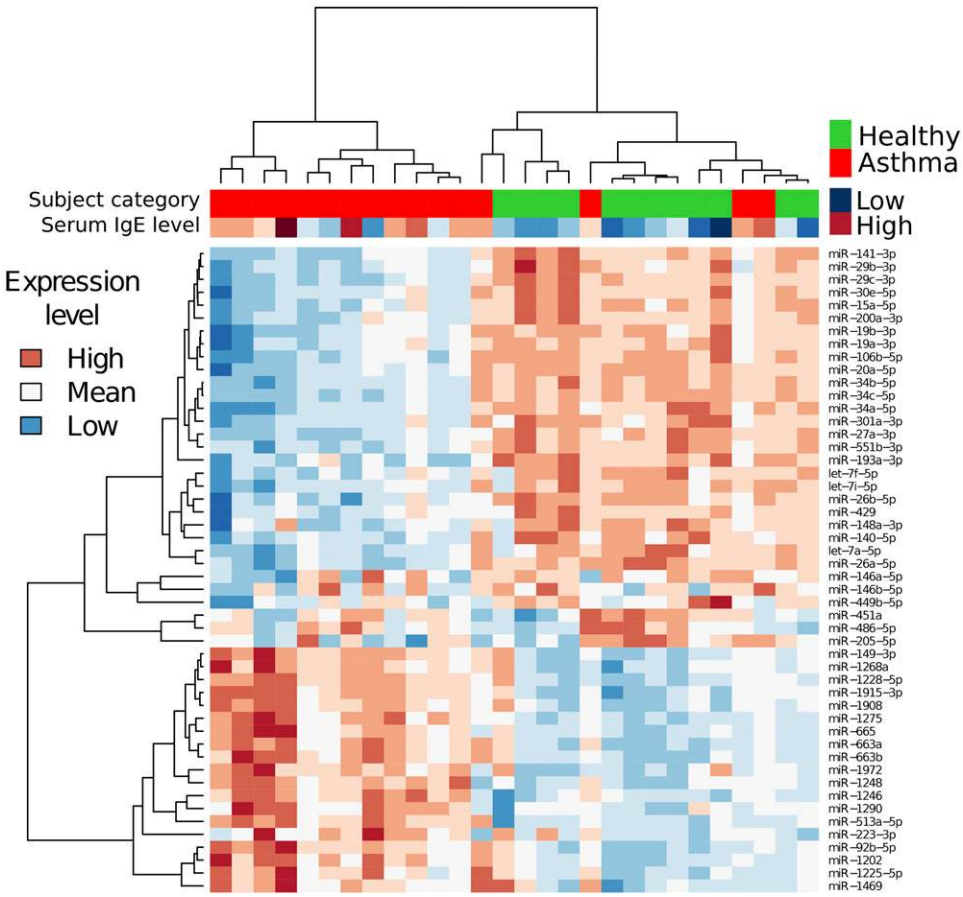

Figure 1. Bronchial epithelial brushings show a strong and consistent difference in micro-RNA (miRNA) expression in steroid-naive asthma compared with healthy control subjects. Unsupervised hierarchical clustering using the 50 most variable miRNAs separates 12 of 16 steroid-naive subjects with asthma from healthy control subjects (*red* denotes expression level higher than the mean across all subjects, *blue* denotes lower than the mean). Among subjects with asthma, no clear relationship between miRNA expression and serum IgE level was observed.

that 12 of 16 steroid-naive subjects with asthma had a substantially different pattern of miRNA expression than all 12 healthy control subjects, whereas four of the steroid-naive subjects with asthma were similar to the healthy control subjects (Figure 1). Similar results were obtained using the 100 most variable miRNAs (*see* Figure E2). Within the 16 steroid-naive subjects with asthma, there was no discernible association between IgE levels and miRNA expression pattern (Figure 1; *see* Figure E2). Using linear models, we identified 217 miRNAs as differentially expressed in the 16 steroid-naive subjects with asthma as compared with the 12 healthy control subjects (FDR $<$ 0.05). Of these, 79 were higher and 138 lower in asthma relative to healthy control subjects (*see* Table E2). These differentially expressed miRNAs were notable for the presence of multiple members of several miRNA families (groups of miRNAs with high sequence similarity that derive from distinct DNA sequences), including members of the miR-34/449 family (*see* Figure E3). Some primary RNA transcripts give rise to multiple miRNAs, which can be identified by their clustering within small genomic intervals (e.g., 10 kb) or within introns from a single coding transcript. We found that 60 of the 217 differentially expressed miRNAs belonged to 20 clusters that contained at least two differentially expressed miRNAs. The miRNAs in a given cluster were differentially expressed in the same direction for all but one of these clusters.

miRNAs Differentially Expressed in Subjects with Asthma Using ICS at Enrollment

Comparing healthy control subjects with steroid-using subjects with asthma who were standardized to 8 weeks of treatment with budesonide, 200 μg twice a day, we found 200 differentially expressed miRNAs (FDR $<$ 0.05) (*see* Table E3). A total of 170 of these were the same as those observed in the healthy versus steroid-naive subjects with asthma. A total of 53 miRNAs were increased in both cohorts and 117 miRNAs, including four members of the miR-34/449 family (Figure 2), were decreased in steroid-naive and steroid-using cohorts compared with healthy control subjects. There were no statistically significant (FDR $<$ 0.05) differences among miRNA expression when steroid-using asthma samples were compared with either pre- or post-ICS treated steroid-naive asthma samples.

Effect of ICS Treatment in Steroid-Naive Asthma

In a paired analysis, we compared miRNA expression in steroid-naive subjects with asthma before and after treatment with budesonide, 200 μg twice a day. After 8 weeks of budesonide there was a statistically significant decline in exhaled nitric oxide levels (68.1 ± 37.3 vs. 24.2 ± 8.8 ppb; $P = 0.0018$) but no statistically significant improvement in FEV_1 (86.7 ± 12.4% vs. 89.5 ± 10.3% of predicted; $P = 0.21$) in this population with relatively mild airway obstruction at baseline. Using daily diary card monitoring, we found a significant improvement in the percentage of subjects who were well-controlled (as defined by the NHLBI Guidelines for the Diagnosis and Management of Asthma [EPR-3]) (28) after 4 weeks of budesonide (29%) and 8 weeks of budesonide (43%) compared with before ICS (0% at Visit 2; $P < 0.05$ for both comparisons). For 207 of the 217 differentially expressed miRNAs in steroid-naive subjects with asthma, the mean absolute fold difference (compared with healthy control subjects) was lower after treatment with ICS (*see* Table E4), with nine miRNAs showing a statistically significant change with ICS treatment (FDR $<$ 0.05) (Figure 3; *see* Table E5). To account for regression to the mean, we also considered miRNAs that were differentially expressed in steroid-naive subjects with asthma after ICS versus healthy control subjects. A total of 126 miRNAs met this criterion, and for 111 of them, the mean absolute fold difference (compared with healthy control subjects) was lower after treatment with ICS than before treatment (also included in Figure 3). Overall, these treatment data reveal a detectable but incomplete restorative effect of ICS on miRNA expression.

TABLE 2. SELECTED DIFFERENTIALLY EXPRESSED miRNAS IN AIRWAY EPITHELIAL BRUSHINGS FROM STEROID-NAIVE ASTHMA*

Mirbase-18 Name	Accession No.	Log_2 Fold Difference (*vs. Healthy Control Subjects*)	FDR Q Value
miR-1246	MIMAT0005898	1.83	1.1×10^{-4}
miR-663a	MIMAT0003326	1.7	3.3×10^{-5}
miR-1275	MIMAT0005929	1.57	6.7×10^{-5}
miR-1228-5p	MIMAT0005582	1.37	4.2×10^{-5}
miR-149-3p	MIMAT0004609	1.35	5.5×10^{-5}
miR-1915-3p	MIMAT0007892	1.32	3.6×10^{-5}
miR-1908	MIMAT0007881	1.29	5.3×10^{-4}
miR-92b-5p	MIMAT0004792	1.2	1.1×10^{-4}
miR-1268a	MIMAT0005922	1	4.9×10^{-4}
miR-638	MIMAT0003308	0.8	2.7×10^{-3}
miR-1207-5p	MIMAT0005871	0.75	9.5×10^{-4}
miR-1972	MIMAT0009447	0.7	2.9×10^{-3}
miR-30d-5p	MIMAT0000245	−0.37	2.5×10^{-3}
miR-107	MIMAT0000104	−0.39	6.7×10^{-3}
miR-93-5p	MIMAT0000093	−0.43	5.2×10^{-3}
miR-30c-5p	MIMAT0000244	−0.46	6.0×10^{-3}
miR-30b-5p	MIMAT0000420	−0.57	5.5×10^{-5}
let-7c	MIMAT0000064	−0.63	1.2×10^{-3}
miR-30a-5p	MIMAT0000087	−0.67	3.8×10^{-5}
miR-24-3p	MIMAT0000080	−0.68	8.7×10^{-4}
miR-31-5p	MIMAT0000089	−0.72	8.3×10^{-4}
miR-99a-5p	MIMAT0000097	−0.74	1.3×10^{-4}
let-7a-5p	MIMAT0000062	−0.77	9.7×10^{-4}
let-7i-5p	MIMAT0000415	−0.79	1.2×10^{-3}
let-7g-5p	MIMAT0000414	−0.83	5.0×10^{-4}
miR-29a-3p	MIMAT0000086	−0.86	6.6×10^{-5}
miR-26a-5p	MIMAT0000082	−0.87	1.2×10^{-4}
miR-449a	MIMAT0001541	−0.9	5.4×10^{-3}
let-7f-5p	MIMAT0000067	−0.91	5.8×10^{-4}
miR-106b-5p	MIMAT0000680	−0.91	1.1×10^{-4}
miR-19b-3p	MIMAT0000074	−0.92	3.4×10^{-4}
miR-27b-3p	MIMAT0000419	−0.96	3.2×10^{-5}
miR-449b-5p	MIMAT0003327	−0.97	7.0×10^{-3}
miR-200a-3p	MIMAT0000682	−1.03	1.5×10^{-4}
miR-27a-3p	MIMAT0000084	−1.16	1.6×10^{-5}
miR-34a-5p	MIMAT0000255	−1.23	3.6×10^{-5}
miR-29c-3p	MIMAT0000681	−1.34	3.3×10^{-5}
miR-141-3p	MIMAT0000432	−1.67	3.3×10^{-5}
miR-34b-5p	MIMAT0000685	−1.83	3.3×10^{-5}
miR-34c-5p	MIMAT0000686	−1.98	3.3×10^{-5}

Definition of abbreviation: FDR = false discovery rate.

*Data are from 16 subjects with steroid-naive asthma and 12 healthy control subjects with no asthma. miRNAs shown here were selected from the larger list in Table E2 based on meeting a more stringent statistical significance cutoff (Benjamini-Hochberg FDR $<$ 0.01) and the finding of a relatively high hybridization signal (mean log_2 signal intensity $>$9).

Confirmation Using Real-Time qPCR

We performed confirmatory qPCR for a subset of 40 miRNAs that were identified as differentially expressed and produced strong signals in the microarray experiments (FDR $<$ 0.01 in steroid-naive asthma vs. control subjects and mean log_2 signal intensity $>$9) (Table 2). Five probes did not yield detectable signal on qPCR, suggesting either array nonspecificity or qPCR assay failure. For the remaining 35 detectable miRNAs, 22 had concordant, statistically significant changes by qPCR, 9 had concordant but nonsignificant changes, 3 had nonsignificant changes in the opposite direction, and 1 had a significant change in the opposite direction (Figure 4). As expected given the greater sensitivity and dynamic range of qPCR, the difference measured by

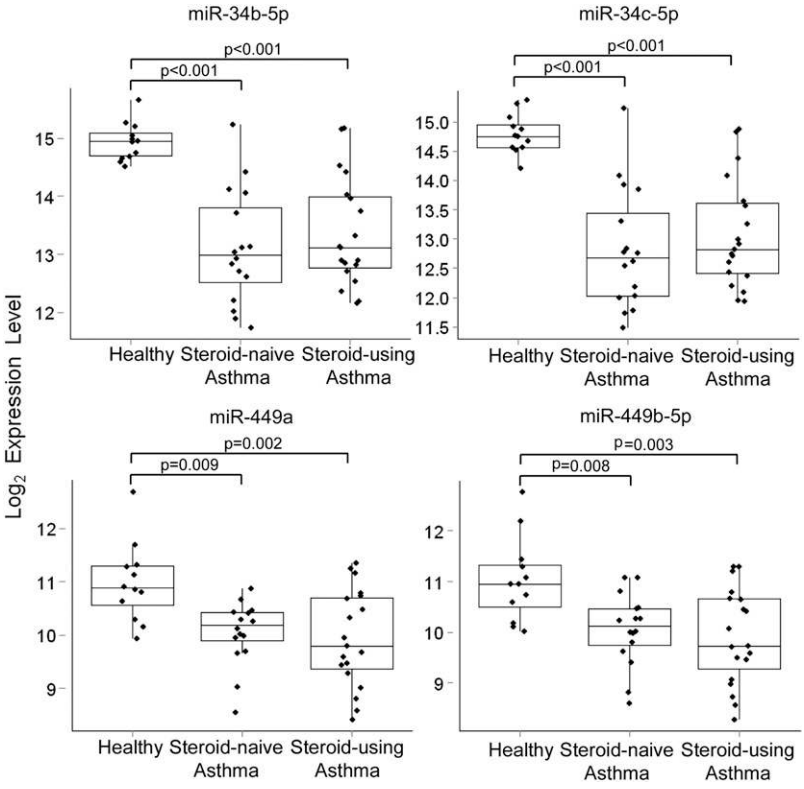

Figure 2. Repression of miR-34/449 family members is found in two asthma cohorts. For steroid-naive asthma and steroid-using asthma, expression levels of miR-34c-5p, miR-34b-5p, miR-449a, and miR-449b-5p is lower than in healthy control subjects.

qPCR was larger than the difference measured by arrays for some miRNAs.

Effects of IL-13 on Airway Epithelial miRNA Expression *In Vitro*

Human airway epithelial cells grown at ALI differentiate over 21 days to recapitulate a pseudostratified airway epithelium. Microarray analysis revealed that miRNA expression in human airway epithelial cells grown at ALI was highly correlated with miRNA expression in the bronchial epithelial brushings (*see* Figure E4). To determine the effect of IL-13 on miRNA expression, we exposed cultured bronchial epithelial cells from three human donors to IL-13 at 10 ng/ml for the final 14 days of culture and analyzed miRNA expression using microarrays. We confirmed that IL-13 induced mucous metaplasia by histologic analysis (not shown) and by quantitative reverse transcriptase PCR ($>10^3$-fold increase in *MUC5AC* mRNA). We identified 262 differentially expressed miRNAs (FDR < 0.05). Of these, 94 were also differentially expressed in steroid-naive subjects with asthma versus healthy subjects, and 91 of these 94 show concordant changes in asthma and with exposure to IL-13 (Figure 5A). Notably, all four members of the miR-34/449 family that we identified as lower in asthma were significantly repressed by IL-13 across the three donors in a time- and dose-dependent manner (Figures 5B–5D). miR-34b-5p and miR-34c-5p were maximally repressed at 10 ng/ml, whereas miR-449a and miR-449b were maximally repressed at 25 ng/ml. These data demonstrate that IL-13 has a strong and consistent effect on miRNA expression in bronchial epithelial cells and recapitulates the repression of miR-34/449 family members that we observed in asthma.

Effects of Corticosteroids on IL-13–induced miR-34/449 Repression *In Vitro*

We performed additional cell culture experiments designed to determine whether corticosteroids can reverse the effect of IL-13 on bronchial epithelial cell miR-34/449 expression. Human bronchial epithelial cells were grown for 21 days in an ALI culture with and without IL-13 (10 ng/ml) and budesonide (10^{-6} or 10^{-7} M) for 14 days. We found that dexamethasone, administered at doses sufficient to alter the expression of a known corticosteroid-responsive gene (FKBP5), did not reverse the repression of the miR-34/449 family members that was caused by IL-13 exposure *in vitro* (Figure 6).

Relationships between Expression of miRNAs and Their Predicted mRNA Targets

Analyses of the relationships between miRNA levels and levels of their predicted mRNA targets have been used to infer how miRNAs contribute to regulation of gene expression in many systems (26, 29, 30). We examined whether changes in miRNAs seen in steroid-naive subjects with asthma were accompanied by changes in mRNA targets predicted by TargetScan 6 (31, 32). mRNA expression data (generated using microarrays) were available for 15 of the 16 steroid-naive subjects with asthma and 11 of the 12 healthy control subjects. The set of mRNAs that were decreased in asthma was consistently enriched in targets of miRNAs that were decreased in asthma (*see* Figure E5A). Analysis of correlations between the expression of each differentially expressed miRNA and each predicted target in the combined population of 15 steroid-naive subjects and 11 healthy control subjects yielded a similar result (Figure 7A), with enrichment of positive correlations. Permutation testing indicated that the increase in positive correlations was highly significant ($P < 0.0001$). This predominance of positive correlations has been observed in other systems (26, 29, 30) and suggests the existence of "incoherent feed forward loops," which are regulatory circuits that confer robustness by buffering changes in gene expression (*see* Figure E5B; see [29, 30]; and Discussion, below). We used pathway analysis (DAVID) to identify groups of genes that are especially likely to be involved in these regulatory circuits (*see*

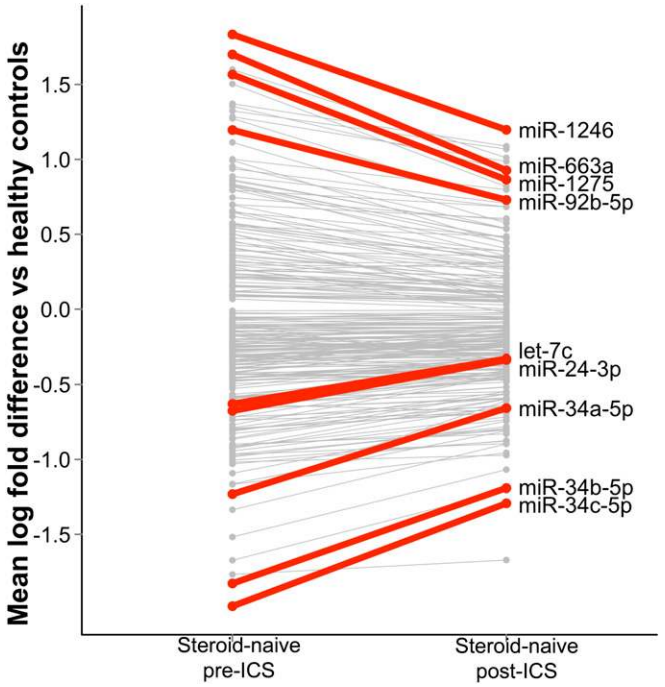

Figure 3. Inhaled corticosteroids (ICS) have modest restorative effects on micro-RNA (miRNA) expression in epithelial brushings. Plotted is the direction of change in 225 miRNAs that were either differentially expressed in steroid-naive asthma versus healthy control subjects (false discovery rate [FDR] < 0.05), or differentially expressed in the same subjects post-ICS compared with healthy control subjects (FDR $<$ 0.05). *Red lines* denote the nine miRNAs showing statistically significant within-subject change after ICS (FDR < 0.05 using a paired analysis). For all nine miRNAs, values returned toward those seen in healthy control subjects after ICS treatment.

Table E6). This analysis implicates many of the differentially expressed miRNAs in regulation of genes involved in gene transcription, chromatin organization, and other key cellular processes. To further explore the characteristics of these regulatory circuits in asthma, we examined miRNA-target correlations within the population of subjects with steroid-naive asthma (excluding healthy control subjects) (Figure 7B). Here we found a strikingly opposite result, with a highly significant predominance of negative correlations ($P < 0.0001$ by permutation analysis). This suggests that the inhibitory effects of miRNAs on gene expression vary within the population of individuals with asthma (see DISCUSSION). One example of a negative correlation was NOTCH1/449.We found evidence for negative correlations between miR-449 and *NOTCH1* mRNA levels ($r = -0.36$, $P = 0.038$ for miR-449b; and $r = -0.33$, $P = 0.062$ for miR-449a, using data from our subjects with asthma), suggesting that asthma-associated changes in miR-449 may affect *NOTCH1* expression in this disease. We further verified targeting of NOTCH1 by miR-449 experimentally by mimicking miR-449 (or negative control mimic) in BEAS-2B cells transfected with a reporter vector bearing either relevant sequences from the NOTCH1 3′-UTR (NOTCH1 sensor) or irrelevant 3′UTR (negative control sensor) (*see* Figure E6). The miR-449 mimic decreased NOTCH1 sensor expression by 38% ($P < 0.01$) but did not decrease expression of the negative control sensor.

DISCUSSION

Our data indicate that airway epithelial cells in asthma have dramatic alterations in miRNA expression levels. The percentage of known miRNAs that we found differentially expressed far exceeds the percentage of coding RNAs (mRNAs) that we found to be differentially expressed in prior studies (21, 33). Furthermore, a consistent pattern of miRNA abnormalities was observed in two cohorts of subjects with asthma, steroid-naive subjects with asthma, and steroid-using subjects with asthma. In our steroid-naive subjects with asthma, standardized treatment with ICS had a modest effect on miRNA expression. Overall, ICS tended to normalize abnormalities in miRNA expression, but this effect was only statistically significant for a relatively small number of miRNAs and did not restore miRNA levels to those found in healthy control samples. Companion experiments performed using cultured bronchial epithelial cells revealed that IL-13, a Th2 cytokine that is strongly linked to epithelial gene mRNA expression changes and mucous metaplasia in asthma, had consistent and substantial effects on bronchial epithelial miRNA expression that recapitulated many of the differentially expressed miRNAs that we observed in asthma. One salient finding is that members of the miR-34/449 family (miR-34c-5p, miR-34c-5p, miR-449a, and miR-449b-5p) are highly repressed *in vivo* in asthma and repressed *in vitro* by IL-13

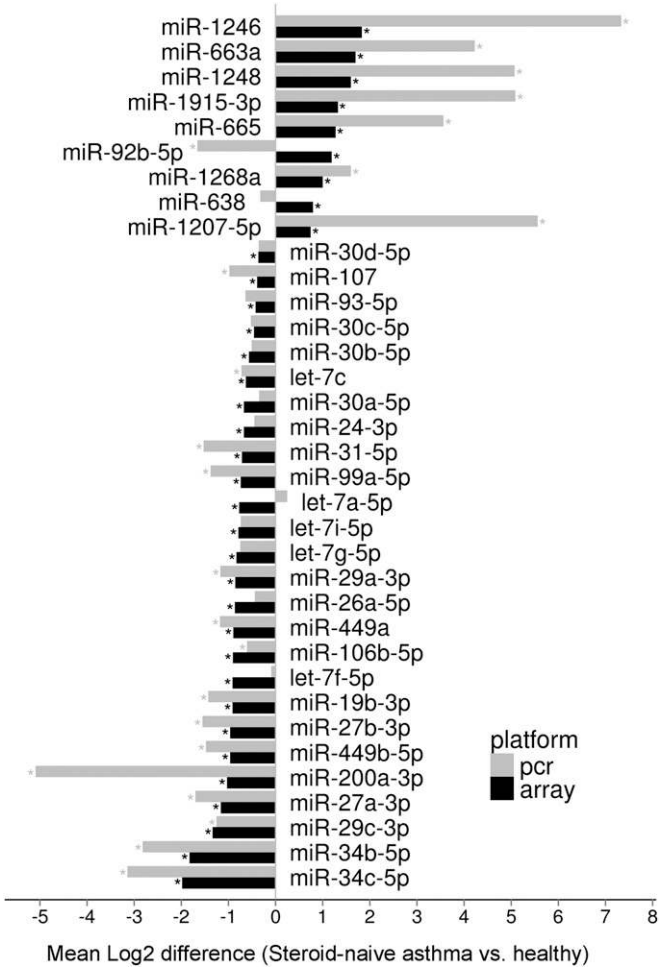

Figure 4. Polymerase chain reaction (PCR) validation of microarray findings in steroid-naive asthma. Quantitative PCR (qPCR) was used to measure 40 micro-RNA (miRNAs) selected for confirmation based on false discovery rate less than 0.01 and mean $\log_2$ signal intensity greater than nine in microarray analyses. Five of these miRNAs did not yield detectable signal on qPCR (omitted from figure). *False discovery rate < 0.05.

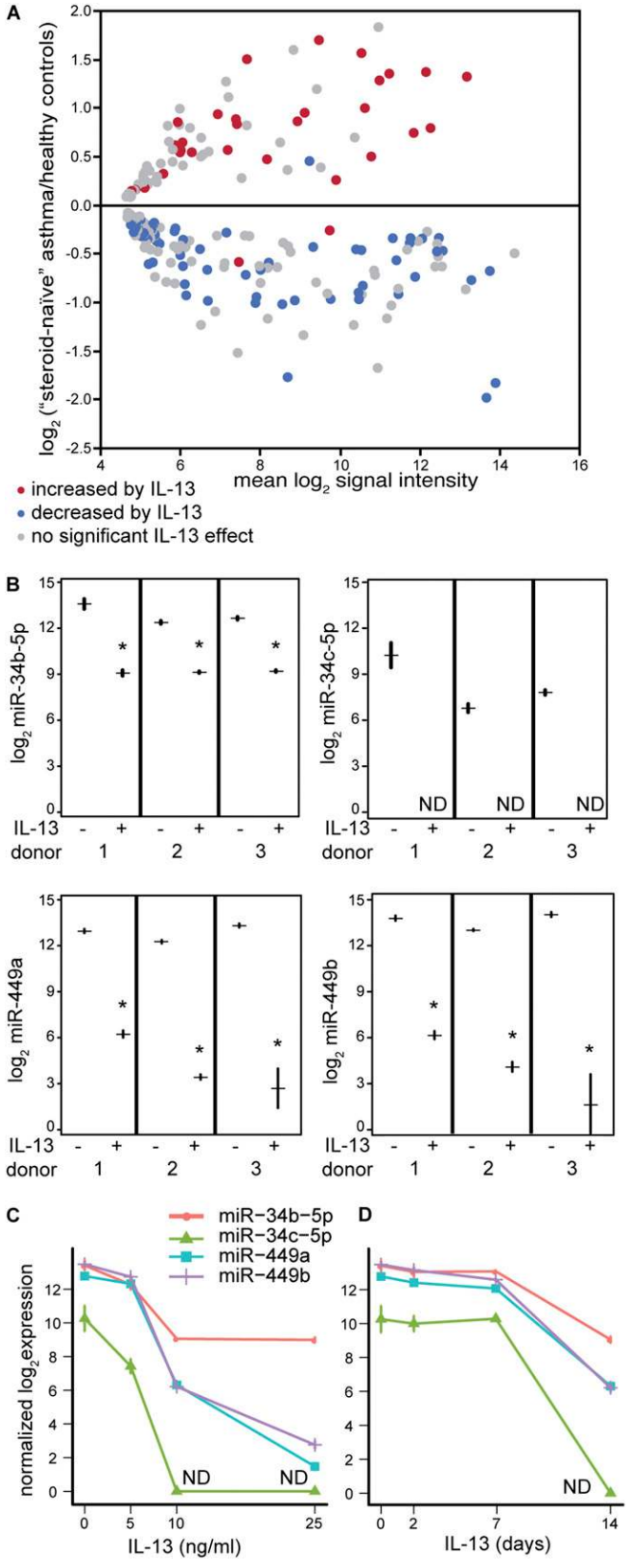

Figure 5. IL-13 stimulation alters micro-RNA (miRNA) expression in cultured human bronchial epithelial cells. (*A*) *In vitro* stimulation with IL-13 induces many miRNA changes similar to those seen in asthma. Each point represents a miRNA that was differentially expressed in steroid-naive asthma versus control samples by microarray analysis. An additional microarray experiment was performed to determine the effects of IL-13 stimulation on cultured cells from three donors and points are color-coded to indicate miRNAs that were significantly increased or decreased (false discovery rate < 0.05) or not significantly altered by IL-13 stimulation. (*B*) Expression of miR-34c-5p, miR-34b-5p, miR-449a, and miR-449b-5p was repressed in epithelial cells from three human donors exposed to IL-13 (10 ng/ml) for 14 days (three to four replicate wells per donor for each condition). The repression of miR-34/449 family members by IL-13 is time-dependent (*C*, 10 ng/ml IL-13 for 0–14 d, single donor) and dose-dependent (*D*, 14 d exposure to IL-13 at 0–25 ng/ml, single donor). All cells were cultured at air-liquid interface for a total of 21 days and exposed to IL-13 during the final 1–14 days of culture.

exposure, and that this repression persists despite corticosteroid treatment.

Members of the miR-34/449 family are closely related (*see* Figure E3) and have been implicated in regulation of epithelial cell proliferation and differentiation. Recently, miR-449 was found to be a critical regulator of differentiation of airway ciliated cells (18). miR-449 expression increased several orders of magnitude during ciliary differentiation *in vitro* (18, 34), localized to ciliated cells by *in situ* hybridization, and promoted centriole multiplication and multiciliogenesis, in part by targeting *NOTCH1* mRNA (18). Notch may play a specific role in airway mucous metaplasia in asthma because Notch antagonists prevent IL-13–induced mucus metaplasia in human airway epithelial preparations (35) and local inhibition of Notch signaling through inhalation of a γ-secretase inhibitor reduces mucous cell production in an animal model of asthma (36). Our analyses of bronchial epithelial miR-34/449 levels in asthma and after IL-13 stimulation are consistent with the hypothesis that IL-13–induced inhibition of miR-34/449 family members results in increased Notch expression and activity and alters mucociliary differentiation to reduce ciliated cells and increase mucous cells. We found some evidence for inverse correlations between miR-449 and *NOTCH1* mRNA levels in asthma, which is also consistent with this hypothesis. Although mucociliary differentiation is a complex process that is regulated at many levels, our data suggest that miR-34/449 family members may contribute to alterations in differentiation seen in asthma.

Individual miRNAs can target large sets of mRNAs, suggesting that the many epithelial miRNA expression changes seen in asthma could have a substantial impact on gene expression. We used computational approaches similar to those previously used by others to search for evidence of various types of regulatory interactions (26, 27). We saw strong evidence that miRNA abnormalities in asthma were associated with changes in expression of predicted targets of these miRNAs. When comparing subjects with steroid-naive asthma with healthy control subjects, we found that miRNA targets were significantly more likely than nontargets to change in the same direction (e.g., decrease in a miRNA and decrease in its target). This would not be expected to result from the direct inhibitory effects of miRNAs. Similar miRNA-target expression associations in other systems have been connected to the existence of incoherent feed forward loops (*see* Figure E5B), which are believed to confer robustness by buffering changes in gene expression (29, 30). Further computational analysis suggested that genes involved in transcriptional regulation (especially zinc-finger proteins) and in chromatin organization were overrepresented in these inferred feed forward loops. We therefore speculate that miRNA-containing feed forward loops buffer complex, multicomponent airway epithelial cell regulatory networks that are altered in

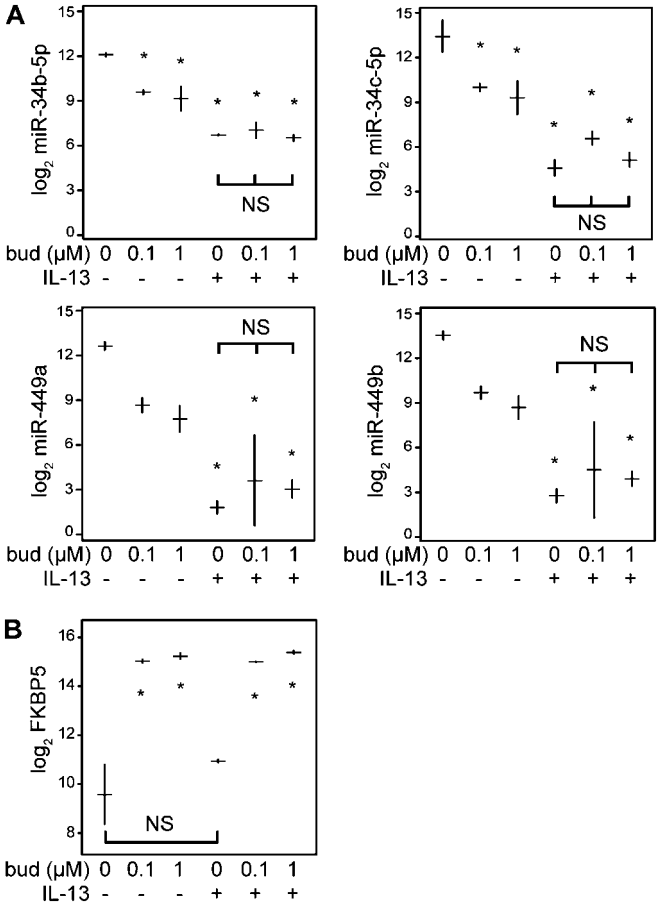

Figure 6. IL-13–induced repression of miR-34/449 family members persists despite corticosteroid treatment. (*A*) Human bronchial epithelial cells were treated with budesonide (bud), IL-13 (10 ng/ml), or both for 14 days and micro-RNA (miRNA) levels were measured by quantitative polymerase chain reaction. (*B*) The corticosteroid-induced mRNA *FKBP5* was measured by quantitative reverse transcriptase polymerase chain reaction in the same samples. $^*P < 0.05$ compared with untreated cells by Tukey-Kramer test. NS = not significant.

asthma. These networks are highly complex and many mechanisms other than miRNA-mediated inhibition can account for altered mRNA levels in disease. When we excluded healthy subjects and focused on analyzing miRNA-mRNA correlations in subjects with steroid-naive asthma, we found that miRNAs and their predicted targets were more inversely correlated than expected by chance ($P < 0.0001$). These results are consistent with the known direct inhibitory effects of miRNAs and suggest that miRNAs play a larger role in buffering asthma-induced gene expression changes in some subjects with asthma than in others. These computational analyses suggest systems-level hypotheses about the role of airway epithelial miRNAs and their targets in asthma, but have many limitations including problems with accurate prediction of miRNA targets and the failure to account for highly complex and diverse regulatory mechanisms. Hence, although correlative analyses suggest widespread effects of altered miRNA levels on gene expression, more work is required to identify specific miRNA:mRNA interactions that are important in asthma pathogenesis.

This study adds substantially to the understanding of airway epithelial miRNA changes in asthma. One prior study of miRNA expression in the lung in asthma found no differences from healthy control subjects (20). That study used bronchial biopsies, which contain a mixed and variable population of cells. We speculate that focusing on a specific cellular compartment of the airway (the epithelium) minimizes the effects of variability in constituent cell populations. A more recent study (19) used microarrays to study miRNAs in cultured primary bronchial epithelial cells from subjects with asthma on a variety of treatment regimens and healthy control subjects. That study identified 66 miRNAs as differentially expressed by array (fold difference >1.5 and unadjusted $P \leq 0.10$) and confirmed three of these changes by qPCR. Our study used freshly isolated (never cultured) cells, separated subjects according to treatment regimen, and involved a larger number of subjects. Despite our use of more stringent statistical significance testing, we identified and verified a substantially larger set of miRNA abnormalities. Furthermore, none of their three qPCR-confirmed miRNA findings were reproduced in our data. One major difference between that study and ours that may account for these differences is that they studied airway epithelial cells that were cultured over time. Freshly isolated cells are more likely to preserve cues from the *in vivo* disease environment (e.g., local IL-13 driven inflammation in asthma). Accordingly, our data further provide new insights into mechanisms of epithelial miRNA regulation by identifying IL-13 as a mediator that can induce many of the miRNA changes seen in asthma.

Our study has some specific limitations. First, our microarray and qPCR analyses identify many abundant and differentially expressed miRNAs in the epithelium in asthma, but do not necessarily measure all potentially biologically important miRNAs. Microarray probes may not detect less abundant miRNAs and may fail to distinguish some miRNAs from other RNAs with similar sequences, including other members of the same miRNA family. To account for this, we used qPCR, which has greater sensitivity and specificity, to validate selected miRNA findings. Second, although our data indicate that miRNA abnormalities we observed in asthma were not fully corrected by ICS, it is possible that more prolonged or intensive ICS treatment could have a larger effect. Third, although our study was adequately powered to detect many differences in miRNA expression, additional larger studies are required to better understand how miRNA expression may differ within subgroups of subjects with asthma. Our finding that 4 of 16 steroid-naive subjects with asthma had miRNA expression patterns similar to those seen in healthy control subjects is intriguing, but further studies are required to determine whether this is a stable characteristic of certain individuals with asthma, and whether this more normal pattern of miRNA expression reflects mechanistic or clinical differences in these individuals with asthma. Fourth, we recognize that even a small percentage of inflammatory cells in our epithelial brushings could yield detectable miRNAs in our analysis of these brushings. For this reason, we have performed cell culture experiments using human airway epithelial cells to increase our confidence that the miRNAs of interest (e.g., the miR-34/449 family) are present and differentially expressed specifically in epithelial cells under relevant conditions (IL-13 exposure). Finally, although we saw similar patterns of miRNA abnormalities in stable steroid-naive subjects (relatively mild asthma) and steroid-using subjects (somewhat more severe disease), our study does not address miRNA expression during asthma exacerbations or in individuals with severe asthma.

In summary, our studies of airway epithelial samples have identified widespread changes in miRNA levels in asthma, including repression of epithelial miRNAs that are known to regulate epithelial cell differentiation. Inhaled steroids tend to normalize these abnormalities in miRNA expression, but their effect is modest. Given the pathogenic role that airway epithelial cells play in asthma and the paucity of available therapeutics that target these

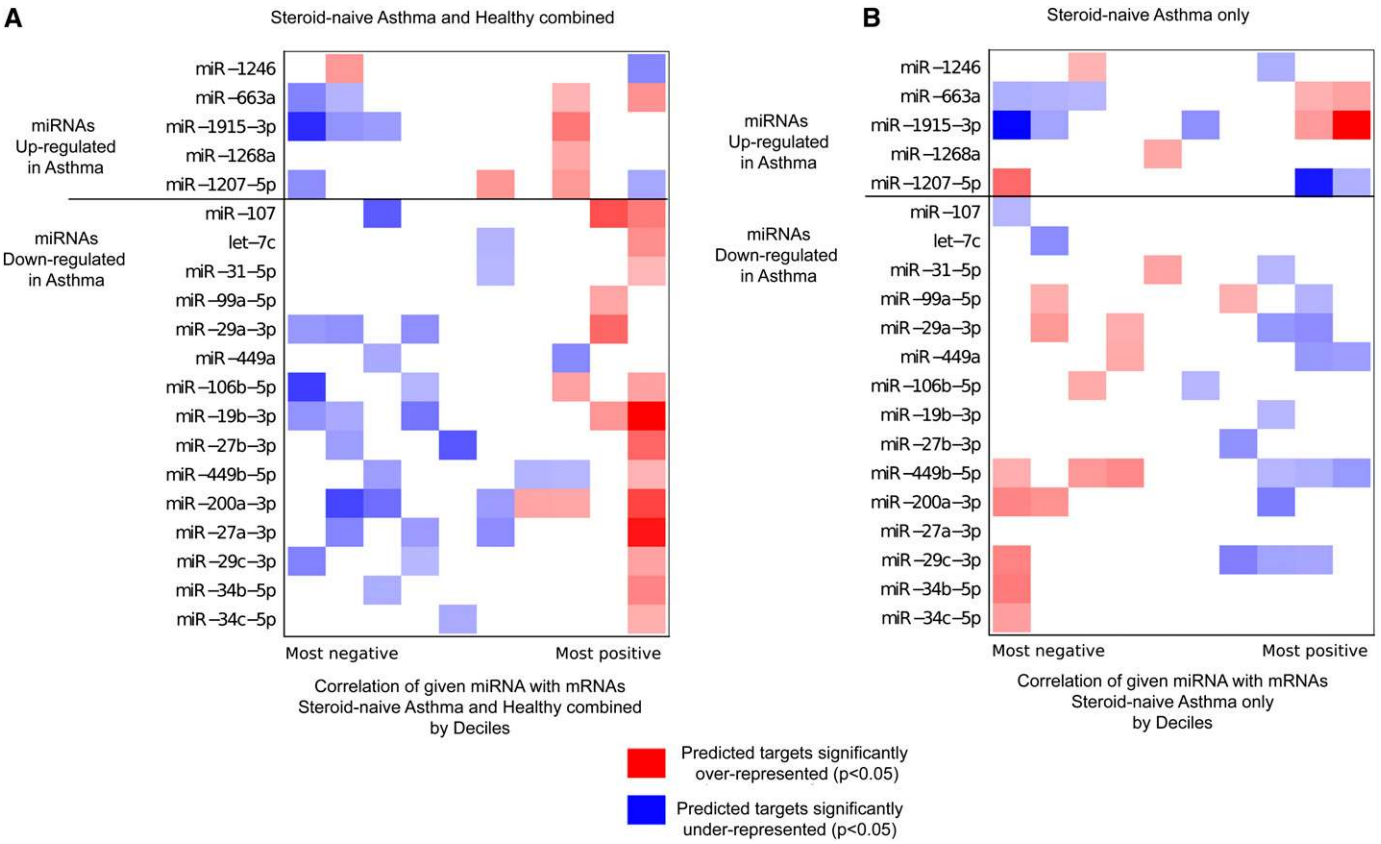

Figure 7. Relationship between expression of asthma-associated micro-RNA (miRNAs) and their predicted target mRNAs. Pearson correlation was performed between expression levels of any given miRNA (*y* axis) and the full complement of epithelial mRNAs from the same subjects, and deciles of correlation coefficients are presented from most negative (*x* axis, *left side*) to most positive (*x* axis, *right side*). Within each decile, the underrepresentation and overrepresentation of mRNAs that are predicted targets of the given miRNA were assessed using chi-square testing (26). (*A*) When steroid-naive subjects with asthma and healthy control subjects are considered together, positive correlations predominate. (*B*) When steroid-naive subjects with asthma are considered in isolation, negative correlations predominate.

cells, epithelial miRNAs or their mRNA targets may be valuable therapeutic targets.

Author disclosures are available with the text of this article at www.atsjournals.org.

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