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MicroRNA-21 is up-regulated in allergic airway inflammation and regulates IL-12p35 expression¹

Thomas X. Lu^{*,†,‡}, Ariel Munitz^{*}, and Marc E. Rothenberg^{*,2}

^{*}Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Ave, Cincinnati, OH, 45229

[†]Graduate Program of Molecular and Developmental Biology, University of Cincinnati College of Medicine, 3333 Burnet Ave, Cincinnati, OH, 45229

[‡]Physician Scientist Training Program, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Ave, Cincinnati, OH, 45229

Abstract

Allergic airway inflammation is characterized by marked *in situ* changes in gene and protein expression, yet the role of microRNAs (miRNAs), a new family of key mRNA regulatory molecules, in this process has not yet been reported. Using a highly sensitive microarray based approach, we identified 21 miRNAs with differential expression between doxycycline-induced lung-specific IL-13 transgenic mice (with allergic airway inflammation) and control mice. In particular, we observed over-expression of miR-21 and under-expression of miR-1 in the induced IL-13 transgenic mice compared to control mice. These findings were validated in two independent models of allergen-induced allergic airway inflammation and in IL-4 lung transgenic mice. While IL-13 induced miR-21 expression was IL-13 receptor alpha 1 dependent, allergen induced miR-21 expression was mediated mainly independent of IL-13 receptor alpha 1 and STAT6. Notably, predictive algorithms identified potential direct miR-21 targets among IL-13-regulated lung transcripts such as IL-12p35 mRNA that was decreased in IL-13 transgenic mice. Introduction of pre-miR-21 dose-dependently inhibited cellular expression of a reporter vector harboring the 3'UTR of IL-12p35. Moreover, mutating miR-21 binding sites in IL-12p35 3'UTR abrogated miR-21 mediated repression. In summary, we have identified a miRNA signature in allergic airway inflammation, which includes miR-21 that modulates IL-12, a molecule germane to T helper cell polarization.

Keywords

Allergy; lung; inflammation; gene regulation

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²Address correspondence to Marc Rothenberg, MD, PhD, Division of Allergy and Immunology, Cincinnati Children's Hospital Medical Center, 3333 Burnet Ave, Cincinnati, OH, 45229. Phone: 513-636-7210; Fax: 513-636-3310; Rothenberg@cchmc.org.

Introduction

Asthma is a chronic inflammatory disease characterized by inflammation of the airways, tissue remodeling, and a decline in respiratory function (1–3). In the United States, 5–10% of the population suffer from asthma, representing a common diagnosis for pediatric hospital admission and a major cause for lost days at work and school (4). Despite intense ongoing research, the incidence of the disease continues to rise, necessitating the need for new scientific inquiry.

MicroRNAs (miRNAs) are single stranded non-coding RNAs of 19–25 nucleotides in length that mediate post-transcriptional silencing of target genes (5, 6). In animals, miRNAs usually bind to complementary sites in the 3' untranslated region (UTR) of target genes and regulate target gene expression by either translational inhibition, mRNA degradation, or both (7). MiRNAs are involved in diverse biological processes, including development, stress response, cancer, and cardiac hypertrophy, implicating them in normal and pathological processes (8). However, to the best of our knowledge, studies relevant to asthma or asthma risk are still lacking, except for a recent report demonstrating that a single nucleotide polymorphism at the 3' untranslated region of HLA-G, an asthma-susceptibility gene, affects the binding of three miRNAs to this gene (9). Allergic airway inflammation may be particularly sensitive to miRNA regulation since it is characterized by marked changes in gene and protein expression in the lung (10–12). For example, lung over-expression of IL-13, a key T helper type 2 (Th2) cell derived effector cytokine in asthma pathogenesis, induces allergic airway inflammation characterized by prominent inflammatory cell accumulation, goblet cell metaplasia (mucus production), smooth muscle hyperplasia, and airway hyperresponsiveness (AHR), processes that are mediated by marked changes in gene and protein expression including cytokines and chemokines (13).

Here, we used both miRNA microarray and qRT-PCR based approaches to assess miRNA expression in murine models of allergic asthma. We define a miRNA signature consisting of 21 differentially regulated miRNAs in IL-13-induced experimental asthma. Focusing on the most highly induced miRNA, we subsequently demonstrate that miR-21 regulates murine IL-12p35, a key cytokine associated with balancing T helper cell polarization.

Material and Methods

Mice

Bitransgenic mice bearing CCSP-rtTA and (tetO)₇CMV-IL-13 transgenes were generated in which IL-13 was expressed in a lung-specific manner that allowed for external regulation of transgene expression (14). Transgene expression was induced by feeding bi-transgenic mice doxycycline-impregnated food for 4 weeks. Constitutive IL-4 lung transgenic mice under the control of the CC10 promoter were kindly provided by Dr. Fred Finkelman (15). The IL-13 receptor alpha 1 and STAT6 deficient mice were described previously (16, 17).

Experimental asthma induction

Experimental asthma was induced by injection with 100 µg ovalbumin (OVA) and 1 mg aluminum hydroxide as adjuvant twice, followed by two 50 µg OVA or saline intranasal

challenges 3 days apart, starting a least 10 days after the second sensitization. Mice were sacrificed 18–24 hours after the second challenge (10). *Aspergillus fumigatus* antigen-associated asthma was induced by challenging mice intranasally three times a week for 3 weeks with 100 µg (50 µl) of *A. fumigatus* extract or 50 µl of saline each time. Mice were sacrificed 48 hours after the last challenge (18). Experimental asthma from IL-13 bitransgenic mice was induced by feeding bi-transgenic mice doxycycline-impregnated food for 4 weeks, as described (19). The control group received no doxycycline. Mice were sacrificed at the end of 4 weeks of IL-13 induction. Intratracheal delivery of IL-13 was performed as previously described (20). 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.

RNA extraction and microarray experiments

Total RNA was isolated from lung tissue using miRNeasy Mini Kit according to manufacturer's protocol (Qiagen, Valencia, CA). RNA quality was assessed by using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) and only samples with RNA integrity number >8 were used. RNA samples from these tissues were subsequently fluorochrome labeled by using the miRCURY Hy3/Hy5 labeling kit and hybridized to Exiqon miRCURY Locked Nucleic Acid (LNA) array (Version 10.0), comprising LNA modified probes for all mouse miRNAs in the release 10.0 of the miRBase microRNA Registry (21, 22). The microarray analysis was conducted in the Genomics and Microarray Core Facility at the University of Cincinnati. Data were normalized to a common reference sample using R statistical software (R Foundation for Statistical Computing) (23). MiRNA expression data were analyzed and displayed using Genesis (version 1.7.2) (24). The microarray data have been submitted to ArrayExpress database in compliance with minimum information about microarray experiment (MIAME) standards (ArrayExpress accession number: E-MEXP-1992, www.ebi.ac.uk/arrayexpress).

Quantitative Assessment of miRNA levels

Levels of miRNA expression were measured quantitatively by using TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) as described following manufacturer's protocol and assayed on the Applied Biosystems 7300 Real-Time PCR System (25). Normalization was performed with snoRNA202 (26). Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated using the comparative Ct method as previously described (27).

miRNA *in situ* hybridization

In situ hybridizations were performed in 8 µm cryosections from the lung of saline and *Aspergillus fumigatus* challenged mice (28). Slides were stained using an automated system, the Discovery XT (Ventana Medical Systems, Tucson, AZ), according to manufacturer's protocols. Slides were pre-treated with 100 µl of RiboPrep (Ventana Medical Systems, Tucson, AZ) for 20 minutes at 37°C. After rinsing the slides with Reaction Buffer (Ventana Medical Systems, Tucson, AZ), slides were then treated with 100 µl of RiboClear (Ventana

Medical Systems, Tucson, AZ) for 12 minutes at 37°C. The slides were rinsed again and then digested with Protease 3 (Ventana Medical Systems, Tucson, AZ) for 12 minutes at 37°C. After protease digestion, the digoxin labeled Locked Nucleic Acid (LNA) scrambled control probe and LNA miR-21 anti-sense probe (Exiqon A/S, Denmark) were hybridized to the slides at 52°C for 6 hours. Following post-hybridization washes with 0.1X SSC buffer at 47°C, 100 µl of rabbit anti-digoxin (Sigma, St. Louis, MO) antibody, diluted 1:2000 in Discovery Antibody Diluent (Ventana Medical Systems, Tucson, AZ), was applied to the slides for 30 minutes at room temperature. The slides were rinsed and then incubated with 100 µl of UltraMap anti-rabbit AP (Ventana Medical Systems, Tucson, AZ) for 16 minutes at room temperature. Color detection was done using a ChromoMap Blue Kit (Ventana Medical Systems, Tucson, AZ). Slides were counterstained with Nuclear Fast Red (Polyscientific, Bayshore, NY), coverslipped and mounted for viewing.

Cell Culture

Raw264.7 (ATCC No.: TIB-71, American Type Culture Collection, Manassas, VA) and NIH 3T3 (ATCC No.: CRL-1658, American Type Culture Collection, Manassas, VA) cells were maintained in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. The murine lung epithelial (MLE) cell line MLE15 and murine fetal lung mesenchyme (MFLM) cell line MFLM4 were kindly provided by Dr. Jeffrey Whitsett (Cincinnati Children's Hospital) and were maintained in HITES media supplemented with 4% FBS and 1% penicillin/streptomycin (29). Type II alveolar cells were isolated and cultured as previously described (30) and were kindly provided by Dr. Timothy Weaver (Cincinnati Children's Hospital). Bone marrow derived macrophages were prepared by culturing cells in medium containing 30% L929 conditioned medium (31). Bone marrow derived dendritic cells were prepared by culturing cells in medium supplemented with 40 ng/mL GM-CSF, 20 ng/mL IL-4, 10% FBS and 1% penicillin/streptomycin (32). Neutrophils were generated by using the thioglycollate-induced peritonitis model according to previously published methods (33). After 4 hours, mice were sacrificed, the peritoneal cavity was rinsed with 10 mL PBS and neutrophil purity was 95% as determined by Diff-Quik staining. For LPS activation of Raw264.7 cells, the cells were stimulated with 1 µg/mL LPS (Strain: 055:B5, Sigma, St. Louis, MO) for 24 hours.

Target Predictions

miRNA targets were predicted by using miRanda (34) and TargetScan (35) algorithms. Targets predicted by both algorithms were intersected with IL-13 down-regulated genes (19) and considered for further analysis. NFIB binding site was searched from the 1KB region upstream of 1st exon of all IL-13 induced genes in the lung using the TraFaC program which analyzes non-coding genomic sequences that are evolutionarily conserved between mouse and human (36).

IL-12p35 expression analysis

Total RNA was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). Samples were analyzed by TaqMan quantitative RT-PCR for IL-12p35 transcripts and normalized to HPRT1 (primer and probe sets from Applied

Biosystems, IL-12p35 assay ID: Mm00434169_m1, HPRT1 assay ID: Mm00446968_m1). Relative expression was calculated using the comparative Ct method.

Luciferase Reporter Plasmid Construction

The full length mL-12p35 3'UTR was amplified with the following primers: mL-12p35 Forward: ggccactagtGAAAGGCTCAAGGCCCTCT, mL-12p35 Reverse: ggccaagcttGAACCACAAAATAAGGTATGTTTCAA, and cloned between the Spe I and Hind III sites of the multi cloning region in the 3'UTR of the firefly luciferase expression vector pMIR-Report (Ambion, Austin, TX) and designated pmIL-12p35. The pMIR-21 vector has a perfect miR-21 binding site cloned into the 3'UTR region of the pMIR-Report vector (Ambion, Austin, TX).

Site-directed mutagenesis of miR-21 binding site in pmIL-12p35

The mutations in the miR-21 binding site of pmIL-12p35 were introduced with the QuikChange II XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol and designated pmIL-12p35 Mut. The mutagenesis primers are as follows: mL-12p35 Mut Forward 5'-GGGTGACTGAGTGTTCATAAACACTTTGGCACAAAAACAATTTCGAATTCAGT TCTTGCTCTTCTGCTAA-3', mL12p35 Mut Reverse: 5'-TTAGCAGAAGAGCAAGAAGTGAATTCGAATTGTTTTGTGCCAAAGTGTTTATG AAAACACTCAGTCACCC-3'. The constructs were sequenced to prove sequence integrity.

Transfection of pre-miRNA expression plasmid and reporter plasmids

293T cells were cotransfected with 500 ng of firefly reporter plasmids, 25 ng of reference renilla luciferase reporter plasmid pGL4.73 (Promega, Madison, WI), and 500 ng of pMIRNA1 – Pre-miR-21 or 500 ng of pMIRNA1 control vector (SystemBiosciences, Mountain View, CA) using Lipofectamine reagents as per the recommended conditions (Invitrogen, Carlsbad, CA). Lysates were prepared at 36 h post-transfection.

Dual-Luciferase Reporter Assays

Transfected cells were lysed in 300 µl of Passive Lysis Buffer (Promega, Madison, WI) for 30 min at room temperature. Firefly and renilla luciferase activity were measured using the Promega Dual Luciferase Assay kit following the manufacturer's instructions and a Veritas Microplate luminometer (Turner Biosystems, Sunnyvale, CA). All measurements were normalized for renilla luciferase activity to correct for variations in transfection efficiencies and non-miR-21-specific effects of miRNA transfection on enzymatic activity.

Results

Expression profiling of miRNA in IL-13 lung transgenic mice

To identify miRNAs differentially expressed after IL-13 induction in IL-13 lung transgenic mice, miRNA expression of total lung tissue was profiled using Exiqon miRCURY LNA array (Version 10.0), comprising LNA modified probes for all mouse miRNAs available in the miRBase microRNA Registry (21, 22). Out of the 579 mouse miRNAs assayed, 131

miRNAs were expressed above background levels. In agreement with previous reports, several miRNAs, including miR-23b, miR-24, miR-30b, miR-451, and members of the let-7 family, were highly enriched in the mouse lung, giving strong hybridization signals on the miRNA arrays (data not shown) (37). Comparing doxycycline-induced IL-13 transgenic mice with control mice that received no doxycycline, 21 miRNAs were found to be differentially expressed at $p < 0.01$, suggesting that they were regulated directly or indirectly by IL-13 (Fig. 1A). Notably, miR-21 was the most up-regulated miRNA on the array and miR-1 was the most down-regulated miRNA. To validate the results of the microarray platform, we determined the expression of a subset of miRNAs by real time RT-PCR (Fig. 1B). We found strong correlation between our microarray profiling and real-time RT-PCR data (Pearson correlation coefficient: 0.99, $p = 0.001$, Fig. 1C).

MiR-21 is induced in three separate models of experimental asthma while miR-1 is repressed in the same models

We aimed to determine levels of miR-21 and miR-1 in three independent asthma models (OVA, *Aspergillus fumigatus* and induced IL-13 bitransgenic mice) using real-time RT-PCR analysis. In the OVA model, mice were sensitized by two i.p. injections of OVA and aluminum hydroxide. The *Aspergillus fumigatus* model involves a unique mucosal sensitization route (intranasal) compared with the OVA model (10, 18, 20, 38, 39). While the methods of experimental asthma induction are different, all three asthma models have similar phenotypes, including Th2-associated eosinophilic inflammation, mucus production, and AHR (10, 18, 19, 40, 41). First, we examined the inducible IL-13 bitransgenic system and indeed validated that dox-treated IL-13 bitransgenic mice had a 4.62-fold increase in miR-21 ($p < 0.01$) and a 0.28-fold repression of miR-1 ($p < 0.05$) compared to control mice that received no doxycycline (Fig. 1B). Second, we examined the OVA-induced asthma model and demonstrated that OVA-challenged mice had a 2.64-fold ($p < 0.0001$) induction of miR-21 and a 0.34-fold repression of miR-1 ($p < 0.001$) compared to saline challenged mice (Fig. 2A–B). Third, we examined the *Aspergillus fumigatus* model of experimental asthma and demonstrated that antigen challenged mice have a 4.01-fold increase in miR-21 level ($p < 0.0001$) and a 0.55-fold repression of miR-1 ($p < 0.01$) compared with control mice (Fig. 2A–B). In these models, the BALF eosinophil levels were $1.15 \pm 0.34 \times 10^4$, $3.39 \pm 0.76 \times 10^6$ and $4.94 \pm 0.88 \times 10^5$ cells following IL-13, OVA, and *Aspergillus fumigatus* challenge, respectively, as reported (39). Finally, to determine if the induction of miR-21 could be mediated by IL-4, we determined miR-21 expression level in IL-4 lung transgenic mice and found that IL-4 transgenic mice had a 5.81 ± 1.06 -fold induction of miR-21 ($p < 0.01$) compared to wild type mice (Fig. 2C).

MiR-21 is induced predominantly by an IL-13 receptor alpha 1 independent pathway

We focused on miR-21 as this was the most markedly changed miRNA and one implicated in processes germane to asthma including cell growth and differentiation, tissue remodeling and myeloid cell function (42–45). Having identified miR-21 as both an IL-13 and allergen induced gene, we were interested in determining if the IL-13 or allergen-induced expression was IL-13R α 1 dependent, as we have recently reported that this receptor mediates some of the key cardinal features of experimental asthma (airway hyperresponsiveness and goblet cell metaplasia), but not leukocyte accumulation (39). First, we delivered IL-13

intratracheally to wild type and IL-13R α 1 gene deficient mice and demonstrated that IL-13 induced miR-21 was IL-13R α 1 dependent (Fig. 3A). We then examined both the OVA-induced model and the *Aspergillus fumigatus* model of experimental asthma using wild-type and IL-13R α 1 gene deficient mice. Notably, this analysis revealed that both OVA and *Aspergillus fumigatus* induced miR-21 expression largely through an IL-13R α 1 independent pathway. OVA-challenged wild type mice had a 3.13 ± 0.22 -fold induction of miR-21, while OVA challenged gene targeted mice had a 2.39 ± 0.13 -fold induction ($p < 0.01$ for saline vs. OVA in both wild type and gene targeted mice). *Aspergillus fumigatus* challenged wild type mice had a 3.11 ± 0.10 -fold induction of miR-21, while *Aspergillus fumigatus* challenged gene targeted mice had a 2.95 ± 0.23 -fold induction ($p < 0.01$ for saline vs. *Aspergillus fumigatus* in both wild type and gene targeted mice) (Fig. 3B–C). We subsequently determined that miR-21 was induced through a STAT6 independent mechanism in OVA challenged mice (Fig. 3D).

MiR-21 is expressed in cells of monocyte/macrophage lineage in the allergic lung

To determine the cell type specific localization of miR-21, *in situ* hybridization (ISH) was carried out on cryosections of *Aspergillus fumigatus* challenged lungs using LNA anti-miR-21 and scrambled control probes (28, 46). MiR-21 was primarily detected in the cytoplasm of mononuclear and multi-nucleated myeloid cells with morphology resembling monocytes/macrophages (Fig. 4A). To determine if these cells are indeed in the monocyte/macrophage lineage (both are CD68+), we performed anti-CD68 staining on serial sections. Serial sectioning revealed that anti-CD68 staining was associated with miR-21 expression, at least in part (Fig. 4B). Staining of doxycycline induced IL-13 bitransgenic mouse lungs showed similar results (Supplementary Fig. 1). We quantified the number of miR21 positive cells as a function of total airway macrophages as determined by CD68+ staining in serial sections and found that 64 ± 6.8 % of (mean \pm S.D, n=3 mice) CD68+ cells were miR-21 positive. In addition, we determined the expression profile of miR-21 in different cell types and found that bone marrow derived macrophages and dendritic cells expressed relatively high levels of miR-21 compared with lung epithelial cells, fibroblasts and neutrophils (Fig. 4C). The cell composition of BALF cells can be found in Supplementary Fig. 2. We subsequently demonstrated that miR-21 is inducible in a murine macrophage cell line by LPS treatment (Fig. 4D).

MiR-21 targets in the allergic lung

We searched for miR-21 mRNA targets using target-prediction software miRanda and TargetScan. It has been reported that integration of miRNA target predictions from multiple algorithms substantially increases the functional correlations and decreases the false positive rate compared to single algorithms (47, 48). Common predicted targets between miRanda and TargetScan algorithms arrived with a list of 37 predicted targets (Table I). While some of the targets of miRNAs are modulated through translational inhibition only, the majority of targets have mRNA level changes inverse to their respective miRNA regulator (49, 50). We thus intersected the 37 predicted targets with IL-13 down-regulated genes and arrived at a list of IL-13 regulated target genes (Table II). Notably, IL-12p35 was predicted to have a strong miR-21 binding site conserved across species (Fig. 5A). We determined the

expression level of IL-12p35 and found that it was indeed down-regulated in all three of the employed asthma models (Fig. 5B).

MiR-21 targets IL-12p35

To determine whether IL-12p35 is a molecular target of miR-21, we constructed a luciferase reporter vector containing the full length mIL-12p35 3'UTR, as well as a positive control vector harboring a perfect miR-21 complementary sequence in the 3'UTR region. Transfecting the miR-21 expression vector inhibited the expression of the luciferase reporter vector containing the mIL-12p35 3'UTR as well as the expression of the positive control vector, while there was no effect in the luciferase vector that did not contain a miR-21 binding site (Fig. 5C). The reduced luciferase activity of mIL-12p35 and pMiR-21 vectors at baseline is likely due to inhibition from both the endogenously expressed miRNAs, as well as other translational inhibition mechanisms such as the 5'-3' circularization disruption by endogenous translational inhibitor proteins that bind to the 3'UTR region (51). The inhibition of mIL-12p35 reporter was dose dependent with an ED50 of 29.84 ± 1.24 ng (7.19 ± 0.30 nM) and a plateau reached at 250 ng (60 nM) (Fig. 5D). Mutation of the seed sequence of the predicted miRNA-binding site abrogated this effect (Fig. 5E-F).

Discussion

Our study has provided a comprehensive global miRNA expression profile of an IL-13 induced asthma model. Nearly 4% of 579 miRNAs assayed displayed differential expression in IL-13 transgenic mice compared to control mice (Figure 1). This level of miRNA change is consistent with other disease states such as breast cancer, leukemia, and myocardial infarction (52–54). Notably, each miRNA has been predicted to potentially target hundreds of genes (35, 55), indicating the potential significance of small changes in miRNAs. We found that miR-21 and miR-1 were the most induced and repressed miRNAs, respectively. We validated the induction of miR-21 and repression of miR-1 in two additional independent models of allergic airway inflammation as well as the induction of miR-21 by chronic over-expression of the IL-4 transgene in the lung. We analyzed the receptor dependency of miR-21 induction and found that IL-13 induced expression of miR-21 was dependent on IL-13R α 1, but allergen induced miR-21 expression was mediated largely by an IL-13R α 1 independent pathway. Although IL-13 alone completely utilizes IL-13R α 1 to induce experimental features of asthma, this receptor has a key but not complete role in the development of allergen-induced experimental asthma. Notably, leukocyte recruitment to the lung occurs independent of IL-13R α 1 (39). As such, the finding that miR21 induction occurs independent of IL-13R α 1, indicates that miR21 induction likely is derived from (or associated) with the sustained leukocyte recruitment and/or activation in IL-13R α 1 deficient mice. Consistent with this, allergen induced miR-21 was demonstrated to occur independent of STAT6, consistent with sustained leukocyte recruitment in these mice (56). This implies that miR-21 induction may be related to the large portion of asthma signature genes that are STAT6 independent (56). The STAT6 and IL-13R α 1 independent genes that correlate with miR-21 include the C3a receptor and several chemokines (Ccl8, Ccl12, Cxcl10, data not shown) (39, 56). Notably, recent studies demonstrate that certain aspects of asthma occur through a STAT6 independent pathway (57, 58). *In situ* hybridization of asthmatic lung

revealed that miR-21 was expressed by inflammatory leukocytes most consistent with myeloid cells and in agreement with recent studies identifying miR-21 in hematopoietic cells (59–61). Cell type expression profile of miR-21 confirmed that macrophages and dendritic cells indeed had the highest level of expression compared with other cell types analyzed. Although the expression of miR-21 was in cells most consistent with macrophages and/or dendritic cells (both CD68+), this does not rule out expression in other cells that may contain less readily detectable RNA (e.g. eosinophils). Notably, miR-21 has been shown to be inducible in a human promyelocytic cell line HL-60 after phorbol 12-myristate 13-acetate (PMA) treatment, which induces macrophage like differentiation (60). We subsequently demonstrated that miR-21 is inducible in the murine macrophage cell line Raw264.7 by LPS treatment. The relationship between the LPS induced pathway and our *in vivo* finding has yet to be determined. In addition, miR-21 has been reported to target the transcriptional repressor NFIB (43), providing a mechanism by which miR-21 induction could up-regulate gene expression. We performed *in silico* analysis of all IL-13 induced genes in the lung, and identified 130 genes with potential NFIB sites in the 1KB region upstream of the 1st exon (Supplementary Table I).

Using bioinformatic approaches, we identified candidate target genes of miR-21. We further intersected this with our previous IL-13-repressed mRNA expression profiling data to identify candidate target genes that were differentially expressed, possibly because of action of these miRNAs. This analysis identified IL-12p35 as a putative target of miR-21. Transfection and reporter assays indeed identified miL-12p35 as a target gene of miR-21, with miR-21 having the ability to reduce IL-12p35 expression via the 3'UTR. The magnitude of miR-21 mediated repression of the miL-12p25 reporter vector was about 40%, similar with the reported effects of miRNA-mediated mRNA repression in other systems (44, 49, 50, 62–67). These results imply that the increased levels of miR-21 in experimental asthma contribute to the observed decrease in IL-12. IL-12 is a key cytokine derived from macrophages and dendritic cells (both CD68 positive and consistent with our *in situ* hybridization results) involved in adaptive immune responses involving Th1 cell polarization (68, 69). The ability of miR-21 to down regulate IL-12 indicates a novel checkpoint for regulating the level of this key immune mediator. These results suggest that strategies designed to deliver miR-21 may prime for Th2 and IL-13 associated responses; conversely, miR-21 inhibition has potential to drive Th1 polarization, promoting cellular responses (and classic adjuvant activity) (70, 71). Down-regulation of IL-12p35 could also affect IL-35 production and Treg cell function, leading to a pro-inflammatory phenotype (72). In addition to regulating IL-12 levels, miR-21 may regulate other processes germane to allergic airway inflammation. Notably, MiR-21 has been shown to regulate the level of the matrix metalloprotease (MMP) inhibitor RECK (42, 73, 74), and indeed we have identified this gene to be down regulated by IL-13 in the lung (Table II); it is interestingly to speculate that this could account for some of increased MMP activity seen in asthma (75, 76).

In our study, we identified miR-1 as the most down-regulated gene in the IL-13 lung transgenic mice. MiR-1 is considered to be a muscle specific miRNA (77–79), implicated in the determination of the differentiated state of muscle cells, in myogenesis and in cardiogenesis (78, 80). Down-regulation of miR-1 has been associated with cardiac and

skeletal muscle hypertrophy (81, 82). Notably, disruption of just one of the two miR-1 family members, miR-1-2, has profound consequences for development and maintenance of the heart, as miR-1-1 did not compensate for loss of miR-1-2 (80). This indicates that a stable level of miR-1 is very important for normal muscle physiology. MiR-1 is down-regulated in all three of our asthma models ranging from 1.82- to 3.57-fold. It is interesting to speculate that down-regulation of miR-1 contributes to the smooth muscle hypertrophy and re-modeling seen in asthma (83).

In conclusion, we report a miRNA signature of experimental asthma. We found miR-21 is the most up-regulated miRNA and the up-regulation is largely through an IL-13R α 1 independent pathway. Using computational and tissue culture-based assays, we identify miL-12p35 as a molecular target of miR-21. As such, miR-21 induction in experimental asthma likely leads to a concomitant decrease in miL-12p35, which likely contributes to polarization of T helper cells toward a Th2 response. This increase in the expression of miR-21 likely contributes to the action of IL-13 in the lung (and possibly asthma pathogenesis). Taken together, these results suggest a key role for miRNA in allergic lung inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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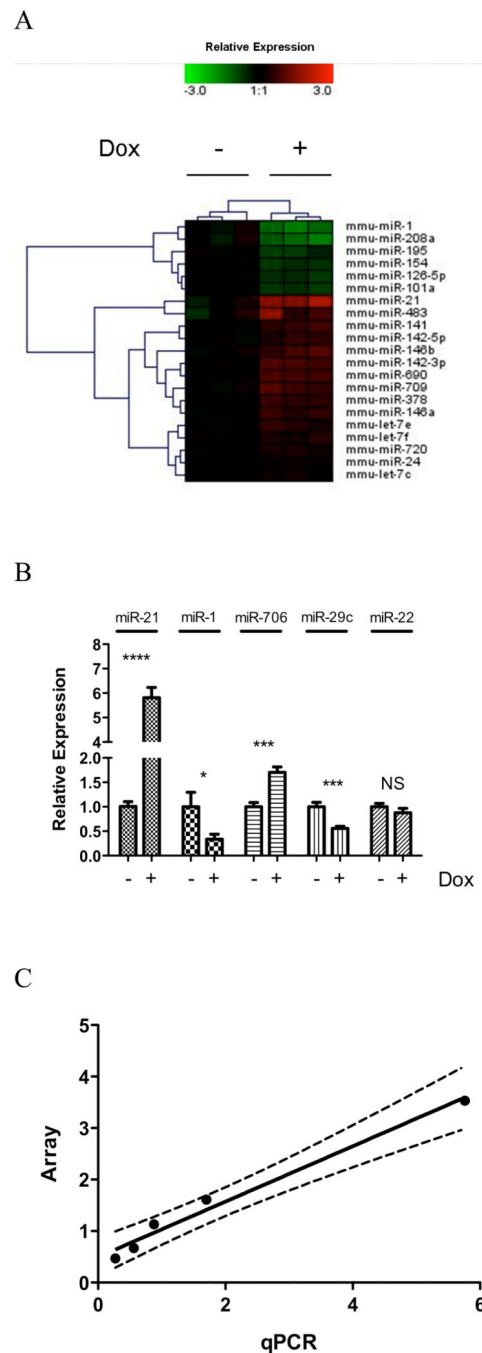


Figure 1. MiRNA expression profile in IL-13 transgenic mice lung

(A) Heat map of 21 differentially expressed miRNAs following control no doxycycline (–) and doxycycline (+) exposure for 28 days. Relative expression is log₂ transformed. (B) A qRT-PCR validation of a selected set of miRNA probes normalized to snoRNA202. NS, not significant; *, $p < 0.05$; ***, $p < 0.001$, ****, $p < 0.0001$. (C) Correlation of miRNA microarray and qRT-PCR validation; dashed line represents 95% confidence interval. Data are represented as mean \pm S.E.M; $n = 5-7$ mice per group; data representative of 3 experiments.

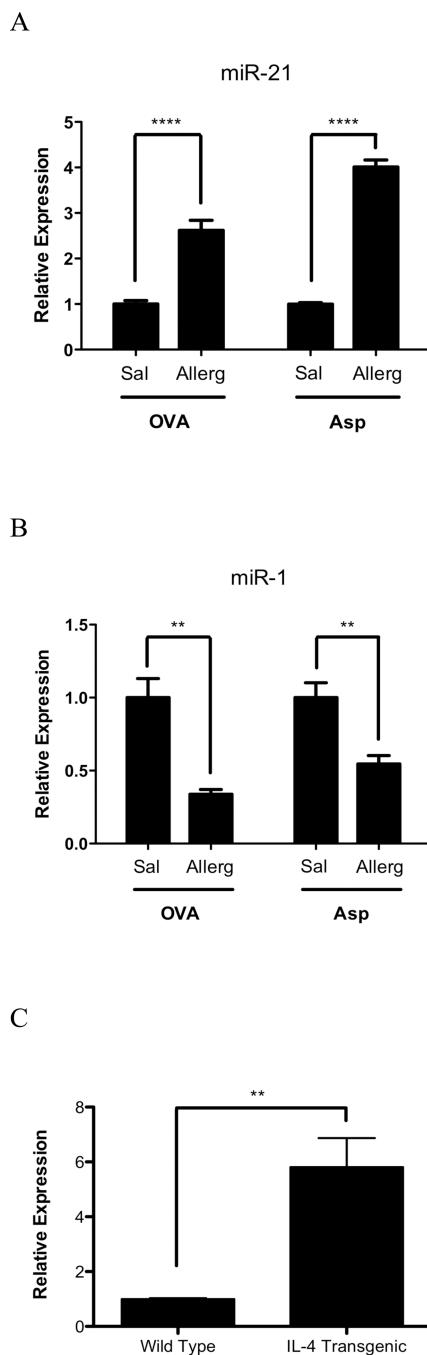
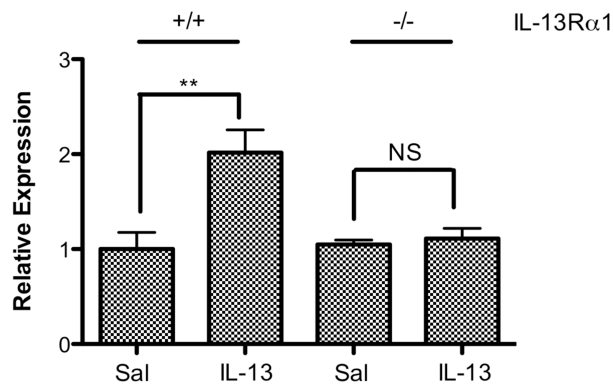


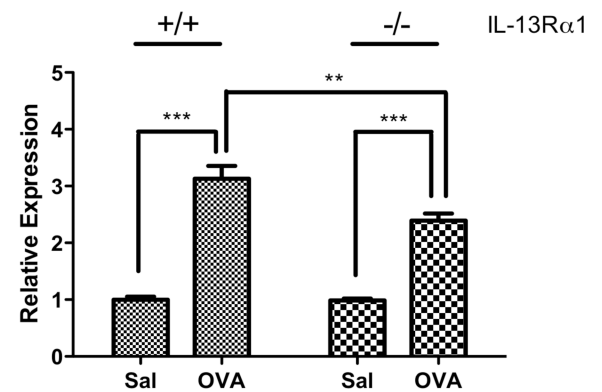
Figure 2. Expression of miR-21 and miR-1 in experimental asthma models

MiR-21 (A) and miR-1 (B) expression were assessed in OVA and *Aspergillus fumigatus* asthma models. (C) MiR-21 expression was determined in IL-4 lung transgenic mice. The relative expression levels were determined by qRT-PCR normalized to snoRNA202; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. Data are represented as mean \pm S.E.M; $n = 3-7$ mice per group; data representative of 3 experiments.

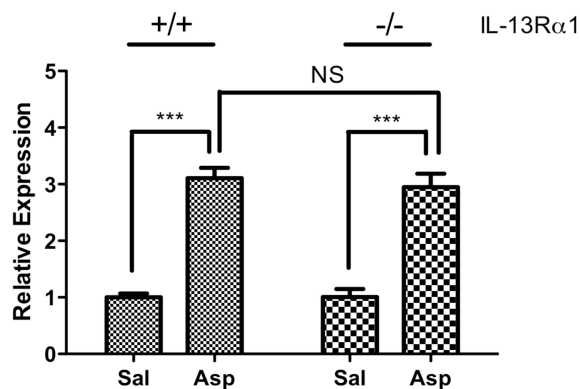
A



B



C



D

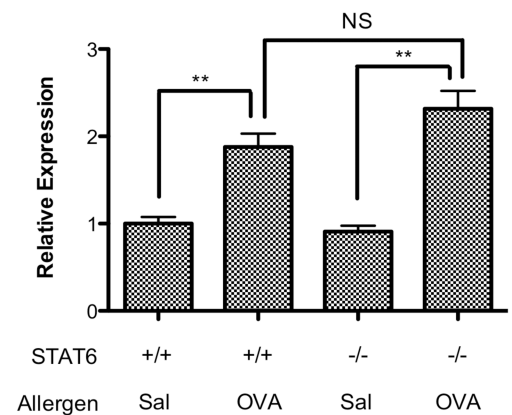


Figure 3. Expression of miR-21 in allergen challenged IL-13 receptor alpha 1 deficient mice
 (A) MiR-21 expression was determined in IL-13 challenged wild type and IL-13 receptor alpha 1 deficient mice. (B–C) MiR-21 expression was determined in OVA (B) and *Aspergillus fumigatus* (Asp) models (C) using IL-13 receptor alpha 1 ($-/-$) mice and wild type ($+/+$) controls. (D) MiR-21 expression was determined in OVA challenged wild type and STAT6 deficient mice. The relative expression levels were determined by qRT-PCR normalized to snoRNA202. NS, not significant; **, $p < 0.01$, ***, $p < 0.001$. Data are represented as mean \pm S.E.M.; $n = 3-8$ mice per group; data representative of 3 experiments.

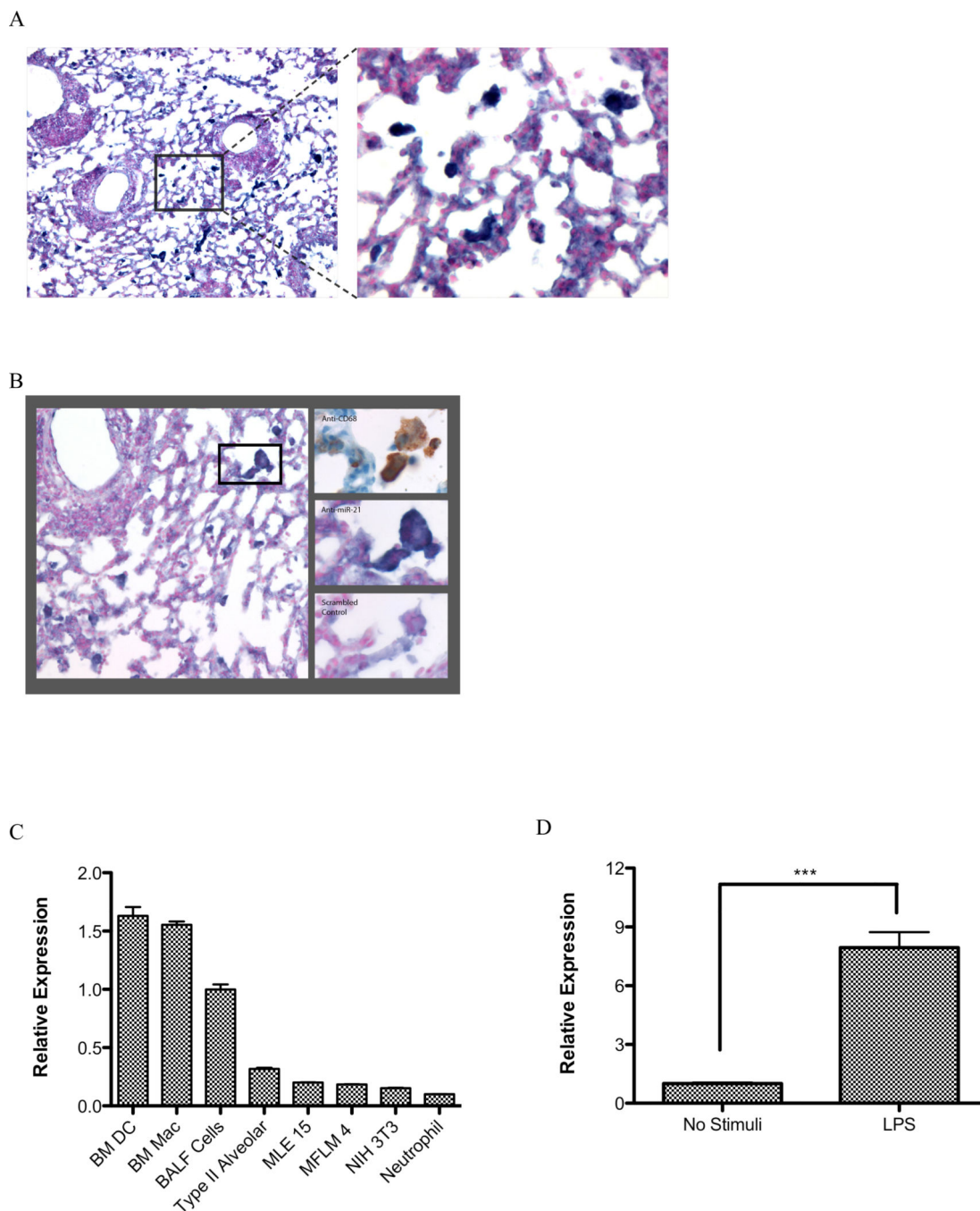


Figure 4. *In situ* hybridization of miR-21 in *Aspergillus fumigatus* challenged wild type mouse lung

Expression of miR-21 in (A–B) *Aspergillus fumigatus* challenged wild type mouse lungs, were determined by LNA-based *in situ* hybridization. (A) LNA-anti-miR-21, left: 100X field; right: 400X field; (B) Left: 200X field, LNA-anti-miR-21. Right: serial sections at 600X; right top: LNA-anti-miR-21, right middle: anti-CD68, right bottom: LNA-scrambled control probe. (C) Relative expression of miR-21 in different cell types; BM DC: bone marrow derived dendritic cells; BM Mac: bone marrow derived macrophages; BALF: bronchoalveolar lavage fluid cells; MLE15: murine lung epithelial cell line; MFLM4:

murine lung mesenchyme cell line; NIH 3T3: murine fibroblasts. (D) Relative expression of miR-21 in LPS stimulated murine macrophage cell line Raw264.7; ***, $p < 0.001$. Data are representative of three experiments.

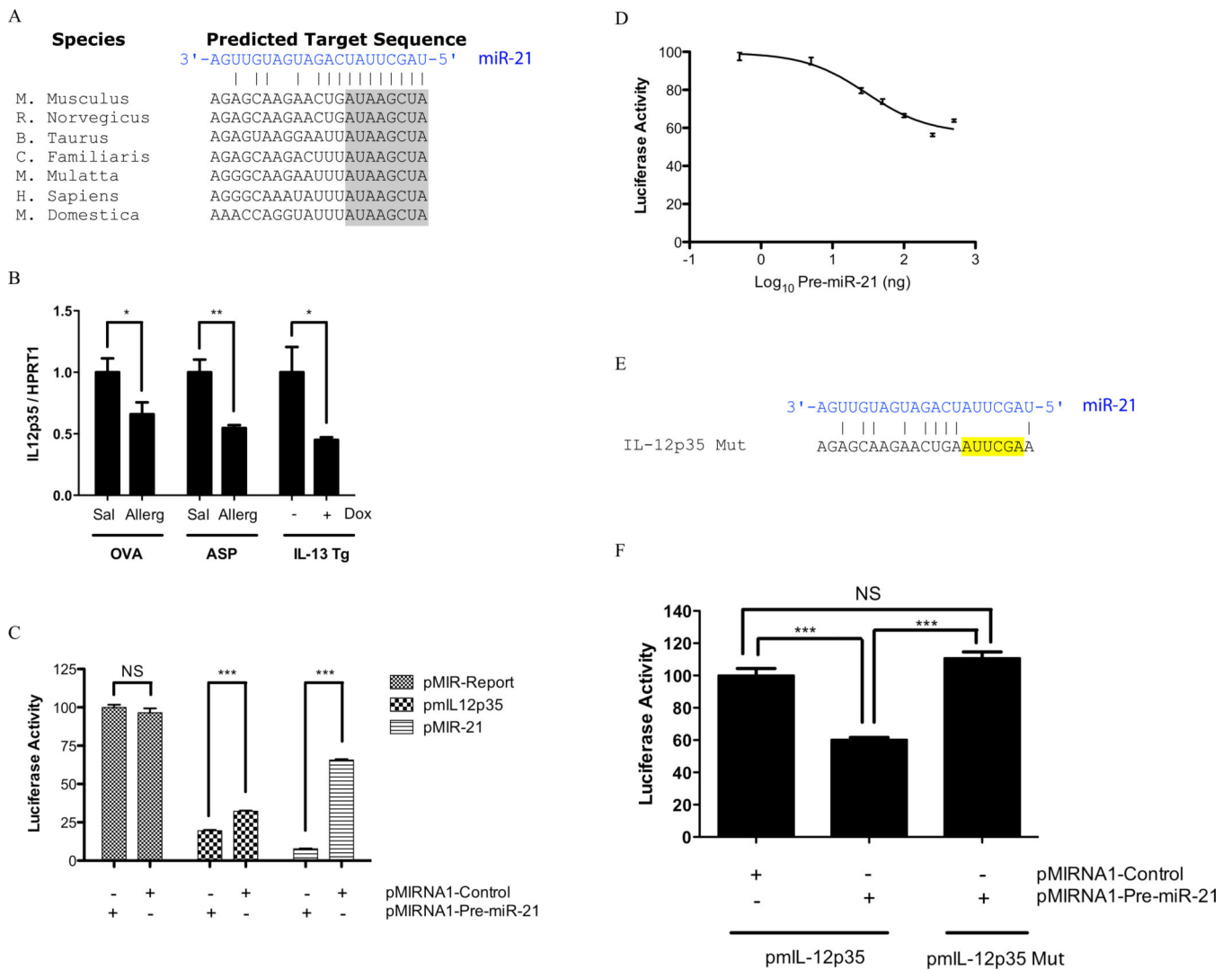


Figure 5. MiR-21 targets IL-12p35

(A) Predicted highly conserved binding site for miR-21 in 3'UTR of IL-12p35. The 8-mer seed sequence is shaded in gray. (B) IL-12p35 expression was determined in OVA, *Aspergillus fumigatus* (Asp), and doxycycline induced IL-13 bitransgenic models. The relative expression levels were determined by qRT-PCR normalized to HPRT1. *, $p < 0.05$; **, $p < 0.01$; $n = 5-7$ mice per group. (C) Relative luciferase activity in 293T cells co-transfected with control firefly luciferase vector (pMIR-Report), or a firefly luciferase reporter vector containing the 3'UTR of IL-12p35 (pmIL12p35), or a firefly luciferase reporter vector with perfect miR-21 binding site in the 3'UTR (pMIR-21), and either the pre-miR-21 expression vector (pMIRNA1-Pre-miR-21) or control vector (pMIRNA1-Control). Firefly luciferase activity was normalized to the renilla luciferase activity then to the average of the control firefly luciferase reporter; NS, not significant; ***, $p < 0.001$; $n = 4$ per group, data representative of 3 experiments. (D) A dose response study of pre-miR-21 expression vector on the luciferase activity of the luciferase vector containing the 3'UTR of IL-12p35; $n = 4$ per group, data representative of 3 experiments. (E) Mutation (yellow-highlighting) in the 3'UTR of mIL-12p35. (F) Relative luciferase activity in 293T cells co-transfected with

reporter plasmid containing either the wild type or mutant mIL-12p35 3'UTR. Firefly luciferase activity was normalized to the renilla luciferase activity then to the average of the wild type mIL-12p35 firefly luciferase reporter; NS, not significant; ***, $p < 0.001$; $n = 4$ per group, data representative of 3 experiments. All data are represented as mean \pm S.E.M.

Table I

Predicted targets of miR-21 that are common between miRanda and TargetScan algorithms

Gene Symbol	Description	Transcript ID
Arhgap24	Rho GTPase-activating protein 24	ENSMUST00000094559
Aspn	Asporin	ENSMUST00000021820
Brd1	Bromodomain containing 1	ENSMUST00000023022
Cbx4	Chromobox homolog 4	ENSMUST00000026665
Ccl1	Chemokine (C-C motif) ligand 1	ENSMUST00000021043
Cntfr	Ciliary neurotrophic factor receptor	ENSMUST00000102962
Dazl	Deleted in azoospermia-like	ENSMUST00000010736
Hnrpu	Heterogeneous nuclear ribonucleoprotein U	ENSMUST00000037748
Il12A	IL-12a	ENSMUST00000029345
Jag1	Jagged 1	ENSMUST00000028735
Kcna3	Potassium voltage-gated channel, shaker-related subfamily, member 3	ENSMUST00000052718
Krit1	KRIT1, ankyrin repeat containing	ENSMUST00000080085
Matn2	Matrilin 2	ENSMUST00000022947
Mrpl9	Mitochondrial ribosomal protein L9	ENSMUST00000029786
Mtap	Methylthioadenosine phosphorylase	ENSMUST00000058030
Nfib	NFIB	ENSMUST00000050872
Ntf3	Neurotrophin 3	ENSMUST00000050484
Pcbpl	Poly(rC)-binding protein 1	ENSMUST00000053015
Pcbp2	Poly(rC)-binding protein 2	ENSMUST00000077037
Pcd4	Programmed cell death 4	ENSMUST00000025931
Peli 1	Pellino 1	ENSMUST00000093290
Pitx2	Paired-like homeodomain transcription Factor 2	ENSMUST00000029657
Plag1	Pleiomorphic adenoma gene 1	ENSMUST00000003369
Ppara	Peroxisome proliferator-activated receptor α	ENSMUST00000057979
Psrl1	Proline/serine-rich coiled-coil 1	ENSMUST00000102630
Reck	Reversion-inducing cysteine-rich protein With kazal motifs	ENSMUST00000030198
Rnf103	Ring finger protein 103	ENSMUST00000064637
Satb1	Special AT-rich sequence-binding protein 1	ENSMUST00000024720
Ski	Sloan-Kettering viral oncogene homolog	ENSMUST00000030917
Sox2	SRY box containing gene 2	ENSMUST00000099151
Spg20	Spastic paraplegia 20, spartin (Troyer syndrome) Homolog (human)	ENSMUST00000044116
Stag2	Stromal Ag 2	ENSMUST00000069619
Tgfb1	TGF, β induced	ENSMUST00000045173
Tiam1	T cell lymphoma invasion and metastasis 1	ENSMUST00000002588
Wwp1	WW domain containing E3 ubiquitin protein Ligase 1	ENSMUST00000035982
Xkr6	X Kell blood group precursor related family Member 6 homolog	ENSMUST00000100485
Zcchc3	Zinc finger, CCHC domain containing 3	ENSMUST00000099207

Table II

Predicted targets of miR-21 that are common between miRanda, TargetScan, and IL-13 down-regulated genes

Name	Transcript ID	TargetScan Prediction	miRanda Prediction
Cntfr	ENSMUST00000102962	AGUUGUAGUCAGACUAUUCGAU CCACCATCAGATT-ATAAGCTC	aGUUGUAGUCAGACUAUUCGAU : cCACCATCAGATT-ATAAGCTc
Il12A	ENSMUST0000029345	AGUUGUAGUCAGAC-UAUUCGAU GAAGAGCAAGAACTGATAAGCTA	agUUGUAGUCAGACUAUUCGAU : agAGCAAGA-ACTGATAAGCTA
Pitx2	ENSMUST0000029657	AGUUGUAGUCAGACUAUUCGAU TTAACATTACT---ATAAGCTT	AGUUGUAGUCAGACUAUUCGAU : : TTAACATTA--CT-ATAAGCTt
Plag1	ENSMUST0000003369	AGUUGUAGUCAGACUAUUCGAU CTAGCCTCTTCG-ATAAGCTT	aGUUGUAGUCAGACUAUUCGAU : : : cTAGCCTC--TTCGATAAGCTt
Psrc1	ENSMUST00000102630	AGUUGUAGUCAGACUAUUCGAU GGTCAATCAGTT-ATAAGCTT	aguugUAGUCAGACUAUUCGAU : ggtcaATCAGTT--ATAAGCTt
Reck	ENSMUST0000030198	AGUUGUAGUCAGAC--UAUUCGAU TGT'TTACAGTTTGAAATAAGCTA	aguuguaGUCAGAC--UAUUCGAU : tgt'tttaCAGTTTGAAATAAGCTA
Satb1	ENSMUST0000024720	AGUUGUAGUCAGACUAUUCGAU TTACTATCATGCAAATAAGCTT	AGUUGUAGUCAGACUAUUCGAU : : TTACTATCATGCAAATAAGCTt
Sox2	ENSMUST0000099151	AGUUGUAGUCAGAC-UAUUCGAU CAAATGTCCATTGTTTATAAGCTG	agUUGUA-GUCA-GACUAUUCGAU : : : caAATGTCCATTGTTTATAAGCTG
Spg20	ENSMUST0000044116	AGUUGUAGUCAGACUAUUCGAU GGATTTTCAGCAG-ATAAGCTA	agUUGUAGUCAGACUAUUCGAU : ggATTTTCAG-CAGATAAGCTA
Wwp1	ENSMUST0000035982	AGUUGUAGUCAGAC-UAUUCGAU GCACAGATAGTGTGATAAGCTG	aGUUGUAGUCAGAC-UAUUCGAU : : : gCACAGATAGTGTGATAAGCTG