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Diagnostic, Functional and Therapeutic Roles of MicroRNA in Allergic Diseases

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Abstract

Allergic inflammation is accompanied by the coordinated expression of a myriad of genes and proteins that initiate, sustain, and propagate immune responses and tissue remodeling. MicroRNAs (miRNAs) are a class of short single-stranded RNA molecules that post-transcriptionally silence gene expression and have been shown to fine-tune gene transcriptional networks as single miRNAs can target hundreds of genes. Considerable attention has focused on the key role of miRNAs in regulating homeostatic immune architecture and acquired immunity. Recent studies have identified miRNA profiles in multiple allergic inflammatory diseases including asthma, eosinophilic esophagitis, allergic rhinitis, and atopic dermatitis. Specific miRNAs have been found to have critical roles in regulating key pathogenic mechanisms in allergic inflammation including polarization of adaptive immune responses and activation of T cells (e.g. miR-21 and miR-146), regulation of eosinophil development (e.g. miR-21 and miR-223), and modulation of interleukin (IL)-13-driven epithelial responses (e.g. miR-375). This review discusses recent advances in our understanding of the expression and function of miRNAs in allergic inflammation, their role as disease biomarkers, and perspectives for future investigation and clinical utility.

Keywords

Allergy; microRNA; miRNA; non-coding RNA; asthma; eosinophilic esophagitis; atopic dermatitis; allergic rhinitis; eosinophils; inflammation; biomarkers

Introduction

Allergic inflammatory diseases encompass a wide range of conditions including asthma, allergic rhinitis, atopic dermatitis, and more recently eosinophilic esophagitis.^{1–6} Each of these diseases involves sustained inflammation that is associated with marked histological changes as well as molecular changes in gene and protein expression. The pathways utilized

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in regulating and fine-tuning these processes represent a particularly attractive area for microRNA (miRNA) studies.

MiRNAs are single-stranded RNA molecules of 19-25 nucleotides in length that mediate post-transcriptional gene silencing of target genes⁷ and are highly conserved throughout evolution.^{8,9} MiRNAs were initially discovered in *C. elegans* in 1993 as silencers of genes that regulate developmental timing.¹⁰ Subsequently, miRNAs were recognized as a distinct class of small regulatory RNAs in multiple species that regulate a wide variety of functions such as cell proliferation, differentiation, apoptosis, stress response, and immune response.¹¹⁻¹³ MiRNAs directly suppress gene expression by base pairing to the 3' untranslated region (UTR) of target mRNA. Depending on the level of complementarity to the target sites, target mRNA degradation and/or translational repression occurs, with imperfect base pairing favoring translational repression.¹⁴ A single miRNA can target hundreds of genes, and individual genes are typically targeted by multiple miRNAs, adding complexity to the network. MiRNAs can also exert global effects on gene expression by either affecting epigenetic mechanisms, such as DNA methylation or histone acetylation, or targeting transcription factors.¹⁵⁻¹⁹ Therefore, miRNAs are a particularly promising class of molecules that may be well positioned to regulate allergic inflammatory processes.

Recently, miRNAs have been shown to be detectable in cell-free body fluids such as serum and plasma samples.^{20,21} The circulating miRNAs are protected from blood RNases either by existing in cell membrane-derived vesicles such as exosomes or by forming a complex with lipid-protein carriers such as high-density lipoprotein.²²⁻²⁴ The majority of miRNAs in plasma form a complex with the AGO2 protein, which is part of the RNA-induced silencing complex responsible for gene silencing; this association protects the miRNAs from degradation.²⁵⁻²⁷ These circulating miRNAs could be ideal blood biomarkers due to their disease-specific dysregulation and their relative stability compared with mRNAs. This possibility is especially attractive for diseases such as eosinophilic esophagitis, for which current modalities for diagnosis and follow-up involve invasive endoscopic procedures.^{28,29}

Herein, we review and discuss the connections between miRNAs and allergic inflammatory diseases including asthma, eosinophilic esophagitis, atopic dermatitis, and allergic rhinitis. We focus on specific miRNAs that are dysregulated in one or more allergic diseases, leading to inappropriate expression of mRNA targets that contribute to disease pathology, and identify a core set of miRNAs involved in the pathogenesis of allergic inflammation.

Allergic Asthma

Asthma is a common chronic disorder of the airways that involves a complex interaction of airflow obstruction, bronchial hyperresponsiveness, and an underlying inflammation. Allergic asthma is the most common form of asthma, with symptoms triggered by inhaled allergens.³⁰

miR-21 regulate polarized adaptive immune responses in allergic asthma models 9

We and others have found that miR-21 is upregulated in multiple experimental asthma models, including the ovalbumin (OVA), *Aspergillus fumigatus*, house dust mite (HDM), and lung-specific interleukin (IL)-13-induced murine models.^{31,32} While the methods of experimental asthma induction are different in each of these systems, all of these models share similar phenotypes including airway eosinophilia, Th2-associated inflammation, mucus production, and airway hyperreactivity.³²⁻³⁹ Notably, miR-21 is among the top overexpressed miRNAs in the inflamed lung tissue.³¹ The highest expression levels of miR-21 are localized to macrophages and dendritic cells.³¹ The IL-12p35 3'UTR harbors a highly evolutionarily conserved target sequence for miR-21 8 (Figure 1).³¹ As IL-12 is a key

molecule involved in Th1 polarization in the adaptive immune responses,⁴⁰ this finding suggests that miR-21 could potentially regulate Th1 vs. Th2 balance by regulating IL-12p35 expression. Indeed, using a luciferase reporter system, IL-12p35 has been identified as a molecular target of miR-21.³¹ Pre-miR-21 dose-dependently inhibits cellular expression of the luciferase reporter vector harboring the 3'UTR of IL-12p35, and mutating miR-21 binding sites in the IL-12p35 3'UTR abrogates miR-21-mediated repression.³¹ Furthermore, miR-21-deficient dendritic cells produce increased levels of IL-12 compared to wild-type dendritic cells after equivalent LPS stimulation. Using the OVA-induced asthma model, miR-21-deficient mice had reduced lung eosinophilia after allergen challenge and significantly increased Th1 cytokine IFN- γ and decreased Th2 cytokine IL-4 in the bronchoalveolar lavage fluid (BALF).⁴¹ Notably, the IL-12/IFN- γ pathway was the most prominently affected pathway in the lungs of miR-21-deficient mice as identified by whole-genome microarray analysis, adding significance to the finding that one of miR-21's major functions is likely to regulate immune polarization, at least in the setting of immune hypersensitivity responses.⁴¹ Additionally, miR-21-deficient CD4⁺ T cells produced increased IFN- γ and decreased IL-4. Conversely, utilizing a Th1-associated delayed-type hypersensitivity model, miR-21 deficiency significantly enhanced the delayed-type hypersensitivity responses.⁴¹ These findings are not just of academic interest as miR-21 is upregulated in multiple human Th2-associated diseases including eosinophilic esophagitis, atopic dermatitis, and ulcerative colitis.⁴²⁻⁴⁴ In addition, miR-21 has been reported to be upregulated in human airway epithelial cells in response to IL-13 treatment and suppresses TLR-2 signaling in an animal model of asthma.⁴⁵ AntagomiRs or anti-miRs are a class of chemically engineered nucleotides that silences miRNA expression *in vitro* and/or *in vivo*.^{46, 47} While a recent report by Collison *et al.* found that intranasal anti-miR-21 administration starting one day before allergen challenge in a HDM model of asthma had no significant effect on eosinophil recruitment or Th2 cytokine production,⁴⁸ the anti-miR-21 was given after the intranasal sensitization phase of the protocol, when the Th1 vs. Th2 balance had likely already been established. This may suggest that miR-21 has its most significant role in the early sensitization stage. Alternatively, the antagomiRs may not recapitulate the phenotype of miR-21^{-/-} mice due to either the type of antagomiR used or the mode and frequency of administration, as indicated by the lack of agreement in recent antagomiR and gene-deficient murine studies.^{49, 50} These results demonstrate that small perturbations in miRNA levels can have profound effects on adaptive immunity.

miR-126 regulates the effector function of Th2 cells and the allergic inflammatory response in experimental asthma

MiR-126 has been found to be upregulated in the airway wall of an acute HDM-induced 7 experimental asthma model.³² The upregulation was dependent on the TLR-4 and MyD-88 pathways. MiR-126 was not upregulated in either TLR-4- or MyD-88-deficient mice after HDM challenge. Inhibition of miR-126 by antagomiRs abrogated the asthmatic response as demonstrated by reduced inflammation, airway hyperreactivity, Th2 cytokines (e.g. IL-5 and IL-13), airway eosinophilia, and mucus production.³² However, in a chronic model of experimental asthma, miR-126 was initially upregulated after 2 weeks of allergen challenge but declined to near baseline levels after 6 weeks of allergen challenge.⁵¹ Inhibition of miR-126 by antagomiRs in the chronic asthma model reduced recruitment of intraepithelial eosinophils in the conducting airways but had no effect on mucus cell hyperplasia or subepithelial fibrosis. The authors concluded that sustained changes in miRNA may not be essential for perturbation of chronic asthma.⁵¹

Let-7 regulates IL-13 expression and the allergic inflammatory response in experimental asthma

The let-7 family includes let-7a through let-7k.⁵² Originally discovered in *C. elegans*, let-7 was subsequently found as the first known human miRNA.⁵² The let-7 family members are highly conserved across species. However, let-7h, -7j, and -7k are not expressed in mice or humans.⁵² An initial report by Polikepahad *et al.* demonstrated that IL-13 is a direct target of let-7 using a luciferase reporter system.⁵³ They subsequently demonstrated that Th1 cells have significantly higher let-7a expression compared to Th2 cells. The authors inhibited let-7a expression by using locked nucleic acid (LNA) antagomiRs, which are short antisense RNAs with a modified ribose moiety resistant to endonucleases and exonucleases.⁵⁴ Inhibition of let-7a by LNA antagomiR significantly upregulated IL-13 mRNA expression in T cells. Using an anti-let-7 LNA antagomiR that targets let-7a, -7b, -7c, and -7d, the authors' *in vivo* findings were opposite of their *in vitro* findings. They found that anti-let-7 LNA antagomiR alleviated experimental asthma *in vivo*, with reduced BALF inflammatory cell infiltration and downregulation of IL-4, IL-5, and IL-13 levels.⁵³ A subsequent report by Kumar *et al.* demonstrated downregulation of let-7a, -7b, -7c, -7d, -7f, -7g, and -7i in the asthmatic lungs after OVA challenge.⁵⁵ They found that let-7 inhibited IL-13 secretion in PMA/PHA-stimulated T-cells. Using intranasal delivery of a let-7 mimic, the authors found that let-7 attenuated experimental asthma, with reduced inflammatory cell infiltration, mucus secretion, airway fibrosis, and airway hyperreactivity.⁵⁵ Several differences could potentially explain the discrepancies between these two reports. First, Polikepahad *et al.* used an antagomiR that inhibited only four members of the let-7 family. It is possible that there is a compensatory upregulation of other let-7 family members. Second, in the study by Polikepahad *et al.*, the mice received two intravenous doses of LNA antagomiRs, the first the day before and the second the day after the first intranasal allergen challenge.⁵³ In contrast, Kumar *et al.* administered 2'-O-Me antagomiR intranasally 30 minutes before every intranasal allergen challenge. The different types of antagomiR and route and frequency of challenge could partially account for the disparate results. Third, both the let-7 mimic and the let-7 inhibitor could have off-target effects that contribute to the observed *in vivo* phenotype. An additional report by Collison *et al.* found upregulation of let-7b in a HDM model of experimental asthma without any effect of anti-let-7b on the asthmatic phenotype.⁴⁸ Given the discrepancies reported in the literature, future studies are needed to better define the role of let-7 in allergic inflammation.

Antagonism of miR-145 inhibits experimental allergic airway inflammation

MiR-145 was recently found to be upregulated in the airway wall of a HDM model of experimental asthma.⁴⁸ The authors found that anti-miR-145 significantly attenuated eosinophil infiltration, mucus production, Th2 cytokine production, and airway hyperreactivity if the anti-miR was given intranasally every other day starting prior to the first HDM challenge. Notably, the effects of anti-miR-145 were comparable to dexamethasone treatment. However, once the inflammation was established, both anti-miR-145 and dexamethasone demonstrated limited attenuation of the asthma phenotype. Administration of a single dose of anti-miR-145 before the last HDM challenge attenuated airway hyperreactivity and mucus production but had no effect on eosinophil infiltration.⁴⁸ Future studies are needed to identify the precise molecular targets of miR-145 that mediate its anti-inflammatory effects.

Antagonism of miR-106a decreases experimental asthma severity

MiR-106a has been found to be upregulated in the lungs of an OVA/Alum model of experimental asthma.⁵⁶ MiR-106a is expressed in macrophages, T cells, B cells and epithelial cells with the highest level of expression in the macrophages.⁵⁷ MiR-106a regulates IL-10 expression by directly targeting the 3'UTR of IL-10.⁵⁷ Anti-miR-106a given

after sensitization and intranasal allergen challenge significantly reduced inflammatory cell infiltration, Th2 cytokine levels (e.g. IL-4, IL-5 and IL-13), OVA-specific IgE, goblet cell metaplasia, airway fibrosis, and airway hyperreactivity.⁵⁶ These findings are notable since the anti-miR-106a decreased experimental asthma severity after the asthma phenotype had been firmly established. Thus, anti-miR-106a could be highly relevant to the treatment of clinical diseases and provide further rationale for the development of miRNA therapeutics.

miRNAs in smooth muscle cells of asthma

Smooth muscle cells are the main effector cells of airway hyperreactivity and have a key role in asthma pathogenesis.⁵⁸ MiR-133a, a muscle specific miRNA, has been found to be downregulated in cultured human airway smooth muscle cells after IL-13 stimulation.⁵⁹ Anti-miR-133a increased the expression of the small GTPase RhoA at baseline, and pre-miR-133a significantly attenuated IL-13-induced RhoA expression.⁵⁹ Since the Rho kinases have been shown to mediate smooth muscle contraction, the authors proposed that synthetic miR-133a could suppress airway hyperresponsiveness by targeting RhoA expression.⁶⁰ A separate study found that miR-140-3p is downregulated in human asthmatic airway smooth muscle cells after TNF- α stimulation but not in non-asthmatic airway smooth muscle cells.⁶¹ MiR-140-3p modulates CD38 expression both by directly targeting the CD38 3'UTR and by indirect activation of p38MAPK and NF- κ B.⁶¹ These findings are of interest since the CD38 pathway is important for calcium mobilization and smooth muscle contractility.⁶²

miRNA expression in human asthmatic subjects

While most of our current understanding of the role of miRNA in asthma pathogenesis comes from experimental allergic asthma models, several studies have attempted to identify the role of miRNA in human asthmatic subjects. An initial study by Williams *et al.* found no significant difference in the expression of 227 miRNAs in the airway biopsies obtained from 8 patients with mild asthma (FEV₁ of $83 \pm 4\%$ of predicted values) compared to 8 normal subjects (FEV₁ values $95 \pm 4\%$).⁶³ Several possibilities could explain the lack of changes in the miRNA expression profile. The authors proposed the possibility that the inflammatory changes were too mild and that changes in miRNA expression may be more evident in patients with more severe asthma. Another possibility is that the samples were not taken during an episode of asthma exacerbation. Studying the airways after an allergen challenge that is known to intensify allergic airway inflammation may have revealed miRNAs that are critical in mediating the allergic inflammatory response. A subsequent study by Liu *et al.* identified upregulation of miR-221 and miR-485-3p in the peripheral blood of 6 pediatric patients with asthma admitted to hospital compared to 6 controls.⁶⁴ They subsequently confirmed the upregulation of miR-221 in the peripheral blood of an additional 4 pediatric patients with asthma compared to 4 additional controls in a separate study.⁶⁵ They also found upregulation of miR-221 and miR-485-3p in the lung of an OVA-induced experimental asthma model. Administration of anti-miR-221 with a nebulizer reduced the total cell and eosinophil numbers in the BALF compared to administration of scrambled control.⁶⁵ The authors proposed that this effect is potentially mediated by targeting SPRED2, which is a regulator of allergen-induced airway inflammation and hyperresponsiveness in murine asthma models.⁶⁴ In a separate study comparing human airway bronchial epithelial cells from 16 patients with asthma to those from 16 healthy controls, the authors found 24 differentially expressed miRNAs, with miR-203 being the most downregulated miRNA.⁶⁶ Since miR-203 has been reported to regulate skin cell differentiation, the authors proposed that miR-203 may be involved in regulating the differentiation of lung epithelial cells.⁶⁶

MiRNAs have been found to be crucial for the development and function of T cells.⁶⁷ CD8⁺ T cell development is dependent upon the miRNA-processing enzyme DICER, and CD4⁺ T cells deficient in DICER preferentially differentiate into Th1 cells.⁶⁷ A recent study compared the miRNA expression in CD4⁺ and CD8⁺ T cells from 12 patients with severe asthma compared to 8 healthy controls. For patients with severe asthma, miR-146a and miR-146b were reduced in both CD4⁺ and CD8⁺ T cells, while miR-28-5p was downregulated in CD8⁺ T cells only.⁶⁸ The downregulation of miR-146a may partially mediate the severe asthma phenotype since T cells lacking miR-146a have been found to be hyperactive in both acute and chronic inflammatory states.⁶⁹ On the other hand, a recent animal study using an experimental OVA-induced asthma model showed that the levels of miR-146a, miR-146b, miR-150, and miR-181a were upregulated in splenic CD4⁺ T cells after allergen challenge.⁷⁰ Further studies are needed to determine whether miR-146a and miR-146b have pro- or anti-inflammatory effects in asthma and whether their roles are context and timing dependent.

Exosomal miRNA expression in BALF of human asthma subjects

Recently, exosomes have been implicated in the pathogenesis of asthma. In a preliminary report, Levanen *et al.* isolated exosomes from the BALF of subjects with mild intermittent asthma and healthy controls and measured the miRNA profile of the exosomes.⁷¹ At baseline, 24 miRNAs were differentially expressed in subjects with asthma compared to controls, with the majority of the altered miRNAs being downregulated. The expression profile of these 24 miRNAs was correlated with the FEV₁ of the subjects ($R^2 = 0.74$). Exposure to air pollution did not significantly alter the exosomal miRNA expression in either group. Pathway analysis showed that the differentially regulated miRNAs are implicated in regulating cytokines and inflammatory responses.⁷¹ Further studies are needed to elucidate the function of differentially expressed exosomal RNAs.

Eosinophilic Esophagitis

Eosinophilic esophagitis is an emerging allergic disease characterized by intense eosinophil infiltration in the esophagus that is unresponsive to acid suppressive therapy. The incidence and prevalence of eosinophilic esophagitis has been steadily on the rise, with the disease now reported in every continent except Africa.^{28, 29} Eosinophilic esophagitis represents a special opportunity to identify which miRNAs truly participate in human Th2-associated inflammatory responses as the allergic tissue is readily available and amenable to molecular analysis as esophageal biopsies are standard procedures during routine endoscopy for disease monitoring. Using esophageal biopsy samples from patients, miRNA signatures that distinguish non-eosinophilic forms of esophagitis from eosinophilic esophagitis have been identified.^{44, 72} The expression of these differentially regulated miRNAs was largely reversible in patients that responded to glucocorticoid treatment.⁴⁴ In two independent studies, miR-21 has been identified as one of the most upregulated miRNAs in human eosinophilic esophagitis and to directly correlate with esophageal eosinophil levels.^{44, 72} Notably, esophageal miR-21 levels inversely correlated with esophageal IL-12p35 levels. Using bioinformatic pathway analysis, co-regulated miR-21 target genes in patients with eosinophilic esophagitis were found to be significantly enriched in the regulation of T-cell polarization and IFN- γ production.⁴⁴ These data provide the first evidence to substantiate a connection between human allergic disease and the IL-12/IFN- γ axis, adding credence to the pre-clinical studies that implicated miR-21 as a key regulator of IL-12 and immune polarization (Figure 1). Analysis of esophageal eosinophil level and a myriad of esophageal transcripts for correlation with miR-21 demonstrated impressive correlations of miR-21 with esophageal eosinophil count, cell-specific markers for eosinophils, and CCL-26 (eotaxin-3), which is functionally involved in eosinophil recruitment.^{44, 73}

Both miR-146a and miR-146b have been found to be upregulated in patients with eosinophilic esophagitis.⁴⁴ A previous report by Lu *et al.* found that miR-146a could selectively suppress Th1 responses.⁷⁴ Utilizing miR-146a-deficient mice, the authors showed that miR-146a deficiency selectively impaired Treg-mediated suppression of Th1 responses, leaving the suppression of Th2 and Th17 responses intact. The inhibition of Treg-mediated suppression of Th1 responses was mediated in part by targeting STAT-1 expression.⁷⁴ Therefore, upregulation of miR-146a could prevent differentiation of IFN- γ -producing Th1 cells. Since IFN- γ promotes Th1 response and inhibits the differentiation of Th2 and Th17 cells, miR-146a-mediated suppression of Th1 responses could lead to unopposed Th2 activation. Using plasma samples from patients with eosinophilic esophagitis, three of the differentially regulated miRNAs in the esophageal biopsies, miR-146a, miR-146b, and miR-223, were also found to be differentially regulated in the plasma.⁴⁴ Since circulating miRNAs often exist in exosomes, which can be taken up by other cells,^{22, 25, 75, 76} the circulating plasma miR-146a could be taken up by the Treg cells and further propagate or help to maintain the Th2 responses by suppressing Th1 activation. In addition, miR-146b remains elevated in patients with eosinophilic esophagitis that responded to pharmacological treatment and is in disease remission. While the specific role of miR-146b in regulating adaptive immune responses has not been investigated, miR-146a and miR-146b have an identical seed sequence that is critical for miRNA-mediated target gene expression. It is plausible that miR-146b could also regulate STAT-1 expression and suppress Th1 responses. Perhaps elevated miR-146b might predispose eosinophilic esophagitis in disease remission to relapse. Current diagnostic criteria for eosinophilic esophagitis is based on esophageal biopsy histology.^{28, 29} An esophageal biopsy needs to be obtained every time a diagnosis is made and on subsequent follow-ups to assess therapeutic response. The circulating plasma miRNAs, including miR-146a and miR-223, could potentially serve as non-invasive biomarkers for diagnosis or assessment of therapeutic response alone or in combination with other biomarkers.⁷⁷

One of the most downregulated miRNA in eosinophilic esophagitis is miR-375.⁴⁴ Notably, miR-375 is downregulated after IL-13 stimulation in both human bronchial and human esophageal epithelial cells.⁷⁸ In addition, miR-375 has been reported to be downregulated in Th2-associated diseases including atopic dermatitis and ulcerative colitis.^{43, 79} Interestingly, in patients with eosinophilic esophagitis, miR-375 expression inversely correlates with the degree of allergic inflammation as measured by esophageal eosinophil levels and the gene expression levels of the Th2 cytokines IL-5 and IL-13 and the mast cell-specific enzymes CPA-3 and TPSAB-1. Notably, when miR-375 is overexpressed in human esophageal epithelial cells, it regulates IL-13-induced genes, particularly in immunoinflammatory pathways. Regulated genes include those involved in extracellular matrix organization, formation of cell junctions, and the inflammatory response. However, it is important to note that Biton *et al.* found that IL-13 stimulation induces miR-375 expression in HT-29 human colon adenocarcinoma cells.⁸⁰ Notably, the induction of miR-375 in these cells was only seen after 2 hours of treatment and subsequently returned to baseline or below-baseline levels. It is interesting that miR-375 regulated production of TSLP in HT-29 cells,⁸⁰ especially since TSLP is an important epithelial-derived cytokine that is involved in the pathogenesis of allergic inflammation and is genetically linked with eosinophilic esophagitis susceptibility.^{81, 82} Despite this interesting mechanistic connection, miR-375 has not been found to regulate TSLP expression in esophageal epithelial cells.⁷⁸ Notably, miR-375 is downregulated, not upregulated, in patient samples from multiple Th2-associated diseases,^{43, 44, 79} as well as from hyperproliferative diseases including esophageal squamous carcinoma.^{83, 84} These data suggest that the activity of miR-375 may be dependent on its cellular context, as indicated by previous reports.^{85, 86} While the collective data already draw strong attention to the role of miR-375 in regulating epithelial cell responses in Th2

immunity, additional studies are needed to define its exact role and importance, especially with regard to TSLP production.

Atopic Dermatitis

Atopic dermatitis is a hyperproliferative cutaneous disorder associated with a defective skin barrier and a mixed Th1/Th2 inflammatory response resulting in susceptibility to cutaneous infections and prominent pruritis.⁸⁷ Notably, miRNAs upregulated in other allergic disorders, including miR-21, miR-146, and miR-223, are also upregulated in the skin of patients with atopic dermatitis.^{42, 88} In addition, Sonkoly *et al.* found that miR-155 was one of the highest upregulated miRNAs in skin biopsies from patients with atopic dermatitis compared to healthy controls.⁷⁹ Topical exposure of non-lesional skin of atopic dermatitis patients with relevant allergens could induce miR-155 expression. MiR-155 is expressed by skin T cells, dendritic cells, and mast cells. Upregulation of miR-155 is observed following T-cell differentiation into both Th1 and Th2 lineages.⁷⁹ Functionally, miR-155 has been shown to suppress CTLA-4, a negative regulator of T-cell function. This CTLA-4 suppression in turn enhances the T-cell proliferative response since CTLA-4 has known anti-proliferative function in activated T cells.⁷⁹ In macrophages, miR-155 has been shown to target IL-13R α 1.⁸⁹ Downregulation of miR-155 favors the development of pro-Th2 M2 type of macrophages and reduces IL-12p70 production in monocyte-derived dendritic cells.^{89, 90} Given the critical role of miR-155 in regulating innate and adaptive immunity,⁹⁰⁻⁹³ more detailed studies on the regulation of miR-155 and its interactions with the immune system in the context of atopic dermatitis are needed to further elucidate its role in pathogenesis and its potential to serve as a novel therapeutic target.

Allergic Rhinitis

The evaluation of miRNA expression and function in patients with allergic rhinitis has received relatively little attention. A report by Shaoqing *et al.* compared the miRNA expression profile of nasal mucosa from patients with allergic rhinitis and non-allergic controls that underwent surgery for nasal obstruction. They found 9 miRNAs with more than a 2-fold change between the allergic rhinitis group and control group.⁹⁴ They subsequently verified the downregulation of miR-143, miR-187, and miR-224 by quantitative RT-PCR. A report by Zhang *et al.* found that miR-125b is overexpressed in the epithelial cells of sinonasal mucosa in patients with chronic eosinophilic rhinosinusitis with nasal polyps.⁹⁵ MiR-125b was found to enhance type I IFN production by targeting eIF-4E binding protein 1.⁹⁵ A preliminary report by Chen *et al.* screened miRNA levels in cord blood samples with elevated cord blood IgE level. Eight miRNAs were downregulated in cord blood samples with elevated IgE.⁹⁶ Downregulation of miR-21 in the cord blood sample was associated with elevated TGFBR-2 expression on cord blood leukocytes. The authors also examined peripheral blood monocytes in 6-year-old children and found that miR-21 and miR-126 remained significantly downregulated in monocytes from children with allergic rhinitis compared to non-allergic controls. The authors proposed that downregulation of miR-21 in peripheral blood persisted from the neonatal stage to childhood and could potentially be an early predictor of allergic rhinitis.⁹⁶ Mechanistically, the association of miR-21 downregulation with the subsequent development of atopy is not consistent with its known role in regulating IL-12.^{31, 41} Further studies are needed to investigate the potential role of miR-21 and other miRNAs in allergic rhinitis.

Eosinophil Development

Significant attention has been focused on the role of miRNAs in regulating eosinophils and their development. Eosinophils differentiate from a common myeloid progenitor and then via an eosinophil lineage-committed progenitor that is CD-45⁺ and IL-5R α ⁺.^{97, 98} The

cytokine IL-5 is particularly important in eosinophil lineage development as it promotes the selective differentiation of eosinophils and the release of mature eosinophils from the bone marrow.⁹⁹ In an *ex vivo* culture model of bone marrow-derived eosinophils, both miR-21 and miR-223 were upregulated during the differentiation of eosinophil lineage-committed progenitors to mature eosinophils. MiR-21 deficiency reduced eosinophil progenitor cell growth, whereas miR-223 deficiency accelerated eosinophil growth (Figure 2)^{100, 101}. MiR-21-deficient eosinophil progenitor cultures have increased apoptosis during differentiation of mature eosinophils from progenitor cells. The miR-21-deficient mice have reduced eosinophil levels in the blood and reduced eosinophil colony-forming unit capacity in the bone marrow.¹⁰¹ While microarray analysis of differentially regulated genes between miR-21^{+/+} and miR-21^{-/-} eosinophil progenitor cultures identified only one gene (*Psrc1*) as a predicted target of miR-21, functional enrichment analysis identified an overall functional effect in the pathways associated with the observed phenotype and known role of miR-21 in other systems.^{102, 103} Thus, it is likely that miR-21 exerts modest effects on direct targets that then synergistically interact to ultimately regulate eosinophilopoiesis.

MiR-223-deficient eosinophil progenitors had the opposite phenotype of the miR-21-deficient eosinophil progenitors. MiR-223 deficiency resulted in increased eosinophil progenitor proliferation compared to wild-type cells. Mechanistically, miR-223 may exert its effect by regulating insulin-like growth factor 1 receptor (IGF1R), at least in part. Indeed, IGF1R is a target of miR-223 and is expressed on eosinophil progenitors.¹⁰⁰ Notably, IGF1R is upregulated in miR-223-deficient eosinophil progenitors. Treatment with an IGF1R inhibitor attenuated the proliferation of both miR-223^{+/+} and miR-223^{-/-} eosinophil progenitors.¹⁰⁰ Whole-genome microarray analysis of miR-223-deficient eosinophil progenitor cultures identified an alteration in eosinophil cell growth and hematological development as the most affected biological functions, substantiating a key role for miR-223 in controlling eosinophilopoiesis.¹⁰⁰ Indeed, miR-223^{-/-} eosinophil progenitors have been shown to have a delay in the upregulation of CCR-3, likely indicating a delayed maturation *in vitro*.¹⁰⁰ It is likely that miR-21 and miR-223 cooperatively regulate the proliferation and differentiation of eosinophil lineage-committed progenitors, with upregulation of miR-21 preventing premature arrest in eosinophil progenitor proliferation and the later upregulation of miR-223 preventing overproduction of eosinophils and promoting eosinophil maturation.

T Helper Cell Differentiation and Activation

T lymphocytes are a major driver of allergic diseases. Early studies utilized a global miRNA deletion in T cells by deleting the miRNA processing enzyme DICER. DICER deficient T helper cells proliferated poorly upon stimulation, and preferentially expressed the Th1 cytokine IFN γ .⁶⁷ More recent studies focusing on the roles of individual miRNAs have shown critical roles of miRNA in regulating the development and activation of T helper cells.

During T cell development, miR-181a has been shown to augment the sensitivity of T cells to peptide antigens. Inhibiting miR-181a expression in immature T cells reduces antigen sensitivity significantly impairs T cell selection.¹⁰⁴ T cell apoptosis is important in regulating both the length of strength of T cell responses. The miR-17-92 cluster has been shown to promote a lymphoproliferative disease phenotype by targeting PTEN and Bim.¹⁰⁵ MiR-21 has been shown to be up-regulated during T cell activation and suppresses apoptosis in activated T cells.¹⁰⁵

The development of polarized T helper cells is central to the pathogenesis of allergic inflammation since allergic inflammation is predominately a Th2 response. Several of the miRNAs up-regulated in allergic inflammation including miR-155, miR-21 and miR-146a

(Table 1) has been shown to regulate the polarized T cell responses. MiR-155 deficient T helper cells exhibit a Th2 bias *in vitro* in part due to up-regulation of the miR-155 target cMaf.^{91, 92} miR-155 positively regulates both Treg and Th17 cell differentiation *in vitro* by targeting SOCS1.¹⁰⁶ The up-regulation of miR-21 has been shown to promote Th2 response both by repressing IL-12p35 expression in dendritic cells and by a T cell intrinsic pathway.^{31, 41, 107} miR-146a has been found to selectively target Treg mediated suppression of Th1 responses by targeting STAT1.⁷⁴ It is likely that additional miRNAs are involved in T cell development and activation during allergic inflammatory responses and this represents an exciting area for future investigation.

Mast Cells

Mast cells are key effector cells in allergic inflammation that release potent inflammatory mediators upon allergen exposure. miRNAs have been shown to regulate the development of mast cells. miR-221 and miR-222 are significantly up-regulated after mast cell activation. Overexpression of miR-221 and miR-222 led to a increased number of cells in the G1/G0 phase and fewer cells in the G2/M phase.¹⁰⁸ In addition, miR-221 over-expression in mast cells led to increased degranulation, decreased migration and increased adherence.¹⁰⁹ Another miRNA up-regulated upon mast cell activation, miR-146a, was shown to increase mast cell apoptosis.¹¹⁰ In a separate study, it was shown that miR-132 was the most up-regulated miRNA upon mast cell activation and regulates the heparin-binding EGF-like growth factor.¹¹¹ During mast cell differentiation, miR-126 has been shown to be down-regulated and it positively regulates mast cell proliferation by targeting the negative regulator of mast cell proliferation Spred1.¹¹² While miRNAs have shown promising roles in regulating mast cell proliferation and activation, the current data are preliminary and future investigation and needed to further define the function of miRNA in mast cells.

Identification of a Core Set of miRNAs Involved in Allergic Inflammation

To date, miRNA profiles have been identified in allergic asthma,^{31, 32, 48, 51, 53, 55, 63, 64, 66, 113} eosinophilic esophagitis,^{44, 72} atopic dermatitis,^{42, 79} and allergic rhinitis.⁹⁴ Excluding allergic rhinitis, which does not have an overlapping miRNA profile perhaps due to the preliminary analysis reported to date, a core set of miRNAs involved in allergic inflammation is becoming apparent. These miRNAs and their direction of regulation are listed in Table 1 which includes miRNAs that are upregulated or downregulated in at least two of the three allergic diseases of allergic asthma, eosinophilic esophagitis, and atopic dermatitis. While several of these miRNAs, such as miR-193b and miR-365, have no known reports on their roles in allergic inflammation, the roles of many of the other major allergy-related miRNAs and their molecular targets have been investigated (Figure 3). These commonly regulated miRNAs could be particularly important for the initiation or maintenance of allergic inflammatory processes.

Future Perspectives

MiRNAs are potentially of critical importance in the pathogenesis of allergic inflammation. While our knowledge about the miRNA regulation of allergic inflammation has considerably advanced over the last several years, multiple areas warrant future investigation. One exciting possibility is the ability of multiple miRNAs to coordinately target a common pathway. In particular, the polarized Th responses could be regulated by multiple miRNAs targeting different components of the T-cell polarization pathways. The miRNAs miR-21 and miR-146a are upregulated and the let-7 family members are downregulated in all of the allergic inflammatory diseases profiled to date. Upregulation of miR-21 appears to promote Th2 and attenuates Th1 responses by targeting IL-12 expression. MiR-146a has been found to be required for Treg-mediated suppression of Th1 responses

but not Th2 responses. Upregulation of miR-146a could potentially enhance the Treg-mediated suppression of Th1 responses and result in unopposed Th2 activation. The let-7 family members appear to target IL-13 expression; downregulation of let-7 could enhance Th2 responses by upregulating IL-13 expression. Since the Th1 and Th2 responses exist in a balanced state, miR-21, miR-146a, and let-7 may work additively or synergistically to initiate and/or maintain an exaggerated Th2 response by targeting different components of the Th-cell polarization pathway. The coordinated regulation of a pathway by multiple miRNAs may lead to much greater activation or repression than that mediated by a single miRNA.

A second area worthwhile of future investigation is the reversibility of established disease phenotype. Most of the studies to date have used either gene-targeted mice or delivery of antagomiRs before allergen challenge. In one study, delivery of antagomiR after allergic inflammation is established resulted in very limited attenuation of disease phenotype compared to delivering the antagomiR before allergen challenge.⁴⁸ The importance of miRNAs in reversing disease phenotype needs to be further investigated, perhaps by delivering several miRNA mimics or inhibitors in combination.

A third area worth future investigation is a stronger focus on the role of miRNA in human studies. Most of our knowledge of the function of miRNA in allergic inflammation to date is based on cell cultures and murine models, except for a few early studies in human allergic diseases. Further elucidating the roles of miRNAs in the human context will likely improve our understanding of miRNA in the pathogenesis, diagnosis, and prognosis of allergic diseases and lay the foundation for the development of miRNA based therapies.

A fourth area worth future investigation is the combinatorial targeting of a key mRNA by several miRNAs or the targeting of multiple mRNAs by a key miRNA. Most studies have focused on targeting of a single mRNA by one miRNA. While this approach has given us important insight into the role miRNAs play in the pathogenesis of allergic inflammation, combinatorial targeting could lead to even greater target mRNA repression or identification of new gene networks and gene-gene interactions.

A fifth area worth future investigation is the interplay between miRNAs and epigenetics. MiRNAs have been reported to be epigenetically regulated and to regulate the epigenetic machinery.¹⁷ DNA methylation and histone modifications have been shown to regulate the expression of multiple miRNAs. Conversely, miRNAs have been shown to regulate epigenetic modulators including DNA methyltransferases and histone deacetylases.¹⁷ Uncovering the role of miRNAs in the epigenetic regulation of allergic inflammation could help us to understand both the chronicity of the disease and the high relapse potential observed in patients.

A sixth area worth future investigation is the relationship between miRNAs dysregulated in helminth infections and the allergy related miRNAs. Helminth infection has been proposed to induce a modified Th2 response that favors both survival of the host and parasites. This modified Th2 response is characterized by high levels of IL-10 expression and is proposed to be protective from development of allergy.¹¹⁴ Currently very little is known about host miRNA response to parasite infection, except that suppression of let-7 gives significantly lower parasite burden.¹¹⁵ Comparing helminth induced miRNAs and allergy induced miRNAs could further refine the role of miRNAs in development of Th2 type inflammation and the development of allergies.

A seventh area worth future investigation is the potential for miRNAs to serve as biomarkers for disease diagnosis, stratification, and monitoring and as an alternative to current invasive measurements. Most of the studies to date are preclinical studies focused on cancer

diagnosis and prognosis.^{116, 117} However, it is likely that miRNAs have both diagnostic and prognostic value in allergic inflammation. The recent discovery of circulating miRNAs in the serum/plasma, particularly in a form that is relatively stable compared with mRNA and even protein, offers the exciting possibility that miRNAs may serve as non-invasive biomarkers. Lastly, the development of improved technologies including new bioinformatic algorithms, proteomics, next-generation sequencing, new miRNA mimics / antagomiRs, and novel small-molecule miRNA inhibitors will help us to define novel pathways regulating or regulated by miRNAs and to facilitate the development of miRNAs as potential therapeutic targets (Table 2).

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Abbreviations

AGO2	argonaute-2
BALF	bronchoalveolar lavage fluid
CCL	chemokine (C-C motif) ligand
CCR	chemokine (C-C motif) receptor
CD	cluster of differentiation
CPA3	carboxypeptidase A3
CTLA-4	cytotoxic T-lymphocyte associated protein 4
HDM	house dust mite
IL	interleukin
IFN	interferon
IGF1R	insulin-like growth factor 1 receptor
LNA	locked nucleic acid
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
miRNA or miR	microRNA
MyD88	Myeloid differentiation primary response 88
NF-κB	nuclear factor κ B
OVA	ovalbumin
PMA	phorbol myristate acetate
PHA	phytohemagglutinin
RT-PCR	reverse transcription polymerase chain reaction
SPRED2	Sprouty related, EVH1 domain containing 2
STAT	signal transducer and activator of transcription

TGFBR2	transforming growth factor, beta receptor II
Th	T-helper
TLR	toll-like receptor
TNF	tumor necrosis factor
TPSAB1	tryptase alpha/beta 1
Treg	T regulatory
TSLP	thymic stromal lymphopoietin
UTR	untranslated region

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What do We Know

- miRNAs have critical roles in regulating pathogenic mechanisms involved in allergic inflammation
- miRNAs have been demonstrated to regulate Th1 vs. Th2 polarization in multiple allergic inflammatory diseases
- miRNAs have been shown to regulate cell types critical to the allergic inflammatory response including eosinophils, T cells, mast cells and basophils
- An esophageal miRNA signature could distinguish eosinophilic esophagitis from non-eosinophilic forms of esophagitis.
- Proof of principle pre-clinical studies with gene knockout strategies and anti-miRNA based therapeutics have demonstrated the potential clinical utility of modifying miRNAs in allergic disease.
- Collectively, these data establish miRNAs as fundamental regulators of allergic inflammation; as such, miRNAs have potential importance as diagnostic and therapeutic targets for disease.

What is Still Unknown

- What is the function of some of the commonly dysregulated miRNAs in allergic diseases?
- Could multiple dysregulated miRNAs cooperatively target a common pathway in allergy?
- Could miRNAs reverse established disease phenotype?
- What is the therapeutic potential of miRNA mimics and inhibitors?
- How does miRNAs regulate the epigenetic machinery and how do the epigenetic machinery regulate miRNA expression in allergic diseases?
- Could miRNAs be developed into biomarkers for diagnosis, stratification and prognosis of allergic diseases?
- What are the cell specific action and mode of action of individual miRNAs?

Figure 1. Schematic showing the regulation of the adaptive immune system by miR-21 in allergic inflammatory responses

Th1 and Th2 cells exist in a balanced state. Allergen or IL-13 upregulates miR-21 expression, which represses target gene IL-12 and in turn inhibits Th1 differentiation. Decreased production of the Th1 cytokine IFN- γ causes unopposed Th2 activation and increased production of Th2 cytokines. The Th2 cytokines may further amplify the miR-21-mediated responses. A highly conserved miR-21 seed sequence in the 3' UTR of IL-12p35 is shown in the insert at the top left corner; the seed sequence is shaded in gray.

Figure 2. Schematic showing miR-21 and miR-223 have opposite effect in eosinophil progenitor proliferation

Relative cell number of eosinophil progenitor cultures derived from wild-type (black line), miR-223^{-/-} (red line) & miR-21^{-/-} (purple line) mice is shown. Targeted ablation of miR-21 decreases eosinophil progenitor proliferation. Targeted ablation of miR-223 increases eosinophil progenitor proliferation.

Figure 3. Schematic showing the known roles of a commonly regulated set of miRNAs and their targets in allergic inflammation

The Th1 and Th2 cells exist in a balanced state and inhibit each other's differentiation. MiR-21 inhibits IL-12 and in turn inhibits Th1 differentiation. MiR-146 promotes Treg-mediated suppression of Th1 cells, likely by targeting STAT-1. The suppression of Th1 response promotes a Th2-dominant response with Th2 cytokine IL-13 production. Let-7 inhibits IL-13 production, and downregulation of let-7 likely further amplifies the Th2 response. In T cells, miR-155 promotes both the Th1 and Th2 response by inhibiting the inhibitor CTLA-4. In macrophages, miR-155 attenuates IL-1-mediated responses by downregulating IL-13 receptor alpha 1 (IL-13Rα1). IL-13 inhibits miR-375 expression in epithelial cells, and miR-375 may both potentiate and inhibit IL-13-mediated responses in epithelial cells. IL-13 inhibits miR-133a in smooth muscle cells, and this increases the expression of the small GTPase RhoA that mediates smooth muscle contraction. The miRNAs and their direct targets are shown in red. DC, dendritic cell.

Table 1

List of a core set of miRNAs involved in allergic inflammation, the direction of their change in expression, and their mRNA targets

miRNA	Asthma	Eosinophilic Esophagitis	Atopic Dermatitis	mRNA Targets in Allergic Inflammation
<u>Upregulated or downregulated in all three diseases</u>				
let-7c	↓	↓	↓	IL-13
miR-21	↑	↑	↑	IL-12p35
miR-142-5p	↑	↑	↑	
miR-142-3p	↑	↑	↑	
miR-146a	↑	↑	↑	STAT1
miR-193b	↓	↓	↓	
miR-223	↑	↑	↑	IGF1R
<u>Upregulated or downregulated in two diseases</u>				
let-7a	↓	ND	↓	IL-13
let-7b	↓	ND	↓	IL-13
let-7d	↓	ND	↓	IL-13
miR-146b	↑	↑	ND	
miR-155	↑	ND	↑	CTLA4
miR-365	ND	↓	↓	
miR-375	ND	↓	↓	

ND, difference in expression not significantly different from control

Table 2

Potential utility of miRNAs in allergic diseases

1.	Coordinately targeting key pathways, such as the polarization of adaptive immune responses, by several miRNAs
2.	Reversal of an established disease phenotype
3.	Combinatorial targeting of a key mRNA by several miRNAs
4.	Regulating the epigenetic machinery
5.	Serving as biomarkers for disease diagnosis, stratification, and prognosis
6.	Development as potential therapeutic targets