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Regulation of airway immunity by epithelial miRNAs

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Summary

The airway epithelium is essential to protect the host from inhaled pathogens and particles. It maintains immune homeostasis and mediates tissue repair after injury. Inflammatory diseases of the airways are associated with failure of epithelial functions, including loss of barrier integrity that results in increased tissue permeability and immune activation; excessive mucus secretion and impaired mucociliary clearance that leads to airflow obstruction and microbial overgrowth; and dysregulation of cellular signals that promotes inflammation and alters tissue structure and airway reactivity. MicroRNAs play crucial roles in mounting appropriate cellular responses to environmental stimuli and preventing disease, using a common machinery and mechanism to regulate gene expression in epithelial cells, immune cells of hematopoietic origin, and other cellular components of the airways. Respiratory diseases are accompanied by dramatic changes in epithelial miRNA expression that drive persistent immune dysregulation. In this review, we discuss responses of the epithelium that promote airway immunopathology, with a focus on miRNAs that contribute to the breakdown of essential epithelial functions. We emphasize the emerging role of miRNAs in regulation of epithelial responses in respiratory health and their value as diagnostic and therapeutic targets.

Keywords

miRNA; airway epithelium; inflammation; respiratory disease; airway immunity

Introduction

The airway epithelium lining the bronchial and nasal mucosa is a functionally diverse barrier equipped with cells specialized to protect against a wide array of environmental insults in

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the form of infectious agents, particles, pollutants, chemicals, and allergens. In addition to forming a physical barrier that regulates water and ion transport, the airway epithelium is immunologically active in initiating host defense, controlling immune cell coordination and tissue remodeling, and conducting mechanical clearance of trapped microbes and particles through the mucociliary apparatus.[1,2] Breakdown of fundamental epithelial functions plays a central role in the development and progression of chronic respiratory diseases. Indeed, epithelial dysfunction is well-documented in asthma and airway allergy, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and in the upper airways in allergic rhinitis (AR) and allergic chronic rhinosinusitis (CRS).[2–4] In chronic inflammatory diseases, persistence of the inflammatory state ultimately reprograms cells to sustain an abnormal response even in absence of immunological triggers.[5,6]

miRNAs shape cellular responses in health and disease by regulating networks of genes that control cellular behavior.[7–9] The very first studies of miRNAs established an essential role in developmental biology,[10] but decades of detailed studies have expanded our understanding of how individual miRNAs are integrated in the control of specific cellular and immunological functions.[11] Dysregulated miRNAs are strongly implicated in human chronic pathology, and it is of critical importance to understand how miRNAs rewire cellular responses as their manipulation could change the course of an inflammatory process and reverse disease progression. The availability of the airway epithelium, at the interface of the external and internal milieu, opens possibilities for development of a new generation of RNA-based therapies that could be administered through an inhaled route for rapid, targeted uptake by epithelial cells that are directly involved in driving airway pathology.

Principles of miRNA regulation

miRNAs are evolutionarily conserved short noncoding RNAs that regulate gene expression.[12] Encoded in our DNA genome, they are often organized in clusters where a single gene gives rise to more than one mature miRNA. Clusters typically contain two or three miRNAs, but larger clusters also exist.[13] Transcription of these genes produces a primary miRNA (pri-miRNA) in which one or several clustered miRNAs appear in the same orientation.[14,15] Pri-miRNAs are generally transcribed by RNA polymerase II, where they fold into stem-loop structures with single-stranded ends that undergo cleavage in two sequential steps. First, the stem-loops containing mature miRNA sequences are excised from the pri-miRNA by a Microprocessor complex consisting of the RNase type III enzyme Drosha and its RNA-binding partner DGCR8. The resulting hairpin precursor miRNA (pre-miRNA) is transported to the cytoplasm, where it is further processed by another RNase III enzyme, Dicer, which removes the loop creating a short double-stranded miRNA duplex. One of the strands of this duplex becomes the mature miRNA, while the passenger strand (often designated miRNA*) is degraded. The mature miRNA can be derived from the 5'- or 3'-end of the pre-miRNA hairpin (termed –5p and –3p, respectively), and for some pre-miRNAs, strand selection varies in different cell types and contexts. In any case, the mature miRNAs is loaded into an Argonaute protein, the core RNA-binding and functional component of the miRNA-induced silencing complex (miRISC).[16,17]

Argonaute proteins, loaded with a miRNA, interact with factors that repress translation and accelerate mRNA decay to accomplish widespread post-transcriptional gene regulation.[18] A six nucleotide, highly conserved seed-sequence (nucleotides 2–7 from the miRNA 5'-end) is primarily responsible for mRNA target recognition through complementary base-pairing. Sequences in the 3'UTR of target mRNAs are the most common and effective sites of miRNA-regulation, although target interaction can also occur in the 5'UTR and coding sequence.[19,20]

MicroRNAs with identical or highly similar seed-sequences are classified into miRNA families.[21] Other factors, such as ancestry and evolutionary conservation of sequence identity, similar motif usage which reflects conservation of secondary structure, and conserved mature miRNA-target relationships also help to define miRNA families. Thus, members of a miRNA family are likely to have overlapping target sets and functional redundancy in cellular processes. However, even small differences between miRNA family members can endow them with target specificity, as target recognition does not require perfect complementary base-pairing within the seed-sequence and further pairing in the 3' half of the miRNA can enhance target repression.[18] Moreover, family affiliation does not guarantee functional redundancy, as miRNA expression is highly cell and context dependent.

Altered expression of specific miRNAs can have dramatic effects to entire cellular pathways even though miRNAs typically accomplish fairly modest repression of individual targets (generally less than two-fold at the mRNA and protein level).[22] One explanation for this phenomenon is the network properties of miRNA regulation. A single miRNA may target hundreds of mRNAs and each mRNA, in turn, may harbor multiple miRNA binding sites.[23] Thus, when functionally related targets converge in a cellular pathway, or when a miRNA hits a critical dose-sensitive regulatory node in a gene expression network, profound biologic effects can result.

Various means of regulation of the expression and function of miRNAs themselves adds another layer of complexity. MicroRNA expression can be controlled by the expression and activity of transcription factors, chromatin modification, and DNA methylation, as well as alterations of the miRNA processing machinery.[24] As such, single nucleotide polymorphisms in human miRNA loci affect miRNA binding, processing and function.[25] In addition, long noncoding RNAs can act in *trans* as "sponges" that limit miRNA interactions with their target mRNAs,[25] and target-directed miRNA degradation can even induce specific miRNA decay[26,27] In summary, the evolutionary conservation of miRNA regulation of complex biologic systems suggests that an undisputed advantage is created by this redundancy. At the same time, it makes prediction and validation of miRNA targets challenging. Bioinformatic tools that aid prediction of miRNA interactions continuously improve, yet high rates of false positive and false negative predictions emphasize the importance of experimental validation to determine true miRNA:target binding and biologic function.

Epithelial miRNAs are altered in airway disease

Asthma, COPD, CF, AR and CRS are highly prevalent airway diseases in which environmental, genetic and epigenetic factors alter epithelial dynamics and disrupt lung homeostasis.[3,28,29] Great efforts to profile miRNAs have revealed a large number of differentially expressed miRNAs specifically in airway epithelial cells in asthma,[30–37] COPD,[34,38–40] CRS,[41] AR[36,37,42] and CF.[43–45] MicroRNAs are also dysregulated in lung cancer, as has been reviewed in detail elsewhere.[46,47] Ex vivo cultures of primary human bronchial epithelial cells (HBECs) and epithelial cell lines treated with disease-relevant stimuli such as IL-13 (asthma, AR, CRS), IL-1β (COPD and CF) and smoke conditioned media (COPD) partly mimic miRNA expression patterns obtained from patient samples.[30,40,48–52] Differences in miRNA expression that have been reported in air-liquid-interface (ALI) cultured primary HBECs compared to samples analyzed directly ex vivo suggest that even the more advanced culture systems created to mimic the native airway epithelium may introduce cellular differentiation and composition discrepancies that are reflected in miRNA expression profiles.[53]

Extracellular miRNAs in biospecimens isolated from the airway lumen such as bronchoalveolar lavage (BAL), induced-sputum and exhaled breath condensate may reflect changes in the epithelial secretome.[54–58] Indeed, a study of mouse lungs demonstrated a large overlap between miRNAs expressed in the airway epithelium and miRNAs secreted in extracellular vesicles (EVs) in BAL fluid, indicating that these EVs are primarily of epithelial origin.[59] However, following allergen challenge, EVs and EV-associated miRNAs derived from immune cells increased, indicating that the cellular source of EVs in the airway lumen differs in health versus airway disease. Several studies have determined miRNA content in EVs from liquid airway biopsies in various respiratory diseases and ex vivo culture supernatants, and these may serve as biomarkers of disease or disease endotype.[56,60–62]

The cellular composition of the airways changes dramatically in chronic airway diseases due to infiltrating immune cells as well as large alterations in the epithelial cell landscape. Shifts in epithelial diversity and cellular activation complicate the functional interpretation of differential miRNA expression detected in bulk biopsies from patients compared to healthy subjects. Single-cell RNA sequencing (scRNAseq) provides mRNA expression profiles for thousands of individual cells. MicroRNAs have not been widely studied at the same scale since most scRNAseq techniques primarily quantify protein-coding mRNAs and other polyadenylated transcripts. Nevertheless, miRNA expression at the single cell-level by small RNA sequencing following extensive cell handling has been achieved,[63,64] and it may be possible to infer miRNA activity from scRNA-seq data using motif enrichment and mRNA expression changes.[65] With current technologies, being able to assign gene expression profiles to distinct epithelial subpopulations brings us one step closer to connecting dysregulated miRNAs to specific cell types, cell states and ultimately distinct pathological functions in chronic airway diseases. In the following sections, we will focus on miRNA regulation of specific epithelial properties and functions that are affected in these diseases.

miRNA regulation of epithelial cell differentiation and proliferation

The importance of miRNA regulation during airway epithelial cell development became evident with the very first study using Dicer knockout mice, where global miRNA deficiency was restricted to the lung epithelium.[66] These mice develop gross anatomical defects and instead of the typical branching pattern of the bronchial tree, large fluid-filled sacs form and increased epithelial cell death appears at a later developmental stage. The interest in miRNA regulation of epithelial cell proliferation and differentiation grew quickly and several early studies focused specifically on the miR-17~92 cluster.[67] miR-17~92 expression declines as lung development progresses during embryogenesis.[68] Overexpression of miR-17~92 in mouse embryonic lung epithelium leads to a lethal phenotype with decreased differentiation into distinct epithelial lineages and accelerated epithelial progenitor cell proliferation that was suggested to occur via miR-17 targeting of the tumor suppressor RBL2.[68] Similarly, miR-17~92 cluster deletion leads to early postnatal lethality due to lung hypoplasia.[69] The large polycistronic miR-17~92 cluster produces six mature miRNAs (miR-17, miR-20a, miR-18a, miR-19a, miR-19b-1 and miR-92a-1) that belong to four miRNAs families.[70] The exact mechanism by which the miR-17~92 cluster controls epithelial cell differentiation is not clear, as each mature miRNA can target hundreds of different genes. Critical transcription factors that regulate epithelial differentiation, CEBPA and GATA6, are among the validated direct targets of miR-17~92 miRNAs.[71] miR-17 and miR-20a target STAT3 and MAPK14, which regulate the expression of E-cadherin, an adhesion molecule with critical roles in epithelial barrier formation.[72] In a study of asthma, miR-19a was upregulated in bronchial epithelial cells from patients with severe asthma compared to mild asthma and healthy controls.[31] Experiments with synthetic miR-19a mimics and miR-19a inhibitors indicated that miR-19a promotes bronchial epithelial cell proliferation by targeting TGF-β receptor 2, affecting downstream signaling through SMAD3.

miR-19a is also upregulated in human airway-infiltrating T cells in asthma.[73] This raises the possibility that a common regulatory circuit coordinates inflammatory responses in both airway epithelial cells and T cells. However, the signal(s) that induce specific upregulation of miR-19 in airway T cells and epithelial cells remain unclear. In T cells, miR-19 directly targets the signaling inhibitors *Pten*, *Socs1* and *Tnfaip3* (A20), and promotes Th2 cytokine production.[73] Mouse T cells lacking the miR-17~92 cluster are deficient in inducing asthma-related pathology including airway hyper-responsiveness, mucus metaplasia, lung inflammation and airway eosinophilia. miR-19a alone can partially restore these functions. Thus, the miR-17~92 cluster encodes a group of highly pleiotropic regulators that control critical epithelial and non-epithelial responses in the lung.

Throughout the conducting airways, the balance between various types of specialized cells is tightly regulated. The major cell types in the epithelial layer of the trachea includes basal cells, ciliated cells and goblet cells, but club cells and neuroendocrine cells are also present.[74] Further down the lower respiratory tract, the bronchi and bronchioles are dominated by club cells, neuroendocrine cells and ciliated cells, and in the alveoli at the very end of the bronchial tree, type I and type II epithelial cells are found. Studies of human fetal lung explants revealed an upregulation of miR-200 family miRNAs (miR-200b and miR-200c) during type II epithelial cell differentiation which

correlates inversely with expression of the transcription factors ZEB1/ZEB2 and TGF-β, known targets of this miRNA family.[75] TGF-β plays an important role in the alveoli by negatively regulating surfactant proteins which are critical to maintain the alveolar structure. Accordingly, transfection with a miR-200 family inhibitor suppresses type II epithelial cell differentiation and surfactant expression. Mouse miR-142 also limits the differentiation surfactant-expressing type II alveolar epithelial cells.[76] As abnormalities in pulmonary surfactant are implicated in both emphysema and chronic bronchitis, two defining pathological features of COPD,[77] miRNA regulation of alveolar epithelial cell differentiation may contribute to the pathophysiology of COPD.

MicroRNAs also regulate the differentiation of bronchial airway epithelial cells, and this process is also critical to the healthy respiratory function. miR-34/449 family miRNAs are highly enriched in ciliated cells,[78–80] and interference with miR-449 targeting activity represses ciliogenesis.[80] NOTCH1 and its ligand DLL1 are critical targets, with Notch signaling undergoing miR-449-mediated inhibition to enable differentiation of ciliated cell progenitors.[80] miR-34/449 family miRNAs share identical seed-sequences and similar expression patterns, and appear to play compensatory roles in lung development. Mice lacking either miR-34a/b/c or miR-449a/b/c develop normally.[81,82] In contrast, mice lacking all miR-34/449 miRNAs exhibit frequent postnatal mortality, infertility and respiratory dysfunction caused by defective mucociliary clearance.[83] CP110, a protein that regulates primary cilia assembly, may contribute to this phenotype since knockdown of CP110 reverses abnormal ciliogenesis in miR-34/449 deficient mice. The expression of all members of the miR-34/449 family are significantly reduced in bronchial epithelial cells in asthmatic subjects, and ex vivo cultures of normal HBECs stimulated with IL-13 largely recapitulates the miRNA expression pattern in asthmatics, including downregulation of the miR-34/449 miRNA family.[30] A recent study using flow cytometric sorting of HBECs differentiated in ALI culture confirmed selective enrichment of miR-34/449 miRNAs in ciliated cells, which decreased upon IL-13 treatment.[84]

Advances in novel sequencing technologies over the past decade have increased our understanding of the cellular heterogeneity of the airway epithelium.[1] Adding to the known cellular communities described above, new populations with unique gene expression signatures and differentiation trajectories have been discovered. Ionocytes were first reported in 2018 and are defined by expression of FOXI1, a transcription factor that regulates the CF transmembrane conductance regulator (CFTR).[85,86] CFTR encodes a chloride-ion transporter that is defective or absent in individuals diagnosed with CF.[87] Ionocytes account for only 1–2% of human airway epithelial cells, but are more enriched in CFTR mRNA than any other airway cell.[85] In addition to identification of new cell types, sequencing studies have improved our knowledge of rare epithelial populations in the airway. Tuft cells are well-characterized in intestinal epithelium, but they are also present at low frequency in the airway. Despite their rarity, tuft cells are an important source of the pro-inflammatory cytokine IL-25, a finding with important implications for type 2-driven airway pathology in AR, CRS and asthma.[88,89] Furthermore, mucous ciliated cells that co-express goblet and ciliated cell gene signatures have been identified as a novel epithelial cell state in the epithelium of asthmatic patients,[90] consistent with previous reports of IL-13-induced trans-differentiation of ciliated cells into the goblet lineage.[91]

Strategies using pseudotime gene expression analysis have enabled detailed studies of epithelial trajectories,[92] however, little is known about miRNA regulation of these lineage decisions. We recently reported that a member of the miR-200 miRNA family, miR-141, promotes goblet cell differentiation and mucus production.[93] Disrupting miR-141 expression by CRISPR/Cas9-targeting in primary HBECs cultured at ALI decreases the frequency of IL-13-induced goblet cells in favor of an enriched basal cell signature. Moreover, miR-141 targets a large network of genes expressed during goblet cell differentiation. Predicted miR-141 target genes are distributed across four distinct transitional states defined by pseudotime gene cluster analysis of human trachea, from basal-like cells, through secretory preparation and club secretory clusters, to mucus producing goblet cells.[92] Prior biochemical analyses of wild type, miR-200 family knockout and miR-200 overexpressing murine epithelial cells confirms direct miRNA binding to many of these target mRNAs.[94] Notably, miR-141 expression is induced in bronchial brushings from allergic asthmatic subjects shortly after allergen challenge, suggesting that miR-141 may regulate goblet cell responses in response to allergens.[93] A study of CRS patients with nasal polyps, a disease also driven largely by IL-13, demonstrated that the CRS nasal epithelium displayed basal cell hyperplasia wherein mucus secretory cells and ciliated cells are trapped in an undifferentiated state causing reduced epithelial diversity.[5] How miRNAs are implicated in the control of distinct epithelial lineages and alterations that occur in specific disease settings remains to be elucidated. Further studies investigating miRNA regulation of epithelial cell differentiation and regeneration to maintain cell fitness and airway health are needed.

miRNA regulation of epithelial barrier integrity

The structural arrangement of closely packed pseudostratified cells effectively prevents the airway epithelium from leaking substances into the submucosa.[95] Contact points between neighboring cells in the form of tight junctions, adherens junctions and desmosomes seal the intercellular space even further to block any flow through. Most apically located, tight junctions are comprised of trans-membrane proteins claudin, occludin and junctional adhesion molecules. They interact with adaptor proteins, zonula occludens, which are anchored to the actin cytoskeleton.[96] Tight junctions are essential to establish an impermeable barrier, however, selective permeability is required to allow hematopoietic cells to translocate between the submucosa and airway lumen as they scan the immune environment. To this end, tight junctions are meticulously regulated. miRNAs have been reported to target tight junctions in several different types of barrier tissues including the blood-brain-barrier, intestinal and skin barrier, urinary tract and bladder epithelium and endothelium.[97] In AR, CRS, asthma and COPD, the airway epithelium is characterized by tight junction defects and increased epithelial permeability,[98–101] where allergens, pathogens, smoke and pollutants have been reported to cleave tight junctions, facilitating interactions with immune cells.[102,103] Tight junction organization may also be dysregulated by mislocalization of CFTR, which alters epithelial permeability in CF.[104]

miR-155 is a miRNA with multiple roles in airway immunity.[105] Adding to its established functions in lymphocytes and myeloid cells, miR-155 was recently reported to limit tight junction formation in epithelial cells.[106] In the human bronchial epithelial cell line,

16HBE14o-, miR-155 is induced in a dose-dependent manner by poly(I:C), a dsRNA viral mimic recognized by Toll-like receptor 3. Transfection with miR-155 mimics increases epithelial permeability and inhibits claudin expression, whereas a miR-155 inhibitor suppresses poly(I:C)-induced barrier disruption. Poly(I:C) stimulation also decreases tight junction protein expression while also upregulating many miRNAs in primary human nasal epithelial cells.[107] One of these miRNAs, miR-146a, is induced via PI3K, JNK and NK-κB signaling, targets TRAF6, and prevents poly(I:C)-induced downregulation of claudin-1, occludin and junction adhesion molecule A, and maintains epithelial barrier integrity as measured by transepithelial electrical resistance.[107]

In various respiratory contexts, disruption of epithelial integrity is characterized by detachment of ciliated cells, creola bodies (i.e. epithelial cell aggregates) and reduced expression of the intercellular adhesion molecule E-cadherin, the major constituent of adherens junctions.[108] Loss of E-cadherin can result in altered epithelial plasticity and repair in a process called epithelial-to-mesenchymal-transition (EMT). Epithelial cells that undergo EMT acquire a mesenchymal phenotype as they gain expression of N-cadherin which provides the cell with migration potential, and metalloproteases, vimentin and fibronectin that remodel the extracellular matrix.[109] Initial induction of mesenchymal traits is essential to enable epithelial repair. With the removal of cellular junctions, the transdifferentiated epithelial cells are free to migrate to damaged areas throughout the tissue and even through the blood stream, a process hijacked by metastasizing tumor cells. Under normal circumstances, the regenerating epithelium differentiates into a pseudostratified epithelial layer, however, inflammatory airway diseases are characterized by abnormal epithelial repair. Indeed, elevated expression of basal cell markers and repair markers suggests that a more proliferative, less differentiated, airway epithelium is present in asthma and CRS.[5,93] miR-155 promotes EMT in nasal epithelial cells from CRS patients, and inhibition of miR-155 leads to upregulation of epithelial markers and a corresponding downregulation of mesenchymal markers.[110] EMT is accompanied by changes in the expression of many miRNAs in human airway epithelial cells.[111] miR-133a is upregulated in epithelial-derived mesenchymal-like cells, and miR-133a overexpression induces spontaneous EMT in airway epithelial cells and inhibits expression of the epithelial transcription factor GRHL2. Loss of GRHL2 promotes downregulation of ESRP1, a protein that modifies adherens junction proteins, leading to degradation of E-cadherin.[111] Several other miRNA candidates have been implicated in EMT control, primarily in cancer, but also in mouse models of airway inflammation.[108,112,113]

Members of the miR-200 miRNA family are among the most highly expressed miRNAs in the human bronchial epithelium.[93] The miR-200 family play a critical role in maintaining the epithelial phenotype, as overexpression of each of the individual miRNAs in this family (miR-200a/b/c, miR-141, and miR-429) or overexpression of one of the two clusters (miR-200c/141 or miR-200b/200a/429) block EMT in murine mammary gland epithelial cells.[114] These miRNAs target ZEB1 and ZEB2, two transcriptional repressors of E-cadherin, which is thereby upregulated in response to miR-200 miRNA overexpression. Epithelial E-cadherin expression is critical to prevent airway pathology, as clearly demonstrated in a recent study of induced E-cadherin deficiency in mouse lung epithelial cells after birth.[115] These mice developed spontaneous progressive epithelial damage which

ultimately mimicked the lung pathology of asthma with goblet cell metaplasia, mucus production, and infiltration of eosinophils.

One important consequence of decreased E-cadherin expression is alteration of the activity of its binding partner β-catenin. Upon dissociation from E-cadherin, unless it is degraded, β-catenin translocates from the cytoplasm to the nucleus where it mediates transcriptional activation of the E-cadherin repressors TWIST, SNAIL and SLUG, and other mesenchymal genes that contribute to epithelial barrier remodeling.[108] Several features observed in relation to decreased E-cadherin expression may be mediated at least in part by activation of β-catenin, as inhibition of signaling events downstream of β-catenin improves barrier function and attenuates airway inflammation, airway hyper-responsiveness and goblet cell metaplasia in mouse models of asthma.[116,117] MicroRNAs targeting β-catenin and other critical WNT signaling molecules have been identified in asthma, CF and COPD.[118–120]

TGF-β is a critical regulator of epithelial cell proliferation and repair, and it induces EMT. By increasing release of growth factors while undergoing EMT, TGF-β signaling is reinforced by transforming epithelial cells.[121] These growth factors, in turn, activate fibroblasts to produce extracellular matrix components, making EMT is a strong driving force behind fibrosis.[122] Excessive collagen deposition below the basement membrane leads to airway wall thickening, and TGF-β signaling is associated with increased smooth muscle mass, which are hallmarks of airway remodeling in asthma and, to a lesser degree, COPD.[108] To date, little is known about miRNAs directly involved in the regulation of pulmonary fibrosis by epithelial cells, but miR-155 has been implicated in human lung fibroblasts in response to TGF-β and in mouse models of bleomycin-induced lung fibrosis.[123] Of note, alterations in extracellular matrix composition have additional consequences in the lung. For instance, stiffening of the extracellular matrix changes the migratory properties of type 2 innate lymphoid cells (ILC2s), impacting the development of airway eosinophilia.[124] Regulation of inflammatory processes by the epithelium has gained much attention in the past decade and is discussed in the following section.

miRNA regulation of epithelial-immune cell crosstalk

The functional constraints resulting from altered epithelial differentiation and proliferation in airway pathologies affect both specialized cellular functions and epithelial communication with mucosal immune cells that drive inflammation. For instance, basal cell hyperplasia in CRS increases the pool of pro-allergic cytokines IL-33 and TSLP which reinforce the allergic milieu, even in absence of an allergic stimulus.[125] IL-33 is constitutively expressed by epithelial cells at barrier sites including the lung,[126] and its release is important in early detection of parasitic or viral infections, and inhalation of toxic substances or protease-containing allergens. IL-33 is a potent inflammatory mediator that is kept under tight regulation through multiple mechanisms, including the action of miRNAs.[127] miR-200b and miR-200c expression in BAL cells inversely correlates with IL-33 production and asthma severity.[128] miR-200b/c may regulate IL-33 production both directly and indirectly. Human *IL33* mRNA contains a functional miR-200b/c binding site, and miR-200 mimics and inhibitors reciprocally regulate IL-33 expression in MRC5 cells, a human lung fibroblast cell line. However, although the miR-200b/c binding site is not conserved in mice, exogenous

administration of miR-200b mimics to lungs of allergen challenged mice also reduces IL-33 levels and resolves airway inflammation. The miR-200 family is expressed at lower levels in asthmatic epithelium at baseline compared to healthy epithelium, with induction following allergen challenge in asthmatics,[30,93] suggesting that miR-200 may modulate elevated IL-33 in the asthmatic epithelium. Similar to the miR-200 family miRNAs, miR-155 is expressed several fold lower in BAL cells isolated from asthmatic patients compared to healthy controls.[128] Indirect or direct miR-155-targeting of IL-33 has not been reported, although mice with germline miR-155 deficiency have significantly reduced IL-33 protein in lung tissue after allergen challenge compared to wild type controls.[129]

IL-33 may be particularly important for susceptibility to allergic diseases in early life as it is dynamically regulated during lung development. In mice, epithelial release of IL-33 increases rapidly at birth due to mechanical damage caused by replacement of liquid with gas inflation at the first breath, a process that was replicated by exposing late-stage murine embryos to vacuum.[130] This burst in IL-33 forms a type 2 cytokine environment in the lung that peaks during alveolarization (day 7–14 in mice) when the lung epithelium undergoes final differentiation, and IL-33-responsive immune cells (ILC2s, mast cells, eosinophils and basophils) accumulate in the lung.[131] Mice sensitized to aeroallergen at day 14 of life exhibited an increased response to allergen challenge later in life compare with those sensitized as adults. This finding indicates that the formation of a type 2 niche during a critical developmental period can influence later susceptibility to allergic inflammation.[131] There is no doubt that miRNA activities are essential during lung development, but their role in shaping the immune environment in the airways in early life remains an important area for further exploration.

The immunological activities of the airway epithelium are extensive. Epithelial cells express an array of innate immune receptor families that are capable of recognizing pathogen-associated molecular patterns from inhaled microbes, parasites and allergens as well as damage-associated molecular patterns released from dying or damage cells.[2] Upon recognition of a potential threat, epithelial cells activate downstream signaling by releasing pro-inflammatory cytokines and chemokines, including IL-6, IL-8, CCL17, CCL20, GM-CSF, IL-33, IL-25 and TSLP, which attract or activate mucosal cells of the innate and adaptive immune system.

IL-8 elevation in the airways in CF patients drives neutrophilic inflammation. Bronchial brushings from CF patients differentially express several miRNAs, including miR-17, which is decreased in CF compared to non-CF control subjects.[132] miR-17 can regulate *IL8* mRNA expression in primary epithelial cells, epithelial cell lines and CFTR knockout mice, and miR-17 overexpression inhibits IL-8 production, potentially dampening airway neutrophilia in vivo.[132] Like CF, COPD is characterized by increased IL-8 levels and airway neutrophilia, but COPD is generally caused by cigarette smoking or long-term exposure to lung irritants. miR-29b expression in both lung and plasma decreases in COPD patients and correlates with pulmonary function and airway inflammation. Functionally, miR-29b reduces cigarette smoke extract-induced IL-8 in the bronchial cell line HBE4-E6/E7.[133] miR-181c expression is also reduced in lung tissue of COPD patients, in primary HBECs, and in mice after exposure to cigarette smoke.[134] Overexpression of miR-181c attenuates the inflammatory

response induced by cigarette smoke as seen by decreased neutrophil infiltration, reactive oxygen species generation, and inflammatory cytokine production. Inhibition of miR-181c produces the opposite result, increasing the inflammatory response.

Bronchial epithelial cell-expressed miR-146a may play a specific role in a subset of asthma cases by regulating the recruitment of neutrophils. miR-146a expression is reduced in bronchial brushings from asthmatic patients compared to non-asthmatic control subjects irrespective of asthma endotype (i.e. neutrophilic, eosinophilic or paucigranulocytic asthma).[135] But BAL neutrophils correlate inversely with bronchial brushing miR-146a expression, and miR-146a is induced in HBEC cultures by IL-17A, a cytokine associated with non-allergic neutrophilic asthma. IL-8 and CXCL1, both chemokines capable of attracting neutrophils, are significantly increased in neutrophilic asthma. miR-146a overexpression in HBECs inhibits the production of these chemokines and reduces the ability of HBEC supernatants to support neutrophil migration in vitro. Together, these findings suggest that lower epithelial miR-146a expression may contribute to neutrophilic asthma.

The most common form of asthma is defined by type 2 inflammation with elevated levels of IL-5 and IL-13 that promotes eosinophilia and goblet cell hyperplasia, respectively.[136] miR-155 overexpression in bronchial epithelial cells specifically suppresses the IL-13-induced eosinophil-recruiting chemokines eotaxin-1 and eotaxin-3.[49] Related to this observation, miR-155 deficient mice have severely impaired airway eosinophilia and eotaxin levels, and lower mucus production in a type 2 model of airway inflammation.[137] Regulation of epithelial mucus production by miRNAs is discussed in the next section.

miRNA regulation of epithelial mucus production

Multiple biological systems work together to ensure protection of the airway mucosa. The outermost defense is the airway mucus produced by goblet cells which traps inhaled pathogens and particles, preventing them from penetrating the tissue. The mucus gel also contains immunomodulatory molecules produced by goblet cells that have been studied extensively in the gastrointestinal tract where they serve essential functions in immune tolerance.[138]

A pathological mucus response is a hallmark feature of CF and affects both the airway and intestinal mucosa. Altered ion homeostasis in the CF airway leads to impaired mucociliary clearance with mucus accumulation and airway surface liquid volume depletion.[139] The thick mucus layer promotes bacterial colonization, which generates a highly proinflammatory environment. High protease burden from infiltrating neutrophils damages the lung tissue, and ultimately leads to bronchiectasis and lung failure. Many miRNAs alter CFTR activity and inflammatory pathways in CF, with several of them regulating CFTR expression directly.[140] Moreover, mutations in the *CFTR* 3'UTR enhance the affinity of miRNA binding causing a decrease in CFTR expression that can contribute to CF pathology.[141]

In addition to CF, chronic inflammatory airway diseases such as COPD and asthma are similarly characterized by mucus hypersecretion and impaired mucus clearance that cause

obstruction of the airways.[142] This condition is associated with poor disease prognosis, increased exacerbation frequency, and mortality. Mucus plugs can persist over long periods of time in distinct sites of the lungs in patients with asthma, suggesting that goblet cells undergo functional reprogramming in localized regions.[143] Distant communication may also play a role as epithelial-derived EVs can induce mucus hypersecretion and alter miRNA expression in recipient epithelial cells.[144] RNA profiling of bronchial biopsies from COPD patients with chronic mucus hypersecretion, defined by self-reported questionnaires, identified 20 miRNAs and over 500 predicted target mRNAs that were differentially expressed in COPD with chronic mucus hypersecretion.[38] However, knowledge of specific miRNAs that are critical in regulating mucus is currently limited. miR-141 expression is important for IL-13-induced goblet cell mucus production, as both miR-141 repression in primary HBECs differentiated at ALI and miR-141-specific antagomir administration to the airways of allergen-challenged mice results in decreased mucus production.[93] The possible roles of other miR-200 family members and the mechanism by which miR-141 regulates mucus-producing goblet cells remain unclear clear and warrant further investigation. In cervical cancer cells, miR-141 targets FOXA2,[145] a transcription factor involved in the transition of club cells to a goblet cell phenotype in the context of mucus cell metaplasia. Targeted deletion of FOXA2 in the airway epithelium results in spontaneous mucus metaplasia in mice.[146] Several other transcription factors coordinate this transcriptional program, and disruption of these mediators leads to mucus cell metaplasia.[147]

SPDEF is the master transcription factor of goblet cells. Mice with a targeted deletion of SPDEF lack goblet cells, and transgenic mice overexpressing SPDEF exhibit goblet cell hyperplasia.[147,148] Moreover, CRISPR/Cas9-targeting of SPDEF efficiently abolishes mucus production in primary ALI-cultured HBECs.[149] miR-125b targets SPDEF in primary HBECs, and miR-125b expression in sputum correlates inversely with SPDEF expression in subjects with asthma.[150] Furthermore, intranasal delivery of miR-125b in mice decreases SPDEF protein levels, goblet cell differentiation and mucus hypersecretion. miR-330 indirectly regulates expression of MUC5AC,[151] the major pathological gel-forming mucin found in mucus plugs in fatal asthma.[152] miR-330 is downregulated in the blood of asthmatic patients, and IL-13-induced MUC5AC production in the cell line HBE16 is accompanied by a dose-dependent decrease in miR-330 expression.[151] The many roles played by epithelial miRNAs in the regulation of airway health and disease are summarized in Figure 1.

Epithelial miRNAs as therapeutic targets in airway disease

A growing body of evidence elucidates the instrumental roles played by the airway epithelium in the onset and progression of pathological responses in chronic inflammatory airway diseases. For instance, in children with respiratory problems, structural changes in the airway epithelium have been observed before the onset of airway inflammation and clinical diagnosis of asthma, indicating that epithelial changes may occur early in asthma pathogenesis, thereby challenging the view that chronic airway inflammation is the primary driver of airway remodeling.[153] Many of the susceptibility genes that have been identified in allergic diseases are expressed by the airway epithelium, however, the exact mechanisms linking susceptibility genes to an altered response to environmental risk

factors are still not clear.[108] Furthermore, studies in recent years suggest that epithelial communication with the immune system shape the airway environment already at birth and affects disease susceptibility in early life,[130,131] and possibly much longer. The idea that the airway epithelium creates a niche that persists over time makes it especially interesting as a pharmacological target. For instance, reprogramming of epithelial cells that contribute to persistent mucus-plugging could improve airflow obstruction in patients with a severe mucus phenotype. Preclinical studies in mice have shown great promise, as specific miRNA antagomir administration can acutely reduce mucus production in the lung.[93] Antagomirs targeting epithelial miRNAs can also dampen airway inflammation and improve clinical features such as airway hyperresponsiveness.[93,154–157]

The accessibility of the airway epithelium which allows administration of antagomirs through an inhaled route makes epithelial miRNAs highly attractive for therapeutic intervention. Non-epithelial cells in the airways, especially macrophages and other phagocytic cells, may also take up therapeutic oligonucleotides. However, studies using fluorescently conjugated antagomirs in mice have demonstrated efficient uptake specifically by the airway epithelium compared to other cellular populations,[158] and technologies to improve delivery of miRNA-directed therapeutics to ensure efficient and safe targeting are being developed.[159] These improvements may be particularly important for targeting cell populations such as lymphocytes that have shown poor uptake in experiments using non-assisted antagomir administration.[158] Further work using miRNA reporter systems to carefully map miRNA activity in target cells is an important direction for future studies.

Currently, over 150 mRNA-based therapeutics and vaccines are being evaluated for treatment of a variety of diseases, with most of these candidates in early stages of development.[160] Airway miRNAs are also being explored as biomarkers of pulmonary disease or specific disease endotypes and have proved capable of distinguishing inflammatory phenotypes.[56,161] With the current SARS-CoV-2-pandemic and the tremendous success of mRNA-based vaccines, the RNA diagnostics and therapeutics market is expected to grow at a rapid pace in the coming years, and will likely include new miRNA-based or miRNA-directed therapeutics.

Conclusion

Future research focusing on unravelling the molecular networks underlying abnormal epithelial responses to environmental exposures and the contribution of genetic factors hold promise for the identification of novel intervention strategies in respiratory diseases (Figure 2). miRNAs clearly play crucial roles in counteracting airway pathology ensuring maintenance of epithelial barrier function, epithelial diversity, appropriate mucus secretion, and lung homeostasis. Detailed insight into these regulatory mechanisms using new technologies such as scRNA-seq will lead to an increased understanding of cellular changes in distinct disease contexts and how to reverse pathological trajectories. Key future goals include development of safe and efficient therapeutic strategies aimed at correcting dysfunctional epithelial biology in chronic disease states, and identification of patients that will benefit from epithelial-focused therapies.

Acknowledgements

Abbreviations:

ALI	Air-liquid-interface
AR	Allergic rhinitis
BAL	bronchoalveolar lavage
COPD	Chronic obstructive pulmonary disease
CRS	Chronic rhinosinusitis
CF	Cystic fibrosis
EV	Extracellular vesicle
HBEC	Human bronchial epithelial cell
scRNAseq	Single-cell RNA sequencing
ILC2	Type 2 innate lymphoid cell

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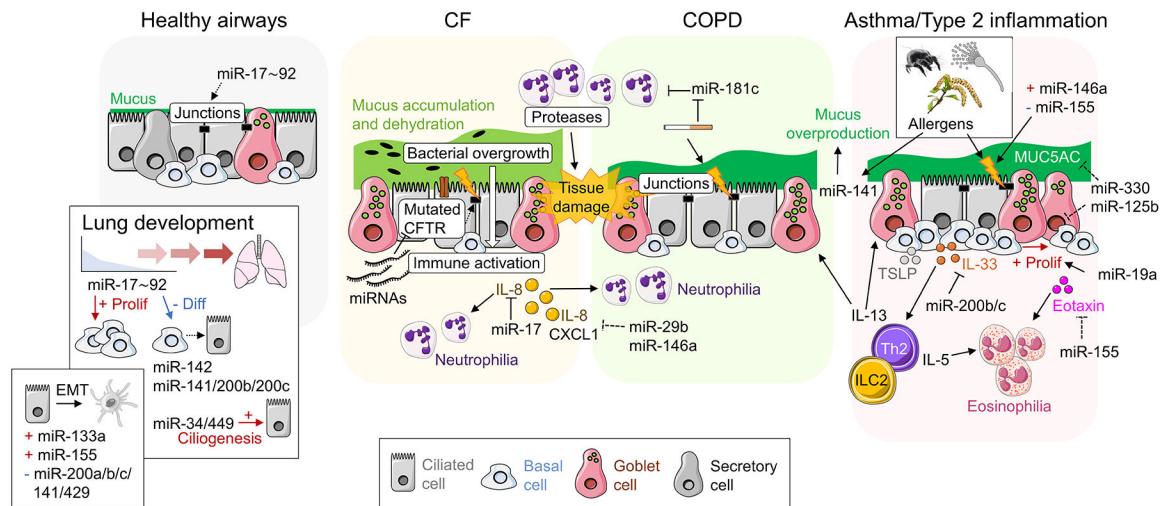
Figure 1.
Summary of miRNA functions in airway epithelium in health and in the chronic respiratory diseases cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and asthma and allergic (i.e. Type 2) inflammation. MicroRNAs regulate epithelial development, structural integrity, mucus secretion, cell differentiation, environmental sensing, and intercellular communication, including crosstalk with inflammatory cells. See the main text for details and references.
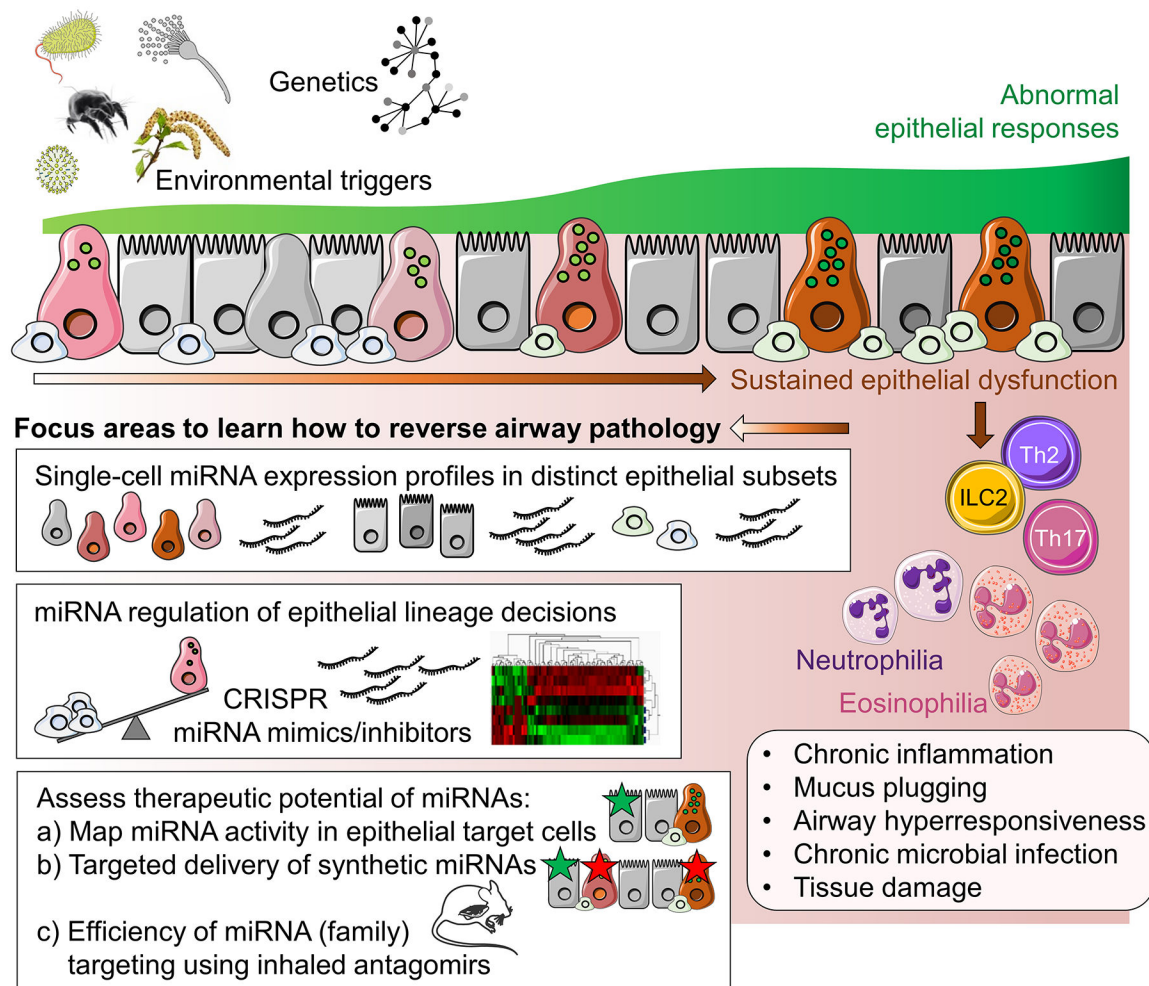
Figure 2.
Summary of conceptual areas of focus and experimental strategies for further research to better understand and ultimately learn how to reverse the miRNA-regulated abnormal epithelial responses that underlie chronic pulmonary diseases.