Review

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The role of microRNAs in chronic respiratory disease: recent insights

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Abstract: Chronic respiratory diseases encompass a group of diverse conditions affecting the airways, which all impair lung function over time. They include cystic fibrosis (CF), idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD) and asthma, which together affect hundreds of millions of people worldwide. MicroRNAs (miRNAs), a class of small non-coding RNAs involved in post-transcriptional gene repression, are now recognized as major regulators in the development and progression of chronic lung disease. Alterations in miRNA abundance occur in lung tissue, inflammatory cells, and freely circulating in blood and are thought to function both as drivers and modifiers of disease. Their importance in lung pathology has prompted the development of miRNA-based therapies and biomarker tools. Here, we review the current literature on miRNA expression and function in chronic respiratory disease and highlight further research that is needed to propel miRNA treatments for lung disorders towards the clinic.

Keywords: chronic disease; lung; microRNA; respiratory disease; therapy.

Introduction

MicroRNAs (miRNAs, miRs) represent a major mechanism of post-transcriptional gene regulation in eukaryotes. These small (~22 nucleotide) non-coding RNAs typically repress gene expression through complementary binding to the 3′ untranslated region (UTR) of a messenger RNA (mRNA). miRNAs were first identified as being involved in *Caenorhabditis elegans* larval development (Lee et al., 1993; Wightman et al., 1993), and today are recognized as important regulators of most evolutionary conserved processes in plants and animals. At present, the human genome contains at least 1500 annotated miRNA genes (with many more predicted; see Kozomara and Griffiths-Jones 2014; Londin et al., 2015), and each is thought to target on average more than 400 individual mRNAs (Friedman et al., 2009). Expression profiles of miRNAs are critically important in defining functional states of specific tissues, and their dysregulation in a number of diseases suggests their dynamic roles in maintaining homeostasis. In chronic respiratory disease, wherein lung function decline results in an inability to breathe, both the pathogenicity and the restorative nature of miRNAs are well studied. More research and better tools, however, are needed to fully understand the functional implications of miRNAs in lung health and disease. In this review, we summarize the current picture of miRNA biology, describe the roles of miRNAs in several chronic lung diseases, and discuss recent advances in the development of miRNAs as disease biomarkers and novel therapeutics.

Biogenesis and function of miRNAs

MicroRNA genes are transcribed by RNA polymerase II, either as single miRNA transcripts, polycistronic transcripts, or located within a larger protein-coding transcript (Lee et al., 2004; Rodriguez et al., 2004). These primary transcripts (pri-miRNAs) are usually at least several hundred base pairs long, are capped and polyadenylated, and form a distinctive stem-loop structure (Cai et al., 2004). The stem-loop and adjacent sequences are recognized by the microprocessor complex, consisting of Drosha (an RNase III enzyme) and DiGeorge syndrome critical region 8 (DGCR8), which crops the stem-loop from the surrounding sequence into a ~70 bp precursor miRNA (pre-miRNA) (Lee et al., 2003; Denli et al., 2004;

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Landthaler et al., 2004; Han et al., 2006). The pre-miRNA is then exported from the nucleus by Exportin 5 and a RAS-related nuclear protein (Ran-GTPase) (Yi et al., 2003; Lund et al., 2004). In the cytoplasm, the pre-miRNA is cleaved by Dicer, a second RNase III enzyme, to create a small double-stranded miRNA duplex averaging 22 nucleotides in length (Zhang et al., 2004). The duplex is recognized by Argonaute (AGO) proteins and quickly unwound such that the non-targeting passenger strand (*) is degraded and/or removed (Meister et al., 2004). The targeting guide strand is usually determined by its lower relative thermostability compared to the passenger strand, though strand selection is not entirely strict and either can be utilized (Khvorova et al., 2003). Either the 3p or 5p versions of miR-142, for example, can be selected based on tissue-context (Wu et al., 2009; Ha and Kim 2014).

Next, the AGO-miRNA complex assembles into a larger structure known as the RNA-induced silencing complex (RISC), located in cytoplasmic P-bodies and stress granules, which targets the miRNA to the 3′ UTR of an mRNA (Hammond et al., 2000; Kawamata and Tomari 2010). The seed site of the miRNA (nucleotides 2–7) typically creates Watson-Crick base pairs with complementary nucleotides in the target, though non-canonical seed site pairing can also occur (Lewis et al., 2005; Bartel, 2009). Computational algorithms based on this pairing, such as TargetScan, miRanda, Pictar and miRDB, among others, were developed as useful tools for predicting miRNA-target interactions (John et al., 2004; Lall et al., 2006; Agarwal et al., 2015; Wong and Wang, 2015). Gene repression is facilitated by direct mRNA cleavage via a special AGO protein called slicer, by mRNA destabilization through decapping or poly(A) tail removal, or by inhibition of protein translation by prevention of ribosome binding (Bartel, 2004; Chekulaeva and Filipowicz, 2009). Conversely, some miRNAs have also been found to up-regulate gene expression by modifying interactions between AU-rich elements (AREs) and ARE-binding proteins within the 3′ UTR (Bhattacharyya et al., 2006; Vasudevan et al., 2007), by binding to the 5′ UTR (Ø rom et al., 2008), or through promoter activation (Place et al., 2008); however, these mechanisms are generally not well understood.

A single miRNA may target multiple sites within a single 3′ UTR and often regulates multiple genes within the same signaling network. Moreover, the high evolutionary conservation of miRNAs across eukaryotes and the redundancy of miRNA genes, as exhibited by families of miRNAs possessing the same targeting sequence, suggests their vital role in controlling biological processes (Lee et al., 2007). It is predicted that evolutionary constraints maintain miRNA-mRNA pairing in greater than 60% of human genes, while more than 84% of protein output is miRNA-controlled (Friedman et al., 2009; Guo et al., 2010). In contrast, a significant proportion of miRNAs in humans are not part of conserved families and have relatively low expression levels (Friedman et al., 2009). The emergence of *de novo* miRNA families has increased by more than three-fold in mammals, and these are overrepresented within introns, likely catalyzed by host gene transcription (Meunier et al., 2013). These miRNAs may be equally as, if not more, important in human-specific diseases, as evidenced by a recent study indicating that profiles of lowly expressed miRNAs significantly enhance cancer phenotyping (Rasnic et al., 2017). Moreover, the effects of most miRNAs on protein expression are very modest (Baek et al., 2008), and the majority of miRNA deletion models do not exhibit any observable defects (Miska et al., 2007). In most studies of chronic respiratory diseases, miRNA knockout models either do not exist or have not been published. Some miRNAs alone can act as drivers of disease, as first evidenced by the deletion of miR-15a/16-1 in chronic lymphocytic leukemia (Klein et al., 2010); however, most data from miRNA research in the lung suggest that their primary function is to fine-tune gene expression and maintain signaling pathway homeostasis. Collectively, the dysregulation of multiple miRNAs and pathways may play a significant role in respiratory disease phenotypes and progression, though the specific mechanism(s) by which this dysregulation occurs remains obscure.

MicroRNA regulation in chronic respiratory diseases

Cystic fibrosis

Cystic fibrosis (CF) is the most commonly inherited disease among Caucasians, affecting about 70,000 people worldwide (Alexander et al., 2016). CF is caused by mutations in the CF transmembrane conductance regulator gene (*CFTR*) (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989). The most common mutation is deletion of a phenylalanine residue at position 508 (F508del), which occurs on approximately 70% of CF chromosomes (Kerem et al., 1990). F508del is a trafficking mutant, which causes misfolding, ER retention and degradation of the CFTR protein. *CFTR* encodes a chloride ion channel, that when defective, impairs chloride, sodium and bicarbonate transport and leads to airway dehydration and buildup

of thick mucus secretions, fostering the development of persistent respiratory infections (Alexander et al., 2016). Because of these dense mucus accumulations, pathogens cannot be cleared efficiently, resulting in chronic host inflammatory responses at sites of infection. This inflammation, due mainly to the infiltration of neutrophils recruited by elevated interleukin 8 (IL-8), eventually leads to structural changes in the lung. Lung injury and tissue remodeling, involving both the epithelium and underlying stroma, promotes further airway obstruction, which may be observed in infants as young as 1 month (Ratjen and Döring, 2003; Courtney et al., 2004). Lung damage worsens with age and becomes the defining cause of mortality (Sly et al., 2009). New treatments that restore CFTR channel function are not currently beneficial for all CF patients. Interestingly, lung disease severity cannot be predicted based on *CFTR* mutation alone, suggesting the existence of modifier genes that control respiratory phenotype independent of the *CFTR* locus (The Cystic Fibrosis Genotype Phenotype Consortium, 1993).

miRNAs have emerged as important modifiers of CF lung disease severity, though early research focused on identifying miRNAs involved directly in *CFTR* regulation. Evaluation of microRNA prediction algorithms and subsequent *in vitro* luciferase-based reporter assays by our group showed that miR-145 and miR-494 directly target the 3′ UTR of *CFTR* and reduce its expression (Gillen et al., 2011). Similar studies by additional groups confirmed the repressive role of miR-145 and miR-494 on *CFTR*, and identified increased expression levels of these miRNAs in airway epithelial cells from CF patients compared to healthy controls (Megiorni et al., 2011; Oglesby et al., 2013; Ramachandran et al., 2013). Only miR-494 expression was found to be directly correlated with *CFTR* levels *in vivo;* however, both miRNAs may act to modify CF lung phenotypes through parallel pro-fibrotic and inflammatory pathways (Oglesby et al., 2013; Megiorni et al., 2013). Additional miRNAs revealed to directly target *CFTR* were miR-144 and miR-223 (Hassan et al., 2012; Oglesby et al., 2013). Some discrepancy has arisen on the role of miR-101, as this miRNA targeted the *CFTR* 3′ UTR in some studies, but not in others (Gillen et al., 2011; Megiorni et al., 2011; Hassan et al., 2012). In addition, CF-causing mutations were identified in the 3′ UTR of *CFTR*, one of which (1043A → C) was recently found to alter the affinity of miR-433 and miR-509, suggesting these miRNAs to also have disease relevance (Amato et al., 2013).

Several groups also identified miRNAs involved in CF lung disease severity independent of CFTR. miRNA expression profiling studies in CF patients and cells identified hundreds of miRNAs that were dysregulated in disease. The first to be investigated, miR-126, was significantly decreased in endobronchial brushings from CF patients (Oglesby et al., 2010). Downregulation of miR-126 increased expression of Target of Myb1 (TOM1), which is involved in intracellular protein trafficking and acts as a negative regulator of innate immune responses in the CF lung. The authors proposed that upregulated TOM1 might reduce the pro-inflammatory burden in CF (Oglesby et al., 2010). In addition, miR-155 was found to be overexpressed in CF cell lines (Bhattacharyya et al., 2011). Increased miR-155 was associated with upregulation of IL-8 activity through direct repression of SHIP1 (an inositol phosphate phosphatase), which led to enhanced PI3K/Akt signaling, an increased inflammatory response, and worsening of disease (Bhattacharyya et al., 2011).

In contrast, several miRNAs also appear to have beneficial properties in CF. miR-17 was found to directly repress *IL-8*, suggesting its use as an anti-inflammatory agent (Oglesby et al., 2015). Ramachandran et al. identified miR-138 to improve F508del CFTR biosynthesis and expression in airway epithelial cells by targeting SIN3 homolog A (SIN3A), a transcriptional regulatory protein involved in CTCF-mediated gene regulation (Ramachandran et al., 2012). Interestingly, miR-138 and SIN3A were both shown to control a subset of genes and pathways important for wild type and mutant CFTR biogenesis. Moreover, miR-16 was shown to rescue the trafficking defects of F508del CFTR, likely through suppression of Heat shock protein 90 (HSP90), a chaperone involved in protein-folding activity (Kumar et al., 2015). We recently characterized a novel microRNA, miR-1343, that represses the receptors for transforming growth factor beta (TGF-β), a cytokine involved in wound-healing responses. TGF-β is often overexpressed in the CF lung and is a known modifier of lung disease severity (Drumm et al., 2005; Harris et al., 2009; Stolzenburg et al., 2016). The *miR-1343* gene is located near a genomic region associated with CF lung disease severity at chromosome 11p13, which also overlaps genetic variants associated with chronic obstructive pulmonary disease (COPD) (Wright et al., 2011; Kim et al., 2012; Corvol et al., 2015), positing a role for miR-1343 in a number of fibrotic respiratory diseases.

Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease most commonly diagnosed in older adults (>50) and characterized by usual interstitial pneumonia and rapid respiratory decline within 5 years (Gross and Hunninghake, 2001; Raghu et al., 2011). Though the cause of

IPF is unclear, several occupational risk factors are known, including cigarette smoke, farming and pollutants (Baumgartner et al., 2000). A small percentage of IPF cases cluster within families, suggesting genetic risk factors may also exist (Marshall et al., 2000). Disease pathogenesis involves airway tissue remodeling through the excess deposition of extracellular matrix (ECM) proteins and the formation of fibroblastic foci, evident by histology (Fernandez and Eickelberg, 2012). These processes are driven mainly by repeated injury to the lung epithelium, followed by basement membrane destruction and activation of myofibroblasts involved in ECM secretion and tissue repair. Myofibroblasts may arise both from resident and circulating fibroblasts, as well as possibly by transdifferentiation of epithelial cells via epithelial-to-mesenchymal transition (EMT) (Jin and Dong, 2011). Signaling through the TGF-β pathway is thought to underlie many of these changes (Leask and Abraham, 2004; Willis et al., 2005). IPF is irreversible and treatment strategies are largely ineffective in preventing disease progression and respiratory failure (Gharaee-Kermani et al., 2009; Olson and Swigris, 2012).

Perhaps the most common experimental model of IPF is bleomycin induction of pulmonary fibrosis in mice. Following intraperitoneal or intratracheal instillation, bleomycin stimulates inflammatory and fibrotic responses within 2 weeks (Adamson and Bowden, 1974). The extremely rapid onset of symptoms and partial reversibility of fibrosis in this model limits the direct applicability of findings to the clinic (Moeller et al., 2008). Regardless, the bleomycin model has proven indispensible for studying biological mechanisms leading to disease and the involvement of miRNAs in these processes. One of the best studied miRNAs associated with pulmonary fibrosis is miR-29. A screen for miRNAs dysregulated in bleomycin-treated mice identified miR-29 as significantly reduced compared to PBS-injected controls (Cushing et al., 2011). Levels of miR-29 inversely correlated with the expression of collagen and directly regulated several pro-fibrotic genes including integrins and ECM remodelers (Cushing et al., 2011). A recent study suggests that decreased miR-29 could be caused by impaired signaling through protein phosphatase 2A (PP2A) and histone deacetylase 4 (HDAC4) following fibroblast interactions with collagen 1 (Khalil et al., 2015). Additionally, miR-29 expression was suppressed by TGF-β via its downstream effector, SMAD3, which likely occurs through PI3K/Akt pathway activation (Cushing et al., 2011; Xiao et al., 2012; Yang et al., 2013b). Transposon-mediated gene transfer of miR-29 or direct injection of miR-29 mimics into bleomycin-treated mice successfully decreased TGF-β signaling and collagen expression, while improving phenotypes of fibrosis (Xiao et al., 2012; Montgomery et al., 2014).

Several other miRNAs with anti-fibrotic functions were also identified in models of IPF. miR-326 was found to target directly the 3′ UTR of *TGFβ1*, reduce the expression of pro-fibrotic genes and proliferation of fibroblasts, and alleviate histological features of bleomycin-induced pulmonary fibrosis in mice (Das et al., 2014). Likewise, miR-200 family members inhibited EMT and reduced the activity of lung fibroblasts isolated from bleomycin-treated mice and IPF patients (Yang et al., 2012). MicroRNA expression profiling revealed let-7d to be significantly decreased in IPF lungs while the activity of this miR negatively correlated with markers of fibrosis and fibroblast phenotypes (Pandit et al., 2010; Huleihel et al., 2014). Likewise, levels of the miR-17 ~ 92 cluster were also decreased in IPF patients, probably caused by a feedback loop involving reduced targeting of DNA methyltransferase (DNMT1) leading to altered DNA methylation patterns at the miR-17~92 promoter (Dakhlallah et al., 2013). Moreover, both miR-9-5p and miR-153 repressed experimental fibrosis in cells and mice by targeting the 3′ UTR of TGF-β receptor 2 (*TGFBR2*), a mechanism similar to that of miR-1343 discussed previously (Fierro-Fernández et al., 2015; Liang et al., 2015; Stolzenburg et al., 2016). Though miR-153 levels were decreased in the cell-based model of IPF, consistent with the anti-fibrotic role of this miRNA hypothesized by Liang and colleagues, miR-9-5p levels were increased in human pulmonary fibrosis samples (Fierro-Fernández et al., 2015; Liang et al., 2015). These findings underscore the complex mechanisms driving miRNA regulation and indicate that miRNA mis-expression may either be a driver or a consequence of disease.

In contrast to those miRNAs highlighted above, a number have also been described as pro-fibrotic in IPF. Pottier and coworkers found that miR-155 was overexpressed in mice treated with bleomycin and identified keratinocyte growth factor (KGF) as a direct target. Following miR-155 transfection, KGF was released at lower levels by lung fibroblasts in response to pro-inflammatory cytokine stimulation (Pottier et al., 2009). Combined with its regulation of IL-8 in CF (Bhattacharyya et al., 2011), these results suggest a multifunctional role of miR-155 in lung inflammation. Furthermore, miR-145 was also upregulated in the lungs of IPF patients, similar to findings in CF lungs (Megiorni et al., 2013; Oglesby et al., 2013; Yang et al., 2013a). In addition to targeting SMAD3, as described earlier (Megiorni et al., 2013), miR-145 also induced myofibroblast differentiation and promoted activation of latent TGF-β, while knockout of *miR-145* protected against bleomycin-induced pulmonary fibrosis (Yang et al., 2013a).

Moreover, the upregulation of miR-21 in lungs from IPF patients and bleomycin-treated mice was reported by several groups, demonstrating its pro-fibrogenic activity in fibroblasts and enhancement of EMT in lung epithelial cells (Liu et al., 2010; Yamada et al., 2013). An interesting finding from Oak et al., is the lower expression of AGO proteins (AGO1 and AGO2) in IPF lungs, indicating that miRNA processing in general may be disrupted in this disease (Oak et al., 2011).

COPD

COPD is one of the leading causes of death worldwide, affecting about 64 million people, and is predicted to become the third most common cause of death by 2030 (Mathers et al., 2006). It is characterized by progressive respiratory obstruction, encompassing both chronic asthmatic bronchitis and emphysema, and is not fully reversible (Decramer et al., 2012). Cigarette smoke is by far the biggest risk factor for developing COPD, and disease occurrence correlates with frequency and duration of smoking (Pauwels and Rabe, 2004). However, genetic causes, such as alpha1 antitrypsin deficiency (AATD), also contribute to disease onset and progression in a small percentage of patients (Decramer et al., 2012). Normally, smoke inhalation irritates the lung epithelium and causes the release of pro-inflammatory cytokines that initiate innate and adaptive inflammatory responses. In patients susceptible to COPD development (approximately 20% of smokers), this inflammatory response is amplified, and eventually leads to squamous metaplasia, fibroblast activation, mucus production and airway remodeling (Pauwels and Rabe, 2004; Hogg and Timens, 2009). Factors contributing to COPD susceptibility are not entirely clear, though a combination of exposure to pollutants, childhood respiratory infections and genetic modifiers are likely involved (Decramer et al., 2012).

To assess the involvement of miRNAs in COPD, many recent studies have sought to identify panels of misregulated miRNAs in patients or in rodents exposed to tobacco smoke. An early investigation found 28 differentially expressed miRNAs in bronchial epithelial cells from current smokers compared to those who never smoked (Schembri et al., 2009). Most of these miRNAs, including miR-128, were downregulated in smokers and inversely correlated with gene expression levels. More recently, Kim et al. identified 12 differentially expressed miRNAs in lung tissue from individuals with COPD compared to those with normal lung function, the majority of which also exhibited downregulation (Kim et al., 2017), though only two of these (miR-181d, miR-30a-3p) overlapped with the first study (Schembri et al., 2009). Decreases in global microRNA abundance were also observed in populations of alveolar macrophages from cigarette smokers (Graff et al., 2012). In contrast, Leuenberger and colleagues found that miR-223 was upregulated in the lungs of smokers (Leuenberger et al., 2016). Increased miR-223 correlated with decreased Histone deacetylase 2 (HDAC2) expression, which led to elevated expression of a pro-inflammatory chemokine, CX3CL1. miR-101 was also increased in respiratory tissue samples following cigarette smoke exposure (Hassan et al., 2012).

Experiments conducted by the De Flora group currently represent some of the most comprehensive studies investigating miRNAs in rodent models of COPD. Twenty-four miRNAs were differentially expressed by three-fold or more in rats exposed to cigarette smoke for 4 weeks (Izzotti et al., 2009a). Consistent with observations in humans, most of these miRNAs (23 out of 24) were downregulated. A majority of miRNAs also showed decreased expression in a similar study conducted in mice (Izzotti et al., 2009b). Higher levels of smoke exposure corresponded to greater alterations in more miRNAs, most of which returned to baseline following smoking cessation, though some did not (Izzotti et al., 2011). An additional study in smoke-exposed mice found that miR-135b abundance increased in the respiratory tract (Halappanavar et al., 2013). miR-135b decreased the expression of interleukin 1 receptor (*IL-1R1*) through its 3′ UTR and was also a target of signaling through the IL-1 pathway, suggesting this miRNA as a critical regulator of IL-1-mediated neutrophilic inflammation (Halappanavar et al., 2013). Furthermore, miR-218-5p was found to be significantly downregulated in lung tissue of smoke-exposed mice and humans (Conickx et al., 2017).

To characterize miRNAs involved in COPD development and severity in humans irrespective of smoking, Ezzie and coworkers analyzed lung tissue samples from smokers with and without COPD (Ezzie et al., 2012). Approximately 70 miRNAs and more than 2500 potential target mRNAs were differentially expressed. The authors found that increased miR-15b levels directly repressed the expression of SMAD7, an inhibitor of the TGF-β signaling pathway, which is implicated in COPD severity (Zandvoort et al., 2006; Ezzie et al., 2012). Meanwhile, reduced levels of miR-146a following cytokine treatment were observed in fibroblasts from COPD patients compared to non-COPD smokers (Sato et al., 2010). Similar studies analyzed lung tissue from patients with varying severity of COPD disease to identify dysregulated miRNAs (Mizuno et al., 2012; Savarimuthu Francis et al., 2014). The former found increased miR-199a-5p expression to strongly associate

with more severe disease and with levels of Hypoxia inducible factor 1 alpha (Hif-1α) (Mizuno et al., 2012). However, in contrast, subsequent studies found decreased levels of miR-199a-5p in monocytes and T-cells from COPD patients (Chatila et al., 2014; Hassan et al., 2014). The latter (Savarimuthu Francis et al., 2014) identified five miRNAs to be significantly downregulated in lung tissue from patients with moderate compared to mild disease. Among these was miR-34c, expression of which correlated with levels of *SERPINE1*, a gene encoding for a protease inhibitor that is TGF-β-responsive and implicated in COPD pathogenesis (Jiang et al., 2010; Savarimuthu Francis et al., 2014). Assaying regions of varying emphysema severity within COPD lungs also revealed differentially expressed miRNAs, with miR-30c, miR-181d and miR-638 found to correlate with levels of their predicted targets (Christenson et al., 2013).

Asthma

Of the chronic respiratory diseases in humans, asthma remains one of the most common, affecting more than 300 million people worldwide (GBD 2015 Disease and Injury Incidence and Prevalence Collaborators 2016). Asthma is defined by recurrent attacks of inflammation, airway hyperresponsiveness, bronchial tube swelling and reversible airway obstruction. During disease progression, changes to the airway occur, including bronchial smooth muscle hypertrophy, epithelial shedding, fibroblast activation, ECM deposition and neovascularization (Nelson et al., 2003). However, significant heterogeneity of asthma phenotypes exist across individuals, and currently comprise five different subtypes characterized by age of onset, presence of T helper type 2 cell (T_H2) inflammation, involvement of eosinophilia, obesity level and whether the attack is exercise-induced (Wenzel 2012). Both genetic and environmental factors are associated with asthma development and exacerbation, though an interaction between the two most likely contributes to disease severity and responsiveness (Martinez, 2006; Miller and Ho, 2008). In contrast to CF, IPF and COPD, asthma has a relatively low fatality rate and can be appropriately managed for most patients with inhaled glucocorticoids. However, there is no preventative strategy or cure for asthma, and many patients still experience side effects and suffer from uncontrolled symptoms (Olin and Wechsler, 2014).

Common models of asthma typically involve induction of asthma symptoms in mice using allergens. Animals are sensitized to allergens, such as ovalbumin or house dust mite, through intraperitoneal injections and are subsequently challenged with a larger dose of the allergen via inhalation. Groups using this method identified 37 and 58 miRNAs, respectively, that were differentially expressed in total lungs from ovalbumin-treated mice (Garbacki et al., 2011; Bartel et al., 2017). Profiling of short RNAs using deep sequencing also revealed numerous dysregulated miRNAs in ovalbumin-challenged compared to naïve mice, and identified let-7 family members to be highly expressed in the lung (Polikepahad et al., 2010). Administration of let-7a targeted the 3′ UTR of *IL-13*, reduced IL-13 levels, and resolved inflammation in the airway (Kumar et al., 2011). However, inhibition of let-7 family members in a similar experiment also attenuated IL-13 expression, implying the existence of complex mechanisms regulating let-7 family expression and cytokine production (Polikepahad et al., 2010). Using a lung-specific IL-13-inducible transgenic mouse, Lu et al., confirmed the increased expression of let-7 members following IL-13 induction, but also identified 21 other miRNAs, including miR-21, that were concurrently differentially expressed (Lu et al., 2009). Ovalbumin-challenge of *miR-21* (-/-) null animals revealed dysregulation of IL-12 and impaired T cell polarization, suggesting a prominent role for this miRNA in T_H1 versus T_H2 responses (Lu et al., 2011).

Several other miRNAs are also implicated in T_H2 inflammation. miR-126, miR-16 and miR-21, were all significantly increased in the lungs of mice exposed to both house dust mite allergen and ovalbumin (Mattes et al., 2009; Collison et al., 2011a). Suppression of miR-126 reduced airway hyperactivity and inflammation, and impaired T_H2 cytokine release (Mattes et al., 2009). Likewise, miR-145 was up-regulated in the lungs of house dust mite-treated mice, and its inhibition caused reduced levels of IL-5 and IL-13 secreted from isolated T_H2 cells (Collison et al., 2011b). In addition, the role of miR-155 in T_H2-mediated inflammation was demonstrated independently by several groups. miR-155 null animals exposed to ovalbumin had reduced T_H2 cell numbers, expression of IL-5/13, and eosinophil influx to the lung (Malmhäll et al., 2014). Airway eosinophilia returned to wild type levels by adoptive transfer of $CD4^+$ T cells into *miR-155*$^{-/-}$ null mice, suggesting that miR-155 acts through a T_H2 cell-dependent mechanism. Similarly, Okoye et al., found that the expression of miR-155 (among other miRNAs) distinguished T_H2 cells from other T cells, and was upregulated in house dust mite-challenged mice (Okoye et al., 2014). miR-155 was specifically required for both T_H2-driven inflammation and activation of dendritic cells (Okoye et al., 2014; Zech et al., 2015), indicating its pathogenic role in allergic asthma.

Despite the prevalence of asthma, to date few studies examined mechanisms of microRNA function in human disease, with the majority completing profiling studies in

patient cells to identify misregulated miRNAs. One report did not identify any differentially expressed miRNAs in lung biopsies from asthmatic patients, though this may have been due to cellular heterogeneity within the samples (Williams et al., 2009). Specifically in bronchial epithelial cells from affected individuals, Jardim and colleagues found 66 miRNAs with significantly altered abundance compared to cells from healthy controls (Jardim et al., 2012). Similarly, in another report 217 miRNAs were differentially expressed in bronchial epithelial cells from asthma patients not using steroids compared to healthy controls, with only nine of those significantly influenced by steroid treatment (Solberg et al., 2012). Profiling of miRNA expression in circulating $CD4^+$ and $CD8^+$ T cells from patients with severe asthma identified miR-146a/b as significantly downregulated (Tsitsiou et al., 2012), though experiments in mouse models showed the opposite (Feng et al., 2012). Airway-infiltrating $CD4^+$ T cells exhibited relatively few changes in miRNA expression between mild/severe asthma patients and healthy controls, except for miR-19a, which was significantly over-expressed and found to regulate IL-13 cytokine production (Simpson et al., 2014). Moreover, lymphocytes from pediatric asthma patients contained 83 differentially expressed miRNAs, including upregulation of miR-221 (Liu et al., 2012). Inhibition of miR-221 in ovalbumin-treated mice reduced eosinophil and leukocyte airway infiltration (Qin et al., 2012), suggesting a role in attenuating inflammatory processes.

Clinical use of miRNAs in respiratory disease

miRNAs as biomarkers

Though research in respiratory cells and tissue has greatly enhanced our understanding of microRNA function in lung disease, translating these findings into clinical applications has proven to be challenging. Mouse and *ex vivo*/*in vitro* models of disease are not always indicative of the human condition, and obtaining lung biopsies from patients is usually problematic. However, recent findings that miRNAs can exist stably in an extracellular state (El-Hefnawy et al., 2004; Chen et al., 2008), and can be detected in body fluids such as blood, sputum, saliva and breast milk, suggest the potential of miRNAs as disease biomarkers. Extracellular miRNAs are usually encapsulated in lipid-based complexes, such as microvesicles or exosomes, and can be taken up by distant cells where they exert regulatory functions (Valadi et al., 2007; Hunter et al., 2008). The differential cellular expression patterns of miRNAs observed in a number of chronic lung diseases suggests that profiling extracellular miRNAs could provide a less invasive diagnostic and prognostic approach.

To identify circulating miRNAs as potential biomarkers for IPF, Li and colleagues performed expression profiling on serum collected from IPF patients and healthy controls. Levels of miR-21 and miR-155 were significantly elevated in IPF patient serum (Li et al., 2013, 2014), consistent with findings from the cell-based mouse and human studies discussed earlier (Pottier et al., 2009; Liu et al., 2010; Yamada et al., 2013). The upregulation of serum miR-21 was confirmed as a predictor of poor prognosis by two other groups (Yang et al., 2015; Makiguchi et al., 2016). In addition, Yang and coworkers identified 47 differentially expressed miRNAs in serum from IPF patients, of which let-7d was significantly downregulated compared to healthy controls (Yang et al., 2015) as observed previously in tissue biopsies (Pandit et al., 2010). Reduced expression of let-7d in plasma, combined with increased levels of miR-25-3p, was also associated with acute exacerbation of IPF, a rapid decline in lung function of unknown cause (Min et al., 2016).

The analysis of circulating miRNAs was also very successful in characterizing potential biomarkers for COPD. Through miRNA profiling, several groups identified panels of miRNAs to be misregulated in serum, exhaled breath condensates, and in peripheral blood mononuclear cells from COPD patients compared to controls (Akbas et al., 2012; Pinkerton et al., 2013; Dang et al., 2017). Significantly higher levels of miR-21 and lower levels of miR-181a were evident in serum from individuals with COPD and asymptomatic heavy smokers, and an increased ratio of miR-21:miR-181a was associated with disease severity, implicating high miR-21 levels as a risk factor for COPD development (Xie et al., 2014). Furthermore, a reduced abundance of let-7c and miR-125b was seen in induced sputum of patients with COPD compared to healthy controls, which was correlated with increased expression of Tumor necrosis factor receptor type II (Van Pottelberge et al., 2011). Plasma miRNAs associated with skeletal muscle weakness in COPD and α-1 antitrypsin deficiency were also identified (Donaldson et al., 2013; Dasi et al., 2014).

To reveal circulating miRNAs that are predictive in asthma, Panganiban and colleagues measured the expression of miRNAs in serum collected from asthmatic patients (Panganiban et al., 2012). Four miRNAs were differentially expressed in comparison to healthy controls, with enhanced levels of miR-1248 and decreased levels of

miR-26a, let-7a and let-7d. Reduced let-7a was also seen in exhaled breath condensates and exosomes isolated from bronchoalveolar lavage fluid from asthmatic patients (Levänen et al., 2013; Pinkerton et al., 2013). This is in contrast to cell-based studies in which there was no differential expression of let-7 family members (Lu et al., 2009; Polikepahad et al., 2010), though it may support the anti-inflammatory hypothesis for this miRNA (Kumar et al., 2011). miR-26a was also shown by Kho and colleagues to be differentially expressed in asthmatic serum and to correlate with lung function, as did several other miRNAs previously implicated in the disease, including miR-203 (Jardim et al., 2012; Solberg et al., 2012; Kho et al., 2016). In addition, miR-223, miR-142 and miR-629 levels in sputum were associated with asthma disease severity (Maes et al., 2016), and decreased miR-192 expression in peripheral blood mononuclear cells was observed 2 h following allergen challenge (Yamamoto et al., 2012). In concordance with data on IPF and COPD, elevated serum levels of miR-21 were also reported in asthma and were predictive of responsiveness to steroid treatment (Elbehidy et al., 2016).

At this time, studies to identify extracellular miRNA biomarkers in CF lung disease have not yet been published. However, several circulating miRNAs, including miR-21 and miR-155, appear to be mis-expressed in CF liver disease and CF-related diabetes, respectively (Cook et al., 2015; Montanini et al., 2016).

miRNAs as therapeutics

Given the critical roles of miRNAs in respiratory disease pathogenesis, but also in promoting lung health, novel therapeutics designed to target miRNA function have potential. Depending on the context of miRNA function in disease, introduction of miRNA mimics to increase expression or inhibitors to suppress miRNA action (anti-miRs) could be utilized. Though the efficacy of naked nucleic acids in rodent models were demonstrated clearly (Wolff et al., 1990; Henry et al., 2011), RNA activity and safety in humans is restricted by stability, cellular uptake, target specificity and immune activation. Therefore, advances in miRNA therapeutics are limited by the availability of specialized delivery technologies. To prevent degradation by RNases and increase targeting, chemical modifications to the RNA can be introduced, including locked nucleic acids (LNA) (Wahlestedt et al., 2000) and the addition of 2′-O-methyl groups (Krützfeldt et al., 2005). The addition of a cholesterol moiety (Krützfeldt et al., 2007), conjugation to N-acetyl-D-galactosamine (GalNAc) (Nair et al., 2014), and peptide attachment (Fabani et al., 2010) were also shown to improve cellular uptake. The stable expression of miRNA mimics or miRNA sponges through viral vectors has been explored, though complications arising from viral toxicity and overloading the miRNA processing machinery have largely stalled this technology (Castanotto et al., 2007; Trang et al., 2010; Aalbers et al., 2011). More recently, packaging of RNA within synthetic systems, such as liposomes, nanoparticles and polymers, appears to enhance delivery (Wu et al., 2011; Babar et al., 2012; Kouri et al., 2015). For all approaches, however, correct tissue targeting remains a considerable challenge.

Though miRNA-based therapeutics specifically for the chronic respiratory diseases discussed here have not yet entered clinical trials, several miRNA mimics/anti-miRs have undergone testing for the treatment of other illnesses (reviewed in Rupaimoole and Slack, 2017). One of the first to reach clinical trials was Miravirsen (Santaris Pharma), an LNA antimiR against miR-122 for the treatment of hepatitis C. miR-122 was found to interact at the 5′ end of the hepatitis C viral genome, which promoted stability and replication of viral RNA (Jopling et al., 2005). Systemically injected LNA-antimiRs naturally accumulate in the liver, and were found to deplete miR-122 levels and improve liver function in both mice and primates (Elmén et al., 2008a,b). In phase I and II clinical trials, Miravirsen treatment proved to be both safe and effective (Janssen et al., 2013), and is currently being pursued in additional phase II studies. A GalNAc-conjugated antimiR against miR-122 (RG-101, Regulus Therapeutics), also for the treatment of hepatitis C, showed promise in phase I trials (van der Ree et al., 2017), but is unlikely to be pursued further due to safety concerns. In addition, development of MRX34 (Mirna Therapeutics), a mimic of miR-34 delivered via a lipid-based carrier targeted to tumor microenvironments (Wiggins et al., 2010; Trang et al., 2011; Beg et al., 2017), was halted due to immune-related adverse reactions.

Therapeutics targeting several miRNAs with known involvements in lung disease are also under development for other illnesses. The findings from these studies could conceivably be applied to respiratory-based delivery systems. For example, miRagen Therapeutics is testing a miR-29 mimic (MRG-201) in pathologic fibrosis and an LNA-antimiR against miR-155 (MRG-106) in hematological malignancies (Rupaimoole and Slack, 2017). A miR-16 mimic (mesomiR-1, EnGeneIC) delivered to lung tumors by epidermal growth factor receptor (EGFR)-conjugated nanoparticles has also entered phase I trials. Systemic injection of miR-21 LNA-antimiRs in mice indicates that targeting to the kidneys and heart is achievable (Thum et al., 2008; Chau et al., 2012), suggesting

that delivery to the lung could also be attained. Intranasal delivery of anti-miR-155 in mice significantly reduced miR-155 levels in the airway wall and parenchymal tissues, but had mixed effects in cells of immunological origin (Plank et al., 2015). Moreover, studies involving intratracheal delivery of siRNAs show mixed results, likely due to the presence of mucus, mucociliary clearance pathways, and complicated branching structure of the lungs that impair uptake (Lam et al., 2012). Ideally, technology allowing for aerosolization would offer the least-invasive delivery method (Jensen et al., 2010), though these have not yet been reported in clinical settings.

Conclusions and perspectives

Over the past decade, the role of miRNAs in both promoting and alleviating chronic respiratory disease has become increasingly apparent. Misregulation of miRNA expression is seen in a number of different lung tissues and inflammatory cell types, and these changes correlate with disease severity/risk in a number of studies (Figure 1). Many of the same miRNAs, including miR-21, miR-155 and members of the let-7 family, appear to be involved in multiple distinct respiratory diseases, suggesting the importance of these miRNAs, and the pathways that they regulate, in maintaining global lung homeostasis. As a result, miRNAs are proposed as novel disease biomarkers and potential therapeutic targets. However, research to optimize delivery and ensure the safety and efficacy of miRNA-based treatments remains in its infancy and additional research is needed to determine whether lung-based treatments are indeed feasible.

Before miRNAs can become effective clinical tools, a number of questions regarding miRNA biology must be addressed. First, there are substantial concerns about reproducibility among studies. Multiple groups employing similar experimental conditions have reported different, and often contrasting, results. Though this may be due to variability among patients, the lack of standard methods for quantifying miRNA expression remains a problem in the field. Better tools and assays are needed to make high-confidence miRNA measurements, especially for low abundance miRNAs. Second, in most studies to date it is not clear whether miRNA misregulation is a direct cause of disease or an indirect readout of disrupted upstream

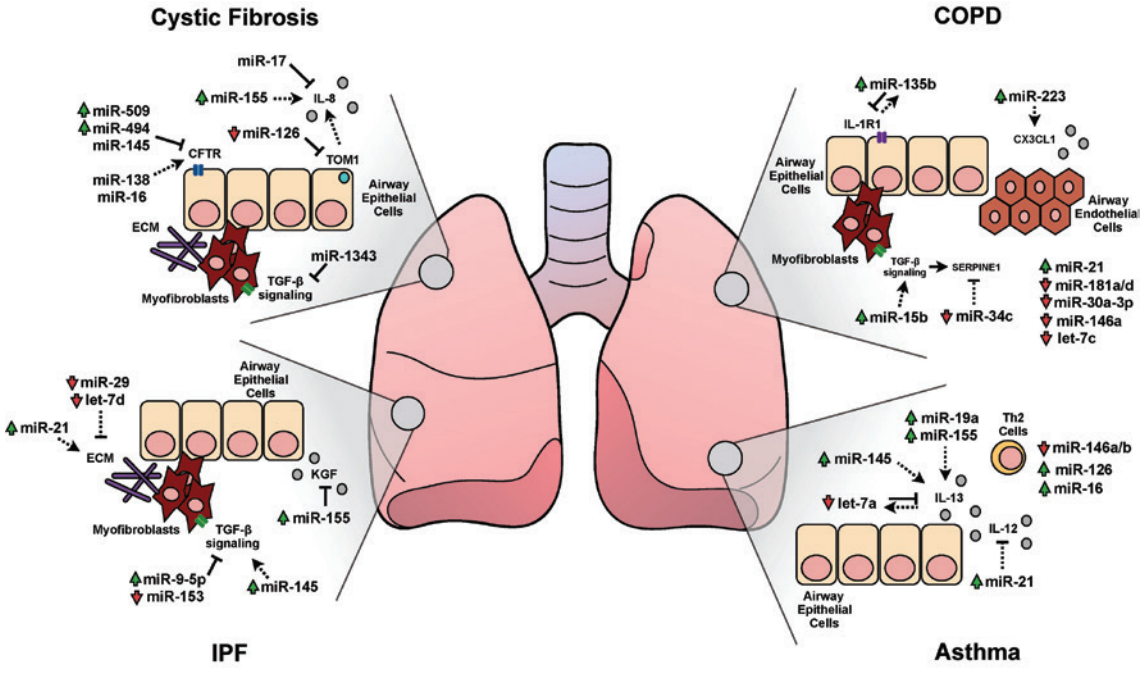

Figure 1: Mechanisms of microRNA action in chronic respiratory diseases.
The best-studied miRNAs and their targets involved in cystic fibrosis, idiopathic pulmonary fibrosis (IPF), chronic obstructive pulmonary disease (COPD), and asthma are shown. Different cell types investigated in disease pathogenesis are illustrated. Green arrows indicate miRNA upregulation in disease, and red arrows indicate downregulation. Flat arrows show negative regulatory effects, pointed arrows show positive regulatory effects, and dotted lines signify an indirect interaction.

gene regulatory processes. In the latter case, treatments targeting the miRNA will likely have no effect, though utilization as a biomarker may still be an option. With the exception of a few reports on miRNA null lines (Lu et al., 2011; Yang et al., 2013a; Malmhäll et al., 2014), most studies either did not attempt or were unable to establish both necessity and sufficiency of the miRNA under investigation. Lastly, the large number of miRNA target genes and variety of cell types involved in each disease has so far impeded identification of their precise involvement in disease mechanisms. Experiments evaluating miRNA incorporation into exosomes indicate that miRNAs can function over long distances within tissues and whole organisms. Therefore, lung epithelial cells and fibroblasts (the main focus of publications reviewed here) might not be pivotal in the role of miRNAs in lung disease. The increasing availability of RNA-sequencing methods (as opposed to microarray- and *in silico*-based tools), together with single cell technologies, should provide more accurate data on miRNA profiles dysregulated in disease and the role of each miRNA in a given cell type. As new tools to assay and manipulate miRNA function are developed, our insight into control of chronic respiratory diseases by miRNAs will continue to improve, with the ultimate goal of creating enhanced disease management strategies and improved patient outcomes.

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