REVIEW

New Insights about miRNAs in Cystic Fibrosis

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The molecular basis of cystic fibrosis (CF) is a mutation-related defect in the epithelial–cell chloride channel called CF transmembrane conductance regulator (CFTR). This defect alters chloride ion transport and impairs water transport across the cell membrane. Marked clinical heterogeneity occurs even among patients carrying the same mutation in the *CFTR* gene. Recent studies suggest that such heterogeneity could be related to epigenetic factors and/or miRNAs, which are small noncoding RNAs that modulate the expression of various proteins via post-transcriptional inhibition of gene expression. In the respiratory system, it has been shown that the dysregulation of miRNAs could participate in and lead to pathogenicity in several diseases. In CF airways, recent studies have proposed that miRNAs may modulate disease progression by affecting the production of either CFTR or various proteins that are dysregulated in the CF lung. Herein, we provide an overview of studies showing how miRNAs may modulate CF pathology and the efforts to develop miRNA-based treatments and/or to consider miRNAs as biomarkers. The identification of miRNAs involved in CF disease progression opens up new avenues toward treatments targeting selected clinical components of CF, independently from the *CFTR* mutation. (*Am J Pathol* 2015, 185: 897–908; <http://dx.doi.org/10.1016/j.ajpath.2014.12.022>)

The most common lethal genetic disorder in whites is cystic fibrosis (CF). It is caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene. More than 1900 pathogenic variants of this gene have been identified. The most common mutation (70%) is an in-frame phenylalanine 508 deletion (F508del-*CFTR*) that induces severe lung disease attributed to impaired trafficking of the mutated CFTR protein to the plasma membrane (Figure 1). The CFTR protein is an apical chloride (Cl[−]) channel that not only participates directly in fluid homeostasis at the epithelium surface, but also regulates other Cl[−] channels, non-Cl[−] channels such as the epithelial Na⁺ channel, and the transport of molecules, such as glutathione.^{1–3} Extensive studies on the *CFTR* gene and its protein have been conducted since its discovery and characterization in 1989.⁴ Nevertheless, the complex mechanisms which regulate *CFTR* expression remain poorly understood, and the sequence of CFTR-channel activation is empirical and unclear. Surprisingly, new treatments designed to correct the mutated CFTR protein defect have clearly focused the attention of some laboratories on defective CFTR

processing and have greatly improved our knowledge of the CFTR channel.^{5,6}

Physiopathology

In the CF pathology, morbidity and mortality are mainly related to self-perpetuating cycles of airway obstruction, infection, and inflammation.⁷ There is a pressing need for research into the sequence of events leading to progressive lung tissue damage, which remains incompletely understood. Initially, CFTR deficiency induces the loss of Cl[−] secretion and the loss of epithelial Na⁺ channel inhibition that leads to excess sodium and water reabsorption, resulting in dehydration of airway surface liquid compared to physiological situation (Figure 1, A and B).⁸ This imbalance

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Figure 1 Diagram comparing the non-cystic fibrosis (non-CF; **A**) and CF (**B** and **C**) pulmonary epithelium. **A:** The CF transmembrane conductance regulator (CFTR) defect causes hydromineral imbalance characterized by a decrease in the output of chloride ions (Cl^-), an increase in the entry of sodium ions (Na^+), and a dehydration of the airway surface liquid (ASL) leading to a viscous mucus. **B:** The alteration of mucociliary clearance induced by viscous mucus promotes cycles of airway obstruction, infection by various pathogens, and inflammation characterized by neutrophil accumulation and IL-8 overproduction. **C:** IL-8 is consensually described as the marker of pulmonary inflammation in CF lungs; excessive neutrophil degranulation with inflammation damages the pulmonary epithelium.

results in the depletion of the airway surface liquid and in the increase of mucus viscosity that induces an impaired mucociliary clearance. In addition, infection and inflammation are observed and become persistent. Clinical data obtained during the past decade suggest that inflammation may precede infection, but this point is still debated.^{9,10} The F508del-*CFTR* mutation causes a massive proinflammatory phenotype in the lungs, characterized by an overexpression of chemoattractant molecules, such as IL-8, a proinflammatory cytokine whose expression is significantly increased in the CF airway and known as a marker of the pulmonary inflammation in CF. IL-8 is a well-known chemoattractant for neutrophil in the lung epithelium, participates in lung defense^{7,9–12} (Figure 1, B and C), and is a reference to evaluate CF airway inflammation. Many studies have demonstrated a crucial role for the transcription factor (TF) NF- κ B in the lung inflammation but have failed to identify a clear link between CFTR and NF- κ B, despite some evidence implicating ionic homeostasis.^{7,13–16} Elucidating the mechanisms that underlie the inflammatory response might result in therapeutic improvements, thereby improving both the quality of life and length of survival of CF patients. In parallel, the lungs of children become chronically infected. Typically, infants are colonized by *Haemophilus influenza* and/or *Staphylococcus aureus* but quickly *Pseudomonas aeruginosa* becomes the predominant organism in the airways.¹⁷ This chronic infection perpetuates a vicious circle of infection and inflammation, leading to destruction and remodeling of the airway epithelium (Figure 1C), bronchiectasis, hypoxemia, hypercarbia, and, finally, pulmonary insufficiency. Despite remarkable advances in the knowledge achieved after the identification of the disease-causing gene, the clinical outcome for CF patients remains dependent on the gradual decline in lung function. Unfortunately, the mechanisms underlying the lung disease progression remain unclear, and the impact of polymorphisms throughout the genome complicates the outcome prediction. To date, minimal correlations have

been found between *CFTR* genotype and disease severity, suggesting a role for environmental, stochastic, and genetic factors in the clinical heterogeneity of patients carrying the same mutation.^{18,19}

Treatments of CF Lung Disease

CF lung treatment strategies target multiple steps in the progression of the pathology, including rehydration of the airway surface, mucolytics, anti-inflammatory agents, anti-infective agents, and, more recently, CFTR protein modulation.¹³

Current Treatments

Until now, CF has no cure, even if treatments have greatly improved life expectancy in past decades.¹³ The current lung disease treatments are symptomatic and proposed to treat CF lung pathology, fundamentally managing the consequences induced by the CFTR failure. One of the main treatments is the chest physical therapy, aiming at loosening and expectorating the excessive and hyperviscous airway mucus. To increase the mucus clearance, pharmacological treatments are also used, such as inhaled recombinant human DNase.¹⁴ The prevention and treatment of bacterial lung infections is deemed as a primary aim in CF. Improved antibiotic treatment strategies against airway infections are regarded as the main reason for the increased life expectancy of the CF patients in the past decade.²⁰ Many CF centers try to eradicate airway bacteria colonization with several antibiotics, even in the absence of symptoms. Oral antibiotics are often used to treat mild lung infections. By contrast, inhaled antibiotics may be used to prevent or control infections due to the mucoid *P. aeruginosa*. For severe or hard-to-treat infections, patients may be given i.v. antibiotics.

As outlined above, inflammation is a hallmark of CF lung pathology,⁷ and can be treated with drugs that have pleiotropic effects on cytokine secretion and/or neutrophils. Therefore, early trials of anti-inflammatory treatment have been done with

corticosteroids, but serious adverse effects, such as glucose intolerance, bone demineralization, and growth delay, were observed.²¹ To improve the risk/benefit ratio, inhaled steroids could be prescribed, but benefits have not been shown clearly.²² Long-term macrolide antibiotic therapies are also prescribed to decrease CF lung inflammation.²³ After evidence of a tremendous efficacy in diffuse panbronchiolitis, a disease that shares similarities with CF lung disease, they have been proposed in CF with great successes. The mechanism of efficacy of macrolides may be related to their antimicrobial, and anti-inflammatory, properties, and specifically in CF, to an activation of the chloride efflux.^{24,25} Among the several macrolides, azithromycin is the most used, with fewer adverse effects than other macrolides and dosing intervals. Other anti-inflammatory therapies proposed in CF are often confidential to a group or to a specific country.²⁶

All these approaches are symptomatic, and do not directly target CFTR protein activity and/or the defective gene. In this context, new therapies that target the mutated CFTR protein to cure CF are under development.

New Approaches to Correct CFTR

Among the many evaluated strategies designed to correct CF abnormalities, only a few drugs targeting the mutated CFTR protein have shown meaningful efficiency to provide hope to the patients. These new drugs are small molecules that correct the trafficking and/or activity of the mutated CFTR. As introduced, several *CFTR* mutations lead to different mutated proteins. The most effective drug designed is a potentiator that targets CFTR gating mutations, by improving the channel opening probability, namely ivacaftor (VX-770; Vertex Pharmaceuticals Incorporated, Cambridge, MA). Although promising, ivacaftor is effective only in a few CF patients worldwide, because it has limited efficacy in patients carrying the F508del mutation.^{5,26–28} Preliminary results from two phase 3 trial studies using a combination of ivacaftor and a CFTR corrector (lumacaftor; VX-809; Vertex Pharmaceuticals Incorporated) have recently shown significant improvements in the lung function of patients homozygous for the *CFTR* F508del mutation, despite absence of proof-of-concept data.²⁶ Some groups have also demonstrated that this strategy, combining potentiator and corrector, could be limited, and new strategies still need to be proposed. In fact, potentiators make CFTR less stable at the apical membrane.^{29,30} In this context, further research is needed to produce an array of strategies appropriate for all *CFTR* mutations. Thus, several groups have suggested that miRNA-targeted therapies might generate benefits by increasing CFTR chloride efflux.^{31–33} Further studies need to be conducted to produce new strategies appropriate for the various stages of CF disease.

miRNA Data

miRNAs are small (20 to 24 nucleotides) noncoding RNAs that regulate post-transcriptional gene expression. Since the

identification of the first miRNA, *lin-4*, in the nematode *Caenorhabditis elegans*, >1800 miRNAs have been found in humans^{34,35} and listed in the database miRbase 21 (<http://www.mirbase.org>, last accessed June 1, 2014). The miRNA biogenesis is well described.^{36–38} The miRNAs constitute a large family of phylogenetically conserved genes expressed in all cell types and tissues. The crucial role for miRNAs in protein regulation was recognized only recently. The full miRNA system may regulate >60% of protein-coding genes in human cells, and miRNA dysregulation is involved in many lung diseases, including CF.^{39–42}

Mechanisms of Action of miRNAs

In numerous cases, miRNAs may provide a pleiotropic mechanism to explain the apparent complexity of different pathologies. Via a canonical pathway, miRNAs predominantly down-regulate the expression of their target genes by pairing with the 3'-untranslated regions (UTRs) of target mRNAs, thereby inducing mRNA degradation or translation inhibition.⁴³ Thus, miRNA down-regulation results in increased protein expression of the targeted genes, whereas miRNA up-regulation represses their expression. This system of regulation, associated with TFs, allows a fine control of protein expression. The different possibilities and the complexity of these regulations are illustrated in Figure 2A. A TF can control the expression of different genes and/or miRNAs. The product of one gene can also control the expression of multiple miRNAs. However, one miRNA can influence the expression of different TFs and/or genes (Figure 2A). Recently, it has been proposed that miRNAs could directly bind DNA and, in the case of synonymous variants, alter a binding for miRNA and thereby modify the expression of the target gene⁴⁴ (Figure 2, B and C). Modifications, such as mutations or polymorphisms, may change miRNA attachment and consequently inhibit expression of the protein of interest (Figure 2, B and C). Conversely, change in the 3'-UTR of an mRNA sequence can inhibit the binding of a miRNA and induce protein overexpression. In physiopathology, few variations could completely change the protein profile expression and therefore alter normal physiology.

Although the mechanisms underlying the effects of miRNAs are now well understood, the origin of miRNA dysregulation in disease is generally unknown. The mechanisms that control miRNA processing in specific cells and tissues need to be elucidated. The function and expression of mature miRNAs are controlled by mechanisms that regulate the processing of pri-miRNAs and pre-miRNAs.³¹ The complexity of the regulation system is important to recognize because each miRNA can target multiple mRNAs (Figure 2A). Each miRNA belongs to a multifactorial system of pathways subjected to many regulation systems. This extraordinary complexity substantially complicates efforts to understand the miRNA system and to offer simple

Figure 2 **A:** Diagram of interaction between miRNAs, genes, and transcriptional factor (TF) to regulate gene/protein expression. A miRNA regulates one or more genes, which are regulated by TFs that can also regulate the expression of other miRNAs. **B** and **C:** Mutation/polymorphism in the noncodant 3'-untranslated region (UTR) of a gene may modify the affinity of a miRNA for the 3'-UTR of the gene and, thus, alter the protein expression.

therapy. Because miRNAs are involved in the regulation of protein-coding gene expression, they play a role in many processes that induce disease states, such as cancer, and participate in CF pathology.^{45,46}

miRNA Target Prediction Tools

Since the discovery of miRNAs in 1993, the view on gene expression regulation has started to change. Studies have been focused on predicting the target genes. This method is empirical and needs to be confirmed by biological experiments, whereas miRNome studies become easier. Two approaches have been used to study RNA regulation by miRNAs to determine the eventual link between miRNAs and RNAs.

First, the gene candidate approach is based on *in silico* analysis with bioinformatic databases predicting a score of miRNAs that could bind to RNAs of interest. It is important to use several bases because the results obtained are

variable, depending on the criteria. Nowadays, different algorithms have been developed by different laboratories to predict miRNA-mRNA interaction, such as DIANA-microT, TargetScan, miRWalk, miRanda, and PicTar. These algorithms are based not only on base pairing between miRNAs and the mRNA 3'-UTR, thermodynamic stability of miRNA-mRNA duplex, conservation of target sites, and free energy, but also on cooperative translational control and multiplicity of miRNA binding sites.^{47,48} The main algorithms used by the resources are artificial neural network, hidden Markov model, Naïve bayes, restricted bayesian, or support vector machine and could be combined to optimize the prediction. For example, TargetScan, one of the famous resources for miRNA-RNA interaction, used a restricted bayesian network model considering target position, conservation between species, and A-U-rich flanking regional accessibility. These different algorithms are used to avoid the many false-positive results obtained with the complementary sequence method.

The common practice for researchers is to look at predictions from several miRNA-target interaction algorithms and focus on their intersection; then, predictive results require validation by biological models. To illustrate this, we have predicted, using MirWalk software,⁴⁹ without specific criteria, miRNAs that could bind CFTR and proteins that could bind these miRNAs (Figure 3A). miRNA targets were predicted by combinatorial use of five different web-based databases (miRanda, miRDB, miRwalk, RNA22, and TargetScan). The results were presented with Cytoscape software version 3.1.1.⁵⁰ More than 49,000 proteins—half of all proteins expressed on human cells—are predicted to be regulated by miRNAs regulating CFTR. This example illustrates the importance of experimental *in vitro* and *in vivo* validation. Figure 3B shows the validated miRNAs regulating CFTR and represents only validated proteins regulated by these miRNAs. In this validated model, only 1132 proteins are presented. This example illustrates that predictions are just a tool before *in vitro/in vivo* validation.

Second, a data-driven prognostic method without a priori knowledge consisted of studying the differential expression of several miRNAs in CF and non-CF models. This comparison could be performed by different methods of miRNA expression profiling as microarray, miRNA sequencing, PCR arrays, and Fluidigm. *In silico* studies were then performed to determine whether the miRNAs overexpressed in CF were predicted to target *CFTR*.

As such, these two approaches are complementary and respond to different needs. Both methods have been used to find miRNAs implicated in the pathophysiology of CF.

miRNAs and Genetic Diseases

Considering that miRNAs can regulate the expression of numerous target genes, miRNAs are expected to regulate various physiological processes, including apoptosis,

Figure 3 **A** and **B**: Network of cystic fibrosis transmembrane conductance regulator (CFTR; red) regulation mediated by miRNAs (yellow) that could influence the expression of other proteins. **A**: miRNAs predicted by *in silico* analysis to target CFTR (red) and other predicted proteins (blue). **B**: miRNAs demonstrated by *in vivo* experiments to regulate CFTR and known to regulate other proteins. **C**: miRNA target sequence location within the 3'-untranslated region (UTR) of the CFTR mRNA.

inflammation, differentiation, and proliferation. It is expected that mutations affecting miRNA function could have a pathogenic role in human genetic disease, similar to protein-coding genes. For example, numerous articles have shown that aberrant miRNA expression is implicated in numerous human cancers, but without a direct link between miRNAs and human genetic diseases.^{45,51,52} To date, there are no examples of mutations that are clearly associated with human mendelian disease because the regulation of miRNAs is redundant and a significant alteration of the function may elicit a minor perturbation of biological processes. Meola et al⁵³ have well summarized and described three main types of mutation mechanisms affecting miRNA function: i) mutations affecting miRNAs directly, ii) mutations in the 3'-UTR of target mRNA, and iii) mutations in genes that participate in the process of miRNA function. In the first case, only two kinds of mutations affecting miRNAs have been described, mutations with a large sequence of genes affecting miRNAs⁵³ or polymorphism in the seed sequence miRNA.⁵⁴ In the case of mutation in the 3'-UTR, the first report identified two different sequences in the binding site of miR-189 in the 3'-UTR of the SLITRK1 mRNA in Tourette syndrome.⁵⁵ Now, many articles have reported this kind of mutation affecting the regulation of mRNAs. In the last case of mutation, only few human mutations were described to participate in miRNA maturation. For example, one of this mutation affects DGGR8, a component of Drosha complex implicated in the maturation of miRNAs, which

participates in DiGeorge syndrome.⁵⁶ A complete loss of function of the core protein components of the miRNA processing is incompatible with life.

Altogether, the role of miRNAs in genetic diseases needs to be investigated more, especially in CF pathology.

miRNAs and the Pathophysiology of CF

Although many studies have focused on the role for miRNAs in regulating *CFTR* gene expression, little attention had been directed to other aspects, such as airway obstruction, inflammation, and infection.

Regulation of *CFTR* by miRNAs

Several groups are investigating the role for miRNAs in molecular pathways involved in the pathogenesis of CF. In particular, the regulation of expression by miRNAs of *CFTR* wild-type and mutated genes has been examined. Furthermore, the mRNA of *CFTR* is low abundant in airway epithelia, and miRNAs may silence low-abundance mRNA targets more efficiently than high-abundance mRNAs.^{32,57}

By using *in silico* analyses, Gillen et al⁵⁸ identified miRNAs that directly repressed *CFTR* expression by binding to the 3'-UTR segment of the transcript. *CFTR* regulation seems to differ across the three cell lines used (Table 1). Interestingly, some miRNAs that repressed *CFTR* expression also targeted the $\text{Na}^+/\text{K}^+/\text{Cl}^-$ cotransporter NKCC1 channel, suggesting a general role for miRNAs in coordinating chloride transport and cell volume across epithelial cell membranes. miR-101 and miR-494 markedly repress *CFTR* expression in human embryonic kidney 293 cells when used alone, and the effect is considerably increased when the two miRNAs are combined.⁶¹ *In silico* analysis established that miR-101 and miR-494 can target other genes known to influence the disease severity of CF patients. A coordinated network of gene expression controlled by miRNAs has been suggested.⁶¹ In addition, air pollutants decrease the expression of *CFTR* and increase the expression of miR-101 and miR-144 in 16HBE14o- bronchial epithelial cells. Interestingly, miR-101 is up-regulated in the lung of patients with chronic obstructive pulmonary disease, a condition characterized by low levels of *CFTR* expression.⁶³

This approach with RNA-miRNA is limited by prediction tools, and next-generation sequencing allows the analysis of different situations (eg, CF versus non-CF) without preconceived ideas. Two groups studied global miRNA expression in CF and non-CF models before performing *in silico* analyses. Interestingly, miR-138 has been predicted to target the TF SIN3A, which is able to bind the CTCF-binding sites of *CFTR*.⁶² SIN3A is a target of miR-138 in airway epithelia, which regulates *CFTR* expression by suppressing SIN3A. Furthermore, miR-138 overexpression increases the expression and number of *CFTR* molecules reaching the cell surface, as well as

Table 1 Deregulated Demonstrated miRNAs in Cystic Fibrosis and Their Targets

Year	miRNA	Direct target	Indirect target	Cell types	Reference
2010	miR-126	TOM1		Bronchial brushings, 16HBE14o-, CFBE41o-, HEK293	42
2011	miR-155 miR-494, miR-384, miR-376b, miR-1246, miR-145, miR-331-3p, and miR-939	SHIP1 CFTR	IL-8	IB3-1, S9 Caco-2	59
2011	miR-600, miR-494, miR-384, miR-1290, miR-1246, miR-145, miR-1827, miR-331-3p, and miR-939	CFTR		PANC-1	58
2011	miR-600, miR-494, miR-607, and miR-384 miR-101, miR-1246, miR-494, and miR-384 miR-449	CFTR SLC12A2 Notch1		16HBE14o- PANC-1 Primary air-liquid interface cultures of human airway epithelia	60
2011	miR-101 and miR-494	CFTR		HEK293	61
2012	miR-138	SIN3A	CFTR	Primary air-liquid interface cultures of human airway epithelia; Calu3, HEK293, HeLa	62
2012	miR-101 and miR-144	CFTR		16HBE14o-	63
2013	miR-145, miR-223, and miR-494	CFTR		Bronchial brushings, 16HBE14o-, CFBE41o-, HEK293	64
2013	miR-145	SMAD3		Nasal epithelium of patients with HEK293	65
2013	miR-509-3p and miR-494	CFTR		Primary air-liquid interface cultures of human airway epithelia; Calu3	32

CFTR, cystic fibrosis transmembrane conductance regulator; SHIP, Src homology-2 domain-containing inositol 5-phosphatase 1 protein; TOM, translocase of the outer membrane.

CFTR activity in cells expressing not only wild-type CFTR, but also CFTR-F508del, thus opening therapeutic avenues for restoring CFTR function.⁶²

A direct link between miRNAs and *CFTR* expression in CF has been reported.⁶⁴ *CFTR* is directly regulated by miR-145, miR-223, and miR-494 when used alone, with a synergistic effect of combinations of these miRNAs. Furthermore, deficient CFTR channel activity is a possible cause of the overexpression of miR-145, miR-223, and miR-494 in CF.⁶⁴ Recently, miR-509-3p and miR-494 have been identified as post-transcriptional CFTR repressors that act synergistically to produce larger effects in CF. There is a reported link between the expression of the two miRNAs and CFTR function.³²

However, the system of *CFTR* regulation by miRNAs seems more complex, with numerous parameters not yet elucidated. The regulation of *CFTR* changes before and after birth; the *CFTR* gene undergoes tissue-specific and temporal changes in expression.⁶⁶ This regulation is tightly controlled by TFs and miRNAs. To increase complexity, gene mutation in miRNA targeting sites of CFTR could be a new pathogenetic mechanism in CF.⁶⁷ Three single-nucleotide polymorphisms, one of which is located in a region predicted to bind miR-433 and miR-509 and causes a small decrease in CFTR protein expression due to enhanced binding of miR-509-3p, have been identified (Figure 2, B and C). Further studies need to be performed to understand the effects of miRNA on the *CFTR* gene. The discussed studies illustrate the complex mechanisms by

which miRNAs convey functional binding to CFTR mRNA targets. We have summarized in Figure 3C demonstrated binding sites of miRNAs regulating CFTR in pulmonary cells. Extensive analysis needs to be conducted before proposing miRNAs as therapeutic targets.

miRNAs, Inflammation, and CF

Chronic airway inflammation is a major component of CF pathophysiology. The genesis of this inflammation remains unclear but could involve miRNAs. Different approaches have been used to assess the role for miRNAs in CF inflammation.

A global approach to identify miRNAs that were dysregulated in CF provided the first set of evidence.⁴² miR-126 was found to be down-regulated in CF compared to the non-CF models *in vivo* and *in vitro*. Results showed that miR-126 targets the IL-1R1 and Toll-like receptor 2 and 4 signaling inhibitor translocase of the outer membrane 1 and the NF- κ B pathway.

An expression screen of a miRNA library in F508del-CFTR and wild-type CFTR lung epithelial cell lines identified miRNAs associated with the proinflammatory phenotype in the CF lung.⁵⁹ miR-155 is up-regulated in CF cells, both *in vitro* and *in vivo*, and there is a link between CFTR activity and miR-155 expression. By using bioinformatics analysis and *in vitro* cell-line experiments, a direct link between the levels of miR-155 and those of Src homology-2 domain-containing inositol 5-phosphatase 1

protein was established. This relationship indirectly affects IL-8 protein expression via phosphatidylinositol 3-kinase/Akt pathway activation and downstream mitogen-activated protein kinase signaling activation. miR-155 overexpression in CF is mediated by the RNA-binding proteins tristetraprolin and KH-type splicing regulatory protein, known to regulate miRNA processing.³¹ In studies evaluating inflammation, IL-8, and the TF NF- κ B in CF, a binding site for NF- κ B at the 5'-UTR segment of two CFTR-regulating miRNAs was identified. CFTR expression and function decrease to a larger extent when NF- κ B is functional.³² Thus, inflammatory processes under NF- κ B control influence miRNA expression, through an unknown mechanism, thereby regulating CFTR expression and function. Another proinflammatory profile in CF is linked to dysregulation of the transforming growth factor- β 1 signaling pathway via SMAD3 proteins. By using a candidate miRNA approach, miR-145 was found to regulate SMAD3 expression in human embryonic kidney 293 cells. miR-145 and SMAD3 mRNA levels negatively correlate in nasal respiratory epithelial cells from CF patients.⁶⁵ SMAD3 is a protein that protects against infections and inflammation. These studies documenting a link between miRNAs and CF inflammation suggest new ways of developing treatment strategies to decrease inflammation in CF patients.

Regulation of CF Lung Obstruction and Infection by miRNAs

Loss of homeostasis of airway surface liquid volume in patients with CF may impair mucus structure, clearance, and airway defenses. In the airway epithelium, CFTR abnormalities cause dehydration of the mucus, which becomes thick and viscous, impairing mucociliary clearance and affecting respiratory function. Thus, elucidating airway mucus regulation is a current research objective. The MUC5AC mucin is a major airway mucus component whose production is stimulated by neutrophil elastase. miR-146a decreases the production of MUC5AC.⁶⁸ Thus, miR-146a may be involved in controlling mucus secretion by human bronchial epithelial cells. The role for MUC5AC expression is highly controversial, although some evidences point to an effect on the severity of CF lung disease.⁶⁹ A better understanding of this protein regulation in physiological context could be essential to adapt the treatment of CF patients.

To investigate the hypothesis that miRNAs are important regulators of multiciliogenesis in vertebrates, Marcet et al⁶⁰ studied the miRNA expression profile before and after multiciliogenesis in both primary cultures of differentiated human airway mucociliary epithelial cells grown at an air-liquid interface, and in the mucociliary epidermis of *Xenopus* embryos. Interestingly, the same miRNA, miR-449, is up-regulated during multiciliogenesis in both models and promotes multiciliogenesis by directly repressing the Delta/Notch pathway known to be

associated with cell proliferation and differentiation. The thick viscous mucus in CF airways encourages local colonization by various pathogens, such as *H. influenza*, *S. aureus*, and *P. aeruginosa*. Only a few groups are working on links between miRNAs and the innate immune response, which is the first line of defense against infections by exogenous pathogens. In CF, the expression of miRNAs targeting CFTR increases^{32,64}; the presence of pathogens in the CF airway epithelium modulates the expression of miRNAs, which, in turn, regulates CFTR. The expression of miR-93 and IL-8 is correlated during *P. aeruginosa* infection in bronchial epithelial cells, and a combined treatment based on the use of different pre-miRNAs has been proposed to achieve high efficiency.⁷⁰

In Table 1, we have summarized the different deregulated miRNAs that have been shown to regulate CFTR and other proteins in the context of CF. Altogether, these data indicate new mechanisms that may influence the expression of CFTR and the development of CF pathology. This first step of research has led to new therapeutics, to specifically target CFTR protein.

Perspectives

miRNAs as Biomarkers

A major clinical problem in CF is the absence of a specific biomarker for monitoring and/or predicting lung disease progression. Spirometry measurements constitute the reference standard to date. The forced vital capacity and forced expiratory volume in 1 second, although widely used to assess disease progression, are affected by multiple lung changes, such as inflammation, infection, and respiratory epithelium damage. The most relevant techniques for assessing inflammation and cell functions are invasive and cannot be used in regular clinical practices. In this context, epigenetics, and particularly miRNAs, as one of the important epigenetic mechanisms that regulate gene expression, should constitute a promising field for the development of biomarkers in the comprehension of CF.

For several diseases, identifying biomarkers and improving the diagnosis are key challenges for researchers and clinicians. Various miRNAs have emerged as crucial post-transcriptional gene expression regulators involved in many normal and pathological processes. Several characteristics of miRNAs suggest their usefulness as biomarkers, despite the uncertain role of these circulating miRNAs in biological functions.

miRNAs are extremely resistant to degradation, so miRNAs are ideal candidates as biomarkers for cancer diagnostics. Thus, different groups have found that miRNAs are highly stable in serum or plasma, despite the presence of high levels of ribonuclease activity in blood. For example, incubation of plasma at room temperature for up to 24 hours led to no decrease in the level of circulating miRNAs; more miRNAs present in serum are insensitive to modification of pH.^{71,72}

The measurement of miRNAs in body fluid has high potential for future noninvasive diagnostic tests and can be detectable in easily accessible body fluid like peripheral blood. Interestingly, miRNAs are also present in other body fluids, such as the sputum, saliva, and urine.⁷³ miRNAs are stably present in sputum, and lung adenocarcinoma patients can be differentiated from healthy individuals by using a panel of four sputum miRNAs.⁷⁴ Furthermore, differences in the serum miRNA expression pattern correctly separate healthy individuals and patients with various diseases, including lung cancer.^{45,75–77}

In human peripheral blood, miRNAs are detectable not only in serum or plasma, but also in the cellular fraction. Consequently, miRNA expression can be assessed using simple and affordable molecular biology tools available to laboratories, such as quantitative PCR, and it could be easy to observe the evolution of the pathology at different stages of the illness.

These arguments have led many groups, especially in the field of cancer, to evaluate miRNAs as diagnostic or prognostic biomarkers.⁷⁸ These studies are numerous and may be partially applied to the CF domain. The use of miRNAs as minimally invasive biomarkers has generated new opportunities for the diagnosis and prognosis of cancer because they exhibit tumor-specific expression in blood of patients with cancer. The first miRNA aberrantly expressed in lung cancer was identified in 2004.⁷⁹ A low level of let-7 expression is significantly associated with poor survival in patients with surgically resected non-small cell lung cancer, suggesting a prognostic role for miRNA assays. A recent multicenter study established that different types of malignant or nonmalignant diseases could be differentiated on the basis of blood-borne miRNA profiles.⁸⁰ The miRNA expression levels may also serve as indicators of drug responses in lung cancer.⁸¹ The miRNA pathways involved in several molecular processes will probably be elucidated in the near future to avoid conflicting studies in terms of the miRNAs associated with patient survival.

Despite promising data, no studies have clearly demonstrated the importance of miRNAs as biomarkers in CF. A decrease in the cost of RNA profiling and increased availability of RNA and miRNA profiling should allow the development of standardized protocols. These new insights and the increasing knowledge about the role for miRNAs will help to develop novel miRNA-based therapeutic approaches.

miRNAs as Therapeutics

It is well accepted that aberrant miRNA expression can occur in various diseases, including lung cancer. Modulators of miRNA expression may, therefore, produce benefits in lung diseases.⁸² There are two main obstacles to this treatment approach. To avoid or minimize adverse effects, one or a few relevant targets must be identified, and therefore the pathophysiology of CF must be fully elucidated. The most appropriate cells must be effectively targeted.

Figure 4 **A:** One miRNA binds to the 3'-untranslated region (UTR) of many mRNAs, and consequently may inhibit mRNA translation or mRNA degradation of these targets. **B:** Specific miRNA inhibition may result in an increased expression of all of the targets. **C:** Specific inhibition of the target site of one miRNA by a modified oligonucleotide may further prevent inhibition of other miRNA targets.

Initial research focused on nucleic acid-based treatments with antisense molecules, including ribozyme, aptamer, and siRNAs. Most of the approaches used to target mRNA sequences rely on RNase H-mediated mRNA degradation on binding to a complementary DNA oligonucleotide.⁸³ In CF, considerable hope was placed for many years in gene therapy to deliver copies of the wild-type *CFTR* DNA coding sequence to target cells. Since the 1990s, however, research into gene therapy has failed to provide clear evidence of clinical benefits, and no gene therapies have been approved by regulatory authorities. The main problems have been limited efficacy of the gene transfer vector, short-lived gene expression, and adverse effects with both viral and nonviral techniques.⁸⁴

miRNAs are more promising methods of gene correction than other molecules, for many reasons. They are smaller, less antigenic, and easier to insert into cells, compared to their protein-coding counterparts. Although DNA-based approaches require delivery to the nucleus, miRNAs only need to be delivered to the cytoplasm, and the inhibition results in subsequent miRNA degradation and, thus, the up-regulation of target that can be maintained for >3 weeks.^{34,75} Another advantage of miRNAs is the limited need for high-strand complementarity.

Modulation of miRNAs is now more currently used in basic science to elucidate the mechanisms underlying mRNA function. To date, *in vivo* therapeutic studies have used only single miRNAs, but combinations of miRNAs will be evaluated in the future. Different studies and patents proposed, with the recent development of advanced nebulizers, airway nebulization of miRNAs to more specifically target airway epithelial cells, but the result is highly carrier dependent and

new technologies are needed to increase the yield.³³ In CF, the ideal target is *CFTR* and corrective effects should occur for all *CFTR* mutation variants, without inducing adverse effects.⁸⁵

Interestingly, low levels of gene transfer of *CFTR* to airway epithelia are sufficient to correct chloride transport *in vitro* and *in vivo*.^{86–89} Recently, a group cultured 10% of wild-type or corrected cells mixed with 90% of CF cells. The authors demonstrated that the 10% of normal cells were sufficient to normalize the main dysregulated parameters, such as Cl^- or Na^+ conductance and IL-8 secretion.⁹⁰ In a concept of gene therapy, this ability to correct the CF parameters by correcting few cells is important, because it is well known that it will be impossible to correct all of the cells of the lungs of CF patients. Furthermore, targeting other Cl^- channels, such as the calcium-activated chloride channel called ANO1, may hold promise to compensate for the loss of *CFTR* Cl^- activity and the abnormal repair process.⁹¹ However, all other dysregulated proteins in CF could also constitute targets to correct imbalances in physiological parameters. Typically, miRNAs regulate gene expression of multiple targets (Figure 4A). Inhibition of the miRNAs will result in derepression of all these targets and increase the possibility of adverse effects in the use of miRNAs as therapeutic targets (Figure 4B). An interesting approach in therapeutics is to use interfering miRNA-target interactions, which are antisense oligonucleotides that bind to the miRNA target site of an mRNA, thereby preventing miRNAs from gaining access to that site (Figure 4C).

A single miRNA-based treatment is currently used in clinical practice and targets the specific liver-expressed miR-122 using the locked nucleic acid–modified antisense oligonucleotide. After a study on chimpanzees showing that locked nucleic acid–modified anti-miRNAs are specific, nontoxic, and stable, miravirsin has been used for treatment of hepatitis C virus infection and shows prolonged dose-dependent reductions in hepatitis C virus RNA levels in patients with chronic hepatitis C virus infection.⁹² Other miRNA-based treatments are being developed.

In CF, it would be interesting to block the target site(s) of miRNA(s) in the 3'-UTR of *CFTR* to avoid miRNA inhibition. Respiratory involvement being predominant, it might be possible to give the miRNA target site blockers to patients by inhalation to avoid adverse effects in other organs. Understanding the mechanism of miRNA function is crucial to identify the best approach for drug design. There is much interest in identifying the role of miRNAs in the modulation of drug responses, but limited studies have been reported. Studies on miRNAs and pharmacogenomics also hold promise for improving the treatment of CF patients.

Conclusion

The latest research shows that miRNAs exert crucial effects on gene regulation and may play a prominent role in cell

physiology. Improved understanding of the sequence of events has already allowed the use of miRNAs for diagnostic and prognostic purposes and, more specifically, for detecting susceptibility to certain cancers. Currently, the clinical use of miRNAs remains limited, but major changes can be expected in the near future. New tools can specifically target sequences of interest and resolve problems associated with the multiplicity of targets. The main hurdle is identification of miRNAs that target proteins or pathways of interest. In CF, *CFTR* mRNA is a promising target, because the first studies have shown that miRNAs can modulate the normal or mutated *CFTR* protein. In contrast, current treatments target only specific *CFTR* mutations. Although many studies have established the usefulness of miRNAs in targeting *CFTR*, further research is needed to identify the miRNA combination that best corrects the abnormalities without inducing adverse effects. Similarly, the best concentrations and dosing schedules need to be determined. Promising data from preclinical studies indicate that miRNA treatments will be evaluated in patients with CF.

Thus, miRNA therapy is a fascinating and dynamic field of research in CF. The future can be expected to establish a major role for miRNAs as therapeutic agents or diagnostic tools for CF patients.

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