Chapter 12

Role of Non-coding RNAs in Cystic Fibrosis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/60449

Abstract

Cystic Fibrosis (CF) is a common autosomal recessive disorder, caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene. *CFTR* gene expression is tightly controlled by transcriptional and post-transcriptional regulatory factors, resulting in complex spatial and temporal expression patterns. Here, we describe an overview of the findings about the contribution of ncRNAs, especially miRNAs, in physiological *CFTR* gene expression and in CF. Determination of mechanisms governing its expression is essential for developing new CF therapies. ncRNAs, including lncRNAs and miRNAs, could also contribute to CF progression and severity and their dysregulation in CF opens new perspectives for patient follow-up and treatment.

Keywords: CFTR gene expression, Cystic Fibrosis, non-coding RNA, lncRNA, miRNA

1. Introduction

Cystic Fibrosis (CF) is a common autosomal recessive disorder. Although in its classical form CF affects several organs, including the pancreas and the gastrointestinal and reproductive tracts, its morbidity is mainly due to pulmonary damages. This disorder is caused by mutations in the Cystic Fibrosis Transmembrane conductance Regulator (*CFTR*) gene. This gene displays great mutational heterogeneity, depending on the ethnic and phenotypic background, with almost 2,000 referenced *CFTR* alterations (genet.sickkids.on.ca/). The most common mutation

is the p.Phe508del, whereas other mutations, located both in coding and non-coding regions, are rare or private. The p.Phe508del mutation induces aberrant protein folding, leading to endoplasmic reticulum (ER)-associated degradation, atypical intracellular trafficking and reduced stability of the CFTR protein at the apical membrane. Dysfunction or lack of the CFTR protein causes an obstructive lung disease characterized by impaired ion transport in the airway epithelium, accumulation of sticky mucus in the air space and chronic airway inflammation. Physiological *CFTR* expression is tightly controlled by transcriptional, post-transcriptional, translational and post-translational regulatory mechanisms, resulting in complex spatial and temporal expression patterns. Notwithstanding the importance of *CFTR* transcriptional regulation [1-3], *CFTR* expression can be modulated through other mechanisms. Indeed, epigenetic changes, such as DNA methylation or histone acetylation, also influence *CFTR* gene expression in different tissues [4-7]. Post-transcriptional controls also regulate its expression, for instance via the usage of upstream open reading frames (uORFs) encoded within the *CFTR* 5'UTR [8] and the 3'UTR that controls *CFTR* mRNA stability through ARE sequences (AU-rich elements) [9]. An emerging area of research is focusing on the role played by non-coding RNAs (ncRNAs), such as microRNAs (miRNAs), in *CFTR* gene expression. Starting from 2011, a few studies have shown the involvement of miRNAs in the physiological control of the complex spatio-temporal expression pattern of *CFTR* mRNA [10,11], including a recent work by our group [3]. Moreover, the implication of long non-coding RNAs (lncRNAs) and miRNAs in human diseases is well documented [12], including in inherited disorders [13] and lung diseases [14-17]. However, only few studies, described below, have hitherto been carried out on the role of ncRNAs in CF. This chapter is an overview of the findings about the role of ncRNAs in physiological *CFTR* gene expression and in CF.

2. What are non-coding RNAs?

Large expanses of the genome are transcribed into RNAs, but only a small portion of these RNAs encode proteins [18,19]. Many fundamental cellular processes rely on conserved ncRNAs, particularly on ribosomal RNAs (rRNAs), the ribosome RNA components that allow mRNA translation into proteins (Figure 1). Other roles are the transport of amino acids via transfer RNAs (tRNAs) and mRNA splicing through the implication of small nucleolar RNAs (snoRNAs). miRNAs and their crucial role as key modulators of post-transcriptional gene regulation were discovered more than 20 years ago [20]. In the last few years, lncRNAs have been identified as new modulators of key biological processes [21-23, 18]. Currently, ncRNAs are divided in two classes, based on their length; long ncRNAs (lncRNAs, > 200 nt) and short ncRNAs (<200 nt), such as miRNAs, small nucleolar RNAs (snoRNAs) and PIWI-interacting RNAs (piRNAs) [24].

Ribosomal RNA (rRNA) and transfer RNA (tRNA) are the most represented ncRNAs in humans. Long non-coding RNAs (lnc or long ncRNA) are longer than 200 nt and are subdivided in five categories based on their genomic localization: pRNA (promoter-associated RNA), eRNA (enhancer-associated RNA), gsRNA (gene body-associated RNA), lincRNA (intergenic RNA) and NAT (Natural Antisense Transcript). Short non-coding RNAs (short

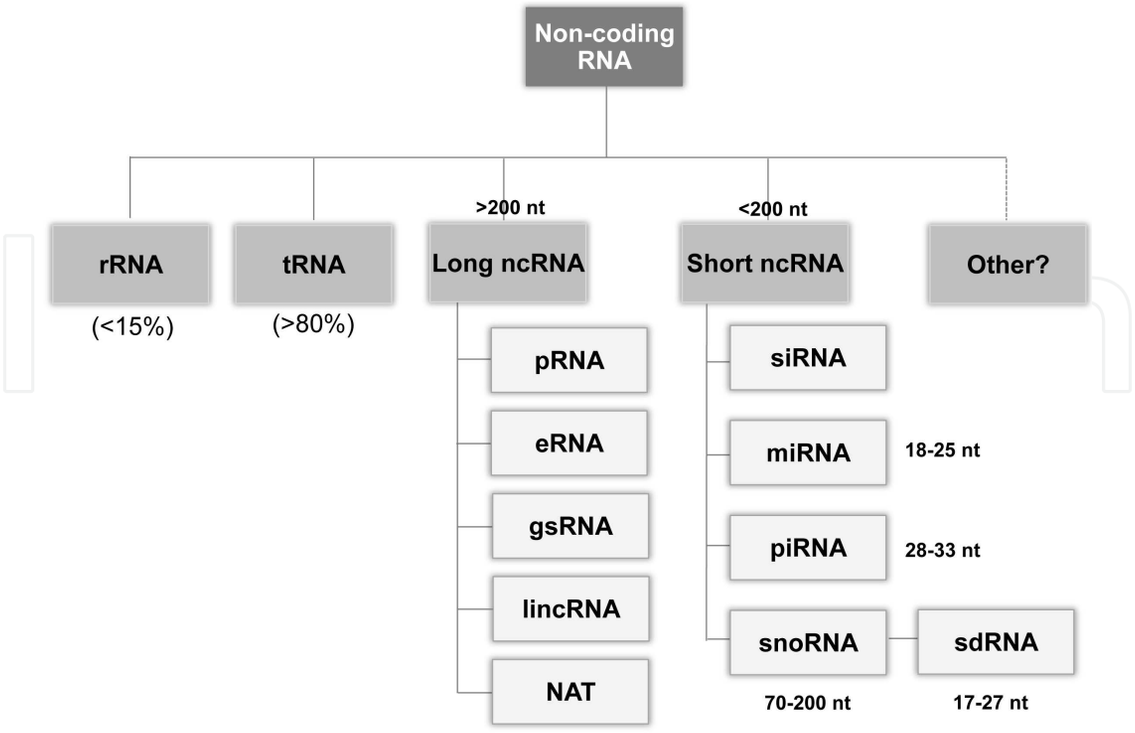

Figure 1. Non-coding RNAs.

ncRNAs) are smaller than 200 nt and are subdivided in four classes based on their size and function: siRNA (small interfering RNA), miRNA (microRNA), piRNA (PIWI-interacting RNA), snoRNA (small nucleolar RNA) and derived snoRNA (sdRNA).

2.1. Long non-coding RNAs

lncRNAs include all ncRNAs longer than 200 nt (except rRNA and tRNA). They constitute the bulk of the non-coding transcriptome [25].

2.1.1. lncRNA biogenesis

It is thought that most lncRNAs originate within a 2-kb region surrounding the Transcription Start Site (TSS) of protein-coding genes (65% of lncRNAs overlap with a promoter and are called pRNAs), or map to enhancer regions (19%; named eRNAs), or derive from antisense transcripts that overlap with annotated gene bodies (5%, called NATs), or are associated with the bodies of protein-coding genes (gsRNA, gene body-associated lncRNAs) [26, 27]. The remaining lncRNAs originate from more distal (>2kb) unannotated regions (11%) and are commonly referred to as long intervening or intergenic ncRNAs (lincRNAs) [28, 29] (Figure 2).

a-Promoter-associated RNAs (pRNA), b-Enhancer-associated RNAs (eRNA), c-Intronic and gene body-associated (sense) RNAs (gsRNA), d-Natural Antisense Transcripts (NAT), e-Long Intergenic RNAs (lincRNA). In the lower part of the figure are described the main lncRNA functions.

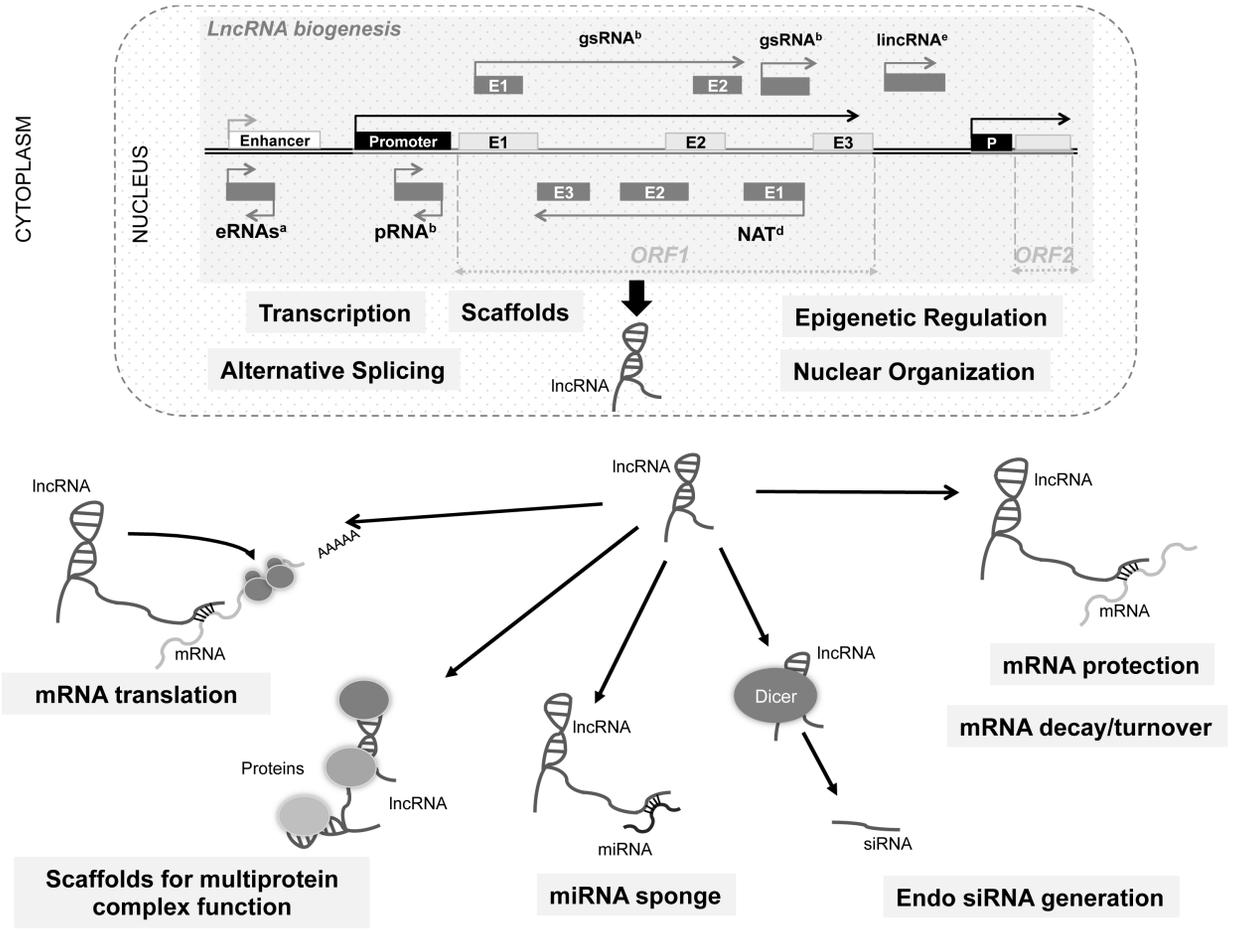

Figure 2. lncRNAs, from biogenesis to functions.

The finding that a large number of lncRNAs arise from loci close to protein-coding genes is consistent with previous genome-wide analyses of lncRNAs [30]. Although all studies agree that the 5′end of lncRNAs, like for mRNAs, is capped by methylguanosine, their splicing status and their 3′-end processing have not been fully defined [26]. It is likely that splice site recognition occurs at low frequency at most lncRNA loci and that lncRNAs may be predominantly mono-exonic and non-polyadenylated [26]. Most lncRNAs are not translated [28] and their localization is predominantly nuclear [25].

2.1.2. lncRNA functions

lncRNAs have regulatory functions in different biological processes (Figure 2). Many of their functions are related to their capacity to bind to RNA, DNA and proteins. The founding member of the lncRNA family is Xist (~17 kb). Xist originates from the silent X chromosome in female cells and coats this chromosome during the early stages of development to establish epigenetic X inactivation [31]. lncRNAs can be used as indicators of the transcriptional activity of a locus or a gene [19]. Their roles as scaffolds for nuclear processes, guides for ribonucleoprotein complexes or decoys have been described in the literature. Similarly to miRNAs, they can act as activators or repressors of protein expression.

lncRNAs are considered to be more species, tissue and developmental stage-specific than mRNAs [32]. A growing number of studies show that lncRNA deregulation has a role in various diseases [33,34; for reviews: 35,36], including pulmonary disorders. Several reviews have discussed the role of lncRNAs and miRNAs in respiratory diseases [16,17,37] and a recent work reported lncRNA involvement in CF (detailed in section 4.2.1).

2.2. MicroRNAs

2.2.1. miRNA localization and biogenesis

Animal miRNAs derive from the nuclear genome. In humans, the majority of canonical miRNAs are encoded by introns of non-coding or coding transcripts, but some miRNAs are encoded by exonic regions (Figure 3). Often, several miRNA loci are in close proximity, thus constituting a polycistronic transcription unit [38]. Most miRNAs use their host gene transcripts as carriers, but separate transcription from internal promoters remains possible. Generally, miRNAs in the same cluster are co-transcribed. Most miRNA genes located in introns of protein-coding genes share the promoter of the host gene [39]. miRNA genes often have multiple transcription start sites [40]. miRNA loci in intergenic regions apparently have their own transcriptional regulatory elements, thus constituting independent transcription units.

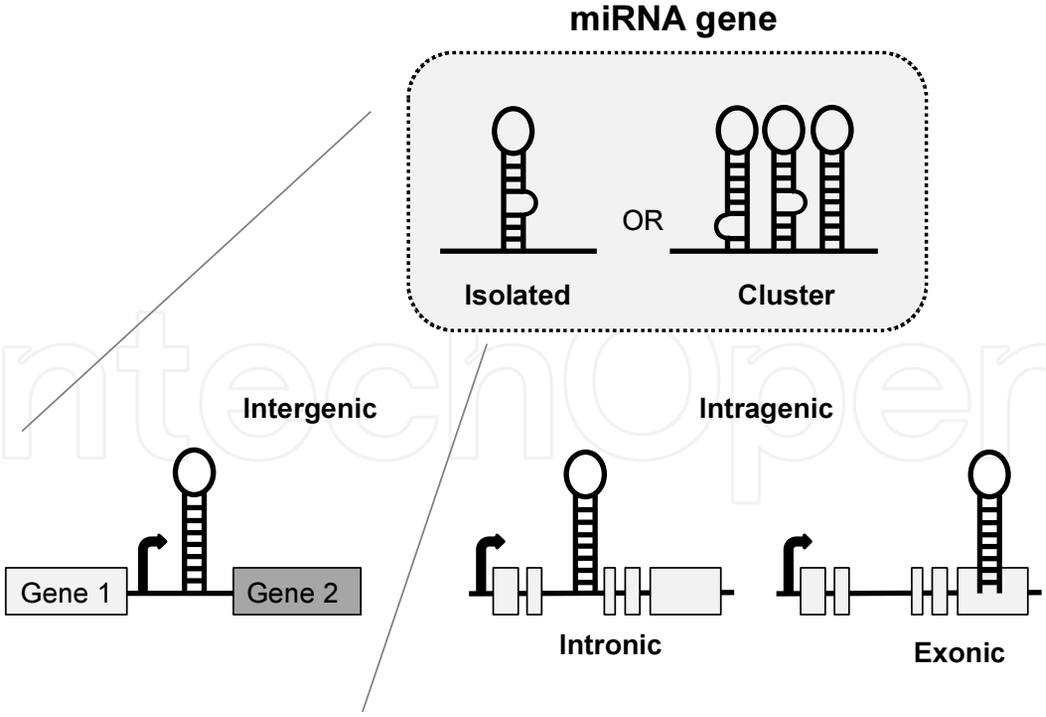

Figure 3. Genomic locations of miRNAs. miRNA genes, isolated or in clusters, are located in intergenic *(ex: miR-494)* or intragenic genome regions, including exons of non-coding *(e.g. miR-155)* or coding (e.g. miR-985) genes and introns of non-coding (e.g. the miR-15a ~16-1 cluster) or coding *(e.g. miR-126)* genes.

miRNA biogenesis includes several steps. First, the gene coding for a given miRNA is transcribed by RNA polymerase II into a long primary transcript (pri-miRNA, ranging from 100 nt to several kilobases). Some miRNA genes, especially those located in Alu elements, are transcribed by RNA polymerase III [41]. When several miRNAs are in a cluster, pri-miRNAs can contain multiple miRNAs. Transcription factors, such as p53, MYC, C/EBP, FOXA positively or negatively regulate miRNA transcription [3,42,43]. Epigenetic control, such as DNA methylation and histone modifications also contribute to miRNA gene regulation [44].

Then, several maturation steps are necessary for miRNA processing. Indeed, the long pri-miRNAs (typically over 1kb) contain stem–loop structures in which mature miRNA sequences are embedded. The nuclear RNase III Drosha acts by cropping the stem–loop to release small hairpin-shaped RNAs of 65 nt in length (pre-miRNA) from the pri-miRNAs. To do this, Drosha, together with its cofactor DiGeorge Syndrome Critical Region 8 (DGCR8), forms the Microprocessor complex. As Drosha cleavage defines one end of the mature miRNA and thereby determines its specificity, it is important that the Microprocessor complex precisely recognizes and cleaves each pri-miRNA. Importantly, Drosha-mediated processing of intronic miRNAs does not affect splicing of the host pre-miRNA [45]. Multiple auxiliary factors could contribute to pri-miRNA maturation [46]. For example, three primary sequence determinants (the basal UG, CNNC and the apical GUG motifs) contribute to efficient processing of human pri-miRNAs. At least one of these three motifs is present in almost 80% of human miRNAs [46]. The splicing factor SRp20 (also called SRSF3) and the RNA helicase DDX17 bind to the CNNC motif and increase processing of human pri-miRNAs by Drosha. Moreover, the terminal loops of miRNA precursors are enriched in cis-elements that recruit regulatory proteins. For example, the splicing factors HNRPA1 and KSRP bind to the conserved terminal loops of some pri-miRNAs and facilitate Drosha-mediated processing [47-49].

Following Drosha processing, pre-miRNAs are exported in the cytoplasm where they are cleaved by Dicer near the terminal loop, liberating a small RNA duplex. Dicer, like Drosha, belongs to a family of RNase III-type endonucleases that act specifically on double-stranded RNA.

RNA duplexes include two mature miRNAs: one derived from the 5' strand and the other one from the 3' strand of the precursor (e.g. miR-27a-5p and miR-27a-3p). One is also called the ‘guide’ (miRNA) and is usually more biologically active than the other one (the ‘passenger’, often referred to as miRNA*). The passenger is normally degraded, but, in some cases, it can be functional [50]. The mature miRNA strand is subsequently incorporated in the RNA-induced silencing complex (RISC, or miRISC for miRNA-containing RISC, or miRNP for microribonucleoprotein), where it directly binds to a member of the Argonaute (AGO) protein family (four AGO members, AGO1 are the most frequently used).

To date, about 1,900 miRNAs (1,881 precursors and 2,588 mature miRNAs; GRCh38 human genome assembly) have been reported in the miRbase database (http://www.mirbase.org/). A substantial number of these miRNAs have dubious annotations and for nearly one-third of miRNA loci, there is no convincing evidence concerning the production of authentic miRNAs (miRbase).

2.2.2. miRNA roles

miRNAs are small ncRNAs that can act in the nucleus and in the cytoplasm [51] through binding to RNA, DNA and proteins. They play an important role in the negative regulation of gene expression by base-pairing to partially complementary sites on the target mRNAs, usually in the 3′ UTR part. Binding of an miRNA to its target mRNA, within the RISC complex, typically leads to translational repression and exonucleolytic mRNA decay. However, highly complementary targets can be cleaved endonucleolytically.

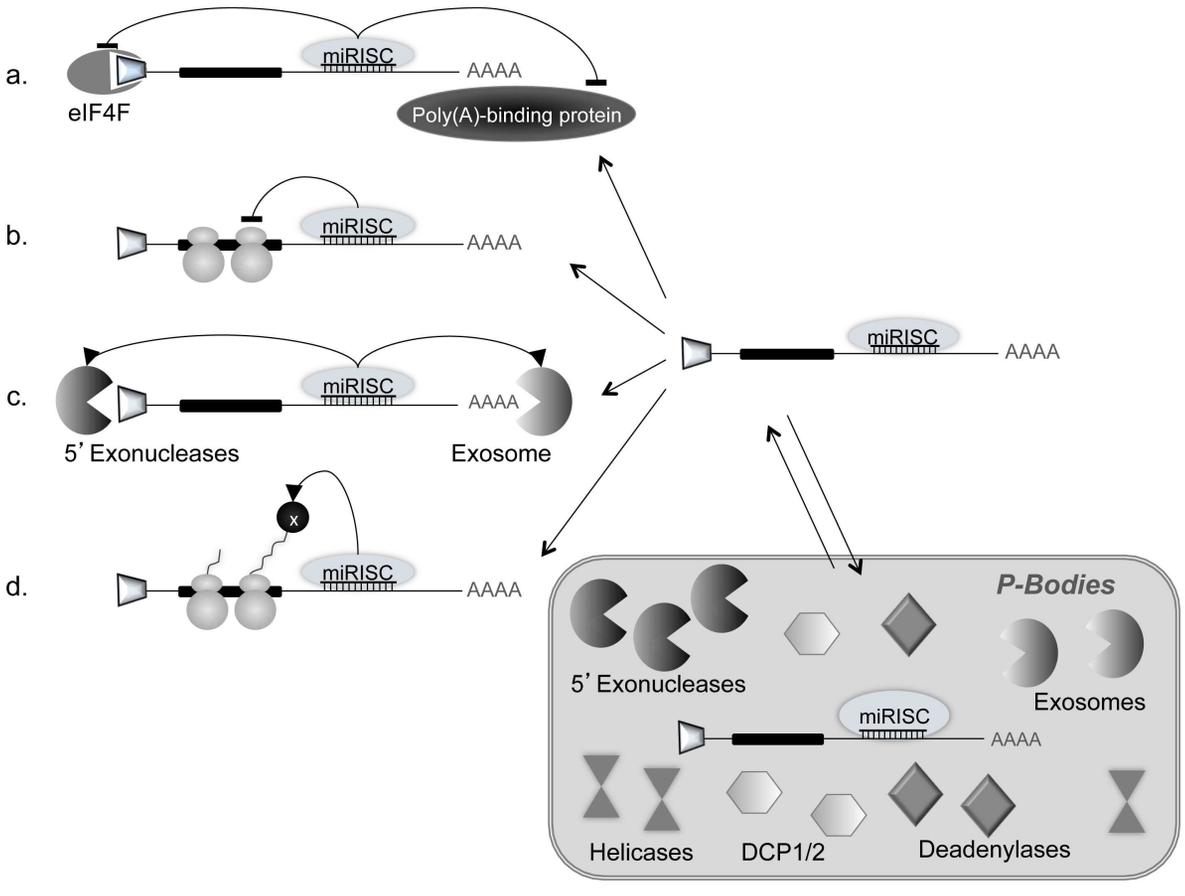

Figure 4. Main miRNA roles. a. Translation block by inhibiting cap and poly(A)-binding protein recognition. b. Elongation inhibition by slowing down elongation or ribosome 'drop-off'. c. Degradation by deadenylation and decapping. d. Proteolysis. Degradation of a nascent peptide. e. mRNA storage in P-bodies that contain exonucleases, RNA helicases, decapping enzymes, DCP1/2, exosomes, deadenylases.

Several miRNAs have a role in lung diseases, such as asthma, chronic obstructive pulmonary disease (COPD) and idiopathic pulmonary fibrosis (see Table 1). The studies reporting the involvement of miRNAs in CF are detailed in section 3.2.2.

Disease	MicroRNAs	Targets	References
Asthma	miR-106a	IL-10	[81]
	miR-21	IL-12	[82]
	miR-133a	RhoA	[83]
	miR-26a	Glycogen synthase kinase 3ß	[84]
	let-7	IL-13	[85]
COPD	miR-181d	Interferon γ, collagen XVI αI	[86]
	miR-30c	Proto-cadherin	[86]
	miR-146a	Prostaglandin E2	[87]
Idiopathic pulmonary fibrosis	miR-21	SMAD7	[88]
	miR-155	KGF	[89]
	miR-let7d	HMGA2	[90]

COPD, chronic obstructive pulmonary disease.

Table 1. Examples of pulmonary diseases in which miRNAs have a role

3. Role of non-coding RNAs in the physiological regulation of *CFTR* gene expression

As the function of lncRNAs has not been studied yet, we only present findings on the miRNA roles in the regulation of *CFTR* gene expression.

3.1. miRNAs and *CFTR* gene expression

CFTR gene expression is spatially and temporally regulated. Several studies have demonstrated the differential use of transcription start sites, depending on the tissue type or the developmental stage [52-55]. In the lung, *CFTR* transcripts can be detected early during embryo development (12th week of pregnancy) and their level progressively increases up to the 24th week of pregnancy. Thereafter, *CFTR* expression in the airways decreases and is repressed until after birth and remains very low during adult life [56,60]. The changes in CFTR protein expression in human foetuses are consistent with *CFTR* mRNA temporal pattern of expression [60]. In a recent study, we showed that miRNAs (miR-101, miR-145, miR-384) regulate the switch from strong foetal to very low *CFTR* expression after birth. Specifically, miR-101 and miR-145 negatively regulate the level of *CFTR* transcripts in adult lung cells, while they have no effect in foetal lung cells. miR-101 directly acts on its cognate site in the 3'UTR-*CFTR* in combination with an overlapping AU-rich element. Other studies showed that *CFTR* expression is also post-transcriptionally regulated by miRNAs, such as miR-145 and miR-494 [10,11]. Gillen *et al.* demonstrated that miR-145 is expressed in primary adult human airway epithelial cells, where *CFTR* expression is low, and directly acts on *CFTR* stability [10]. In addition to its

specific role in mature lung cells, miR-101 decreases luciferase activity in an embryonic kidney cell line [11], whereas it does not affect *CFTR* mRNA stability in pancreatic cell lines [10], suggesting a potential role as a tissue-specific factor.

3.2. Methods to investigate miRNA role in the regulation of *CFTR* gene expression

Different approaches can be employed to investigate miRNA role in the regulation of *CFTR* gene expression, as depicted in Figure 5A.

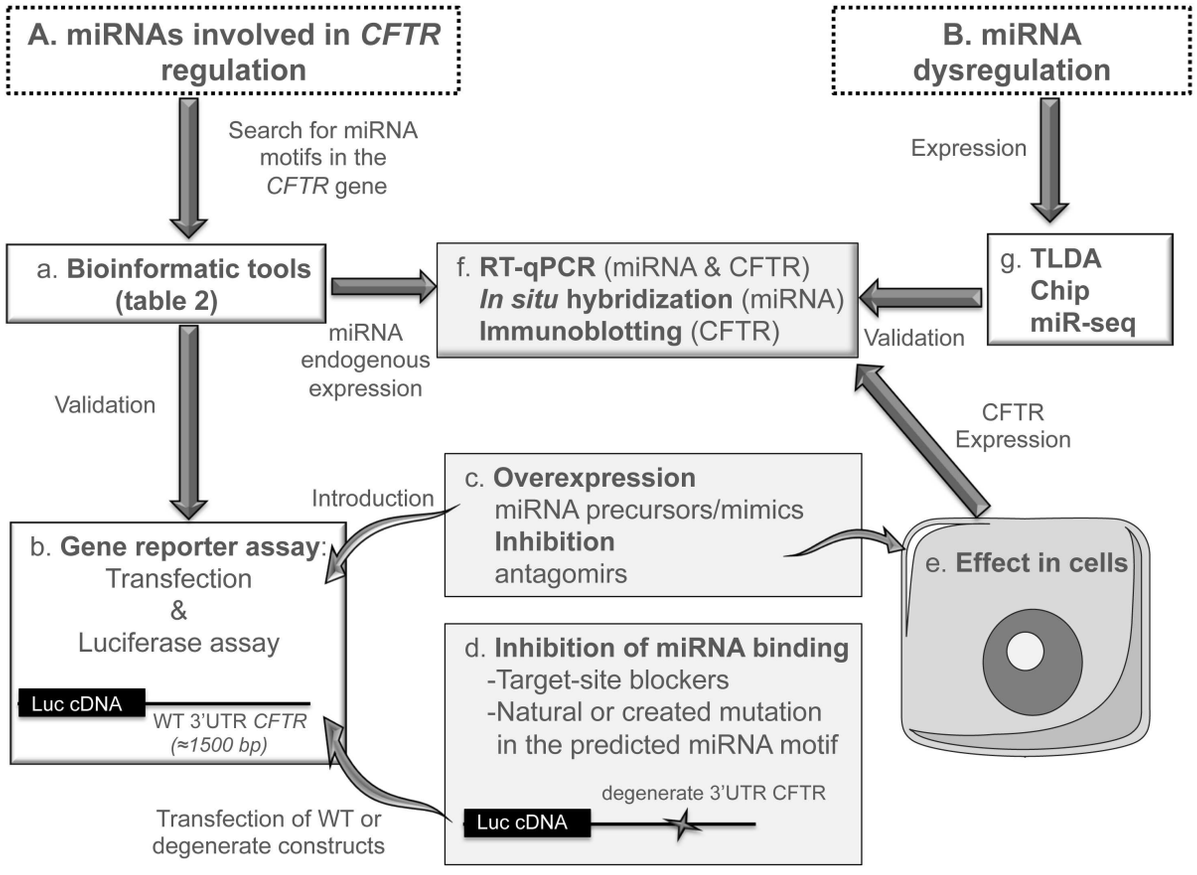

Figure 5. Approaches for miRNA study. A. Strategies used to study the involvement of miRNAs in the regulation of *CFTR* gene expression. B. Strategies to identify miRNAs that are deregulated in CF samples compared to non-CF samples. Chips for global miRNA profiling are commercialized by Agilent, Affimetrix and Exiqon. miR-seq (miRNA sequencing) is usually performed by Illumina platforms. Luc, luciferase; TLDA, TaqMan Array Micro Fluidic Cards (Applied Biosystems); WT, wild type.

3.2.1. Predictive tools are freely available

Predictive tools are necessary to assess the putative presence of miRNA binding motifs. A non-exhaustive list, including information about each program, is proposed in Table 2. Databases collecting all information on miRNAs are listed in Table 3. Although miRBase (http://www.mirbase.org/) is the most used database and collects links for several predictive pro-

grams, others exist. These tools have been developed to predict miRNA targets or the miRNAs that putatively bind to a selected gene. Some of them propose the possibility to input several miRNAs and/or several genes to identify integrated networks.

Name	Website	Characteristics	References
TargetScan	http://www.targetscan.org/	Target site prediction for mammals. Secondary structure taken into account.	[91]
miRanda	http://microrna.org/	Target site prediction for human, mouse, rat, fruitfly and nematode. Thermodynamic stability of RNA duplexes taken into account.	[92]
PicTar	http://www.pictar.org/	Algorithm for target-site prediction based on the alignment of 3'UTR with predicted sites. Several databases are used for vertebrates, flies, nematodes.	[93]
RNAhybrid	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	Tool for finding the minimum free energy hybridization for long and mainly short RNAs, such as microRNAs to one or more given targets.	[94]
PITA	http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html	Target-site prediction for human and other species that evaluates the microRNA targets accessibility as a component analysis.	[95]
miRDB	http://mirdb.org/miRDB/	Tool for miRNA target-site prediction and functional annotation based on the mirTarget algorithm for all known human, dog, rat, and chicken transcripts.	[96]
DIANA-microT	http://diana.cslab.ece.ntua.gr/microT/	Target-site prediction program taking intro account both conserved and non-conserved miRNA regulatory elements (MREs) and providing scores as an indication of the expected fold change in protein production.	[97]
miRBase Target	http://www.mirbase.org/	Prediction of target sites based on alignment and conservation. Provides links to other mains programs for miRNA target prediction (e.g., TargetScan, PicTar).	[98]
miRTar	http://mirtar.mbc.nctu.edu.tw/human/	Web-based system based on the analysis of conserved sequences (seed or 3' miRNAs) using external prediction tools (TargetScan, miRanda, PITA, RNAhybrid). Analyzes miRNA biological functions using the KEGG pathway to draw a miRNA target interaction network.	[99]
miRNAMap	http://mirnamap.mbc.nctu.edu.tw/index.php	Uses the computational tools, miRanda, RNAhybrid, and TargetScan to identify miRNA targets in the 3'UTR of target genes.	[100]

Name	Website	Characteristics	References
MiRonTop	http://www.microarray.fr:8080/miRonTop/index	Identification of miRNAs from DNA microarrays and high-throughput sequencing data. Uses several existing miRNA target prediction approaches.	[101]
MIR@nt@n	http://maia.uni.lu/mironton.php	Integrative approach for searching miRNAs target sites, to build networks including motifs as feedbacks and feedforward loops from lists of molecular actors.	[102]
ProMiR	http://bi.snu.ac.kr/ProMiR/	Prediction of potential conserved and non-conserved microRNAs in a query sequence from 60 to 150 nucleotides and clusters near known or unknown miRNAs in various species.	[103]

Table 2. Free bioinformatics tools for miRNAs

Name	Website	Characteristics	References
miRBase	http://www.mirbase.org/	Collection of annotation, literature, genomic coordinates for human miRNAs and from several other species. From deep sequencing datasets, uses the pattern of mapped reads to assess the confidence in each miRNA annotation.	[98]
miRGen	http://diana.cslab.ece.ntua.gr/mirgen/	Integrated database collecting relationships between animal miRNAs, genomic annotation sets and miRNA targets to a combination of used target prediction programs.	[104]
miRNAMap	http://miRNAMap.mbc.nctu.edu.tw/	Resource for collecting experimentally verified microRNAs and verified miRNA target genes in human and other metazoan genomes reducing the false positive prediction rate of miRNA target sites. Expression profiles by RT-qPCR of 224 human miRNAs in 18 normal tissues.	[100]
CoGemiR	http://cogemir.tigem.it/	Database offering an overview of the genomic organization and conservation of microRNAs in different metazoan species during evolution.	[105]
miRanda	http://microrna.org/	Atlas of miRNA expression in human, mouse and rat tissues based on small RNA library sequencing.	

Table 3. Free databases for miRNAs

3.2.2. Functional approaches

To evaluate *in vitro* the impact of miRNAs on *CFTR* gene regulation, several approaches can be used (Figure 5 A). a) First of all, *in silico* analysis by using bioinformatics tools can be performed (see 3.2.1). b) The role of specific miRNAs could be assessed *in vitro* by using airway cell lines and luciferase gene reporter assays, in which the luciferase coding sequence is under the control of the 3' UTR of *CFTR*, as previously described [11,3]. c) By transfecting mimics or precursors, miRNAs can be overexpressed and the relative level of luciferase expression reflects the effect of a given miRNA on the 3'UTR-*CFTR*. Inhibitors may also be used to confirm the specific effect of the miRNA under study. d) To validate the direct effect of the identified miRNAs (for instance, miRNA-494 and miRNA-101), the cis element for miRNAs binding in the 3'UTR-*CFTR* can be mutated by site-directed mutagenesis (created mutation). Target-site blockers may also be used to inhibit binding to the tested motif [3]. e) To validate these effects, the endogenous level of *CFTR* mRNA following miRNA overexpression or down-regulation can be assessed using different techniques.

4. Role of non-coding RNAs in CF

4.1. What is the impact of mutations in microRNA target sites on the *CFTR* gene?

4.1.1. Mutations in the 3'UTR of the CFTR gene

Assessing the putative impact of single nucleotide polymorphisms (SNPs) in the 3'UTR of *CFTR* is essential to define the pathological importance of these cis-regulatory motifs in non-coding regions, especially after the development of Next-Generation Sequencing (NGS) technologies. Recent work identified the SNP c.*1043A>C on the 3'UTR of the *CFTR* gene in one patient with a CFTR-related disorder and congenital bilateral absence of vas deferens [61]. This SNP is located in a region predicted to interact with miR-433 and miR-509-3p. Expression analysis demonstrated that the c.*1043A>C mutation increases the affinity for miR-509-3p and slightly decreases that for miR-433. *In vitro*, these two miRNAs reduces CFTR protein expression. The authors suggested that the very low expression of miR-509-3p in normal human bronchial epithelial (NHBE) cells could explain the mild phenotype caused by this mutation. Thus, the c.*1043A>C mutation, by acting as a mild *CFTR* mutation that enhances the affinity for an inhibitory miRNA, could represent a novel pathogenic mechanism in CF.

4.1.2. Methods to investigate the impact of CFTR gene mutations

Bioinformatics tools, such as miRNA binding site prediction programs (listed in Table 2), could be used to predict whether at a mutated position there is a cis-regulatory motif and whether it corresponds to an miRNA binding site. Moreover, tools like RNAhybrid could allow predicting the binding energy of the mutated motif. *In vitro* luciferase reporter gene assays together with mutagenesis approaches to create degenerate 3'UTR-*CFTR* sequences can be used to study the different variants (Figure 5Ad).

4.2. Deregulation of non-coding RNAs in CF

Clinical manifestations of CF are various including chronic pulmonary inflammation and infection that strongly contribute to the morbidity and mortality of these patients [62]. Over-inflammation precedes chronic infection that is then amplified by pathogens. Notably, the protease–antiprotease balance, which is responsible for lung remodelling, is disrupted in CF airways early in life and then this imbalance is chronically maintained [63]. Pulmonary tissues in patients with CF are usually infected by antibiotic-resistant *Pseudomonas aeruginosa*. Interleukin-8 (IL-8), IL-6 and Tumor Necrosis Factor alpha (TNF-alpha) are pro-inflammatory cytokines that are highly expressed in CF lung epithelial cells and allow the recruitment of neutrophils [62]. Lipopolysaccharide (LPS) and IL-1beta, which bind to Toll-like receptor 4 (TLR4) and IL-1R, respectively, are also involved. The identification of the molecular events involved in lung epithelium injury and repair is essential for understanding CF physiopathology. Expression levels of miRNAs physiologically vary greatly among tissues. Recent advances explored their effects on influencing signaling pathways in CF. Identification of deregulated miRNAs may offer possible future directions for clinical applications.

4.2.1. Deregulated lncRNAs in CF

To date, only one publication has reported aberrant expression of specific lncRNAs in CF bronchial epithelium *in vivo* [64]. To establish the lncRNA profiles of CF and non-CF bronchial epithelium, 10 CF and 12 non-CF (controls) bronchial brushing samples were analyzed using a human lncRNA Array v3.0 (ArrayStar). In this way, more than 30586 lncRNAs and 26109 protein coding transcripts were evaluated. Overall, 1063 lncRNAs, most of which were intergenic, were differentially expressed in non-CF and CF bronchial brushing samples. RT-qPCR analysis of the differential expression of well-known ncRNAs (XIST, MALAT1, HOTAIR, and TLR8 antisense) did not confirm the down-regulation of MALAT1 and HOTAIR in CF samples compared to controls. Interestingly, MALAT1 (lnc-SCYL1-1*) and HOTAIR (lnc-SMUG1-7*) have been described as oncogenic lncRNAs in lung cancer (*asterisks meaning the existence of several isoforms).

4.2.2. Deregulated miRNAs in CF

miRNA profiling studies identified various miRNAs with altered expression in CF (summarized in Figure 6). For instance, *in vivo* the expression of miR-145, miR-223 and miR-494 is increased in CF bronchial brushing samples (individuals with at least one p.Phe508del CFTR allele) compared to non-CF controls and correlates with decreased p.Phe508del *CFTR* expression. Moreover, these three miRNAs inhibit *CFTR* mRNA expression [65]. These authors also highlighted a relationship between their regulation and CFTR chloride channel activity. Specifically, they showed that treatment with inh-172, a specific inhibitor of the CFTR chloride channel, significantly increases the level of miR-145, miR-223 and miR-494 in 16 HBE14o- respiratory epithelial cells. These data are in agreement with the hypothesis by Wenming Xu *et al*. [66] that CFTR chloride channel alteration affects the miRNA profile. Another study reported that miR-509-3p and miR-494 are increased in well-differentiated primary cultures of human CF but not of non-CF airway epithelia, [67]. Other miRNAs have been found to be

deregulated in CF, such as miR-155, miR-126 and miR-31 [68]. miR-155 is overexpressed in IB3-1 CF cells compared to IB3-1 control cells and in *ex vivo* CF cells (bronchial brushing samples versus normal human bronchial epithelial cells and CF neutrophils CD66+ versus control cells). Moreover, in IB3 cells, miR-155 up-regulation is related to defective CFTR chloride channel activity (following exposure to inh-172). This leads to repression of SHIP1, a well-known effector in the regulation of inflammation, and consequently to activation of the PI3K/AKT pathway that stabilizes IL-8 mRNA through MAPK. Thus, miR-155 up-regulation contributes to the maintenance of a pro-inflammatory phenotype. Furthermore, miR-155-antagomir leads to IL-8 down-regulation in IB3-1CF cells and could represent a candidate treatment for CF. The biogenesis of miR-155 has been in part elucidated and involves the inflammatory RNA binding proteins KSRP and TTP [69]. KSRP promotes miR-155 production, while TTP down-regulates, via miR-1, miR-155 mature expression in CF lung epithelial cells.

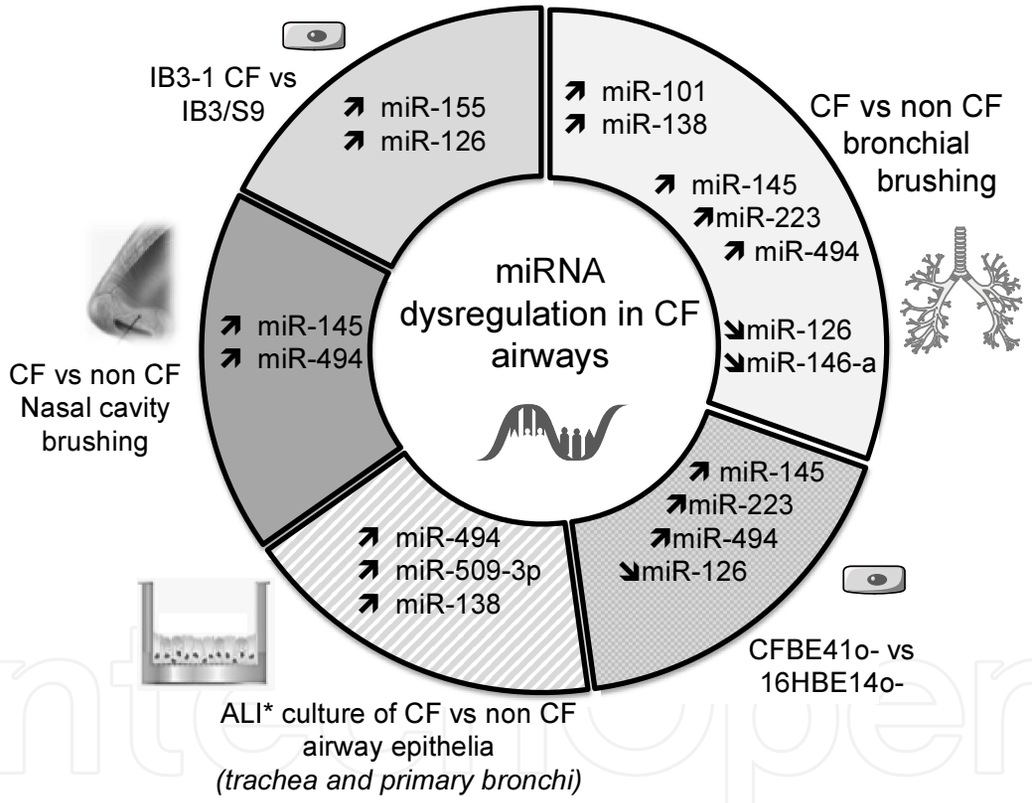

Figure 6. Deregulated miRNAs in different CF models. The schematic lists miRNAs that have been reported to be up-regulated or down-regulated in CF (tissues, cell lines, primary cultures) versus (vs) non-CF samples. *ALI: Air–Liquid Interface.

Conversely, miR-126 down-regulation has an anti-inflammatory role to compensate the immunity response. Oglesby *et al.* [70] reported that miR-126 is consistently decreased in CF compared to non-CF airway epithelial cells and this reduction correlates with up-regulation of TOM1, a negative regulator of TLR2, TLR4, IL-1, IL-1β and TNF-alpha [71,72]. TOM1, which also negatively regulates NF-κB, may play an anti-inflammatory role in CF lung. The authors

hypothesized that the observed reduction in miR-126 expression in CF cells may be due to ER stress induced by accumulation of misfolded p.Phe508del CFTR proteins. A complete analysis of the pathways triggered in CF cells seems essential to identify/develop novel treatments for CF.

Another study assessed the miRNA profile, by using Agilent microarray, of CF IB3-1 cells infected or not with *Pseudomonas aeruginosa*, a model mimicking the inflammatory response observed in pulmonary tissues of CF patients [73]. Two miRNAs (miR-93 and miR-494) were found to be strongly deregulated in infected cells. These two miRNAs are predicted to interact with the 3'UTR of *IL-8* mRNA. Down-regulation of miR-93 was confirmed in two other bronchial epithelial cell lines (CF CuFi-1 and non-CF Nuli-1 cells). In non-infected CF cells, miR-93 is strongly expressed and is involved in IL-8 down-regulation combined with low NF-κB recruitment to the IL-8 promoter. In CF cells infected with *Pseudomonas aeruginosa*, the combined effects of high NF-κB recruitment to the *IL-8* gene promoter and miR-93 down-regulation lead to high IL-8 expression [73].

Finally, miR-31 is down-regulated in CF bronchial brushing cells compared to non-CF cells [74]. In CF epithelial cells, miR-31 negatively modulates the expression of IRF1, a transcription factor that regulates the level of cathepsin S (CTSS). CTSS is overexpressed in CF airways cell lines, such as bronchial (CFBE), tracheal (CFTE) and CF primary bronchial epithelial cells (CF-PBECs) and has been detected in CF lung secretions. CTSS activates the epithelial sodium channel and cleaves and inactivates antimicrobial proteins such as surfactant A, lactoferrin and members of the β-defensin family, thus contributing to lung inflammation in patients with CF [63-66]. Moreover, in a cohort of paediatric patients with CF, it was found that CTSS level correlates with the decline of lung function. Thus, miR-31 is a potential regulator of CTSS expression via IRF1 in CF epithelial cells [74].

4.2.3. Methods to study deregulated non-coding RNA

Methods to quantify miRNAs in CF and non-CF samples are depicted in Figure 5B. Approaches to identify dysregulated lncRNAs in CF samples are detailed in Figure 7 and databases for lncRNAs are listed in Table 4.

Name	Website	Characteristics	References
LNCipedia	http://www.lncipedia.org/	Integrated database of human lnc transcripts. Specific annotation of lncRNAs. Algorithms to assess the protein-coding potential of transcripts. LncRNA gene conservation between human, mouse and zebrafish.	[106,107]
lncRNA2Target	http://www.lncrna2target.org/	Curated database with human lncRNA-to-target gene. Target genes based on overexpression and knockdown studies.	[108]

Name	Website	Characteristics	References
lncRNAWiki	http://lncrna.big.ac.cn/index.php/Main_Page	Component of ScienceWiki for community curation of human lncRNAs.	[109]
lncRNAdb	http://www.lncrnadb.org/	Database for functional eukaryotic lncRNAs. Allow blast search of putative lncRNAs. Gene expression data, evolutionary conservation, structural information, genomic context, subcellular localization, functional evidence.	[110,111]
NONCODE	http://www.noncode.org/	Integrated knowledge database dedicated to ncRNAs. NONCODE specific ID for each ncRNA with a conversion tool to RefSeq and Ensembl. Includes all ncRNAs, except transfer RNAs and ribosomal RNAs, ncRNA sequences and relative information (expression, cellular location, chromosomal information...). More than 80% of entries are based on literature data.	[112,113]
NRED : ncRNA expression database	http://nred.matticklab.com/cgi-bin/ncrnadb.pl	Gene expression repository for human and mouse lncRNAs. Includes both microarrays and in situ hybridization data. Includes evolutionary conservation, secondary structure, genomic context.	[114]
GENCODE	http://www.gencodegenes.org/	Human lncRNAs catalog from manually annotated genes Data available via the UCSC Genome Browser	[119]
Human Body Map lincRNAs	http://www.broadinstitute.org/genome_bio/human_lincrnas/	Human reference catalog for lincRNAs Expression data from RNAseq accross 24 tissues and cell types. lincRNA features (sequence, structure, transcriptional and orthology features).	[115]
fRNAdb	http://www.ncrna.org/frnadb	Database containing a large collection on non-coding transcripts including annotated and non-annotated sequences from the H-inv database, NONCODE and RNAdb databases.	[116]
NPInter	http://www.bioinfo.org/NPInter/index.php	Database integrating the diverse body of experimental knowledge on functional interactions between ncRNAs (except tRNAs and rRNAs) and protein-related biomacromolecules such as proteins, mRNA of genomic RNAs. Functional interactions (both physical interactions and other forms of interactions) eliciting a cellular reaction.	[117]

Name	Website	Characteristics	References
lnC*e*DB	http://gyanxet-beta.com/lncedb/	Database of lncRNAs acting as competing endogenous RNAs. Putative interactions between lncRNAs and mRNA targets using algorithms and *in silico* tools such as StarBase.	[118]
lncRbase	http://bicresources.jcbose.ac.in/zhumur/lncrbase/	Comprehensive database of human and mouse lncRNAs. Contains genomic location, overlapping small ncRNAs, associated repeat elements and imprinted genes and lncRNA promoter information.	[119]

Table 4. Free databases for lncRNAs

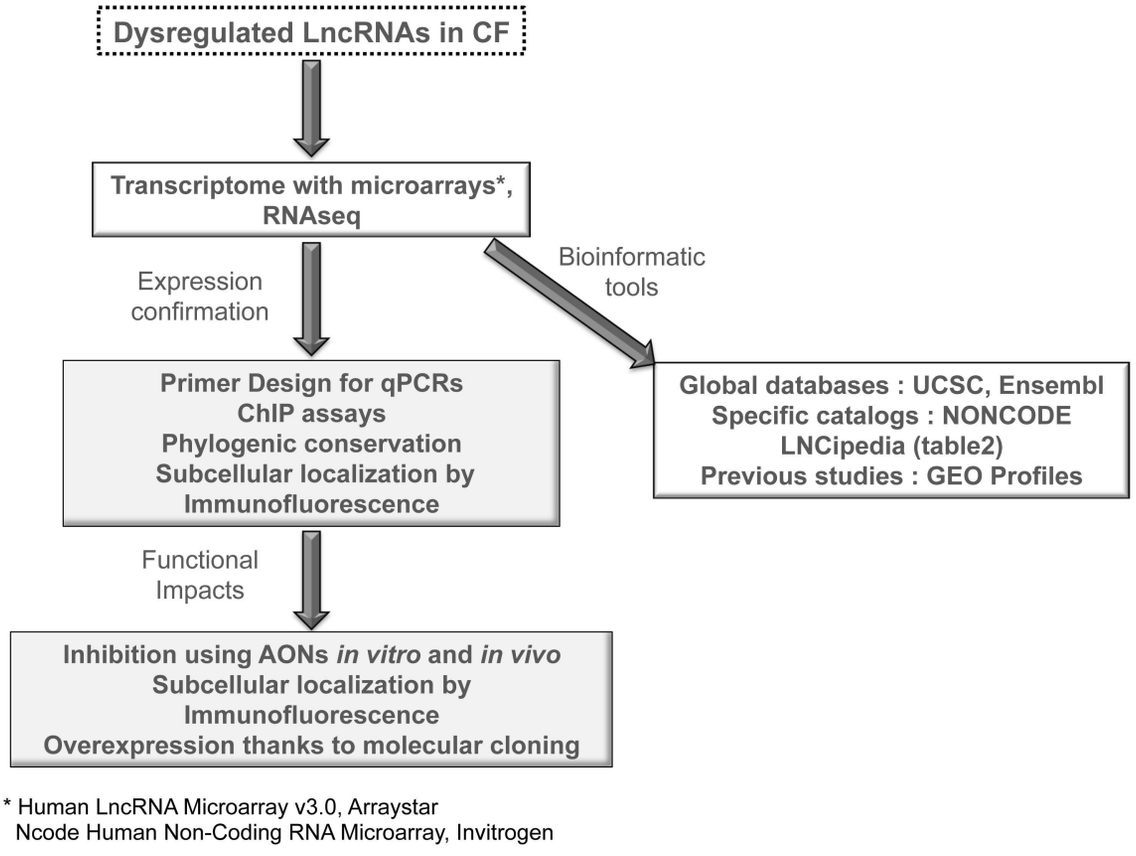

Figure 7. Strategies to study lncRNAs in CF and non-CF samples. Few techniques allow the quantitative analysis of lncRNAs in biological samples. RNAseq: global sequence screening is currently an easy option thanks to NGS and small RNA sequencing. This method allows detecting and quantitating in a biological sample all non-coding transcripts. However, as lncRNAs are very weakly expressed, this strategy might not be fully appropriate. To obtain a significant profile of ncRNAs, more than 180 million reads are required, whereas for protein-coding transcripts, less than 30 million are needed. Therefore, microarray analysis remains a powerful tool for global profiling of lncRNAs. In total, more than 30,586 lncRNAs have been analyzed based on the last lncRNA databases. AONs: antisense oligonucleotides.

4.3. Impact of mutations in lncRNA or miRNA genes

To date, no ncRNA mutation has been described in CF. However, alterations in RNA sequence and/or structure can affect the synthesis, maturation and turnover of ncRNAs. Changes in RNA molecules can be introduced in different ways. For instance, SNPs may affect miRNA biogenesis. miRNA tailing can modify pre-miRNAs and mature miRNAs. RNA editing can modify nucleotide sequences of RNA transcripts. NGS technologies, including exome sequencing and complete re-sequencing of the *CFTR* gene, could reveal mutations in lncRNA sequences that may affect CF severity and outcome.

5. Targeting miRNA as new putative therapeutic tool

5.1. Could miRNAs help in improving CF treatment?

A recent work demonstrated that miR-138 mimics might restore CFTR-Phe508del expression and functional chloride transport. However, the authors stressed that miR-138 mimics may also have undesired effects, because miR-138 targets SIN3A, a highly conserved transcriptional repressor that regulates many genes [67]. Another anti-miRNA agent has been exploited as inhibitor of miR-509-3p, which is involved in the regulation of the *CFTR* gene. Recently, we reported that miRNA function can be blocked by targeting the *CFTR* gene with blockers. We designed blockers to prevent the binding of several miRNAs specifically to the 3'UTR-*CFTR* and tested them in well-differentiated primary human nasal epithelial cells from healthy individuals and patients with CF carrying the p.Phe508del *CFTR* mutation. These molecules rescued CFTR chloride channel activity by increasing *CFTR* mRNA and protein levels. This is in agreement with previous studies showing that complementation of just 6–10% of CFTR transcripts leads to the production of enough CFTR to maintain normal chloride transport in epithelia [75]. These data are supported by findings that the presence of a naturally occurring sequence variation in the *CFTR* promoter, in cis of a severe mutation, increases transcription. This allows the production of enough CFTR protein to reach the apical membrane cells and partially restore CFTR channel function, thus leading to a moderate CF phenotype despite the presence of a severe disease-causing mutation [76]. Similarly, stabilization of p.Phe508del CFTR protein has been associated with increased p.Phe508del CFTR channel activity [77].

5.2. Assays and molecules

As depicted in Figure 5Ad, inhibitors or target-site blocker oligonucleotides have been previously used to restore CFTR expression. Tests have been performed by incorporating inhibitors that induce degradation of the targeted endogenous miRNA or with oligonucleotides that block miRNA binding to the 3'UTR of *CFTR* in cell lines, primary cultures and reconstituted epithelium

6. Conclusion and remarks

The identification of *cis*- and *trans*-regulators and pathways involved in *CFTR* gene expression is essential for developing new CF targeted therapies. Over the past four decades, therapies for CF have focused entirely on symptoms to improve patients' quality of life. The first treatment (VX-770) targeted the basic defect in p.Gly551Asp-CFTR (1.6% of patients with CF worldwide) [78]. The new molecule VX-809 has been evaluated in patients carrying the p.Phe508del CFTR mutation; however, on its own it does not have clear effects [79] and clinical trials testing the combination of different molecules are in progress. The mechanisms responsible for the phenotype severity are not well understood yet. Mutational heterogeneity and complex alleles influence CF severity. Moreover, the role of few modifier genes has been established [80]. ncRNAs could also contribute to CF progression and severity and their dysregulation in CF opens new perspectives for patient follow-up and treatment.

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