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ABSTRACT The *CFTR* gene displays a tightly regulated tissue-specific and temporal expression. Mutations in this gene cause cystic fibrosis (CF). In this study we wanted to identify *trans*-regulatory elements responsible for *CFTR* differential expression in fetal and adult lung, and to determine the importance of inhibitory motifs in the *CFTR*-3′UTR with the aim of developing new tools for the correction of disease-causing mutations within *CFTR*.

We show that lung development-specific transcription factors (FOXA, C/EBP) and microRNAs (miR-101, miR-145, miR-384) regulate the switch from strong fetal to very low *CFTR* expression after birth. By using miRNome profiling and gene reporter assays, we found that miR-101 and miR-145 are specifically upregulated in adult lung and that miR-101 directly acts on its cognate site in the *CFTR*-3′UTR in combination with an overlapping AU-rich element. We then designed miRNA-binding blocker oligonucleotides (MBBOs) to prevent binding of several miRNAs to the *CFTR*-3′UTR and tested them in primary human nasal epithelial cells from healthy individuals and CF patients carrying the p.Phe508del *CFTR* mutation. These MBBOs rescued CFTR channel activity by increasing CFTR mRNA and protein levels.

Our data offer new understanding of the control of the *CFTR* gene regulation and new putative correctors for cystic fibrosis.

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## Introduction

Most transcription factors are part of multi-protein complexes that recruit other transcription factors and cofactors to mediate local and long-range chromatin changes through physical modifications [1]. Within the transcriptional and post-transcriptional *trans*-regulatory elements that make up gene regulatory networks, transcription factors often interact with microRNAs (miRNAs) to control gene expression [2, 3]. miRNAs are post-transcriptional regulators that are expressed in a tissue-specific or developmental stage-specific manner, thereby greatly contributing to cell/tissue-specific protein expression profiles, including during lung organogenesis [4, 5].

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*; MIM# 602421) gene are responsible for cystic fibrosis (MIM# 219700), a common recessive lethal disorder. Chronic lung disease is the major cause of mortality and morbidity in patients with cystic fibrosis. *CFTR* gene expression is spatially and temporally regulated and several studies have demonstrated the differential use of transcription start sites, depending on the tissue type or the developmental stage [6–9]. 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 [10–14]. The changes in CFTR protein expression in human fetuses are consistent with *CFTR* mRNA temporal pattern [14]. This developmental regulated expression suggests that CFTR is involved in lung organogenesis, possibly by participating in the mechanico-sensory process that is essential for the regulation of Wnt/β-catenin signalling during lung development [15].

Several repressors and activators have been implicated in the regulation of *CFTR* transcription [16–22]. For instance, CCAAT-enhancer-binding protein (C/EBP)δ positively regulates *CFTR* transcription by binding to an inverted CCAAT element in the promoter [16]. In addition, C/EBPβ binds to a DNase I-hypersensitive site that is present only in tissues expressing *CFTR* [23] and we recently demonstrated its binding to *CFTR* minimal promoter [24]. Chromatin structures that facilitate long-range interactions between various regulatory elements cluster specifically to the *CFTR* promoter exclusively in *CFTR* expressing cells [25, 26]. Recently, it was reported that *trans*-interacting Forkhead Box A (FOXA) factors induce *CFTR* activation through binding to a key *CFTR cis*-regulatory element located in introns 10 and 11 [27–29]. *CFTR* expression is post-transcriptionally regulated also by miRNAs, such as miR-145 and miR-494 [30–32]. Indeed, several miRNAs, including miR-145, are expressed in primary adult human airway epithelial cells where *CFTR* expression is low [30]. Moreover, increased expression of some miRNAs has been reported in bronchial epithelium and primary culture of airway epithelia from patients with cystic fibrosis compared to healthy controls [33, 34].

Nevertheless, the developmental control of *CFTR* expression is still poorly understood in humans and the identification of *cis*- and *trans*-acting factors responsible for this complex spatio-temporal regulation is challenging. The aims of this study were 1) to identify *trans*-regulatory elements responsible for the differential expression of *CFTR* in fetal and adult lung; and 2) to determine the importance of inhibitory motifs in the 3′UTR of *CFTR* in order to develop new tools for *CFTR* mutation correction in patients with cystic fibrosis.

## Materials and methods

### *Gene reporter vectors, expression plasmid constructs and directed mutagenesis*

The pGL3b-CFTR-WT vector has been previously described [35]. The 3′UTR of the *CFTR* gene (1.7 kb from the termination codon to the poly-adenylation signal) was subcloned in the pGL3-control vector (Promega, Charbonnieres, France) downstream of the *Luciferase* gene (pGL3C-CFTR-3UTR). The expression plasmids used are listed in supplementary table S1. Point mutations in *cis*-motifs to abolish binding were introduced by direct mutagenesis using the QuickChange®II site-directed mutagenesis kit (Stratagene, Massy, France). All constructs were verified by direct sequencing.

Short-interfering RNAs (siRNAs), miRNA precursors and miRNA-binding blocker oligonucleotides (MBBOs) are described in supplementary table S2.

### *Cell culture*

The cell lines and culture media used for this work are described in table 1.

Nasal cells from healthy individuals or patients with cystic fibrosis and homozygous for the p.Phe508del *CFTR* mutation were obtained by scratching the inferior turbinate epithelium with ASI Rhino-Pro® curettes (Arlington Scientific, Springville, UT, USA). All signed informed written consent and this research project received the agreement by the French ethical research committee (N°ID-RCB 2011-A01520-41). Nasal cells were cultured in an air–liquid interface (ALI) culture system as previously described [37] with

TABLE 1 Description of cells used

| Cells | Providers[#] | Characteristics | Stage | Donor | Type | Coating | Medium | Culture |
|---|---|---|---|---|---|---|---|---|
| **A549** | ATCC | Human pulmonary epithelial cell line | Adult | | Cell line | No | As previously described [35] | 37°C, 5% $CO_2$ |
| **Beas-2B** | ATCC | Human bronchial epithelial cell line | Adult | | Cell line | No | As previously described [35] | |
| **T84** | ATCC | Human colic epithelial cell line | Adult | | Cell line | No | As previously described [35] | |
| **HBEpic** | ScienCell, Clinisciences | Human fetal bronchial epithelial cells | Fetal | Fetus, age 20 weeks, female Fetus, age 21 weeks, male Fetus, age 22 weeks | Primary culture | Collagen I | Bronchial epithelial cell medium (BEpiCM, ScienCell, Clinisciences) | |
| **Human whole lung** | DV Biologics, Cambridge Bioscience | Human whole lung cells | Fetal | Fetus, second trimester | Primary culture | Collagen I | Fibroblast cellutions media (DV Biologics, Cambridge Bioscience) | |
| **CF BE41o-** | D.C. Gruenert (San Francisco, CA, USA) | Human pulmonary epithelial cell line (p.PHE508del/ p.PHE508del) | Adult | | Cell line | No | As previously described [36] | |
| **16HBE14o-** | | Human pulmonary epithelial cell line (N/N) | Adult | | Cell line | No | As previously described [36] | |

[#]: full details of the providers are as follows. ATCC, Manassas, VA, USA; ScienCell, Carlsbad, CA, USA; Clinisciences, Montrouge, France; DV Biologics, Costa Mesa, CA, USA; Cambridge Bioscience, Cambridge, UK.

minor modifications. All the media used were supplemented with antibiotics and all the supports were coated with collagen I. After 3 weeks of growth in monolayer, 300 000 cells per well were plated in collagen I-coated 12 mm Transwell-Clear® supports, 0.4 µm pore size (Corning Inc., Corning, NY, USA). The ALI medium in the upper compartment was removed after confluence and the medium in the lower compartment was changed every 2–3 days. Experiments were performed when epithelial cells were well differentiated by visual inspection (at least 28 days).

### *Transient transfections*

All the details of the transient transfection assays are in supplementary table S3.

### *RNA extraction and RT-qPCR*

Total RNA was extracted, reverse transcribed and amplified as previously described [38]. miRNAs were purified with the miRNeasy Mini Kit and the RNeasy MinElute Cleanup kit (Qiagen, Courtaboeuf, France). Reverse transcription was performed using 40 ng of miRNA and the miRCURY LNA™ Universal cDNA Synthesis Kit (EXIQON, Vedbaek, Denmark) and qPCR was performed with a 1:10 dilution of first strand DNA and microRNA LNA™ primers specific for each miRNA (EXIQON). The relative expression levels were calculated using the comparative DDCt method with *SNORD44* and *SNORD48* small nucleolar RNAs as endogenous controls.

### *Quantitative chromatin immunoprecipitation assays*

Quantitative chromatin immunoprecipitation (Q-ChIP) was carried out as previously described [24]. Purified cross-linked chromatin was immunoprecipitated with 3 µg of each antibody (Santa Cruz, Heidelberg, Germany; Clinisciences, Montrouge, France). As a control for nonspecific DNA binding, 3 µg of anti-immunoglobulin G antibodies were used (Santa Cruz, Clinisciences). Results were expressed relative to the input signal and to nonspecific immunoprecipitated chromatin.

### *Electromobility shift assay*

Electromobility shift assay (EMSA) was performed as previously described [17].

### *Reporter assay*

Cells were harvested 48 h after transfection and the activity of firefly luciferase and Renilla luciferase was measured using the Dual-Glo® Luciferase Assay System (Promega).


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### *Labelling, detection and analysis for miRNA profiling*

Total RNA (100 ng) from A549 and HBEpiC cells was labelled using the Agilent miRNA Complete Labeling and Hybridization Kit (Agilent Technologies, Massy, France) and then hybridised to the Agilent Human miRNA Microarray (V2, Agilent) that contains probes for 723 mature human miRNAs. Arrays were scanned using an Agilent scanner and features were extracted with the Agilent Feature Extraction software (version 10.5.1.1). Expression data were initially normalised to the 75th percentile and then averaged among the groups using the GeneSpring GX (Agilent) software. The Kruskall–Wallis test was used for group comparisons and the Benjamini–Hochberg correction was applied to adjust for multiple comparisons. Unsupervised hierarchical clustering was performed using the GeneSpring GX software.

### *Western blotting*

Whole proteins were extracted using 1X Laemmli buffer. Proteins were separated on 7% or 10% SDS-PAGE gels and transferred to PVDF membrane (Westran® Clear Signal Whatman®; Dominique Dutscher, Issy les Moulineaux, France). Antibodies and concentrations used are described in the captions to the figures.

### *CFTR activity*

CFTR activity was assessed by iodide-mediated quenching of the halide-sensitive yellow fluorescent protein (YFP), as previously described [36], using the Premo Halide sensor technology (Invitrogen, Villebon sur Yvette, France). 40 h after incubation with MBBOs or negative control, CFTR conductance was stimulated with an agonist mixture (forskolin, 3-isobutyl-1-methylxanthine, apigenin) for 10 min. Then, CFTR-mediated iodide efflux was measured in each individual well by recording the fluorescence emission continuously (400 ms per point) for 2 s (baseline) and after addition of 50 µL of 140 mM iodide solution.

### *Statistical analysis*

Q-ChIP, luciferase and RT-qPCR assays were performed at least three times and samples were analysed at least in triplicate. The t-test was employed for paired comparisons using InStat (GraphPad Software, version 3.0, Instat 3 folder). For assessing the effect of MBBOs, the Wilcoxon signed rank test was used with the R software to generate box plots with significances.

## Results

### *Transcription factors involved in the regulation of CFTR temporal expression*

To identify the regulatory elements that participate in *CFTR* downregulation in human lung after birth, we used primary human fetal bronchial epithelial cells (HBEpiC, three primary cultures from three different donors, gestational age in table 1) and whole lung cells as well as the A549 and Beas-2B cell lines (from adult human lung). *CFTR* transcript level in fetal HBEpiC and whole lung cells was comparable or even higher than in T84 cells, in which *CFTR* is constitutively expressed (fig. 1a) [39]. Moreover, *CFTR* expression in HBEpiC and whole lung cells was more than four-fold higher than in A549, Beas-2B cells and in adult lung RNA from healthy individuals. Based on the well-known *CFTR* expression profile in fetal and adult lung [12, 13], we considered that HBEpiC cells are a representative model of fetal lung cells and that *CFTR* expression in A549 and Beas-2B cells is comparable to that of adult lung.

To identify putative *cis*-regulatory motifs, by using open-source bioinformatics software we then performed an *in silico* analysis of the *CFTR* 5′UTR. We thus focused on the transcription factors FOXA and C/EBP, based on two criteria: 1) their high score in predicted transcription factor binding and 2) their involvement in lung morphogenesis. First, we confirmed FOXA and C/EBP binding to the *CFTR* minimal promoter by Q-ChIP (fig. 1b). Moreover, EMSA showed that C/EBPβ and FOXA2 bind directly to the targeted region (fig. 1c).

Next, we investigated the role of these transcription factors in the regulation of *CFTR* promoter activity. When overexpressed in A549 and Beas-2B cells (adult lung), FOXA1, FOXA2 and C/EBPα had a repressive effect on *CFTR* transcription (fig. 2a and b). In line with this result, *FOXA1*, *FOXA2* and *C/EBPα* silencing using specific siRNAs increased *CFTR* expression (fig. 2a and b). As control, qPCR and western blot showed that endogenous transcription factor levels were strongly reduced in the presence of each specific siRNA (fig. S1). Conversely, in fetal HBEpiC cells, FOXA1 and FOXA2 did not have any effect, while C/EBPα strongly induced *CFTR* transcription (fig. 2c). Finally, C/EBPβ overexpression increased endogenous *CFTR* transcript level in A549 (2×) and Beas-2B (3×) cells and even more in HBEpiC cells (30 ×) (fig. 2a–c). Analysis of the endogenous level of each transcription factor showed a homogeneous expression in the different cell lines (fig. S2).

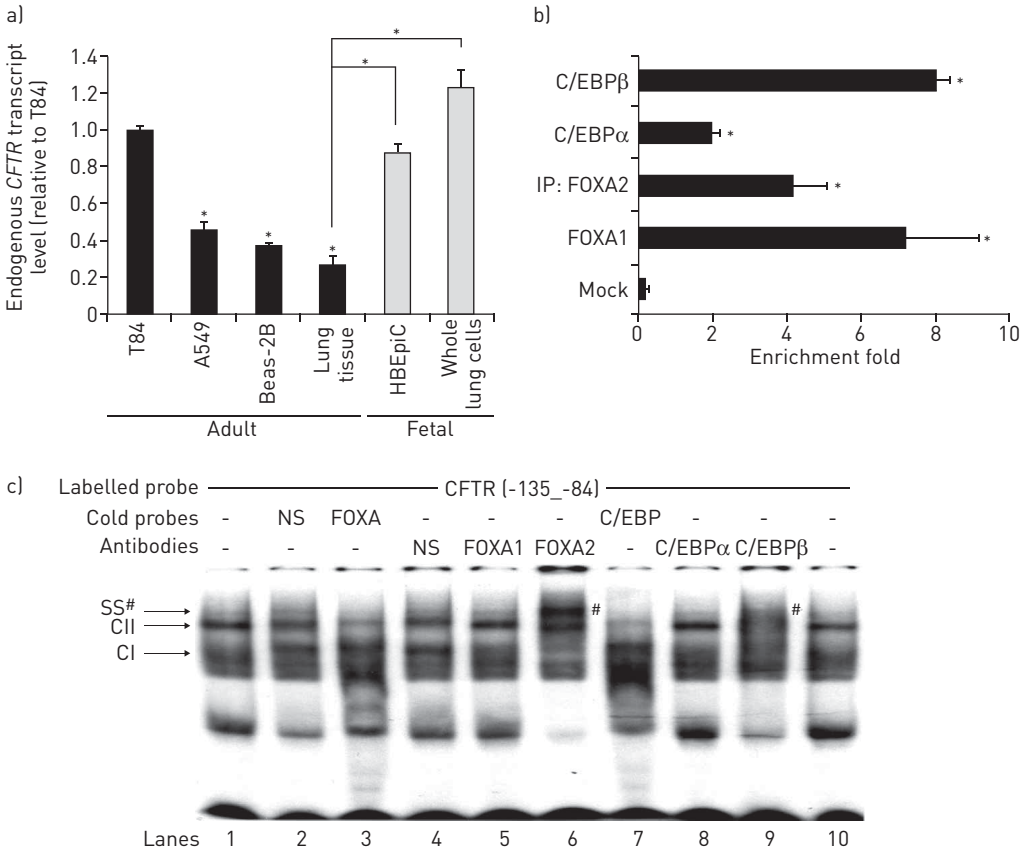


FIGURE 1 C/EBP and FOXA transcription factors bind to the *CFTR* promoter. a) Quantification of endogenous *CFTR* transcript level in lung cells compared to the T84 cell line by RT-qPCR. Data were normalised to *β-actin* transcript level. b) Binding affinity of the FOXA1, FOXA2, C/EBPα and C/EBPβ transcription factors for the *CFTR* promoter. Chromatin from A549 cells was immunoprecipitated (IP) with specific antibodies against FOXA1, FOXA2, C/EBPα and C/EBPβ or with nonspecific antibodies (IP mock). Data were normalised to non-immunoprecipitated chromatin. c) FOXA and C/EBP transcription factors bind to the –135 to –84 bp region of the *CFTR* promoter in A549 cells. Electromobility shift assay was performed with specific radiolabelled probes (CFTR-135_-84) incubated with A549 nuclear proteins. Specificity was assessed by competition with an excess of (FOXA and C/EBP) cold probes compared to a nonspecific probe (NS). Supershift (SS) assay was performed with specific anti-FOXA1, -FOXA2, -C/EBPα and -C/EBPβ antibodies compared to a nonspecific antibody (NS). Arrows highlight specific complexes (CI and CII). *: $p<0.0001$.

As FOXA and C/EBP factors do not act alone, we re-examined the *in silico* analysis data by using a lower cut-off in order to select other transcription factors that may contribute to *CFTR* regulation. Possible candidates of this transcription factor network included RREB-1 (the transcription factor with the highest score), several transcription factors with a known effect on *CFTR* gene expression, such as USF2, SRF and YY1 [17, 18, 35], and transcription factors involved in lung morphogenesis, but with a lower score than C/EBPs and FOXAs (SOX17, FOXF1 and NKX2.1) [40]. We then used reporter assays to investigate whether these regulatory elements could participate in the temporal regulation of *CFTR* expression. Co-transfection of the pGL3b-CFTR-WT reporter vector (wild-type minimal CFTR promoter) with the ubiquitously expressed USF2 and SRF or the developmental-specific NKX2.1 induced luciferase activity in adult and fetal lung cells (fig. 2d). A forced expression of YY1 protein caused a strong decrease in reporter activities (~50% of the control luciferase value). Conversely, RREB-1 inhibited *CFTR* transcriptional activity in the adult pulmonary A549 and Beas-2B cell lines but not in primary fetal HBEpiC cells. Similarly, the activating effect of SOX17 and FOXF1 in fetal HBEpiC cells was reduced or even abolished in A549 and Beas-2B cells.

These results show that FOXA1, FOXA2 (directly) and C/EBPα negatively regulate *CFTR* transcription in a specific manner in mature lung cells, while C/EBPβ induces *CFTR* transcription through direct binding to the promoter, regardless of the temporal stage. Other transcription factors, such as SOX17, RREB-1 and FOXF1, play also a role in the temporal regulation of *CFTR* expression in fetal and adult lung.


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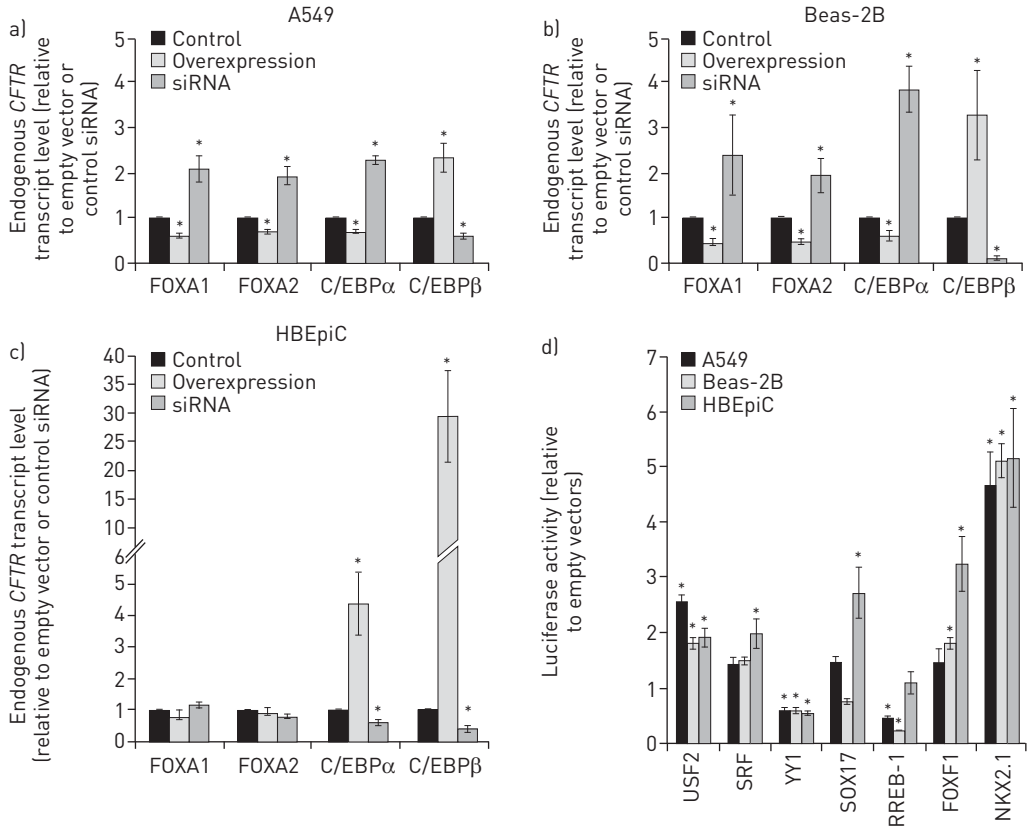


FIGURE 2 Several factors control *CFTR* transcription. a–c) Effect of FOXA1, FOXA2, C/EBPα or C/EBPβ overexpression or silencing with specific siRNAs on the endogenous *CFTR* transcript level in adult pulmonary cells (A549 (a) and Beas-2B cells (b)) and c) fetal cells (HBEpIC cells). RT-qPCR data were normalised to *β-actin* transcript level. d) Role of different transcription factors in *CFTR* transcriptional activity regulation. Luciferase activity was measured in the different pulmonary cell lines following co-transfection of the pGL3b-CFTR-WT reporter vector (wild-type minimal *CFTR* promoter) and of constructs expressing the different transcription factors. Data were normalised to the luciferase activity level following transfection of empty vector. *: $p<0.0001$.

### *A complex pattern of cis- and trans-acting elements in the 3′UTR of CFTR is involved in the temporal regulation of its expression*

To evaluate the effect of the 3′UTR on the post-transcriptional regulation of *CFTR*, we then transfected A549, Beas-2B and HBEpiC cells with the pGL3C-CFTR-3UTR reporter vector (*CFTR* 3′UTR) or vector alone. The 3′UTR of *CFTR* strongly repressed luciferase activity in all cell types, indicating that this region contains *cis*-repressive elements (fig. 3a). Using the bioinformatic tool AREsite (http://rna.tbi.univie.ac.at/cgi-bin/AREsite.cgi), we identified four new putative AU-rich elements (ARE) in the 3′UTR of the *CFTR* gene (ARE-4816, ARE-5533, ARE-5698 and ARE-6074) in addition to those previously described [41] and that we renamed ARE-4585, ARE-4760 and ARE-4891, according to their nucleotide position (fig. 3b). To determine the role of these motifs in the regulation of *CFTR* expression, we transfected A549, Beas-2B and HBEpiC cells with pGL3C-CFTR-3UTR reporter vectors in which each of these motifs was mutated and then measured luciferase activity. Only ARE-4760 appeared to be implicated in mRNA stabilisation because mutation of this motif was associated with a decrease in luciferase activity compared to cells transfected with pGL3C-CFTR-3UTR (wild-type sequence) (fig. 3b). ARE-4585, ARE-5533, ARE-5698 and ARE-6074 seemed to be involved in mRNA destabilisation in A549 and/or Beas-2B cells, whereas they had no significant effect in HBEpiC cells (fig. 3b). The strongest effect was obtained using ARE-5698, which *in silico* was identified as the most conserved ARE motif in the *CFTR* 3′UTR. Other *cis*-acting elements might explain the repressive activity of the 3′UTR of *CFTR* in adult cell lines. Computational predictions detected 13 putative miRNA-binding motifs in the *CFTR* 3′UTR (fig. 3c). Among the previously studied miRNAs, miR-145 has been involved in the regulation of *CFTR* expression in colonic and pancreatic cell lines [30]. We then assessed the role of miRNAs in the post-transcriptional control of *CFTR* in pulmonary cells by using luciferase reporter assays after transfection with miRNA precursors and the pGL3C-CFTR-3UTR reporter vector. MiR-942, miR-665, miR-383, miR-1290 and miR-1246 did not induce any significant effect in any cell type, whereas miR-600 reduced luciferase activity in all cell lines

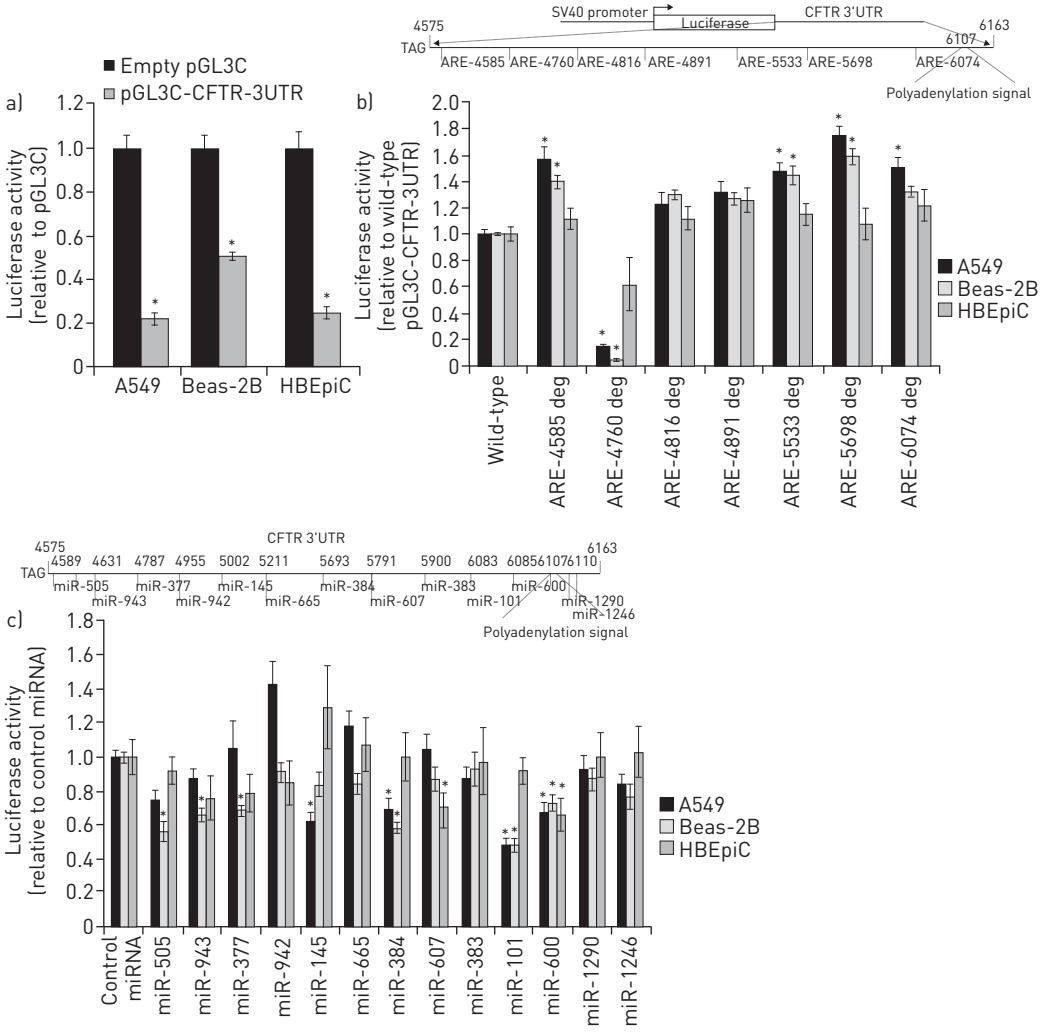


FIGURE 3 *Cis*- and *trans*-acting elements in the 3′UTR are involved in the temporal regulation of *CFTR* gene expression. a) Role of the *CFTR* 3′UTR. Luciferase activity was measured in the different lung cell lines after transfection of the reporter vector containing the 3′UTR of the *CFTR* gene downstream of the *Luciferase* gene (pGL3C-CFTR-3UTR). Data were normalised to those obtained with empty pGL3C vector. b) Importance of AU-rich element (ARE) motifs in the post-transcriptional regulation of *CFTR* expression. Each putative ARE site within the *CFTR* 3′UTR in the pGL3C-CFTR-3UTR vector was mutated and then the effect of their mutation was evaluated by measuring luciferase activity in the different pulmonary cell lines. Data were normalised to the values obtained with the wild-type reporter vector (pGL3C-CFTR-3UTR). c) Role of miRNAs in the post-transcriptional regulation of *CFTR* expression. Precursors of the miRNAs predicted to bind to the *CFTR* 3′UTR were transfected in the different pulmonary cell lines and then luciferase activity was measured. Data were normalised to the LNA™ miRNA inhibitor negative control oligonucleotide (control miRNA; EXIQON, Vedbaek, Denmark). *: $p<0.0001$.

compared to control miRNA (fig. 3c). MiR-505, miR-943, miR-377, miR-145, miR-384 and miR-101 decreased luciferase activity in A549 and/or Beas-2B cells, but not in HBEpiC cells (fig. 3c).

As the strongest repressive effect on *CFTR* post-transcriptional regulation in A549 and Beas-2B cells was induced by miR-101, we next focused on this miRNA and confirmed its negative impact on endogenous *CFTR* transcript level after transfection in adult pulmonary cells (fig. 4a). After transfection of the miR-101 precursor, miR-101 overexpression was verified in the three cell lines (fig. 4b). We also confirmed the endogenous expression of miR-101 and its differential expression in A549, Beas-2B (adult) and HBEpiC cells (fetal) (fig. 4c).

Previous studies demonstrated that miRNA-mediated regulation might require the presence of an ARE sequence [42–44]. As the miR-101 and miR-600 binding sites overlap with the ARE-6074 motif and the miR-384 binding site overlaps with the ARE-5698 motif (fig. 4d), we asked whether the effect of these miRNAs following binding to the 3′UTR of *CFTR* is dependent on the integrity of the ARE motifs. To this aim we co-transfected the miR-101, miR-600 and miR-384 precursors with reporter vectors


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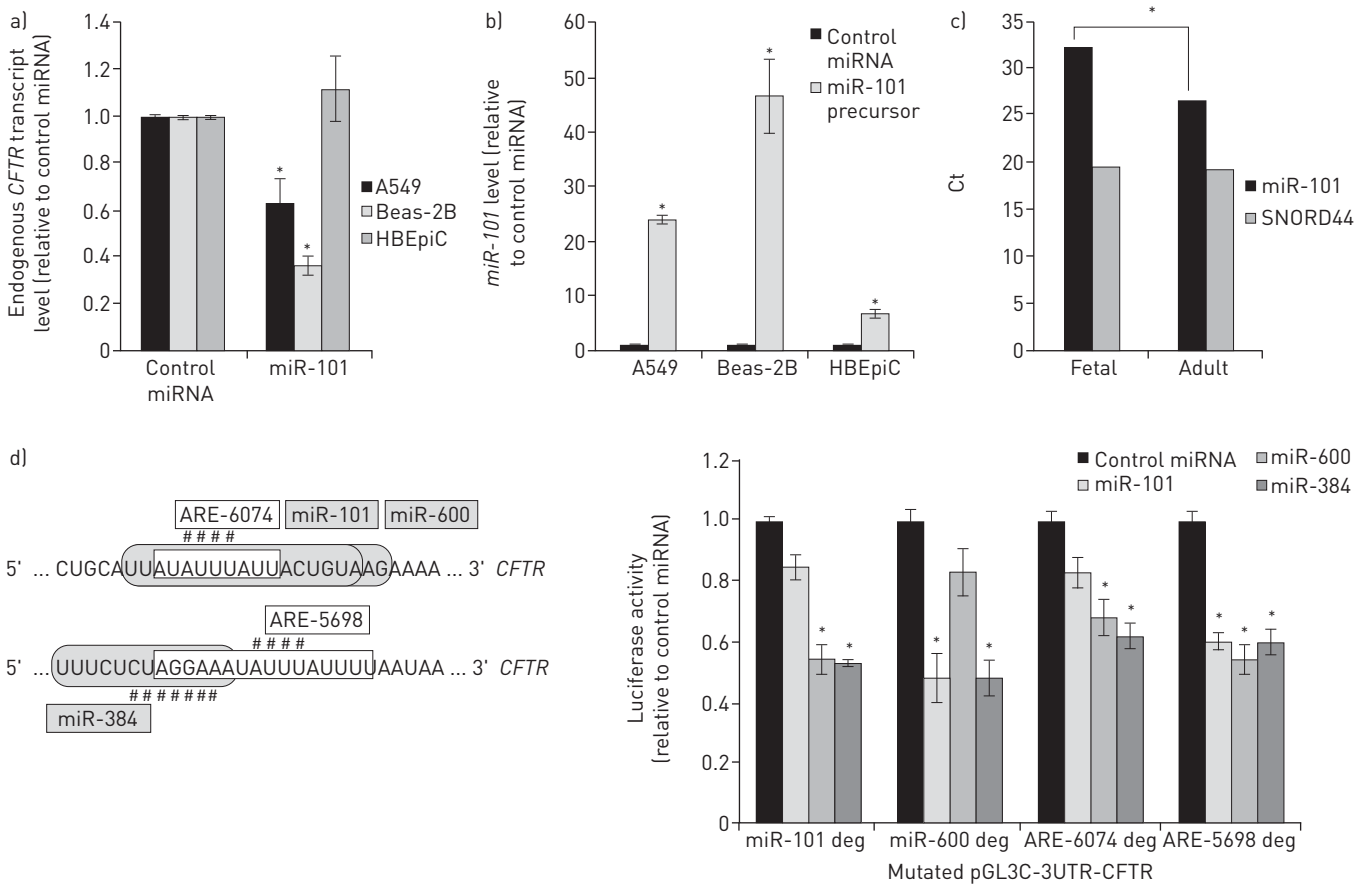


FIGURE 4 Role of miR-101. a) Effect of miR-101 over-expression on the endogenous *CFTR* transcript level in the different pulmonary cell lines. Data were normalised to *β-actin* transcript level. b) Control of miR-101 over-expression in the three cell lines. Hsa-miR-101 level was quantified by RT-qPCR (EXIQON, Vedbaek, Denmark) after transfection of miR-101 precursor or control miRNA in pulmonary cell lines. Data were normalised to *SNORD44* transcript level. c) Endogenous miR-101 expression level in fetal (HBEpiC) and adult (A549) lung cells. Data were compared to the expression of the internal control *SNORD44*. d) Overlapping AU-rich element (ARE) and miRNA binding sites in *CFTR* 3′UTR. AREs are represented by white squares, miRNA binding sites by grey squares. #: mutated nucleotides. Involvement of *cis*-elements in miRNA effect on *CFTR* post-transcriptional activity. The role of miR-101, miR-600 and miR-384 was evaluated by measuring luciferase activity in A549 cells after co-transfection of each miRNA precursor with a reporter vector containing the *CFTR* 3′UTR in which the binding site for miR-101 or miR-600 (miR-101-deg and miR-600-deg), or the ARE-5698 or ARE-6074 motif (ARE-5698-deg and ARE-6074-deg) was mutated. Data were normalised to the luciferase activity in cells transfected with control miRNA. *: p<0.0001.

containing wild type or mutated *CFTR* 3′UTR. Only miR-101 lost its repressive effect on luciferase activity following the mutation of its binding site within the *CFTR* sequence or abrogation of the ARE-6074 (fig. 4d). Mutation of ARE-6074 and ARE-5698 did not affect the activity of miR-600 and miR-384, respectively.

As miRNAs have been previously described in lung development mainly in mice, we investigated their differential expression in adult human lung tissue and fetal primary whole lung cells using Agilent DNA microarrays. Analysis of the microarray data showed that 65 miRNAs had the highest expression variability between adult and fetal lung. Among the 30 probes with the strongest expression difference between adult and fetal lung (supplementary table S4), we found that miR-451, miR-150 and miR-145 were specifically upregulated in adult lung (fig. 5a). We confirmed the endogenous expression of miR-145 and its differential expression in A549, Beas-2B (adult) and HBEpiC cells (fetal) (fig. 5b).

These data demonstrate the implication of miRNAs in the tightly controlled developmental regulation of *CFTR* expression and, more particularly, of miR-101 and miR-145, the expression of which is higher in adult than in fetal lung. Moreover, they show that miR-101 directly acts on its cognate site in combination with an overlapping ARE motif.

### *From identifying crucial regulators of CFTR expression to testing new potential therapeutic tools for cystic fibrosis*

The region encompassing the miR-101 binding site and ARE-6074 is critical for the miR-101 role in the regulation of *CFTR* expression. Based on this observation, we designed MBBOs to prevent binding of

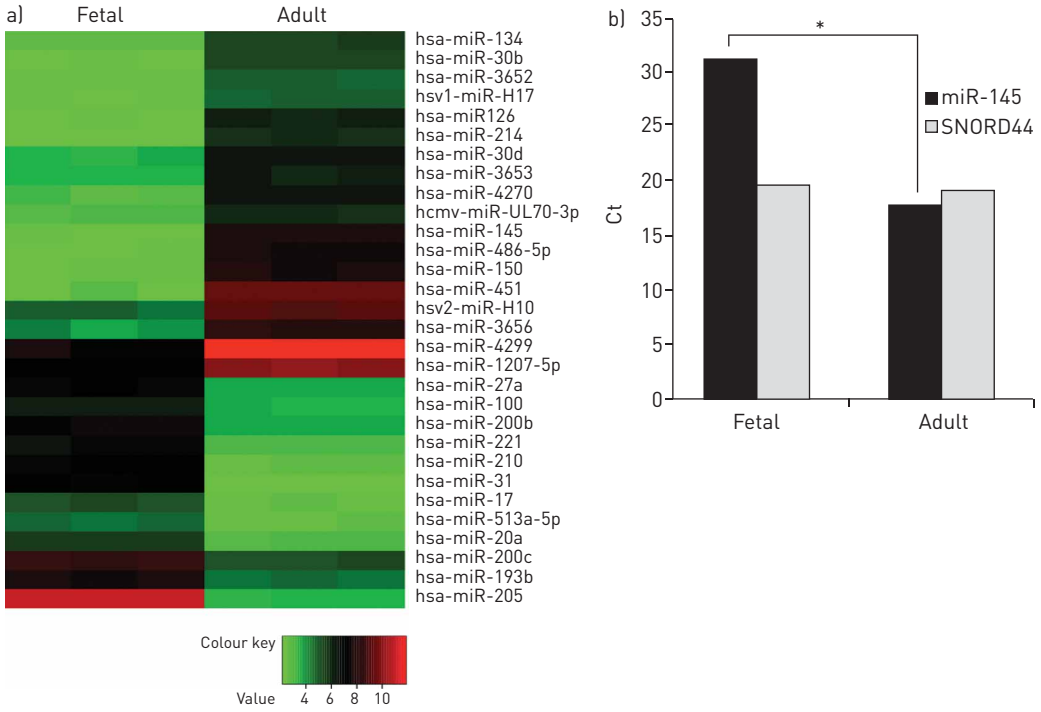


FIGURE 5 miRNA profiling revealed overexpression of miR-145 in adult lung tissues. a) Hierarchical cluster analysis of the miRNAs that are differentially expressed in adult *versus* fetal lung tissue. Microarray was performed using total miRNA from fetal (HBEpiC) and adult pulmonary (A549) cells. b) Endogenous miR-145 expression level in fetal (HBEpiC) and adult (A549) lung cells. Data were compared to the expression of the internal control *SNORD44*. *: p<0.0001.

several miRNAs, including miR-101, miR-600, miR-145 and miR-384, to the 3′UTR of *CFTR*. Co-transfection of these MBBOs with the pGL3C-CFTR-3UTR reporter vector led to a 1.5- to 6-fold increase of luciferase activity in Beas-2B and A549 cells, respectively (fig. 6a). The positive effect on endogenous *CFTR* expression upon MBBO-1 transfection was confirmed in these cells (fig. 6b).

Next, we evaluated the effect of the MBBOs *ex vivo* because mutant mice do not develop the characteristic manifestations of human cystic fibrosis. To this aim, we added medium containing control oligonucleotide, MBBO-1 or MBBO-3 without any transfection reagent to the upper compartment of Transwell-Clear® supports in which reconstituted ALI epithelial cells obtained from human nasal cells of control individuals (n=8) and CF patients homozygous for the p.Phe508del mutation (n = 6) were cultured. After 2 h at 37°C, the medium was removed from the upper compartment to restore the ALI. Freshly prepared control oligonucleotide or MBBOs were added every 2 days and *CFTR* expression was assessed 24 h post-treatment. MBBO effect was even stronger in epithelial cells from control individuals. Indeed, MBBO-1 induced a 2- to 6-fold increase of the endogenous *CFTR* expression in the epithelium derived from healthy individuals (fig. 6c) compared to the 2- to 3-fold increase in cells from patients with CF (fig. 6d). This effect was not significantly improved by repeated incubation with MBBO-1.

MBBO-1 and MBBO-3 significantly increased *CFTR* mRNA (fig. 7a) and protein expression (fig. 7b) in cystic fibrosis epithelia compared to control oligonucleotide. We next investigated MBBO effect on CFTR channel activity by using a functional assay (iodide-mediated quenching of the halide-sensitive YFP variant) and the human bronchial epithelial cell lines CFBE41o- (derived from a patient with cystic fibrosis) and 16HBEo- (normal phenotype). We first confirmed the absence of CFTR-dependent anion transport in CFBE41o- cells (cystic fibrosis) compared to 16HBEo- (non-cystic fibrosis) cells in which iodide entered and quenched YFP fluorescence (fig. 7c). Addition of Inh-172 (a CFTR inhibitor) in non-cystic fibrosis cells led to results comparable to those obtained in cystic fibrosis cells, confirming that the assay measures CFTR-dependent anion transport. Incubation with MBBO-1 and MBBO-3 for 2 h, significantly increased anion transport in cystic fibrosis cells compared to untreated cells (fig. 7d) and fluorescence quenching was proportional to the amount of CFTR detected in the cells by immunoblotting.

These data support the importance of the regions encompassing the miR-101 and miR-145 binding sites in *CFTR* regulation and suggest that MBBOs could represent a new therapeutic option for CF.


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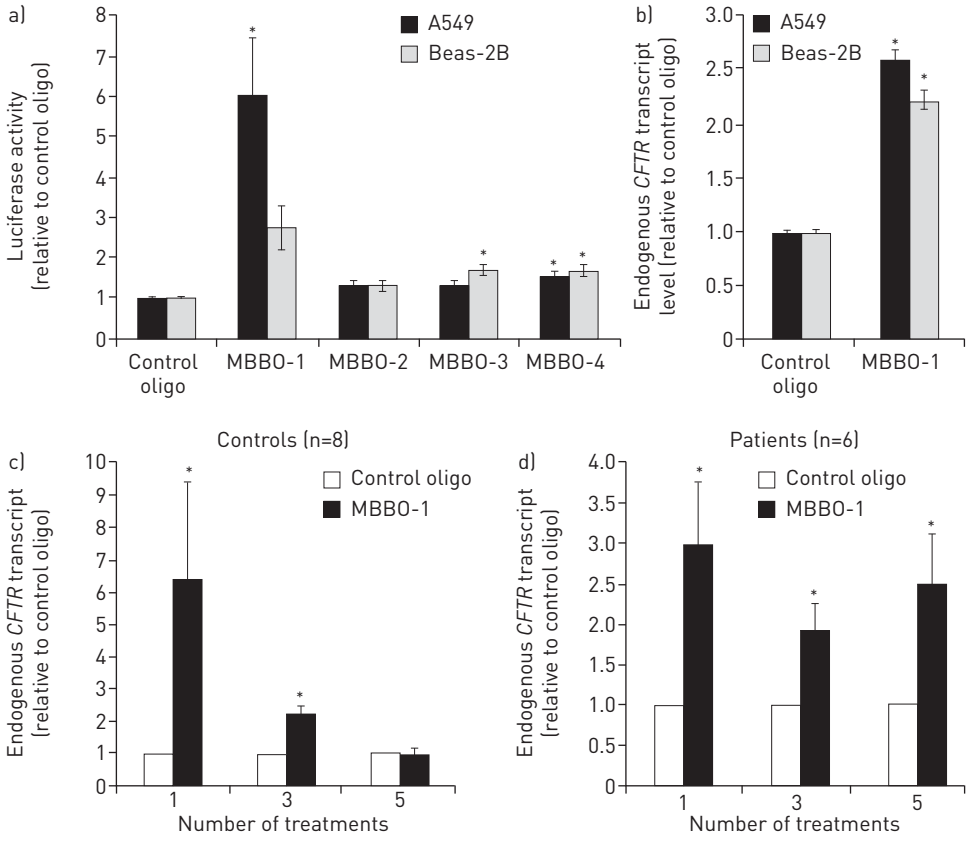


FIGURE 6 Effect of miRNA-binding blocker oligonucleotides (MBBOs) on *CFTR* expression in pulmonary cells. a) Effect of different MBBOs on *CFTR* post-transcriptional regulation. MBBOs (100 nM) were transfected together with the pGL3C-CFTR-3UTR reporter vector in A549 and Beas-2B pulmonary cells and luciferase activity data were normalised to miRCURY LNA™ microRNA Inhibitor Negative Control (control oligonucleotide; EXIQON, Vedbaek, Denmark). b) Effect of MBBO-1 on the endogenous *CFTR* transcript level in A549 and Beas-2B pulmonary cells. *CFTR* mRNA level was assessed by RT-qPCR following the transfection of MBBO-1 or control oligonucleotide. Data were normalised to *β-actin* transcript level. c, d) Effect of MBBO-1 on the endogenous *CFTR* transcript level in nasal epithelial cells from c) healthy controls (n=8), or from d) CF patients homozygous for the p.Phe508del mutation (n=6). *CFTR* mRNA level was assessed by RT-qPCR 24 h after one, three or five treatments with MBBO-1 or control oligonucleotide. Data were normalised to *β-actin* transcript level. *: p<0.0001.

## Discussion

Expression studies carried out in humans, mice and goats have revealed that the *CFTR* gene is developmentally regulated [10, 12–14, 45]. The most well-known site of developmentally regulated *CFTR* expression is the airway surface epithelium, with relatively high expression during embryonic and fetal development, followed by a marked decrease in expression after birth [45]. Despite extensive studies, the mechanisms accounting for this switch in *CFTR* expression remain unknown.

In a critical region of the *CFTR* gene that contains several naturally occurring variants [18, 35, 46], we found many ubiquitous [17, 18] and tissue- or lung developmental-specific transcription factors [40] involved in coordinating the switch from strong to very low *CFTR* expression in lungs after birth. The specific occupancy of these factors on the promoter may, in interaction with others factors, including FOXA1 and C/EBPβ that bind to other part of the *CFTR* gene depending on the nucleosome positioning [28], influence the particular pattern of expression of this gene. We also show that miRNAs, including miR-101 and miR-145, negatively regulate the level of *CFTR* transcripts in adult lung cells, whilst having no effect in fetal lung cells. In addition to its specific role in mature lung cells, miR-101 decreases luciferase activity in an embryonic kidney cell line [31], whereas it does not affect *CFTR* mRNA stability in pancreatic cell lines [30], suggesting a potential role as a tissue-specific factor. We then demonstrate the implication of miRNAs in the tightly controlled developmental regulation of *CFTR* expression and more particularly we show that miR-101 acts on its cognate site in combination with an overlapping ARE motif.

Finally, we demonstrate the benefit of characterising regulatory factors to identify novel therapeutic targets. Early studies indicated that complementation of just 6–10% of *CFTR* transcripts generate enough *CFTR*

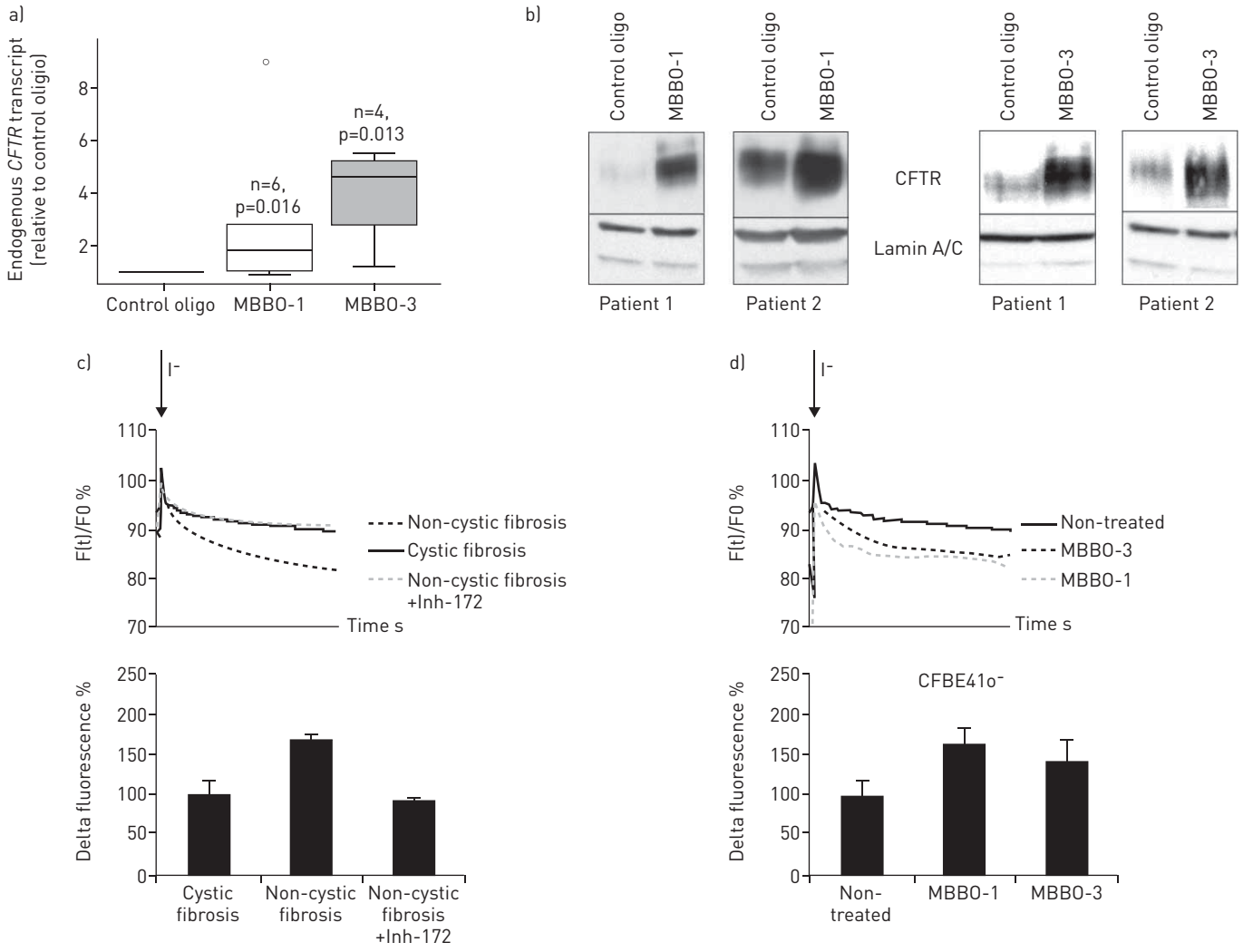


FIGURE 7 Effect of miRNA-binding blocker oligonucleotides (MBBOs) on CFTR expression and activity in epithelial cells from cystic fibrosis (CF) patients. a) Effect of MBBO-1 and MBBO-3 on endogenous *CFTR* transcript level in nasal epithelial cells from CF patients who are homozygous for the p.Phe508del mutation (24 h after the first treatment with 100 nM MBBO-1, MBBO-3 or control oligonucleotide at 37°C for 2 h). Data were normalised to *β-actin* transcript level. b) Effect of MBBO-1 and MBBO-3 on endogenous CFTR protein level in nasal epithelial cells from CF patients who are homozygous for the p.Phe508del mutation. Immunoblots were performed with an anti-CFTR antibody (1:400, clone MM13-4; Millipore, Molsheim, France) using total protein extracts prepared 24 h after the first treatment with MBBO-1, MBBO-3 or control oligonucleotide. Lamin A/C protein level (1:10 000, anti- Lamin A/C antibody; Sigma Aldrich, Saint-Quentin Fallavier, France) was used as loading control. c, d) Representative cell fluorescence recordings from bronchial cells that transiently express halide-sensitive yellow fluorescent protein (YFP) (the scale bar reports the percentage of total cell fluorescence). CFTR activity was measured in c) 16HBEo- (non-cystic fibrosis) and CFBE41o- (cystic fibrosis) cells, and in d) non-treated or MBBO-treated CFBE41o- cells. Extracellular addition of iodide (arrow) caused YFP quenching at a rate that is proportional to iodide influx and CFTR activity. Channel opening is signalled by a decrease of the probe fluorescence. Quenching is directly proportional to the chloride efflux. Graphs show the quantification of the obtained data. *: p<0.0001.

levels to maintain normal chloride transport in epithelia [47]. 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 can allow the production of enough CFTR protein to reach the apical membrane cells and restore partial CFTR channel function, thus inducing a moderate cystic fibrosis phenotype despite the presence of a severe disease-causing mutation [35]. Similarly, stabilisation of p.Phe508del CFTR protein has been associated with increased p.Phe508del CFTR channel activity [48]. Recent work demonstrated that miR-138 mimics might restores CFTR-Phe508del expression and a functional chloride transport [32]. However, the authors underlined the fact that miR-138 mimics may have undesirable effects because miR-138 targets SIN3, a highly conserved transcriptional repressor which regulates many genes [32]. Over the past four decades, therapies for cystic fibrosis have focused entirely on symptoms to improve the patients' quality of life. The first treatment (VX-770) targeted the basic defect in p.Gly551Asp-CFTR (1.6% of patients with cystic fibrosis worldwide) [49]. The new molecule VX-809 has been investigated for patients carrying the p.Phe508del CFTR mutation; however, alone no clear improvement has been reported [50] and clinical trials testing the combination of different molecules are


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in progress. Herein, we tested a new putative therapeutic tool that specifically targets the *CFTR* gene [51]. Focusing on miR-101 and miR-145, we designed MBBOs that target the miRNA binding sites in the *CFTR* 3′UTR instead of the miRNA itself. This blockage led to the correction of CFTR channel activity through stabilisation of *CFTR* mRNA and increase in the protein level in nasal epithelial cells from patients homozygous for p.Phe508del, the most frequent *CFTR* mutation. As miR-101 and miR-145 knock-down is associated with deregulation of epigenetic pathways resulting in cancer progression [52] and lung cancer [53], our approach in which MBBOs block only their binding to their cognate *CFTR* mRNA motif may have therapeutic benefits by stabilising *CFTR* transcripts and ultimately providing enough functional protein to improve the patients' phenotype without disturbing other signalling cascades. These findings underline the importance in the continued understanding of pathways that are targeted in the lung after birth, which could ultimately lead to new targets in lung disorders, especially in cystic fibrosis.

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