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The HDAC inhibitor SAHA does not rescue CFTR membrane expression in Cystic Fibrosis

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ABSTRACT

The development of suitable Cystic Fibrosis (CF) models for preclinical bench tests of therapeutic candidates is challenging. Indeed, the validation of molecules to rescue the p.Phe508del-CFTR channel (encoded by the *Cystic Fibrosis Transmembrane conductance Regulator* gene carrying the p.Phe508del mutation) requires taking into account their overall effects on the epithelium. Suberoylanilide Hydroxamic Acid (SAHA), a histone deacetylase inhibitor (HDACi), was previously shown to be a CFTR corrector *via* proteostasis modulation in CFTR-deficient immortalized cells. Here, we tested SAHA effects on goblet cell metaplasia using an *ex vivo* model based on the air-liquid interface (ALI) culture of differentiated airway epithelial cells obtained by nasal scraping from CF patients and healthy controls. *Ex vivo* epithelium grew successfully in ALI cultures with significant rise in the expression of *CFTR* and of markers of airway epithelial differentiation compared to monolayer cell culture. SAHA decreased CFTR transcript and protein levels in CF and non-CF epithelia. Whereas SAHA induced lysine hyperacetylation, it did not change histone modifications at the *CFTR* promoter. SAHA reduced *MUC5AC* and *MUC5 B* expression and inhibited goblet epithelial cell differentiation. Similar effects were obtained in CF and non-CF epithelia. All the effects were fully reversible within five days from SAHA withdrawal. We conclude that, *ex vivo,* SAHA modulate the structure of airway epithelia without specific effect on CFTR gene and protein suggesting that HDACi cannot be useful for CF treatment.

1. Introduction

Cystic Fibrosis (CF) is a genetic disease caused by the absence or dysfunction of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) cAMP chloride channel (Riordan et al., 1989; Welsh and Smith, 1993). In CF, attenuated chloride and bicarbonate secretion affect the transepithelial ion flow across the apical membrane of airway epithelial cells, causing hyperviscosity of the airway surface liquid (ASL) (Matsui et al., 1998; Derichs et al., 2011). Structural changes in the airway epithelium phenotype, such as goblet cell hyperplasia (Burgel et al., 2007), may also contribute to ASL hyperviscosity. The sticky mucus acts as a trap for microorganisms, thus favoring bacterial and fungi colonization. A vicious circle of infection/inflammation in the respiratory airways leads to lung failure and, often, to the death of CF patients. An excess of pro-inflammatory cytokines, such as IL-8 (Cohen and Prince, 2012), combined with a lack of anti-inflammatory molecules, such as lipoxin A4 (Karp et al., 2004; Chiron et al., 2008) or secretoglobin 1A1 (Starosta et al., 2006), may promote this phenomenon.

Patients with CF require numerous therapies to manage these

Abbreviations: ALI, air-liquid interface; ALOX15A, 15-LO1: 15-Lipoxygenase-1; ALOX15B, 15-LO-2: 15- Lipoxygenase-2; AMP, adenosine monophosphate; ASL, airway surface liquid; BSA, bovine serum albumine; cDNA, complementary desoxyriboNucleic acid; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ChIP, chromatin immunoprecipitation; DMSO, dimethyl sulfoxide; FEV1, forced expiratory volume in 1 seconde; FOXJ1, forkhead box protein J1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H3K4me3, histone H3 lysine 4 trimethylation; H3K27me3, histone H3 lysine 27 trimethylation; H4Kac, histone H4 lysine acetylation; HDACi, histone deacetylase inhibitor; IL-8, interLeukine-8; MC, monolayer primary culture; MUC5AC, mucine 5AC; MUC5B, mucine 5B; PBS, phosphate buffered saline solution; RNA, riboNucleic acid; ROS, reactive oxygen species; SAHA, suberoylanilide hydroxamic acid; TEER, trans epithelial electrical resistance; HDACi, histone deacetylases inhibitors

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symptoms (Farrell et al., 2008), including mucolytic and antibiotic agents and chest physiotherapy to treat the airway disease and digestive enzymes to replace the loss of exocrine pancreatic function. These and other interventions have increased life expectancy dramatically, but improvement is needed to reduce the high treatment burden and increase survival (Sawicki et al., 2009). A CFTR potentiator (VX-770) (Ramsey et al., 2011) has been recently approved for the treatment of CF patients carrying the p.Gly551Asp mutation (2–5% of CF patients). To date, the drug given alone has failed for CF patients with the most common mutation p.Phe508del. A corrector (VX-809) that partially alleviate the folding defect and allows some p.Phe508del to reach the apical membrane, showed low efficacy (Van Goor et al., 2011; Clancy et al., 2012). Although VX-809 yields only limited benefits, it shows greater efficacy in combination with VX-770: 25% of patients showed a > 10% increase in FEV1 and to a lesser extent for majority (5% increase in FEV1 for 55% of CF patients). For non-responders an alternative or a complementary therapeutic has to be developed. Several CFTR correctors have been previously reported to be active *in vitro* (Hutt et al., 2010; Verkman and Galietta, 2009) but further characterization of therapeutics modulating transcriptional gene expression may be highly desirable. We recently showed a benefit from an increase in p.Phe508del-*CFTR* transcript level to CFTR protein rescue and channel activity improvement (Viart et al., 2015).

Histone deacetylase inhibitors (HDACi) are potential candidates as proteostasis modulators and transcriptional activators and some can partially improve CFTR activity (Boinot et al., 2014). These molecules inhibit class I, IIa, IIb and IV HDAC through the zinc-binding domain located inside their catalytic site. Thus, HDACi prevent lysine deacetylation in the N-terminal tail of histones and inhibit chromatin condensation. SAHA (Suberoylanilid Hydroxamic Acid; Vorinostat®, Merck), the first FDA-approved HDACi, is indicated in the treatment of cutaneous T-cell lymphoma (Marks, 2004). A previous study showed that, *in vitro*, SAHA could partially restore CFTR chloride channel activity (equivalent to 28% of wild type) in the CFBE41o- cell line (immortalized human airway epithelial cells homozygous for the p.Phe508del *CFTR* mutation) (Hutt et al., 2010). However, differences in the baseline chromatin state between *in vivo* airway epithelial cells/tissues and immortalized cells, notably at the *CFTR* promoter (Ott et al., 2012; Bergougnoux et al., 2014), could critically alter the response to SAHA treatment *in vivo*.

Cell model selection is critical for interpretable experiments. A limitation in the understanding of the CF pathogenesis is the difficulty in studying the link between the mutant CF gene expression and the associated molecular and cellular effects in the absence of a optimal animal model for this disease. We hypothesized that nasal epithelium ALI cultures are relevant preclinical models suitable to test CF therapies, because this culture system allows the generation of a polarized airway epithelium with a basolateral and an apical compartments that are optimal for lab-bench testing. We obtained an air-liquid interface (ALI) culture with nasal epithelial cells from healthy controls (non-CF) and CF patients. In the present work, we wanted to pursue the investigation to determine whether SAHA, which rescue CFTR activity in CFBE41o- bronchial cells, can also rescue it in an *ex vivo* model of the human airway epithelium. First, we thoroughly analyzed the features of the CF and non-CF nasal epithelium ALI cultures. Then, we tested whether SAHA affects chromatin condensation and improves CFTR membrane expression in these airway epithelia. We evaluated SAHA effects on goblet cell metaplasia and on the expression of MUC5AC and MUC5B genes, coding for two major secreted mucins that are components of the mucus layer that covers the airway epithelium.

Finally, to assess whether these effects are reversible, all parameters were measured again after SAHA withdrawal. Our results indicate that the effects of candidate molecules for CF therapies have to be assessed in primary cell models before clinical testing.

Table 1
Characteristics of patients with CF and healthy controls (non-CF).

	Patients with CF	Healthy controls
Number	8	10
Demographic data		
Sex (M/F)	6/2	1/9
Age (yr, median [IQR])	21.5 [18.5–24.5]	27.50 [24.50–32.20]
CFTR genotype	p.Phe508del/ p.Phe508del	NA
Nutritional data		
BMI (median [IQR])	20.93 [20.29–21.61]	21.45 [21.22–21.49]
Spirometric data		
FEV_1 (L, median [IQR])	2.85 [1.29–3.00]	3.48 [3.44–3.54]
FEV_1 (% theoretical, median [IQR])	68.20 [42.28–75.75]	108.00 [105.25–114.15]
FVC (L, median [IQR])	3.66 [2.63–4.86]	4.12 [3.86–4.20]
FVC (% theoretical, median [IQR])	96.00 [65.00–97.60]	109.00 [104.50–112.55]
FEV_1/FVC ratio (median [IQR])	0.66 [51.25–66.00]	0.85 [0.84–0.88]
Bacteriological data		
P. aeruginosa (n)	7	0
S. aureus (n)	4	0

Abbreviations. *IQR*, InterQuartile Range; *BMI*, Body Mass Inde; *FEV_1*, Forced Expiratory Volume (in 1 s); *FVC,* Forced Vital Capacity; *NA,* Not applicable.

2. Experimental procedures

2.1. Patients and cell cultures

Nasal cells were obtained by nasal scrapes from sixteen adult patients with CF (p.Phe508del homozygous) and ten healthy volunteers recruited at the Respiratory Department of the Montpellier University Hospital (France) from 2012 to 2014. The study was approved by the local ethic committee (CPP #2011.12.08ter Sud Méditerranée III; n°ID-RCB: 2011-A01520-41). All volunteers gave their written informed consent. *Ex vivo* ALI cultures were done as previously described (Viart el al., 2015). Fifty percent of success for the *ex vivo* culture of nasal scraping samples from CF patients (8 out of 16 cultures) was observed. Causes of failure were fungal or bacterial contamination (twice) and, in most cases (6 samples), epithelial cells did not adhere at the beginning of the culture. No difficulty (100% success) was observed with the healthy volunteers' nasal scraping samples. Before each experiment, the trans epithelial electrical resistance (TEER) was checked and was higher than 300 mΩ, thus confirming the epithelium cohesion. Clinical data were collected at sampling time (Table 1; only data on the eight patients with CF with successful ALI cultures are shown). The CFBE41o- bronchial epithelial cell line was a generous gift from D. Gruenert (UCSF, CA) and grown in MEM EARLES (11504506, Fisher Scientific) in 5% CO2 37 °C.

2.2. Treatment

SAHA (Cayman Chemical®, Ann Arbor, Michigan, USA) stock solution (20 mg/ml) was prepared in DMSO and stored at −20 °C. For all experiments with the exception of the cytotoxicity assays, 5 μM SAHA (final concentration) or DMSO (vehicle) was added to the basolateral surface of well-differentiated ALI epithelial cell cultures. The treatment protocol is described in ref (Bergougnoux et al., 2017, submitted).

2.3. Cytotoxicity assays

SAHA cytotoxicity was evaluated in apical surface wash samples using the Cytotoxicity Detection Kit (ROCHE®).

2.4. Transfections assays

The wild-type (WT) and p.Phe508del *CFTR* cDNA were heterologous expressed in CFBE41o- cells. We used the p-TRACER-CFTR expression vector for transiently transfection using JET PEI agent and pCEP-CFTR with a 6.2 Kb WT *CFTR* cDNA for stably transfection (maintained by hygromycin treatment) in CFBE41o- cells.

2.5. RNA extraction and quantitative reverse transcription − PCR

Cells were harvested in 350 µL RLT buffer (QIAGEN®) and stored at −80 °C until extraction. RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN®) according to the manufacturer's protocol. 300 ng of total RNA was reverse transcribed and PCR-amplified as previously described (19) using *GAPDH* to normalize quantification. T84 cell cDNA (for *CFTR*) and human stomach cDNA (for *MUC5AC* and *MUC5B*) were used as calibrators (obtained from Clontech®). Primers are listed in Table S-1.

2.6. Western blot assays

Cells were lysed in 1X Laemmli buffer and stored at −80 °C. Proteins were separated on 7% SDS-PAGE gels and transferred to PVDF membranes (Westran® Clear Signal Whatman®, Dutsher SAS). Monoclonal antibodies (mAb) against CFTR (clone L12B4 and clone MM13-4, Millipore, 1:400) and proteins acetylated on lysine residues (#06-933, Millipore®, 1:500) were diluted in 5% skimmed milk. Protein levels of lamin A/C (clone 14, Millipore®, 1:10000) were assayed as internal control of protein loading. Membranes were incubated with primary mAbs at 4 °C overnight (for CFTR) or 1 h at room temperature (RT). Proteins were revealed by chemiluminescence (Millipore, #WBKLS0500) after 1 h incubation with the secondary antibodies (anti-rabbit #sc-2004, 1:20000; anti-mouse #sc-45042 1:40000, Santa Cruz®).

2.7. Immunofluorescence assays and histological assessment of epithelia

For immunofluorescence assays, ALI nasal epithelia on filters were fixed in 10% formalin for 15 min. All washes and a 20-min permeabilization were done with 1X PBS/0.1%Triton both at the apical and basolateral side. To reduce unspecific background, epithelia were saturated with a PBS/2%BSA solution at RT for 30 min. 150 µL of mAb/PBS/2%BSA solution was added to the apical pole and incubated at 4 °C overnight. Primary antibodies against MUC5AC (mouse [45M1] ab3649 Abcam® 1:100) and β-tubulin (#T7941 Sigma-Aldrich® 1:400) were used. Secondary antibodies (Alexa anti-mouse 568 (red) and 488 (green), 1:2000 in PBS-2%BSA) were added at RT for 1 h. DAPI (1:5000 in PBS/2%BSA) was added for 1 min. After 3 washes in 1X PBS, filters were mounted on microscope slides with fluoroshield mounting medium (Abcam® #ab104135) and observed under a fluorescence microscope (Zeiss Axio Imager). The goblet/ciliated cell ratio was calculated using the ImageJ software.

For histological assessment of the epithelium structure, ALI nasal epithelia on filters were rinsed with 1X PBS, fixed with 10% formalin, routinely processed and paraffin-embedded. Serial sections (4 µm) were cut and mounted on glass slides (Superfrost/Plus, Fisher®), dried at RT and then boiled in a water bath at 98 °C in citrate buffer pH6 for 10 min. After deparaffinization, sections were stained with Hematoxylin-Eosin.

2.8. Native chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed in triplicate using three non-CF ALI epithelial cell cultures as previously described (Bergougnoux et al., 2014). Quantification of H4KAc, H3K4Me3 and H3K27Me3 enrichment was performed by quantitative PCR as previously described (Bergougnoux et al., 2014) with primers listed in Table S-1.

2.9. Statistical analysis

Mann Whitney or Wilcoxon tests were used depending on the non-normality of the distribution assessed using the Shapiro-Wilk test. As each variable was evaluated several times, one patient's epithelium was evaluated with repeated measures. The variables were thus compared in univariate analyses using a generalized linear mixed-effects model for repeated measures. Two-sided statistical tests were performed using a 0.05 (5%) significance level. R® 3.1.1 software was used for all statistical analyses.

3. Results

3.1. An ex vivo pseudo-stratified epithelium optimal for CFTR study

We obtained *ex vivo* well-differentiated airway epithelial cultures from nasal scraping samples of ten controls (non-CF) and eight patients with CF (p.Phe508del homozygous) (Fig. S1). To validate our *ex vivo* model, we demonstrated, based on the detection of β-tubulin and MUC5AC expression by immunofluorescence staining, that ciliated cells (β-tubulin-positive) and goblet cells (MUC5AC-positive) were both present in day-28 ALI epithelia (Fig. 1A). Hematoxylin-eosin staining of paraffin-embedded day-28 ALI epithelium sections revealed a well-differentiated, pseudo-stratified mucociliary epithelium (Fig. 1B). *CFTR* gene expression was assessed at each culture step, from the monolayer primary cell cultures (MC) derived from the nasal scraping up to the differentiated epithelia (ALI) (D28: after 28 days of culture). *CFTR* mRNA level increased significantly in D28 ALI cultures compared to MC (Fig. 1C, $p = 0.03125$) to reach a level similar to that measured in fresh nasal epithelial cells (NEC) from non-CF donors ($n = 6$, p-value = 0.22), confirming that the model is optimal for the study of endogenous CFTR expression.

3.2. Comparison of non-CF and p.Phe508del CF ALI epithelia

CFTR transcript levels were significantly increased in the p.Phe508del CF ALI epithelia compared to non-CF (Fig. 1D), notably after D10+ of ALI culture without treatment (D28 plus 10 days post differentiation herein designated D10+).

In CF, differentiation markers including *FOXJ1*, *MUC5AC* and *MUC5B* are increased at D10+ compared to D5+ post-differentiation (D28 plus 5 days post differentiation herein designated D5+); in non-CF these markers are stable or decreased (Fig. 2A–C). These data suggest that a delay in CF epithelium differentiation exists between CF and non-CF cultures. *ALOX15A*, *ALOX15B* and *IL-8* were comparably expressed in non-CF and CF ALI nasal epithelial cultures (Fig. 2D–F). *ALOX15A* and *IL-8* mRNA levels were increased from D5+ to D10+, for both CF and non-CF ($p < 0.00001$).

3.3. SAHA reduced CFTR mRNA expression and protein

First, to validate SAHA impact on CFTR, we assessed SAHA on the bronchial epithelial cell line CFBE41o- stably or transiently transfected by wild-type (WT) or p.Phe508del *CFTR* cDNA. The choice of SAHA concentration was based on three previous publications where 1, 5 or 10 µM were used (Table-S2). We decided to use 5 µM because of it is the concentration the most used. We observed an increase in mature (band C) and immature (band B) WT CFTR expression compared to vehicle (DMSO) or control (ALI medium) in two independent experiments (Fig. 3A and B), that is consistent with previous studies (Hutt et al., 2010).

On the contrary, in both CF and non CF epithelia, incubation with 5 µM SAHA decreased mature (band C) and immature (band B)

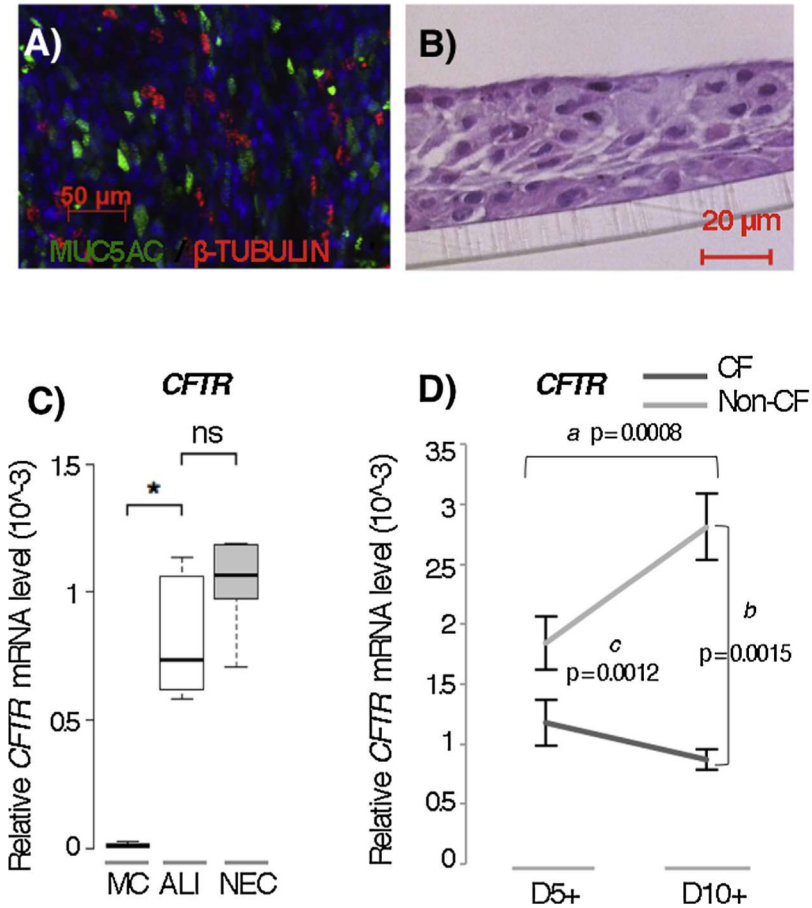

Fig. 1. *Ex vivo* pseudo-stratified nasal epithelium is optimal for CFTR study.**(A)** Immunofluorescence imaging of *ex vivo* nasal epithelium obtained from a healthy control (non-CF) (top view) after 28 days of ALI culture (D28). MUC5AC, *green*; Beta-tubulin, *red*. Scale bar, 50 µm.**(B)** ALI culture of nasal cells allows obtaining a well-differentiated pseudostratified epithelium, as indicated by classical Hematoxylin & Eosin staining. Scale bar, 20 µm. Representative images of $n = 3$ non-CF and CF epithelia.**(C)** Boxplots show *CFTR* mRNA levels normalized to *GAPDH* mRNA levels in monolayer (MC) and D28 ALI cultures (ALI) from primary human nasal cells and in fresh nasal epithelial cells (NEC; from scraping of six healthy subjects). Transcripts were assessed by non-specific quantitative Reverse Transcription and PCR analysis; *ns*: not significant; $^*P < 0.05$.**(D)** Graph shows *CFTR* transcript levels normalized to *GAPDH* in non-CF and CF epithelia at D5+ and D10+ without treatment (D28 + 5 or 10 days post differentiation, CONTROL condition); $n_{non\text{-}CF} = 10$; $n_{CF} = 8$. (a) Comparison between D5+ and D10+, (b) comparison between non-CF and CF samples, (c) interaction between CF and non-CF sample behaviors.

p.Phe508del and WT CFTR expression compared to vehicle ($p = 0.007$) or control ($p = 0.034$) (Fig. 3C and D). Reverse Transcription-qPCR analysis showed no difference in *CFTR* steady-state mRNA abundance between untreated cells and cultures incubated with vehicle. Following an incubation with SAHA, *CFTR* transcript level at D5+ was significantly decreased in both non-CF and CF ALI epithelia compared to vehicle ($p_{non\text{-}CF} = 0.0101$, $p_{CF} = 0.00109$) (Fig. 3E). This reduction was reversible after five days of SAHA withdrawal (D10+; $p_{non\text{-}CF} = p_{CF} = 0.99$). We checked that DMSO and 5 µM SAHA did not have any effect on *GAPDH* transcript level compared to untreated cells ($p = 0.70$ and $p = 0.34$ for vehicle and SAHA at D5+, respectively).

These results indicate that SAHA decreases endogenous *CFTR* expression at the transcriptional level in primary CF and non-CF nasal epithelia contrary to the observed increase in a heterologous cellular context. Based on these results, we conclude that, in a more complex model, SAHA is not an efficient CFTR corrector.

To determine whether CFTR reduction was due to inhibition of transcription in primary CF and non-CF nasal epithelia, we performed native ChIP before and after SAHA incubation (D5+) using antibodies against three histone modifications associated with the *CFTR* promoter: H4KAc (a marker of opened chromatin), H3K4Me3 (a marker of active transcription) and H3K27Me3 (a marker of transcription repression) (Fig. 3F). We focused on non-CF epithelia because of the elevated cell numbers required for these experiments (10 million cells, *i.e.*, 20 wells per antibody). SAHA did not significantly affect H4KAc ($p = 0.36$), H3K4Me3 ($p = 0.86$) and H3K27Me3 ($p = 0.97$) level compared to untreated cells (CONTROL), whereas it increased whole lysine acetylation in non-CF ($n = 7$, $p = 0.017$, Fig. 3G) and in CF ALI epithelia ($n = 8$, 0.0347, Fig. 3H). Actually, the *CFTR* promoter was enriched in H4KAc and H3K4Me3 before incubation with SAHA (ratio of 1.02 and 1.20 compared to the transcriptionally active *GAPDH* promoter). This result indicates that, even if SAHA induced acetylation, the chromatin at the *CFTR* promoter is not condensed by SAHA treatment.

3.4. SAHA affected airway epithelial MUC5AC and MUC5B gene expression and the number of secretory cells

Quantification of *MUC5AC* and *MUC5B* gene expression showed that incubation with 5 µM SAHA for five days almost completely abrogated airway mucin gene expression in both non-CF and CF ALI epithelia (*MUC5AC*: $p_{non\text{-}CF} = 0.0000433$, $p_{CF} = 0.000311$; *MUC5B*: $p_{non\text{-}CF} = 0.00024$, $p_{CF} = 0.00015$) (Fig. 4A and B). This effect was fully reversed at D10+ after five days without SAHA. Immunofluorescence assays of MUC5AC expression showed that SAHA decreased the number of (MUC5AC+) goblet cells but not that of (β-tubulin+) ciliated cells (reduction of the goblet cell/ciliated cell ratio; $p = 0.03125$). This effect also was reversible upon SAHA withdrawal (Fig. 4C and D).

3.5. SAHA has not cytotoxic effect on epithelia

To determine whether the decrease of *CFTR*, *MUC5AC* and *MUC5B*

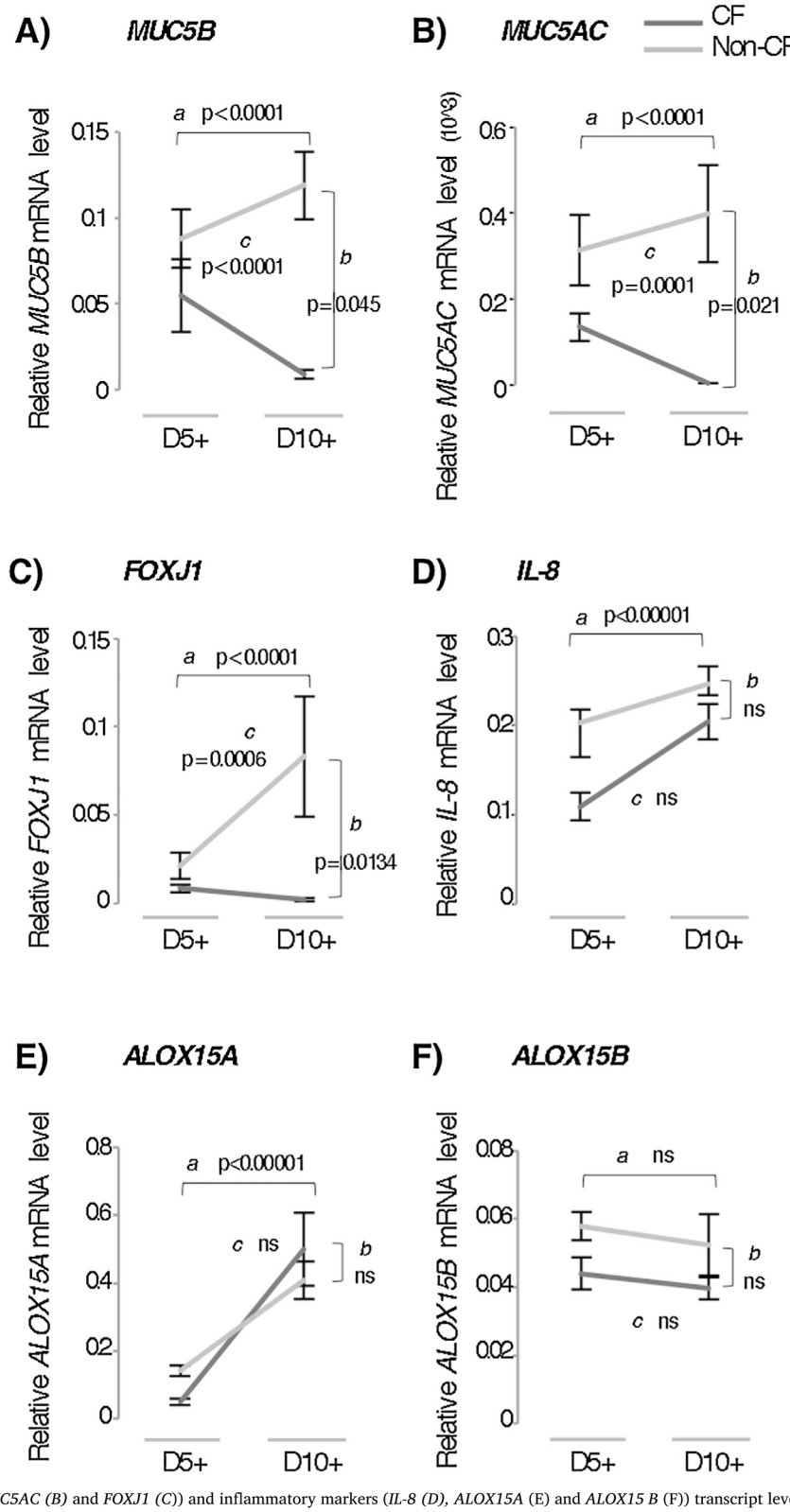

Fig. 2. Epithelial (*MUC5 B (A), MUC5AC (B)* and *FOXJ1 (C)*) and inflammatory markers (*IL-8 (D), ALOX15A* (E) and *ALOX15 B* (F)) transcript levels in untreated non-CF and CF ALI *ex vivo* nasal epithelia.Graphs show transcript levels normalized to *GAPDH* mRNA level in untreated CF (dark grey) and non-CF epithelia (light grey) at D5+ ($n_{non\text{-}CF}$ = 10; n_{CF} = 8) and D10+ ($n_{non\text{-}CF}$ = 5; n_{CF} = 4). Statistical analysis based on mixed regression model showing (a) the date effect (comparison between D5+ and D10+); (b) the group effect (comparison between non-CF and CF samples); (c) the interaction date*group (behavior of CF and non-CF samples depending on the date). *ns*: not significant.

mRNA and loss of goblet cells induced by SAHA treatment is due to a secondary effect on ALI nasal epithelia, we incubated D28 ALI cultures with 1–10 μM SAHA or vehicle alone (DMSO) in ALI medium (basolateral treatment) for 5 days (as described in Bergougnoux et al., 2017, in press, Data in Brief). DMSO did not have any cytotoxic effect compared to control (ALI medium alone) (ns; p = 0.14) (Fig. 4E). Conversely, after five days (D5+), cell death was significantly decreased in ALI cultures incubated with SAHA compared to vehicle-

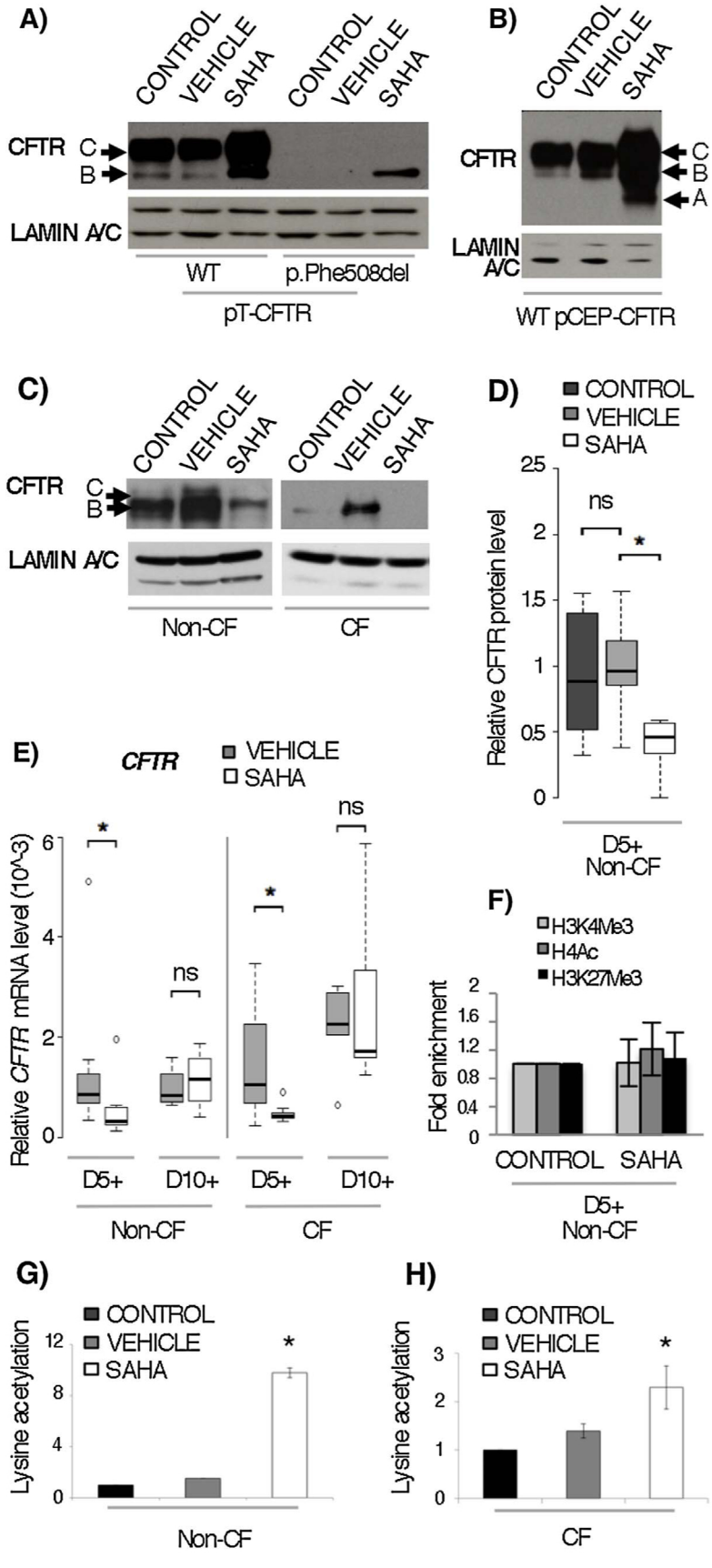

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Fig. 3. SAHA increased WT and p.Phe508del CFTR protein quantity in CFBe41o- overexpressing CFTR (A and B) but reduced CFTR protein (C and D) and mRNA expression (E) in non-CF and CF epithelia, without changing histone acetylation/methylation at the *CFTR* promoter (F) even if it induced lysine hyperacetylation (G and H).After five days of incubation with SAHA, DMSO or without treatment (CONTROL), CFBE41o- cell line transfected by WT or p.Phe508del pTRACER-CFTR expression vector plasmids **(A)**, stably transfected by WT pCEB-CFTR expression vector plasmids **(B)** or primary non-CF and CF epithelia were lysed **(C)**. CFTR (MM13-4 and L12B4 antibodies) and Lamin A/C expression was assessed by western blotting. Representative images of n = 3 experiments for CFBE41o-. **(D)** Boxplots show CFTR protein levels normalized to lamin A/C levels in seven non-CF epithelia. As CFTR was detected only in two CF samples, meaningful comparisons were not possible.**(E)** Boxplots show *CFTR* mRNA levels normalized to *GAPDH* levels at D5+ and D10+ for SAHA or VEHICLE conditions (D5+: $n_{non\text{-}CF}$ = 10 and n_{CF} = 8; D10+: $n_{non\text{-}CF}$ = 4 and n_{CF} = 5). **(D)** Histone modifications associated with the *CFTR* promoter in three non-CF epithelia after incubation with SAHA or without treatment (CONTROL). Bars represent the relative DNA enrichment (mean ± SEM calculated on ≥3 independent replicates for each sample) normalized to the control condition (=1). The amount of immunoprecipitated DNA was expressed relative to the amount of histone modifications at the *GAPDH* locus (H4KAc and H3K4Me3) or at the sequence tagged site JB10 (H3K27Me3). *ns*: not significant; *P < 0.05.

treated ALI cultures (p = 0.004). This apparent protective effect disappeared after treatment withdrawal (D10+, shown for 5 μM SAHA, Fig. 4E).

Altogether, these results suggest that SAHA has non-specific effects on airway epithelia.

4. Discussion

In this study, we assessed the effect of the pan-HDACi SAHA on CFTR expression and on some CF features, such as inflammation and goblet cell hyperplasia, using an *ex vivo* airway pseudo-stratified

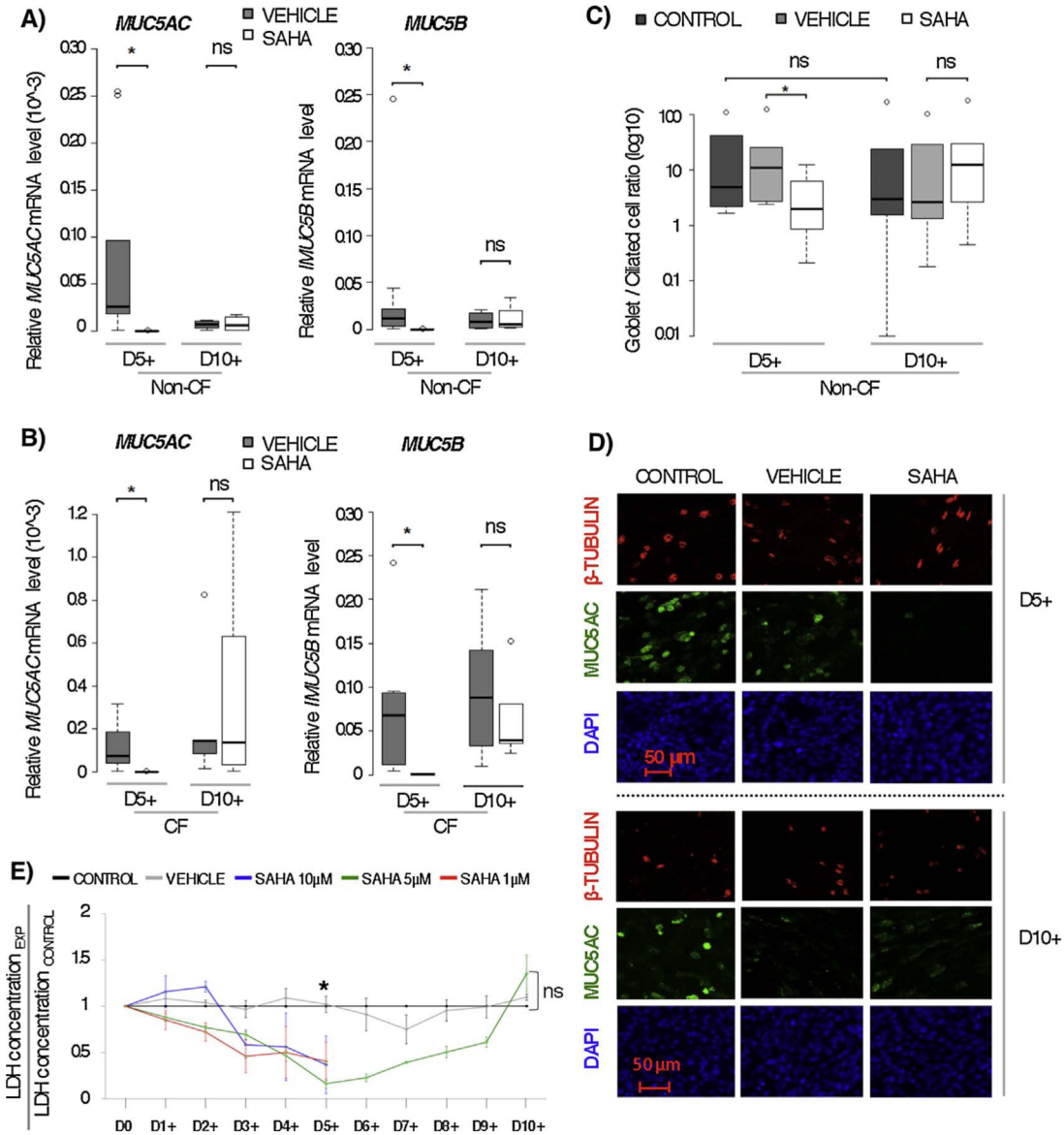

Fig. 4. SAHA treatment affects the expression of the airway epithelial mucins *MUC5AC* and *MUC5 B* (A and B) and the number of secretory cells (C and D) without cytotoxicity (E).**(A** and **B)** Boxplots show *MUC5AC* and *MUC5 B* transcript levels normalized to *GAPDH* in non-CF **(A)** and CF **(B)** epithelia at D5+ and D10+ for VEHICLE (DMSO) or SAHA conditions (D5+: $n_{non\text{-}CF}$ = 10 and n_{CF} = 8; D10+: $n_{non\text{-}CF}$ = 4 and n_{CF} = 5).**(C)** Boxplots show the MUC5AC-positive (goblet)/beta-tubulin-positive (ciliated) cell ratio normalized (log_{10}) to the cell number (DAPI) calculated with the ImageJ software in six non-CF epithelia at D5+ after incubation with VEHICLE (DMSO), SAHA or nothing (control). *ns*: not significant; *P < 0.05.**(D)** Immunofluorescence analysis (top view) of MUC5AC (green) and beta-tubulin (red) expression in CONTROL (untreated), Vehicle (DMSO) and SAHA-treated non-CF epithelia at D5+ and D10+. Nuclei were stained with DAPI. Scale bar, 50 μm. Representative images from six non-CF epithelia.**(E)** SAHA cytotoxicity in ALI epithelia was assessed using the Cytotoxicity LDH detection kit and optical density at 420–690 nm was measured in the supernatants of three non-CF epithelia (200 μL per ALI well, corresponding to 1 cm2 of epithelium, collected every 24 h) from D0+ to D5+ (D28 + D0 to D5 post differentiation) of treatment with VEHICLE (DMSO), SAHA (1, 5 or 10 μM) or without treatment (CONTROL) and also from D5+ to D10+. LDH concentration was calculated using a concentration range and normalized to the control concentration (=1). Data are the mean ± SEM. *P < 0.05.

epithelium model obtained from nasal cells of patients with CF or healthy controls.

HDACi are expected to be good candidates for CF therapy because they act both as transcriptional activators and proteostasis modulators. Previous studies, summarized in Supplementary Tables S-2 and S-3 (Boinot et al., 2014; Hutt et al., 2010; Sondo et al., 2011; and Supplementary references E1–E13), reported variable effects of HDACi on CFTR expression/activity *in vitro* and in patients with CF. The large heterogeneity of different cellular models used to determine HDACi mechanism of action does not allow concluding on a clear effect of these molecules on CFTR expression and function.

While a first study showing an up-regulation of *CFTR* expression following SAHA treatment has been carried out in primary bronchial cells and in CFBE41o- cells (Hutt et al., 2010), another study showed results close to our present findings since SAHA decreased CFTR membrane expression and function in lung primary cells obtained from one p.Phe508del homozygous patient (Sondo et al., 2011). Despite these controversial results, more recent published work taken for granted SAHA as a valuable therapy for CF (Pankow et al., 2015).

Here, we evaluated SAHA effect in an *ex vivo* model, where scrapings of nasal epithelial cells from patients with CF were cultured using an ALI system to mimic as much as possible the *in vivo* conditions. Reconstituted epithelium from ten non-CF controls and eight patients with CF (homozygous for the p.Phe508del mutation) were used. We show that SAHA decreased *CFTR* transcript and protein levels in CF ALI epithelia. In this study, we confirmed that SAHA increases CFTR mature protein expression in immortalized CFBE41o- cells but not in CF *ex vivo* epithelium. Of note, CFBE41o- cells were immortalized using the SV-40 virus and SAHA could enhance the transcriptional activity of viral elements and up-regulation of *CFTR* expression observed in transfected cells may also be attributed to the activation of the cytomegalovirus promoter driving *CFTR* heterologous expression and transduction to stably CFTR expression generates lines with variable numbers of integrated sequences expressing exogenous *CFTR* at high levels.

In the primary model used herein, the reduction of *CFTR* mRNA level induced by SAHA was not due to chromatin condensation. Indeed, opened chromatin modifications H4KAc and H3K4Me3 (before or after SAHA treatment) levels were five-fold higher than in adult lung and the closed one H3K27Me3 was twice as low; this correlates with the higher *CFTR* expression in nasal cells (NEC or *ex vivo* ALI epithelium) than in lung (Bergougnoux et al., 2014). Targeted ChIP experiments and measure of the stability of *CFTR* mRNA (using an inhibitor of transcription) should be performed to determine whether CFTR down-regulation after SAHA treatment is due to the absence/inhibition of specific transcription factors (through modification of their post-translational acetylation, for example) or to the alteration of *CFTR* transcript stability. However, the third and the more likely hypothesis is a loss of epithelial integrity after SAHA treatment that is supported by the reduction of the CFTR expression in both non-CF and CF *ex vivo* epithelia. SAHA has anti-proliferative (Richon et al., 2000), pro-apoptotic (Rikiishi, 2011) and pro-oxidative (Ungerstedt et al., 2005) effects in cancer cells. CFTR has been also shown to play an essential role for the glutathione transmembrane export (Linsdell and Hanrahan, 1998), a major anti-oxidant in lung. Although our results showed that normal cells are more resistant to SAHA treatment (no cytotoxic effect), the down-regulation of CFTR could be a part of a cyto-protective response to oxidative stress generated by SAHA.

In CF *ex vivo* epithelium, SAHA reduced the expression level of the inflammation resolution markers *ALOX15A* and *ALOX15B* without change in apical and basolateral secretion of the pro-inflammatory cytokine IL-8 (Bergougnoux et al., 2017, in press, Data in Brief). In contrast, in non-CF epithelium, the inflammatory mediators balance is re-equilibrated by the reduction of the Il-8 secretion. Moreover, the apical secretion of the pro-inflammatory cytokine IL-8 was significantly higher in CF than non-CF cultures at D10+. This abnormal inflammatory signaling in CF could be the result of the effect of CFTR anion channel dysfunction on ASL composition and volume, which secondarily alter cellular signaling as previously suggested by Machen (2006). Nevertheless, long-term *ex vivo* culture can also be a source of inflammation in primary epithelial cells and modify the injury/repair imbalance. The absence of crosstalk with neutrophils in our model could also have altered CF inflammatory response.

SAHA effect on goblet epithelial cells is more clear-cut. SAHA reduced *MUC5AC* and *MUC5B* transcript level and the number of goblet cells in a reversible manner. These results suggest that SAHA induces dedifferentiation of epithelial cells, maybe specifically of goblet cells and of CFTR-expressing cells. As HDACi pro-differentiation activity has been previously described (Minucci and Pelicci, 2006; Göttlicher, 2004), the hypothesis of a dedifferentiation effect may appear surprising. A recent study showed that secretory cells could dedifferentiate after basal stem cell ablation (Tata et al., 2013). It also could be hypothesized that SAHA induce basal cell injury in our primary culture and damage epithelial integrity. Previous studies suggested that dedifferentiation and remodeling of primary airway epithelium affected CFTR protein expression (Dupuit et al., 1995; Brezillon et al., 1995). The morphological modifications observed in the airway epithelium after SAHA treatment may in part explain the alteration of *CFTR* gene expression and CFTR protein processing. Considering the possible role of CFTR in airway cells differentiation, the reduction of CFTR expression after SAHA treatment may also alter the epithelial differentiation in a vicious circle.

Considering the absence of CFTR correction and the non-specific effects of SAHA on CF and non-CF airway epithelia, cautions should be taken to consider SAHA as a drug for CF treatment. Use of primary nasal epithelial cells may bring important knowledge on the mechanism of action of HDACi drugs and their potential side effects in non-cancerous tissues/cells using an innovative *ex vivo* model of the airway epithelium.

Preclinical bench-testing of candidate drugs needs optimal cellular models that are specific for the pathology under study and provide enough biological material to assess drug efficacy and toxicity. Most of the previous studies on CF drugs used immortalized cell lines (cancer cells or virus-immortalized cells) or cells that overexpress mutated *CFTR* using a heterologous expression vector. CF research laboratories use a variety of cell types as a model of the human respiratory system: immortalized respiratory cell lines *e.g.* Calu-3, 16HBE14o-, BEAS-2B and A549 cells, and primary human cells. Although the use of immortalized cell lines is common, data generated using these models are not directly applicable to the human system. Submerged culture of primary human bronchial epithelial cells is possible; however, cells in this system fail to undergo mucociliary differentiation. Due to the changes in the cellular and molecular environment, the response to the tested molecule might have been biased. In order to recapitulate the pseudostratified mucociliary phenotype observed *in vivo*, primary human bronchial epithelial cells can be cultured at the air-liquid interface but procurement of freshly human bronchial samples is invasive. Conversely, nasal scraping is a minimally invasive procedure that can be performed by a trained nurse and is suitable for studies in children. Moreover, the histological analogy between nasal and bronchial epithelial cells (Bergougnoux et al., 2015) and transcriptomic studies (Clarke et al., 2013) confirm that nasal cells are an appropriate biological material for CFTR studies. Nasal ALI cultures also offer advantage to have detectable CFTR endogenous mRNA and protein and do not require overexpression of CFTR protein to non-physiological levels.

Histological and molecular characterization of our *ex vivo* model of non-CF and CF nasal epithelia before drug assessment suggested that i) the CF profile could be maintained in ALI cultures and ii) CF cell differentiation may be slightly delayed in culture, as previously shown in hyper-proliferating CF polyps (Hajj et al., 2007).

The presence of many ciliated and goblet cells, the integrity of pseudostratified airway epithelium, the weak inflammatory imbalance

and the high endogenous CFTR expression in this model (compared to monolayer cultures) indicate that *ex vivo* nasal epithelia from patients with CF represents an appropriate preclinical alternative model to assess new CF therapies when *in vivo* models are inadequate.

Conflict of interest

The authors declare they have no conflict of interest with the contents of this article.

Author contributions

ABe, RC, AS, MC, NM, IV, MTC and AB participated in research design. ABe, AFP, LK, EB performed the experiments. ABe, NM, IV, MTC and AB analyzed the results. RC and AB included patients and controls, recorded clinical parameters and collected biological samples. ABe wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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