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Anoctamin 1 dysregulation alters bronchial epithelial repair in cystic fibrosis

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ABSTRACT

Cystic fibrosis (CF) airway epithelium is constantly subjected to injury events due to chronic infection and inflammation. Moreover, abnormalities in CF airway epithelium repair have been described and contribute to the lung function decline seen in CF patients. In the last past years, it has been proposed that anoctamin 1 (ANO1), a Ca^{2+}-activated Cl^- channel, might offset the CFTR deficiency but this protein has not been characterized in CF airways. Interestingly, recent evidence indicates a role for ANO1 in cell proliferation and tumor growth. Our aims were to study non-CF and CF bronchial epithelial repair and to determine whether ANO1 is involved in airway epithelial repair. Here, we showed, with human bronchial epithelial cell lines and primary cells, that both cell proliferation and migration during epithelial repair are delayed in CF compared to non-CF cells. We then demonstrated that ANO1 Cl^- channel activity was significantly decreased in CF versus non-CF cells. To explain this decreased Cl^- channel activity in CF context, we compared ANO1 expression in non-CF vs. CF bronchial epithelial cell lines and primary cells, in lung explants from wild-type vs. F508del mice and non-CF vs. CF patients. In all these models, ANO1 expression was markedly lower in CF compared to non-CF. Finally, we established that ANO1 inhibition or overexpression was associated respectively with decreases and increases in cell proliferation and migration. In summary, our study demonstrates involvement of ANO1 decreased activity and expression in abnormal CF airway epithelial repair and suggests that ANO1 correction may improve this process.

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disease that is due to mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene encoding an essential cAMP-dependent chloride (Cl^-) channel. Defective CFTR function in the airway epithelium leads to lung alterations, the main cause of morbidity and mortality of CF patients.

In CF airway epithelial cells, CFTR impairment results in decreased Cl^- secretion and increased sodium (Na^+) influx, leading to an increase in water absorption that translates into greater viscidity of the airway surface liquid. In advanced CF, defective ciliary clearance and mucus hypersecretion cause airway obstruction and produce a favorable environment to chronic infection and inflammation, resulting in epithelial injury and lung function impairments [1]. In addition, abnormalities in epithelial injury repair in CF airways have been described [2] and induce characteristic epithelial remodeling [3]. Several studies have established involvement of interleukin-8, some metalloproteinases, and CFTR Cl^- activity in the epithelial repair process, but the underlying mechanisms remain unclear [2,4,5].

Chloride ion movements across the cell membrane are tightly regulated by Cl^- channels and transporters, which remain poorly characterized in the lungs, the only exception being the CFTR channel. CFTR is the main gateway for Cl^- secretion in the human airway epithelium. However, alternative Cl^- conductance associated with channels called calcium-activated chloride channels (CaCCs) has been identified in several cell types, including those of secretory epithelia [6,7]. Different authors have compared CaCC activities in human nasal cells and have observed an increased in CF compared to non-CF group [8–11]. For

Abbreviations: ANO1, anoctamin 1; CaCC, calcium-activated chloride channel; CF, cystic fibrosis; CI, normalized cell index; F508del, phenylalanine 508 deletion; RTCA, real time cell analysis; TMEM16a, transmembrane 16a protein; WGA, wheat germ agglutinin

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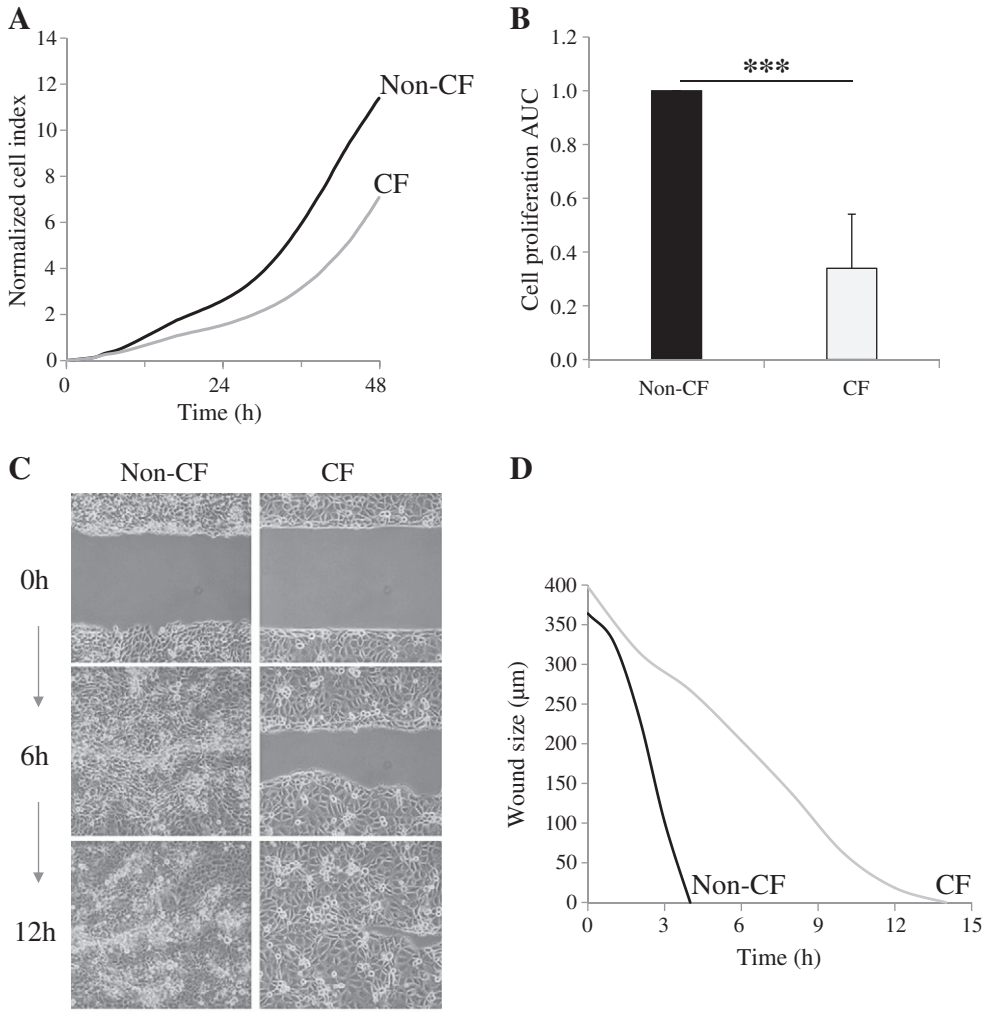

Fig. 1. Cell proliferation and migration of non-CF and CF bronchial epithelial cell lines in basal conditions. A) Original traces of non-CF and CF cell line proliferation monitored for 48 h under basal conditions using real time cell analysis ($n = 8, p < 0.001$). B) Quantification of cell proliferation within 48 h performed with area-under-the-curve (AUC) values normalized to non-CF cells. C) Representative photographs taken every 6 h during 12 h of wound closure of non-CF and CF cell lines under basal conditions. D) Wound closure kinetics of non-CF and CF cell lines under basal conditions showed in C.

this reason it has been proposed that CaCCs might compensate for CFTR function impairment in CF patients [11] but the molecular identity of CaCCs remained unknown for many years. CaCCs are activated by purinergic signaling through the binding of ATP/UTP to $P2Y_2$ receptor that induces an increase of intracellular Ca^{2+} concentration. Based on this mechanism, a $P2Y_2$ agonist (denufosol tetrasodium) has been developed and tested in a clinical trial for CF patients. Despite the positive effects on lung function that have been observed in phase II of the clinical trial, phase III failed to show a significant difference in lung function of patients treated with denufosol or placebo [12,13]. This study outcome might be due to the short half-life and duration of action of the molecule [14]. Design of specific CaCC activators could improve stimulation; therefore CaCC molecular identification was necessary.

In 2008, three research teams identified one potential CaCC candidate called ANO1 (anoctamin 1) or TMEM16a (transmembrane protein 16a) [15–17]. This protein exhibits all the functional characteristics of CaCCs, especially regarding the anion permeability sequence and Ca^{2+} dependence. ANO1 is a transmembrane protein expressed in secretory epithelia including the airway surface epithelium [18–20]. Mice lacking ANO1 exhibit decreased Ca^{2+}-activated Cl^- secretion and impaired mucociliary clearance, indicating a substantial physiological role for ANO1 in the airways [21,22]. Moreover, ANO1 is involved in mucin production [23], HCO_3^- permeability [24] and cytokine secretion [25]. Taken together, these results indicate that ANO1 is a CaCC and may be capable to restore Cl^- efflux and other important functions dysregulated in CF airways and could be in a relationship with CFTR protein [26].

Furthermore, ANO1, which is located on the amplified region 11q13, is overexpressed in many cancer cells [27–29]. Thus, two different groups showed that ANO1 inhibition decreased the proliferation of a human pancreatic cancer cell line [30] and suppressed the growth and invasiveness of human prostate cancer cells [31]. Moreover, a recent study showed that ANO1 is a marker of poor prognosis in head and neck squamous cell carcinoma and implies cell migration [32]. The exact mechanism by which ANO1 modulates proliferation and migration of cancer cells is still unknown but Duvvuri and colleagues have recently reported that ANO1 might influence cell proliferation by activating the RAS–RAF–MEK–ERK1/2 pathway [33]. These data suggesting the involvement of ANO1 in cell proliferation and migration led us to hypothesize that ANO1 might be involved in airway epithelium repair.

Here, our main objectives were to study non-CF and CF airway epithelial repair and to assess ANO1 involvement in this process. We evaluated epithelial repair using proliferation and wound healing experiments in non-CF versus CF bronchial epithelial cells. We then compared ANO1 activity and expression between many non-CF and CF airway epithelial models. To assess the potential role for ANO1 in airway epithelial repair we tested the effect of ANO1 inhibition on proliferation

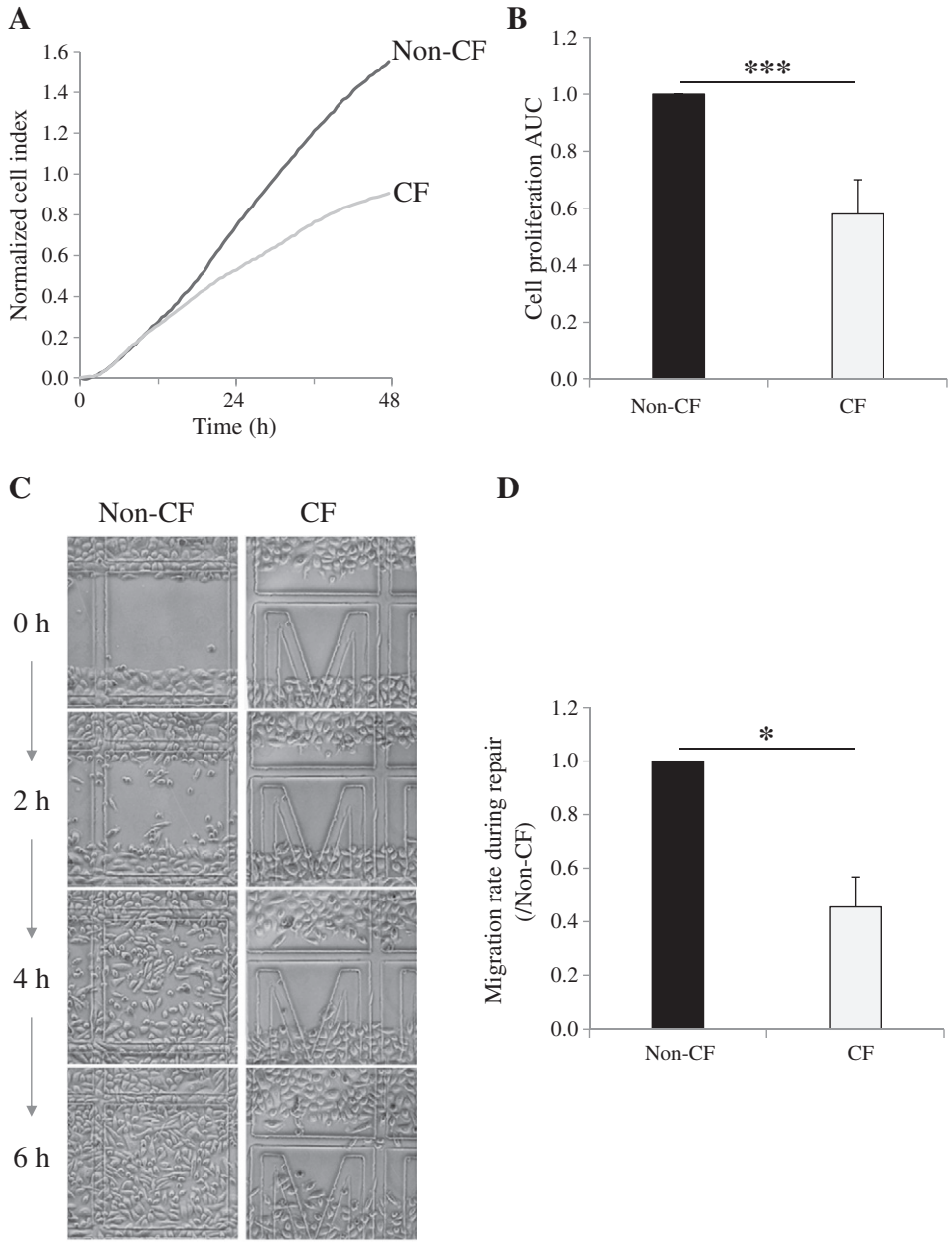

Fig. 2. Cell proliferation and migration of non-CF and CF primary bronchial epithelial cells in basal conditions. A) Original traces of cell proliferation of non-CF and CF primary cells monitored for 48 h under basal conditions using real time cell analysis. B) Quantification of primary non-CF (n = 5) and CF (n = 4) cell proliferation within 48 h performed with area-under-the-curve (AUC) values normalized to non-CF cells ($p < 0.001$). C) Representative photographs taken every 2 h during 6 h of wound closure of primary non-CF and CF cells under basal conditions. D) Migration rates during repair of primary CF (n = 4) compared to non-CF (n = 6) bronchial epithelial cells ($p < 0.05$).

and wound healing. Finally, we investigated the effect of ANO1 overexpression on CF bronchial epithelial repair.

2. Materials and methods

2.1. Models

Human bronchial epithelial cell lines 16HBE14o− (non-CF) and CFBE41o− (CF, F508del/F508del) were provided by Dr. D.C. Gruenert (San Francisco, CA, USA) and cultured as previously described [34]. Similar passage numbers have been used for each cell type.

Primary human bronchial epithelial cells (hAECB) and fully differentiated human bronchial air–liquid-interface cultures (MucilAir™), isolated from bronchial biopsies from non-CF (no pathology reported) and CF (F508del/F508del) patients, were purchased from Epithelix SARL (Geneva, Switzerland) and cultured according to the provider's recommendations.

Animal model studies were performed on $Cftr^{tm1Eur}$ adult mice homozygous for the F508del mutation and their wild-type littermates as previously described [35]. All animal procedures were performed in accordance with our institution's guidelines on the care and use of laboratory animals.

Human lung explants were collected and processed in compliance with the current French public health legislation (articles L.1235-2 and L.1245-2, code de la santé publique, www.legifrance.gouv.fr). Each participating institution informed the patients and made sure that they were not opposed to the use of surgical samples, removed during a medical act, for research purpose. Samples and cell cultures were discarded after use. Lung fragments were obtained from 7 non-CF patients who underwent surgery (48 ± 26 years) and from 7 CF

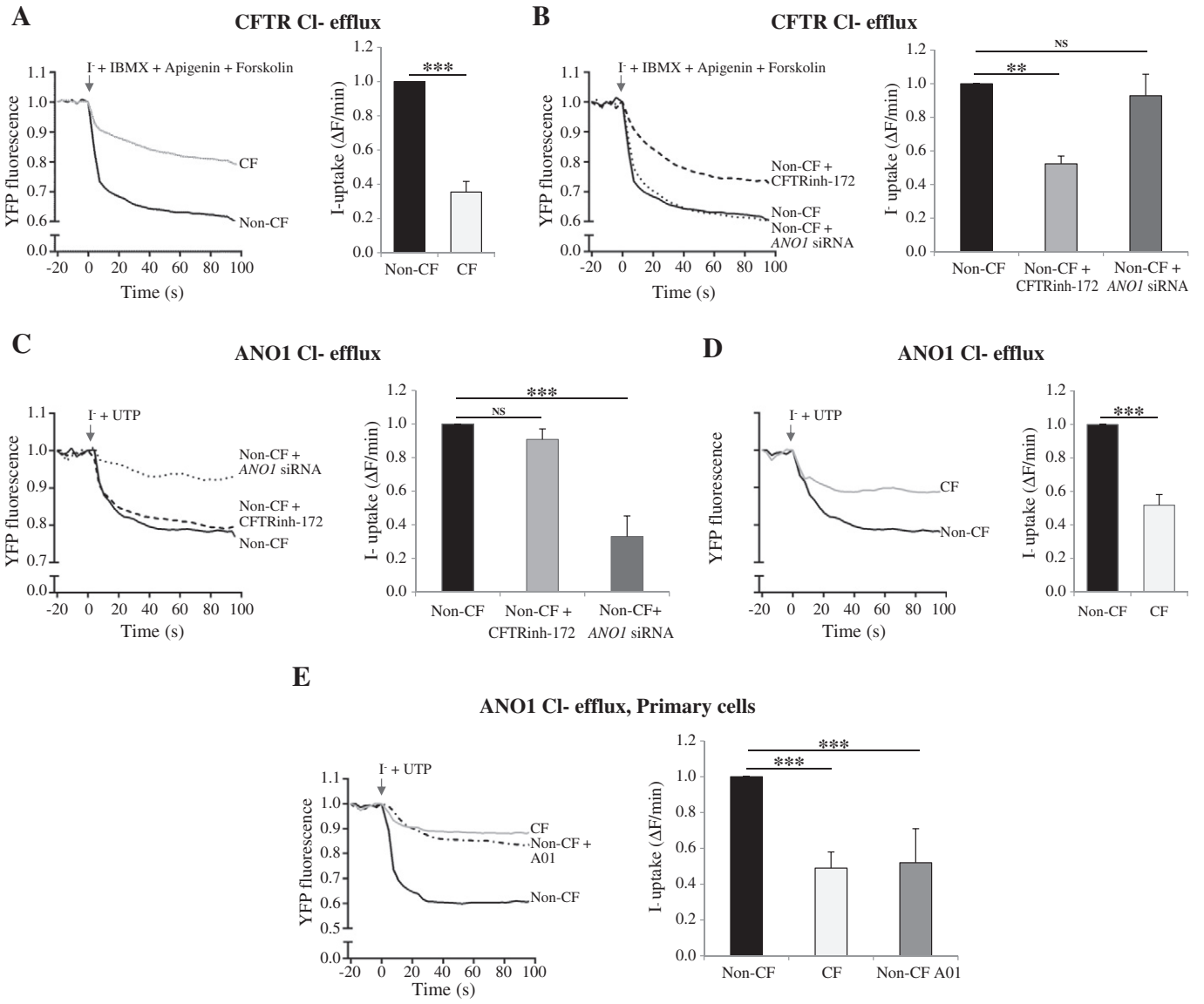

Fig. 3. Chloride channel activity in non-CF and CF bronchial epithelial cells. A) Representative and original traces (left) and quantification (right) of CFTR Cl^- efflux measured in non-CF and CF cells under basal conditions ($n = 5$, $p < 0.01$). B) Representative and original traces (left) and quantification (right) of CFTR Cl^- efflux measured in non-CF cells transfected with *ANO1* siRNA or treated for 48 h with CFTR(inh)-172 ($n = 5$, scrambled vs. *ANO1* siRNA: p = ns, control vs. CFTR(inh)-172: $p < 0.01$). C) Representative and original traces (left) and quantification (right) of ANO1 Cl^- efflux measured in non-CF cells transiently transfected with *ANO1* siRNA or treated with CFTR(inh)-172 (10 μM) ($n = 8$, $p < 0.01$). D) Representative and original traces (left) and quantification (right) of ANO1 Cl^- efflux measured in non-CF and CF cells under basal conditions ($n = 55$, $p < 0.0001$). E) Representative and original traces (left) and quantification (right) of ANO1 Cl^- efflux measured in primary human non-CF and CF cells treated with the pharmacological ANO1 inhibitor (A01, 10 μM) ($n = 5$; $p < 0.001$).

patients homozygous for the F508del mutation (37 ± 13 years) who underwent lung transplantation. For non-CF patients, samples were obtained in a non-pathologic area without inflammatory cells. After dissection of the tissue, samples were directly frozen in liquid nitrogen before extraction.

2.2. Cell proliferation monitoring

Cell proliferation was first analyzed by counting. Non-CF and CF cells were seeded and cultured within 48 h. Cells were dissociated with trypsin–EDTA then counted using a hemocytometer.

Cell proliferation was also analyzed using real-time cell analysis (RTCA). Cells were pelleted, re-suspended, and nucleo-transfected (Lonza, Amboise, France) in transfection buffer in a concentration of 10^6 cells/100 μL. After plating, cell growth was monitored every 30 min for 48 h using the xCELLigence RTCA MP instrument (Roche Diagnostics, Meylan, France) as previously described [36]. The impedance signal was analyzed by normalizing the data from each well for the first value obtained after treatment initiation: $CI_{normalized} = CI_{time\ x} / CI_{time\ 0}$; the normalized impedance signal is designated "normalized cell index" hereafter.

2.3. Wound closure assay

Wound closure assays were performed using specific wound assay chambers (Ibidi®, Biovalley, Marne la Vallée, France) that provided uniform wounds between two monolayers. A constant number of cells producing a confluent layer within 72 h were seeded in each well of Ibidi® silicone culture-inserts. The cells were incubated at 37 °C and 5% CO_2. After 24 h, cells were treated with T16Ainh-A01 (Tocris Bioscience, Bristol, United Kingdom), transfected with scrambled siRNA/*ANO1* siRNA (Life Technologies, Saint Aubin, France) or control vector/ANO1-GFP vector (CliniSciences, Nanterre, France) (see below). The culture-insert was removed 48 h later, leaving a cell-free gap (or wound) for cell colonization. Wound closure was observed for 6 or 12 h under an Axiovert 200 microscope (Zeiss). Mean migration rates during wound

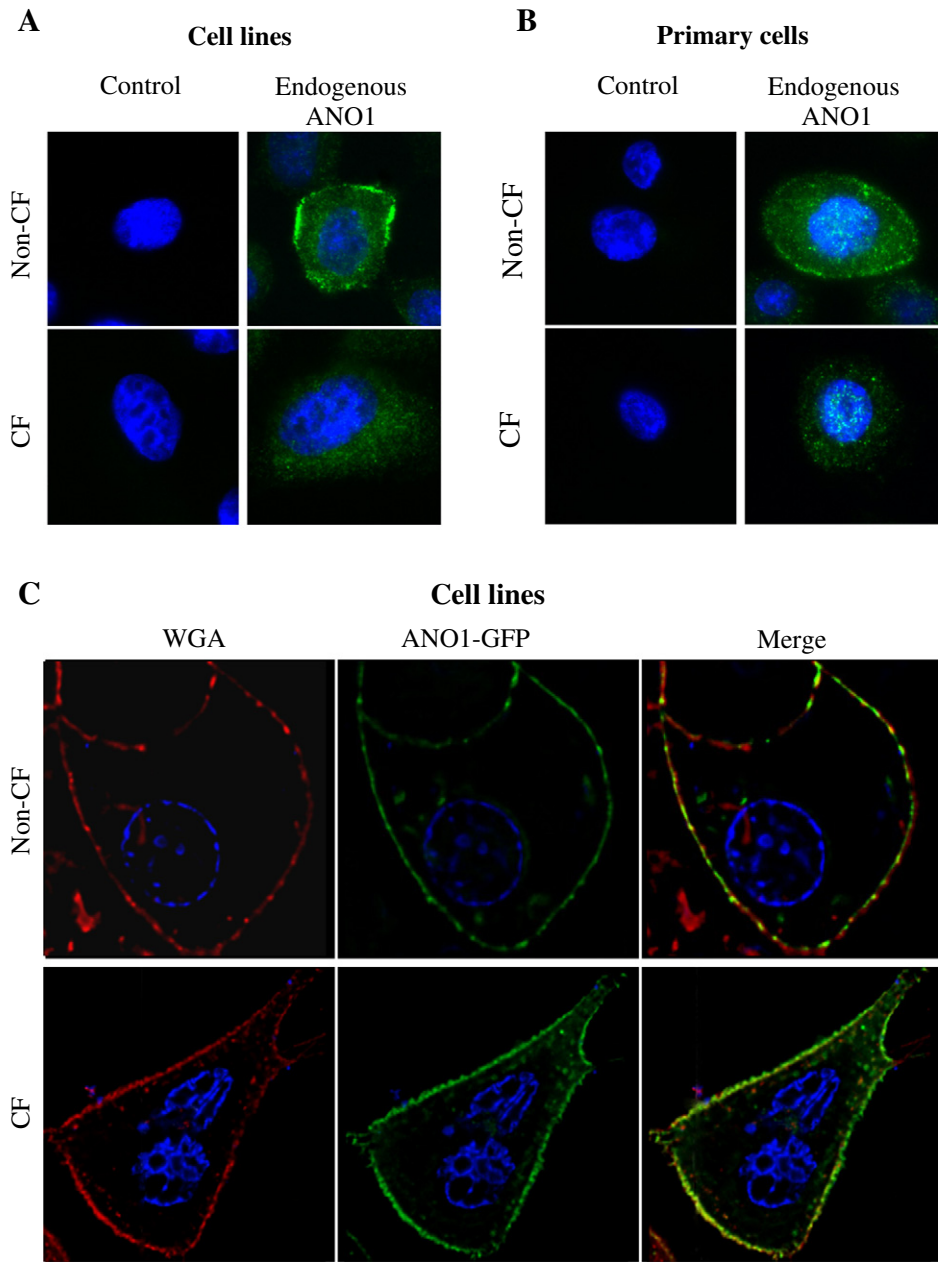

Fig. 4. Intracellular localization of ANO1 protein in bronchial epithelial cells. A) Immunocytochemical detection of endogenous ANO1 protein (green) in non-CF (top images) and CF (bottom images) bronchial epithelial cell lines. Control: lack of ANO1 staining when the cells were exposed to secondary antibody only. Nuclei were stained with DAPI (blue). B) Immunocytochemical detection of endogenous ANO1 protein (green) in primary non-CF (top images) and CF (bottom images) cells. Control: lack of ANO1 staining when the cells were exposed to secondary antibody only. Nuclei were stained with DAPI (blue). The images are representative of more than six experiments. C) Intracellular localization of overexpressed ANO1 protein (green) in non-CF (top images) and CF (bottom images) cell lines transfected with ANO1-GFP vector. Confocal immunofluorescence images, magnification: ×630. Wheat germ agglutinin (WGA) was used to stain the plasma membrane (red). Merge of ANO1-GFP and WGA images (right). Nuclei were stained with DAPI (blue). The images are representative of more than six experiments.

closure were assessed in three areas of the gap. At each time point and in each area, five lengths were measured using AxioVision Rel software (Zeiss).

2.4. ANO1 and CFTR inhibition

To induce transient ANO1 inhibition, we transfected non-CF cell line using TransIT-TKO® transfection reagent (Euromedex, Souffelweyersheim, France) with a set of three *ANO1* siRNAs or with scrambled siRNA, 24 to 48 h before the experiments.

A pharmacological inhibitor of ANO1 channel, T16Ainh-A01 (A01), was used to inhibit ANO1 channel activity [37]. A01 stock solution (10 mM) was made in dimethyl sulfoxide (DMSO). A01 was diluted to a final concentration of 10 μM. Control experiments were performed with DMSO at 1:1000 dilution.

CFTR activity was inhibited using CFTR(inh)-172 (Merck, Molsheim, France) as previously described [38].

2.5. ANO1 immunocytochemistry

Cells were cultured on glass coverslips and fixed in 4% paraformaldehyde in culture medium supplemented with 10% FBS for 15 min at room temperature and then permeabilized with PBS containing 0.1% Triton X-100 for 15 min. Cells were incubated with ANO1 primary antibody (Abcam, Paris, France) overnight in a humidified chamber at 4 °C. For control experiments, the primary antibody was omitted. After being

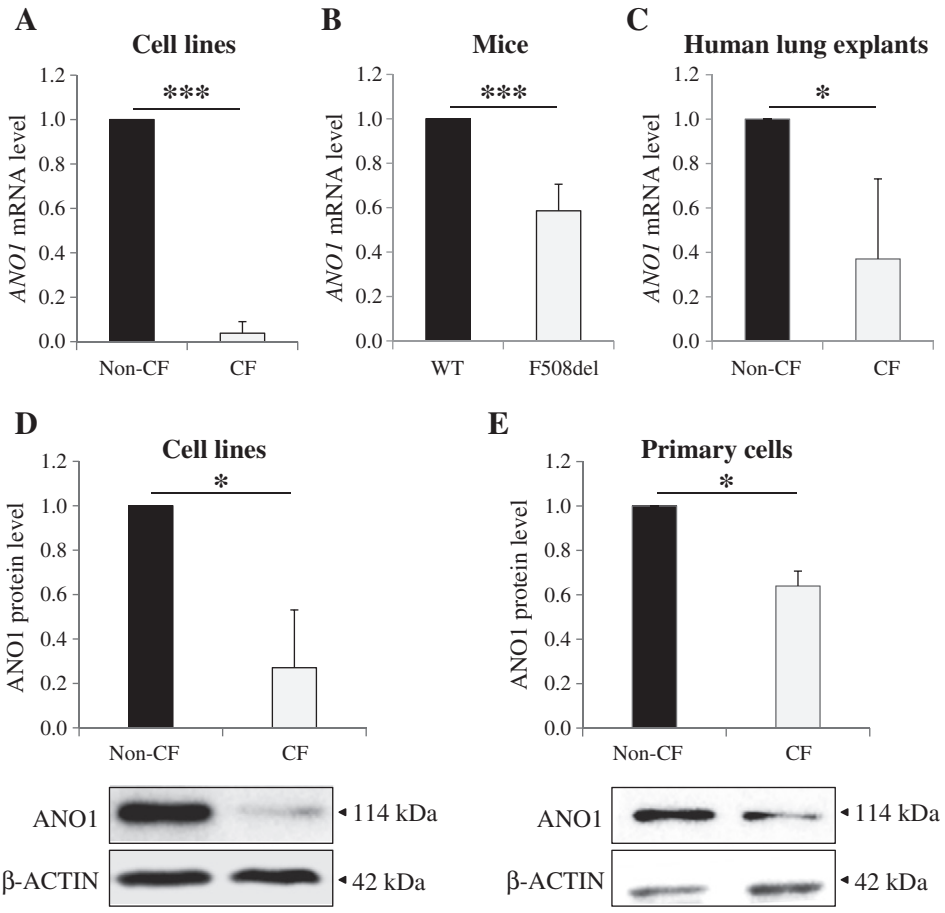

Fig. 5. Quantification of ANO1 expression in different non-CF and CF models. A, B and C) Relative quantification of *ANO1* mRNA under basal conditions respectively in non-CF and CF bronchial epithelial cells ($n = 9$; $p < 0.0001$), in lungs of wild-type (WT) and CF (F508del) mice ($n = 9$; $p < 0.001$) and in lung explants from non-CF and CF (F508del/F508del) patients ($n \geq 7$; $p < 0.05$) performed using RT-qPCR. *GAPDH* was used as a reporter gene. D) Western-blot showing ANO1 protein expression in non-CF and CF bronchial epithelial cells under basal conditions ($n = 6$; $p < 0.05$). ANO1 expression was detected using anti-ANO1 antibody (top panel). Equal loading was verified using anti-β actin antibody (bottom panel). E) Western-blot showing ANO1 expression in primary cultures of bronchial epithelial cells (MucilAir™) from non-CF ($n = 5$) and CF ($n = 3$) patients cultivated on an air–liquid interface ($p < 0.05$).

washed with PBS, cells were subsequently incubated with goat-anti-rabbit IgGAlexa Fluor 488 (Life Technologies) secondary antibody (1:2000 dilution) for 1 h at room temperature. After being washed again in PBS, the coverslips were mounted using a Vectashield mounting medium with DAPI (CliniSciences). For each cell model (cell lines or primary cells) images were acquired using the same exposure time with a 63× oil objective on an Axiovert 200 microscope (Zeiss). DAPI and secondary antibody were visualized using 405 and 488 nm laser lines, respectively.

2.6. ANO1 overexpression and fluorescence detection

To induce ANO1 overexpression and monitor ANO1 localization, a human GFP-tagged ANO1 vector was used (CliniSciences). The full-length open reading frame (NM_018043) was subcloned into pCMV6-AC-GFP vector with C-terminal GFP tag. GFP-tagged ANO1 vector was transiently transfected into non-CF or CF cells using Lipofectin® transfection reagent (Life Technologies) according to the manufacturer's instructions.

The intracellular localization of the ANO1-GFP protein was determined using an Axiovert 200 microscope (Zeiss, Le Pecq, France). Live cells were incubated with DAPI (10 μL/mL, 30 min) and Wheat Germ Agglutinin (WGA) (5 μg/mL, 10 min) (Life Technologies) to label respectively the nucleus and the plasma membrane. Images were deconvoluted using Huygens software (Hilversum, Netherlands).

2.7. Fluorescence assay of CFTR- and ANO1-dependent chloride channel activities

CFTR and ANO1 Cl^- activities were assessed by I^- quenching of halide-sensitive YFP-H148Q/I152L protein (Life Technologies). The probe was transfected into the cells and, after 48 h of culturing, conductance was stimulated for CFTR and ANO1 respectively with cAMP agonist cocktail and UTP (10 μM). I^- solution (140 mM) was added, and fluorescence was recorded into a plate reader as previously described [39]. The initial I^- influx rate following the addition of each solution was computed from changes in YFP fluorescence data using non-linear regression and represents the original and representative traces. For quantitative analysis, slope for fluorescence quenching was performed using a linear regression and correlates to the size of the chloride conductance (I^- uptake). The rate of change (ΔF/min) is used for bar graph representation.

2.8. Total mRNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA extraction and reverse transcription were performed as previously described [40]. Quantitative PCR was performed using an ABI StepOnePlus™ (Life Technologies) and TaqMan technology. For relative quantification, the *ANO1* mRNA level, calculated using the $2^{-\Delta\Delta Ct}$ method as published previously [41], was normalized for *GAPDH* and

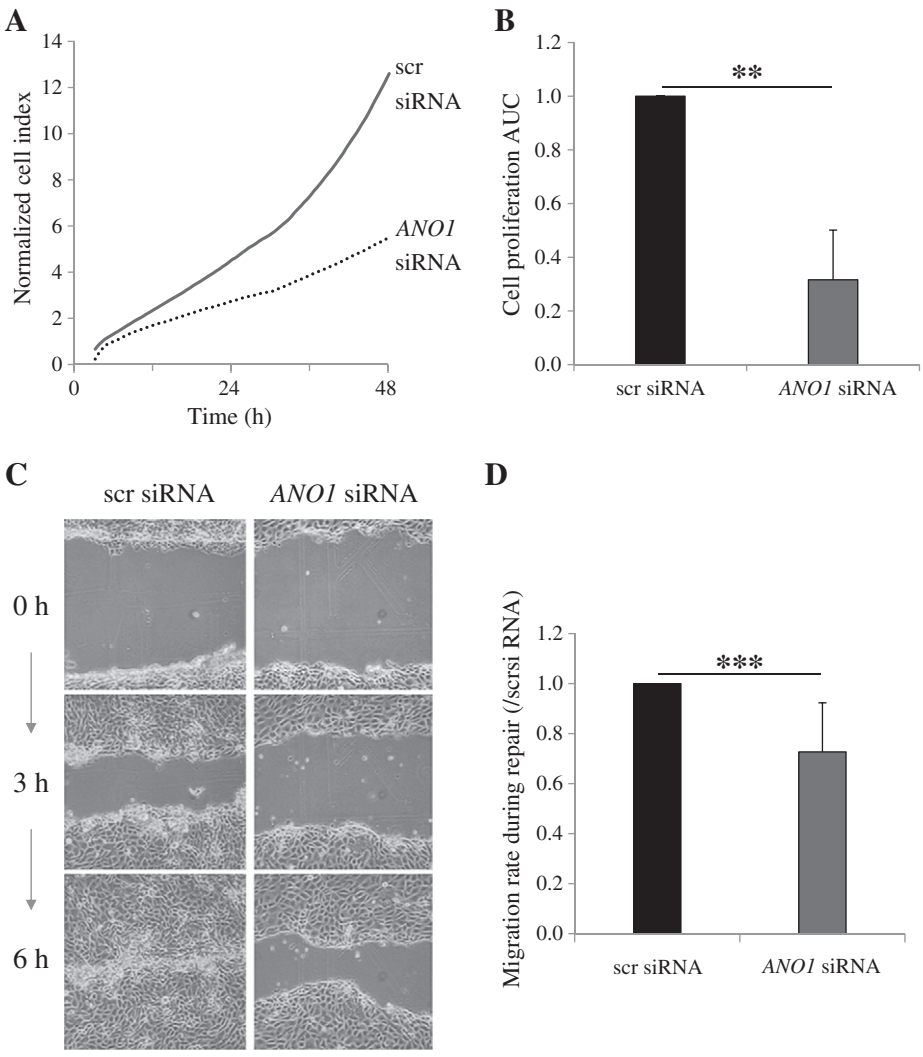

Fig. 6. Effect of ANO1 inhibition on proliferation and migration of non-CF bronchial epithelial cell line. A) Original traces of cell proliferation of non-CF cells transfected with scrambled siRNA (scr siRNA) or *ANO1* siRNA measured using an RTCA instrument. B) Quantification of cell proliferation performed using area-under-the-curve (AUC) values normalized to control (scr siRNA) ($n = 6$; $p < 0.01$). C) Representative photographs taken every 3 h during 6 h of wound closure of non-CF bronchial epithelial cell line transfected with scrambled siRNA (scr siRNA) or *ANO1* siRNA. D) Migration rate during repair of non-CF cells transfected with scrambled siRNA (scr siRNA) or *ANO1* siRNA ($n = 13$; $p < 0.001$).

for the expression levels of respective non-CF models. Each sample was assessed in triplicate to insure experiment quality.

2.9. Total protein extraction and Western-blot analysis

As previously detailed [42], 20 μg of total proteins extract was reduced and size-separated on 8% SDS-polyacrylamide gels then transferred to PVDF membranes (Bio-Rad, Marnes-la-Coquette, France), blocked in 5% BSA (PAA, Les Mureaux, France) and incubated with specific primary antibodies against ANO1 [43] (Abcam) and β-actin (Sigma, Saint Quentin Fallavier, France). The proteins of interest were detected, imaged, and quantified as previously described [40].

2.10. Statistical analysis

All data are described as mean ± SD. In the figure legends, n indicates the number of repeated experiments. Between-group differences were tested using the unpaired Mann–Whitney test. Values of p lower than 0.05 were considered significant; in the figures, statistically significant differences with $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***) are indicated.

3. Results

3.1. Proliferation and migration are delayed in CF bronchial epithelial cells

We first compared proliferation of non-CF and CF bronchial epithelial cell lines under basal conditions for 48 h using real time cell proliferation (Fig. 1A). After quantification, we found significantly lower proliferation of the CF cells compared to the non-CF cells (Fig. 1B). Measuring non-CF and CF cell proliferation using a Malassez hemocytometer produced similar results (data not shown).

We next evaluated cell migration by performing wound closure assays with non-CF and CF cell lines. Cell migration of non-CF and CF cells was first followed until full covering of the cell-free areas. Representative images of wound closure assays showed marked reduced migration rate in CF compared to non-CF cells (Fig. 1C). As illustrated in Fig. 1D, the mean time needed to achieve full coverage of the cell-free gap was significantly increased in CF compared to non-CF cells (12 h

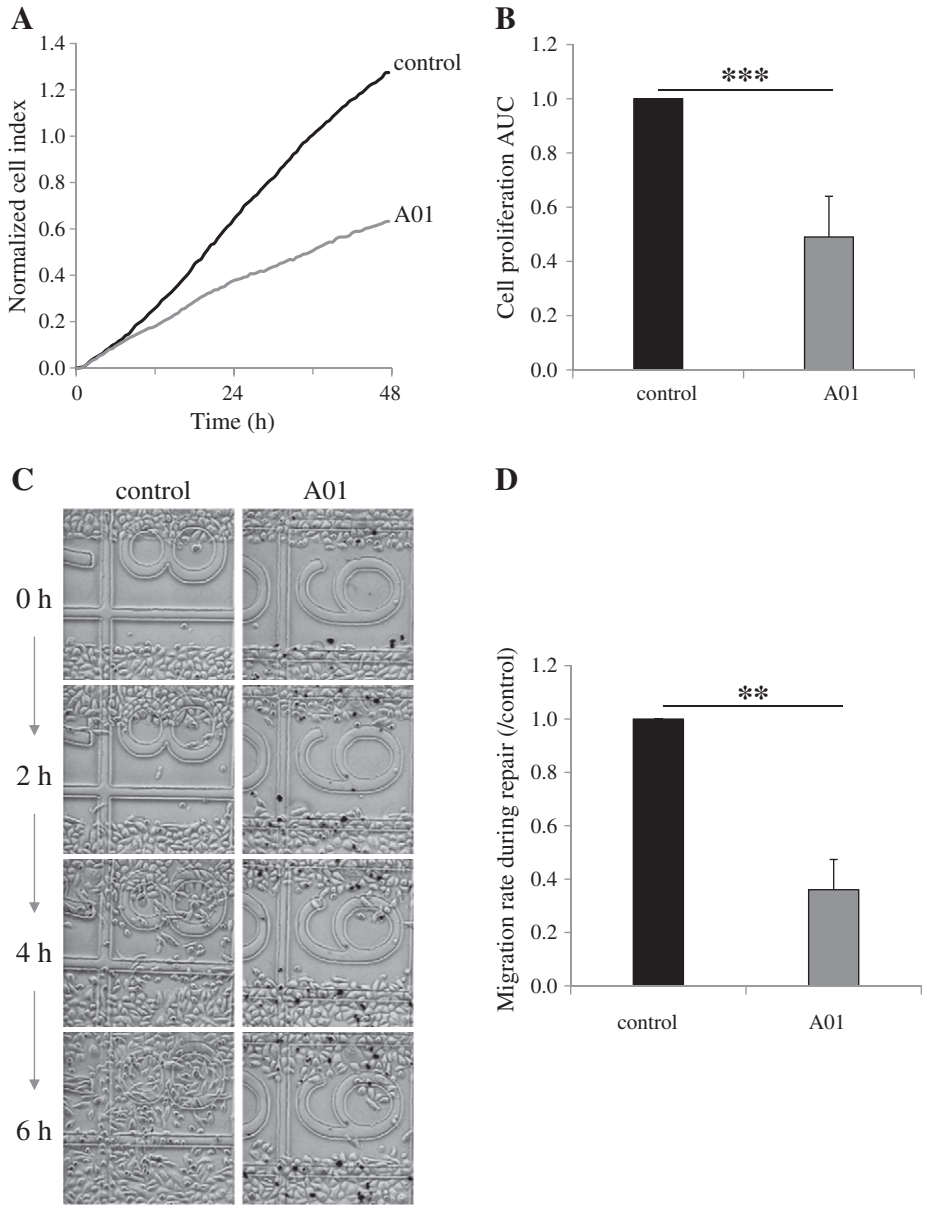

Fig. 7. Effect of ANO1 channel activity inhibitor on proliferation and migration of primary non-CF bronchial epithelial cells. A) Original traces of cell proliferation of non-CF primary cells treated with DMSO (control) or A01 (10 μM) measured using an RTCA instrument. B) Quantification of cell proliferation performed using area-under-the-curve (AUC) values normalized to control ($n = 5$; $p < 0.001$). C) Representative photographs taken every 2 h during 6 h of wound closure of non-CF primary cells treated with DMSO (control) or A01 (10 μM). D) Migration rate during repair of non-CF primary cells treated with DMSO (control) or A01 (10 μM) ($n = 6$; $p < 0.01$).

vs. 4 h). Based on the kinetic study, we decided to perform further wound closure assays within 6 h. Migration rate of CF cells (14.6 μm/h) was significantly lower than non-CF cells (61.9 μm/h) ($n \geq 5$; $p = 0.004$). To be sure and to avoid any significant cell proliferation, we have also calculated cell migration rate during repair of non-CF and CF cells during the first 2 h following the start of the experiment. The results show that migration rate during this short period is significantly reduced, 2.3 fold lower ($p < 0.05$) in CF compared to non-CF cells ($p < 0.05$; data not shown).

To confirm these findings, we performed proliferation and wound closure assays with primary bronchial epithelial cells from non-CF and CF patients (Fig. 2). Fig. 2A and B showed that proliferation of CF cells was significantly reduced compared to non-CF cells. Migration rate during repair was 2 fold lower in primary CF cells compared to non-CF cells (Fig. 2C and D).

Taken together, these results indicated a delayed proliferation and a reduced migration rate in CF compared to non-CF cells.

3.2. ANO1 chloride channel activity is decreased in CF bronchial epithelial cells

We then evaluated ANO1 Cl^- activity in non-CF compared to CF bronchial epithelial cells.

To first characterize CFTR activity, we measured CFTR Cl^- efflux under basal conditions in both cell lines (Fig. 3A). CFTR Cl^- activity was assessed by I^- quenching of a pre-transfected protein which is fluorescent and halide-sensitive. Fluorescence quenching by I^- influx correlates directly with the Cl^- channel activity. CFTR was stimulated with a cocktail of cAMP agonists prior to recording. As expected, YFP fluorescence was significantly greater in CF than in non-CF cells indicating lower CFTR Cl^- activity in CF cells. To ensure that we succeeded in separating CFTR and ANO1 Cl^- efflux, we measured Cl^- efflux stimulated with either CFTR (Fig. 3B) or ANO1 (Fig. 3C) activators in non-CF cells treated with CFTR or ANO1 inhibitors. CFTR Cl^- efflux was significantly decreased by CFTR(inh)-172 but not by *ANO1* siRNA and the opposite

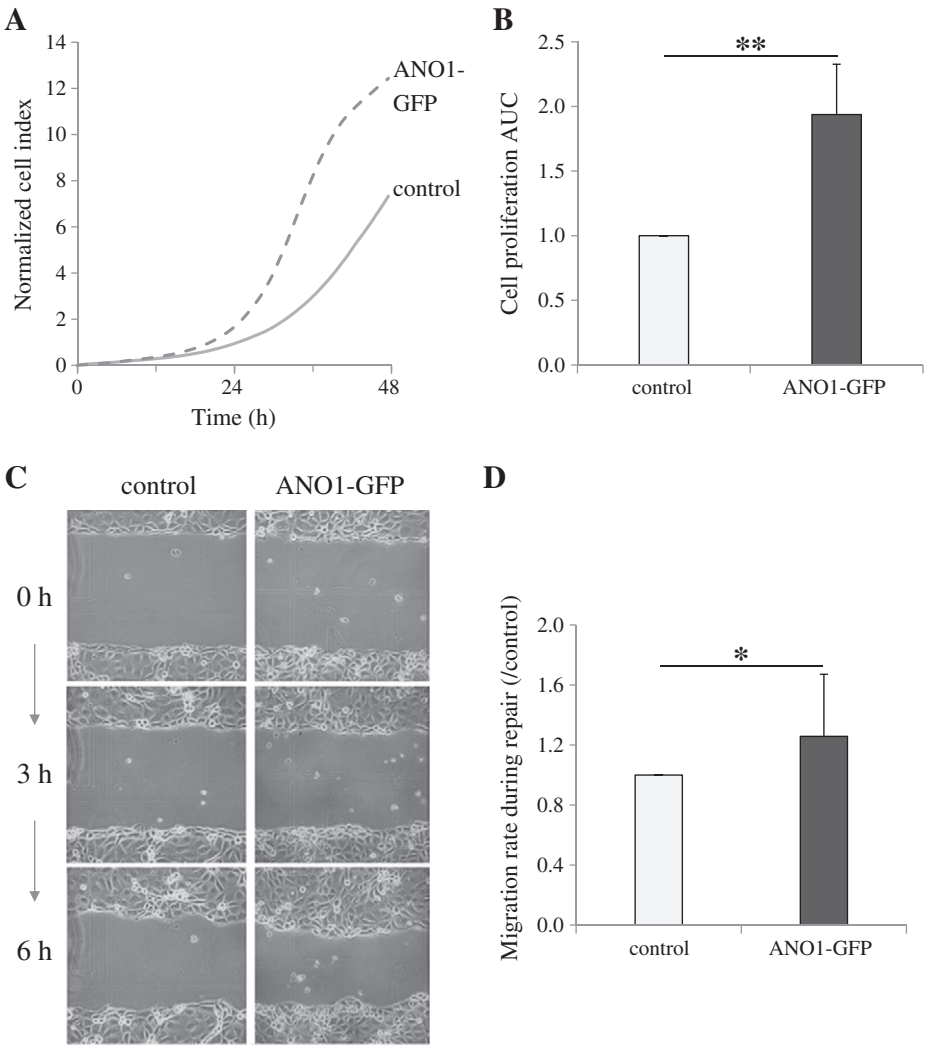

Fig. 8. Effect of ANO1 overexpression on proliferation and migration of CF bronchial epithelial cell line. A) Original traces of cell proliferation of CF cells transfected with control or ANO1 overexpression vector (ANO1-GFP) measured using an RTCA instrument. B) Quantification of cell proliferation performed using area-under-the-curve (AUC) values normalized to control ($n = 4$; $p < 0.01$). C) Representative photographs taken every 3 h during 6 h of wound closure of CF cells transfected with control vector or ANO1 overexpression vector. D) Migration rates during repair of CF cells transfected with control vector or ANO1 overexpression vector ($n = 13$; $p < 0.05$).

result was observed when ANO1 Cl^- efflux was measured, confirming the specificity of our techniques.

We compared ANO1 Cl^- activity under basal conditions in non-CF and CF cells (Fig. 3D). YFP fluorescence was significantly less reduced by purinergic stimulation in CF than in non-CF cells implying that ANO1-dependent Cl^- efflux was significantly reduced in CF compared to non-CF cell lines. Similar results were obtained on human primary cells, and significantly inhibited by a pharmacological inhibitor of ANO1 (A01) (Fig. 3E).

3.3. Endogenous ANO1 protein is mainly localized at the plasma membrane of non-CF bronchial epithelial cells

We then hypothesized that the impairment in ANO1 Cl^- activity in CF cells might be ascribable to abnormal intracellular localization linked to CF context. We investigated localization of endogenous ANO1 protein in bronchial epithelial cells. First, we performed immunocytochemistry experiments on non-CF and CF cell lines using a specific ANO1 antibody. Fig. 4A showed that ANO1 protein is localized at the plasma membrane in non-CF cells but not exclusively because ANO1 was also detected in the cytoplasm of these cells. Interestingly, we cannot detect ANO1 at the plasma membrane in CF cells using the same exposure time. ANO1 appeared to be predominantly localized in the cytoplasm of CF cells. With primary non-CF and CF cells, ANO1 was detectable at the plasma membrane in non-CF cells but not in CF cells confirming previous results (Fig. 4B).

To determine whether ANO1 trafficking was normal in CF cells, we induced ANO1 overexpression by transiently transfecting a GFP-tagged ANO1 vector into non-CF and CF cell lines. The confocal immunofluorescence images demonstrated that ANO1 protein co-localized with the plasma membrane marker (WGA) (Fig. 4C) in both non-CF and CF transfected cells. We observed, by halide sensor method, a 4.1-increase of ANO1 activity when non-CF cells were transfected with this ANO1-GFP plasmid ($p < 0.001$; data not shown). Thus, ANO1 trafficking to the plasma membrane was correct in CF bronchial epithelial cells.

3.4. ANO1 protein expression is decreased in CF models

We then investigated whether the decreased ANO1 Cl^- activity in CF cells might be due to a decreased ANO1 expression. We first compared *ANO1* mRNA expression under basal conditions using real-time quantitative PCR (Fig. 5A). Interestingly, we found a strong significant decrease in *ANO1* mRNA expression in CF compared to non-CF cells. Western-blot analysis showed that ANO1 protein expression was 3.5-fold lower in CF cells compared to non-CF cells (Fig. 5D).

To confirm these findings, we used other relevant non-CF and CF models. We compared *ANO1* mRNA expression in lungs from non-CF-like and CF-like (homozygous for the F508del mutation) mice having the same genetic background (Fig. 5B). *ANO1* transcripts were significantly decreased in lungs from CF-like mice. We also evaluated *ANO1* mRNA expression in lung explants from non-CF and CF (F508del/F508del) patients, and found significantly lower mRNA levels in the CF explants (Fig. 5C). Finally, we measured ANO1 protein levels in primary bronchial epithelial cells cultured in air–liquid interface obtained from non-CF and CF (F508del/F508del) patients. ANO1 protein was significantly reduced in CF compared to non-CF human primary cells (Fig. 5E). Thus, ANO1 expression was decreased in all CF models carrying the homozygous F508del mutation compared to corresponding non-CF models.

3.5. ANO1 is involved in delayed proliferation and migration of CF cells

To investigate the involvement of ANO1 in epithelial repair and to test whether decreased in ANO1 activity and expression in CF models might explain the difference in proliferation and migration rate, we evaluated the consequences of ANO1 inhibition on cell proliferation and migration. To decrease ANO1 expression in non-CF cell line, we transfected either a specific *ANO1* siRNA or scrambled siRNA. We checked *ANO1* siRNA efficiency by analyzing ANO1 mRNA and protein levels after transfection. *ANO1* mRNA levels were 2-fold decreased in cells transfected with specific *ANO1* siRNA (data not shown). We then measured cell proliferation in these conditions. The normalized cell index of non-CF cells transfected with *ANO1* siRNA was significantly lower compared to control condition (Fig. 6A). Fig. 6B shows the areas under the cell-proliferation curves. In addition, wound closure assays showed that the migration rate during repair was significantly decreased in non-CF cells transfected with *ANO1* siRNA compared to cells transfected with scrambled siRNA (Fig. 6C and D).

To determine whether the channel activity of ANO1 was required for cell proliferation and migration, we performed proliferation and wound closure assay with primary non-CF cells treated or not with a pharmacological blocker of ANO1 channel i.e., T16Ainh-A01 or A01. Fig. 7A and B show that primary non-CF cell proliferation was significantly decreased in the presence of A01. Wound closure assay with previously described conditions showed that A01 induced a significantly marked decrease in cell migration (Fig. 7C and D).

Finally, to determine whether ANO1 overexpression may improve CF cell proliferation and epithelial repair, we analyzed the consequences of ANO1 overexpression on proliferation and migration rate of the CF cell line. We transfected ANO1-overexpression or control vector in CF cells and checked transfection efficiency by comparing *ANO1* mRNA expression in CF cells transfected with ANO1 overexpression vector or control vector. As expected, *ANO1* mRNA levels were 70-fold increased with ANO1-overexpression vector (data not shown). We measured also ANO1 Cl^- activity in cells transfected with ANO1 overexpression vector and showed that ANO1 overexpression increased ANO1 Cl^- efflux (data not shown).

Real time proliferation experiments showed that proliferation was significantly increased in CF cells overexpressing ANO1 compared to control (Fig. 8A and B). Although the migration defect was not completely corrected, the migration rate of CF cells was significantly increased by ANO1 overexpression (Fig. 8C and D).

Thus, these data established that ANO1 is involved in cell proliferation and in the abnormal repair observed in CF bronchial epithelial cell monolayers and that ANO1 overexpression improves these two parameters in CF cells.

4. Discussion

Although the existence of chloride channels other than CFTR was known for more than 30 years [6], their possible contribution to the progression of CF pathology remains unclear. Here, after confirming that CF bronchial epithelial cells displayed an abnormal repair process, we report the first evidence that ANO1 expression is significantly decreased in various models of CF bronchial epithelia homozygous for the F508del mutation compared to non-CF models. The decrease in ANO1 expression resulted in decreased ANO1 Cl^- secretion by CF bronchial epithelial cells. We also show that ANO1 is involved in cell proliferation and migration and that ANO1 overexpression in CF cells leads to improve bronchial epithelial repair. All together, our data support a role for defective ANO1 expression and activity in the delayed repair of CF airway epithelium.

We find decreased ANO1 Cl^- activity in CF compared to non-CF bronchial epithelial cells. Several groups have assayed ANO1 activity in non-CF cells, but none compared CF and non-CF models. However, before the molecular identification of ANO1 as a CaCC candidate, global CaCC currents were described as normal or increased in nasal epithelial cells from CF patients [8–11]. Several hypotheses may explain the discrepancies between these findings and our results. First, ANO1 may be only a minor component of Ca^{2+}-activated Cl^- conductance [37], although this is still debated [44]. The ANO1-specific Cl^- efflux measured in our study may not represent global Ca^{2+}-activated Cl^- conductance recorded in previous studies. Other unidentified molecular CaCCs expressed in the airway could be up-regulated in CF thereby increasing the total Ca^{2+}-activated Cl^- conductance. Second, supporting our results, a more recent study showed significantly decreased ATP-induced Cl^- currents in cultures of bronchial and bronchiolar epithelial cells from CF compared to non-CF patients [45]. Most studies comparing global CaCC activity between non-CF and CF models were done using only nasal epithelium cells and not bronchial cells. We propose that CaCC distribution and regulation may differ between the upper and lower airways. This hypothesis is supported by a study reporting considerable differences in global gene expression patterns between the nasal and the bronchial CF epithelium [46], suggesting that the nasal epithelium may be a poor surrogate for the lower airway epithelium.

Our data indicate that the decreased Cl^- channel activity in CF cells is ascribable to decreased ANO1 mRNA and protein expression. To our knowledge, a single previous study showed also significantly decreased *ANO1* mRNA levels in CF compared to non-CF bronchial epithelial cells [47], which the authors ascribed to clonal drift. In our work, the confirmation that ANO1 mRNA and protein levels are low in other relevant and non-immortalized CF models suggests instead true down-regulation of ANO1 expression in CF that may participate to the worsening of the disease. Our first hypothesis to explain such decreased expression involves the unfolded protein response (UPR). This cell recovery mechanism was described as activated in CF airways notably by the presence of F508del CFTR to regulate protein load of the endoplasmic reticulum [48]. Activation of the UPR induces transcriptional regulations and for example a decreased *CFTR* mRNA expression [49]. ANO1 decreased expression in CF bronchial epithelial models may be related to this mechanism. This hypothesis may be linked to the results of Kunzelmann and collaborators [26]. These authors show that currents generated by ANO1 are inhibited by CFTR suggesting a link between both proteins. Moreover, a previous study documented that a marked decrease in *ANO1* mRNA expression in sino-nasal epithelium by hypoxia exposure may be through a decrease in steady-state mRNA [50]. These data suggest a role for oxygen restriction, present in advanced CF lung disease, which could explain the decreased ANO1 expression in CF human lung explants in our study. The exact mechanisms responsible for the decreases in ANO1 mRNA and protein in CF lower airways remain to be identified.

The second major finding from our work is the involvement of ANO1 in the abnormal proliferation and repair of CF bronchial epithelial cells. Our study first confirms that CF bronchial epithelial cells show less proliferative potential and decreased migration rate during epithelial repair compared to non-CF cells. Indeed, similarly, marked delays in wound closure have been documented in another pair of CF and non-CF bronchial epithelial cell lines and also in CF versus non-CF human primary

cells [5,51]. Several mechanisms may be involved, including an inherent defect of the CF airway epithelia due to *CFTR* mutation [5], effects of bacterial infections [52] and effects of the chronic inflammatory context seen in CF airways [2]. The mechanisms that underlie wound repair resemble those involved in tumor cell migration: in both cases, ion channels regulating cell volume and membrane potential play a crucial role [53]. In the present study, we demonstrate that specific ANO1 inhibition in non-CF cells induces a decrease of cell proliferation and migration during repair. These results corroborate cancer studies indicating involvement in cell proliferation of Cl^- channels in general and of ANO1 in particular [30,31,54]. In an earlier study, it has been shown that loss of CFTR Cl^- activity directly impaired wound closure by decreasing the lamellipodium surface area, thereby impairing cell migration [55]. Conceivably, the diminished ANO1 Cl^- activity observed in CF bronchial epithelial cells may have a similar effect on lamellipodia.

In conclusion, our study highlights for the first time a default of both ANO1 activity and expression in CF airways and supports a major role for ANO1 deficiency in abnormal epithelial repair observed in CF airways. These findings indicate the potential interest to develop drugs targeting this protein to prevent the progression of lung disease in cystic fibrosis patients.

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